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Original article

# Characterization of murine monoclonal anti-M and anti-N blood group reagents produced by national Blood Centre, Thai Red Cross Society

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## Abstract

***Background:*** This study is serologic testing of anti-M and anti-N murine monoclonal antibodies which secreted from five murine monoclonal hybridoma clones composed of NBC-M2($7H_3 2B_7$), NBC-N1($139G_3G_2$), NBC-N2 ($9A_3D_9$), NBC-N3($9A_5B_7$), and NBC-N4($9D_3D_5$). All the clones were produced by the Antiserum and Standard Cells Production Section, National Blood Centre, Thai Red Cross Society.
***Objective:*** To study serologic characteristics of anti-M and anti-N murine monoclonal antibodies products.
***Methods:*** The serologic characteristics were performed for potency and specificity of antibodies. Effect of proteolytic enzymes including Papain, Trypsin, Chymotrypsin, and Neuraminidase to monoclonal antibodies were done. Testing temperature and pH effected antigen-antibody binding reaction. Our anti-M and anti-N were compared to commercial products. Type of known and unknown M and N antigen red blood cells were determined and also the unknown was compared to polyclonal anti-M and anti-N. Antibodies stability testing by preserving at 4°C, 22°C - 24°C, and room temperature for every four months until two years. Isotype were tested by using commercial kits.
***Results:*** Our anti-M and anti-N had the best potency and specificity to both normal red blood cells and enzyme treated red blood cells. Antigen-antibody binding reaction was better in low temperature as 4°C and at room temperature than at 37°C. Effect of pH showed antigen-antibody binding was better at pH 5.5 and 7.0 than at pH 8.5. Our anti-M and anti-N potency were stronger than the commercial products and rabbit polyclonal antibodies. Known M and N antigen red blood cells typing results showed correctly in every sample. Unknown M and N antigen red blood cells typing results showed both rabbit polyclonal and monoclonal antibodies corresponding in every sample. Stability testing showed anti-M and anti-N were stable more than one year when preserved at 4°C. As for the isotype testing, it showed anti-M and anti-N are $IgG_1$.
***Conclusion:*** Anti-M and anti-N murine monoclonal antibodies were appropriate for producing blood group phenotyping reagents to replace the polyclonal rabbit immunized serum.

***Keywords:*** Anti-M, anti-N, monoclonal, serology.

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MNS blood group is a complex human blood group system. M and N antigens are located on glycophorin A molecule on erythroid surface.[1] We have been using polyclonal rabbit anti-M and anti-N for red blood cells (RBCs) typing at the National Blood Center, Thai Red Cross Society (TRCS). However, the antibodies have low specificity and cross-reactivity with Mi series antigens in the Miltenberger (Mi) subsystem.[2, 3]

The National Blood Centre, TRCS has collaborated with the Japanese Red Cross regarding human and murine monoclonal hybridoma production technique for rare blood group reagents project. With the collaboration, the National Blood Centre, TRCS has successfully created murine monoclonal antibodies specific to M antigen (NBC-M2), NBC-M2($7H_3 2B_7$), and N antigens: NBC-N1($139G_3G_2$), NBC-N2 ($9A_3D_3$), NBC-N3 ($9A_5B_7$), NBC-N4 ($9D_3D_5$) using human erythroid cells as immunizing agent fused to generate hybridoma cell lines.[4] In this study, we characterized the anti-M and anti-N murine monoclonal antibodies and compared their sensitivity and specificity with commercially available antisera for M and N antigens phenotyping.

This study has been approved by the Ethics Human Research Committee, the National Blood Centre, Thai Red Cross Society, Bangkok, Thailand (COA no. NBC 6/2016).

## Materials and methods

### *Characterization of murine monoclonal anti-M and anti-N products*

We characterized murine monoclonal antibodies anti-M (NBC-M2), and anti-N (NBC-N1, N2, N3, and N4)[4] serological reactivities for red blood cells MN phenotyping against M and N antigens red blood cells for specificity, potency, proteolytic enzyme effect, effect of temperature, pH, stability, and sensitivity.

### *Specificity and potency testing*[5, 6]

Anti-M from hybridoma clone NBC-M2 ($7H_3 2B_7$) and anti-N from five hybridoma clones: NBC-N1 ($139G_3G_2$), NBC-N2 ($9A_3D_9$), NBC-N3($9A_5B_7$), and NBC-N4 ($9D_3D_5$) were tested with known samples of group O RBCs: MM, MN and NN by standard tube technique. Serial two-fold dilution of anti-M and anti-N were done in 1.0% mixture of phosphate buffer saline and bovine serum albumin (PBS-BSA, pH 7.2). For each dilution, 100 µl antibodies were mixed with 2.0% cell suspension, the cell-serum mixture was incubated at room temperature (RT, 22 - 24°C) for 10 minutes, centrifuged at 3,400 rpm for 15 seconds, and then examined for agglutination sum score.[5]

### *Effect of proteolytic enzymes*[6]

Four proteolytic enzymes, namely; papain, trypsin, chymotrypsin, and neuraminidase were used to treat group O MM, MN and NN RBCs. The enzymes treated RBCs were tested for reactivity with Anti-M NBC-M2($7H_3 2B_7$) and Anti-N NBC-N1 ($139G_3G_2$).

