nuclera

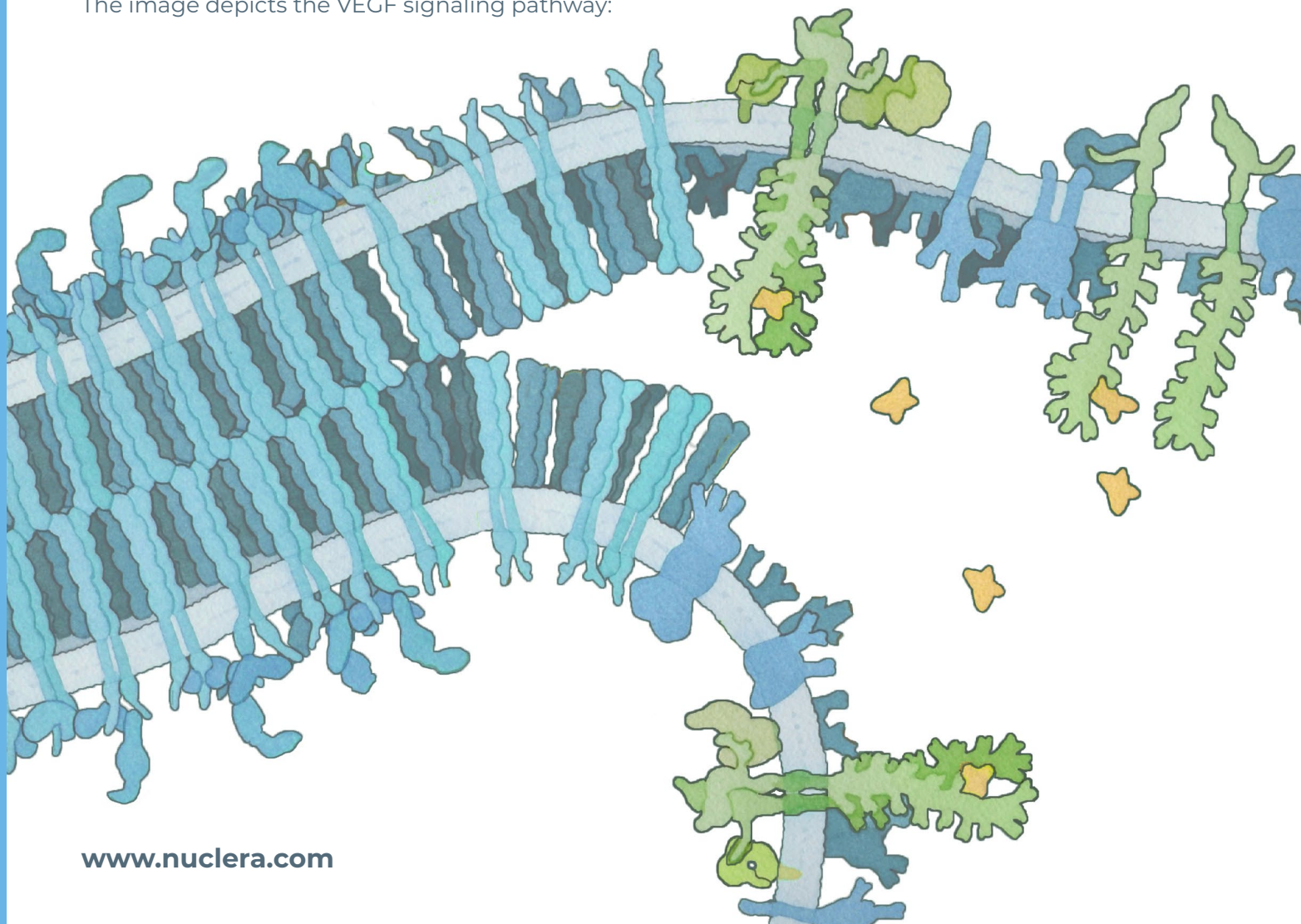
Application Note

Rapid cell-free expression and solubility screening to obtain active human VEGF with eProtein Discovery™ platform

Marco Manni, Sunidhi Shetty and Eleonora Bassu

VEGF is a key regulator of angiogenesis and plays a central role in the process of tumor growth, making it an appealing target for anticancer therapeutics. Using the eProtein Discovery™ platform, we demonstrated that the ideal construct and conditions for expressing soluble VEGF can be determined within 24 hours. This platform demonstrates time-saving for both soluble construct identification and micrograms of active proteins obtained in 48 hours.

