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# An antibody-free evaluation of an mRNA COVID-19 vaccine

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## Abstract

This manuscript describes the use of an analytical assay that combines transfection of mammalian cells and isotope dilution mass spectrometry (IDMS) for accurate quantification of antigen expression. Expired mRNA COVID-19 vaccine material was stored at 4 °C, room temperature (~25 °C), and 56 °C over a period of 5 weeks. The same vaccine was also exposed to 5 freeze-thaw cycles. Every week, the spike protein antigenic expression in mammalian (BHK-21) cells was evaluated. Housekeeping proteins, β-actin and GAPDH, were simultaneously quantified to account for the variation in cell counts that occurs during maintenance and growth of cell cultures. Data show that vaccine stored at elevated temperatures results in reduced spike protein expression. Also, maintaining the vaccine in ultracold conditions or exposing the vaccine to freeze-thaw cycles had less effect on the vaccine's ability to produce the antigen in mammalian cells. We describe the use of IDMS as an antibody-free means to accurately quantify expressed protein from mammalian cells transfected with mRNA vaccine.

## 1. Introduction

In 1989, Robert Malone performed a lipofectamine transfection into mammalian cells using RNA molecules and successfully expressed the genes encoded by the RNA transcript. He stated that this showed the possibility for future researchers to "Treat RNA as a Drug" [1,2]. Malone's work was an early step towards the research that would result in the modern mRNA vaccine. mRNA vaccines transport mRNA transcripts into the host's cell cytoplasm via a lipid nanoparticle (LNP) vector [3]. The transcripts are then translated into an antigen that will induce immune activity [3].

mRNA vaccine development introduces new challenges since the vaccine doesn't contain antigenic proteins. Thus, traditional analytical methods used to evaluate the quality of the

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vaccine protein are no longer applicable [4]. New analytical techniques are needed to address this gap as the mRNA vaccine technology is broadly implemented.

The World Health Organization (WHO) considers vaccine stability an important criterion for ensuring vaccine quality. Quality is defined as consistently meeting appropriate levels of purity, potency, safety, efficacy and reproducibility over the course of a shelf-life study [5]. The United States Food and Drug Administration (U.S. FDA) published guidance in 2015 stating the need for quantitative assays that are stability indicating [6]. A shelf-life or vaccine stability study is performed by subjecting the pharmaceutical product to various conditions and evaluating the quality at set time points [5,7]. The stability of mRNA vaccines is of particular interest since the structural integrity of mRNA must be maintained during transport and storage [8]. Even slight degradation could have significant effects on vaccine potency and efficacy [9]. To prevent degradation during the COVID-19 pandemic, commercial mRNA vaccines required ultracold storage conditions [9]. Keeping the vaccines frozen during transport and prior to administration proved to be challenging [10]. Having analytical techniques to evaluate the stability of the mRNA vaccine material is not only integral for WHO guidelines, but it is critical for managing cold chain supply challenges presented by this new vaccine technology [11,12].

Using one or more of the following methods, mRNA vaccine composition and purity are analyzed prior to and during stability studies: sequencing complementary DNA, UV spectroscopy, and fluorescent assays for quantification and purity; real-time quantitative reverse transcription PCR; chromatographic assays; light scattering assays; or gel-, capillary-, and laser doppler electrophoresis [4,9,13]. These assays analyze the quality and purity of both the RNA and LNP present in the initial vaccine material. However, they cannot evaluate the transfection efficiency or the production of antigenic protein. A prerequisite of any potency or stability assay for new vaccine technologies would need the vaccine material to be transfected into an appropriate cell line to ensure that an antigenic protein is expressed.

Transfection efficiency is evaluated by measuring protein expression from cells transfected with mRNA. Transfection efficiency assays may use Western blot, flow cytometry, and fluorescent microscopy of antigen modified with fluorescent tags [3,14]. Quantitative ELISA [15–17] and flow cytometry with fluorescent antibodies tags, are used to quantify antigenic protein produced in cells [18,19]. These methods, however, require an extensive amount of antibody characterization and validation. In addition, the antibodies also need to be re-evaluated regularly to determine whether they are appropriate for use with each new variant of disease [20,21].

Recently, we described an antibody-free analytical method to quantify the amount of protein expressed in mammalian cells transfected with an expired commercial mRNA vaccine [22]. The method uses isotope dilution mass spectrometry (IDMS) [23,24] for precise and accurate quantification of proteins expressed by commercially available vaccines. Here, we describe an extension of this method to study mRNA vaccine stability.

## 2. Materials and methods

### 2.1. Growth, maintenance, and transfection of baby hamster kidney cells (BHK-21)

For these experiments BHK-21 (CCL-10, ATCC, Manassas, VA USA) cells were used. A description of the growth and maintenance of this cell line was recently described in detail [22]. Monolayers of BHK-21 cells were trypsinized to generate a $2 \times 10^6$ cells/mL suspension in Opti-MEM™.

### 2.2. mRNA vaccine material

Expired commercial vaccine vials from the same manufacturing lot were received frozen and stored at manufacturer specifications until ready for use. The vials were used without modification and without additional transfection reagents. No attempt was made to quantify mRNA or evaluate quality of the mRNA or LNP prior to transfection experiments. Additional vaccine material of a different manufacturing lot was received thawed and stored at 4 °C. This material had not expired based on manufacture expiration date (MED) but had exceeded its beyond-use date (BUD). The material remained at 4 °C until use.

### 2.3. Forced degradation of mRNA vaccine material

To prepare the vaccine material for the stability study, the vials were removed from the freezer and allowed to thaw at room temperature. To ensure that vial-to-vial variations were not observed in these experiments, multiple vaccines vials were pooled into one 50 mL conical tube for a final volume of more than 45 mL. These 45 mL of material were separated into 9 mL aliquots in 15 mL conical tubes. One milliliter of pooled vaccine was then aliquoted into 45 individual 2-mL Protein LoBind® Eppendorf tubes with snap caps to prevent sample loss. Nine replicate aliquots were stored at each of the following temperatures: 25 °C, 4 °C, room temperature (~25 °C), and 56 °C. The aliquots were stored at these conditions for a maximum of 5 weeks. Each week before transfection, the nine aliquots that had been stored at each temperature were brought to room temperature and combined into a 15 mL conical tube. The volume of vaccine required for the transfection experiment was removed and the remaining vaccine was divided into 1 mL aliquots and returned to their respective storage temperature. For the vaccine stored at 56 °C, a shorter time study was also performed. Five 1-mL vaccine aliquots were prepared and stored at 56 °C in a heat block. One vial was removed from the thermomixer at 1 h. The others were removed after 4 h, 8 h, 24 h, and 63 h. Samples were immediately stored at 4 °C until transfection experiments were performed. A vial that was freshly thawed and not exposed at 56 °C was used as a 0 h timepoint.