To prepare papain treated RBCs, working papain solution was prepared by mixing one part of stock papain and 2 g of powdered papain was added to 100 mL of PBS (pH 5.4). The enzyme solution was agitated for 15 minutes at RT. Clear fluid was collected by filtration or centrifugation. L-cysteine hydrochloride was added and incubated solution at 37 °C for one hour. PBS (pH 5.4) was added to the final volume of 200 ml and stored aliquots at - 20 °C or cold.)[5] in nine parts of 0.15 M (PBS), pH 5.5. Then and one part of packed RBCs was mixed with four parts of working papain solution, incubated at 37°C for 15 minutes, washed four times with PBS. The papain treated RBCs was resuspended to be 2.0% (V/V,), in PBS, pH 7.2.

To prepare trypsin and chymotrypsin treated RBCs, trypsin (Sigma, type XI) and chymotrypsin (Sigma, type II) were prepared as 10 mg/ml in 0.1 M PBS, pH 7.7. One part of packed RBCs was mixed with ten parts of trypsin or chymotrypsin solution, incubated at 37°C for 30 minutes, and then washed four times with PBS. The trypsin or chymotrypsin treated RBCs were suspended, 2.0% (V/V), in PBS, pH 7.2.

To prepare neuraminidase treated RBCs, neuraminidase from *Vibrio cholera* was prepared as 5 units per ml in normal saline solution (NSS) with 1 mM $CaCl_2$. One part of packed RBCs was mixed with twenty parts of neuraminidase, incubated at 37°C for 30 minutes, and then washed 4 times with PBS. The neuraminidase treated RBCs were suspended, 2.0% (V/V), in PBS, pH 7.2.

Anti-M and anti-N murine monoclonal antibodies were diluted by serial two-fold dilution in 1.0% PBS, pH 7.2. 100 µl of enzyme treated RBCs was mixed with of 100 µl of anti-M or anti-N in a test tube, incubated at RT for 10 minutes, centrifuged 3,400 rpm for 15 seconds, and then examined for results by sum score.[4]

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***Effect of temperature***[6]

Anit-M and anti-N murine monoclonal antibodies were tested for agglutination potency to group O: MM, MN and NN RBCs at 4°C, RT, and 37°C. Anti-M and anti-N were diluted by serial two-fold dilution in 1.0% PBS, pH 7.2. : 100 µl of 2.0% RBCs suspension was mixed with 100 µl of anti-M and anti-N in a test tube incubated at specific temperature: 4°C, RT, and 37°C for 30 minutes each, then centrifuged at 3,400 rpm for 15 seconds, and examined results as agglutination sum score.[4]

***Effect of pH testing***[6]

Anti-M and anti-N murine monoclonal antibodies were diluted by serial two-fold dilution in 1.0% PBS with specific pH: pH 5.5, pH 7.0, and pH 8.5. For each pH diluted antibody 100 µl of 2.0% suspension of group O: MM, MN and NN in 1.0% PBS: BSA pH 7.2 were mixed with100 µl of anti-M and anti-N in a test tube, incubated at RT for 10 minutes, centrifuged at 3,400 rpm for 15 seconds, and then examined for agglutination sum score.[4]

***Comparison with different manufactures Anti-M and Anti-N reagents***

Anti-M and anti-N murine monoclonal antibodies were tested for potency in comparison with Epiclone™ Anti-M and anti-N (bioCSL, Parkville, Australia), Seraclone® mouse IgM monoclonal Anti-M and anti-N (Bio-Rad, Dreieich, Germany), anti-M and anti-N (CE Immundiagnotika GmbH, Eschelbronn, Germany), and rabbit polyclonal anti-M and anti-N antibodies (National Blood Center, TRCS, Bangkok, Thailand). The testing methods were done according to recommendation of the manufacturer.

***Typing of antigen M and antigen N in donated blood samples***

Repeat MN antigens typing on 225 group O donors with know MNS antigens were done with produced monoclonal anti-M and anti-N In addition, random sampling of 559 blood donor sample at the National Blood Center, TRCS were also tested for M and N typing. There were 124, 171, 208, and 56 blood donor sample of group A, B, O, and AB, respectively with unknown MN antigen type.

Each blood sample was typed for M and N antigens using two types of reagents, anti-M and anti-N murine monoclonal antibodies and rabbit polyclonal antibodies by standard tube technique. Briefly, anti-M and anti-N were diluted by serial two-fold dilution in 1.0%, PBS, pH 7.2. RBCs samples were diluted to 2.0% suspension in 1.0% PBS-BSA, pH 7.2. For each sample, 100 µl of 2.0% RBCs suspension was mixed with 100 µl of diluted anti-M and anti-N in a test tube, incubated at RT for 10 minutes, centrifuged at 3,400 rpm 15 seconds, and then examined for agglutination sum score.

