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Seed amplification assay for the detection of pathologic alpha-synuclein aggregates in cerebrospinal fluid

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Abstract

Misfolded alpha-synuclein (αSyn) aggregates are a hallmark event in Parkinson's disease (PD) and other synucleinopathies. Recently, αSyn seed amplification assays (αSyn-SAAs) have shown promise as a test for biochemical diagnosis of synucleinopathies. αSyn-SAAs use the intrinsic self-replicative nature of misfolded αSyn aggregates (seeds) to multiply them in vitro. In these assays, αSyn seeds circulating in biological fluids are amplified by a cyclical process that includes aggregate fragmentation into smaller self-propagating seeds, followed by elongation at the expense of recombinant αSyn (rec-αSyn). Amplification of the seeds allows detection by fluorescent dyes specific for amyloids, such as thioflavin T. Several αSyn-SAA reports have been published in the past under the names 'protein misfolding cyclic amplification' (αSyn-PMCA) and 'real-time quaking-induced conversion'. Here, we describe a protocol for αSyn-SAA, originally reported as αSyn-PMCA, which allows detection of αSyn aggregates in cerebrospinal fluid samples from patients affected by PD, dementia with Lewy bodies or multiple-system atrophy (MSA). Moreover, this αSyn-SAA can differentiate αSyn aggregates from patients with PD versus those from patients with MSA, even in retrospective samples from patients with pure autonomic failure who later developed PD or MSA. We also describe modifications to the original protocol introduced to develop an optimized version of the assay. The optimized version shortens the assay length, decreases the amount of rec-αSyn required and reduces the number of inconclusive results. The protocol has a hands-on time of ~2 h per 96-well plate and can be performed by personnel trained to perform basic experiments with specimens of human origin.

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Author contributions

L.C.-M., C.M.F, S.P. and M.S. contributed to the development of the protocols. With the help of S.P., C.M.F. and M.S., L.C.-M. wrote the first draft of the article. C.S. is the principal investigator who provided funding and supervision for this research. C.S. produced the final version of the article.

Competing interests

L.C.-M. and C.M.F. are employees of Amprion Inc., a biotechnology company that focuses on the commercial use of SAAs (PMCA and RT-QuIC) for high-sensitivity detection of misfolded protein aggregates. C.S. is a Founder, Chief Scientific Officer and Member of the Board of Directors of Amprion Inc. The University of Texas Health Science Center at Houston has licensed patents and patent applications to Amprion. The other authors declare no competing interests.

Additional information

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Introduction

The lack of validated biomarkers and approved tests for the biochemical diagnosis of Parkinson's disease (PD) and related synucleinopathies has been a major shortcoming in the handling of these diseases. Currently, the only widely accepted way to confirm a clinical PD diagnosis is by postmortem analysis. The histopathological hallmarks of PD include the loss of nigral dopaminergic neurons and the presence of nigrostriatal Lewy bodies and Lewy neurites, mostly composed of misfolded alpha-synuclein (αSyn) aggregates[1,2]. A body of evidence indicates that αSyn aggregates play a central role in the etiology of PD[3]. Moreover, the misfolding of αSyn into pathological species and subsequent neuronal spread might be the key event in the disease[4].

Recently, many publications have described similarities in certain biological features between misfolded αSyn aggregates and prions[5], which are proteinaceous, infectious agents that self-propagate in vivo, causing diseases such as Creutzfeldt–Jakob disease (CJD), bovine spongiform encephalopathy and chronic wasting disease[6]. Protein misfolding cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC) tests exploited this self-replicating nature of prions for diagnostic purposes, achieving impressive performances approaching 100% sensitivity and specificity with various biological fluids[7–12]. Because misfolded αSyn aggregates also self-propagate in vitro, PMCA and RT-QuIC methods were adapted for the amplification and detection of αSyn aggregates in cerebrospinal fluid (CSF). Although both methodologies have converged to a very similar protocol, the two names continue to be used in literature to reference the αSyn assay[13–16]. To avoid confusion between PMCA and RT-QuIC assays that were initially developed to replicate infectious prions, the consensus name 'seed amplification assay (SAA)' has emerged to collectively refer to assays that exploit self-replication of misfolded proteins by means of fragmentation and elongation cycles[17–19]. In this protocol, we describe SAA conditions that we initially published under the name αSyn-PMCA[14,19–21]. We also describe modifications to increase throughput and reliability for detection of PD and dementia with Lewy bodies (DLB) and recount experimental settings reported by other groups to produce similar results.

Development of αSyn SAAs

Protein misfolding is a pathological process involved in various human diseases that results in the accumulation of misfolded protein aggregates rich in β-sheet conformation[22,23]. Most proteins that aggregate in vivo and cause disease can propagate in vitro under specific conditions. Protein misfolding in vitro follows a seeding nucleation mechanism, in which an aggregated protein with a β-sheet–rich conformation (seed) can template the conformational change of an identical protein in its native state to acquire the same pathological structure as the seed[23,24,25] (Fig. 1a). The seed can be generated in vitro by primary nucleation (spontaneous or de novo). This is energetically disfavored compared to misfolding driven by a preformed seed (e.g., from a biological sample)[25]. Both seeded and spontaneous aggregation follow a sigmoidal pattern, presenting an initial lag phase with no observable increase in aggregation, followed by a growth phase with rapid incorporation of monomers

to polymers and a final stationary phase with no observable change in the aggregation state of the polymers (Fig. 1a).

The development of SAAs started in the prion field. In 1994 it was first described that aggregated, pathogenic prion protein (PrP^{Sc}) could directly interact and convert the normally folded prion protein (PrP^{C}) into PrP^{Sc} in a cell-free setting[24]. This landmark publication used recombinant purified radiolabeled protein and partial denaturation conditions to induce the conversion of PrP^{C} into PrP^{Sc}. The method was limited by its high complexity and the low conversion rate, because the PrP^{C}/PrP^{Sc} ratio was ≤1:50. The first method for efficient seeded conversion of PrP^{C} by small amounts of PrP^{Sc} was the PMCA assay, first described in 2001[26]. This method used sonication for seed fragmentation and brain homogenate (BH) containing PrP^{C} as substrate. PMCA was the first technology to incorporate the templated conversion of PrP^{C} to PrP^{Sc} into a cyclical process of fragmentation and elongation, which is a critical feature of all SAAs. Both fragmentation and agitation were used in the original publication[26], indicating that the PMCA principle is not restricted to the use of sonication. This publication is considered a breakthrough development because the final PMCA product comprised >97% in vitro–generated PrP^{Sc}. In 2006 PMCA was performed by using sonicators that could execute the fragmentation/elongation cycles automatically and was shown to amplify aggregates containing ~26 monomers (10^{-12} BH dilution), a quantity understood to be in the range of a single prion aggregate[27]. This unprecedented billion-fold level of amplification was 4,000 times more sensitive than the gold standard bioassay. PMCA was then used to detect prions in blood of infected animals at the clinical and pre-clinical stages of the disease[28,29], which was the first indication that the technology could be used for diagnostic applications. Subsequently, PMCA was adapted to detect human prions and enabled detection of PrP^{Sc} in the urine of patients with variant Creutzfeldt–Jakob disease (vCJD)[7]. This method was then quickly expanded to achieve highly accurate detection of prions in the blood of patients with vCJD[8,30] and later improved to achieve even lower detection limits compatible with preclinical detection of the vCJD agent[9,30].

In 2007, the PMCA assay was modified to use recombinant PrP (rPrP) instead of BH and was called ‘rPrP-PMCA’[31]. Compared to using BHs, production of large amounts of recombinant protein is easier and allows the possibility of spectroscopical labeling of the rPrP-PMCA–generated material. Although thioflavin T (ThT) fluorescence of the PrP aggregates generated in rPrP-PMCA was shown in this initial publication, western blot and digestion of proteinase K was the preferred readout, in agreement with the gold standard in the field. In 2008 rPrP-PMCA was performed with shaking instead of sonication, and the assay received the new name ‘quaking-induced conversion’[32]. These modified conditions were very sensitive and quite fast, because a 10^{-8} dilution of BH could be amplified in just 18 h. In this publication, quaking-induced conversion was used in combination with digestion of proteinase K and western blot. In 2011 the assay switched from western blot analysis to fluorescence readings and achieved sensitivity above 80% and 100% specificity in the detection of sporadic CJD by using human CSF samples[33]. With the introduction of periodic fluorescence readings, the name of the assay was changed to its current version, RT-QuIC. Both PMCA and RT-QuIC are now used to assist clinical diagnosis of sporadic CJD and vCJD.