The image depicts the VEGF signaling pathway:



Protein Details

Human vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis and plays a central role in the process of tumor growth and metastatic dissemination. VEGF also has involvement with neovascular age-related macular degeneration and rheumatoid arthritis[1,2]

VEGF protein has previously been recombinantly produced *in vivo* using yeast, insect and mammalian cell expression systems. Expressing this protein in *E.coli* has proven to be challenging due to the inherent cysteine-knot motif in human VEGF proteins that can result in protein misfolding and the formation of inclusion bodies[3]. These challenges have been illustrated in one study, where human recombinant VEGF was expressed as inclusion bodies, solubilized in buffer containing urea, purified and then subjected to protein refolding and renaturation.[4] Expressing soluble VEGF in *E.coli* is only possible in the presence of solubility fusion tags such as maltose-binding protein (MBP). Moreover, MBP-VEGF proteins can only express successfully in *E.coli* Origami 2 (DE3) strains which carry specific gene mutations that facilitate proper disulfide bond formation[5]. Optimizing protein expression in *E.coli* cells can be laborious and time consuming, taking days to weeks to discover the ideal condition to express and purify soluble proteins. *E.coli* protein expression involves cloning the protein of interest in various expression plasmids encoding different solubility tags, trying expression in different *E.coli* cell lines and optimizing different expression conditions.

In this eProtein Discovery™ application note, we aim to show that the ideal construct and conditions for expressing soluble active VEGF can be determined within 24 hours using a nanoliters scale cell-free protein expression and purification system. The conditions for soluble expression are validated through scale up expression and purification in tubes. This platform demonstrates time-saving for both soluble construct identification and micrograms of active proteins obtained in 48 hours.

An Overview of Cell-Free Protein Synthesis

Cell-free protein synthesis (CFPS) shown in Figure 1, is a technique which enables protein expression *in vitro* without the need for living cells. The eProtein Discovery™ system uses CFPS to produce proteins. By combining DNA constructs (eGenes) with CFPS reagents and optional additives, functional target proteins can be generated in a matter of hours.

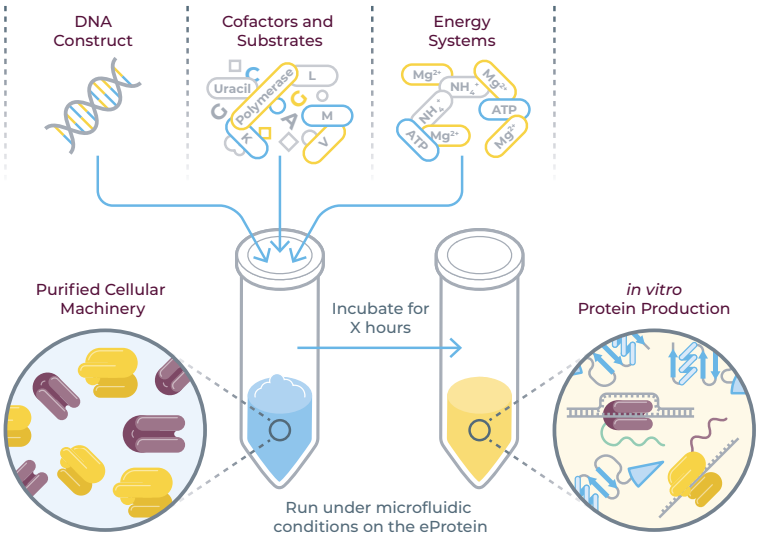


Figure 1. Overview of cell-free protein synthesis within the eProtein Discovery™ System

eProtein Discovery™ platform and experimental design

The eProtein Discovery™ system is an integrated platform that enables rapid protein prototyping by:

(a) Solubility assessment of protein constructs with varying solubility fusion tags
(b) Cell-free protein expression in customizable conditions
(c) Soluble expression yield quantitation
(d) Bead-based protein purification
(e) Purified protein yield determination

This platform enables quick solubility and purified yield assessment of proteins with different solubility tags.

