

# Stability of Hybridoma Cell Line Clone 4C91F9 Secreting Anti-E Monoclonal Antibody

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**Received** November 7, 2023  
**Accepted** December 12, 2023  
**Published** December 31, 2023

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## ABSTRACT

**Introduction:** Since 2018, the Antiserum and Standard Cell Preparation Section, National Blood Center, Thai Red Cross Society (NBC) have succeeded in producing a hybridoma cell line secreted anti-E with a good antibody titer and suitable for blood grouping reagent anti-E production. At first, we established 4 different human monoclonal hybridoma IgM cell lines stably expressing anti-E, namely 4C91A2, 4C91B9, 4C91F9, and 4C91G5, respectively. After that, 4 different cell lines were selected only the perfect one with good growth rate and high titer antibody character. Finally, a clone namely 4C91F9 was selected for culture on a large scale and its supernatant was collected for blood grouping reagent anti-E production. So, we must ensure the 4C91F9 cell line has good antibody titer production stability in the long run. The study aimed to detect the stability of hybridoma cell line clone 4C91F9 secreting anti-E monoclonal antibody from 1st to 3rd generation.

**Methods:** The cell line clone 4C91F9 secreting 1st generation anti-E was thawed from liquid nitrogen and selected to be the best single cell line with a good growth rate and high titer antibody secretion tested by limiting dilution method. Afterward, the selected cell line will be frozen in liquid nitrogen for 7 days. In addition, the 1st generation cell line that separated from freezing was cultured to harvest supernatant subjected to further serologic testing. The 2nd generation cell line was thawed after being frozen for 7 days for the limit dilution method again until 3rd generation. Serological testing for supernatant from all generation cell lines consisted of antibody identification with panel cells and antibody titration (potency) with R1R2, R1Rz, and r'r red blood cell group O. Also, a specificity test with antigen E positive and antigen E negative red blood cells was performed.

**Results:** Anti-E antibodies from all three generations of clone 4C91F9 showed perfect stability with high titer. Titers of 512, 1024, and 1024 were obtained at room temperature, and 512, 512, and 512 were obtained at the indirect antiglobulin test (IAT), respectively.

**Conclusion:** The characteristics of all generations of anti-E production are comparable to the American Association of Blood Banks (AABB) standard. This result confirms the quality of the anti-E monoclonal antibody suitable for being a blood typing reagent produced by Nation Blood Center, Thai Red Cross Society to ensure the safety of the blood donation and transfer process.

**Keywords:** Anti-E, Human monoclonal, Hybridoma cell, Limiting dilution

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## Introduction

Since, 1975 when monoclonal antibody was successfully developed by using the mouse monoclonal hybridoma technique continuous development of monoclonal antibody production technique has been ongoing until now [1, 2]. In Thailand the production of murine monoclonal blood grouping reagent succeeded

around 2001, the first blood grouping reagent that succeeded in producing from murine monoclonal antibody is anti-A and anti-B by Antiserum, and standard cell preparation section, National Blood Center, Thai Red Cross Society (NBC) [3, 4].

Afterward, the production of monoclonal for blood grouping reagent production in Thailand has

continuously developed until the present we use both murine and human monoclonal techniques. According to some types of cell lines in the Rh system establishing can only use human monoclonal techniques such as anti-E, anti-C, anti-e, anti-c, etc. Because mice cannot be immunized with all types of antigens. In addition, mice must respond only to nontoxic antigens and especially, only antigen that has protein which involved with the evolutionary sequence of mice can immunize its immune system [5]. However, some cell lines can use both murine and human monoclonal techniques such as anti-A, anti-B, anti-M [6, 7], etc.

After Antiserum and standard cell preparation section staff back from human monoclonal training at the Japanese Red Cross Society in 2012-2014, we succeeded in establishing many antibodies secreting cell lines, especially human monoclonal anti-E. In the past anti-E supernatant was imported from abroad with high cost. Including anti-E is crucial for blood grouping reagent used in red blood cell antigen testing before a blood transfusion to ensure safety. This is particularly significant due to variation in the frequency of E antigen with Caucasians having a frequency of 29%, Blacks is 22% and Asians is 39% [8, 9]. Therefore, a stable antibody-secreting cell line is one requirement because if the cell line is not stable that means the quality of the blood grouping reagent was not good, either. This study aimed to search for 3 generations of stable expressing anti-E clone 4C91F9 cell lines by using the limiting dilution method.