Most αSyn-SAAs use recombinant protein, shaking and fluorescence readings to monitor the amplification of αSyn seeds. Many instruments can automatically perform shaking and fluorescence readings, making the transition to high-throughput settings more straightforward, whereas sonication mostly relies on manual fluorescence readings. Nevertheless, both PMCA and RT-QuIC assays amplify seeds on the basis of the same principle of cyclic fragmentation-elongation, and conditions published under both names are practically the same. Thus, to avoid confusion with the names originated from the prion field, the consensus name 'SAA' has been adopted to describe assays that rely on amplification by elongation-fragmentation cycles[17–19].

Development of the protocol

The first and most critical step in developing our protocol was the production of recombinant αSyn (rec-αSyn) to be used as substrate. Purification protocols must yield pure rec-αSyn (>85%) free of preformed seeds. Subsequently, the assay was optimized by using in vitro–generated misfolded αSyn aggregates (synthetic seeds). Synthetic seeds can be created de novo under particular in vitro conditions, such as constant agitation of high concentrations of rec-αSyn for a few days. However, the relevance of synthetically generated αSyn aggregates compared to endogenous αSyn aggregates is highly contested[34,35]. Different quantities of synthetic seeds were added to the monomeric rec-αSyn substrate, and conditions were selected to detect seeded aggregation rather than spontaneous aggregation[14].

The intermittent agitation used in αSyn-SAAs accelerates the seeded aggregation by mechanically fragmenting aggregates in the biological sample, which generate many smaller aggregates that can act as seeds (Fig. 1b). These seeds then interact with the rec-αSyn substrate and incorporate the monomeric protein into the aggregate, propagating the misfolded conformation and increasing the number of aggregates in the reaction. Once the aggregates have sufficient time to elongate, they can then be fragmented again (generating many smaller seeds) in the next cycle. Thus, repeated fragmentation-elongation cycles substantially amplify the amount of αSyn aggregates in the reaction at the expense of rec-αSyn. By the end of the reaction, most of the rec-αSyn protein is converted to αSyn aggregates, and the original αSyn aggregates in the biological sample represent a minute proportion of the final product (Fig. 1b). In biological samples without αSyn aggregates, the seeding reaction does not occur, and thus aggregation is delayed until some of the protein can spontaneously aggregate under the experimental conditions used, which usually takes a much longer time. The absence of self-aggregation in the αSyn-SAA protocol described here allows the faithful self-replication of the endogenous αSyn aggregates. Aggregates generated in vitro by αSyn-SAA usually maintain the structural, biochemical and seeding features of the CSF seeds[21].

Once the assay is optimized by using rec-αSyn seeds and can detect aggregates in the femtogram range, human biological samples containing aggregates are added to the rec-αSyn substrate, and the sensitivity and specificity of the assay are evaluated. Kinetic parameters can be calculated from the aggregation curves and correlated to clinical and biochemical information[20]. Theoretically, a greater number of seeds produces faster aggregation[14,36], but it is still unclear if the same can be achieved for endogenous αSyn

aggregates in biological samples. The kinetic parameter that best correlates with the seed concentration is the time to reach 50% of the maximum fluorescence(T_{50}). Assay parameters not only correlate with the number of seeds in a sample, but also with the intrinsic ultrastructure of the αSyn aggregates. In particular, high maximum fluorescence (F_{max}) and T_{50} values are normally produced by CSF samples from patients with PD, whereas low F_{max} and T_{50} values have been observed in patients with multiple-system atrophy (MSA)[21].

Comparison with other approaches

Much effort has been devoted to identifying biomarkers in biological fluids for the objective, early, sensitive and non-invasive biochemical diagnosis of PD and related synucleinopathies[37–39]. Some of the biomarkers studied so far in CSF and/or plasma include various forms of αSyn (monomeric, oligomeric, phosphorylated, etc.), neurofilament light chain, neuromelanin antibodies, lysosomal enzymes, dopamine metabolites, micro-RNAs, amyloid-β and tau[39,40]. Studies showing different patterns of gene expression, metabolomics and protein profiling have also been done comparing PD and healthy controls (HCs)[40]. Because this article focuses on the detection of αSyn species associated with disease by SAAs, we limit our discussion to the work done on αSyn as a biomarker.

The presence and levels of various forms of αSyn in biological fluids has been extensively studied. Several assays measuring total αSyn levels, such as western blots, ELISAs, mass spectrometry and Luminex[41], have been published. Total αSyn levels in CSF appear to be significantly decreased in PD groups compared to HCs[41–43]. In an evaluation of various studies, the level of αSyn in CSF discriminated patients with PD versus HCs with a sensitivity ranging from 61% to 94% and specificity ranging from 25% to 64% (refs. [41,42]). The substantial overlap between PD and HC groups precludes the use of these methods for diagnosis of individual patients[44]. Moreover, several studies of smaller cohorts have not found significant differences[45].

αSyn forms that are considered pathological, including phosphorylated αSyn (p-αSyn) and oligomeric αSyn, have been studied as disease-specific biomarkers as well. αSyn phosphorylation is one of the principal pathological modifications and is indeed present in >90% of Lewy bodies[46]. Some studies have reported that patients with PD have higher concentrations of p-αSyn in CSF compared to HCs[37,47]. The low concentration of p-αSyn in CSF, which makes quantitation more challenging, and the low number of independent studies reproducing these results make the diagnostic accuracy of this marker unclear[37].

Detection of soluble oligomeric forms of αSyn is also an interesting biomarker, considering that oligomerization precedes protein aggregation and deposition in Lewy bodies, so oligomers are probably circulating in biological fluids before the onset of the disease[22]. Furthermore, several studies have shown that oligomeric αSyn might be the most neurotoxic species in synucleinopathies[48,49]. There are some preliminary reports showing higher levels of αSyn oligomers in CSF from patients with PD[50,51]. However, the diagnostic accuracy of studies measuring αSyn oligomers in CSF is low, with a pooled sensitivity of 71% and specificity of 64% (ref. [37]). It is possible that a combination of various αSyn-related

biomarkers may increase the diagnostic performance of the assay[37], as recently reported by Majbour and colleagues[52].

Various SAAs have been reported to detect αSyn seeds in numerous tissues and biological fluids, including CSF, skin, gastrointestinal tissues, submandibular glands and olfactory mucosa of patients with PD or other synucleinopathies[13,14,19,21,36,53–67]. αSyn-SAAs have been referenced in previous publications under the names 'PMCA', 'RT-QuIC' and 'HANABI'[68], and they all use the same concept but slightly different assay conditions to achieve amplification of αSyn seeds. Most of the studies using αSyn-SAAs have been performed with CSF by using shaking as the force of fragmentation and a fluorometric assay based on ThT binding to monitor the formation of the aggregate products. In Table 1, we describe some of the alternative conditions used in different αSyn-SAAs that have been published to date. These publications were selected because the SAA protocol used was substantially different. There are many more papers, which use slight variations of some of the protocols listed here. In addition, we did not include SAAs using other forms of aggregate fragmentation or distinct readouts of the aggregation signal.

Reported diagnostic accuracies for PD, DLB and MSA versus HCs and controls with other neurodegenerative diseases are quite remarkable, usually describing sensitivity and specificity between 85% and 95% (refs. [13,14,16,17,19,21,36,55,66]). Importantly, recent reports indicate that SAAs can detect αSyn seeds during prodromal stages, as in the case of people affected by isolated rapid eye movement sleep behavior disorder[58,61]. In addition, SAAs have been shown to detect αSyn seeds in the CSF of patients with pure autonomic failure and predict the phenoconversion to PD or MSA on the basis of the kinetics of αSyn aggregation and the maximum fluorescence[69].

SAAs using tissues as biological samples have also shown interesting results and offer an opportunity for detection in samples other than CSF. However, the sensitivity and specificity for detection in these tissues is often lower than CSF. In addition, it is unclear if the amplified material from these tissues represents the same αSyn aggregates in the brain, as has been shown for CSF[21]. Peripheral tissue samples are, in principle, less invasive than CSF collection, but the current approaches require more than one biopsy collection from submandibular glands[54] or several skin punches[63]. Nevertheless, detection of αSyn aggregates in peripheral samples, particularly skin[57,63], have produced good results and offer an interesting alternative to CSF. Obviously, the future of αSynSAAs is to achieve detection of αSyn aggregates in easily collectable biological fluids, such as blood, urine or saliva. However, working with these fluids is challenging because of their complexity and the expected very low concentration of aggregates in them.

Lastly, some studies have described αSyn-SAA protocols including other forces for aggregate fragmentation and changing the readouts of the assay (e.g., ELISA and western blot)[63–65,68]. It remains unknown if these changes in the protocol could provide advantages for αSyn seed detection in complex biological samples.

