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Case study on human α1-antitrypsin: Recombinant protein titers obtained by commercial ELISA kits are inaccurate.

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Abbreviations: BLI, biolayer interferometry; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HRP, horse radish peroxidase; IMDM, Iscove's Modified Dulbecco's Medium; \( \text{pl} \alpha1 \text{AT} \), human plasma-derived α1-antitrypsin; \( \text{r} \alpha1 \text{AT} \), recombinant human α1-antitrypsin; RP-HPLC, reversed-phase high-performance liquid chromatography; SPR, surface plasmon resonance; \( \text{rEPO} \), recombinant human erythropoietin; VCD, viable cell density
Abstract

Accurate titer determination of recombinant proteins is crucial for evaluating protein production cell lines and processes. Even though enzyme-linked immunosorbent assay (ELISA) is the most widely used assay for determining protein titer, little is known about the accuracy of commercially available ELISA kits. We observed that estimations of recombinant human α1-antitrypsin (rα1AT) titer by Coomassie-stained SDS-PAGE gels did not correspond to previously obtained titers obtained by a commercially available ELISA kit. This prompted us to develop two independent quantification assays based on biolayer interferometry and reversed-phase high-performance liquid chromatography. We compared the rα1AT titer obtained by these assays with three different off-the-shelf ELISA kits and found that the ELISA kits led to inconsistent results. The data presented here show that recombinant protein titers determined by ELISA kits cannot be trusted per se. Consequently, any ELISA kit to be used for determining recombinant protein titer must be validated by a different, preferably orthogonal method.
1 Introduction

Product titer of secreted recombinant proteins is the key optimization parameter during industrial bioprocessing and selection of clonal cell lines, thus choosing the right method to monitor titer is of utmost importance. Different protein quantification techniques can be employed depending on requirements toward accuracy, sensitivity, dynamic range, reproducibility, time-to-results, cost, and throughput. Enzyme-linked immunosorbent assay (ELISA) has been widely adopted, as it in many cases offers a ready-made method that requires little optimization before it can be implemented into a workflow. Alternative methods include quantification by densitometric analysis of Coomassie-stained SDS-PAGE gels or Western blots, reversed-phase high-performance liquid chromatography (RP-HPLC) or biosensor-based methods using surface plasmon resonance (SPR) [1], resonant mirror technology [2], or biosensor-based methods using surface plasmon resonance (SPR) [1]. Each of these methods has advantages or disadvantages that are dependent on assay design and the instrumentation used. In this study, we sought to quantify recombinant human α1-antitrypsin (rα1AT) secreted from Chinese hamster ovary (CHO) cells using a variety of the aforementioned quantification techniques.

Human α1-antitrypsin is a 52 kDa glycoprotein with three N-glycans [4] and its primary biological function is the inhibition of neutrophil elastase and proteinase 3 [5]. Currently, only human plasma-derived α1AT (plα1AT) is available as treatment of α1AT-deficiency in humans [6]. rα1AT can be produced in transgenic sheep [7], but an immune response to endogenous sheep α1AT in the purified product has been observed [8]. An attractive alternative is to produce rα1AT in CHO or human cells and efforts have been undertaken to achieve this [4,9–14]. N-glycosylation patterns of rα1AT produced from CHO and human cells have been shown to be similar but not identical to plα1AT [4,15]. In addition, serum half-life and activity of rα1AT have been shown to be equivalent to plα1AT [4,10,16]. Thus, rα1AT produced in mammalian cell lines is a promising candidate to replace plα1AT as treatment and future studies are warranted to investigate whether this can be done safely.

During our ongoing investigations into CHO-produced rα1AT, we found it essential to obtain accurate absolute titers of rα1AT. In a recent report [13], we noticed that our estimations of rα1AT titer obtained from Coomassie-stained SDS-PAGE protein gels seemed to be lower than rα1AT titers obtained from ELISA (unpublished observations). To investigate this potential discrepancy in detail,
we established two independent α1AT quantification methods and compared those with three off-the-shelf ELISA kits. In addition, to compare CHO-produced rα1AT with pα1AT, we removed N-glycans and performed an activity assay, showing that rα1AT and pα1AT behave identical. Quantification of CHO-produced rα1AT by Coomassie-stained SDS-PAGE gels, RP-HPLC and BLI resulted in similar titers. On the other hand, quantification of α1AT by ELISA led to inconsistent titers with up to a six-fold difference between ELISA and the aforementioned methods, and up to a 17-fold difference between ELISA kits. Our case study on rα1AT demonstrates the necessity of validating commercially available ELISA kits when determining recombinant protein titer.

