Sample preparation of brain tissue for extraction of β-amyloid and other neuronal markers

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Introduction

Alzheimer's disease (AD) is characterized by regional neuronal degeneration, synaptic loss, the presence of intracellular neurofibrillary tangles (NFTs), and the progressive deposition of senile plaques in the extracellular space. While neurofibrillary tangles are composed of hyperphosphorylated Tau, the senile plaques are formed by aggregation and deposition of β-amyloid (Aβ) peptides. Many investigations conducted in last decade have pointed to the soluble circulating Aβ oligomers as the building blocks for these insoluble plaques and tangles. The increased concentration of soluble Aβ oligomers has also been linked to synaptic dysfunction and cognitive decline. Aβ peptides are a group of varying length oligomers originating through degradation of the amyloid precursor protein (APP); the two most common oligomers, Aβ 1-40 and Aβ 1-42, both have disease implications. Aβ 1-40 is the predominant form found in biological fluids such as blood and cerebrospinal fluid (CSF). Aβ 1-42 is highly hydrophobic and tends to aggregate at much lower concentrations than Aβ 1-40. It is the main component of plaques and thus plays a significant role in the pathogenesis of AD.

The isolation of amyloid peptides continues to present a challenge. While there are many protocols reported in the literature for the purification of amyloid peptides, a simplified, universal method remains elusive. In general, the majority of reported protocols propose a sequential approach. In first step, aqueous buffers are used to extract hydrophilic components. In the following step(s), strong detergents, acids, or strong chaotropes are applied to extract difficult to solubilize components. These steps are severely hampered by not only precipitation and protein loss but also incompatibility with many common downstream assays such as ELISA, Immunoprecipitation (IP), Western blotting (WB), or Mass Spectrometry (MS).

Here we present a study comparing relative extraction efficiency for a variety of buffers. The presented results demonstrate that, for most applications, the use of Tris-buffered Saline (TBS) for extraction of soluble Aβ fractions and commercial lysis buffers for extraction of "insoluble" Aβ fractions can effectively obviate the need for formic acid-based extraction.

Table 1. The most common protocols and buffers used for extraction of brain proteins.

Reference	Method	Buffers used
B. Kaplan *et. al.* J Clin Pathol 2003 (Review)	Sequential extractions	From TBS through guanidine hydrochloride or formic acid (FA) to MeCN/TFA
G.M. Shankar *et. al.* Nature Medicine (2008)	Sequential extractions (Ultracentrifugation)	a. TBS (20 mM Tris, 150 mM NaCl pH 7.4) b. TBS + 1% TX-100 c. TBS + 5 M guanidine chloride pH 8
A. Rostagno & J. Ghiso Curr. Protoc. Cell Biol. (2009)	Sequential extractions Ultracentrifugation (112,000 xg 1 hr. 10C) Centrifugation 14,000 xg Ultracentrifugation/sucrose gradient with SDS 220,000 xg/20 hr	a. PBS b. 20 mM Tris + 2% SDS c. 70% FA d. 99% FA
E. Portelius *et. al.* Acta Neuropathol. (2010)	Sequential extractions (Centrifugation; Sonication)	a. TBS b. 70% FA
H. Schieb *et. al.* JBC (2011)	Sequential extractions Centrifugation (16,000 xg)	a. 50 mM HEPES, 150 mM NaCl, 1% Nonident, 0.5% Na Deoxycholate, 0.1% SDS b. PBS c. 70% FA
G.M. Shankar *et. al.* Methods Mol. Biol. (2011)	Sequential extractions (Centrifugation; Sonication)	a. TBS (15 mM Tris, 40 mM NaCl, 3 mM KCl) b. TBS with 1% TX-100 c. 88% FA (sonication)
G. Shevchenko *et. al.* J. Proteome Res. (2012)	Sequential extractions (Centrifugation; Delipidation)	10 mM Tris, 150 mM NaCl, 1 mM EDTA and PBS with: a. 40-60% organic solvent b. 1% detergent (also other additives) c. 1% FA
M. Izco *et. al.* J Alzheimers Dis. (2013)	Sequential extractions (Ultracentrifugation)	a. TBS (20 mM tris, 150 mM NaCl pH 7.4) b. TBS + 1% TX-100 c. 70% FA
S. Musunuri *et. al.* J. Proteome Res. (2014)	Direct extraction (Centrifugation @ 10,000 xg; Delipidation)	10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA and PBS with 1% N-octyl-β-D-glucopyranoside

