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ABO 및 D형 혈액형 판정용 동결 적혈구 국가표준품 제조 및 확립

Development of Cryopreserved Red Blood Cell Panels as Biological Reference Standards for Performance Evaluation of ABO and D Blood Grouping Reagents

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Background: Accurate blood typing is essential for blood transfusions, and requires the constant evaluation and maintenance of ABO and D blood grouping reagents. In the present study, we developed cryopreserved red blood cell (RBC) panels and evaluated their feasibility as a standard reference material to verify the quality of ABO and D blood grouping reagents in Korea.
Methods: RBC units obtained from healthy donors were cryopreserved using a high-glycerol method. A total of 400 sets of RBC panels were prepared, composed of blood group A (N=5), B (N=5), O (N=10), AB (N=4), Rh D-positive (N=4), Rh D-negative (N=5), and weak-D (N=1), and 200 sets of RBC subgroup panels composed of A_2, A_2B, A_2B_3, A_1B_3, and B_3, and A_2, A_2B, A_2B_3, A_1B_3, and A_3B (N=1, each). Quality assessment of the cryopreserved RBC panels before and after cryopreservation was performed by measuring their sensitivity, specificity, avidity, and potency titers.
Results: Our cryopreserved ABO and D RBC panels had a sensitivity and specificity of 100% to existing monoclonal blood grouping reagents, regardless of blood type and cryopreservation time. There were no significant differences in the avidity time and potency titers of the cryopreserved RBCs before and after 6 or 12 months of cryopreservation.
Conclusions: The quality parameters measured here suggest that our newly developed cryopreserved RBC panels were reliable for use as a standard reference material for the performance evaluation of anti-A, -B, and -D blood grouping reagents.

Key Words: Cryopreservation, Red blood cells, Blood grouping reagent, High-glycerol method

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INTRODUCTION

Accurate identification of the blood group of pre-transfusion blood specimens is essential for performing blood transfusions. Despite advances in profiling the molecular genetics of blood groups, a conventional serological grouping method is still widely used as a screening test in transfusion medicine [1-3]. As errors in blood grouping may lead to severe complications, such as a hemolytic transfusion reaction, and even death [4], several countries have developed individual standards for the quality assessment of blood grouping reagents to ensure safe and effective transfusion. In the United States of America, the standards are specified in the Code of Federal Regulations, (CFR) Title 21, Chapter I, Subchapter F. Biologics, part 660, subpart C [5]. In European Union (EU)

countries, the ABO and Rh blood grouping reagents are classified as *in vitro* diagnostic medical devices, which comply with regulations stipulated in the common technical specifications for *in vitro* diagnostic medical devices (2009/108/EC) [6]. The Government of India provides guidance materials for quality control of ABO and Rh blood grouping reagents [7].

In Korea, as in the United States of America or EU, medical devices, including *in vitro* diagnostic reagents, are categorized into classes 1 to 4 according to the level of regulatory control required to ensure the safety and effectiveness of medical practices [8]. ABO and D blood grouping reagents are classified as class 4 medical devices, and a performance test report for the quality control of domestically produced or imported ABO and D blood grouping reagents is therefore required for the validation of a brand new reagent or a new batch of existing reagents, according to the approved and reviewed guidelines for ABO blood grouping reagents [9]. As outlined in Supplementary Table 1, the report should include the sensitivity, specificity, potency titer, and avidity of anti-A and -B sera with ABO subgroups, including A_2 and A_2B, and anti-D sera with weak-D and Rh D-negative groups [9]. However, the availability of qualified blood that can be used to test the quality of the reagents, and at the same time meet the national regulatory testing standards, remains an issue. The United States Food and Drug Administration (FDA) recommends using fresh or frozen red blood cells to prepare cell suspensions for testing blood grouping reagents [10]. However, from 2007 to 2009 in Korea, only 0.052% of 3,397,983 healthy blood donors had diverse ABO subgroup phenotypes, and the frequencies of A_2 and A_2B were only 0.002% and 0.015%, respectively [11]. In addition, unlike in Caucasians, the prevalence of D-negative and weak-D blood groups is relatively low in the Korean population (only 0.15% and 0.01%, respectively) [12]. Due to the rarity of certain ABO and D subgroups in Korea, and the Korean Blood Management Act, which prohibits the use of voluntary non-remunerated blood for a different purpose other than transfusion, many reagent companies in Korea have struggled to obtain qualified red blood samples for testing the quality of blood grouping reagents and submitting performance test reports.

To address this issue, in the present study we aimed to prepare standard cryopreserved RBC panels using a high-glycerol method to verify the quality of monoclonal ABO and D blood grouping reagents, and to evaluate the suitability of the panels for use as a standardized reference, in cooperation with the Ministry of Food and Drug Safety (MFDS) of Korea.

MATERIALS AND METHODS

1. Sample collection

All participants submitted written informed consent prior to the acquisition of whole blood samples from 61 eligible volunteers who had undergone several screening tests (ABO/Rh D blood grouping, complete blood count, routine chemistry, and serological tests for hepatitis B virus, hepatitis C virus, HIV, and syphilis) at Severance Hospital, Seoul, Korea in August 2018. The samples were collected into blood collection bags containing citrate phosphate dextrose adenine (CPDA)-1 anticoagulant solution. The collected whole blood units were from the following blood groups: A (N=10), B (N=10), O (N=20), AB (N=8), Rh D-positive (N=8), and Rh D-negative (N=10). Subsequently, RBC concentrate was produced from each whole blood unit using standard operating procedures. Whole blood units of weak-D (N=2) and ABO subgroups (N=8), including A_2B (N=2), A_2B_3 (N=2), A_1B_3 (N=2), B_3 (N=1), and A_3B (N=1), were obtained from the Korean Red Cross Central Blood Center, Seoul, Korea, with the approval of the Institutional Review Board of the Ethics Committee of Korean Red Cross. Due to the unavailability of A_2 subgroup donors in Korea, two units of A_2 subgroup blood, produced by the Continental Services Group, Inc. (Miami, FL, USA), were imported by Mirr Scitech Corp. (Seoul, Korea). This study was approved by the Institutional Review Board and Ethics Committee of Severance Hospital (IRB No.: 4-2018-0143).

