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Establishment of the first Chinese national standard for protein subunit SARS-CoV-2 vaccine

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ABSTRACT

A reference standard is needed for quality control of protein subunit SARS-CoV-2 vaccines to meet urgent domestic needs. The Chinese National Institutes for Food and Drug Control (NIFDC) launched a project to establish the first reference material for the protein subunit SARS-CoV-2 vaccine to be used for calibration of antigen testing. The potency and stability of the national candidate standard (CS) were determined by collaborative calibration, and accelerated and freeze–thaw degradation studies. Moreover, a suitability study of the CS was performed. Eight laboratories in mainland China were asked to detect antigen content of CS using a common validated enzyme-linked immunosorbent assay (ELISA) kit established by NIFDC and in-house kits in the collaborative study. Six laboratories returned valid results, which established that the antigen content of the CS was 876,938 YU/mL, with good agreement across laboratories. In the suitability study, the CS exhibited excellent parallelism and a linear relationship with four samples produced by different expression systems and target proteins. In addition, good stability in the accelerated and freeze–thaw degradation study was observed. In conclusion, the CS was approved by the Biological Product Reference Standards Sub-Committee of the National Drug Reference Standards Committee as the first Chinese national standard for determining antigen content of protein subunit SARS-CoV-2 vaccines, with an assigned antigen content of 877,000 U/mL (Lot. 300050–202101). This standard will contribute to a standardized assessment of protein subunit SARS-CoV-2 vaccine in China and may provide experience for developing reference materials for antigen content detection of SARS-CoV-2 vaccine in other countries.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection causes coronavirus disease 2019 (COVID-19) [1]. In contrast to severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS), a large number COVID-19 cases manifest as mild and asymptomatic infections and this disease is characterized by rapid transmission and high infection rates [2]. Vaccines are expected to control the spread of this infectious disease, and therefore, will have an enormous global impact. Researchers have launched multiple technical approaches for vaccine research and development worldwide, including the development of nucleic acid (DNA, RNA), viral vectors, protein subunits, inactivated viruses, live attenuated viruses, and virus-like particle vaccines [3]. Of these, protein subunit vaccines represent the

Abbreviations: (SARS-CoV-2), Severe acute respiratory syndrome coronavirus 2; (COVID-19), Coronavirus disease 2019; (SARS), Severe acute respiratory syndrome; (MERS), Middle East respiratory syndrome; (WHO), World Health Organization; (Longcom), Anhui Zhifei Longcom Biopharmaceutical Co., Ltd; (NTAb), Neutralizing antibodies; (NIFDC), National Institutes for Food and Drug Control; (CS), Candidate standards; (CHO), Chinese hamster ovary; (S), Spike; (RBD), Receptor-binding domain; (ELISA), Enzyme-linked immunosorbent assay; (RM), Reference material; (95% CI), 95 percent confidence interval; (OD), Optical density; (CV), Coefficient of variation; (HRP), Horseradish peroxidase; (QC), Quality control; (NHPs), Non-human primates; (GMTs), Geometric mean titers.

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largest group in the clinical phase. As of July 20, 2021, and according to data released by the World Health Organization (WHO), the landscape of novel coronavirus candidate vaccine development worldwide comprises 36 protein subunit vaccines that have entered the clinical stage, accounting for 33% (36/108) of all candidate vaccines [3].

The receptor-binding domain (RBD) is a universally accepted active domain of protein subunit SARS-CoV-2 vaccines. Several manufacturers are urgently developing protein subunit SARS-CoV-2 vaccines with RBD or spike (S) proteins as antigen [3] (Table S1). Novavax evaluated two pivotal phase 3 trials: a trial in the U.K. that demonstrated an efficacy of 96.4% against the original virus strain, 86.3% against the Alpha (B.1.1.7) variant and 89.7% efficacy overall [4,5]; and the trial in the US and Mexico that demonstrated 100% protection against moderate and severe disease and 90.4% efficacy overall [5]. In mainland China, seven protein subunit vaccines have been introduced in clinical trials [3,6]. The protein subunit vaccine (CHO-expressing RBD) developed by Anhui Zhifei Longcom Biopharmaceutical Co., Ltd (Longcom) was approved for emergency use on March 10, 2021 [7]. The protein subunit vaccine (GH-CHO-expressing RBD) developed by Clover Biopharmaceuticals Inc. [8], and the one (Sf9-expressing RBD) developed by WestVac Biopharma Co., Ltd. [9], have been introduced in phase III clinical trials (Table S1).

