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1 **Identification of antigenic peptides derived from B-cell epitopes of nucleocapsid protein**
2 **of mouse hepatitis virus for serological diagnosis**

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22

1 **Abstract**

2

3 Mouse hepatitis virus (MHV) infection is found commonly in laboratory mice and
4 this virus has been known to cause various diseases such as subclinical infection, enteritis,
5 hepatitis, and encephalitis. Serological tests are used commonly to diagnose MHV infection.

6 Complete MHV virions have been used primarily as antigens for serological diagnosis to date.

7 To develop an antigen that is more specific, more sensitive, and easier to prepare for

8 serological diagnosis, the antigenic sites in the MHV nucleocapsid (N) protein were screened

9 in this study. Sixteen antigenic linear sequences in the N protein were found using antisera

10 obtained from mice infected naturally with MHV and a peptide array containing overlapping

11 10-mer peptides covering the entire N protein. From these antigenic sequences, two

12 synthesized peptides, ILKKTWADQTERGL and RFDSTLPGFETIMKVL, which were

13 consistent with positions 24–38 and 357–372 of the N protein respectively, were used as

14 antigens in ELISA. Evaluation of ELISA with these peptides revealed that both peptides were

15 specific to anti-MHV antisera. Furthermore, ELISA performed using these peptides was more

16 sensitive than commercial ELISA used for a screening sera from mice infected accidentally to

17 MHV maintained in cages, suggesting that these peptides are useful for serological diagnosis

18 of MHV infection.

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20 **Keywords:** diagnosis, ELISA, epitope mapping, Mouse hepatitis virus, nucleocapsid protein

21

1 **1. Introduction**

2

3 Mouse hepatitis virus (MHV) is an enveloped positive-stranded RNA virus, which
4 belongs to the *Coronaviridae* (de Vries et al., 1997). MHV infection causes various diseases
5 such as subclinical infection, enteritis, hepatitis, and encephalitis (Compton et al., 2004;
6 Haring and Perlman, 2001; Matthews et al., 2002). MHV strains are classified as enterotropic
7 or polytropic. Polytropic strains disseminate from the initial site of replication in the
8 respiratory epithelium to neural, hepatic, vascular, and lymphatic tissues. Replication of
9 enterotropic strains is limited generally to replication within the intestinal tract and these
10 strains cause subclinical infection in adult mice. In contrast, polytropic strains are more likely
11 to cause more severe diseases with clinical symptoms (Baker, 1998). Since MHV is one of the
12 most prevalent viruses in laboratory mice (Jacoby and Lindsey, 1997), it is important to detect
13 this virus in mice colonies by appropriate and sensitive screening tests during routine health
14 surveillance programs.

15 Serological assays detecting antibodies against pathogens in sera are the most
16 popular screening tests for diagnosis of infection (Compton and Riley, 2001). ELISA and the
17 indirect immunofluorescent assay are the most common serological assays for diagnosis of
18 MHV infection (La Regina et al., 1988; Peters et al., 1979). To date, infected cells or purified
19 viruses are used usually as antigens in serological tests for detecting anti-MHV antibodies.
20 Conversely, cross-reactivity between antibodies present in sera and nonspecific antigens used
21 in the test may yield a false positive result. Therefore, the use of purified antigens such as
22 recombinant proteins and synthesized antigenic peptides derived from pathogens will make
23 serological assays more specific and sensitive. MHV comprises at least three proteins: the

1 envelope glycoprotein designated as the spike (S) protein, the envelope membrane (M)
2 protein, and the nucleocapsid (N) protein (de Vries et al., 1997). The S protein is involved in
3 cellular receptor binding and membrane fusion (Gallagher and Buchmeier, 2001). The N
4 protein is associated with genomic RNA and plays a role in virus assembly through
5 interactions with the viral RNA and the M protein (Hurst et al., 2005; Verma et al., 2006).
6 The N protein consists of three conserved domains among the strains, which are
7 separated by two variable regions (Parker and Masters, 1990). Several monoclonal antibodies
8 against the S, N, and M proteins were reported previously, indicating that these proteins are
9 immunogenic (Fleming et al., 1983; Kubo et al., 1994; Nelson et al., 2000; Stohlman et al.,
10 1994; Stühler et al., 1991). Therefore, these proteins are potential antigens for serological
11 diagnosis.

12 In this study, the antigenicity of the peptides derived from the MHV N protein was
13 demonstrated by using anti-MHV antisera and peptide array membrane, on which a set of
14 overlapping peptides covering the entire N protein was synthesized. The results showed that
15 16 antigenic linear sequences existed in the N protein. In these antigenic sequences, two sites
16 coding amino acids 24–38 (ILKKTWADQTERGL) and 357–372
17 (RFDSTLPGFETIMKVL) of the N protein are strongly antigenic to anti-MHV antisera
18 derived from mice infected naturally. ELISA performed using these peptides was specific and
19 could detect anti-MHV antisera with higher sensitivity, than a commercial ELISA. These
20 results indicate that these two peptides are useful for serological diagnosis of MHV infection
21 in laboratory mice colonies.

