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Expression and purification of a novel single-chain diabody (scDb-hERG1/β1) from *Pichia pastoris* transformants

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ABSTRACT

In the last decades, protein engineering has developed particularly in biotechnology and pharmaceutical field. In particular, the engineered antibody subclass has arisen. The single chain diabody format (scDb), conjugating small size with antigen specificity, offers versatility representing a gold standard for a variety of applications, spacing from research to diagnostics and therapy. Along with such advantages, comes the challenge of optimizing their production, improving expression systems, purification procedures and stability. All such parameters are detrimental for protein production in general and above all for recombinant antibody expression, which has to be fine-tuned, choosing a proper protein-expression host and adjusting expression protocols accordingly. In the present paper, we present data regarding the production and purification of a single chain diabody directed against the macromolecular complex hERG1/β1 integrin. We focus on the expression of clones deriving from the transformation of *Pichia pastoris* yeast cells. In particular, we compare two different clones arose from two separate transformation processes, demonstrating that both are suitable for proper protein expression. Moreover, we have set up an expression protocol and compared the yields obtained using two purification machines: Akta Pure and Akta Start, with a positive outcome.

1. Introduction

Recombinant protein production has emerged in the last forty years as a possible path to overcome limitations due to the exploiting of natural resources. Above all, pharmaceuticals, such as human insulin and growth hormone have been produced thanks to genetic engineering techniques, using *Escherichia coli,* as host [1]. So far, recombinant protein production is considered a milestone for drug discovery. In this scenario, a subclass belonging to recombinant protein is represented by engineered antibodies. Among them, bispecific antibodies (bsAbs), such as single chain diabodies (scDb) have recently raised a lot of attention, considering their possible biotechnological and medical applications. In fact, in addition to the small size, bsAbs can redirect cells and simultaneously block two different targets in different pathways that carry out unique or overlapping functions in pathogenesis [2]. Moreover, they are able to penetrate tissues efficiently, offering advantages from a therapeutic point of view. However, the development of bsAbs has experienced many difficulties, mainly due to manufacturing problems, poor yields, instability and immunogenicity [3]. So far, it is reasonable to propose that bAbs represent a new era of improved medical treatment options. Nevertheless, several critical points still remain to be solved, such as the simplification of the structure and, among all, the production procedures. One of the detrimental factors that affects antibody production is the choice of a proper expression system. Different kinds of hosts have been used, including prokaryotes, such as *Escherichia coli* [4] and *Bacillus subtilis*, and eukaryotes, including *Saccharomyces cerevisiae* [5], *Pichia pastoris* [6], insect cells [7], plant cells [8] and mammalian cells [9]. The application of bAbs for both therapeutics and diagnostics is strictly linked to the development of platforms for an efficient and cost-effective production of these molecules [10]. Such goal is influenced by many factors, as protein expression is mutable and yields vary, being affected by protein size, solubility, stability and amino acid sequence; thus the expression of each protein must be optimized. Despite the undeniable returns, the genetic approach to increase

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recombinant protein yields, based on the engineering of a host organism can be expensive and time-consuming, particularly if we consider a transcriptomic approach analysis. In order to overcome such hindrances, an alternative, and potentially cheaper option is to optimize the experimental culture conditions, for example adjusting media composition [11].

The optimal expression system depends on the fragment variability, as well as the required purity and quantity of the final product. Recombinant antibody production in yeast and fungi combines the advantages of eukaryotic expression, including protein processing, folding, and post translational modifications. In yeasts, proteins are expressed into the culture supernatant allowing faster and easier purification, which results in better quality purified proteins, with less contaminating endotoxins as compared to the bacterially expressed counterparts [12].

Along with challenges linked to the choice of the most suitable expression system, the development of an efficient purification pattern still remains a crucial point. Affinity chromatography represent the gold standard for antibody purification, despite requiring a continuous effort in protocol tuning and refining, including the choice of the most suitable resin and the setup of parameters, such as retention time and elution steps [13].

In this scenario, the present work focuses on fine-tuning the production and purification of a bispecific antibody in a single chain diabody (scDb) format. The latter, previously patented (Patent: "Novel antibodies". Inventors: Annarosa Arcangeli, Claudia Duranti, Silvia Crescioli, Laura Carraresi. Patent Ref: ReWO2019/015936 (University of Florence)), it is proposed as an effective molecular diagnostic tool with therapeutic potential. In fact, it is directed against the molecular complex formed by hERG1 ion channel and β1 integrin. Such complex is selectively expressed in cancer cells, thus representing a real oncogenic target from both a therapeutic and diagnostic point of view [14,15]. Due to the antibody format, a particular effort has been necessary for the development of a good production system. Therefore, we have focused on the comparison of different antibody preparations, in order to identify a "gold standard" protocol for its production, capable of avoiding problems related to the yield and stability of the protein itself. Such protocol would guarantee the overcoming of distortions, including sub-optimal culture temperature, instruments' malfunction (shaker, incubator for yeast cultures, etc.) and inconstant storage temperature of frozen yeasts, major responsible of consequent reduction in cellular efficiency.

