Chemiluminescent BetaMark™ x-40 ELISA Kit Components and Protocol

Kit Components

• Capture Antibody Coated Plate*	1 plate
• 1-40 Standard	(1) 20µg vial
• 5X Wash Buffer	125mL
• Standard Diluent	3mL
• 2X Incubation Buffer	12mL
• HRP Detection Antibody	40µL
• Chemiluminescent Substrate A	6mL
• Chemiluminescent Substrate B	6mL
• Plate Sealer	1
• 1.5mL Microcentrifuge Tubes	10

* The strips in this plate are not readily removable; it is intended to be used as a whole plate. Attempting to remove the strips will result in damage for which BioLegend is not responsible.

Kit Protocol

Assay Preparation

Note: Equilibrate all kit components to room temperature before use

I. Preparation of Standard Intermediates (Refer to Table 1)
   A. Label (2) 1.5mL microcentrifuge tubes as intermediate #1 & 2 (use enclosed tubes).
   B. Add 990µL of standard diluent to intermediate tubes #1 & 2.
   C. Reconstitute 20µg vial of 1-40 standard with 80µL of Standard Diluent. Mix well by inversion, do not vortex. Concentration will be 250µg/mL.

Once reconstituted, standard must be used within the same day.

   D. Add 10µL from the vial of reconstituted 1-40 standard to 990µL of standard diluent in intermediate tube #1. Mix well by inversion, do not vortex.
   E. Remove 10µL from intermediate #1 tube and add to 990µL standard diluent in intermediate #2 tube. Mix well by inversion, do not vortex.
   F. The final concentration of intermediate tube #2 will be 25ng/mL.

TABLE 1.

Tube Number	Volume of Standard	Volume of Standard Diluent (µL)	Final Concentration (ng/mL)
Reconstituted Standard	0	80	250,000
Intermediate #1	10 µL of reconstituted standard	990	2,500
Intermediate #2	10 µL of intermediate #1	990	25

II. Preparation of 1X Incubation Buffer

A. Label a 50mL centrifuge tube as "1X Incubation Buffer".

B. Dilute 2X incubation buffer to 1X by adding 10mL of 2X incubation buffer to 10mL of lab grade water* in the 50mL tube labeled "1X Incubation Buffer".

C. Mix well by vortexing. This will be diluent for the standard curve and samples.

III. Preparation of 1X Wash Buffer

A. Label a 1L container as "1X Wash Buffer".

B. Dilute 5X wash buffer to 1X for use. Mix 125mL of 5X wash buffer with 500mL of lab grade water* for a total volume of 625mL.

C. Mix well.

*Note: Lab grade filtered water such as injection grade, cell culture grade, Reverse Osmosis De-Ionization (RODI).

IV. Preparation of Standard Curve (Refer to Table 2)

A. Label (8) 1.5mL microcentrifuge tubes as #1-8 (use enclosed tubes).

B. Aliquot 240µL of the 1X incubation buffer to each of the standard curve tubes (#2-8) and 494µL to tube #1 (standard curve top point).

C. Remove 6µL from intermediate #2 and add to 494µL of 1X incubation buffer in tube #1 (this will be the top point of the standard curve, final concentration will be 150pg/mL).

D. Mix well by inversion, do not vortex.

E. Continue making 1.8 fold serial dilutions by adding 300µL of the previous dilution to 240µL of 1X incubation buffer in tubes #2-7. Mix well by inversion between each dilution.

TABLE 2

Tube Number	Volume of Standard (µL)	Volume 1X Incubation Buffer (µL)	Intermediate Concentration (pg/mL)
1	6µL intermediate #2	494	300.0
2	300µL of #1	240	166.6
3	300µL of #2	240	92.6
4	300µL of #3	240	51.4
5	300µL of #4	240	28.6
6	300µL of #5	240	15.8
7	300µL of #6	240	8.8
8	0	240	0

V. Sample Preparation

A. Dilute samples in 1X incubation buffer to 2X concentration. Mix well by inversion.

For example, if the final sample dilution should be 1:10 dilute the sample 1:5 in 1X incubation buffer. The sample will then be diluted 1:2 in step VII for a final dilution factor of 1:10.