Limitations

The high amplification level achieved by αSyn-SAA increases the possibility for false-positive results due to cross-contamination. This has been observed in some of our previous experiments, but careful applications of methodologies and strategies to minimize cross-contamination have successfully reduced or eliminated the rate of false positives. Because the pathogenesis of PD and other synucleinopathies start years before the onset of clinical symptoms[4,22], it is likely that a proportion of age-matched HCs may have misfolded oligomers circulating in biological fluids. Conversely, clinically misdiagnosed cases of synucleinopathies will be negative in αSyn-SAA. These difficulties complicate the clinical validation of the technology.

Although the assay is quantitative in nature, as shown by the dose response observed when amplifying different levels of synthetic αSyn seeds, it remains qualitative for CSF samples. The current limitation is the lack of a standard that could be used as a calibration curve. Synthetic αSyn aggregates generated de novo do not faithfully mimic the endogenous aggregates found in CSF. Moreover, endogenous αSyn seeds do not aggregate within the linear range of the synthetic seeds. In our experience, their use as calibrators would lead to gross underestimation of the endogenous seeds in CSF. Interestingly, the aggregates generated by the αSyn-SAA method described here have an ultrastructure very similar to the endogenous seeds[21], which could be used in the future to generate a standard calibrator. In addition to the lack of calibrator, preliminary results indicate that other CSF components influence the kinetics of aggregation, further complicating quantitation. The αSyn-SAA described here can differentiate PD and MSA by means of F_{max}. It is unknown if synucleinopathies like DLB or PD dementia can be distinguished by means of a single kinetic parameter or a mixture of kinetic parameters. Another limitation of αSyn-SAA is that it detects only seeding-competent aggregates. Although most of the biologically relevant αSyn aggregates can probably seed aggregation, we cannot rule out the presence of off-pathway aggregates that could play an important role in disease pathogenesis. In practical terms, the main limitation of the protocol described here is the length of the assay and the relatively high concentration of substrate needed. We have recently developed an optimized, high-throughput version of our original assay that shortens the assay length and uses less substrate, overcoming two key limitations of the original assay[16,17]. Nonetheless, SAAs generally require more time than other simpler assays, such as ELISA or Luminex.

Experimental design

The flowchart in Fig. 2 shows the steps to perform the assay described in this protocol. Acquisition or purification of rec-αSyn and its characterization are perhaps the most relevant steps to ensure that the procedure will perform optimally. Good results have been reported by using both commercially available and in-house–produced substrates, including wildtype and mutant rec-αSyn. The protocol described here uses wildtype rec-αSyn with a C-terminal histidine tag, and the purification protocol uses a single IMAC (immobilized metal affinity chromatograpy) step. The purification protocol for in-house production of rec-αSyn is described in Reagents, although we suggest using highly purified rec-αSyn quality-controlled for αSyn-SAA, which is commercially available from Amprion (cat. no. S2020). The plasmid to express rec-αSyn contains a mutation in codon 136 to avoid cysteine

misincorporation during the orthologous expression of human rec-αSyn in *Escherichia coli*. Substrate characterization includes various SDS-PAGE analyses (Coomassie and western blot), size exclusion chromatography, dynamic light scattering and mass spectrometry. Nevertheless, the final test for substrate quality is the αSyn-SAA assay itself. For that, CSF samples from patients with known PD and HCs are the best option, although buffer only and synthetic seeds in buffer can be used as an approach for negative and positive controls, respectively[20]. The αSyn-SAA buffer system is simple, just PIPES and NaCl. Although the assay does not need sterile conditions, all buffers are filtered with 0.22-μm filters to eliminate undissolved material or dust acquired during preparation. Buffer and reagent stability depends on the laboratory conditions (temperature changes, humidity, etc.) and handling. We recommend preparing fresh buffers on a quarterly basis. The equipment required for the simplest αSyn-SAA is minimal: a plate shaker and a fluorometer. More advanced instruments offer various shaking modes (orbital, double orbital, linear, etc.) and can automatically shake, read and incubate a 96-well plate. The rest of the necessary equipment is typically found in a biochemistry laboratory, including a spectrophotometer, a fluorometer, a pH meter, biosafety cabinets and an electrophoresis rig. Once the buffers are prepared, the recombinant protein tested and the equipment programmed for the shaking/incubation cycles, the assay requires only the addition of CSF samples to a plate followed by the addition of the substrate mix. This process can take 1.5–2 h per plate and does not require special equipment other than regular micropipettes and perhaps multichannel micropipettes to add the substrate mix. Samples are analyzed in replicates, typically triplicates or duplicates, depending on the objective of the experiment and sample availability.

The assays described in this article have been optimized for the His-tagged version of the protein, which is easier to purify than the untagged protein and can be manufactured with high yields. We have done a limited number of experiments with the untagged protein, and the results indicate higher levels of spontaneous aggregation, suggesting that the assay would require modification to accommodate this version of the substrate.

In this article, we include two procedures, the original and the optimized (automated) versions of the assay. Each version has advantages and disadvantages. For example, the original assay is not limited to a specific instrument, and it has been shown to distinguish αSyn seeds from patients with PD versus those from patients with MSA. On the other hand, the optimized assay takes less time and is more precise because it reads fluorescence almost continuously but is limited to one type of instrument, and only one plate can be used at the same time. Nevertheless, the optimized assay is compatible with automation, which can increase the throughput substantially. Sensitivity and specificity have been reported to be similar for both assays.

Materials

Reagents and biological materials

- Recombinant αSyn with C-terminal His-tag (substrate). Produced in-house as described below in Reagent setup or purchased from Amprion (cat. no. S2020) or equivalent. Substrate can be purchased by direct message in the following link:

https://ampriondx.com/contact/. The nominal concentration is 6.5 mg/ml, but a certificate of analysis is issued with the exact concentration of each lot.

- HyClone PBS 1× (Cytiva, cat. no. SH30256.01). Store at room temperature (RT) and discard on the expiration date.
- HyClone PBS 10× (Cytiva, cat. no. SH30258.01). Store at RT and discard on the expiration date.
- Deionized water (18.2 mΩ·cm; Growcells, cat. no. NUPW-0500). Store at RT and discard on the expiration date.
- PIPES (MilliporeSigma, cat. no. 80635). Store at RT and discard on the expiration date.
- AccuGENE 5 M NaCl (Lonza, cat. no. 51202). Store at RT and discard on the expiration date.
- ThT (MilliporeSigma, cat. no. T3516–25G). Store at RT and discard on the expiration date.
- Optimized assay: 3/32-inch Si_3N_4 beads (Tsubaki Nakashima)
- Optimized assay: BSA (Fisher, cat. no. BP9706100). Store at 4–8 °C and discard on the expiration date.
- Patients' CSF samples. These were collected as described in our previous publications[14,19–21]. For sample collection, we recommend using the procedure described in detail in the Parkinson's Progression Markers Initiative website (https://www.ppmi-info.org/study-design/research-documents-and-sops). CSF samples should be kept frozen at −80 °C in aliquots of ≥0.125 ml until use and avoid freeze-thaw cycles, although we know that the assay tolerates a small amount of freeze-thaw cycles (two to five).

Equipment

- COSTAR 96-well plates (Corning, cat. no. 3916)
- Applied Biosystems MicroAmp optical adhesive film (ThermoFisher, cat. no. 4311971)
- TipOne RPT pipette tips, 100, 200 and 1,000 µl (USA Scientific, cat. nos. 1180–1840, 1180–8810 and 1182–1830, respectively)
- 1.5-ml Eppendorf LoBind Tubes (Eppendorf, cat. no. 022431081)
- 50-ml conical tubes (Thermo Scientific, cat. no. 339653)
- Thermomixer R (Eppendorf)
- Gemini SpectraMax EM fluorometer (Molecular Devices)
- MaxQ4000 incubator (ThermoFisher)
- Optimized assay: FLUOstar Omega fluorometer (BMG)

Reagent setup

PIPES—500 mM PIPES should be prepared in deionized water, and pH should be adjusted to 6.50 ± 0.05 (6.45–6.55) by using 1 M NaOH. The pH-adjusted solution should be filtered through 0.22-μm filters. After filtration, store at 4 °C for ≤2 months.

ThT—ThT is prepared to a 5 mM concentration in deionized water and filtered through 0.22-μm filters. Prepare 500-μl aliquots and store them at −20 °C. A single aliquot can undergo many freeze-thaw cycles and last many years.

Si_3N_4 beads (optimized assay)—3/32-inch Si_3N_4 beads are blocked with 1% (w/v) BSA in 100 mM PIPES pH 6.50 for 1 h, washed twice with 100 mM PIPES pH 6.50 and dried overnight. Blocked beads are stable for ≥1 year at RT.

Recombinant αSyn with C-terminal His-tag

Expression of 6xHis-tagged α-syn in E. coli.

