Protocol Booklet

Product Code(s)	HB9805, HB8758, HB8524, HB9159
Product Name	β-Amyloid Peptide (1-42) (human)
	β-Amyloid Peptide (1-40) (human)
	β-Amyloid Peptide (25-35) (human)
	β-Amyloid Peptide (42-1) (human)
Purpose	Handling and Aggregating Amyloid Beta

Please note: This product is for RESEARCH USE ONLY and is not intended for therapeutic or diagnostic use. Not for human or veterinary use

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1. Background

Amyloid Beta (Aß) is a family of peptides that form the main constituent of senile plaques in Alzheimer's disease and has been extensively investigated for its role in the aetiology of neurodegenerative diseases. Aß is produced from the proteolytic cleavage of amyloid precursor protein (APP), a membrane glycoprotein found extensively in synapses throughout the brain (see Figure 1) to result in a family of peptides ranging in size from 37 to 49 amino acids long with the main species being Aß1-40 and Aß1-42.

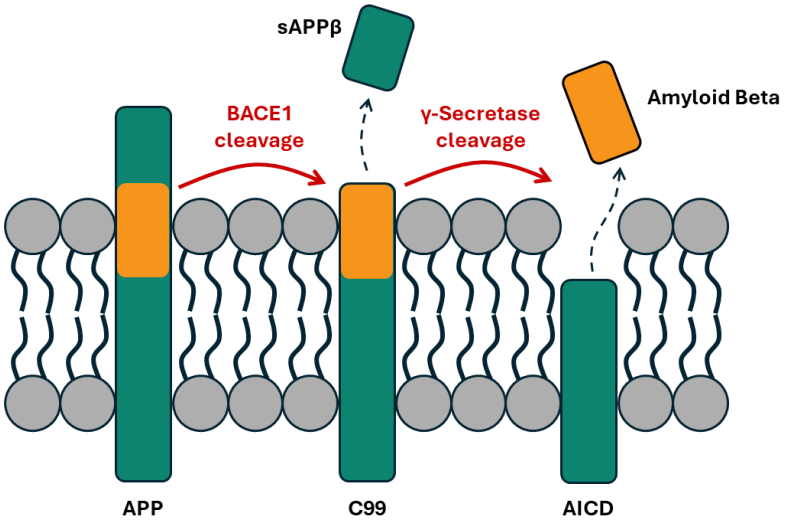

Figure 1. Generation of amyloid beta through APP proteolytic cleavage. Amyloid precursor protein (APP) is cleaved by ß-secretases such as BACE1 to give a 99 residue C-terminal fragment (C99) which is then cleaved again by the γ-secretase complex to liberate free amyloid beta which mostly takes the form of either Aß1-40 or Aß1-42.

Monomeric Aß has multiple roles in normal physiology where it is involved in trafficking of synaptic vesicles, regulating the excitation / inhibition balance at synapses and activating pathways regulating BDNF release. However Aß isoforms are prone to aggregation where they form larger and larger complexes and aggregates which are the main driver of their toxicity. Aß monomers start to coalesce into small soluble oligomers (complexes of 2-20 monomers) which are now believed to be the major toxic species and can diffuse throughout the brain. Aß oligomers have an array of toxic effects including disruption of synaptic plasticity, promotion of tau hyperphosphorylation and triggering of oxidative stress. Oligomers can coalesce into soluble protofibrils which have additional toxic effects such as being endocytosed by glial cells and being associated with inflammation. These protofibrils can then further aggregate into insoluble fibrils when have a characteristic cross-ß sheet pattern which then further accumulate into the plaque deposits found in the brain's of patients with Alzheimer's disease.

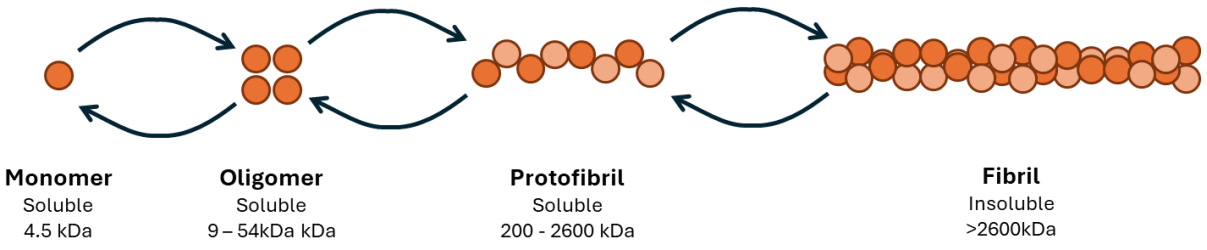

Figure 2. Aggregation of soluble Aß monomers into insoluble fibrils is a dynamic and concentration dependent process in equilibrium.

