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13. ABSTRACT (Maximum 200 Words)

The detection of the pathologic prion protein that is implicated in transmissible spongiform encephalopathies (TSEs) is necessary to diagnose the disease. Presently, the Western Blot or ELISA are used to test the brain stem in cattle for the presence of pathologic prion (PrP^{Sc}) after Proteinase K (PK) digestion of normal, cellular prion (PrP^{C}) before admission of these animals into the food chain. An animal in the end stages of disease (40 - 72 weeks after infection) will be detected by these methods; however, an infected animal will not be detected by these methods during preclinical stages of prion infection (1-20 weeks). The RNA-polymerase immunodetection method (RAPID) is a technique whereby the exponential amplification ability of the PCR is coupled to the detection of proteins by antibodies in an enzyme linked immunosorbent assay (ELISA) format using magnetic beads. It is similar to the immuno-PCR method except that the final step of nucleic acid amplification is by RNA polymerase during isothermal incubation. For the IPCR, the final step of nucleic acid amplification is by Taq polymerase using a 2-temperature cycle incubation. As a starting platform using microwell plates as the solid format, we have been able to show that real-time immuno-PCR (IPCR) detects recombinant hamster PrP^{C} down to 0.1-1.0 femtogram/mL concentrations. Recombinant hamster PrP^{C}, as well as PK-digested scrapie infected hamster brain homogenates diluted from 10^{-1} to 10^{-8} exhibited a quantitative dose response. The methods we use in real-time IPCR will now be modified for use with a magnetic bead solid format and RNA polymerase isothermal amplification. Our recent publication describing the use of our modified real-time IPCR method for detection of HIV-1 p24 antigen, as well as the data obtained for PrP/scrapie analyses described in this report distinguishes IPCR (and potentially RAPID) as a method capable of detecting of PrP^{Sc} in samples from infected animals and humans in the pre-clinical phase of infection.

14. SUBJECT TERMS
Detection of prion in blood, PrP^{Sc}, PRP^{C}, isothermal, amplification, PCR, RAPID

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INTRODUCTION:

The detection of the pathologic prion protein that is implicated in transmissible spongiform encephalopathies (TSEs) is necessary to diagnose the disease. Presently, the Western Blot or ELISA are used to test the brain stem in cattle for the presence of pathologic prion (PrP^{Sc}) after Proteinase K (PK) digestion of normal, cellular prion (PrP^{C}) before admission of these animals into the food chain. An animal in the end stages of disease (40 - 72 weeks after infection) will be detected by these methods; however, an infected animal will not be detected by these methods during preclinical stages of prion infection (1-20 weeks). The RNA-polymerase immunodetection method (RAPID) is a technique whereby the exponential amplification ability of the PCR is coupled to the detection of proteins by antibodies in an enzyme linked immunosorbent assay (ELISA) format using magnetic beads. It is similar to the immuno-PCR method except that the final step of nucleic acid amplification is by RNA polymerase during isothermal incubation. For the IPCR, the final step of nucleic acid amplification is by Taq polymerase using a 2 temperature-cycle incubation. As a starting platform using microwell plates as the solid format, we have been able to show that real-time immuno-PCR (IPCR) detects recombinant hamster PrP^{C} down to 0.1-1.0 femtogram/mL concentrations. Recombinant hamster PrP^{C}, as well as PK-digested scrapie infected hamster brain homogenates diluted from 10^{-1} to 10^{-8} exhibited a quantitative dose response. The methods we use in real-time IPCR will now be modified for use with a magnetic bead solid format and RNA polymerase isothermal amplification. Our recent publication describing the use of our modified real-time IPCR method for detection of HIV-1 p24 antigen, as well as the data obtained for PrP/scrapie analyses described in this report distinguishes IPCR as a method capable of detecting of PrP^{Sc} in samples from infected animals and humans in the pre-clinical phase of infection. Thus, we have shown proof of principle that the combination of serologic testing and molecular diagnostic techniques can detect prion protein at levels unmatched by all current methods. Efforts will now be extended to modify this method to the rapid strategy.

BODY:

Our specific aims were to: (1) Develop a prototype, ultra-sensitive RAPID method for prion protein detection and define its increased sensitivity over currently used prion assays; (2) challenge the method for its femtogram/ml or below sensitivity in prion-spiked blood and urine, and in blood from infected animals, and (3) standardize the method for reproducibility and readiness for transfer to a commercial entity.

In previous experimental studies, our laboratory has spent several years modifying the immuno-PCR method (IPCR) (Sano et al., 1992) for the detection of HIV-1 p24 Ag as well as prion protein. We have now published on these results using HIV-1 p24 Ag from infected culture supernatants and diluted patient plasma samples (Barletta et al., 2004). The modifications which we have applied to the standard IPCR method work reproducibly in the majority of experimental runs although the test is not yet validated to the standards of a clinical laboratory test.

We have accomplished specific aim #1 using IPCR in a microwell solid format for the detection of HIV-1 p24 antigen (see Appendix: Barletta et al., 2004) and prion protein (paper to be submitted within 2 weeks for publication, see Appendix) and are in the process of translating this

protocol for use in the RAPID protocol using magnetic beads as the solid format. Many of the parameters which are required to be optimized for success in the amplification strategies have been completed.

We have partially accomplished specific aim #2 with data by showing the detection of PrP^{Sc} in PK-digested scrapie infected hamster brain homogenates down to 10,000 PrP^{Sc} molecules. These data are included in a draft of a paper to be submitted within 2 weeks for publication entitled: "Detection of 1000 Infectious Doses of Pathologic Prion Protein (PrP^{Sc}) from Scrapie Infected Hamster Brain Homogenates Using Real-Time Immuno-PCR" (see Appendix).

We have not accomplished specific aim #3 as we have not yet tested the sensitivity of the assay in prion-spiked blood and urine, or in blood from infected animals.

Several of our modifications which we have shown to be significant improvements to the standard IPCR method are now being implemented in the RAPID protocol.

These modifications include:

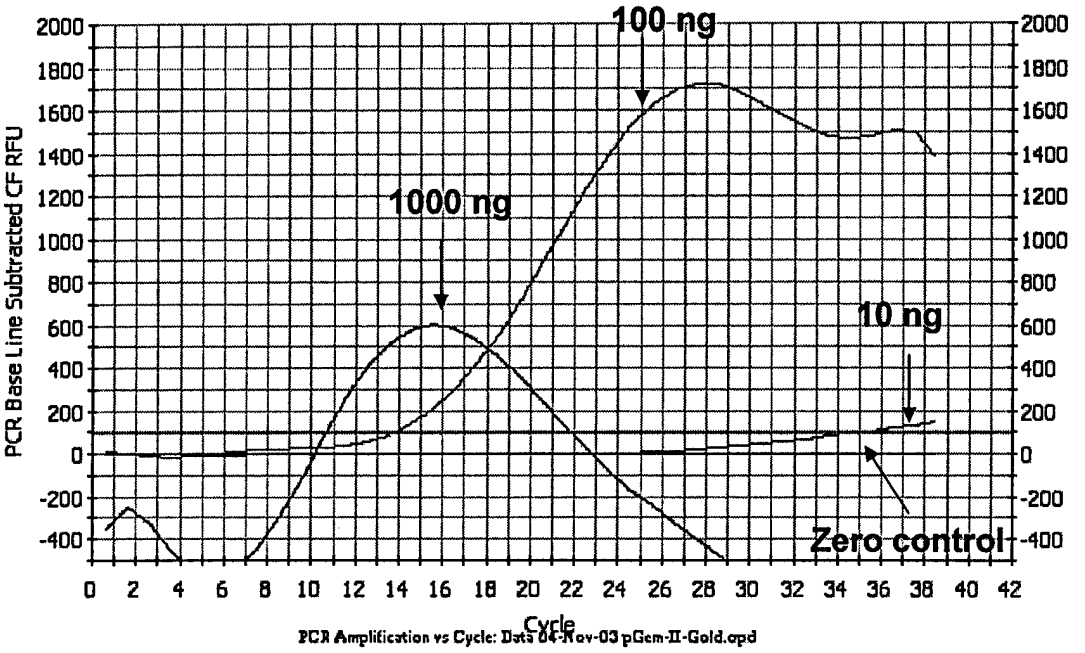
1. Use of the same animal species capture and secondary biotinylated antibody (to decrease animal inter-species non-specific cross reactivity),
2. Use of a DNA:Stabilcoat blocking reagent composed of a "DNA Blocking Reagent" (Roche Diagnostics; Indianapolis, IN) combined 1:1 with "Stabilcoat" (Surmodics, Eden Prairie, MN) designed to minimize non-specific protein and DNA interactions in nucleic acid hybridizations (Roche Diagnostics; Indianapolis, IN). This blocking buffer was tested in the magnetic bead assay and found to be superior to Seablock, Superblock (Pierce Co., Rockford, IL), and 5% BSA for the reduction of non-specific background.
3. Addition of "FcR Blocking Reagent" (Miltenyi Biotec;Auburn, CA) to the secondary antibody dilution to block antibody Fc receptor non-specific interactions,
4. Addition of 10 mM EDTA and 5 Units/mL sodium heparin to all buffers and wash reagents to minimize non-specific binding caused by molecular ionic interactions,
5. Addition of 2 extra blocking steps (one after SA-HRP addition) with DNA:Stabilcoat blocking reagent and pre-PCR (described by Nunc-Nalge Corp. in a TechNote described at (http://www.nunc.nalgenunc.com/resource/technical/nag/DP0031.htm).
6. Multiple biotinylation of the DNA template to increase efficiency of binding to the linker streptavidin molecule.

Details of these modifications are described in "Lowering the Detection Limits of HIV-1 Viral Load Using Real-Time Immuno-PCR for HIV-1 p24 Antigen" (Barletta et al., 2004; See Appendix).

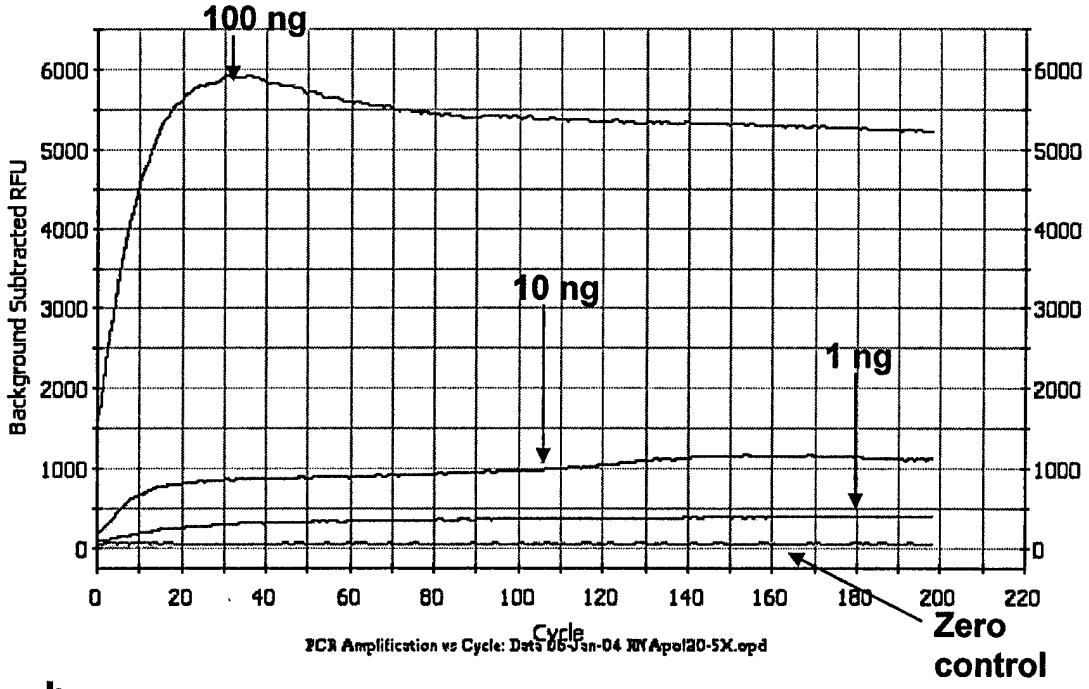
Research accomplishments associated specifically with the RAPID protocol include:

1. Determination of the most sensitive fluorescent dye (SYBR Green I, SYBR Green II, or SYBR Gold) detection of transcription products using real-time technology.

In these experiments, 3 different fluorescent dyes: SYBR Green I, SYBR Green II, and SYBR Gold (Molecular Probes; Eugene, OR) were tested at different dilutions using a pGEM control vector plasmid (Promega; Madison, WI) as a DNA template (2743 bp) which contains the T7 promoter. Reagents from the T7 Megascript Kit (Ambion Inc.; Austin, TX) were used in these studies. Ambion, Inc. claims that the reagents in this kit will produce mg amounts of RNA products from ug quantities of DNA template. Fig. 1 shows that SYBR Green II exhibited higher relative fluorescence units (RFU) than SYBR Gold (i.e., 500-6,000 vs 200-1,700 RFU, respectively) of transcript products detected in real-time by the iCycler system (Bio-Rad Laboratories; Hercules, CA). SYBR Green I exhibited extremely low fluorescence in comparison to both SYBR Gold and SYBR Green II and was not considered for further studies (data not shown). In later experiments SYBR Green II exhibited even higher RFU than SYBR Gold ranging from 1,000-10,000 when used at lower dilutions (1:5,000) for detection of transcript products (data not shown). SYBR Green II was therefore selected as the optimal fluor for further experimentation using the RAPID method.



a.



b.

Fig. 1. Real-time amplification plots of flourescent transcripts generated by T7 RNA polymerase using various amounts of pGEM template (1-1000 ng) for T7 transcription with either SYBR Gold **(a)** or SYBR Green II **(b)** at a 1:20,000 dilution.

Fluorescent transcripts were verified by gel electrophoresis and the results were identical to real-time data with detection of transcripts down to 1 ng using SYBR Green II at 1:20,000 and 1:5,000 dilutions (Fig. 2: a and b, respectively). Additionally, fluorescence was visually detectable by UV illumination from transcript products in the reaction tube (Fig. 3: a and b, respectively). The ability to detect fluorescence directly in the reaction tube may allow the test to be performed without the need for sophisticated detection equipment.

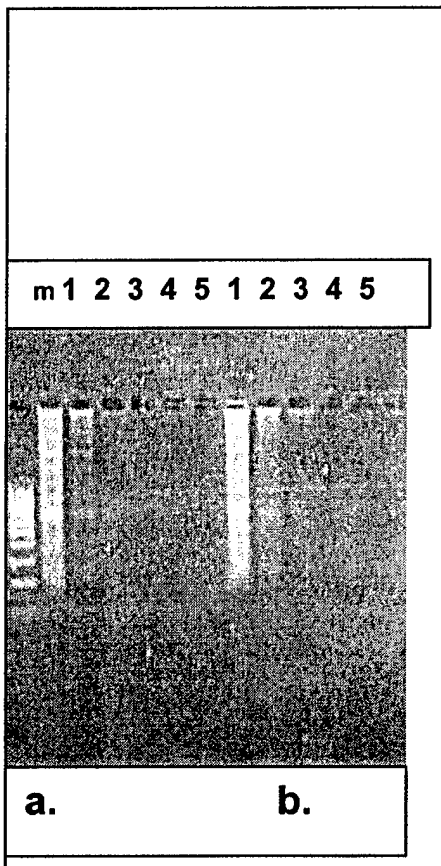


Fig. 2.

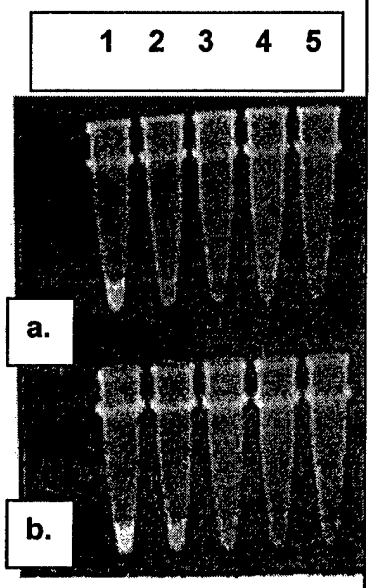


Fig. 3.

Fig. 2. Electrophoresis of RNA Transcripts. Starting pGEM template amounts were 100, 10, 1, 0.1, and 0 ng in Lanes 1, 2, 3, 4, and 5, respectively. SYBR Green II was used at 1:20,000 **(a)**, and 1:5,000 **(b)** dilutions, respectively. (m= molecular weight marker)

Fig. 3. UV illumination of RNA Transcripts. Starting pGEM template amounts were 100, 10, 1, 0.1, and 0 ng in Lanes 1, 2, 3, 4, and 5, respectively. SYBR Green II was used at 1:20,000 **(a)**, and 1:5,000 **(b)** dilutions, respectively. (m= molecular weight marker)

2. Development of the magnetic bead indirect ELISA protocol using HIV-1 anti-p24 antibodies and HIV-1 p24 Ag as the target analyte.

Our laboratory has already developed a standard indirect ELISA assay for HIV-1 p24 Ag using microwell plates. The magnetic bead indirect ELISA protocol was developed using HIV-1 p24 Ag as the analyte because the reagents used in the method are readily available in our laboratory. Now that the basic reagents and procedures for the HIV-1 p24 magnetic bead assay are delineated and all parameters optimized, we will convert the system for use with anti-prion antibodies and hamster recombinant PrP^C. The optimized protocol for the magnetic bead ELISA assay is described below:

a. Incubate equal amounts of biotinylated (rabbit) secondary anti-p24 Ab (Perkin Elmer Life Sciences, Inc., Boston, MA) with various dilutions of quantified HIV-1 p24 Ag frp, infected culture supernatants (diluted in lysis buffer: 0.5% Triton-X, PBS) for 1 hr, RT;
b. Add 50 uL of the pre-incubated (Ab-Ag from a) to 50 uL of 10^7 Dynal paramagnetic beads (Dynal Biotech, Oslo, Norway) coated with 50 ug each of 13B6 and 13G4 mouse monoclonal anti-p24 antibodies (Institute of Human Virology, Baltimore, MD) for 1 hr, RT. Wash magnetic beads 3X.
c. Add 50 uL streptavidin-HRP (KPL: Gaithersburg, MD) diluted to 0.2 ug/mL in 1:10 IPCR block buffer (Barletta et al., 2004) for 1 hr, RT. Wash magnetic beads 3X. Wash 3X with PBS, 0.5% Tween-20 (WB).
d. Add 50 uL TMB (Perkin Elmer Life Sciences, Inc., Boston, MA) for 30 min, RT. Remove solution from magnetic beads and read optical density.

Sensitivity of detection was down to 150 pg/mL with a threshold signal to noise (S/N) ratio of $\geq$2.0. The average S/N ratio from replicate experiments for 15 ng/mL, 1.5 ng/mL, 150 pg/mL, and 15 pg/mL was 14.9, 6.7, 2.2, 1.2, respectively (data not shown).

KEY RESEARCH ACCOMPLISHMENTS:

Key research accomplishments are listed below:

- Successful implementation of a protein amplification protocol (IPCR) for the detection of HIV-1 p24 Ag (Barletta et al., 2004) using microwell plates as a solid format. The IPCR method, similar in methodology to the RAPID test, was used to optimize many of the parameters. We are using information gained from the IPCR experiments to accelerate work with development of the RAPID protocol using magnetic beads as a solid format. Use of the same blocking buffers and wash reagents has already been applied to the RAPID protocol and sensitivity of detection is down to 150 picogram/mL level. This sensitivity of detection is comparable or higher than that described in most protocols in the literature (Kiselev et al., 1999; Kourilov et al., 2002) which typically attain nanogram/mL levels of analyte detection.

- Successful implementation of real-time detection of amplified transcripts using the iCycler (Bio-Rad Laboratories, Inc., Hercules, CA).
- Selection of a fluorescent dye (SYBR Green II) which increases the detection of amplified transcripts 3.5 times (6,000 RFU/1,700 RFU for 100 ng pGEM template) over SYBR Gold and SYBR Green I fluorescent dyes.
- Successful design of a magnetic bead indirect ELISA assay using a model HIV-1 p24 system.

REPORTABLE OUTCOMES:

1. Publications: Barletta JM, Edelman DC, Constantine NT. Lowering the Detection Limits of HIV-1 Viral Load Using Real-Time Immuno-PCR for HIV-1 p24 Antigen. *Amer J Clin Pathol.* 2004:122; 20-27. (See Appendix).

2. Presentation at the Cambridge Healthtech Institute's Eighth Annual Transmissible Spongiform Encephalopathies, (February 23-23, 2004). "Detecting Attogram Levels of Prion Protein". (See Appendix).

3. Patent: A provisional application for patent (#60/546,204) entitled "Immuno-PCR Method for the Detection of a Biomolecule in a Test Sample" was submitted February 23, 2004 by Barletta JM, Constantine NT, and Edelman D. Confirmation number 7366.

CONCLUSIONS:

The identification of prion diseases prior to clinical symptoms or death would address several critical issues in the prion arena. Firstly, blood targeted for transfusion could be made safer through the application of a screening method to detect infection. Scientific reports have indicated that blood from infected animals, and now humans (Llewelyn et al., 2004), can be transfusion-transmitted many months prior to the appearance of symptoms. Secondly, there is public concern about the safety of the food supply, knowing that infected beef has been implicated as the cause of variant Creutzfeldt-Jakob Disease (vCJD) in Europe. An effective screening test for cattle (and deer/elk) would ease such concern. Thirdly, there has been an enormous, negative economic impact on the food industry in Europe due to the slaughter of thousands of cattle because of concern of infection. One case of mad cow disease has now been identified in the US (Llewelyn et al., 2004), and it is certain that a similar and substantial economic impact may be realized if more cases should occur. The USDA has already mandated testing an increase in the testing of the cattle population using a recently approved Bio-Rad Laboratories Test. It is envisioned that a suitable blood or urine screening test to detect prion infection in cattle, deer, elk, and other species, would eliminate the need to slaughter uninfected animals and would increase the efficiency of screening large numbers of cattle easily. Fourthly, the US military and other US organizations have concern about possible undetected prion infection in

US personal and their families who have been stationed in Europe during the prion epidemic that occurred in the 1980s. The pre-symptomatic identification of infected persons would allow for increased vigilance of exposed individuals by health care workers and the consideration for the institution of experimental preventative or treatment measures, if clinically indicated.

Our rationale for suggesting that amplification techniques be applied to the early detection of prion diseases can be equated to past experience with HIV diagnostics. In HIV infection, prior to the development of nucleic acid tests, HIV infection could not be detected by antibody tests at less than 3 weeks post infection. However, the use of nucleic acid amplification techniques proved that low levels of a marker for the agent could indeed be detected, and their use has revolutionized the field for the early detection of a number of infectious agents. Consequently, the nucleic acid tests for HIV (and HCV) are recognized by the US FDA and are now used to test essentially all blood units targeted for transfusion. The situation for prion may not be dissimilar; i.e., markers are present but cannot be identified due to the lack of tests with sufficient analytical sensitivity. This is supported by the facts that the infectious prion protein can be detected serologically in tissues (brain) that have high levels of prion, and that blood has been shown to contain the infectious unit, but prions cannot be detected using the same tests. Accordingly, it is more than reasonable that a lack of sensitivity of currently used tests is the explanation.

Much of the background work involving optimized reagents for the RAPID has been determined by our experimental work in the optimization of a similar amplification method (IPCR). We have applied a standard IPCR protocol for detection of both HIV-1 p24 Ag and PrP^{C} and PrP^{Sc} with reproducible success although the IPCR is not yet validated to the standards of a clinical diagnostic test. Further, use of the same antibodies, blocking reagents, and wash buffers have already been translated to the ELISA portion of the RAPID test using magnetic beads as the solid format with success.

