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Improving protocols for α-synuclein seed amplification assays: analysis of preanalytical and analytical variables and identification of candidate parameters for seed quantification

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Abstract

Objectives: The effect of preanalytical and analytical factors on the α-synuclein (α-syn) seed amplification assay's (SAA) performance has not been fully explored. Similarly, there is limited knowledge about the most suitable assay protocol and kinetic parameters for misfolded α-syn seed quantification.

Methods: We studied the effect of centrifugation, repeated freeze-thaw cycles (up to seven), delayed freezing, detergent addition, and blood contamination on the performance of the cerebrospinal fluid (CSF) α-syn SAA real-time quaking-induced conversion (RT-QuIC). Moreover, we analysed the inter- and intra-plate variability, the recombinant protein batch effect, and the RT-QuIC parameters' variability when multiple samples were run in controlled conditions. Finally, we evaluated the assay potential of quantifying α-syn seed by assessing kinetic curves in serial CSF dilutions.

Results: Among tested preanalytical variables, a ≥0.01 % blood contamination and adding detergents significantly affected the RT-QuIC kinetic parameters and the number of positive replicates. Increasing the number of replicates improved result reproducibility. The number of positive replicates in serially diluted CSF samples improved discrimination between samples with high and low seeding activity, and the time to threshold (LAG) was the most reliable kinetic parameter in multiple experiment settings.

Conclusions: Preanalytical variables affecting α-syn RT-QuIC performance are limited to blood contamination and detergent addition. The number of positive replicates and the LAG are the most reliable variables for quantifying α-syn seeding activity. Their consistent measurement in serial dilution experiments, especially when associated with an increased number of sample replicates, will help to develop the α-syn RT-QuIC assay further into a quantitative test.

Keywords: seed amplification assay; alpha-synuclein; real-time quaking-induced conversion; Parkinson's disease; biomarker; Lewy body disease

Introduction

Seed amplification is a novel *in vitro* approach exploiting the seeded polymerization properties of prion-like amyloidogenic proteins for their ultrasensitive detection in biofluids and other tissues [1]. Assays based on seed amplification (SAA) as the real-time quaking-induced conversion (RT-QuIC) have recently led to a significant advancement in the diagnostics of neurodegenerative disorders [2]. After being established as a clinically validated diagnostic test for Creutzfeldt–Jakob disease [3], the RT-QuIC and other similar SAAs have been exploited for the detection of Lewy body (LB)-associated misfolded α-synuclein (α-syn), becoming a laboratory tool with enormous diagnostic potential for synucleinopathies, a highly prevalent group of disorders that includes Parkinson's disease and dementia with Lewy bodies [4–18]; however, there is still a lack of deep knowledge about the effect of

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preanalytical and analytical factors that may significantly impact the assay outcomes. Therefore, systematically identifying those potentially influencing the α-syn RT-QuIC results can improve the standardisation of handling protocols among laboratories and, consequently, the reproducibility and clinical value of this already robust assay.

The RT-QuIC readout is currently analysed to provide a qualitative dichotomous (positive/negative) response. Nonetheless, increasing evidence indicates that the analysis of kinetic curves of the RT-QuIC reaction might also contribute quantitative data indirectly revealing the number of protein seeds in the biosamples and, eventually, the LB pathology burden [19]. However, assessing the reproducibility of the qualitative and, especially, quantitative data provided by α-syn RT-QuIC represents another unresolved issue, significantly limiting the impact in clinical practice where a quantitative SAA could also help to evaluate disease severity and progression.

In the present work, focusing on cerebrospinal fluid (CSF), the most used biomatrix for α-syn RT-QuIC, we systematically investigated the effect of preanalytical and analytical variables potentially affecting the assay's outcome. Moreover, aiming to evaluate its repeatability and potential as a quantitative assay, we tested the reproducibility of qualitative and quantitative α-syn RT-QuIC results by analysing in parallel multiple samples in controlled conditions and assessing the α-syn seeding activity in serial CSF dilutions.

Materials and methods

If not otherwise specified, the α-syn RT-QuIC assay was performed as previously described [20], using CSF samples obtained, aliquoted and stored according to standard protocols (for details, see Supplementary Methods). The kinetics of the fluorescent curve of each positive replicate was analysed through the following parameters: 1) the time to reach the threshold (LAG, expressed in hours), 2) the maximum fluorescence peak (Imax) corresponding to the highest relative fluorescent units (RFU) reached during the 30 h test run, 3) the area under the fluorescence curve (AUC), and 4) the slope (curve rise from 10,000 RFU up to Imax, expressed as RFU/min).

The LAG, Imax, AUC, and slope were expressed as the median of the positive replicates of each sample per plate, and the variation across different plates was calculated as per cent interquartile coefficient of variation (QVC %).

