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Cell-culture growth conditions resulting in the oxidation of a recombinant antigen-binding fragment

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Abstract

Use of Quality-by-Design (QbD) tools is becoming an important part of the bioprocessing industry when developing a process for manufacturing operations to ensure the robustness and reproducibility of the biologic product. In the present study, a QbD tool, Design of Experiments (DOE), was utilized to optimize a bioprocess for the production of a CHO recombinant antigen-binding fragment (rFab) in small-scale bioreactors. DOE studies evaluated percent dissolved oxygen, temperature, and feeding strategy specific to this Chinese Hamster Ovary (CHO) clone. It was determined that these factors influenced cell viability, yield of the recombinant protein, and metabolic byproduct formation. To ensure the quality of the target molecule in the cell-culture process, small-scale purifications and analytical evaluation of the target molecule were completed prior to cell-culture scale-up to ensure that oxidation of the rFab, presence of free light chain, and truncation of thiol group were not observed. Analysis of the purified rFab by mass spectrometry indicated that rFab oxidation occurred under poor cell-culture conditions. PCR profile array results also revealed increased transcription of the oxidative genes Superoxide Dismutase 3, Myeloperoxidase, Dual Oxidase Like 2, Nuclear Receptor Coactivator 7, NADPH Oxidase Organizer 1, Mitochondria Uncouple Protein 3, Eosinophil Peroxidase, Lactoperoxidase Like, Serum Albumin Like, and Glutathione *S*-Transferase Pi 1 in this CHO strain. The present study suggests a mechanism and pathway for the oxidation of an rFab molecule during cell-culture bioprocess optimization. The present study also demonstrated the importance of utilizing the QbD tool of DOE to optimize the cell-culture bioprocess prior to scaling up into the large-scale production bioreactor.

Keywords: Quality by Design (Qbd), Design of Experiment (DOE), Recombinant antigen-binding fragment (rFab), Protein oxidation

Introduction

Use of Quality-by-Design (QbD) tools leads to the identification of both critical process parameters (CPPs) and critical quality attributes (CQAs) and is becoming an important part of the bioprocessing industry when developing and executing a process in manufacturing operations (Nagashima et al. 2013; Torkashvand and Vaziri 2017; Lipsitz et al. 2016; Horvath et al. 2010). To comply with FDA regulations, QbD tools are gaining wider acceptance and are used to establish quality along with maintaining the quality of a product between batches resulting in reduced manufacturing process variability. Application of QbD tools has been documented in the development and manufacture of recombinant proteins, monoclonal antibodies (mAbs) and antigen-binding fragments (Fabs) (Nagashima et al. 2013; Torkashvand and Vaziri 2017; Lipsitz et al. 2016; Horvath et al. 2010). Each of these molecules present different challenges to achieve the desired level of quality and maintain the level of quality required for the downstream application of the target protein. For example, Fabs are affected to a lesser extent than a mAb to proteolytic cleavage due to their small size and ability to be expressed in both mammalian

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and bacterial systems, but suffer when over expressed resulting in aggregation leading to lower product quality (Karu et al. 1995; Nelson and Reichert 2009). In addition to aggregation, both molecules are subject to oxidation, another major concern for maintaining the quality of the product (Karu et al. 1995; Nelson and Reichert 2009; Yan et al. 2009; Moritz and Stracke 2017).

Oxidation of protein molecules has been shown to occur during extended cell-culture bioprocessing along with under storage and triggered by oxidative stress (Yan et al. 2009; Moritz and Stracke 2017). During cell culture, oxidative stress has been associated with high rates of aeration, mixing speeds, temperature, osmolality, pH, and overexpression of the target protein resulting in the production of reactive oxygen species (ROS) inside the mammalian cells (Filomeni et al. 2015; Schieber and Chandel 2014). Common reactive oxygen species are hydrogen peroxide, superoxide radicals, peroxynitrite and hydroxide radicals. The presence of these ROSs results in DNA damage and oxidation of the expressed target molecule. It has been reported that there are several enzymes associated with generation of ROSs, such as NAPDH oxidases (NOXs) and dual oxidase (DUOX), that are highly expressed in cells under oxidative stress (Filomeni et al. 2015; Schieber and Chandel 2014).

In this study, we describe the development of a Chinese Hamster Ovary (CHO) expressed rFab cell-culture bioprocess leading to a purified molecule that is used in a diagnostic product. During bioprocess development, we applied the QbD tool of DOE to identify the production CPPs. DOE study results identified those cell-culture process parameters that effected the quality of the purified rFab. The data presented in this study demonstrate that growth temperature and percent dissolved oxygen (DO) caused physiological changes resulting in the oxidation of the target molecule. Upon optimization at small-scale and following scale-up to production, the developed CHO bioprocess produced the target molecule free of oxidation.

The present study emphasized the importance of utilizing the QbD tool of DOE to reveal the process parameters that influence the quality of a recombinant protein. In addition, the present study suggests a possible mechanism and pathway for the oxidation of a recombinant Fab molecule under non-optimal growth conditions.

Materials and methods

CHO cell culture

Chinese Hamster Ovary cells expressing the recombinant antigen-binding fragment (rFab) target molecule were grown in EX-CELL CD-CHO Fusion animal component free without L-glutamine, hypoxanthine, and thymidine medium (product number 14365C, Sigma-Aldrich, St Louis, MO). Cell-culture DOE studies were performed in 0.3 L bioreactors (Dasbox; Eppendorf AG) and scaled-up into a 5 L bioreactor (BioFlo320; Eppendorf AG) for production growths. Two supplementation strategies were evaluated during the DOE study; Strategy one fed BalanCD CHO Feed 1 (Catalog number 91127, Irvine Scientific, Santa Ana, CA) on days 2 and 4 and BalanCD CHO Feed 1and Cell Boost 5 (Catalog number SH30865, HyClone, Logan, UT) on day 6, while Strategy 2 fed BalanCD CHO Feed 1 on days 3 and 5 and BalanCD CHO Feed 1 and Cell Boost 5 on day 7. Both supplements have been shown by the vendors to increase viable cell number and enhance target molecule production. Supplement formulations are proprietary to the vendor. Proportion of supplements used, and final feeding strategy used in the final production process are proprietary to Abbott. During growth, viable cell number and viability were measured using a ViCell automated cell counter and analyzer (Beckman Coulter, CA, USA). Metabolites were analyzed using a BioProfile 100 Plus Bioanalyzer (Nova Biomedical, Waltham, MA, USA).

rFab quantitation

High-performance liquid chromatography (HPLC) was used to determine the rFab concentration from the cell-culture harvests. One hundred microliters of each standard, control of known rFab concentration, and sample were injected with phosphate buffered saline (PBS; 10 mM $NaPO_4$, 150 mM NaCl, pH 7.2) mobile phase onto a Poros G/20 column (4.6 mm D × 50 mm L Column; Life Technologies Corporation, Bedford, MA; Ref 1-5122-24) using a Waters 2695 HPLC Separation Module (Waters Corporation, Milford, MA). The rFabs were then eluted with 12 mM HCl and detected at 280 nm with a Waters 2996 HPLC PDA Detector. The concentration of rFabs in the controls and the samples was calculated based on a curve generated from the standards using Empower3 software (Water Corporation, Milford, MA).

Design of Experiments

Design of experiments studies were performed using eight small-scale Dasbox bioreactors. A DOE study evaluating a combination of three process parameters was designed using JMP statistical software (version 12, SAS institute; Table 1). The DOE data were analyzed using the custom Design-Fit least square model of the JMP software.

rFab purification

rFab cell-culture harvest (50 mL) was subjected to centrifugation at 1000 rpm for 10 min at 4 °C (JS-4.2, Beckman Coulter, Indianapolis, IN, USA) and filtration (0.2 μM capsule filter, GE Healthcare, Sweden) followed

Table 1 DOE evaluation of three cell-culture process parameters

Bioreactor	DO%	Temperature (°C)	Supplement at day
1	50	37	2,4,6
2	30	37	3,5,7
3	30	32 (day 6 shift from 37 to 32)	3,5,7
4	50	32 (day 6 shift from 37 to 32)	2,4,6
5	30	37	2,4,6
6	50	37	3,5,7
7	30	32 (day 6 shift from 37 to 32)	2,4,6
8	50	32 (day 6 shift from 37 to 32)	3,5,7

by the application of the clarified harvest onto a 1.0 mL HiTrap Protein L column (GE Healthcare; Sweden) per the manufacturer's protocol.