A sensitivity of detection of 150 pg/mL is comparable or greater to other magnetic bead ELISA tests published in the literature (Kiselev et al., 1999; Kourilov et al., 2002). We hope to increase the level of detection at least 2 logs by using one of 3 different amplification protocols (e.g., isothermal RNA polymerization or DNA polymerization) using RNA polymerase or Phi 29 DNA polymerase, respectively, or multithermal DNA polymerization using Taq polymerase.

The remaining work to be done is the attachment of the target template (pGEM plasmid) to the streptavidin bridge which links to the biotinylated detector antibody. We are presently in the process of exploring the optimal methods of biotinylation which include incorporation of biotinylated nucleotides during PCR amplification using either specific primers, or random priming (PCR or Random Priming DNA Biotinylation Kit; KPL, Gaithersburg, MD); or alternatively, intercalation of psoralen-biotin into the pGEM DNA template by long-wave UV irradiation (Brightstar Psoralen-Biotin Labeling Kit; Ambion, Austin, TX).

At this point, isothermal (or some other method of nucleic acid amplification) would be performed using the Ambion Megascript reagents. If the sensitivity of detection is at the low picogram to high femtogram/mL, the method will be challenged using recombinant PrP^{C} and

infectious prion from infected animal tissues and blood. Finally, extensive validation and standardization of the method for the detection of prion will be performed when transferred to a commercial company for manufacturing. .

In summary, the development of an effective, pre-mortem or ante-mortem test to identify infectious prions in humans and animals is pertinent to a number of health and economic concerns in the US. Further, the availability and application of such a screening or confirmatory tool will help to ease public sector concerns and proclaim the US commitment to maintaining safe blood and food.

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APPENDICES:

1. Publications: Barletta JM, Edelman DC, Constantine NT. Lowering the Detection Limits of HIV-1 Viral Load Using Real-Time Immuno-PCR for HIV-1 p24 Antigen. *Amer J Clin Pathol.* 2004:122; 20-27.

2. Draft of paper to be submitted to *J Clin Microbiol.* Barletta JM, Edelman DC, Highsmith WE, Constantine NT. Detection of 1000 Infectious Units of Pathologic Prion Protein (PrPSc) from Scrapie Infected Hamster Brain Homogenates Using Real-Time Immuno-PCR.

3. Presentation at the Cambridge Healthtech Institute's Eighth Annual Transmissible Spongiform Encephalopathies, (February 23-23, 2004). "Detecting Attogram Levels of Prion Protein". (Powerpoint Presentation)

Lowering the Detection Limits of HIV-1 Viral Load Using Real-Time Immuno-PCR for HIV-1 p24 Antigen

Janet M. Barletta, PhD, Daniel C. Edelman, MS, and Niel T. Constantine, PhD

Key Words: Immuno-PCR; IPCR; Polymerase chain reaction; p24 antigen; HIV-1; Viral load; ELISA; Enzyme-linked immunosorbent assay; Real-time PCR

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Abstract

Presently, the assay that attains maximal sensitivity and dynamic range of HIV-1 viral copy number (50 copies per milliliter) is nucleic acid amplification of HIV RNA in plasma. Enzyme-linked immunosorbent assay (ELISA) methods for quantification of HIV-1 p24 antigen have been relatively insensitive. In this report, we show data that indicate real-time immuno–polymerase chain reaction (IPCR), a combination of the ELISA and PCR techniques, is more sensitive for HIV-1 p24 antigen detection than other currently reported methods. When derived from an IPCR standard curve, a dose response was observed from patient samples with known viral loads diluted within a 3-log range (1.68-6,514 viral RNA copies per milliliter). IPCR detected 42% [22/52] of patient samples that had fewer than 50 viral RNA copies per milliliter by reverse transcriptase–PCR. IPCR shows the potential to become the most analytically sensitive test available for determination of HIV-1 viral load by the detection of HIV-1 p24 antigen.

The sensitivity for detecting HIV-1 in HIV-1–infected people has been increasing systematically by the use of sophisticated molecular techniques. Presently, the assay that attains maximal sensitivity and dynamic range of viral copy number is nucleic acid amplification of HIV-1 RNA in plasma: the Roche Amplicor HIV-1 Monitor Test (versions 1.0 and 1.5; Roche Molecular Systems, Basel, Switzerland). The linear range of version 1.5 is from 400 to 750,000 HIV-1 RNA copies per milliliter when using the Standard Specimen Processing Procedure (200 µL of sample), but HIV-1 RNA can be quantitated from 50 to 100,000 copies per milliliter when using the Ultrasensitive Specimen Processing Procedure, which requires 500 µL of sample centrifuged at 23,600*g* for 1 hour to concentrate virions.

A modified version 1.5 of the Ultrasensitive RNA assay was reported to detect 12 viral copies per milliliter.[1-3] In comparison with the Ultrasensitive procedure, the modified version requires twice as much sample (1.0 mL) and an increased centrifugation time (80 minutes). The Amplicor HIV-1 Monitor Test is used as an aid in assessing viral response to antiretroviral treatment measured by changes in HIV-1 RNA. For the Ultrasensitive Specimen Processing Procedure (version 1.5) the precision (coefficient of variation) for the linear range of the assay is between 32% and 102% for 25 to 100,000 HIV-1 RNA copies per milliliter. The coefficient of variation for the modified version has not yet been determined. The Amplicor tests (versions 1.0 and 1.5) have a sensitivity of 100% and a specificity of 97.4%.[4] Other nucleic acid testing methods, such as the Procleix HIV-1/HCV assay, a transcription-mediated, amplification-driven assay (Gen-Probe, San Diego, CA), detect HIV-1 and hepatitis C virus RNA down to 10 to 13 copies of HIV-1 per milliliter with 95% sensitivity.[5]

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In addition to nucleic acid testing for the determination of HIV-1 viremia, several serologic assays to detect the HIV-1 p24 antigen have been developed. The HIV-1 p24 antigen is detectable in the blood of infected people during the acute phase of infection and late in the disease. The methods presently available for the quantification of HIV-1 p24 antigen have been relatively insensitive and, thus, have limited clinical usefulness. The sensitivity and specificity of the HIV-1 p24 antigen assay recently were determined to be 88.7% and 100%, respectively.[4] However, it has been documented by some investigators that HIV-1 p24 antigen testing was even less sensitive than 88.7%, with positive results in fewer than 50% of patients who had primary HIV infection.[6]

Ledergerber et al[7] have shown that the detection of HIV-1 p24 antigen by use of the ELAST ELISA Amplification System (tyramide) (Perkin-Elmer Life Sciences, Boston, MA), after heat-mediated immune complex dissociation[8] detects down to 0.5 pg/mL of HIV-1 p24 antigen (a level comparable to the detection of viral RNA at copy numbers of >8,333/mL)[9] because 50 HIV-1 RNA copies are equivalent to 3.0 fg of HIV-1 p24 antigen (see calculations in the next paragraph). The signal amplification "boosted" ELISA method (ELAST ELISA Amplification System), performed with diluted heat-denatured plasma samples, confirmed HIV-1 p24 antigen positivity in 97.8% vs 95.7% HIV-1 RNA–positive plasma samples[10] and is effective in monitoring response to antiretroviral therapy.[11]

It is likely that HIV-1 p24 antigen is present in the plasma of patients with HIV-1 with viral loads of fewer than 50 viral copies per milliliter but is undetectable by the boosted ELISA immunologic method and by molecular tests. This is based on the following premise that HIV-1 virions are likely to be present at levels detectable by immuno-polymerase chain reaction (IPCR) but not PCR: 50 RNA copies of HIV-1 are equal to 25 virions, and there are approximately 3,000 molecules of p24 antigen per HIV-1 virion.[12] It can be estimated that 75,000 HIV-1 p24 molecules are available for detection at 50 viral RNA copies. Therefore, to meet or exceed the sensitivity of detection of the HIV-1 reverse transcriptase–polymerase chain reaction (RT-PCR) or the modified version of the assay, at least 75,000 HIV-1 p24 antigen molecules (3.0 fg of HIV-1 p24 antigen or 50 RNA copies) must be detected by the IPCR method.

Our objectives for this study were 2-fold: (1) to verify that IPCR is a method capable of detecting extremely low levels of viremia using diluted patient samples with known viral RNA copy numbers, and (2) to detect HIV-1 p24 antigen in patient samples containing fewer than 50 RNA copies per milliliter. Our experimental data demonstrate that IPCR, a method that combines the specificity of protein detection (for HIV-1 p24 antigen) with the exponential amplification of PCR is a more sensitive method for the determination of early HIV-1 infection than HIV-1 RNA detection by RT-PCR.

Materials and Methods

The ELISA for HIV-1 p24 antigen was performed as described in the HIV-1 p24 antigen ELISA kit (Zeptometrix, Buffalo, NY) using TopYield strips (Nalge Nunc, Naperville, IL). Several technical modifications were applied to decrease nonspecificity in the negative control samples. Briefly, the IPCR assay consisted of the following steps: an ELISA for antigen detection using an immobilized mouse monoclonal capture antibody and a biotinylated secondary human antibody, followed by streptavidin–horseradish peroxidase (HRP) **Figure 1**. The procedure was identical to the ELISA procedure except for the addition of 10 pg/mL of biotinylated DNA (500 base pairs) after the addition of streptavidin-HRP followed by real-time PCR using the iCycler iQ instrument (Bio-Rad Laboratories, Hercules, CA). The PCR cycling parameters were an initial 6 minutes at 95°C followed by 20 cycles of 1 minute at 95°C and 2 minutes at 68°C in TopYield strips. We then placed 5-µL from each reaction into new PCR reagents for the second amplification of 50 cycles. Fluorescence was detected during PCR amplification by hydrolysis of a hybridization probe labeled with a 5'-reporter dye, 6-carboxyfluorescein, and the 3'-Black Hole Quencher dye (Biosearch Technologies, Novato, CA). A diagram of real-time IPCR with fluorescent probe detection is shown in Figure 1.

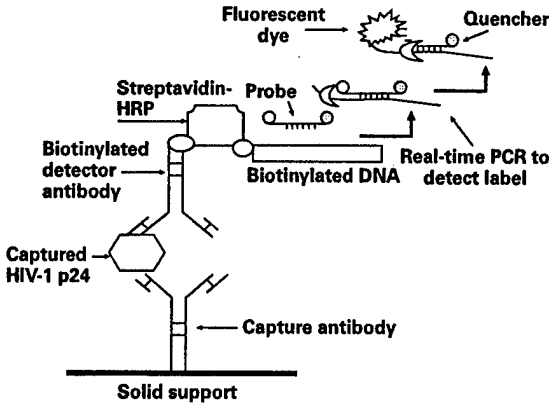

Figure 1 The immuno–polymerase chain reaction (IPCR) technique. Capture anti–HIV-1 p24 antibody, adsorbed to the microwell plate, is used to capture antigen. Streptavidin–horseradish peroxidase (HRP) bridges between a biotinylated detector anti–HIV-1 p24 antibody and biotinylated reporter DNA. The reporter DNA is amplified by PCR using a fluorescent probe for real-time analysis

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All plasma samples were diluted 1:6 to 1:600 in 0.5% Triton X-100 (Sigma-Aldrich, St Louis, MO) lysis buffer and heat denatured at 100°C for 5 minutes.[7] Technical modifications of the procedure included using 5 U/mL of heparin and a 0.5-mol/L concentration of EDTA in all wash and diluent buffers, additional blocking steps after addition of streptavidin-HRP and before PCR, the use of reagents that block nonspecific immunologic reactions between antibody molecules (FcR blocking reagent, Miltenyi Biotec, Auburn, CA), and 2 rounds of PCR amplification (an initial 20 cycles followed by 50 cycles in new PCR reagents).

To determine the detection limit of IPCR for HIV-1 p24 antigen, we generated a standard curve by IPCR using dilutions of HIV-1 p24 antigen from HIV-1–infected cell culture supernatants quantified by ELISA. These dilutions encompassed log-fold differences (10^0-10^8 HIV-1 p24 molecules). The IPCR method involves several unknown variables in the quantification of molecules that are related to the ratio of DNA reporter molecules to antigen (eg, the number of biotins per antibody molecule, the number of biotins bound to avidin, and the number of biotinylated DNA reporter molecules bound to the tetravalent avidins). Thus, the standard curve generated by IPCR (in contrast with a PCR standard curve using template DNA) would be the only valid method of extrapolation of molecular numbers. *Threshold* was defined as 10 times the mean SD of fluorescence in all wells over the baseline cycles (according to the manufacturer's instructions for the iCycler iQ). For some runs, the threshold was raised farther above the fluorescence level of the negative controls. *Cycle threshold* (Ct) was the cycle at which the sample's fluorescence intersected the background fluorescent threshold.

To verify performance of the IPCR, 14 to 37 replicates from HIV-1 antibody–positive patients with known HIV-1 RNA viral loads (determined by using the Amplicor HIV-1 Monitor Test) were diluted (1:6-1:60) within groups of 3 logs (1.68-6,514 viral RNA copies) and then analyzed by real-time IPCR. We reasoned that the dilution of patient samples with known HIV-1 viral loads within the ranges of 1.68 to 43.7, 60.7 to 607, and 5,179 to 6,514 viral RNA copies would be the experimental parallel to the in vivo situation of low to very low levels of HIV-1 p24 antigenemia. The specificity of this method was established by screening more than 2 times the number of replicates of negative control samples as positive control samples. Each sample was analyzed minimally in triplicate, and samples were determined to be positive when the relative fluorescence units (RFUs) of 50% or more of the test replicates were above the threshold set for replicates of 6 to 8 normal control (HIV-1 antibody–negative) patient samples. All reactions were checked by gel electrophoresis because nonspecific background fluorescence might raise the setting of the background fluorescence threshold level, thereby decreasing the sensitivity of detection, resulting in the elimination of low-positive samples that actually were amplified by IPCR (and determined positive only by gel electrophoresis). In addition to the patient samples with known viral loads that were diluted to determine the lower limit of detection by IPCR, 52 HIV-1–infected (antibody-positive) samples below the limit of detection of the Amplicor HIV-1 Monitor Test (<50 viral RNA copies) were tested at dilution 1:6 by using real-time IPCR.

Results

We developed an optimized real-time PCR method for amplification of a 500-base-pair DNA molecule that is coupled to the detection of HIV-1 p24 antigen in the IPCR. The PCR standard curve (using only template DNA) displayed a correlation coefficient of 0.997, and 1 to 10 copies of DNA were able to be detected (data not shown). To generate a standard curve correlating with HIV-1 p24 antigen quantification, an HIV-1–infected cell culture supernatant, previously quantified by ELISA, was diluted serially and analyzed by real-time IPCR. The IPCR standard curve was performed 5 times in replicate, and the sensitivity of detection was seen to vary depending on the background fluorescence in the negative control sample. However, in 1 of 5 replicate IPCR standard curve assays, the sensitivity of detection was 10^2 copies of HIV-1 p24 antigen (data not shown). The remaining IPCR standard curves consistently attained a sensitivity of detection of 10^3 copies of HIV-1 p24 antigen.

The mean Cts after real-time IPCR for 10^8, 10^7, 10^4, and 10^3 molecules of HIV-1 p24 antigen were 17.65, 20.02, 25.43, and 31.15, respectively **Figure 2A**. The IPCR standard curve displayed a correlation coefficient of 0.977 **Figure 2B**. For the real-time IPCR standard curve, sensitivity was limited owing to the elevated RFUs exhibited by the negative (0 HIV-1 p24 antigen molecules) control (Figure 2A). Thus, fewer than 10^3 HIV-1 p24 antigen molecules could not be detected above the background fluorescence of the negative control sample. Note that 3 replicates of the 10^3 dilution of HIV-1 culture supernatant are shown displaying the variability seen when testing higher dilutions of HIV-1 p24 antigen.

As shown in **Table 1**, when patient samples with known viral RNA copies (by RT-PCR determination) were diluted in 3 groups of 1.68 to 43.7, 60.7 to 607, and 5,179 to 6,514 copies per milliliter, a dose response relative to the Ct was observed. Under optimal conditions, the Ct should increase 3.3 cycles for every log decrease in the number of molecules. When interpreting the data, it is important to distinguish HIV-1 RNA copy numbers from HIV-1 p24 antigen molecules. Based on the estimate of 3,000 HIV-1 p24 molecules per HIV-1 virion,[12] patient samples diluted to

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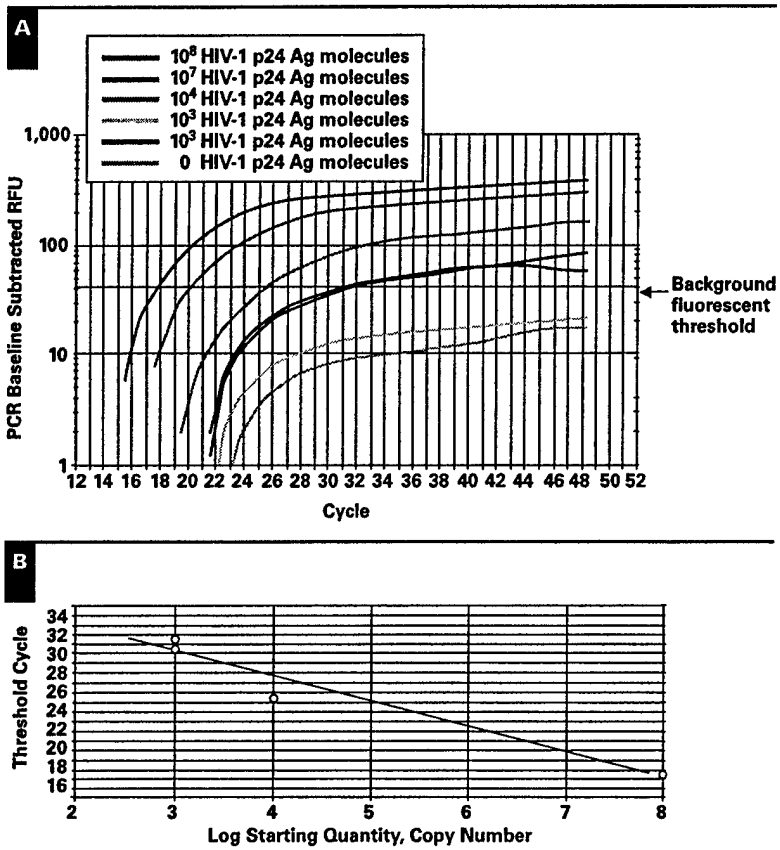

Figure 2 Immuno–polymerase chain reaction (IPCR) standard curves. **A**, IPCR was performed on serial dilutions of quantified HIV-1–infected cell culture supernatants with known concentrations of HIV-1 p24 antigen equaling log-fold dilutions from 10^8 to 10^3 copies. Note that 3 replicates (green lines) of the 10^3 dilution are shown. **B**, Correlation coefficient of the IPCR standard curve with the corresponding regression equation. The mean cycle threshold was derived from 5 replicates of the IPCR standard curve. Correlation coefficient, 0.977; slope, –2.593; intercept, 38.048; y = –2.593x + 38.048; PCR efficiency, 143.0%. Circles, standards; Ag, antigen; RFU, relative fluorescence unit.

Table 1
Median Cycle Threshold by Immuno–Polymerase Chain Reaction of Patient Samples With Known Viral Loads*

	HIV-1 RNA copies (/mL)*		
	1.68-43.7†	60.7-607†	5,179-6,514†
Predicted median HIV-1 p24 antigen molecules (/mL)‡	3.4×10^4	5.0×10^5	8.7×10^6
Median cycle threshold	29.3	26.8	21.1
Empirically determined median HIV-1 p24 antigen molecules (/mL)§	2.5×10^3	19.9×10^4	3.1×10^6
No. of determinations	36	20	14
No. (%) of samples detected	17 (47)	10 (50)	8 (57)

* Determined by the Amplicor HIV-1 Monitor Test, version 1.5 (Roche Molecular Systems, Basel, Switzerland).
† Diluted to these approximate numbers.
‡ Based on an estimated 3,000 HIV-1 p24 antigen molecules per virion.
§ Calculated from the regression equation of the immuno–polymerase chain reaction standard curve using the median cycle threshold of each range of patient samples.

5,179 to 6,514 viral copies will equal a predicted approximate average of 5,846 viral copies divided by 2 RNA copies per virion, which equals 2,923 virions times 3,000 HIV-1 p24 antigen molecules per virion, which equals 8,769,750 (8.8×10^6) HIV-1 p24 antigen molecules. Likewise, for the patient samples diluted to 60.7 to 607 and 1.68 to 43.7, the numbers of HIV-1 p24 antigen molecules will equal a predicted approximate average of 5.0×10^5 and 3.4×10^4 HIV-1 p24 antigen molecules, respectively.

Between samples within the range of 5,179 to 6,514 viral copies and samples within the range of 60.7 to 607 viral molecules, the median Ct increased 5.7 cycles (from 21.1 to 26.8). Between samples within the range of 60.7 to 607 viral molecules and samples within the range of 1.68 to 43.7 viral copies, the median Ct increased 2.5 cycles (from 26.8 to 29.3) (Table 1). By using the regression equation of the IPCR standard curve to calculate HIV-1 p24 antigen molecules, the median Ct of 21.1 for the specimens diluted to

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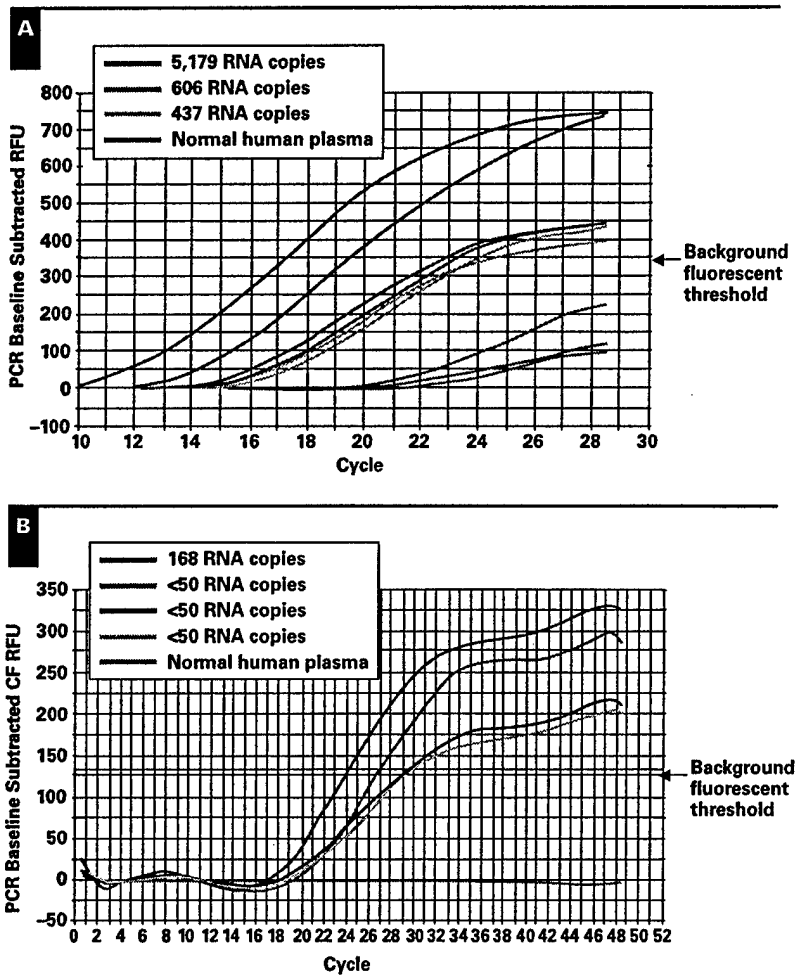

Figure 3 Immuno–polymerase chain reaction (IPCR) of plasma dilutions from HIV-1–infected patients having known viral RNA copy number and normal plasma control samples. Replicate (2-3) dilutions of HIV-1–infected and normal human plasma were analyzed by real-time IPCR using the iCycler thermocycler (Bio-Rad Laboratories, Hercules, CA). **A**, Diluted HIV-1–infected patient samples corresponding to 5,179 (black lines), 606 (green lines), and 437 (blue lines) viral RNA copies (7.7×10^6, 9.1×10^5, 6.5×10^5 HIV-1 p24 antigen molecules, respectively). Normal human plasma is depicted by red lines. **B**, Individual HIV-1–infected patient plasmas with low (168) to undetectable (<50) viral RNA copies (2.5×10^5 and $<7.5 \times 10^4$ HIV-1 p24 antigen molecules, respectively). RFU, relative fluorescence unit.

