Reproducible and Easy Production of Mammalian Proteins by Transient Gene Expression in High Five Insect Cells

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Running head: Transient plasmid-based protein production in High Five

Abstract

The expression of mammalian recombinant proteins in insect cell lines using transient plasmid-based gene expression enables the production of high-quality protein samples. Here, the procedure for virus-free transient gene expression (TGE) in High-Five insect cells is described in detail. The parameters that determine the efficiency and reproducibility of the method are presented in a robust protocol for easy implementation and set-up of the method. The applicability of the TGE method in High Five cells for proteomic, structural and functional analysis of the expressed proteins is shown.

Key words: Transient Gene Expression, TGE, High Five, Insect cells, Expression vector

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1 Introduction

High quality protein samples are essential for structural, proteomic and functional analysis of biological processes [1, 2, 3]. Especially, the current 2019-CoV pandemic shows the importance of reliable recombinant expression systems that are able to produce ample amounts of correctly folded viral and host proteins. These proteins may be used as tools in diagnostic screening, establishing assays for drug-screening, vaccinology, structural analysis at atomic level using crystallisation or cryo-EM [4]. For functional biologic studies it is essential to produce these proteins in their native state. Therefore, the choice of the appropriate expression system is of upmost importance [5, 6].

Many viral and mammalian proteins required for host-pathogen interaction studies depend on specific post-translational modifications to be biologically active [5, 6]. Others form multimers or assemble as part of multi-protein complexes for full functionality. Proper assembly and folding of the target proteins is only possible using sophisticated eukaryotic expression systems (yeast, insect, mammalian and plant), which all have their specific advantages and disadvantages [1].

Recombinant protein expression requires a template for the target gene, an expression vector and a suitable method to introduce and maintain the recombinant expression vector in the producer cell line [5]. Most of the available expression vectors require substantial (re)cloning of the desired target gene into individual vectors, each specific for a particular host system or versatile vectors which can be used in multiple expression systems [2]. The current cloning procedures like e.g., Golden Gate [7], Molecular Cloning [8] and “SLIC-Fusion” [9] are highly efficient. In combination with the available commercial synthesis of custom-made genes there are almost no limitations to generate required expression constructs [5]. Therefore, the bottleneck has changed from cloning to fast expression and screening systems.

In industry, the requirement for a GMP controlled process and an optimised yield both determine the choice of stable cell lines as the expression strategy. However, in contrast to transient expression systems, development of stable cell lines is very time-consuming and cost intensive, which is not suited for high-throughput expression analysis [2, 5]. The viral and plasmid-based transient expression systems in mammalian HEK cells or High-Five insect cells (Hi5 cells) both allow scale-down to 2 ml cultures for automation and high-throughput screening [10, 11]. This is essential to develop the initial optimal construct for expression.

The transient gene expression (TGE) in either HEK293-6E or Expi293F cell lines is well established but requires a license and/or expensive transfection materials as well as specific growth media, which make this system expensive and difficult to implement. Since 2015, virus-free transient gene expression in Hi5 cells was optimized and improved substantially [11-15]. This system uses affordable media, is easy to establish, robust and reproducible in performance. Growth is possible in simple incubators without the need of special CO_2 aeration. In comparison with HEK293 and CHO TGE systems we have shown for many tested proteins that the yield in the Hi5 TGE was more than sufficient to provide the required amounts of high-quality protein.

In this Chapter, we present an optimized and robust TGE protocol for Hi5 insect cells exemplified by the production and characterisation of the S1 fragment of the SARS-CoV-2 Spike surface protein. The TGE method is applicable to both single molecule expression from a single plasmid and multi-protein complex expression using a large set of vectors in parallel. TGE in Hi5 cells is especially suited for fast, inexpensive and simple screening using multi-well or chamber bioreactors, as well as for large scale production of recombinant mammalian proteins in shake flasks or bioreactors. In conclusion, plasmid-based transient expression in Hi5 insect cells simplifies eukaryotic protein expression to a point where it is superior to using prokaryotic systems [2].

2. Materials

2.1 Expression Vectors and cell lines

1. pOpIE2-C series (C-terminal tagged) and pOPIE2 N series (N-terminal tags) are available from the authors on request.
2. The High Five (Hi5) insect cell line (officially called BTI-Tn-5B1-4) was isolated by the *Boyce Thompson Institute* for Plant Research, Ithaca, USA. The cell line can be acquired from Thermo Fisher Scientific (*see* **Note 1**).

