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Testing inhibitors of α -synuclein amyloid fibril growth in a ThT-based kinetic assay

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Protocol status: Working

This protocol was systematically used and tested in several works

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Abstract

This protocol is designed to determine activity of inhibitors of α -synuclein amyloid fibril growth. It is based on the measurements of the fibrillization rate in a seeded assay in a plate reader using fluorescence signal of ThT. The protocol was optimized protein- and peptide-based inhibitors with activity 5 nM – 5 μ M, and using of 384-well plate. Duration of the test is about 2 days.

Materials

Devices

Plate reader capable to read fluorescence from a bottom, perform shaking, and maintain temperature of 37°C (Tecan Spark or analogue).

Black 384-well plates with transparent bottom ("Nunc", Thermo Fisher Scientific, ref# 242764). Plate seals (ideally silicone).

Bath sonicator (for preparation of "seeds" – fragmented fibrils).

Pipets: 1000, 200, 100, 20, 2 μ L (it is better to test precision of pipets using balances before measurements to avoid problems)

Vortex

Reagents

Pre-formed amyloid fibrils (~200 μ L of 50 μ M solution)

Solution of α -synuclein in a low-salt buffer (9600 μ L of 100 μ M solution per plate or 1200 μ L per inhibitor)

Solution of inhibitors: approximately 50 μ L of 20 μ M and 50 μ L of 2 μ M

"2x Buffer": 12 mM pH 7.2 Na-PO₄ buffer containing 280 mM NaCl, 20 mM NaN₃, 2 mM EDTA, and 10 μ M ThT (to yield 6 mM pH 7.2 Na-PO₄ buffer containing 140 mM NaCl, 10 mM NaN₃, 1 mM EDTA, and 5 μ M ThT after dilution i.e. "1x Buffer")

Milli-Q water.

Troubleshooting



Safety warnings

- ⚠ α -Synuclein amyloid fibrils are capable of autocatalytic replication by prion-like mechanism. All manipulations before plate sealing should be performed in an isolated area. Sealed plates after experiment have to be decontaminated before disposal.

Preparation

1 **Preparation of seeds.**

Prepare 200 μL of 50 μM solution of pre-formed fibrils in 1x buffer in a 1500 μL Eppendorf tube and sonicate them in a bath sonicator for 15 min.

Note: Sonication times of 5 min to 30 min will provide approximately the same results. If you

would like to use more diluted fibrils as seeds longer sonication times can be needed.

2 **Set up measurements program on plate reader** that includes:

- Temperature control: 37°C

- Fluorescence bottom reading, excitation 430 nm slits 5 nm, emission 480 nm slits 20 nm (emission slits are wider than excitation ones to reduce photodegradation of dye.

Excitation is shifted to blue comparing to the absorption maximum of the dye in order to reduce light scattering signal). Optimally, the gain should be set up to the value at which 50 μM α -synuclein fibrils yield signal 20-50% of the maximal device range. (For Tecan Spark with 32-bit ADC it corresponds to signal levels of 10000-30000.)

- Kinetic measurements, 10 minutes per cycle (any value of 5 to 20 minutes should be ok also). Shaking

each cycle before measurements, 5s, preferably double orbital (this is necessary to prevent formation of clumps of fibrils at the bottom of the well and making the signal more homogeneous). Duration of measurements about 50 hours (300 cycles of 10 minutes).

*Be sure that the computer connected to plate reader is configured not to sleep/update during the measurements

3 **Pre-run testing of seeds and set-up.**

Amyloid fibrils have moderate storage stability and can degrade/aggregate over time that will lead to seeds of too low activity for efficient measurements. If activity is slightly lower than expected the problem can be solved by adding higher amount. However, when activity is >10x lower, it is better not to start experiment at all and use another batch of pre-formed fibrils.

3.1 Mix in Eppendorf tube

55 μL of 2x buffer,

55 μL of 100 μM α -synuclein solution

1.1 μL of 50 μM seeds solution prepared during Step 1.

Briefly vortex resulted solution and add to two wells of 384-well plate 50 μL of the formed solution.

If this is the first time new measurements conditions (gain, slits, filters) are used it is better to add to the third well 45 μL of 1x buffer and 5 μL of 50 μM seeds (control to test

what is the expected value of the signal upon 10% protein fibrillization)
(for this step is better to use partially used plate from one of previous experiments than the plate that will be used for the main measurements).