2. Cryopreservation and thawing of RBC panels

A modified version of a high-glycerol method that is generally used in clinical applications was used for cryopreservation of RBCs, as previously described [13-16]. Within 7 days of collection, the RBC concentrates were glycerolized using Glycerolyte 57 solution (Baxter Healthcare, Deerfield, IL, USA) to a final glycerol concentration of 40%. Aliquots of the glycerolized RBCs (1 mL) were dispensed into separate cryotubes (SPL Life Sciences, Gyeonggi-do, Korea) using an automatic dispenser (DOSE IT, Integra Biosciences, Tokyo, Japan), and the cryotubes were frozen and stored at −70°C in a controlled-rate cryo-freezer (Thermo Fisher Scientific, Waltham, MA, USA), which freezes cells by re-

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ducing the temperature by approximately 1°C per minute, with a continuous temperature monitoring system. In total, 400 sets of RBC panels (Supplementary Fig. 1A), composed of group A (N=5), B (N=5), O (N=10), AB (N=4), Rh D-positive (N=4), Rh D-negative (N=5), and weak-D (N=1), and 200 sets of RBC subgroup panels (Supplementary Fig. 1B), composed of A_2, A_2B, A_2B_3, A_1B_3, B_3, and A_2, A_2B, A_2B_3, A_1B_3, A_3B (N=1, each), were produced.

After 6 or 12 months of storage, the cryopreserved RBCs were thawed in a 37°C water bath for 2–3 minutes with gentle mixing, and then transferred into test tubes. One milliliter of 9% NaCl was then added dropwise into the test tubes, and the test tubes were incubated at room temperature for at least 1 minute. The thawed RBCs were centrifuged at 3,400 rpm (about 1,000-1,020×g) for 20 seconds, and the supernatant was removed. The process of mixing with 1 mL of 2.5% NaCl, centrifuging, and removing the supernatant was repeated several times until a clear supernatant was obtained. The thawed RBCs were finally stored at 4°C.

3. Evaluation of RBC panels

1) Quality of frozen RBC panels

To evaluate the stability of the ABO/D antigens in newly produced RBC panels before and after 6 or 12 months of cryopreservation, a traditional agglutination method was performed using both slides and tubes [1, 11]. The two anti-A, -B, and -D monoclonal reagents used for ABO/D grouping in the present study were Sihdia (Shinyang Chemicals, Seoul, Korea), designated as reagent 1, and Bioclone (Ortho Clinical Diagnostics, Raritan, NJ, USA), designated as reagent 2, as they were the most easily obtained reagents. All reagents and blood grouping tests were performed according to the manufacturer's instructions.

The sensitivity and specificity of the RBC panels were evaluated to determine the presence or absence of antigen A, B, and/or D, using both the test tube and the slide methods. The potency titers were measured using both the test tube and the column agglutination technology methods by determining the reciprocal of the greatest dilution of the reagent that showed visible agglutination [3]. Additional inter-laboratory studies were performed to confirm the quality of the newly developed RBC panels, in terms of sensitivity, specificity, potency titer, and avidity time at 6 and 12 months after cryopreservation at the Catholic University of Korea Eunpyeong St. Mary's Hospital, Seoul, Korea and the Asan Medical Center, Seoul, Korea.

2) Performance of two commercial blood grouping reagents

The diagnostic performances of blood grouping reagents 1 and 2 were evaluated using our post-thawed RBC panels, comprised of group A (N=2), B (N=2), O (N=1), AB (N=2), Rh D-positive (N=2), and Rh D-negative (N=1) samples, at 6 and 12 months after cryopreservation. The potency titers determined using the two selected reagents were compared with those determined using the WHO International Standard of blood grouping reagents (NIBSC codes for anti-A: 03/188, anti-B: 03/164, and anti-D: 99/836).

3) Effect of storing RBC panels in preservatives after thawing

The effect of Alsever's solution (Sigma-Aldrich Inc., St. Louis, MO, USA) on the preservation of post-thawed RBCs was compared with that of normal saline. The cryopreserved RBC panels, including group A (N=2), B (N=2), AB (N=2), and Rh D-positive (N=2) samples stored for 12 months, were thawed and suspended in either Alsever's solution or normal saline. The potency titers of the freeze-thawed RBCs stored at 4°C in the refrigerator before testing were then evaluated on the day of thawing, and at 1, 3, and 7 days after thawing.

4) Effect of transportation using different storage media and time on RBC panels

An accelerated deterioration test was performed for different transport times with either dry ice or ice packs as the transport media. The RBC panels that were cryopreserved for 6 months, comprising group A (N=2), B (N=2), AB (N=2), O (N=1), Rh D-positive (N=2), and Rh D-negative (N=1) samples, were placed in one of two styrofoam boxes, one filled with dry ice and the other filled with ice packs, mimicking the actual transportation conditions across Korea. After 6 and 24 hours of storage, the RBC panels were thawed, and their potency titers were measured and compared.

RESULTS

1. Quality evaluation of freeze-thawed RBC panels

Our newly developed RBC panels had a sensitivity of 100% and a specificity of 100% to the two selected anti-A, -B, and -D blood

grouping reagents, regardless of the cryopreservation duration. All the group A and AB samples reacted with anti-A sera, and all the group B and AB samples reacted with anti-B sera. All D-positive samples agglutinated well with anti-D sera. Group B and O, group A and O, and Rh D-negative and weak-D samples did not react with anti-A, -B, or -D sera, respectively. The strength of agglutination ranged from +2 to +4, fulfilling the Korean batch release criteria outlined in the Supplementary Table 1 [9].

The time required for agglutination expressed in seconds for each blood type, also known as the avidity time, using the two selected reagents is shown in Table 1. The avidity time for each RBC panel also fulfilled the batch release criteria, and were therefore considered to be acceptable.

The potency titers of the RBC panels for each blood group before and after 6 and 12 months of cryopreservation according to the two selected blood grouping reagents are shown in Table 2. Changes in potency titers that would not satisfy the batch criteria listed in the Supplementary Table 1 were not observed in any of the blood groups tested.

An additional quality assessment of the 6 or 12 month-cryopreserved RBC panels was performed at two different laboratories in Korea using the two selected blood grouping reagents (data not shown). Although there were some inter-laboratory variations in potency titers, all the RBC panels demonstrated a sensitivity and specificity of 100% regardless of the test center, blood groups of thawed RBCs, blood grouping reagents used, and cryopreservation time. The avidity time measured was always less than 120 seconds, which is satisfactory according to the manufacturer's instructions for blood grouping reagents.