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23 **2. Materials and Methods**

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2.1. Mouse sera.

Sera from C57BL/6 mice infected accidentally with MHV were provided by Prof. Ichiro Miyoshi from the Center for Experimental Animal Science, Graduate School of Medical Sciences, Nagoya City University. As per the guidelines of the Institutional Animal Care and Use Committee (IACUC), Nagoya City University, for screening and eradication of MHV infection, the mice were euthanized and sera were obtained. Normal mice sera were acquired from BALB/c, C57BL/6N, and DBA/2 mice obtained from SLC (Shizuoka, Japan). These sera were derived from the samples obtained for routine screening during a health surveillance program in the animal facility as per the guidelines of IACUC of Graduate School of Veterinary Medicine, Hokkaido University.

2.2. MHV-N peptide array

A set of overlapping 10-mer peptides, obtained by shifting one amino acid from the amino terminus to the carboxy terminus of the N protein that was derived from MHV-JHM strain (GenBank ID: X00990), were synthesized on derivatized cellulose membranes (INTAVIS Bioanalytical Instruments AG, Köln, Germany) using the Auto Spot Peptide Synthesizer ASP-222 (INTAVIS Bioanalytical Instruments AG) according to the manufacturer's protocol (Frank, 2002). Four hundred forty-six peptides were synthesized on the membrane, which are consistent with the peptides coding amino acids from 1–10 to 446–455 of the N protein.

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2.3. Detection of antibodies in mouse sera binding to peptides on the peptide array membrane

Sera were diluted to 1:1,000 with 5% skim milk in PBS-Tween 20 (137 mM NaCl, 81 mM Na₂HPO₄, 15 mM KH₂PO₄, 27 mM KCl, and 0.1% Tween 20) and incubated with the peptide array membrane for 2 h at room temperature. The peptide array membrane was washed with PBS-Tween 20 three times and incubated with the HRP-conjugated anti-mouse IgG (GE Healthcare BioSciences, Uppsala, Sweden) diluted to 1:10,000 with 5% skim milk in PBS-Tween 20 for 2 h at room temperature. After reaction with the HRP-conjugated anti-mouse IgG, the membrane was washed with PBS-Tween 20 three times. The antibodies bound specifically to the peptides on the membrane were detected by the ECL Advance Western Blotting Detection Kit (GE Healthcare Biosciences). Visualization and calculation of chemiluminescence of each spot were performed using LAS-3000 imaging system (Fuji film, Tokyo, Japan).

2.4. ELISA

A commercial ELISA, MONILISA MHV (Wakamoto Pharmaceutical Co., Ltd., Tokyo, Japan), was used in this study. Sera used in this ELISA were diluted to 1:40. Evaluation of whether sera were MHV positive or negative was confirmed by comparison with immunoreactivity of positive control sera provided by the manufacture. The peptides coding amino acids 24–38 (ILKKTWADQTERGL) and 357–372 (RFDSTLPGFETIMKVL) of the MHV-N protein were synthesized by Invitrogen (Carlsbad,

1 CA, USA). In a 96-well plate, 200 μ l of 0.5 μ M peptide in 0.1 M sodium
2 carbonate-bicarbonate buffer (pH 9.6) was seeded, and the plate was incubated overnight at 4
3 $^{\circ}$ C. The wells were washed three times with PBS-Tween 20 and incubated with 200 μ l of 1%
4 BSA in PBS-Tween 20 (blocking buffer) for 1 h at 37 $^{\circ}$ C. The wells were washed three times
5 with PBS-Tween 20, and 200 μ l of sera diluted to 1:200 with blocking buffer was added and
6 the plate was incubated for 1 h at 37 $^{\circ}$ C. After washing the well three times with PBS-Tween
7 20, 200 μ l of the HRP-conjugated anti-mouse IgG diluted to 1:10,000 was added to the wells
8 and incubated for 1 h at 37 $^{\circ}$ C. After washing the well three times with PBS-Tween 20, 200 μ l
9 of 1.5 mg/ml *o*-phenylenediamine with 0.01% H₂O₂ was added to each well and incubated for
10 10 min at 37 $^{\circ}$ C. Following this, 50 μ l of 6 N H₂SO₄ was added and OD was measured at 450
11 nm using a plate reader.

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13 *2.5. Statistics*

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15 For statistical analyses, Student's t-test, Fisher's exact test, and chi square test were
16 performed using Statview 5.0 software (SAS Institute Inc., Cary, NC, USA).