2 Materials and Methods

2.1 Plasmids
Plasmids encoding codon-optimized human erythropoietin and human α1AT as well as empty vector control plasmid (pcDNA3.1/Zeo(+)) have previously been described [3,13]. Purified plasmid was obtained using the Nucleobond® Xtra Midi Kit (Machery-Nagel, Düren, Germany) according to the manufacturer’s instructions.

2.2 Cell cultivation and transient transfection
CHO-S suspension cells (Life Technologies, Thermo Scientific, Rockford, IL) were grown in CD CHO medium (#10743029, Life Technologies) supplemented with 8 mM L-glutamine (#LONZ17-605F, Lonza Group AG, Basel, Switzerland) and 2 µL/mL anti-clumping agent (Life Technologies). Cells were expanded in Corning vent cap shake flasks (Sigma-Aldrich, St. Louis, MO) in a humidified incubator at 120 rpm (25 mm orbit), 37°C, and 5% CO₂. Transfection was performed essentially as previously described [13]. In brief, 3x10^7 (recombinant human erythropoietin; rEPO) or 5x10^7 (empty vector and α1AT) cells were transfected using FreeStyle™ MAX Reagent (Life Technologies) in 30 mL (EPO) or 50 mL (empty vector and α1AT) complete CD CHO medium without anti-clumping agent according to manufacturer’s instructions. Transfected cells were incubated in Corning vent cap shake flasks (Sigma-Aldrich) at 120 rpm (25 mm orbit), 37°C, and 5% CO₂. 3 hours post-transfection, anti-clumping agent was added to reach a 2 µL/mL final concentration. Viable cell density (VCD) and viability were measured every day (day 0 – 3) and supernatant samples were obtained from day 1 to day 3.
Supernatant samples were obtained by centrifugation (2000g, RT, 5 min) and supernatants were recovered, aliquoted and stored at -80°C. Spent medium was harvested in the same manner from CHO-S cells seeded at a concentration of 1x10^6 cells/mL and cultivated for 4 days. VCD measurements were performed in duplicates on a NucleoCounter NC-200 Cell Counter (ChemoMetec, Allerod, Denmark) using Via1-Cassettes™ using a 'Viability and Cell Count Assay Method 2 Assay' (NucleoView software ver. 1.1.18.7) according to the manufacturer’s instructions.

2.3 Plasma-derived human α1-antitrypsin

Lyophilized \( \text{plα1AT} \) obtained from Athens Research & Technology (#16-16-011609, Athens, GA, USA) was reconstituted in de-ionized H\(_2\)O. Aliquots were made and stored at -80°C. The concentration of \( \text{plα1AT} \) in the aliquots was determined by measuring absorbance (triplicate measurements) at 280 nm on a NanoDrop 2000 (Thermo Scientific) blanked with 30 mM sodium phosphate, 300 mM sodium chloride (pH 6.5) buffer (lyophilisation buffer) using the extinction coefficient 4.33 (A\(_{280\text{nm}}\), 1% solution, 1 cm path length) [17].

2.4 \( \alpha1 \)-antitrypsin quantification by Coomassie protein stain

10 µL samples were subjected to reducing (25 mM dithiothreitol in 1xNuPAGE LDS Sample Buffer) SDS-PAGE on NuPAGE® Novex® 4-12% Bis-Tris Protein Gels (Life Technologies) in MOPS running buffer (#NP000102, Life Technologies). Proteins were stained with InstantBlue (Expedeon Inc., San Diego, CA) according to manufacturer’s instructions. Protein bands were quantified by densitometric analysis [18] using Image] 1.48v [19]. Total densitometric intensity was measured in a defined area comprising the \( \alpha1\)AT band and background intensity was subsequently subtracted. Background for \( \text{plα1AT} \) was a sample only containing media and background for supernatants samples containing \( \text{rα1AT} \) were supernatant samples from the empty vector control. Linear regression of samples with known concentration of \( \text{plα1AT} \) (0, 5, 15, 30 and 50 µg/mL) was performed (concentration vs densitometric intensity) and the slope of the linear regression was used to calculate \( \text{rα1AT} \) titer.

