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# Development of a Monoclonal Antibody-based Immunohistochemical Method for Detecting Newcastle Disease Virus

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## Abstract

In this study, an immunohistochemical (IHC) technique for detecting Newcastle disease (ND) virus (avian avulavirus 1) using paraffin sections of formalin-fixed tissues was developed by production of novel monoclonal antibodies. Mice were immunized with ND virus isolate APMV1/chicken/Japan/Fukuoka-1/2004 (genotype VII), and 14 mouse hybridoma clones producing IgG antibodies against the ND virus were established. Among these antibodies, only three reacted with the viral antigen on the paraffin sections, while one (22C483) exhibited the highest sensitivity in IHC analysis. Results of immunoprecipitation, western blot analysis, and indirect enzyme-linked immunosorbent assay showed that 22C483 reacted with the nucleoprotein of ND virus. Thus, IHC analysis using 22C483, in combination with enzyme digestion treatment for antigen retrieval, can be used for elucidating the pathogenesis of ND.

**Discipline:** Animal health
**Additional key words:** chickens, immunohistochemistry, monoclonal antibody, Newcastle disease virus

## Introduction

Newcastle disease (ND) in poultry is an important infectious disease caused by ND virus (Suarez 2013). Currently, ND virus is taxonomically classified as avian avulavirus 1, which was formerly referred to as avian paramyxovirus 1 (Amarasinghe 2017). ND viruses are classified as lentogenic (mild), mesogenic (moderately virulent), and velogenic (highly virulent) according to their virulence (Suarez 2013). ND caused by velogenic viruses can induce a high mortality rate in infected poultry and is always included in the differential diagnosis of highly pathogenic avian influenza.

ND virus has a wide host range; it has been detected in more than 200 bird species (Kaleta & Baldauf 1988). Some reports have documented that the possible epidemiological interaction between poultry and wild birds is responsible for outbreaks of ND (Heckert et al. 1996, Kinde et al. 2005). Therefore, investigation of the pathogenesis of ND in poultry and wild birds is necessary to minimize the risk of viral transmission between wild birds and poultry.

Immunohistochemical (IHC) analysis, which is performed to detect the antigen on histological sections of formalin-fixed, paraffin-embedded tissues, can be a useful research tool for elucidating the pathogenesis of infectious diseases. However, IHC analysis for detecting ND virus is not commonly performed worldwide, probably because of the lack of commercially available suitable primary antibodies. Establishing an IHC method for ND virus detection would facilitate the progress in this research field. In this study, we developed a monoclonal antibody (mAb) applicable to IHC analysis for ND virus detection.

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## Materials and methods

### 1. Preparation of ND virus antigen

Japanese velogenic ND virus strain APMV1/chicken/Japan/Fukuoka-1/2004 (ck/Fukuoka/2004) was selected for viral antigen preparation. This strain is classified as genotype VII of class II, which was the common lineage responsible for the recent velogenic ND outbreaks in Asia (Mase et al. 2009).

This viral strain was propagated in embryonated chicken eggs for 1-2 days for antigen preparation. Infectious allantoic fluids were collected from these eggs and treated with beta-propiolactone for viral inactivation. The virus was purified using 20%-50% sucrose gradient ultracentrifugation. The viral antigen solution was prepared by suspending the pellet of purified viruses in phosphate-buffered saline. The antigen concentration was calculated using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc., USA). The viral antigen solution was dispensed (1 mg/mL) and stored at −80°C until use.

The viral antigen solution was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with MOPS buffer using the NuPAGE system (Thermo Fisher Scientific, Inc.) to confirm whether the electrophoresis pattern of the prepared antigen was consistent with that of previously reported ND virus (Alexander & Collins 1981).

### 2. Development of mAb screening methods

Indirect enzyme-linked immunosorbent assay (ELISA) was developed to detect the antibody against ck/Fukuoka/2004 antigen. The antigen solution (0.5 μg/mL) was mixed with the same amount of 0.1% Triton X-100 solution and then applied onto 96-well ELISA plates. A Block Ace solution (DS Pharma Biomedical Ltd., Japan) as a blocking agent was applied to reduce nonspecific reactions. In mAb screening, the supernatant in individual cell culture plate wells was used as the primary antibody, whereas horseradish peroxidase-labeled rabbit anti-mouse IgG (H+L or gamma chain-specific; Zymed Laboratories Inc., USA) was used as the secondary antibody. 2,2′-Azino-di-(3-ethylbenzthiazoline sulfonic acid) (Sigma-Aldrich Inc., USA) was used as a chromogen. The reaction was spectrophotometrically measured at 405 nm using a microplate reader SPECTRAFluor Plus (Tecan Trading AG, Switzerland). The serum of mice immunized with ck/Fukuoka/2004 was used as the positive control.