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18 **3. Results**

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20 *3.1. Epitope mapping of the MHV N protein*

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22 To determine antigenic peptides for detecting anti-MHV antibodies, B-cell epitope
23 mapping of the MHV N protein was performed using the peptide array membrane. This array

1 membrane comprised 446 spots, which consisted of 10-mer peptides obtained by a single
2 amino acid shift, to produce a complete overlapping set of the N protein. Sera from mice that
3 were infected accidentally with MHV were incubated with the peptide array membrane. These
4 mice were confirmed to be positive using commercial ELISA for detecting anti-MHV
5 antibodies. As shown in Fig. 1, the IgG antibodies in the sera, bound specifically to the
6 peptides on the spots, were detected. The serum used in Fig. 1 contained antibodies that
7 recognized at least four rows of spots, positioned at 27–29, 84–86, 183–187, and 362–366.
8 This result indicates the presence of four antigenic sites in the MHV N protein that are B-cell
9 linear epitopes. In contrast, no significant intensity was observed when sera from control mice
10 were used (data not shown). Sera from seven mice infected with MHV were then analyzed
11 with the same method. The chemiluminescence intensity of each spot was measured, and the
12 mean values are shown in the graph (Fig. 2). Highest intensity was observed around the 28th
13 spot, on which a peptide consistent with amino acids 28–37 of the MHV N protein was
14 synthesized. A similar intensity was observed at the 362nd spot consistent with the intensity
15 observed at the sequence position 362–371. The peptides on the spots with an intensity higher
16 than 2.0 in Fig. 2 were defined as antigenic, and therefore, as B-cell linear epitopes. Sixteen
17 B-cell epitopes in the MHV N protein were found, and their position and sequence are shown
18 in Table 1.

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20 *3.2. Development of ELISA to detect anti-MHV antibodies using antigenic peptides derived*
21 *from the MHV-N protein*

22

23 The B-cell epitopes of the MHV N protein from MHV seen in Fig. 2 and Table 1

1 were expected to be antigens for serological diagnosis of MHV infection. To examine
2 whether these epitopes were appropriate for diagnosis, we developed an ELISA using
3 synthesized peptides corresponding to the B-cell epitopes of the MHV N protein. Two
4 16-mer peptides corresponding to the amino acids 24–39 (MHV-N 24–39) and amino acids
5 357–372 (MHV-N 357–372) of the N protein were employed as antigens for this ELISA. The
6 sensitivity and specificity of ELISA using the peptide-antigens were examined by normal or
7 MHV-positive mouse sera. When using the MHV-N 24–39 peptide, the OD values of
8 1:50-diluted MHV-positive sera were higher than those of the diluted MHV-positive sera, and
9 the values decreased as the dilution increased (Fig. 3, upper graph). In contrast, the OD values
10 of normal sera were almost the same as those without sera. In the case of the MHV N 357–
11 372 peptide, a similar result was seen (Fig. 3, lower graph). These results indicate that both
12 the MHV N peptides could be employed as specific antigens for detecting anti-MHV
13 antibodies.

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15 *3.3. Evaluation of ELISA using the MHV-N peptides for serological diagnosis of MHV* 16 *infection*

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18 To evaluate whether ELISA using the MHV-N peptides could be employed for
19 diagnosis, its immunoreactivity was examined using sera from the MHV-contaminated colony
20 that comprised mice infected accidentally with MHV and their cage mates. When sera from
21 eight control mice were used, the mean and the SD were as follows: 0.113 and 0.025 for the
22 MHV-N 24–39 peptides, and 0.181 and 0.023 for the MHV–N 357-372 peptides (Table 2).
23 The means and SD of OD values from the 15 mice maintained in the MHV-contaminated

1 colony are also given in Table 2. The mean OD values of sera from mice in
2 MHV-contaminated colony were significantly higher than that from control mice.
3 Furthermore, these sera were evaluated by both commercial ELISA and ELISA using the
4 MHV-N peptides. As shown in Table 2, the cutoff OD values of ELISA using MHV-N
5 peptides for MHV-positive sera were determined to be the mean + 2 SD from the control mice.
6 All control samples were negative in each ELISA (Table 3). When sera from mice in the
7 MHV-contaminated colony were used, eight of the 15 samples were positive in commercial
8 ELISA. In contrast, 11 samples were positive in the MHV-N 24–39 and 12 samples were
9 positive in the MHV-N 357–372 ELISA. All samples that were positive in commercial
10 ELISA were also positive in ELISA performed using the two peptides (data not shown).
11 Furthermore, the positive/negative frequency was significantly different between the mice in
12 the control and MHV-contaminated colonies when using ELISA using each MHV-N peptides.
13 In contrast, according to the chi-square test results, the positive/negative frequency of sera
14 from mice in the MHV-contaminated colony was not significantly different between
15 commercial ELISA and ELISA using the MHV-N peptides. These results indicate that the
16 ELISA performed using peptides from the N protein is as specific as the commercial ELISA
17 for the detection of MHV infection.