2.5 \( \alpha1\)AT quantification by biolayer interferometry

Biolayer interferometry was performed using an Octet RED96 (Pall, Menlo Park, CA, USA). Streptavidin kinetic grade biosensors (18-5021, Fortebio, Pall) were hydrated in PBS (600 s) on the Sidekick offline biosensor immobilization station. After a baseline was reached in PBS, biosensors were functionalized
with CaptureSelect biotin anti-α1AT conjugate (Thermo Fisher) at 5 µg/mL in PBS, and blocked in PBS containing 1 µg/mL biocytin (120, 600, and 300 s incubation steps, respectively). After equilibration in spent CHO-S medium (120 s), samples and standards were measured for 300 s with a shaking speed of 1000 rpm at 30 °C. A dilution series was prepared in spent CHO-S medium using commercially available pla1AT (Athens Research & Technology) at 40, 20, 10, 5, 2.5, 1.25 and 0.625 µg/mL. Thawed CHO-S supernatants were diluted two-fold and all samples contained 0.1% BSA (w/v), 0.1% tween-20 (v/v), and 500 mM NaCl as end concentrations. Regeneration was performed using three cycles of 5 s incubation in regeneration solution (20 mM TRIS, 2 M MgCl₂, pH 7.0) and 5 s in neutralization solution (PBS). Assays were performed in 96-well black microplates (655209, Greiner Bio-One, Kremsmünster, Austria) at 1000 rpm shaking speed. Octet System Data Analysis 7.1 software was used to calculate binding rates and absolute α1AT concentrations (standard curve equation: Dose response 4PL; binding rate equation: Initial binding, 120 s).

2.6 α1AT quantification by RP-HPLC

α1AT from thawed supernatant samples was quantified by RP-HPLC on a Ultimate 3000 (Dionex, Thermo Scientific) using a Discovery BIO Wide Pore C5 (150 x 2.1 mm, 3.0 µm) column (Sigma Aldrich), operated at 40°C and a flow rate of 0.5 mL/min. Buffer A was composed of 0.1% TFA in milliQ water and buffer B was composed of 0.07% TFA in acetonitrile. The equilibration phase consisted of 95% buffer B for 7 min, a gradient to 35% buffer B over 0.5 min, and 35% buffer B for 3.5 min. The elution phase consisted of a gradient from 35-75% buffer B over 12 min and a gradient from 75-95% buffer B over 0.2 min. Protein detection was performed by UV light absorption at 214 nm and α1AT titer was determined using a dilution series of pla1AT (Athens Research & Technology) in spent CHO-S medium at 40, 20, 10, 5, 2.5, 1.25 and 0.625 µg/mL aided by the Chromeleon 7 software package (Dionex, Thermo Scientific).

2.7 α1AT quantification by ELISA

The α1AT titer in supernatant samples was determined using three different commercially available human α1AT ELISA kits. Kit #1: SimpleStep sandwich ELISA (#ab189579, Abcam, Cambridge, UK) was performed according to manufacturer’s instructions. Readings obtained from the pla1AT standard from the kit were subjected to linear regression (log(concentration) versus log(absorbance)). Kit #2:
Competitive ELISA (#KA0458, Abnova, Taipei City, Taiwan) was performed according to manufacturer’s instructions. Readings obtained from the \( p\alpha1AT \) standard from the kit were subjected to linear regression (\( \log(\text{concentration}) \) versus \( \log(\text{absorbance}) \)). Kit #3: Conventional sandwich ELISA (#OKIA00048; Aviva Systems Biology, San Diego, CA) was performed according to the manufacturer’s instructions. Readings obtained from the lyophilized serum \( \alpha1AT \) standard from the kit were subjected to a non-linear ‘Sigmoidal, 4PL, \( X \) is \( \log(\text{concentration}) \)’ fit in GraphPad Prism (version 6.05 for Windows, GraphPad Software, La Jolla, CA). Three different types of medium were used as diluent for \( p\alpha1AT \) in the ELISA experiments: 1) CD CHO medium supplemented with 8 mM L-glutamine and 2 \( \mu \)L/mL anti-clumping agent; 2) EX-CELL® ACF CHO Medium (#C5467-1L, Sigma-Aldrich) supplemented with 8 mM L-glutamine; 3) Iscove’s Modified Dulbecco’s Medium (IMDM; #ATCC-30-2005, LGC Standards, Teddington, UK) supplemented with 10% fetal bovine serum (FBS; #ATCC-30-2020, LGC Standards), 4 mM L-glutamine, 1x HT Supplement (#11067030, Life Technologies) and 1.5 mg/mL sodium bicarbonate (#13433, Sigma-Aldrich). The dilution factor for supernatant samples as well as media samples containing spiked \( p\alpha1AT \) was kept constant for each kit.