As an additional method, we developed a modified immunoperoxidase technique using cell culture plates to efficiently screen mAbs, which can react with the formalin-fixed antigen. Chicken fibroblast DF-1 cells in 96-well cell culture plates were infected with ck/Fukuoka/2004. The cells exhibiting viral-induced cytopathic effects were fixed in 10% neutral buffered formalin for 1-2h. The plates were then rinsed with phosphate-buffered saline, dried, and stored. In mAb screening, cells were incubated with each mAb, followed by the application of the secondary antibody Histofine Simple Stain Max PO(M) reagent (Nichirei Biosciences Inc., Japan). The positive reaction was visualized using the DAB substrate kit (Nichirei Biosciences Inc.).

IHC technique was also used, as required, for mAb screening, and its protocol is later described.

### 3. Production of hybridomas producing anti-ND virus IgG mAb

Two young adult BALB/c mice were intraperitoneally immunized with ck/Fukuoka/2004 antigen (10 μg/time) mixed with aluminum adjuvants (2 mg in 100 μL/time) twice at ≥2-week intervals. The final immunization was performed 3 days before cell fusion by intravenously injecting the antigen (10 μg/time) diluted in saline (400 μL) without adjuvants. Thereafter, the mice were euthanized by intraperitoneal injection of pentobarbital sodium (200 mg/kg body weight), and the spleens were collected. The minced spleens were filtered through a 40-μm mesh. Hybridomas were produced by cell fusion of the spleen cells and mouse myeloma P3U1 cells using dimethyl sulfoxide Hybri-Max reagent (Sigma-Aldrich Inc.). Hybridomas secreting anti-ND virus antibody were selected by mAb screening and were cloned twice using the single-cell sorting method. To select for hybridomas producing IgG, the cloned mAbs were subtyped using the rapid mouse antibody isotyping kit (Thermo Fisher Scientific Inc.). After screening, three mAbs (22C483, 2E5-11, and 8E9A9) were selected for further analysis.

### 4. Reactivity of mAbs to Japanese ND virus isolates

Indirect immunofluorescence assay was performed to evaluate the reactivity of the three mAbs against five Japanese ND virus isolates and two vaccine strains. The ND viruses used were velogenic viruses isolated from chicken (ck/Fukuoka/2004, APMV1/chicken/Japan/Okayama-1/2002, and APMV1/chicken/Japan/Ibaraki/254/2001 of genotype VII), a mesogenic virus derived from a feral pigeon (APMV1/pigeon/Japan/Niigata/2007 of genotype VI), a lentogenic virus isolated from a duck (APMV1/duck/Japan/Hyogo/2002 of genotype I), and vaccine strains (B-1 and VG/GA of genotype II). Primary chicken kidney cell culture plates were prepared, and cells were infected with each ND virus. Each mAb was used as the primary antibody. Alexa

Fluor 488-conjugated goat anti-mouse IgG (H+L; Thermo Fisher Scientific Inc.) was used as the secondary antibody. Nuclear staining was performed using a Cellstain DAPI solution (Dojindo Laboratories Co., Ltd., Japan). The reaction was observed under a fluorescence microscope.

In addition, the three mAbs were tested by routine virus neutralization test and hemagglutination inhibition test to examine the neutralizing capacity and hemagglutination inhibition activities against ck/Fukuoka/2004, respectively.

**5. Production of mouse mAb ascites**

The three mAbs were produced in ascites of mice pre-primed intraperitoneally once with 0.3 mL of 2,6,10,14-tetramethylpentadecane. Hybridomas ($10^6$ cells) excreting each mAb were injected into the peritoneal cavity of young adult BALB/c mice. After 2-3 weeks, ascites was collected from the abdominal cavity, after which the mice were euthanized as previously described. Furthermore, ascites was semipurified using ammonium sulfate precipitation. Antibody concentration was calculated using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.).