2. Materials and Methods

2.1. Expression and purification of scDb-hERG1/β1 antibody

The scDb-hERG1/β1 was developed starting from two single chain Fragment variable (scFv) antibodies, one directed against hERG1 (scFv-hERG1from mAb-hERG1) and one against the β1 integrin (scFv-β1 from mAb-β1 TS2/16). The V_H and V_L sequences of the two scFv(s) were joined by three peptide linkers (A, M and B), in the following order: V_HhERG1-linker A-V_L β1 - linker M − V_H β1-linker B-V_LhERG1. Linker M was 20 amino acid long, to allow the proper assembly of the protein in a Diabody format. scDb-hERG1/β1 was transformed in GS115 *Pichia pastoris* yeast strain according to the spheroplasting technique. The construct was digested with SalI restriction enzyme and transformed into the *Pichia pastoris* strain GS115 by spheroplasting, generating Mut^+ transformants [Duranti C. et al., submitted to Molecular Cancer Therapeutics].

After two days of growth at 30 °C, six best grown clones from the 15 mg/ml G418 plates were picked up and evaluated for their capacity to express the protein of interest, setting up a small-scale liquid culture, according to Pichia Expression Kit protocol (Invitrogen).

After three days of induction with 0.5% final concentration of 100% methanol, supernatants were harvested and purified using AKTA Pure (Ge Healthcare).

2.2. ÄKTA purification

Purification of scDb-hERG1/β1 was performed by Affinity Chromatography, using ÄKTA Protein Purification System (Ge Healthcare Life Sciences) and ÄKTA Start with HisTrap HP 1 ml columns. Wash steps and equilibration were performed according to the manufacturer's instructions, using Wash buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.3); elution was performed utilizing a linear gradient of Elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.3). Analysis was accomplished using UNICORN 7.0 software.

2.3. Antibody concentration and dialysis

Dialysis has been performed using Slide-A-Lyzer Dialysis Cassette, 10K cut off exchanging the media in at least 1 to 10 ratio with PBS 1X for 12 h in agitation at 4 °C.

2.4. SDS-PAGE

Each sample was applied with the same volume of 15 μl to a stacking gel (400 μl acrylamid (40%)-bisacryamide (0.8%), 1 ml 0.5 M Tris-HCl, pH 6.8, 40 μl 10% SDS, 20 μl 10% ammonium persulfate, 4 μl TEMED, 2,54 ml H2O). Stacking gel were added on the resolving gel (2,6 ml acrylamid (40%)-bisacryamide (0.8%), 1,75 ml 1.5 M Tris-HCl, pH 8.8, 70 μl 10% SDS, 35 μl 10% ammonium persulfate, 3,5 μl TEMED, 2,55 ml H2O). Electrophoretic runs were performed at 150 V. Gels were either stained with Coomassie Brilliant Blue or transferred to PVDF membranes for western blotting analysis to assess the presence of the protein (around 30 KDa).

2.5. Western blot

After SDS-PAGE gels were transferred to PVDF membrane (Amersham) in transfer buffer (14,4 g, 3,03 g TrisHCl, 200 ml methanol, 800 ml H2O) at 100 V for 1 h. Membranes were washed in T-PBS (PBS 0.1% Tween) and then blocked with T-PBS 5% BSA O/N. Membranes were exposed to primary antibody peroxidise-coupled (Sigma) diluted in T-PBS 5% BSA for 1h at room temperature. After washing the membranes three times for 10 min, signals were visualized using ECL reagent (Amersham).

WB were performed by using the following antibodies: anti-6xHis-HRP conjugated antibody (Sigma).