5,179 to 6,514 viral copies per milliliter equaled $10^{6.5}$ (3,162,277) HIV-1 p24 antigen molecules; the median Ct of 26.8 for the specimens diluted to 60.7 to 607 viral RNA copies per milliliter equaled $10^{4.3}$ (19,952) HIV-1 p24 antigen molecules; and the median Ct of 29.3 for specimens diluted to 1.68 to 43.7 viral copies per milliliter equaled $10^{3.4}$ (2,511) HIV-1 p24 antigen molecules (Table 1). Thus, the empirically determined number of HIV-1 p24 antigen molecules was within 1 log of the predicted number of molecules for all patient sample groups.

Figure 3 shows IPCR results of diluted patient samples across a 3-log range of viral RNA copies compared with the negative control sample (normal human plasma). These examples were selected from replicates performed during a single run. A dose response represented by a decrease of approximately 3 to 5 Ct cycles (1-1.5 log difference) was observed between the samples containing 5,179 and 606 or 437 viral RNA copies (Figure 3A). A dose response represented by a decrease of approximately 3 to 5 Ct cycles also was observed between the samples containing 168 and fewer than 50 viral RNA copies (Figure 3B).

In addition to the patient samples with known viral loads that were diluted to determine the lower limit of detection by IPCR, 52 HIV-1 infected (HIV-1 antibody–positive) samples below the limit of detection of the Amplicor Monitor Test (<50 viral RNA copies) were tested by IPCR **Table 2** Note that the median Ct for 22 of these patient samples was 26.5 and is predicted to equal $7.5 \times 10^{4.5}$ (or fewer) HIV-1 p24 antigen molecules. For these samples, the number of HIV-1 p24 antigen molecules calculated from the regression equation of the IPCR standard curve equaled $10^{4.5}$ (31,622) HIV-1 p24 antigen molecules (approximately 10.5 virions). The IPCR detected 42% of these samples as positive (Table 2), indicating the presence of HIV-1 p24 antigen in a 22 of 52 samples that were below the level of detection

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Table 2
Assessment of HIV-1 p24 Antigenemia in Patients With Fewer Than 50 RNA Copies per Milliliter*

Median cycle threshold	26.5
Predicted median HIV-1 p24 antigen molecules (/mL)†	$<7.5 \times 10^4$
Empirically determined median HIV-1 p24 antigen molecules (/mL)‡	3.1×10^4
No. of determinations	52
Median viral load copies (/mL)§	10.5
No. (%) of samples detected	22 (42)

* Determined by the Amplicor HIV-1 Monitor Test, version 1.5 (Roche Molecular Systems, Basel, Switzerland).
† Based on an estimated 3,000 HIV-1 p24 antigen molecules per virion.
‡ Calculated from the regression equation of the immuno–polymerase chain reaction standard curve using the median cycle threshold of all positive patient samples.
§ Calculated from the empirically determined number of HIV-1 p24 antigen molecules.

by RT-PCR. The true immunological status of these samples cannot be determined because there is no molecular or immunologic method presently available as a clinical test that can definitively ascertain that any of these specimens contained any viral copies.

Discussion

Because several published studies show that patients with symptomatic primary infection have a relatively aggressive clinical course with earlier onset of immunodeficiency and AIDS than those with asymptomatic HIV-1 seroconversion,[13-16] it is important to diagnose primary HIV-1 infection and begin antiretroviral treatment as early as possible. Suspected primary HIV-1 infection can be verified by testing for HIV-1 RNA or HIV-1 p24 antigen. An antibody test also should be performed, although the results might be uniformly negative during the early period of infection (0-45 days).[17] This interval, the serologic "window period," is thought in some cases to last as long as 6 months[18,19] and is characterized by seronegativity, occasionally detectable antigenemia, viremia (as measured by RNA), and variable CD4 lymphocyte levels. Detection of specific HIV-1 antibody signals the end of the window period and labels the person as seropositive. HIV-1 p24 antigen, although transient, is thought to be significant during the 2 periods at each end of the spectrum of HIV-1 seropositivity[20,21]; that is, as early as 2 weeks after infection (before and shortly after the appearance of HIV-1 antibodies) and at the terminal stages of infection with the development of AIDS. However, HIV-1 p24 antigen might be detected in small quantities within the vast period of the asymptomatic seropositive state.

There is a widespread opinion that quantification of the HIV-1 viral load using HIV-1 p24 antigen is unsuitable for the management of patients as a predictor of CD4 decline, progression to AIDS, and survival.[22] However, Schupbach et al[23] argue that since "most mechanisms of viral pathogenesis involve proteins rather than nucleic acids or virus particles as the mediators of disease, one might expect that the concentration of viral proteins would be a better marker of disease activity or progression than the particle-associated viral RNA." We find this to be a valid concept in addition to their further suggestion that the inferiority in the correlation of HIV-1 p24 antigen testing with indicators of disease progression has been due to "technical inadequacies of the procedures presently in use."[23]

The detection of HIV-1 p24 antigen is considered a simple and inexpensive alternative to HIV-1 RNA testing for monitoring treatment and protecting the blood supply.[24] Schupbach[25] has repeatedly made the case that simple modifications (eg, heat-mediated destruction of test-interfering antibodies and increased sensitivity achieved by signal amplification [ELAST]) have shaped the HIV-1 p24 antigen test into a tool that rivals nucleic acid testing. In fact, in a study of 169 adult patients representing all stages of an HIV-1 chronic infection whose HIV-1 p24 antigen and viral RNA concentrations were assessed for CD4-adjusted Cox proportional hazard models, both viral RNA and HIV-1 p24 antigen were significant predictors of progression to AIDS. HIV-1 p24 antigen was superior to RNA in the model for survival, was a significant predictor of the CD4 decline, and was superior or equivalent to viral RNA depending on the group analyzed.[7] In this report, we show data that indicate IPCR is a method capable of higher sensitivity for the detection of HIV-1 p24 antigen than currently reported methods (ELISA and ELAST) and might represent the next generation of test methods capable of supporting Schupbach's findings.[25]

The pretreatment of samples by the use of acid or heat to dissociate immune complexes has shown a substantial increase in the level of HIV-1 p24 antigen detected in patient samples with high-titered antibody (from 12.4% to 50.7% for acid-dissociated pretreatment and from 26% to 60% for heat-dissociated pretreatment).[8,26] After heat denaturation of patient samples, HIV-1 p24 antigen levels predicted subsequent clinical disease progression in early-stage HIV-1 infection and correlated with CD4 lymphocyte count and HIV-1 RNA level.[27] These studies indicate that the presence of HIV-1 p24 antigen in HIV-1–infected patients might be more significant than previously thought owing to the fact that methods for HIV-1 p24 antigen detection have been inferior to nucleic acid testing. For IPCR, there is no significant sample preparation or concentration required. Extremely low (<10 μL) sample volumes are used as dilutions from 1:6 to 1:600. Because the IPCR method still is able to detect positive samples at these high dilutions without virion concentration emphasizes the exquisitely powerful amplification potential of the method in the determination of extremely HIV-1 p24 antigen levels in clinical

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specimens containing ultralow numbers of HIV-1 virions. In addition, it is logical to deduce that an HIV-1 p24 antigen–based method is a more sensitive indicator for the detection of virus given that the target protein of the test (HIV-1 p24 antigen) is present in the virion at much higher numbers than viral RNA copies (approximately 3,000 HIV-1 p24 antigen molecules vs 2 RNA copies per virion).

The IPCR standard curve displayed a correlation coefficient of 0.977 (Figure 2B), which is acceptable but not optimal. However, the IPCR protocol involves the primary step of antibody recognition of HIV-1 p24 antigen, which results in an indirect estimation of the target, a variable in the test protocol that might account for a loss of accuracy and precision compared with a standard curve generated by PCR of a DNA template alone.

A dose response represented by increasing Ct with decreasing HIV-1 p24 antigen molecules was observed between all dilution groups. The IPCR protocol is not standardized to the level at which the linear quantification of HIV-1 p24 antigen molecules is ideal, and consequently we believe that analysis of more replicates to eliminate samples that are erratic outliers and increased standardization of the IPCR method will significantly improve linear quantification so that a wider dynamic range is attained.

It previously has been shown that one of the most common problems associated with the use of IPCR is its nonreproducibility owing to high background (noise) in the negative control samples.[28-30] In the performance of the ELISA, if more than 33% of the negative control samples react as false-positive samples (a typical standard of US Food and Drug Administration–approved ELISA tests (eg, Coulter HIV-1 p24 Antigen Assay, Coulter, Miami, FL), then the test results are invalid. We enforced a more stringent criterion for the acceptance of false-positive control reactions in the present study; ie, a run was not accepted if more than 12.5% (1/8) of the negative control samples reacted as a false-positive. When nonspecific background fluorescence is present in IPCR, the negative threshold is raised, effectively and artifactually decreasing the sensitivity of detection. This effect was seen with the IPCR standard curve in which the lower limit of detection of 10^3 HIV-1 p24 antigen molecules was due to the higher relative RFU exhibited by the negative control standard.

We modified the reported IPCR protocols in several ways to substantially reduce the occurrence of false-positive results (see the "Materials and Methods" section). Our (modified) IPCR protocol significantly reduced false-positive results in the negative control samples, but it is likely that these technical modifications could compromise the sensitivity of the IPCR (ie, 47.2%-57%; Table 1).

A low level of sensitivity also might occur because of reasons other than technical. For example, with such borderline low levels of viral copy number, it is likely that a lower percentage of true-positive results will be detected by IPCR or any other method. The modified Amplicor HIV-1 Monitor Ultrasensitive RNA assay shows a 90% sensitivity for a 10-copy number specimen using 1 mL of specimen for analysis. Presently, because the IPCR standard curve will detect 1,000 HIV-1 p24 molecules, the limit of detection for our modified IPCR protocol is 0.66 viral RNA copies per reaction divided by 2, which equals 0.33 virions per reaction (ie, 0.33 virions is equal to approximately 1,000 HIV-1 p24 antigen molecules). This number of HIV-1 p24 antigen molecules is equal to 40 attograms per reaction. Furthermore, the specificity of the method approaches 100%, as positive samples were defined only if higher than the threshold setting for the negative control samples. Thus, the exponential capabilities inherent in the IPCR method permit the detection of ultralow levels of HIV-1 p24 antigen molecules. We propose that with further refinements, the IPCR method might be the most sensitive method to confirm the diagnosis of HIV-1 at the earliest time of infection (<1 week), monitor antiretroviral treatment by HIV-1 p24 antigen levels, and screen the blood supply for the enhanced detection of the presence of HIV-1.

Our study sought to confirm and is the first to demonstrate that ultralow detection of less than 1 virion per reaction is theoretically possible with IPCR without the use of large sample volumes, sample concentration methods, or extensive sample processing. The combination of PCR coupled to the serologic detection of HIV-1 p24 antigen (a protein present at 1,500 times the levels of HIV-1 RNA per virion) further increases the probability of HIV-1 detection as free HIV-1 p24 antigen circulates in the blood and/or is sequestered in immune complexes and tissues in the absence of virions. In fact, in a study of 55 patients whose viral RNA in plasma previously had been suppressed for 6 months during antiretroviral therapy, it was found that HIV-1 p24 antigen detected in these samples was not associated with viral RNA–containing particles, suggesting the source of HIV-1 p24 antigen might be located in other compartments of virus expression.[31]

Our primary intent was to demonstrate the exquisitely sensitive amplification of the IPCR technique and to show the potential of IPCR to provide clinicians with the earliest possible detection of HIV-1 and more specific information of patient response to therapy. We have shown this potential with continued modifications to completely eliminate nonspecific background in approximately 80% of assay runs and, thereby, increase the sensitivity of the method. IPCR has the potential to become the most sensitive method available for the determination of HIV-1 infection by determination of the presence of HIV-1 p24 antigen at an earlier time than is attainable by any serologic or molecular method presently in use.

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Detection of 1000 Infectious Units of Pathologic Prion Protein (PrP^{Sc}) from Scrapie Infected Hamster Brain Homogenates Using Real-Time Immuno-PCR

Running Title: IPCR Detection of Pathologic PrP^{Sc} from Scrapie Homogenates

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(For everybody's information: The manuscript is being submitted to the Journal of Clinical Microbiology. The guidelines are ...every portion must be double-spaced, have line numbers, minimum font size 12, running title not to exceed 54 characters and spaces, and an Abstract of 250 words).

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Abstract: The detection of the pathologic prion protein that is implicated in transmissible spongiform encephalopathies (TSEs) is necessary to diagnose prion diseases. Presently, the Western Blot or enzyme linked immunosorbent assay (ELISA) are used to test the brain stem in cattle for the presence of pathologic prion (PrP^{Sc}) after proteinase K (PK) digestion of normal, cellular prion (PrP^{c}) before admission of these animals into the food chain. An animal in the end stages of disease (40 - 72 weeks after infection) will be detected by these methods; however, an infected animal will not be detected by these methods during preclinical stages of prion infection (1-20 weeks). The immuno-polymerase chain reaction (IPCR) is a technique whereby the exponential amplification Ability of the PCR is coupled to the detection of proteins by antibodies in an ELISA format. In this report, we describe a modified real-time IPCR method capAble of detecting recombinant hamster PrP^{C} down to 100 attogram/mL concentrations. Recombinant hamster PrP^{C}, as well as PK-digested scrapie infected hamster brain homogenates diluted from 10^{-1} to 10^{-8} exhibited a semi-quantitative dose response. This level of detection is up to 10 million-fold more sensitive than the sensitivity of detection by standard Western Blot or ELISA methods and distinguishes IPCR as a method capAble of detecting of PrP^{Sc} in samples from infected animals and humans in the pre-clinical phase of infection. Further, these studies show that unless PK-digestion of the brain homogenate is verified to remove PrP^{C}, highly sensitive assays such as IPCR may incorrectly define a sample as positive.

INTRODUCTION:

Transmissible spongiform encephalopathies in mammals are mediated by an Abnormal conformer of the normal prion protein (PrP^{C}). The abnormal conformer, termed PrP-scrapie (PrP^{Sc}) or PrP-resistant (PrP^{Res}) is characterized by insolubility, a high proportion of beta-pleated sheet tertiary structure, and resistance to degradation by PK (McKinley et al., 1991). In humans, the clinical forms of TSE include a sporadic form: Creutzfeld-Jacob disease (CJD); genetic forms: Gerstmann-Straussler-Scheinker Syndrome (GSS) and Fatal Familial Insomnia (FFI); and, infectious forms: kuru. In animals, a variety of TSE's have been described, including scrapie in sheep and goats and bovine spongiform encephalopathy (BSE) in cattle {for reviews, see Prusiner (1991)

and Prusiner and DeArmond (1994)}. In 1996, a variant of CJD (vCJD) was described which occurs in young adults, has atypical clinical features and neuropathology (Will et al., 1996), and results from the consumption of tissue from cattle contaminated by the BSE infectious agent (Bruce et al.,1997; Scott et al., 1999; Collinge, 1999).

These diseases are not commonly diagnosed prior to the occurrence of clinical symptoms because PrP^{Sc} is typically identified only during the clinical stages of infection when it is present in high quantities in the brain. In humans, during the clinical stage of TSE, PrP^{Sc} has been directly detected in a variety of tissues including CSF, spleen, tonsils, lymph glands, retina, proximal optic nerve, rectum, adrenal gland, thymus and muscle (Hill et al.,1997; Hilton et al., 1998; Bieschke et al., 2000; Bruce et al., 2001; Wadsworth et al., 2001: Glatzel et al., 2003) and in appendix (Hilton et al., 1998) during the preclinical stage of infection. One study attempted the detection of PrP^{Sc} in the blood of humans infected with various forms of CJD using immunocompetitive capillary electrophoresis (ICCE), but determined that the method as presently performed was unsatisfactory for use as a screening test in human TSE (Cervenakova et al., 2003).

In contrast, it has been conclusively shown that infectivity is present in the blood of infected animals both during and preceding symptomatic disease {i.e., animals which are injected intracranially (IC) or intravenously (IV) with blood from an infected animal will develop BSE} (Casaccia et al., 1989; Brown et al., 1999; Taylor et al., 2000; Houston et al., 2000; Hunter et al., 2002; and Cervenakova et al., 2002). That infectivity has been consistently recovered in blood, but the infectious prion has never been directly detected in blood from naturally or experimentally induced infections by a diagnostic method (Brown et al., 2001) implies the transmissible prion agent is present, but below the level of sensitivity of detection of current serological methods. Further, in studies describing the detection of PrP^{Sc} in urine from scrapie-infected hamsters, no PrP^{Sc} could be identified in the kidney tissue of the animal suggesting that the PrP^{Sc} originated from other organs and was filtered into the urine from the blood (Shaked et al., 2001).

Thus, there is compelling evidence that infectious PrP^{Sc} is present in blood of experimentally infected animals prior to the onset of symptoms. In humans, this possibility has recently been supported by a single case of vCJD that appeared to be transmitted by a blood transfusion from an individual who later succumbed to vCJD

(Llewelyn et al., 2004). These observations imply that the inAbility of current methods to detect ultra-low levels of PrP^{Sc} in blood (or other biologic samples) is the most likely limitation to the development of effective ante-mortem screening tests for TSEs. This recent report of a transfusion-transmitted case of vCJD has raised significant concern for the safety of the blood supply, and underscores the importance of developing a highly sensitive preclinical blood test for prion detection.

The demonstration that BSE is transmissible to humans, albeit at a low frequency, has lead to the European Union mandate (EU Regulation 999/2001) for screening of cattle for BSE prior to admission of individual carcasses to the food supply. Presently, there are 5 BSE testing kits that are approved by the Commission of the European Communities for testing of bovine brain material in Europe (Moynagh and Schimmel, 1999: EC,2003) and include the Platelia™ BSE test (Bio-Rad LAboratories; Hercules,CA); the Enfer TSE Test (Enfer Scientific; Newbridge, Ireland); the InPro aCDI Test (InPro Biotechnology Inc., San Francisco, CA); and the Prionics®-Check WESTERN and Prionics®-Check LIA (Prionics AG; Schlieren, Switzerland). These test kits utilize either the Western blot or a variation of the standard ELISA (i.e., enhanced chemiluminescent or sandwich immunoassay) for detection of PrP^{Sc} from the obex region of the bovine spinal cord. Except for the aCDI Test ,which does not require PK digestion of brain homogenates, all other commercial BSE Western blot and ELISA kits require preliminary homogenization and PK digestion of the obex sample to remove normal prion protein (PrP^{c}) from the specimen before analysis. Results are reported as qualitative (Western Blot) or semi-quantitative (i.e., positive if Above a non-specific background threshold OD or negative if below this threshold OD). These tests, by nature of their methodologies, lack sensitivity in the detection of PrP^{Sc}. The highest sensitivities for these tests are 30 pg/mL, 1 ng/mL, and a limit of a 10^3 dilution of BSE infected brain homogenate for the Prionics Check LIA test (Biffiger et al., 2002), the aCDI test (Safar et al., 2000), and the Bio-Rad Platelia TeSeE® Detection Kit (Grassi et al., 2001), respectively.

The IPCR is a method that combines the specificity of immunologic detection methods with the exponential amplification of PCR. First developed by Sano et al., (1992), it has shown detection of less than 100 molecules of analyte corresponding to a 10,000-fold enhancement in sensitivity over standard serological methods for several

target antigens (Sano et al., 1992; Chang and Huang, 1997; Case et al., 1999). We have designed a modified real-time IPCR method for the detection of PrP^{Sc} after appropriate PK digestion to remove PrP^{C}.

Our objectives for this study were two-fold: 1) to assess the maximum sensitivity of IPCR for the detection of decreasing concentrations (down to 10^4 molecules) of recombinant hamster PrP^{C} and for for PrP^{Sc} in scrapie infected hamster brain homogenates; and 2) to compare IPCR with the ELISA to determine the increase in sensitivity for estimation of infectious units (IUs) detectAble in scrapie infected hamster brain homogenates.

In these studies, we demonstrate that IPCR is a method that shows unmatched sensitivity of detection when compared to other standard methods such as the Western Blot and ELISA. We further demonstrate that when using a highly sensitive assay to detect PrP^{Sc} (such as IPCR), complete PK-digestion is critical to avoid detection of residual PrP^{C} when no specific anti-PrP^{Sc} Ab is availAble.

Materials and Methods:

PK digestion of brain homogenates: Normal and scrapie hamster brain homogenates {10% homogenates titered at 10^9 infectious units (IU)} were obtained from the LAboratory of Robert Rohwer (University of Maryland, Baltimore) and recombinant hamster PrP was obtained from Prionics A.G., Schlieren, Switzerland). PK digestion was performed as described by Kang et al., (2003). Briefly, 200 uL of 10% brain homogenate was mixed with 100 uL of 8 M guanidine hydrochloride (GdHCl) and 500 uL of PBS was added for a final concentration of 1 M GdHCl. The solution was mixed and incubated at room temperature for 10 min with agitation, and then centrifuged at 13,800x g for 10 min. The pellet was resuspended in 100 uL of 8 M GdHCl and 700 uL of PBS was added. 400 uL of this solution was incubated with or without PK (50 ug/mL) for 30 min at 37°C. After PK digestion, 1.6 mL of methanol was added to the mixture and the mixture was incubated at -20°C for 1 hr. The sample was centrifuged at 15,800 g for 15 min and the supernatant was removed from the pellet. The supernatent and pellet (which contains the PrP^{Sc}) were both analyzed by Western Blot.

Western Blot: Untreated and PK-digested sample pellets were resuspended in 30 uL sample loading buffer (ref) and heated at 100°C for 5 min before separation on a 12% polyacrylamide gel (Novex, Invitrogen, Carlsbad, CA). After gel electrophoresis, the proteins were transferred to a nitrocellulose membrane (Bio-Rad Inc, Hercules, CA) and the membrane was blocked in 10% Blotto {non-fat milk in PBS with 0.1% Tween 20 (PBST)} before probing with 3 ug/mL 8B4 or 7A12 antibody diluted in 10% Blotto (both 7A12 and 8B4 mouse monoclonal antibodies were provided by Dr. Man-Sun Sy; Case Western Reserve University, Cleveland, OH). The membrane was washed using PBST and anti-mouse HRP (0.2 ug/mL) diluted in 10% Blotto was added. The membrane was then washed and visualized with the LumiGLO Western Blotting Kit (KPL, Gaithersburg, MD).

