DRG® Human β Amyloid 1 – 42 Colorimetric (COL-3995)

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1 NAME AND INTENDED USE

The DRG Human β Amyloid (hAβ42) ELISA is for determination of hAβ42 in samples (e.g., serum, tissue culture medium, etc.). The assay will recognize both natural and synthetic forms of hAβ42. The anti-human Aβ42 antibody used in this kit is capable of selectively detecting Aβ42 and not Aβ40/Aβ43 (4-6).

This kit has been configured for research use only and is not to be used in diagnostic procedures.

2 PRINCIPLE OF THE TEST

The DRG Human Aβ42 kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for the NH_2 terminus of hAβ has been coated onto the wells of the microtiter strips provided. Samples, including standards of known hAβ content, control specimens and unknowns are pipetted into these wells, followed by the addition of a rabbit antibody specific for the 1-42 sequence of hAβ. Bound rabbit antibody is detected by the use of a horseradish peroxidase-labeled anti-rabbit antibody.

After removal of excess anti-rabbit antibody, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of hAβ (1-42) present in the original specimen.

3 WARNINGS AND PRECAUTIONS FOR USERS

3.1 General Remarks

- This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.
- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing infectious agents.

3.2 Remarks for the Test

1. When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
2. Microtiter plates should be allowed to come to room temperature before opening the foil bags. Once the desired number of strips has been removed, immediately reseal the bag and store at 2 - 8°C to maintain plate integrity.
3. Samples should be collected in pyrogen/endotoxin-free polypropylene tubes.
4. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely, microfuge and mix well with diluent prior to analysis.
5. When analyzing samples, add a protease inhibitor cocktail with AEBSF (a serine protease inhibitor) and prepare the standard dilutions using the same diluent as used with the biological samples. Serine proteases can rapidly degrade Aβpeptides, thus using AEBSF (water soluble and less toxic than PMSF) at a 1 mM final concentration is very helpful. Keep samples on ice until ready to apply to plate.
6. When possible, avoid use of badly hemolyzed or lipemic sera. If large amounts of particulate matter are present, centrifuge prior to analysis.
7. Standards can be dispensed into a reservoir and transferred to the plate using a multi-channel pipette. Be sure to aspirate all previous standard solution prior to adding the next standard (in ascending order) to the reservoir.
8. It is recommended that all standards and samples be run in duplicate. Prepare standards and samples in 5 mL polypropylene tubes. We recommend diluting samples 1:2 to 1:5 for Aβ42.
9. Sample matrix has a dramatic impact on Aβrecovery. To ensure accurate quantitation, the standard curves must be generated in the same diluent as the samples.
10. Avoid use of sodium azide, thymol or thimerosal as sample preservatives. These agents inhibit measurement of Aβpeptide.
11. The *Standard/Sample Diluent* is a sterile fluid and should be dispensed and used to avoid contamination. Use immediately after opening or store frozen.
12. Samples that are >1000 pg/mL should be diluted with Standard/Sample Diluent.
13. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
14. Do not mix or interchange different reagent lots from various kit lots.
15. Do not use reagents after the kit expiration date.
16. Read absorbances within 2 hours of assay completion.
17. We recommend covering plate with adhesive plate covers during incubation steps.

18. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. **Never** insert absorbent paper directly into the wells.
19. Because Stabilized Chromogen is light sensitive, avoid prolonged exposure to light. Also avoid contact between Stabilized Chromogen and metal, or color may develop.