SDS-PAGE analysis

rFabs were reduced in sample buffer containing 2-mercaptoethanol (Bio-Rad, Hercules, CA). Two micrograms of reduced rFab were loaded in duplicate on a 10–20% TGX Criterion gel (Bio-Rad, Hercules, CA) along with single lanes of non-reduced samples and Precision Plus Dual Xtra Standards (Bio-Rad, Hercules, CA). The gel was run at 200 V using a Bio-Rad Power Pac HC System, and stained in Coomassie Blue (Bio-Rad, Hercules, CA) for 2 h followed by destaining in 50% methanol/10% acetic acid for 2 h. The gel was then transferred into 10% methanol/10% acetic acid for 18 h followed by incubation in deionized water for 30 min before imaging on the Bio-Rad GS800 Imaging Densitometer (Bio-Rad, Hercules, CA).

ESI–MS analysis

The rFab samples were reduced using *tris*(2-carboxyethyl) phosphine (TCEP, catalog number 77720, Thermo Scientific, Rockford, IL), and both reduced and non-reduced samples were desalted using Amicon® Ultra-10 K filters (Millipore, Burlington, MA). Desalted samples were analyzed using a TripleTOF® 5600 mass spectrometer (Sciex, Framingham, MA) coupled to a Eksigent MicroLC 200 HPLC (Sciex, Framingham, MA) using a C18 OPTI-TRAP™ cartridge (Optimize Technologies Inc., Oregon City, OR) (Li et al. 2017; Yan et al. 2007). The oxidative peak intensity percentage was calculated by dividing the peak intensity of the oxidized rFab at 48,844 Da by the peak intensity of rFab at 48,813 Da times 100%.

RT^2 profiler PCR array to detect gene transcription

RNA was isolated from the cell-culture samples using the RNeasy Plus mini kit (Qiagen, Hilden, Germany) per the manufacturers recommendations. cDNA was synthesized using an RT^2 First Strand Kit (Qiagen, Maryland, USA) per the manufacturers protocol. RT^2 profiler PCR array was performed per manufacturer protocol using 96-well plates containing oxidative stress genes from Chinese Hamster Ovary cells (Qiagen, Catalog number 330231 PAJJ-065ZA, Maryland, USA). SYBR Green was used as the detection probe for the RT^2 Profiler PCR array (Qiagen, Maryland, USA). After performing the RT^2 Profiler PCR array experiment, the delta C_T (ΔC_T) for each oxidative pathway-focused gene in each plate was calculated using the formula $\Delta C_T = C_T^{GOI} - C_T^{AVG\ HKG}$ (GOI is gene of interest and HKG is housekeeping genes for normalization). The Delta Delta C_T ($\Delta\Delta C_T$) method was used for quantifying RT^2 Profiler PCR Array data. To calculate the $\Delta\Delta C_T$ for each gene across RT^2 PCR profiler PCR array, the following formula for $\Delta\Delta C_T$ was used; $\Delta\Delta C_T = \Delta C_T$ (oxidative condition) $- \Delta C_T$ (non-oxidative condition). In this study, genes from non-oxidative condition were used as control.

Results

DOE optimization study

Analysis of the DOE study as outlined in Table 1 indicated that shifting the growth temperature from 37 to 32 °C at 50% DO improved both cell growth and viability. Bioreactors four and eight achieved the highest cell growth and viability when compared to the other bioreactors in this study even though they had different feeding schedules (Fig. 1a, b). The integrated viable cell density (IVCD) data indicated that bioreactors four and eight exhibited similar growth patterns achieving 69.0×10^6 cells × day/mL and 71.2×10^6 cells × day/mL, respectively (Fig. 1a). In addition, these two bioreactors also demonstrated the highest cell viability and viable cell number following 18 days of culture as compared to other bioreactors in the study (Fig. 1b, c).

The metabolic profile of each bioreactor was followed throughout the DOE study. A trend in glucose utilization was observed in all the bioreactors with all eight bioreactors consuming glucose across the 18-day culture (Fig. 1d). In addition to glucose, lactate and ammonium concentrations were also followed throughout the culturing period. Bioreactors four and eight (32 °C growth temperature at 50% DO) demonstrated the lowest concentrations of lactate and ammonium following 18 days of culture (Fig. 1e, f). The other bioreactors grown at 37 °C at either 30% or 50% DO had higher levels of lactate and ammonium upon culture termination (Fig. 1e, f). These data would suggest that bioreactors four and eight potentially utilized both lactate and ammonium to support growth toward the end of culture period.

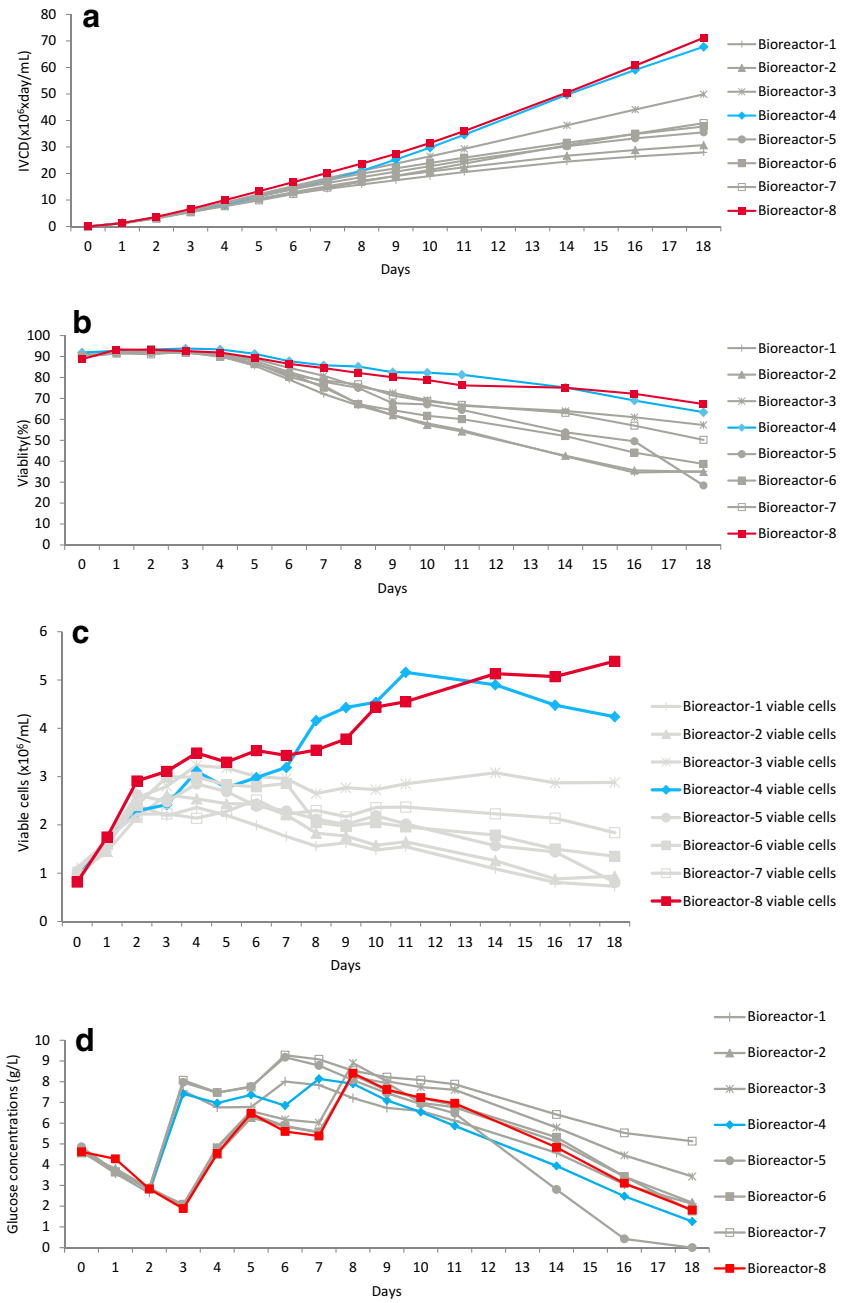


Fig. 1 **a** DOE optimization study evaluating three process parameters; integrated viable cell density of CHO cells in 0.3 L bioreactors shown over 18 days of growth. **b** DOE optimization study evaluating three process parameters; cell viability of CHO cells in 0.3 L bioreactors shown over 18 days of growth. **c** DOE optimization study evaluating three process parameters; Viable cell density of CHO cells in 0.3 L bioreactors over 18 days of growth. **d** DOE optimization study evaluating three process parameters; Glucose concentrations during growth of CHO cells in 0.3 L bioreactors shown over 18 days of growth. **e** DOE optimization study evaluating three process parameters; Lactate concentrations during growth of CHO cells in 0.3 L bioreactors shown over 18 days of growth. **f** DOE optimization study evaluating three process parameters; Ammonium concentrations during growth of CHO cells in 0.3 L bioreactors shown over 18 days of growth. **g** DOE optimization study evaluating three process parameters; rFab titer in 0.3 L bioreactors shown over 18 days of growth

