Korean J Physiol Pharmacol
Vol 13: 373-378, October, 2009
DOI: 10.4196/kjpp.2009.13.5.373

Optimized Immunohistochemical Analysis of Cerebellar Purkinje Cells Using a Specific Biomarker, Calbindin D28k

Byung Joo Kim[1,*], So Yeon Lee[2,*], Hyung Woo Kim[1], Eun-Jung Park[2], Jun Kim[2], Sang Jeong Kim[2], Insuk So[2], and Ju-Hong Jeon[2]

[1]School of Korean Medicine, Pusan National University, Yangsan 626-870, [2]Department of Physiology, College of Medicine, Seoul National University, Seoul 110-799, Korea

Cerebellar Purkinje cells (PCs) play a crucial role in motor functions and their progressive degeneration is closely associated with spinocerebellar ataxias. Although immunohistochemical (IHC) analysis can provide a valuable tool for understanding the pathophysiology of PC disorders, the method validation of IHC analysis with cerebellar tissue specimens is unclear. Here we present an optimized and validated IHC method using antibodies to calbindin D28k, a specific PC marker in the cerebellum. To achieve the desired sensitivity, specificity, and reproducibility, we modified IHC analysis procedures for cerebellar tissues. We found that the sensitivity of staining varies depending on the commercial source of primary antibody. In addition, we showed that a biotin-free signal amplification method using a horseradish peroxidase polymer-conjugated secondary antibody increases both the sensitivity and specificity of ICH analysis. Furthermore, we demonstrated that dye filtration using a 0.22 μm filter eliminates or minimizes nonspecific staining while preserving the analytical sensitivity. These results suggest that our protocol can be adapted for future investigations aiming to understand the pathophysiology of cerebellar PC disorders and to evaluate the efficacy of therapeutic strategies for treating these diseases.

Key Words: Purkinje cell, Cerebellum, Calbindin D28K, Immunohistochemical analysis

INTRODUCTION

Purkinje cells (PCs) are crucial elements in the cerebellum that control motor functions, such as movement, motor learning, and body balance (Ito, 2002). They are the sole output neurons in the cerebellar cortex, sending inhibitory signals to target neurons of the cerebellar nuclei (Ito, 2002). PC activity is coordinately modulated by complex cellular networks involving parallel fibers, climbing fibers, basket cells, and stellate cells across three layers of the cerebellar cortex (Ivan, 2007). PC somas and dendritic arbors are mainly found in the Purkinje cell layer (PCL) and molecular layer (ML), respectively. PC axons pass through the granular layer (GL) and white matter (WM) and terminate at deep cerebellar nuclei (Sotelo, 2004; Douyard et al., 2007; Cheron et al., 2008). Many insults, such as ischemia, toxins, infections, and genetic changes, directly or indirectly lead to destructive disturbances of PCs, which are associated with spinocerebellar ataxias (Koeppen, 2005; Millen and Gleeson, 2008). Thus, a pathophysiological understanding of the cellular changes of PCs can provide clues to therapeutic strategy developments for treating cerebellar disorders.

Morphological or density changes of PCs in the cerebellum are primarily determined by immunohistochemical (IHC) analysis using PC-specific biomarkers that discriminate PCs from other types of cells in the cerebellum (Haworth et al., 2006; Whitney et al., 2008a). Calbindin D28k (CaB), a member of calcium-binding protein superfamily, is a PC-specific protein in the cerebellum (Bastianelli, 2003). CaB has six EF-hand motifs, serves as a cytosolic calcium buffer, and plays an important role in motor functions (Schwaller et al., 2002; Simons and Pellionisz, 2006; Whitney et al., 2008b). Although CaB gene ablation did not affect normal development, CaB null mice were impaired in motor coordination, which is associated with changes in synaptically-evoked postsynaptic calcium transport (Airaksinen et al., 1997; Schwaller et al., 2002).

