# Simplified ultrasensitive prion detection by recombinant PrP conversion with shaking

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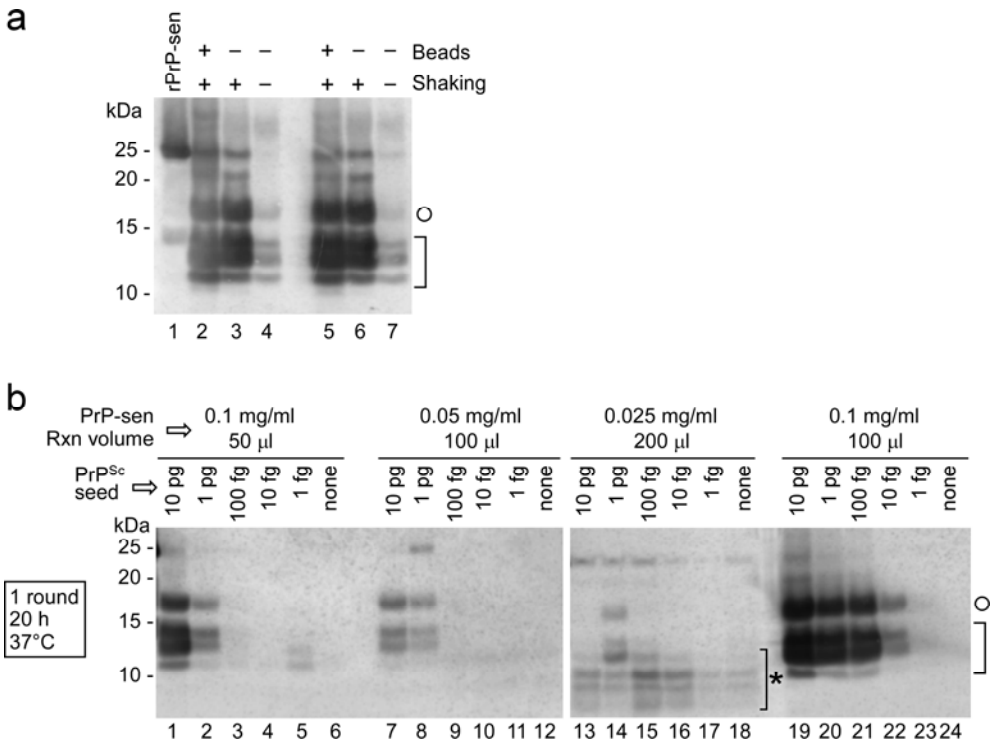
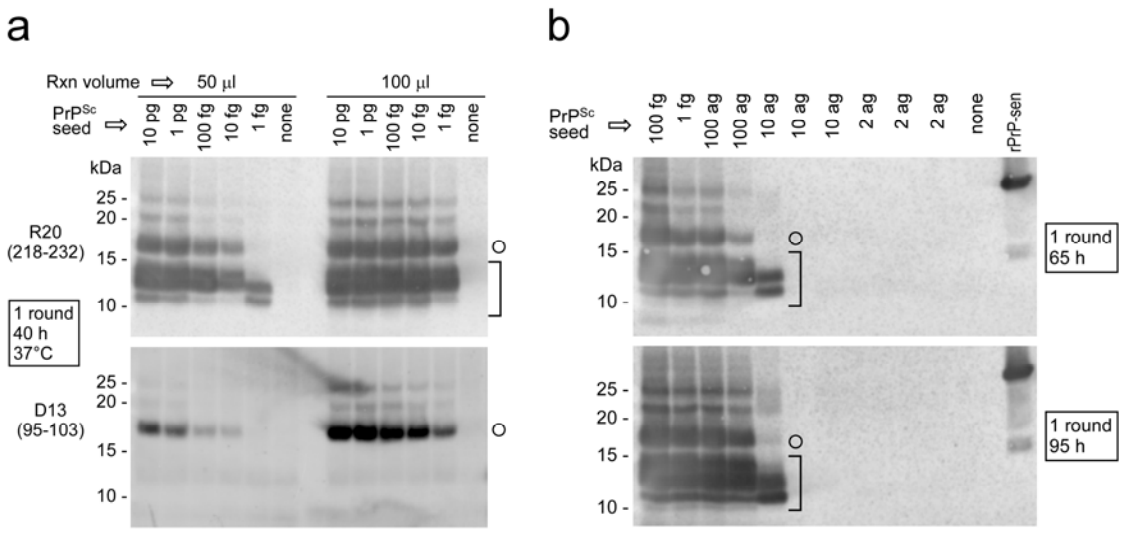
**Supplementary Results**