As there is currently no international standard for antigen content of protein subunit SARS-CoV-2 vaccine available, the potency units of protein subunit SARS-CoV-2 vaccines from most developers in mainland China have been determined using the developer's internal references expressed as μg, YU or CU (Table S1). This results in different potency units and difficulties in horizontal comparisons of dose and immunogenicity across different preparations, and has become a key problem in quality control (QC) of protein subunit SARS-CoV-2 vaccines. Therefore, a national standard to quantify the content of protein subunit SARS-CoV-2 vaccines is urgently needed in China.

The Chinese National Institutes for Food and Drug Control (NIFDC) has established an antigen content detection kit for protein subunit SARS-CoV-2 vaccines. On this basis, the recombinant RBD expressed in CHO cells was selected as the national candidate standard to carry out collaborative calibration, suitability, and stability studies. Using this approach, we aim to establish the first national standard for protein subunit SARS-CoV-2 vaccine.

2. Materials and methods

2.1. Participating laboratories

A total of eight laboratories participated in this study, including four that had developed protein subunit SARS-CoV-2 vaccine, three that had developed inactivated SARS-CoV-2 vaccines, and a national control lab (Table S2). These participants were randomly anonymized as Labs 1 to 8.

2.2. Reagents and methods

Enzyme-linked immunosorbent assay (ELISA) is a well-accepted method for quantifying antigen content. The NIFDC extensively screened monoclonal antibody libraries to obtain antibodies suitable for establishing a double antibody sandwich ELISA-based kit as a feasible method for antigen detection, which showed good suitability in vaccines developed from different expression systems and target proteins [10]. We designated this established antigen detection method as the "common kit".

Recombinant protein S (200ug) and complete Freund's adjuvant were thoroughly mixed to produce an emulsion. Goat was injected intramuscularly with the mixture. Boosting immunizations were administered at day 14, 21 and 28 post primary immunizations using the same method with an exception of replacing Freund's complete adjuvant with incomplete adjuvant. Fourteen days after the last immunization, blood was collected by artery puncture. The common kit was coated with goat polyclonal antibody which was purified by Protein G Sepharose 4 fast flow (Cytiva, 17061806)[11]. The detection antibody was monoclonal antibody 20D8. Horseradish peroxidase (HRP) was conjugated with 20D8 by using Lightning-Link® (Abcam, ab102890) kit[12]. In brief, 96-well microtiter plates were coated with goat polyclonal antibody. The plates were then incubated with the antigen at appropriate serial dilutions according to the linearity range (0.156–2.500 YU/mL). Detection was performed using HRP-20D8, followed by the addition of tetramethylbenzidine substrate. The absorbance was measured at 450/630 nm using an ELISA plate reader (SpectraMax® iD3, Molecular Devices, USA). All participants in this study were asked to use this common kit to detect antigen content. Moreover, two laboratories (Lab 2 and 7), responsible for the development of the recombinant protein vaccine, used their own antigen detection methods (defined as the "in-house kit", double antibody sandwich ELISA-based kit) to measured synchronously.

3. Reference material and national candidate standard

The reference material (RM, assigned 630,900 YU/mL), used as an internal control of antigen content by Longcom (Longcom is subordinate to Chongqing Zhifei Biological Products Co., Ltd.), and national candidate standard (CS, Lot. M20201003-2) were both the purified protein subunit SARS-CoV-2 vaccine bulks (without adjuvant) provided by Longcom (Table S3). The coding sequence for the RBD of SARS-CoV-2 (S protein residues 319–541, GISAID accession No. EPI_ISL_402119) was codon-optimized for mammalian cell expression. The signal peptide sequence of the MERS-CoV S protein (S protein residues 1–17) was added to the protein N-terminus for protein secretion, and a *hexa*-His tag was added to the C terminus to facilitate further purification processes. To increase the immunogenicity, two copies of RBD from R319 to K537 were attached as a tandem repeat dimer [13]. Sterile aliquots of purified RBD were prepared in 4000 vials, closed with rubber stoppers, and sealed with aluminum caps; CS was in liquid form. The mean volume was 0.57 mL per vial [coefficient of variation (CV) = 1.2%]. The antigen content was 732,593 YU/mL detected against RM using Longcom's in-house kit (n = 15), and the CV value of the antigen content homogeneity was 2.4%.