18

19 **4. Discussion**

20

21 MHV infection is found commonly in laboratory mice and causes various diseases
22 such as subclinical infection, enteritis, hepatitis, and encephalitis (Compton et al., 2004;
23 Haring and Perlman, 2001; Matthews et al., 2002). Therefore, it is important to monitor

1 laboratory mice colonies for MHV infection. Serological assays are used generally for the
2 diagnosis of MHV infection (Compton and Riley, 2001; La Regina et al., 1988; Peters et al.,
3 1979). ELISA is used commonly for diagnosis of MHV infection. In the present study,
4 several B-cell epitopes in the MHV N protein were identified using sera from MHV-positive
5 mice and the MHV-N peptide array membrane. In addition, it was demonstrated that ELISA
6 using the antigenic peptides derived from B-cell epitopes of the MHV N protein is useful for
7 detection of anti-MHV antibodies in mouse sera.

8 The N protein is one of the structural proteins of *Coronaviridae* (deVries et al.,
9 1997). Previous studies indicated that the N protein of *Coronaviridae* is conserved relatively,
10 immunogenic, and expressed abundantly during infection (Daginakatte et al., 1999; Fleming
11 et al., 1983; King and Brian, 1982). Furthermore, a recent study demonstrates that the whole
12 N protein is suitable as an antigen for serological diagnosis using ELISA (Kunita et al., 2011).
13 Therefore, in this study, it was examined whether the peptides derived from the MHV N
14 protein were suitable antigens for diagnosis. SPOT peptide array was used for screening of
15 antigenic epitopes (Frank, 2002; Reineke et al., 2001; Reineke et al., 2002). In this study, the
16 peptide array membrane was prepared that contained a set of nine amino acids overlapping
17 the 10-mer peptides covering the entire N protein. This array was expected to clarify
18 accurately the comprehensive antigenic linear B-cell epitopes in the N protein. In addition,
19 sera from mice infected naturally with MHV were used for identification of antigenic sites in
20 the N protein in live MHV. As expected, several antigenic sites were detected in the N protein
21 (Figs. 1 and 2 and Table 1). Two sites positioned from amino acids 24 to 39 and from amino
22 acids 357 to 373 were more antigenic than the other sites in the N protein. Previous reports
23 demonstrated the location of epitopes of monoclonal antibodies against the N protein

1 (Stohlman et al., 1994). The epitopes were classified into five regions: amino acids 1–133,
2 171–196, 231–249, 249–277, and 374–465. These antigenic regions corresponded, in part, to
3 those in the present results in Table 1. Because sera used for epitope mapping in this study
4 were derived from MHV-infected C57BL/6 mice, the same strain used in the study by
5 Stohlman et al., this result is considered to be reasonable. In addition, some epitopes that have
6 not been reported as yet were found in the present result, e.g., amino acids 150–159, 307–325,
7 335–346, and 357–373. In particular, the 357–373 amino acid segment was a strong antigenic
8 site in this study. The present results indicate that the approach using peptide array was very
9 efficient for screening the antigenic sites in the entire N protein to identify B-cell epitopes. In
10 addition, these sites are not the same as the RNA binding site in the central domain and the
11 interactive sites of the M protein in the highly conserved carboxy-terminal domain among the
12 strains of MHV (Hurst et al., 2005; Nelson et al., 2000; Parker and Masters, 1990).

13 In this study, two sites corresponding to amino acids 24–39 and 357–372 of the N
14 protein were selected as ELISA antigens. As shown in Fig. 3 and Table 2, ELISA using the
15 MHV-N 24–39 or MHV-N 357–372 peptides showed no nonspecific cross-reaction to normal
16 sera and was able to show specific reaction to anti-MHV antisera. The positive/negative
17 frequency was significantly different between mice from the control and MHV-contaminated
18 colonies when ELISA was performed with each MHV-N peptides (Table 3). Furthermore,
19 from this ELISA, not only all MHV-positive sera but also some MHV-negative sera
20 diagnosed by commercial MHV ELISA were detected to be positive (data not shown). In
21 contrast, the antigen in commercial ELISA used in this study was the complete MHV virus
22 (Kagiyama et al., 1990). These results indicate that the MHV-N 24–39 and MHV-N 357–372
23 peptides are as specific as the complete MHV virus for serological diagnosis of MHV

1 infection, and suggest that the ELISA using these peptides is more sensitive than commercial
2 ELISA. In addition, the utilization of peptides as antigens, compared to the complete MHV as
3 used in commercial ELISA, gives us the advantage in quality control, preparation, and
4 economy of ELISA products. However, the positive/negative frequency of sera from mice in
5 the MHV-contaminated colony was not significantly different between commercial ELISA
6 and ELISA using the MHV-N peptides. In addition, the reactivity of sera from various mice
7 strains was not examined by ELISA using the MHV-N peptides. Further studies are necessary
8 for validation of sensitivity and specificity of the two peptides to sera from mice in the
9 MHV-contaminated colony.

10 The sequence of the three carboxyl-terminal residues in the MHV-N 24–39 peptide
11 (GLN) is polymorphic among the strains of MHV. For example, this sequence is substituted
12 into GPN in polytropic MHV-1, MHV-3, and MHV-A59 strains and into AGN in polytropic
13 MHV-S and enterotropic MHV-Y strains (Parker and Masters, 1990). Therefore, it is
14 necessary to examine whether this peptide is reactive to antisera against various MHV strains.
15 In contrast, the amino acids in the MHV-N 357–372 peptide are conserved completely among
16 all MHV strains that protein sequence data were registered in GenBank database, suggesting
17 that the MHV-N 357–372 peptide can detect infection caused by various strains of MHV.