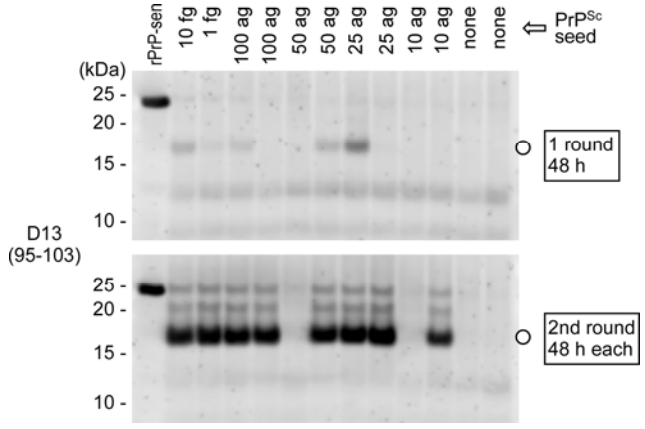
**Supplementary Methods**



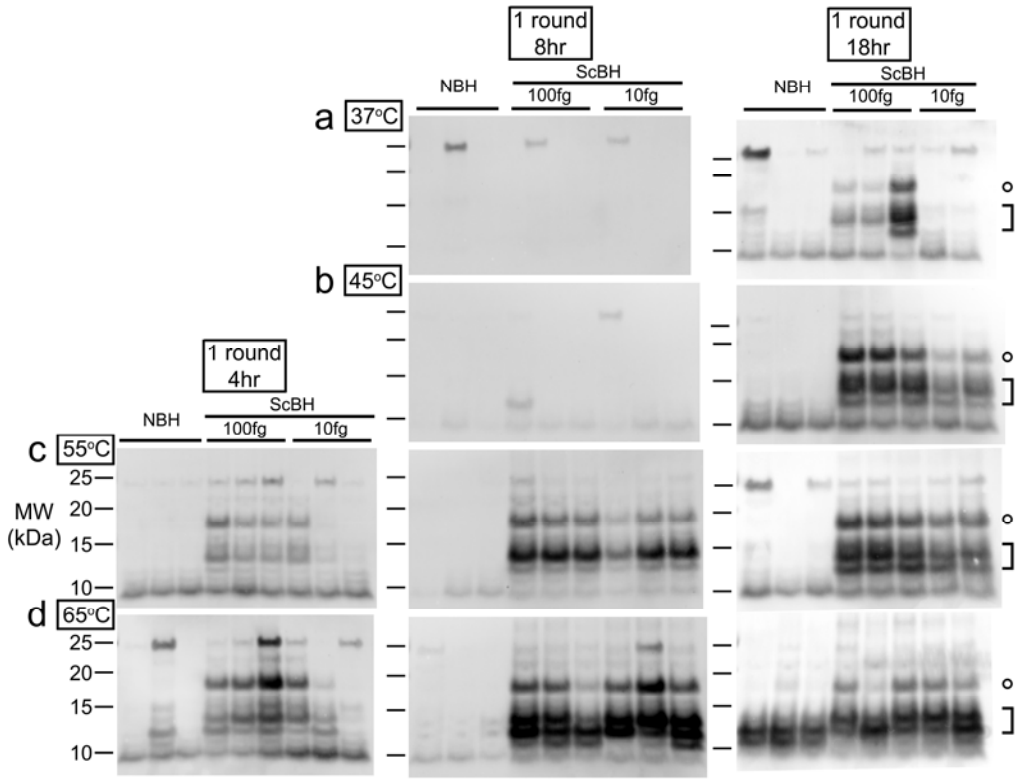

**Supplementary Figure 1. Tube shaking supports ultrasensitive prion-seeded conversions of rPrP-sen.** We used either purified $PrP^{Sc}$ (**a**) or scrapie brain homogenate (**b**) to seed the conversion of rHaPrP-sen to protease-resistant forms in QUIC reactions performed in 0.1 % sodium dodecyl sulfate (SDS) and 0.1 % Triton X-100, in PBS. PK digestions and immunoblotting of reaction aliquots were performed as described in **Supplementary Methods**. We used the C-terminal polyclonal antibody R20 in the immunoblots. Circles designate the 17-kDa rHaPrP-$res^{(Sc)}$ band and brackets designate the position of the ≤13 kDa rHaPrP-$res^{(Sc)}$ bands. (**a**) Comparison of PK-resistant QUIC reaction products from duplicate 24-h unshaken reactions and reactions shaken with or without 0.1 mm glass cell disruption beads (Scientific Industries). We seeded 50 µl reactions containing 0.1 mg/ml (4 µM) hamster rPrP-sen with 10 ng of purified hamster $PrP^{Sc}$ and subjected the tubes to cycles of 2 min of shaking and 28 min without shaking at 37 ˚C. Note enhanced rHaPrP-$res^{(Sc)}$ formation in the shaken reactions, but the lack of influence of the beads. 100 ng of rPrP-sen without PK-treatment is shown in lane 1. (**b**) 20-h QUIC reactions performed with the designated rPrP-sen concentrations, reaction volumes, and seed amounts. The seed amounts indicate the estimated quantity of $PrP^{Sc}$ added in 2-µl aliquots of scrapie brain homogenate diluted in 1 % normal brain homogenate. Lanes 6, 12, 18, and 24 received aliquots of only 1% normal brain homogenate. We subjected the tubes to cycles of 10 s of shaking and 110 s without shaking. The asterisk marks the position of rHaPrP-$res^{(spon)}$ bands.