Assessment of the effect of preanalytical variables

Multiple freeze-thaw cycles: A 1.5 mL aliquot of CSF was thawed for 1 h at room temperature (RT), vortexed for 10 s, split into six aliquots (#2 to 7) in 0.5 mL tubes, and frozen in dry ice. Aliquots #3 to 7 were thawed and frozen in dry ice for the second freeze and thaw cycle. The procedure was repeated to complete six freeze-thaw cycles. Six processed aliquots and a never-thawed one (#1) were thawed on the day of the analysis. Aliquots #1 to 7 were loaded in the same RT-QuIC plate and run on the same plate reader to allow intra-experiment comparison between freeze-thaw cycles.

Delayed (at RT or +4 °C) freezing of freshly collected CSF: At CSF collection (≤30 min at RT), three 150 µL CSF aliquots were stored at +4 °C and subsequently frozen at −80 °C after 4, 8, or 24 h, respectively, while three aliquots were kept at RT and frozen at −80 °C after 4, 8, or 24 h.

Blood contamination: Each CSF sample was thawed and spiked with 1 % of whole blood from a healthy donor and then progressively diluted 1:10 up to a 0.0001 % blood concentration. To establish the level of blood contamination in the original and spiked CSF samples, we applied the Combur10-Test® strips (Roche, Switzerland), a urine reagent test strip considered adequate for detecting CSF blood contamination [21]. The test strips can distinguish between five blood contamination levels: negative (i.e., no blood contamination), 1+, 2+, 3+, and 4+, where 0, 10, 25, 50 and over 250 red blood cells per µL are expected. Among the 12 reference samples analysed, seven were negative, three were 1+ and two were 2+. Samples graded as 3+ after blood spiking had a 0.001 % blood concentration, while all samples with spiked blood levels from 1 to 0.01 % were classified as grade 4+.

Centrifugation at RT or +4 °C of freshly collected CSF: At CSF collection (≤30 min at RT), two 150 µL CSF aliquots were centrifuged at 2000×*g* for 10 min at RT or 4 °C, respectively. The supernatant (140 µL) was collected in a new 0.5 mL tube and stored at −80 °C.

Addition of non-ionic detergents to freshly collected CSF: Immediately after CSF collection (≤30 min at RT), 150 µL CSF aliquots were added with 0.05 % Tween-20, 0.05 % Triton-X, or 0.05 % NP-40, respectively, gently mixed and stored at −80 °C.

Assessment of analytical factors

Analysis of inter- and intra-plate variability and recombinant protein batch effect: To test the inter-plate variability and the α-syn batch effect, we used 14 RT-QuIC positive samples and three batches of recombinant α-syn (A, B, and C). For the intra-plate variability analysis, we selected 12 RT-QuIC positive samples. The same aliquot of CSF, recombinant α-syn batch B and the same optical reader were used in all experiments.

Repeatability of the number of positive replicates: To this aim, we selected 32 CSF samples showing 1/4 (n=1), 2/4 (n=9), 3/4 (n=10), and 4/4 (n=12) positive replicates at screening. CSFs were run six times in quadruplicate in consecutive experiments. Repeatability was assessed by comparing a) the number of positive replicates, b) the sum of positive replicates from two experiments (out 8 wells) (n=15 random combinations), and c) the sum of positive replicates from three experiments (out 12 wells) (n=20 random combinations).

Reliability of positive/negative case classification: To compare the reliability of the dichotomic (positive/negative) RT-QuIC result in repetitive analyses we used the same 32 CSFs selected for the

analysis of positive replicates, considering the following criteria for positivity: a) at least 2 of 4 positive replicates according to the original protocol [22]; b) at least 3 of 8 positive replicates; c) at least 4 of 12 positive replicates.

Quantitative assessment of seeding activity: Using CSFs run six times consecutively, we evaluated the association between the number of positive replicates and the kinetic parameters. In a subgroup of cases (see Supplementary Methods), we also analyzed serial samples dilutions prepared using a CSF from a patient with normal pressure hydrocephalus who tested negative by α-syn RT-QuIC. We ran each "dilution" in octuplicates and stopped the dilution series when the α-syn RT-QuIC yielded less than 3 (out of 8 loaded wells) positive replicates.

Evaluation of the reliability of the "negative" RT-QuIC results in replication experiments: To this aim we selected 50 CSF samples from individuals without LB pathology at autopsy and 50 CSF samples from patients clinically diagnosed with parkinsonism who tested negative (i.e., 0/4 positive wells) at screening.

Statistical analysis

Statistical analysis was performed on raw data. A two-sided $p<0.05$ was considered to indicate statistical significance. Analyses were performed using GraphPad Prism 9 (GraphPad Software, USA) and SPSS 21 (IBM, USA). The LAG, Imax, AUC and slope were compared using the Friedman test (non-parametric for multiple matched groups) or the Kruskal–Wallis test (non-parametric for multiple not-paired groups) with post hoc Dunn's multiple comparison test. The Wilcoxon matched-pairs signed rank test was performed to compare kinetic parameters' inter- and intra-plate variability. The intraclass correlation coefficient (ICC) was used to assess the repeatability of positive replicates, while Fleiss' kappa was used for the reliability of dichotomic (positive/negative) results across multiple experiments.