4 KIT COMPONENTS

Reagents	Quantity 96 Test kit	Preparation and Storage
hAβ42 Standard, synthetic peptide Refer to vial label for quantity and reconstitution volume.	1 vials	Lyophilized 2 – 8°C
Standard/Sample Diluent; Sterile filtered.	1 x 60 mL	2 – 8°C
Antibody -Coated Wells 96 wells per plate. Plate pre-coated with mAb to NH_2 terminus of Aβ	1 plate	2 – 8°C
Detection Antibody Diluent. Contains 15 mM sodium azide.	1 x 11 mL	2 – 8°C
Detection Antibody. Rabbit anti-hAβ42. Contains 15 mM sodium azide.	1 x 50 µL	2 – 8°C
Secondary Antibody (100x). Anti-Rabbit IgG (horseradish peroxidase labeled). Contains 3.3 mM thymol.	1 x 125 µL	Concentrate 2 – 8°C
Secondary Antibody Diluent: Contains 3.3 mM thymol.	1 x 25 mL	2 – 8°C
Wash Buffer (25x)	1 x 100 mL	Concentrate 2 – 8°C
Stabilized Chromogen, Tetramethylbenzidine (TMB);	1 x 25 mL	2 – 8°C
Stop Solution	1 x 25 mL	2 – 8°C
Plate Covers, adhesive strips	3	2 – 8°C

NOTE: Once opened, standard, standard diluent, detection and secondary antibodies should be stored at -20 to -80°C for improved shelf life.

5 MATERIALS REQUIRED BUT NOT SUPPLIED

- Standard Reconstitution Buffer [55 mM Sodium Bicarbonate Buffer ($NaHCO_3$, ultrapure grade), pH 9.0].
- Calibrated adjustable precision pipettes, preferably with disposable plastic tips (a manifold multi-channel pipette is desirable for large assays), beakers and graduated cylinders.
- Reagent reservoirs for dispensing standards, antibody solutions and substrate.
- Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).
- Deionized or distilled H_2O.

- Microtiter plate reader capable of measurement at or near 450 nm.
- Shaking platform (for low to moderate shaking) with plate and tube vortex adapter, or a mini-orbital shaker.
- Disposable 12 x 75 mm polypropylene tubes for diluting standards and samples.
- 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) or protease inhibitor cocktail containing AEBSF.
- Absorbent paper towels.
- Graph paper: linear (Cartesian), log-log, or semi-log, as desired.

Assay Procedure

5.1 Directions for Washing

Incomplete washing will adversely affect the test outcome. All washing must be performed with *Wash Buffer* provided.

Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) into the bottom of each well. Take care not to scratch the inside of the well.

After aspiration, fill the wells with at least 0.4 mL of diluted wash solution. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under **Assay Procedure**. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

Alternatively, the wash solution may be put into a squirt bottle. If a squirt bottle is used, flood the plate with wash buffer, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

If using an automated washer, we suggest 4 wash cycles for all wash steps except the last wash step where 5 wash cycles are recommended.

5.2 Preparation and Storage of Reagents

This assay has been calibrated against the mass determination of highly purified native Aβ where mass was corrected for peptide content by amino acid analysis.

5.2.1 Preparation of Standard Reconstitution Buffer

Dissolve 2.31 grams of sodium bicarbonate in 500 mL of distilled or deionized water.
Add 2 N sodium hydroxide until pH is 9.0. Filter solution through a 0.2 µm filter unit.

5.2.2 Reconstitution and Dilution of hAβ42 Standard

Note: Polypropylen tubes may be used for standard dilutions.

1. Remove the *hAβ42 Standard* vial from storage and let equilibrate to room temperature (RT). Reconstitute the *hAβ42 Standard* to 1.0 µg/mL with Standard Reconstitution Buffer (55 mM sodium bicarbonate, pH 9.0). Refer to the standard vial label for instructions. Swirl or mix gently and allow vial to sit for 5 minutes at room temperature then transfer to ice for 90 minutes with occasional mixing. Briefly vortex prior to preparing standards.
2. Generation of the standard curve using the Aβ peptide standards provided in the kit must be performed using the same composition of buffers used for the diluted experimental samples. For example, if brain extracts are diluted 1:10 with *Standard/Sample Diluent*, then the buffer used to dilute standards should be 90% *Standard/Sample Diluent* and 10% brain extraction buffer (including AEBSF at a final concentration of 1 mM).

