

Evaluation of Alternative Signal Sequences

Lonza

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INTRODUCTION

Optimal antibody expression is the result of a combination of many factors ranging from the choice of expression system to the media used for cell culture fermentation. Lonza's GS Gene Expression System™ (GS:glutamine synthetase) has been frequently used to generate cell lines capable of high-level antibody production in bioreactor processes. A key goal for the biopharmaceutical industry is to reduce the cost of goods of biological therapeutics. This goal can be achieved by increasing the productivity of cell lines by enhancing all aspects of the process of recombinant protein production. This poster focuses upon improving secretion of recombinant antibodies.

When generating GS vectors for expression of recombinant antibodies, Lonza routinely uses antibody derived signal sequences to direct recombinant protein to the secretory pathway. To investigate if manipulation of this pathway can enhance antibody expression, nineteen alternative signal sequences were evaluated for their ability to increase antibody production in both a transient expression system and from stable cell lines. Antibody generated by stable cell lines was characterised by SDS-electrophoresis and electrospray mass spectroscopy (ESI-MS) to determine the impact of the alternative signal sequences on the final antibody product and whether the alternative signal sequences were likely to be appropriately processed.

MATERIALS AND METHODS

Vector Construction

- GS expression vectors encoding a model IgG₄ antibody were generated. The features of the vectors are shown in Figure 1.
- Two control vectors were generated using either non gene-optimised (WT) or gene-optimised cDNA sequences (WT_{opt}) using the antibody derived signal sequences routinely used by Lonza. Two controls were required to determine the contribution of gene-optimisation to any observed benefit resulting from the use of an alternative signal sequence.
- Nineteen constructs were generated using gene-optimised sequences employing the alternative signal sequences under evaluation (V1-V19).
- Currently a different signal sequence is used on the heavy or light chain gene (WT or WT_{opt}), in the case of the alternative signal sequences (V1-V19), the same sequence was used for both genes.

Transient Transfection

- CHOK1SV cells were transiently transfected in 24-well plates using Lipofectamine-2000 (Invitrogen) with the control vectors (WT and WT_{opt}) and vectors containing the 19 alternative sequences (V1-V19).
- Cells were transfected with each vector six times and the antibody concentration in the culture medium was determined 72 hours after transfection by ELISA for assembled antibody.
- Vectors demonstrating increased mean antibody concentrations compared to the controls were then selected for analysis by stable transfection.