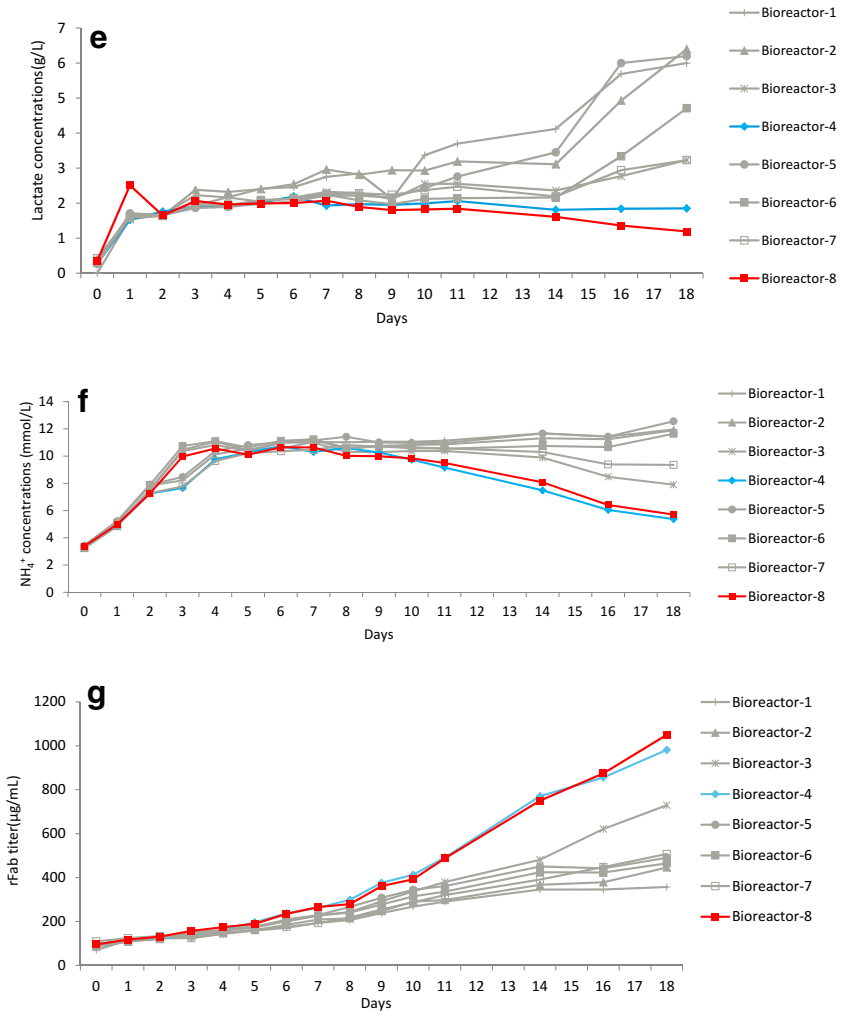


Fig. 1 (continued)

The rFab titer was followed throughout the DOE study. It was determined that both bioreactors 4 and 8 demonstrated the highest rFab titers as compared to other bioreactors in the study, 0.981 g/L and 1.05 g/L, respectively (Fig. 1g).

Utilizing JMP statistical software custom Design-Fit least square modeling, the DOE study results were analyzed. The Prob > F value (P value for the model) and the R^2 value of the model, 0.0091 and 0.929494, respectively, indicate that the design of model is significant. The strength of the model suggests that a shift in temperature from 37 to 32 °C along with an interaction between temperature and DO has a significant effect on rFab production as indicated by the probability > $|t|$ values of 0.0044 and 0.0250, respectively (Additional file 1: Fig. S1). The prediction profiler also supports the reduction in growth temperature to 32 °C along with increasing the percent DO to improve product yield (Fig. 2). The interaction between DO and temperature is further demonstrated in the interaction profiler plots, as shown in Fig. 3. Interactions between terms that are significant show up as

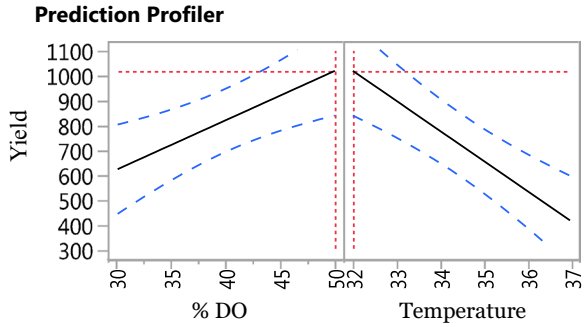


Fig. 2 Prediction profiler from DOE optimization study evaluating three process parameters indicating reduction in growth temperature to 32 °C along with increasing the percent DO improve product yield

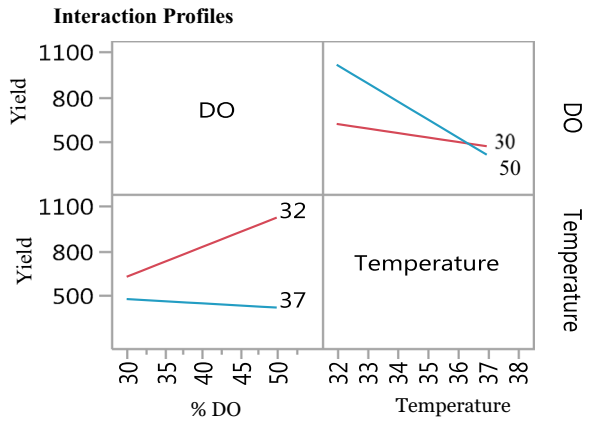


Fig. 3 Interaction profiler within design-fit model created by JMP software. Interactions between terms show up as crossed lines or lines of different slopes. No interaction is shown as parallel lines. In this figure, DO and temperature interact as indicated by lines of different slopes and lines that will cross each other

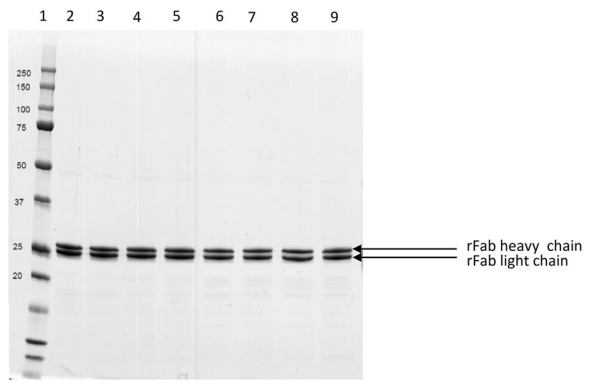


Fig. 4 SDS-PAGE analysis of rFabs from each of the eight bioreactors in the DOE optimization study. The SDS-PAGE samples were collected at day 18 of the cell culture. Lane 1 shows the molecular weight markers. Lanes 2 through 9 represent bioreactors 1 through 8

crossed lines or lines of different slopes, confirming that 50% DO and 32 °C temperature have significant impact on yield (Fig. 3).

Analysis of rFab quality

The target molecule from each of the DOE bioreactors was purified and the quality assessed by SDS-PAGE/densitometry and mass spectrometry. Reduced SDS-PAGE analysis demonstrated two distinct bands, heavy and light chains, with a purity as determined by densitometry of greater than 95% for all samples (Fig. 4). Non-reduced SDS-PAGE analysis demonstrated one band of the correct molecular weight, 48.8 kD (Additional file 1: Fig. S2).