3.1. Stability studies

Accelerated degradation of CS was investigated by storing the samples at 37℃ for 0, 3, 5, 7, 14, 21, 28, 35, 42, and 56 d. Sample vials were stored at 2-8℃ and used for short-term laboratory manipulation for a period of 7, 14, 21, 28, 35, 42, and 56 d. As it was the first-generation national standard and an optimum storage temperature had not been determined, two temperatures intended for storage (-20 and −80℃) were selected to measure real-time stability. Moreover, a program to measure real-time stability is currently ongoing by regularly measuring the potency of samples stored at −20 and −80℃. The antigen content of the vials was measured against RM using Longcom's in-house kit. Vials at all temperatures and storage time points were evaluated using at least three independent assays. The baseline (732,593 YU/mL) was obtained from the result of antigen content homogeneity measured using the in-house kit described above. The degradation rate of antigen content was also calculated at each temperature. For freeze–thaw stability, the antigen content of CS following 1, 3, and 5 freeze–

thaw cycles was expressed against the baseline (732,593 YU/mL). Vials with all freeze–thaw cycles were evaluated in at least three independent assays.

3.2. Collaborative calibration

For the calibration of the CS, each participant laboratory performed 4–6 independent assays. Each assay included freshly thawed RM and CS samples. At least four dilutions were required per sample, and each dilution was tested in duplicate for each independent assay. Participants reported the raw data [optical density (OD), 450/630 nm] and calculated RBD antigen content.

3.3. Suitability study

To assess the suitability of the candidate as a standard for the bulk, four samples were included in this study. Samples 1–4 were representative of the bulk of protein subunit SARS-CoV-2 vaccine produced by laboratories 2 to 5. Laboratories 2 to 5 separately tested their own samples (1,2,3, and 4) using a common kit. In addition, laboratories 2 and 4 tested samples 1 and 3 using their in-house kits. Each participant performed 4–6 independent assays and reported both raw data (OD 450/630 nm) and calculated results. The details of these samples are presented in Table S4.

3.4. Statistical analysis

The raw data were submitted to NIFDC and a parallel line assay was run to analyze the log-transformed relative OD values (OD 450/630 nm) of the raw data. Parallelism and linearity were analyzed using multiple parallel-line comparisons, and the preparations, regression, parallel, linearity, relative potency, and 95% confidence intervals were calculated by the statistical software "Statistic". Statistical validity was assessed using analysis of variance (ANOVA) tests. When the F-value $< F(DF, n)_{0.05}$, it indicated a P-value > 0.05, and there was no significant deviation from linearity and parallelism between the two lines; thus, the result was reported as 'valid'. From valid results, the relative potency (antigen content) could be estimated; however, estimated potency with associated 95% confidence intervals (CI) out of 80–120% were rejected, despite no significant deviations from linearity and/or parallelism. This was because the difference in OD values between two wells were large at the same dilution, resulting in inaccurate results. Combined antigen contents were obtained by averaging all similar results from all assays from each laboratory, and the overall antigen contents were calculated. The antigen content was expressed as the 95% CI. Estimates of intra-laboratory (between assay) and inter-laboratory (between laboratory) variability were expressed as CV (%) of antigen content estimates.

4. Results

4.1. Quality control of the CS

The major QC tests for raw material of CS including, the ratio of antigen content to protein content, protein content, identity, sterility, residual host cell DNA and protein, purity, bacterial endotoxin, and molecular weight, met both the specifications of the Chinese Pharmacopeia (CP, version 2020) and the Manufacturing and Control Procedures from Longcom (Table S5).