2.2 Cell Culture

1. 125 mL up to 5 L vented polycarbonate shake flasks (Corning).
2. Orbitron™ platform shaker with 50 mm orbit (Infors) in a 27°C climatized room with 50 % humidity (*see* **Note 2**).
3. Complete Cultivation Medium for Hi5 insect cells: EX-CELL 405 with addition of glutamin (Sigma) (*see* **Note 3**)

2.3 Transfection Reagents and Additional Chemicals for Protein Production

Prepare all solutions using ultrapure water and analytical grade reagents. All reagents will be sterile filtered and stored at 4°C (unless otherwise mentioned).

1. 1 mg/mL Polyethylenimine 40 kDa, linear (Polysciences): Dissolve 0.05 g of PEI in 50 mL of MilliQ pH 7 (*see* **Note 4**)
2. Expression plasmid (*see* **Note 5**) best at a concentration of 500-1000 ng/µL highly pure in MilliQ or TE buffer.

3 Methods

3.1 Construction of expression vectors

The efficiency of TGE depends on a highly active promoter that can promote transcription by the RNA polymerase II. The immediate early promoter OpIE2 from the baculovirus *Orgyia pseudotugata* was identified as the currently strongest promoter of this type in Hi5 insect cells [10, 11]. This promoter was cloned into the backbone of pIEX/bac5. We generated a series of variants of this expression vector pOpIE2-C1-C5 for easy fusion of the gene of interest (GOI) to purification tags. An overview of the constructs is shown in Fig 1. We preferably use the restriction sites: NheI, SpeI, XbaI and AvrII, which all have the same overhang of nucleotides for generating C-terminal fusion. This allows easy recombineering of the cassettes into new variants of the available elements as well as integrating synthetic genes with individual preferred tag sequences. For example, version C5 was generate from C2 by removal of the GFP11 ß-strand sequence by simple digestion with XbaI and AvrII followed by ligation of the vector (*see* **Note 6**).

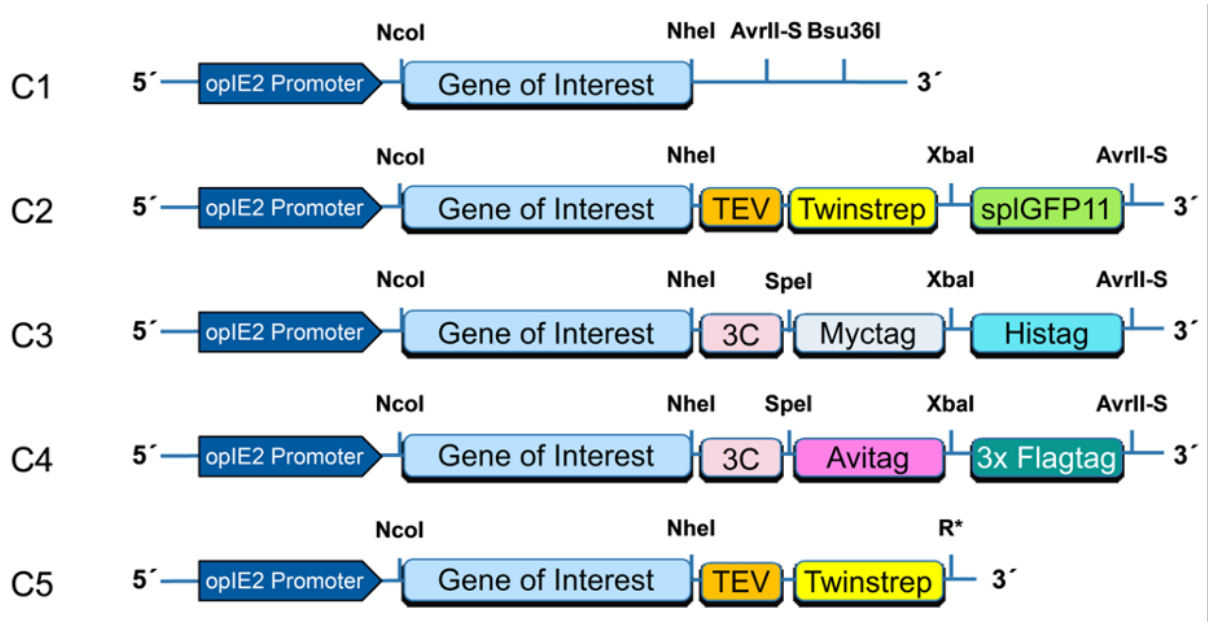

Fig. 1 Schematic representation of the pOpIE2-C-series of constructs.