Our detection system is based on an improved split-green fluorescent protein (GFP) system. Within the Smart Cartridge system, each eGene™ (DNA construct) is designed to encode a small detection tag (17 amino acids, 1.5 kDa). Upon soluble expression of the protein of interest, the co-expressed detection tag will bind to the complementary detector protein, (Figure 2) which is added at the end of the cell-free protein expression process. The complementation of detector tag with detector protein will result in emission of fluorescent signals, captured by the eProtein Discovery instrument. The fluorescent intensity emission is proportional to the amount of soluble proteins present.

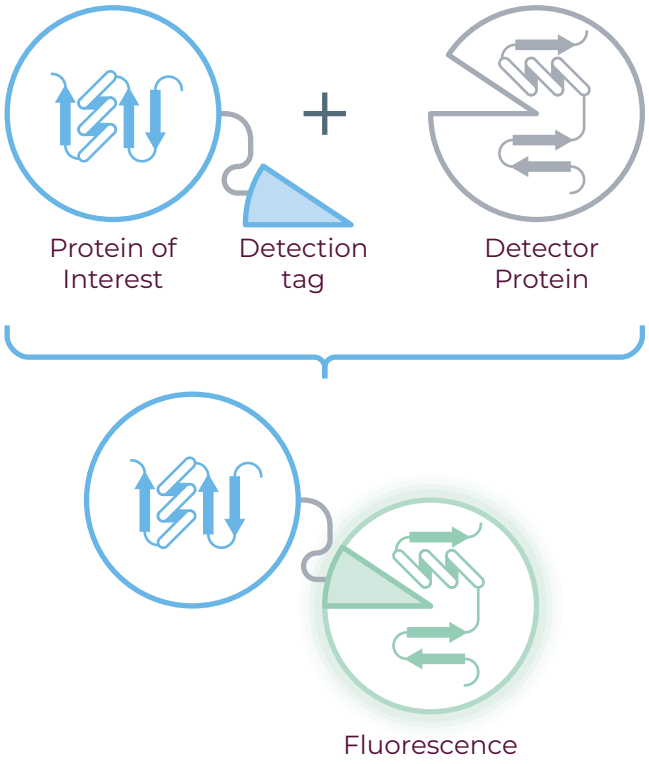


Figure 2. A schematic showing fluorescence complementation technology used within the Smart Cartridge to detect soluble protein expression.

eProtein Discovery™ workflow

The workflow timeline in Figure 3 outlines the process involved to obtain soluble, active protein. The workflow begins with the design of DNA constructs on our proprietary eProtein Discovery™ software, followed by on-cartridge protein solubility screening and purifiable yield determination. The winning constructs will then be selected for larger scale expression off-platform using the optimal expression conditions discovered from the on-cartridge screen.