**6. Validation of mAbs in IHC analysis for ND viral antigen detection**

IHC analysis was performed to evaluate the applicability of the three mAbs. Tissue sections of lymphoid organs were prepared using the archived paraffin block of a chicken experimentally infected with ck/Fukuoka/2004. For blocking the endogenous peroxidase activity, deparaffinized sections were treated with 0.3% $H_2O_2$ in methanol for 20 min at room temperature. Two antigen retrieval methods, heat-induced antigen retrieval (HIAR) and enzyme digestion, were tested for each mAb, together with the sample without any antigen retrieval. Sections in citrate buffer (pH 6.0) were heated in a microwave oven (500 W) for 15 min for HIAR. Enzyme digestion was performed by applying 0.1% Actinase E solution (Kaken Pharmaceutical Corp., Ltd., Japan) for 10 min at 35°C. This was followed by blocking using 5% skim milk solution (Wako Pure Chemical Industries Ltd., Japan) for 20 min at room temperature, followed by incubating the sections with different dilutions of each mAb (mouse ascites) at 4°C overnight. After incubation using a Histofine Simple Stain Max PO(M) kit (Nichirei Biosciences Inc.) as the secondary antibody for 1 h at room temperature, IHC reaction was visualized using the DAB substrate kit (Nichirei Biosciences Inc.). The reaction time for visualization was determined using microscopic observation. Hematoxylin was used as the counterstain. The reaction intensity of the IHC method was scored using microscopic observation. Systemic organs of uninoculated chickens were also tested to examine nonspecific reactions of mAbs to normal chicken tissues. The reactivity of mAbs to avian avulavirus 2 was tested by IHC analysis using a sample of chicken embryo that was infected with chicken/Yucaipa/California/1956.

**7. Determination of the viral protein that binds with 22C483**

As 22C483 exhibited the most promising results in the IHC analysis, immunoprecipitation and subsequent western blotting (WB) were performed to investigate the viral protein to which 22C483 binds. First, ck/Fukuoka/2004 antigens were immunoprecipitated by 22C483 (cell culture supernatant) using Dynabeads Protein A/G kit (Thermo Fisher Scientific, Inc.) for semipurification of the antigen. Then, the ND viral protein immunoprecipitated by 22C483 was electrophoresed on SDS-PAGE gel under reduced condition with MOPS buffer using the NuPAGE system (Thermo Fisher Scientific, Inc.) and was then transferred onto a nitrocellulose membrane. In WB, 22C483 (cell culture supernatant) and a mouse anti-ND virus nucleoprotein antibody (clone NP401; courtesy of Dr. Nagai at Nagoya University, Japan) (Nishikawa et al. 1987) were used as the primary antibodies to compare the results of the two antibodies. A horseradish peroxidase-conjugated anti-mouse TrueBlot secondary antibody (Rockland Inc., USA) was used as the secondary antibody because it does not react with the reduced SDS-denatured antibody used in the immunoprecipitation step. A laboratory-made mAb anti-bovine interleukin 6 (cell culture supernatant), which does not bind to ND viral antigens, was used as the negative control in the immunoprecipitation step.

As a separate approach, an N-terminal FLAG-tagged recombinant nucleoprotein of ND virus was produced by silkworm protein expression system ProCube (Sysmex corp., Japan) using baculovirus containing the full gene of ND virus APMV1/chicken/Japan/Ibaraki/254/2001 nucleoprotein (accession number AB124599.1). Routine indirect ELISA was performed to investigate the reaction between the recombinant nucleoprotein and three mAbs, along with the serum of mice immunized with ck/Fukuoka/2004 as a positive control and normal mouse serum as a negative control.

**8. Animal welfare**

All experiments using living animals were approved by the Ethics Committee of the National Institute of Animal Health, Japan (authorization numbers 11-066, 11-093, 13-019, and 13-066).

## Results

### 1. mAb production and screening

ND viral antigen derived from ck/Fukuoka/2004 was successfully developed and used for antibody detection and mAb production. A total of 14 hybridomas secreting anti-ck/Fukuoka/2004 IgG mAbs were established (Table 1), and eight mAbs reacted with formalin-fixed antigen in modified immunoperoxidase tests. Preliminary IHC tests revealed that three mAbs (22C483, 2E5-11, and 8E9A9) could detect ck/Fukuoka/2004 antigens on paraffin sections obtained from infected chicken tissues. These three mAbs were selected for further evaluation.

### 2. Reactivity of mAbs to Japanese ND virus isolates

Indirect immunofluorescence assay using cultured cells showed that 22C483 reacted with all the ND viruses, including vaccine strains. The positive reaction by 22C483 was observed in the cytoplasm, indicated by the granular appearance (Fig. 1). The other two mAbs (2E5-11 and 8E9A9) reacted with six ND viruses, but not with the virus isolated from a pigeon. The positive reactions by 2E5-11 and 8E9A9 were found in the cytoplasm and were occasionally intense along the cell membrane. None of the three mAbs showed neutralizing and hemagglutination inhibition activities against ck/Fukuoka/2004.