18 In summary, 16 linear B-cell epitopes were identified in the MHV N protein in this
19 study. The present results indicate that two peptides, ILKKTWADQTERGLN and
20 RFDSTLPGFETIMKVL, are useful for serological diagnosis of MHV infection using
21 serological assays such as ELISA. Immunological assay with peptide array membrane and
22 antisera is an effective method for identifying B-cell epitopes in the proteins derived from the
23 pathogenic agents and for the development of specific antigens for serological diagnosis.

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Table 1. Antigenic sequences in the MHV N protein

Position of spots (Intensity > 2.0 in Fig. 2)	Position of amino acids	Peptide sequences
1, 2, 5	1–14	MSFVPGQENAGSRS
24–30	24–39	ILKKTWADQTERGLN
49–55	49–64	PKQTATTQPNSGSVVP
72, 73	72–82	ITQFQKGKEFQ
81, 83	81–92	FQFAQGQGVPIA
130	130–139	YLGTGPYAGA
150	150–159	WVASQQAETR
165	165–174	VERDPSSHEA
184–186	184–195	VLPQGFYVEGSG
193	193–202	GSGRSAPASR
240–244	240–253	LVLAKLGKDAGQPK
307–316	307–325	SDPQFPILAELAPTAGAFF
335, 337	335–346	KNSGGADGPTKD
347	347–356	VYELQYSGAV
356–364	356–373	VRFDSTLPGFETIMKVLN
384	384–393	GGADVVSPPK

Table 2. Detection of anti-MHV antibody from mice sera by ELISA using the MHV-N peptides

	MHV-N 24–39 peptide			MHV-N 357–372 peptide		
	OD ₄₅₀			OD ₄₅₀		
	mean	SD	mean + 2 SD	mean	SD	mean + 2 SD
Control (n = 8)	0.113	0.025	0.164	0.181	0.023	0.226
MHV-contaminated colony (n = 15)	0.326*	0.205		0.388*	0.219	

* $p < 0.05$ vs control by Student's t-test.

Table 3. Evaluation of ELISA using the MHV-N peptides for detection of MHV infection

	Positive samples/total samples		
	Commercial ELISA	ELISA using MHV-N 24–39	ELISA using MHV-N 357–372
Control	0/8	0/8	0/8
MHV-contaminated colony	8/15*	11/15*	12/15*

* $p < 0.05$ vs control by Fisher's exact test.

1 **Figure Legends**

2

3 **Fig. 1.** Detection of antigenic sites in the MHV N protein using a peptide array membrane.
4 Antibodies in mouse sera bound to each peptide on a peptide array membrane were visualized
5 by chemiluminescence. A typical image from seven MHV-positive sera samples is shown.

6

7 **Fig. 2.** Antigenicity of the MHV N protein. Peptide-binding antibodies in sera from mice
8 infected naturally were detected as shown in Fig. 1. The intensity of chemiluminescence at
9 each spot was quantified. The values are relative to the background and are presented as mean
10 \pm SEM of seven sera. The values on X-axis indicate the position of the spots.

11

12 **Fig. 3.** ELISA using the MHV-N peptides. MHV-positive or -negative sera were diluted and
13 used for ELISA using the MHV-N peptides as described in Materials and Methods. The upper
14 graph is a plot of OD₄₅₀ from ELISA using the MHV-N 24–39 peptide. The lower graph is a
15 plot of OD₄₅₀ from ELISA using the MHV-N 357–372 peptide. Open circles indicate
16 MHV-negative sera, and closed squares indicate MHV-positive sera. (-) indicates the sample
17 without serum. The plots are representatives from three independent experiments.