B. Run samples in duplicate or triplicate.

Note: All sample matrices will perform differently in the kit. It is important that you determine the ideal dilution for your particular sample type. It is good practice to run 2-3 dilutions per sample to ensure at least 1 dilution falls within the range of the standard curve. For most sample types, a good starting dilution would be 1:5 - 1:10. Due to the format of the assay, samples cannot be run neat.

VI. Preparation of diluted HRP Detection Antibody

A. Label a 15mL tube as "Diluted HRP Detection Antibody"

B. Add 6mL of 1X Incubation buffer to the tube labeled "Diluted HRP Detection Antibody"

C. Add 12µL of HRP Detection Antibody and mix well by vortexing

VII. Running the Assay

Day 1

A. Remove plate from foil pouch.

B. Add 300µL per well of 1X wash buffer to the plate (Prepared in step III above).
Note: Use of automated plate washer is highly recommended.

C. Dump out wash buffer and pat dry.

D. Add 50µL of each prepared standard to the plate in duplicate or triplicate. Follow the plate layout below.
Note: Wells E1-E3 contains the zero or blank sample (Std #8).

E. Add 50µL of each sample to the plate in duplicate or triplicate.

F. Add 50µL of diluted HRP Detection Antibody to all wells.

G. Cover with plate sealer.

H. Mix the plate gently on a plate shaker for 1.0 minute to ensure detection antibody is well mixed with the samples/standards.

I. Incubate overnight at 2-8°C, 18 hours.

Note: Once diluted with 50µL of diluted HRP Detection Antibody, the final standard curve concentrations will be oulined in Table 4. Please use these concentrations to generate your standard curve.

TABLE 3.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 1	Std 1	Sample 1	Sample 1							
B	Std 2	Std 2	Std 2	Sample 2	Sample 2							
C	Std 3	Std 3	Std 3	Sample 3	Sample 3							
D	Std 4	Std 4	Std 4	Sample 4	Sample 4							
E	Std 8	Std 8	Std 8	Sample 5	Sample 5							
F	Std 5	Std 5	Std 5	Sample 6	Sample 6							
G	Std 6	Std 6	Std 6	Sample 7	Sample 7							
H	Std 7	Std 7	Std 7	Sample 8	Sample 8							

Day 2

A. Remove plate from refrigerator and dump contents.
B. Wash plate by adding 300µL 1X wash buffer per well.
C. Dump out wash buffer and pat dry.
D. Repeat steps B & C 4 more times for a total of 5 washes.
E. Mix chemiluminescent substrates for use:
    i. Add 5.5mL of substrate A to a 15mL centrifuge tube.
    ii. Add 5.5mL of substrate B to the same 15mL centrifuge tube.
    iii. Mix well by vortexing.
F. Add 100µL of mixed substrate per well.

Note: If reading multiple plates add substrate one plate at a time, do not add substrate to all plates at the same time. Add substrate to each plate immediately before reading.