IHC analysis using CaB antibodies has been widely used in cerebellar PC studies (Nakamura et al., 1999; Haworth et al., 2006; Whitney et al., 2008a). Several signal amplification methods have been developed to increase the sensitivity of IHC analysis, particularly the avidin-biotin complex (ABC) method. However, this method often produces a

Received September 2, 2009, Revised September 18, 2009, Accepted October 7, 2009

Corresponding to: Ju-Hong Jeon, Department of Physiology, Seoul National University College of Medicine, 28, Yongon-dong, Chongno-gu, Seoul 110-799, Korea. (Tel) 82-2-740-8399, (Fax) 82-2-763-9667, (E-mail) jhjeon2@snu.ac.kr
*This first two authors contributed equally to this article.

ABBREVIATIONS: PC, Purkinje cell; CaB, calbindin D28k; IHC, immunohistochemistry; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; RT, room temperature; OCT, optimal cutting temperature; AEC, aminoethylcarbazole; ABC, avidin-biotin complex; ML, molecular layer; PCL, Purkinje cell layer; GCL, granular cell layer; WM, white matter region.

serious pitfall in diagnostic IHC approaches. (Green et al., 1992; Iezzoni et al., 1999). Moreover, IHC analysis results can vary substantially with experimental conditions such as antibody specificity, IHC protocol, and dye impurity (O'Leary, 2001; Lyon, 2002; Goldstein et al., 2007). Method validation is needed to ensure the reliability of IHC analysis results. In this study, we provide a sensitive, specific, and reproducible IHC method that can be adapted for assessing cerebellar PC pathophysiology.

METHODS

Materials

Primary antibodies to CaB were supplied by Sigma (Cat. No. C9848) or Chemicon (Cat. No. AB1778). Horseradish peroxidase (HRP)-conjugated secondary antibody or HRP polymer-conjugated secondary antibody (UltraVision LP detection system, Ready-To-Use) were purchased from Chemicon (Cat. No. AP342P) or Thermo Fisher Scientific (Cat. No. TL-125-HL). AEC solution was obtained from Zymed (Cat. No. 00-2007). All other reagents, if not specified, were purchased from Sigma.

Preparation of cerebellar slices

The cerebellar tissues were isolated from anesthetized 6~7 weeks old Wistar rats (body weight 250~300 g) and immediately fixed with ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS) for 16 h at 4°C. The fixed tissues were sequentially dehydrated by incubation with PBS containing 12~18% sucrose at room temperature (RT), then embedded in optimal cutting temperature (OCT) medium and frozen in liquid nitrogen. The frozen tissues were cut with a cryostat at 10 μm thickness into parasagittal slices at −20°C and slices were placed on glass slides.

Immunohistochemical analysis

Cerebellar tissue specimens were fixed with 4% paraformaldehyde in PBS for 30 min at RT and permeabilized with 0.1% Triton X-100 for 10 min at RT. Endogenous peroxidase activity was removed by treatment with 0.3% hydrogen peroxide for 10 min at RT. The specimens were blocked with 5% goat serum (Chemicon) in PBST for 1 h at RT and then incubated with commercial antibodies to CaB (Sigma or Chemicon) for 1 hr at RT or for 16 h at 4°C. The specimens were further incubated with HRP-conjugated (Chemicon) or HRP polymer-conjugated (Thermo Fisher Scientific) secondary antibody for 1 h at RT. After the addition of 0.22 μm syringe-filtered aminoethylcarbazole (AEC) solution (Zymed) with gentle rocking for 5 min, the specimens were mounted using aqueous mounting medium (ScyTek laboratories) and examined by microscope (Olympus DP 71). Further details of experimental procedures are given in Table 1.

RESULTS

Determining the sensitivity and specificity of IHC methods

ABC methods have been widely used to increase the sensitivity of IHC analysis. However, avidin directly reacts with endogenous carboxylases (pyruvate, 3-methylcrotony, propionyl, and acetyl-coA carboxylases) that utilize biotin as a prosthetic group (Alon et al., 1990; Alon et al., 1992; Alon et al., 1993). This causes a serious pitfall in diagnostic IHC approaches (Green et al., 1992; Iezzoni et al., 1999).