4.2. Stability studies

The antigen content of the CS stored at −80, −20, 2–8, and 37℃ from days 0–56 were determined against RM using Longcom's in-house kit. In the accelerated degradation study, the residual antigen content decreased to 78% of the base point at 37℃ after 35 d (Fig. 1 A). Optimum stability was observed at 2–8℃ for short-term laboratory manipulation, and the residual antigen content decreased to 78% after 56d (Fig. 1 B). Moreover, we found that CS was stable at temperatures used for long-term storage (-20 and −80 °C) (Fig. 1 C and D). For freeze–thaw stability (Table 1), the antigen contents of CS after 1,3, and 5 freeze–thaw cycles were analyzed concurrently, and were expressed against the vials

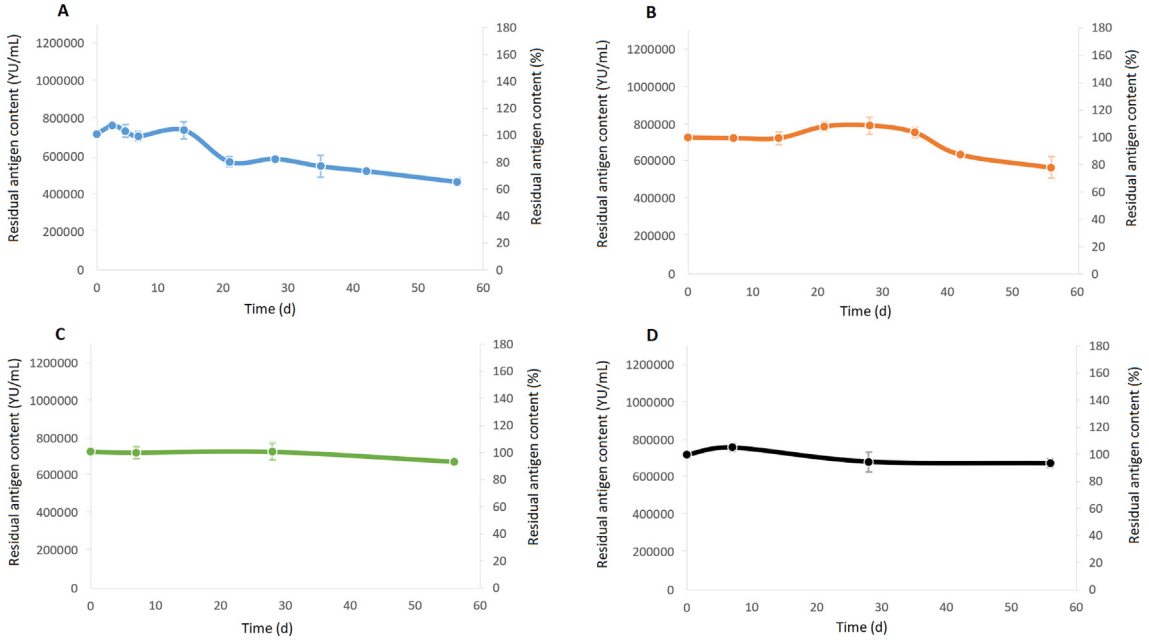

Fig. 1. Results of the analysis of stability at different temperatures. **A**, Accelerated degradation study of CS stored at 37℃. **B**, Stability of short-term laboratory manipulation (2-8℃). **C** and **D**, Stability at the temperature intended for storage (-20 and −80℃). Antigen content of the CS stored at each temperature from 0 to 56 d was determined against RM using the in-house kit. A program to measure real-time stability is ongoing by regularly measuring the potency of samples stored at −20 and −80℃.

Table 1
Summary of results from freeze-thaw stability study.

Freezing and thawing cycles	Residual antigen content (YU/mL) [CV [a] (%)]	Residual antigen content (%)	Degradation rates (%)
0	716,100 [3.1] *n* = 3	/	/
1	711,900 [6.4] *n* = 3	98	2
3	714,100 [4.0] *n* = 3	97	3
5	716,100 [3.1] *n* = 3	97	3

[a] CV (%) = intra-laboratory variability. [b] n = assay number. Degradation rate (%) = 100% - residual antigen content (%).

without freeze–thaw cycles (base point). The degradation rates were 2, 3, and 3%, respectively.