## Methodology

### *Limiting dilution and cell culture* [10]

The 1<sup>st</sup> generation single cell line, name 4C91F9 was limited dilution and culture in 96 well plates (1 cell per well) with RPMI 1640 medium and incubated in CO<sub>2</sub> incubate (37°C, 5% CO<sub>2</sub>) until 7-14 days cell line was grown as a single colony. When the colony area was about 2 out of 3 parts of the well bottom, the supernatant was collected for the serological test (specificity test with E antigen-positive cell) and 10 of good growth rate, high antibody titer was selected to culture in 24 well plates. Then, supernatant from 24 well plates was collected for the serological test again (specificity, titration, and identification) but in this step, only one stable cell line was selected for transfer to a 25 cm<sup>2</sup> tissue culture flask. Finally, the cell line in a 25 cm<sup>2</sup> tissue culture flask was divided into 2 parts, part 1 was re-frozen in liquid nitrogen for 7 days and it was called 2<sup>nd</sup> generation, name 4C91F93F9. In part 2 cell line was continuously cultured in the same 25 cm<sup>2</sup> tissue culture flask which the first cell concentration was adjusted to 2.5 x 10<sup>5</sup> cells/ml approximately and cultured till death. Then, the supernatant was harvested for serological test (identification, potency test or antibody titration, specificity test, and specificity test by using enzyme technique) The 2<sup>nd</sup> generation cell line namely

4C91F93F9 was thawed after being frozen for 7 days and selected a good cell line by limiting dilution method again until supernatant of 3<sup>rd</sup> generation (4C91F93F93D3) was harvested and 4<sup>th</sup> generation cell line was got.

### *Serological test*

#### *(i) Antibody Identification* [11, 7]

All 3 generations of supernatant were identified with panel cell of NBC Lot.62060 Exp. 15 JUL 2019 by using the tube technique method 100 µl of supernatant and 100 µl of panel cell were dropped into the test tube and incubated at room temperature for 5 minutes. Then, centrifuged at 3000 rpm for 15 seconds, and grading (w,0-4) agglutination was observed and recorded. Next step, incubated at 37°C for 30 minutes centrifuged at 3000 rpm for 15 seconds and grading (w,0-4) agglutination was observed and recorded. Finally, washed 3 times with 0.9% normal saline solution and 100 µl of anti-human globulin serum was dropped and centrifuged at 3000 rpm for 15 seconds and grading (w,0-4) agglutination was observed and recorded. Also, blood grouping anti-E reagent of NBC was used as a positive control.

#### *(ii) Potency test (antibody titration)*

3 generations of supernatant were tested with 3% group O red blood cell R1R2 (genotype DCcEe), R1Rz (genotype DCEe) and r''r (genotype dcEe) phenotype suspension with two-fold serial dilution method by using tube technique method that 100 µl of supernatant and 100 µl of 3% red blood cell suspension were dropped into the test tube and incubated at room temperature for 5 minutes. Then, centrifuged at 3000 rpm for 15 seconds, and the score (w,0-12) agglutination was observed and recorded. Next step, incubated at 37°C for 30 minutes centrifuged at 3000 rpm for 15 seconds, and score (w,0-12) agglutination was read and recorded. Finally, washed 3 times with 0.9% normal saline solution and 100 µl of anti-human globulin serum was dropped and centrifuged at 3000 rpm for 15 seconds and score (w,0-12) agglutination was observed and recorded. Blood grouping anti-E reagent of NBC was used as a positive control.

#### *(iii) Specificity test*

The 3 generations of supernatant were tested with 100 samples group O red blood cell that has E antigen positive, phenotype R1R2 (genotype DCcEe), R1Rz (genotype DCEe), R2R2 (genotype DcE), R2r (genotype DdcEe) and r''r (genotype dcEe). Also, tested with 100 samples group O red blood cell that has E antigen negative, phenotype R1R1 (genotype DCE), R1r (genotype DdCce), rr (genotype dce) and r'r (genotype dCce). This step was incubated at room temperature for 5 minutes, centrifuged at 3000 rpm for 15 seconds and results were interpreted by grading (w,0-4) agglutination. Also, blood grouping anti-E reagent of NBC was used as a positive control.

*(iv) Specificity test by using enzyme technique [12, 13]*

Papain enzyme was used with a two-stage enzyme technique for the specificity test with group A, B, O, and AB red blood cells, 182 known antigen samples which have Ee, EE, and ee antigen and 195 unknown antigen samples compared with blood grouping reagent anti-E of NBC.

In addition, bromelain enzyme was used with a one-stage enzyme technique for the specificity test with group A, B, O, and AB red blood cells, 182 known antigen samples that have Ee, EE, and ee antigens, and 195 unknown antigen samples compared with blood grouping reagent anti-E of NBC.