***Stability testing***[6]

Anti-M and anti-N murine monoclonal antibodies were stored at 4°C, RT, and 37° C, and were tested for potency every four months for two years. Interval potency testing of the antibodies in difference temperature storage was examined by titration of monoclonal anti-M and anti-N with corresponding antigen as stated in potency testing.

***Anti-M and anti-N isotyping classes testing***

Isotyping for mouse: $IgG_1$, $IgG_{2a}$, $IgG_{2b}$, $IgG_3$, IgA, and IgM were performed using Pierce rapid isotyping kits-mouse (Thermo scientific, USA) according to the manufacturer recommendation.

***Assistance from Japanese Red Cross***

Our anti-M and anti-N monoclonal product was sent to Kanto-Kosshinetsu Block Blood Centre, Japanese Red Cross for assisting the testing with some rare blood group red cell.

## Results

***Specificity and potency of anti-M and anti-N monoclonal antibodies***

Anti-M from hybridoma clone NBC-M2 ($7H_32B_7$), and anti-N from hybridoma clones: NBC-N1 ($139G_3G_2$), NBC-N2 ($9A_3D_9$), NBC-N3 ($9A_5B_7$), and NBC-N4 ($9D_3D_9$) were tested with known samples of group O RBCs: MM, MN and NN. Anti-M NBC-M2 ($7H_32B_7$) demonstrated specific positivity with MM and MN and negative with NN RBCs. Anti-N NBC-N1 ($139G_3G_2$) was the only one clone that demonstrated no cross reactivity with MM red cells and yielded higher agglutination titer with RBCs group O genotype NN and MN **Table 1**. Therefore, we performed further testing on monoclonal anti-M: NBC-M2 ($7H_32B_7$) and only one monoclonal anti-N: NBC-N1 ($139G_3G_2$).

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**Table 1.** Specificity and potency of anti-M and anti-N from hybridoma clones.

| Type of antibodies | Name of hybridoma | Sum scores of antibodies titration | | |
|---|---|---|---|---|
| | | RBCs | | |
| | | O,MM | O,MN | O,NN |
| Anti-M | NBC-M2 ($7H_3 2B_7$) | 54 | 42 | 0 |
| Anti-N | NBC-N1 ($139G_3G_2$) | 0 | 78 | 86 |
| Anti-N | NBC-N2 ($9A_3D_9$) | 75 | 125 | 135 |
| Anti-N | NBC-N3 ($9A_5B_7$) | 75 | 124 | 86 |
| Anti-N | NBC-N4 ($9D_3D_5$) | 75 | 125 | 133 |

**Table 2.** Proteolytic enzymes effect.

| Type of antibodies | RBCs | Non-treated RBCs | Sum scores of antibodies titration | | | |
|---|---|---|---|---|---|---|
| | | | Enzyme treated RBCs | | | |
| | | | Papain | Trypsin | Chymotrypsin | Neuraminidase |
| | O, MM | 54 | 0 | 0 | 16 | 0 |
| **Anti-M** | O, MN | 42 | 0 | 0 | 6 | 0 |
| | O, NN | 0 | 0 | 0 | 0 | 0 |
| | O, MM | 0 | 0 | 31 | 0 | 0 |
| **Anti-N** | O, MN | 78 | 0 | 36 | 47 | 0 |
| | O, NN | 86 | 0 | 34 | 53 | 0 |

***Proteolytic enzymes susceptibility***

Anti-M failed to react with papain, trypsin and neuramidase treated O, MM and O, MN RBCs indicating that these enzymes destroyed M antigen on RBCs surface completely but not chymotrypsin. Anti-N also failed to react with papain and neuramidase treated O, MN and O, NN RBCs, while trypsin and chymotrypsin had only partial effect as shown in **Table 2.** Unexpectedly, anti-N reacted with some $O_{MM}$ RBCs that were treated with trypsin.

***Anti-M and anti-N monoclonal antibodies are cold reacting***

The anti-M and anti-N are cold reacting antibodies. The best reaction of anti-M was at 4°C, declined by RT, and the least was at 37°C. Anti-N showed comparable reactivity at 4°C and RT, and yielded lower reactivity at 37°C **(Figure 1).**