2. Performance evaluation of selected blood grouping reagents in comparison to the WHO International Standard Reagents

The diagnostic performance of two selected blood grouping reagents in comparison with that of WHO standard reagents on the RBC panels at 6 and 12 months after cryopreservation is shown in

Table 1. Avidity time of thawed RBC panels in seconds before and after 6 and 12 months of cryopreservation using two selected blood grouping reagents (reagent 1 and 2) for (A) anti-A and anti-B, and (B) anti-D

(A)

ABO blood group	Anti-A reagent						ABO blood group	Anti-B reagent					
	Reagent 1			Reagent 2				Reagent 1			Reagent 2		
	Pre-CP	Post-CP		Pre-CP	Post-CP			Pre-CP	Post-CP		Pre-CP	Post-CP	
		6 M	12 M		6 M	12 M			6 M	12 M		6 M	12 M
A (N=10)	2 (2)*	2 (2)*	2 (2)*	2 (2)*	3 (2-3)*	3 (3)*	B (N=10)	2 (2)*	2 (2)*	2 (2)*	2 (2)*	3 (3)*	3 (3)*
AB (N=8)	2 (2)*	2 (2)*	2 (2)*	2 (2)*	3 (3-4)*	2.5 (2-3)*	AB (N=8)	2 (2)*	2 (2)*	2 (2)*	2 (2)*	3 (3-4)*	2.5 (2-3)*
A_2_01	2	2	2	2	3	3	A_2_01	-	-	-	-	-	-
A_2_02	2	2	2	2	3	3	A_2_02	-	-	-	-	-	-
A_2B_01	2	2	2	2	3	3	A_2B_01	2	2	2	2	2	3
A_2B_01	2	2	2	2	3	3	A_2B_01	2	2	3	2	2	3
A_1B_3_01	2	3	2	2	3	3	A_1B_3_01	5	3	3	5	3	4
A_1B_3_02	2	2	2	2	3	3	A_1B_3_02	3	3	3	5	3	4
A_2B_3_01	2	3	3	2	3	3	A_2B_3_01	5	3	3	5	3	4
A_2B_3_02	2	3	2	2	3	3	A_2B_3_02	5	3	3	5	3	5
A_3B_01	2	3	3	2	3	5	A_3B_01	2	3	2	2	3	3
B_3_01	-	-	-	-	-	-	B_3_01	5	3	3	5	3	5

(B)

Rh blood group	Anti-D reagent					
	Reagent 1			Reagent 2		
	Pre-CP	Post-CP		Pre-CP	Post-CP	
		6 M	12 M		6 M	12 M
DP (N=8)	2 (2-3)*	3 (3)*	3 (3)*	2 (2-5)*	5 (5)*	4.5 (4-5)*

*Data are medians (min-max).
Abbreviations: CP, cryopreservation; M, months; DP, D-positive.

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Table 2. Potency titers (median (min-max)) of RBC panels before and after 6 and 12 months of cryopreservation using two selected blood grouping reagents (reagent 1 and 2) for (A) anti-A and B, and (B) anti-D

(A)

ABO blood group	Anti-A reagent						ABO blood group	Anti-B reagent					
	Reagent 1			Reagent 2				Reagent 1			Reagent 2		
	Pre-CP	Post-CP		Pre-CP	Post-CP			Pre-CP	Post-CP		Pre-CP	Post-CP	
		6 M	12 M		6 M	12 M			6 M	12 M		6 M	12 M
A (N=10)	4,096 (512-16,384)	8,192 (2,048-16,384)	4,096 (1,024-16,384)	2,048 (512-8,192)	2,048 (512-8,192)	2,048 (512-4,096)	B (N=10)	8,192 (512-16,384)	8,192 (2,048-16,384)	8,192 (1,024-16,384)	1,024 (512-8,192)	2,048 (512-4,096)	1,024 (512-8,192)
AB (N=8)	4,096 (1,024-16,384)	4,096 (2,048-16,384)	4,096 (1,024-16,384)	2,048 (512-4,096)	2,048 (1,024-4,096)	1,024 (512-4,096)	AB (N=8)	4,096 (1,024-16,384)	4,096 (2,048-16,384)	4,096 (512-16,384)	512 (256-2,048)	1,024 (512-2,048)	512 (512-4,096)
A_2 (N=2)	4,096 (1,024-16,384)	4,096 (1,024-8,192)	512 (1,024-16,384)	1,024 (512-4,096)	1,024 (512-4,096)	1,024 (256-4,096)	A_2 (N=2)	-	-	-	-	-	-
A_2B (N=2)	4,096 (1,024-16,384)	2,048 (1,024-16,384)	3,072 (1,024-16,384)	1536 (512-4,096)	768 (256-2,048)	1,024 (256-4,096)	A_2B (N=2)	8,192 (2,048-16,384)	8,192 (2,048-16,384)	4,096 (1,024-16,384)	1,024 (1,024-4,096)	2,048 (256-4,096)	1,024 (256-4,096)
A_2B_3 (N=2)	6144 (2,048-16,384)	8,192 (2,048-8,192)	6,144 (1,024-8,192)	2,048 (512-8,192)	2,048 (512-4,096)	2,048 (512-8,192)	A_2B_3 (N=2)	1,024 (512-2,048)	2,048 (512-8,192)	1,024 (256-2,048)	256 (128-512)	256 (128-1,024)	192 (64-1,024)
A_2B_3 (N=2)	2,048 (512-8,192)	2,048 (1,024-8,192)	2,048 (1,024-8,192)	1,024 (512-2,048)	1,024 (256-2,048)	768 (256-2,048)	A_2B_3 (N=2)	128 (64-256)	96 (1-512)	16 (1-512)	128 (32-128)	48 (8-128)	24 (4-128)
A_2B (N=1)	3072 (2,048-4,096)	3,072 (512-8,192)	768 (512-1,024)	768 (512-1,024)	768 (512-2,048)	768 (256-1,024)	A_2B (N=1)	6,144 (4,096-16,384)	8,192 (2,048-16,384)	5,120 (1,024-16,384)	1,536 (512-2,048)	2,048 (512-8,192)	1,536 (1,024-4,096)
B_3 (N=1)	-	-	-	-	-	-	B_3 (N=1)	1,024 (128-2,048)	1,536 (1,024-2,048)	512 (128-1,024)	128 (32-256)	160 (64-256)	96 (32-256)

(B)

Rh blood group	Anti-D reagent					
	Reagent 1			Reagent 2		
	Pre-CP	Post-CP		Pre-CP	Post-CP	
		6 M	12 M		6 M	12 M
DP (N=8)	4,096 (256-16,384)	1,024 (256-4,096)	1,024 (128-2,048)	1,536 (128-8,192)	512 (64-2,048)	256 (64-2,048)
DW (N=2)	6 (2-16)	2.5 (1-256)	9 (1-128)	6 (2-16)	4 (4)	8 (1-8)

Abbreviations: CP, cryopreservation; M, months; DP, D-positive; DW, weak-D.