**Results**

Cell line expression anti-E clone name 4C91F9 representative of 1<sup>st</sup> generation was thawed from liquid nitrogen with good condition, does not have any dead cell, and have good morphology (round shape). Also, they can produce 2 good generations of clone names 4C91F93F9 and 4C91F93F93D3 with perfect characters like a mother cell.

For serological testing results, antibody identification with panel cell Lot.62060 (product of NBC) compared with blood grouping reagent anti-E (product of NBC) shows that all 3 generation cell lines perfectly secrete anti-E (Table 1).

**Table 1** Antibody identification of supernatant from 3 generations of human monoclonal hybridoma anti-E, compared with anti-E from National Blood Center, Thai Red Cross Society (NBC) (Lot.61020 Exp. 23 JAN 20)

| Antibodies Identification Results |       |   |   |   |   |   |    |    |    |   |    |    |             |
|-----------------------------------|-------|---|---|---|---|---|----|----|----|---|----|----|-------------|
| Anti-E                            | Temp. | 1 | 2 | 3 | 4 | 5 | 6  | 7  | 8  | 9 | 10 | 11 | Specificity |
| 4C91F9                            | RT    | 0 | 0 | 0 | 0 | 0 | 4+ | 4+ | 4+ | 0 | 0  | 4+ | anti-E      |
| 4C91F93F9                         |       | 0 | 0 | 0 | 0 | 0 | 4+ | 4+ | 4+ | 0 | 0  | 4+ | anti-E      |
| 4C91F93F93D3                      |       | 0 | 0 | 0 | 0 | 0 | 4+ | 4+ | 4+ | 0 | 0  | 4+ | anti-E      |
| Anti-E of NBC                     |       | 0 | 0 | 0 | 0 | 0 | 4+ | 4+ | 4+ | 0 | 0  | 4+ | anti-E      |
| 4C91F9                            | 37°C  | 0 | 0 | 0 | 0 | 0 | 4+ | 4+ | 4+ | 0 | 0  | 4+ | anti-E      |
| 4C91F93F9                         |       | 0 | 0 | 0 | 0 | 0 | 4+ | 4+ | 4+ | 0 | 0  | 4+ | anti-E      |
| 4C91F93F93D3                      |       | 0 | 0 | 0 | 0 | 0 | 4+ | 4+ | 4+ | 0 | 0  | 4+ | anti-E      |
| Anti-E of NBC                     |       | 0 | 0 | 0 | 0 | 0 | 4+ | 4+ | 4+ | 0 | 0  | 4+ | anti-E      |
| 4C91F9                            | IAT   | 0 | 0 | 0 | 0 | 0 | 4+ | 4+ | 4+ | 0 | 0  | 4+ | anti-E      |
| 4C91F93F9                         |       | 0 | 0 | 0 | 0 | 0 | 4+ | 4+ | 4+ | 0 | 0  | 4+ | anti-E      |
| 4C91F93F93D3                      |       | 0 | 0 | 0 | 0 | 0 | 4+ | 4+ | 4+ | 0 | 0  | 4+ | anti-E      |
| Anti-E of NBC                     |       | 0 | 0 | 0 | 0 | 0 | 4+ | 4+ | 4+ | 0 | 0  | 4+ | anti-E      |

*Remark: This table use grading agglutination test while 4+ is agglutination suggested the perfectly secretion anti-E 4 is one solid agglutinate background is clear.*

*3 is several large agglutinates; background is clear.*

*2 is medium sized agglutinates; background is clear.*

*1 is many small agglutinates; background is turbid.*

*0 is no agglutination or hemolysis of red cells. Cells float freely.*

The potency test (antibody titer) of 3 generations with R1R2 phenotype red blood cells compared with blood grouping reagent anti-E (product of NBC) at room temperature are 512, 1024, 1024, and 128. At 37°C step antibody titer are 1024, 1024, 1024, and 256. Also, antibody titer at IAT is 512, 512, 512, and 256, respectively (Table 2). In addition, potency tests with cell R1Rz and r'r phenotype of all 3 generations of the cell line are according to the standard of anti-E titer (Tables 3 and 4). In conclusion the potency test, 3 generation cell lines of the 4C91F9 clone are anti-E(IgM) antibodies and have a strong reaction at room temperature, 37°C, and IAT step.

