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Collaborative Study to Establish National Reference Standards for Anti-HIV-1 Antibody

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Background: National reference standards for anti-HIV-1 antibody are needed to evaluate the performance and maintain the quality control of anti-HIV-1 antibody assays. The aim of this study was to prepare a mixed-titer performance panel and assess its suitability as a national reference standard for anti-HIV-1 antibody according to stability, collaboration, and other studies.

Methods: Nineteen serum samples from different HIV patients were obtained, along with 15 units of fresh frozen plasma samples with negative anti-HIV-1 antibody results. Ten anti-HIV-1 antibody-positive candidate standards and two negative candidate standards were prepared based on the reactivity in the Alinity i HIV Ag/Ab combo assay (Abbott Laboratories, Wiesbaden, Germany). A collaborative study was conducted across eight laboratories using five anti-HIV-1 antibody assays. Real-time and accelerated stability were evaluated to assess the long-term stability.

Results: In the collaborative study, results of all five anti-HIV-1 antibody assays were positive for all 10 candidate standards prepared using HIV patient samples. The CV of each assay for every candidate standard was within 10%, except for one assay result. No real-time and accelerated stability change trend was observed at –70°C or –20°C, supporting that the reference standards were maintained in a stable state at –70°C for long-term storage.

Conclusions: The overall results suggest that the 12 candidate standards could serve as national reference standards for anti-HIV-1 antibody.

Key Words: Anti-HIV-1 antibody, HIV, Reference standard

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INTRODUCTION

According to a WHO report, approximately 37.7 million people were living with HIV worldwide in 2020, with an estimated 680,000 deaths [1]. In Korea, the incidence of HIV infection has increased steadily since 1985, when the first two cases of HIV infection were diagnosed [2]. More than 1,000 cases have been reported annually over the last eight years. According to the Korea Centers for Disease Control and Prevention, there were 1,016 new HIV infection cases in 2020 [2].

Detection of HIV-infected blood is one of the most critical *in vitro* diagnostic (IVD) screening assays performed for donated blood. Many Korean companies have developed anti-HIV-1 antibody assay devices. Approximately 13% (79/590) of the laboratories participating in external quality assessments for anti-HIV-1 antibody detection use assay devices manufactured in Korea [3]. National reference standards for anti-HIV-1 antibody are needed to evaluate the performance and maintain quality con-

trol of IVD assays. The first national reference standard for anti-HIV-1 antibody (Anti-HIV 1, Mixed Titer Performance Panel, code number: 12/038) was established and distributed in 2012 [4]. As all of these first standards have been used with none remaining, we prepared a new mixed-titer performance panel for anti-HIV-1 antibody, which required validation as a candidate national reference standard [5, 6].

This study aimed to assess the suitability of the new mixed-titer performance panel as a national reference standard through the precision of fill, homogeneity, and stability, along with a collaborative study design. These results could establish the second Korean national reference standard for anti-HIV-1 antibody to replace the depleted first standard.

MATERIALS AND METHODS

Acquisition of materials and selection of candidate standards

Nineteen serum samples with volumes between 100 mL and 200 mL, obtained from different HIV-infected patients, were provided by the High-Risk Human Serum Bank of Chung-Ang University. Fifteen units of fresh frozen plasma samples with negative anti-HIV-1 antibody assay results were obtained from Chung-Ang University Hospital Blood Center. This two-year retrospective study was approved by the Institutional Review Board of Dongguk University Ilsan Hospital, Korea (DUIH 2019-02-023). Because the samples were collected from the High-Risk Human Serum Bank and Blood Center, informed consent for use of samples from patients and blood donors was waived by the ethics committee of our hospital.

To select suitable materials as candidate standards, samples were screened for anti-HIV-1 antibody with the Alinity i HIV Ag/Ab combo (Alinity i, Abbott Laboratories, Wiesbaden, Germany), HIV combi PT (cobas e601, Roche Diagnostics GmbH, Penzberg, Germany), and SD Bioline HIV 1/2 3.0 (SD, Yongin, Korea) devices, as the three most commonly used assays. Ten samples with positivity in all three screening assays and sufficient volumes (>130 mL) were selected as the candidate standards. The Alinity i HIV Ag/Ab combo was used to select samples with various concentrations. Two negative standards were selected.