4.3. Calibration of candidate standard

Laboratories 1 to 8 used the common kit to measure antigen content of CS in the collaborative study, while laboratories 2 and 7 used their own in-house kit. Laboratory 4 provided suitability data, but not collaborative calibration data because they found that if the RM distributed from NIFDC was not stored properly, its

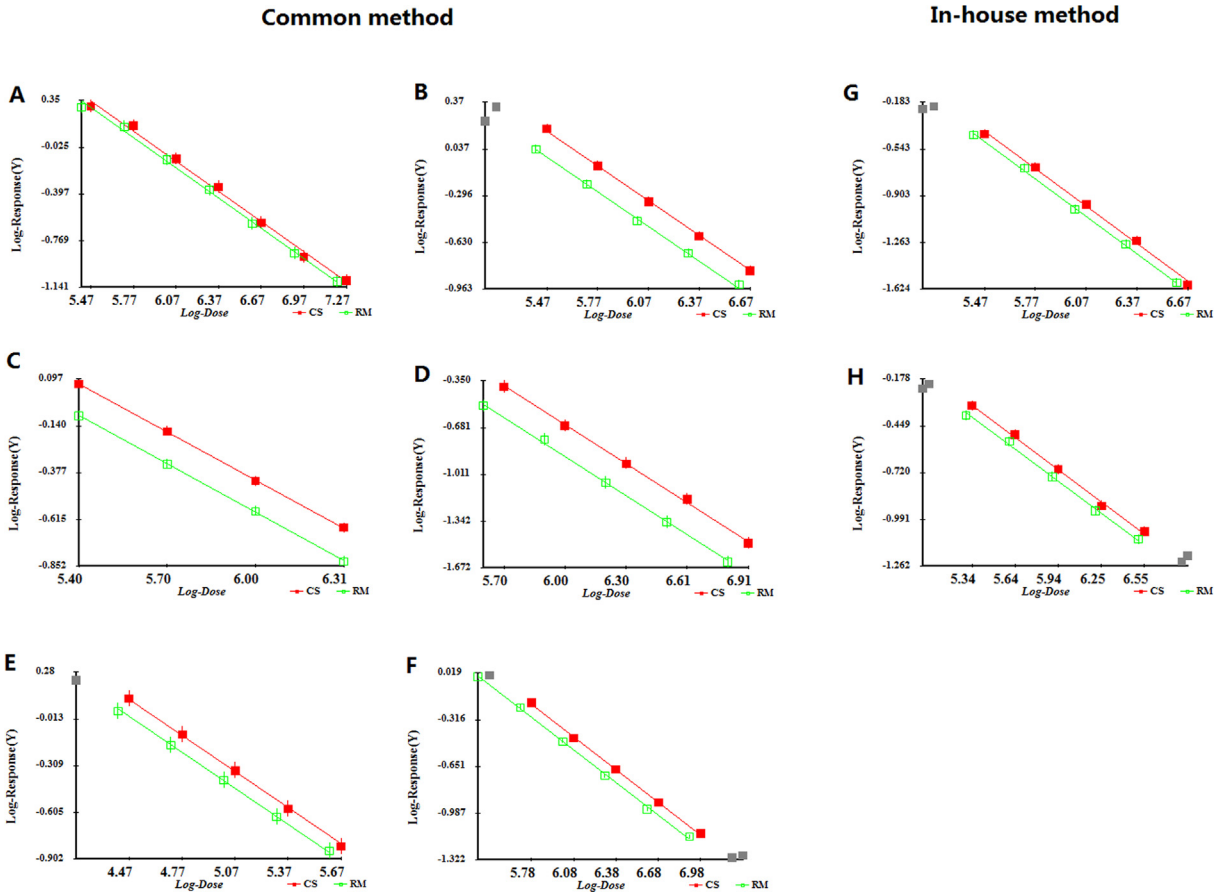

Fig. 2. Linearity and parallelism in calibration study. Antigen content in CS was compared with that of the RM. **A-F,** Representative parallelism and linearity results of CS detected by Labs 1, 2, 3, 5, 6, and 7 using the common kit. **G** and **H**, Representative parallelism and linearity results of CS detected by Labs 2 and 7 using their in-house kit. Parallelism and linearity were analyzed by multiple parallel line comparisons and the statistical validity was assessed by analysis of variance tests. All data of parallelism and linearity showed P > 0.05.

Table 2
Results of the calibration of the candidate standard.