1. Clone the full-length C-terminally 6xHis-tagged human α-syn (NM_000345) into the ampicillin-resistant bacterial expression vector pET-21b (NdeI and HindIII cloning sites). To avoid cysteine misincorporation at codon 136 in bacterially expressed αSyn, change the codon at 136-TAC to 136-TAT by site-directed mutagenesis.
2. Transform competent *E. coli* BL21(DE3)-pLysS according to the manufacturer's instructions (Invitrogen, cat. no. 44–0054). Plate 50 μl of the final mixture onto an agar plate with 100 μg/ml carbenicillin (Chem-Impex, cat. no. 00049) and incubate overnight at 37 °C to select transformed cells.
3. To prepare a starter culture, inoculate a single colony of the transformed cells to a 250-ml flask (Fisher Scientific, cat. no. 10–090B) containing 50 ml of autoclaved Terrific Broth (Fisher Scientific, cat. no. BP2468500) with 100 μg of carbenicillin (TB-100C).
4. Incubate the flask overnight at 37 °C with shaking at 200 rpm. On the next day, transfer the 50-ml culture into a Falcon tube and centrifuge at 5,000g for 10 min at RT.
5. To grow cells for purification, add 50 ml of TB-100C to the bacterial pellet under sterile conditions and resuspend thoroughly. Inoculate a 2-liter flask (Fisher Scientific, cat. no. NC0343699) containing 500 ml of TB-100C with 25 ml of the resuspended cells.
6. Incubate the culture at 37 °C with 200-rpm agitation. Monitor the bacterial growth by measuring the OD_{600}. Induce the culture with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (SigmaAldrich, cat. no. I5502–10G) once the OD_{600} reaches 0.6–0.7. Use 52.5 μl of 1 M IPTG for 525 ml of culture. After the induction starts, incubate the culture for 6 h at 25 °C with shaking at 150 rpm.

7. Centrifuge the culture for 30 min at 3,000g and 4 °C. Collect the pellet in a 50-ml conical tube (Thermo Scientific, cat. no. 339653). Wash the bacterial pellet with STE washing buffer (10 mM Tris-Cl pH 7.5 (Fisher Scientific, cat. no. BP152–10), 100 mM NaCl (Sigma-Aldrich, cat. no. S3014) and 1 mM EDTA (Promega, cat. no. V4231)) by using 15 ml per liter of culture. Centrifuge for 15 min at 5,000g at 4 °C. Discard the supernatant and store the pellet at −80 °C until use. In our experience, pellets are optimal for 1 year from generation.

Purification of 6xHis-tagged α-syn.

8. Resuspend the pellets in 20 ml of lysis buffer per liter of culture (50 mM NaH_2PO_4 pH 8.0 (Sigma-Aldrich, cat. no. S5011), 300 mM NaCl, 10 mM imidazole (Fisher Scientific, cat. no. O3196–500),0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) (Acros Organics, cat. no. 215740050) prepared in isopropanol and 0.1 mM tris(2-carboxyethyl)phosphine (TCEP) (Sigma Aldrich, cat.no. C4706) added right before use.

9. Sonicate the lysate with a 0.5-inch probe tip by using the Misonix sonicator for 5 min (30-s pulse on, 30-s pulse off) at 60% intensity on ice.

10. Centrifuge the lysate at 12,000g for 15 min at 4 °C in 50-ml Falcon tubes. Collect the supernatant and centrifuge again at 100,000g for 30 min at 4 °C.

11. Collect the supernatant and filter through a 0.45-μm filter (Sigma-Aldrich, cat. no. SLHVR33RS). Store the supernatant on ice.

12. Prepare a 1.5-cm × 20-cm glass chromatography column (Bio-Rad, cat. no. 7374152) with 10 ml of Ni Sepharose 6 Fast Flow slurry (Cytivia, cat. no. 17531802).

13. Wash the column with 1 column volume (CV) of elution buffer (50 mM NaH_2PO4 pH 7.4, 250 mM imidazole, 300 mM NaCl and 0.1 mM TCEP) by using gravity chromatography.

14. Wash the column with 5 CVs of equilibration buffer (50 mM NaH_2PO_4 pH 7.4, 10 mM imidazole, 300 mM NaCl and 0.1 mM TCEP).

15. Load the filtered bacterial supernatant onto the column. Wash the column with 5 CVs of washing buffer (50 mM NaH_2PO_4 pH 7.4, 20 mM imidazole, 300 mM NaCl and 0.1 mM TCEP).

16. Elute the bound protein with elution buffer (50 mM NaH_2PO_4 pH 7.4, 250 mM imidazole, 300 mM NaCl and 0.1 mM TCEP). Collect 1.5 ml of fractions in 2-ml microcentrifuge tubes (Eppendorf, cat. no. 022431102) kept on ice.

17. Check the elution fractions with Bradford reagent to detect total protein. Determine fractions containing αSyn by SDS-PAGE analysis and pool them.

18. Dialyze the pooled αSyn fractions by using a dialysis cassette (7,000 MWCO; Thermo Scientific, cat. no. 66710) against 1× PBS (prepared with 10× HyClone PBS (Cytivia, cat. no. SH30258.02) and deionized water). Perform two dialyses

at 4 °C (first dialysis, 10 ml-sample in 4,000 ml of 1× PBS for 16 h; second dialysis, 10-ml samples in 2,000 ml of 1× PBS for 4 h).

19. After the dialysis, if there is noticeable precipitation, centrifuge the sample at 10,000g for 10 min at 4 °C. Filter the resultant supernatant through a 0.22-μm filter (Sigma-Aldrich, cat. no. SLGPR33RS).

20. Filter the supernatant through a 100-kDa MWCO filter (MilliporeSigma, cat. no. UFC910024) by using a swinging bucket rotor at 3,220g and 4 °C for 25 min.

21. Filter the sample through a 0.22-μm filter and determine the protein concentration by using the bicinchoninic acid (BCA) kit (ThermoFisher, cat. no. 23225).

22. Finally, make 500-μl aliquots in 1.5-ml low-binding tubes (Eppendorf, cat. no. 022431081). Snapfreeze in liquid nitrogen and store at −80 °C until use. In our experience, the presence or absence of endotoxin does not change the quality of the protein for the amplification assay.

Equipment setup

Thermomixer—Turn off the temperature control of the Thermomixer R and place it inside the MaxQ4000 incubator set to 37 °C. Using the automatic program option of the Thermomixer, create a program that shakes the plate at 500 rpm for 60 s and incubates it for 29 min. Set this program for 400 h. Set the Gemini Spectra EM fluorometer to 37 °C before each reading and shake the plate for 5 s before fluorescence reading. Use $435_{Ex}/485_{Em}$ (excitation (Ex)/emission (Em)) filters and set the photomultiplier sensitivity to auto.

FLUOstar Omega fluorometer (optimized assay)—Set the FLUOstar Omega fluorometer to 37 °C. Create a protocol in plate mode to read fluorescence. Use 440–10/490–10 filters, set the gain to 900 and specify top optics. Set the cycle time to 1,800 s, the number of cycles to 300 and the number of flashes per well to 3. Set the shaking conditions to orbital shaking at 800 rpm and shake for 60 s before each cycle. Set the idle movement to the ‘wait at incubation position’ setting.

Procedure

Below, we describe both the original SAA and the newly optimized SAA. A comparison of the two versions can be found in Experimental design

Procedure 1: original αSyn-SAA

▲**CRITICAL** 200 μl of αSyn-SAA reaction mix is prepared for each CSF sample by combining 40 μl of CSF and 160 μl of substrate mix. The final reaction mix contains 1 mg/ml rec-αSyn in 1× PBS, 100 mM PIPES pH 6.50, 500 mM NaCl and 5 μM ThT. The concentration of rec-αSyn determines the volume of NaCl needed to reach 500 mM, because the 1× PBS buffer contains 154 mM NaCl.

1. Place a COSTAR 96-well plate on ice and add 200 μl of water to the outer wells to minimize the potential evaporation of the reaction mix. Thus, only the 60 inner

wells are used to test samples. Cover the plate with the lid while preparing the reaction mix.

2. As an example, if using a 5-mg/ml stock concentration of rec-αSyn, mix 2,520 µl of 500 mM PIPES pH 6.50, 3,920.6 µl of deionized water, 1,106.8 µl of 5 M NaCl and 12.6 µl of 5 mM ThT in a 50-ml conical tube. This provides enough substrate mix for 20 CSF samples, allowing a 5% excess for multichannel pipetting. Swirl the tube to mix the contents and keep it on ice. Prechill a reservoir compatible with multichannel pipetting.