### 2.8 Elastase inhibition assay

\( r\alpha1AT \) was purified by CaptureSelect \( \alpha1AT \) Affinity Matrix (Thermo Fisher) according to the manufacturer’s instructions. Purified protein was buffer exchanged into PBS using a PD-10 desalting column (GE Healthcare), snap frozen in liquid nitrogen and stored at -80°C. The concentration of purified \( r\alpha1AT \) was determined by absorption at 280 nm as described for \( p\alpha1AT \). The \( \alpha1AT \) inhibitory activity was determined using an Elastase inhibition assay (EnzChek Elastase Assay Kit, Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. In short, purified \( r\alpha1AT \) and \( p\alpha1AT \) (1.0, 0.5, 0.25, 0.125, 0.0625, 0.0312, 0.0156, and 0.0078 \( \mu \)M) were incubated with purified active porcine pancreatic elastase followed by the addition of fluorescently labelled substrate (DQ-elastin). The reaction was allowed to occur at room temperature, and the measurement of fluorescence was performed after 20 min (Excitation: 485 nm, slit width 9.0 nm; Emission: 530 nm, slit width 13.5 nm).
2.9 Other methods

Anti-α1AT Western blotting was performed essentially as previously described [13] with the exception that MES buffer was used instead of MOPS buffer. Deglycosylation with PNGase F was performed according to the manufacturer's instructions (New England Biolabs, Ipswich, MA). Samples in the PNGase F experiment were TCA-precipitated before SDS-PAGE.

3 Results

3.1 CHO-produced human α1-antitrypsin is N-glycosylated and enzymatically active

CHO-S was transiently transfected with either empty vector or a plasmid encoding human α1AT. VCD and viability were monitored over a three-day period and were found to be similar (Fig. 1A). Supernatant samples were taken every day and aliquots were stored until further analysis. Throughout this study, plα1AT was used as a reference. According to the manufacturer's instructions, plα1AT was reconstituted in deionized water to a concentration of 2.34 mg/mL. This value corresponded well to our own measurements of protein concentration based on absorbance at 280 nm (2.27±0.07 mg/mL).

To compare the quality and integrity of the CHO-produced rα1AT with plα1AT, we analysed samples on a Western blot probed with a polyclonal anti-α1AT antibody. plα1AT migrated as one species, whereas rα1AT was detected primarily as two main species migrating in a smear, a hallmark of heterogeneous glycosylation (Fig. 1B, lane 5 and 6). Upon removal of N-glycans by PNGase F treatment, rα1AT collapsed into one band that co-migrated with plα1AT (Fig. 1B, lane 1 and 2), showing that the polypeptide of rα1AT was intact. Moreover, the Western blot (Fig. 1B) shows that rα1AT and plα1AT are differentially N-glycosylated, as previously reported [4]. No bands were detected in the empty vector and rEPO control supernatants, both before and after PNGase F treatment (Fig. 1B, lane 3, 4, 7, 8). The presence of rEPO was confirmed by probing the blot with a polyclonal anti-EPO antibody (data not shown).

To confirm that CHO-produced rα1AT had retained its inhibitory activity, we compared rα1AT and plα1AT in a porcine elastase inhibitory assay. We used a fluorescent substrate to monitor the inhibition of elastase protease activity in the presence of different concentrations of rα1AT and plα1AT.

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Both α1ATs showed very similar inhibitory activities, as reported before [4,16]. This shows that \textit{in vitro} biological activity of rα1AT was not perturbed, despite differences in glycosylation.