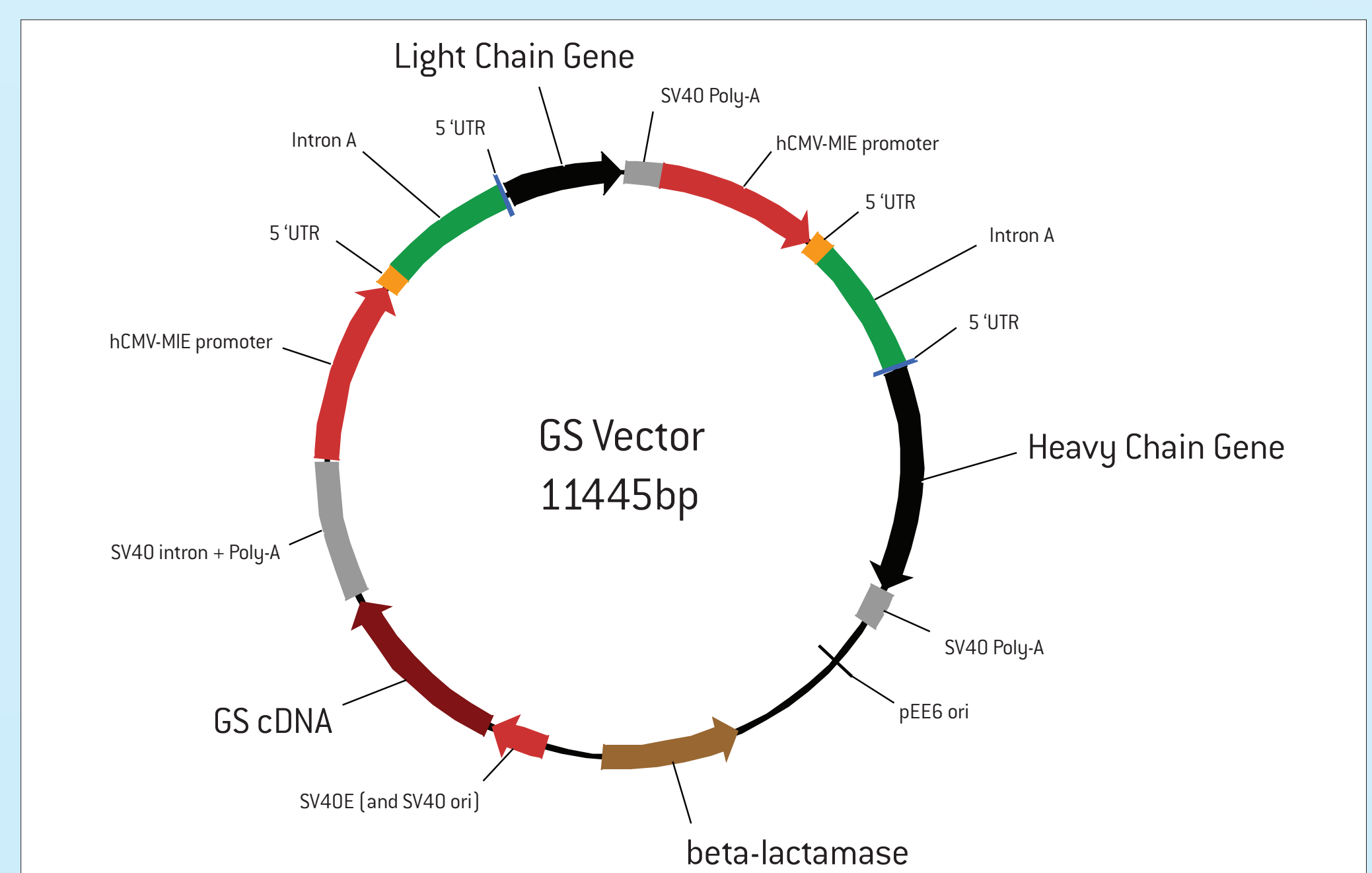
Stable Transfection

- CHOK1SV cells were transfected with the vector constructs using standard electroporation methods.
- Transfected cells were plated out across 96-well plates. The following day, selective medium containing methionine sulphoximine (MSX) was added such that the final concentration in each well was 50 μM MSX.
- Plates were screened for developing colonies at 3-4 weeks post-transfection. For each transfected vector, 100 colonies were transferred to 24-well plates in medium containing 25 μM MSX. Cultures were allowed to 'overgrow' for 2 weeks, after which the cell culture medium from each well was collected and analysed by Protein A HPLC to determine the antibody concentration.
- Mean antibody concentrations were compared by ANOVA and Tukey's method. A p value of ≤0.05 was considered significant.

Evaluation of Product Quality

- Antibody from stable cell lines was purified using Protein A affinity chromatography.
- Purified antibody was analysed by SDS-electrophoresis under both non-reducing and reducing conditions using the Agilent 2100 Bioanalyser.
- Purified antibody was also deglycosylated and analysed by ESI-MS to determine the molecular weight of the antibody product.

Figure 1: Representation of the expression vector used for signal sequence evaluation. Lonza's GS Gene Expression System™ uses a single vector encoding both the heavy and light chain antibody genes, with expression of each chain driven by the strong human cytomegalovirus major intermediate early promoter. The GS gene is driven by the weaker SV40 promoter which is thought to bias for selection of integration into transcriptionally active sites in the genome.