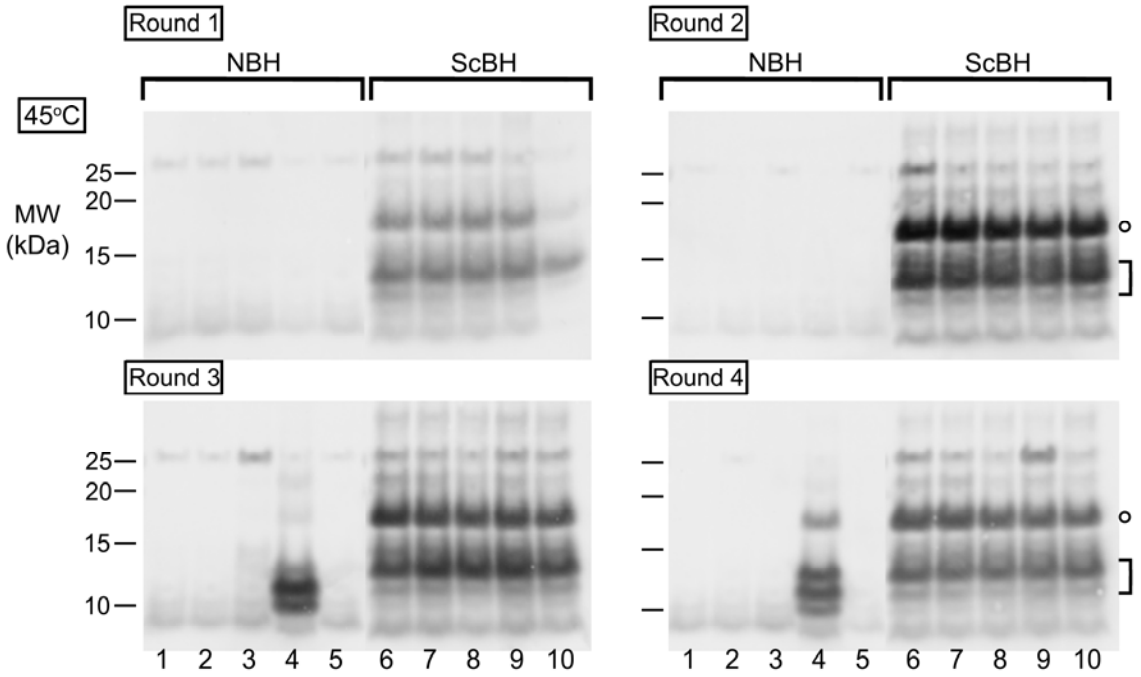

**Supplementary Figure 2. Extended reactions can enhance QUIC sensitivity to small amounts of scrapie brain homogenate seed.** (**a**) We performed 40-h QUIC reactions with 0.1 mg/ml rPrP-sen and the designated reaction volumes and seed amounts using the shaking cycle and buffer conditions described for **Supplementary Fig. 1b**. The upper and lower panels show immunoblots performed using antibody R20 and D13, respectively (PrP epitope residues shown in parentheses). (**b**) We performed 65-h (upper blot) and 95-h (lower blot) QUIC reactions as in panel a using 100-µl reaction volumes and dilutions of scrapie brain homogenate containing the designated amount of $PrP^{Sc}$. The lanes marked 'none' received comparable amounts of normal brain homogenate only. We used antiserum R20 for these blots. Open circles designate the 17-kDa rHaPrP-res$^{(Sc)}$ band and brackets designate the positions of the 10-13 kDa rHaPrP-res$^{(Sc)}$ or rHaPrP-res$^{(spon)}$ bands. The positions of molecular mass markers are designated in kDa on the left.