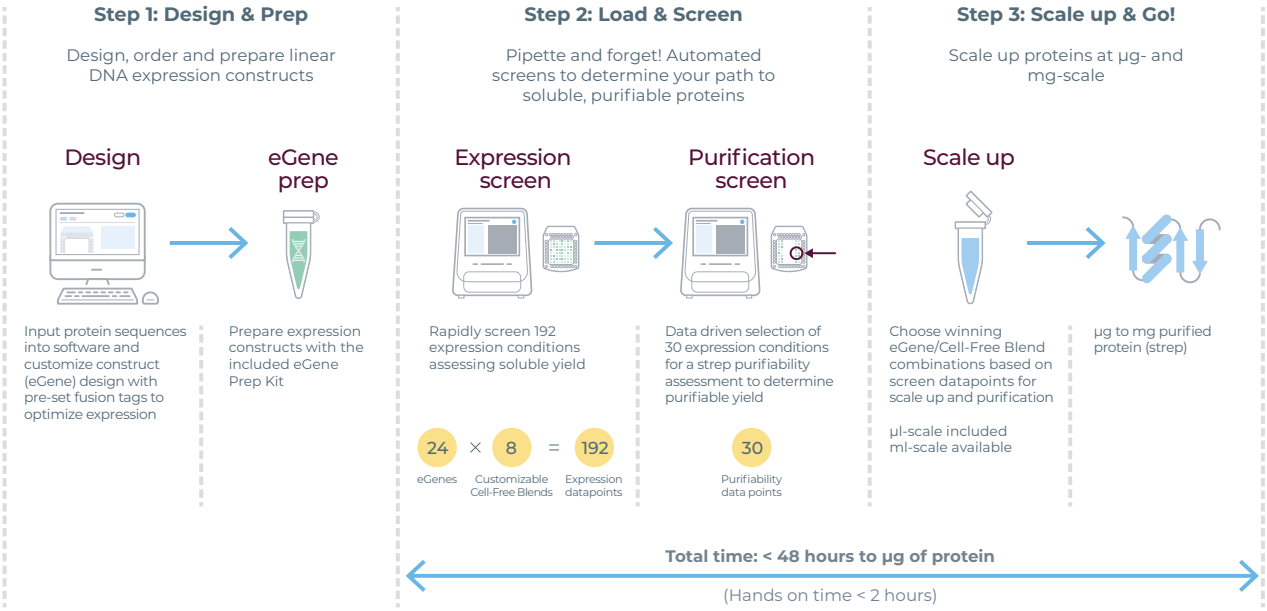


Figure 3. eProtein Discovery™ workflow. An illustration of the steps involved from eProtein Discovery™ construct design to protein production.

The eProtein Discovery™ system allows you to optimize protein expression by creating different combinations of 24 DNA constructs (eGenes™) and 8 Cell-Free Blends (Cell-Free Core reagents + additives) to get your protein in hand quicker. The eProtein Discovery system will automate the screen of 24 different eGene™ constructs vs. 8 Cell-Free Blends and determine soluble yield in each of the 192 conditions. A customizable subset of the combinations will be further screened for purifiability. With solubility and purifiability metrics in hand, you can select combinations to scale up on the bench, next day.

Output results are clearly displayed by the eProtein Discovery™ software which facilitates the correct choice of eGenes™ and Cell-Free Blends with results shown in the form of protein expression predictive yield, solubility and purifiability.

In this case study with human VEGF, 8 protein constructs were designed using an iterative cloud based platform. These 8 protein constructs were generated with different solubility tags attachment in either the N- or C-terminus. STREP fusion was included for purification purposes (Table 1).

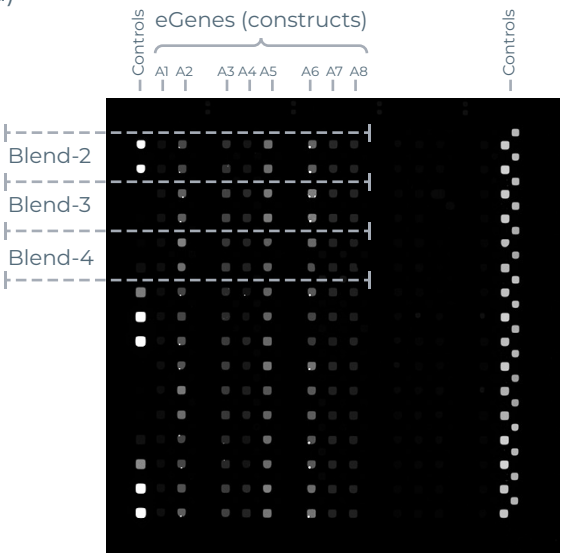
Label	Description	Label	Description
A1	STREP_VEGF_DET	A5	SUMO_VEGF_DET
A2	DET_VEGF_STREP	A6	TRX_VEGF_DET
A3	DET_STREP_VEGF	A7	VEGF_STREP_SUMO_DET
A4	VEGF_STREP_DET	A8	VEGF_STREP_TRX_DET

Table 1. DNA constructs corresponding to VEGF with various solubility and detector (DET) tags in the N- and C- terminus.