3. Add 0.100 mL of the reconstituted standard to a tube containing 0.900 mL *Standard/Sample Diluent* or as otherwise dictated by the treatment of experimental samples. Label as 100,000 pg/mL hAβ42. Mix.
4. Add 0.100 mL of the 100,000 pg/mL standard to a tube containing 0.900 mL *Standard/Sample Diluent*. Label as 10,000 pg/mL.
5. Add 0.200 mL of 10,000 pg/mL standard to a tube containing 1.8 mL of *Standard/Sample Diluent*. Label as 1000 pg/mL.
6. Add 1.0 mL of *Standard/Sample Diluent* to each of 6 tubes labeled 500, 250, 125, 62.5, 31.25, 15.63, 0 pg/mL hAβ42.
7. Make serial dilutions of the standard as described in the dilution table on the next page. Mix thoroughly between steps.

Dilution table:

Standard:	**Add:**	**Into:**
1000 pg/mL	Prepare by adding 0.2 mL of 10,000 pg/mL standard to 1.8 mL of Diluent Buffer.	
500 pg/mL	1.0 mL of the 1000 pg/mL std.	1.0 mL of the Diluent Buffer
250 pg/mL	1.0 mL of the 500 pg/mL std.	1.0 mL of the Diluent Buffer
125 pg/mL	1.0 mL of the 250 pg/mL std.	1.0 mL of the Diluent Buffer
62.5 pg/mL	1.0 mL of the 125 pg/mL std.	1.0 mL of the Diluent Buffer
31.25 pg/mL	1.0 mL of the 62.5 pg/mL std.	1.0 mL of the Diluent Buffer
15.63 pg/mL	1.0 mL of the 31.25 pg/mL std.	1.0 mL of the Diluent Buffer
0 pg/mL	1.0 mL of the Diluent Buffer	An empty tube

Remaining reconstituted hAβ42 standard may be stored at -80ºC for up to 4 months.
Return the Standard Diluent Buffer to the freezer.

5.2.3 Preparation of Samples

Prepare one or more dilutions of each sample. These dilutions should be made in Standard/Sample Diluent, although the exact dilution must be determined empirically (e.g., 1:2 and 1:5 represent a reasonable range). This dilution must be performed because certain components in samples can interfere with the detection of the Aβ peptides and/or Aβ levels are too high. AEBSF should be added to the diluted samples and the standards at a final concentration of 1 mM in order to prevent proteolysis of the Aβ peptides.

5.2.4 Preparation of Detection Antibody Solution

Prepare Detection Antibody Solution by thoroughly mixing contents of the Detection Antibody vial with the entire contents of the Detection Antibody Diluent (11 mL).

5.2.5 Preparation of Secondary Antibody Solution

Please Note: The Anti-rabbit IgG-HRP 100x concentrate is in 50% glycerol. This solution is viscous. To ensure accurate dilution, allow Anti-rabbit IgG-HRP concentrate to reach room temperature. Gently mix. Pipette Anti-rabbit IgG-HRP concentrate slowly. Remove excess concentrate solution from pipette tip by gently wiping with clean absorbent paper.

1. Dilute 10 µl of this 100x concentrated solution with 1 mL of Secondary Antibody Diluent for each 8-well strip used in the assay. Label as Anti-rabbit IgG-HRP Working Solution.

   For Example:

# of 8-Well Strips	Volume of Secondary Antibody (HRP 100x conc.) Concentrate	Volume of Diluent
2	20 µL solution	2 mL
4	40 µL solution	4 mL
6	60 µL solution	6 mL
8	80 µL solution	8 mL
10	100 µL solution	10 mL
12	120 µL solution	12 mL

2. Return the unused Secondary Antibody (HRP 100x concentrate) to the refrigerator.

5.2.6 Preparation of Wash Buffer

Allow the 25x concentrate to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of the 25x wash buffer concentrate with 24 volumes of deionized water (e.g., 50 ml may be diluted up to 1.25 liters, 100 ml may be diluted up to 2.5 liters). Label as Working Wash Buffer.