### 2.4. Transfection of cells with mRNA vaccine material

The transfection protocol was recently described in detail [22]. Briefly, the starting concentration of mRNA was calculated based on the amount of mRNA in a respective dose as specified by the manufacturer. The volume needed to provide the expected μg amount of mRNA was added to a 1.5 mL Protein LoBind® Eppendorf microcentrifuge tube (Eppendorf, Hamburg, Germany). To each tube, $5 \times 10^5$ cells were added. The cells and vaccine material were mixed and transferred to a Fisherbrand™ 6-well surface-treated sterile

tissue culture plate (Fisher Scientific, Waltham, MA USA) containing 2 mL of outgrowth media/-well. Outgrowth media consists of DMEM containing 1 % FBS, 1 % L-glutamine, and 1 % antibiotic-antimycotic solution. The plate was gently rocked by hand and placed in the incubator at 37 °C with 5 % $CO_2$ for 24 h. The outgrowth media was then removed by aspiration, replaced with complete DMEM, and further incubated at 37 °C with 5 % $CO_2$ for an additional 24 h. After transfection, the total time that cells were allowed to grow and multiply was 48 h. The complete DMEM was aspirated off the cells that were adhered to the bottom of the well. Finally, the wells were washed with 2 mL of 1× PBS before being prepared for cell lysis and protein extraction.

### 2.5. Preparation of working stock, calibration, and labeled solutions

Target peptides were chosen based on general characteristic rules that have been described elsewhere [23,24]. The housekeeping (HK) and SARS-CoV-2 spike protein (S) light/native peptides and corresponding $C^{13}N^{15}$ heavy/labeled peptides were synthesized by Biosynth (formerly Vivitide, Gardner, MA USA). A peptide calibration curve was generated for spike protein that ranged from 1 to 250 fmol/μL. The peptide calibration curve for the HK proteins ranged from 10 to 3600 fmol/μL. Each of these standards also contained 50 fmol/μL of labeled peptides. Five μL of a 1 pmol/μL solution of labeled peptides was added to all unknown samples so that they also contained 50 fmol/μL of labeled peptides. Having a constant amount of labeled peptides in both the calibration curves and unknown samples is a requirement for absolute quantitation by IDMS.

### 2.6. Preparation of BHK-21 cells and IDMS quantification

To lyse the cells, 600 μL of Pierce™ RIPA lysis buffer (Thermo Scientific, Waltham, MA USA) was added to each well. The RIPA buffer was washed over the cells several times with a pipette. The cell lysate was transferred to a 1.5 mL Protein LoBind® Eppendorf tube and 2 μL of (~678 U) of deoxyribonuclease 1 (DNase 1) (Invitrogen, Carlsbad, CA USA) was added. The samples were briefly vortexed, transferred to a AQUASONIC™ model 150D sonicating water bath and sonicated for 30 min. After sonication, each sample was split by transferring 300 μL to a second 1.5 mL tube. Proteins were precipitated by adding 900 μL of cold Optima™ acetone (Fisher Scientific, Waltham, MA USA) to each tube. For samples intended for Western blot analysis, cell lysis was performed using 300 μL of RIPA to lyse the cells and 900 μL of acetone to precipitate the proteins. All samples were stored overnight at −25 °C.

The samples containing precipitated proteins were removed from the freezer and centrifuged in a Microfuge 16 for 30 min (Beckman Coulter Indianapolis, IN USA). The supernatant was removed from the protein pellet. Cold absolute (200 proof) ethanol (Fisher Scientific, Waltham, MA USA) was added to wash the pellet [25]. The samples were then centrifuged at 16,162×*g* for 15 min and the ethanol removed. Seventy-five μL of 0.05 % solution of *Rapi*Gest™ SF Surfactant (Waters Corporation, Milford, MA USA) in 50 mM ammonium bicarbonate was added and the samples were incubated at 100 °C for 5 min. After returning to room temperature, 10 μL (~172 pmol) of Promega Sequencing Grade Modified Trypsin (Promega, Madison, Wisconsin, USA) was added. Samples were incubated for 4 h in a thermomixer set at 37 °C with shaking at 250 rpm. Ten μL of HCL was added and the

samples allowed to incubate at room temperature to degrade the *Rapi*Gest™ [26]. Five μL of the 1.0 pmol/μL combined S and HK labeled working stock mixture was added. Samples were vortexed and centrifuged at 16,162×*g* for 10 min. The 100 μL sample was then transferred to a glass autosampler vial for analysis.

### 2.7. LC/MS instrumentation parameters

The LC/MS parameters have been previously described in detail [22]. Briefly, a Thermo Scientific Vanquish Horizon was used with a 150 mm × 2.1 mm i. d. Hypersil GOLD™ Vanquish reversed phase C18 UHPLC column (1.9 μm particle size, Thermo Scientific) as the analytical column. Optima™ LC/MS grade water with 0.1 % formic acid (v/v) and Optima™ LC/MS grade acetonitrile with 0.1 % formic acid (v/v) were used as the aqueous and organic mobile phases. A 5 μL injection volume was used with a constant flow rate of 200 μL/min. To detect the peptides eluting from the LC column, a Thermo Scientific TSQ Altis™ triple quadrupole tandem mass spectrometer with an electrospray interface was run in positive ion mode. Thermo Scientific TraceFinder™ was used for instrument control, data processing, and data reporting.

### 2.8. Western blot analysis

Western blot analysis has been previously described [22]. Briefly, protein cell lysates from vaccine stability-transfected BHK-21 preparations were resuspended in sample buffer and denatured at 70 °C for 10 min. Proteins were gel loaded, electrophoresed at 200V for 45 min, and transferred to a polyvinylidene fluoride (PVDF) membrane. An affinity purified primary SARS-CoV-2 spike protein (receptor binding domain) monoclonal antibody (Invitrogen, 1 μg/μL; 1:100 final dilution) was probed to confirm spike protein expression. Subsequently, to examine equal protein gel loading, the membrane was washed with PBS tween and re-probed with mouse-β-actin monoclonal antibody (1 μg/μL, 1:1000 final) (Novus™ Biologicals, LLC, Centennial, CO USA). Precision plus protein™ (PP) dual color standards were used as a size reference (Bio-Rad Laboratories, Hercules, CA USA).

## 3. Results and discussion

The specifications of the spike peptide mixture have been previously described [22,23]. In 2020, five peptides were chosen that lacked the methionine, cysteine, and tryptophan that cover the S1 and S2 regions of the spike protein. These peptides were chosen from the sequence of the ancestral Wuhan strain of SARS-CoV-2 and remain unchanged in the 2023 Omicron variants. The sequences of Omicron variants BA.4, BA.5, BQ.1, BQ.1.1, and XBB.1.5 were obtained from the Global Initiative on Sharing Avian Influenza Data (GISAID) [27] and aligned to ensure that the target peptides were conserved (sequences not shown).