FIGURE 1

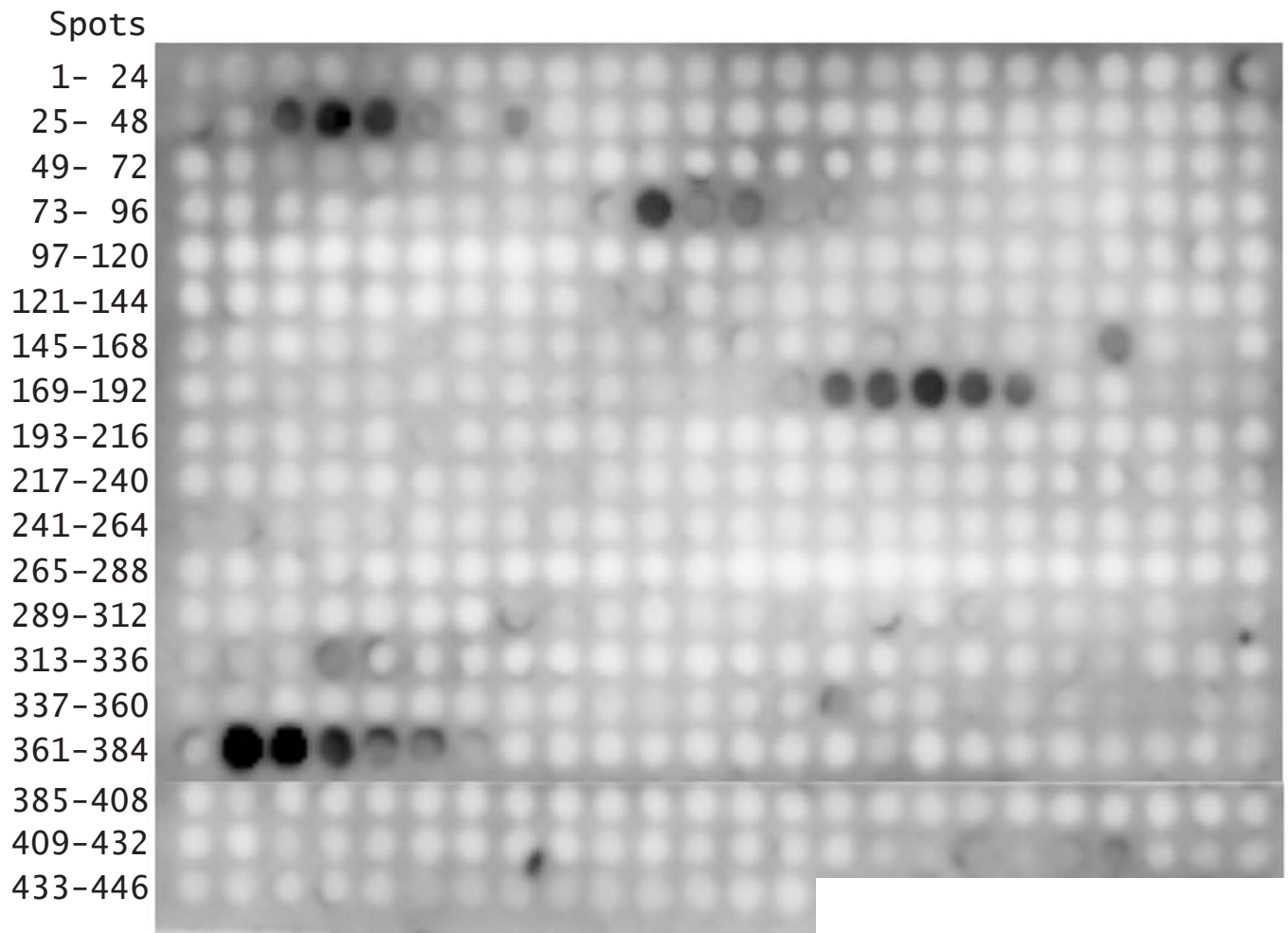


FIGURE 2

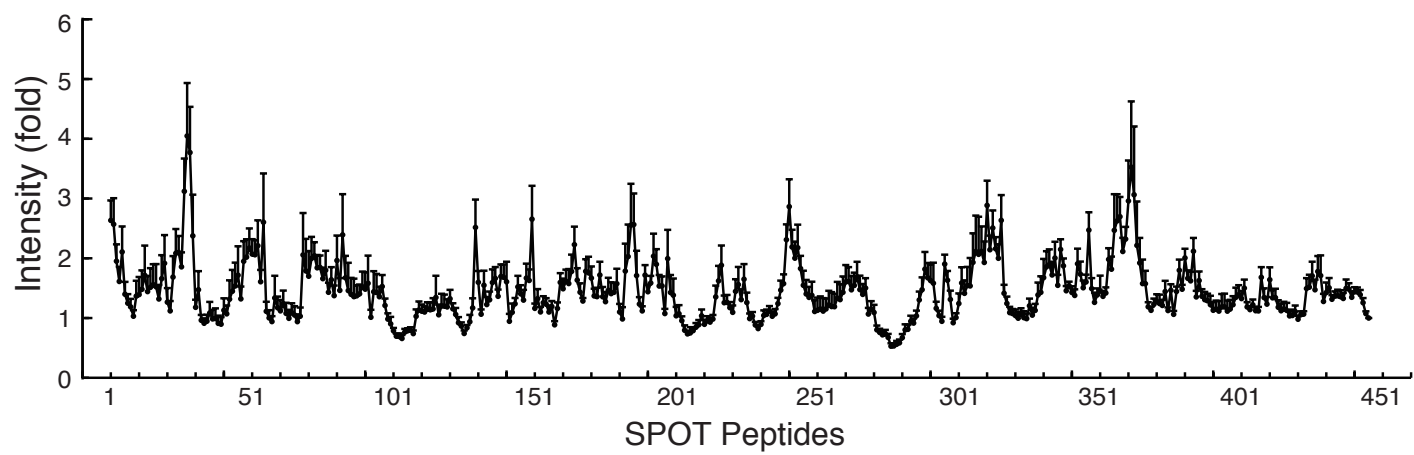
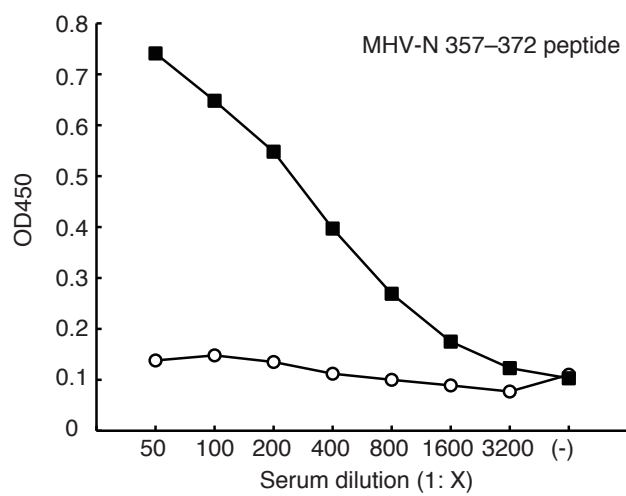
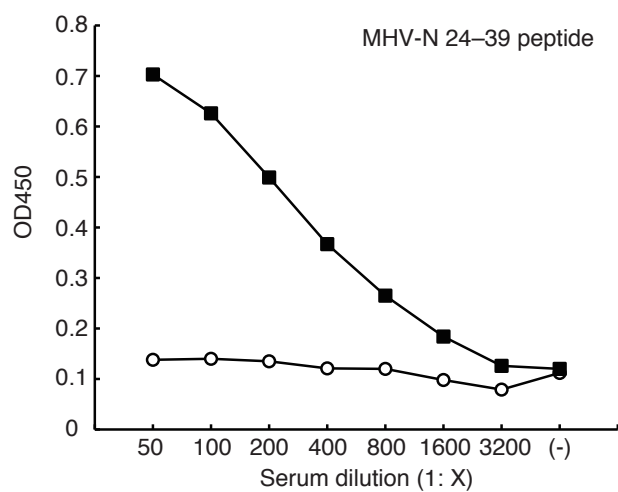