Materials:

Human brain tissue (Analytical Biological Services Inc.)
- Gray matter from 3 non-Alzheimer's donors (healthy "H") and 3 from Alzheimer' patients ("A")

Reagents & Buffers
- RIPA lysis buffer, 10X (Merck Millipore cat # 20-188)
- CytoBuster™ Protein Extraction Reagent, "Cyto" (Novagen cat # 71009-50mL)
- Triton X®-100 Reduced, "TX-100" (Sigma-Aldrich® cat # X100RS)
- Triton X®-114, "TX-114" (Sigma-Aldrich® cat # X114)
- Acetonitrile, "MeCN" or "ACN" (Merck Millipore cat # AX0145-1)
- Formic Acid, "FA" (Fluka® cat # 56302-1L-GL-F)
- Tris-Buffered Saline, 10X, "TBS," final 1x: 50 mM Tris, pH 7.4, 150 mM NaCl (Boston BioProducts cat # BM-301)
- Halt™ Proteases and Phosphatase inhibitor cocktail, 100x (Thermo Scientific™ cat # 78440)
- Detection reagent: Luminata™ Forte western HRP Substrate (Merck Millipore cat # WBLVF0500)

Antibodies (Merck Millipore):
- Anti Alzheimer's precursor protein A4 (cat # MAB348)
- Anti Tau phosphoSerine 396 (cat # AB9658)
- Anti amyloid β clone WO-2 (cat # MABN10)
- Goat anti mouse (cat # AP132P)
- Anti Tau 4-repeat isoform RD4 (cat # 05-804)

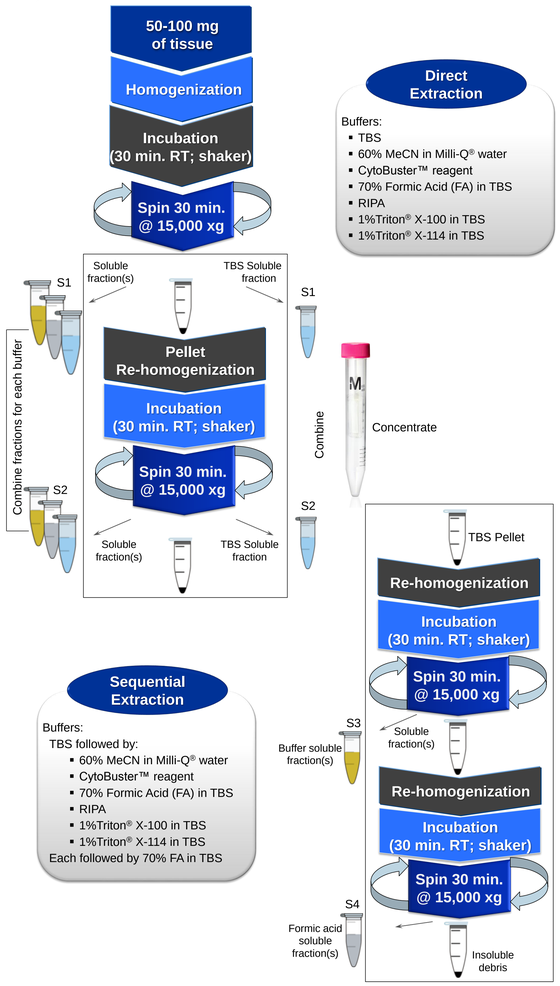
Concentrators (Merck Millipore):
- Amicon® Ultra-4 ml (cat # UFC800308)
- Amicon® Ultra-0.5 (cat # UFC500308)

ELISA and Milliplex® kits (Merck Millipore):
- High sensitivity Human Amyloid β-40 ELISA 96 well kit (cat # EZHS40)
- High sensitivity Human Amyloid β-42 ELISA 96 well kit (cat # EZHS42)
- Human Amyloid Beta (β) & Tau magnetic bead panel 96 well plate assay (cat # HNABTMAG-68K)