3. Results

3.1. Optimal protocol for the expression of the scDb-hERG1/β1 antibody

scDb-hERG1/β1 antibody is directed against hERG1 ion channel and β1 integrin [Duranti et al., submitted to MCT, 2021]. The scDb-hERG1/β1construct was developed engineering two single chain Fragment variable (scFv) antibodies, one directed against hERG1 (scFv-hERG1 from mAb-hERG1) and one against the β1 integrin (scFv-β1 from mAb-β1 TS2/16). The scFv-hERG1 was developed in the format V_H-linker-V_L, while the scFv-β1 was developed in the format V_L-linker-V_H and the strategy adopted to obtain the final scDb-hERG1/β1construct was that of cloning the scFv-β1 in place of the scFv-hERG1 linker. The construct expressing the antibody has been cloned in a vector suitable for *Pichia pastoris* expression, which is pPIC9K vector. The final construct has been mutagenized in order to restore a Cys amino acid, fundamental for the disulfide bond formation, as described in detail in Duranti et al., 2018 [15]. Subsequently, GS115 *Pichia pastoris* strain was transformed, according to the spheroplasting protocol.

Briefly, frozen stocks colonies have been striated and let grown in YPD plates for 48 h, afterwards a methanol based induction protocol (modified from *Pichia Expression kit*) using glycerol, BMGY and methanol, BMMY media has been performed. Methanol has been added every

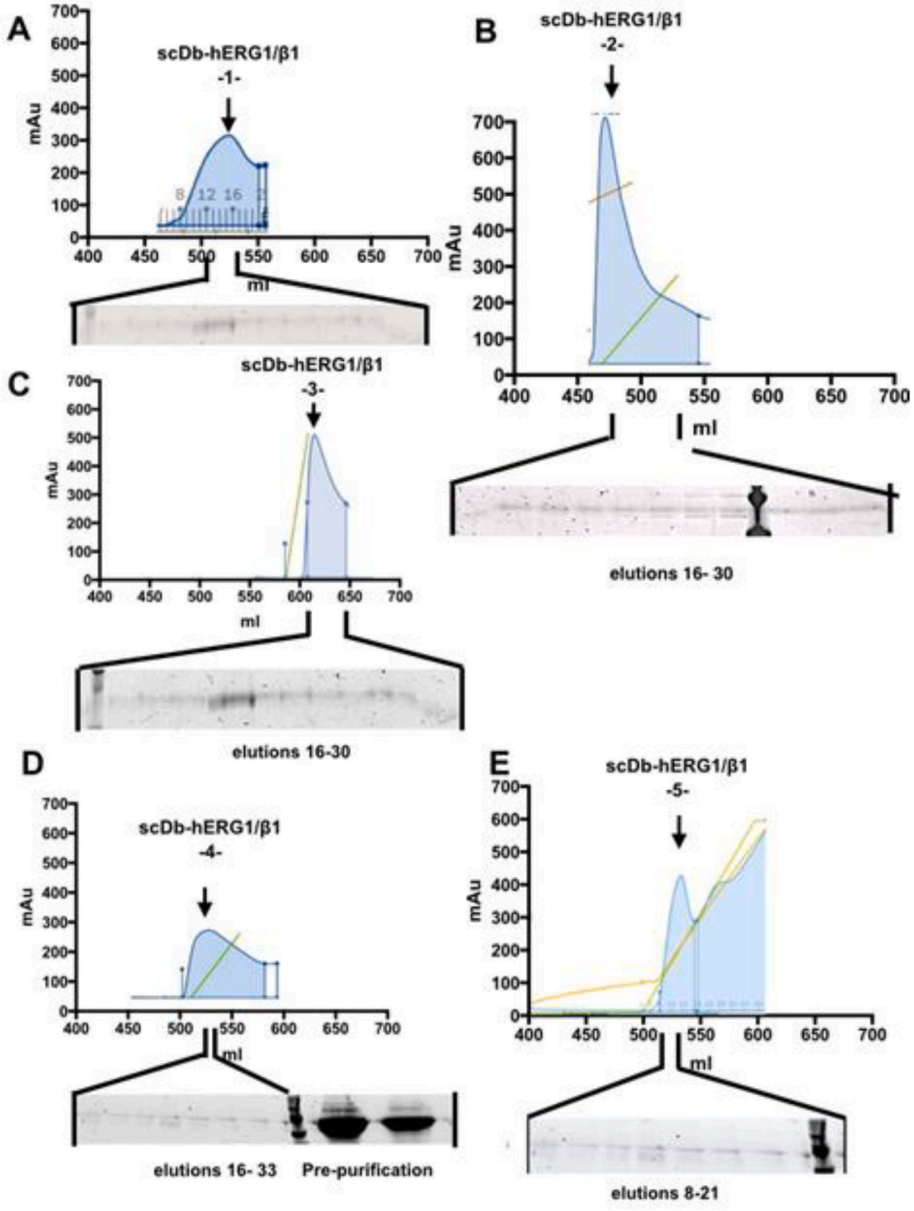

Fig. 1. (A–E) Chromatogram and SDS-Comassie Brilliant blue staining of scDb-hERG1/β1-G5 clone among the five different preparations analyzed.