The C represent the series of C-terminal tags which are cloned into the backbone and can be directly chosen to generate the required fusion construct. Optional constructs containing two possible protease sites (TEV and Rhinovirus 3C) in line with three optional tag combinations having either of the purification tag sequences (Twinstrep, Histag, Avitag and Flagtag) are available. For antibody detection by western blot two variants carry the sensitive detection sequences (Myctag, Flagtag). The specific *GFP*11 ß-stand can be used as part of the split*GFP* detection system (GFP fluorescence) [11]. The Avitag can be specifically labeled with biotin using the BIR ligase. This allows immobilization of the GOI to streptavidin linked materials (Western blotting, SPR, BLI and other biophysical analytic techniques).

The same strategy is also applied for N-terminal fusion constructs (pOpIE2-N-series, data not shown). For cloning of the N-terminal sequence of the GOI we use the NcoI site which contains the AUG start codon as well as an upstream BamHI site for easy cloning into the vector. Additionally, seamless new fusion tags can be cloned using Golden Gate cloning or SLIC fusion method.

3.2 Transient expression in Hi5 cells

The described protocol is for a 120 mL expression in Hi5 cells. The employed vectors have an important impact on the yield. The scale of the transfection experiment can be easily adapted by linear decrease of the components to a final volume of 2 ml as well as increasing to large scale (2 L).

1. **[Day -3/-2, e.g., Friday]** Prepare a 40 mL culture containing 0.4 x 10^6 c/mL 72 h prior transfection or prepare a **4**0 mL culture containing 0.5 x 10^6 c/mL 48 h before transfection and incubate the culture 72 h at 27 °C and 90 rpm (*see* **Note 7**).

2. **[Day 0, e.g., Monday]** Count the cells (viability should be above 95 %) and prepare a 30 mL culture containing 4 x 10^6 c/mL by centrifuging the required volume of the cell suspension at 180 x *g* for 4 min. Discard the supernatant and resolve the cell pellet in 30 mL fresh EX-CELL 405.

3. Pipette 120 µg of your DNA directly to the prepared cells and mix gently (*see* **Note 8**).

4. Immediately pipette 480 µL PEI of the 1 mg/mL 40 kDa PEI stock solution to the cells and mix gently.

5. Incubate the culture at 27 °C and 90 rpm for 4 up to 20 h (*see* **Note 9**).

6. Add 90 mL fresh EX-CELL 405 media.

7. **[Day 2, e.g., Tuesda**y] 48 h after transfection feed the cells with 120 mL fresh EX-CELL 405 media.

8. **[Day 3-5, e.g., Thursday]** Take samples daily, count the cells and determine transfection efficiency in the cytometer or/and determine target protein expression by a suitable technique (SDS-PAGE, slot blot or western blot). If viability of the cells or quality of the recombinant protein starts to drop, harvest the culture.

9. For intracellular proteins, carefully centrifuge the cells at 180 (up to max. 500) x *g* for 4 (up to max. 10) min and freeze the cell pellet at -20 °C until cell lysis and purification. Secreted target proteins are first centrifuged at 180 x *g* for 4 min, followed by a centrifugation of the supernatant at 2000 x *g* for 20 min. Afterwards, the supernatant is filtered with 0.2 µm filters and stored at 4°C until purification (*see* **Note 10**).

3.3 Production of the SARS-CoV-2 S1-Opt-delFurin-hFc

The example presented here is the S1 fragment of the SARS-CoV-2 Spike surface protein. It represents an antigen that can be used as optional candidate for generation of vaccines and a protein highly relevant to get insight into the viral infection mechanism. Viral surface proteins often have higher order structures (homo- or hetero-multimers) and are substantially glycosylated. Here we show the result of the protein purification, binding activity and the subsequent analysis of the glycosylation that shows to be specific for lepidopteran insect cells.