**Table 2** Comparison of anti-E titer from 3 generation stable cell lines supernatant and anti-E reagent of National Blood Center, Thai Red Cross Society (NBC) (Lot. 61020 Exp. 23 JAN 20) with R1R2 (genotype DCcEe) cell

| Agglutination strength of each dilution |                      |               |    |    |    |    |    |    |    |     |     |     |      |
|-----------------------------------------|----------------------|---------------|----|----|----|----|----|----|----|-----|-----|-----|------|
|                                         |                      | Anti-E        | N  | 2  | 4  | 8  | 16 | 32 | 64 | 128 | 256 | 512 | 1024 |
| RT                                      | 5 minutes plus spin  | 4C91F9        | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 10  | 8   | 5   | 3    |
|                                         |                      | 4C91F93F9     | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 10  | 8   | 5   | 5    |
|                                         |                      | 4C91F93F93D3  | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 10  | 8   | 8   | 5    |
|                                         |                      | Anti-E of NBC | 12 | 12 | 12 | 12 | 10 | 8  | 5  | 5   | 3   | 0   | 0    |
| 37°C                                    | 30 minutes plus spin | 4C91F9        | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 10  | 8   | 5   | 5    |
|                                         |                      | 4C91F93F9     | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 10  | 8   | 8   | 5    |
|                                         |                      | 4C91F93F93D3  | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 10  | 8   | 8   | 5    |
|                                         |                      | Anti-E of NBC | 12 | 12 | 12 | 12 | 10 | 8  | 5  | 5   | 3   | 0   | 0    |

| Agglutination strength of each dilution |               |    |    |    |    |    |    |   |   |   |   |   | Titer |
|-----------------------------------------|---------------|----|----|----|----|----|----|---|---|---|---|---|-------|
| IAT                                     | Anti-E of NBC | 12 | 12 | 12 | 10 | 10 | 8  | 8 | 5 | 5 | 3 | 1 | 256   |
|                                         | 4C91F9        | 12 | 12 | 12 | 12 | 10 | 8  | 8 | 5 | 5 | 5 | 3 | 512   |
|                                         | 4C91F93F9     | 12 | 12 | 12 | 12 | 12 | 10 | 8 | 8 | 5 | 5 | 3 | 512   |
|                                         | 4C91F93F93D3  | 12 | 12 | 12 | 12 | 12 | 10 | 8 | 8 | 5 | 5 | 3 | 512   |
|                                         | Anti-E of NBC | 12 | 12 | 12 | 12 | 12 | 10 | 8 | 5 | 5 | 3 | 3 | 256   |

**Remark:** This table use score 1-12 agglutination while 12 is one solid agglutinate background is clear. 10 is several large agglutinates; background is clear. 8 is medium sized agglutinates; background is clear. 3-5 is many small agglutinates; background is turbid. 0 is no agglutination or hemolysis of red cells. Cells float freely.

**Table 3** Comparison of anti-E titer from 3 generation stable cell lines supernatant and anti-E reagent of NBC, TRCS (Lot. 61020 Exp. 23 JAN 20) with R1Rz (genotype DDEe) cell

| Agglutination strength of each dilution |               |    |    |    |    |    |    |    |     |     |     |      | Titer |
|-----------------------------------------|---------------|----|----|----|----|----|----|----|-----|-----|-----|------|-------|
| RT 5 minutes plus spin                  | Anti-E        | N  | 2  | 4  | 8  | 16 | 32 | 64 | 128 | 256 | 512 | 1024 |       |
|                                         | 4C91F9        | 12 | 12 | 12 | 12 | 12 | 12 | 10 | 8   | 5   | 5   | 3    | 512   |
|                                         | 4C91F93F9     | 12 | 12 | 12 | 12 | 12 | 12 | 10 | 8   | 8   | 5   | 3    | 512   |
|                                         | 4C91F93F93D3  | 12 | 12 | 12 | 12 | 12 | 12 | 10 | 8   | 8   | 5   | 3    | 512   |
| 37°C 30 minutes plus spin               | Anti-E of NBC | 12 | 12 | 12 | 12 | 10 | 10 | 5  | 5   | 1   | 0   | 0    | 128   |
|                                         | 4C91F9        | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 10  | 8   | 8   | 5    | 1024  |
|                                         | 4C91F93F9     | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 10  | 8   | 8   | 5    | 1024  |
|                                         | 4C91F93F93D3  | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 10  | 8   | 8   | 5    | 1024  |
| IAT                                     | Anti-E of NBC | 12 | 12 | 12 | 12 | 10 | 8  | 5  | 5   | 3   | 3   | 1    | 128   |
|                                         | 4C91F9        | 12 | 12 | 12 | 12 | 10 | 10 | 8  | 5   | 5   | 3   | 3    | 256   |
|                                         | 4C91F93F9     | 12 | 12 | 12 | 12 | 12 | 10 | 10 | 8   | 8   | 5   | 3    | 512   |
|                                         | 4C91F93F93D3  | 12 | 12 | 12 | 12 | 12 | 10 | 10 | 8   | 8   | 5   | 3    | 512   |
|                                         | Anti-E of NBC | 12 | 12 | 12 | 12 | 10 | 10 | 8  | 8   | 5   | 3   | 3    | 256   |

**Remark:** This table use score 1-12 agglutination while 12 is one solid agglutinate background is clear. 10 is several large agglutinates; background is clear. 8 is medium sized agglutinates; background is clear. 3-5 is many small agglutinates; background is turbid. 0 is no agglutination or hemolysis of red cells. Cells float freely.