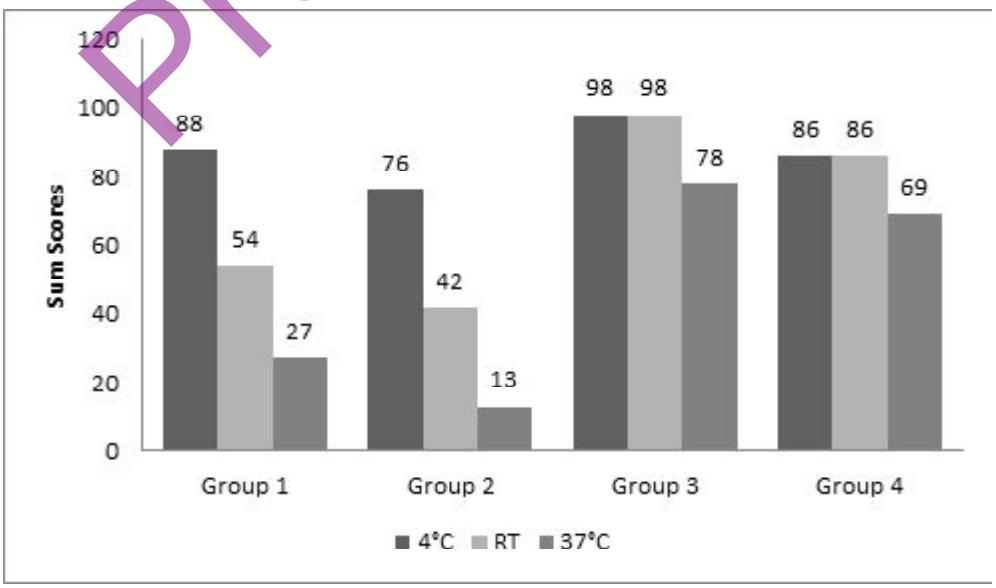


**Figure 1.** Variation of temperature affected MN antigens-antibodies binding reaction. Group 1 to be anti-M with MM; Group 2 to be anti-M with MN; Group 3 to be anti-N with NN; and Group 4 to be anti-N with MN.

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**Table 3**. Effect of pH on anti-M and anti-N binding with RBCs.

| pH | Sum scores of antibodies titration | | | | | |
|---|---|---|---|---|---|---|
| | Anti-M | | | Anti-N | | |
| | O,MM | O,MN | O, NN | O,MM | O, MN | O, NN |
| 5.5 | 38 | 26 | 0 | 0 | 78 | 90 |
| 7.0 | 26 | 26 | 0 | 0 | 78 | 90 |
| 8.5 | 5 | 3 | 0 | 0 | 59 | 74 |

**Table 4.** Titers of anti-M and anti-N from various sources.

| | Anti-M | | | | Anti-N | | |
|---|---|---|---|---|---|---|---|
| Source of antibodies | Method | RBCs | Titer | Source of antibodies | Method | RBCs | Titer |
| CSL | 5min, RT | O, MM | 8 | CSL | 5 min, RT | O, MM | 4 |
| LM 100/140 (IgG) | centrifuged | O, MN | 4 | MH 1(IgG) | centrifuged | O, MN | 2 |
| Bio-Rad | 30 min, RT | O, MM | 32 | Bio-Rad | 30 min, RT | O, MM | 32 |
| BS 57 (IgM) | no centrifuged | O, MN | 16 | BS 41 (IgM) | no centrifuged | O, MN | 16 |
| CE Immunediagnostika 11H2 (IgG) | 5 min, RT centrifuged | O, MM | 8 | CE Immunediagnostika 1432C7 (IgM) | Immediate centrifuged | O, MM | 32 |
| | | O, MN | 4 | | | O, MN | 16 |
| TRC mouse monoclonal $7H_3 2B_7$(IgG1) | 10 min, RT centrifuged | O, MM | 16 | TRC mouse monoclonal $139G_2E_{11}$(IgG1) | 10 min, RT centrifuged | O, MM | 32 |
| | | O, MN | 8 | | | O, MN | 16 |
| TRC rabbit polyclonal | 10 min, RT centrifuged | O, MM | 16 | TRC rabbit polyclonal | 10 min, RT centrifuged | O, MM | 8 |
| | | O, MN | 8 | | | O, MN | 4 |

TRC, National Blood Center, Thai Red Cross Society; RT, incubated at room temperature (22°C - 24°C).

***Optimal pH for anti-M and anti-N agglutinating***

Anti-M and anti-N attained optimal reactions at pH 5.5 and 7.0. Anti-M was more sensitive to alkaline pH than anti-N as shown in **Table 3.**

***Comparing potency of TRC anti-M and anti-N murine monoclonal antibody with other antisera sources***

Anti-M and anti-N produced by National Blood Centre were compared to commercial products as shown in **Table 4**. Anti-M (NBC-M2) had comparable antibodies titer to rabbit polyclonal and 11H2 (CE Immunodiagnostika). While anti-M had higher titer than LM 100/140 (CSL), but lower than BS 57 (Bio-Rad). Anti-N (NBC-N1) had comparable antibodies titer to BS 41 (Bio-Rad) and 1432C7 (CE Immunodiagnostika). Anti-N also had higher titer than MH 1 (CSL) and rabbit polyclonal antibodies. Both TRC murine monoclonal anti-M and anti-N were thus comparable to most commercially available reagents.

***M and N antigens phenotyping by murine monoclonal anti-M and anti-N***

A total of 225 blood samples with known M, N antigens were tested using murine monoclonal anti-M and anti-N under standard tube test conditions were found to be highly accurate phenotyping as show in **Table 5.** There were 105 samples of O, MM, 94 O, MN and 26 O, NN. All O, MM were positive with anti-M and negative with anti-N while O, MN RBCs were positive with anti-M and anti-N. Regarding all of 26 O, NN genotypes, they were negative with anti-M and positive with anti-N.

