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Protocol status: Working
We use this protocol and it's working

Created: May 22, 2022

Real time-quaking induced conversion assay (RT-QUIC)

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ABSTRACT

Seeded-amplification assay (SAA) method for the detection of pathology-associated aggregation-templating competent forms of alpha-synuclein and other interacting factors.

ATTACHMENTS

RT-QUIC outline.xlsx

PROTOCOL MATERIALS

Tweezers **TEDPELLA Catalog #534** Step 7

Corning® 384-well Black/Clear Bottom Low Flange Ultra-Low Attachment Microplate Bulk Packed **Corning Catalog #4588**

Step 7

BioSpec Products 2.3 mm Zirconia/Silica Beads 1 lb bottle **Fisher Scientific Catalog #NC0451999**

Step 7

Amicon Ultra-0.5 Centrifugal Filter Unit 24 pack **Merck Millipore (EMD Millipore) Catalog #UFC505024**

Step 4

250g Guanidine hydrochloride **G-Biosciences Catalog #BC85** Step 5

Thioflavin T **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T3516** Step 9

PCR Plate Heat Seal foil piercable **Bio-Rad Laboratories Catalog #1814040**

Step 11

Last Modified: Jan 15, 2024

PROTOCOL integer ID:
63010

Keywords: ASAPCRN

Funders Acknowledgement:
Aligning Science Across Parkinson's
Grant ID: ASAP-020527

Plate preparation

30s

1 Thaw down a-syn monomer and sonicated fibril aliquots On ice, do not generate bubbles by pipetting

2 Measure monomer concentration via Nanodrop
Add 3 µL of 10x diluted aliquot in PBS onto nanodrop piedestal;
Parameters: other proteins; coefficient extinction: 5.98; MW: 14.4 kDA
Perform two measurements and confirm <10% standard error between two measurements
If necessary, prepare 20X and 30X dilutions to confirm findings.

Equipment

NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer	NAME
UV-Vis Spectrophotometer	TYPE
Thermo Scientific	BRAND
ND-ONE-W	SKU

3 Calculate the volume of monomer need following: [M] 0.3 mg/mL monomer in a reaction mix X 30 µL per well

*30 ul (reaction volume) * 3 (replicates) * ____ (different conditions) = _____ul;*

Dilute the monomer preparation with 1X PBS (Phosphate-buffered saline) to [M] 2.5 mg/mL in order to filter the protein preparation for a high efficiency

4 Use Amicon Ultra-0.5 Centrifugal Filter Unit 24 pack **Emd Millipore Catalog #UFC505024** to filter out possible aggregates or HMW contaminates

5 Meantime measure concentration of sonicated fibrils: prepare serial dilutions: 2x; 4x; 8x in [M] 3 Molarity (M) Guanidine HCL, volume: 10 µL
250g Guanidine hydrochloride **G-Biosciences Catalog #BC85**
Add 3 µL onto nanodrop piedestal;
Parameters: other proteins; coefficient extinction: 5.98; MW: 14.4 kDA
Blank against [M] 3 Molarity (M) Guanidine HCL;
Perform two measurements for each dilution and confirm <10% standard error between two measurements

Equipment

NanoDrop™ One UV-Vis Spectrophotometer	NAME
spectrophotometer	TYPE
Thermo Scientific	BRAND
ND-ONE-W	SKU
https://www.thermofisher.com/order/catalog/product/ND-ONE-W	LINK
Sample Volume (Metric): Minimum 1µL; Spectral Bandwidth: ≤1.8 nm (FWHM at Hg 254 nm); System Requirements: Windows™ 8.1 and 10, 64 bit; Voltage: 12 V (DC); Wavelength Range: 190–850 nm	SPECIFICATIONS

6 Measure DLS data for sonicated fibrils

Protocol

NAME

Dynamic Light Scattering measurements

CREATED BY

andrew.west

PREVIEW

7 Place single zirconia bead into each well of the

Corning® 384-well Black/Clear Bottom Low Flange Ultra-Low Attachment Microplate Bulk Packed **Corning Catalog #4588**

BioSpec Products 2.3 mm Zirconia/Silica Beads 1 lb bottle **Fisher Scientific Catalog #NC0451999**

Tweezers **TEDPELLA Catalog #534**

8 **Prepare serial dilutions of sonicated fibrils:**

Prepare solution: [M] 1 mg/mL measured PFFs (20 µL volume);