VEGF expression and purification on eProtein Discovery

An expression and solubility screen was first performed to determine the optimal expression of human VEGF within the eProtein Discovery™ platform using three different Cell-Free Blends (Cell-Free Core reagents + additives) in combination with constructs containing 8 different tag variations. These three Cell-Free Blends consist of different cell-free protein synthesis reagents. Blend-3 is designed to promote disulfide bond formation while Blend-4 contains chaperones that promote proper protein folding. Blend-2 contains the core component of the cell-free synthesis reagents without any additional components. (Figure 4a).

(a)



Label	Description
A1	STREP_VEGF_DET
A2	DET_VEGF_STREP
A3	DET_STREP_VEGF
A4	VEGF_STREP_DET
A5	SUMO_VEGF_STREP_DET
A6	TRX_VEGF_STREP_DET
A7	VEGF_STREP_SUMO_DET
A8	VEGF_STREP_TRX_DET

(b)

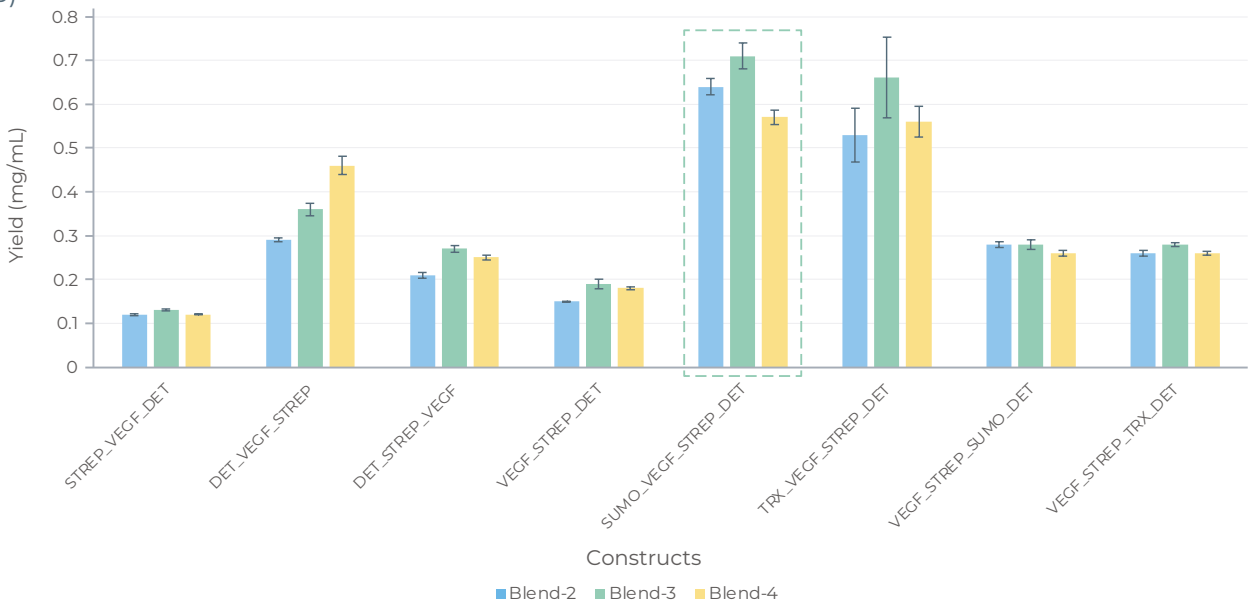


Figure 4. Expression screen data. (a) The adjoining table illustrates the DNA constructs corresponding to VEGF with various solubility tags in the N- and C- terminus. Each construct was run in duplicate. The dotted lines denote the Cell-Free Blends run with the corresponding constructs. The position of the detector (DET) controls are also noted. Bright white spots are droplets with expression of soluble proteins. (b) Yield of proteins expressed is calculated based on fluorescent intensity measured. Construct A5 (SUMO_VEGF_STREP_DET) produced the highest expression in Blend-3. Constructs with the highest yield (mg/mL) were selected for on-cartridge purification (highlighted in green dashed box).