### 3.2 Quantification by Coomassie stain

In order to get an initial estimation of the rα1AT titer, any proteins contained in supernatant as well as in \textit{pla}1AT reference samples were separated on SDS-PAGE gels and subsequently stained with Coomassie. A single, well-defined band was observed in the \textit{pla}1AT samples (Fig. 2A, lane 7-10), demonstrating that the \textit{pla}1AT protein standard was pure and that no detectable degradation had occurred. An rα1AT specific band between the 50 and 65 kDa marker bands was observed only in the supernatant samples containing rα1AT (Fig 2A, lanes 1, 3 and 5), enabling quantification of rα1AT by densitometry. Absolute concentrations of rα1AT were calculated by comparison with a standard curve generated from the dilution series of \textit{pla}1AT (Fig. 2B). The rα1AT titers were estimated to be 7.3, 33.6 and 44.7 µg/mL for day 1, day 2 and day 3 samples, respectively.

### 3.3 Quantification by biolayer interferometry and RP-HPLC

Efforts to quantify rα1AT have mostly been limited to ELISA-based methods. To independently confirm the densitometric estimation of rα1AT titer, we developed both optical biosensor and RP-HPLC-based quantification methods. Analogous to efforts to quantify rEPO by BLI [3], we developed a direct, one-step binding assay using a camelid single-domain antibody fragment directed against human α1AT (anti-α1AT \textit{V}_{\text{H}}). As this method is affinity-based, it could potentially suffer from the same drawbacks as ELISA [20]. Therefore, we also developed a chromatographic quantification method using RP-HPLC. This technique for protein quantification has become well established in industry and offers an antibody-independent method to quantify α1AT [21].

To construct an immunosensor, an anti-α1AT \textit{V}_{\text{H}} biotin conjugate was immobilized onto streptavidin biosensors. Functionalization of the tips with the \textit{V}_{\text{H}} reproducibly resulted in a shift of 3.6 nm (data not shown). To assess the sensitivity of the immunosensors, we prepared a dilution series of \textit{pla}1AT. Incubation for 300 s resulted in a maximum shift of about 1.0 nm. The maximum shift decreased with decreasing \textit{pla}1AT concentrations, but even at the lowest concentration of \textit{pla}1AT used (0.63 µg/mL), we still detected a shift of about 0.05 nm (Fig. 3A). Absolute concentrations of rα1AT were calculated by comparison with a calibration curve generated from three separate dilution series of \textit{pla}1AT (Fig.
The $\alpha_1$AT titers were determined to be 0.7, 29.9 and 47.7 µg/mL for day 1, day 2 and day 3 samples, respectively.

The RP-HPLC method consisted of an equilibration and an elution stage. Mobile phase A was water containing 0.1% (v/v) TFA. Mobile phase B was acetonitrile containing 0.07% (v/v) TFA. The equilibration stage was found to be essential to prevent clogging of the column and subsequent loss of sensitivity. Our gradient optimization efforts focused on obtaining a chromatographic peak of $\alpha_1$AT well separated from host cell protein peaks. The elution stage consisted of a gradient from 35-75% buffer B over 12 minutes (Fig. 3C, insert). Representative chromatograms of pl$\alpha_1$AT, r$\alpha_1$AT, and spent CHO-S medium show that pl$\alpha_1$AT and r$\alpha_1$AT display the same retention time and that they are separated well from CHO host cell proteins (Fig. 3C). The retention time of $\alpha_1$AT was estimated from 35 chromatograms of a pl$\alpha_1$AT standard sample obtained at different concentrations and on different days. The average retention time calculated in this way was 6.642±0.002 minutes. Absolute titers of r$\alpha_1$AT were calculated by comparison with a calibration curve generated from three separate dilution series of pl$\alpha_1$AT (Fig. 3D). The r$\alpha_1$AT titers were determined to be 7.7, 29.9 and 44.3 µg/mL for day 1, day 2 and day 3 samples, respectively.

### 3.4 Commercially available $\alpha_1$AT ELISA kits report up to a 17-fold difference in titer

We wanted to determine the r$\alpha_1$AT titer by ELISA using three commercially available kits from different manufacturers that employ different assay formats, i.e., SimpleStep sandwich ELISA (Abcam, kit #1), competitive ELISA (Abnova, kit #2), and conventional sandwich ELISA (Aviva systems biology, kit #3). The $\alpha_1$AT standards included in the kits were used to generate a standard curve (kit #1, Fig. 4A; kit #2, Fig. 4B; kit #3, Fig. 4C) and absolute titers of r$\alpha_1$AT were calculated using these standard curves. The r$\alpha_1$AT titers from the three different ELISA kits varied up to 17-fold, 13-fold, and 15-fold for day 1, day 2, and day 3 supernatant samples, respectively (Fig. 4D).