24h, to a final concentration of 0.5% for 72h, to induce antibody expression. At the end of 72h, the entire volume was centrifuged in order to precipitate the yeast cells and recover the supernatant where the protein of interest is located, since the construct coding for the scDb-hERG1/β1 antibody was cloned in frame with the sequence for the secretion signal (α-factor). This peptide allows the translocation of the protein in the endoplasmic reticulum and the subsequent cutting by a peptidase in the reticulum lumen. Then the protein, after having undergone folding processes, is conveyed into the Golgi apparatus and from here, into the extracellular space.

A crucial point was the refresh of the scDb-hERG1/β1 frozen stocks. To this purpose, colonies capable of growing in the presence of 15 mg/ml Geneticin were selected, grown in 50 ml liquid culture (in the presence of Geneticin) and screened for protein secretion by dot blot. The scDbs from either G5 or 3G9 colonies were stable at +4 °C for at least 10 weeks.

3.2. Expression of the two best clones resulting from spheroplasting transformation

The G5 clone has been tested in five different preparations, starting from 1L culture, hereafter named scDb-hERG1/β1G5 with a progressive number from 1 to 5. While for the 3G9 clone, 12 different preparations, starting from different volumes, hereafter named scDb-hERG1/β1-3G9 with a progressive number from 1 to 12 have been tested. Within the latter, we have pointed out a comparison between two different purification machines, Akta Pure and Akta Start, giving rise to a gold standard protocol not only for protein expression, but also for purification.

3.3. Purification of G5 and 3G9 clones

For the purification of the scDb-hERG1/β1G5 protein, ÄKTA Pure chromatography system has been used, based on the application of different elution buffers with increasing imidazole concentrations. Washing steps were programmed in the AKTA apparatus with an imidazole-free wash buffer, while the elution was performed with a