Results

Variability of kinetics parameters of fluorescence curves in standard conditions

First we analysed the variability of the RT-QuIC kinetic parameters LAG, Imax, AUC, and slope in standard analytical conditions (i.e., protocol as in [20]). To this aim we used three positive control samples run in 72 plates, using 17 different α-syn recombinant batches and 3 plate readers (Fluostar OMEGA, BMG, Germany). The LAG QVC % was below 10 %, whereas the QVC % of Imax, AUC and slope were between 10 and 20 % (Figure 1). There was a significant difference (p=0.0395) between the LAG and AUC QVC % values.

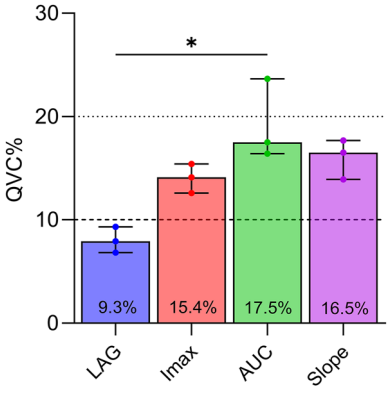

Figure 1: Variability of kinetic parameters of fluorescence curves in 72 consecutive experiments. The graph shows the QVC % values of four RT-QuIC kinetics parameters across 72 experiments (i.e., plates) performed with the standard assay protocol [20], 17 different α-syn recombinant batches and three plate readers (Fluostar OMEGA). For each kinetic variable the median value of the positive replicates was used to calculate the QVC %. Bars indicate the median and interquartile range (the raw percentage refers to the median QVC % of the three controls). QVC, quartile variation coefficient; LAG, time to the threshold; Imax, maximum intensity; AUC, area under the curve. *p≤0.05.

Assessment of the effect of preanalytical variables

To test the effect of repeated thawing and freezing, we run aliquots of 11 CSF samples subjected to up to seven freeze/thaw cycles. The treatment did not significantly affect the assay's results, as all samples remained consistently positive (n=7) or negative (n=4), and showed no significant variations in the kinetic parameters of the fluorescence curves (Figure 2A–C).

We also evaluated the effects of delayed freezing by keeping the CSF samples (n=12) at 4 °C or RT for 4, 8 and 24 h. Delayed freezing at 4 °C for 4 h prolonged the LAG compared to non-treated samples (Figure 2D), indicating a slower reaction. We observed the same trend in the delayed freezing at 4 °C for 8 and 24 h. In contrast, the delayed freezing at RT for up to 24 h did not affect the kinetic parameters (Figure 2D–F).

Next, we tested the effect of blood contamination using serial tenfold dilutions of blood in CSF (n=12) up to a concentration of 0.0001 %. We found that at 1 % and 0.1 % blood concentrations, the α-syn RT-QuIC reaction was completely inhibited. Differently, the blood concentration of 0.01 % did not inhibit the seeding reaction but affected the kinetics of the response by significantly reducing the Imax, AUC and slope compared to the reference samples and those with a 0.0001 % blood

