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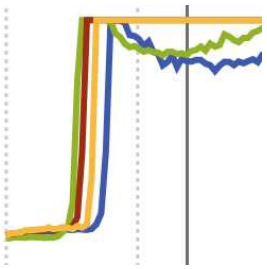
RT-QuIC Assay for the Detection of Chronic Wasting Disease in Rectal Mucosa of White-Tailed Deer



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We use this protocol and it's working

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Abstract

This protocol details a Real-Time Quaking-Induced Conversion (RT-QuIC) assay to detect the prion-seeding activity associated with Chronic Wasting Disease prions in rectal mucosa samples from white-tailed deer. The authors tested this protocol as part of an agreement between USDA-APHIS, USDA-ARS, and USGS.

Note

*The authors appreciate and acknowledge the initial critiques and refinements made to this protocol as were provided by Christina Orru, Andrew Hughson, Natália do Carmo, and Byron Caughey at the NIH/NIAID Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, Hamilton, MT.

**The protocol described herein is a unification of the general method described by Orru, et al. (2017) with additional details and modifications supported in studies reporting sensitivity for CWD in cervid species. Notable publications included:

Henderson, et al. (2015). Quantitative assessment of prion infectivity in tissues and body fluids by real-time quaking-induced conversion. *J Gen Virol, 96*(Pt 1), 210-219. doi:10.1099/vir.0.069906-0

Haley, Siepker, Walter, et al. (2016). Antemortem Detection of Chronic Wasting Disease Prions in Nasal Brush Collections and Rectal Biopsy Specimens from White-Tailed Deer by Real-Time Quaking-Induced Conversion. *J Clin Microbiol, 54*(4), 1108-1116. doi:10.1128/JCM.02699-15

Haley, Siepker, Hoon-Hanks, et al. (2016). Seeded Amplification of Chronic Wasting Disease Prions in Nasal Brushings and Recto-anal Mucosa-Associated Lymphoid Tissues from Elk by Real-Time Quaking-Induced Conversion. *J Clin Microbiol, 54*(4), 1117-1126. doi:10.1128/JCM.02700-15

Orru, et al.. (2017). RT-QuIC Assays for Prion Disease Detection and Diagnostics. *Methods Mol Biol, 1658*, 185-203. doi:10.1007/978-1-4939-7244-9_14

Haley, Donner, et al. (2020). Cross-validation of the RT-QuIC assay for the antemortem detection of chronic wasting disease in elk. *Prion, 14*(1), 47-55. doi:10.1080/19336896.2020.1716657

Haley, Henderson, et al. (2020). Management of chronic wasting disease in ranched elk: conclusions from a longitudinal three-year study. *Prion, 14*(1), 76-87. doi:10.1080/19336896.2020.1724754

Henderson, et al. (2020). Progression of chronic wasting disease in white-tailed deer analyzed by serial biopsy RT-QuIC and immunohistochemistry. *PLoS One, 15*(2), e0228327. doi:10.1371/journal.pone.0228327

Guidelines

Protocol Notes

1. SDS is included only in the Resuspension Buffer due to reports that long-term storage in the presence of SDS diminishes the seeding activity of homogenate samples (Caughey Lab, unpublished).
2. N-2 media supplement is included to buffer adsorption of seeding material to the walls of dilution vessels in diluted homogenate samples. N-2 is not included in initial homogenization buffers as the quantity of total protein in these samples far exceeds the binding capacity of the vessels.

Materials

Required Laboratory Equipment:

- Balance capable of mg accuracy
- Bead beating grinder*
- Pipettes 2 µL - 10 mL
- Centrifuge capable of 1.5 mL tubes at 21,000 x g
- Centrifuge capable of 0.2 mL tubes (single speed benchtop for PCR tubes)
- Water-bath sonicator with fittings for 0.2 mL tubes*
- Vortex mixer
- Rocking/rotating incubator compatible with 1.5 mL tubes
- RT-QuIC capable microplate reader*

Note

*Equipment models used to develop this protocol

Equipment

MP Biomedicals™ FastPrep -24™ Classic Instrument	NAME
Benchtop homogenizer	TYPE
Fisher Scientific	BRAND
12079310	SKU
https://www.fishersci.co.uk/shop/products/mp-biomedicals-fastprep-24-instrument/12079310	LINK

Equipment

Q700	NAME
Sonicator	TYPE
Qsonica	BRAND
Q700-110	SKU
https://www.sonicator.com/	LINK

Equipment

Q700 Microplate Horn	NAME
Sonicator Accessory	TYPE
Qsonica	BRAND
431MPXH	SKU

Equipment

FLUOstar Omega	NAME
Microplate Reader	TYPE
BMG Labtech	BRAND
FLUOstar Omega	SKU

Note

Optional Backup Power Supply:

This assay includes multi-day run-times with computer-controlled instruments. Consider using an uninterruptible power supply (UPS) system to avoid premature assay termination due to power outages/irregularities.