There are a range of different Aß species but the most widely studied in neurodegenerative research are:

- Amyloid ß 1-40 - comprises about 90% of the total Aß formed in the brain and while more soluble than Aß1-42 still forms a major constituent of senile plaques in Alzheimer's disease.
    - Available from Hello Bio as HB8758 - β-Amyloid Peptide (1-40) (human), a high purity lyophilised monomer in a 1mg pack size.

- Amyloid ß 1-42 - comprises only around 10% of Aß in the brain, however it is less soluble, more neurotoxic and quicker to aggregate than Aß1-40 due to a higher propensity to form ß-sheet structures.
    - Available from Hello Bio as HB9805 - β-Amyloid Peptide (1-42) (human), a high purity lyophilised monomer in packs ranging from 100µg to 1mg.

- Amyloid ß 25-35 - produced from the cleavage of Aß1-40 and is another component of senile amyloid plaques in the brain. Aß25-35 is also able to aggregate into fibrils and has been found to have cytotoxic effects.
    - Available from Hello Bio as HB8524 - β-Amyloid Peptide (25-35) (human), a high purity lyophilised monomer in a 1mg pack size.

- Amyloid ß 42-1 - a reverse form of the Aß1-42 peptide that is used as a control peptide in experiments using Aß1-42 as, while it has been found to aggregate, it is much less toxic to cells than Aß1-42
    - Available from Hello Bio as HB9159 - β-Amyloid Peptide (42-1) (human), a high purity lyophilised monomer in a 1mg pack size.

2. Handling Monomers

Amyloid ß monomers supplier by Hello Bio are provided as a lyophilizate that has been previously treated with HFIP. We recommend to dissolve peptides following the below NH_4OH protocol although others are provided in section 3.1.

1) Dissolve Aß peptide in 1% ammonium hydroxide (NH_4OH) using a volume of around 70-80µl per 1mg of peptide. Briefly spin the vial at >1000g to bring all the solution to the bottom of the vial.
    a. 1% ammonium hydroxide is conveniently available preformulated from Hello Bio as HB9790 - 1% NH4OH solution for dissolving Beta-Amyloid peptides
    b. Do not store the peptide in 1% NH_4OH

2) Immediately dilute the Aß solution in ice-cold PBS to a concentration of ≤1mg/ml. Pipette up and down to ensure the solution is well mixed.

3) Either use immediately or snap-freeze the solution in aliquots and store at -80°C.
    a. Before use it is recommended to spin the Aß aliquot at full speed in a benchtop centrifuge to remove any small insoluble aggregates that have formed.

We recommend following the following tips to avoid undesired aggregation of Aß peptides:

- Use clean tools to avoid introducing contaminants into the solution that could be seeds for aggregation.
- Avoid extensively vortexing Aß samples as this can promote aggregation.
- Be careful to avoid introducing bubbles into Aß solutions as this can be another source of seeds for aggregation.
- Avoid freeze-thaw of Aß solutions as this has been found to promote aggregation.

3. Aggregation of Amyloid Beta

There are a diverse range of methods used to generate different oligomerisation species of Aβ each with their own range of advantages and disadvantages. We have a protocol based upon the work of Stine et al., 2011. It is important to start with a solution of pure Aβ monomers to maximise the probability of resulting in a uniform aggregation state at the end.

3.1 Starting with Pure Monomeric Aβ

There are a few different methods that are used to ensure a solution of pure monomeric Aβ.

HFIP Treatment

1. Dissolve the Aß peptide in HFIP (1,1,1,3,3,3-hexafluoro-2-propanol, CAS: 920-66-1) and vortex briefly to mix.
2. Dry the solution under a stream of nitrogen
3. Redissolve the peptide in HFIP at 1mg/ml, sonicate in a bath sonicator for 5 minutes.
4. Dry the solution under a stream of nitrogen
5. Redissolve the peptide in HFIP at 1mg/ml, sonicate in a bath sonicator for 5 minutes.
6. Dry the solution under a stream of nitrogen
7. Redissolve the peptide in HFIP at 1mg/ml, aliquot into smaller volumes appropriate for a single experiment then sonicate in a bath sonicator for 5 minutes.
8. Dry the solution under a stream of nitrogen followed by drying under vacuum for 1-2 hours until the peptide is completely dry.
9. Store the Aß aliquots at -80°C until further use.