**Table 5.** Phenotyping of known antigen M and N.

| RBCs | Samples | Detectable samples | |
|---|---|---|---|
| | | Anti-M | Anti-N |
| O, MM | 105 | 105 | 0 |
| O, MN | 94 | 94 | 94 |
| O, NN | 26 | 0 | 26 |
| **Total** | **225** | **199** | **120** |

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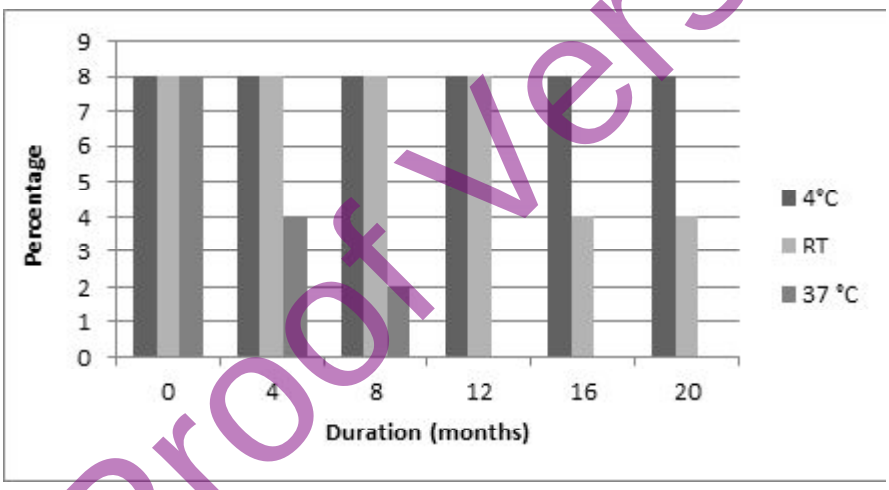
**Table 6.** Phenotyping in unknown MN antigen blood donor samples.

| RBCs ABO | Number tested | Anti-M | | Anti-N | | MN antigen detected |
|---|---|---|---|---|---|---|
| | | Rabbit polyclonal antibodies | Monoclonal antibodies | Rabbit polyclonal antibodies | Monoclonal antibodies | |
| A | 51 | 51 | 51 | 0 | 0 | **MM** |
| A | 63 | 63 | 63 | 63 | 63 | **MN** |
| A | 10 | 0 | 0 | 10 | 10 | |
| B | 86 | 86 | 86 | 0 | 0 | |
| B | 63 | 63 | 63 | 63 | 63 | |
| B | 22 | 0 | 0 | 22 | 22 | |
| O | 96 | 96 | 96 | 0 | 0 | |
| O | 90 | 90 | 90 | 90 | 90 | |
| O | 22 | 0 | 0 | 22 | 22 | |
| AB | 23 | 23 | 23 | 0 | 0 | |
| AB | 30 | 30 | 30 | 30 | 30 | |
| AB | 3 | 0 | 0 | 3 | 3 | **NN** |
| **Total** | **559** | **502** | **502** | **303** | **303** | |



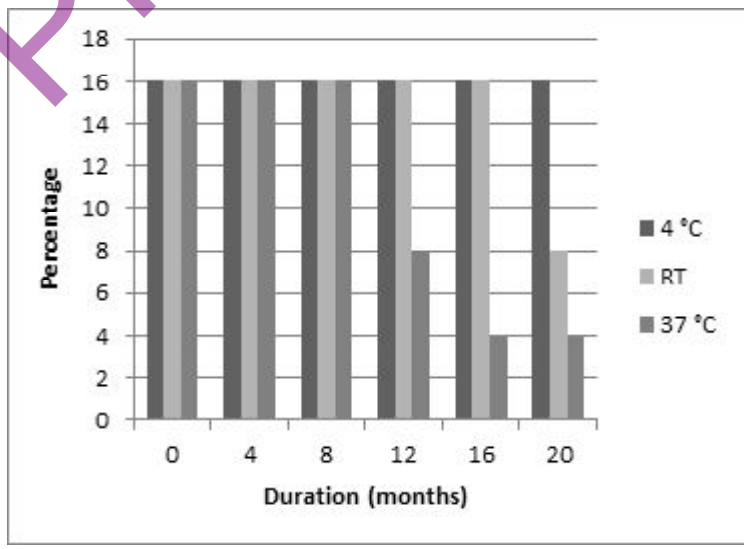




**Figure 2.** Stability of murine monoclonal (A) anti-M (B) anti-N.

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M and N phenotyping also performed in additional 559 unknown MN blood group blood donor samples, using murine monoclonal and rabbit polyclonal antibodies. M and N phenotyping result by both types of antibodies demonstrated agreement results as shown in **Table 6.** From blood donors, there were blood donors: 256 MM (45.8%) 246 MN (44.0%) and 57 NN (10.2%). Therefore, among Thai MM and MN blood group are more common than NN. The calculated genotype alleles, frequency of M allele and N allele was 0.679 and 0.321, respectively. The distribution of MN phenotype was consistent with Hardy-Weinberg principle. The type of ABO and MN alleles expected population study ($P = 0.164$).