**Table 1. Screening results of mAbs against ck/Fukuoka/2004** [a)]

| mAb | Isotype | Screening method | | |
|---|---|---|---|---|
| | | ELISA | IP | IHC |
| 22C483 | IgG1 | +[b)] | + | + |
| 9H1-11 | | + | + | − |
| 13C162 | | + | + | − |
| 13D111 | | + | + | − |
| 3B8-7 | | + | − | − |
| 6H101 | | + | − | NT |
| 2E5-11 | IgG2a | + | + | + |
| 8E9A9 | | + | + | + |
| 5B9-7 | | + | + | − |
| 1F439 | IgG2b | + | + | − |
| 22C412 | | + | − | − |
| 1A103 | | + | − | NT |
| 19E65 | | + | − | NT |
| 26A512 | | + | − | NT |

[a)]mAb: monoclonal antibody, ELISA: enzyme-linked immunosorbent assay, IP: modified immunoperoxidase technique, IHC: immunohistochemistry, NT: not tested.
[b)]+: positive, −: negative.

### 3. IHC analysis using mouse mAb ascites

mAbs from mouse ascites were successfully developed; their concentrations were 4.21 mg/mL for 22C483, 7.04 mg/mL for 2E5-11, and 5.40 mg/mL for 8E9A9. IHC analysis using 22C483 detected ck/Fukuoka/2004 antigens in association with the histological necrotic lesions of lymphoid organs and exhibited higher sensitivity than those of other mAbs (2E5-11 and 8E9A9) (Table 2 and Fig. 2). Moreover, when we repeated the IHC analysis using paraffin blocks after several months of storage, the positive reactions by 2E5-11 and 8E9A9 were weaker than those in the previous trial (data not shown).

Nonspecific reactions to the nuclei of neurons and ganglion cells of normal chickens were observed in IHC analysis using 22C483 with HIAR and without treatment for antigen retrieval (Table 3). Enzyme digestion treatment prevented nonspecific reactions. Taken together, we considered that the optimal condition for 22C483 (mouse ascites) in IHC analysis was the antibody dilution of 1:3,200 to 1:12,800 with enzyme digestion treatment. 2E5-11 reacted with the brush border of the normal renal tubules. Nonspecific reactions by 8E9A9 were observed in plasma cells and unidentified mononuclear cells in the connective tissue throughout the body and in the tubular brush border of the kidney. All the three mAbs did not react with the chicken embryo tissues infected with avian avulavirus 2.

These results suggested that 22C483 can be considered as the most promising mAb for IHC analysis and it was therefore selected for further analysis.

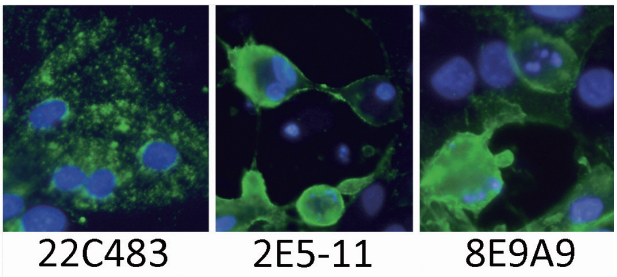


**Fig. 1. Typical indirect immunofluorescence assay pictures using three mAbs (22C483, 2E5-11, and 8E9A9) and primary chicken kidney cells infected with ND virus ck/Fukuoka/2004.**
The positive reaction by 22C483 was observed in the cytoplasm of multinucleated cells (syncytium) with granular staining pattern and was occasionally strong in perinuclear areas. The assays using 2E5-11 and 8E9A9 were positive in the cytoplasm and often exhibited strong reactions along the cell membrane. ND viral antigens were visualized in green (Alexa Fluor 488). The cell nucleus was stained in blue (DAPI).