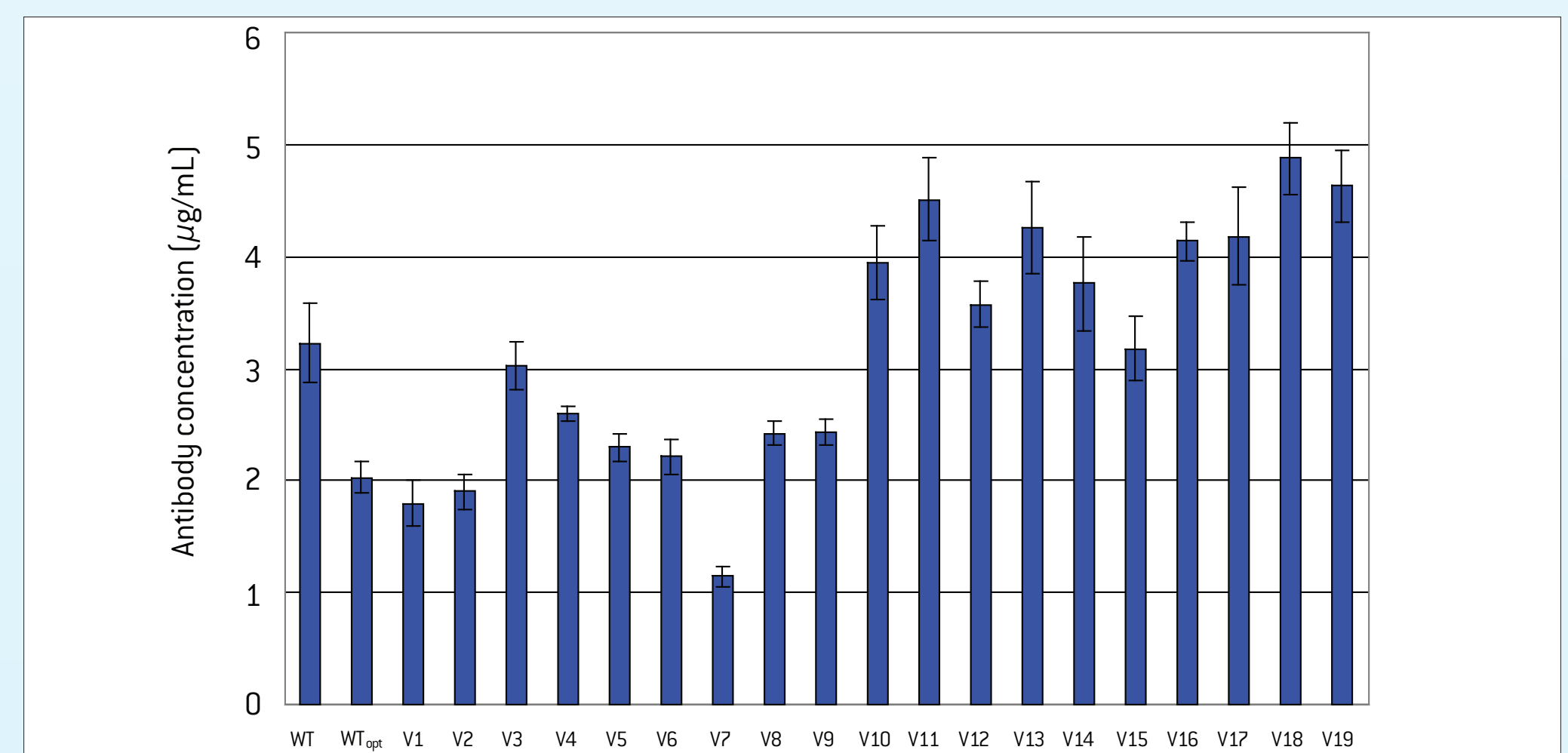


RESULTS

Transient Transfection

- Signal sequence constructs were screened in a transient transfection system for their ability to increase the secretion of antibody from CHOK1SV cells.
- Mean antibody concentrations generated by transient transfection of each signal sequence construct were compared to those generated by transient transfection of vectors WT and WT_{opt} (Figure 2).
- Mean antibody concentrations of 3.2 μg/mL and 2.0 μg/mL were observed for the WT and WT_{opt} controls respectively. Transient transfection of constructs employing the alternative signal sequences resulted in mean antibody concentrations ranging from 1.1 μg/mL for V7 to 4.9 μg/mL for V18.
- When the experiment was repeated, a similar result was obtained (data not shown).
- The results of the transient transfection experiment were used to select which vectors were to be assessed in stable transfections. Vectors V11, V13, V16 and V18 were chosen as they produced high mean antibody concentrations in both rounds of transient transfection. Vector V15 was selected as an internal control as it gave an equivalent antibody concentration to the WT control.
- The difference in mean antibody concentration between control vectors was unexpected; in independent stable transfections more antibody is reproducibly produced from the WT_{opt} vector¹. This indicates that analysis of constructs in stable cell lines is essential to confirm improvements in antibody expression.
- Differences were observed in the concentration of antibody secreted from transiently transfected CHOK1SV cells between the different signal sequences, indicating that choice of signal sequence can affect the level of antibody secretion.
- Screening nineteen signal sequence constructs using a transient transfection system has identified four constructs for further evaluation.