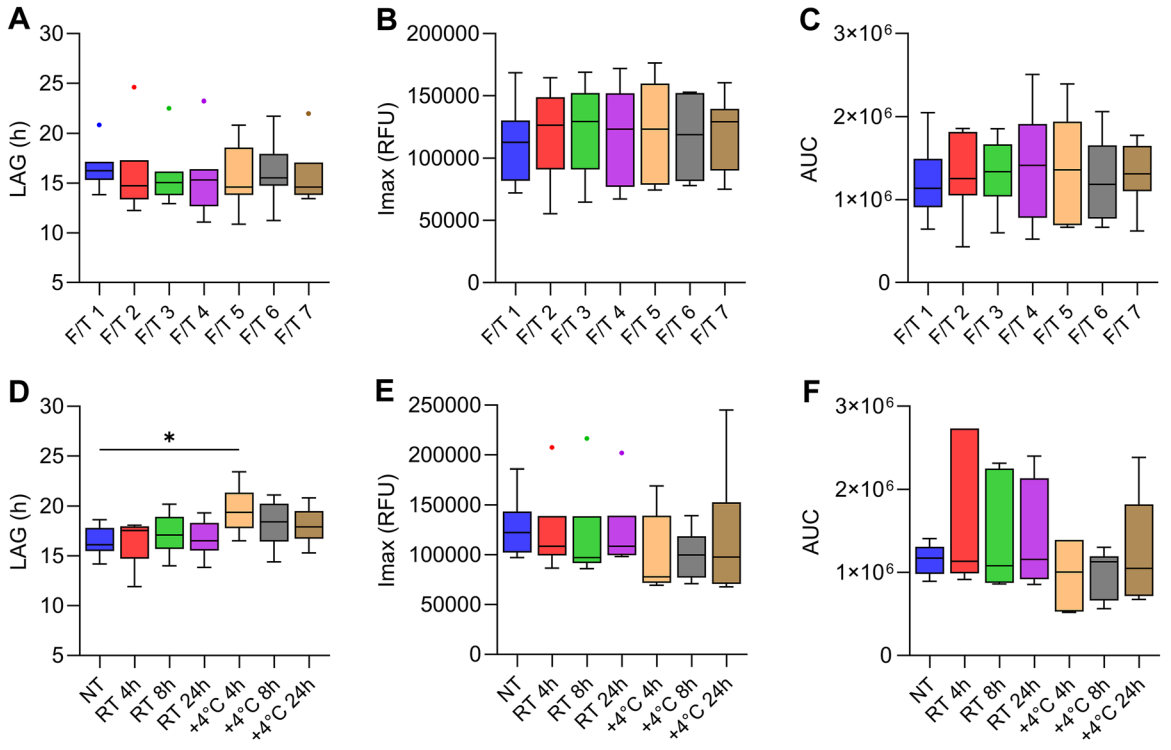

Figure 2: Effect of multiple freeze-thaw cycles and delayed freezing on RT-QuIC kinetic parameters. Seven RT-QuIC positive and 4 RT-QuIC negative CSF samples were run seven times each after 1 to 7 freeze/thaw cycles and seven additional times with no or delayed (4, 8, and 24 h) freezing at RT or 4 °C. Panels (A–C) show the effect of the treatment on the LAG, Imax and AUC, panels (D–F) the effect of delayed freezing. Bars indicate the median and interquartile range. LAG, time to the threshold; Imax, maximum intensity; AUC, area under the curve; RFU, relative fluoresce units; F/T, freeze-thaw cycle; NT, not treated; RT, room temperature. *p≤0.05.

contamination (or no contamination) (Figure 3B and C, Supplementary Figure S1). Accordingly, the LAG showed a slight trend towards higher values in the 0.01 %-contaminated group, confirming the overall lower efficiency of the seeding reaction (Figure 3A). Blood contamination did not affect the fluorescence readings generated by negative samples.

The comparison of the kinetic parameters between non-centrifuged and centrifuged CSF revealed a tendency to a shorter LAG and higher Imax in the former group, with differences reaching statistical significance for samples centrifuged at 4 °C (Figure 3D–F). Negative samples remained unmodified (data not shown).

Adding non-ionic detergents to freshly collected CSF (n=12) resulted in a shorter LAG, a lower Imax, and a prolonged plateau phase (as demonstrated by the higher AUC), indicating a profound alteration of the reaction kinetics (Figure 3G–I). Notably, adding non-ionic detergents also affected the kinetics of the negative samples, resulting in “false” positive curves (Supplementary Figure S2).

Assessment of the effect of intra- and inter-assay variability and recombinant protein batch

To test the intra-assay variability of kinetic parameters (as median QVC %), we ran 12 CSF samples three times each in the same RT-QuIC plate using the same reaction conditions. The QVC % was 13.6 % (IQR 10.6–18.2 %) for the LAG, 11.9 % (9.9–16.6 %) for the Imax, 22.9 % (15.5–29.6 %) for the AUC, and 16.3 % (11.5–21.7 %) for the slope (Figure 4A–C, Supplementary Figures S1 and S3). Next, we tested the interassay variability by running the same samples thrice on different plates. The results showed that only the LAG QVC % remained below 20 %, indicating lower robustness of the other parameters when comparing samples analysed in different plates, even using the same recombinant protein batch.

For the batch effect analysis, we compared three different batches of recombinant α-syn (A, B, and C). The LAG QVC % was <20 %, with no significant difference among the three batches (Figure 4D). In contrast, the QVC % of Imax