Preparation of the mixed-titer performance panel for anti-HIV-1 antibody

Thawed serum samples that had been stored at −70°C were filtered through 0.22-μm micropore vacuum filters (500 mL Vacuum Filter/Storage system, Corning, NY, USA). Bronidox (0.05% final concentration; Sigma-Aldrich, St. Louis, MO, USA) was then added to each sample.

Plasma samples were converted into serum samples using a defibrination procedure with some modifications [7]. $CaCl_2$ was added to the plasma for seroconversion. After continuous mixing for 12–18 hours at 4°C with the aid of magnetic bars, centrifugation was performed at 3,000 *g* for 30 minutes at 4°C. The upper layer was collected. Fibrin was removed through filtration with a 0.22-μm micropore vacuum filter. The serum was frozen at −70°C, thawed in a 4°C refrigerator, and centrifuged at 3,000 *g* for 30 minutes at 4°C to collect the supernatant. Bronidox (0.05% final concentration) was then added to each serum sample.

The candidate standards were stored at −70°C in screw-cap microtubes (Sarstedt, Numbrecht, Germany) at 500 μL/tube. Prior to storage, candidate standards were tested for known blood-borne virus markers, including the Alinity i HBsAg (Abbott Laboratories) and Alinity i Anti-HCV (Abbott Laboratories) assays. The presence of anti-HIV-1 antibodies was confirmed using western blot assay (HIV BLOT 2.2; MP Biomedicals, Solon, OH, USA).

Precision of fill

Ten microtubes were randomly selected from each of the 12 candidate standards and weighed before and after filling to check the variation in the amount filled into each tube. A CV of <1% for a tube with an expected 500-μL volume was considered to be acceptable according to WHO guidelines [6].

Homogeneity

Between-unit homogeneity was assessed for 10 tubes (the minimum number for a simple randomized design) selected from each of the 12 candidate standards. The samples were tested in triplicate in random order on a single run. Between-unit standard deviations were calculated using one-way ANOVA. The F-test was used to determine whether between-unit deviations were significant at a 95% probability level according to the ISO35 guidelines [5].

Collaborative study

Eight laboratories with certification in laboratory excellence were selected to participate in this collaborative study. Two sets of 12 candidate standards coded as mixed-titer performance panels (MP/1 to MP/12) were mailed to these laboratories. Each laboratory performed one or more of the five most commonly used anti-HIV-1 antibody assays in Korea (Table 1). Each assay was conducted in a total of three laboratories. The first set of candidate standards was assayed in duplicate on the first day of the

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Table 1. Assays used in the collaborative study

Reagent product name	Instrument	Manufacturer
HIV Ag/Ab Combo	Alinity i	Abbott Laboratories, Wiesbaden, Germany
HIV combi PT	cobas e 601, cobas e 602	Roche Diagnostics GmbH, Penzberg, Germany
HIV 1/O/2 Enhanced	ADVIA Centaur XPT	Siemens, Erlangen, Germany
HIV DUO Ultra	Vidas	BioMérieux, Craponne, France
SD BIOLINE HIV 1/2 3.0		SD, Yongin, Korea

study, and the second set was assayed in duplicate on the second day.

For the four assays (Abbott Alinity i, Roche cobas e, Siemens ADVIA Centaur XPT, and BioMérieux Vidas) that were qualitative but displayed numerical values (e.g., the Alinity i HIV Ag/Ab combo assay provides the sample to cut-off index ratio [S/CO] value), the overall mean estimate for each assay was calculated as the mean of all six datasets from three individual laboratories. The CVs of the means of the six datasets were also calculated.

Long-term stability

Real-time and accelerated stability of the panel were evaluated to assess the long-term stability. Real-time stability was examined at –70°C for 0, 3, 6, 9, and 12 months. At a given time point, tubes were tested using the Alinity i HIV combo assay in triplicate. The recovery rate was calculated at each time point and compared to that at time point 0. After linear regression assumptions were confirmed, Microsoft Excel (Microsoft, Redmond, WA, USA) was used to calculate the slope *P*-value.

