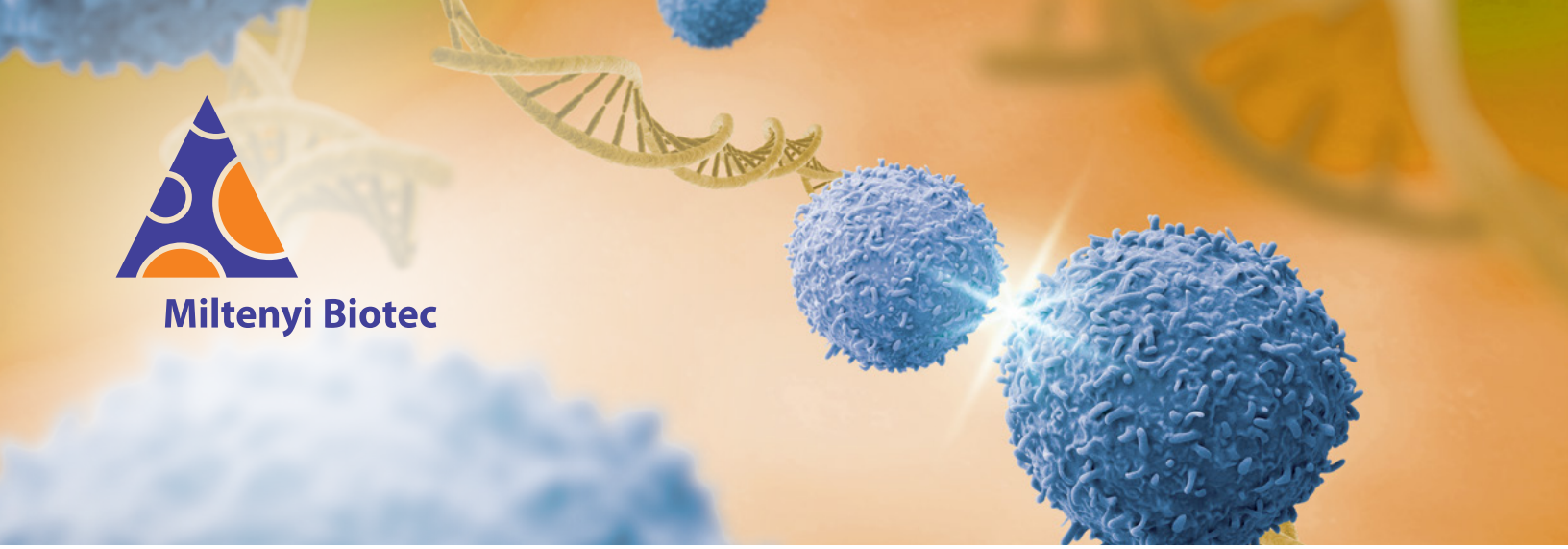

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An improved protocol for extracting protein from FFPE for the seed amplification assay (SAA) to study alpha-synuclein aggregates

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Background

Parkinson's Disease (PD), PD with dementia (PDD) and dementia with Lewy bodies (DLB) – neuropathologically categorized as Lewy body disease (LBD)—are neurodegenerative disorders affecting millions of people worldwide, primarily older individuals. With a growing aging population, the cost of care, both medically and emotionally, is expected to increase exponentially, and there are currently no cures for these diseases. LBDs can severely affect cognitive and motor function, contribute to neuropsychological dysfunction, and interfere with basic daily activities.

LBD (together with multiple system atrophy (MSA)) are considered synucleinopathies, characterized by the deposition of the misfolded alpha-synuclein (αSyn) protein as well as neuronal loss. To this end, misfolded αSyn can spread from cell-to-cell and promote conversion of normal αSyn to the pathogenic misfolded form of αSyn in a recipient cell. Traditionally, immunohistochemistry (IHC) has been used to detect misfolded αSyn, but recent advances allow detection of minute amounts of the misfolded protein, which may not have