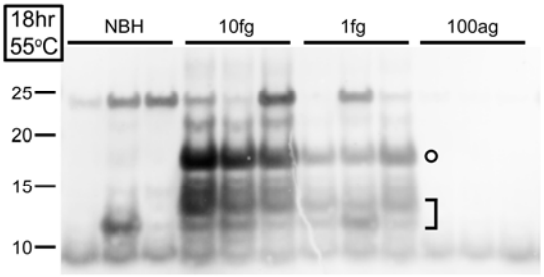

**Supplementary Figure 3. Serial QUIC reactions**. For the first round, we performed QUIC reactions under the conditions described in the **Supplementary Fig. 2b** legend and **Supplementary Methods** except for the use of 48-h reaction times and reduced detergent concentrations (0.05 % SDS and 0.05 % Triton X-100). For the second round, we diluted 10% of the volume of the first round reaction products into 9 volumes of reaction buffer containing fresh rPrP-sen. We immunoblotted PK-digested products using D13 primary antibody. Open circles designate the 17-kDa rHaPrP-res$^{(Sc)}$ band. The positions of molecular mass markers are designated in kDa on the left.




**Supplementary Figure 4. Effect of temperature on QUIC reaction products and kinetics**. We seeded QUIC reactions at different temperatures and reaction times with scrapie brain homogenates (diluted in N2) containing the designated amount of $PrP^{Sc}$ or normal brain homogenate (NBH) at the dilution used for the 100-fg scrapie brain homogenate sample. We immunoblotted the PK-digested products with antibody R20. Rows (**a**), (**b**), (**c**), and (**d**) show reactions performed at 37 ˚C, 45 ˚C, 55 ˚C and 65 ˚C, respectively. Successive columns of blots show reactions run for 4, 8 and 18 h. We ran all of the QUIC reactions in 0.1% SDS and 0.1 % Triton X-100 in PBS with 0.1 mg/ml rPrP-sen with 60 s shaking at 1500 rpm and 60 s rest. We digested the reaction products with PK under the Sarkosyl-containing conditions described in step-by-step protocol below. The positions of molecular mass markers are designated in kDa on the left in the first column or by corresponding tick marks by the other columns. The open circles designate the position of the 17 kDa band and the bracket the 10-13 kDa bands.




**Supplementary Figure 5**. **Continuity of rPrP-res$^{(Sc)}$ formation four serial QUIC reactions**. We seeded 45 ˚C QUIC reactions with scrapie brain homogenates (diluted in N2) containing 100 fg of $PrP^{Sc}$ or the same dilution of normal brain homogenate (NBH). We used the 10 s on and 110 s off shaking cycle and the buffer conditions described in **Supplementary Fig. 4**. For each subsequent round, we diluted the previous round's reaction products 1:1000 into reaction buffer containing fresh rPrP-sen and repeated the QUIC reaction sequence. We performed PK digestions (Sarkosyl conditions) and immunoblotting of reaction products from each round as described in the step-by-step protocol below using antiserum R20. The positions of molecular mass markers are designated in kDa on the left.




**Supplementary Figure 6. Sensitivity of 18-h QUIC reaction at 55 ˚C**. We seeded QUIC reactions with scrapie brain homogenates (diluted in N2) containing the designated amount of $PrP^{Sc}$ or normal brain homogenate (NBH) at the dilution used for the 10-fg scrapie brain homogenate sample. Reaction buffer constituents, PK-digestion conditions, and immunoblotting were as described in **Supplementary Fig. 4** and the step-by-step protocol below. The positions of molecular mass markers are designated in kDa on the left. The open circles designate the position of the 17 kDa band and the bracket the 10-13 kDa bands.

**Supplementary Results**:

*Tube shaking versus sonication*. With sonication of PMCA reaction tubes in cuphorn probes, the delivery of vibrational energy to samples can vary substantially with tube position, tube construction, probe age, bath volume, and the redistribution of samples within the tubes by sonication-induced atomization and condensation. Although we have found that these variations can be overcome with sufficient experience and equipment, it would be much simpler to substitute shaking for sonication as has been done with another type of *in vitro* PrP conversion reaction [1]. When a group of sample tubes are shaken laterally in a rack, each tube is subjected to the same motion, making it easier to treat all reactions equivalently.