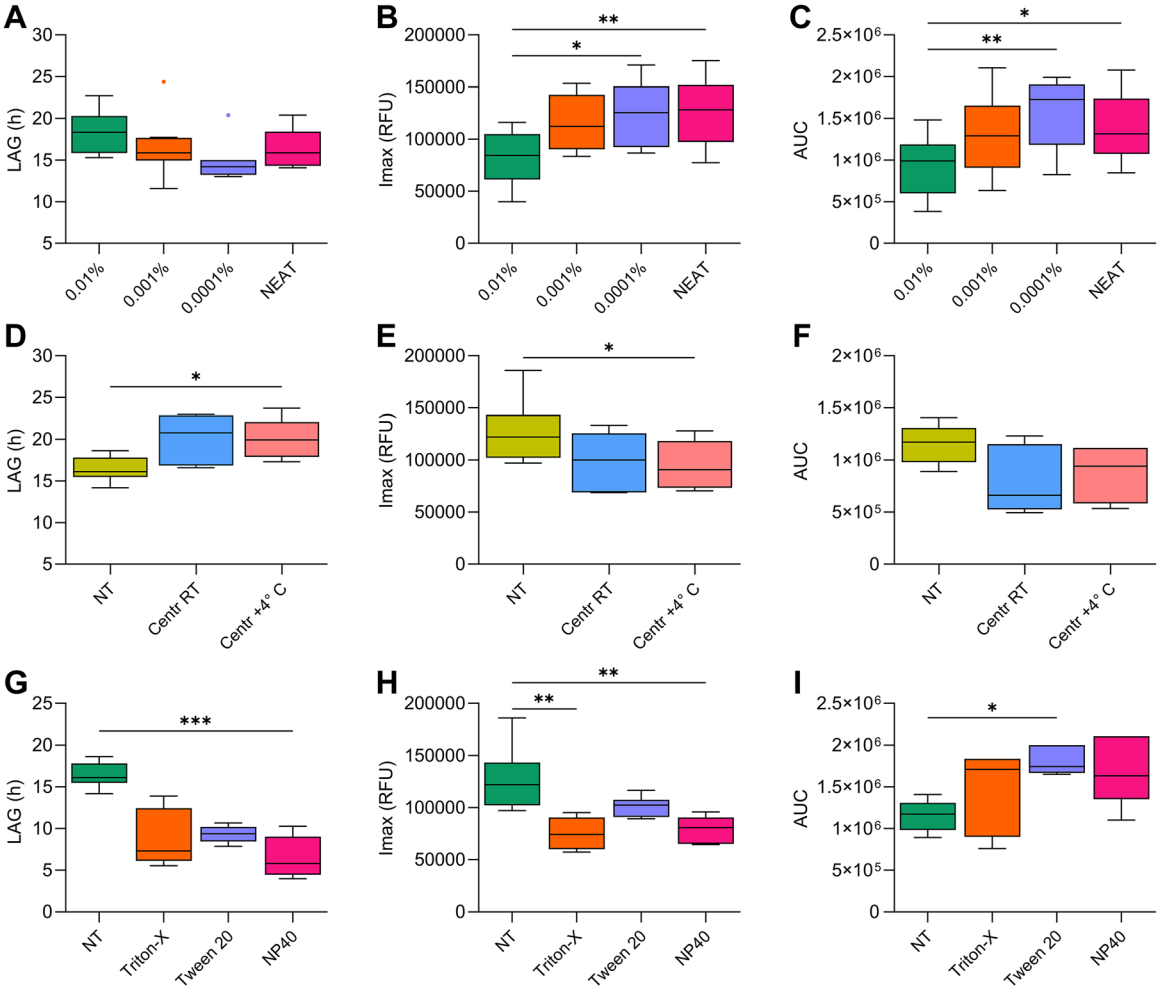

Figure 3: Effect of blood contamination, centrifugation, and addition of non-ionic detergents on RT-QuIC kinetic parameters. Twelve CSF samples (8 positive and 4 negative by RT-QuIC) were (1) spiked with 1 % of whole blood from a healthy donor and tenfold serially diluted up to a 0.0001 % blood concentration, (2) centrifuged at 2000×*g* for 10 min at RT or +4 °C, and (3) added with 0.05 % Tween-20, 0.05 % Triton-X, or 0.05 % NP-40, respectively. Panels (A–C) show the effect of blood contamination on the LAG, Imax and AUC, panels (D–F) the effect of delayed freezing, and panels (G–I) the effect of detergent addition. Bars indicate the median and interquartile range. LAG, time to the threshold; Imax, maximum intensity; AUC, area under the curve; RFU, relative fluoresce units; NEAT, not contaminated; NT, not treated; Centr, centrifugation; RT, room temperature. *p≤0.05, **p≤0.01, ***p≤0.001.

showed substantial variability between batches A and C. Still, the inter-batch QVC % was below 20 % (Figure 4E). Finally, despite the non-significant variation between batches, the QVC % was significantly higher for both AUC and slope (Figure 4F, Supplementary Figure S1).

Repeatability of the number of positive replicates

To test the reliability of the parameter "number of positive replicates" we used 32 CSFs run six times in quadruplicate (12 plates, 4 batches). An ICC of 0.658 revealed only moderate reliability of single-run (i.e., 4 wells) comparisons. However, the ICC value increased to 0.853 and 0.922 (excellent reliability) when we randomly sum up the number of positive replicates obtained in two (8 wells) and three (12 wells) experiments.

Reliability of positive/negative case classification

Next, we evaluated the test-retest reliability of the positive/negative outcome according to the original RT-QuIC protocol developed for the diagnosis of Creutzfeldt–Jakob disease [22], which considers the CSF sample as "positive" when at least 2 of 4 replicates cross the threshold (i.e., 2/4, 3/4, 4/4).

