Increased antibody yield due to modification of LC and HC expression by gene regulatory elements

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Abstract

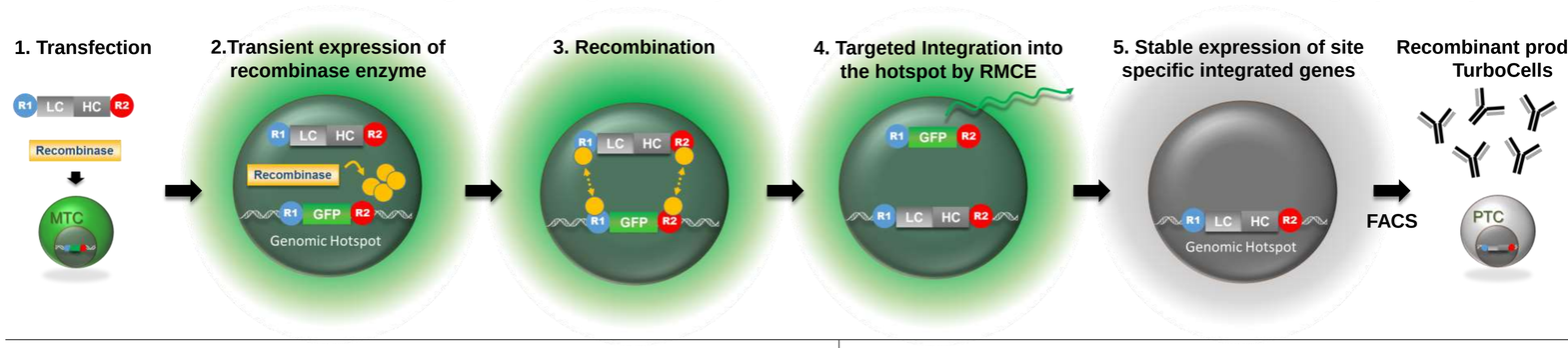
The amount of antibodies secreted from a CHO cell, is strongly dependent on the transcription and translation level of light (LC) and heavy chains (HC) as well as the ratio of both synthesized polypeptides inside the cell. In literature, it was shown that an excess of intracellular LC over HC improves the antibody production, whereas the opposite ratio results in decreased yields of product. In single-vector expression systems composed of two distinct LC and HC expression cassettes, the transcription of both genes and finally the polypeptide ratio and antibody production can be influenced by the arrangement and orientation of both units and promoters to each other, because of e.g. promoter interferences. Gene regulatory elements can prevent these interactions and could allow an adjustment of transcription levels and an increase of titers.

Based on this assumption, an antibody expressing vector containing two expression units for LC and HC, was modified by insertion of insulators, S/MAR elements and non-functional, spacing sequences in a kind of flanking or separating both parts. Afterwards, the newly generated vectors were stably integrated in a previous tagged, high active genomic locus of CHO-K1 TurboCells™ by recombinase mediated cassette exchange (RMCE) technology. In contrast to random integration resulting in variable, locus dependent expression (position effect), targeted integration at a defined, characterized genomic locus allows a direct comparison of the vectors in terms of expression and genetic modification. Because of consistent chromatin environment around the identical integration site, the integrated vectors are influenced in equal measure. Differences in expression between vectors are solely related to their modifications. Targeted producer cells were analyzed in fed batch experiments for antibody production. Additionally, the amounts of LC and HC mRNA and polypeptides were quantified by RT-qPCR and CE-protein analysis, respectively.

Dependent on the type of the inserted sequences and their positions inside the vector, the transcription of LC and HC was differently affected resulting in both different amounts and ratios of the transcripts. These variations, in turn, were associated with different amounts of LC and HC polypeptides and also different yields of secreted antibody. The majority of modifications inside the expression vector achieved an improved antibody expression in CHO cells. By insertions of gene regulatory elements between the LC and HC expression units, transcription of both mRNA was significantly enhanced resulting in an up to 2-fold increased titer.

Results

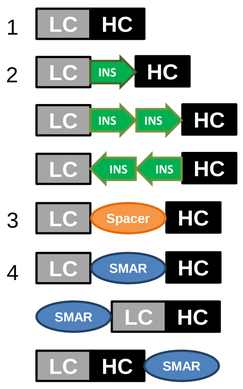
1 - TurboCell™ Platform – Targeted Integration by Recombinase Mediated Cassette Exchange (RMCE)



The TurboCell™ platform as tool for cell line development, is based on so called Master TurboCells (MTC) in which high active loci (hotspots) are tagged with the fluorescent marker GFP and heterologous recombinase recognition sites (R1 and R2). By recombinase mediated cassette exchange (RMCE), any GoI (gene of interested) can be integrated at these pre-characterized genomic loci resulting in so called producer TurboCells (PTC) with predictable, stable and high expression of the GoI.