1. 10 ug/ml: 10 ul PFFs + 990 ul of PBS;
2. 1000 ng/ml: 10 ul PFFs + 90 ul of PBS;
3. 100 ng/ml: 10 ul PFFs + 90 ul of PBS;
4. 10 ng/ml: 10 ul PFFs + 90 ul of PBS;
5. 1 ng/ml: 10 ul PFFs + 90 ul of PBS;
6. 100 pg/ml: 10 ul PFFs + 90 ul of PBS;
7. 10 pg/ml: 10 ul PFFs + 90 ul of PBS;
8. 1 pg/ml: 10 ul PFFs + 90 ul of PBS;
9. 100 fg/ml: 10 ul PFFs + 90 ul of PBS;
10. 10 fg/ml: 10 ul PFFs + 90 ul of PBS;

9 Prepare reaction mix:

[M] 0.3 mg/mL monomer in a reaction mix + [M] 10 micromolar (μM) Thioflavin T **Sigma Aldrich Catalog #T3516** in PBS (account for 10% volume to add) = __ 30 μL reaction * triplicates * sample number (include standard curve of PFFs serial dilutions, 10 reactions in triplicates)

[total volume * 0.1% = total volume - (total volume * 0.1%)] - [volume of filtered monomer at __ mg/mL after filter] - [ThT volume] = __ volume of PBS

10 Prepare a standard curve using PFFs dilutions from go to step #8

1. 100 ng/ml: 10 ul of **#2 (from step 8)** + 90 ul of reaction mix;
2. 10 ng/ml: 10 ul of **#3 (from step 8)** + 90 ul of reaction mix;
3. 1 ng/ml: 10 ul of **#4 (from step 8)** + 90 ul of reaction mix;
4. 100 pg/ml: 10 ul of **#5 (from step 8)**+ 90 ul of reaction mix;
5. 10 pg/ml: 10 ul of **#6 (from step 8)** + 90 ul of reaction mix;
6. 1 pg/ml: 10 ul of **#7 (from step 8)** + 90 ul of reaction mix;
7. 100 fg/ml: 10 ul of **#8 (from step 8)** + 90 ul of reaction mix;
8. 10 fg/ml: 10 ul of **#9 (from step 8)** + 90 ul of reaction mix;
9. 1 fg/ml: 10 ul of **#10 (from step 8)** + 90 ul of reaction mix;
10. PBS: **10 ul of PBS** + 90 ul of reaction mix;

11 Transfer reaction to the plate: start filling the plate with a standard curve samples (seal the portion wi 30s sealing foil)

PCR Plate Heat Seal foil piercable **Bio-rad Laboratories Catalog #1814040**

Proceed the experimental samples and cover the whole plate with a sealing foil. Spin down the plate

500 x g
for 00:00:30

Equipment	
Eppendorf™ 022620572	NAME
Microcentrifuges with a plate rotor	TYPE
Eppendorf	BRAND
13-690-003	SKU

Example of the plate layout:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
		DMV										Cortex									Matrix			
A																			PFFS					
B	A1			C1			E1			A1			C1			E1			100					
C	A2			C2			E2			A2			C2			E2			10					
D	A3			C3			E3			A3			C3			E3			1					
E	A4			C4			E4			A4			C4			E4			0.1					
F	A5			C5						A5			C5						0.01					
G	A6			C6						A6			C6						0					
H	A7									A7									0					
I																			0					
J	B1			D1			F1			B1			D1			F1			0					
K	B2			D2			F2			B2			D2			F2			0					
L	B3			D3			F3			B3			D3			F3								
M	B4			D4			F4			B4			D4			F4								
N	B5									B5														
O	B6									B6														
P																								

12 Set up the program on a plate reader

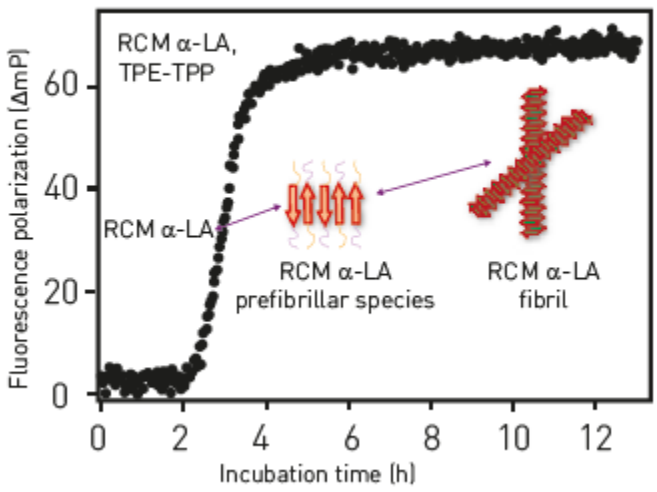
Equipment	
Omega - RT-QuIC / PRION Version - Fluorescence Base	NAME
BMG Fluostar plate reader	TYPE
BMG	BRAND
0415-102P	SKU