It is recommended to use a UPS that supplies continuous power through a battery to avoid interruptions due to power switching.

Example UPS:

Equipment

SMART UPS SRT 1500VA 120V	NAME
Uninterruptible Power Supply	TYPE
APC	BRAND
SRT1500XLA	SKU

Consumables:

- Single-use scalpel and cutting surface (e.g., weigh boat) for each sample
- 2 mL tubes compatible with bead beating grinder
- 0.7mm Zirconia beads **BioSpec Products Catalog #**11079107ZX
- 0.2 and 1.5 mL tubes for tissue processing and aliquoting
- 15 and 50 mL conical tubes for buffer preparation
- 0.5 mL 100 kDa molecular weight cut-off (MWCO) centrifugal filters
- 96-well optical bottom black plate **Thermo Fisher Scientific Catalog #**265301
- Nunc™ Sealing Tapes **Thermo Fisher Scientific Catalog #**232702
- Serological pipettes 5-10 mL
- Micro-pipette tips 2-1000 µL

Chemical Reagents:

- 10X PBS (Phosphate-buffered Saline) pH 7.4
- NaPTA (Sodium phosphotungstic acid)
- $MgCl_2$ (anhydrous*; m.w. = 95.21)
- SDS (Sodium dodecyl sulfate)
- N-2 Media Supplement **Gibco - Thermo Fisher Catalog #**17502048
- Na_2HPO_4 (anhydrous*, dibasic; m.w. = 142)
- $NaH_2PO_4 \cdot H2O$ (monohydrate*, monobasic; m.w. = 138)
- NaCl
- EDTA (Ethylenediaminetetraacetic acid) solution 0.5 M, pH 8
- ThT (Thioflavin T)

*specific hydration states of the listed reagents are not required; however, differences will impact the molecular weights of the reagents and change the buffer preparation calculations provided below.

Prion Protein Substrate:

- recombinant truncated Syrian hamster prion protein: Ha(90-231) $rPrP^C$
- Substrate were shipped on dry ice and stored at -80°C without being allowed to thaw

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Solutions and Buffers:

4% NaPTA Solution

	A	B	C	D
		Stock concentration	amount	Final concentration
	NaPTA	powder	2 g	4% (w/v)
	MgCl2 (m.w. = 95.21)	powder	809 mg	170 mM
	water	n/a	to 50 mL	

Resuspension Buffer

	A	B	C	D
		Stock concentration	amount	Final concentration
	PBS (pH 7.4)	10X	5 mL	1X
	SDS	powder	50 mg	0.1 % (w/v)
	N-2 media supplement (Gibco-Fisher)	100X	0.5 mL	1X
	water	n/a	to 50 mL	

RT-QuIC Assay Buffer

- prepare fresh for each assay run
- see below for stock solution recipes
- see Step 15 for $rPrP^{C}$ substrate preparation instructions

To prepare 10 mL RT-QuIC Assay Buffer, combine:

	A	B	C	D
		Stock concentration	Volume (µL)	Final concentration
	water	n/a	to 10 mL total	n/a
	NaPO4 buffer pH 7.4	100 mM	1000	10 mM
	NaCl	2 M	1500	300 mM
	EDTA	0.5 M	20	1 mM
	ThT	1 mM	100	10 µM
	Filtered rPrPC substrate	X mg/mL	1000 (µL/mL) / X	0.1 mg/mL

	A	B	C	D
			(mg/mL)	

Stock solution recipes for RT-QuIC Assay buffer:

100 mM NaPO4 buffer pH 7.4

- 3.1 g $NaH_2PO_4 \cdot H_2O$ (monobasic; m.w. = 138)
- 10.9 g Na_2HPO_4 (dibasic; m.w. = 142)
- water to 1 L

2 M NaCl

- 5.8 g NaCl (m.w. = 58.44)
- water to 50 mL

1 mM ThT

- 16 mg ThT (m.w. = 318.86)
- water to 50 mL

0.5 M EDTA

- purchased as stock solution (see above)

Filtered $rPrP^C$ substrate

- see Step 15

Sample Preparation: Rectal Mucosa Homogenization – 10% w/v

6m 15s

1 For each sample, prepare a 2-mL screw cap tube containing 1 g of 0.7 mm Zirconia beads (BioSpec 11079107zx) and label with sample/animal ID.