The expression of spike protein was accurately quantified by IDMS. β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were simultaneously quantified in the same analytical run. This allowed normalization of the results based on the number of cells. The housekeeping genes are proportionate to the number of cells in a sample and thus, can be used as an alternative to cell counting [28,29]. This method's accuracy requires

the complete digestion of the proteins of interest in the region of the target peptides. In 6-well plates, cell culture produces many cellular proteins beyond the ones quantified in this method. We determined that the increased amount of protein in the sample would require parameters, such as trypsin concentration or the duration of the digest, to be adjusted. To avoid changes to our digestion parameters and to achieve complete digestion of the target proteins, the sample was split into two 1.5 mL Protein LoBind® Eppendorf tubes prior to protein precipitation. Each resulting sample was treated independently throughout the remaining process. This ensured that no sample was lost and that the digestion parameters were suitable for the amount of protein in the sample. Following IDMS analysis, data from the two corresponding IDMS results were combined to report the results of protein expression from that well.

In previous stability experiments, cells were harvested after 48 h because cells harvested after 24 h have been shown to have significantly lower amounts of spike protein [22]. While the IDMS method is sensitive enough to quantify spike protein after 24 h, the incubation time was extended to increase the amount of protein expressed. That allowed the forced degradation experiments to be adequately characterized. Allowing cells to grow to 72 h resulted in cell overgrowth and cell death. Floating cell debris was visibly observed and a reduction of β-actin and GAPDH was quantified by IDMS. Thus, cells that might have expressed spike protein were lost before they could be analyzed. The authors expected that concentrations of β-actin and GAPDH would remain constant across all experiments that were harvested at the same time. Especially if all the wells were seeded with the same initial number of cells and if the cells grew and multiplied at the same rate.

We first demonstrated the precision of the method under conditions in which the minimal number of variables associated with cell culture is required. Vaccine from three separate vials of the same manufacturing lot was transfected in triplicate into cells that were of the same passage. Fig. 1 shows the repeatability of this analytical method within a cell passage and within the same lot of vaccine. The relative standard deviation (RSD) was as low as 3 % for cells that were transfected with 10 μg of vaccine. The RSD did not exceed 12 % for any vaccine concentration. This figure also shows the linearity of the results as increasing amounts of vaccine were used for cell transfection. The limit of quantitation (LOQ) of the method is 1 pmol and thus, this experiment demonstrates that spike protein expression could be quantified from as little as 10 μg of this mRNA vaccine.

A challenge arises because cell culture variables, such as how many times the cells have been passaged may affect the results of experiments that are conducted over a period of weeks. This is where it is crucial to use HK proteins to normalize any variability that might be occurring during the cell culture process. The assumption would be that cells harvested at the same time (i.e., 48 h) should have the same amount of housekeeping protein if the cell growth rate is the same among all passages. Thus, these proteins serve to normalize variations that may occur in the cell culture growth and maintenance. In previous work, it was shown that there is repeatable expression between cell passages when normalized to housekeeping proteins [22]. While both β-actin and GAPDH can be used for normalization, GAPDH is present in lower abundance than β-actin and would not require the second, higher

concentration calibration curve. Thus, for these experiments where the expressed antigen is at lower concentrations, analyzing only GAPDH was sufficient.

The vaccine available for this analytical method was expired and slated for disposal. The absolute amount of antigen expressed would likely be greater from material that was not expired, but this material was useful for demonstrating trends in degradation studies. Our lab has previously performed degradation studies to demonstrate an alternative potency assay for influenza vaccine material [30,31]. The vaccine was received and stored at manufacturer-specified conditions until ready for use. No attempt was made to quantify mRNA or evaluate the quality of the mRNA prior to transfection experiments. The purpose of this experiment was to evaluate antigen expression and not the integrity or composition of the vaccine material itself.

Each week, the vaccine material was removed from its respective storage condition, pooled in a 15 mL tube and cell transfections were performed. The pooled sample was then split into 1 mL volumes and placed into 2 mL Protein LoBind® Eppendorf tubes. These tubes ensure that no protein is retained on the walls of the tube and its snap cap ensures that there is no samples loss due to evaporation. Fig. 2A depicts the absolute quantitation results of expressed spike protein, β-actin, and GAPDH of vaccine material stored at room temperature over the course of 5 weeks. The amount of expressed spike protein decreases as the number of weeks increase. It was not clear whether it was the mRNA or the vaccine delivery system that was degrading under this storage condition, resulting in the decreased expression of the antigen. The amount of β-actin and GAPDH remained relatively consistent over the 5 weeks, indicating that the cell culture conditions were stable and that the decrease in spike protein expression was due solely to the vaccine. To report the amount of protein expressed, variation in the number of cells present in each sample must be considered as this experiment required multiple cell passages. For example, in week 1, it appears that spike protein expression dropped significantly. This data taken alone might result in one concluding that the vaccine degrades rapidly at room temperature. However, at week 1, both GAPDH and β-actin were also present at low amounts indicating the number of cells analyzed in that experiment had decreased. Thus, both spike protein and house-keeping proteins must be monitored for accurate quantitation of spike protein expression. Normalization of the data was previously discussed in detail [22]. Normalization with either β-actin or GAPDH is performed by taking the average of all the β-actin values or all the GAPDH values, respectively. The individual housekeeping values of each sample were then divided by the respective housekeeping average, resulting in the normalization factor for that sample. The value for the expressed spike protein was then divided by the normalization factor for either β-actin or GAPDH, resulting in the normalized value of expressed spike protein for that sample. The normalized data of the room temperature condition are overlaid with the absolute amount of spike protein quantified in Fig. 2B. The normalized data demonstrates the need to use house-keeping proteins to account for fluctuations in cell propagation. As expected, a decrease in the expression of spike protein was observed over the weeks that the vaccine was subjected to room temperature conditions.

Fig. 3A shows the normalized spike protein data for vaccine stored at 4 °C. Per manufacturer-approved storage and handling, some mRNA vaccines are thawed and can be

refrigerated for a period of several weeks prior to vaccine administration to individuals. This experiment reflects this storage condition scenario. The data were normalized to GAPDH. Notably, the results were the same when the data were normalized to β-actin (data not shown). While a decreasing trend was observed in the amount of antigen expressed over the 5-week period, it was not as pronounced as when the vaccine was stored at room temperature.

Fig. 3B shows the data from cells that were transfected with vaccine stored at −25 °C and subjected to consecutive freeze-thaw cycles during the 5 weeks. These data were also normalized to GAPDH. The trend shows a decrease in spike protein expression resulting from consecutive freeze-thaw cycles, but it is not as pronounced as the decrease observed when the vaccine is stored at room temperature. Thus, it can be concluded that the vaccine was more stable after consecutive freeze-thaw cycles than it was being continuously stored at room temperature. Such data could be useful when evaluating storage procedures for vaccines in development.