**Table 4** Comparison of anti-E titer from 3 generation stable cell lines supernatant and anti-E reagent of National Blood Center, Thai Red Cross Society (NBC) (Lot. 61020 Exp. 23 JAN 20) with r''r (genotype dcEe) cell

| Agglutination strength of each dilution |               |    |    |    |    |    |    |    |     |     |     |      |      | Titer |
|-----------------------------------------|---------------|----|----|----|----|----|----|----|-----|-----|-----|------|------|-------|
| RT 5 minutes plus spin                  | Anti-E        | N  | 2  | 4  | 8  | 16 | 32 | 64 | 128 | 256 | 512 | 1024 |      |       |
|                                         | 4C91F9        | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 10  | 10  | 8   | 5    | 1024 |       |
|                                         | 4C91F93F9     | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 10  | 10  | 8   | 5    | 1024 |       |
|                                         | 4C91F93F93D3  | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 10  | 10  | 8   | 5    | 1024 |       |
| 37°C 30 minutes plus spin               | Anti-E of NBC | 12 | 12 | 12 | 12 | 12 | 10 | 8  | 8   | 5   | 3   | 1    | 256  |       |
|                                         | 4C91F9        | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 10  | 8   | 8   | 5    | 1024 |       |
|                                         | 4C91F93F9     | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 10  | 8   | 5   | 5    | 1024 |       |
|                                         | 4C91F93F93D3  | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 10  | 8   | 5   | 5    | 1024 |       |
| IAT                                     | Anti-E of NBC | 12 | 12 | 12 | 12 | 12 | 10 | 8  | 5   | 5   | 3   | 3    | 256  |       |
|                                         | 4C91F9        | 12 | 12 | 12 | 12 | 12 | 12 | 10 | 8   | 8   | 5   | 5    | 1024 |       |
|                                         | 4C91F93F9     | 12 | 12 | 12 | 12 | 12 | 12 | 10 | 8   | 8   | 5   | 5    | 1024 |       |
|                                         | 4C91F93F93D3  | 12 | 12 | 12 | 12 | 12 | 12 | 10 | 8   | 8   | 5   | 5    | 1024 |       |
|                                         | Anti-E of NBC | 12 | 12 | 12 | 12 | 12 | 10 | 8  | 5   | 5   | 3   | 3    | 256  |       |

**Remark:** This table use score 1-12 agglutination while 12 is one solid agglutinate background is clear. 10 is several large agglutinates; background is clear. 8 is medium sized agglutinates; background is clear. 3-5 is many small agglutinates; background is turbid. 0 is no agglutination or hemolysis of red cells. Cells float freely.

Specificity testing results, all 3 generations of supernatant reacted with 100 samples antigen E positive group O red blood cell phenotype R1R2 (genotype DCcEe), R1Rz (genotype DCEe), R2R2 (genotype DcE), R2r (genotype DdcEe) and r''r (genotype dcEe) and did not react with 100 samples antigen E negative group O red blood cell phenotype R1R1 (genotype DCE), R1r (genotype DdcEe), rr (genotype dce) and r'r (genotype dCce) (Table 5).

**Table 5** Specificity from 3 generation stable cell lines supernatant and anti-E reagent of National Blood Center, Thai Red Cross Society (NBC) (Lot. 61020 Exp. 23 JAN 20) with antigen E positive red blood cell which have phenotype R1R2, R1Rz, R2R2, R2r and r''r phenotype and E negative red blood cell which have phenotype R1R1, R1r, rr and r'r

| Antigen E positive cells |           |      |      |      |     |      | Antigen E negative cells |     |    |     |
|--------------------------|-----------|------|------|------|-----|------|--------------------------|-----|----|-----|
| Anti-E                   | Phenotype | R1R2 | R1Rz | R2R2 | R2r | r''r | R1R1                     | R1r | Rr | r'r |
| 4C91F9                   |           | 58   | 6    | 24   | 8   | 4    | 0                        | 0   | 0  | 0   |
| 4C91F93F9                | Number of | 58   | 6    | 24   | 8   | 4    | 0                        | 0   | 0  | 0   |
| 4C91F93F93D3             | positive  | 58   | 6    | 24   | 8   | 4    | 0                        | 0   | 0  | 0   |
| Anti-E of NBC            | results   | 58   | 6    | 24   | 8   | 4    | 0                        | 0   | 0  | 0   |
| 4C91F9                   |           | 0    | 0    | 0    | 0   | 0    | 68                       | 18  | 8  | 6   |
| 4C91F93F9                | Number of | 0    | 0    | 0    | 0   | 0    | 68                       | 18  | 8  | 6   |
| 4C91F93F93D3             | negative  | 0    | 0    | 0    | 0   | 0    | 68                       | 18  | 8  | 6   |
| Anti-E of NBC            | results   | 0    | 0    | 0    | 0   | 0    | 68                       | 18  | 8  | 6   |