Table 3. Comparison of potency titers of RBC panels measured by either one of the selected blood grouping reagents or the WHO standardized reagents for (A) anti-A and anti-B reagents, and (B) anti-D reagents, at 6 and 12 months after cryopreservation

(A)

Blood group	Manufacturer	Anti-A		Anti-B	
		6 M	12 M	6 M	12 M
A 1	Reagent 1	2,048	4,096	-	-
	Reagent 2	1,024	2,048	-	-
	WHO standard	128	128	-	-
A 2	Reagent 1	2,048	4,096	-	-
	Reagent 2	2,048	2,048	-	-
	WHO standard	128	128	-	-
B 1	Reagent 1	-	-	4,096	8192
	Reagent 2	-	-	2,048	4,096
	WHO standard	-	-	1,024	1,024
B 2	Reagent 1	-	-	8,192	8,192
	Reagent 2	-	-	1,024	2,048
	WHO standard	-	-	1,024	1,024
AB 1	Reagent 1	2,048	1,024	4,096	4,096
	Reagent 2	1,024	1,024	1,024	1,024
	WHO standard	64	128	512	512
AB 2	Reagent 1	2,048	2,048	8,192	4,096
	Reagent 2	1,024	1,024	2,048	1,024
	WHO standard	64	128	512	1,024
O 1	Reagent 1	-	-	-	-
	Reagent 2	-	-	-	-
	WHO standard	-	-	-	-
DN 1	Reagent 1	-	-	-	-
	Reagent 2	-	-	-	-
	WHO standard	-	-	-	-

(B)

Blood group	Manufacturer	Anti-D	
		6 M	12 M
DP 1	Reagent 1	1,024	1,024
	Reagent 2	256	512
	WHO standard	64	128
DP 2	Reagent 1	1,024	1,024
	Reagent 2	256	256
	WHO standard	64	64

Abbreviations: M, month; DN, D-negative; DP, D-positive.

Table 3. The potency titers of the RBC panels measured using the selected reagents were always equal to or higher than those measured using the WHO standardized reagents, suggesting promising diagnostic performance for the selected reagents.

3. Effect of using preservatives on ABO/D antigen stability

The potency titers of freeze-thawed RBC panels preserved either in Alsever's solution or in normal saline on the day of thawing, and at 1, 3, and 7 days after thawing, were compared and are shown in Fig. 1. Some of the RBC panels preserved in both preservatives showed a slight decrease in potency titers over time, but not in more than 2 titers, except in one sample.

4. Effect of transportation conditions on ABO/D antigen stability

The potency titers of freeze-thawed RBC panels stored for 6 and 24 hours, either on dry ice or ice packs, were measured and are shown in Fig. 2. All the RBC panels showed a maximum decrease of 1 titer in potency titers, regardless of the transportation time and media used.

DISCUSSION

Since the discovery of ABO blood groups by Karl Landsteiner in the early 1900s, serological tests using agglutination have been the standard for ABO grouping and D typing [17, 18]. With the evolution of molecular genotyping technology in blood grouping systems, certain parts of the conventional serological methods have been replaced in limited clinical settings; however, serological methods are still widely used and are continuously improved for the accurate identification of blood groups [2, 3, 18, 19]. In conventional serological methods, performing quality assurance checks of blood grouping reagents before they are routinely used is extremely important. However, because of the limited number of RBC samples available for quality checks in Korea, further research is needed to identify high-quality testing alternatives. In 1950, it was first demonstrated that blood mixed with glycerol could be frozen, and then retained its viability after thawing [20]. Since then, several studies have been carried out to establish protocols for RBC cryopreservation, and cryopreserving RBC units for future use has been a routine procedure in blood banks worldwide [21]. To address the need for standardized RBC panels for controlling the quality of blood grouping reagents in Korea, in the present study we developed cryopreserved RBC panels using a high-glycerol method and evaluated their qualities for use as a standardized panel for assessing blood grouping reagents. Despite the FDA's recommendation of using frozen red blood cells for testing blood grouping reagents [10], to the best of our knowledge, no other researchers elsewhere have carried out equivalent