A forced degradation study was performed to emulate a situation in which a refrigeration system failed during transport and vaccine was subjected to elevated temperatures. In this study, 56 °C was used to represent the vaccine being left in a hot vehicle during transport. Previously, this temperature condition was used to demonstrate the loss of potency of influenza vaccines under thermal stress [30,31]. By week 1, the spike protein expression of the material stored at 56 °C was undetectable. To properly observe the degradation of the vaccine, a shorter time study was performed. Since it was anticipated that the spike protein expression would decrease rapidly once exposed to the high temperature, the concentration of vaccine was increased to 100 μg to ensure that the signal would be above the LOQ for several time points. In this study, five 1-mL aliquots of vaccine were placed in a heat block held at 56 °C. The aliquots were removed from the elevated temperature after 1 h, 4 h, 8 h, 24 h, and 63 h, respectively. The IDMS results normalized to GAPDH are shown in Fig. 4A. A significant decrease in spike protein expression was observed as the time of exposure to the elevated temperature increased. This experiment was performed in duplicate. A Western blot analysis was performed on the replicate set of cells transfected with these vaccine materials. The Western blot image is presented in Fig. 4B. These results show that the vaccine antigen expression is significantly decreased at higher temperatures and even brief exposure could have detrimental effects on the vaccine’s efficacy.

Currently, available COVID-19 vaccines have two expiration dates. The first is the date in which the vaccine must be discarded, even if the material was never removed from the manufacturer’s specified cold storage condition (e.g., −70 °C, −20 °C, etc.). This expiration date is based on the date of manufacture and will be referred to, herein, as the manufacture expiration date (MED). Once removed from the low temperature storage conditions, the vaccine is considered stable in the refrigerator for a manufacturer’s specified amount of time (e.g., 3 days, 4 weeks, 10 weeks, etc.). This will be referred to as the beyond-use date (BUD). The vaccine used for all the previous studies discussed in this manuscript remained at the temperature storage conditions but exceeded the MED. Vaccine material from the same manufacturer was obtained. The new material was from a different lot number and the MED had not passed. However, this material had been removed from the freezer, thawed,

and stored at 4 °C until it was considered past the BUD. Cells were transfected with 50 μg of a vaccine in which the MED had passed, but it had never been thawed prior to analysis. This sample is represented as MED(X)_BUD (0) where the X indicates which expiration date has passed. Cells were also transfected with 50 μg of a vaccine that had not passed the MED but had been thawed. These were represented by MED (0)_BUD(X). Fig. 5A compares the amount of spike protein that was expressed from both vaccine materials. Similarly, cells were transfected with 50 μg of a MED sample that had been incubating at 4 °C for the same length of time as the BUD sample. Fig. 5B shows the amount of spike protein expressed from MED(X)_BUD(X) and MED (0)_BUD(X). Without information on how much spike protein would be expressed from an unexpired vaccine, conclusions cannot be reached as to whether the vaccine retained its full potency even when it has reached the MED but continued to be stored under those specified conditions. However, it is clear from Fig. 3A that storage at 4 °C had a significant impact on the antigen production, and that in Fig. 5 the BUD had a more significant effect on protein expression than the MED.

## 4. Conclusions

The evolving vaccine landscape includes mRNA vaccines, self-amplifying mRNA (smRNA) vaccines [32,33], and both replicating and non-replicating viral vector vaccines [34,35]. These vaccine platforms are also being investigated for protection against diseases other than COVID-19 [36–39]. In addition, there is interest in developing multicistronic vaccines that are intended to produce more than one protective antigen [40,41]. As these new vaccine platforms become more prevalent, the methods used to evaluate them should be updated. We present a method that quantifies proteins expressed from an expired commercial mRNA COVID-19 vaccine. The method is well-suited to be adapted to different vaccine platforms and modified for vaccines of different diseases. Modifications in the mRNA construct or changes in the vaccine delivery system would likely affect antigen expression. New candidate vaccines that incorporate these changes in order to produce a more effective or stable vaccine could be evaluated using this method prior to *in vivo* studies. This analytical method can accurately quantify the amount of antigen or multiple antigens produced in cell culture and may aid in the development and assessment of an important new class of vaccines.

This manuscript presents an antibody-free method to evaluate vaccine stability. Antibody-based analytical methods require an extensive amount of method and antibody validation. These methods require modification when the antigen changes and need to be updated for each new variant of disease. In addition, these antibody-based methods are limited in their ability to accurately quantify the protein expression of multiple proteins from cell culture and in their ability to correct for cell counts by including housekeeping proteins. Mass spectrometry, however, can simultaneously quantify many proteins in one analytical run.

The stability experiments presented are those in which storage conditions were shown to affect protein expression. An expired commercial COVID-19 vaccine was used to demonstrate the capability of the analytical method to accurately monitor how temperature changes can affect protein expression over time. This development of an *in vitro* model and the capability of extracting quantitative results that are accurate, precise, and repeatable

requires understanding of where potential sample losses can occur throughout the entire analytical workflow. The required cell culture translates into a lengthy (~3 day) analysis time. However, throughput can be increased by automating many of the downstream sample preparation steps with the use of a liquid handler. This manuscript also describes how to quantify and use housekeeping proteins in a mass spectrometric assay to quantify protein expression from these newest vaccines in which the cellular functions of the host are used to produce the antigen.

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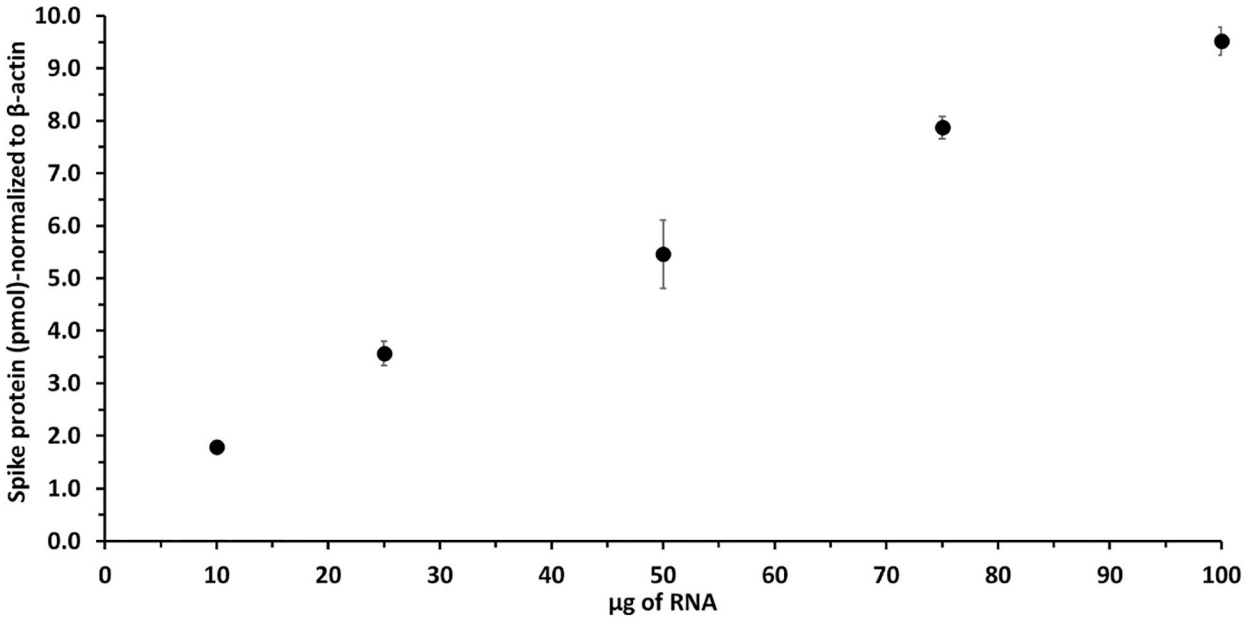


