

Short Communication

Optimized Delivery of Small Regulatory RNAs into Industrial CHO Cell Lines Enables High-Throughput microRNA Screening

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally control mRNA abundance and thus critically regulate cellular phenotypes. In addition, efforts were made to engineer Chinese hamster ovary (CHO) cells which represent the most frequently applied host cell type for commercial production of therapeutic proteins. In the present study, we established a high-throughput screening approach which enabled us to assess the power of miRNAs in CHO cell line engineering approaches. To this end, a robust cultivation method was developed mimicking appropriate cultivation conditions for CHO manufacturing cell lines in micro-scale format. Different chemical transfection reagents were tested for their capability to efficiently introduce small RNAs such as siRNAs and miRNA mimics into CHO cells. We found that the novel cationic lipid Screen Fect®A plus exhibits excellent transfection efficiency and functionality in various CHO cell lines cultivated in complex production media. Recent approaches using miRNAs in CHO cells were limited to model cell lines that were far away from industry standard or produced standard IgG molecules. The experiments described here were performed using industrial production CHO cell lines stably expressing different monoclonal antibodies as well as novel antibody-derived molecules at various expression levels. This study demonstrates that the identification of a suitable transfection reagent and the careful optimization of the transfection conditions in combination with a micro-scale cultivation process led to the development of a high-throughput miRNA screening platform. A feasibility study using known pro-productive miRNAs rendered this approach suitable for future attempts aiming at the identification of novel miRNA candidates for innovative CHO cell engineering strategies.

INTRODUCTION

While microRNAs (miRNAs) were already discovered in the early 1990s [1], they have only very recently been rendered suitable for optimizing Chinese hamster ovary (CHO) production cell lines [2-5]. Due to the possibility to regulate multiple target genes without raising the translational burden of the cell [6], miRNAs could no longer escape notice with regard to CHO cell engineering. There is a plethora of conceivable pathways to be exploited by miRNAs such as the protein production machinery, secretory pathways, cell cycle or apoptosis [5]. To study phenotypic changes caused by a given effector miRNA, stabilized small RNA duplexes called miRNA mimics can be introduced into cultured mammalian cells by transient transfection [7,8]. However, with regard to CHO cells this approach represents a considerable challenge since CHO production cells are grown in complex cultivation media that usually hamper nucleic acid transfer using cationic lipids and polymers. Due to continuous cell division transiently transfected small regulatory RNAs are only available in the cell for a limited period of time [9]. Chemical transfection methods including cationic polymers,

calcium phosphate and cationic lipids are the most commonly used methods for introduction of nucleic acids such as plasmid DNA, mRNAs, small interfering RNAs (siRNAs) and miRNAs into cultured mammalian cells [10]. These positively charged chemicals form complexes with anionic nucleic acids resulting in positively charged lipoplexes which interact with the negatively charged cell surface. The exact uptake mechanism of the lipoplexes is still not fully understood but it is believed that endocytosis and phagocytosis are involved in this process [11-13]. Although transfection of DNA in CHO cell cultures mediated by polyethylenimine (PEI) and calcium phosphate (CaPO) has been proven feasible [14,15], these transfection methods seem to be unsuitable for a successful delivery of small ribonucleic acids such as siRNAs and miRNAs into CHO cells [7]. Recently, a chemical transfection reagent termed ScreenFect® has been identified to be capable of efficiently delivering active miRNA mimics into pharmaceutical CHO production cell lines [7].

In the present study, we evaluated and optimized the application of two novel ScreenFect® derivatives (ScreenFect®Aplus and ScreenFect®AsiRNA) for its ability to

transfer functional miRNA mimics into industrial CHO cells cultivated in complex production media. Towards this end, we transfected various CHO cell lines growing in complex production media and which produced different forms of recombinant proteins. We compared these two novel transfection reagents to their parental form (ScreenFect®A) as well as to other frequently used cationic lipids. We could demonstrate that ScreenFect®Aplus was able to efficiently introduce functional miRNA mimics into all CHO cell lines tested. Optimization of the miRNA transfection procedure in conjunction with the development of a micro-scale cultivation process using agitated 96-well plates enabled the generation of a high-throughput miRNA screening platform for CHO cells. The feasibility of the established workflow was finally demonstrated using known productivity enhancing miRNAs.