Figure 2: The mean antibody concentration in medium from CHOK1SV cells transiently transfected with control vectors (WT and WT_{opt}) was compared to that obtained from cells transiently transfected with the vectors employing the alternative signal sequences (V1-V19). n=6. Error bars represent standard deviation.



Stable Transfection

- Stable transfection was used to evaluate five alternative signal sequences (Figure 3).
- Analysis of secreted antibody in the culture medium from 100 cell lines generated per construct demonstrated that only constructs V16 and V18 showed a statistically significant increase in mean antibody concentration compared to the WT_{opt} control (Table 1).
- Comparison of vectors WT and WT_{opt} by stable transfection demonstrated that gene optimisation leads to an increase in mean antibody concentration. This result is similar to that reported by Kalwy *et al.*¹. These data indicate that the use of an alternative signal sequence alongside gene optimisation can result in a further increase in mean antibody concentration.
- Antibody generated using constructs V16 and V18 was analysed to investigate the impact of the alternative signal sequences on the recombinant antibody product.
- SDS-electrophoresis of Protein A purified antibody under reducing conditions showed that there was no observable difference in molecular weight of the heavy or light chain polypeptides when either the control or alternative signal sequences were used. Under non-reducing conditions, the pattern of bands associated with assembled antibody were almost identical when either the control or alternative signal sequences were used. These results indicate that alternative signal sequences are likely to be appropriately processed during antibody secretion (Figure 4).
- Analysis of Protein A purified antibody by ESI-MS demonstrated no difference in the molecular weight of antibody product when expressed using the control or alternative signal sequences. This provides further evidence that it is likely that each signal sequence is appropriately processed (Figure 5).

Figure 3: Stable transfection of signal sequence constructs. Stable cell lines were generated for each vector under evaluation. Cells were cultured in 24-well plates for 14 days at which point antibody concentration was determined by Protein A HPLC. Box plots show distribution of antibody concentrations in media derived from 100 stable cell lines per vector. * indicates constructs demonstrating a statistically significant increase in mean antibody concentration over the WT_{opt} construct (p ≤ 0.05).