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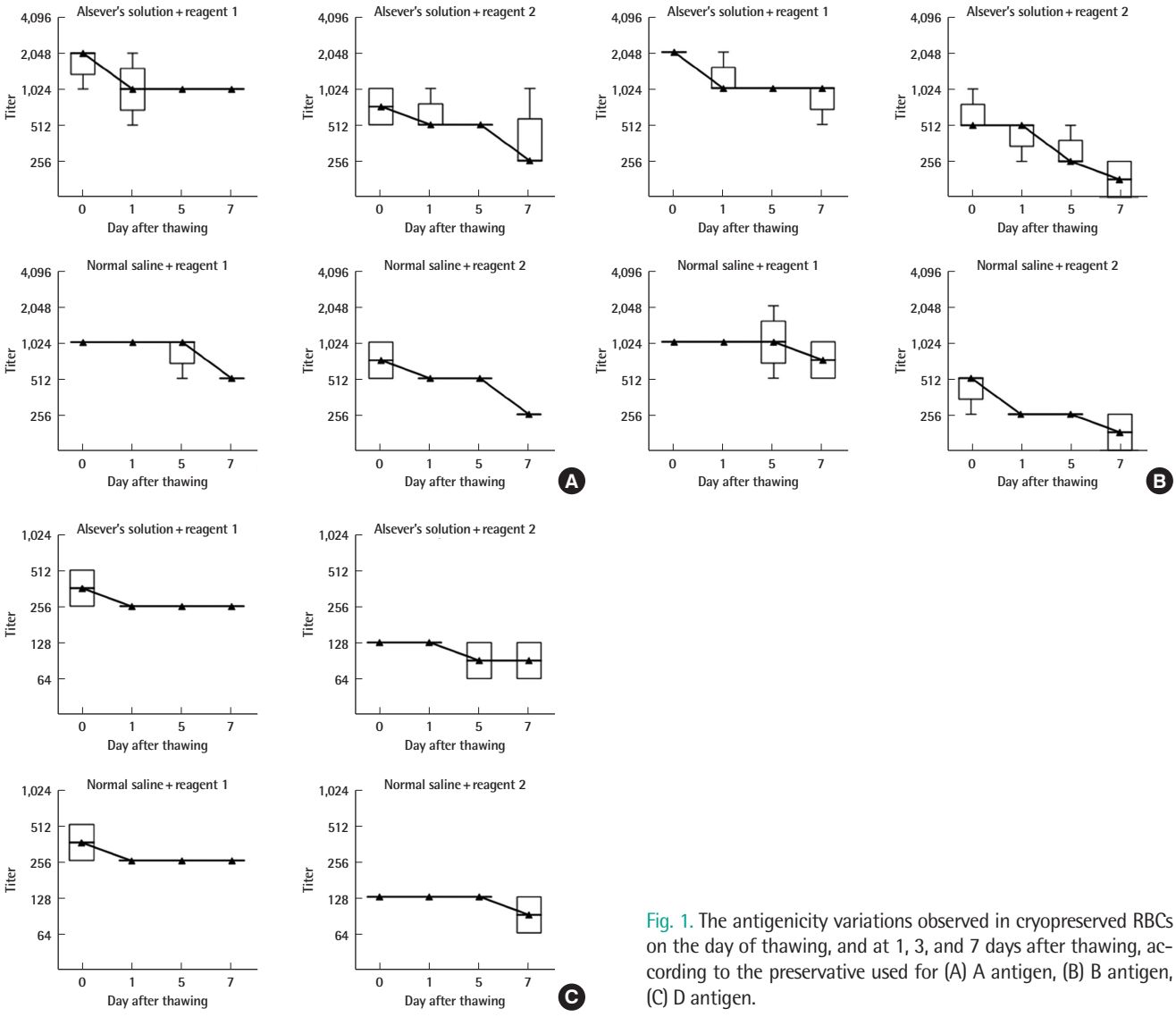

Fig. 1. The antigenicity variations observed in cryopreserved RBCs on the day of thawing, and at 1, 3, and 7 days after thawing, according to the preservative used for (A) A antigen, (B) B antigen, (C) D antigen.

experiments.

In the present study, 400 sets of cryopreserved RBC panels comprising ABO/D groups, and 200 sets of cryopreserved RBC panels comprising various ABO subgroups, were prepared using a high-glycerol method. According to the Korean batch release criteria, the quality of these newly developed panels before and after 6 or 12 months of cryopreservation was evaluated and found to be satisfactory. These criteria included positive reactions with reagents bearing the corresponding antibodies, negative reactions with reagents lacking the corresponding antibodies, and shorter agglutination times compared to those specified in the manufacturer's instructions. In addition, the measured potency titers were equal to, or higher than, those listed in the batch release criteria. Therefore, our newly developed RBC panels are suitable for use as a standardized reference panel for quality assurance of blood grouping reagents.

Even though several blood grouping reagents are available in Korea, we tested the diagnostic performance of two reagents that are more easily accessible in Korea than the WHO standardized reagents on our newly developed RBC panels. We verified the quality and performance of the selected reagents in terms of sensitivity, specificity, avidity, and potency titer. Based on these results, we propose that any available blood grouping reagent can be used on our RBC panels depending on the hospital conditions.

In the present study, we tested the effects of two preservatives, Alsever's solution and normal saline, and the effects of transporta-