We then wanted to examine whether the discrepancy between the titers obtained by ELISA originated from compatibility issues with media composition. We therefore tested whether using different growth media affected the determination of spiked $\alpha_1$AT concentration. We chose CD CHO, EX-CELL, and IMDM+FBS to represent protein-free media, plant hydrolysate-containing media, and serum-containing media, respectively. Overall, $\alpha_1$AT concentration established by ELISA did not seem to be
affected by media composition, as only minor differences could be observed between media types (Fig. 4E). The only exception was kit #2, where \( \text{pl} \alpha 1 \text{AT} \) spiked in IMDM+FBS gave rise to a 1.3±0.2-fold higher concentration as compared to CD CHO media.

3.5 Titer comparison: a spiked \( \text{pl} \alpha 1 \text{AT} \) standard leads to better agreement of ELISA kit #3 with reference methods

Finally, we collected the titers from all assays to compare their performance. In addition, we not only compared the titers obtained by using the standard contained in the different ELISA kits, but also to a spiked \( \text{pl} \alpha 1 \text{AT} \) standard (Fig. 5). We observed good agreement between the titers obtained by Coomassie-staining, BLI, and RP-HPLC for day 2 and 3 samples (Fig. 5B and 5C). The titer obtained by BLI for day 1 samples was markedly lower than the other assays (Fig. 5A). When using the protein standards contained in the ELISA kits, none of the three kits tested gave protein titers similar to the three independent assays BLI, Coomassie-staining and RP-HPLC. For all three time points, kit #2 was closest with an average fold difference of 1.4±0.1 when compared to the RP-HPLC-based assay, whilst kit #3 overestimated by 2.5±0.2-fold and kit #1 underestimated by 5.9±0.2-fold. Consistent with our preliminary data, kit #3 gave higher titers than determined by Coomassie-staining. When ELISA titers were adjusted to a known concentration of spiked \( \text{pl} \alpha 1 \text{AT} \), kit #3 gave approximately two-fold lower titer values, giving rise to only a 1.2±0.1-fold higher titer than RP-HPLC. In contrast, kit #1 and kit #2 remained largely unaffected. It thus seems that the aforementioned overestimation of titer from kit #3 seems to originate from the protein standard included in the kit.

4. Discussion

In this study, we have explored several different quantification methods to achieve accurate titers of CHO-produced \( \text{r} \alpha 1 \text{AT} \). Absolute protein titer is a key figure to report during clonal selection, bioprocessing, and downstream processing phases of the recombinant protein production process [22]. In our recent work on the development of a microscale screening platform for improving recombinant protein productivity in CHO cells [13], we reported a \( \text{r} \alpha 1 \text{AT} \) titer of approximately 72 µg/mL as established by ELISA. However, subsequent estimates of titer by Coomassie-stained SDS-PAGE gels were considerably lower. We therefore performed a densitometric analysis of Coomassie-
stained SDS-PAGE gels and developed two independent quantification assays (BLI and RP-HPLC). Except for day 1 titers obtained by BLI, we observed good agreement between these assays. The aberrant time point may suggest a sensitivity issue of BLI in CHO supernatant samples of low product titer. Between the ELISA kits with the lowest and highest titer, we observed a 17, 13 and 15-fold difference for day 1, day 2 and day 3 samples, respectively (Fig. 4D). It thus seems that quantification of α1AT by ELISA leads to inconsistent absolute titers. It is important to note that all three ELISA kits gave consistent results when used for relative quantification of rα1AT, as the increase in titer from day 1 to day 3 was found to be similar.

The overestimation bias of kit #3 was found to originate from the protein standard included in the kit, as the use of spiked pα1AT as a standard led to better agreement with the titers obtained by RP-HPLC. According to the manufacturer, the protein standard is lyophilized serum containing pα1AT, which has been quantitated against a proprietary human α1AT source. The quality and concentration of α1AT in the lyophilized serum from kit #3 could not be analysed by SDS-PAGE and Coomassie-staining due to the presence of human serum albumin (data not shown), which is highly abundant in serum and has roughly the same molecular weight as α1AT [23,24] and therefore masks α1AT.