Store both the concentrate and the Working Wash Buffer in the refrigerator.

The diluted buffer should be used within 14 days.

5.3 Assay Procedure

Be sure to read the *Warnings and Precautions for Users* section before carrying out the assay.

Note: The protocol described below has been developed to provide sensitive and reproducible detection of Aβ peptides. Although the assay uses a standard double antibody ELISA approach combined with colorimetric detection, the inherent complexity in handling/detecting Aβ peptides must be recognized.

1. Reconstitute hAβ42 Standard.
2. Prepare samples and standards with appropriate diluents.
3. Wash plate 4x with Working Wash Buffer, then pat dry on a paper towel. See DIRECTIONS FOR WASHING.
4. Add 100 µL/well of Aβ peptide standards, controls, and dilutions of samples to be analyzed. Incubate for 2 hours while shaking on an orbital plate shaker at RT **or** overnight at 4°C without shaking.
5. Wash plate 4x with Working Wash Buffer and pat dry on a paper towel. See DIRECTIONS FOR WASHING.
6. Pour Detection Antibody Solution into reservoir; immediately add 100 µL/well of Detection Antibody Solution to the plate. Incubate at RT for 2 hours while shaking.

7. Wash plate 4x with Working Wash Buffer, then pat plate dry on a paper towel. See DIRECTIONS FOR WASHING.
8. Pour Secondary Antibody Solution into reservoir; immediately add 100 µL/well of Secondary Antibody Solution to the plate. Incubate plate at RT for 2 hours with shaking.
9. Wash plate 5x with Working Wash Buffer, pat dry on a paper towel. See DIRECTIONS FOR WASHING.
10. Add 100 µL of Stabilized Chromogen to each well. The liquid in the wells will begin to turn blue.
11. Incubate for **30 minutes at room temperature and in the dark.** ***Please Note*****: Do not cover the plate with aluminum foil or metallized mylar.** The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limits of the instrument. The O.D. values at 450 nm can only be read after the *Stop Solution* has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.
12. Add 100 µL of Stop Solution to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.
13. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µL each of Stabilized Chromogen and Stop Solution.
    Read the plate within 2 hours after adding the Stop Solution.

6 CALCULATION OF THE RESULTS

1. Plot on graph paper the absorbance of the standards against the standard concentration. (Optimally, the background absorbance may be subtracted from all data points, including standards, unknowns and controls, prior to plotting.) Draw the best smooth curve through these points to construct the standard curve. If using curve fitting software, the four parameter algorithm provides the best curve fit.
2. Read the hAβ1-42 concentrations for unknown samples and controls from the standard curve plotted in step 1. **Multiply value(s) obtained for serum/plasma sample(s) by the appropriate factor to correct for the sample dilution in section 7.2.3** (Samples producing signals higher than that of the highest standard (1000 pg/mL) should be further diluted in *Standard/Sample Diluent Buffer* and reanalyzed, multiplying the concentration found by the appropriate dilution factor.)

6.1 Typical Data

The following data were obtained for the various standards over the range of 0 to 1000 pg/mL hAβ42.

Standard hAβ42 (pg/mL)	Optical Density (450 nm)
0	0.221
15.63	0.241
31.25	0.257
62.5	0.316
125	0.385
250	0.601
500	1.059
1000	1.864

7 LIMITATIONS OF PROCEDURE

Do not extrapolate the standard curve beyond the 1000 pg/mL standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute samples >1000 pg/mL with Standard/Sample Diluent Buffer, reanalyze these and multiply results by the appropriate dilution factor.

The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of serum samples have not been thoroughly investigated. The rate of degradation of native hAβ in various matrices has not been investigated. The immunoassay literature contains frequent references to aberrant signals seen with some sera, attributed to heterophilic antibodies. Though such samples have not been seen to date, the possibility of this occurrence cannot be excluded.

This kit is for research use only. Not for human therapeutic or diagnostic use.

8 REFERENCES

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