3. Thaw the CSF samples in cold tap water to accelerate the thawing process. Samples can also be thawed on the bench without water.

4. Vortex the CSF samples to mix the contents and briefly centrifuge the samples to spin down the CSF attached to the bottom of the tube lid.

5. Remove the lid of the plate and add 40 µl of each CSF sample to three wells in the plate to analyze samples in triplicate. Change the low retention tip for each of the wells/replicates.

6. Once all the CSF samples are loaded in the plate, thaw the rec-αSyn protein in tap water.

7. Finish making the reaction mix by adding rec-αSyn. For one plate, a 5-mg/ml stock concentration of rec-αSyn and a 5% excess for multichannel pipetting, add 2,520 µl of rec-αSyn to the prechilled mix. Swirl the tube to mix the contents.

   ▲CRITICAL STEP Avoid bubble formation by excessive agitation.

8. Distribute the reaction mix in the prechilled reservoir and add 160 µl to each of the wells with CSF samples in them by using a multichannel pipette. Mix the solution by pipetting up and down a few times in each well.

9. Remove the plate from the ice and seal it with a MicroAmp film by using a rubber roller.

   ▲CRITICAL STEP Make sure to apply sufficient force to properly seal the wells.

10. Remove the side tabs of the film and measure time zero fluorescence by using the Gemini Spectra EM fluorometer with the above-mentioned settings. Export the readings as an Excel file for subsequent analysis.

? TROUBLESHOOTING

11. Place the plate on the Thermomixer R inside the incubator and cover the plate with aluminum foil to protect the reaction mixture from direct light.

12. Start the reaction by starting the shaking/incubation cycle in the Thermomixer R.

13. Read the plate every 12 h for 360–400 h (15–17 d). Preheat the fluorometer to 37 °C before every reading. Pause the Thermomixer R right before the shaking part of the cycle, remove the plate from the Thermomixer R and place it between

two plates filled up with water and sealed and store it at 37 °C to maintain the temperature of the plate during reading.

14. Read fluorescence at 485 nm, place the plate back in between the two plates at 37 °C and place it back onto the Thermomixer R. Resume the shaking/incubation cycle. Export each reading as an Excel file for subsequent analysis.

15. Determine the F_{max} for each replicate. This can be easily determined by using the function = MAX() in Excel.

16. Compare F_{max} to a positivity fluorescence threshold that must be determined on the basis of the fluorometer used. In the case of the Gemini EM fluorometer set up to Auto photomultiplier sensitivity, we have used thresholds of 50 a.u.[14], 1,000 a.u.[19] and 150 a.u.[62,69], depending on whether all forms of synucleinopathies are being studied or only PD. Thus, if F_{max} > threshold, the replicate is considered positive. The information for all three replicates is integrated as follows:

Experimental result	αSyn-SAA result
Three positive replicates	αSyn-SAA-positive sample (PD or MSA)
Two positive replicates	αSyn-SAA inconclusive
Zero or one positive replicate	αSyn-SAA-negative sample (HC or below detection)

? TROUBLESHOOTING

17. Further analyze results for αSyn-SAA–positive samples to determine if they correspond to PD or MSA. This determination is performed by using the average measured F_{max} of the three replicates.

The average of the estimated F_{max} calculated by fitting a model to the aggregation curve can be used as well (see kinetic analysis).

Experimental result	αSyn-SAA result
50 a.u. < average F_{max} < 1,800 a.u	MSA
Average F_{max} > 2,000 a.u	PD

Kinetic analysis requires all the datapoints collected during the experiment. Prepare a time versus fluorescence table for each sample. Using GraphPad Prism, fit the following Boltzmann exponential model to the data:

$$F(t) = F_{min} + \frac{F_{max} - F_{min}}{1 + e^{\frac{T_{50} - t}{slope}}}$$

Because fluorescence readings are performed manually, estimated F_{max} is more representative than the measured F_{max}. Estimated F_{max}, F_{min}, T_{50} and slope, can be used for correlation studies.

T_{50}has been shown to depend on the mass of synthetic αSyn seeds and was shown to correlate with the Hoehn and Yahr Scale Score in a small cohort study[14].

Procedure 2: optimized αSyn-SAA

▲CRITICAL 200 µl of αSyn-SAA reaction mix is prepared for each CSF sample by combining 40 µl of CSF and 160 µl of substrate mix. The final reaction mix contains 0.3 mg/ml rec-αSyn in 1× PBS, 100 mM PIPES pH 6.50, 500 mM NaCl and 5 µM ThT. The concentration of rec-αSyn determines the volume of NaCl needed to reach 500 mM, because the 1× PBS buffer contains 154 mM NaCl.

1. Place one blocked Si_3N_4 bead in each of the 96 wells of the COSTAR 96-well plate. This step should be performed in a clean area, ideally an area never exposed to αSyn seeds. All 96 wells can be used to analyze samples under these conditions.
2. Place the plate on ice and cover it with a lid while preparing the reaction mix.
3. As an example, if using a 5-mg/ml stock concentration of rec-αSyn, mix 4,032 µl of 500 mM PIPES pH 6.50, 8,988.3 µl of deionized water, 1,857.8 µl of 5 M NaCl and 40.3 µl of 5 mM ThT in a 50-ml conical tube. This provides enough substrate mix for 32 CSF samples, allowing a 5% excess for multichannel pipetting. Swirl the tube to mix the contents and keep it on ice. Prechill a reservoir compatible with multichannel pipetting.
4. Thaw the CSF samples in cold tap water to accelerate the thawing process. Samples can also be thawed on the bench without water.
5. Vortex the CSF samples to mix the contents and briefly centrifuge the samples to spin down the CSF attached to the bottom of the tube lid.
6. Remove the lid on the plate and add 40 µl of each CSF sample to three wells in the plate to analyze samples in triplicate. Change the low retention tip for each of the wells/replicates.
7. Once all the CSF samples are loaded in the plate, thaw the rec-αSyn protein in tap water.
8. Make the substrate mix by adding rec-αSyn to the reaction mix. For one plate, a 5-mg/ml stock concentration of rec-αSyn and a 5% excess for multichannel pipetting, add 1,209.6 µl of rec-αSyn to the prechilled reaction mix. Swirl the tube to mix the contents. Avoid bubble formation by excessive agitation.
9. Distribute the generated substrate mix in the prechilled reservoir and add 160 µl to each of the wells with CSF samples in them by using a multichannel pipette. Mix the reaction mixture by pipetting up and down a few times.
10. Remove the plate from ice and seal it with a MicroAmp film by using a rubber roller.

    ▲CRITICAL STEP Make sure to apply sufficient force to properly seal the wells.

11. Remove the side tabs of the film. Place the plate in the plate carrier of the FLUOstar Omega fluorometer and start the reaction. The plate will continuously be shaken/read for 150 h (~6.5 d), and no further steps are necessary until the end of the experiment.

? TROUBLESHOOTING

12. After 150 h, export the results file by using Mars, the BMG analysis software. Using Mars, determine the estimated F_{max} (relative fluorescence units (RFU)), estimated F_{min} (RFU), slope (RFU/h) and T_{50} (h) by fitting the following four-parameter model to the RFU:

$$F(t) = F_{min} + \frac{F_{max} - F_{min}}{1 + \left(\frac{T_{50}}{t}\right)^{Slope}}$$

13. Select R2 to add the coefficient of determination $\left(R^2\right)$ for each fitting result to the working file.

14. Select Kinetic Calculations and use the predetermined calculation of the time to threshold. Select the raw data as input. Time to threshold is the time in hours at which the fluorescence of a replicate crosses a given threshold (5,000 RFU in our case).

15. Export all the calculated parameters into Excel for further analysis.

16. Determine the sample average F_{max} of the three replicates.

17. Determine the sample coefficient of variation of the F_{max} of the three replicates. The optimized assay uses a probabilistic algorithm to make a final determination on the status of the CSF sample. The probability of each replicate being positive is calculated with the following probability function:

$$P_{pos} = \frac{e^{A + B * F\max_{5000} + C * R\text{square}_{93}}}{1 + e^{A + B * F\max_{5,000} + C * R\text{square}_{93}}}$$

where P_{pos} is the probability of a replicate being positive, $A = -4.02$, $B = 2.98$, $C = 1.87$ and $F\max_{5,000}$ and $R\text{square}_{93}$ are binary values depending on a threshold. If the F_{max} of a given replicate is >5,000 RFU, then $F\max_{5,000} = 1$; otherwise, it is 0. If the R^2 for the fitting of the four-parameter model to the fluorescence data is >0.93, then $R\text{square}_{93} = 1$; otherwise, it is 0. If the probability for positivity (P_{pos}) is >0.12, then the replicate is determined to be positive; otherwise, it is determined to be negative. The information for all the replicates is integrated as follows for final call:

Experimental result	Optimized αSyn-SAA result
Three positive replicates	αSyn-SAA positive
Zero or one positive replicate	αSyn-SAA negative
Two positive wells and average $F_{max} < 5,000$ RFU	αSyn-SAA negative
Two positive wells and coefficient of variation of $F_{max} > 110$	αSyn-SAA negative
Two positive replicates	αSyn-SAA inconclusive (re-test recommended)

? TROUBLESHOOTING—A probabilistic algorithm has been developed to deem samples as positive (αSyn seeds detected) or negative (αSyn seeds not detected). Parameters to differentiate PD and MSA have not been introduced yet.