**Fig. 1.**

Triplicate transfections were performed with three separate vials of the same manufacturing lot of expired vaccine. Concentrations ranged from 10 µg to 100 µg. Spike protein expression increased as the concentration of vaccine material increased. Repeatability of the experiment is demonstrated as the RSD was less than 12 % for transfections at each vaccine concentration.

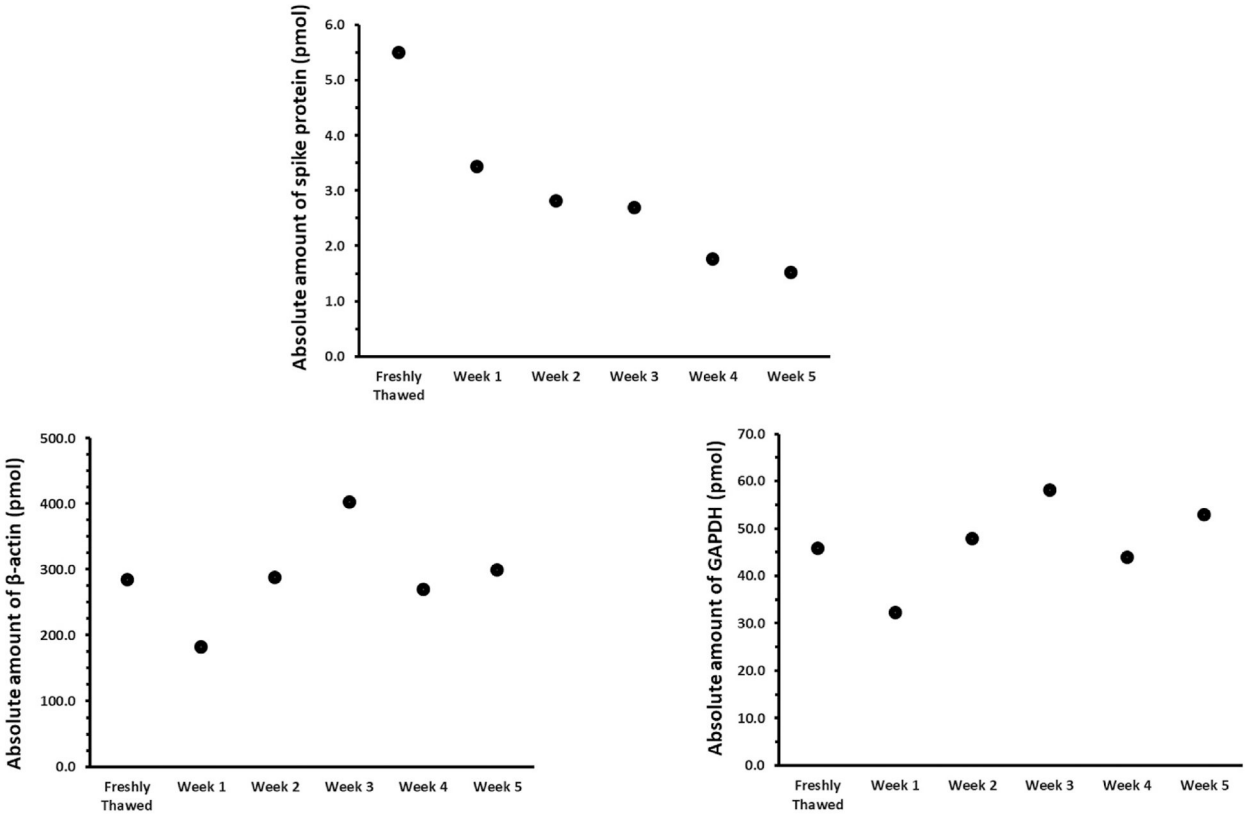


**Fig. 2A.**

The absolute amount in pmol of spike protein (top), β-actin (bottom left) and GAPDH (bottom right) for BHK-21 cells transfected with 50 μg of expired vaccine material stored at room temperature.