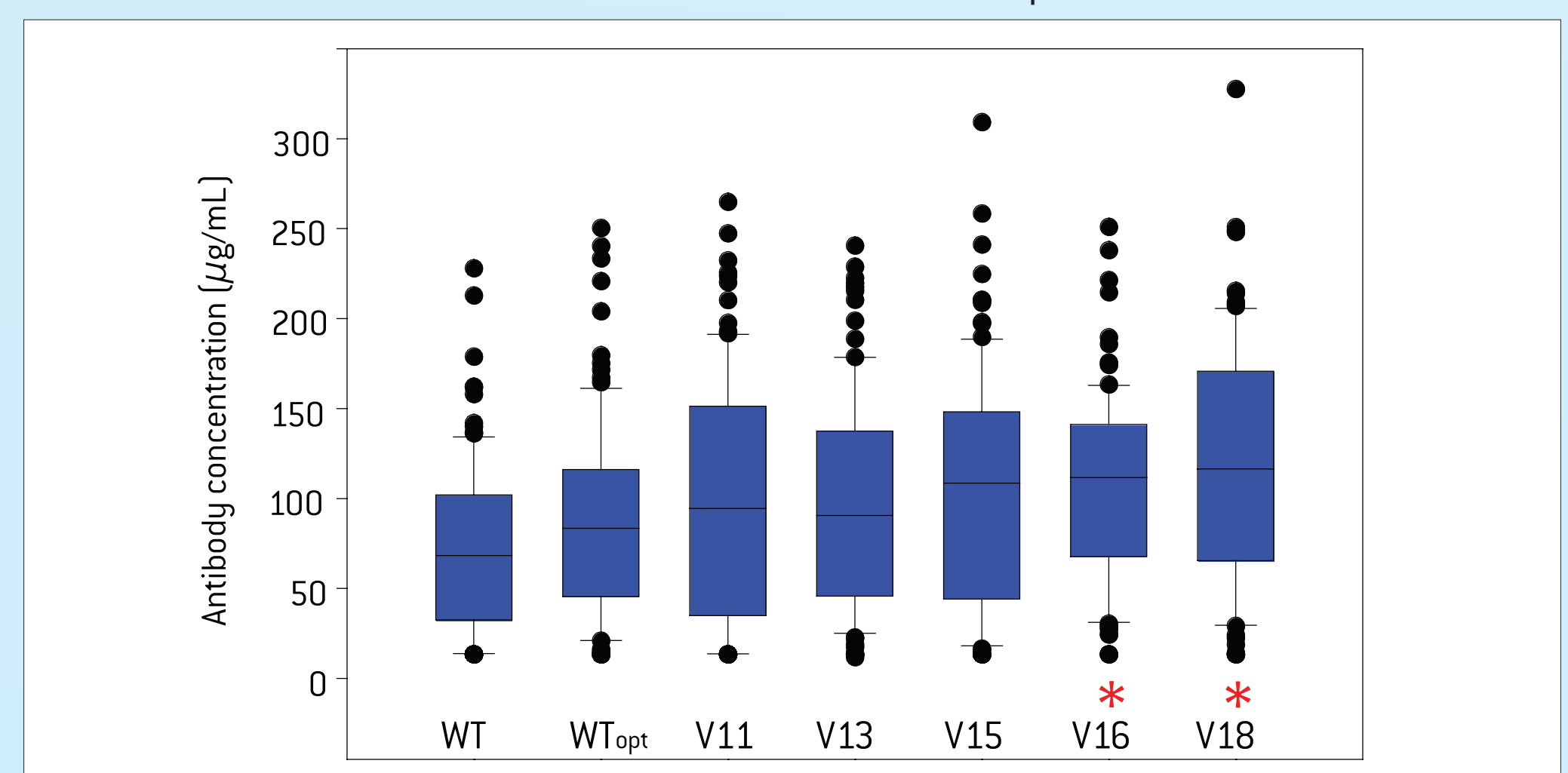


Table 1: Statistical analysis of 24-well plate data generated during stable cell line construction. n=100 per construct.

Vector ID	Mean (μg/mL)	Range ¹ (μg/mL)	Percent increase in mean compared to WT _{opt}	p values ²
WT	73.0	≤13.4 - 228.0	-	-
WT _{opt}	89.3	≤13.4 - 250.4	-	-
pV11	99.8	≤13.4 - 264.8	12	0.846
pV13	97.8	≤13.4 - 240.6	10	0.429
pV15	105.1	≤13.4 - 309.2	18	0.294
pV16	106.1	≤13.4 - 251.0	19	0.024
pV18	118.2	≤13.4 - 327.6	32	0.011

⁽¹⁾ Limit of the quantitation for the Protein A HPLC assay is 13.4 μg/mL.
⁽²⁾ p values calculated by comparison against vector WT_{opt}.

Figure 4: Analysis of purified antibody by SDS-electrophoresis. Figure showing non-reduced (panel A) and reduced (panel B) electropherogram images of samples taken from stable cell lines generated using construct WT_{opt}, V16 or V18.