Troubleshooting

Troubleshooting advice can be found in Table 2.

Timing

Hands-on time for the assay is quite short compared to the total time of the assay. Thawing CSF samples on tap water takes 5–10 min, while carefully adding 40 μl of CSF to each well can take up to 1.5 h. Thawing of the substrate and reconstituting the substrate mix takes 5 min, while careful addition of the substrate mix and sealing of the plate can take up to 20 min. Because the original αSyn-SAA is performed in a shaker that does not read fluorescence, periodic manual readings are required every 12–24 h for 300–400 h. Data analysis is not highly demanding, especially because it can be easily automatized by using Excel.

The hands-on time for setting up a plate for the optimized assay is the same as for the original assay, but the rest of the experiment is much quicker (150 h instead of 400 h), and the fragmentation-elongation cycles and fluorescence reads are performed automatically by the FLUOstar Omega fluorometer. Data analysis is easily automatized by using both Mars and Excel, depending on personal preference.

We and others are improving published protocols to make them quicker and more reliable. The assay can be easily accelerated, but self-aggregation of the substrate presents a challenge. The quality and reproducibility of the substrate are key when implementing changes in αSyn-SAAs. One alternative is to accelerate the assay and cut it short, so that there is no time for self-aggregation to occur. This is a common approach, but the higher propensity for self-aggregation produces more variability, and a larger number of replicates are usually needed to reach the same results. The protocols described here practically eliminate self-aggregation, with some reactions staying flat for up to 1 month when seeded with HC CSF. This allows a significant window for amplifying very low amounts of aggregates.

If rec-αSyn expression and purification are performed in house, this process will be the most time demanding part of the assay. The production of the bacterial pellets expressing rec-αSyn takes up to 4 d, including transformation, selection of individual clones, generation of a starter culture and induction of protein expression in the final culture. Protein purification takes 2 d, with a break overnight during dialysis and resuming the next morning with filtration of the dialyzed material.

Anticipated results

Original assay

CSF samples from patients with PD, DLB or MSA should aggregate within 200–400 h when using the original αSyn-SAA, while CSF samples from HC should not aggregate (Fig. 3a). PD and MSA CSF samples carrying misfolded αSyn aggregates present distinctive aggregation curves. Most MSA aggregation curves present low F_{max} (<1,000 a.u.), whereas PD CSF samples present high F_{max} (>2,000 a.u.) (Fig. 3b). The lack of aggregation in HC CSF samples, even after 350 h of reaction, is demonstrated by F_{max} values <50 a.u. The second distinctive feature between PD and MSA in the original assay is the speed of aggregation (Fig. 3c). MSA CSF samples present a lower T_{50}between 50 and 100 h), whereas PD samples take longer to aggregate (usually >150 h). T_{50}cannot be calculated for HC samples, given the lack of aggregation.

Optimized assay

Aggregation curves are similar to the original assay, but they occur faster (Fig. 4). Most PD CSF samples start aggregating between 50 and 70 h. In fact, the optimized assay could be performed for 100 h without loss of sensitivity. F_{max}values for PD CSF samples vary between 50,000 and 150,000 RFU, whereas MSA samples often show F_{max} between 2,000 and 20,000 RFU, and HC samples present F_{max} below 1,000 RFU.

The assay conditions reported here have shown high sensitivity for PD (88.5% (ref. [14]), 95.2% (ref. [19]) and 93.6% (ref. [21])) and high specificity as well (96.9% (ref. [14]), 89.9% (ref. [19]) and 100% (ref. [21])), depending on the study. MSA has also been detected with high sensitivity (80.0% (ref. [14] and 84.6% (ref. [21])) and high specificity (96.9% (ref. [14]) and 100% (ref. [21])), but the detection levels seem to be lower than for PD. The discrimination between MSA and PD or DLB samples can also be achieved by αSyn-SAA in patients without definite diagnosis, such as patients with pure autonomic failure. In these patients, αSynSAA predicted future phenoconversion of patients with pure autonomic failure to MSA with 100% sensitivity and specificity[69]. The accuracy of the optimized assay has been reported for PD and HC samples, showing 96% sensitivity and specificity for baseline samples[16]. These results were similar to two other αSynSAA methods[17]. Overall, the αSyn-SAA conditions reported here can amplify minute amounts of misfolded αSyn aggregates in CSF from patients with synucleinopathies, without self-aggregation of the substrate and with high reproducibility, sensitivity and specificity.

Data availability

The authors declare that the main data discussed in this protocol are available in the supporting primary research papers[16,21]. The raw datasets are available for research purposes from the corresponding authors upon reasonable request.

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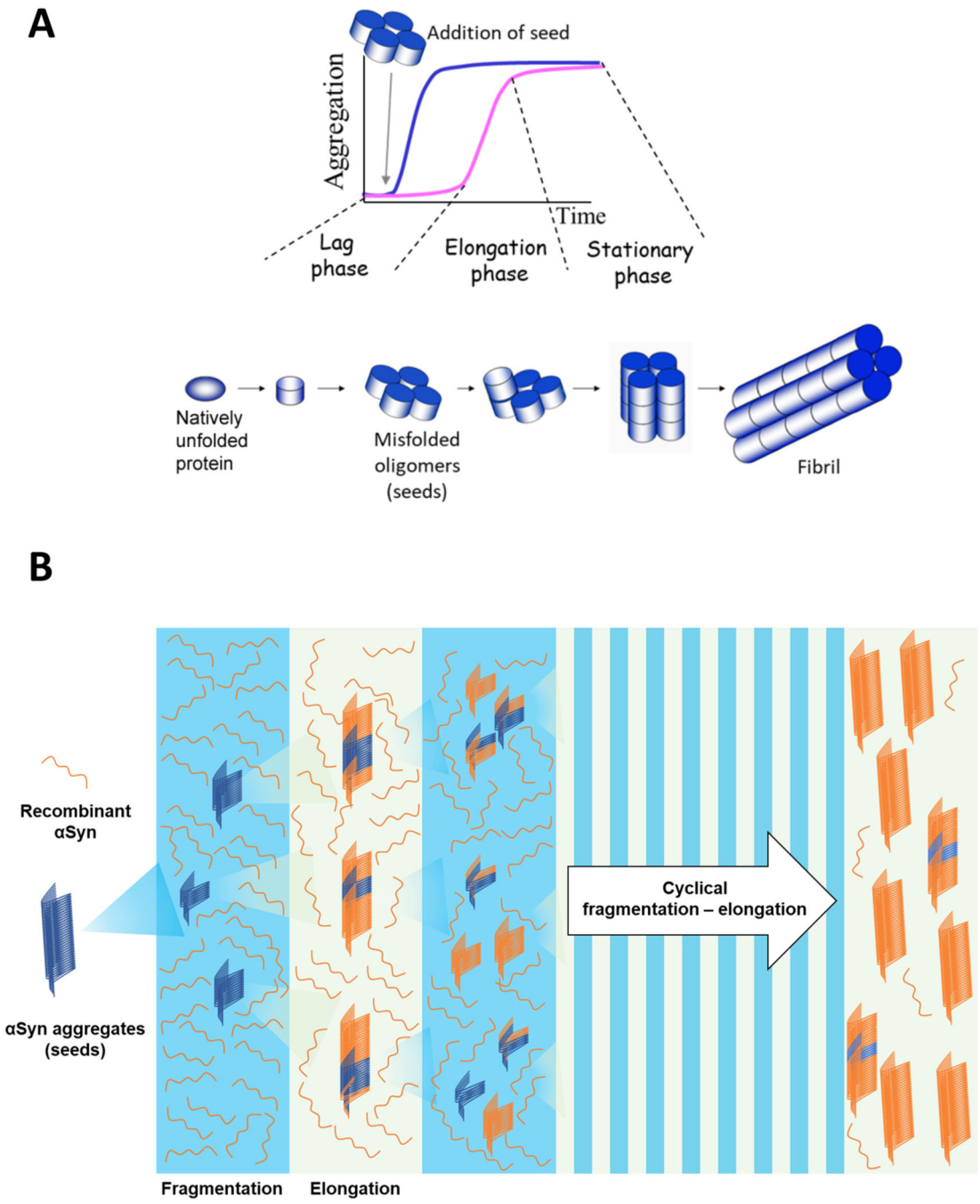