ELISA: Serial dilutions of untreated and PK-digested normal and scrapie infected hamster brain pellets (reconstituted to the original volume of 400 uL) were made in 0.5% Triton-X/PBS and used for both ELISA and IPCR analyses. Two different ELISA methods were used for the analysis of recombinant hamster PrP^{C}, normal, and scrapie infected hamster brain homogenates. We developed an (in-house) prion ELISA, which is also the serologic portion of the IPCR, using TopYield stripwells (Nalge Nunc Corp., Naperville, IL) as previously described for HIV-1 p24 antigen (Barletta et al., 2004). Briefly, the assay consisted of prion detection using one of two different immobilized capture mouse monoclonal antibodies (Kang et al., 2003): 1) 8B4: which recognizes the N-terminal portion of PrP^{C} (35-45 AA); or 2) 7A12: which recognizes the central region of PrP^{C} and PrP^{Sc} (143-155 AA). The specificities of all antibodies used in these studies are illustrated in Figure 1. Biotinylated mouse monoclonal antibody 3F4 (Signet Pathology Systems, Inc; Dedham, MA) was used as the detector Ab, followed by streptavidin-HRP for colorimetric detection with TMB substrate in ELISA. We compared the in-house ELISA with a commercial ELISA (Enzyme Immunoassay Kit for the Determination of PrP^{C}, cat # A05201, SPIbio Inc., Massey Cedex, France). The SPIbio ELISA was performed exactly as specified by the manufacturer and is an indirect ELISA method with acetylcholinesterase attached to the detector antibody. The cutoff for positive samples for the in-house ELISA was an OD ratio for signal/noise (S/N) of > 2.0. The cutoff for positive samples for the SPIbio ELISA was the average OD of the dilution buffer, which is

nonspecific background (NSB) plus 3 SD (as recommended by the manufacturer). All samples were tested in duplicate and the mean OD of replicates is shown in Figure 3.

IPCR: The procedure for IPCR was identical to the ELISA procedure except for the addition of 10-100 pg/mL biotinylated reporter DNA (500 bp of lambda DNA sequence with 25% incorporated biotinylated dCTP) after the addition of streptavidin-HRP. Real-time PCR was then performed directly in TopYield stripwells using the iCycler iQ™ instrument (Bio-Rad LAboratories, Hercules,CA). The PCR cycling parameters were an initial 6 min at 96°C, followed by 20 cycles of 1 min at 95°C and 2 min at 68°C. After 20 cycles of PCR, 5 uL from each reaction was aliquoted into new PCR reagents in standard PCR tubes for a second amplification round of 50 cycles. Fluorescence was detected during PCR amplification by hydrolysis of a hybridization probe lAbeled with a 5'-reporter dye: 6-carboxyfluorescein, and a 3'-Black Hole Quencher dye (Biosearch Technologies, Inc; Novato, CA).

Technical modifications which we added to the original IPCR protocol (Sano et al., 1992) included using 5 U/mL heparin and 0.5 M EDTA in all wash and diluent buffers, additional blocking steps after addition of streptavidin-HRP and before PCR, the use of a reagent in antibody diluents which blocks non-specific immunologic reactions between antibody molecules (FcR Blocking Reagent, Miltenyi Biotec, Aurora, CA), and 2 rounds of PCR amplification (an initial 20 cycles followed by 50 cycles in fresh PCR reagents in standard PCR tubes). An automated plate washer with individual probes for each microwell was used after the addition of DNA reporter template to decrease the possibility of (well-to-well) cross-contamination.

To determine the detection limit of IPCR for PrP^{Sc}, we generated an IPCR standard curve for dilutions ranging from 10^2 to 10^{11}molecules of recombinant hamster PrP. The specificity of this method was estAblished by screening >2X the number of replicates of negative controls as positive control samples. All samples were tested in 3-4 replicates and the negative controls (normal hamster brain homogenates) were run in replicates of 8-12. The criterion for acceptance of a run was that 75% or greater of the negative controls must fall below the fluorescent threshold. Samples were determined to be positive when the relative fluorescence units (RFUs) of at least 50% of the test replicates

were Above the threshold set for 75% or more of the negative control replicates. Additionally, serial dilutions of recombinant hamster PrP^c standards or scrapie infected hamster brain homogenates had to exhibit a semi-quantitative dose response for the run to be accepted.

The IPCR method involves several unknown variables for the quantification of molecules which are related to the ratio of DNA reporter molecules to antigen (e.g., the number of biotins per antibody molecule, the number of biotins which bind to streptavidin during the reaction, and the number of biotinylated DNA reporter molecules which bind to the tetravalent streptavidins during the reaction. Thus, the standard curve generated by IPCR (in contrast to a PCR standard curve using template DNA) would be the only valid method of extrapolation for molecular numbers. The fluorescent threshold was automatically set by the iCycler instrument and was defined as the mean standard deviation of fluorescence in the sample well over baseline cycles (Bio-Rad LAboratories, iCycler iQ Operating Instructions, Hercules, CA). For some runs, the threshold was manually raised Above the fluorescence level of the negative controls. Cycle threshold (Ct) was the cycle at which the sample's fluorescence intersected and exceeded the background fluorescent threshold (BFT) during continued rounds of amplification. A diagram of real-time IPCR with fluorescent probe detection is shown in Figure 2.

RESULTS:

We determined the level of sensitivity of detection for recombinant hamster PrP^C and PK-digested normal and scrapie infected hamster brain homogenates for both the in-house ELISA and the SPIbio ELISA. The NSB of the SPibio ELISA was 0.114 + 0.009 (3SD) = 0.123. Using logfold dilutions, the lowest concentration of recombinant hamster PrP^C detected was 500 pg/mL (OD = 0.192). PK-digested normal hamster brain homogenate was detected at a 1:100 dilution, slightly above the NSB threshold (OD = 0.207), but not at higher (1:1,000 or 1:10,000) dilutions (ODs = 0.119 and 0.114, respectively). PK-digested scrapie infected hamster brain homogenate was detected at a 1:100 dilution (OD = 0.984), and at a 1:1000 dilution (OD = 0.145) but not at a higher (1:10,000) dilution (OD = 0.118) (Figure 3).

Two different mouse monoclonal antibodies with different specificities for PrP^C were used as the capture antibody in the in-house ELISA and IPCR. 8B4 recognizes the N-terminal end of PrP^C (35 – 45 AA) which is cleaved by PK treatment and thus will detect normal PrP^C (if present) in scrapie infected material after PK digestion. If PrP^C is completely digested, the OD will be $\leq$ NSB in the ELISA and no PrP^C specific (33-35 kDa) band will be detected by Western Blot. 7A12 recognizes the central region of PrP^C and PrP^{Sc} (143 -155 AA) that is retained after PK treatment (Kang et al., 2002) and is specific to a 20-25 kDa band detected by Western Blot (Figures 1 and 4). The lowest concentration of recombinant hamster PrP^C detected by the in-house ELISA using 7A12 was equal to the detection limit of the SPIbio ELISA at 500 pg/mL (S/N = 2.0) (data not shown). PK-digested scrapie infected hamster brain homogenate was detected at 1:100 and 1:1000 dilutions (S/Ns = 40.9 and 4.9, respectively), but not at higher (1:10,000) dilutions by 7A12, and only at a 1:100 dilution (S/N = 4.3) by 8B4 (data is not shown for 1:1000 and 1:10,000 dilutions). Note that 7A12 showed a 9.5 - fold greater reactivity at the 1:100 dilution of PK-digested scrapie infected hamster brain homogenate than 8B4 (S/N = 40.9 versus 4.3, respectively). Additionally, 7A12 showed an approximate 2.3 – fold greater reactivity at the 1:100 dilution of PK-digested scrapie infected hamster brain homogenate than the SPIbio ELISA kit antibody (Figure 3).

Regardless of the higher reactivity of 7A12 for PrP^{Sc}, a low but significant (2X above threshold OD value) difference was still observed (in 50% of experiments performed) using 8B4 for the detection of PrP^C in the 1:100 dilution of PK-digested scrapie infected versus normal hamster brain homogenate (S/N = 4.3) indicating the residual presence of PrP^C in the scrapie infected brain homogenate. Thus, even though 8B4 does not recognize PK-digested PrP^{Sc} (because the N-terminal end of the molecule which includes the recognition site for 8B4 is truncated) the ratio of PK-digested scrapie infected hamster brain to PK-digested normal hamster brain was still interpretable as positive by both the in-house (S/N = 4.3) and SPIbio ELISA (OD = 0.207) where the designation of a positive sample was either a S/N $\geq$ 2.0 or an OD $\geq$ 0.123, respectively

Western Blots were performed using 8B4 and 7A12 to demonstrate the specificity of these two antibodies in the detection of PrP^C versus PrP^{Sc} in supernatant and pellet

preparations of normal and scrapie infected hamster brain homogenates. Both 8B4 and 7A12 detect recombinant hamster PrP^C (Figures. 4a and 4b; Lane 1). However, truncated recombinant human PrP^C is only detected by 7A12 but not 8B4 (Figures 4a and 4b; Lane 2). Non-PK digested normal hamster brain homogenate (supernatant and pellet) is weakly detected by 8B4 and strongly detected by 7A12 (Figures 4a and 4b: Lanes 3 and 4). PK-digested scrapie infected hamster brain homogenate (pellet only) was weakly detected by 7A12, but not 8B4 (Figures 4a and 4b, Lane 6). Note that a shift in molecular weight (from 33 to 20 kDa) indicates the truncated form of PrP^{Sc} is detected by 7A12 (Figure 4b, Lane 6). The specificity differences between 8B4 and 7A12 are most clearly demonstrated using the pellet preparations of PK-digested scrapie infected and normal hamster brain homogenates. Neither Ab detects PK-digested normal hamster brain homogenate (Figures 4a and 4b, Lane 9), but both detect PK-digested scrapie infected hamster brain homogenate (Figures 4a and 4b, Lane 8). However, bands of lower mw (from 20-25 kDa) are present when probed with 7A12, but not 8B4 (Figure 4b, Lane 8 versus Figure 4a, Lane 8, respectively). These data indicate that both 8B4 and 7A12 are detecting residual PrP^C which remain undigested in PK-digested scrapie infected hamster brain homogenates, but the reduction in mw (from 33 to 20-25 kDa) detected only by 7A12 (but not 8B4) indicates the presence of pathologic PrP^{Sc}.

We developed an optimized PCR method for use with the IPCR assay that displayed a correlation coefficient of 0.997, an efficiency of 99.6%, and was able to detect down to

1 molecule of DNA template alone in a standard curve (data not shown). The IPCR standard curve displayed mean Cts of 19.23, 19.02, 25.26, 37.89, 42.86 for 10^{11}, 10^9, 10^7, 10^3, and 10^2 molecules of recombinant hamster PrP^C, respectively (Figure 5a) with a correlation coefficient of 0.961, and an efficiency of 123.3% (Figure 5b). 1 fg/mL (approximately 10^2 molecules/mL) recombinant hamster PrP^C was consistently detectAble when analyzing 2 - 4 replicates of each standard dilution, and 100 ag/mL (10^1 molecules/mL) was detectable in approximately 50% of experiments performed (data not shown). Is this expected using the Poisson distribution? Find out. Note that the standards with higher concentrations of input recombinant hamster PrP^C (10^9 and

309 10^{11} molecules per reaction) are indistinguishable by IPCR and did not display a dose
310 response but instead exhibited nearly identical Cts (i.e., 19.23 and 19.02, respectively)
311 (Figure 5a).
312 As shown in Figure 5a, one of the negative controls (0 PrP molecules) exhibits a
313 linear (not exponential) increase in fluorescence. The source of this sporadic, linear
314 increase in fluorescence is unknown and is currently under investigation. However, the
315 non-exponential nature of the increase in fluorescence makes it unlikely to be due to
316 amplification of non-specifically bound reporter DNA. When erratic or gradually
317 increasing background fluorescence occurs, the BFT must be raised above that level.
318 Raising the BFT will decrease sensitivity of detection as well as (in some cases), skew
319 the dose response. Similar levels of sensitivity of detection (i.e., $10^2 - 10^3$
320 molecules/mL) were observed using a real-time IPCR method specific for HIV-1 p24 Ag
321 (Barletta et al., 2004).
322 Dilutions of PK-digested normal and scrapie infected hamster brain homogenates were
323 tested by IPCR using either 8B4 or 7A12 as the capture Ab. A semi-quantitative dose
324 response relative to the Ct was observed. Under optimal conditions the Ct should
325 increase 3.3 cycles for every log decrease in molecular number. When using 8B4 as
326 the capture Ab, the mean Cts of multiple (2 - 4) determinations for 10^{-4} and 10^{-5} dilutions
327 of PK-digested scrapie infected hamster brain homogenates were 23.4 and 27.1,
328 respectively. Note that one of the 3 replicates of the 10^{-5} dilution was not detected as
329 positive (i.e., below BFT) (Figure 6a). Additionally, the mean Cts of the 10^{-2} and 10^{-3}
330 dilutions were indistinguishAble from the 10^{-4} dilution (e.g., 23.5 and 23.6 versus 23.4)
331 (data not shown). We have repeatedly observed that when high concentrations of PrP
332 are present (i.e.1 ug/mL - 10 ng/mL), quantitative discrimination between adjacent log
333 dilutions does not consistently occur using IPCR.
334 When using 7A12 as the capture antibody, the mean Cts of multiple determinations
335 for the 10^{-3}, 10^{-5}, and 10^{-7} dilutions of PK-digested scrapie infected hamster brain
336 homogenates were 24.9, 37.3, and 45.4, respectively. Note that 2 of the 8 replicates of
337 normal hamster brain homogenates exhibited a linear (not exponential) increase in
338 fluorescence. As previously described, gradually increasing background fluorescence
339 in the negative controls necessitates raising the BFT. In this case, the BFT was raised

to 20 RFU (see BFT #1) (Figure 6b) which skewed the expected dose response. When using BFT #1, the dose response for the PK-digested scrapie infected hamster brain homogenates is not ideal (i.e., equivalent to 3.3 cycles per log decrease in molecular number). Rather in this case, there are approximately 8 -12 cycles between the 2 groups of log dilutions when the appropriate number of cycles should be 6.6 (for the 2 log decrease between each dilution). However, after lowering the BFT to 7.5 RFU (see BFT #2) (Figure 5b), the mean sample Cts correlate well with the dilution series. That is, the mean Cts for 10^{-3}, 10^{-5}, and 10^{-7} dilutions of PK-digested scrapie infected hamster brain homogenates were now 16.6, 23.9, and 29.4 (or an increase of approximately 6 cycles for the 2 log decrease between each dilution). In this case, the Cts of the normal hamster brain homogenates exhibit Cts that are still significantly different (i.e., $\geq$ 6 cycles higher than the mean Ct for the 10^{-7} dilutions of scrapie infected hamster brain homogenate samples).

Finally, the mean Cts correlate well with the estimated prediction of the amount of PrP^{Sc} present in the sample. For instance, it has been noted previously that 1 LD_{50} is defined to be equal to approximately 10 IU or 1.0 pg/mL (Brown et al., 2001). The scrapie infected hamster brain homogenate used was titered at 10^9 IU/ID; therefore, a 10^{-8} dilution of the homogenate would be predicted to equal 10^1 LD_{50} (which is therefore equivalent to 10^2 IU, or approximately 10 pg/mL of PrP^{Sc} (according to Brown et al., 2001). Using the in-house ELISA test with 7A12 as the capture antibody, a 10^{-8} dilution of the homogenate equaled approximately 70 fg/mL to 0.7 pg/mL (which is 14 times less than the predicted concentration (based on information from Brown et al., 2001).

We have observed that grouping two adjacent log dilutions together for analysis often displayed increased accuracy in the Ct quantitative dose response, i.e. the assay, in its current cofiguration, performs best in the semi-quantitative mode. This may be due in part, to the fact that the final exponential amplification process of IPCR disproportionately accentuates small differences in captured molecules. In order to calculate the number of PrP^{Sc} molecules, we grouped the average Ct from two adjacent logfold dilutions of the scrapie infected hamster brain homogenates generated from IPCR (e.g., the average Ct data from the combined a)10^{-3} and 10^{-4}; b) 10^{-5} and 10^{-6}; and c)10^{-7} and 10^{-8} logfold dilutions). The average Cts for each dilution group were 27.8 $\pm$

3.3, 33.0 ± 5.6, and 35.9 ± 5.8, respectively. The regression equation of the recombinant hamster PrP^C standard curve (Figure 5b) was used to calculate the PrP^{Sc} log number of molecules from the optimized mean Cts (Figure 6b, using BFT #2) for the 3 dilution groups (a,b, and c) of PK-digested scrapie infected hamster brain homogenates. These extrapolated number of PrP^{Sc} molecules were then compared to the estimated number of PrP^{Sc} molecules generated from the standard curve of the in-house ELISA using 7A12 as the capture antibody for analysis of recombinant hamster PrP^C. The calculated number of PrP^{Sc} molecules derived from the in-house ELISA standard curve was $8.6 \times 10^{8-9}$, $8.6 \times 10^{6-7}$, and $8.6 \times 10^{4-5}$ for the a, b, and c dilution groups, respectively. Using the IPCR regression equation of the hamster recombinant standard curve, the mean Cts of 27.8, 33.0, and 35.9 for the a, b, and c dilution groups calculated to log 7.5 (antilog = 31,622,777 or 3.1×10^7 molecules), log 5.7 (antilog = 501,187 or 5.0×10^5 molecules), and log 4.7 (antilog = 50,118 or 5.0×10^4 molecules), respectively. These calculated PrP^{Sc} molecular numbers derived from the regression equation of the IPCR standard curve (Figure 5b) are all within 1.5 logs of the estimated number of PrP^{Sc} molecules generated from the in-house ELISA (Figure 3). Brain homogenate sample dilutions, approximate concentrations derived from the in-house ELISA, and calculated PrP^c molecular numbers are described in more detail in Table 1.

DISCUSSION:

Using IPCR, we have detected PrP^C at 100 ag/mL levels (recombinant) and at the equivalent of 1,000 IU of scrapie infected hamster brain homogenate. As with PCR, IPCR incorporates an amplification process (signal amplification) that allows a exponential increase in sensitivity of detection of analyte. The IPCR method for protein detection presently exists as a non-standardized, semi-quantitative analytical test which (when appropriately controlled values are generated) reports greater sensitivity of detection than any test presently available. We emphasize that although the IPCR method is not yet reproducible to the standards required by clinical laboratory testing, there is no doubt that it has the potential (with continued modifications) to become a routine method, similar to PCR.

For the diagnosis of prion infected animals, the units of infectivity (e.g., LD_{50}, IU) as well as the detection limits necessary to obtain valid diagnoses must be well defined. Different literature sources may cite slightly different values for equivalent PrP^{Sc} concentrations to LD_{50} and these sources are often used to estimate the number of LD_{50} which must be detected for pre-clinical diagnosis of BSE and human infection. However, the number of PrP^{Sc} molecules which define an estimate of LD_{50} unit from different sources usually do not exceed a 2-log range. Prusiner et al., (1998) estimates 1 LD_{50} to be equivalent to 10^5 molecules or 5 fg PrP^{Sc}. In contrast, Brown et al., (2001a) estimates that 1 LD_{50} is equivalent to 10 IU, which is approximately equal to 100 fg/mL PrP^{Sc}. The limit of sensitivity of the Western blot is 1,000-3,000 IU/mL (Lee et al., 2000, 2001; Brown P, 2001b; MacGregor, 2001), or approximately 100 pg - 1 ng/mL PrP^{Sc}. Since the levels of infectivity in brain of pre-clinical rodents vary from $10^5 - 10^7$ IU, MacGregor et al., (2001) estimates the sensitivity of detection of the Western Blot and most serology assays (3,000 IU) are at least 33-fold = (10^5/3,000 IU) Above the level required for detection of pre-clinical infected animals.

Other tests described in the literature which report greater sensitivity of detection ($\leq$ 100 pg/mL) than the Western Blot include a paraffin-embedded tissue blot that can detect PrP^{Sc} in mice earlier than the Western Blot (30 days after infection) (Schulz-Schaffer et al., 2000); and a quantitative sandwich ELISA using time-resolved dissociation-enhanced fluorescence technology (DELFIA, EG&G Wallac, Turku, Finland) which states a detection limit in plasma samples of approximately 50 pg/mL (1.4 pM) for PrP^{C} in vCJD patients (i.e., when PK-digestion was performed, no PrP^{Sc} was detected) (Völkel et al., 2001). There is one report of detection of PrP^{Sc} aggregates in CSF down to approximately 0.1 pg/mL (2 fM) by dual-color fluorescent confocal scanning (Bieschke et al., 2000). This level of detection is greater than two orders of magnitude more sensitive than Western blot analysis. The conformation-dependent immunoassay (Safar et al., 1998) also reports a sensitivity which rivals the Western Blot at 1 ng/mL (28 pM). Another diagnostic approach described by Saborio et al., (2001) and Soto et al., (2002) reports amplification of PrP^{Sc} *in vitro* by a cyclical process involving alternate phases of incubation and sonication of the sample. In these experiments a 10,000 fold dilution of scrapie infected hamster brain homogenate generated 6 -12 pg in a 10% normal brain

homogenate (3.4 pM) of dectectable PrP^{Sc} after 10 amplification cycles. All of these procedures appear promising and offer greater sensitivity than currently available methods, but (similar to IPCR) none have been standardized for use in the clinical laboratory.

Although transmission of infectivity has been documented in either naturally or experimentally infected animals, and there is now one report of a likely transmission of vCJD from donor to a blood recipient (Llewelyn et al., 2004), the infectious agent has not yet been detected in the blood of infected cattle or humans. Only Schmerr et al., 1999, report the detection of PrP^{Sc} in sheep and elk blood before clinical symptoms of disease occur (3 months after exposure to infectious PrP^{Sc}) with a sensitivity of detection of 0.5 – 13.5 pg/mL (MacGregor, 2001) using ICCE. In contrast, Cervenakova et al. (2003) were unable to distinguish between extracts from leucocytes from healthy and CJD-infected chimpanzees, or between healthy human donors and patients affected with various forms of CJD using ICCE. However, inconsistencies in the detection of PrP^{Sc} in the blood of pre-clinical animals may be due to differences between the various animal and human model systems. Indeed, there may be other reasons why infectious prion protein is undetectable in blood or pre-clinical specimens by present methodologies. For instance, PrP^{C} may be bound and blocked from molecular interactions by other (ill-defined) protein chaperone molecules in the blood (Telling et al., 1995: DebBurman et al., 1997) or takes on a different conformation in the blood which is unrecognizable by antibodies presently available and used in serological tests. However, it is highly probable that at least one of the reasons PrP^{C} has been undetectable in blood is due to a lack of sensitivity of currently available methods.

The two ELISA methods (the in-house and the SPIbio ELISA) used in our studies to analyze recombinant hamster PrP^{C} as well as PK-digested normal and scrapie infected hamster brain homogenates were comparable in sensitivity, exhibiting limits of detection to 500 pg/mL for recombinant hamster PrP^{C}. Additionally, PrP^{Sc} in PK-digested scrapie infected hamster brain was detected at both 1:100 and 1:1000 dilutions (10^{7} to 10^{8} IU) using both ELISA methods.

The IPCR standard curve for recombinant PrP^{C} displayed a correlation coefficient of 0.961 (Figure 5b) which is not as precise as an optimized PCR amplification of a DNA

template alone. However, unlike PCR, the IPCR protocol involves the primary step of antibody recognition of recombinant hamster PrP^C, which results in an indirect estimation of the target: a variable in the IPCR test protocol which may account for a loss of precision when compared to a standard curve generated by PCR of DNA template alone. A semi-quantitative dose response represented by increasing Ct with decreasing recombinant hamster PrP^C molecule numbers or increasing dilutions of scrapie infected hamster brain homogenates was consistently observed.