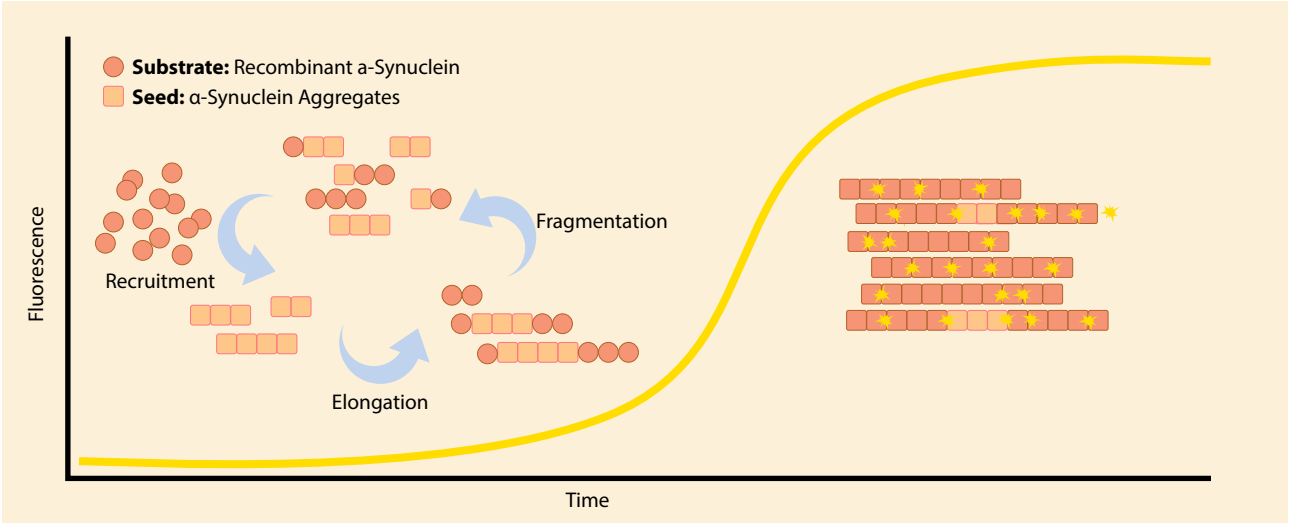

Figure 1. Diagram of the alpha-synuclein seed amplification assay (αSyn SAA). Misfolded αSyn seeds recruit recombinant αSyn substrates for elongation into fibrils and protofibrils. Cyclic amplification of the misfolded protein occurs through intermittent shaking, which induces fragmentation thereby increasing the number of seeds to recruit substrate, and incubation cycles to allow elongation. The amplification of misfolded protein is detected in real time with ThT, a fluorescent dye that binds to β-sheet rich structures of the misfolded protein.

been detected by IHC, using a sensitive seed amplification assay (SAA). The SAA uses misfolded αSyn seeds to recruit and amplify αSyn substrates (fig. 1).[1] This assay was initially performed with fresh or frozen brain samples, which can be both hard to obtain and expensive to store, and ultimately, lack pathological confirmation that IHC can provide. Therefore, post-mortem formalin-fixed paraffin-embedded (FFPE) samples are not only more accessible but can also provide both pathological and biochemical characteristics in the same sample. Because of this, there is an increasing need to adapt more sensitive and robust assays for FFPE samples.[2] However, protein extraction from FFPE tissues remains challenging as various cross-links exist from fixation process, significantly decreasing the amount of proteins that can be used for analysis.

Previous studies have used the FFPE Tissue Dissociation Kit (Miltenyi Biotec) for cell separation and DNA analysis. This application note summarizes a study published by Kim *et al.* establishing a protein extraction protocol that can be used for downstream αSyn SAA, enabling disease-specific detection of LBD and MSA, using low amounts of FFPE tissue input. The established FFPE SAA is comparable to the gold-standard SAAs using frozen brain tissue.[3]

Materials and methods

Case selection
Six subjects with LBD, two subjects with MSA, and one control subject were selected from the University Health Network-Neurodegenerative Brain Collection (UHN-NBC, Toronto, ON, Canada). Brain tissues were fixed in 10% neutral buffered formalin for two weeks followed by paraffin embedding.