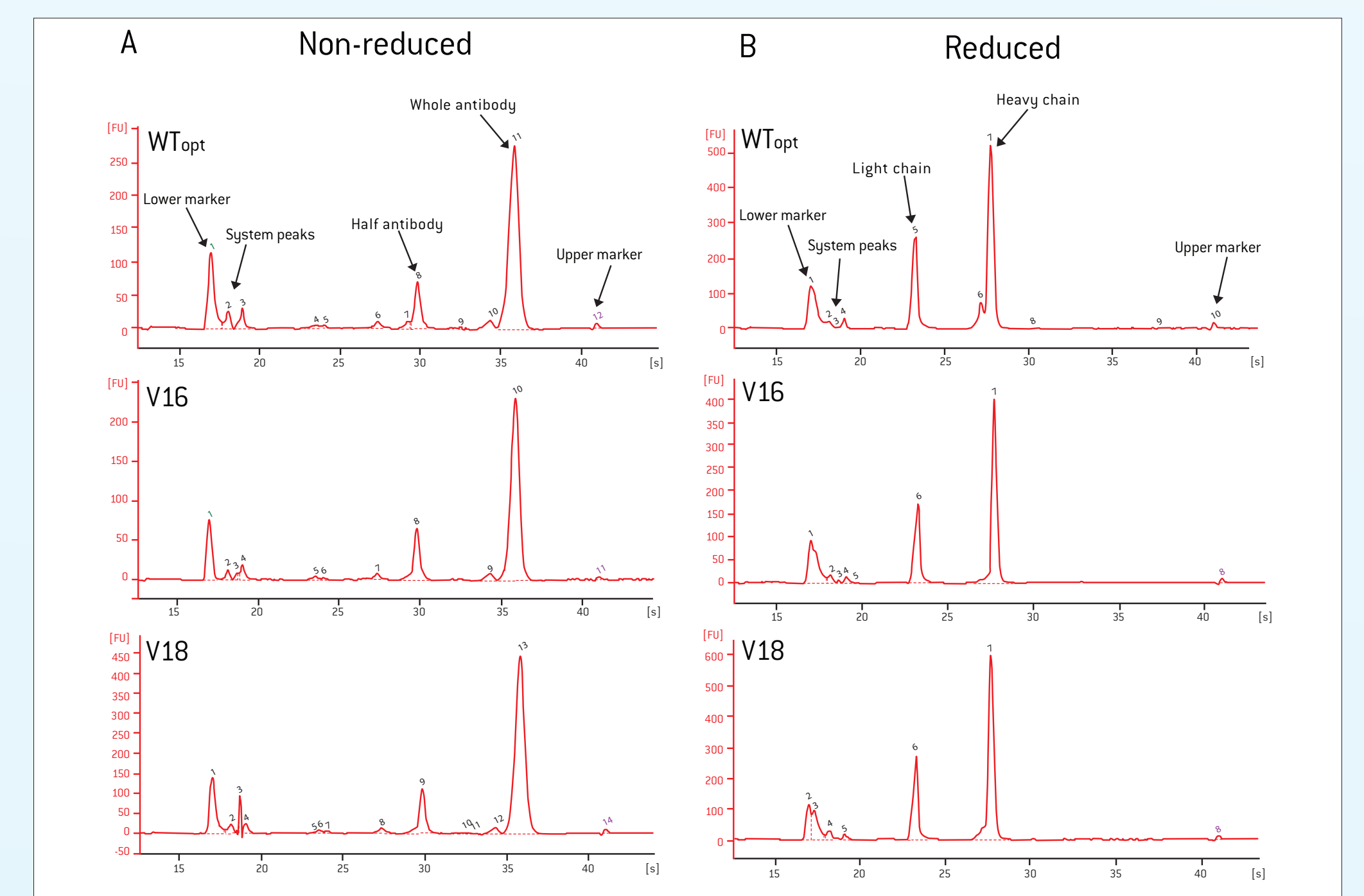
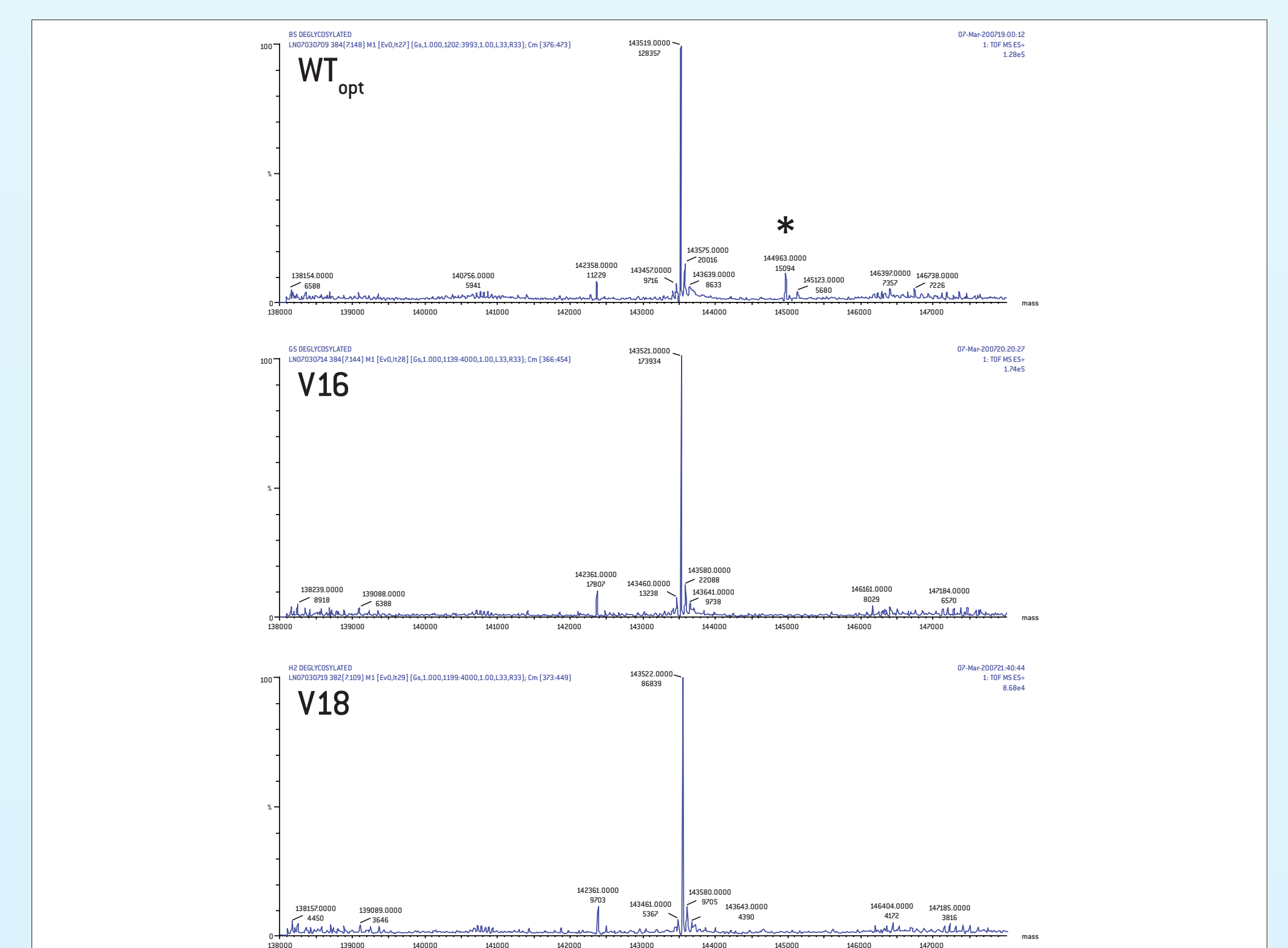


Figure 5: Analysis of purified antibody by ESI-MS. Figure shows mass spectroscopy profiles from deglycosylated material. Note: * = residual glycosylation in WT_{opt} derived sample.



CONCLUSION

- Evaluation of a panel of alternative signal sequences in stable cell lines has identified sequences which result in increased antibody concentrations in media compared to the antibody derived signal sequences routinely used at Lonza.
- Two lead candidate sequences were identified for further evaluation by additional experimentation.
- Analysis of antibody product generated using these sequences shows no change in antibody product by SDS-electrophoresis or ESI-MS. This suggests that no alteration in antibody product has been introduced when expressed using these different signal sequence and that the signal sequences used are processed appropriately. Antibody product quality is therefore unlikely to be adversely affected by use of the alternative signal sequences.
- To further characterise the benefit of the lead signal sequence, stable cell lines will be constructed using construct V18 and evaluated in bioreactors run in fed-batch mode.
- Choice of signal sequence is likely to be a key contributor towards improved antibody expression.

ACKNOWLEDGMENTS

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REFERENCES

1. Kalwy S, Rance J, Young R. Mol Biotechnol. 2006 Oct;34(2):151-6.

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