### *Stability of anti-M and anti-N*

**Figure 2A** presents the results of storage in difference temperature and time interval. Anti-M reactivity was stable for 12 months in 4°C and RT storage. The activity declined by RT storage at 16 months and 20 months with equal reactivity. As for 37°C storage, the activity was declined at 4 months and eight months to be no datable since 12 months storage.

**Figure 2B** presents the results of storage in different temperature and time intervals. Anti-N reactivity was stable for 16 months at 4°C and RT storage. The activity declined by RT storage at 20 months. As for 37°C storage, the activity was declined at 12 month and 16 month and 20 month storages with equal reactivity.

## Discussion

The MNS blood group system consists of 4 major antigens M, N, S and s. M and N antigens are located on glycophorin A, while the S and s are on glycophorin B molecules of the RBCs membrane. This system, included many variant alleles, is called Miltenberger series in the past or "variant MNS" at the present. The prevalence of variant MNS, around 10.0% of Southeast Asian population, is among the highest in the world. Anti-M, anti-N and anti-Mi$^a$ are clinically significant antibodies, particularly anti-Mi$^a$, which is highly prevalent antibodies in East Asia.[7 - 11]

The distribution of M and N antigens in this study (n = 559) is the second largest as reported from Thai population. The proportion of MM (45.8%), MN (44.4%) and NN (10.4%) are consistent with previous studies from the National Blood Center (n = 948) that the distribution MM (36.9%), MN (46.7%), and NN (16.4%), respectively.[8] And from two earlier reports (n = 200) the first one also reveal that MM (50.5%), MN (37.5%), and NN (12.0%)[12] and another one said that MM (43.2%), MN (48.5%), and NN (8.3%).[11] The report in Thai Sikhs were; MM (47.5%), MN (39.4%) and NN (13.1%).[13]

Hemolytic transfusion reactions from M and N incompatibility are rare. Hemolytic disease of the newborn from anti-M has emerged from recently reports. Several cases of mother with M-negative blood type that developed anti-M causing hemolysis followed by prolonged anemia due to toreticulocytopenia in their newborns have been reported from India[14], Japan[15], and Iran.[16] Severity varies from mild hemolytic anemia to Hydropsfetalis.[17, 18] Several fetal deaths from anemia have also been reported.[19] It is still unexplainable why a normally clinically insignificant antibodies causes hemolysis in a small proportion of cases. Anti-N, on the other hand, is rarely reported as a cause of hemolytic disease of the newborn. Anti-N has been detected as autoantibody in a few cases of autoimmune hemolytic anemia[20 - 22] or chronic hemodialysis.[23]

In this study, we have anti-M sum scores 54 with O, MM and 42 with O, MN and anti-N sum scores 86 with O, NN and 78 with O, MN is high potency and need dilution to produce anti-N reagent. Our anti-M has no cross-reactivity with NN and Mi RBCs while anti-N has some cross-reactivity with Mi RBCs so we must determine absolute dilution for each lot of production to decrease cross-reactivity. Cross-reactivity was observed that titer of rabbit anti-N decreased after was absorbed with MM RBCs. Repeated MM RBCs can absorb anti-N from rabbit human and lectin. The Miltenbeger subsystem was reported that there was cross-reactivity with anti-M, anti-N and, anti-Mi$^a$.[10] Moreover anti-M and anti-N reacted with Tn and T activated cells.[24] Although anti-M NBC-M2 ($7H_32B_7$) and anti-N NBC-N1 ($139G_3G_2$) were selected for specificity with RBCs genotype O, MM and O, NN. Result of trypsin treatment result in cross-reactivity of $O_{NN}$ RBCs can indicate clearly that both antibodies have specificity to which parts of M and N antigens, because of difference type of enzyme destroys difference parts of glycophorin molecule on RBCs. Trypsin can destroy activity of glycophorin A but cannot destroy activity of glycophorin B. On the other hand chymotrypsin can destroy activity of glycophorin B but cannot destroy activity of glycophorin A. Papain

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can destroy both glycophorin A and glycophorin B. Neuraminidase destroys N-acetyl neuraminic acid on RBCs that effect to activity of M and N antigens.[6] Our results as shown in **Table 2** reveal that both anti-M and anti-N are negative with treated papain RBCs and neuraminidase, are correct. Anti-M is negative with treated trypsin RBCs and positive with treated chymotrypsin RBCs show so anti-M is specific with M antigens on glycophorin A. Anti-N is positive with treated trypsin RBCs because there is N-like activity on glycophorin B, while positive with treated chymotrypsin RBCs which shows specific of our anti-N with N antigens on glycophorin A same. Although IgG usually works the best at 37°C but both TRC monoclonal antibodies seem to work better in low temperature. This observation is also seen with anti-M and anti-N from other sources, which anti-N LM 17/19 and LM 17/20 (CSL) optimal reaction occurred at RT or below.[25] In contrast, Anti-M 7/18/C8-G8 reacted with MM RBCs over the entire temperature range 4°C - 45°C and have shown only small inverse relationship to the temperature.[26]