The accelerated stability was examined at –20°C, 4°C, 24°C, and 37°C for 0, 7, 14, 21, and 28 days. At a given time point, tubes were placed at –70°C. All tubes were then subject to the Alinity i HIV combo assay simultaneously in triplicate on the final day (28 days after storage). The recovery rate compared to time point 0 was calculated for each temperature. The log transformation of the typical first-order degradation kinetics was assumed as the instability model. The Arrhenius equation was applied to the resulting data to predict the stability duration of each candidate standard. After linear regression assumptions were confirmed, *t*-tests for slopes that were significantly different from zero were performed using Microsoft Excel. Microsoft Excel was also used to calculate the degradation rate constant (k) and other parameters according to the CLSI EP25 and ISO35 guidelines [5, 8].

Table 2. Results of anti-HIV-1 antibody screening assays and western blot assay for selected candidate reference standards

Reference standard	Antibody screening			Western blot
	Abbott Alinity i* (S/CO)	Roche cobas e* (COI)	SD Bioline HIV 1/2 3.0	MP HIV BLOT 2.2
MP/01	41.11	641.90	Positive	Positive
MP/02	156.54	829.80	Positive	Positive
MP/03	396.89	430.70	Positive	Positive
MP/04	609.60	288.40	Positive	Positive
MP/05	167.11	581.20	Positive	Positive
MP/06	0.07	0.31	Negative	Negative
MP/07	91.12	961.90	Positive	Positive
MP/08	121.56	772.80	Positive	Positive
MP/09	345.61	406.60	Positive	Positive
MP/10	901.91	897.70	Positive	Positive
MP/11	655.61	333.10	Positive	Positive
MP/12	0.09	0.28	Negative	Negative

*Cut-off is 1.00.
Abbreviations: MP, mixed-titer performance panel; S/CO, sample to cut-off index ratio; COI, cutoff-index.

RESULTS

Prepared mixed-titer performance panel for anti-HIV-1 antibody

The results of the three screening assays and the western blot assay for the selected 12 candidate reference standards are presented in Table 2. All candidate standards, except for the two negative candidate standards, were positive in the western blot assay. The standards showed varying results in the Alinity i assay according to the S/CO ratio.

Precision of fill and homogeneity

The CV on a 500-μL fill was 0.56%, which was within the acceptable range. CVs of the between-unit homogeneity evaluation of the 12 candidate standards ranged from 0.0% to 1.1%. The F-test showed no significant between-unit heterogeneity ($P \geq 0.05$).

Collaborative study

All five assays performed by the eight laboratories in the collaborative study were qualitative assays for anti-HIV-1 antibody. The results reported by participating laboratories for every positive candidate standard (MP/01–05, MP/07–11) were positive, and those for negative candidate standards (MP/06, MP/12) were negative. For the four qualitative assays that showed numerical

Table 3. Estimated values of candidate reference standards per assay in the collaborative study

Reference standard	Abbott Alinity i*		Roche cobas e601, e602*		Siemens ADVIA Centaur XPT*		BioMérieux Vidas*		SD Bioline HIV 1/2 3.0
	Mean (S/CO)	CV (%)	Mean (COI)	CV (%)	Mean (index)	CV (%)	Mean	CV (%)	
MP/01	40.47	3.9	581.51	3.2	>12		23.55	4.3	Positive
MP/02	159.49	7.3	753.73	4.0	>12		22.04	4.0	Positive
MP/03	382.58	4.2	394.37	3.7	>12		25.05	3.8	Positive
MP/04	644.24	4.6	282.97	3.9	>12		21.06	4.6	Positive
MP/05	153.83	2.7	556.22	3.7	>12		19.69	5.1	Positive
MP/06	0.06	11.9	0.28	5.5	<0.05		0.04	5.7	Negative
MP/07	93.91	2.8	888.19	4.8	>12		24.27	4.2	Positive
MP/08	127.93	7.2	717.35	3.9	>12		21.62	4.2	Positive
MP/09	372.38	7.0	373.48	5.4	>12		22.26	4.4	Positive
MP/10	953.96	3.0	927.92	6.0	>12		25.28	2.8	Positive
MP/11	683.99	1.5	269.65	2.8	5.00	24.0	24.07	6.3	Positive
MP/12	0.08	35.6	0.31	11.1	<0.05		0.04	8.4	Negative

*Qualitative assays that showed numerical values.
Abbreviations: MP, mixed-titer performance panel; S/CO, sample to cut-off index ratio; COI, cutoff-index.

values, the overall mean estimates and CVs were calculated. Aside from the ADVIA Centaur XPT assay, all CVs of 10 positive candidate standards were <10% (Table 3).

