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Eric M. Nicholson

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Enrichment of PrP\textsuperscript{Sc} in formalin-fixed, paraffin-embedded tissues prior to analysis by Western blot

Eric M. Nicholson\textsuperscript{1}

Abstract. Diagnosis of prion disease is primarily through immunodetection of the infectious agent. Typically, 2 distinct procedures are recommended for a definitive diagnosis, with immunohistochemistry and Western blot providing the most information as to the specific isolate in question. In the past, these approaches required formalin-fixed, paraffin-embedded tissue and fresh or frozen tissue, respectively; however, methods have been developed that allow for use of fixed tissue for Western blot. The present study describes a method of enriching PrP\textsuperscript{Sc} in formalin-fixed, paraffin-embedded tissues prior to Western blot analysis for the detection of PrP\textsuperscript{Sc}. With this modified procedure, 5 times the previously reported sample size may be used for analysis, greatly enhancing the sensitivity of this procedure.

Key words: Paraffin-embedded tissue; prion; PrP; spongiform encephalopathy; transmissible spongiform encephalopathy; Western blot.

Transmissible spongiform encephalopathies (TSE) or prion diseases result from a conformational change in the host-encoded normal cellular form of the prion protein (PrP\textsuperscript{C}) to a disease-associated form termed PrP\textsuperscript{Sc}. PrP\textsuperscript{Sc} is widely accepted to be the causative agent of TSE.\textsuperscript{4} There are no pathognomonic clinical signs of a TSE, and specific immune responses have not been detected in TSE-infected organisms, therefore, serological tests for the presence of PrP\textsuperscript{Sc} are not available. Due to the fact that the infectious agent is a protein, nucleic acid-based detection methods are similarly unavailable. Currently, diagnosis of TSE is primarily conducted via immunodetection, including immunohistochemistry, Western blot (WB), and enzyme-linked immunosorbent assay (ELISA)-based approaches.\textsuperscript{5,6}

Western blot analysis of PrP\textsuperscript{Sc} is important not only as a confirmatory test for the ELISA rapid tests but also as a primary means to discriminate TSE isolates based upon the specific molecular weight of the resultant PrP\textsuperscript{Sc} bands on the blot. While typical WB procedures employ fresh or frozen tissues, recent advances allow WB analysis of PrP\textsuperscript{Sc} from tissues that had been fixed in formalin expanding the opportunity to analyze TSE isolates for which no fresh or frozen tissues were available.\textsuperscript{1,3} Using this approach, formalin-fixed, paraffin-embedded tissues have been used in WB-based molecular profiling differentiating TSE strains.\textsuperscript{2} The method presented in the current study enhances the sensitivity of these methods using centrifugation to pellet all detectable PrP\textsuperscript{Sc}, allowing substantially more tissue to be analyzed by WB. This allows analysis of samples containing either too little tissue or too little PrP\textsuperscript{Sc} to otherwise be used for formalin-fixed, paraffin-embedded tissue WB.

The present study utilizes paraffin-embedded tissue samples of brainstem (obex region) from sheep euthanized as part of scrapie research conducted at the National Animal Disease Center–U.S. Department of Agriculture–Agricultural Research Service (Ames, Iowa). The brainstem samples were collected in 10% buffered formalin prior to processing in paraffin blocks, with time in formalin ranging from 7 days to 1 year. Studies of formalin-fixed tissues report a marked sensitivity decrease for tissues left in formalin for 2 or more years.\textsuperscript{3}

The method described herein is an extension of previously published methods for WB of formalin-fixed samples for the purposes of detecting the disease-associated form of the prion protein.\textsuperscript{1,3} From each paraffin block, 4 tissue sections (each 5-µm thick) were collected into 1.5-ml microfuge tubes. To each tube, 150 µl of a tissue resuspension buffer (50 mM Tris, 1 mM ethylenediamine tetra-acetic acid, 0.5% Tween 20 [pH 7.5]) was added. The tube was then placed in a 100°C heat block for 10 min then immediately placed on dry ice. The process was repeated once and then followed by an additional 10 min at 100°C. Upon conclusion of the 100°C incubation, the samples were immediately centrifuged at 16,000 × g for 10 min to allow separation of paraffin from the buffer and tissue suspension. Removal of paraffin is essential to downstream WB, and if the separation was incomplete at this step, an additional 10 min at 100°C incubation followed by...
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Figure 1. Representative Western blot of paraffin-embedded tissues. Molecular weight markers on left and right sides of the blot are identical, with the weight (in kD) labeled to the left of the blot. Animal numbers are shown for each group of lanes with the unenriched lane (U), supernatant (S), and pellet (P) labeled for each animal. The 2 inoculated animals, 1077 and 1322, exhibit PrPSc in both the U and P lanes, with the P lanes showing markedly high amounts in both cases while the S lanes exhibit no detectable PrPSc. Animal 1269 is a noninoculated control animal, and no PrPSc is observed in the U, S, or P lanes.

Using the method as described, approximately 4 times the amount of tissue previously used for WB analysis of formalin-fixed, paraffin-embedded tissue1 can be readily loaded into a single well of an SDS-PAGE, greatly enhancing the sensitivity. This allowed for one-fifth of the total sample to be kept for comparison purposes. If the entire sample was pelleted and loaded, the increase in loading would be 5 times what was previously possible.

A representative WB is shown in Figure 1. The 3 bands corresponding to un-, mono-, and di-glycosylated PrPSc between 20 and 30 kD were clearly present in the enriched (pellet) fraction. The nonenriched portion of the sample is clearly lower in intensity for all bands than the enriched portion. The supernatant from the enrichment centrifugation step has no detectable PrPSc, indicating that all the detectable PrPSc is in the pellet following the centrifugation at 186,000 × g.

Using mouse anti-PrP monoclonal antibody P42 at a 1:10,000 dilution (0.1 µg/ml) as the primary antibody. A biotinylated sheep anti-mouse secondary antibody at 0.05 µg/ml and a streptavidin–horseradish peroxidase conjugate were used with a commercial detection system and were visualized using either chemiluminescence or chemifluorescence.
As previously reported, there are numerous higher molecular weight species present for PrPSc-containing samples. These higher weight species are most likely PrPSc irreversibly cross-linked in the formalin fixation process. Estimates of the amount of PrPSc present, as higher order species vary from sample to sample, can approach 50%. The presence of PrPSc as multiple higher weight bands results in a corresponding reduction in the amount of PrPSc that would otherwise be observed in the 20–30 kD range. The method reported herein allows for up to 5 times the amount of material to be loaded, decreasing the likelihood that the reduction in band intensity in the 20–30 kD range will prevent analysis of the molecular weight profile of those bands. Centrifugation for less than 1 hr at 186,000 × g can readily be incorporated into any diagnostic or research protocol utilizing formalin-fixed, paraffin-embedded tissues for detection of PrPSc, thus greatly enhancing the sensitivity of the approach.

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c. Invitrogen Corp., Carlsbad, CA.
d. GE Healthcare Technologies, Piscataway, NJ.
e. Sigma-Aldrich, St. Louis, MO.
f. R-Biopharm AG, Southmarshall, MI.

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