Lab[a]	Common kit			In-house kit		
	N[b]	Antigen contents (YU/mL)	CV (%)[c]	N	Antigen contents (YU/mL)	CV (%)[c]
1	6	792,726	8	/	/	/
2	6	932,681	13	6	813,756	12
3	4	1,025,370	15	/	/	/
5	5	948,621	10	/	/	/
6	6	814,594	9	/	/	/
7	6	855,116	2	6	832,639	3
Inter-lab CV(%)[d]			10			2
Antigen contents (YU/mL)		894,851			823,198	
Overall antigen contents (YU/mL)				876,938		
95% CI[e] (% of overall antigen content)				±57,194 (±7%)		

[a] Lab = laboratory code. [b] *n* = assay number. [c] CV (%) = intra-laboratory variability. [d] Inter-lab CV (%) = inter-laboratory variability. [e] 95% CL = 95 percent confidence limit.

potency decreased significantly. All data from laboratory 8 were rejected because none of the values met the requirements for reliable statistical analysis. Thus, the data submitted from six of the eight laboratories (Labs 1, 2, 3, 5, 6, and 7) met the validation acceptance criteria consisting of tests of parallelism, linearity (Fig. 2), and 95% CI. These laboratories carried out 4–6 independent assays with CS against RM. The results indicated that the intra-laboratory variability of CS ranged from 2 to 15% using the common kit, but was 3 and 12%, respectively, using in-house kits (Table 2 and Fig. 3). The inter-laboratory variability was 10% and 2%, respectively. The overall average value of all pooled results was 876,938 YU/mL with 95 %CI ± 57,194 (7% of the overall average value).

4.4. Suitability of the candidate as the national standard

To ensure that the national standards could be used reliably in different laboratories, laboratories 2 to 5, which produced protein subunit SARS-CoV-2 vaccines, carried out four to six assays with their bulks of protein subunit SARS-CoV-2 vaccines (samples 1–4) against the CS using the common kit. Laboratories 2 and 4 also tested samples 1 and 3 using their in-house kits. As shown in Fig. 4, samples 1–4 were all in excellent parallelism and linearity relationships with CS using the common kit, and samples 1 and 3 also showed good parallelism and linearity relationships using in-house kits, suggesting that the CS was suitable for different expression systems and target proteins. The characteristics of the samples are presented in Table S4.

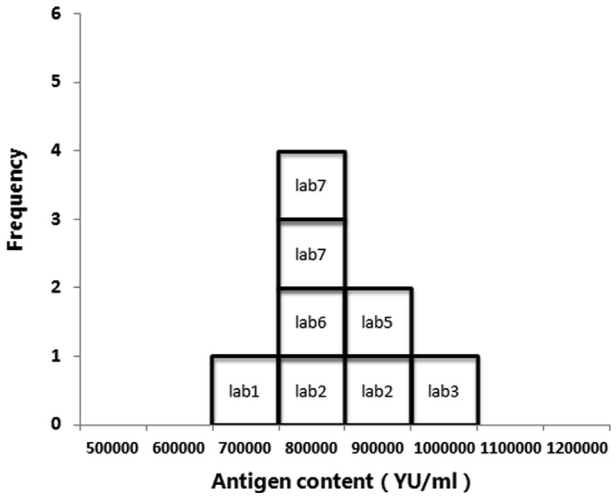

Fig. 3. Distribution of antigen content of the CS. Labs 1, 2, 3, 5, 6, and 7 represent the six participating laboratories.

CS was assumed as 877,000 U/mL to calculate the antigen content of the samples. Samples 1 and 3 were 844,362 U/mL and 2,358,107 U/mL, respectively, when using a common kit, and 911,126 U/mL and 2,661,987 U/mL, respectively, when using in-house kits. The ratios of the results of these two samples using different methods were 108% and 113%, respectively (the average value was 111% with a CV of 3%), suggesting the difference between the various kits compared was minimal when CS was used as the reference for detecting protein subunit SARS-CoV-2 vaccines Table 3.

5. Discussion

Reference materials are required to ensure the safety, effectiveness, and consistency of vaccines. The use of reliable, stable, and uniform standard materials is necessary to ascertain whether the internal and external regulatory QC requirements are met [10]. Hou *et al.* employed convalescent sera of COVID-19 to develop a Chinese national standard for SARS-CoV-2 neutralizing antibodies (NtAb) with an assigned unit of 1000 U/mL on September 16, 2020 [14]. This standard provides a reference for the comparison of NtAb titers induced by different vaccines. The WHO established the first WHO international standard for anti-SARS-CoV-2 immunoglobulin (human) (20/136) on December 17, 2020, which was assigned a standard concentration of 250 IU/ampoule [15]. However, there is currently no international standard for antigen content of protein subunit SARS-CoV-2 vaccine available.