ESI mass spectrometry analysis was performed under reduced and non-reduced conditions (structural detail presented in Additional file 2: Table S1), and demonstrated oxidized target molecules in bioreactors 1, 2, 3, 5, 6, and 7. The ESI–MS profiles from these bioreactors were identical, and a representative non-reduced and reduced profile is shown in Fig. 5a, b for bioreactor 1. The percent of oxidation observed in the rFab molecules from bioreactors 1, 2, 3, 5, 6, and 7 ranged from 12 to 42%, while the rFab molecules from bioreactors 4 and 8 demonstrated no oxidation (Table 2). Non-reduced and reduced ESI–MS profiles for bioreactors 4 and 8 are shown in Fig. 5c–f with no oxidation of the rFab observed in the non-reduced profiles.

Gene transcription analysis of oxidative stress pathways

RT^2 profiler PCR array was used to analyze 84 oxidative stress-related genes from Chinese Hamster Ovary cells. Two bioreactors were evaluated in this study: bioreactors 6 and 8. Bioreactor 6 was chosen due to its high rFab titer and demonstrated high oxidation of the target molecule (Fig. 1g and Table 2), while bioreactor 8 was chosen, since it demonstrated the highest titer of the bioreactors exhibiting no oxidation of rFab (Fig. 1g and Table 2). Bioreactor 8 also served as the oxidative gene control; meaning that those genes transcribed at a level greater than twofold of the control were considered as a notable change in gene transcription in this study. It was determined that 12 genes were highly transcribed under the growth conditions, where the target molecule was oxidized; transcription ranged from 2.02 to 7.04-fold over the control (Fig. 6). In addition, under the same growth conditions, two oxidative protective genes, Glutathione Synthetase (GSS) and Thyroid Peroxidase (TPO), were downregulated exhibiting transcription levels 0.426 and 0.46-fold less than control (Fig. 6).

Bioreactor scale-up

The rFab cell-culture process was scaled-up to 5 L using the growth conditions and feeding strategies utilized in bioreactor 8 of the DOE study (Table 1). The 5 L bioreactor growth pattern mimicked that observed in the small-scale DOE study for bioreactor 8 achieving an IVCD of 86.5×10^6 cells × day/mL at day 16 (Fig. 7a) while maintaining a lactate concentration below 2.0 g/L with ammonium utilized toward end of the culture (Fig. 7b). rFab was produced at a significant level achieving 1.15 g/L and 1.5 g/L of rFab at days 14 and 16, respectively (Fig. 7c). The target molecule was analyzed by mass spectrometry under both reduced and non-reduced conditions, and no oxidized rFab was detected (Fig. 8a, b).

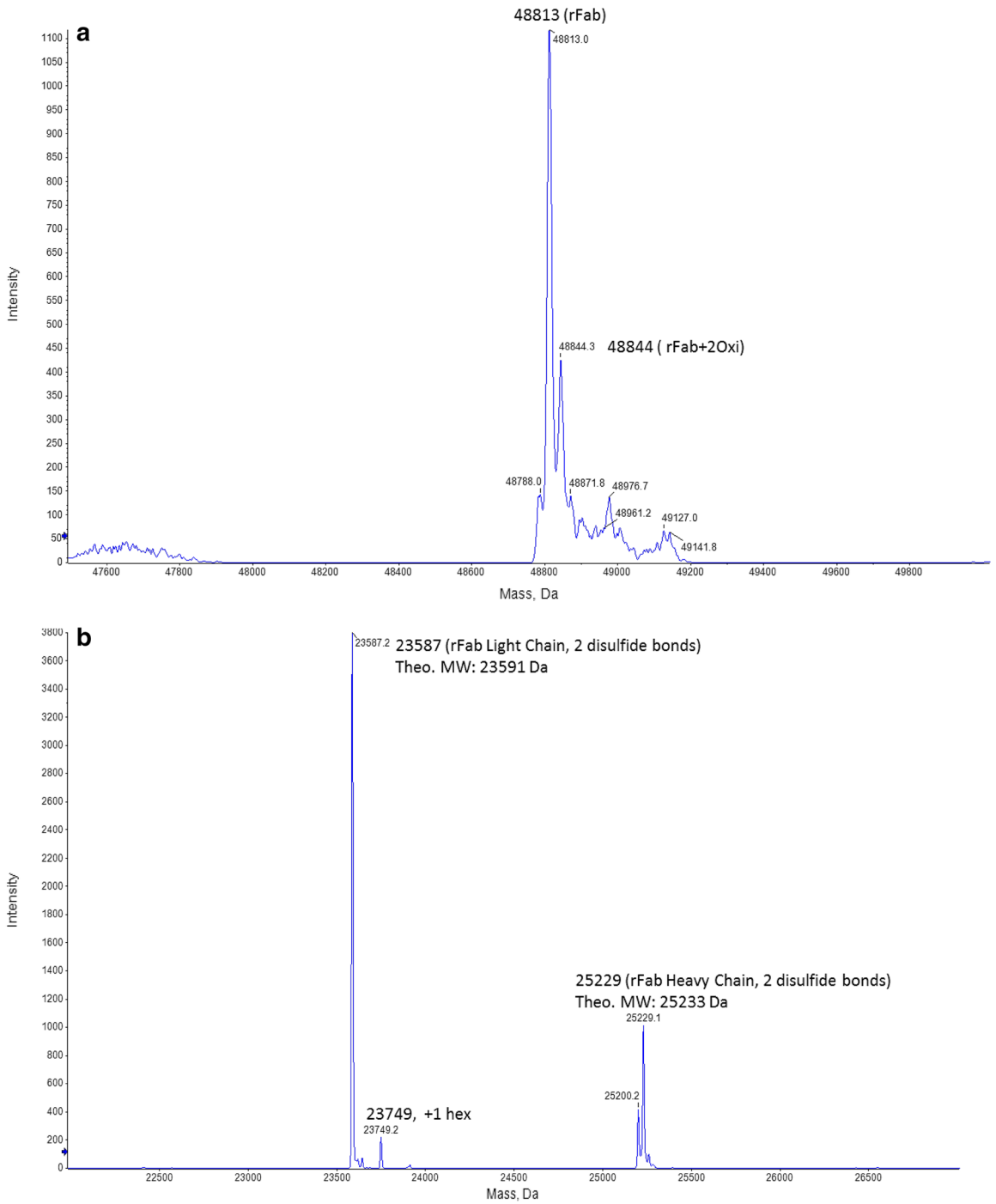
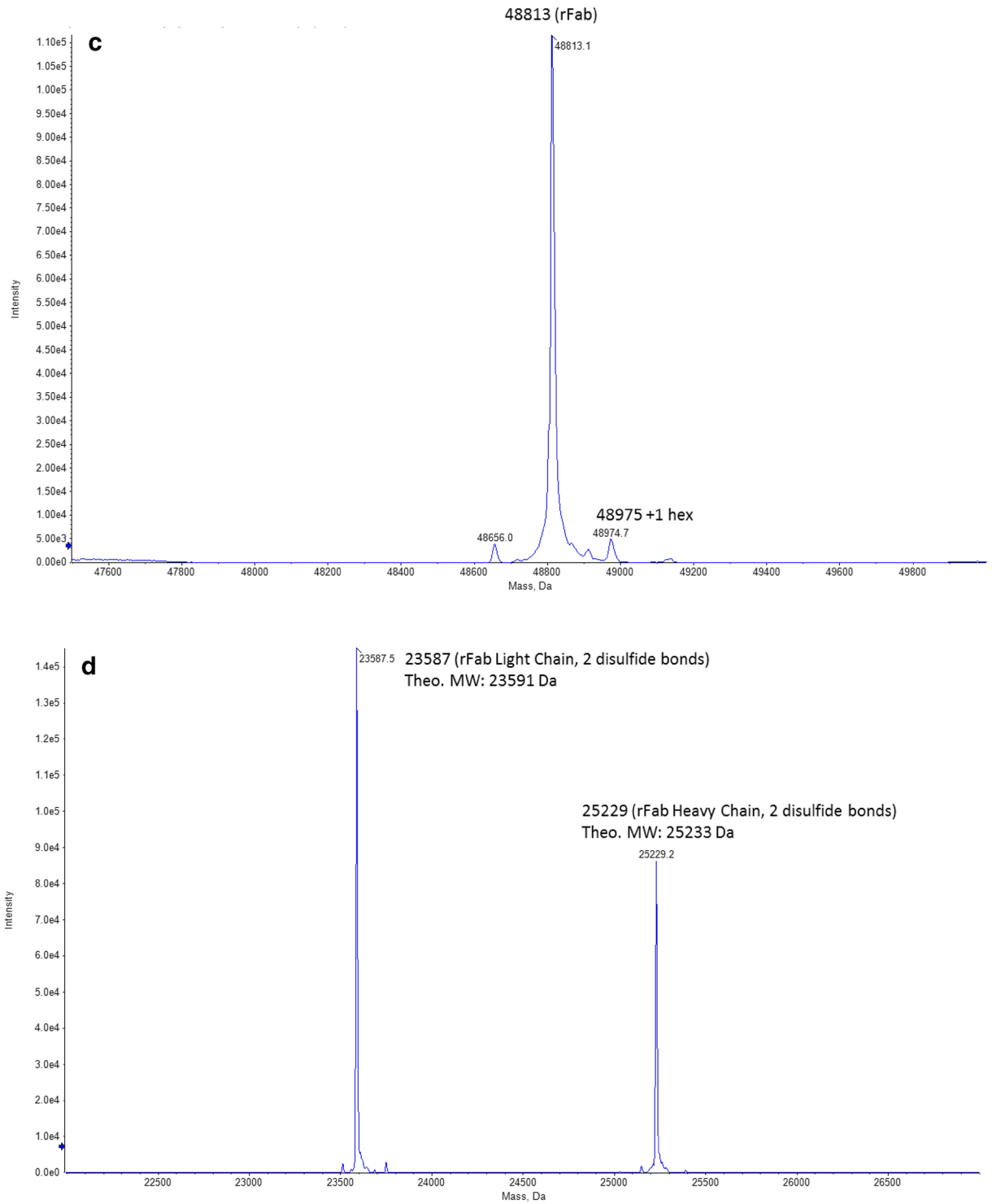