One caveat of any test which uses PK digestion (and not an antibody specific to PrP^{Sc}) as the defining criteria for determination of scrapie infected versus normal tissues is that normal PrP^C may not be completely digested and will be detected by the non-specific antibody. The fraction of PrP^C remaining in scrapie infected brain from terminally ill animals is estimated to be lower than 0.1% of the original amount of PrP^C (European Commission; March 2002, p. 69) and thus has not been a serious problem with less sensitive testing methods where the cutoff range for negative (i.e., mean NSB + 3SD) will exclude 99% of the normal (uninfected) population. Frequently, 5 times the standard deviation is added to the mean of the NSB samples to insure 100% exclusion of samples which may be designated as falsely positive (European Commission, March 2002, p. 29). Therefore, it is likely that if dilutions of brain homogenates are not optimized for complete PK digestion, highly sensitive assay methods (such as IPCR) will detect residual PrP^C in the PK-digested sample resulting in a false positive result. An example of this was shown in our studies when using low dilutions of the brain homogenate (such as 1:100) and an antibody (8B4) which only detects the truncated portion (N-terminus) of PrP^C after PK digestion. Although slightly above the NSB threshold cutoff, the ratio of PK-digested scrapie infected hamster brain to PK-digested normal hamster brain was interpreted as positive by both the in-house and SPIbio ELISA at the 1:100 dilution, but not at higher dilutions (Figure 3). This occurred in 50% of the experiments performed; whereas, in all other experiments the PK digestion was complete and PrP^C was not detected by 8B4 for any dilutons or scrapie infected brain homogenates. We therefore believe, this may have been an artefactual occurrence resulting from variations in reagents and not due to lack of optimization of the established PK digestion protocol (refs). It is likely that these ratios which designate a positive sample for PrP^{Sc} accounted for the positive amplification

observed in the IPCR using 8B4 with scrapie infected hamster brain versus normal hamster brain homogenates at both 10^{-4} and 10^{-5} dilutions of the scrapie infected hamster brain homogenate (Figure 6a).

It has been documented that the level of PrP^{Sc} in the brains of clinically ill animals exceeds that of PrP^{C} by 3-10 fold (Safar et al., 1998) and therefore is not likely to pose a problem after optimized PK digestion. Various studies have shown that hamster PrP^{C} is expressed at levels of approximately 70 ug/gm in brain tissue (Pan et al.,1992; Groschup et al., 1997). Safar et al; (1998) have stated that the PrP^{C} concentration in 8 different prion strains was $\leq$ 5 ug/mL. We have not found data in the literature quantifying the exact ratio of PrP^{C} to PrP^{Sc} in the same sample of scrapie infected hamster brain homogenate. The reason for this may be that the determination of PrP^{C} would be problemmatic if a PrP^{Sc} specific antibody is not available for use. That is...PK digestion must be performed to remove the majority of PrP^{C} leaving PrP^{Sc} intact; however, without PK digestion and/or a PrP^{Sc} specific antibody for detection, the presence of PrP^{C} alone cannot be ascertained as all commercial anti-prion antibodies will detect both PrP^{C} and PrP^{Sc}. Another possibility for this effect is that highly folded conformations of PrP^{Sc} may exist which retain the N-terminus even after extensive PK digestion and are therefore detected by N-terminus specific antibodies such as 8B4. Clearly, the use of a PrP^{Sc} specific antibody would be preferable to the use of PK digestion. Several monoclonal antibodies specific for PrP^{Sc} have been reported, but they have not yet been widely distributed (Korth et al., 1997; Paranithiotis et al., 2003; Serbac et al., 2004).

In order to address these issues, we used the Western Blot to verify that the PrP^{Sc} detected by IPCR was the abnormal conformation which exhibits a 20-25 kDa band on the blot. Using 2 antibodies with distinct specificities for either the non-truncated (8B4) versus the truncated (7A12) version of PrP^{Sc}, we were able to verify that PrP^{C} could be detected in pellet preparations of PK-digested scrapie infected hamster brain homogenate by 8B4. The concomitant presence of PrP^{Sc} (detected by 7A12 but not 8B4) was identified by a shift in molecular weight from 33-35 kDa to 20-25 kDa. Further, using 8B4, the highest dilution of the same PK-digested scrapie infected hamster brain homogenate detected by IPCR was 10^{-5}. However, a 10^{-7} dilution of this homogenate was detected by 7A12, indicating that PK digestion for residual PrP^{C} was complete (and not detected by

8B4) somewhere between the 10^{-5} and 10^{-7} dilutions (See Figures 6a and 6b). Thus, it is important when using a highly sensitive test to insure the removal of all PrP^{C}. To accomplish this, it may be necessary to perform PK digestion on several dilutions of the sample with both 8B4 and 7A12 (or a N-terminus versus a C-terminus specific antibody) to assess the presence of PrP^{Sc}. Only when the N-terminus specific antibody is non-reactive with the sample and the C-terminus specific antibody is reactive should the sample be considered suitable for testing with highly sensitive methods such as the IPCR.

Using IPCR, only a semi-quantitative dose response was observed when analyzing increasing dilutions of PK-digested scrapie infected hamster brain homogenates. Various reasons for this observation include the fact that the IPCR methodology is not yet well standardized and many variables exist within the test format impact upon the final accuracy and reproducibility of the assay. These variables include the ratio of DNA reporter molecules to antigen, which may in turn be affected by several other variables (e.g., the number of biotins per DNA molecule, the number of biotins per antibody molecule, the number of biotins which bind to avidin during the reaction, the number of biotinylated DNA reporter molecules which bind to the tetrvalent streptavidins during the reaction), as well as the exponential amplification of the PCR method which accentuates the downstream effects of any of the above variables. Another reason that a quantitative dose response is not always observed in the IPCR analysis is that the PrP^{Sc} molecule may be present as aggregates in solution and difficult to emulsify. If these aggregates are not adequately dispersed during the initial preparation of the sample, variations or sampling error between dilution series may result. In fact, more precise dose responses were often observed when grouping the data from 2 adjacent dilutions of homogenates. Finally, pipette inaccuracies may account for sporadic variations in results. Pipettor CVs of as low as 5% may result in deviations of _____% (e.g., the Amplicor RT-PCR test for HIV-1 detection has CVs which range from ____to 100% for samples which contain_____ viral copies.

It has previously been shown that one of the most common problems associated with the use of IPCR is the reduction of sensitivity due to high background (non-specific

amplification) in the negative controls (McKie et al., 2002a; 2002b, 2002c). In the performance of the ELISA, if more than 33% of the negative controls react as false positives (a typical standard of FDA approved ELISA tests: Coulter HIV-1 p24 Antigen Assay, Coulter Corp., Miami, FL), then the test results are invalid. We enforced a more stringent criteria for the acceptance of false positive control reactions in this study; i.e., a run was not accepted if > 25% (2 out of 8) of the negative controls reacted as false positive. When higher levels of non-specific background fluorescence are present in the IPCR, the fluorescent threshold may need to be raised, effectively and artefactually decreasing the sensitivity of detection. Higher background RFU was seen sporadically with the IPCR and when this occurred, the sensitivity of detection was reduced. High RFU levels displace the optimal setting of the BFT which, in turn, directly affects placement of Ct values (Figures. 5a and 6b).

We have modified the reported IPCR protocols in several ways to substantially reduce the occurrence of false positives (See Materials and Methods). Our modified IPCR protocol significantly reduces false positives in the negative controls down to a range where 75-80% of normal controls remain below the BFT. The specificity of the modified IPCR method approaches 100%, as positive samples were defined only if above the threshold setting for the negative controls. It is likely that these technical modifications may compromise the sensitivity of the IPCR. However, detection down to 1 molecule/mL of PrP^{Sc} in a biological specimen may not be a prerequisite for diagnosis of pre-clinical prion infection. Presently, based on the IPCR standard curve, the limit of detection for scrapie infected hamster brain homogenate using the modified IPCR protocol is $8.6 \times 10^{4\text{-}5}$ PrP^{Sc} molecules/mL, or X number of molecules of PrP^{Sc} per reaction. [I think its important to distinguish between concentration per ml, and the amount of stuff that goes into each reaction] This number of PrP molecules is equal to 70 – 700 fg/mL when estimated from the ELISA experimental data, and 1.0 – 0.1 pg/mL (50,118 PrP^{Sc} molecules/mL) when calculated from the standard curve using recombinant hamster PrP^{C}. A lower limit of detection using the modified IPCR was attained for recombinant hamster PrP^{C} (e.g., $10^2 – 10^3$ PrP^{C} molecules); however, greater sensitivity of detection is typically attained for the detection of a purified protein (such as recombinant hamster PrP^{C}).

For IPCR, the test sample requires extremely low sample volumes (uL) for optimal amplification by the use of high sample dilutions (ranging from 10^{-2} to 10^{-8}). Although PCR amplification is required, technically advanced equipment is no longer required as portable and inexpensive thermal cycler units are now readily available. PCR reagents may be lyophilized and packaged for long-term stability so that highly trained personnel and controlled laboratory conditions are less a requirement for its performance. The exquisitely powerful amplification potential of the IPCR is exemplified in its ability to detect extremely low molecular numbers of target molecules without sample concentration.

Our study sought to confirm, and is the first to demonstrate, that ultra-low detection of PrP^{Sc} is possible with IPCR without the use of large sample volumes, sample concentration methods, or extensive sample processing. We have shown that with modifications non-specific background in the IPCR method may be substantially reduced, if not completely eliminated, and thereby increase the sensitivity of the method. Although there are relatively few publications describing IPCR protocols (approximately 60 are available from PubMed dating back to Sano et al., 1992), several other groups have performed real-time IPCR (Sims et al., 2000; McKie et al., 2002b, 2002c; Adler et al., 2003) and continue to make novel modifications to the standard IPCR method which have shown dramatic improvements in the sensitivity and specificity of the method. Many of these modifications involve a sophisticated design of reagents with strategic approaches to attain consistent accuracy and reproducibility. These methodological changes exceed the typical and routine approaches which are usually applied to optimize the ELISA portion of the IPCR, such as testing different blocking agents, washing protocols, and reagent titration. Some of these novel approaches include the attachment of the DNA template directly to the detector antibody (Hendrickson et al., 1995) or the synthesis of pre-complexed reagents before addition to the reaction mix (Hendrickson et al., 1995; Niemeyer et al., 1998, 1999, 2003) as well as novel designs of the reporter molecule and/or molecules to insure that only specifically bound and not extraneous or non-specifically adherent DNA reporter molecules are amplified (Schweitzer et al., 2000; Nam et al., 2003). Another interesting approach has been to use 2 detector antibodies with attached DNA reporter molecules which must be ligated in the reaction in order to be

amplified by PCR as a specific (versus non-specific) reporter (Baez et al., 2003; patent #6,511,809).

Thus, given the exponential ability of the PCR method, it is logical to presume IPCR is one method that has the potential to detect ultra-low levels of protein in a sample. In fact, the evolution of IPCR may be closely compared to the history of development of the PCR. At least 10 years of gradual technical improvements to the PCR now define the method as the gold standard for a highly quantitative and sensitive assay. Today, with these refinements, it has replaced all other quantitative assays for nucleic acid targets, possessing a dynamic range of 6 logs and a sensitivity of detection to 1 molecule. Yet, at one time, PCR was proclaimed to be only a qualitative, or at best, semi-quantitative method (refs). Because of the possibility that PrP^{Sc} is present at extremely low concentrations (fg-ag/mL) in preclinical specimens, we believe that with continued modifications and improvements to the method, IPCR is the most likely method to detect PrP^{Sc} at the earliest timepoint in the course of TSE disease. This could address pre-clinical identification of prion disease, thereby offering a method for further protecting the blood supply and the food-chain.

Our primary intent was to demonstrate the exquisitely sensitive amplification ability of the IPCR technique. These studies are the first to show that PrP^{Sc} is able to be detected by real-time IPCR in biological samples of PK-digested scrapie brain homogenates at dilutions which are one million-fold higher than those detected by Western Blot and ELISA. We have shown that IPCR possesses the potential with continued modifications to completely eliminate non-specific background in approximately 80% of assay runs, and thereby increase the sensitivity of the method to detect fg/mL to ag/mL levels. Although not standardized to the level of performance required by routine clinical laboratory tests or certification by a national accrediting agency, IPCR has been shown to be successful by over 50 documented publications in the literature. Since the original protocol was first developed by Sano (1992). IPCR has repeatedly and conclusively been validated by many different scientific groups who have concluded that data generated from the IPCR method is valid when extracted from experimental runs where the appropriate controls are acceptable.

Finally, we emphasize that it is critical that continued research and development of the IPCR method be pursued by the scientific community and manufacturers in order to attain the goal of unparalleled sensitivity for protein detection. We predict that with further standardization, IPCR has the potential to provide the most accurate mechanism to screen the blood supply, monitor animal food and commercial products, and diagnose asymptomatic persons for the presence of pathologic prion.

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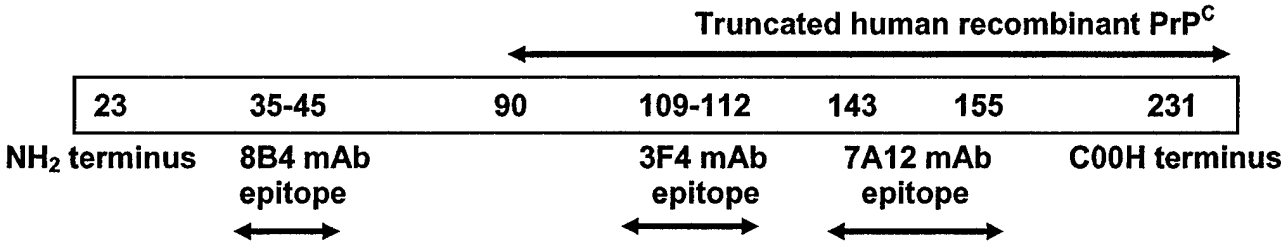
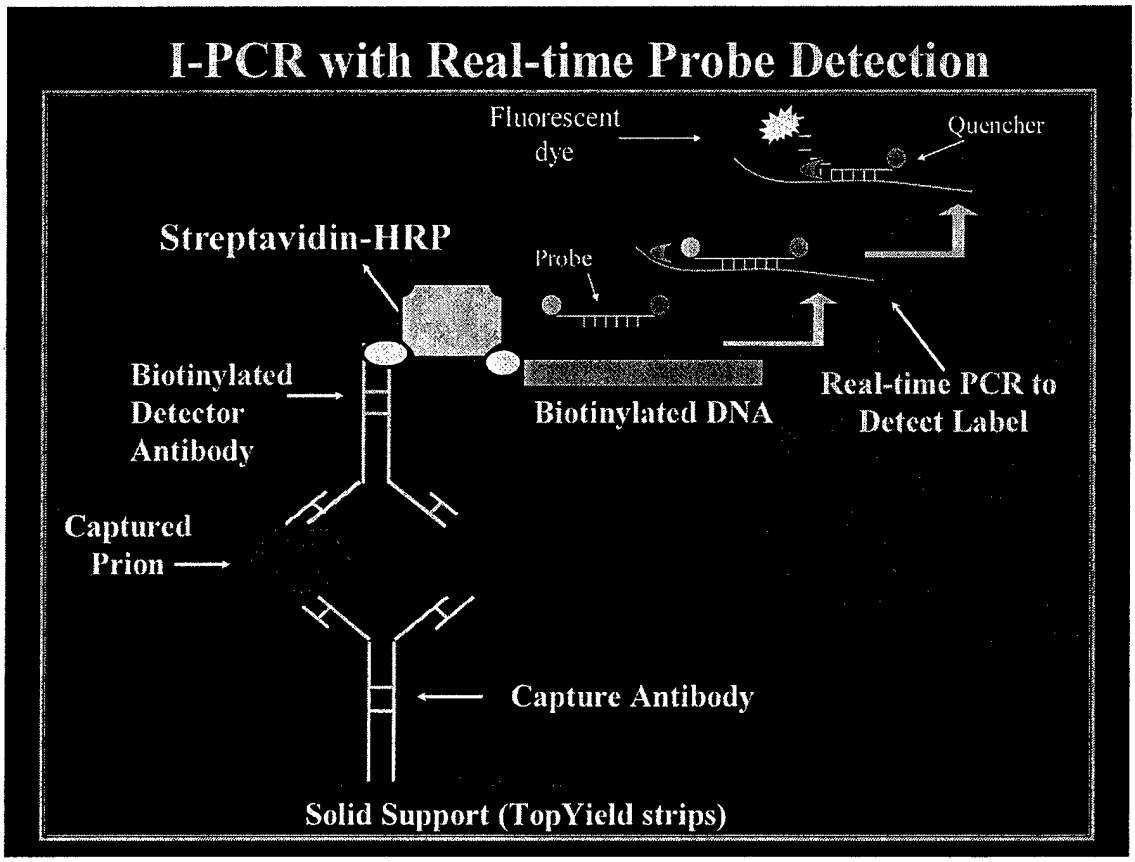

Truncated human recombinant PrP^C
23
35-45
90
109-112
143
155
231
NH$_2$ terminus
8B4 mAb epitope
3F4 mAb epitope
7A12 mAb epitope
C00H terminus

Figure 1.



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1028 **Figure 2.**
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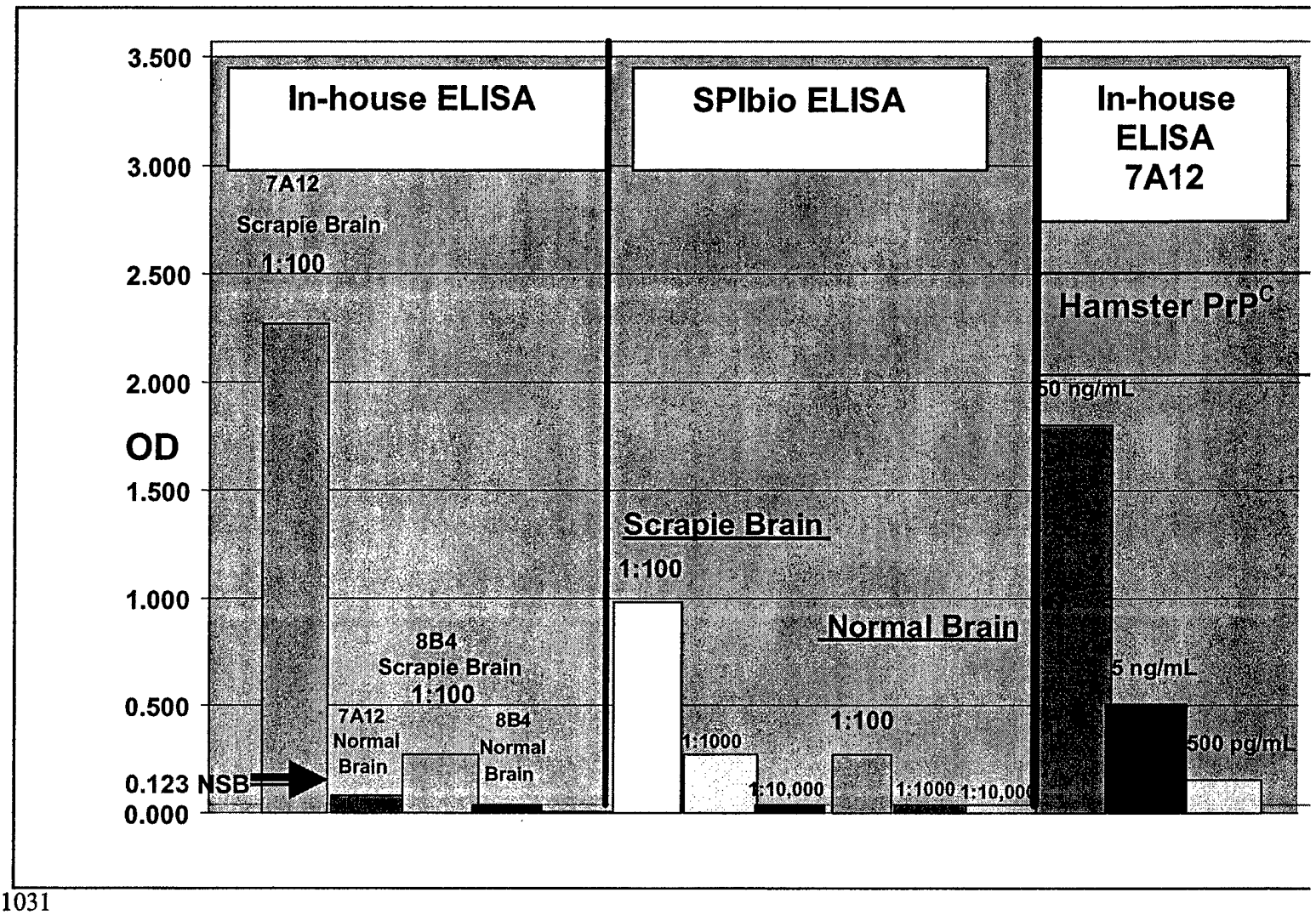
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.123 NSB
0.000
OD
In-house ELISA
SPIbio ELISA
In-house ELISA 7A12
7A12
Scrapie Brain
1:100
8B4
Scrapie Brain
1:100
7A12
Normal
Brain
8B4
Normal
Brain
Scrapie Brain
1:100
Normal Brain
1:100
1:1000
1:10,000
1:1000
1:10,000
Hamster PrP^C
50 ng/mL
5 ng/mL
500 pg/mL

Figure 3.

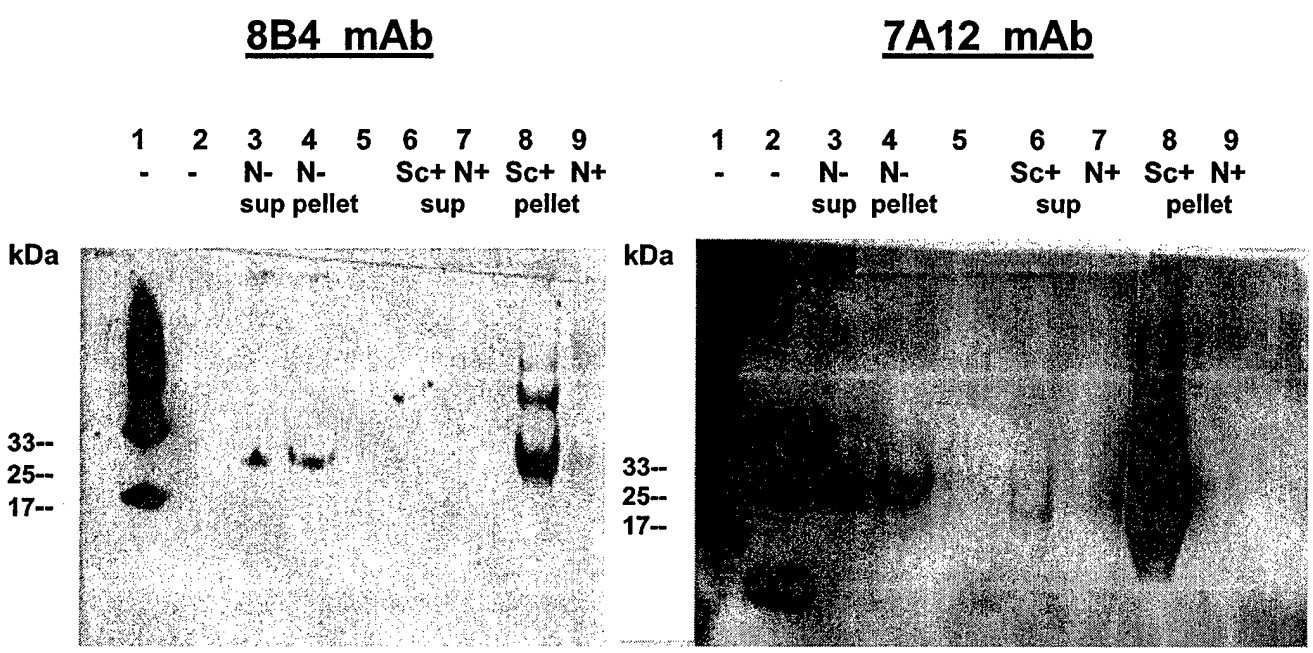
8B4 mAb
1 2 3 4 5 6 7 8 9
- - N- N- Sc+ N+ Sc+ N+
sup pellet sup pellet
kDa
33--
25--
17--
7A12 mAb
1 2 3 4 5 6 7 8 9
- - N- N- Sc+ N+ Sc+ N+
sup pellet sup pellet
kDa
33--
25--
17--

Figure 4a. 4b.