Kit #2 seemed to be affected by the presence of serum spiked into CHO media (Fig. 4E). However, no significant difference in background was observed between samples containing only CD CHO media and empty vector control supernatant samples (data not shown). This suggests that the overestimation bias of kit #2 did not originate from unspecific binding to host cell proteins. Thus, the reason for the unreliable results obtained with kit #1 and #2 is currently unknown. It was recently reported that kit #1 yielded a two-fold difference as compared to an SPR-based assay [25]. This inconsistency could be alleviated by using the protein standard used in their SPR experiments (histagged rα1AT expressed in NS0 cells) instead of the pα1AT standard included in the kit. The mouse polyclonal capture antibody and rabbit polyclonal detector antibody for kit #1 were raised against pα1AT and a synthetic, proprietary peptide, respectively (personal communication from the manufacturer). It thus seems plausible that the observed difference in glycosylation between rα1AT and pα1AT (Fig. 1B) could result in reduced affinity of the capture antibody for rα1AT. Whether this could also be the case for Kit #2 is unknown, because information about the antigens used for
immunization for Kit #2 and #3 could not be obtained from the manufacturers. In addition, two of three capture antibodies (from Kit #2 and #3) could not be provided in solution from the manufacturers, rendering a comparison of the specificity of the three kits impossible. Whether a specific ELISA assay format systematically distorts absolute $\alpha_1$AT titers cannot be inferred from this study.

Since ELISA is the most widely used assay for monitoring protein titer, it seems likely that a considerable number of reported recombinant protein titers is inaccurate. Recently, a $\alpha_1$AT titer of 1.15 g/L was reported [12], which according to the authors is the highest titer of any recombinant protein in shake flasks batch culture of stable mammalian cells. A commercially available ELISA kit was used for determining protein titer and validation of the ELISA kit by an orthogonal assay was not described. Based on the inaccuracy of the $\alpha_1$AT ELISA kits reported in the present study, the validity of the 1.15 g/L $\alpha_1$AT titer appears questionable. Moreover, our recently reported $\alpha_1$AT titer and specific productivity of 72 µg/mL and 13 pg per cell per day based on kit #3 [13] should be corrected to 34 µg/mL and 6 pg per cell per day, respectively.

In the clinical field, there are a multitude of studies that compare and validate commercially available ELISA kits for various target molecules [26–28]. To our best knowledge, a study comparing different off-the-shelf ELISA kits and validating them by alternative quantification methods, has not been performed before on recombinantly produced proteins. Our study demonstrates the necessity of validating commercially available ELISA kits when used for determination of absolute recombinant protein titer. Although manufacturers of ELISA kits have an interest in validating their kits, it seems impractical to validate the kits for every type of recombinant protein. Instead, the onus of validation lies with the user, who should take up the responsibility of providing accurate protein titer measurements. As relative quantification was found to be correct, the tested ELISA kits could be employed during certain phases of the recombinant protein production process, e.g., during clonal selection. Any ELISA kit to be used for determining absolute recombinant protein titer must be validated using a protein standard of known purity and origin. In the present study, a pure $p\alpha_1$AT standard was available, but we recognize that this unfortunately may not be the case for all biopharmaceutical products.
The validation method should preferably be a genuinely orthogonal method, i.e., a non-antibody based method. An HPLC-based method seems to be the optimal choice for validation due to its accuracy, facile sample preparation, and direct read out. However, development of such assays can for some proteins be a labour-intensive and tedious process. As an alternative, densitometric analysis of Coomassie-stained SDS-PAGE gels is an inexpensive solution that rapidly determines recombinant protein titer, if protein titers are above the limit of detection in cell-free supernatants. Despite the observed sensitivity issue, the newly developed BLI-based quantification assay offers a rapid and high-throughput method, which can applied at several phases of the recombinant protein production process. Once validated, however, the advantages of ELISA are manifold due to its throughput, sensitivity and ease of automation.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