Fig. 5 **a** ESI–MS spectrum of rFab from bioreactor 1 under non-reduced condition. The MW profile of rFab was observed at 48,813 Da, and the MW profile of oxidized rFab with two oxidations was detected at 48,844 Da with 37% peak intensity percentage (Table 2). **b** ESI–MS spectrum of rFab from bioreactor 1 under TCEP-reduced condition. The MW at 23,587 Da matched with the light-chain theoretical MW with two disulfide bonds, and the MW at 25,229 Da matched with the heavy chain theoretical MW with two disulfide bonds. **c** ESI–MS spectrum of rFab from bioreactor 4 under non-reduced condition. The MW profile of rFab was observed at 48,813 Da, and no oxidized rFab was detected from bioreactor 4. **d** ESI–MS spectrum of rFab from bioreactor 4 under TCEP-reduced condition. The MW at 23,587 Da matched with the light-chain theoretical MW with two disulfide bonds, and the MW at 25,229 Da matched with the heavy chain theoretical MW with two disulfide bonds. **e** ESI–MS spectrum of rFab from bioreactor 8 under non-reduced condition. The MW profile of rFab was observed at 48,813 Da, and no oxidized rFab was detected from bioreactor 8. **f** ESI–MS spectrum of rFab from bioreactor 8 under TCEP-reduced condition. The MW at 23,587 Da matched with the light-chain theoretical MW with two disulfide bonds, and the MW at 25,229 Da matched with the heavy chain theoretical MW with two disulfide bonds



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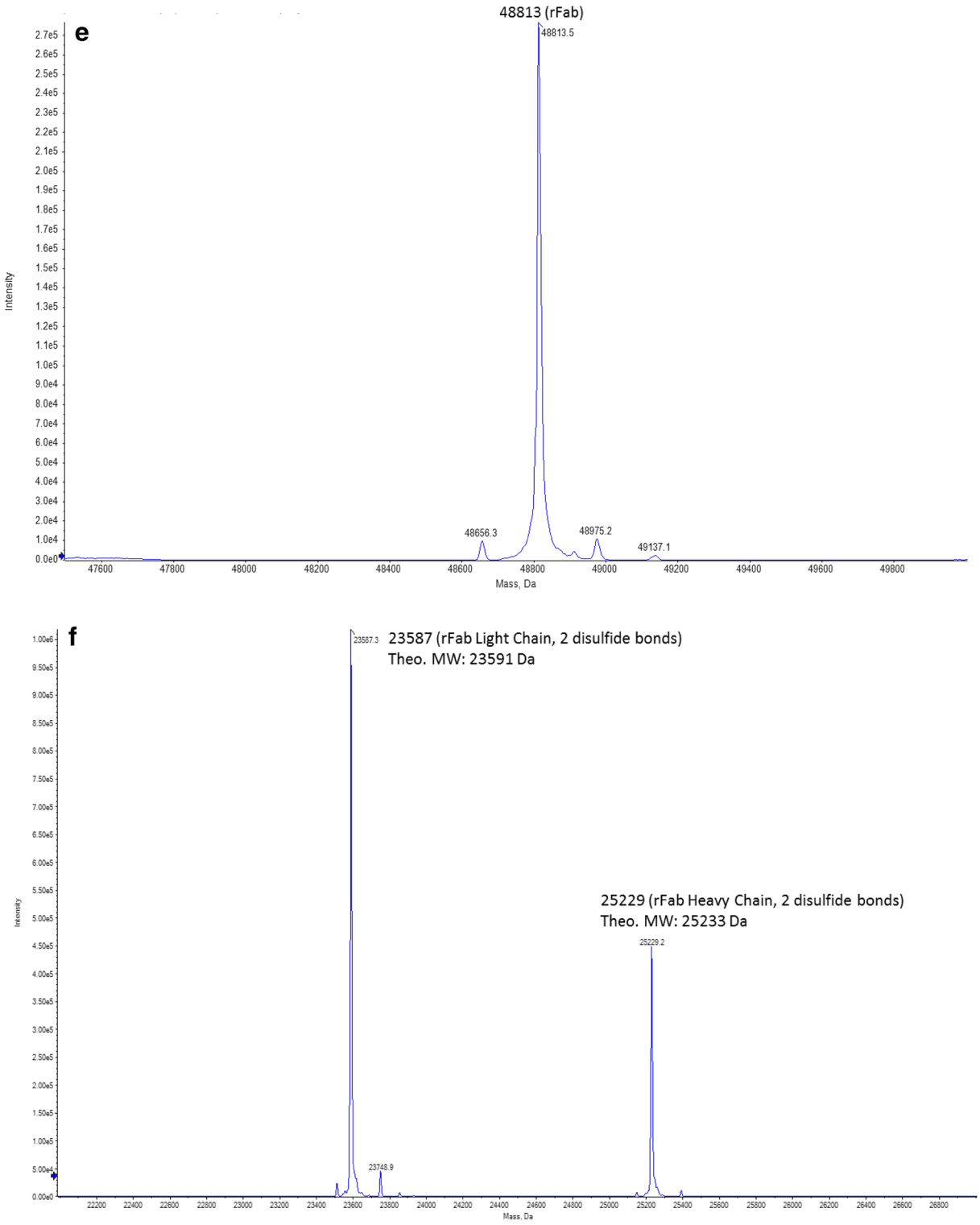

Fig. 5 (continued)

Table 2 ESI–mass spectrometry study results

Sample	Presence of oxidized molecule	Oxidative peak intensity percentage
Bioreactor-1	Yes	37%
Bioreactor-2	Yes	42%
Bioreactor-3	Yes	12%
Bioreactor-4	No	Not detected
Bioreactor-5	Yes	35%
Bioreactor-6	Yes	36%
Bioreactor-7	Yes	26%
Bioreactor-8	No	Not detected

Discussion

Reduction of temperature during production of recombinant proteins in CHO cell culture is widely practiced in the bioprocessing industry (Tait et al. 2013). Multiple studies have demonstrated that during cell culture, this downward temperature shift affects the cell cycle and transcriptome levels of protein synthesis (Tait et al. 2013; Moore et al. 1997; Oguchi et al. 2003). A temperature shift delays the onset of apoptosis, arresting cells in the G1 phase of the cell growth cycle resulting in improved cell viability and productivity of the expressed recombinant protein (Tait et al. 2013; Moore et al. 1997; Oguchi et al. 2003). In the present study, a temperature shift from 37 to 32 °C resulted in both improved cell viability and yield of the expressed rFab.