As a raw material of national standards from a CHO-expressed tandem-repeat dimeric RBD protein (without adjuvant) [13], its immunogenicity and efficacy should also be investigated. Protein subunit SARS-CoV-2 vaccine (with alum-based adjuvant) from the same source as CS could induce high levels of RBD-binding and SARS-CoV-2 neutralizing antibodies in both mice and non-human primates (NHPs) [13]. Two doses (10 μg) protected Ad-hACE2-transduced mice against SARS-CoV-2 infection, as detected by reduced viral RNA titers and improvements in lung injuries. In NHPs, the vaccine (25 and 50 μg) prevented SARS-CoV-2 infection in the lungs, trachea, and bronchi, leading to milder lung lesions [13]. The results of phase 1/2 clinical trials [16] showed that this protein subunit SARS-CoV-2 vaccine was well tolerated, with no serious vaccine-related adverse events. Three immunizations at 0, 30, and 60 d achieved 93–100% seroconversion of neutralizing antibodies, with the GMTs exceeding the magnitude of the convalescent samples. Currently, this protein subunit SARS-CoV-2 vaccine is being evaluated in ongoing international multi-center phase 3 trials (NCT04646590) and has been approved for emergency use in Uzbekistan [16] and in mainland China [7]. Currently, >100 million doses are available in China. Therefore, this protein subunit vaccine bulk proved to have good immunogenicity and was proposed as the raw material for CS.

Table 3
Results of the suitability study for the protein subunit SARS-CoV-2 bulks.

Method	Antigen contents (U/mL) [CV(%)[a]]			
	Sample 1	Sample 2	Sample 3	Sample 4
Common kit	844,362 [4.1] *n*[d] = 6	2,226,652 [11.7] *n* = 4	2,358,107 [4.8] *n* = 4	240,976 [11.1] *n* = 5
In-house kit	911,126 [6.8] *n* = 6	/[b]	2,661,987 [10.2] *n* = 3	/
Ratio[c]	108%	N/A	113%	N/A

[a] CV = coefficient of variation. [b]/=not test. [c] Ratio = ratio between the results of the in-house kit and that of the common kit. [d]n = assay number. [e]N/A = not available.