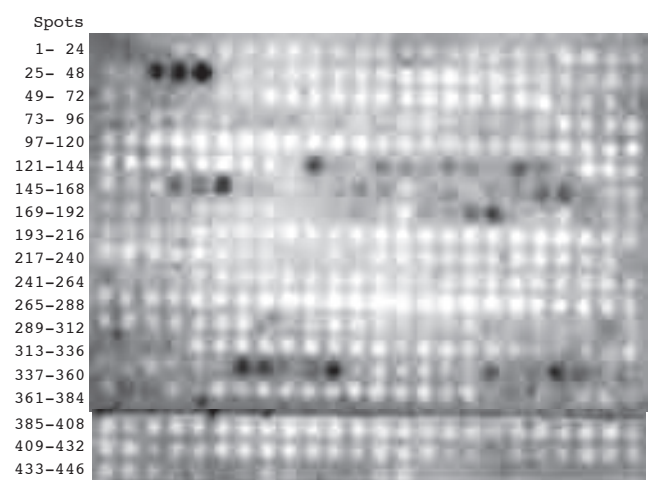
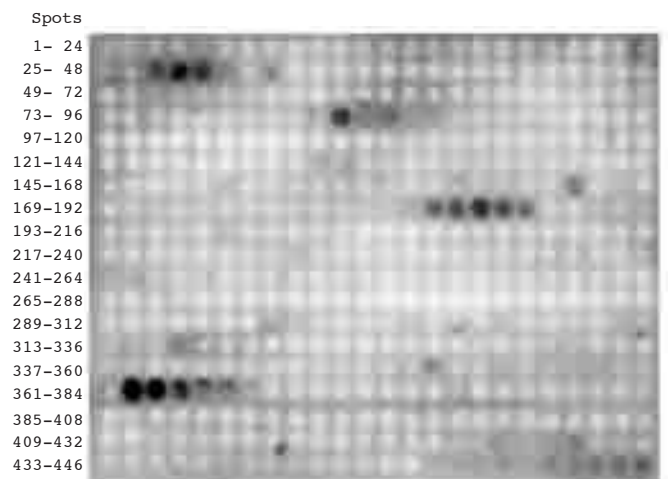
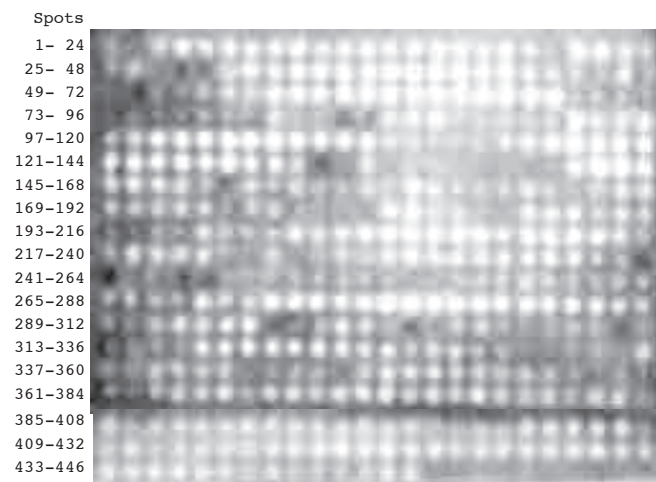
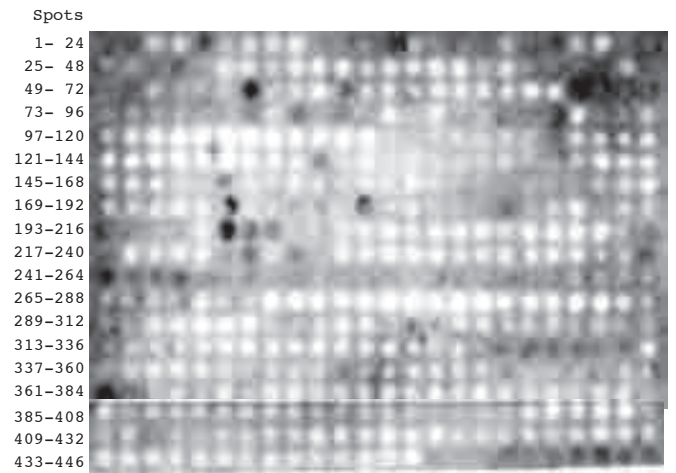