(1) To establish IgG expressing cells, a vector containing genes for light (LC) and heavy chain (HC), as well as R1 and R2, is co-transfected into the MTC with a plasmid encoding the recombinase enzyme. (2) After entering of vectors into the MTC's nucleus, the recombinase enzyme is expressed and (3) initiates the site specific recombination between homologous R1 and R2, respectively (RMCE), positioned at both the genomic hotspot and the IgG expressing vector. (4) This recombination step results in an integration of the IgG expressing vector into the tagged locus whereas the GFP expressing cassette is excised and finally lost by cell division as episomal DNA. (5) After a couple of days, cells express solely IgG (PTC). Because these cells are GFP-negative, they can be easily separated from GFP-positive MTCs by FACS.

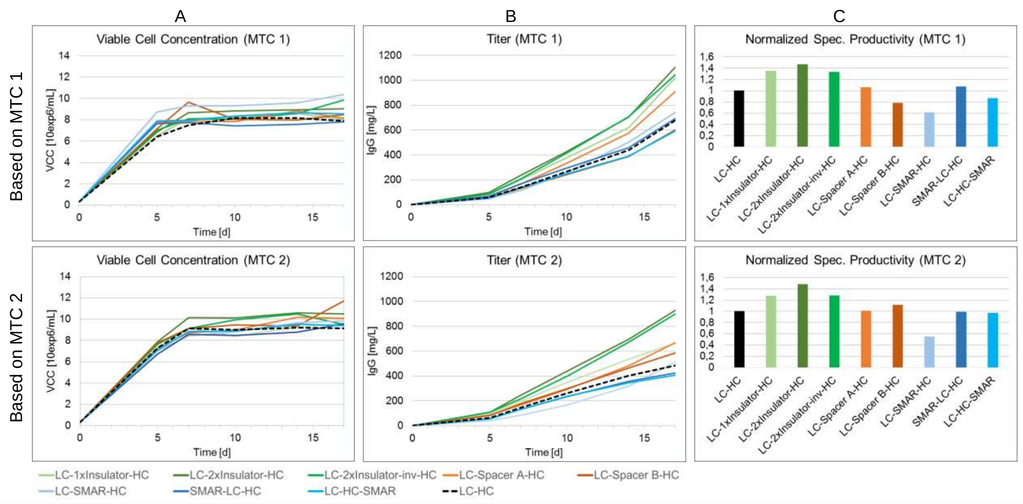
2 - IgG1 expressing vectors



(1) The standard IgG1 expressing vector contains two expression units with separated promoters, polyA and genes encoding light (LC) and heavy chain (HC), respectively. Based on this vector, new IgG expressing plasmids were generated equipped with (2) insulators (INS), (3) non-functional gene sequences (Spacer) and (4) SMAR elements at different positions, respectively.

It was supposed that the genetic elements can influence differently the transcription of the LC and HC gene causing a change in amount and balance of LC and HC peptides resulting in an improvement or impairment of IgG1 titer. To compare exclusively the effects of the genetic elements on transcription, all vectors were site specifically integrated in the same locus of MTCs (RMCE) keeping the influence of the genomic environment (hotspot) constant. To answer the question, if the effects of the genetic elements are universal or locus dependent, PTC pools were established from two differently tagged MTCs (1 and 2).

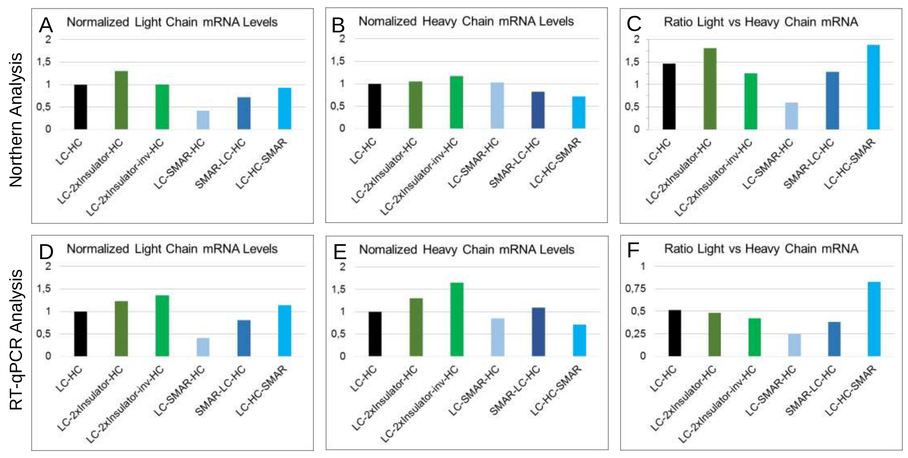
3 - Fed batch of IgG1 expressing TurboCell™ pools



PTC pools established by site specific integration of the different IgG1 expressing vectors into the tagged locus of both MTC 1 and 2, and subsequent cell sorting (FACS), were cultured in shake flasks (fed batch with temperature shift). Viable cell densities (Fig. A) and IgG1 concentrations (Fig. B) were measured (up to day 17). Specific productivities of the recombinant cells were calculated and normalized with respect to data generated from cells carrying the standard vector without genetic elements LC-HC (Fig. C).