We examined CaB expression in cerebellar PCs using biotin-free IHC methods. When CaB captured by anti-CaB antibody (Sigma) was probed with HRP-conjugated secondary antibody (HRP method), the cerebellar specimens were positively but weakly stained in the ML (Fig. 1B). In this method, little staining was observed in the PCL, GCL, and WM. HRP method specificity was assessed by omitting the incubation step with primary antibody. Nonspecific staining was particularly observed in the ML (Fig. 1A), which indicates that HRP-conjugated secondary antibody directly reacts with unrecognized proteins in the cerebellum or staining reagents nonspecifically precipitated.

To enhance the sensitivity of IHC analysis, we analyzed commercially available HRP polymer-conjugated secondary

Table 1. Proposed IHC analysis protocol

	Step	Procedure
1	Washing	PBS once at RT
2	Fixation	4% paraformaldehyde in PBS for 30 min at RT
3	Washing	PBS-containing 0.1% Tween 20 (PBST) three times at RT
4	Permeabilization	0.1% triton X-100 for 10 min at RT
5	Washing	PBST three times at RT
6	Inactivation	Hydrogen peroxide for 10 min at RT
7	Washing	PBST three times at RT
8	Blocking	Goat serum (Chemicon) for 30 min at RT
9	Washing	PBST three times at RT
10	1° Ab	1 : 500 diluted anti-CaB antibody (Sigma) for 60 min at RT
11	Washing	PBST three times at RT
12	2° Ab	No diluted HRP Polymer-conjugated secondary antibody (Thermo Fisher Scientific or Zymed) for 30 min at RT
13	Washing	PBST three times at RT
14	Staining	An appropriate volume of 0.22 μm syringe-filtered AEC solution (Zymed) for 5 min at RT
15	Washing	Distilled water threetimes at RT
16	Mounting	Aqueous mounting solution (ScyTek laboratories)

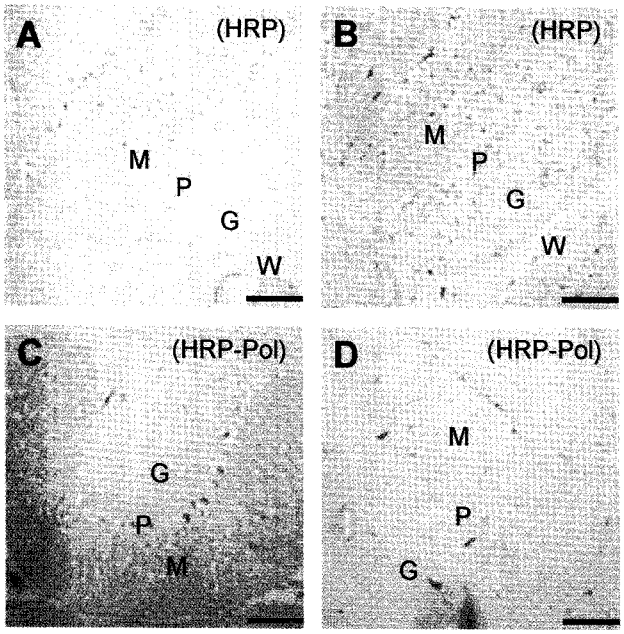

Fig. 1. Sensitivity and specificity of IHC methods. The expression of CaB in cerebellar specimens was analyzed by the HRP method without (A) or with (B) 1 : 500-diluted anti-CaB antibody (Sigma) and stained using unfiltered AEC solution. CaB was captured by two different commercial source antibodies, one from Sigma (C) and another from Chemicon (D), for 1 h at RT. Antibodies were probed by HRP polymer-conjugated secondary antibody (HRP-Pol). M, molecular layer; P, Purkinje cell layer; G, granular cell layer; W, white matter region. Scale bar=100 μm. The data shown are representative results of three to five independent experiments.

antibody staining (i.e., HRP-Pol method). As shown in Fig. 1C, the HRP-Pol method produced a higher signal than the HRP method. However, the nonspecific signals obtained by omitting the incubation step with primary antibody were similar for both methods (data not shown).