The eProtein Discovery system houses a detector that is able to accurately measure the yield of proteins produced from the screen for each of the constructs (Figure 4b). VEGF construct with N-terminus SUMO fusion tag exhibits the highest expression yield in Blend-3; a cell-free expression condition that promotes disulfide bond formation. This result is in agreement with a prior publication showing that VEGF expresses well with a solubility fusion tag, in an *E.coli* strain that helps disulfide bond formation.

Following soluble expression detection, eGene construct and Cell-Free Blends that yield the highest expression were automatically selected by the eProtein Discovery™ system for an on-cartridge purification assessment.

Three soluble candidates that presented the highest expression yield were automatically selected and taken forward for an on-cartridge purification step. Among these candidates were SUMO-VEGF expressed in Blend-2, Blend-3 and Blend-4, in duplicate. These expression candidates were purified on the Smart Cartridge using strep beads. Figure 5 shows the location of SUMO_VEGF_STREP_DET purified on the Smart Cartridge. Fluorescent intensity measurement was used to determine yield of the purified proteins.

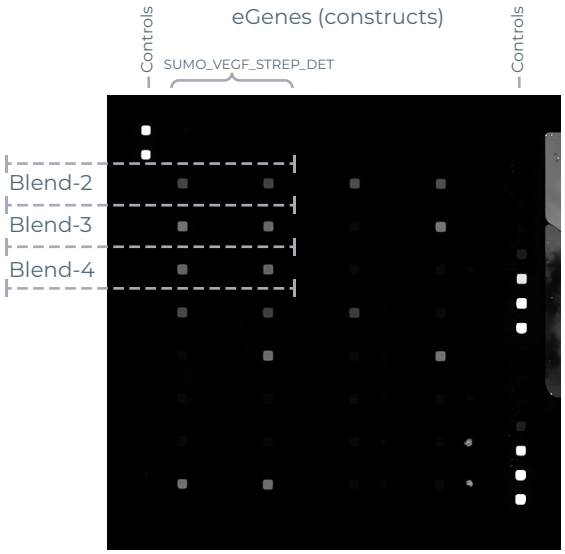


Figure 5. On-cartridge purification. Purification assessment was performed in duplicates. Proteins expressed in cell-free reagents were purified using strep beads. Purified proteins were highlighted in dotted boxes.

The 'winning' construct was SUMO_VEGF_STREP_DET which was shown to produce reproducible expression levels between the duplicates run.

Figure 6 shows a side by side comparison of the expression yield and purified yield for SUMO_VEGF_STREP_DET. The results show that this construct, expressed in all three Cell-Free Blends, is soluble and can be purified. Blend-3 yields the highest protein expression and purified yield and therefore is chosen for off-cartridge scale up expression.

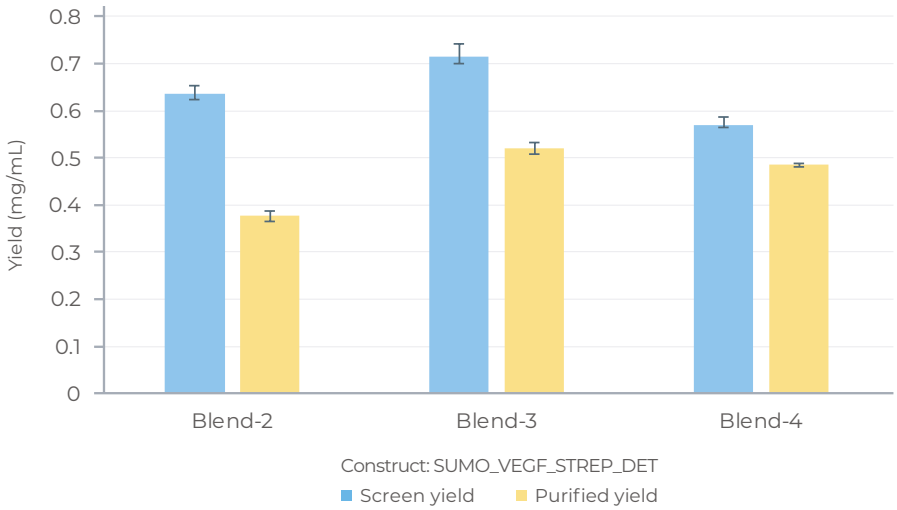


Figure 6. On-cartridge screen and purification yield comparison. This graph shows the yields measured on the Smart Cartridge at the Expression Screen and Purification Screen stages in three different Cell-Free Blends.