MATERIALS AND METHODS

Cell culture

An overview on the recombinant CHO production cell lines used for transient miRNA transfection experiments are listed in Table (1). Boehringer-Ingelheim proprietary serum-free, chemically-defined and animal component free cell culture medium was used for cultivation. Seeding cell density of stock cultures was set to 0.3×10^6 viable cells per milliliter and cells were passaged in a 4/3 day rhythm. Cell concentration and viability during routine stock culture cultivation was assessed using a CedexAnalyzer™ (Roche Diagnostics, Mannheim, Germany) by means of trypan blue exclusion.

Transfection

The delivery of miRNA mimics or siRNAs (Qiagen, Hilden, Germany) was achieved using the cationic lipid transfection reagent ScreenFect® A plus (InCella, Eggenstein-Leopoldshafen, Germany). A volume of 8.62 µL ScreenFect® Dilution Buffer (InCella) was initially added to 0.18 µL ScreenFect®Aplus for each transfection approach before mixing the complex with the respective miRNA/siRNA mimic (final concentration 50 nM) and incubating the transfection complexes for 20 minutes at room temperature. The cationic lipid: miRNA complex was transferred to a 96-deepwell U-bottom plate (Enzyscreen, Haarlem, Netherlands) for small-scale transfection in biological triplicates. Exponentially growing proprietary CHO cell lines were pelleted, resuspended in fresh culture medium and seeded onto the transfection complex solution with a viable cell density of 0.5×10^6 viable cells per milliliter. For agitated cultivation of the transfected cells, 96-deepwell culture plates were placed onto a Mini-Orbital digital shaker (Bellco, Vineland, USA) located inside of a Multitron® incubator (Infors, Bottmingen, Switzerland) and cultivated at 37°C, 5% CO₂, 90% relative humidity and 800 rpm (1.6 mm orbit). MiScript miRNA mimics, All Stars Negative Control siRNA (non-targeting siRNA), fluorescence labeled non-targeting control (siRNA-AlexaFluor®647) and CHO-specific cell death control siRNAs were purchased from Qiagen (Qiagen, Hilden, Germany) in lyophilized form (1.0 nmol/miRNAmimic). Transfections with reagents other than ScreenFect® were performed according to the appropriate protocols of the respective vendors (Table 2).

Focusing on industrial CHO cells, the chosen miRNAs were

Table 1: Overview on recombinant CHO cell lines used for transient miRNA transfection studies and cultivation experiments.

Cell line ID	Selection system	Type of protein	mAbexpressionlevel
CHO-mAb1	GS	IgG antibody	High
CHO-mAb2	GS	Bispecific antibody	Medium
CHO-mAb3	DHFR	IgG antibody	High
CHO-mAb4	GS	BispecificIgG-scFv fusion	Medium
CHO-mAb5	GS	IgG antibody	Low

Table 2: Commercial transfection reagents used for transient transfection of a fluorescence labeled siRNA (siRNA-NT-AlexaFluor®647).

Transfection reagent	Supplier	FC transfection reagent per well	FC siRNA per well	Complex formation time
FuGene HD®	Promega Corp. ¹	3.0 µL	50 nM	10 min
SuperFect®	Qiagen GmbH ²	2.0 µL	50 nM	10 min
ScreenFect® siRNA	InCella GmbH ³	1.5 µL	50 nM	20 min
ScreenFect® A	InCella GmbH ³	1.5 µL	50 nM	20 min
ScreenFect® A plus	InCella GmbH ³	1.5 µL	50 nM	20 min

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picked from relevant literature dedicated to non-coding RNA screening studies in CHO cells. Both members of the miR-30 family (*mmu*-miR-30a-5p and *mmu*-miR-30c-5p) were identified to boost recombinant protein production in CHO cells in a functional high-content miRNA screen. The miRNA mimics *hsa*-miR-1287-5p and *hsa*-miR-557 originate from a genome wide functional miRNA screen in CHO cells performed with a human miRNA library [8]. The *mmu*-miR-483 mimic was included in the investigation since recent studies showed that miR-483 is a key regulator of cellular productivity in CHO cells [16].