**Remark:** phenotype R1R2 (genotype DCcEe), R1Rz (genotype DCEe), R2R2 (genotype DcE), R2r (genotype DdcEe) and r''r (genotype dcEe) phenotype and E negative red blood cell which have phenotype R1R1 (genotype DCE), R1r (genotype DdCce), rr (genotype dce) and r'r (genotype dCce)

For specificity test by using two-stage and one-stage enzyme technique, papain and bromelain were used with two-stage and one-stage enzyme technique, respectively. Both papain and bromelain were tested for 3 generations supernatant compared with blood grouping reagent anti-E of NBC, they are 100% accuracy reacted with known samples which have antigen E positive red blood cell. In the same way, they are also 100% accuracy reacted with unknown samples that is antigen E positive red blood cells. On the other hand, they do not react with both known and unknown antigen E-negative red blood cells (Tables 6-9).

**Table 6** Specificity from 3 generation stable cell lines supernatant and anti-E reagent of National Blood Center, Thai Red Cross Society (NBC) (Lot. 61020 Exp. 23 JAN 20) by using two-stage enzyme technique (papain) with known antigen E positive red blood cell and E negative red blood cell samples

| Cell | Number of        | Anti-E 4C91F9 |    |    | Anti-E 4C91F93F93D3 |    |    | Anti-E4C91F93F93D3 |    |    | Anti-E of NBC |    |    |
|------|------------------|---------------|----|----|---------------------|----|----|--------------------|----|----|---------------|----|----|
|      |                  | Ee            | EE | ee | Ee                  | EE | ee | Ee                 | EE | ee | Ee            | EE | ee |
| A    | Positive results | 10            | 11 | 0  | 10                  | 11 | 0  | 10                 | 11 | 0  | 10            | 11 | 0  |
|      | Negative results | 0             | 0  | 28 | 0                   | 0  | 28 | 0                  | 0  | 28 | 0             | 0  | 28 |
| B    | Positive results | 12            | 10 | 0  | 12                  | 10 | 0  | 12                 | 10 | 0  | 12            | 10 | 0  |
|      | Negative results | 0             | 0  | 23 | 0                   | 0  | 23 | 0                  | 0  | 23 | 0             | 0  | 23 |
| O    | Positive results | 17            | 8  | 0  | 17                  | 8  | 0  | 17                 | 8  | 0  | 17            | 8  | 0  |
|      | Negative results | 0             | 0  | 25 | 0                   | 0  | 25 | 0                  | 0  | 25 | 0             | 0  | 25 |
| AB   | Positive results | 5             | 8  | 0  | 4                   | 8  | 0  | 4                  | 8  | 0  | 4             | 8  | 0  |
|      | Negative results | 0             | 0  | 25 | 0                   | 0  | 25 | 0                  | 0  | 25 | 0             | 0  | 25 |

**Table 7** Specificity from 3 generation stable cell lines supernatant and anti-E reagent of National Blood Center, Thai Red Cross Society (NBC) (Lot. 61020 Exp. 23 JAN 20) by using two-stage enzyme technique (papain) with unknown antigen E positive red blood cell and E negative red blood cell samples

| Cell | Number | Anti-E<br>4C91F9 |       | Anti-E<br>4C91F93F93D3 |       | Anti-E<br>4C91F93F93D3 |       | Anti-E of NBC |       |
|------|--------|------------------|-------|------------------------|-------|------------------------|-------|---------------|-------|
|      |        | E (+)            | E (-) | E (+)                  | E (-) | E (+)                  | E (-) | E (+)         | E (-) |
| A    | 50     | 12               | 38    | 12                     | 38    | 12                     | 38    | 12            | 38    |
| B    | 50     | 19               | 31    | 19                     | 31    | 19                     | 31    | 19            | 31    |
| O    | 50     | 8                | 42    | 8                      | 42    | 8                      | 42    | 8             | 42    |
| AB   | 45     | 15               | 30    | 15                     | 30    | 15                     | 30    | 15            | 30    |