Scale up expression and purification

On-cartridge expression and purification revealed that SUMO_VEGF_STREP_DET expression in Blend-3 led to the highest purification yield, therefore this reagent was chosen for off-cartridge production and purification (Figure 7).

Linear DNA construct corresponding to SUMO_VEGF_STREP_DET was added to Blend-3 expression reagent in tubes and incubated overnight. Expressed proteins were purified using beads. Sumo-tagged VEGF proteins were eluted in 100 µL elution buffer and the eluted proteins were >95% pure as seen in figure 7 SDS-PAGE gel analysis. The final purified yield of SUMO-VEGF is 52 µg of proteins obtained.

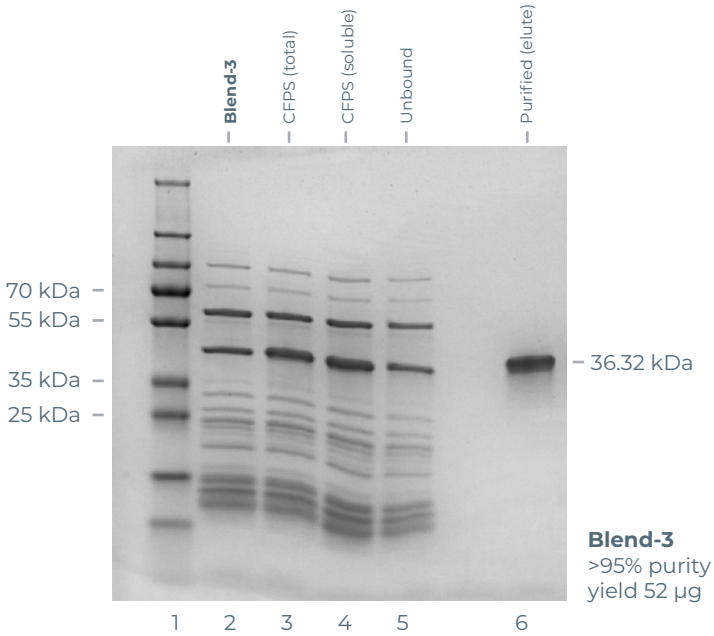


Figure 7. Off-cartridge, scale-up expression and purification of SUMO_VEGF_STREP_DET. SDS-PAGE illustrates the VEGF protein with a molecular weight slightly higher than the predictive weight of 36.32 kDa as the SUMO tag normally shows 6-10 kDa higher than its calculated molecular weight on an SDS-PAGE gel. Lane 1: Molecular weight ladder, Lane 2: Blend-3 negative control, Lane 3: CFPS total after expression, Lane 4: CPFS (soluble) obtained following centrifugation and supernatant retained, Lane 5: Unbound; flow through sample not bound to beads, Lane 6: Purified (elute); sample eluted off of the functionalized beads.

Measurement of VEGF protein activity

Purified SUMO_VEGF_STREP_DET protein was used in the cell-based PathHunter® Bevacuzimab Bioassay to determine the activity of VEGF. The ability of VEGF to bind VEGFR2 receptors on the cell surface resulted in dimerization of the two receptor subunits. This process triggers downstream β-galactosidase (β-gal) enzyme activity and subsequently the release of fluorescent signals. Signals detected were proportional to the presence of active VEGF (Figure 8). SUMO_VEGF protein was found to be active with an EC50 read out of 12.49 ng/mL.