NaOH Treatment

1. Dissolve the Aß peptide in 10mM NaOH to 1mg/ml and sonicate for 30 minutes using a sonicator bath.
2. Aliquot and snap freeze before storing at -80°C
3. When ready to use, thaw rapidly at 37°C then remember to account for the high pH of the Aß solution when using it.

NH_4OH Treatment

1. Dissolve Aß peptide in 1% ammonium hydroxide (NH_4OH) using a volume of around 70-80µl per 1mg of peptide. Briefly spin the vial at >1000g to bring all the solution to the bottom of the vial.
    a. 1% ammonium hydroxide is conveniently available preformulated from Hello Bio as HB9790 - 1% NH4OH solution for dissolving Beta-Amyloid peptides
    b. Do not store the peptide in 1% NH_4OH
2. Immediately dilute the Aß solution in ice-cold PBS to a concentration of ≤1mg/ml. Pipette up and down to ensure the solution is well mixed.
3. Either use immediately or snap-freeze the solution in aliquots and store at -80°C.
    a. Before use it is recommended to spin the Aß aliquot at full speed in a benchtop centrifuge to remove any small insoluble aggregates that have formed.

3.2 How to Monitor Aggregation

Being able to monitor the progression of Amyloid Beta aggregation is critical to understand what species are being used in experiments where Aß is aggregated then used as an *in-vitro* or *in-vivo* manipulation. There are multiple ways of measuring Aß aggregation with it being recommended to use more than one method for a more accurate determination of Aß aggregation status.

Fluorescent Assays

There are a range of fluorescent molecules that differentially bind to Aß fibrils and fluoresce compared to monomers which are available from Hello Bio:

- HB5134 - Thioflavin S (ThS)
- HB17774 - Thioflavin X (ThX)
- HB7143 - Thioflavin T (ThT)
- HB5252 - Methoxy-X04
- HB0737 - Congo Red

Of these dyes Thioflavin X and Thioflavin T are suitable for quantitative measurement of Aß aggregation with Thioflavin X being approximately 5x brighter with 7x higher affinity to Aß compared to Thioflavin T.

1. Prepare Aß in 20mM phosphate buffer containing 0.2mM EDTA, 1mM NaN_3 and 20µM Thioflavin T or 10µM Thioflavin X at pH 8. Aß concentrations of over 1µM exhibit aggregation within a 24 hour timespan.

2. Incubate for 24 hours at 37°C regularly measuring fluorescence at either:
    a. Thioflavin X: Excitation: 420, Emission: 494nm
    b. Thioflavin T: Excitation: 450nm, Emission: 485nm

Transmission Electron Microscopy (TEM)

Aß fibrils are large enough to be visualised using transmission electron microscopy (TEM) with uranyl acetate counterstaining. A standard protocol from Xiao et al., 2015 is:

1. Load 10µl of a fibril sample onto a 300 mesh copper formvar / carbon grid and incubate for 1 minute before blotting off any excess.
2. Counterstain the sample with 10µl of 2% uranyl acetate for 90 seconds before blotting off any excess and drying the grid in a desiccator chamber.
3. Image using a TEM at 80kV and a magnification of approximately 120,000x

Dynamic Light Scattering

Dynamic Light Scattering (DLS) is a technique that uses the scattering of a laser beam off small particles in solution to work out their size distribution (for an example see Jeon et al., 2023). This can be applied to monitoring Aß aggregation as DLS is able to non-invasively measure Aß aggregation in a timecourse and distinguish between small soluble oligomers and larger insoluble fibrils.

Atomic Force Microscopy (AFM)

Atomic Force Microscopy (AFM) is a technique where a nanoscale probe is dragged across a sample then the deflection measured to map the surface topology of a sample. This has been successfully applied to monitor the aggregation of Aβ protofibrils into fibrils (see Harper et al., 1997) and can directly visualise the structure of aggregated Aβ and even has the resolution to visualise the helical structure of mature fibrils.

3.3 Aggregation Protocols

Depending on the size of aggregate you require for your experiments there are different protocols which bias their conditions towards making fibrils assemble to a certain range of sizes.

3.3.1 Preparing Oligomers

1. Dissolve monomeric Aß in DMSO to a 5mM concentration at room temperature (22.6mg/ml for Aß1-42)
2. Dilute the Aß down to 100µM (0.45mg/ml for Aß1-42) using ice-cold F-12 cell culture media (phenol free and containing 146mg/L L-glutamine)
3. Vortex for 15 seconds then incubate at 4°C for 24 hours.