*Enhancement of conversion with shaking*. To test the effect of tube shaking on the $PrP^{Sc}$-seeded conversion of rPrP-sen, we seeded a solution of 0.1 mg/ml bacterially expressed hamster rPrP-sen (residues 23-231) with 10 ng of purified hamster $PrP^{Sc}$ (263K strain) and incubated duplicate reactions for 24 h with or without periodic shaking (**Supplementary Fig. 1a** and **Supplementary Methods**). Treatment of the reaction products with proteinase K (PK) and immunoblotting using an antiserum (R20) raised against a C-terminal PrP epitope revealed $PrP^{Sc}$-seeded PK-resistant conversion products (rPrP-$res^{(Sc)}$). Consistent with our previous observations with sonicated (rPrP-PMCA) reactions[2], QUIC reactions produced prominent rPrP-$res^{(Sc)}$ bands of 17, 13, 12 and 11 kDa. Without shaking, the same rPrP-$res^{(Sc)}$ bands were produced, but were less intense. This provided the evidence that periodic shaking could substitute for sonication in promoting rPrP-$res^{(Sc)}$ formation.

*Reaction volumes and rPrP-sen concentrations*. We then tested dilutions of scrapie brain homogenate in normal brain homogenate as seeds and varied the rPrP-sen concentration and reaction volume. In 20-h reactions, we obtained the most intense rPrP-$res^{(Sc)}$ bands using as little as 10 fg $PrP^{Sc}$ as seed with 100 µl volumes and 0.1 mg/ml rPrP-sen (**Supplementary Fig. 1b**). Reactions seeded with normal brain homogenate produced either no PK-resistant products or a spontaneously arising product(s), rPrP-$res^{(spon)}$, that gave a set of ≤13 kDa PK-resistant bands. The latter were similar to those observed previously in unseeded rPrP-PMCA assays[2] and were clearly distinct from the overall rPrP-$res^{(Sc)}$ banding profile. With 40-h incubations at 0.1 mg/ml rPrP-sen, still smaller amounts of scrapie brain homogenate seeded detectable rPrP-$res^{(Sc)}$ in both 50- and 100-µl reactions, with the latter requiring only 1 fg of $PrP^{Sc}$ (**Supplementary Fig. 2a**). Consistent with previous findings with rPrP-PMCA reactions [7], we found that when blots of $PrP^{Sc}$-seeded reaction products were probed with an antibody to PrP residues 95-103 (D13)[8], the 17-kDa rPrP-$res^{(Sc)}$ band was stained preferentially (**Supplementary Fig. 2**). This indicated that the smaller 11-13 kDa bands that reacted with the C-terminal antibody R20 were C-terminal fragments lacking the N-terminal portion of PrP containing the D13 epitope. With 65- and 95-hr incubations of 100 µl reactions, seed dilutions containing as little as 100 ag $PrP^{Sc}$ produced strong rPrP-$res^{(Sc)}$ signals (**Supplementary Fig. 2b**).

*2-round QUIC reactions*. To seek further gains in sensitivity, we performed two serial rounds of QUIC reactions at 37˚C in which products of a first 48-h round were diluted into fresh rPrP-sen for a second-round reaction (**Supplementary Fig. 3** and **Supplementary Methods**). In this experiment, seeds nominally containing as little as 25-50 ag of $PrP^{Sc}$ were frequently at least weakly positive for the 17-kDa rPrP-$res^{(Sc)}$ band after the first round. It should be noted that the reactions seeded with 1-10 fg $PrP^{Sc}$ were weaker than those observed with such seed levels in earlier 1-round experiments under these same conditions (compare to **Supplementary Fig. 2a**). In this case, second-round reactions seeded with 10 % of the volume of the first round reactions provided more consistent and stronger rPrP-$res^{(Sc)}$ bands seeded with sub-femptogram amounts of $PrP^{Sc}$ (**Supplementary Fig. 3b**).

*Elevated temperatures and QUIC kinetics*. Because temperature elevation is known to promote an earlier type of cell-free PrP conversion reaction[3], we tested QUIC reactions at temperatures higher than 37 ˚C. 46-h reactions at 45 ˚C are described in the main text and **Fig 1**. To investigate the effects of temperature on the relative kinetics of QUIC reactions comparisons of 4-, 8- and 18-h reactions seeded with 10 or 100 fg $PrP^{Sc}$ were performed at 37 ˚C, 45 ˚C, 55 ˚C and 65 ˚C (**Supplementary Fig. 4**). The results show that elevated reaction temperatures decreased the time required to produce detectable rPrP-$res^{(Sc)}$ in the reactions, with 100 fg of $PrP^{Sc}$ seed being detected in as little as 4 h at both 55 and 65 ˚C. At 65 ˚C, formation of rPrP-$res^{(spon)}$ was apparent at 18 h in all three reactions that were seeded with normal brain homogenate. In one of the latter reactions a faint 17-kDa band was also observed, however, the overall profile of PK-resistant bands from these control reactions remained distinct from the rPrP-$res^{(Sc)}$ profile in the $PrP^{Sc}$-seeded reactions. A similar product was occasionally observed in multiple-round QUIC reactions at 45 ˚C (see below). The relatively slow spontaneous generation of such a PK-resistant form of rPrP under

these conditions is not surprising because Baskakov and colleagues have reported similar observations at high temperatures in the presence of detergents [4].