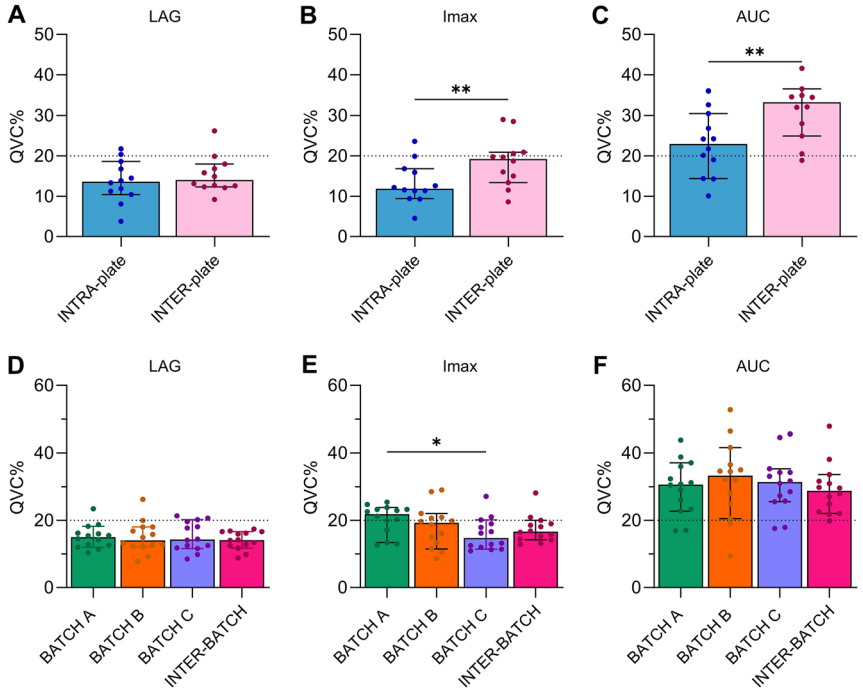

Figure 4: Analysis of intra- and inter-plate variability, and batch effect. Twelve CSF samples were run three times each in the same RT-QuIC plate or in different plates using the same reaction conditions. The same 12 samples were also run with three different batches of recombinant α-syn (in the same plate). Panels (A–C) show the absolute QVC % values for the LAG, Imax, and AUC in the intra-plate and inter-plate conditions. Panels (D–F) show the QVC % values for the kinetic parameters in the intra-batch and inter-batch conditions. Bars indicate the median and interquartile range. LAG, time to the threshold; Imax, maximum intensity; AUC, area under the curve; QVC, quartile variation coefficient. *p≤0.05, **p≤0.01.

The analysis showed a fair agreement for result classification (Fleiss' kappa=0.371). However, the consensus on the final classification significantly improved considering the sum of positive replicates from two (8 replicates) and three (12 replicates) experiments in all random combinations (Fleiss' kappa=0.796 using the criterion of at least 3 of 8 positive replicates, and Fleiss' kappa=0.949 using the criterion at least 4 of 12 positive replicates).

Quantitative assessment of seeding activity

Based on preliminary results [19] showing a positive association between the RT-QuIC parameters "number of positive replicates" and LAG and the LB pathology load evaluated neuropathologically, we compared the median values of kinetic parameters between samples grouped according to the number of positive replicates. To this aim, we considered either all positive replicates (i.e., 2, 3 or 4) or the first two positive replicates crossing the threshold. The latter criterion may allow a more even comparison among samples, given that those showing "negative" replicates may theoretically show late positive curves that are excluded due to the 25 h cut-off. While the former analysis did not show significant differences between groups (Figure 5A and B), the criterion of the first two positive replicates revealed a positive association between the number of positive replicates and a shorter LAG and higher Imax (Figure 5, boxes), AUC, and slope (data not shown) indicating a more efficient seeding reaction in the CSFs with 3 of 4 positive replicates and especially in those 4/4.

The relationship between the number of positive replicates and kinetic parameters was even more evident using the sum of two (8 replicates) or three (12 replicates) experiments, and the differences were further accentuated by selecting the three (when 3/8 is the proposed cut-off of positivity) or four (when 4/12 is the proposed cut-off of positivity) kinetic curves with the shorter LAG, respectively (Supplementary Figures S4 and S5).

Regarding the dilution experiments, CSF samples from "high" seeders showed a significantly higher percentage of positive curves than those of "low" seeders at each dilution (Figure 5C). A trend toward a longer LAG and lower Imax in the low seeding group was also evident, particularly by selecting the three kinetic curves (of 8) with shorter LAG (Supplementary Figure S6). Finally, an arbitrary score based on the number of positive replicates (npr) across the serial dilutions with an incremental correction factor at each dilution (neat=npr*1; dilution 1:1=npr*1.5; dilution 1:3=npr*2; dilution 1:7=npr*2.5) clearly distinguished the "high" and "low" seeding groups (Figure 5D).