2 Weigh and add biopsy sample (up to 150 mg *) to each tube.

Note

*Biopsy samples larger than 150 mg may be processed in multiple pieces/tubes and homogenates pooled prior to freezing.

Note

This protocol is designed for mucosa-only samples. Skin, muscle layers, or excess connective tissue should be removed if evident.

3 Add 9 volumes 1X PBS pH 7.4 .

Note

Example: 100 mg biopsy sample + 900 µL 1X PBS

4 Homogenize using bead beating grinder.

4.1 3 cycles of 00:00:45 at speed 5.5 with 00:05:00 rest On ice between cycles.

5m 45s

5 Centrifuge at 3000 x g, 00:00:30 .

30s

5.1 Collect supernatant*, pooling tubes from larger biopsy samples if necessary.

Note

*Un-disrupted connective tissue will likely remain following homogenization; do not collect.

6 Prepare 50 µL aliquots in 0.2-mL snap cap tubes and reserve remainder in one 2-mL tube. Store at -80 °C .

Note

We suggest preparing enough 50 µL aliquots for any planned experiments plus a few extra before storing the remainder. Though not fully characterized, it is likely that excessive freeze/thaw cycling may degrade the seeding activity present in sample homogenates.

Sample Preparation: NaPTA Precipitation and Resuspension of Rectal Mucosa Homogenate

1h 31m 30s

7 Thaw a 50 µL aliquot of 10% homogenate from each sample to be tested.

8 Sonicate for 00:00:30 in a water bath sonicator at a power output of 180-200 W. 30s

Note

Sonication is performed in a 0.2 mL tube using a sonicator equipped with a microplate horn. Other sonication methods have not been evaluated.

9 Centrifuge ~ 00:01:00 in a benchtop centrifuge at ~2,000 x g (or in a single speed 0.2-mL tube centrifuge) to pellet tissue debris. 1m

9.1 Supernatant will be added to the NaPTA reaction in the next step* .

Note

*Pipette carefully to avoid disturbing the pellet.
*Ensure the supernatant is free of insoluble material before adding it to the NaPTA reaction.

10 In a 1.5-mL centrifuge tube, combine:

	A	B
	15 µL	Homogenate supernatant from Step 9
	1380 µL	1X PBS pH 7.4
	105 µL	4% NaPTA solution

11 Incubate with gentle rocking or rotation 01:00:00 at Room temperature . 1h

Note

*Tubes should be rocked/rotated in a horizontal position; adequate agitation can be visualized as the movement of the air bubble within the tube.

12 Pellet by centrifugation 21000 x g, 00:30:00 . 30m

13 Discard supernatant.*

Note

*It is important to remove the supernatant that contains NaPTA so that it does not interfere with resuspension in the subsequent step.
*We recommend using a vacuum flask with fresh 200-µL pipette tips on the suction line for each sample.
*The vacuum line should be protected from aerosol contamination following applicable regulations but minimally including an in-line High-Efficiency Particulate Air (HEPA) filter.

14 Resuspend the pellet* in 150 µL **Resuspension Buffer****.

Note

*The pellet can be difficult to resuspend. As needed to resolubilize fully, transfer initial resuspension with all undissolved material to 0.2-mL tube and alternate vortexing and sonication (00:00:30 in water bath sonicator at 180-200 W).

Note

****Resuspension Buffer** is prepared fresh. N2 media supplement contains protein components that may degrade if stored for extended periods.

rPrP^{C} Substrate Preparation

15m

15 **Substrate Preparation Instructions:** 15m

- prepare fresh for each assay run
- rPrP^{C} substrate was shipped as frozen aliquots and **filtered before use**.

To filter substrate:

1. Thaw aliquot(s) completely at room temperature.
2. Add substrate to 100 kDa centrifugal filter(s).
3. Centrifuge at 3,000 x g for 10 min or until all liquid has passed through the filter
4. Collect filtrate
5. Determine protein concentration*

Note

*A loss of approximately 10-15% of protein concentration is expected following filtration.

rPrP concentration of the substrate may be measured by absorbance at 280 nm. A mass extinction coefficient (Abs 0.1% (=1 g/L)) of 1.4 is commonly used for truncated hamster (Ha90) substrate.