*Shaking cycles*. Testing of different 1500 rpm shaking cycles showed that a variety of shaking and rest intervals could enhance rPrP-res$^{(Sc)}$ formation, ranging from 10 s shaking with 110 s rest, to 2 min shaking with 28 min rest, to nearly continuous shaking. A direct comparison of different shaking-rest cycles at 45 ˚C revealed that alternating shaking and rest periods of 60 s produced somewhat stronger rPrP-res$^{(Sc)}$ signals than cycles of 10 s shaking and 110 s rest (data not shown). At the same time, nearly continuous shaking (cycles of 110 s shaking and 10 s rest) enhanced rPrP-res$^{(Sc)}$ formation but was less consistent than the other conditions, for reasons that we do not understand.

*PK digestion in Sarkosyl*. The use of N-lauroyl sarcosine (Sarkosyl) during the PK-digestion increased the intensity ratio between the 17 kDa and 10-13 kDa bands (data not shown). Thus, this step has been included in the recommended step-by-step protocol below.

*Continuity of rPrP-res$^{(Sc)}$ amplification through 4 serial QUIC reactions*. When products of PrP$^{Sc}$-seeded reactions were diluted 1000-fold into fresh rPrP-sen to seed the subsequent reaction rounds, strong propagation of rPrP-res$^{(Sc)}$ through at least 4 serial reactions was observed (**Supplementary Fig. 5**). In one of five reactions seeded with normal brain homogenate, rPrP-res$^{(spon)}$ formation was observed by the third 48-h round. Interestingly, in the fourth round of this reaction series, we observed rPrP-res products that displayed the bands of both the typical rPrP-res$^{(spon)}$ and rPrP-res$^{(Sc)}$ patterns (lane 4). We observed a similar result in another similar 4-round series of QUIC reactions as well (data not shown). Although inadvertent contamination of this series of reactions with PrP$^{Sc}$ cannot be conclusively excluded, these results raise the possibility that with extended series of QUIC reactions under these conditions, the formation of a rPrP-res$^{(Sc)}$-like product might sometimes arise spontaneously over multiple rounds or at higher temperatures, e.g. at 65 ˚C, as noted above. However, it is not yet clear whether additional rounds of amplification would allow the products shown in lane 4 of the fourth round of **Supplementary Fig. 5** to "evolve" further toward a product with a pattern typical of pure rPrP-res$^{(Sc)}$. Such a result would be consistent with the apparent spontaneous generation of PrP$^{Sc}$ in PMCA reactions [5].

*Sensitivity of 18-h QUIC reaction at 55 ˚C*. Using this protocol, and a 55 ˚C QUIC reaction, we readily detected ~1 fg PrP$^{Sc}$, which typically corresponds to slightly less than a lethal dose of 263 K scrapie by the most efficient route of inoculation (intracranial) into hamsters (**Supplemental Fig. 6**).

## Supplementary Methods

*Recombinant PrP expression and purification*
rPrP-sen equivent in sequence to residues 23-231 of the Syrian golden hamster sequence was expressed in *E. coli* Rosetta cells (EMD Biosciences) and purified essentially as described previously [2].

*QUIC reactions*
Unless indicated otherwise, we prepared 100 µl reactions in 0.5 ml conical microcentrifuge tubes (Fisher 02-681-334). The final concentrations of reaction buffer components were 130 mM sodium chloride, 20 mM sodium phosphate, pH 7.4, and the designated concentrations [0.05 or 0.1 % (w/v)] of sodium dodecyl sulfate (SDS) and Triton X-100 (TX-100). Both of these detergent concentrations work well in the QUIC reactions. rHaPrP-sen was 0.1 mg/ml (4 µM). The reactions were seeded with brain homogenate from normal Syrian golden hamsters or those affected with the 263K scrapie strain (ScBH) prepared as described previously [2]. Alternatively, purified $PrP^{Sc}$ from the latter animals [6] was used. The $PrP^{Sc}$ concentration in the ScBH was estimated by semiquantitative immunoblotting against purified $HaPrP^{Sc}$ standards [2]. Reaction tubes were incubated for the designated temperatures in a temperature-controlled shaker (Eppendorf Thermomixer R with a 24x0.5 ml tube block), and subjected to periodic shaking at 1500 rpm with the designated on/off cycles.