Long-term stability

There was no significant difference in the results of assays performed for 12 months from the 0-month results (Fig. 1). The recovery rate was 90%–110%. Student's *t*-test for the regression slope of each candidate standard showed]that the slope was not significantly different from zero ($P \geq 0.05$), indicating that the stability was maintained for 12 months.

All candidate standards stored at –20°C showed a recovery rate of 95%–105%, indicating no stability reduction after cold storage for 28 days (Table 4). At 37°C, the recovery rate was the highest at 1 week, which then gradually decreased, suggesting denaturation of the antibody. The resulting slope, estimated by linear regression over time, provided the degradation rate constant (k) (Table 4). For MP/1, the stability duration calculated using first-order degradation kinetics and the Arrhenius equation was 5,759 days. For the other candidate standards, two or fewer slopes of the regression for the four temperature conditions were significantly different from zero. This prevented calculating the exact degradation rate constant (k) at the planned storage temperature or the stability duration using the Arrhenius equation.

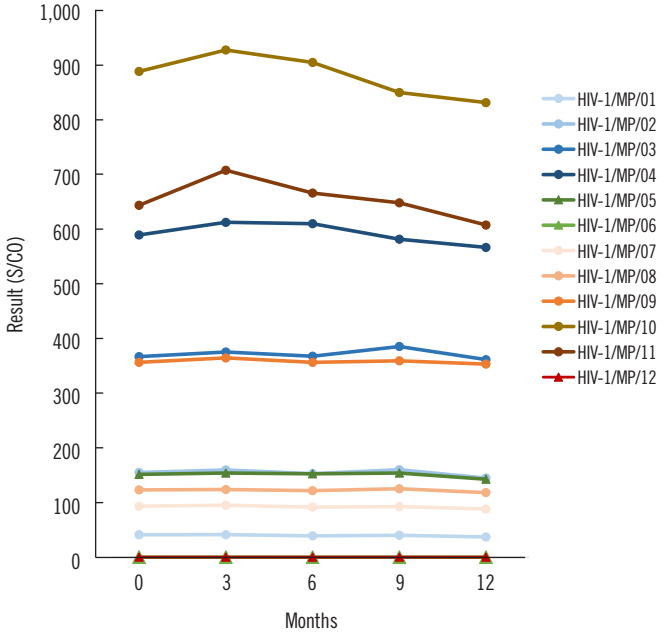

Fig. 1. Real-time stability study results of candidate reference standards after storage at –70°C for 12 months.
Abbreviations: MP, mixed-titer performance panel; S/CO, sample to cut-off index ratio.

DISCUSSION

The WHO is the main organization establishing international biological reference standards that are widely used in the develop-

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Table 4. Accelerated stability study results of candidate reference standards

Reference standard	Temperature (°C)	Recovery rate (%)				k	*P* of slope *t*-test
		1 week	2 weeks	3 weeks	4 weeks		
MP/01	–20	103	103	104	104	0.001	<0.05
	4	108	116	124	124	0.008	<0.05
	24	118	126	130	131	0.009	<0.05
	37	138	134	125	117	0.003	0.66
MP/02	–20	99	100	99	104	0.001	0.32
	4	111	113	114	117	0.005	<0.05
	24	103	106	115	114	0.005	<0.05
	37	116	109	103	94	–0.004	0.40
MP/03	–20	97	99	101	100	0.001	0.49
	4	101	101	103	105	0.002	<0.05
	24	104	110	116	120	0.007	<0.05
	37	122	122	122	117	0.004	0.32
MP/04	–20	101	100	102	100	0.000	0.91
	4	101	96	98	101	0.000	0.79
	24	97	96	96	95	0.002	<0.05
	37	102	99	93	88	0.005	<0.05
MP/05	–20	99	100	99	100	0.000	0.93
	4	100	103	105	105	0.002	<0.05
	24	106	108	107	111	0.003	<0.05
	37	121	121	116	118	0.004	0.32
MP/07	–20	100	102	99	100	0.000	0.93
	4	103	101	100	100	0.000	0.67
	24	98	101	105	111	0.004	0.04
	37	118	116	111	107	0.001	0.76
MP/08	–20	96	98	96	97	0.001	0.41
	4	98	97	101	101	0.001	0.48
	24	95	100	103	105	0.003	0.13
	37	115	109	98	87	–0.006	0.23
MP/09	–20	99	97	99	96	0.001	0.15
	4	100	102	102	102	0.001	0.08
	24	98	103	107	112	0.005	<0.05
	37	112	110	105	99	–0.001	0.70
MP/10	–20	99	99	99	99	0.000	<0.05
	4	100	97	98	100	0.000	0.68
	24	97	99	99	101	0.001	0.47
	37	102	102	101	102	0.000	0.52
MP/11	–20	105	105	103	103	0.001	0.45
	4	103	104	108	110	0.003	<0.05
	24	115	116	119	119	0.006	0.08
	37	119	119	118	120	0.005	0.18