3.3.1 Expression and purification of S1-Opt-delFurin-hFc

The synthetic gene for S1-Opt-delFurin-hFc was codon optimized for mammalian expression and designed according to Wrapp et al. [4]. The sequence was fused to an hFc tag or His tag for easy purification by protein A/ Ni-NTA chromatography. The expression of the protein S1-Opt-delFurin-hFc was done for up to 72 to 96 hours. The transfection efficiency determined by the fraction of GFP fluorescent cells reached up to 60 % at a vitality of 98%. The supernatant of the hFc tagged protein was purified on a 1 ml rProtA Hitrap column using the standard protocol of the supplier (Cytiva). The eluted S1-Opt-delFurin-hFc protein was pooled and concentrated before loading on a HiLoad 16/600 Superdex 200 pg column (Cytiva). A homogenous peak was isolated after size exclusion chromatography using TBS (20 mM Tris pH 7.4, 150 mM NaCl) as equilibration buffer. The eluted fractions were analyzed by SDS-PAGE (Fig. 2). Homogeneous samples were pooled and concentrated to 1 mg/ml and stored at -80°C after snap freezing.

Fig. 2 SDS-PAGE analysis of the samples purified by size-exclusion chromatography

Samples of fractions separated by SEC on a HiLoad 16/600 Superdex 200 pg were analyzed on a Biorad Any kD Gel using denaturing sample buffer. The gel was stained with Instant Blue. Lane 1 Pageruler Plus prestained Molecular Weight Standard (Thermo Fisher Scientific), Lane 2-10 represent successive fractions separated by SEC. The S1-Opt-delFurin-hFc with an estimated size of 130 kD is indicated by the arrow.

This sample was further analyzed by SEC-MALS to determine the conformation of the protein and the glycan content of the protein sample. The S1-Opt-delFur-His tagged protein used for ELISA analysis was purified in a similar way using a 1 ml HisTrap Excel column, followed by SEC using PBS (10 mM phosphate pH 7.4, 2.7 mM KCl and 135 mM NaCl) as equilibration buffer.

3.3.2 SEC-MALS analysis of the glycosylated S1-Opt-delFurin-hFc protein

The protein conformation and amount of glycosylation was analyzed by analytical size exclusion chromatography in combination with multi angle light scattering (SEC-MALS) [18] using an Agilent 1260 Infinity II system with an UV detector connected to a Wyatt TREOS II MALS detector and an Optilab 505-rEX refractive index (RI) detector. 100 µg of the S1-Opt-delFurin-hFc protein sample was separated on a Superdex 200 increase 10/300 column (Cytiva) with TBS running buffer (20 mM Tris pH 7.4, 150 mM NaCl, 0.1 µm microfiltered). The data were analyzed using the Protein Conjugate Analysis method with the ASTRA 7.3.2.19 software. The calculations are dependent on a good estimate of the refractive index increment (dn/dc) of the sample. For proteins this value is near 0.185 ml/g. Glycan modifications have an index of 0.14-0.15 ml/g. The fractional mass of the protein and the glycan were deconvoluted using the signal of the UV detector and the RI detector simultaneously recorded to the MALS data.

SEC-MALS analysis of S1-hOpt-delFurin-hFc (Fig. 3) shows that the protein migrates as a homodimer with an overall molecular mass or 235.0 kDa. The protein content is 201.9 kDa with

a glycan composition of 33.1 kDa. This correlates to a homodimer of the protein with a calculated mass of 101 kDa. (Table 1B).