G. Shake plate on either a plate shaker or using the shaking mechanism within the luminometer for 15 seconds.

H. Read plate immediately.

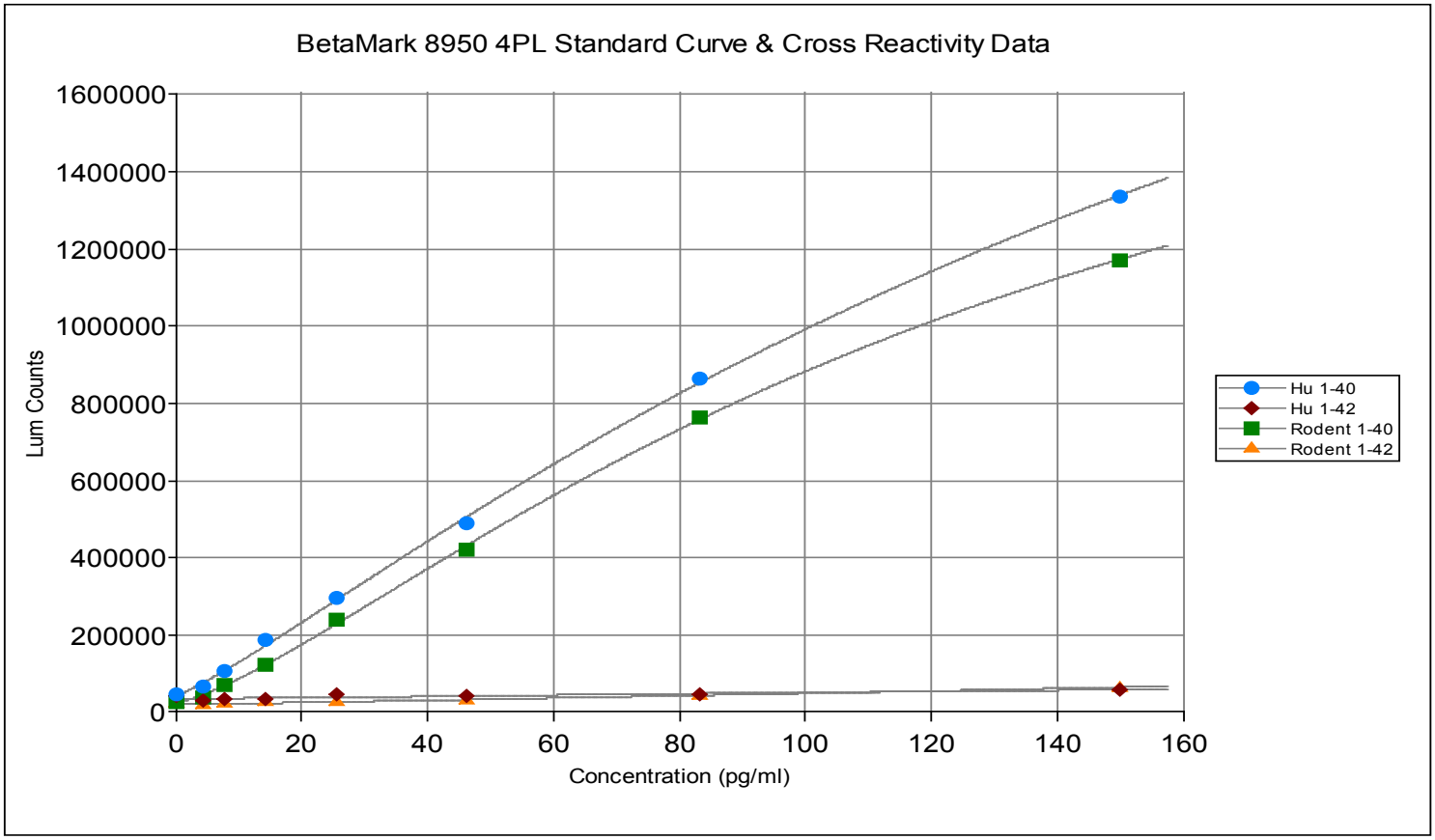
Plates must be read within 5 minutes of adding substrate.

Notes: The recommended luminometer settings are to read at a mid-range sensitivity level for 1 second per well. These settings will vary between plate reader manufacturers, please consult your owner's manual prior to performing this assay.

TABLE 4.

Standard	Final Concentration (pg/mL)
1	150.0
2	83.3
3	46.3
4	25.7
5	14.2
6	7.9
7	4.4
8	0

BetaMark x-40 ELISA Protocol
Catalog No.: 842101



STD Curve pg/mL	Human 1-40	Human 1-42	Rodent 1-40	Rodent 1-42
150.0	1337085	57939	1172348	64246
83.3	864035	47058	764019	44075
46.3	489947	41302	422303	31771
25.7	296467	44426	239681	25165
14.3	188001	33497	122148	26788
7.9	105355	33232	72198	22608
4.4	67544	28756	39310	17785
0	45183	43359	27731	20713

Preparation of Brain Samples for BetaMark™ Beta Amyloid ELISA

Procedure

I. Soluble and insoluble tissue fractionation.

This protocol generates soluble and insoluble subcellular fractions for analysis of Aβ partitioning in tissues.