Run protocol

Basic settings	
Measurement type:	Fluorescence (FI)
Microplate name:	COSTAR 384
Plate mode settings	
No. of cycles:	100
Cycle time [s]:	1800
No. of flashes per well and cycle:	12
Scan mode:	orbital averaging
Scan diameter [mm]:	4
Optic settings	
Excitation:	448-10
Emission:	482-10
Gain:	1000
Shaking settings	
Shaking 1	
Shake:	between readings
Movement:	orbital shaking
Frequency [rpm]:	700
On time [s]:	60
Off time [s]:	60

13 Finish the experiment once standard curve reached the plateau
EXAMPLE: Plateau is reached at7 hours.

a



Data analysis

14 Convert collected data from plate reader into .xlsx type

EXAMPLE:

Sample X44	Sample X45	ample X67	ample X68	ample X69	ample X91	ample X92	ample X93	mple X115	mple X116	mple X117	mple X139	mple X140	mple X
B20	B21	C19	C20	C21	D19	D20	D21	E19	E20	E21	F19	F20	
417	439	227	229	228	130	260	274	244	255	295	223	221	
492	492	163	160	160	154	171	157	164	172	184	149	148	
815	752	149	147	146	135	156	153	146	159	167	132	140	
1145	1024	144	149	157	143	153	152	154	147	147	124	130	
1492	1322	145	150	154	141	142	178	139	150	163	125	135	
1890	1619	145	149	168	146	153	154	146	143	170	130	130	
2011	1875	150	153	168	150	147	138	137	139	176	128	131	
2449	2053	154	149	168	150	147	168	139	151	184	129	128	
2585	2259	153	157	176	180	142	159	140	145	184	130	128	
2782	2439	167	164	191	198	154	167	149	141	190	126	135	
2837	2595	167	158	193	247	147	160	138	135	191	134	132	
3204	2839	172	168	200	274	150	175	149	152	220	129	127	
3367	2823	183	164	198	407	144	159	137	153	202	125	132	
3324	3099	192	179	222	464	152	162	139	148	242	132	138	
3464	3136	209	186	240	504	154	177	141	148	226	131	135	
3746	3284	227	190	246	776	149	175	148	161	263	140	134	
3885	3327	232	204	260	928	151	170	150	147	217	147	139	
3901	3470	259	222	281	1144	150	188	145	146	277	156	150	
3906	3607	281	196	307	876	150	192	145	147	225	177	153	
4026	3624	300	207	337	1477	159	207	150	147	275	198	179	
4188	3888	304	235	379	1530	174	197	151	164	308	233	191	
4321	3816	346	234	414	1432	167	219	162	167	292	227	210	
4503	3883	380	266	473	2147	178	223	156	164	280	243	226	
4433	3979	443	263	516	1724	172	239	162	179	318	270	239	
4153	4011	492	268	608	2214	174	254	162	170	391	299	258	
4365	4032	507	296	667	2851	186	283	164	189	388	314	309	
4689	4218	611	314	742	2326	204	293	160	203	396	358	313	

15 The main advantage of qQuiC analysis is capability to calculate exact amount of fibril forming units in pathological samples relatively to an appropriate standard curve. The approach of calculation FFUs consists of using standard curve with a range of serial dilutions of evaluated ssFibrils spiked in original matrix of the sample. Obtained ThT FL units for each time point and dilution are needed to define an appropriate threshold value needed to define C_T values. To find a threshold value we built a program that can find an increase in the rates of changes greater than X present for fluorescence values in each replicate. The program looks at three points at a time and compares the rate of change from the first point to the second point against the second point to the third point. Once it finds the point where the rate of change increases by X present from one jump to the next the program stores that value to calculate the threshold value for each sample at X percent as the cutoff difference in the rates of change. The code loops through all of the replicates >30 times to check every percent threshold for X from 10% to 30% and returns the threshold value that yield the highest R-squared in a standard curve that is made from the mean C_T values for each replicate. When threshold value is found C_T value for each dilution can be determined as a time when ThT fluorescence reaches to a threshold. If R-square is higher than 0.8 than efficacy of the reaction is enough to be able to calculate FFUs. To define FFUs of experimental samples we applied an extracted equation from linear regression curve. **Code for a program https://github.com/west-lab/FibrilOptimization/blob/master/FibrilPaperCode_main.py**

This step is required to QC the reaction by evaluating the standard curve and finding the Ct values for experimental conditions

16 Use mean half-time of the reaction by analyzing the Ct values of the standard curve

17 Extract RFU values for each condition
EXAMPLE:

	Replicates		
	1	2	3
Experimental	1500	1600	1600
Control	100	200	150

18 Use the extracted data to generate a plot or a group analysis via GraphPad