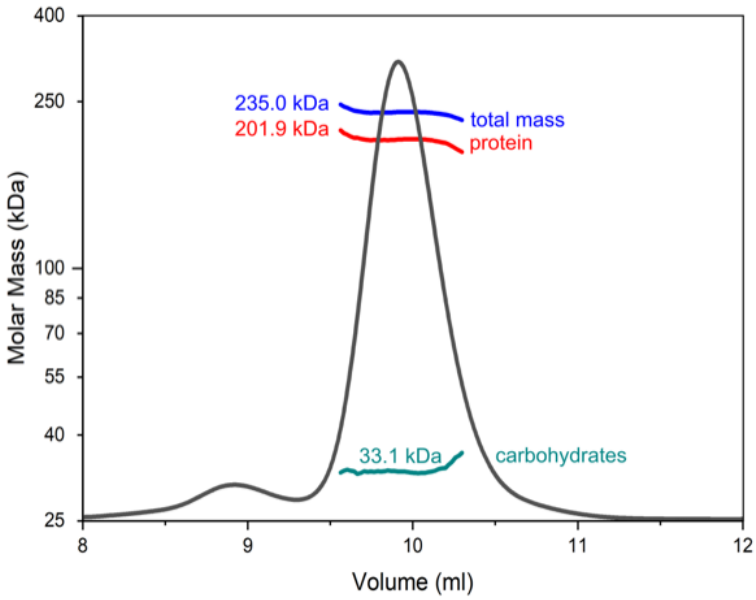

Fig. 3 SEC-MALS Analysis of the S1-Opt-delFurin-hFc protein

The SEC-MALS analysis of S1-Opt-delFurin-hFc shows a main peak fraction with an overall molecular mass or 235.0 kDa (blue line as determined from the RI detector). The protein content was 201.9 kDa (red line as determined from the UV signal) with a glycan composition of 33.1 kDa (carbohydrates). This correlates to a homodimer of the protein with a calculated mass of 101 kDa. The void volume of the column is 8 ml.

3.3.3 Enzyme-Linked Immuno Sorbent Assay (ELISA)

The major function of the SARS-CoV-2 Spike protein is the recognition and binding to the angiotensin-converting enzyme hACE2, priming the internalization of the virus into the human host cell. The functional binding activity of the purified S1-Opt-delFurin-His fragment of the SARS-CoV-2 spike protein to the extracellular domain of hACE2 was tested by ELISA (Fig. 4**)**. Hereto, S1-Opt-delFurin-His was immobilized on a Costar high binding 96 well plate (200 ng/well, blocked with 2 % skimmed milk powder in PBST (PBS 1x with 0.05 % Tween20) followed by incubation with the indicated concentrations of its binding partner hACE2-mFc.

ACE2-mFc binding was detected using goat-anti-mIgG(Fc)-HRP (1:42000, A0168, Sigma) antibody and visualized by tetramethylbenzidine (TMB) substrate. After stopping the reaction by addition of 1 N H_2SO_4, absorbance at 450 nm with a 620 nm reference was measured in an ELISA plate reader (Epoch, BioTek). EC_{50} value was calculated with by GraphPad Prism Version 6.1, fitting to a four-parameter logistic curve, resulting in an EC_{50} value of 2.7 nM.