The present study also monitored the metabolism of the CHO cells during the production cycle to evaluate the effect of the temperature shift on metabolic byproduct formation, and how these metabolites affected productivity and quality of the rFab. Upon shifting the temperature at day 6, no significant difference in glucose utilization among the bioreactors was observed, and some of the bioreactors demonstrated low to no lactate accumulation. The low lactate accumulation may be due to the cells mimicking the stationary phase of the cell cycle as supported by recent studies, which have reported lactate consumption during the stationary phase in fed batch cultures (Bedoya-López et al. 2016; Trummer et al. 2006; Furukawa and Ohsuye 1998; Young 2013; Mulukutla et al. 2012; Cruza et al. 2000; Lao and Toth 1997).

In addition to lactate, ammonium is another metabolic byproduct that has a significant effect on cell growth and viability (Young 2013; Mulukutla et al. 2012; Cruza et al. 2000; Lao and Toth 1997; Yang and Butler 2000). In the present study, all bioreactors had a rapid accumulation of ammonium during the initial days of culture, but only bioreactors 4 and 8 demonstrated a significant use of ammonium towards the end of culturing. These two reactors were subjected to the temperature shift to 32 °C while maintaining DO at 50%. The utilization of ammonium by the CHO cells might be due to amino

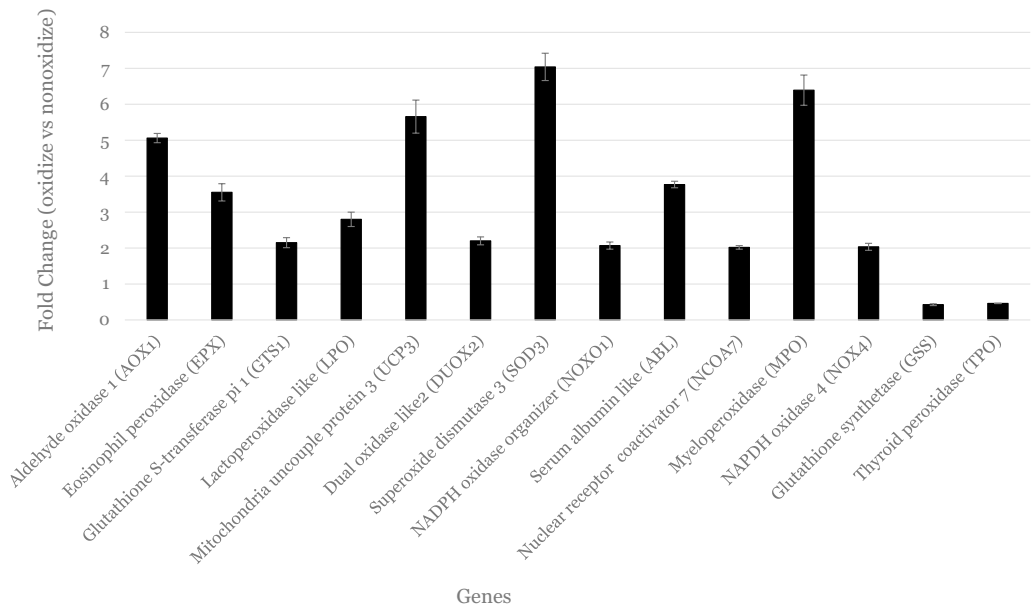

Fig. 6 Gene transcription study was performed using RT^2 Profiler PCR array. A total of 84 genes were analyzed of which 12 were highly transcribed under the growth conditions, where the target molecule was oxidized. Transcription ranged from 2.02 to 7.04-fold over the oxidative gene control, bioreactor 8, which demonstrated no oxidation of the rFab molecule. Under similar growth conditions two oxidative protective genes glutathione synthetase and thyroid peroxidase were down regulated; transcription levels 0.426 and 0.46-fold less than control

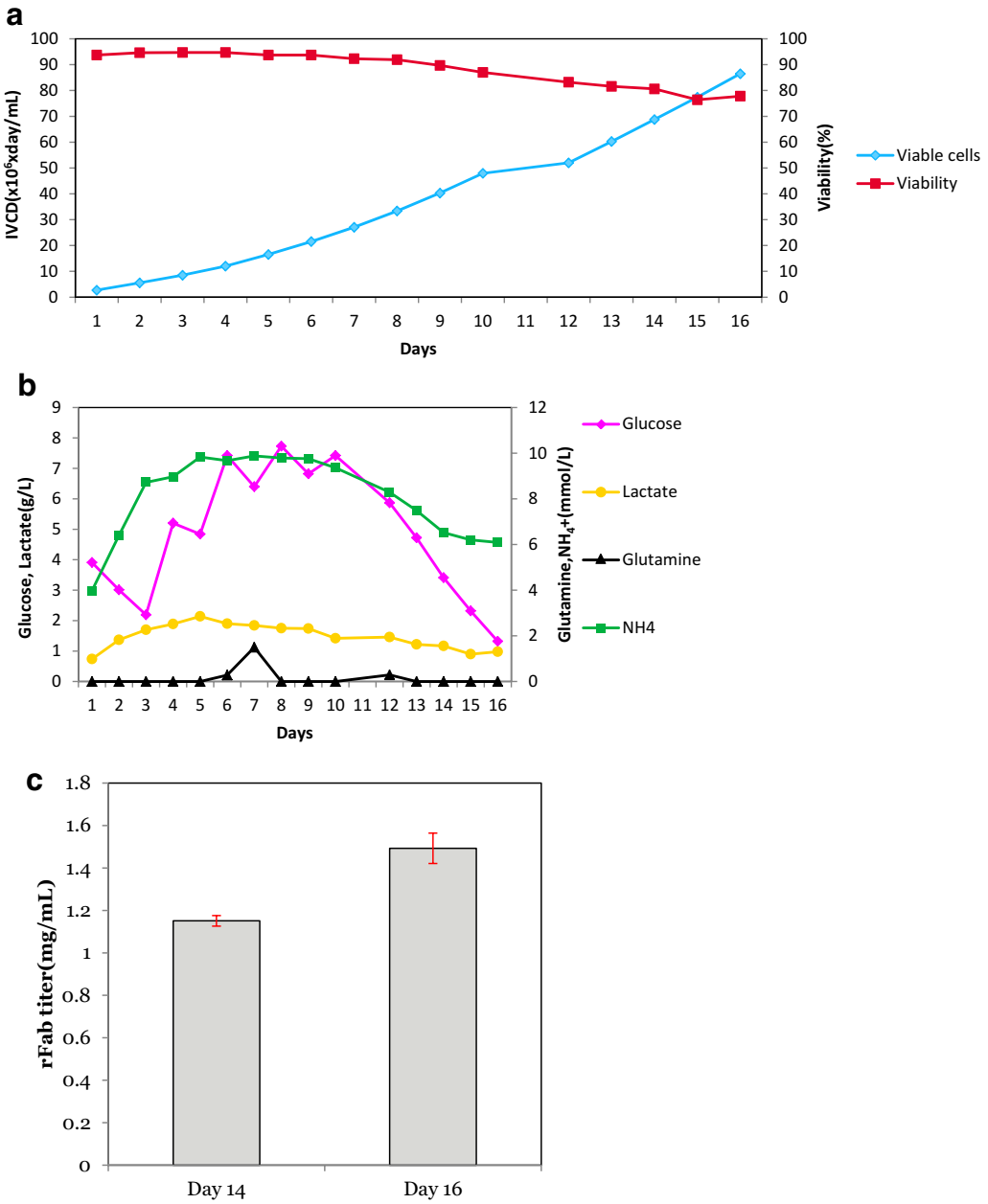

Fig. 7 **a** Integrated viable cell density and cell viability of CHO cells in the 5 L bioreactor over the 16-day growth period. **b** Glucose, lactate, glutamine, and ammonium concentrations during the 16-day growth period in the 5 L bioreactor. **c** rFab titers from the 5 L bioreactor determined at day 14 and day 16 when growth was terminated

acid synthesis needed for recombinant protein production, which correlates to the increase rFab production observed toward end of cell culture for both bioreactors 4 and 8. Similar observations have been made in other studies were excess ammonium was utilized by alpha-keto acids in the TCA cycle for synthesis of amino acids (Young 2013; Mulukutla et al. 2012; Cruza et al. 2000; Lao and Toth 1997; Yang and Butler 2000). The data presented in this study suggests that shifting the temperature from 37 to 32 °C, while maintaining DO at 50% reduces the accumulation of toxic metabolic byproducts, which ultimately improved the cell growth, viability, and yield of the rFab.