References


Figure 1. Characterization of CHO-produced human α1-antitrypsin. CHO-S cells were transiently transfected with either empty vector (e.v.) or plasmid encoding human α1AT. A) Viability and VCD measurements of the transiently transfected cultures from day 0 to day 3. Mean of two measurements (technical replicates) are shown. B) Western blot analysis of 0.5 µg plα1AT (lane 1 and 5), and 10 µL of supernatants (SN) from CHO-S transfected with empty vector (lane 4 and 8), or a plasmid expressing rα1AT (lane 2 and 6) or rEPO (lane 3 and 7). Samples were treated with PNGase F (+) or mock-treated (-). Blots were developed using anti-α1AT IgG. C) The inhibitory activity of plα1AT (circles) on porcine elastase was compared to rα1AT (squares). Maximum proteolytic activity of porcine elastase was set to 100 %. Error bars denote standard deviations from three technical replicates.
Figure 2. Quantification of $\alpha_1$AT by Coomassie stain. Supernatant (SN) samples from transiently transfected cultures described in Fig. 1 and plasma-derived $\alpha_1$AT ($pl\alpha_1$AT) were used. A) Coomassie-stained SDS-PAGE gel of 10 µL samples of day 1 to day 3 supernatant (SN) samples (D1-D3; lane 2-6) and of $pl\alpha_1$AT diluted in complete CD CHO medium (lane 7-10). The gel is representative of three gels and the rectangle exemplifies the area that was used in densitometric analysis. B) Standard curve derived from the densitometric analysis of the $pl\alpha_1$AT gel bands shown in (A). The correlation coefficient ($R^2$) is shown.
Figure 3. Establishing BLI and RP-HPLC-based α1AT quantification assays. Supernatant samples from transiently transfected cultures described in Fig. 1 and plasma-derived α1AT (plα1AT) were used. A) Representative BLI sensorgrams showing immobilized anti-α1AT VH binding of plα1AT at the indicated concentrations diluted in spent CHO-S medium. All sensorgrams were baseline-subtracted using a sensorgram generated in spent CHO-S medium. B) BLI standard curve of plα1AT. Error bars denote standard deviations from three independent dilution series of plα1AT and the correlation coefficient (R²) is shown. C) Representative RP-HPLC chromatograms of 40 μg/mL plα1AT spiked into spent CHO-S medium (solid line), α1AT day 3 CHO-S supernatant (dashed line), and spent CHO-S medium (dotted line). The insert shows the equilibration and elution stage used and the dark grey region indicates the general area where α1AT is retained. D) HPLC standard curve of plα1AT. Error bars denote standard deviations from three independent dilution series of plα1AT and the correlation coefficient (R²) is shown.
Figure 4. Quantification of α1AT by commercially available ELISA kits. Supernatant samples from transiently transfected cultures described in Fig. 1, plasma-derived α1AT (p/α1AT) as well as α1AT standards from the ELISA kits were used. Standard curves generated from the α1AT standard proteins found in the A) SimpleStep sandwich ELISA kit from Abcam (kit #1), B) Competitive ELISA kit from Abnova (kit #2), and C) Conventional sandwich ELISA kit from Aviva Systems Biology (kit #3). Mean of two measurements (technical replicates) and the correlation coefficients ($R^2$) are shown. D) α1AT titers of day 1, day 2, and day 3 were determined by ELISA kit #1 (white bars), #2 (grey bars), and #3 (striped bars). The fold difference between the highest and the lowest titers are indicated for the different time points. E) The concentration of spiked pla1AT was determined by ELISA in three different types of media: CD CHO (white bars), EX-CELL (grey bars) and IMDM+FBS (striped bars). The concentration obtained by ELISA was normalized to values obtained in CD CHO medium. The titer values obtained from technical triplicates are indicated as circles in (D) and (E).
Figure 5. Comparison of \( \alpha \text{1AT} \) protein titer assays. Collected titer values for all assays are shown for A) day 1, B) day 2, and C) day 3 samples. In addition, ELISA protein titer values using plasma-derived \( \alpha \text{1AT} \) (pl\( \alpha \text{1AT} \)) spiked into CD CHO medium as a standard are shown. #1-3 refer to kit #1-3 and the dotted line indicates the mean of the titer values determined by RP-HPLC analysis. The titer values obtained from technical triplicates are indicated as circles in (A), (B), and (C).