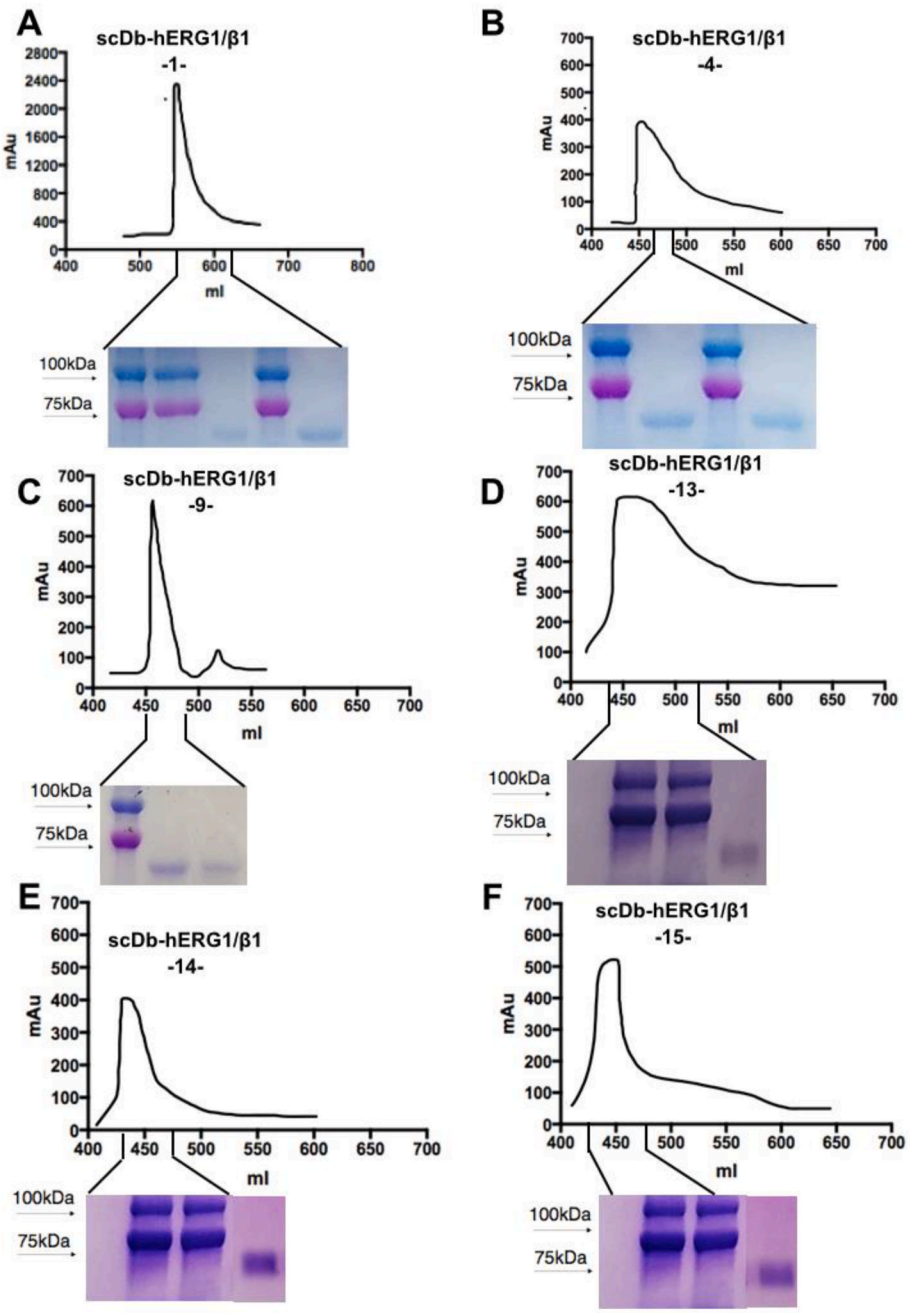

Fig. 2. (A–F) Chromatogram and SDS-Comassie Brilliant blue staining of scDb-hERG1/β1-3G9 clone among five different preparations analyzed. (A) Chromatogram obtained from 1L supernatant purification (upper panel) from induction 1 reported in Table 2 and Coomassie staining analyzing reunited fractions underneath the peak, scDb-hERG1/β1 molecular weight around 60 KDa (lower panel). (B) Chromatogram obtained from 4L supernatant purification (upper panel) from induction 4 reported in Table 2 and Coomassie staining analyzing reunited fractions underneath the peak, scDb-hERG1/β1 molecular weight around 60 KDa (lower panel). (C) Chromatogram obtained from 1L supernatant purification (upper panel) from induction 9 reported in Table 2 and Coomassie staining analyzing reunited fractions underneath the peak, scDb-hERG1/β1 molecular weight around 60 KDa (lower panel). (D) Chromatogram obtained from 1L supernatant purification (upper panel) from induction 13 reported in Table 2 and Coomassie staining analyzing reunited fractions underneath the peak, scDb-hERG1/β1 molecular weight around 60 KDa (lower panel). (E) Chromatogram obtained from 1L supernatant purification (upper panel) from induction 13 reported in Table 2 and Coomassie staining analyzing reunited elutions underneath the peak, scDb-hERG1/β1 molecular weight around 60 KDa (lower panel). (F) Chromatogram obtained from 1L supernatant purification (upper panel) from induction 15 reported in Table 2 and Coomassie staining analyzing reunited fractions underneath the peak, scDb-hERG1/β1 molecular weight around 60 KDa (lower panel).

buffer having increasing imidazole concentrations. Chromatogram analysis were carried out using UNICORN 7.0 software.

For all the preparations, the eluted antibody has been dialyzed by replacing the solution containing imidazole with a physiological solution PBS 1X (using Slide-A-Lyzer™ Dialysis Cassettes, Thermo Fisher), an essential step, since imidazole is an interferer that may alter subsequent applications in which the antibody is used. For each induction, we report the Coomassie brilliant blue gel and chromatogram, Fig. 1 A-E and Fig. 2 A-F and we summarize in Table 1 and Table 2 the quantification using Nanodrop spectrophotometer. Hereafter, we report data obtained first with scDb-hERG1/β1G5 and then with scDb-hERG1/β1-3G9. As shown by the chromatograms in Fig. 1A, scDb-hERG1/β1G5-1- has an elution peak at 280 nm of 150 mAu. The scDb-hERG1/β1 antibody was dialyzed by replacing the solution containing imidazole with a physiological solution PBS 1X (using Slide-A-Lyzer™ Dialysis Cassettes, Thermo Fisher).

The eluate was subsequently analyzed by SDS page and the gel revealed with Coomassie Blue.

From the image, it is possible to observe the scDb-hERG1/β1G5 protein, weighing about 60 kDa, begins to be present in the 8th elution and is traceable in the subsequent elution fractions, up to the 18th. The protein was quantified by Nanodrop spectrophotometer measurement and the value obtained was $A_{280} = 0.724$, used in the Lambert-Beer equation for the calculation of the concentration of the protein of interest: $0.724/98210\ M^{-1}\ cm^{-1} * 1\ cm * 67740.56\ g/mol = 0.499\ \mu g/\mu l$.