*CSF collection*
Hamsters were heavily sedated with isofluorane then exsanguinated using cardiac puncture. Skin and muscles at the back of the neck were dissected away avoiding blood vessels and meninges. A small hole was made at the medial aperture in the meninges using a 26 ¾ G needle and a Drummond micropipette was quickly inserted into the hole. CSF filled the micropipette by capillary action. Rocky Mountain Laboratories is an AALAC-accredited facility, and all animal procedures were approved by the institution's Animal Use and Care Committee.

*Proteinase K digestion, SDS-PAGE and Western blotting*
We used a few different PK (Calbiochem) digestion conditions. For **Fig. 1** and **Supplementary Figs. 1-3**, we diluted 5 µl of reaction product five-fold in PBS with 0.1 % SDS and digested for 1 hr at 37 ˚C with either 1 µg/ml PK (**Fig. 1c-d** and **Supplementary Figs. 1-3**) or 3 µg/ml PK (**Fig. 1a-b**). Pefabloc (Roche) was added to a final concentration of 4 mM to stop the reaction. We then added 20 µg of thyroglobulin and precipitated the proteins with 4 volumes of methanol at -20 °C prior to centrifugation, aspiration of the methanol, and solubilization in SDS-PAGE sample buffer for immunoblotting. In the rest of the experiments (in which the methanol precipitation step was omitted), QUIC reaction products were PK-digested and processed for immunoblotting as described in the step-by-step protocol below.

**Step-by-step Protocols for QUIC (Quaking-Induced Conversion) using hamster 263K scrapie seed and recombinant hamster PrP-sen substrate**

**MATERIALS:**

**A. Reaction tubes**: 0.5 ml conical microcentrifuge tubes with screw caps (Fisher 02-681-334, non-siliconized)

**B. Normal or 263K scrapie brain homogenates (NBH and ScBH, respectively)**:

As described previously [2].

**C. Hamster rPrP-sen**:

As described previously [2], except that, as noted below, the protein solution should be filtered through a 100 kD microtube filter (PALL) immediately before use in QUIC reactions.

**D**. **4X QUIC buffer recipe** (Final composition: 0.4 % SDS, 0.4 % TritonX-100, 4X PBS)

10 % SDS stock (40ul/ml)
10 % TritonX-100 stock (40ul/ml)
10X PBS stock (400 ul/ml):

| | |
|---|---|
| $Na_2HPO_47H_20$ | 26.8 g/L |
| $NaH_2PO_4H_20$ | 13.8 g/L |
| NaCl | 75.9 g/L |
| pH 6.9 | |

$H_2O$ (520 ul/ml)

**<u>QUIC PROTOCOL</u>:**

**1st QUIC Round:**

1) Make up 1ml of 0.1 % SDS in 1X PBS.
2) Thaw aliquots of 10 % NBH & 10 % ScBH.
3) Make up 1ml of 1X N2 supplement (Invitrogen) by dilution into 0.1 % SDS/PBS.
4) Prepare NBH and 263K BH seed diluted in 1X N2 (see dilution series below)

**263K BH Seed Dilution Series:**

| | | |
|---|---|---|
| a) | dilute 10 % BH 1:20 into 1X N2 (5ul stock + 95ul 1X N2) | → 500 pg/1 µl |
| b) | further dilute 1:10 (5ul previous + 45ul 1X N2) | → 50 pg/1 µl |
| c) | further dilute 1:10 (5ul previous + 45ul 1X N2) | → 5 pg/1 µl |
| d) | further dilute 1:10 (5ul previous + 45ul 1X N2) | → 500 fg/1 µl |
| e) | further dilute 1:10 (5ul previous + 45ul 1X N2) | → 50 fg/1 µl |
| f) | further dilute 1:10 (5ul previous + 45ul 1X N2) | → 5 fg/1 µl |
| g) | further dilute 1:10 (5ul previous + 45ul 1X N2) | → 500 ag/1 µl |
| h) | further dilute 1:10 (5ul previous + 45ul 1X N2) | → 50 ag/1 µl |
| i) | further dilute 1:10 (5ul previous + 45ul 1X N2) | → 5 ag/1 µl |
| j) | further dilute 1:10 (5ul previous + 45ul 1X N2) | → 0.5 ag/1 µl |

(1 fg = 2 µl of g)
(100 ag = 2 µl of h)
(30 ag = 6 µl of i)
(10 ag = 2 µl of i)

5) Filter recombinant PrP with a 100kD microtube filter (PALL) by spinning at 3000 X g for 12min.
6) Dilute 1:10 in 0.1 % SDS/PBS and measure OD at 280nm.
   [Protein] mg/mL = (280 nm reading / PrP Extinction Coefficient (2.6)) * Dilution Factor = **X** mg/mL
   *Want 0.1 mg/mL rPrP in 100 µL reaction = 10 µg / X = Y µL rPrP per reaction
   *Amount of water in reaction = 100 – Y – 2 – 25 = Z µL Water per reaction

7) Prepare reaction mix in reaction tubes as described above (add in the order specified).