The results have shown anti-N work better at acidic and neutral pH that is different from other studies. Anti-N, LM 17/19 and LM 17/20 optimal reaction occurred at pH 8.5, it suggests that anti-N generally has its greatest affinity for N antigen at the pH, but give weak or negative reactions at pH 7.0 or below.[11] On the other hand some monoclonal antibodies have shown a broad peak of activity with optimal pH reactions occurring where as anti-B NB1/19.112.28 produced, in Cambridge and Glasgow. The LM30 - 31.5 monoclonal reacts evenly from pH 4.0 to pH 7.5.[12] This phenomenon may be due to the different structure of each kind monoclonal antibodies that may alter their apparent specificity with changing of pH.[27, 28]

Anti-M and anti-N have been produced by the National Blood Centre were compared to commercially available reagents. Antiserum and standard cell preparation section has done phenotyping M and N antigens about 2,400 tests per year. Anti-M and anti-N stability preserved at 4°C for 20 months to have enough for production because our expiry date of anti-M and anti-N reagents is 18 months preserved.

Isotyping classes testing show that results of anti-M $7H_32B_7$ and anti-N $139G_3G_2$ were the as same IgG1 isotype as some commercials that are shown in **Table 5.** Testing results from Japanese Red Cross showed that our anti-M could not detect M variant RBCs type $M^c$ (MNS8) but could detect He (MNS6). Our anti-N also could detect variant RBCs type $St^a$ (MNS15) as shown in **Table 7.** However, we still think that our anti-M can be used for production because variant RBCs type $M^c$ is low prevalence antigen.[29, 30]

**Table 7.** Results from Japanese red cross.

| Antibodies | Rbcs | Grading |
|---|---|---|
| Anti-M | $M^c$ | 0 |
| NBC-M2 ($7H_32B_7$) | M-He (+) | 4+ |
| | M+N+ | 4+ |
| | M-N+ | 0 |
| Anti-N | M+N-$St^a$(+) | $3+^s$ |
| NBC-N1 ($139G_3G_2$) | M+N+ | 4+ |
| | M+N- | 0 |

As you may have well known, anti-N has false positive reaction with N-negative RBCs as there are N-like antigens on glycophorin B (the S/s sialoglycoprotein). The hybrid sialoglycoprotein such as Mi series antigens in the Miltenberger (Mi) subsystem[2], which there are five subclasses in Thais about 9.1%[9]: MiI(Gp.Vw), MiII (GP.Hut), MiIII (GP. Mur), MiVI (GP. Bun) and a new phenotype that we are studying its molecular genotyping in collaboration with Australian Red Cross. We have necessary teasted for our murine monoclonal anti-N with RBCs that are Mi series antigens and N negative additionally. The results have assessed the murine monoclonal antibodies are appropriate for production anti-M and anti-N blood group phenotyping reagents to replace the polyclonal anti-M and anti-N which have been produced from rabbit immunize serum.

## Conclusion

All of our serologic characteristic results demonstrate that the TRC murine monoclonal anti-M and anti-N is suitable reagents for anti-M and anti-N blood group phenotyping.

## Acknowledgements

We wish to thank Uchikawa M, Head of Blood Group; Toyoda C. and his team of scientists at the Kanto-Koshinetsu Block Blood Center of Japanese Red Cross for helpful training in the project on human and murine monoclonal hybridoma technique for rare blood group reagents production.

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## Conflicts of interest statement

Each of the authors has completed an ICMJE disclosure form. None of the authors declare any potential or actual relationship, activity, or interest related to the content of this article.

## Data sharing statement

All data generated or analyzed during the present study are included in this published article. Further details are available for noncommercial purposes from the corresponding author on reasonable request.