Fig. 1 |. Seeding nucleation mechanism and the principle behind the amplification of misfolded αSyn aggregates.

a, Schematic representation of the seeding/nucleation model of protein misfolding and aggregation. The seeding nucleation mechanism identifies three phases for formation of highly organized protein aggregates: the lag or nucleation phase, the growth or elongation phase and the stationary phase. During the lag phase, there is no observable aggregation, and the early misfolding events leading to formation of nuclei take place. Once enough nuclei have been formed, there is a rapid increase in the amount of observable aggregates, resulting

in the formation of protofibrils and fibrils. In the stationary phase, the consumption of substrate slows down the aggregation, and the solution enters an equilibrium in which there is no further change in aggregation. If a seed is added to the aggregation process, the lag phase is decreased significantly, because the reaction bypasses the formation of nuclei. This figure aims only to be a schematic representation of the process and not to indicate the exact sizes of seeds, which are still unknown. **b**, Scheme for the αSyn-SAA reaction. Accelerated aggregation in the absence of self-aggregation is achieved by means of fragmentation and elongation of the endogenous αSyn seeds present in biological samples. Fragmentation effectively increases the number of active seeds, which is followed by elongation induced by quiescent incubation at 37 °C. During elongation the recombinant protein is converted into more aggregates, which are then fragmented again cyclically to amplify the misfolded protein biomarker. By the end of the reaction, the in vitro–generated aggregates represent the vast majority of the aggregates present in the solution, and most of the recombinant protein is consumed and incorporated into aggregates. The cyclic amplification of the biomarker allows its detection by conventional methods, such as thioflavin T fluorescence.

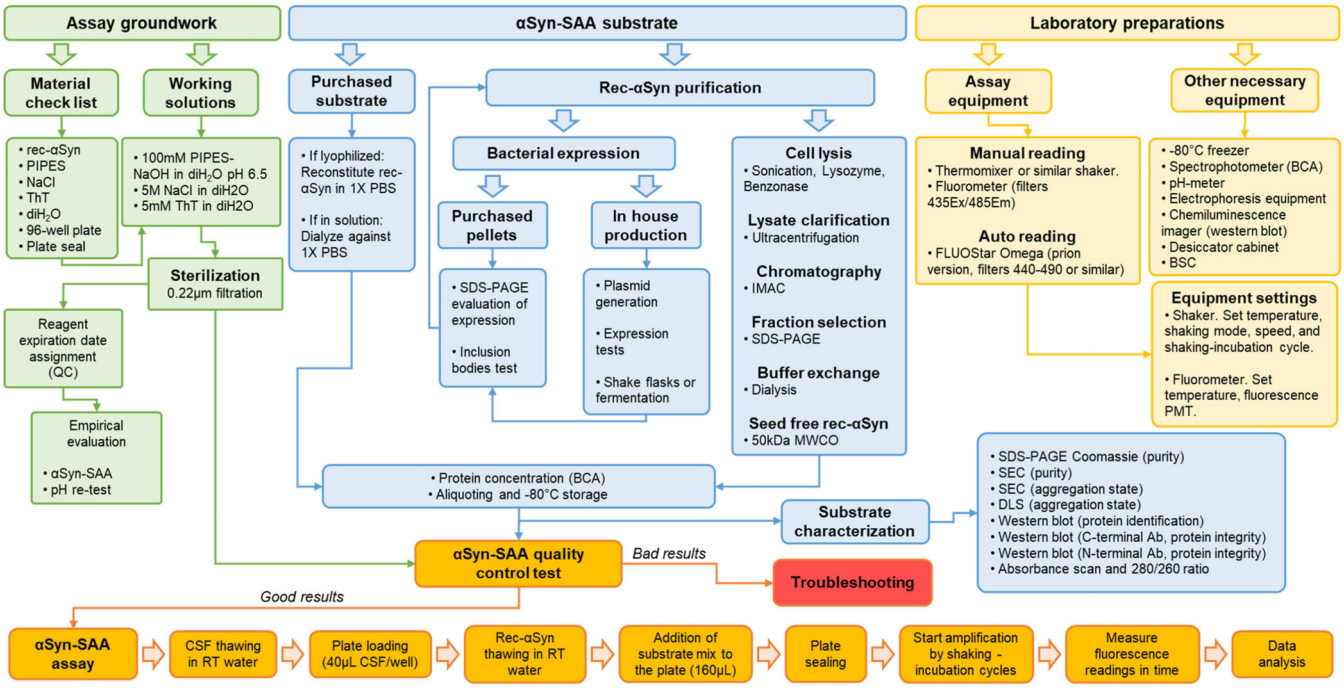

Fig. 2 |. Flowchart of the protocol.

The protocol depicted here consists of three main phases: preparation of basic assay reagents and consumables (green), acquisition or production of rec-αSyn substrate (blue) and implementation of the laboratory equipment used for the assay (yellow). Once those procedures and equipment are implemented, the protocol for the assay is very straightforward (orange). BSC, biosafety cabinet; DLS, dynamic light scattering; IMAC, immobilized metal affinity chromatography; QC, quality control; RT, room temperature; SEC, size-exclusion chromatography.

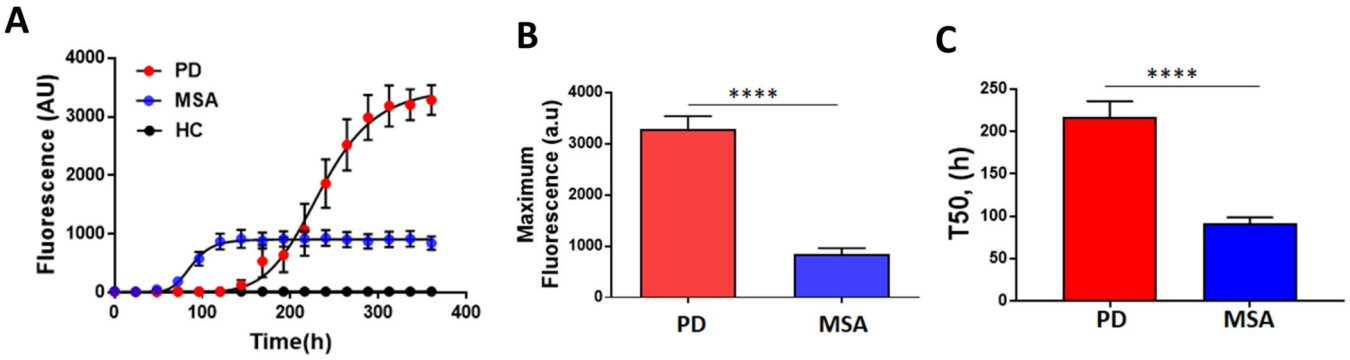

Fig. 3 |. Performance of the original αSyn-SAA for the detection of αSyn aggregates in CSF samples from patients with PD or MSA.

CSF samples (40 μl) from 10 donors with MSA, 10 donors with PD and 10 HC donors were analyzed in triplicate in a 96-well plate. The seed amplification assay was started by adding rec-αSyn monomers (1 mg/ml) and ThT (5 μm) in 100 mM PIPES pH 6.5 containing 500 mM NaCl. The plate was incubated at 37 °C with intermittent shaking at 500 rpm for 1 min every 30 min. The extent of aggregation was monitored by the increase in ThT fluorescence (excitation of 435 nm and emission of 485 nm). **a**, The graph illustrates the kinetics of aggregation measured by ThT fluorescence for a typical αSynSAA reaction in the presence of seeds coming from PD or MSA CSF, as well as HC samples. Data are displayed as the mean ± SEM for 10 patients. **b**, F_{max}measured at the plateau of aggregation. This parameter provides information about the amount of aggregates at the end of the assay as well as about structural differences between them, because different aggregates may have different modes of interaction with ThT, resulting in distinct fluorescence values. **c**, T_{50}corresponds to the time to reach 50% of maximum fluorescence. This parameter reflects the speed of the aggregation reaction. In a seeded assay, T_{50}provides information about the number of seeds present in the biological sample. Differences were analyzed by Student's *t* test (****, P < 0.0001).