**Table 2. Reactivity of mAbs (mouse ascites) in IHC analysis using paraffin sections of lymphoid organs derived from a chicken infected with ck/Fukuoka/2004 [a)]**

| | | Dilution of mAb | | | | | |
|---|---|---|---|---|---|---|---|
| mAb | AR | 1:200 [b)] | 1:800 [b)] | 1:3,200 | 1:12,800 | 1:51,200 | 1:204,800 |
| 22C483 | HIAR | + [c)] | +++ | +++ | +++ | +++ | + |
| | E | + | +++ | +++ | +++ | +++ | + |
| | None | + | +++ | +++ | +++ | +++ | + |
| 2E5-11 | HIAR | + | ++ | + | + | + | + |
| | E | + | ++ | + | + | + | − |
| | None | + | + | + | + | + | − |
| 8E9A9 | HIAR | + | + | + | + | + | − |
| | E | + | + | + | + | + | − |
| | None | + | + | + | + | − | − |

[a)]mAb: monoclonal antibody, IHC: immunohistochemistry, AR: antigen retrieval method, HIAR: heat-induced antigen retrieval, E: enzyme digestion

[b)]Nonspecific background staining was observed at these dilutions of mAbs.

[c)]IHC reaction intensity −: negative, +: weak, ++: moderate, +++: strong

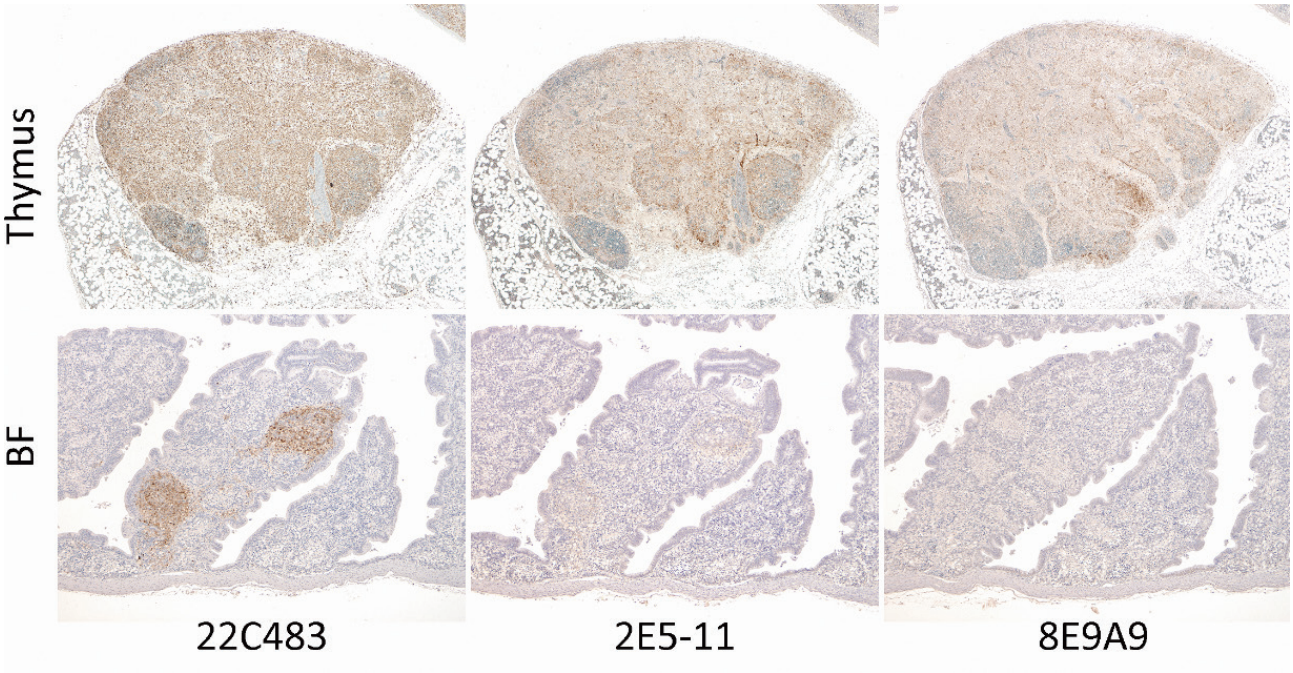


**Fig. 2. IHC pictures of lymphoid organs derived from a chicken infected with ND virus ck/Fukuoka/2004 using mAbs (22C483, 2E5-11, and 8E9A9) with their optimal staining conditions.**
22C483 exhibited the stronger reaction in necrotic lymphoid follicles of the bursa of Fabricius (BF) than it did in the other two mAbs.