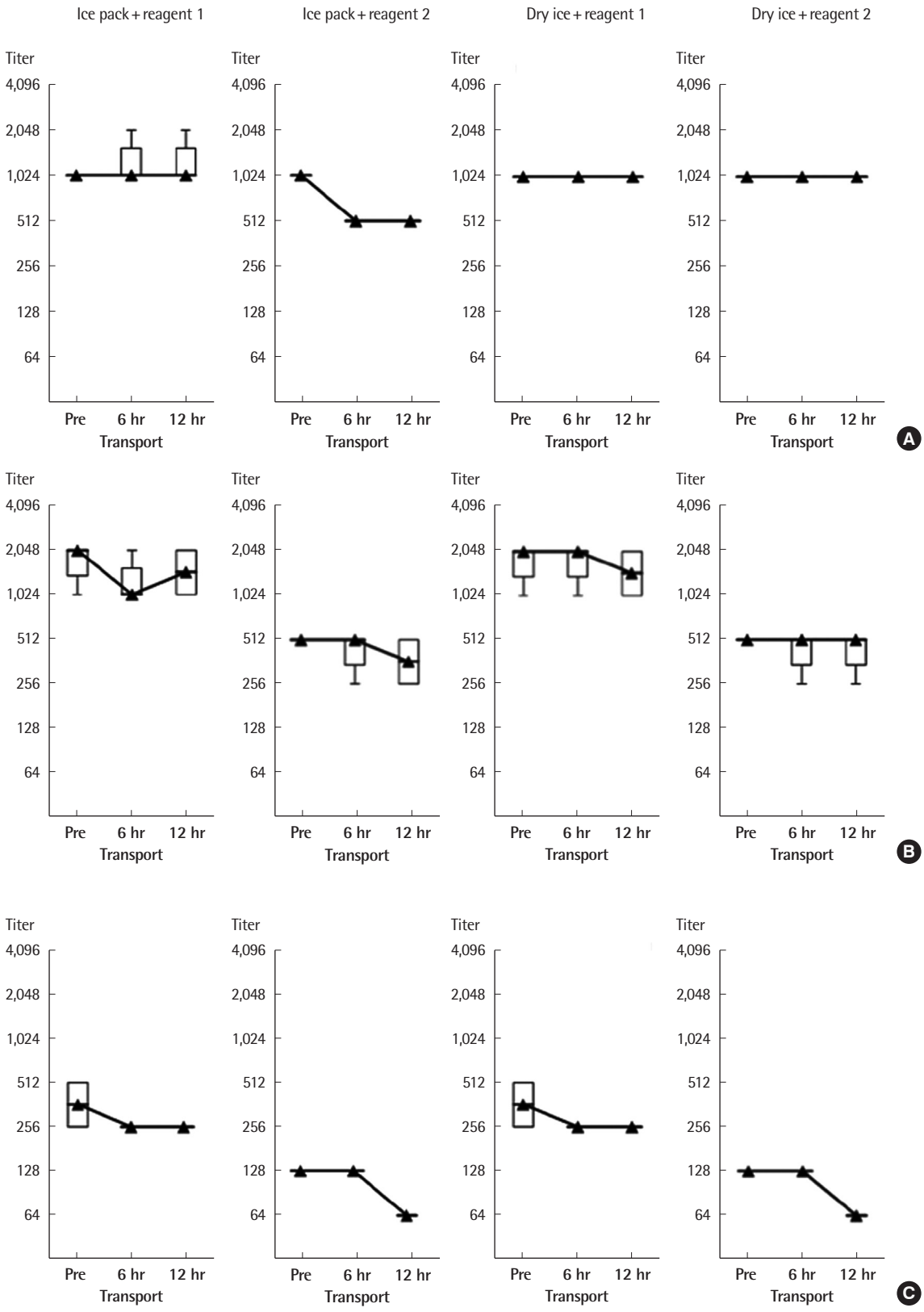

Fig. 2. The antigenicity variations observed in cryopreserved RBCs on the day of thawing, and at 1, 3, and 7 days after thawing, according to the transportation medium used for (A) A antigen, (B) B antigen, (C) D antigen.

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tion conditions on the stability of ABO/D antigens. There was no significant difference between the two preservatives based on the potency titers of the RBC panels cryopreserved for 12 months. However, using normal saline as a preservative increases the number of fragmented RBCs and causes changes in RBC morphology [22], and Alsever's solution is therefore a better preservative for post-thawed RBC panels. The cryopreserved RBC panels stored on both ice packs and dry ice yielded similar potency titers, regardless of the transportation time, indicating the stability and safety of the RBC panels if transported within Korea, and the applicability of both media for transporting blood components. However, a temperature range from 1°C to 10°C is recommended for transporting frozen blood components, including frozen RBCs and fresh frozen plasma, which is safer and easier to achieve using an insulated packaging system with dry ice [23].

The main limitation of the present study was our inability to evaluate the stability of ABO/D antigens and the safety of cryopreserved RBC panels for longer than a year. However, several studies have shown that frozen RBCs stored at –80°C are safe and effective for 21 years [24] and up to 37 years [25]. Therefore, it is reasonable to believe that the quality of our newly developed RBC panels would be acceptably maintained for decades.

In conclusion, we successfully evaluated the performance of cryopreserved RBC panels that can be used as a standardized reference material for evaluating the quality of blood grouping reagents. We believe that these RBC panels will alleviate the issues faced by domestic reagent companies in Korea regarding the difficulty of obtaining blood samples for quality control of blood grouping reagents, and thus improve the quality of reagents for more accurate blood grouping in the near future (Supplementary Fig. 2).

요 약

배경: 정확한 혈액형 판정을 위한 ABO/D 혈액형 판정용 시약의 품질관리는 안전한 수혈을 위해 필수적이다. 이에 본 연구에서는 ABO/D 혈액형 판정용 적혈구 패널을 동결 적혈구 표준품으로 제작하였으며, 항혈청 시약의 품질을 검증하기 위한 표준 물질로서의 타당성을 평가하였다.

방법: 고농도 글리세롤법을 이용해 건강한 기증자로부터 얻은 농축적혈구로부터 A형 5개, B형 5개, O형 10개, AB형 4개, Rh-D 양성 4개, Rh-D 음성 5개, Rh-D 약양성 1개로 구성된 적혈구 패널 400세트와, 각각 한 개의 A_2, A_2B, A_2B_3, A_1B_3, B_3 그리고 A_2, A_2B, A_2B_3, A_1B_3, A_3B로 구성된 적혈구 패널 200세트를 제작하여 민감도, 특이도, 응집력과 응집소 역가를 평가하였다.

결과: 제작된 적혈구 패널 모두 혈액형이나 보존기간에 상관없이 100%의 민감도와 특이도를 보였다. 동결 전과 후에 응집력과 응집소 역가 평가에서도 유의미한 변화는 확인되지 않았다.

결론: 고농도 글리세롤법을 이용한 동결 적혈구 표준품의 품질은 신뢰할 수 있는 것으로 나타났으며 ABO/D 혈액형 판정용 시약의 품질을 검증하기 위한 표준 물질로 공급, 배포될 수 있을 것으로 평가되었다.

Conflicts of Interest

None declared.

Acknowledgements

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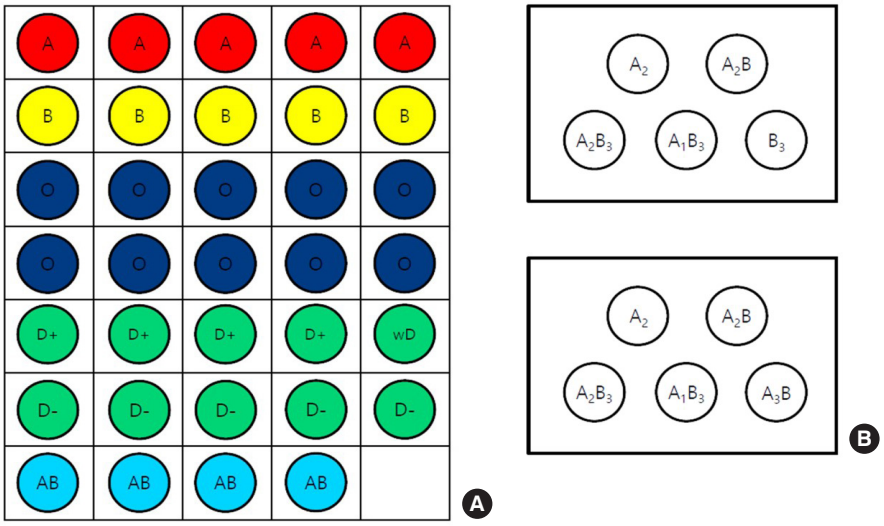
Supplementary Table 1. Batch release criteria for blood-grouping reagents in Korea [9]