3.2 Measure the kinetics of fibrillization for 60-100 minutes.

Seeds can be considered proper if they increase the fluorescence intensity by at least 20% during one hour.

	A	B	C	D
	Value, that correspond to 50uM fibrils (measured separately)	Starting value	Value after 60 min	Decision and reason
	10 000	500	700	Good quality seeds
	10 000	500	550	Low quality seeds. Prepare new batch or increase experiment time to 100 hours and double amount of seeds added
	1000	500	505	Too low gain, and too high scattering check settings of wavelength/slits/filters
	1000	70	100	Too low gain, but otherwise nice measurements
	10 000	1000	1300	Good quality seeds, suboptimal but acceptable conditions

Examples of starting, initial and saturation values during seed test and possible interpretations

Please note that if kinetic curves obtained from two experimental wells with identical solution is different more than 30% the seeds are likely aggregated and have to be resonicated

Preparation of plate

- Testing of each inhibitor will be performed at 10 different concentrations each in 4-repeats. Together with control sample (no inhibitor) it will require 48 wells (2 rows on 384-well plate).



The goal is to make exactly the same concentration of seeds and protein in all wells.
Solution for 4 technical repeats for each concentration will be prepared in one eppendorf (4 repeats of 50 μ L per well and 20 μ L reserve volume = 220 μ L)

Prepare 12 Eppendorf tubes marked 0, 0, 5, 10, 20, 40, 80, 160, 320, 640, 1280, 2650 nM and add to each of them necessary amount of inhibitor solution starting from 2 μ M or 20 μ M inhibitor solution.

	A	B	C	D
	Final [inh], nM	V(2 μ M)	V(20 μ M)	V(water)
	0	-	-	20
	0	-	-	20
	5	0.55		19.5
	10	1.1		19
	20	2.2		18
	40	4.4		16
	80	8.8		11
	160	17.6		2.5
	320	-	3.52	16.5
	640	-	7.04	13
	1280	-	14.1	6
	2560	-	28.2	0

Amount of 2 μ M and 20 μ M inhibitor solution to add to each inhibitor samples

► Starting from this point all operations should be performed as fast as possible
(it will take ~20 min per inhibitor)

*be sure that plate reader is ready and set to 37C at this stage

Prepare 2.5 mL of the mixture containing

- 55 μ M synuclein
- 550 nM seeds
- 1.375 mL of 2x buffer



Vortex it.

(this is the time point when the fibrillization starts)

Pipette 200 μL of this mixture to each of 12 previously prepared eppendorfs and vortex each of them for $\sim 3\text{s}$ (be sure that the inhibitor solution is properly mixed with the added solution)

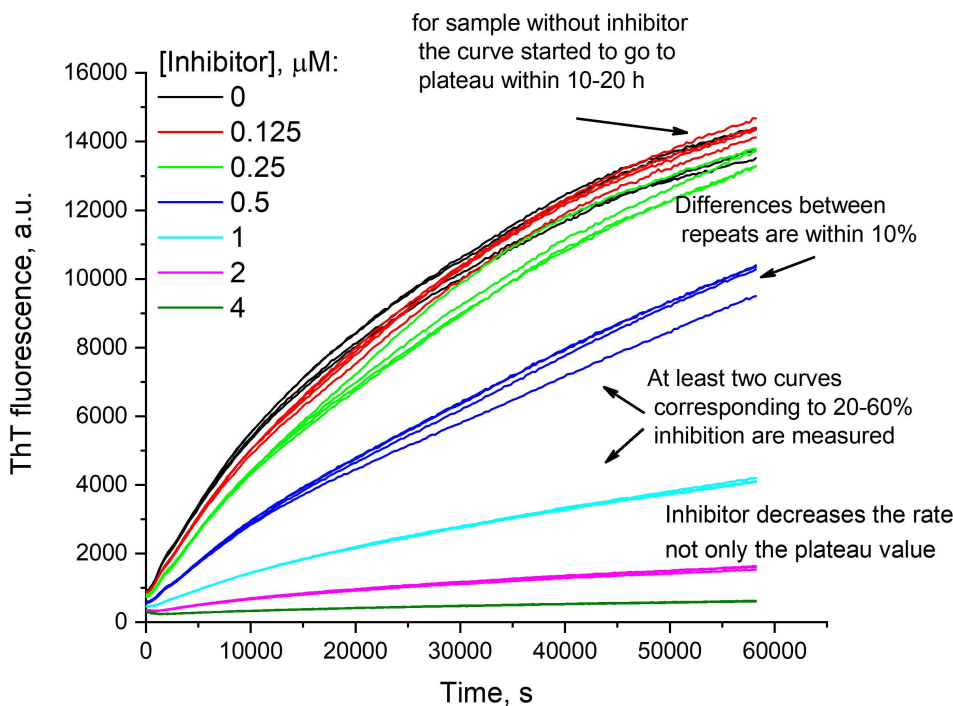
Then pipette samples from eppendorfs to the plate. 50 μL per well. Vortex each eppendorf one more time before pipetting ($\sim 2\text{s}$). Try to touch the inner wall of well when moving solution to ensure the absence of bubbles in wells.