Optimized protocol for protein extraction
Formalin-fixed paraffin-embedded (FFPE) tissues were prepared as either ten 4.5 µm-thick sections of each sample on slides or with a 2-mm or 4-mm micropunch of the FFPE block obtained with a disposable biopsy punch plunger. To ensure that FFPE blocks were re-usable after micropunching, paraffin was melted prior to the micropunch collection and the rest of the tissue was re-embedded. Tissue punches were deparaffinized in gentleMACS™ C Tubes using xylene, followed by rehydration in serial concentrations of ethanol and distilled water. Supernatants were carefully removed and replaced with subsequent solution. Sections on slides were deparaffinized and rehydrated on the slides then scraped into the gentleMACS™ C Tubes.

The FFPE Tissue Dissociation Kit was used for antigen retrieval and dissociation. Briefly, antigen retrieval was performed by incubating deparaffinized tissue in buffer provided in the kit in a 80 °C water bath for 75 minutes, then cooled. The supernatants were removed and the samples were dissociated in gentleMACS C Tubes on the gentleMACS Octo Dissociator with Heaters using the buffer and enzymes provided in the kit. The dissociated tissues were transferred to low protein-binding tubes and centrifuged. The supernatants were discarded, resuspended in phosphate buffered saline (PBS), and centrifuged again. The supernatants were again discarded, and the resulting pellets were weighed and used for protein extraction.

Tissue pellets were resuspended in ice-cold 10% w/v PBS before being transferred to the homogenizing tubes. Homogenization was performed in a bead beater with zirconium oxide beads and PBS spiked with protease inhibitor. The homogenates were centrifuged and the supernatants containing the extracted protein were collected. The bicinchoninic acid assay (BCA) was used to determine protein concentration.

αSyn SAA protocol
Using misfolded αSyn seeds extracted from biological samples, the seeds can recruit recombinant αSyn substrates for elongation into protofibrils and fibrils (fig. 1). The SAA reaction mixture consisted of phosphate buffer, human recombinant αSyn, and Thioflavin T (ThT), which is used to detect real-time cyclic amplification as it binds to β-sheet-rich structures of misfolded αSyn.

Intermittent shaking induces fragmentation of protofibrils and fibrils and increases the number of seeds which can further recruit additional substrates, amplifying the misfolded protein. At the end of the reaction, all the recombinant substrates are incorporated into fibrils, which is represented by a plateau in fluorescence readings. To test the serial propagation of αSyn seeding differences, 2 µL of the SAA end product was used. To evaluate the differential seeding behavior between LBD and MSA subjects, an LBD-favoring SAA protocol and MSA-favoring SAA protocol were used and ThT fluorescence measurements were taken every 15 minutes for 90 hours.