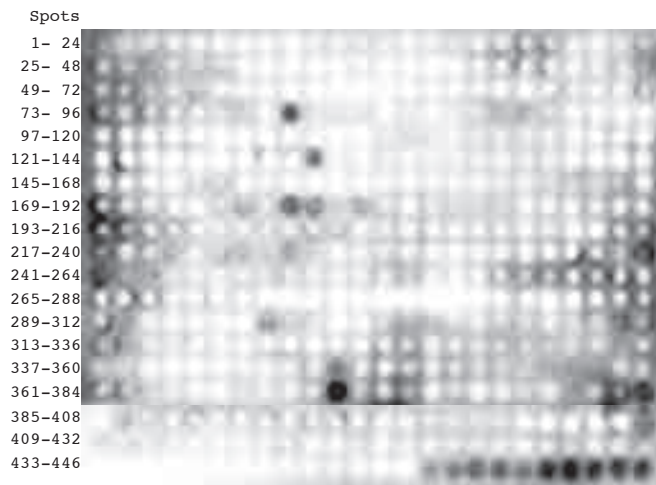
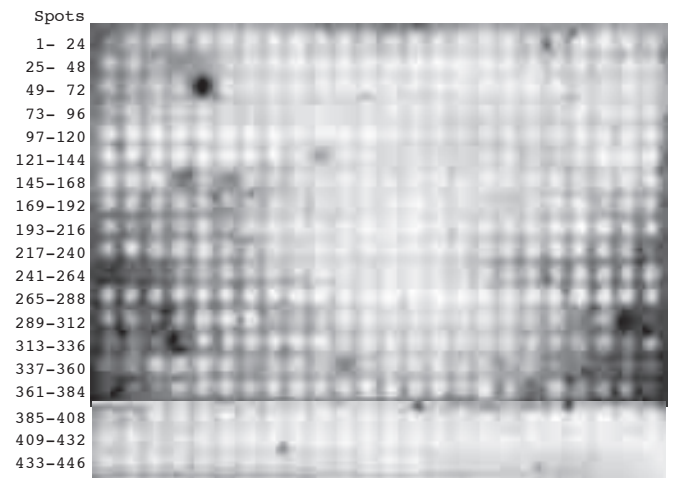
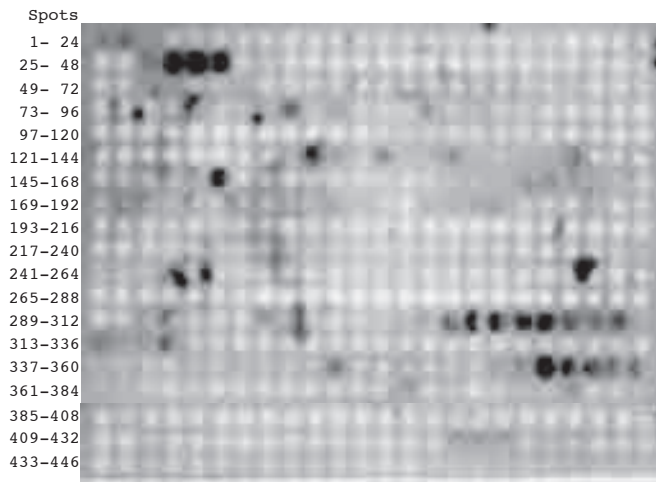


FIGURE 3





Supplemental Figure