Note

Do not re-freeze rPrP^{C} substrate

rPrP^{C} loses sensitivity and shows increased propensity for spontaneous misfolding over time once thawed or when subject to repeated freeze/thaw cycles.

RT-QuIC Assay

3d 13h 16m

16 Prepare **RT-QuIC Assay Buffer**

- must be prepared fresh for each assay run
- see materials for buffer recipe and Step 15 for substrate handling instructions

Note

Prepare enough **RT-QuIC Assay Buffer** to test samples in quadruplicate.

(For full 96-well plate, prepare 10 mL **RT-QuIC Assay Buffer**.)

17 Add 98 µL **RT-QuIC Assay Buffer** to each well of a 96-well optical bottom black plate (Thermo Scientific Nunc 265301).

18 Add 2 µL prepared sample from **Step 14** to each reaction well.

18.1 The following microplate layout is suggested for testing samples in quadruplicate and allows use of a 12-well multichannel pipette for reaction seeding:

	A	B
	Wells	Sample ID
	Column 1 Rows A-D	Sample 1
	Column 2 Rows A-D	Sample 2
	Column 3 Rows A-D	Sample 3
	Column 4 Rows A-D	Sample 4

	A	B
	Column 5 Rows A-D	Sample 5
	Column 6 Rows A-D	Sample 6
	Column 7 Rows A-D	Sample 7
	Column 8 Rows A-D	Sample 8
	Column 9 Rows A-D	Sample 9
	Column 10 Rows A-D	Sample 10
	Column 11 Rows A-D	Sample 11
	Column 12 Rows A-D	Sample 12
	Column 1 Rows E-H	Sample 13
	Column 2 Rows E-H	Sample 14
	Column 3 Rows E-H	Sample 15
	Column 4 Rows E-H	Sample 16
	Column 5 Rows E-H	Sample 17
	Column 6 Rows E-H	Sample 18
	Column 7 Rows E-H	Sample 19
	Column 8 Rows E-H	Sample 20
	Column 9 Rows E-H	Sample 21
	Column 10 Rows E-H	Sample 22
	Column 11 Rows E-H	Sample 23
	Column 12 Rows E-H	Sample 24

19 Seal the plate with film (Thermo Scientific Nunc 232702).

20 Insert the sealed plate into the microplate reader.

21 **Incubation and Fluorescence Measurement Conditions**:

3d 13h 59m

Temperature: 42 °C

Shaking: cycles of 700 rpm, 00:01:00 double orbital followed by 00:01:00 rest.

Measure: at 00:43:00 (or 00:15:00)* intervals: Bottom Read, 20 flashes/well.

Fluorescence: excitation: 450 ± 10 nm, emission: 480 ± 10 nm.

Gain: (manual) 1800.**

Assay length: 85:00:00

Note

*This protocol was optimized using a 43-minute measurement interval, which was the setting of an older plate reader program/script. We currently use and suggest 15-minute measurement intervals when running the protocol. This improves the estimation of baseline ThT fluorescence, which is generally used to calculate reaction threshold values. This also provides a more precise estimation of the time-to-threshold, an informative measure of reaction kinetics.

**Ideal gain settings may vary between individual plate readers and may be adjusted to allow better visualization of fluorescence curves with no impact on the assay reaction itself. A gain set too high will result in excessive baseline signal noise and/or saturated readings for positive reactions. A gain set too low may make it difficult to distinguish a positive signal from the baseline.

Export to datafile

22 For data analysis, we suggest exporting the data in a table format consisting of ThT relative fluorescence units corresponding to each Well/Sample ID at each Measurement Time.

Instructions for data export using BMG MARS software (version ####) are provided below:

22.1 Times can be recorded in hour decimal time to facilitate compatibility with downstream calculations:

1. In the MARS software, open the **Formats and Settings tab**
2. Select the **Number Format Settings button**
3. Open the **Number Formats tab**
4. Under **Global Time Format Options**, select the **middle bubble** and choose **In hours** from the drop down menu

22.2 To generate an Excel export table:

1. In the MARS software, **open the assay file**
2. Open the **Table View tab**
3. Open the **<Select a Cycle group>** drop-down **menu**
4. Select **All Cycles**

In the **top left of the test run window**, select the **Excel Export button**

1. Save the exported file in Excel