**1st Round Reaction Mix:**

| | |
|---|---|
| Z µl | H2O |
| 25 µl | 4X QUIC buffer |
| 2 µl | ScBH seed diluted in 1% NBH |
| <u>Y µl</u> | <u>rPrP-sen</u> |
| 100 µl total volume | |

   Note: Vortex first three components for 5 s prior to adding the rPrP-sen. Add the rPrP-sen gently, as not to create bubbles. Cap reaction tubes, but do not vortex.
8) Place tubes in Eppendorf Thermomixer R with 24 x 0.5ml tube block.
9) Incubate tubes in Thermomixer R for the desired time at 55 ˚C, alternating between 60 s of shaking at 1500 rpm and no shaking for 60 s, unless designated otherwise.

10) Spin the tubes briefly to bring any solution down out of the caps.
11) Remove aliquot for 2nd QUIC round and/or prepare for PK digestion and immunoblot analysis (see below).

**2nd QUIC Round:**

1) Prepare reaction mix in fresh reaction tubes similar to 1st round above. Note: gently vortex sample tubes to evenly suspend any pellet just prior to transferring volume to the 2nd round reaction tube.
2) Filter and measure OD rPrP as stated in Steps 5 and 6 above.
   [Protein] mg/mL = (280 nm reading / 2.6) * 10 = X mg/mL
   *Want 0.1 mg/mL rPrP in 100 uL reaction = 10 ug / X = Y µL rPrP per reaction
   *Amount of water in reaction = 100 – Y – 10 – 25 = Z µL water per reaction

3) Prepare reaction mix in reaction tubes as described above (add in the order specified).

**2nd Round Reaction Mix:**

| | |
|---|---|
| Z µl | H2O |
| 25 µl | 4X QUIC buffer |
| 10 µl | sample volume from 1st round reaction |
| Y µl | rPrP-sen |
| 100 µl total volume | |

Note: Vortex first three components for 5 s prior to adding the rPrP-sen. Add the rPrP-sen gently, as not to create bubbles. Cap reaction tubes, but do not vortex.

4) Proceed as with steps 8-11 of 1st QUIC round.

**PK-digestion and SDS-PAGE sample preparation**:

1) Prepare 1 % N-lauroylsarcosine sodium salt (Sarkosyl) in 1X PBS
2) Dilute stock proteinase K (PK) (10mg/ml) 100-fold into PK storage buffer (final concentration will be 100 µg PK/ml).

PK storage buffer:
50 % glycerol
1 mM $CaCl_2$
50 mM Tris, pH 8.5

3) Combine components for PK digestion reaction and mix by pipetting action.

4 µL PK Digestion buffer (1% Sarkosyl/PBS)
1 µL PK (100 ug/mL)
10 µL Sample
15 µL Total

Note: vortex sample to evenly suspend any pellet just prior to transferring volume

4) Incubate at 37 °C for 1 hour.
5) Add 15 µl 2X SDS-PAGE sample buffer containing 4 M urea to each tube
6) Vortex samples in SDS-PAGE sample buffer for 1 min and boil tubes for 10 min.
7) Zip spin and load samples onto 10 % NuPAGE gel (Invitrogen) with MES buffer (Invitrogen) & run.

**Immunoblotting**:

1) Pre-incubate membranes in methanol for 3 min to activate PVDF membrane and rinse quickly with $dH_2O$.
2) Dry transfer using Invitrogen iGel System and Immobilon-P PVDF membrane (Millipore IPSN07852) for 7 min.
3) Block in 5 % milk/TBST over night at 4 °C or at room temperature for 1 h.

4) Primary antibody: R20 [*J. Virol.* **65**, 6597-6603 (1991), rabbit polyclonal] at 1:20,000 (0.5uL / 10 mL 5 % milk / TBST) for 45-60 min at room temperature.
5) Wash 3X in ~30 mL TBST (500uL Tween 20 / 1L 1X TBS) for 5 min per wash.
6) Secondary antibody –goat anti-rabbit-AP (alkaline phosphatase) conjugate (1:20,000 in 5 % milk / TBST or 0.5 μL / 10mL) (Jackson) for 45-60 minutes.
7) Wash 3X in TBST for 5 min per wash.
8) Add 1.5 mL Attophos AP Fluorescent Substrate System (Promega) to plastic container and lay gel face down onto it for ~2 min. Pull out gel and leave on edge to dry.
9) Visualize gel on Typhoon system (GE).

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