Because the antibody affinity to CaB can vary with the commercial source, we examined whether another commercial anti-CaB antibody (Chemicon) could increase IHC sensitivity. Under the same conditions as the Sigma anti-CaB antibody (1 h treatment time), CaB staining was not observed with the antibody from Chemicon, which recommends a two day incubation time (Fig. 1D). These results demonstrate that the HRP-Pol method using anti-CaB antibody from Sigma is a time-saving and sensitive IHC method. However, nonspecific staining is still problematic with the current method.

Increased IHC specificity by filtrating dye solution

Dye impurities have been recognized as a main problem in establishing standardized IHC methods (Lyon, 2002). Filtration with a 0.22 μm syringe filter is widely used to enhance reagent purity or homogeneity in biochemical experiments. We thus examined whether dye filtration could reduce the nonspecific staining of the HRP-Pol method. As illustrated in Fig. 2A, dye filtration remarkably reduced nonspecific staining and did not affect staining intensity. Specific staining was evident in the PCL, ML, and WM (Fig. 2B). The GCL was also stained weakly but

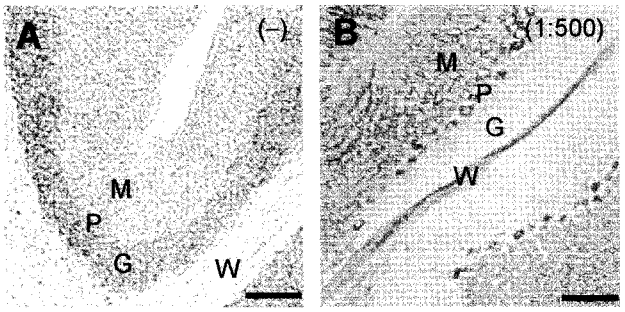

Fig. 2. Increased sensitivity of IHC analysis by filtrating AEC solution. CaB expression in cerebellar specimens was analyzed by the HRP-Pol method without (A) or with (B) 1 : 500-diluted anti-CaB antibody (Sigma). AEC solution was filtrated with a 0.22 μm syringe filter before adding to tissue specimens. Scale bar=100 μm. The data shown are representative results of three to five independent experiments.

specifically (Fig. 2). These staining patterns correlate well with the regional distribution of PC soma, dendrite, and axon regions (Sotelo, 2004; Douyard et al., 2007; Cheron et al., 2008). We also found that dye solutions from other commercial sources, including Sigma or Thermo Fisher Scientific, also have improved specificity after dye filtration (data not shown). Comparable results were obtained when the PicturePlus system (another commercial source of HRP polymer-conjugated secondary antibody, Zymed) was employed in IHC analysis (data not shown). These results show that the HRP-Pol method with dye filtration increases both sensitivity and specificity of IHC analysis.

Optimization of experimental conditions of IHC analysis

To optimize the experimental conditions of the HRP-Pol method, we first determined the optimum titer of anti-CaB antibody by serial dilution (1 : 500 to 1 : 4,000). Of the titer tested, the 1 : 500 dilution produced the highest signal in all cerebellar regions (Fig. 3A~E). Dilutions of up to 1 : 2,000 were capable of positively staining all cerebellar regions except the GCL. We then investigated the optimum incubation time for 1 : 500-diluted primary antibody. Staining intensity with 16 h incubation of the antibody at 4°C (Fig. 3F) was similar to that with 1 h of incubation at RT (Fig. 3B).