A. Homogenize in TBS with protease inhibitors (Pierce sells excellent solid tablets for use with homogenization buffers) at 5 mLs per 1 g tissue. Teflon/glass homogenizer with 5-6 passes on ice. EDTA[1] (2 mM) can be a useful addition to homogenization buffers (see notes below).

B. Spin for 20 minutes x 350,000 g (or equivalent)

C. Remove supernatant (S1 or soluble fraction) - usually contains < 5 % of the total brain Aβ pool in young mice and decreases with age.

D. Resuspend pellet (agitate to break up) with: (use only one method below)

   i. Detergent[2] - 1% triton OR 0.6% SDS (see notes) in TBS/inhibitor buffer using same volume as homogenization step (15 minutes incubation).

   ii. 70% Formic acid[2], 40-50% of homogenization volume (incubate 30 minutes).

E. Spin a second time (20 minutes x 350,000 g or equivalent) and remove supernatant - detergent or formic acid extract fraction (E1).

F. OPTIONAL - if detergent extract was prepared above, then remaining pellet can be further extracted with formic acid - 70% formic acid, half homogenization volume, spin down 10 minutes in microfuge full speed (10 minutes x 14,000 g). Remove formic acid extract (E2).

G. S1 and E1 fractions are best assayed immediately but can be stored at -20°C (including non-neutralized formic acid samples)

II. Whole tissue extraction

This protocol generates a single fraction containing the total Aβ tissue load.

A. Homogenize tissue as above in TBS with protease inhibitors and EDTA.

B. To homogenate add concentrated Triton X-100, SDS (to final 1 or 0.6% respectively) or formic acid (to final 70%). Agitate by pipetting up and down to mix. Detergents and formic acid are added after tissue homogenization to reduce foaming and other potential problems.

C. Spin for 20 minutes x 350,000 g (or equivalent).

D. Remove supernatant for assay (whole brain extract).

NB High salt and biomolecule concentrations in formic acid whole tissue extracts can sometimes result in visible precipitants forming in later steps. These samples retain strong Aβ signal but we have not established to what degree signal attenuated under these conditions.

Notes on Preparation and Assaying of Samples

[1]EDTA in homogenization buffer helps solubilize Aβ and increases recovery of Aβ in the soluble fraction by as much as 50% for mild β-amyloid loaded brain (Cherny, 1999, JBC). HOWEVER, it is unclear if EDTA-solubilized material is free in solution or a precipitate in vivo. Mice at different ages/stages give different soluble/chelator yields (presumably because of shifts in quantity/quality of chelator-soluble Aβ forms with age). Hence, caution should be exercised if experiments are comparing Aβ levels in S1/EDTA fractions ("soluble" Aβ) from different aged mice.

[2]Choice of type of detergent or formic acid will depend on experimental circumstance.

- Non-ionic Triton X-100 extracts a portion of insoluble Aβ. As with chelators, the portion of insoluble Aβ extracted by non- ionic detergents is dependent on age/stage of mice. For early stage of β-amyloid deposition (i.e. mice >12 months) up to 80% of total mouse brain Aβ is extracted by Triton X-100. Proportion drops rapidly 20% or less in old animals (i.e. mice >18 months) with heavy mature β-amyloid deposition. Biolegend Aβ ELISA's (and most other ELISA's) are compatible with non- ionic detergents.
- The ionic detergent SDS gives more complete Aβ extraction from insoluble pellets (>90% with young and ≈ 50% for older mice). HOWEVER, SDS is not compatible with many ELISA systems. Biolegend Aβ kits are resistant to SDS and samples with ≤ 0.2% SDS can be used with minimal loss in sensitivity (< 10%). Hence, E1 fractions with SDS should have a final in assay dilution of at least 3-fold.
- Early protocols used formic acid to extract Aβ from AD brain. For mature β-amyloid deposits in human and old mice brain the highest extraction yields are still observed with this buffer. HOWEVER, a difficult multi-step process is required to prepare formic acid fractions for assay (both E1 and whole tissue extracts). Normalization between samples is also problematic.