Product quantification and cell specific productivity

Concentration of standard mAbs and fusion proteins possessing an Fc part was determined using the bio layer interferometer Octet® QK384 (Pall Life Science, Dreieich, Germany).

The cell-specific product secretion rate qP (pg x cell⁻¹ x d⁻¹) was calculated according to Equation I where D represents the dilution rate, VCD (cells x mL⁻¹) is the viable cell density and C_p (µg x mL⁻¹) the product concentration.

$$q_p = D \times \frac{C_p}{VCD} \times 10^6 \left[\frac{\text{pg}}{\text{cell} \times \text{day}} \right] \quad (\text{Eq. I})$$

Flow cytometry

Fluorescence assisted cell analysis was performed using a MACSQuant® Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany) equipped with a violet (405 nm), blue (488 nm) and red (635 nm) excitation laser. Cell concentration, viability and necrosis were set as readout parameters. For sample preparation,

100 μ L cell suspension was pelleted and resuspended in a multiplex staining solution composed of Boehringer Ingelhe proprietary cell culture medium supplemented with 3 μ g/mL CellTrace™-Calcein Violet-450-AM (Thermo Fischer Scientific, Waltham, MA) and 5 μ g/mL propidium iodide (Carl Roth, Karlsruhe, Germany). Cells were protected from light and incubated for 25 minutes at 37°C for staining. The sample plate remained cooled down to 4°C until measurement. About 25 μ L (approximately 30.000 cells) were analyzed per sample at a flow rate of 100 μ L per minute. Calcein Violet-450-AM positive cells were considered as viable and propidium iodide positives were seen as necrotic.

Fluorescence microscopy

For fluorescence microscopy, the Cellavista™ high-end RS cell imager (Synentec bio Services GmbH, Münster, Germany) was used. For the measurement, 20 μ L of the sample were diluted 1:4 in 1XDPBS (Life Technologies, Carlsbad, CA USA) in a 96-well flat bottom plate (Greiner bio-one, Frickenhausen, Germany). Hoechst® 33342 (BD Pharmingen™, Franklin Lakes, USA) was applied at a final concentration of 2.7 μ g/mL. The incubation was conducted in the absence of light at room temperature for 20 minutes. Filters used: Ultraviolet (395/452 nm), Green (530/585-640 nm), Red (620/692-740 nm). Microscopy was conducted with 10 x magnification on auto-focal mode.

Statistics

Trials were calculated, unless otherwise stated, as mean value \pm standard deviation of mean value ($\bar{x} \pm SD$) of n independent experiments. All experiments were conducted in biological triplicates ($n = 3$). Differences among data sets were tested on significance in a two-tailed Student's t-test assuming an error probability of $\alpha = 0.05$. The one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison post-test was conducted for comparing more than two data sets and for many-to-one comparisons. Significance was assumed for p-values less than or equal to 0.05. Data normalization for product titer and specific productivity fold change diagrams was performed by dividing each data point by the arithmetic mean of the respective on-plate control (non-targeting siRNA).

RESULTS

High-throughput transfection approaches for screening purposes using mammalian production cell cultures require miniaturization of cultivation processes. To develop a representative high-throughput applicable micro-scale cultivation format for suspension growing CHO cells, different microplate cultivation systems were evaluated. A recombinant antibody producing CHO cell line (CHO-mAb4) was cultivated in a batch cultivation process for four days without additional nutrient supply and constant cultivation volume either using a standard shake flask (250 mL), a 48-well FlowerPlate® or aDuetz®-96-deepwell plate. Cells were grown at 37°C and 5 % CO₂ and agitation parameters were optimized for each culture format in order to ensure optimal cell growth (data not shown). Agitation speed was set to 125 rpm (50 mm orbit) for shake flasks, 220 rpm (50 mm orbit) for 48-well FlowerPlates® and 800 rpm (1.6 mm orbit) for Duetz®-96-deepwell plates.