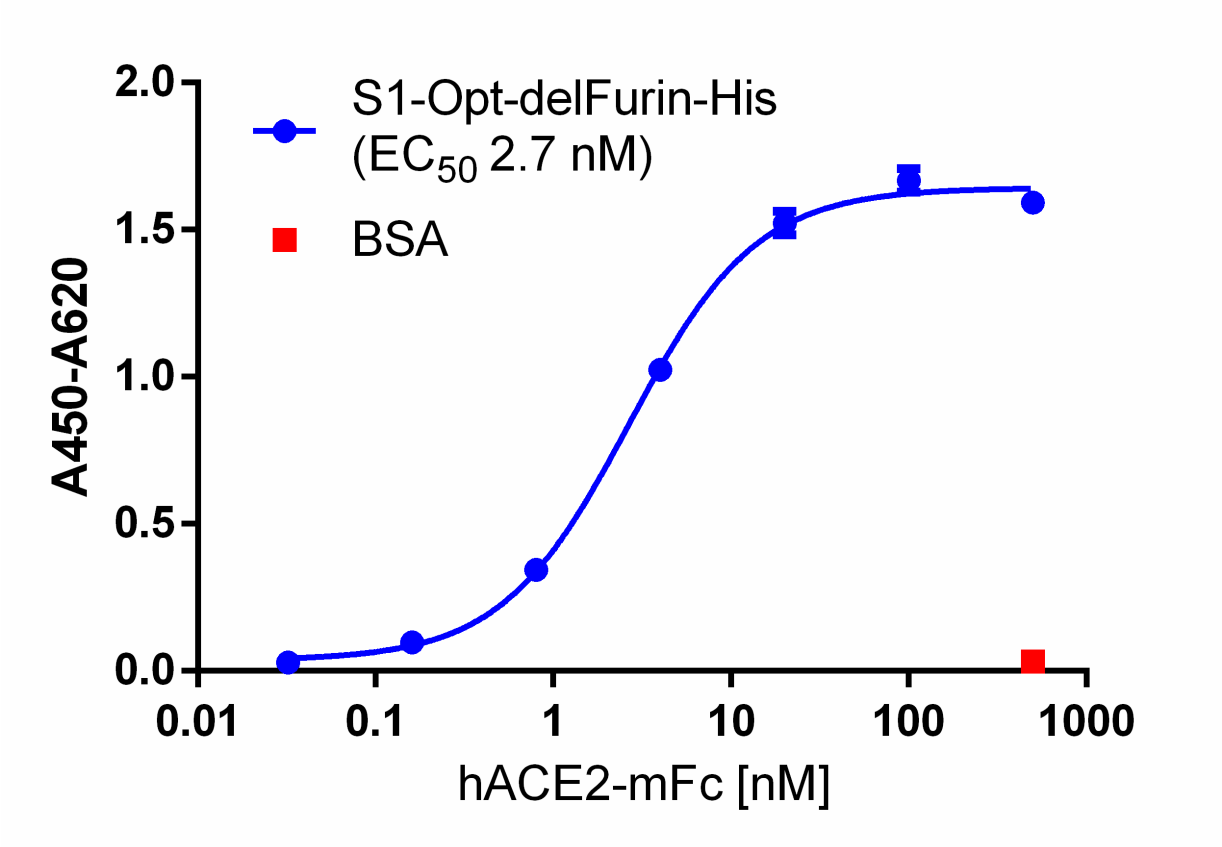

Fig. 4 ELISA analysis of the interaction of S1-Opt-delFurin-His with hACE2.

The S1-Opt-delFurin-His tagged protein was coated to ELISA plates and after blocking non-specific binding sites the wells were incubated with different concentrations of purified hACE2-mFC. The A_{450} and A_{620nm} (reference) were measured after incubation with anti mFC-conjugated with HRP and staining with the substrate (TMB). The calculated EC_{50} value was 2.7 nM

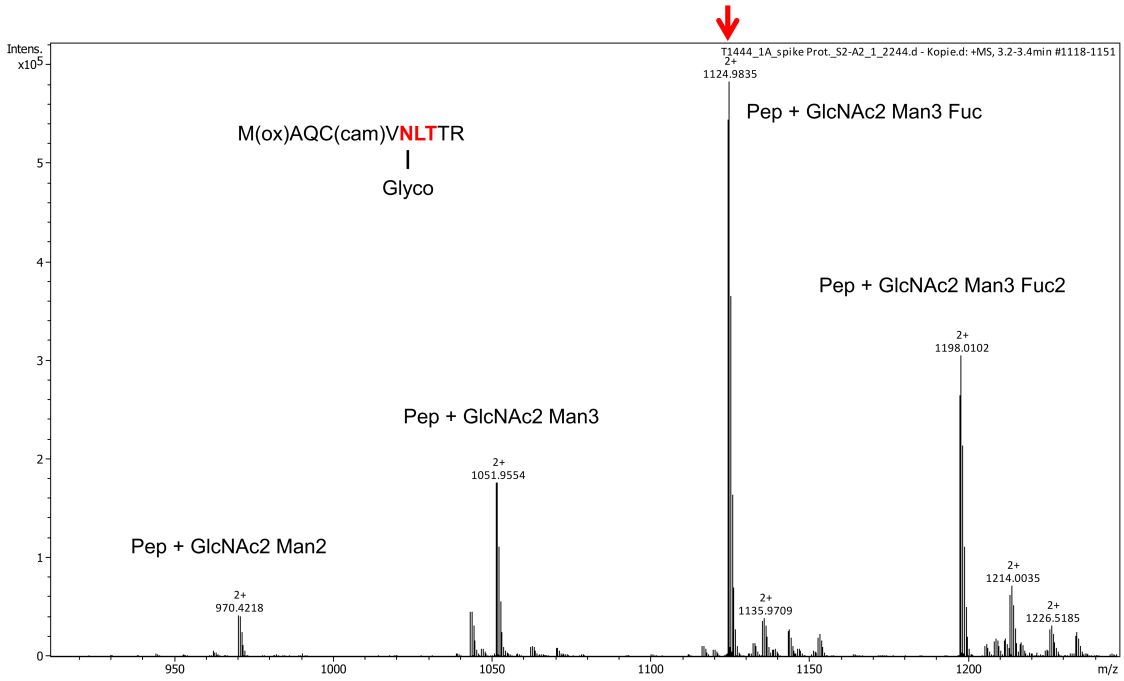

Fig. 5 Representative MS analysis of the glycosylation of isolated tryptic fragment of S1-Opt-delFurin-hFc

The isolated and characterized fragments all carry an equal distribution of three major glycans GlcNAc2 Man3 + GlcNAc2 Man3 Fuc + GlcNAc2 Man3 Fuc2 as shown in this example of the first N-terminal glycosylation site at position 6 of the sequence presented in Table 1B.