Subsequently, the antibody was subjected to concentration through the use of Centricon: 0.5 mg/ml putting together all the fractions reported in Coomassie for a total of 2.5 ml brought to a final volume of approximately 1.85 ml per 0,950 mg.

For scDb-hERG1/β1G5-2, we have obtained a larger amount of protein, as it is also possible to infer from the Coomassie with thicker bands, containing a larger amount of protein. The protein has been

Table 1
The comparison of the five different preparations showed a significant improvement from the concentration steps using Centricon.

Name of preparation	Absorbance 280 nm	Use of	Total amount
	A_{280}	Centricon/Volume after concentration	
scDb-hERG1/β1G5 -1-	0.724	Yes From 2.5 ml to 1.85 ml	0.950 mg
scDb-hERG1/β1G5 -2-	1.15	Yes From 5 ml to 2.8 ml	2.8 mg
scDb-hERG1/β1G5 -3-	0.87	Yes From 2.5 ml to 1.8 ml	1 mg
scDb-hERG1/β1G5 -4-	0.65	No	1 mg
scDb-hERG1/β1G5 -5-	1.06	Yes From 8 ml to 4 ml	3.2 mg

processed using Centricon. Also the stability is deeply affected by concentration, as it is satisfactory only after Centricon processing.

In Fig. 1A-E we report the chromatograms and the SDS-Comassie Brilliant blue staining, concerning five different preparations, regarding which we consider the absorbance A_{280} obtained after the dialysis, the concentration rate using Centricon devices and finally the total amount of protein obtained from each induction. All these data are collected in Table 1 reported below.

Among the different preparations, scDb-hERG1/β1-5-, with a concentration of 3.2 mg/ml, resulted to be the highest in terms of yield. The other preparations, besides resulting in lower amount of protein (Table 1) were not kept sufficiently concentrated, giving rise to antibody degradation processes.

We have also purified 15 different inductions deriving from 3G9 clone, as reported in Table 2.

We have shown that the amount of protein obtained with Akta Pure is not only comparable with that obtained using Akta Start, but even better. Moreover, we report the comparison between the chromatograms obtained with Akta Pure and Akta Start, showing comparable peaks (Fig. 2, A-F).

Chromatograms obtained with Akta Start show a less spiky peak, having the characteristics of a more round-shaped peak, consistent with the fact that the amount of protein underneath the peak is higher (e.g. Figs. 2C and 1 A). Coomassie staining shows neat bands (with a molecular weight of roughly 60 KDa), for all the preparations analyzed. Band thickness is proportional to the amount of protein from each preparation. Also in this case, the stability is deeply affected by concentration, as it is satisfactory only after Centricon processing, as shown in Fig. 3.

4. Discussion

The present work focuses on the development and optimization of a protocol for the expression and purification of a single chain diabody, scDb-hERG1/β1, which targets the hERG1/β1 oncogenic unit, selectively expressed in tumors [13,14].

The data we present are tuned on the expression of different products from two colonies, both producing the antibody scDb-hERG1/β1, which derive from independent *Pichia pastoris* yeast cell transformations. The final goal was the setting up of a gold standard expression protocol and the comparison of the yields obtained using two different purification machines: Akta Pure and Akta Start.

In particular, the first achievement was to determine a proper Expression protocol, which was favored by a continuous yeast culture and by an optimal starting volume, which has turned out to be roughly 1L *Pichia pastoris* culture.

A major problem of bAbs production is the set up of a valuable expression and purification protocol. In the present paper, we have produced two different clones G5 and 3G9, which resulted both in giving good protein yields. It is possible to infer from Table 1 an average of 1,2 mg/L for G5 clone, while data reported in Table 2 show an average production of the 3G9 antibody of roughly 2 mg/L. Nevertheless, some obstacles have emerged, as for the purification of G5 clone, Table 1

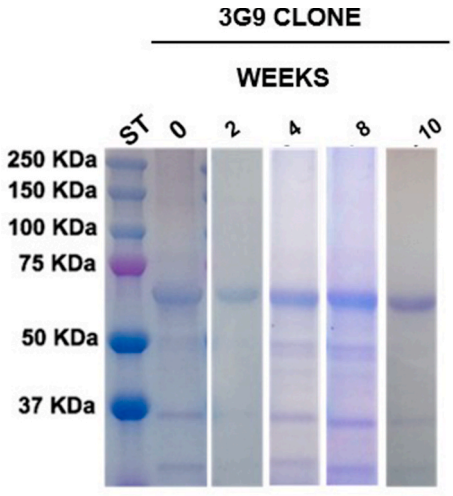

Fig. 3. Coomassie showing Coomassie Brilliant Blue of the 3G9-scDb-hERG1/β1, showing stability rate after 2, 4, 8, 10 weeks at 4 °C.