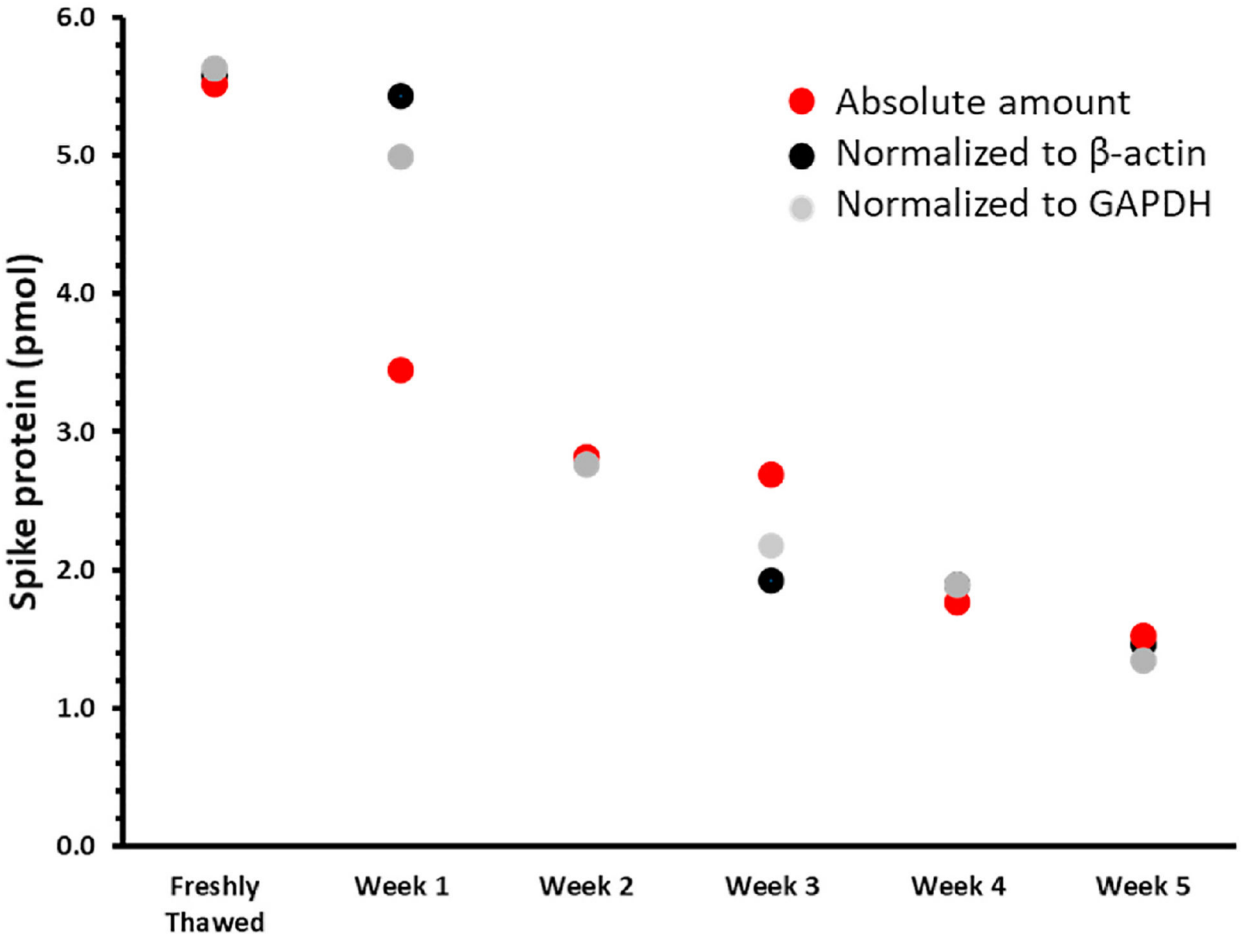


**Fig. 2B.**

The amount of spike protein expressed from cells transfected with 50 μg of expired vaccine that had been stored at room temperature is shown both as absolute amount (●) and normalized to β-actin (●) and GAPDH (●).

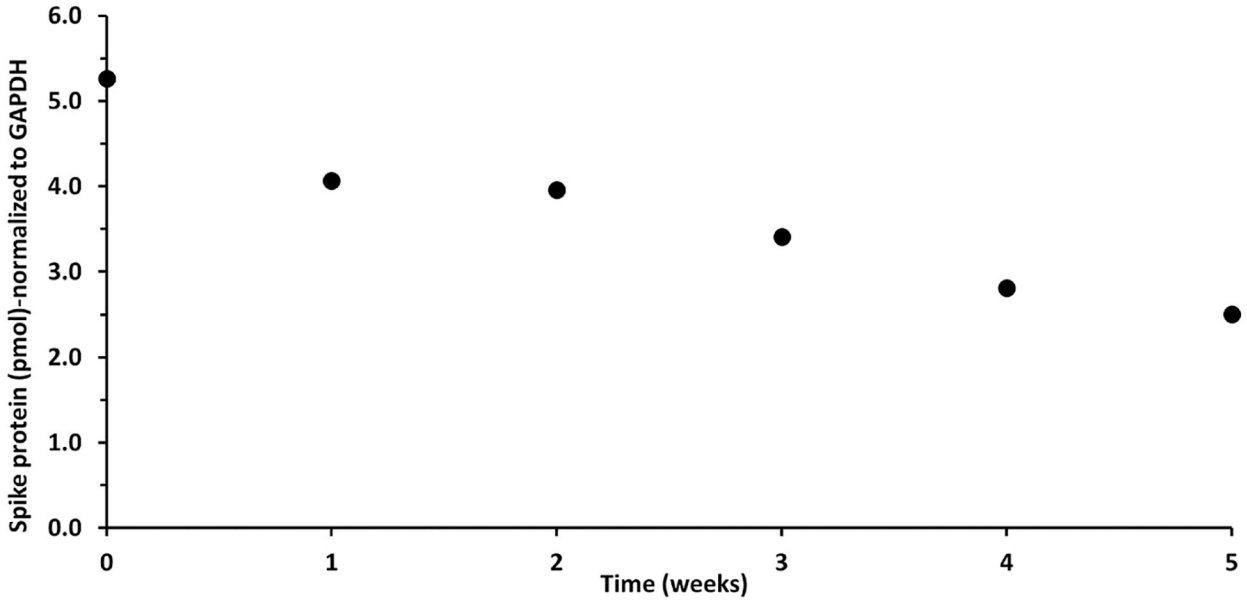


**Fig. 3A.**

The expression of spike protein normalized to GAPDH from BHK-21 cells transfected with 50 μg of vaccine material stored at 4 °C.

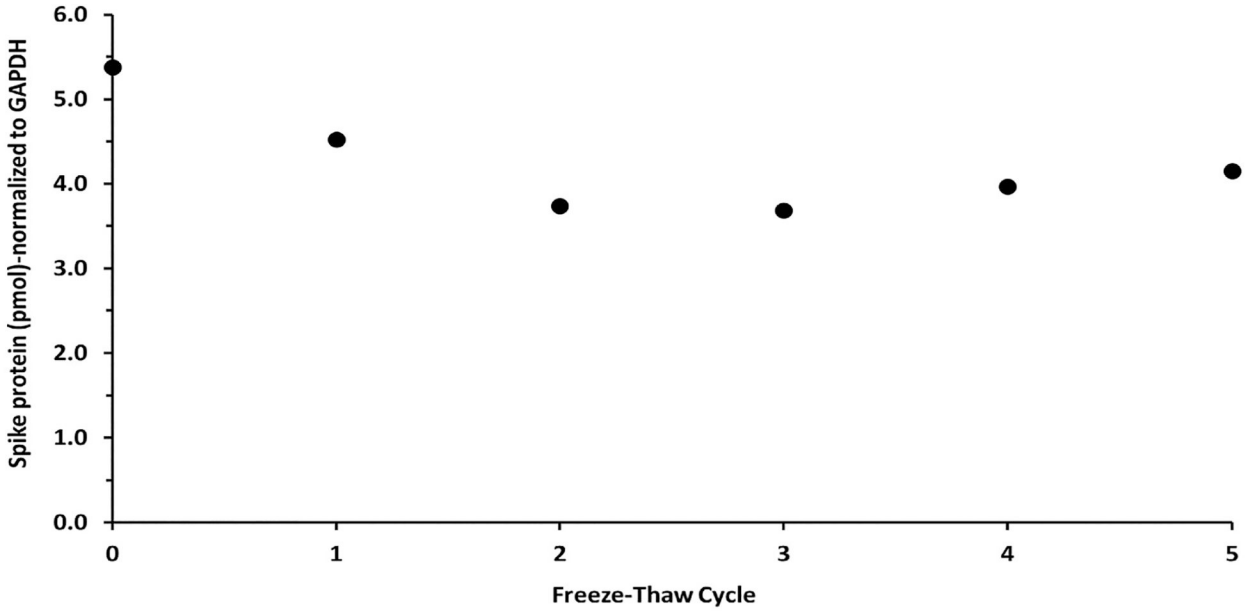


**Fig. 3B.**

Five consecutive freeze/thaw cycles were evaluated over a period of five weeks. 50 μg of vaccine material was transfected into BHK-21 cells and the amount of expression of spike protein measured.