3.4 Analysis of glycosylation

The type of glycosylation was determined by mass spectroscopic analysis of peptides isolated from the protein bands after separation by SDS-PAGE and subsequent tryptic digestion as shown in Fig 5. The (glyco-)peptides extracted after tryptic in-gel digestion of the relevant bands from gel bands stained with Instant Blue were analyzed on an Evosep LC system coupled to a tims/TOF Pro mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany).

Table 1: Characterized glycosylation sites of S1-Opt-delFurin-hFc

A: Analyzed tryptic peptides

The isolated and characterized fragments all carry equal distribution of three major glycans as shown by the analysis presented in **Figure 5**: GlcNAc2 Man3 + GlcNAc2 Man3 Fuc + GlcNAc2 Man3 Fuc2.

Bold: modified amino acids; Bold and red: glycosylated amino acids

Site I:	$_{1}$**M**AQ**C**V**N**LTTR$_{10}$	
	$_{1}$**M**AQ**C**V**N**LTTRTQLPPAYTNSFTR	
Site VI:	$_{148}$VYSSAN**N**C**T**FEYVSQPFL**M**DLEGK$_{171}$	
Site VII:	$_{204}$DLPQGFSALEPLVDLPIGI**N**ITR$_{226}$	
Site VIII:	$_{268}$YNE**N**GTITDAVD**C**ALDPLSETK$_{289}$	
	$_{263}$TFLLKYNE**N**GTITDAVD**C**ALDPLSETK$_{289}$	
Site XII:	$_{601}$YQDV**N**C**T**EVPVAIHADQLTPTWR$_{623}$	Semitryptic peptide!
Site XIII:	$_{636}$AG**C**LIGAEHV**N**N$_{647}$	Semitryptic peptide!
Site XIV:	$_{747}$TKPREEQY**N**STYR$_{759}$	

B: S1-Opt-delFurin-hFc protein sequence

The underlined amino sequence represents the tryptic fragment. Bold amino acids represent the glycosylation recognition site N-X-S/T. The glycosylation N-residue is marked in bold and red. The italic sequence represents the hFc tag.

MAQCV**N**L**T**TRTQLPPAYTNSFTRGVYYPDKVFRSSVLHSTQDLFLPFFSNVTWFHAIHVSGTNGT
KRFDNPVLPFNDGVYFASTEKSNIIRGWIFGTTLDSKTQSLLIVNNATNVVIKVCEFQFCNDPFL
GVYYHKNNKSWMESEFRVYSSAN**N**C**T**FEYVSQPFLMDLEGKQGNFKNLREFVFKNIDGYFKIYSK
HTPINLVRDLPQGFSALEPLVDLPIGI**N**I**T**RFQTLLALHRSYLTPGDSSSGWTAGAAAYYVGYLQ
PRTFLLKYNE**N**G**T**ITDAVDCALDPLSETKCTLKSFTVEKGIYQTSNFRVQPTESIVRFPNITNLC
PFGEVFNATRFASVYAWNRKRISNCVADYSVLYNSASFSTFKCYGVSPTKLNDLCFTNVYADSFV
IRGDEVRQIAPGQTGKIADYNYKLPDDFTGCVIAWNSNNLDSKVGGNYNYLYRLFRKSNLKPFER
DISTEIYQAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQPYRVVVLSFELLHAPATVCGPKKST
NLVKNKCVNFNFNGLTGTGVLTESNKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGVS
VITPGTNTSNQVAVLYQDV**N**C**T**EVPVAIHADQLTPTWRVYSTGSNVFQTRAGCLIGAEHV**N**N**S**YE
CDIPIGAGICASYQTQTNSPGSAS*AAASDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTP*
*EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY**N**S**T**YRVVSVLTVLHQDWLNGKEYKCK*

VSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

The acquisition of mass and tandem mass spectra was done with an average resolution of 60 000. To enable the parallel accumulation-serial fragmentation (PASEF) method, precursor m/z and mobility information was first derived from full scan TIMS-MS experiments. Singly charged precursors were excluded by their position in the m/z -ion mobility plane. The collision energy for fragmentation varied between 31 and 52 eV depending on precursor mass and charge [19]. Protein identification was performed against the CoV-2 Spike protein sequence (S1-Opt-delFur-hFc, Table 1) using the Peaks 10.5 software (BSI, Toronto, Canada). Variable observed amino acid modifications were: oxidized methionine and the main N-glycans GlcNAc2Man3Fuc and GlcNAc2Man3Fuc2 typical paucimannose-type glycosylation for insect host cells. Additionally, carbamidomethylation of cysteine was selected as a fixed modification. Trypsin was selected as the proteolytic enzyme, with a maximum of two potential missed cleavages. To ensure optimal identification of glycopeptides, the data were additionally searched manually for sets of precursors ions producing the N-glycan specific fragment at m/z 204.0872 [GlcNAc+H]+ upon high collision energy induced dissociation (HCD) (Fig. 4 and Table 1).

In total 6 out of 13 glycosylation sites could be identified compared to the analysis of the glycan shield of the CoV-2 spike protein by Watanabe et al. [17]. The glycosylation in HEK293 shows three different types of glycosylation from oligomannose, hybrid to complex glycosylation. In contrast, all glycopeptides analyzed from the S1 protein expression in Hi5 insect cells have a comparable distribution with mainly mono or bi-fucosylated GlcNAc2-Man3 representing the typical paucimannose type of glycosylation of lepidopteran cells. This type of glycosylation is advantageous for crystallographic structural analysis of membrane-bound or secreted mammalian and viral proteins.

4. Notes

1. Important to note is that it seems to improve the yield if the cells have been passaged in the EX-CELL 405 medium over a hundred times [14].
2. Cultivation with humidity is preferred but not absolute necessary. Smaller volumes than 15 mL are best cultivated in 50 mL TPP TubeSpin bioreactor vented tubes (Merck) at a higher shaking speed (120 rpm).
3. Other media might not work for the described method and inhibit the transient plasmid transfection [14].
4. Linear 40 kDa PEI proved to be more reliable then linear 25 kDa PEI. It is soluble in water and can be stored at 4°C.
5. The optimal expression plasmid in our facility comprises the OpIE2 promoter, the IE1 terminator and a FlashBac compatible backbone. The expression cassette is flanked by baculoviral sequences (orf 603 and orf 1629) which can be used for integration into the baculovirus from the Flashbac system [16]. These sequences are used to enhance transient gene expression [13].
6. Re-ligation will result in removal of both the XbaI and SpeI site. This results in a final construct having just the TEV protease site and the Twinstrep affinity tag. This cloning strategy requires that the synthetic genes will be designed without further internal NheI, SpeI, XbaI and AvrII sites.
7. This step ensures that the cells are in the optimal growth phase. Overgrown cells (~5-6 x 10^6 c/mL) or cells not yet in the exponential growth phase (e.g. passaged only a few hours before) do not reach maximum transfection yields.

8. The DNA concentration should be in a range of 0.2-1.0 µg/µl. Replacing 5 % of the total DNA amount with a control plasmid expressing e.g. eGFP will help to monitor transfection efficacy. The overall yield is only slightly affected by eGFP-expression. Additionally one can perform co-expression of a multiprotein complex by distributing the amount of DNA among the expression vectors of the individual subunits.
9. Incubating the cells at high density for 4 h ensures higher transfection rates. Feeding after 20 h leads to a decreased viability and thereby low transfection rate, as the cells will suffer from depletion of medium components by that time.
10. The two-step centrifugation ensures that the cells are removed and not disrupted, preventing to get a lot of intracellular protein contaminating the supernatant. For His-Tag purification it is important to add 0.5 M NaCl to the supernatant to prevent unspecific binding. His Trap™ Excel resin material (Cytiva) can be used to purify His-tag proteins directly from the supernatant. Other NiNTA-resins require re-buffering to pH 7.5 and dialysis of the supernatant to conditions avoiding stripping the Ni^{2+} -ions of the column.

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