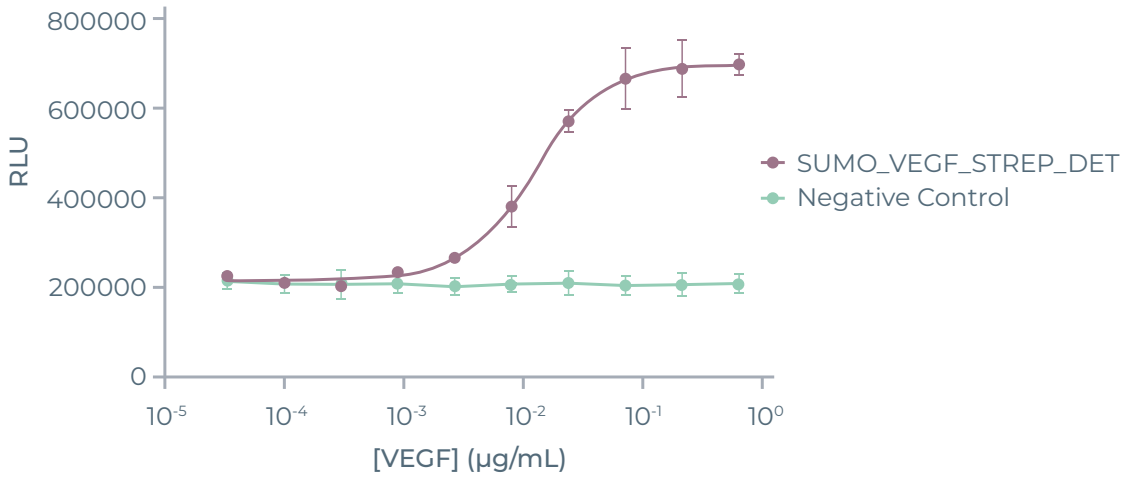


Figure 8. Activity testing of VEGF protein produced with eProtein Discovery. The cell-based PathHunter® dimerization assay was used to observe a change in substrate presence, which was reported in relative light units (RLU) indicating the presence of active protein. Two biological replicates, shown here as a mean, were carried out on two separate occasions. The VEGF protein displayed an EC50 of 12.49ng/mL. PathHunter® is a registered trademark of Eurofins DiscoverX as used in US and/or in other countries.

VEGF protein validation data summary and conclusions

VEGF is a difficult candidate to express in the E.coli expression system[2,3,4,5]. Expression of tag-less VEGF tends to end up with inclusion body formation. To obtain soluble proteins, expressing VEGF with multiple combinations of solubility tags is required to determine the right construct for successful expression and purification. The conventional cell-based expression and purification approaches can be time consuming and laborious, considering the variety of constructs that need to be tested. We have demonstrated that the eProtein Discovery™ platform enables rapid construct expression screening and selection of soluble, purifiable candidates. VEGF with N-terminus SUMO tag gave the highest expression yield in Blend-3. Results generated on the platform were validated through an off-cartridge cell-free expression. The expressed proteins were purified to high purity and yield; comparable to VEGF proteins produced in cell-based expression platforms. The purified SUMO_VEGF protein demonstrated high functional activity within a commercial validated assay. The eProtein Discovery™ is an essential tool for rapidly obtaining active proteins in hand for use in downstream applications.

References

1. Carneliet, P. Angiogenesis in health and disease. *Nature. Medicine* 9, 663-660 (2003).

2. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other diseases. *Nature. Medicine* 1, 27-31 (1995).

3. Lee, S. *et al*. Overproduction of recombinant human VEGF (Vascular Endothelial Growth Factor) in Chinese Hamster Ovary Cells. J. *Microbiol. Biotechnol* 18 (1), 183-187(2008).

4. Lee, I. *et al*. Prokaryotic expression, refolding, and purification of functional human vascular endothelial growth factor isoform 165: Purification procedures and refolding conditions revisited. *Protein Expression and Purification* 76, 54-58 (2011).

5. Nguyen, M. *et al*. Prokaryotic Soluble Overexpression and Purification of Human VEGF165 by Fusion to a Maltose Binding Protein Tag. PLOS ONE 11(5): e0156296. doi:10.1371/journal.

About us

Nuclera is making it possible to obtain active proteins from DNA in 48 hours through its benchtop eProtein Discovery™ platform. Headquartered in Cambridge, UK, Nuclera was founded in 2013 by four PhD students at the University of Cambridge who were motivated to make proteins accessible.

nuclera

Cambridge, UK
Boston, MA USA
Email: hello@nuclera.com

www.nuclera.com