Effect of repeated analysis on the assay accuracy of negative readouts

To test the reproducibility of the negative assay readouts, first we tested CSF samples from 50 Lewy body disease

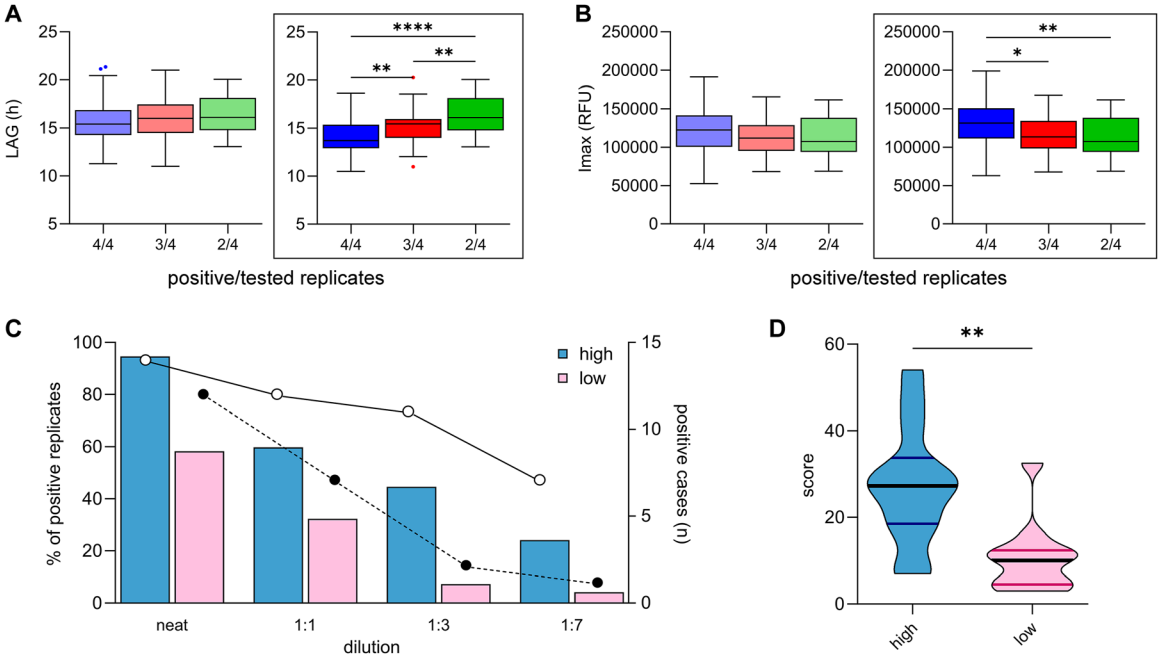

Figure 5: Evaluation of LAG and Imax according to the number of positive replicates, and effect of serial CSF dilutions. (A, B) Comparison of LAG (A) and Imax (B) values between samples showing 4, 3 or 2 positive replicates, based on the analysis of 32 CSFs each run 6 times in quadruplicate. The second (framed) graph in each panel shows the values obtained considering only the first two positive fluorescent curves for each positive sample independently of the total number of positive replicates (2, 3, or 4). (C) Effect of serial 1:2 CSF dilutions on the number and percentage of positive replicates. "High" and "low" seeders are compared. (D) Comparison of "high" and "low" seeders based on an arbitrary score based on the number of positive replicates (npr) across the serial dilutions with an incremental correction factor at each dilution (neat=npr*1; dilution 1:1=npr*1.5; dilution 1:3=npr*2; dilution 1:7=npr*2.5). LAG, time to the threshold; Imax, maximum intensity; AUC, area under the curve; RFU, relative fluoresce units. *p≤0.05, **p≤0.01, ****p≤0.0001.

Table 1: Accuracy of α-syn RT-QuIC in neuropathologic controls negative for LB pathology and individuals with parkinsonism negative at screening, according to the number of tested replicates.

	n	Criterion 1 (positive=2 out of 4 wells)	Criterion 2 (positive=3 out of 8 wells)	Criterion 3 (positive=4 out of 12 wells)
Neuropathologically confirmed α-syn –, negative/tested, %	50	148/150 (98.7)	100/100 (100)	50/50 (100)
Clinically diagnosed parkinsonisms, negative/tested, %	50	144/150 (96.0)	98/100 (98.0)	48/50 (96.0)
Parkinson's disease	20	56/60 (93.3)	38/40 (95.0)	18/20 (90.0)
Multiple system atrophy-parkinsonian subtype	10	30/30 (100)	20/20 (100)	10/10 (100)
Progressive supranuclear palsy	10	29/30 (96.7)	20/20 (100)	10/10 (100)
Others[a]	10	29/30 (96.7)	20/20 (100)	10/10 (100)

[a]Include Parkinsonian syndromes due to vascular, iatrogenic, or other neurodegenerative disorders. α-syn, alpha-synuclein.

(LBD)-negative individuals three times (i.e., 150 analyses). One hundred thirty-two (88.0 %) CSF samples gave 0/4, 16 (10.7 %) 1/4, and only 2 (1.3 %) 2/4. The 2 CSF samples that tested 2/4 once showed 0/4 in the other two analyses (Table 1). Next, we re-analysed 50 individuals diagnosed with parkinsonism who tested negative at the first RT-QuIC, representing a group enriched for potential false negative results. In the second run, 119 (79.3 %) samples gave 0/4, 25 (16.7 %) 1/4, 5 (3.3 %) 2/4, and 1 (0.7 %) 3/4. Notably, 4 of 6 samples showing at least one 2/4 or a 3/4 on test repetition had a clinical diagnosis of Parkinson's disease (Table 1). Overall, these results indicate that increasing the number of replicates to 8 slightly improve the assay accuracy.