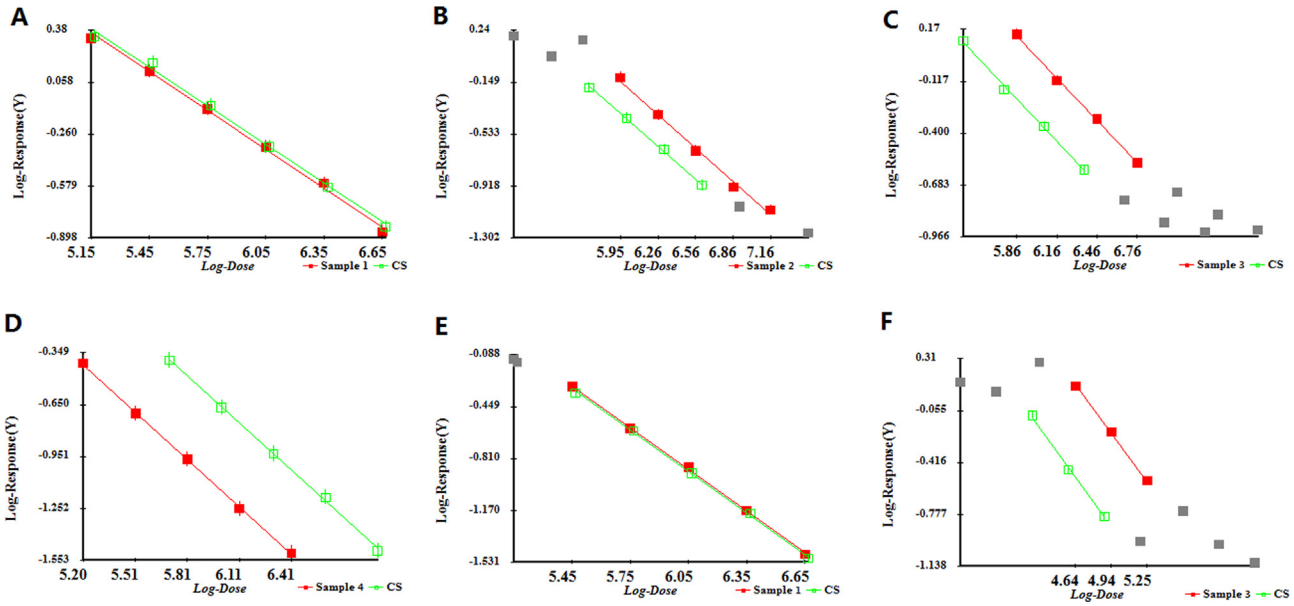

Fig. 4. Suitability of the CS as reference material for antigen content of vaccines. The antigen contents in Samples 1–4 were compared against the CS. **A-D**, Representative parallelism and linearity results of Samples 1, 2, 3, and 4 detected by Labs 2, 3, 4, and 5 using the common kit. **E-F**, Representative parallelism and linearity results of Samples 1 and 3 detected by Labs 2 and 4 using their in-house kits. Parallelism and linearity were analyzed by multiple parallel line comparisons and the statistical validity was assessed by analysis of variance tests. All parallelism and linearity data showed $P > 0.05$.

The results of calibrations showed that the CS was linear and parallel with the RM, and the 95% CIs of antigen contents were within 80%–120%. Variability between laboratories based on the estimates relative to RM was 10% using the common kit and 2% using the in-house kit. In the suitability study, CS exhibited excellent parallelism and a linear relationship with the four samples. In a previous study [10], the raw material was distributed to 11 different laboratories to investigate more extensive suitability. The CS showed good parallelism and linearity with either S protein expressed in CHO cells and *Pichia pastoris*, or RBD protein expressed in CHO cells, *Pichia pastoris, E. coli*, and Sf9 cells. In addition, CS exhibited good accelerated and freeze–thaw stability. These results demonstrate that CS can be used as a uniform antigen standard for protein subunit vaccines.

The premise of establishing a reference material is to design a reliable, stable and suitable detection reagent. Developers have established different methods for detecting antigen content using a variety of expression systems and target proteins. However, only two laboratories provided results using an in-house kit in this collaborative study. This is because some in-house kits are unable to detect CS, possibly because the plate coating or detecting antibody is unable to recognize the RBD protein. In this study, a goat polyclonal antibody against recombinant protein S and a monoclonal antibody 20D8 were used for coating and detecting antibodies using the common kit, respectively. Our results showed that 20D8 could recognize the linear RBD (Supplemental Fig. 1) and had strong neutralization activity against both the pseudovirus and authentic SARS-CoV-2 (Table S6). The most valuable finding was that 20D8 exerted similar neutralization activity against the mutant strain compared with the original strain (Table S6). Therefore, CS combined with this common kit may be suitable for accurately quantifying next-generation protein subunit vaccines designed from mutant strains. However, this needs to be further investigated. The common kit has the advantage of high precision, short detection time, convenient operation, and fewer influencing factors, which are in line with the 3R principles [17]. More importantly, the common kit, characterized by neutralizing monoclonal antibodies, is of great significance to ensure the consistency of *in vitro* antigen content and *in vivo* potency, to improve vaccine production and QC, and ensure to vaccine consistency. Thus, the common kit can be considered for distribution to developers in mainland China to improve the QC of protein subunit SARS-CoV-2 vaccine.

6. Conclusions

Based on the requirements of the WHO manual for the establishment of national and other secondary standards for vaccines [18] and CP (2020 version) regarding the preparation of national standards and the calibration of biological products, CS was approved by the Biological Product Reference Standards Sub-Committee of the National Drug Reference Standards Committee as the first Chinese national standard for antigen content of protein subunit SARS-CoV-2 vaccine in July 2021, with an assigned potency of 877,000 U/mL (Lot. 300050–202101). The storage temperature was temporarily set at −20 and −80℃. The real-time stability of the national standard needs to be monitored periodically by testing samples stored at −20 and −80℃. This national standard will be used for the detection and evaluation of antigen content of protein subunit SARS-CoV-2 vaccine in China and will encourage the development of preventive vaccines against SARS-CoV-2.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2022.02.048.

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