Whereas the viable cell densities of the different cell pools, are similar to each other, the IgG1 titers are partially different. Cells containing vectors with insulators, achieve the highest titers (Fig. B, green lines) and have increased specific productivities (Fig. C, green bars) compared to cells with integrated standard vector (Fig. B and C, black dotted lines, black bars). Non-functional sequences (Spacer A and B) inside the vector lead to increased titers (Fig. B, brown lines) but have less impact on specific productivity (Fig. C, brown bars). SMAR elements can not improve the IgG1 production (Fig. B and C, blue lines, blue bars). Quite the opposite, the vector with a SMAR element positioned between LC and HC expression units, causes a distinct reduction in specific productivity (Fig. C, light blue bars). In terms of relation between integration site and expression, only slight differences between cells derived from MTC 1 and 2 can be observed. Independently from the integration site in MTC 1 and 2, the vectors retain their properties concerning improvement, impairment or stabilization of IgG1 expression. It is concluded that the different expressions are solely caused by the inserted genetic elements and not by the genomic environments of loci.

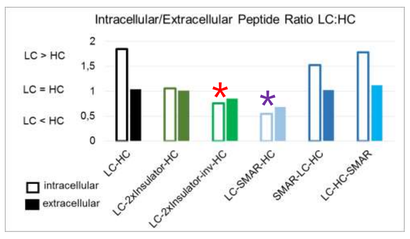
4 - Analysis of LC and HC transcription by Northern blotting and RT-qPCR



RNA from chosen PTC pools derived from MTC 1, was isolated and quantified by Northern (Fig. A-C) and RT-qPCR (Fig. D-F). Levels of mRNA were subsequently normalized with respect to mRNA levels of cells with integrated standard vector (Fig. A, B, D and E, black bars). Furthermore the ratios between LC and HC mRNA were calculated (Fig. C and F).

The integration of insulators between the LC and HC expression units, can enhance the transcription of both mRNA (Fig. A, B, D and E, green bars). Cells containing a vector with a SMAR element, have mainly reduced levels of both LC and HC mRNA (Fig. A, B, D and E). Dependent on the position of the SMAR inside the vector, the effects on transcription are different (blue bars). A SMAR positioned between both expression units or at the downstream end of the vector, influences negatively the transcription of the upstream located LC (light blue bars) and HC gene (bright blue bars), respectively, resulting in significant changes in LC:HC mRNA ratios (Fig. C and F, light and bright blue bars).

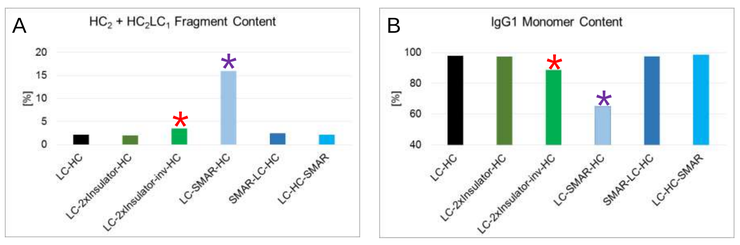
5 - Analysis of LC and HC peptide levels by CE-SDS



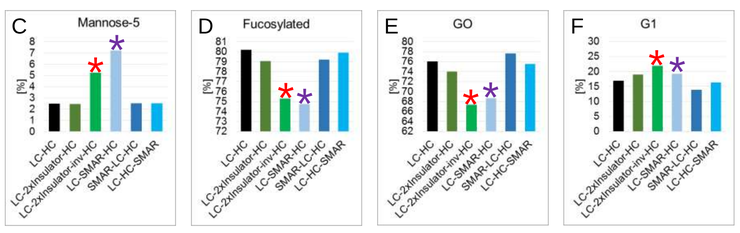
To determine the extra- and intracellular LC:HC peptide ratios, the supernatants and cell lysates from chosen PTC pools based on MTC 1, were batch purified using a mixed anti-Fc/anti-kappa resin. Purified samples were analyzed by reduced CE-SDS. LC:HC ratios were calculated as the half percentage peak area of the HC divided by the percentage peak area of the LC.