	Anti-A			Anti-B			Anti-D		
	Blood type	No. of tests required	Acceptance criteria	Blood type	No. of tests required	Acceptance criteria	Blood type	No. of tests required	Acceptance criteria
Sensitivity	A_1	3	≥ 2+	B	3	≥ 2+	DP	4	≥ 2+
	A_2	1	≥ 2+	A_1B	3	≥ 2+	DW	1	Positive reaction
	A_2B	1	≥ 2+						
Specificity	B	5	Negative reaction	A	5	Negative reaction	DN	5	Negative reaction
	O	10		O	10				
Potency (Titer)	A_1	3	≥ 256	B	3	≥ 256	DP	4	≥ 64
	A_2	1	≥ 128	A_1B	3	≥ 128			
	A_2B	1	≥ 128						
Avidity	A_1	3	As recommended by manufacturer	A	3	As recommended by manufacturer	DP	4	As recommended by manufacturer
	A_2	1		A_1B	3				
	A_2B	1							

Abbreviations: DP, D-positive; DW, weak-D; DN, D-negative.



Supplementary Fig. 1. Composition of cryopreserved RBC panels (A) RBC panels composed of blood groups A, B, O, AB, Rh D-positive, Rh D-negative, and weak-D, and (B) RBC panels composed of A_2, A_2B, A_2B_3, A_1B_3, B_3 and A_2, A_2B, A_2B_3, A_1B_3, A_3B blood types.

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체외진단용 의료기기 표준품

ABO 및 D 형 혈액형판정용 동결적혈구
ABO & D Frozen Red Blood Cell Panel (ABO & D Panel 34 종)
코드번호: MFDS-I-18-005

1. 사용목적

이 표준품은 ABO 혈액형 및 D 혈액형 판정용 검사 시약과 검사 시스템의 성능을 평가하기 위한 목적으로 제조되었으며 진단목적으로 사용해서는 안된다.

2. 구성

이 표준품은 사람에게서 채혈된 전혈에서 분리된 적혈구를 동결하여 구성하였다. 각 vial 에는 아래의 물질을 포함하였다.

- 주성분: Human red blood cells
- 부성분: 40% glycerol (동결보존제)

Table 1. 표준품의 구성

혈액형	Vial 수
A 형	5
B 형	5
O 형	10
AB 형	4
D 양성	4
약 D	1
D 음성	5

3. 동결적혈구 해동방법

동결적혈구 해동은 첨부한 Table 2 의 방법대로 시행한다.

4. 주의사항

이 표준품은 인체에 투여해서는 안된다.

이 표준품은 감염의 우려가 있으므로 취급 시 주의해야 하며 검사실 안전관리지침을 따라 사용하고 처리해야 한다.

5. 보관

-70 ℃ 이하에서 보관한다.

해동 후 즉시 검사에 사용하되, 불가능한 경우 해동한 적혈구를 Alserver's 용액에 부유하여 약 7 일간 4℃에 냉장 보관한다.

해동한 적혈구는 다시 동결하여 보관 및 사용할 수 없다.

6. 제조

이 표준품은 연세대학교에서 제조되었다.

7. 참고문헌

김현옥. 체외진단용의료기기 국가표준품(ABO 및 D 형 혈액형판정용 동결 적혈구) 제조 및 확립 연구.
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Supplementary Fig. 2. Biological standards for in vitro diagnostics. ABO & D Frozen Red Blood Cell Panel (MFDS-I-18-005).

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Table 2. 동결적혈구 해동방법

1	동결적혈구 해동방법	9% NaCl 용액: 멸균증류수 1,000 mL + NaCl 분말 90g을 실온에서 녹인다.
2		2.5% NaCl: 멸균증류수 2,000 mL + NaCl 분말 50g을 실온에서 녹인다.
3		동결 적혈구 vial을 37℃ 온수조에서 천천히 흔들면서 해동 (2~3분 이내 소요)
4		1) 해동된 적혈구를 곧바로 테스트 튜브(13x100mm)로 옮김 2) 9% NaCl 1 mL을 **1 drop씩 천천히** 넣은 후 입구를 파라필름으로 막고 충분히 혼합 (2~3회 정도 튜브를 inverting하면 충분히 혼합됨) 3) 테스트 튜브를 실온에서 최소 1분간 방치
5		3,400 rpm (약 1,000~1,020g), 20초간 원심분리 후 상층액 제거 (적혈구가 충분히 가라앉지 않고 일부 상층액에 부유되어 있는데, 그대로 상층액으로 생각하고 제거함)
6		1) 2.5% NaCl 1 mL 넣은 후 충분히 혼합 (적혈구가 튜브 바닥에 일부 응집되어 있는데, inverting으로 충분히 혼합이 안되므로, tapping을 다소 강하게 하더라도, 튜브 바닥에 적혈구 응집이 남아있지 않도록 충분히 혼합함) 2) 3,400 rpm (약 1,000~1,020g), 20초간 원심분리 후 상층액 제거 (적혈구가 충분히 가라앉지 않고 일부 상층액에 부유되어 있는데, 그대로 상층액으로 생각하고 제거함)
7		6의 과정을 1회 더 반복
8		1) 0.9% 생리식염수 1 mL 넣은 후 혼합 (이 과정에서는 앞의 7)과정 때문에, 적혈구가 튜브 바닥에 일부 응집되어 있는데, inverting으로 충분히 혼합이 안되므로, tapping을 다소 강하게 하더라도, 튜브 바닥에 적혈구 응집이 남아있지 않도록 충분히 혼합함) 2) 3,400 rpm (약 1,000~1,020g), 1분간 원심분리 후 상층액 제거 (상층액에 일부 적혈구가 부유되어 있는데, 그대로 상층액으로 생각하고 제거함)
9		상층액이 깨끗해질 때까지 8)의 과정을 2~3회 반복 (세척을 진행해도 상층액에 일부 적혈구가 부유되어 있는데, 상층액의 색이 깨끗하다면 일부 적혈구가 부유되어 있다 하더라도 세척 종료)

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Supplementary Fig. 2. Continued

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체외진단용 의료기기 표준품

ABO 및 D 형 혈액형판정용 동결적혈구
ABO & D Frozen Red Blood Cell Panel (ABO 아형-1, Panel 5 종)
코드번호: MFDS-I-18-006

1. 사용목적

이 표준품은 ABO 혈액형 판정용 검사 시약과 검사 시스템의 성능을 평가하기 위한 목적으로 제조되었으며 진단목적으로 사용해서는 안된다.