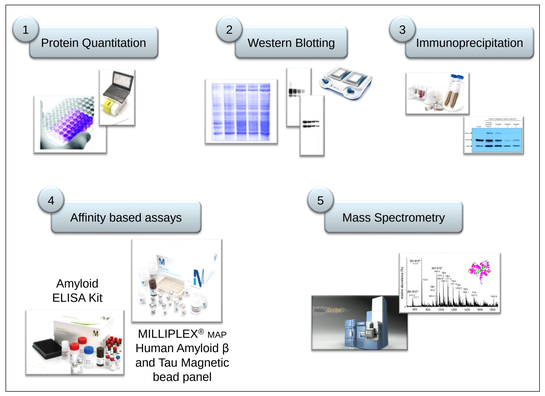
Equipment and supplies
- Acquity UPLC coupled with Xevo® G2-S mass spectrometer (Waters)
- Centrifuge with swinging buckets rotor (Beckman Coulter Allegra™ 25R; 15 mL tube adapter)
- Direct Detect® spectrometer (Merck Millipore cat # DDHW000-10-WW)
- Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BioRad cat # 170-3940).
- SNAP i.d.® 2.0 Protein detection system (Merck Millipore cat # SNAP2BASE)
- NuPage® 4-12% SDS PAGE gels (Thermo Scientific™)
- Immobilon®-P membrane (Merck Millipore cat # IPVH08130)

Methods

A) Extraction



B) Analysis

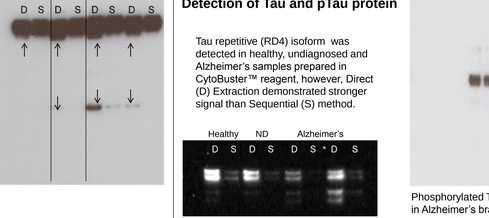


Results

Total protein concentration measured by Direct Detect® spectrometer

Analysis of total protein concentration in the lysates was used to assess efficiency of the applied extraction method. Results, presented below, show that application of a single detergent or a mixture of ionic detergents allows for efficient protein extraction in a single step (Direct Extraction).

	70% FA (mg/mL)	60% MeCN (mg/mL)	TBS (mg/mL)	1% TX-100 (mg/mL)	1% TX-114 (mg/mL)	CytoBuster™ Reagent (mg/mL)	RIPA (mg/mL)
Direct Extraction	0.3 ± 0.5	0.7 ± 0.9	1.8 ± 0.7	2.1 ± 0.2	2.3 ± 1	3.0 ± 0.3	3.6 ± 0.9
Sequential Extraction	Not detected	Not detected	Not Applicable	2.1 ± 1	1.3 ± 0.3	1.3 ± 0.3	2.7 ± 0.2
	n=6	n=6	n=14	n=8	n=6	n=10	n=6

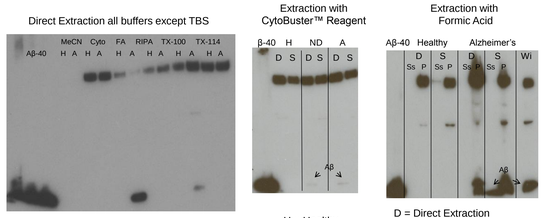


Gel electrophoresis of protein extracted from human brain tissue with various buffers and extraction methods

There were clear differences in protein profiles depending on buffer used for the extraction. The arrows point at some of the more significant differences between types of preparation.

Detection of neuronal markers by SNAP i.d.® Western Blotting system

Detection of Amyloid β by monoclonal antibody MABN10 (recognized residues 4-10)



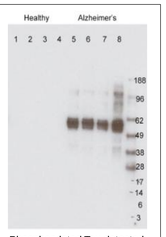
Alzheimer's precursor (APP) was detected in all lysates, however higher content was observed in samples prepared using the Direct (D) Extraction method.