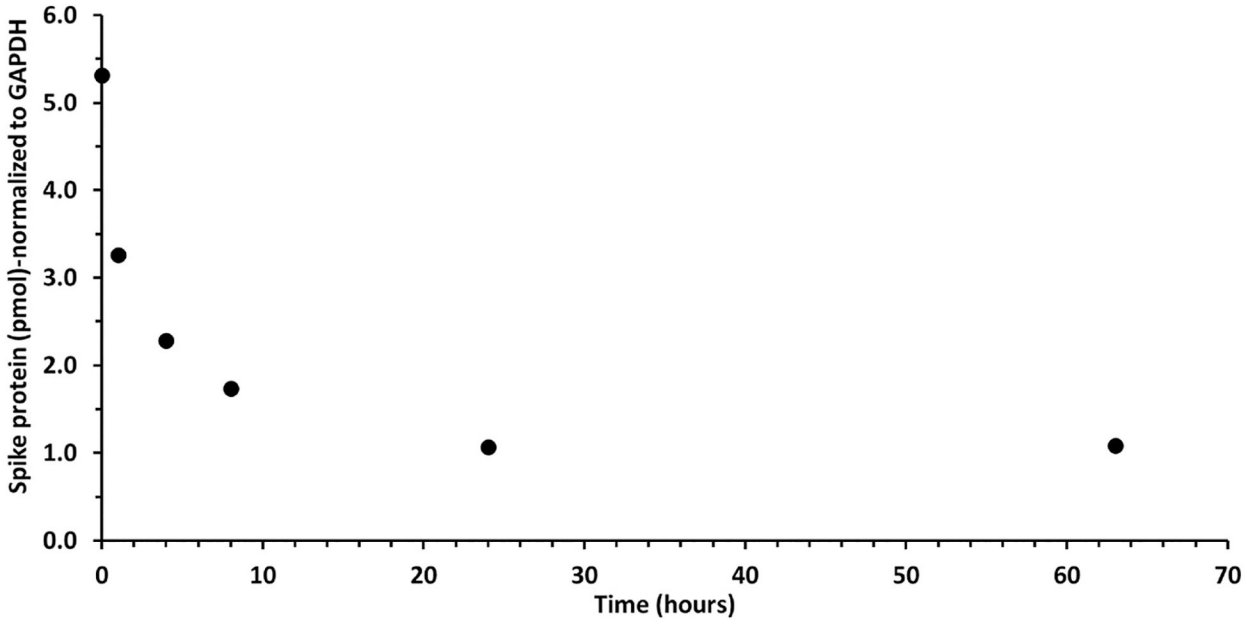


**Fig. 4A.**

The expression of spike protein normalized to GAPDH from BHK-21 cells transfected with 100 μg of vaccine material stored at 56 °C.

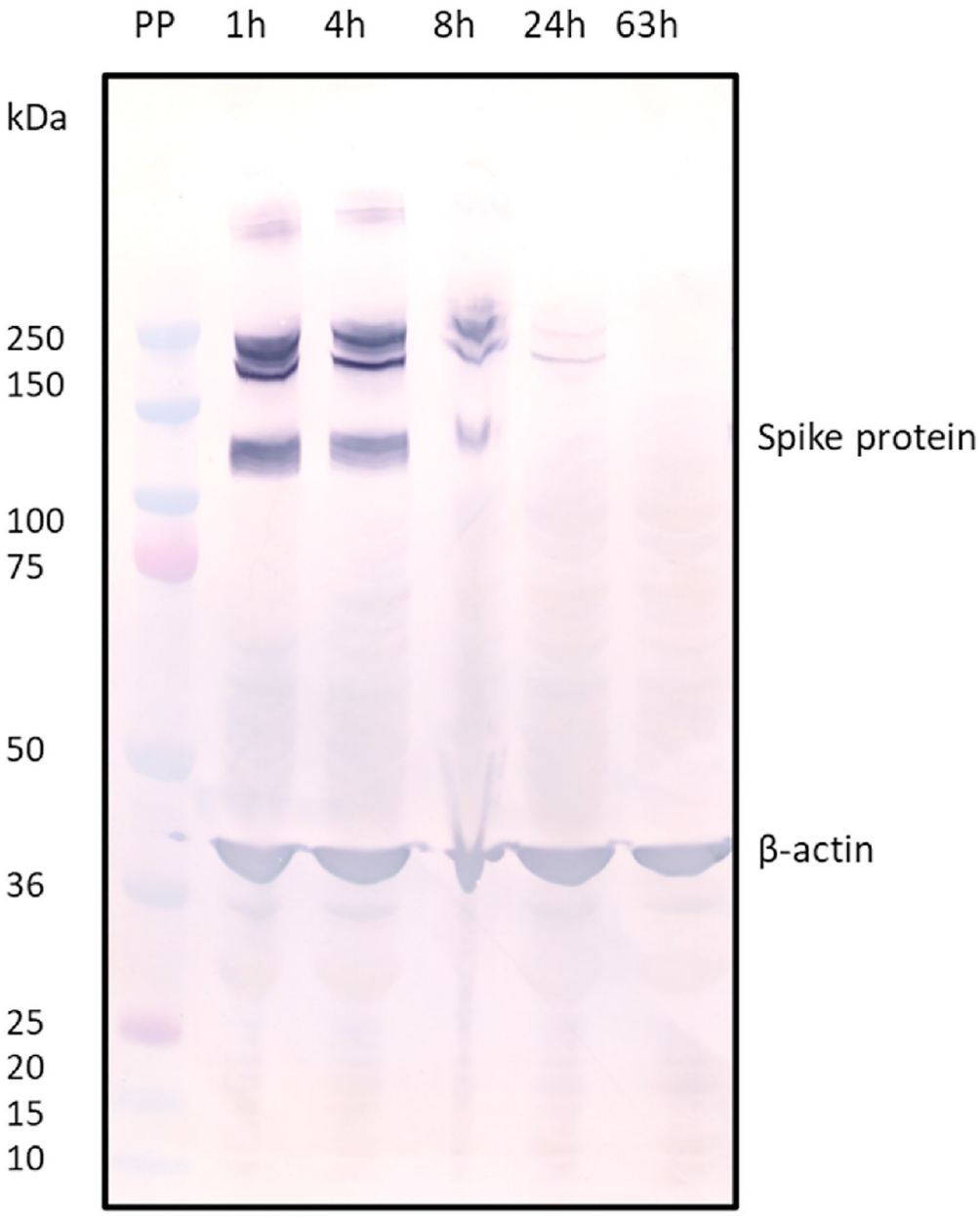


**Fig. 4B.**

Western blot analysis confirms the decrease in spike protein expression observed when BHK-21 cells are transfected with 100 μg of vaccine material stored at 56 °C for extended periods of time. The blot was also treated with anti- β-actin. The β-actin bands are observed and verify equal loading of the gel.