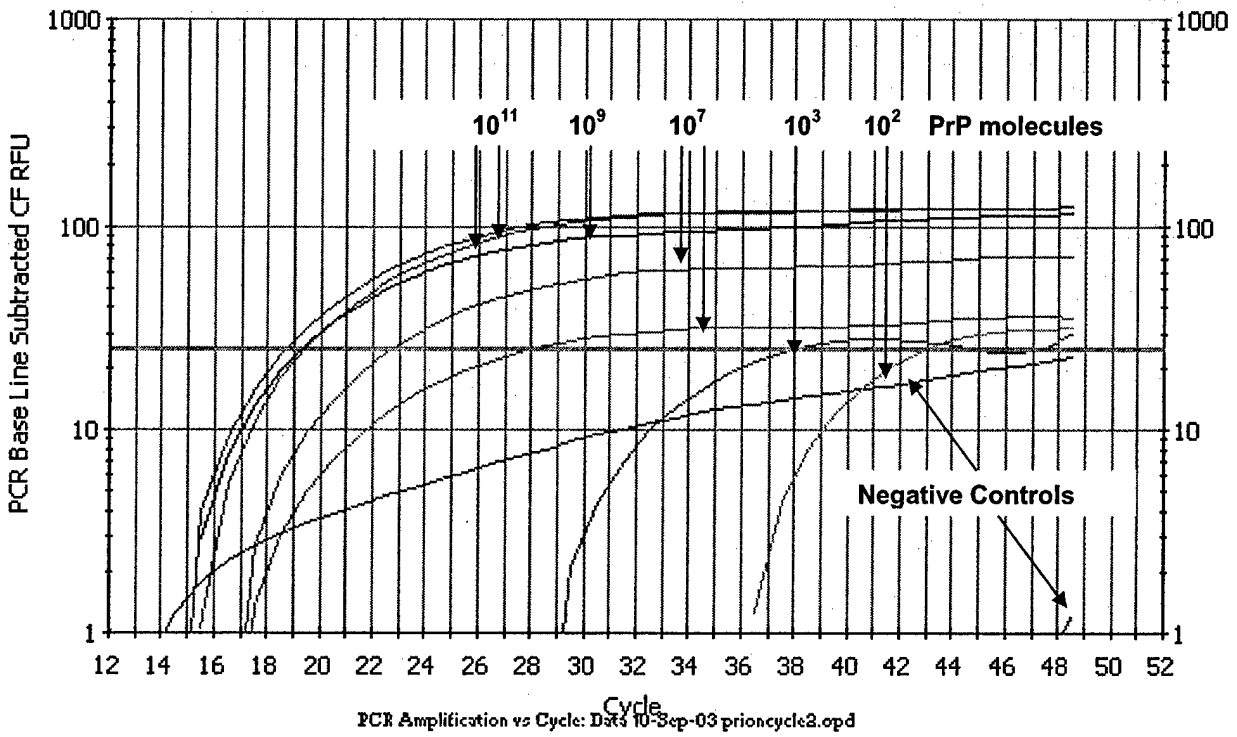

Figure 5a.

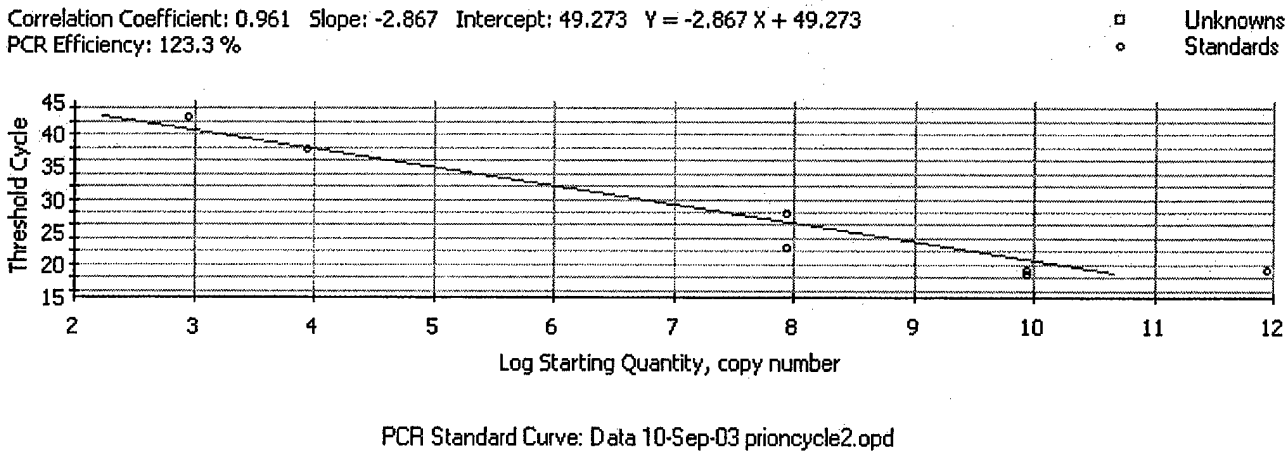

Figure 5b.

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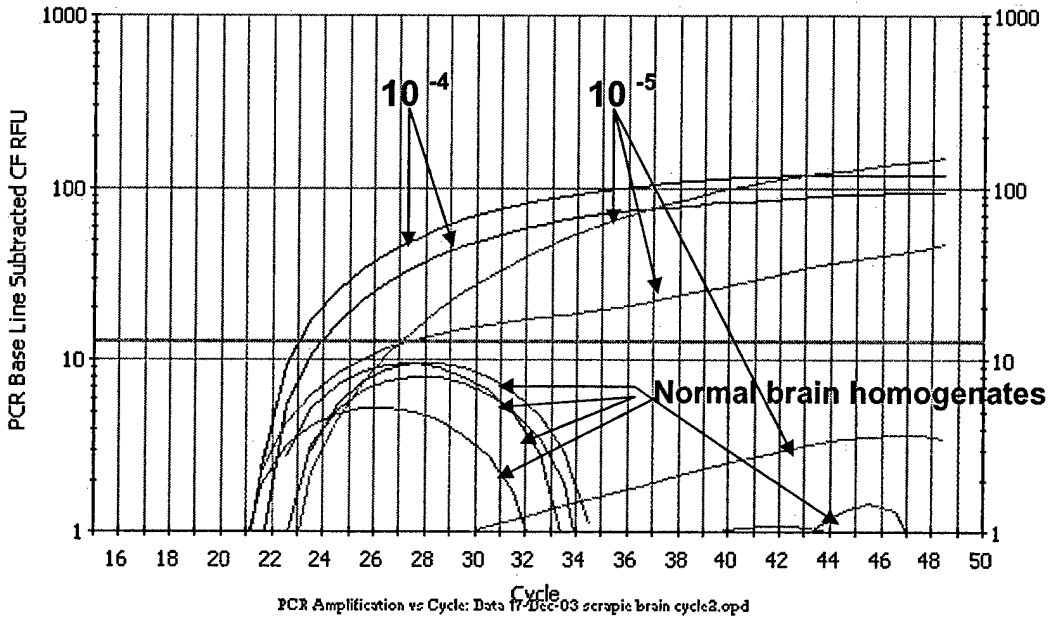
Dilutions of PK-digested
Scrapie brain homogenates (detected with 8B4 mAntibody)



1078
1079 **Figure 6a.**
1080
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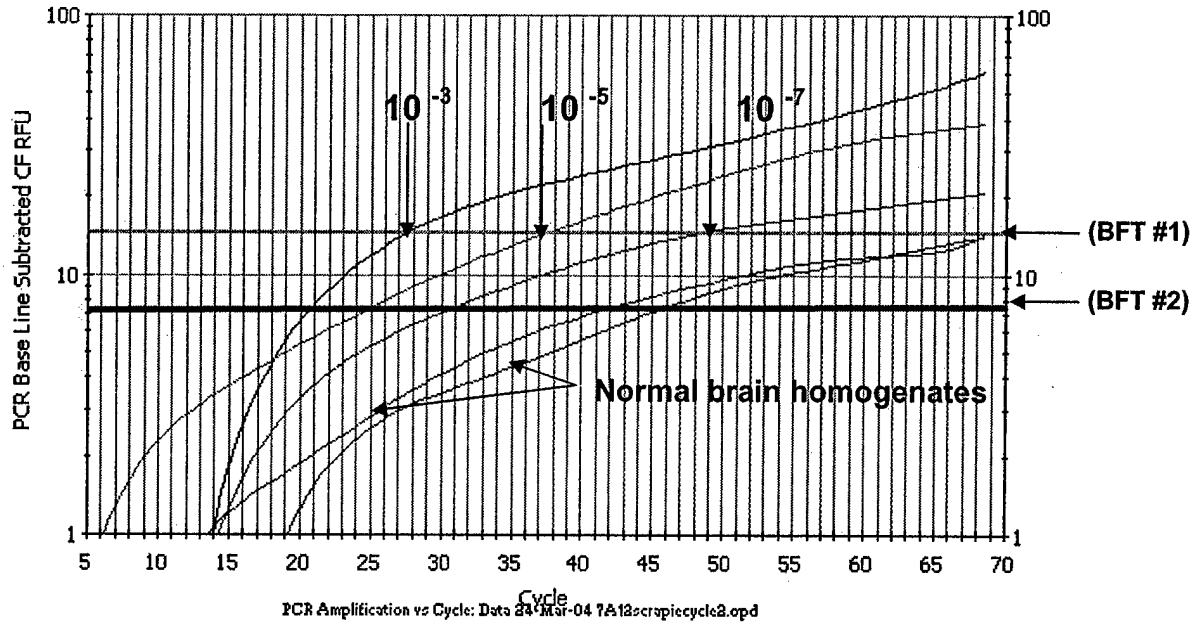
1082

Dilutions of PK- digested
Scrapie brain homogenates (detected with 7A12 mAntibody)



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1085 **Figure 6b.**
1086

Table 1: Determination of LD_{50}, IU, PrP^{Sc} Concentration, and Number of PrP^{Sc} Molecules in Serial Dilutions of Scrapie Infected Hamster Brain Homogenates Derived from IPCR Average Ct Using 7A12

Dilutions of Scrapie Infected Hamster Brain Homogenates	Average Ct[a]	LD_{50}[b]	IU[c]	Approximate Concentration[d] of PrP^{Sc} in Scrapie Infected Hamster Brain Homogenates	Calculated Number of PrP^{Sc} molecules/mL[e]
10^{-2}	THTD[f]	10^{7}	10^{8}	70 ng/mL	8.6×10^{10}
10^{-3} - 10^{-4}	27.8 ± 3.3	10^{5-6}	10^{6-7}	0.7 - 7 ng/mL	$8.6 \times 10^{8-9}$
10^{-5} - 10^{-6}	33.0 ± 5.6	10^{3-4}	10^{4-5}	7 - 70 pg/mL	$8.6 \times 10^{6-7}$
10^{-7} - 10^{-8}	35.9 ± 5.8	10^{1-2}	10^{2-3}	0.7 - 70 fg/mL	$8.6 \times 10^{4-5}$

a. Average Ct ± SE was determined from 9-12 replicates from 5 experiments.

b. LD_{50} is the amount of infectivity that will transmit disease to 50% of a group of animals.

c. Infectious unit or infectious dose (IU) is the amount of infectivity that will transmit disease to one animal. The estimate of IU is based on LD_{50} is described by Brown et al. (2001).

d. Extrapolated from the in-house ELISA test using recombinant hamster PrP^{C} as a standard and 7A12 as the capture antibody. A 1:100 dilution of the PK-digested scrapie infected hamster brain homogenate is approximately equal to 70 ng/mLrecombinant hamster PrP^{C} (See Figure 3).

e. Calculated from the equation: $\frac{PrP^{Sc} \text{ concentration (weight/mL)}}{35{,}000 \text{ mw}} \times \frac{(6 \times 10^{23}) \text{ molecules}}{\text{mole}}$

f. THTD: Too high to determine.

Figure 1. Schematic representation of human PrP. Numbers represent the amino acid (AA)sequence. The underlined regions represent the AA recognition sites of the 8B4, 3F4, and 7A12 antibodies and truncated human recombinant PrP^C.

Figure 2. Diagram of the IPCR technique. Capture anti-PrP antibody, adsorbed to the microwell plate, is used to capture PrP antigen. Streptavidin/HRP bridges between a biotinylated detector anti-PrP antibody and biotinylated 500 bp reporter DNA. The reporter DNA is amplified by PCR using a fluorescent probe for real-time analysis.

Figure 3. In-house ELISA and SPIbio ELISA on PK-digested Scrapie Infected and Normal Hamster Brain Homogenates, and recombinant hamster PrP^C. The in-house ELISA (using 7A12 or 8B4 as capture Ab) or the SPIbio ELISA were performed on dilutions of a 10% homogenate of scrapie infected or normal hamster brain digested with 50 ug/mL PK at 37° for 30 min. The non specific background (NSB) threshold of the in-house ELISA was a mean OD 0.056 $\pm$ 0.003. Positive samples are defined as signal to background (S/N) of $\geq$ 2.0. The NSB threshold of the SPIbio ELISA is the mean OD of 4 replicates of NSB controls + 3SD (0.114 + 0.009= 0.123). Samples Above this threshold were considered positive. recombinant hamster PrP was used as a standard control and not PK-digested. Therecombinant hamster PrP^C was detected with 7A12. (All tests were performed in duplicate in multiple experiments. For all replicates, the SE of the replicates was too small to display in the graph).

Figure 4. Immunoblot Analysis of Prion Protein Expression of Scrapie Infected and Normal Hamster Brain Homogenates Using 8B4 and 7A12 Antibodies. 400 uL of a 1:4 dilution of brain homogenate was incubated with or without PK (50 ug/ml) for 30 min at 37° C. This preparation was centrifuged and the supernatant (sup) or pellet was used for Western Blot detection of PrP^C or PrP^{Sc} by 8B4 (**a**) or 7A12 (**b**) as the detector antibodies. (**-, +**) denotes non PK-digested and PK-digested; and (**N, Sc**) denotes normal versus scrapie infected hamster brain homogenates, respectively.

Lanes: 1: 5.0 ng of recombinant hamster PrP^C;
2: 2.5 ng of truncated human recombinant PrP^C (90-231 AA);
3 and 4: Normal hamster brain homogenate supernatant and pellet;
6 and 7: Scrapie infected and normal hamster brain homogenate supernatants;
8 and 9: Scrapie infected and normal brain homogenate pellets. After probing blot with 8B4, the membrane was stripped and re-probed with 7A12 .

Figure 5. IPCR Standard Curve. **a.** IPCR was performed on serial dilutions of recombinant hamster PrP ranging from 10^{11} to 10^2 molecules for the standard curve. The threshold setting is placed at 25 RFU to exclude one negative control with low levels of fluorescence. **b.** The correlation coefficient of the IPCR standard curve with the corresponding regression equation. The mean Ct was derived from 2-4 replicates.

Figure 6. IPCR of PK-digested Scrapie Infected and Normal Hamster Brain. A 10% homogenate of normal or scrapie infected hamster brain was digested with 50 ug/mL PK at 37° C for 30 min. IPCR was performed on serial dilutions **a.** (10^4 and 10^5) and, **b.** (10^3, 10^5, and 10^7) of scrapie infected hamster brain (PK-digested) homogenates using 8B4 (**a**) or 7A12 (**b**) as the capture antibody. A 10^6 dilution was below the level of detection for 8B4 but a 10^7 dilution was detectable by 7A12 indicating that all residual PrP^c had been digested. Samples shown in graph are representative of 3-4 replicates where 50% or more of the replicates were Above the fluorescent threshold. 80% of all negative controls (8 replicates) were below the fluorescent threshold. Not all replicates of the normal brain homogenates are visible on the graph. The 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-7} dilutions are approximately 7 ng/mL, 0.7 ng/mL, 70

pg/mL, and 700 fg/mL (or 10^7, 10^6, 10^5, and 10^3 ID), respectively. **BFT #1 or BFT #2** = background fluorescent threshold #1 setting versus #2 setting.

Detecting Attogram Levels of Prion Protein

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Janet Barletta, Ph.D.
University of Maryland
School of Medicine

Need for an Ultra-Sensitive Method for Prion Protein Detection

- **Detection in the Blood of Live Humans and Animals**
- **Detection in Blood Products**
- **Detection in Environmental Materials**
- **Detection in Food**

Blood Transmission of Prion

- Blood from experimentally infected animals with scrapie or CJD transmits by IC inoculation.

- Blood from 4/37 clinical CJD cases transmits via IC to mice.

* Transfused blood from infected sheep to naive sheep transmitted disease 300 days prior to the occurrence of symptoms.

Rationale

The Prion Infectious Unit Is Present in Blood

Markers for the demonstration of the infectious unit cannot be detected in blood, but can be in tissues where infectious units are in high quantities

The lack of marker demonstration in blood is due to extremely low levels of the marker, and detection can be addressed using exquisitely sensitive methods

Objective

To Apply a Novel Amplification Method to Increase the Sensitivity for Detection of Prion Protein

Current Prion Diagnostics

Accepted:

- Immunohistochemistry on tissue
- Western blot for diagnosis on tissue (Prionics)
- ELISA on Tissue (Enfer, BioRad, Others)
- Brain Markers in CSF (14-3-3)

Research:

- Rapid Brain Tests
- Time Resolved Fluorescence
- Capillary Electrophoresis
- PMCA, EDRF, MUPS, Others

Calculations and Conversions

- Infectious Units
- Concentration in Grams
- Number of Molecules

Infectious Unit

The minimum amount of infectious material capable of transmitting disease

Concentration

Units of Detection

Microgram	(ug)	10^{-6}
Nanogram	(ng)	10^{-9}
Picogram	(pg)	10^{-12}
Femtogram	(fg)	10^{-15}
Attogram	(ag)	10^{-18}
Zeptogram	(zp)	10^{-21}
Yactogram	(yg)	10^{-24}

Concentration versus Molecules

1 Femtogram = 17,000 molecules

6 Attograms = 100 Molecules

58 Zeptograms = 1 Molecule

Infectious Unit / Concentration / Molecules in Scrapie Brain Homogenate

Dilution	IU	PrP Conc.	No. PrP molecules/mL
1:100 =	**10^7 IU**	**5 ng/mL**	**8.6 x 10^{10}**
1:10,000=	10^5 IU	50 pg/mL	8.6 x 10^8
1:100,000=	**10^4 IU**	**5 pg/mL**	**8.6 x 10^7**
1:1,000,000=	10^3 IU	0.5 pg/mL	8.6 x 10^6
1:10,000,000=	**10^2 IU**	**50 fg/mL**	**8.6 x 10^5**
1:100,000,000=	10^1 IU	5 fg/mL	8.6 x 10^4

Scrapie Infectivity in Blood

- Preclinical (5-13 weeks): 6-12 IU/mL
- Onset of clinical signs (18 weeks):
  Plasma: 20 IU/mL*
- Terminal Stages (20 weeks): 10 ID/mL**

"10 pg/mL of blood represents the level of sensitivity required of any assay for the detection of PrP in blood and would almost certainly need to be increased to the range of 1 pg/mL for use as a preclinical screening test."*

*Brown et al., 1999; Brown et al., 2001.
**Holada et al., 2002.

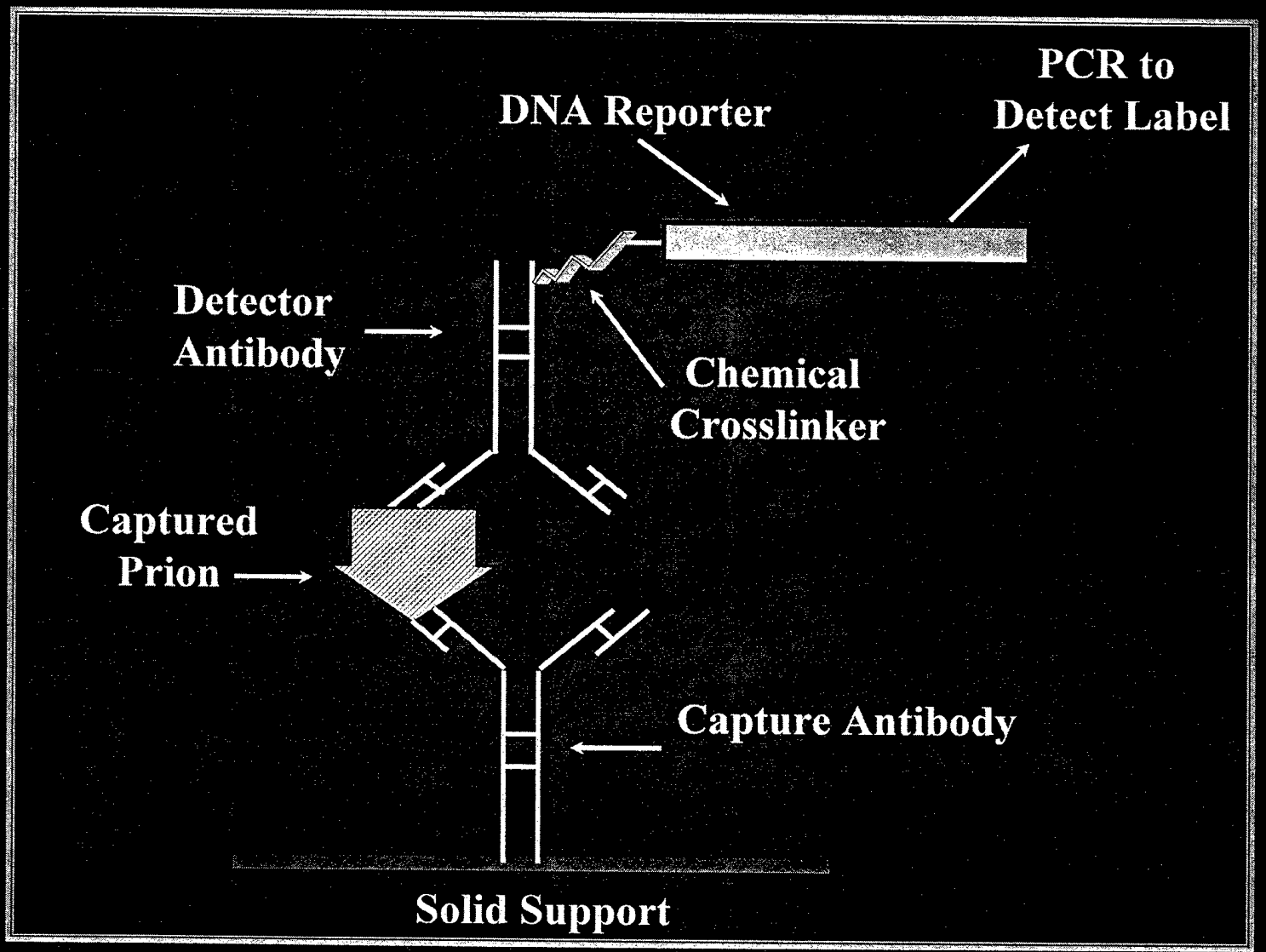
Analytical Detection by Immunologic Assays

Immunofluorescence	ng/ml
ELISA	ng-pg/ml
Western blot	ng-pg/ml
Chemiluminescence, RIA	pg/ml
DELFIA	fg/ml