► Repeat preparations for the next inhibitors in necessary

Seal the plate.

Kinetic measurements

- 5 Start measurement program in plate reader.
Check in $\sim 40\text{ min}$ if the measurements go as expected and samples without inhibitor show increase similar to ones observed in pre-test (Step 3)
Check the progress again in 8-16 hours. If possible save temporary back-up measurement files.

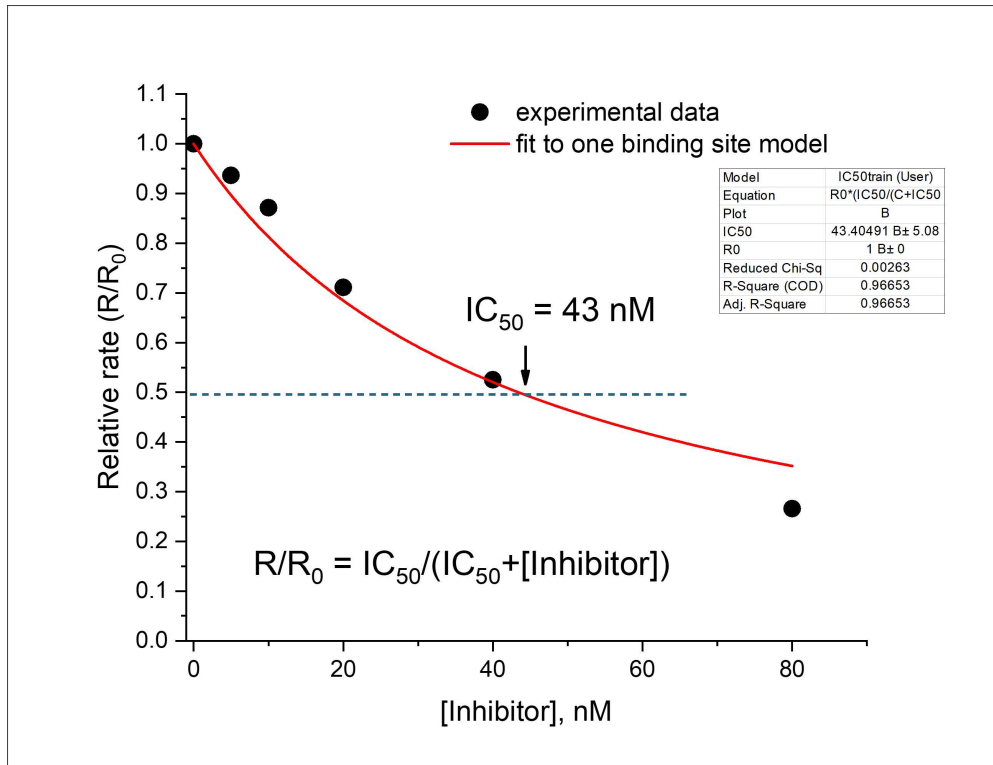


- Example of good quality measurement curves (Afitska et al, JMB 2020, doi:[10.1016/j.jmb.2019.11.019](https://doi.org/10.1016/j.jmb.2019.11.019))

Data analysis

- As the result of kinetic measurements one is expected to obtain excel file with set of kinetic curves containing about 300 time points each.
 - In many cases at the initial part of the curves (first 20-30 min) the curves would show strange behavior (decrease or slower increase of intensity than expected) it is a result of changing the temperature of the plate from room temperature to 37°C that leads to the decrease of fluorescence quantum yield of ThT. This experimental points have to be discarded.
 - Calculate the reaction rate at the beginning of the reaction for each sample using first linear part of the curves that correspond to ~30% of the total fluorescence increase. The simplest way to do this:
 - calculate averages of 3 experimental points close to the beginning of the experiment (for example 30,40,50 min = I_{40_min}) and of 3 points close to 30% conversion (for example 730,740,750 min = I_{740_min}). The difference $I_{740_min} - I_{40_min}$ for each sample will be proportional to the reaction rate (see detailed calculations in the attached example file)