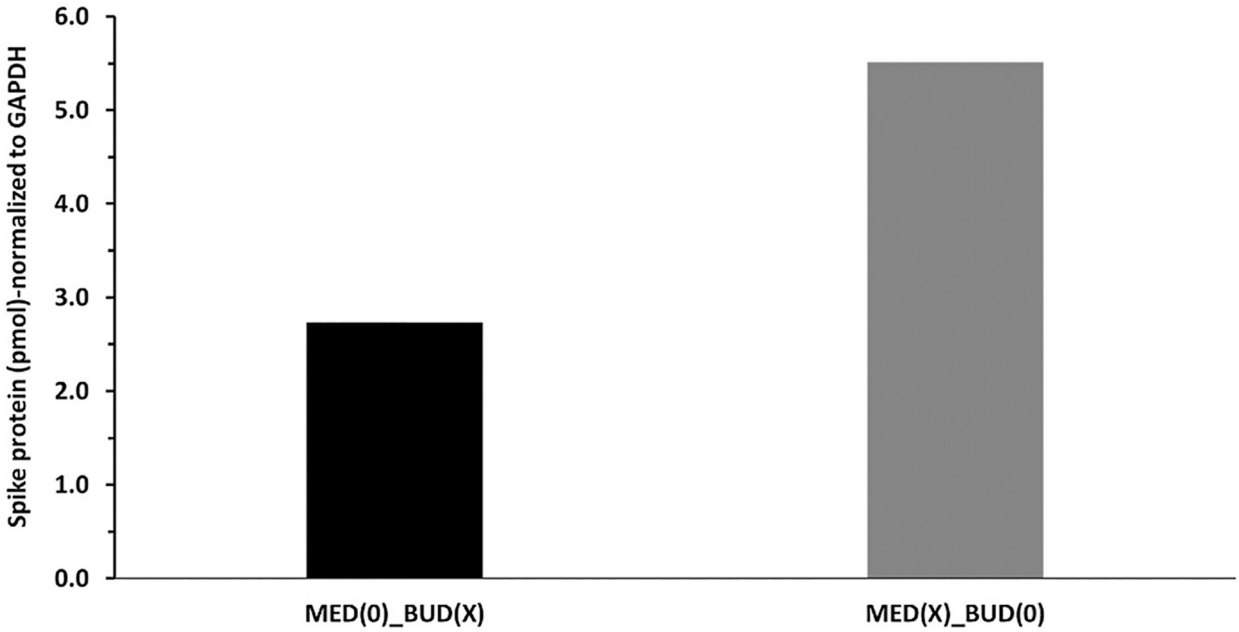


**Fig. 5A.**

A comparison of the expression of spike protein normalized to GAPDH from BHK-21 cells transfected with expired vaccine where the X indicates which expiration date has passed. Spike protein expressed from cells that were transfected with 50 μg of vaccine that had not exceeded the manufacture expiration date (MED) but had been thawed and was past the beyond-use date (BUD), MED (0)_BUD(X) was compared to the amount of spike protein expressed from cells transfected with vaccine material that had passed the manufacture expiration date but had not been thawed, MED(X)_BUD (0).

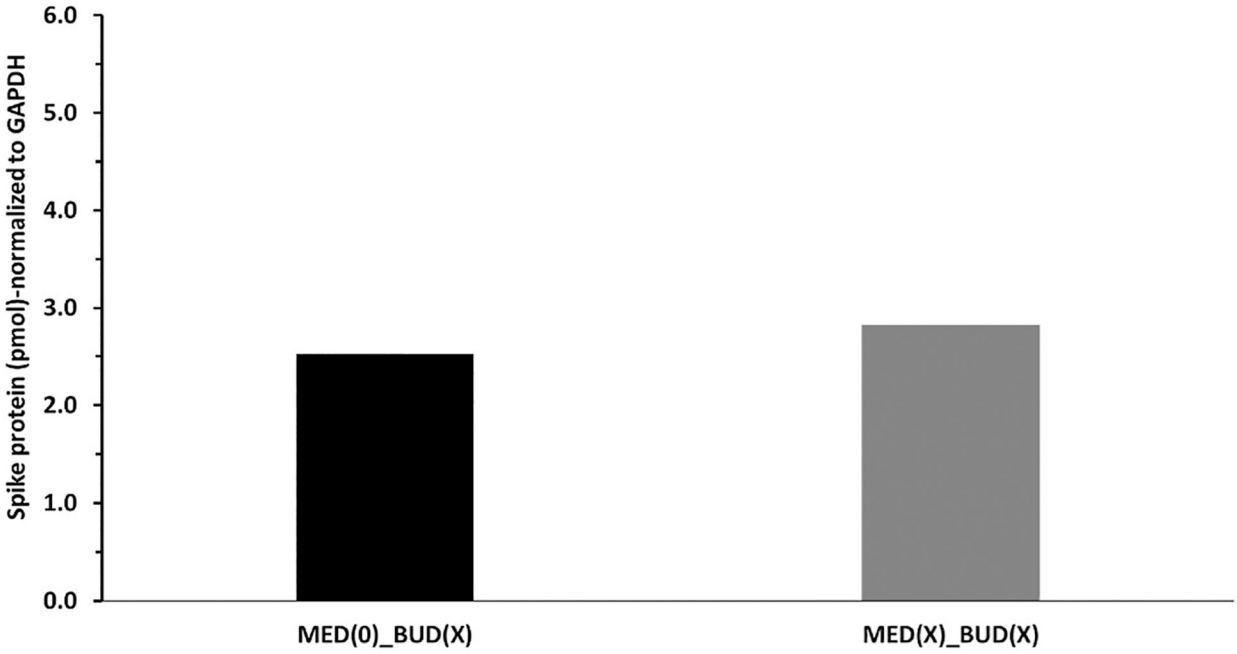


**Fig. 5B.**

A comparison of the expression of spike protein normalized to GAPDH from BHK-21 cells transfected with expired vaccine where the X indicates which expiration date has passed. Spike protein expressed from cells that were transfected with 50 μg of vaccine that had not exceeded the manufacture expiration date (MED) but had been thawed and was past the beyond-use date (BUD), MED (0)_BUD(X), was compared to the amount of protein expressed from cells transfected with vaccine material that had exceeded the manufacture expiration date and incubated at 4 °C for the same duration so that the BUD had also been exceeded, MED(X)_BUD(X).