**Table 3. Nonspecific reaction of 22C483 (mouse ascites) to the nucleus of the neuronal cells in normal chicken brain in IHC analysis [a)]**

| | | Dilution of 22C483 | | | | | |
|---|---|---|---|---|---|---|---|
| mAb | AR | 1:200 [b)] | 1:800 [b)] | 1:3,200 | 1:12,800 | 1:51,200 | 1:204,800 |
| 22C483 | HIAR | ++ [c)] | ++ | ++ | ++ | ++ | − |
| | E | − | − | − | − | − | − |
| | None | ++ | ++ | + | + | + | − |

[a)]IHC: immunohistochemistry, mAb: monoclonal antibody, AR: antigen retrieval method, HIAR: heat-induced antigen retrieval, E: enzyme digestion.

[b)]Nonspecific background staining was noticed at this dilution of 22C483.

[c)]Intensity of nonspecific reaction −: negative, +: weak, ++: moderate

**4. ND viral protein detected by 22C483**

WB showed that the primary antibody NP401, which could bind to the ND viral nucleoprotein, reacted with the ND viral antigen immunoprecipitated by 22C483, indicating that 22C483 and NP401 could react with the same viral antigen, i.e., the nucleoprotein of ck/Fukuoka/2004 (Fig. 3). Although two adjacent bands emerged at approximately 50 kDa on WB, the lower smaller band was considered as the cleavage product of the viral nucleoprotein reported previously (Hamaguchi et al. 1983, Nishikawa et al. 1987). When 22C483 was used as the primary antibody on WB, the same band pattern was reproduced (Fig. 3). The negative control did not yield the electrophoresed band at approximately 50 kDa.

Indirect ELISA using the recombinant ND viral nucleoprotein revealed that 22C483, NP401, and mouse immune serum reacted with the recombinant nucleoprotein. Other mAbs (2E5-11 and 8E9A9) and normal mouse serum did not react with the recombinant nucleoprotein.

## Discussion

In our research, we established an IHC method to detect ND viral antigens using paraffin sections and mAbs developed in the present study. We found that mAb 22C483 exhibited higher sensitivity than the other two mAbs (2E5-11 and 8E9A9). The IHC method using 22C483 would be useful for research on the pathogenesis of ND viral infection.

The mAbs 2E5-11 and 8E9A9 showed reduced staining intensity of the positive reaction, which can be attributed to the prolonged storage time of the paraffin block used for preparing the histological sections. During this storage time, the viral protein's epitope may have been degraded, thereby implying that the epitope to which 2E5-11 and 8E9A9 bind may be vulnerable to the regular storage conditions of paraffin blocks.

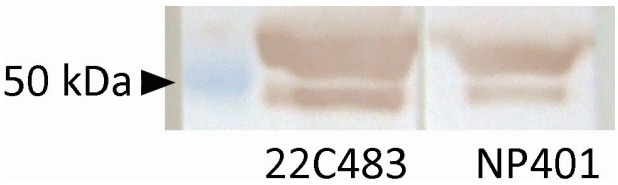


**Fig. 3. WB analysis targeting the ND viral protein that is immunoprecipitated by 22C483**
When two primary antibodies (22C483 and mouse anti-ND viral nucleoprotein NP401) were used, the same band pattern emerged at approximately 50 kDa.

Paramyxoviruses complete their replication cycles in the cytoplasm of infected cells (Harrison et al. 2010, Suarez 2013). Therefore, the positive reaction in IHC methods for ND virus detection should be based on cytoplasmic staining. However, nuclear staining of paramyxovirus antigen has often been reported in IHC studies using canine distemper virus (Alldinger et al. 1993) and measles virus (Hatanpaa & Kim 2014). For those two viruses, nuclear translocation of the viral proteins associated with the viral nucleocapsid was considered the cause of nuclear staining in IHC analysis (Alldinger et al 1993, Hatanpaa & Kim 2014). This nuclear translocation of nucleocapsid-associated proteins has also been ultrastructurally observed for ND virus (Granzow et al. 1999, Tajima 1977). Therefore, nuclear staining could be possible in IHC analysis when a primary antibody against nucleocapsid-related proteins of the ND virus is available. As 22C483 can cause nonspecific reactions in the nuclei of neuronal cells in IHC analyses depending on the staining condition, special attention needs to be paid when the neural tissues are evaluated by IHC techniques using 22C483. Enzyme digestion using Actinase E solution for antigen retrieval prevents this nonspecific reaction by probably degrading the protein that nonspecifically reacts with 22C483 in the nucleus of the neuronal cell, but does not affect the ND viral protein to which 22C483 specifically binds, thus resulting in successful ND virus detection in IHC analysis.

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