Cultivations in microplate format were cultivated at 90 % relative humidity to prevent evaporation of cell culture media. Cells cultivated in 48-well FlowerPlates® and Duetz®-96-deepwell plates demonstrated similar growth behavior (Figure 1A) and cell viability (Figure 1B) as compared to cells grown in standard shake flasks. Furthermore, pH profiles (Figure 1C) and lactate production rates (Figure 1D) were highly similar between the different cultivation conditions. This indicates that cultivation of CHO cells using our optimized micro-scale formats is highly comparable to standard shake flask cultivation but additionally opens up possibilities for high-throughput applications.

For a reliable interpretation of phenotypic changes induced by ectopically introduced miRNAs, it is crucial to prove the effective delivery and functionality of miRNA mimics into the desired cell types. Different CHO cell lines producing different recombinant proteins (Table 1) were transfected with commercially available cationic lipids (Table 2) and subsequently analyzed for positively transfected cells 24 h post transfection via automated fluorescence microscopy (Figure 2). Cells transfected using ScreenFect®*Aplus* exhibited the highest transfection efficiency visualized by discrete red fluorescent spots in immediate vicinity to the cell nuclei. In contrast, only few red spots were detected in cells transfected using SuperFect® (Figure 2). Furthermore, SuperFect® mediated transfection resulted rather in fluorescent precipitates than co-localized with the blue cell nucleistain (Figure 2). Cells transfected using ScreenFect® *siRNA* and ScreenFect® *A* revealed also increased levels of AlexaFluor®647-fluorescence but at substantially lower extent compared to ScreenFect® *A plus* (Figure 2). Slight differences in transfection efficiency can be observed for most transfection reagents in CHO-DHFR^{-/-} and CHO-GS^{-/-} cells. However, ScreenFect®*Aplus* generally demonstrated the highest AlexaFluor®647 fluorescence levels regardless of the transfected cell line. Using an optimized miRNA transfection procedure, reproducible transfection efficiencies close to 100 % were achieved in all CHO cell lines and cultivation media (Figure 3), as shown by quantification of AlexaFluor®647-positive cells using flow cytometry as well as substantial induction of cell death after transfecting a cell death inducing siRNA (siTOX). Transfection of AlexaFluor®647 labeled or unlabeled non-target siRNA did not show negative effects on cell viability but necrosis rates increased substantially for siTOX transfection confirming transfection functionality (Figure 3).

We then selected ScreenFect® *A plus* and transfected several pro-productive miRNA mimics into CHO production cell lines followed by analysis of changes in recombinant protein production. As a reference, a non-targeting negative control siRNA (siRNA-NT) was used. All miRNA mimics and control siRNAs were transfected in 96-well plates and cultivated for 96 hours until transfected cells were analyzed for changes in cell proliferation and viability via flow cytometry and using Bio-Layer Interferometry for determination of changes in antibody concentration in the culture supernatant (Figure 4A). As shown in Figure (4B), over 75 % of the miRNA mimics transfection approaches resulted in an increase in volumetric productivity of greater than 20 % compared to siRNA-NT transfected control cells. Furthermore, more than 45% of the miRNA mimics transfection approaches were able to increase cell-specific productivity. While miR-30a-5p and miR-483-3p appear to