We further determined the optimal conditions of HRP polymer-conjugated secondary antibody, which is presented as a ready-to-use reagent. Serial dilutions resulted in gradually decreasing staining signals. Undiluted reagent produced the highest and clearest signal in all cerebellar regions (Fig. 4). The 1 : 4 to 1 : 16 dilutions produced weak signals in the ML and WM regions, but none in the PCL or GCL. Thus, these data demonstrate that undiluted HRP polymer-conjugated secondary antibody produces the optimum result for IHC analysis with cerebellar splices.

DISCUSSION

The optimization of experimental conditions in IHC analysis is critical to attain the desired sensitivity, specificity, and reproducibility. In this study, we performed a series of experiments to eliminate nonspecific signals and concurrently

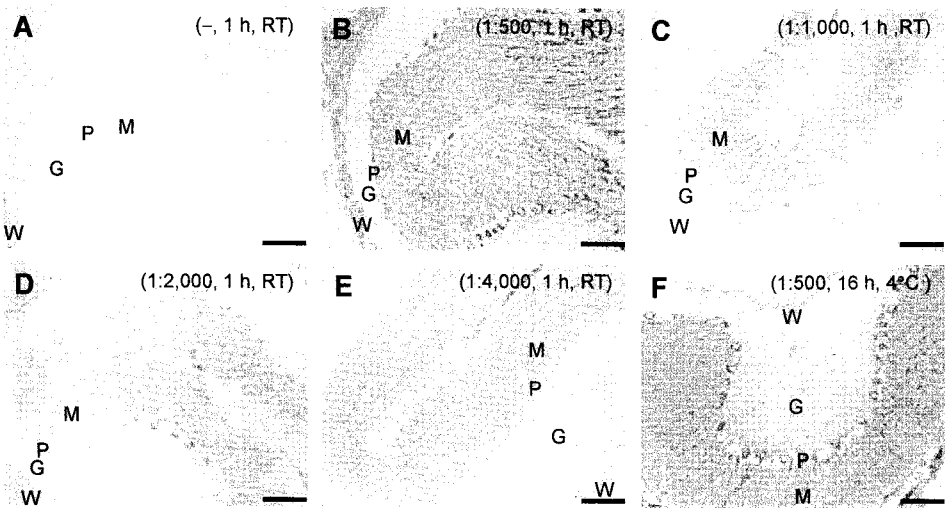

Fig. 3. Determination of the optimal titer of primary antibody. Serially diluted primary antibodies to CaB were used to determine the optimum titer (B~E). The HRP-Pol method with filtrated dye was employed for IHC analysis. Staining specificity was verified by omitting the incubation step with primary antibody (A). Cerebellar specimens were incubated with primary antibody for 1 h at RT (A~E) or for 16 h at 4°C (F). Scale bar=100 μm. The data shown are representative results of three to five independent experiments.