Cells containing a vector either with inverse oriented insulators or a SMAR element positioned in between the LC and HC expression units, show significantly reduced intracellular/extracellular peptide ratios (LC:HC ratio < 1) (asterisks) which are related to reduced levels of LC peptides or/and increased levels of HC peptides (HC excess). In cells carrying other vectors, the intracellular level of LC peptides is higher or equal compared to the one of HC peptides (LC:HC ratio ≥ 1). The extracellular levels of both peptides produced by these cells, seem to be in balance (LC:HC ratio app. 1).

6 - Evaluation of product quality

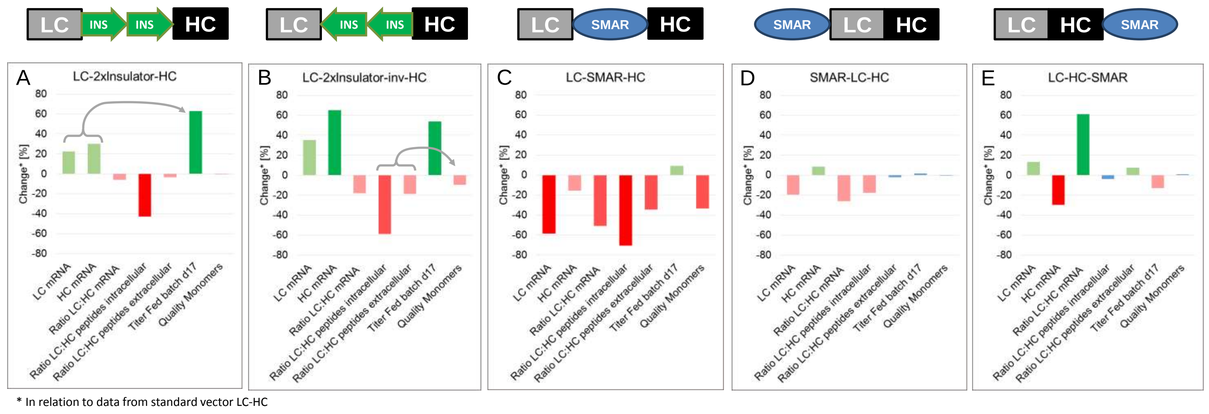


Non-reducing CE-SDS analysis of Protein A purified supernatants after fed batch cultivation of the chosen PTC pools derived from MTC 1, was used to quantify the content of mAb fragments missing one (HC_2LC_1) or both (HC_2) light chains (Fig. A). Antibodies produced by cells with low LC:HC peptide ratios (Chap. 5, asterisks) show increased levels of mAb fragments with missing LCs (Fig. A, asterisk). Especially cells with the construct LC-SMAR-HC produce antibody fragments with a tremendous absence of LC (Fig. A, light blue bar). Furthermore, the content of monomers in purified supernatants was analyzed by SE-HPLC. Cells with reduced ratio of LC and HC peptides (Chap. 5) show significantly decreased monomer contents (Fig. B, asterisks) presumable due to the limited number of LC peptides (Chap. 5) and the aggregation of the HC_2 / HC_2LC_1 –fragments (Fig. A).



N-Glycan profiling assay (Perkin Elmer) was used to determine the N-glycan distribution of the Protein A purified IgG1 from fed batch cultured cell pools. Antibodies produced by cells with vectors causing LC:HC ratios above 1 (Chap. 5), are low Mannose-5 glycosylated (Fig. C, 2%). In contrast, cells carrying vectors with insulators (inverted) or a SMAR element between both expression units leading to a decreased LC:HC ratio, produce antibodies which are higher glycosylated with Mannose-5 (Fig. C, asterisks, 5-7%) and less fucosylated (Fig. D, asterisks). They also show an altered level of galactosylation (Fig. E and F, asterisks). These changes in glycosylation are probably caused by an overload of the cell's folding and quality control machinery related to an excess of HC.

Conclusion



* In relation to data from standard vector LC-HC

Insulators between the LC and HC expression units of the vector promote the transcription of both LC and HC resulting in increased titers (Fig. A and B) whereas a SMAR element at the same position negatively influences the transcription without any effect on IgG production (Fig. C). However, an excess of HC peptides observed in application with vectors containing inverted insulators or a SMAR in between the expression units , has negative effects on protein quality (Fig. B and C). This is revealed by decrease of monomers (Chap. 6, Fig. B) and increase of fragments (Chap. 6, Fig. A) as well as increase of Mannose-5 glycosylation (Chap. 6, Fig. C), decrease of fucosylation (Chap. 6, Fig. D) and alteration in galactosylation of the products (Chap. 6, Fig. E and F). A downstream located SMAR impairs the HC mRNA transcription leading to a reduction of titer because of missing HC peptides for IgG formation despite sufficient amounts of LC mRNA/peptides (Fig. E). However, the quality of the product is not affected by the change in LC and HC transcription due to the SMAR. A SMAR element in the upstream region of the vector seems to have no significant impact on transcription and translation levels of LC and HC as well as on titer and product quality (Fig. D).

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