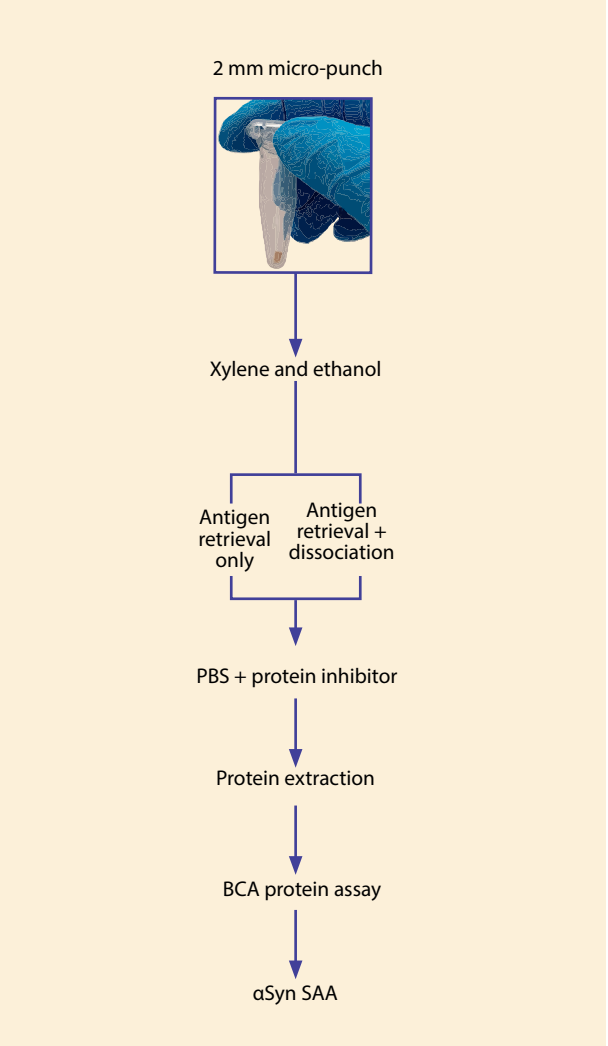

Figure 2. Experimental workflow. Comparison of αSyn seeding using tissues processed with antigen retrieval only or both antigen retrieval and dissociation. The final optimized protocol can be found in the supporting information.[3]

Results

Optimizing FFPE protein extraction and αSyn seeding activity to evaluate αSyn disease-specificity in tissue archived for <2 years and >20 years

A variety of conventional protocols for tissue preparation, deparaffinization, and protein extraction were tested. While 4 mm micropunches yielded consistently higher protein concentrations, 2 mm micropunches yielded enough protein for αSyn seeding. Sections yielded less protein than micropunches, so 2-mm micropunches were used for further validation. Xylene-ethanol was selected for deparaffinization and a solution of PBS spiked with protease inhibitor was selected for protein extraction.

Lack of antigen retrieval and dissociation combined resulted in inconsistent αSyn seeding, and it was further tested if only antigen retrieval, which acts as de-fixation step, is sufficient to optimize protein yield (fig. 2). Combining antigen retrieval with dissociation increased protein yield in 2 mm FFPE micropunches from control, LBD, and MSA (fig. 3A). Increase in protein yield is important, however, recapitulation of the seeding patterns observed in the SAA using frozen brain tissues is crucial. Using the LBD-favoring SAA protocol, antigen retrieval and dissociation resulted in disease-specific seeding (short lag phase and low maximum ThT in MSA while longer lag phase and higher maximum ThT in LBD), as demonstrated previously in SAAs using frozen brain tissue (fig. 3B). Notably, αSyn SAA in LBD FFPE tissue was negative when antigen retrieval was used in the absence of dissociation, emphasizing the importance of dissociation coupled with antigen retrieval for efficient protein extraction and accurate downstream SAA evaluation.

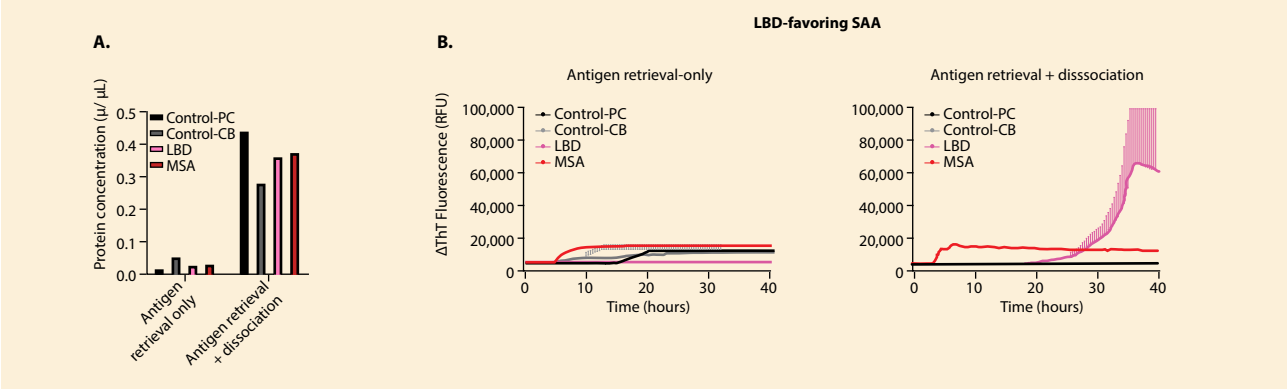

Figure 3. Comparing protein extraction efficiency and αSyn-seeding activity of αSyn extracted from tissues processed with and without dissociation. (A) Total protein amount obtained from 2-mm FFPE micro-punch of the control, LBD, and MSA samples, processed with antigen retrieval only or both antigen retrieval and dissociation prior to the protein. (B) Comparison of disease-specific αSyn seeding activities of αSyn extracted from tissues using antigen retrieval only and both antigen retrieval and dissociation.