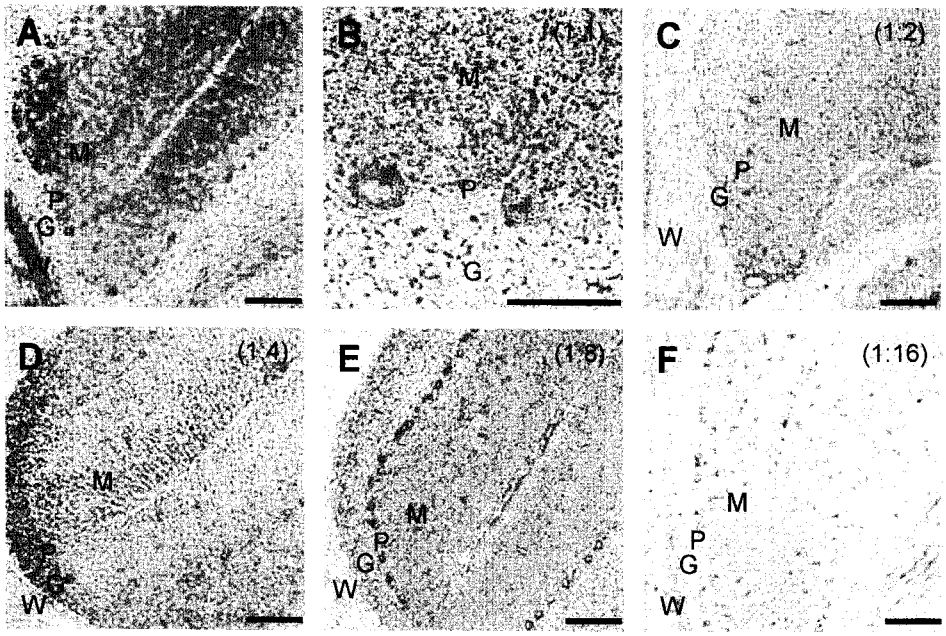

Fig. 4. Determination of the optimal titer of HRP polymer-conjugated secondary antibody. Serially diluted HRP polymer-conjugated secondary antibodies were used to determine the optimum titer (A~F). The cerebellar specimens were incubated with primary antibody to CaB for 1 h at RT. Scale bar=100 μm. The experimental data shown are representative results of three to five independent experiments.

preserve specific staining. In addition, we established a user-friendly and time-saving protocol for IHC analysis applicable to cerebellar PC research (Table 1). Notably, we showed that a dye filtration step is crucial for minimizing nonspecific staining. Also, the appropriate commercial source of primary antibody can substantially reduce experiment times. Finally, the HRP-Pol method can produce enhanced staining sensitivity and specificity.

Spinocerebellar ataxias, degenerative diseases of the mature cerebellum, are caused by the progressive degeneration of PCs (Millen and Gleeson, 2008). Thus, histological assay systems can be used to monitor the efficacy of therapeutic agents or to understand the pathophysiology of PCs. Because we optimized and validated the sensitivity and specificity of IHC analysis, our protocol can be adapted for assessing histological changes of cerebellar PCs.

The ABC method of IHC analysis has been used to amplify staining signals. However, avidin reacts with endogenous biotin-containing proteins (e.g., carboxylases), which could lead to misinterpretation of experimental results (Green et al., 1992; Iezzoni et al., 1999). Thus, blocking endogenous biotin by pretreatment with avidin is often employed to minimize false-positive signals, but this method is not robust under various experimental conditions. Streptavidin, a commonly used non-glycosylated avidin, can bind nonspecifically to fibronectin, vitronectin, and von Willebrand factor via its RYD sequence, which is similar to the RGD motif of cell adhesion molecules (Alon et al., 1990; Alon et al., 1992; Alon et al., 1993). Thus, alternative types of avidin, such as Neutravidin (Pierce) or Extravidin (Sigma) are needed to eliminate nonspecific signals. Nonetheless, these avidins retain the ability to react with endogenous bio-

tin-containing proteins. Therefore, sensitive biotin-free methods are favored in IHC analysis.

Because HRP is generally conjugated to secondary antibody with a 1 : 1 molar ratio, the binding stoichiometry between primary and secondary antibody is crucial for determining analytical sensitivity. Thus, the HRP method has limited signal amplification, due to the number of HRPs coupled to target antigen. By contrast, HRP polymer technology conjugates many HRPs and secondary antibodies to dextran polymers. Therefore, this technology has been used as a biotin-free signal amplification method in many immunological analyses, such as IHC analysis, immunocytochemical assays, enzyme-linked immunosorbent assays, and in situ hybridization (Fukuda et al., 2000; Wiedorn et al., 2001; Jeon et al., 2006; Jeon et al., 2007). In addition, many commercially available HRP polymer products include Fab fragments rather than full-length antibodies as secondary antibodies, which minimize background signals by inhibiting nonspecific binding of the antibody tail region (Fc) to cellular proteins. In this study, we demonstrated that the HRP-Pol method is readily applicable to IHC analysis of cerebellar PCs. Our data showed that the ML, PCL, GCL, and WM are positively stained and that the staining patterns correlate well with the distribution of PC soma, dendrite, and axon regions (Fig. 2A, Fig. 3B, and Fig. 4A), showing the specificity and sensitivity of our IHC method.