The accumulation of lactate and ammonium in culture has been shown to significantly impact the quality of recombinant proteins; for example, affecting the

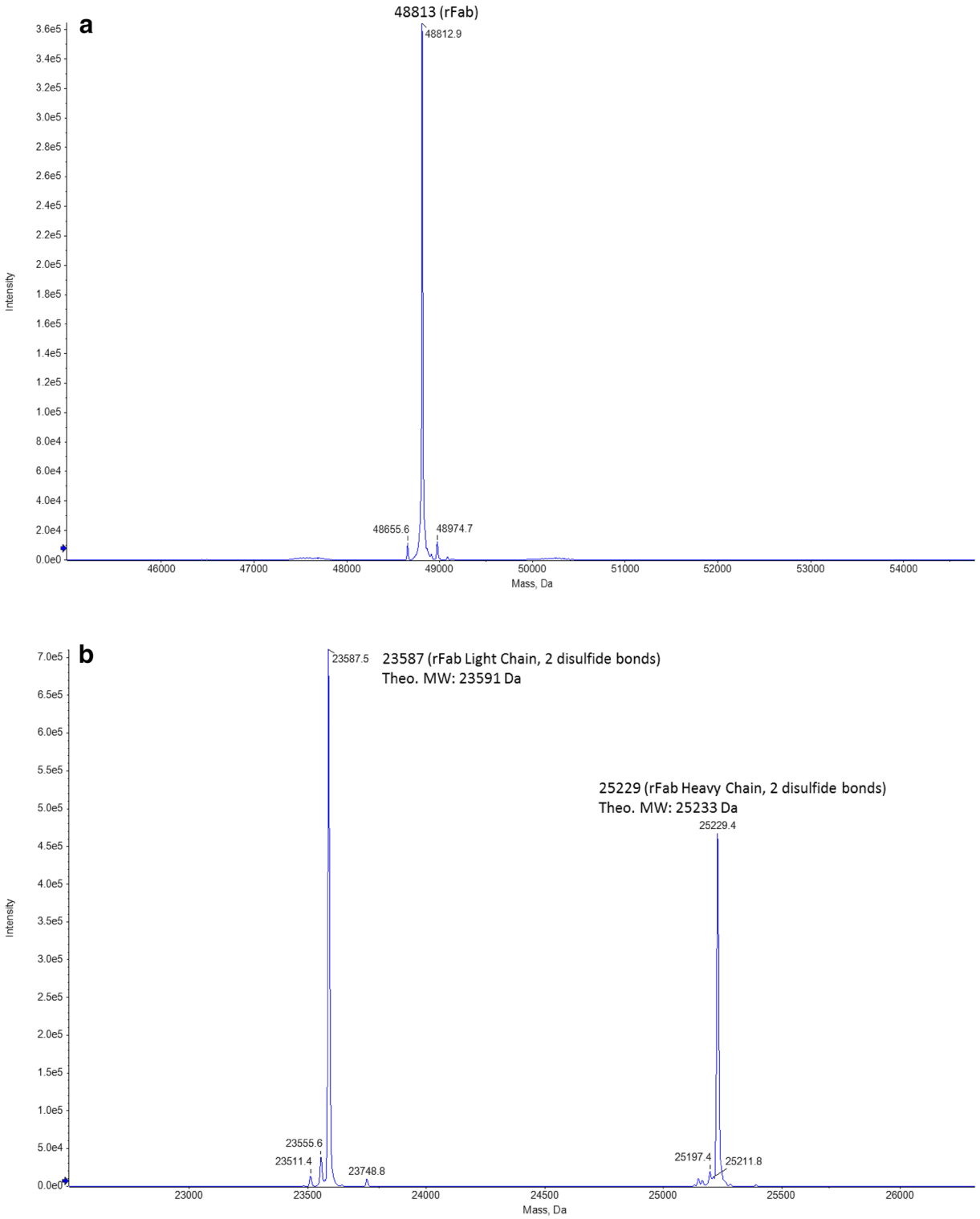

Fig. 8 **a** ESI–MS spectrum of rFab from 5 L bioreactor under non-reduced condition. The MW profile of rFab was observed at 48,813 Da, and no oxidized rFab was detected from 5 L bioreactor. **b** ESI–MS spectrum of rFab from 5 L bioreactor under TCEP-reduced condition. The MW at 23,587 Da matched with the light-chain theoretical MW with two disulfide bonds, and the MW at 25,229 Da matched with the heavy chain theoretical MW with two disulfide bonds

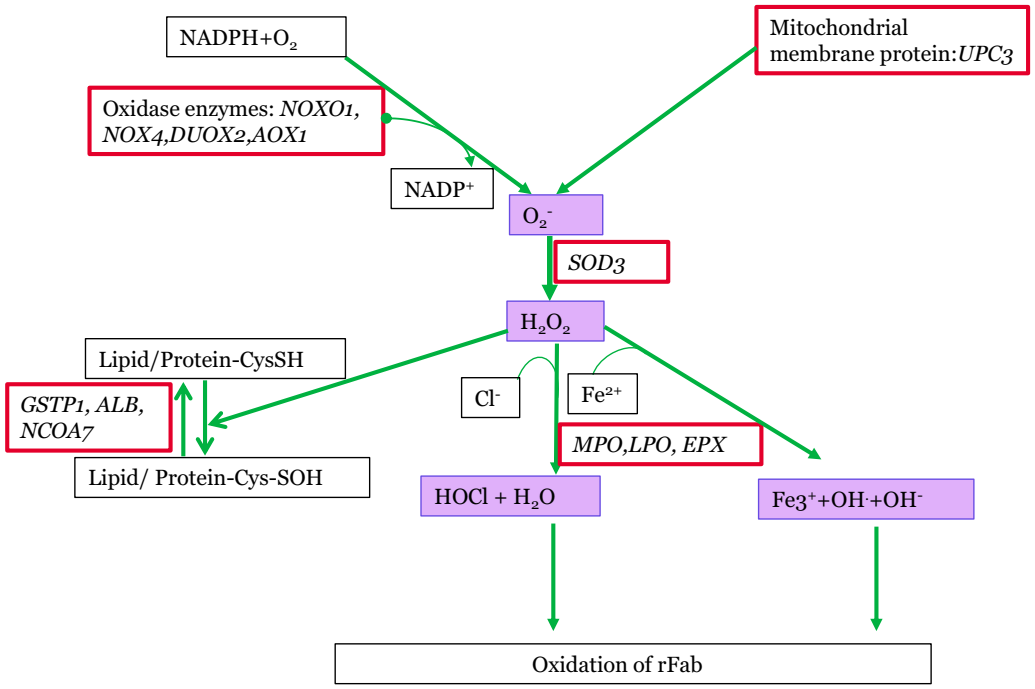

Fig. 9 Schematic diagram illustrating a proposed pathway for rFab oxidation for cells under oxidative stress. The 12 genes identified in the red boxes were found to be highly transcribed in this study; AOX1, Aldehyde Oxidase 1; NOXO1, NADPH Oxidase Organizer 1; EPX, Eosinophil Peroxidase; NOX4, NADPH Oxidase 4; DUOX2, Dual Oxidase 2; UPC3, Uncoupling Protein 3; SOD3, Superoxide Dismutase 3; LOP, Lactoperoxidase; MPO, Myeloperoxidase; GSTP1, Glutathione *S*-Transferase; ALB, Serum Albumin Like; and NCOA7, Nuclear Receptor Coactivator 7. NOXO1, NOX4, DUOX2, AOX1, SOD3, MPO, LPO and EPX genes are all involved with the formation of ROSs (highlighted in the purple boxes), while UPC3 is responsible for pumping ROSs out of the mitochondria. GSTP1, ALB and NCOA7 are protective; their main function is to protect thiol containing proteins and lipids from oxidation by acting as a reducing agent and are not involved in reducing the level of H_2O_2 found within the cells

glycosylation, acetylation, sialylation, and branching of amino sugars of antibodies (Young 2013; Mulukutla et al. 2012; Cruza et al. 2000; Lao and Toth 1997; Yang and Butler 2000). To evaluate the quality of recombinant molecule produced in this study, the purified rFabs from the DOE bioreactors were analyzed by SDS-PAGE and mass spectrometry. The SDS-PAGE data showed that the rFabs produced in the eight bioreactors had a similar purity demonstrating only two distinct light and heavy chain bands of the correct molecular weight. However, mass spectrometry data indicated that six out of the eight bioreactors produced rFab molecules that were oxidized.