- as each concentration of inhibitor was measured in 4 technical repeats take **median** of them (it is better than average because presence of bubble or aggregate can lead to outliers and their probability does not depend on the difference from the mean value)
- divide median values at each concentration to the median value for the sample without an inhibitor to obtain relative initial reaction rates (R/R_0)
- they can be analyzed graphically or fitted to an equation $R/R_0 = IC_{50}/(IC_{50}+[Inhibitor])$ that assume 1 to one interaction.



Example of IC50 calculation

- this file contain example of experimental data and detailed description of the calculation steps

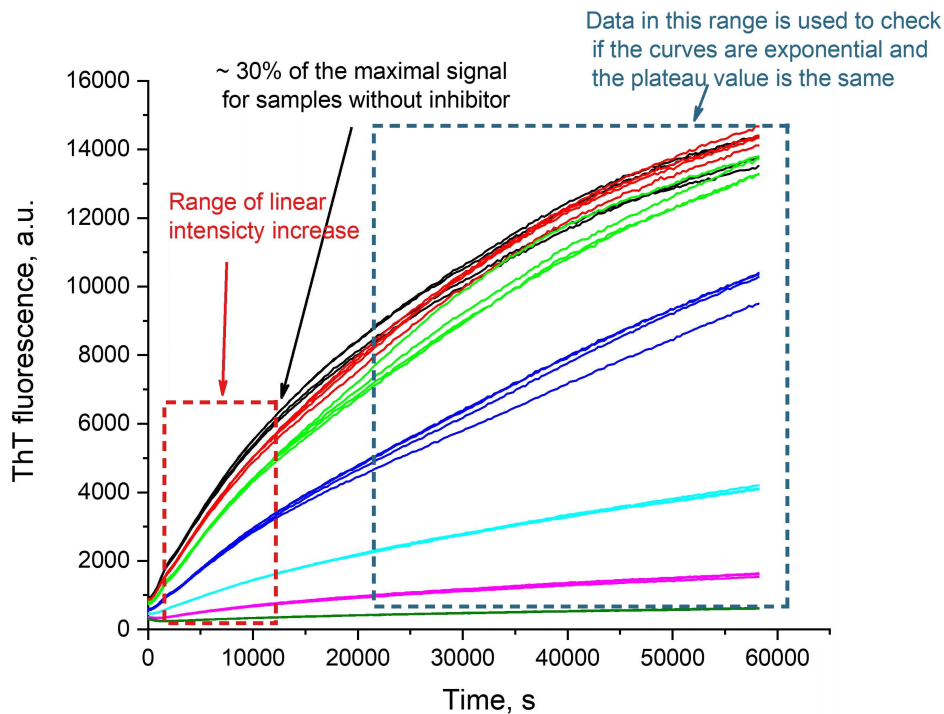


Example of output file from the dev... 254.9KB

- Critical point. For proper data interpretation it is necessary to consider also the value of plateau.

If the plateau intensity significantly decreases at high inhibitor concentrations then there is a risk that tested compound directly interacts with ThT or competes for the same binding sites that would lead to decrease of ThT signal but not to the real inhibition. In

such cases more advanced data processing protocol including fitting individual curves to monoexponential decay curves and analyzing obtained apparent rate constants is necessary ($k_{app}/k_{app_no_inh} = IC_{50}/(IC_{50}+[Inhibitor])$)



Protocol references

- Afitska, K., Priss, A., Yushchenko, D. A., Shvadchak, V. V. (2020) Structural optimization of inhibitors of α synuclein fibril growth: affinity to the fibril end as a crucial factor, *J Mol Biol* 432, 967-977.
DOI: [10.1016/j.jmb.2019.11.019](https://doi.org/10.1016/j.jmb.2019.11.019) - example of application of the protocol
- Galkin, M., Priss, A., Kyriukha, Y., and Shvadchak, V. (2024) Navigating α -Synuclein Aggregation Inhibition: Methods, Mechanisms, and Molecular Targets. *Chem. Rec.* 24, DOI: [10.1002/tcr.202300282](https://doi.org/10.1002/tcr.202300282) - this review contains chapter describing common misinterpretations of the data during measurements activities of fibril growth inhibitors in ThT-based kinetic assays