3.3.2 Preparing Fibrils

1. Dissolve monomeric Aß in DMSO to a 5mM concentration at room temperature (22.6mg/ml for Aß1-42)
2. Dilute the Aß down to 100µM (0.45mg/ml for Aß1-42) using room temperature 10mM HCl.
3. Vortex for 15 seconds then incubate at 37°C for 24 hours.

3.3.3 Preparing Larger Insoluble Aggregates

1. Dissolve monomeric Aß in DMSO to a 5mM concentration at room temperature (22.6mg/ml for Aß1-42)
2. Dilute the Aß down to 100µM (0.45mg/ml for Aß1-42) using room temperature 10mM HCl + 150mM NaCl.
3. Vortex for 15 seconds then incubate at 37°C for 24 hours.

3.4 Separating Aβ Aggregates

Depending on the experimental requirements, the Aβ aggregate mixture can be used directly or purified to isolate specific species. There are a few different methods used to separate Aβ species following an aggregation protocol:

- Filtration is an easy method to remove larger Aβ fibrils and aggregates with spin-column filters being available with a wide range of molecular weight cut offs (MWCO), ranging from as little as 1kDa up to over 100kDa. Spin filtration is extremely quick, however is relatively crude as it only removes Aβ species above the MWCO, leaving a mixture of smaller species remaining.

- Size exclusion chromatography (SEC, see Nichols et al., 2015) is the main method used to separate Aβ species where Aβ species are separated by hydrodynamic size as they move through the resin bed with larger species eluting before smaller oligomers and monomers. SEC is an extremely powerful technique however does require expensive columns and machinery to carry out.

- Aβ species can also be separated using centrifugation (see Ward et al., 2000). Mixed Aβ samples can be added to a density gradient and spun at extremely high g-force (approx. 350,000 g_{av}) to separate the Aβ

species by their molecular weight. This does however require expensive specialised centrifuges and long run-times making this method non-trivial.

4. Uses of Amyloid Beta Aggregates

Creation of high-purity and defined aggregation state amyloid beta species is critical to enable research into the specific molecular mechanisms underlying Aß toxicity *in-vivo* which leads to Alzheimer's disease. Some of the most common uses for Aß oligomeric species include:

- Screening for either small molecule and peptide inhibitors of Aß aggregation or disaggregators of Aß fibrils using high-throughput screening assays. Dyes such as Thioflavin X (ThX) and Thioflavin T (ThT) increase fluorescence in response to the formation of aggregated Aß species therefore can be readily adapted into a high-throughput format.

- *In-vitro* toxicity assays where Aß species are added to cells (e.g. primary or iPSC derived) neurons and the toxicological and molecular mechanisms of cell damage are assayed using tests such as H_2DCFDA to measure reactive oxygen species, Annexin V assays to measure apoptosis or MTT to measure cell proliferation.

    - HB7375 - DCFDA / H2DCFDA - Cellular ROS Assay Kit
    - HB9623 - Annexin V-FITC Apoptosis Staining / Detection Kit
    - HB8164 - Annexin V-PE Apoptosis Staining / Detection Kit
    - HB5283 - MTT

- *In-vivo* models where animals are injected with toxic Aß species to study their impact on a systems level. This is often carried out on genetically modified animals that overexpress human Aß isoforms which then have Aß fibrils injected to trigger further aggregation of host Aß which produces an Alzheimer's like phenotype in a greatly shortened timespan compared to the natural disease process. Researchers commonly carry out immunohistochemical analyses to identify the specific pathology on different cell types such as:

    - Neurons (e.g. Anti-βIII Tubulin antibody ValidAb™, Anti-NeuN antibody ValidAb™, Anti-MAP2 antibody ValidAb™)
    - Glia (e.g. Anti-GFAP antibody ValidAb™, Anti-IBA1 antibody ValidAb™)
    - Oligodendrocytes (e.g. Anti-Myelin Basic Protein (MBP) Antibody ValidAb™)

    Fluorescent dyes can also be used to visualise Aß plaques in histological samples from animal models such as:

    - HB17774 - Thioflavin X (ThX) – next generation dye that is 5x brighter than ThT
    - HB7143 - Thioflavin T (ThT)
    - HB5134 - Thioflavin S (ThS)
    - HB5252 - Methoxy-X04
    - HB0737 - Congo Red

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