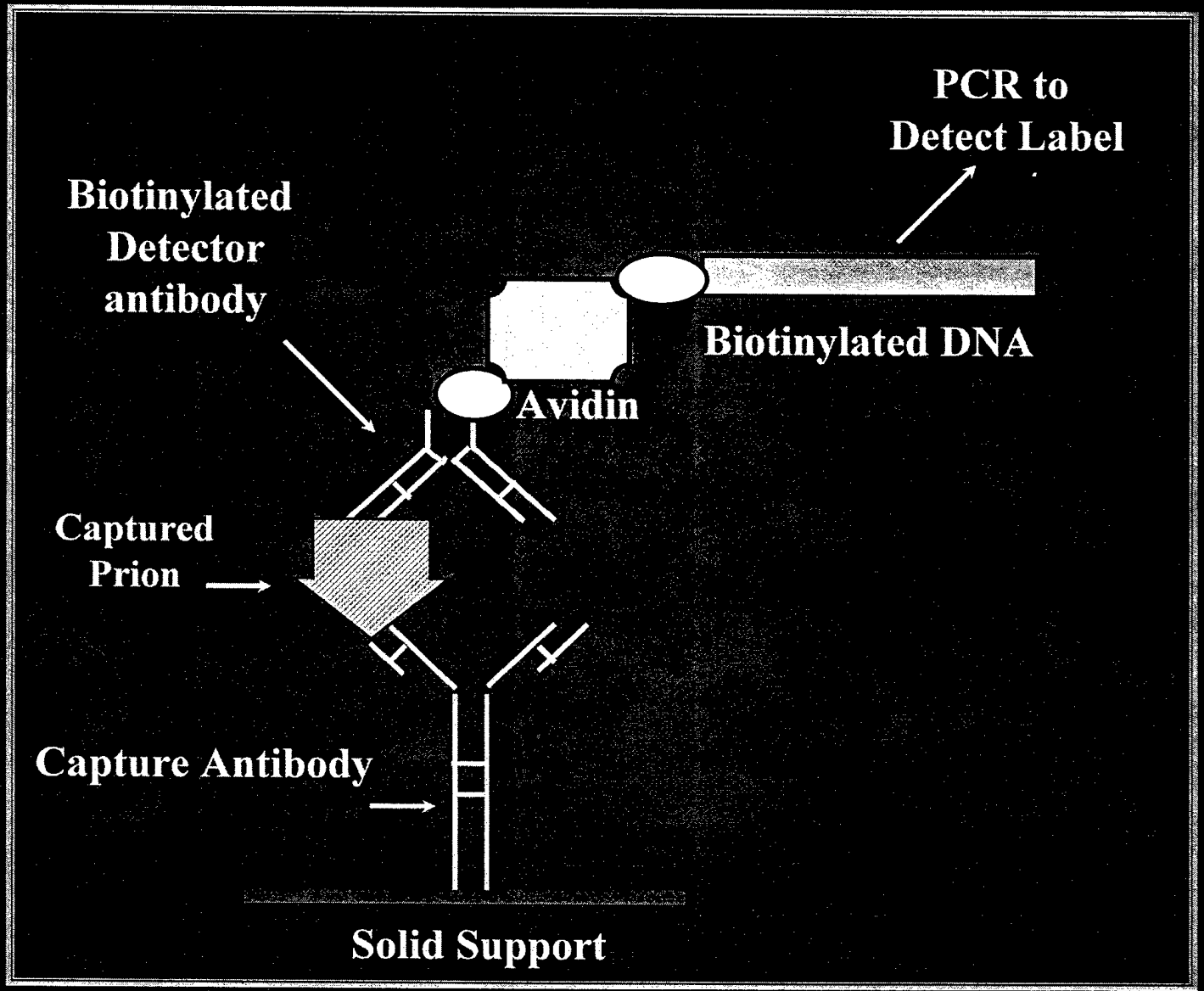
Method

Immuno-
Polymerase Chain Reaction
(I-PCR)

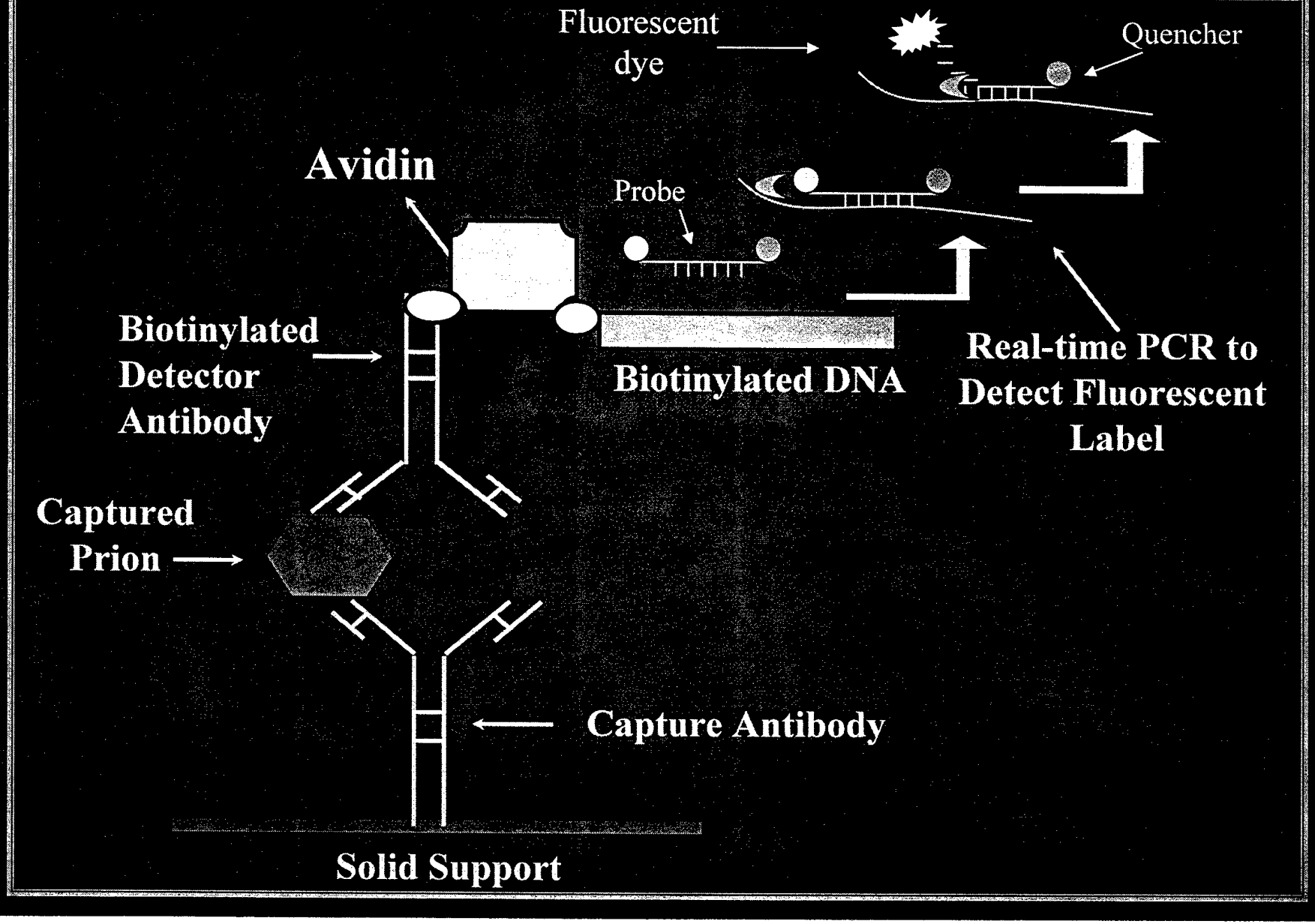
I-PCR with Ab-DNA Conjugate



I-PCR with Biotin/Avidin Modification



I-PCR with Real-time Probe Detection

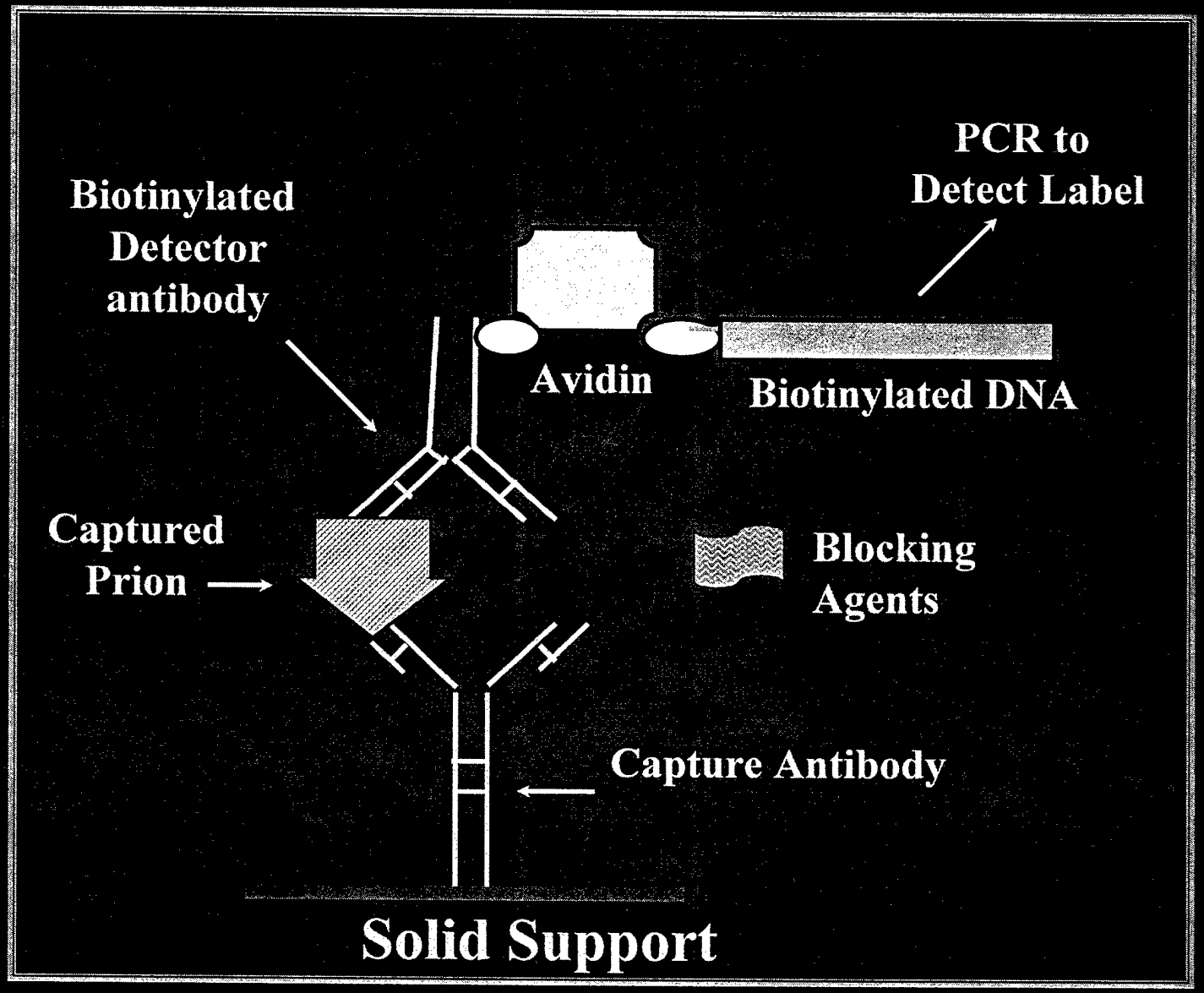


Immuno – PCR

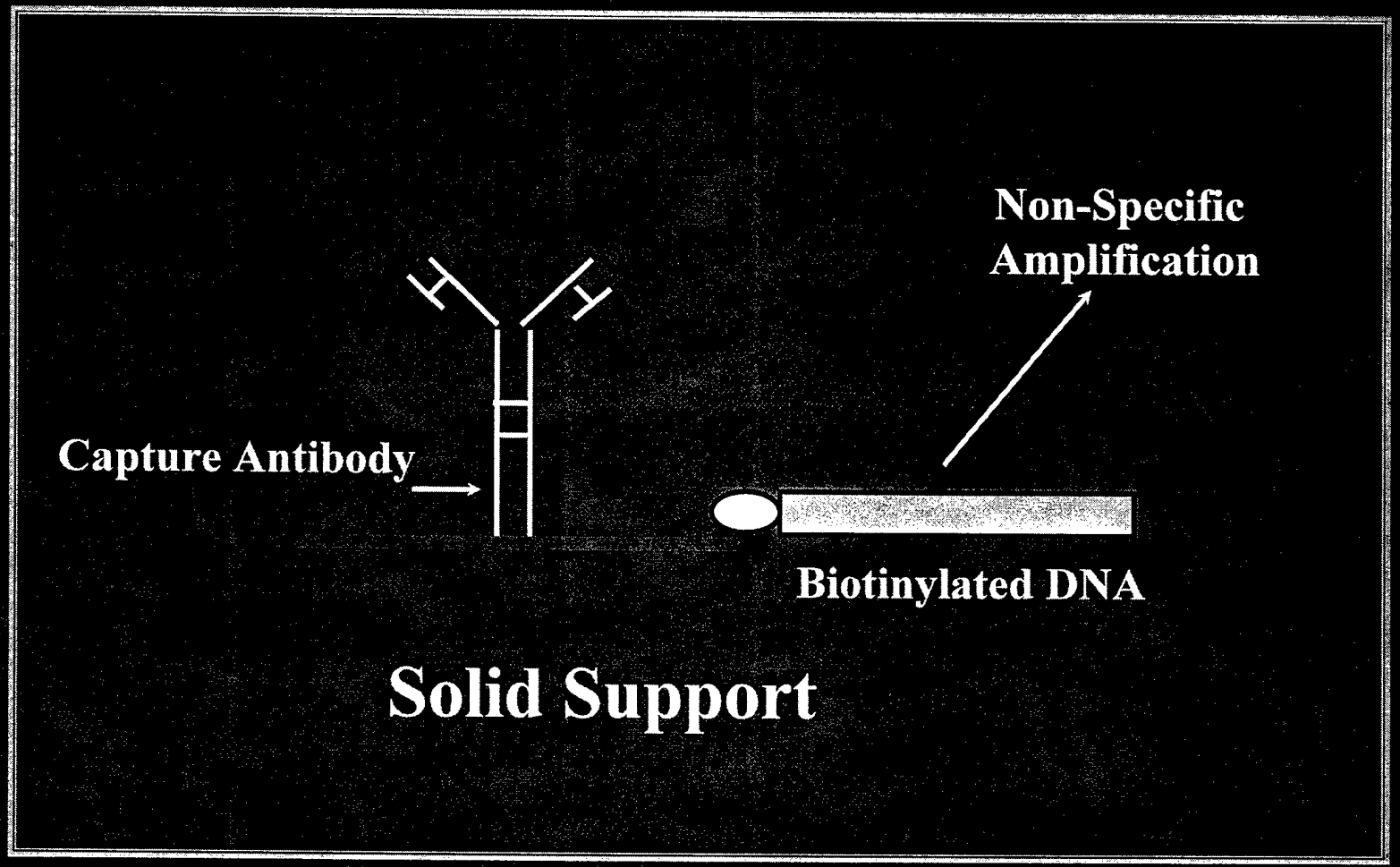
Major Challenge

Background Noise

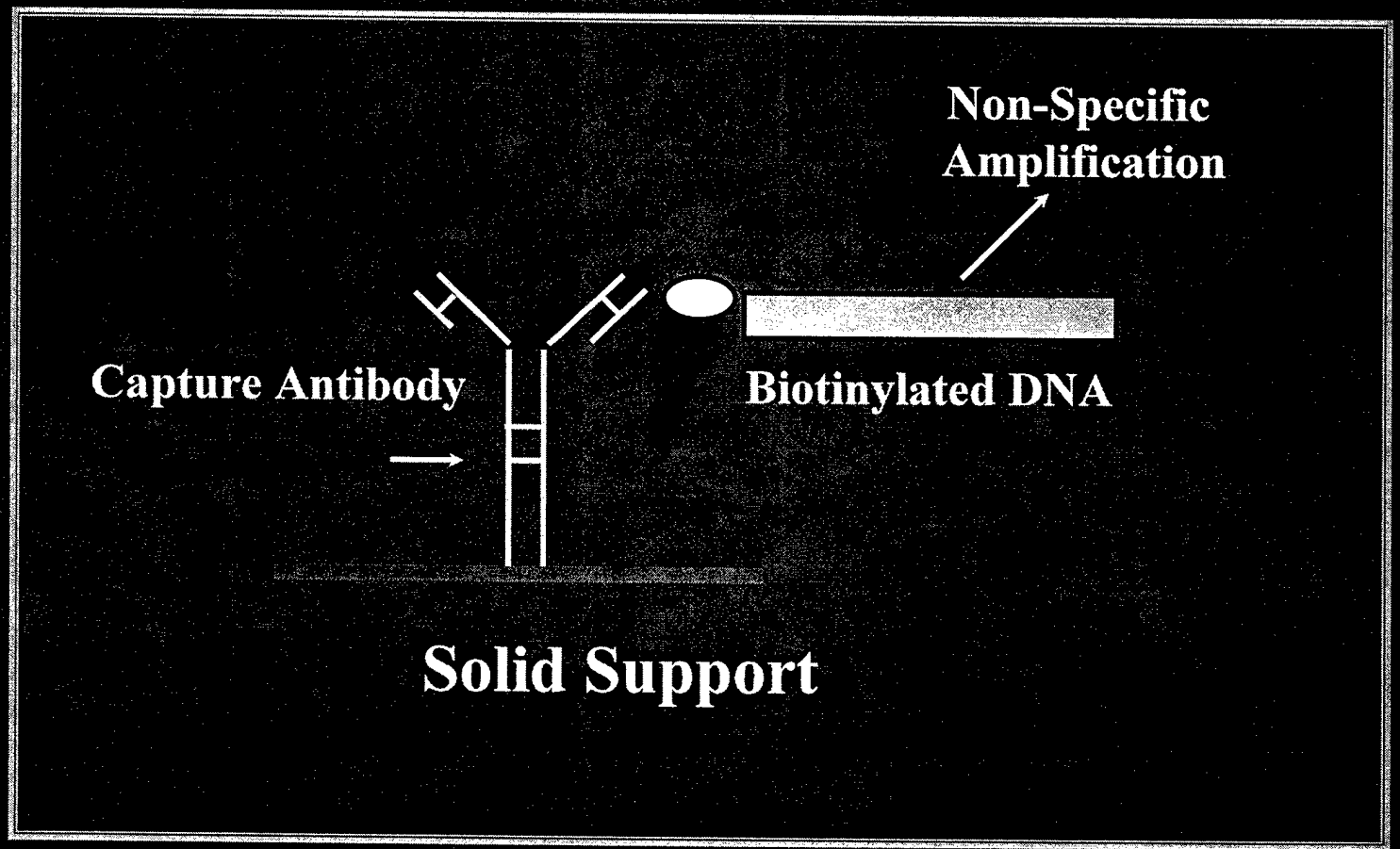
I-PCR


PCR to
Detect Label
Biotinylated
Detector
antibody
Avidin
Biotinylated DNA
Captured
Prion
Blocking
Agents
Capture Antibody
Solid Support

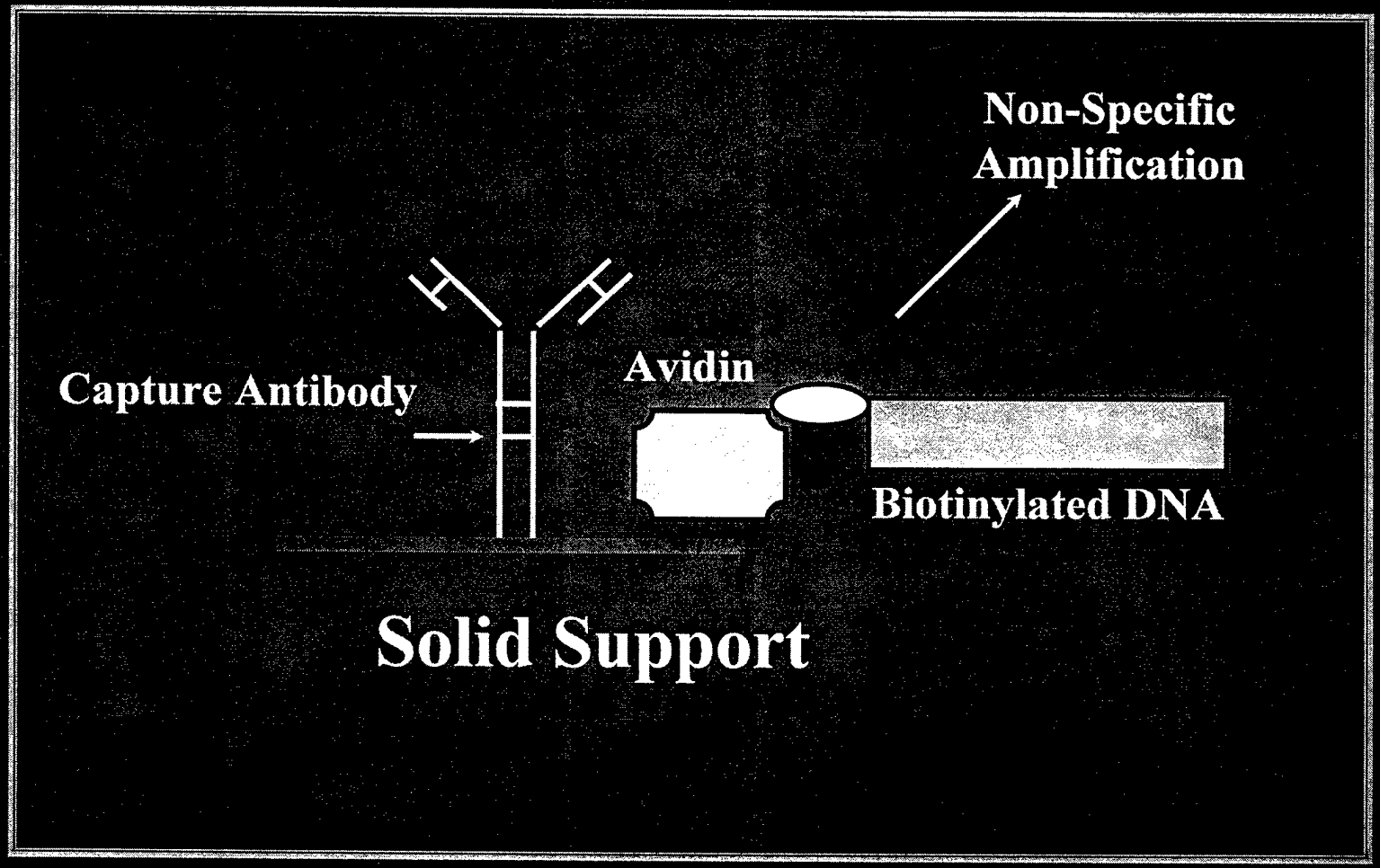
Background:
DNA Reporter to Solid Support