We found that dye filtration with a 0.22 μm syringe filter markedly improves the specificity of IHC analysis, while preserving a maximum level of specific staining. Filtration may remove the particulate dye components that precipitate nonspecifically and/or bind to tissue specimens (Dapson, 2005). Particularly, the nonspecific precipitation of dye is often observed in mucin-rich specimens (e.g., cervical smear specimens; Jeon et al., 2007). Thus, our filtration method can alleviate the problems of IHC approaches using sticky or mucous specimens. Unfortunately, it remains unknown whether filtration is equally effective in IHC analysis using other dyes that act as HRP substrates or alkaline phosphatases.

We found that the Chemicon antibody to CaB fails to produce specific signals in cerebellar specimens under the analysis conditions (1 h of incubation time at RT). It may be that the antibody epitope is modified during sample preparation processes or masked by CaB-interacting proteins, or that the antibody has a low affinity for CaB. On the other hand, Sigma antibody to CaB readily generates a signal under the same conditions. Thus, our findings show that Sigma antibody is desirable in IHC approaches for cerebellar PCs.

In summary, we present a specific, sensitive, and reproducible IHC method. Our protocol can provide the basic tool for future investigations aimed at understanding the pathophysiology of cerebellar PC disorders and evaluating the efficacy of therapeutic strategies.

ACKNOWLEDGEMENTS

This work was supported by a Korea Science and Engineering Foundation (KOSEF) grant funded by the Korean government (R01-2006-000-10990-0) and the Pusan National University Research Grant, 2009. P.E.J. was supported through a graduate program for the BK21 project from the Korean government (MEST).

REFERENCES

Airaksinen MS, Eilers J, Garaschuk O, Thoenen H, Konnerth A, Meyer M. Ataxia and altered dendritic calcium signaling in mice carrying a targeted null mutation of the calbindin D28k gene. *Proc Natl Acad Sci USA* 94:1488−1493, 1997.

Alon R, Bayer EA, Wilchek M. Cell adhesion to streptavidin via RGD-dependent integrins. *Eur J Cell Biol* 60: 1−11, 1993.

Alon R, Bayer EA, Wilchek M. Cell-adhesive properties of streptavidin are mediated by the exposure of an RGD-like RYD site. *Eur J Cell Biol* 58: 271−279, 1992.

Alon R, Bayer EA, Wilchek M. Streptavidin contains an RYD sequence which mimics the RGD receptor domain of fibronectin. *Biochem Biphys Res Commun* 179: 1236−1241, 1990.

Bastianelli E. Distribution of calcium-binding proteins in the cerebellum. *Cerebellum* 2: 242−262, 2003.

Cheron G, Servais L, Dan B. Cerebellar network plasticity: from genes to fast oscillation. *Neuroscience* 153: 1−19, 2008.

Dapson RW. Dye-tissue interactions: mechanisms, quantification and bonding parameters for dyes used in biological staining. *Biotech Histochem* 80: 49−72, 2005.

Douyard J, Shen L, Huganir RL, Rubio ME. Differential neuronal and glial expression of GluR1 AMPA receptor subunit and the scaffolding proteins SAP97 and 4.1 N during rat cerebellar development. *J Comp Neurol* 502: 141−156, 2007.

Fukuda T, Tani Y, Kobayashi T, Hirayama Y, Hino O. A new Western blotting method using polymer immunocomplexes: detection of Tsc1 and Tsc2 expression in various cultured cell lines. *Anal Biochem* 285: 274−276, 2000.