The optimized protocol also included testing archived FFPE brain tissue (> 20 years). Using the FFPE Tissue Dissociation Kit followed by SAA on LBD tissues archived for over 20 years resulted in positive seeding that differed across brain regions, although maximum ThT was lower compared to proteins extracted from more recently collected FFPE tissues (figs. 4A, B).

Validation of disease-specific αSyn seeding patterns in FFPE human brain tissues

To further validate the disease-specific αSyn seeding, additional LBD (temporal cortex) and MSA (cerebellum) cases were processed for protein extraction, using the optimized protocol with the FFPE Tissue Dissociation Kit. With LBD-favoring SAA, LBD showed higher αSyn seeding (i.e., longer lag phase and higher maximum ThT) compared to MSA, and the control was negative, demonstrating robust disease-specific αSyn seeding (fig. 5A).

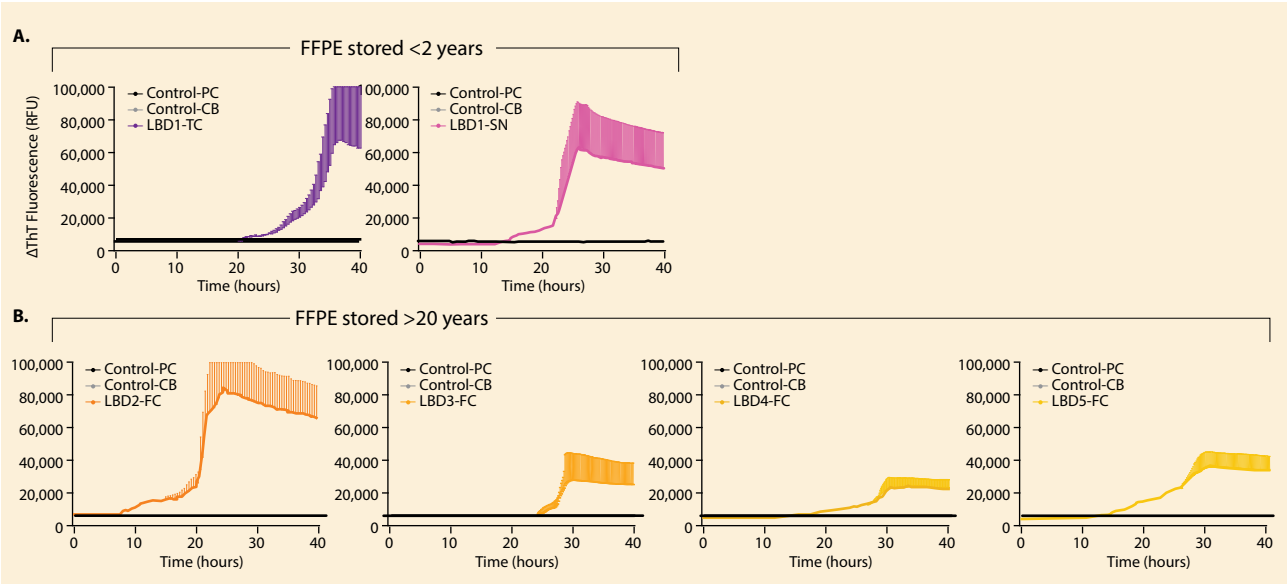

Figure 4. Inter- and intra-subject heterogeneity of αSyn seeding activity in FFPE LBD human brain tissues. (A) αSyn seeding observed using control and LBD FFPE tissues archived for <2 years and (B) αSyn seeding observed using control and LBD FFPE tissues archived for >20 years.