Discussion

In the present work, we investigated the effect of several preanalytical and analytical variables on α-syn RT-QuIC outcome. Moreover, we evaluated the impact of the number of tested replicates and serial dilution on the assay's reliability and reproducibility.

We found that standard centrifugation protocols at RT, repeated freeze-thaw cycles, delayed freezing at RT, and blood contamination up to 0.001 % have no significant effect on the kinetic parameters of the reaction and the number of positive replicates. Remarkably, kinetic parameters were not modified by freeze-thaw up to 7 cycles, not confirming the initial evidence of a delayed Lag in CSFs undergoing five or more freeze-thaw cycles [23].

Regarding CSF handling and preservation before aliquoting, samples centrifuged and temporarily stored at +4 °C instead of RT showed a less efficient kinetics of seeding reaction (i.e., increased LAG and lower Imax), which supports the leading standard operating procedure of processing and aliquoting CSF rapidly at RT [24].

Concerning the well-known negative effect of blood contamination [23, 25, 26], we found an association between its extent and the RT-QuIC readouts. Blood contamination up to 0.001 % did not affect RT-QuIC kinetics; samples spiked with 0.01 % of blood showed a longer LAG and lower Imax and AUC, while blood concentrations greater than 0.1 % fully inhibited the reaction. Given that the blood contamination affecting the assay kinetics is not necessarily recognized at visual inspection, we recommend ruling out blood contamination through cytological routine assessment and, as already suggested for biomarker studies, avoiding using the first 2 mL of CSF collected where contamination most frequently occurs.

Non-ionic detergents, such as Triton-X, Tween 20, and NP-40, dramatically impacted the reaction's kinetic and fostered false positive results, thus negatively affecting the assay specificity. Their addition, likely promoting a self-aggregation of the recombinant α-syn substrate, also determines a shorter Lag (faster reaction) combined with a lower exponential increase of fluorescence signal (lower Imax) and a prolonged plateau phase.

The RT-QuIC protocols currently available assess the seeding activity of each CSF sample in a variable number of wells and use different cut-offs regarding the number of positive replicates to define assay positivity. Despite these differences, two ring trials showed an overall high agreement on the assay accuracy in distinguishing positive vs. negative samples [27, 28]. In the present study, we ran the CSF samples in quadruplicate following the standard protocol of the prion RT-QuIC [22]. Notably, we remarkably increased the repeatability of the total number of positive replicates by testing each CSF sample quadruplicate two or three times. However, the definition of positivity cut-off based on 8 or 12 wells increased the reliability of the final positive/negative case classification only slightly. As a positive effect, however, developing a classification based on 8 or 12 wells instead of 4 eliminated the "unclear" (i.e., 1/4) category that intrinsically reduces reliability.

Regarding the critical issue of the assay's potential to estimate the amount of α-syn seeding activity in the sample, our results show that the number of positive replicates and the LAG are the preferred variables for this purpose, given their higher stability over other RT-QuIC kinetics parameters. In particular, the LAG showed the lowest variability in the inter-plate and inter-batch assessments. We also found a trend toward higher Imax in CSFs showing a shorter LAG. However, the Imax, despite the good intraplate QVC %, showed significant variability in the inter-plate assessment, making this parameter less suitable for large studies requiring multiple plates and recombinant protein batches for analyses.

Preliminary evidence indicates that both the number of positive replicates and LAG are closely related to the α-syn burden in the CNS [10, 19]. Therefore, we tested the capacity of the RT-QuIC to identify cases with high or low seeding activity. Our results show that comparing the kinetics parameters using the same number of replicates across α-syn RT-QuIC positive cases (i.e., the first two curves crossing the threshold) provides a much better correlation between kinetic parameter values and the total number of positive replicates. Moreover, we found that the number of positive replicates in non-diluted CSF samples is positively associated with the likelihood of positive RT-QuIC results at increasing sample dilutions, further supporting the correlation between the number of positive replicates and α-syn seed concentration in CSF. Based on these results, we generated a new score accounting for the overall number of positive replicates at different dilutions that discriminates between "high" and "low" seeding samples. Further work is needed to explore whether the proposed score is a reliable quantitative tool for estimating CSF α-syn seed concentrations in neuropathological and clinical LBD cohorts.

In summary, our results indicate that the α-syn RT-QuIC is a rather robust SAA regarding the effect of preanalytical variables since only blood contamination and adding detergent may significantly influence the outcome. Regarding the potential use of the SAA beyond the dichotomic outcome, we identified the number of positive replicates and the LAG evaluated in serially diluted samples as the two most reliable

variables for this scope, laying the groundwork for developing the RT-QuIC further to a quantitative test.

Research ethics: Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013) and has been approved by the local Ethics Committee (approval number AVEC:18025, 113/2018/OSS/AUSLBO).

Informed consent: Informed consent was obtained from all individuals included in this study.

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