Table 2
The comparison of yields and machine used for the purification of fifteen different preparations.

Induction	Elution (ml)	Conc. Nanodrop (μg/μl)	Volume Post conc.	Conc. Post centricon	Amount	Akta Machine used
1 (1L)	21 ml	0.121 μg/μl	420 μl (50x)	5.87 μg/μl	2.46 mg	AKTA Pure
2 (1L)	13 ml	0.057 μg/μl	129 μl (100x)	5.7 μg/μl	735 μg	AKTA Pure
3 (2L)	14,4 ml	0.28 μg/μl	720 μl (20x)	5.6 μg/μl	4 mg	AKTA Pure
4 (4L)	**90 ml**	**0.053 μg/μl**	**900 μl (100x)**	**5.3 μg/μl**	**4.7 mg**	**AKTA Pure**
5 (3L)	35 ml	0.045 μg/μl	1750 μl (20x)	1 μg/μl	1.75 mg	AKTA Pure
6 (1L)	4 ml	0.024 μg/μl	200 μl (20x)	0.48 μg/μl	960 μg	AKTA Pure
7 (1L)	1,5 ml	0.032 μg/μl	75 μl (20x)	0.64 μg/μl	48 μg	AKTA Start
8 (1L)	3 ml	0.048 μg/μl	150 μl (20x)	0.96 μg/μl	144 μg	AKTA Start
9 (1L)	**32,5 ml**	**0.072μg/μ**	**1625 μl (20x)**	**2.34 μg/μl**	**3.80 mg**	**AKTA Pure**
10 (1L)	20 ml	0.038 μg/μl	1000 μl (20x)	0.76 μg/μl	760 μg	AKTA Start
11 (1L)	16 ml	0.081 μg/μl	800 μl (20x)	1.62 μg/μl	1.3 mg	AKTA Start
12 (1L)	22 ml	0.079 μg/μl	1100 μl (20x)	1.58 μg/μl	1.74 mg	AKTA Pure
13 (1L)	**22 ml**	**0.187 μg/μl**	**1100 μl (20x)**	**3.74 μg/μl,**	**4.11 mg**	**AKTA Start**
14 (1L)	**22 ml**	**0.185 μg/μl**	**1100 μl (20x)**	**3.7 μg/μl**	**4.07 mg**	**AKTA Start**
15 (1L)	**22 ml**	**0.175 μg/μl**	**1100 μl (20x)**	**3.5 μg/μl**	**3.85 mg**	**AKTA Start**