## References

1. Moulds MK. Review: monoclonal reagents and detection of unusual or rare phenotypes or antibodies. Immunohematology 2006;22:52-63.
2. Figur AM, Rosenfield RE. The cross-reaction of anti-N with Type M erythrocytes. Vox Sang 1960;5:169-76.
3. Jaœkiewicz E, Czerwinski M, Syper D, Lisowska E. Anti-M monoclonal antibodies cross-reacting with variant Mg antigen: an example of modulation of antigenic properties of peptide by its glycosylation. Blood 1994;84:2340-5.
4. Kerdkaewngam K, Ponsen S, Phonimit S, Tingtoy U, Phikulsod S. Generating the hybridoma cell line by murine monoclonal hybridoma technology for producing Anti-M and Anti-N blood group Phenotyping Reagents. J Hematol Transfus Med 2014;24:361-9.
5. Combs MR. Method section 1 serum dilution procedure and method section 2 red cell typing. In: Roback JD, Combs MR, Grossman BJ, Hillyer CD, eds. AABB Technical Manual 16th ed. Bethesda, MD: AABB press, 2008:867-97.
6. Sakuldamrongpanich T. Production and characterization of mouse monoclonal antibodies to blood group N antigen. J Hematol Transfus Med 1993;3:213-22.
7. Chandanayingyong D, Pejchandra S. Studies on the Miltenberger Complex Frequency in Thailand and Family Studies. Vox Sang 1975;28:152-5.
8. Fongsarun J, Nuchprayoon I, Yod-in S, Kupatawintu P, Kidprasirt C. Blood groups in Thai Blood Donors. J Hematol Transfus Med 2002;12:277-86.
9. Kerdkaewmgam K, Tubrod J. The differences of nine blood groups system in the three major ethnicities of human. J Med Tech Assoc Thailand 2015;3:5127-40.
10. Chandanyingyong D. Transfusion Medicine. Faculty of Medicine Siriraj Hospital Mahidol University. Thailand;1998:95-100.
11. Chandanayingyong D, Sasaki TT, Greenwalt TJ. Blood groups of the Thais. Transfusion 1967;7:267-76.
12. Na-thalang O, Kuvanont S, Punyaprasiddhi P, Tasaniyanonda C, Sriphaisal TA preliminary study of the distribution of blood group systems in Thai blood donors determined by the gel test. Southeast Asian J Trop Med Public Health 2001;32:204-7.
13. Kerdkaewngam K, Nuchprayoon I, Tingtoy U. Blood types of minorities in Bangkok: A study in Thai Sikhs and the Burmese blood donors. Chula Med J 2015; 59:619-29.
14. Arora S, Doda V, Maria A, Kotwal U, Goyal S. Maternal anti-M induced hemolyticdisease of newborn followed by prolonged anemia in newborn twins. Asian J Transfus Sci 2015;9:98-101.
15. Ishida A, Ohto H, Yasuda H, Negishi Y, Tsuiki H, Arakawa T, et al. Anti-M antibody induced prolonged anemia following hemolytic disease of the newborn due to erythropoietic suppression in 2Siblings. J Pediatr Hematol Oncol 2015;37:e375-7.
16. Duro EA, Desalvo L, Kuret S. Severe hemolytic disease of the newborn caused by anti-M antibodies. Iran J Pediatr 2013;23:607-8.
17. Yasuda H, Ohto H, Nollet KE, Kawabata K, Saito S, Yagi Y, et al. Hemolytic disease of the fetus and newborn with late-onset anemia due to anti-M: A case report and review of the Japanese literature. Transfus Med Rev 2014;28:1-6.
18. Gao XY, Huang H, Li LD. Hemolytic disease of neonates due to anti-M: report of one case and review of reports of 21 cases. Zhonghua Er Ke Za Zhi 2009;47:648-52.
19. Wikman A, Edner A, Gryfelt G, Jonsson B, Henter JI. Fetal hemolytic anemia and intrauterine death caused by anti-M immunization. Transfusion 2007;47:911-7.
20. Singh L, Malhotra S, Kaur G, Basu S, Kaur R. Autoimmune hemolytic anemia dueto auto anti-N in a patient with sepsis. Transfus Apher Sci 2012;47: 269-70.
21. Combs MR, Telen MJ, Hall SE, Rosse WF. A case report: IgGautoanti-N as acause of severe autoimmune hemolytic anemia. Immunohematology 1990;6:83-6.
22. Cohen DW, Garratty G, Morel P, Petz LD. Autoimmune hemolytic anemiaassociated with IgG auto anti-N. Transfusion 1979;19:329-31.
23. Kaur P, Basu S, Kaur G, Kaur R, Das B. Anti N antibody in a patient of renalfailure on hemodialysis. Indian J Hematol Blood Transfus 2014;30 (Suppl 1):174-6.
24. Harris T, Lukasavage T. Anti-M and anti-N reactivity with Tn and T activated cells. Transfus Clin Biol 1997;4:87-8.
25. Fraser RH, Munro C, Williamson AR, Barrie EK, Hamilton EA, Mitchell R. Mouse monoclonal Anti-N II. Physicochemical characterization and assessment for routine blood grouping. J Immunogenet 1982;9: 303-9.
26. Munro AC, Fraser RH, Barrie EK, Bruce M, Mitchell R. Specificity and behaviour of monoclonal antibodies in the MN system. Biotest Bull 1983;1:309-14.
27. Duk M, Lisowska E. Subspecificities of anti-M and anti-N antibodies tested with chemically-modified antigens. Transfus Clin Biol 1997;4:69-71.
28. Fraser RH, Inglis G, Mitchell R. pH effect on anti-M and anti-N reactivity. Transfusion 1986;26:118-9.
29. Palacajornsuk P. Review: molecular basis of MNS blood group variants. Immunohematology 2006;22:171-82.
30. Reid ME. MNS blood group system: a review. Immunohematology 2009;25:95-101.

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