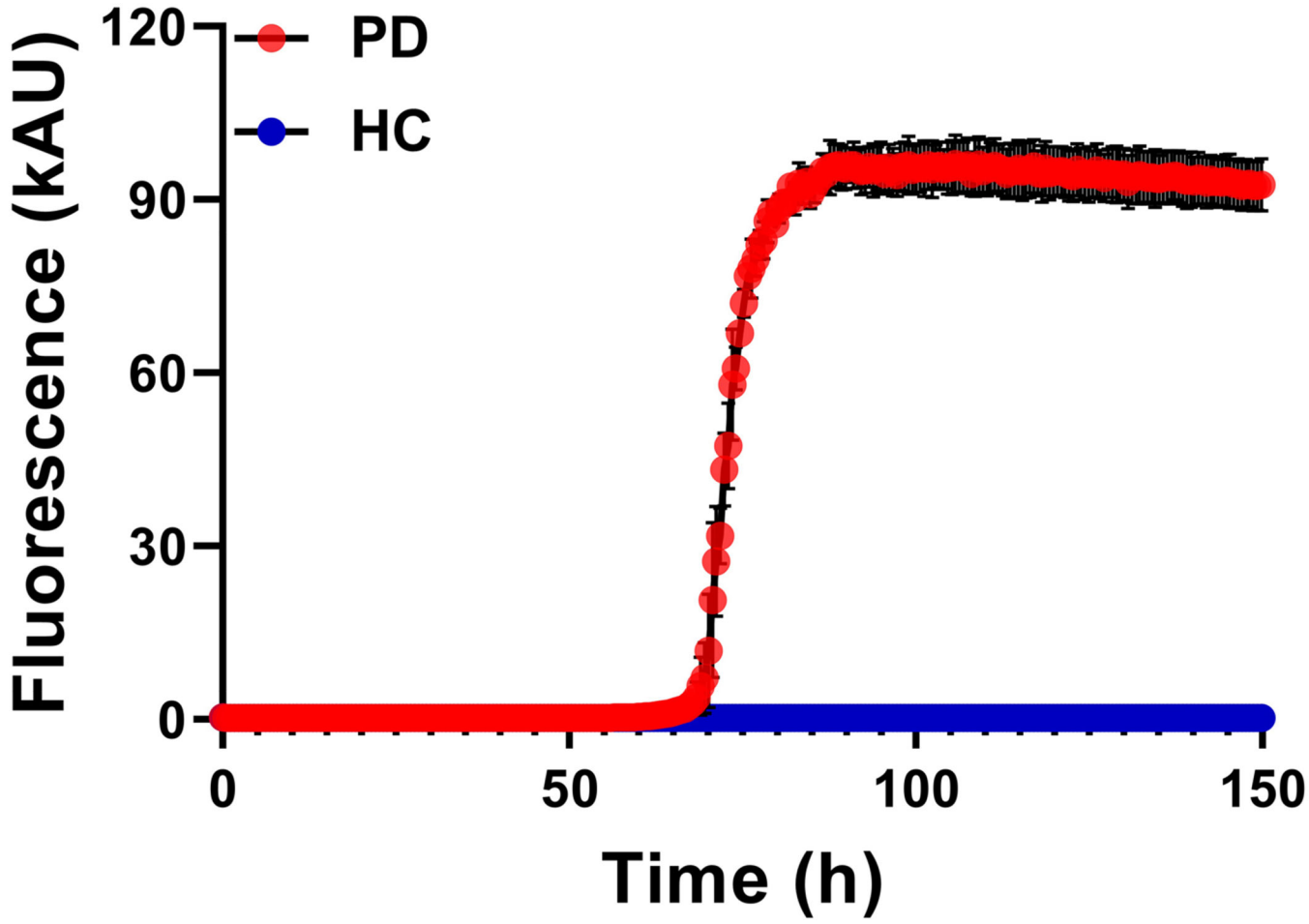

Fig. 4 |. Performance of optimized αSyn-SAA for the detection of αSyn aggregates in CSF samples of patients with PD.

CSF samples (40 μl) from three donors with PD and three HC donors were analyzed in triplicate in a 96-well plate. The seed amplification assay was performed as described in the text. The extent of aggregation was monitored by the increase in ThT fluorescence. Data are displayed as the mean ± SEM for three patients.

Table 1 |

SAA conditions reported in selected publications for the detection of αSyn aggregates in human CSF

Ref.	Biological sample	Substrate (source)	Reaction mix	Reaction volume (μl)	Replicates (CSF volume, μl)	Assay instrument	Assay length (h)	SAA cycle (min)	Shaking	Incubation time (min), temperature (°C)	Bead type (no.)
Fairfoul et al.[13]	CSFand BH	0.1 mg/ml rec-αSyn WT, no tag (Stratech)	100 mM phosphate buffer pH 8.2, 10 μm ThT	100	2 (15)	OPTIMA FluoSTAR Omega fluorometer	120	15	Double orbital 1 min, 200 rpm	14, 30	0.5-mm zirconium silica (37 mg)
Shahnawaz et al.[14] (original αSyn-SAA)	CSF and BH	1 mg/ml rec-αSyn WT+6hist (in house)	100 mM PIPES pH 6.5, 500 mM NaCI, 5 μm ThT	200	3 (40)	Thermomixer R Gemini Spectra EM fluorometer	350–400	30	Orbital 1 min, 500 rpm	29, 37	No beads
Groveman et al.[15]	CSF and BH	0.1 mg/ml rec-αSyn K23Q +6hist (in house)	40 mM phosphate buffer pH 8, 170 mM NaCI, 10 μm ThT, 0.0015% (m/v) SDS	100	4(15)	FLUOstar Omega fluorometer	50	2	Double orbital 1 min, 400 rpm	1, 42	0.8-mm silica (6)
Bargar et al.[57]	CSF, brain, skin, salivary gland and colon	1 mg/ml rec-αSyn WT, no tag (rPeptide)	40 mM phosphate buffer pH 8, 170 mM NaCI, 20 μm ThT	100	4(2)	FLUOstar Omega fluorometer	60	2	Double orbital 1 min, 400 rpm	1, 42	0.8-mm silica (6)
Concha-Marambio et al.[16] (optimized αSyn-SAA)	CSF	0.3 mg/ml rec-αSyn WT +6hist (in house)	100 mM PIPES pH 6.5, 500 mM NaCI, 5 μm ThT	200	3 (40)	FLUOstar Omega fluorometer	150	30	Double orbital 1 min, 700 rpm	29, 37	3/32-inch Si3N4 bead (1)
Russo et al.[17] (Abbvie protocol)	CSF	0.1 mg/ml rec-αSyn WT, no tag (in house)	100 mM phosphate buffer pH 8.2,10 μM ThT	100	8 (5)	Not specified	70	15	Double orbital 1 min, 200 rpm	14, 37	0.10-mm zirconia/ silica beads (25 mg)
Perra et al.[60]	CSF and olfactory mucosa	0.05 mg/ml rec-αSyn WT, no tag (in house)	100 mM phosphate buffer pH 8.2, 10 μm ThT, 0.0075% (m/v) SDS	100	4(15)	FLUOstar Omega fluorometer	80	15	Double orbital 1 min, 200 rpm	14, 30	0.5-mm glass beads (37 mg)

Table 2 |

Troubleshooting table

Step	Problem	Possible reason	Solution
10 (Procedure 1), 11 (Procedure 2)	Blank fluorescence reading	The instrument does not have the right filters	Replace the filters to $435_{Ex}/485_{Em}$, $450_{Ex}/480_{Em}$ or similar
		The reading optics do not match the type of plate The plate sealer is not transparent at the needed wavelength	Clear-bottom plates are compatible with top and bottom optics, but black-bottom plates are compatible only with top reading Use the recommended plate seal (cat. no. 4311971) or use a clear- bottom plate
16 (Procedure 1), 17 (Procedure 2)	Spontaneous self-aggregation	PIPES buffer	Prepare new PIPES and use NaOH only for pH adjustment. If too much NaOH is added, start again and prepare a new solution. Do not add HCl or other acids
		Undissolved PIPES	Pass the buffer through 0.22-μm filters to remove undissolved PIPES
		Hydrated PIPES powder	Buy new reagent and maintain it inside a desiccator cabinet
		Substrate contains pre-aggregated αSyn	Re-filtrate the substrate by using a 100-kDa-cutoff filter
		A shaking mode other than orbital shaking was selected	Program the shaker for orbital shaking. Protocols that use double orbital shaking use different shaking speeds and overall assay conditions
		Poor-quality substrate	Confirm the protein concentration of the stock. Underestimation could lead to a higher concentration of the protein in the assay than desired Buy or purify a new batch of substrate
		Substrate dimerization	Make sure that the plasmid encodes tyrosine at codon 136 by using TAT nucleotides[70] Analyze the substrate by using DTT or 2-Mercaptoethanol (BME). If dimers were formed by disulfide bonds, after DTT, the protein should appear monomeric. If this is the problem, the plasmid needs to be modified as above If the dimers remain (confirmed by western blot), get a new batch of rec-αSyn