**Table 8** Specificity from 3 generation stable cell lines supernatant and anti-E reagent of National Blood Center, Thai Red Cross Society (NBC) (Lot. 61020 Exp. 23 JAN 20) by using one-stage enzyme technique (bromelain) with unknown antigen E positive red blood cell and E negative red blood cell samples

| Cell | Number | Anti-E<br>4C91F9 |       | Anti-E<br>4C91F93F93D3 |       | Anti-E<br>4C91F93F93D3 |       | Anti-E of NBC |       |
|------|--------|------------------|-------|------------------------|-------|------------------------|-------|---------------|-------|
|      |        | E (+)            | E (-) | E (+)                  | E (-) | E (+)                  | E (-) | E (+)         | E (-) |
| A    | 50     | 12               | 38    | 12                     | 38    | 12                     | 38    | 12            | 38    |
| B    | 50     | 19               | 31    | 19                     | 31    | 19                     | 31    | 19            | 31    |
| O    | 50     | 8                | 42    | 8                      | 42    | 8                      | 42    | 8             | 42    |
| AB   | 45     | 15               | 30    | 15                     | 30    | 15                     | 30    | 15            | 30    |

**Table 9** Specificity from 3 generation stable cell lines supernatant and anti-E reagent of National Blood Center, Thai Red Cross Society (NBC) (Lot. 61020 Exp. 23 JAN 20) by using one-stage enzyme technique (bromelain) with unknown antigen E positive red blood cell and E negative red blood cell samples

| Cell | Number | Anti-E<br>4C91F9 |       | Anti-E<br>4C91F93F93D3 |       | Anti-E<br>4C91F93F93D3 |       | Anti-E of NBC |       |
|------|--------|------------------|-------|------------------------|-------|------------------------|-------|---------------|-------|
|      |        | E (+)            | E (-) | E (+)                  | E (-) | E (+)                  | E (-) | E (+)         | E (-) |
| A    | 50     | 12               | 38    | 12                     | 38    | 12                     | 38    | 12            | 38    |
| B    | 50     | 19               | 31    | 19                     | 31    | 19                     | 31    | 19            | 31    |
| O    | 50     | 8                | 42    | 8                      | 42    | 8                      | 42    | 8             | 42    |
| AB   | 45     | 15               | 30    | 15                     | 30    | 15                     | 30    | 15            | 30    |

A total of 266 individuals were recruited into the analysis; there were 103 males and 163 females; aged 30-90 years, with an average age of (62.27±9.63) years. A large proportion of family history and comorbidities were hypertension. BMI within 18.5–24.9 (Normal weight) and 25.0–29.9 (Pre-obesity). A large proportion of blood lipids; 40.60% were TC <200 mg/dL, 36.84% were TG <150 mg/dL, 33.83% were LDL-C <100 mg/dL and 49.62% were HDL-C 40-59 mg/dL (Table 1).

## Discussion

Cell line anti-E clone 4C91F9 is appropriate to use as a master cell for the large-scale culture that is about 10 liters per year or more depending on blood bank and hospital demand because they have stable antibody titer till 3rd generation. The serological testing results of supernatants of all 3 generations are according to the American Association of Blood Banks (AABB) standard [14] Wherefore, they are suitable for use to be a raw material of blood grouping reagent anti-E production when compared with related anti-E human monoclonal research [15].

Nowadays cell line 4C91F9 has been used as a master cell of blood grouping reagent anti-E production for a while. The main reason why we must do a limiting dilution method to select some perfect cell line before

large-scale culture is hybridoma cell is a hybrid of B lymphocyte and myeloma cell fusion that can mutate all the time. The effect of mutation is cell line may have an antibody titer-secreting decrease or loss of antibody-secreting ability. Sometimes, the secreting and non-secreting antibody cell lines were mixed and nobody was known by the naked eye.

When the mix of secreting and non-secreting antibody cell lines were cultured on a large scale, normally non-secreting antibody cell lines will grow rapidly and use all nutrients. Finally, when insufficient nutrients all cell lines must die but secreting cell lines do not grow enough to secrete antibodies, and the direct effect is low antibody titer and inability to detect all weak antigens leading to false negative results. Production staff will resolve low titer antibody problem by concentration which means high-cost production

occurs. On the other hand, a high antibody titer has more benefits such as can be diluted with 0.9% normal saline solution to an antibody titer according to the standard of each antibody in the production process. In addition, blood grouping reagent which is high titer antibody can be diluted with 0.9% normal saline solution or phosphate buffered saline (PBS) to be applied on an Automated Pretransfusion Blood Testing Analyzer (PK7300/PK7400) for donor screening [16]. Therefore, limiting dilution must be done every time after the cell line is thawed from liquid nitrogen or a 6-month check once in a while using the cell line.