H = Healthy
ND = Not Diagnosed
A = Alzheimer's

D = Direct Extraction
S = Sequential Extraction
Ss = Suspension
P = Precipitated fraction

Detection of Tau and pTau protein

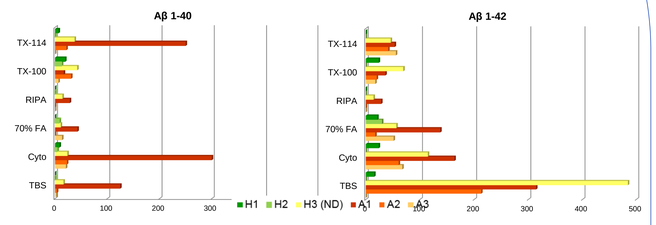
Tau repetitive (RD4) isoform was detected in healthy, undiagnosed and Alzheimer's samples prepared in CytoBuster™ reagent, however, Direct (D) Extraction demonstrated stronger signal than Sequential (S) method.



Phosphorylated Tau detected in Alzheimer's brain samples prepared in RIPA buffer.

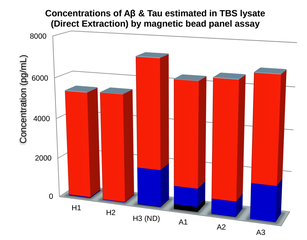
Quantitation of Aβ peptides by ELISA and MILLIPLEX® MAP multiplex assay

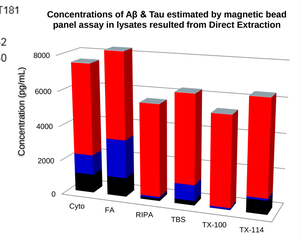
Total concentration of Aβ 1-40 and Aβ 1-42 (pg/mL) in human brain tissue lysates prepared with different buffers using the Direct Extraction Method.



In Direct Extraction, CytoBuster™ reagent ("Cyto"), TBS and Triton® X-114 appeared to be more efficient in solubilizing Aβ 1-40 fragment than Formic Acid (FA).

In Direct Extraction, TBS appeared to be the most efficient in solubilizing Aβ 1-42 fragment. CytoBuster™ reagent ("Cyto") and Formic Acid (FA) showed similar extraction efficiency.

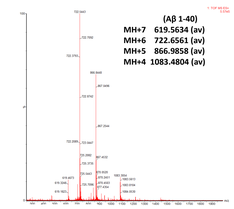


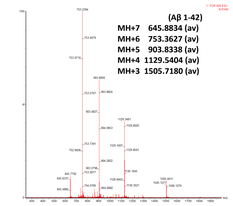


In agreement with ELISA results, application of "Aβ & Tau magnetic bead panel assay" showed comparable efficiency between Formic acid (FA) and CytoBuster™ reagent ("Cyto").

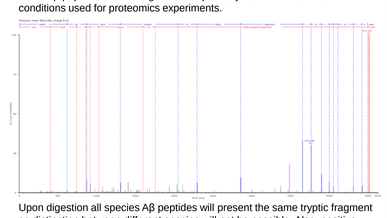
Mass spectrometry

Ionization pattern of synthetic Aβ fragments:

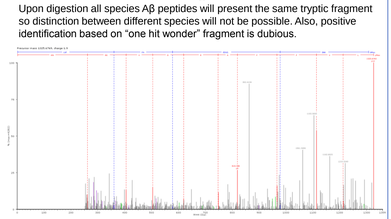




Intact Aβ peptides do not fragment exceptionally well under standard conditions used for proteomics experiments.



Upon digestion all species Aβ peptides will present the same tryptic fragment so distinction between different species will not be possible. Also, positive identification based on "one hit wonder" fragment is dubious.



Summary

- The challenge in efficient isolating amyloid β (Aβ) peptides is identifying methods which are ultimately compatible with downstream analyses.
- Simple, Direct Extraction using TBS or ionic detergent cocktails and centrifugation provides a reasonable choice for the preferential recovery of the soluble fraction of Aβ oligomers.
- The resulting soluble fractions which require no neutralization steps, are amenable to filtration-based concentration, and are compatible with downstream applications such as western blotting, ELISA, and multiplex bead-based assays.
- Sequential Extraction using TBS then CytoBuster™ reagent further improved protein recovery from diseased brain lysates as indicated by detection of Amyloid β1-40, 1-42, and Tau.
- 70% Formic acid (FA) extraction resulted in the highest yields of insoluble Aβ and is thus required for recovery of highly phobic species. It also showed the greatest inter-prep variability; this result is likely due to issues with precipitation during neutralization or inefficient re-solubilization.

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