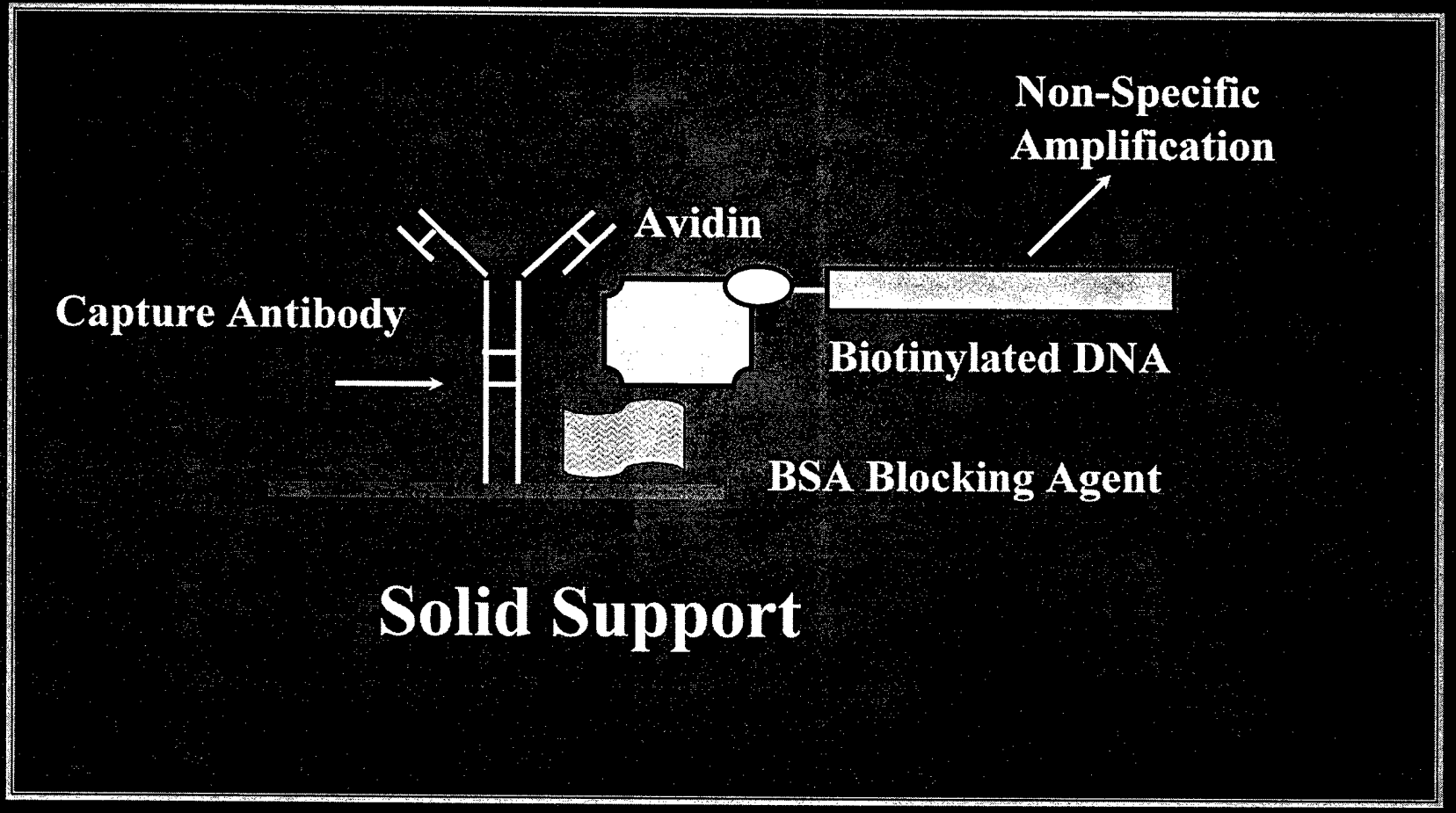
Background:
DNA Reporter to Capture Antibody



Background:
Avidin to Solid Support
DNA to Avidin



Background:
BSA to Solid Support Avidin to BSA
DNA to Avidin



Background Reduction Strategies

- Titration of all reactants simultaneously.
- Optimization of blocking cocktails, wash buffers, and wash cycles.
- Modification of amplification procedures, DNA, primer lengths, and probes.
- Modification of solid support surface chemistry.
- Others (Confidential)

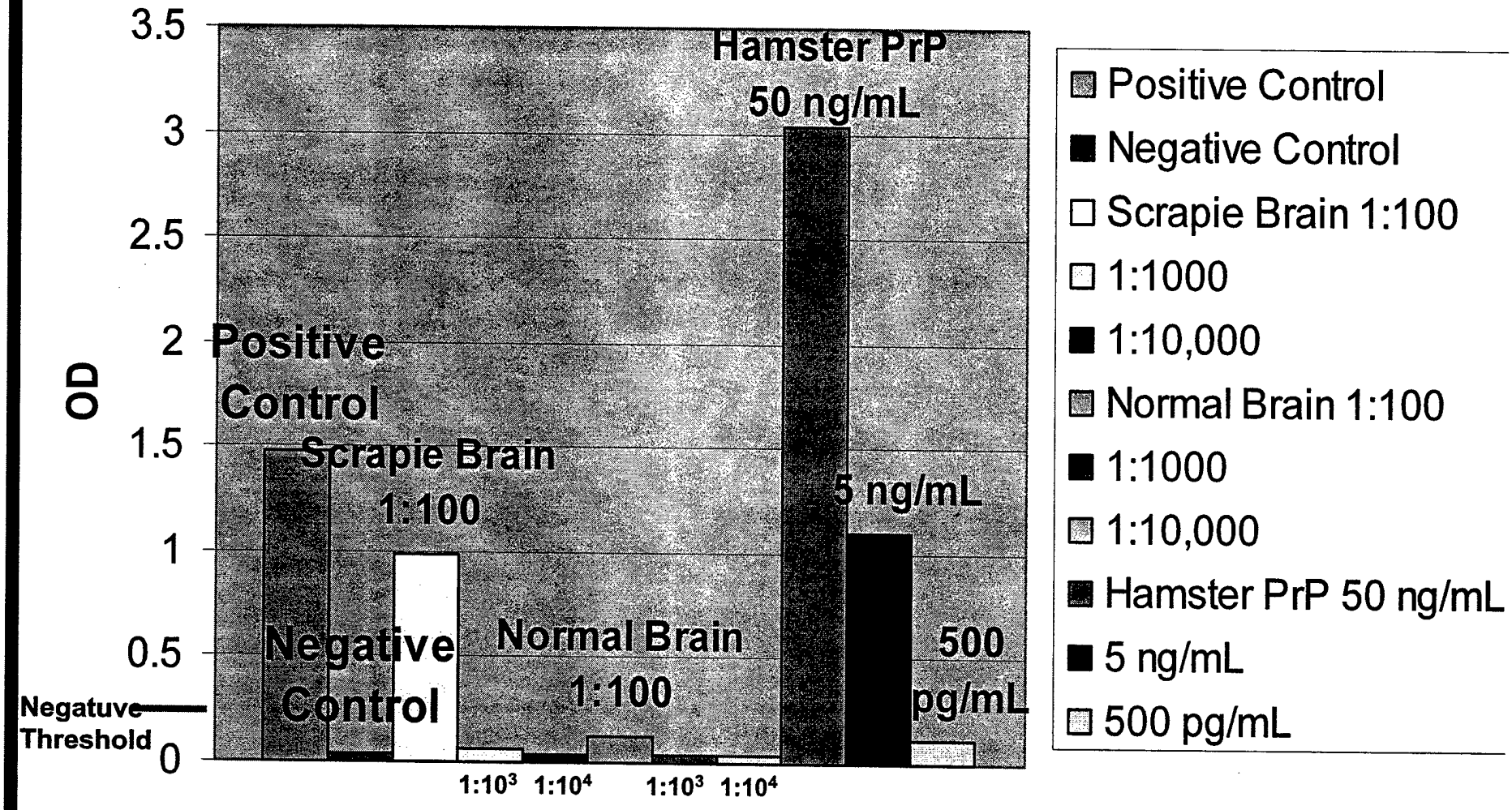
Results

Testing of Recombinant Hamster PrP

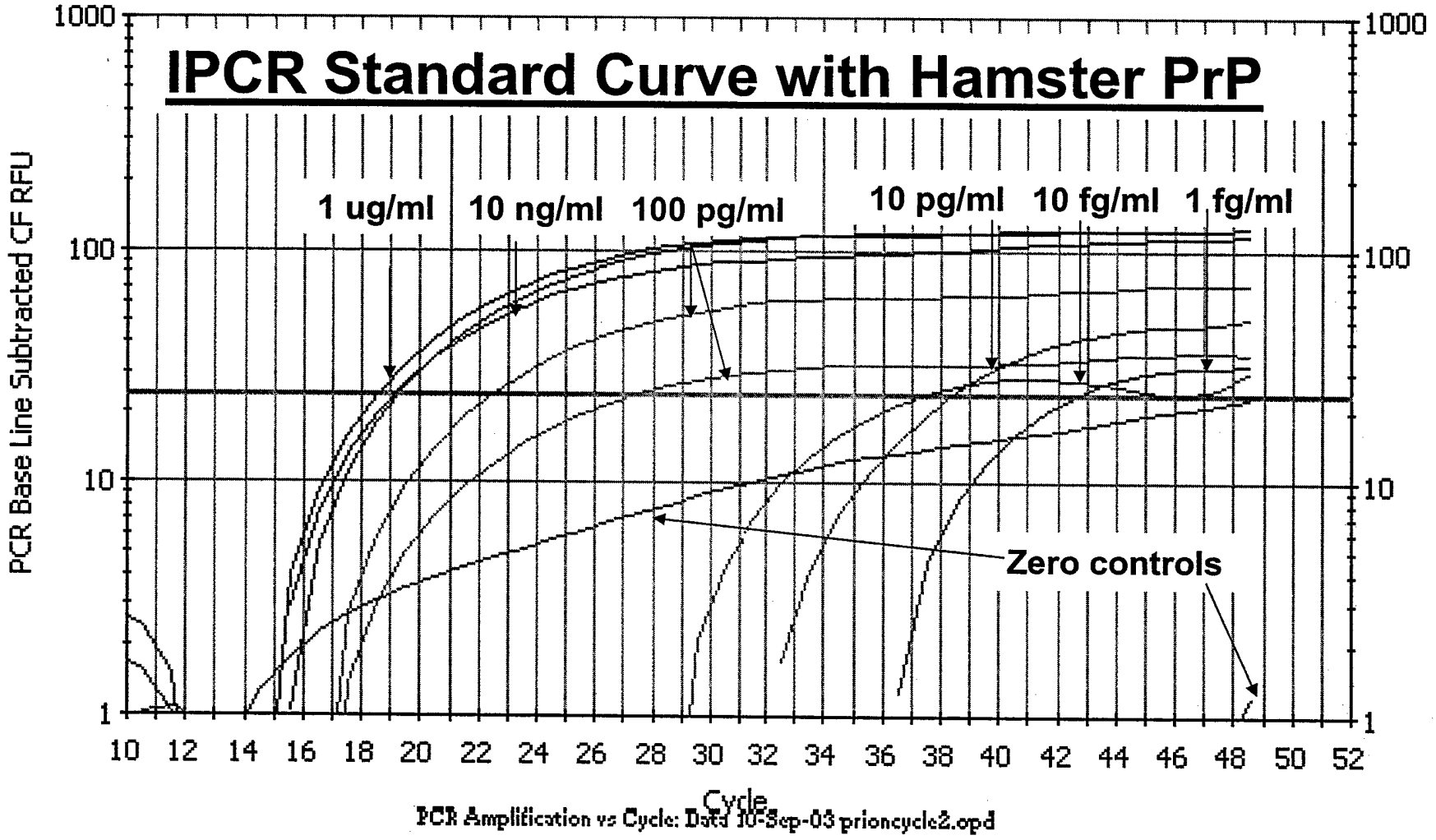
Assessment of Hamster Scrapie Brain

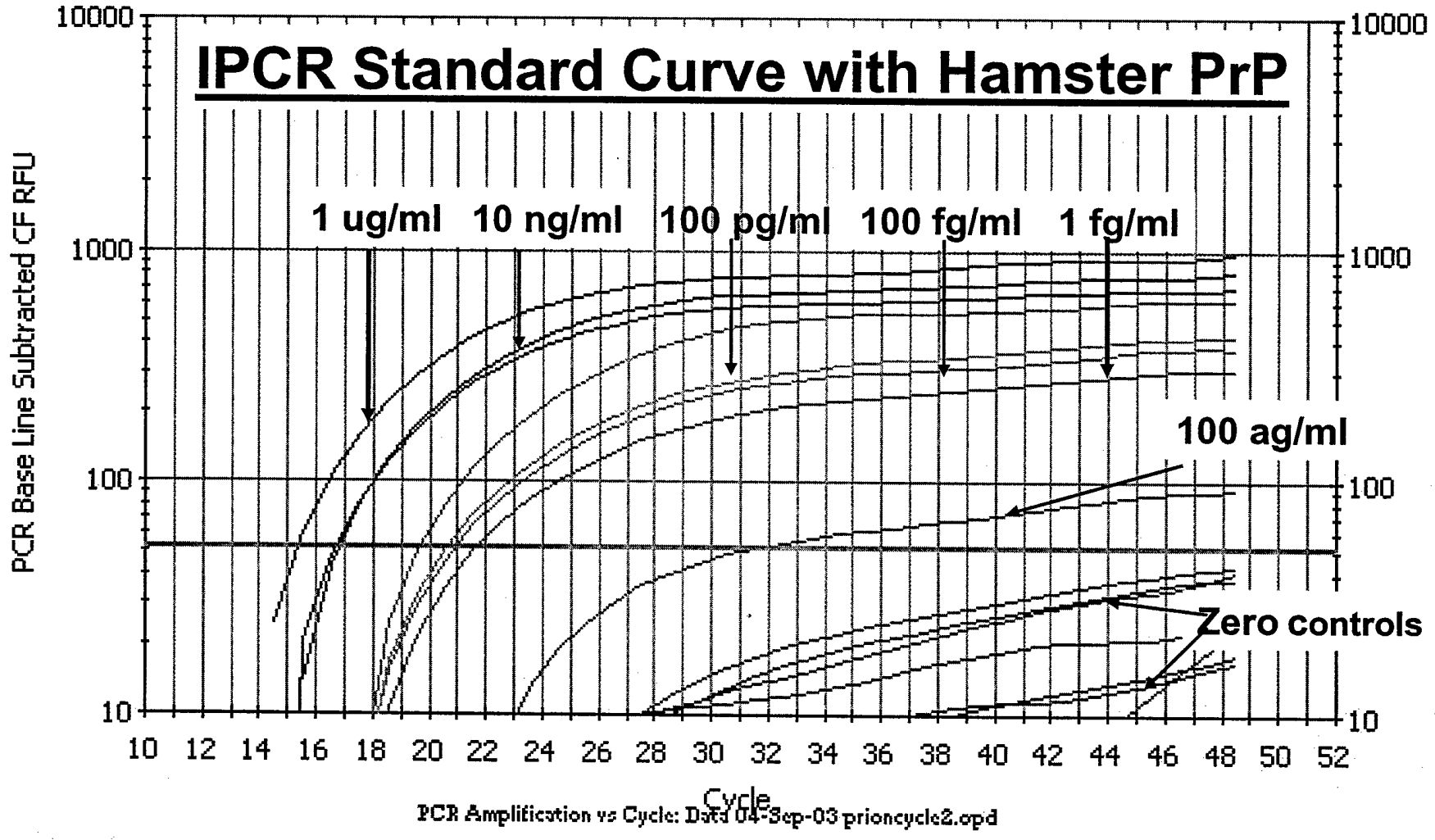
Comparison of ELISA with I-PCR

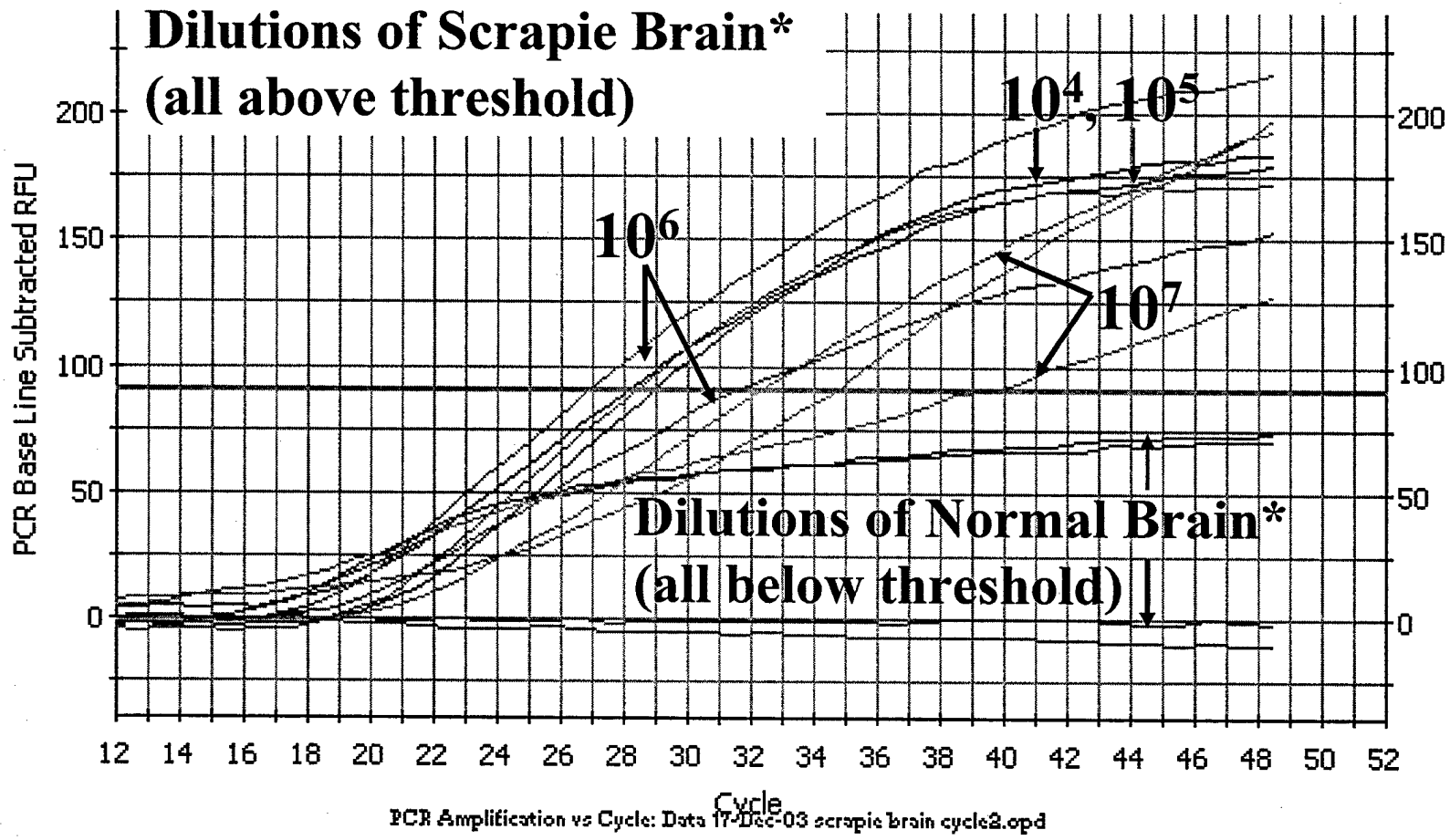
SPIbio ELISA Kit for PrPc



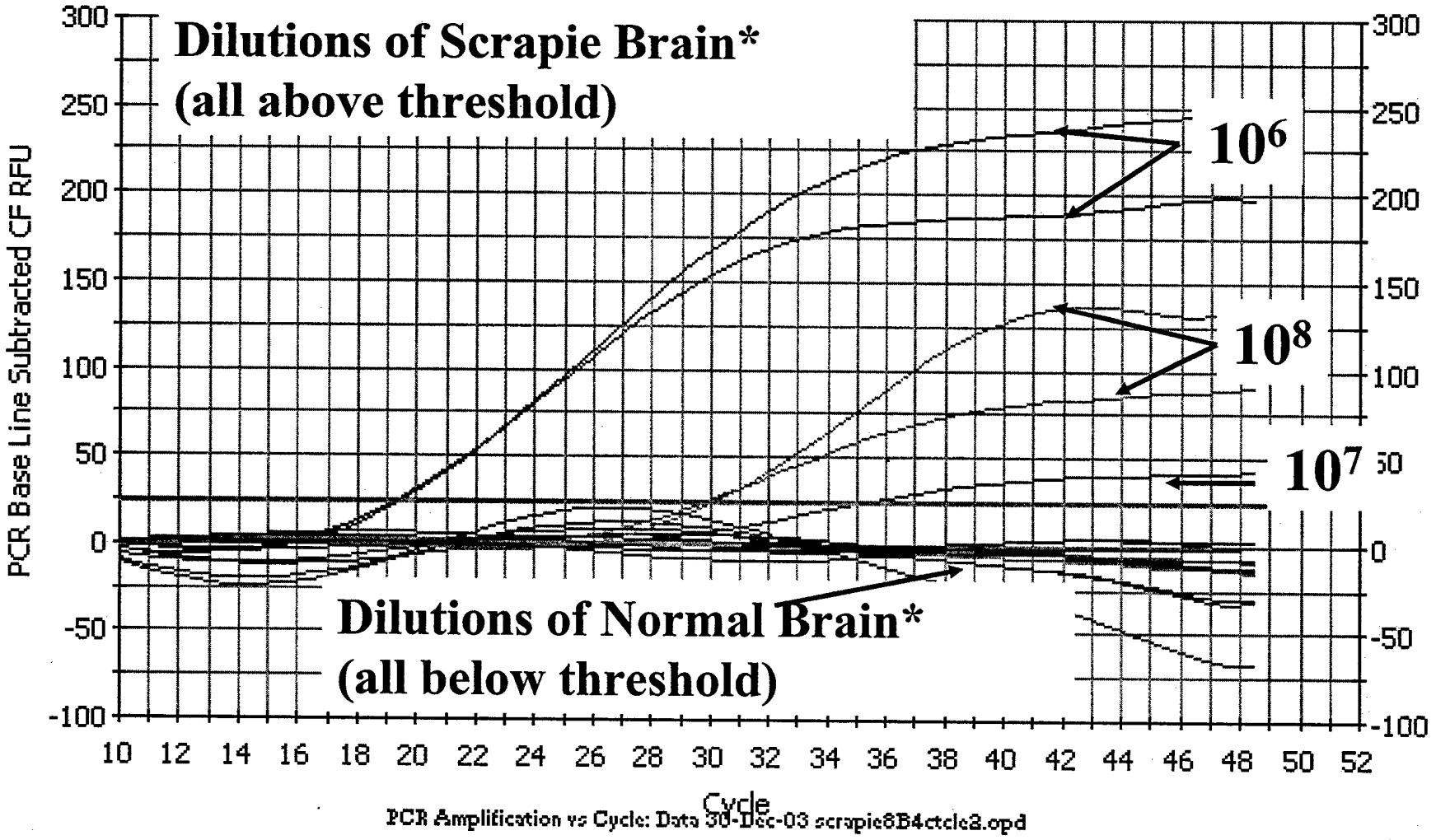
Note: All brain homogenates were PK-treated.


IPCR Standard Curve with Hamster PrP
1 ug/ml
10 ng/ml
100 pg/ml
10 pg/ml
10 fg/ml
1 fg/ml
Zero controls
PCR Base Line Subtracted CF RFU
1000
100
10
1
10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52
Cycle
PCR Amplification vs Cycle: Data 10-Sep-03 prioncycle2.opd


IPCR Standard Curve with Hamster PrP
1 ug/ml
10 ng/ml
100 pg/ml
100 fg/ml
1 fg/ml
100 ag/ml
Zero controls
PCR Base Line Subtracted CF RFU
10000
1000
100
10
10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52
Cycle
PCR Amplification vs Cycle: Data 04-Sep-03 prioncycle2.opd



* All brain homogenates were PK-treated.



* All brain homogenates were PK-treated.

Detection Limits: ELISA versus I-PCR for Scrapie Brain Homogenate

ELISA detected a 10^2 dilution (10^7 IU or 5 ng/mL)

I-PCR detected a 10^8 dilution (10 IU or 5 fg/mL)

Assay Characteristics

Assay Time:

Total time: 22 hours

Hands-on time: 2 hours

Cost per assay (materials): $6.08

Expertise: Same as for viral load tests

Conclusions

- **I-PCR for recombinant prion protein detection has exhibited an analytical sensitivity of 100 attograms/mL, 10 million times lower than ELISA.**
- **I-PCR for homogenates of scrapie brain has exceeded the sensitivity offered by ELISA by 6 logs (1,000,000 times lower than ELISA).**
- **The I-PCR method can identify prion protein at levels unmatched by other current methods.**