## Conclusion

The anti-E clone name 4C91F9 is appropriate to use as a master cell for producing supernatant as raw material for blood grouping reagent anti-E and good quality of them will reduce financial cost in medical services because supernatant transportation or commercial anti-E blood grouping reagent cost from abroad is expensive. Sometimes, may take a long time to wait. But at present, we have our cell line so we can immediately produce as much as desired.

## Acknowledgements

I would like to acknowledge Dr. Soisaang Phikulsod for initiating the murine and human monoclonal hybridoma technique for the rare blood group reagent project. Finally, I sincerely thank Dr. Makoto Uchikawa, Mrs. Chizu Toyoda, and Miss Sayaka Kaito from the Japanese Red Cross Kanto-Koshinetsu Block Blood Center, Japan who transmit human monoclonal knowledge and skills.

## References

- [1] Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined Specificity. *Nature*. 1975;256:495-7.
- [2] Ponsen S, Premprayoon N. Application of monoclonal antibody in medicine. *J Med Biosci*. 5(1). 2023;89-96.
- [3] Phikulsod S, Poltlen R, Uthid K, Tubrod J. Monoclonal blood grouping reagents prepared by National Blood Centre, Thai Red Cross Society: I mouse response to immunization with different B antigens quantity and sources. *J Hematol Transfus Med*. 1991;1:299-307.
- [4] Phikulsod S, Poltlen R, Kaewkitiroj P, Uthid K, Tingtoy U, Tubrod J. Monoclonal anti-A reagent using hybridoma technique prepared by National Blood Centre, Thai Red Cross Society. *J Hematol Transfus Med*. 1992; 2:373-81.
- [5] Sakuldamrongpanich T. Monoclonal Antibody;Blood Grouping reagent. *Thai J Hematol Transfus Med*. 2002; 12: 235-43.
- [6] Boonhai S, Aiemumporn K, Tingtoy U. Production of a new anti-M monoclonal reagent using human hybridoma technology. *Chula Med J* 2016;60:101-13.
- [7] Kerdkaewngam K, Posen S, Phonimit S, Tingtoy U, Phikulsod S. Generating the hybridoma cell line by murine monoclonal hybridoma technology for production anti-M and anti-N blood group phenotyping reagent. *J Hematol Transfus Med*. 2014; 24: 361-9.
- [8] Dean L. Blood groups and red cell antigens: the Rh blood group. [Internet]. 2005 [cited 2023 Nov 2]. Available from: [www.ncbi.nlm.nih.gov/books/NBK2269/](http://www.ncbi.nlm.nih.gov/books/NBK2269/)
- [9] Ponsen S, Phonimit S, Premprayoon N, Kerdkaewngam K, Tingtoy U. Production of human hybridoma secreting monoclonal antibody against Rh(E) antigen. *J Hematol Transfus Med*. 2019; 29: 83-90.
- [10] Ponsen S, Phonimit S, Premprayoon N, Tingtoy U. Production of anti-D human monoclonal antibody (IgM) using human monoclonal hybridoma technique. *Chula Med Bull*. 2019; 1: 147–54.
- [11] Yeela T, Buathong W. Screening and identification of irregular antibodies in blood donation. *Songkla Med J*. 2004; 22: 89-93.
- [12] Outrakoolpoonsuk K, Bejrachandra S, Saipin J, Leehaphaiboonsakun W, Suratanarungsun V, Plubjuice P. Detection of red cell antibody by enzyme technique. *J Hematol Transfus Med*. 1999; 9: 103-10.
- [13] Ponsen S, Premprayoon N, Tingtoy U. Antibody screening and identification in transfusion medicine and benefits of antibodies in development of human monoclonal hybridoma for blood group reagent production. *Chula Med Bull*. 2020; 2(1): 87-96.
- [14] John D. Roback, ed. Technical Manual. 16th ed. Bethesda: American Association of Blood Banks; 2008.
- [15] Thompson K, Barden G, Sutherland J, Bel-don I, Melamed M. Human monoclonal antibodies to C, c, E, e, and G antigens of the Rh system. *Immunology*. 1990; 71: 323-7.
- [16] Meng-Hua Y, Jen-Wei C, Kaito S, Makoto U, Nelson H. T, Sheng-Tang W, Sheng-Mou H, and Yann-Jang C. Universal Detection of Mia Antigen and Frequencies of Glycophorin Hybrids among Blood Donors in Taiwan by Human Monoclonal Antibodies against Mia (MNS7), Mur (MNS10), and MUT (MNS35) Antigens. *Diagnostics*. 2021;11:806.