2. 구성

이 표준품은 사람에게서 채혈된 전혈에서 분리된 적혈구를 동결하여 구성하였다. 각 vial 에는 아래의 물질을 포함하였다.

- 주성분: Human red blood cells
- 부성분: 40% glycerol (동결보존제)

Table 1. 표준품의 구성

혈액형	Vial 수
A_2 형	1
A_2B 형	1
A_2B_3 형	1
A_1B_3 형	1
B_3 형	1

3. 동결적혈구 해동방법

동결적혈구 해동은 첨부한 Table 2 의 방법대로 시행한다.

4. 주의사항

이 표준품은 인체에 투여해서는 안된다.
이 표준품은 감염의 우려가 있으므로 취급 시 주의해야 하며 검사실 안전관리지침을 따라 사용하고 처리해야 한다.

5. 보관

-70 ℃ 이하에서 보관한다.
해동 후 즉시 검사에 사용하되, 불가능한 경우 해동한 적혈구를 Alserver's 용액에 부유하여 약 7 일간 4℃에 냉장 보관한다.
해동한 적혈구는 다시 동결하여 보관 및 사용할 수 없다.

6. 제조

이 표준품은 연세대학교에서 제조되었다.

7. 참고문헌

김현옥. 체외진단용의료기기 국가표준품(ABO 및 D 형 혈액형판정용 동결 적혈구) 제조 및 확립 연구.
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Supplementary Fig. 2. Continued

(Continued to the next page)

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Table 2. 동결적혈구 해동방법

1	동결적혈구 해동방법	9% NaCl 용액: 멸균증류수 1,000 mL + NaCl 분말 90g을 실온에서 녹인다.
2		2.5% NaCl: 멸균증류수 2,000 mL + NaCl 분말 50g을 실온에서 녹인다.
3		동결 적혈구 vial을 37℃ 온수조에서 천천히 흔들면서 해동 (2~3분 이내 소요)
4		1) 해동된 적혈구를 곧바로 테스트 튜브(13x100mm)로 옮김 2) 9% NaCl 1 mL을 **1 drop씩 천천히** 넣은 후 입구를 파라필름으로 막고 충분히 혼합 (2~3회 정도 튜브를 inverting하면 충분히 혼합됨) 3) 테스트 튜브를 실온에서 최소 1분간 방치
5		3,400 rpm (약 1,000~1,020g), 20초간 원심분리 후 상층액 제거 (적혈구가 충분히 가라앉지 않고 일부 상층액에 부유되어 있는데, 그대로 상층액으로 생각하고 제거함)
6		1) 2.5% NaCl 1 mL 넣은 후 충분히 혼합 (적혈구가 튜브 바닥에 일부 응집되어 있는데, inverting으로 충분히 혼합이 안되므로, tapping을 다소 강하게 하더라도, 튜브 바닥에 적혈구 응집이 남아있지 않도록 충분히 혼합함) 2) 3,400 rpm (약 1,000~1,020g), 20초간 원심분리 후 상층액 제거 (적혈구가 충분히 가라앉지 않고 일부 상층액에 부유되어 있는데, 그대로 상층액으로 생각하고 제거함)
7		6의 과정을 1회 더 반복
8		1) 0.9% 생리식염수 1 mL 넣은 후 혼합 (이 과정에서는 앞의 7)과정 때문에, 적혈구가 튜브 바닥에 일부 응집되어 있는데, inverting으로 충분히 혼합이 안되므로, tapping을 다소 강하게 하더라도, 튜브 바닥에 적혈구 응집이 남아있지 않도록 충분히 혼합함) 2) 3,400 rpm (약 1,000~1,020g), 1분간 원심분리 후 상층액 제거 (상층액에 일부 적혈구가 부유되어 있는데, 그대로 상층액으로 생각하고 제거함)
9		상층액이 깨끗해질 때까지 8)의 과정을 2~3회 반복 (세척을 진행해도 상층액에 일부 적혈구가 부유되어 있는데, 상층액의 색이 깨끗하다면 일부 적혈구가 부유되어 있다 하더라도 세척 종료)

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Supplementary Fig. 2. Continued

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체외진단용 의료기기 표준품

ABO 및 D 형 혈액형판정용 동결적혈구
ABO & D Frozen Red Blood Cell Panel (ABO 아형-2, Panel 5 종)
코드번호: MFDS-I-18-007

1. 사용목적

이 표준품은 ABO 혈액형 판정용 검사 시약과 검사 시스템의 성능을 평가하기 위한 목적으로 제조되었으며 진단목적으로 사용해서는 안된다.

2. 구성

이 표준품은 사람에게서 채혈된 전혈에서 분리된 적혈구를 동결하여 구성하였다. 각 vial 에는 아래의 물질을 포함하였다.

- 주성분: Human red blood cells
- 부성분: 40% glycerol (동결보존제)

Table 1. 표준품의 구성

혈액형	Vial 수
A_2 형	1
A_2B 형	1
A_2B_3 형	1
A_1B_3 형	1
A_3B 형	1

3. 동결적혈구 해동방법

동결적혈구 해동은 첨부한 Table 2 의 방법대로 시행한다.

4. 주의사항

이 표준품은 인체에 투여해서는 안된다.

이 표준품은 감염의 우려가 있으므로 취급 시 주의해야 하며 검사실 안전관리지침을 따라 사용하고 처리해야 한다.

5. 보관

-70 ℃ 이하에서 보관한다.

해동 후 즉시 검사에 사용하되, 불가능한 경우 해동한 적혈구를 Alserver's 용액에 부유하여 약 7 일간 4℃에 냉장 보관한다.

해동한 적혈구는 다시 동결하여 보관 및 사용할 수 없다.

6. 제조

이 표준품은 연세대학교에서 제조되었다.

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Supplementary Fig. 2. Continued

(Continued to the next page)

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Supplementary Fig. 2. Continued

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