Abbreviations: k, degradation rate constant; MP, mixed-titer performance panel.

ment, evaluation, standardization, and control of products in the medical industry [6]. The WHO recommends preparing and establishing national reference standards owing to the limited availability of international standards [6]. The Korean IVD reference standards were established in 2012 by the Ministry of Food and Drug Safety (MFDS) [9]. The first national reference standards for anti-HIV-1 antibody (MP panel) were prepared and distributed as of 2012, which have now been depleted. In this study, serum samples were obtained from HIV-infected patients to establish new national reference standards for anti-HIV-1 antibody. Ten candidate reference standards were selected based on the positive results of three screening assays, and two candidate standards with negative results in the three screening assays were selected.

Evaluating between-unit homogeneity is important to confirm that each tube has the same quantity of anti-HIV-1 antibody. We confirmed that the variation between tubes was sufficiently small for the candidate reference standards.

The laboratories participating in this collaborative study performed one or more of the five quantitative assays. The results reported by the laboratories for positive candidate standards (MP/01–05, MP/07–11) agreed with the known positivity, and the results for the negative candidate standards (MP/06, MP/12) agreed with the known negativity status. Different assays showed different numerical results for the four qualitative assays with numerical values. Except for ADVIA Centaur XPT, all CVs of the 10 positive candidate standards were <10%. Because the WHO anti-HIV-1 antibody international reference standard is not designated in the form of international units, internationally used HIV antibody assay devices are qualitative assays [10, 11]. The aim of this multi-center collaborative study was not to assign specific values (property values) of candidate standards but rather to confirm the presence of anti-HIV-1 antibody and measure the values of candidate standards using different assays.

According to ISO35 guidelines, when no degradation is found at least 20°C above the planned storage temperature, the degradation rate under planned storage conditions may be assumed to be negligible, subject to confirmation by appropriate monitoring arrangements [5]. Our accelerated stability study results indicated that all candidate standards were stable after storage at –20°C for 1 month. The stability duration of MP/1, for which the typical Arrhenius model could be applied, was 5,759 days. We also determined that these candidate standards were stable at our planned storage temperature of –70°C. We recommend that these reference standards be monitored once a year according to the MFDS guidelines. This is the first report of an accelerated study aimed at applying the Arrhenius equation to Korean IVD reference standards. Previous data for WHO international standards showed stability but without using the Arrhenius model [12], or that it is impossible to use the Arrhenius model because there is no observed drop in the potency of standards [13, 14]. Real-time stability studies on national reference standards should be regularly conducted to ensure quality [15]. Our real-time stability study confirmed that the candidate standards remained stable for 1 year.

In conclusion, we successfully established 12 national reference standards for anti-HIV-1 antibody. The 12 candidate national reference standards presented consistent results across laboratories using five assays and exhibited stability under long-term frozen storage. These candidate standards can be used as a national reference panel for anti-HIV-1 antibody.

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AUTHOR CONTRIBUTIONS

Huh HJ conceived and designed this study and conducted the experiments. Chae SL and Cha YJ supervised the study. Kim SK and Chung JW analyzed the data. Huh HJ prepared the initial draft. Yoo SJ, Roh KH, Chae SL, and Cha YJ reviewed and revised the initial draft. All authors read and approved the final draft.

CONFLICTS OF INTEREST

None.

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