During aerobic metabolism cells utilize glucose and glutamine rapidly to provide energy in the form of ATP to synthesize nucleic acids, proteins, and building blocks of the cell membrane. As a part of this rapid metabolism, cells produce an excessive amount of ROSs due to oxidative stress. The excessive amount of ROSs alters the cells redox balance resulting in protein oxidation and apoptosis (Yan et al. 2009; Filomeni et al. 2015; Schieber and Chandel 2014; Levine and Puzio-Kuter 2010; Lambeth 2004).

Based on the gene transcription data presented in this study, a suggested pathway for rFab oxidation in the cytoplasm is being presented for cells under oxidative stress (Fig. 9). The gene transcription data presented in this study indicated high transcription of 12 oxidative stress-related genes in the bioreactor, where the rFab was determined to be oxidized. The NOXO1, NOX4, DUOX2, AOX1, SOD3, MPO, LPO, and EPX genes are all involved with the formation of ROSs, such as superoxide radicals (O_2^-) and H_2O_2, while UPC3 is responsible for pumping ROSs out of the mitochondria (Yan et al. 2009; Filomeni et al. 2015; Schieber and Chandel 2014; Levine and Puzio-Kuter 2010; Lambeth 2004; Alfaro et al. 2009; Garvey 2003; Zelko et al. 2002). ROSs need to be removed as soon as possible from inside the cells, since high accumulation of ROSs can alter the redox balance and triggered the formation of hydroxy radical-related metabolites, which ultimately leads to oxidized proteins or protein-related molecules inside the cell.

The gene transcription data presented in this study also demonstrated that MPO, EPX and LPO were also highly transcribed in the cultures, where rFab was determined to be oxidized. These genes are responsible for

the production of hypohalous acids and hydroxy radicals from hydrogen peroxide in the presence of metal ions (Yan et al. 2009; Filomeni et al. 2015; Schieber and Chandel 2014; Yang and Butler 2000; Levine and Puzio-Kuter 2010; Lambeth 2004; Alfaro et al. 2009; Garvey 2003; Zelko et al. 2002; Kohler and Jenzer 1989; Davies 2011; Jong and Klebanoff 1980; Ten et al. 1989; Oliver et al. 2011; Rosas-Díaz et al. 2015). The higher transcription of these genes suggests the presence of hypohalous acids and hydroxy radicals inside the CHO cells. The gene transcription data also revealed the over transcription of some oxidative stress genes that are protective in nature: GSTP1, NCOA7, and ALB. The main function of these genes is to protect thiol containing proteins and lipids from oxidation by acting as a reducing agent and are not involved in reducing the level of H_2O_2 found within the cells (Yan et al. 2009; Filomeni et al. 2015; Schieber and Chandel 2014; Levine and Puzio-Kuter 2010; Lambeth 2004; Alfaro et al. 2009; Oliver et al. 2011; Rosas-Díaz et al. 2015). Finally, two oxidative stress protective or ROS-scavenging genes, GSS and TPO that remove or convert ROS to nonreactive oxygen species, were shown to be down regulated in bioreactor, where rFab was oxidized (Banerjee 2007; Li et al. 2003; Ruf and Carayon 2006; Kessler et al. 2008). Taking a closer look at the suggested pathway, NOXO1, NOX4, DUOX2, AOX1, SOD3, and UPC3 are responsible for the formation and accumulation of H_2O_2, while MPO, LPO, and EPX are responsible for the production of hypohalous acids and hydroxy radicals from hydrogen peroxide in the presence of metal ions leading to the oxidation of the target molecule (Yan et al. 2009; Filomeni et al. 2015; Schieber and Chandel 2014; Levine and Puzio-Kuter 2010; Lambeth 2004; Alfaro et al. 2009; Garvey 2003; Zelko et al. 2002; Kohler and Jenzer 1989; Davies 2011; Jong and Klebanoff 1980; Ten et al. 1989; Oliver et al. 2011). The high transcription of these genes could result in the generation of ROSs inside the cells due to rapid oxidative metabolism leading to oxidation of the rFab via the suggested pathway.

Utilizing the results from the DOE study along with an understanding of the suggested oxidative pathway, the CHO rFab bioprocess was scaled-up into a 5 L bioreactor. The 5 L bioreactor growth pattern replicated that observed in the DOE optimization study, maintaining a steady lactate concentration with ammonium utilized toward end of the culture. rFab was produced at a significant level (1.5 g/L), and most importantly, no oxidation of the rFab molecule was observed.

Conclusions

The present study clearly demonstrates that the quality of the recombinant protein is closely associated with those growth conditions placing oxidative stress upon the CHO culture. Utilizing the QbD tool of DOE allowed for an experimental design, analysis and prediction of growth conditions with the statistical power to develop and optimized a cell-culture bioprocess leading to the quality and quantity of a recombinant protein to fit a desired application, as in this case, use in a diagnostic immunoassay.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40643-019-0270-8.

Additional file 1: Fig. S1. The DOE study results were analyzed by JMP statistical software custom Design-Fit least square modeling. The Prob > *F* value, under the Analysis of Variance is the *P* value for the model, along with R^2 value of the model, 0.0091 and 0.929494, suggest a significant model. Model analysis indicates that a shift in temperature from 37 to 32 °C along with a combination of DO and temperature shift have a significant effect on rFab production as indicated by the Probability > |*t*| values of 0.0044 and 0.0250, respectively. **Fig. S2.** SDS-PAGE analysis of rFabs from each of the eight bioreactors in the DOE Optimization study under non-reduced condition. The SDS-PAGE samples were collected at day eighteen of the cell culture. Lane M is molecular weight markers. Lanes 1 through 8 represent bioreactors 1 through 8.

Additional file 2: Table S1. ESI–MS analysis summary for all eight bioreactors in the DOE study.

Abbreviations

Qbd: Quality by Design; DOE: Design of Experiment; rFab: recombinant antigen-binding fragment; CHO: Chinese Hamster Ovary; CPPs: critical process parameters; CQAs: critical quality attributes; DO: dissolved oxygen; TCEP: *tris*(2-carboxyethyl) phosphine; IVCD: integrated viable cell density; ROS: reactive oxygen species; SOD3: Superoxide Dismutase 3; MPO: Myeloperoxidase; DUOX: Dual oxidase; DUOX2: Dual Oxidase Like 2; NCOA7: Nuclear Receptor Coactivator 7; NOXs: NAPDH oxidases; NOX4: NAPDH oxidase 4; NOXO1: NADPH Oxidase Organizer 1; UPC3: Mitochondria Uncouple Protein 3; EPX: Eosinophil Peroxidase; LPO: Lactoperoxidase; ALB: Serum Albumin Like; GSTP1: Glutathione *S*-Transferase Pi 1; GSS: Glutathione Synthetase; TPO: Thyroid Peroxidase; AOX1: Aldehyde Oxidase 1.

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Authors' contributions

KS, CZ, and MS performed the experiments and analyzed the data. KS and SA designed the experiments. KS and SA wrote the paper. SA, BZ, and JF revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data sets presented in this manuscript are open for discussion from the corresponding author except where noted. The cell line and purified materials are intellectual property of Abbott Laboratories and cannot be shared.

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Not applicable.

Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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