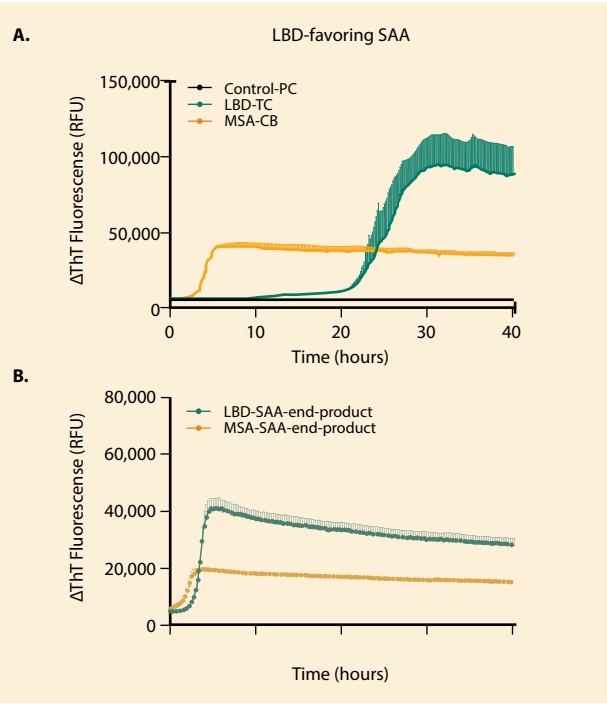

Figure 5. Biochemical validation of disease-specific seeding of αSyn using additional LBD and MSA FFPE brain tissue. (A) Disease-specific αSyn seeding was observed using the LBD-favoring SAA, where LBD has higher seeding activity (i.e., longer lag phase and higher maximum ThT) compared to that of MSA. (B) αSyn seeding of LBD and MSA SAA end-products in subsequent SAA using LBD-favoring SAA shows replicability of disease-specific αSyn seeding.

It was tested if αSyn seeding differences between FFPE LBD and MSA are replicable by templating using SAA end-products because if the seeds do not maintain the disease specific seeding, that would indicate ineffective extraction of the original disease-specific protein. Seeding of LBD and MSA SAA end-products in subsequent SAA reaction using LBD-favoring SAA showed replicability of αSyn seeding differences, highlighting that the antigen retrieval and dissociation step helps to recapitulate disease-specific αSyn seeding as observed in SAA with frozen brain (fig. 5B).

Conclusion

Misfolded αSyn is prevalent in synucleinopathies such as LBD and MSA and can spread cell-to-cell promoting conversion of normal αSyn to the pathogenic, misfolded form. SAAs are useful for studying small amounts of αSyn aggregates, but it can be challenging to extract sufficient amount of protein from FFPE tissue and successfully perform biochemistry on FFPE brain tissues that have been stored for long periods of time.

This study established a protocol using the FFPE Tissue Dissociation Kit to remove paraffin residues, reverse crosslinks, and dissociate, leaving behind a pellet of dissociated tissue which can be used for downstream protein extraction and αSyn seeding assays. Using a standardized kit and automated dissociation is highly beneficial because it makes assays more reproducible. Notably, this protocol requires low input material – 2 mm FFPE micropunches or ten 4.5 um sections on slides are sufficient to yield protein and analyze seeding activities. The resulting tissue pellet can be resuspended in different buffers, allowing applicability to other SAA protocols or biochemical analysis. In addition, protein extracted from FFPE human brain tissue that was fixed and paraffin-embedded over 20 years ago still retain disease-specific seeding. While this protocol was only tested on synucleinopathies, it may also prove effective for performing the tau SAA on large repositories of FFPE Alzheimer's disease (AD) brain samples. These findings highlight the potential of this approach to unlock valuable biomarker and mechanistic insights from archival FFPE tissues across a broader range of neurodegenerative diseases.

In summary:

- Sufficient levels of high-quality protein are hard to extract from FFPE tissue stored for long periods of time.
- The FFPE Tissue Dissociation Kit provides a streamlined way to obtain ample protein from low input, requiring only 2 mm FFPE micropunches or or ten 4.5 um sections on slides for disease-specific αSyn seeding.
- The resulting pellet can be resuspended in any buffer, providing flexibility for downstream applications.

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