Goldstein NS, Hewitt SM, Taylor CR, Yaziji H, Hicks DG. Members of Ad-Hoc Committee On Immunohistochemistry Standardization, Recommendations for improved standardization of immunohistochemistry. *Appl Immunohistochem Mol Morphol* 15: 123−133, 2007.

Green M, Sviland L, Taylor CE, Peiris M, McCarthy al., **Pearson AD, Malcolm AJ.** Human herpes virus 6 and endogenous biotin in salivary glands. *J Clin Pathol* 45: 788−90, 1992.

Haworth R, McCormack N, Selway S, Pilling AM, Williams TC. Calbindin D-28 and microtubule-associated protein-2: Their use as sensitive immunohistochemical markers of cerebellar neurotoxicity in a regulatory toxicity study. *Exp Toxicol Pathol* 57: 419−426, 2006.

Iezzoni jc, Mills SE, Pelkey TJ, Stoler MH. Inhibin is not an immunohistochemical marker for hepatocellular carcinoma. An example of the potential pitfall in diagnostic immunohistochemistry caused by endogenous biotin. *Am J Clin Pathol* 111: 229−234, 1999.

Ito M. Historical review of the significance of the cerebellum and the role of Purkinje neurons in motor learning. *Ann NY Acad Sci* 978: 273−288, 2002.

Ivan GJ. Synaptic signaling in cerebellar plasticity. *Biol Cell* 99: 363−378, 2007.

Jeon JH, Kim CW, Shin DM, Cho SY, Jang GY, Lee HJ, Kim IG. Colorimetric transglutaminase assays combined with immunological signal amplification. *Anal Biochem* 348: 327−329, 2006.

Jeon JH, Shin DM, Cho SY, Song KY, Park NH, Kang HS, Kim YD, Kim IG. Immunocytochemical detection of HPV16 E7 in cervical cancer. *Exp Mol Med* 39: 621−628, 2007.

Koeppen AH. The pathogenesis of spinocerebellar ataxia. *Cerebellum* 4: 62−73, 2005.

Lyon HO. Dye purity and dye standardization for biological staining. *Biotech Histochem* 77: 57−80, 2002.

Millen KJ. Gleeson JG, Cerebellar development and disease. *Curr Opin Neurobiol* 18: 12−19, 2008.

Nakamura R, Kurita K, Kawanami T, Kato T. An immunohistochemical study of Purkinje cells in a case of hereditary cerebellar cortical atrophy. *Act Neuropathol* 97: 196−200, 1999.

O'Leary TJ. Standardization in immunohistochemistry. *Appl Immunohistochem Mol Morphol* 9: 3−8, 2001.

Schwaller B, Meyer M, Schiffmann S. New function for old proteins: the role of the calcium-binding proteins Calbindin D-28k, calretinin and parvalbumin, in cerebellar physiology. Studies

with knockout mice. *Cerebellum* 1: 241−258, 2002.

Simons MJ, Pellionisz AJ. Genomics, morphogenesis and biophysics: Triangulation of Purkinje cell development. *Cerebellum* 5: 27−35, 2006.

Sotelo C. Cellular and genetic regulaton of the development of the cerebellar system. *Prog Neurobiol* 72: 295−339, 2004.

Whitney ER, Kemper TL, Bauman ML, Rosene DL, Blatt GJ. Cerebellar Purkinje cells are reduced in a subpopulation of autistic brains: a stereological experiment using calbindin D-28k. *Cerebellum* 7: 406−416, 2008a.

Whitney ER, Kemper TL, Rosene DL, Bauman ML, Blatt GJ. Calbindin-D28k is a more reliable marker of human Purkinje cells that standard Nissl stains: a stereological experiment. *J Neurosci Methods* 168: 42−47, 2008b.

Wiedorn KH, Goldmann T, Henne C, Kuhl H, Vollmer E. EnVision+, a new dextran polymer-based signal enhancement technique for in situ hybridization (ISH). *J Histochem Cytochem* 49: 1067−1071, 2001.