Steps to prepare formic acid extracts for Aβ assay;

- The bulk of the formic acid buffer needs to be evaporated off (down to 10-20% of original vol) with a nitrogen stream. If dried completely formic acid brain samples form a plastic-like pellet that is highly resistant to resolubilization.
- Aliquots of the remaining material must be neutralize (to pH 7) with 5 M NaOH in 1M Tris. Indicator should be added to the neutralization buffer to signal when pH 7 is reached.
- Assay immediately - DO NOT FREEZE neutralized samples (precipitates of highly enriched insoluble hydrophobic species will form that interfere with assay signal).

Care should be taken to have equivalent pH for all samples. Neutralized samples should have a final in assay dilution of at least 10- fold. Normalizing between samples is problematic. Residual formic acid interferes with most protein assays and protein levels may not be proportional to starting materials in any case. Careful attention to maintaining equivalent sample volumes is often the most practical approach.

TBS = Tris, pH 7.4 (50 mM) + NaCl (150 mM)

Helpful Hints

- Washing is critical in this assay. To ensure proper washing, a plate washer is highly recommended for all washes. Pipette washing is not vigorous enough and can result in high background and poor sensitivity.
- Beta Amyloid is a highly hydrophobic protein and is very sticky. Mixing of the standards and samples is also a key step. Mix tubes by quickly inverting or shaking them for 10-15 seconds per tube before removing material for the next serial dilution. This will help ensure good linearity with your standard curve and samples. Also, minimize the number of times a pipette goes in and out of the standard tubes, this will reduce any loss of peptide.

Frequently Asked Questions

- What wavelength do I use to read the plate?

  Since this is a chemiluminescent assay, there is no wavelength to read the plates. The plate reader should have a hole setting in the cartridge where the light emitted by the substrate can pass through and be detected. Wavelength settings are for colorimetric assays only.

- I am getting very high background and my standard curve is not linear at the low end. What is causing this?

  This is typically a washing issue. Inadequate washing will lead to high background and poor sensitivity at the low end of the curve. To fix the issue, either use a plate washer or squirt bottle for all washing steps. Pipette washing will not be vigorous enough. This should help reduce the background and in turn give better sensitivity at the low end.

- I have a plateau at the top end of my standard curve, what could be causing this?

  Typically this is caused by not preparing your standard curve from intermediate #2 and using the reconstituted standard instead. The concentration is too high to be detected by the reader and will cause a plateau of the first few points on the standard curve.

- My samples are undetectable in the assay, what is causing this?

  Generally there are 2 causes. First, ensure that you added the diluted HRP Detection Antibody to the plate prior to the over-night incubation. Omission of this step will result in no signal. Another reason could be due to very low levels of A_Beta in your samples. Due to the limitations of the kit, the samples must be diluted a minimum of 1:2 in the assay and cannot be run undiluted.

- What are the recommended dilutions for my samples?

  This is going to vary based on your specific samples. We recommend running 3 different dilutions for each sample, which helps ensure one of the dilutions will fall within the range of the standard curve. If you are testing a large number of samples, consider doing a trial run with a subset of samples to optimize the dilution factors.

- What types of samples will work with this assay?

  Currently, this assay works with TCS, plasma, CSF and tissue homogenate samples. It is not recommended for use with serum samples.samples, consider doing a trial run with a subset of samples to optimize the dilution factors.

- What type of curve fit should I use?

  We recommend using a 4 parameter curve fit to generate the standard curve for this assay.

- What if I have further questions?

  Please call our technical support at +1.800.223.0796 or send e-mail to ab.technical@biolegend.com. We will be more than happy to answer any questions you may have.