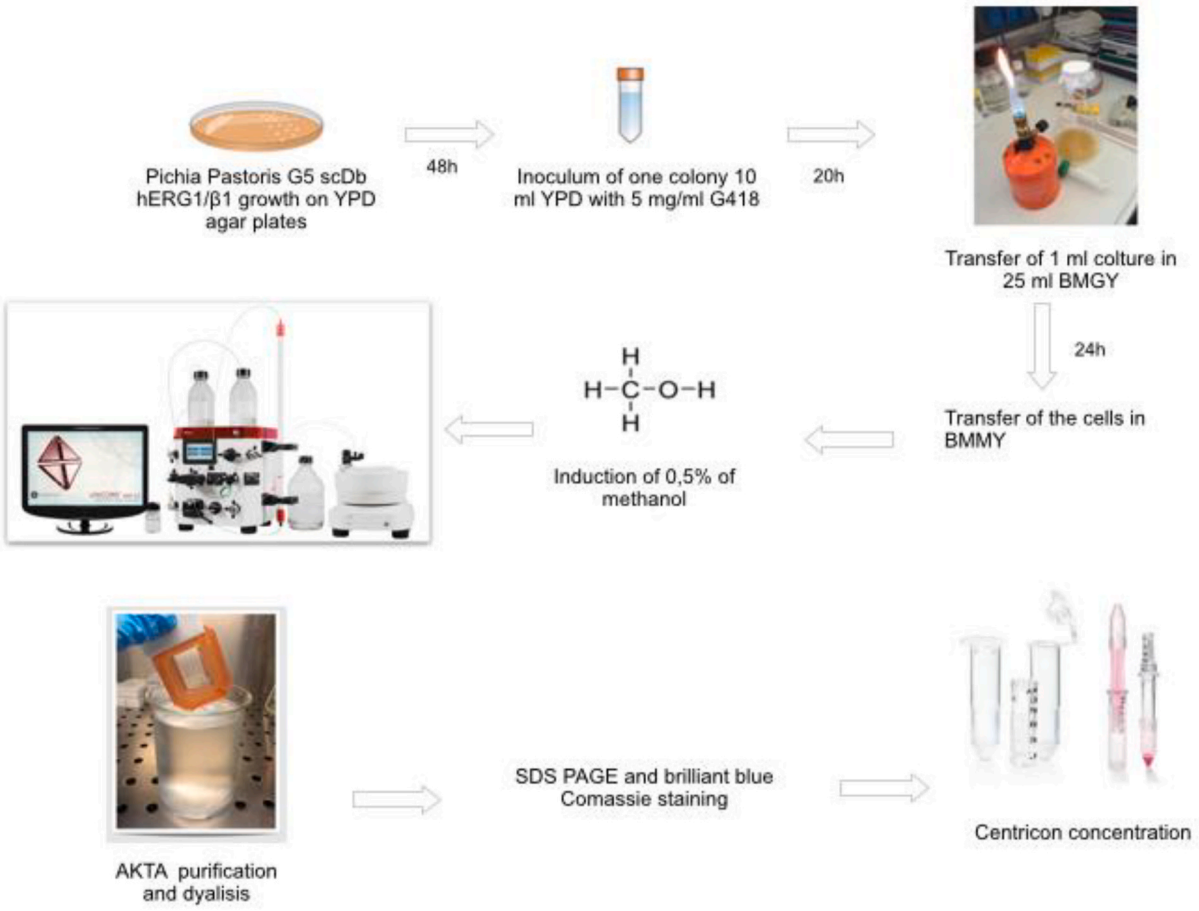

Fig. 4. Flow chart reporting the *gold standard* protocol optimized for the induction of *Pichia pastoris* scDb hERG11/β1 clone, showing the different steps starting from the YPD plate inoculum to the transfer in BMGY and subsequently in BMMY, addiction of methanol, AKTA purification, dialysis, SDS PAGE with Comassie staining and Centricon concentration.

shows improved yields for the preparations processed with Centricon devices in comparison with Preparation 4 which was not concentrated. Also Coomassie staining show better bands for the 3G9 clone. The latter not only has been concentrated using Centricon, but was also purified using Akta Start machine. In our experience, in fact, it is worth noting that in Table 2 we have compared different purifications using Akta Pure and Akta Start showing that yield is not affected by the use of one of the two machines. In particular, the use of Akta Start allows to maintain yield rates, a single peak chromatogram and all the characteristics shown by the preparations purified using Akta Pure, demonstrating to be a completely efficient machine for antibody purification.

Phenomena implicated in such degradation could be ascribed to suboptimal culture temperature, instrument's malfunction (shaker, incubator for yeast cultures etc.), inconstant frozen conditions with a consequent reduction of cellular efficiency. In fact, storing proteins at room temperature can lead to protein degradation, inactivation, or microbial growth. Particularly stable proteins may be kept at 4 °C in a simple storage buffer, as it occurs for the scDb-hERG1/β1 antibody, for both clones. As, at this temperature, the sample is more easily dispensed avoiding thawing and refreezing cycles. Moreover, we have given evidences that maintaining the antibody concentrated, using Centricon devices, is crucial for its stability, which we have demonstrated to be remarkable, up to 10 weeks (Fig. 3).

Given the obtained results, we thus highlight a protocol shown in the flow-chart reported in Fig. 4, as a possible "gold standard" since it places emphasis on overcoming any of the phenomena listed above and in particular on the conservation of a sufficiently concentrated protein, through the use of Centricon devices.

CRediT authorship contribution statement

Claudia Duranti: Investigation, Formal analysis, Methodology, Visualization, Writing – original draft, Supervision. **Elena Lastraioli:** Investigation, Formal analysis, Data curation, Supervision. **Jessica Iorio:** Investigation, Formal analysis, Data curation, Supervision. **Chiara Capitani:** Investigation, Formal analysis. **Laura Carraresi:** Investigation. **Leonardo Gonnelli:** Investigation, Resources. **Annarosa Arcangeli:** Investigation, Formal analysis, Methodology, Visualization, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

C.D., L.C. and A.A. are named inventors on a patent covering the scDb-hERG1-β1 antibody and derivatives thereof. A.A. is co-founder of MCK Therapeutics Srl, spin off of the University of Florence, that owns the license of the patent. All other authors declare no potential conflicts of interests.

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