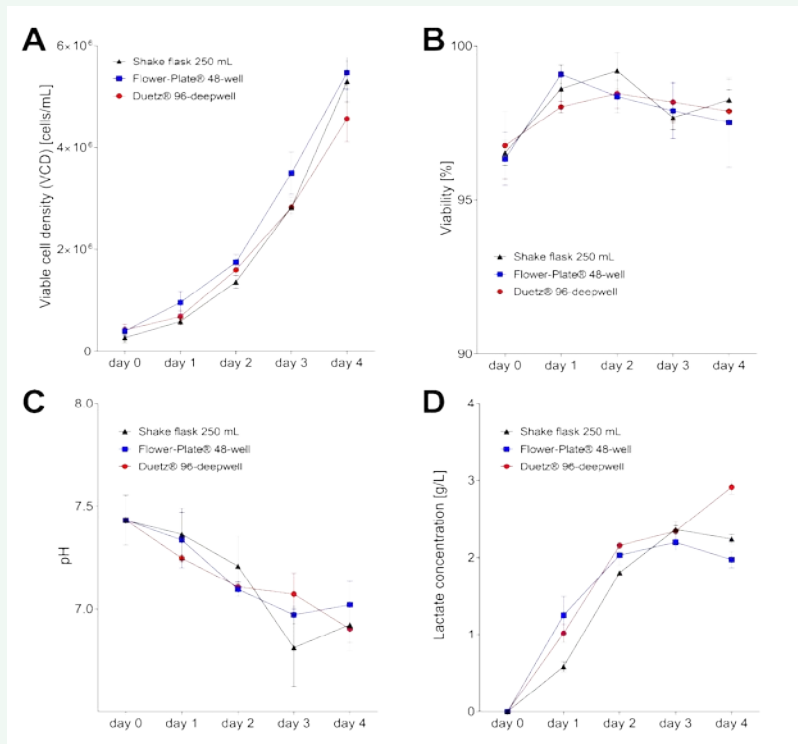


Figure 1 Batch cultivation of CHO-mAb4 cells in different cultivation formats. (A) Time-resolved determination of viable cell density (VCD) and (B) viability during the cultivation period. (C) pH and (D) lactate concentration during cultivation.

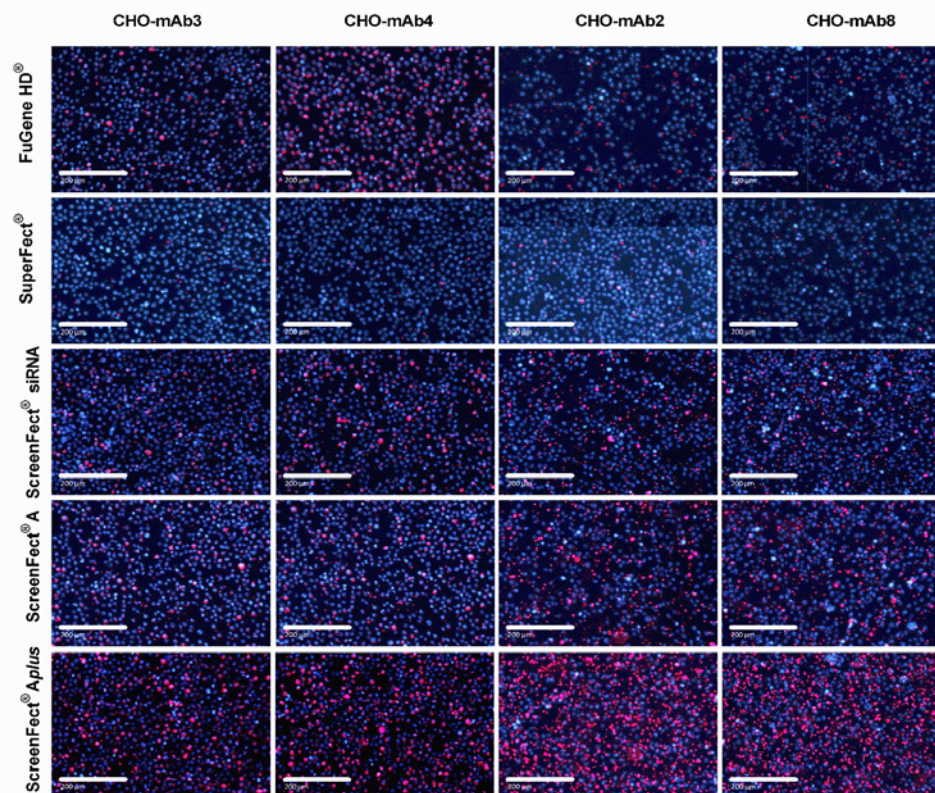


Figure 2 Fluorescence micrographs (10X magnification) of CHO cell lines transiently transfected with an AlexaFluor®647 coupled siRNA (red dots) using several commercially available transfection reagents. Cell nuclei were counterstained with Hoechst® 33342 (blue). Scale bars indicate 200 µm.

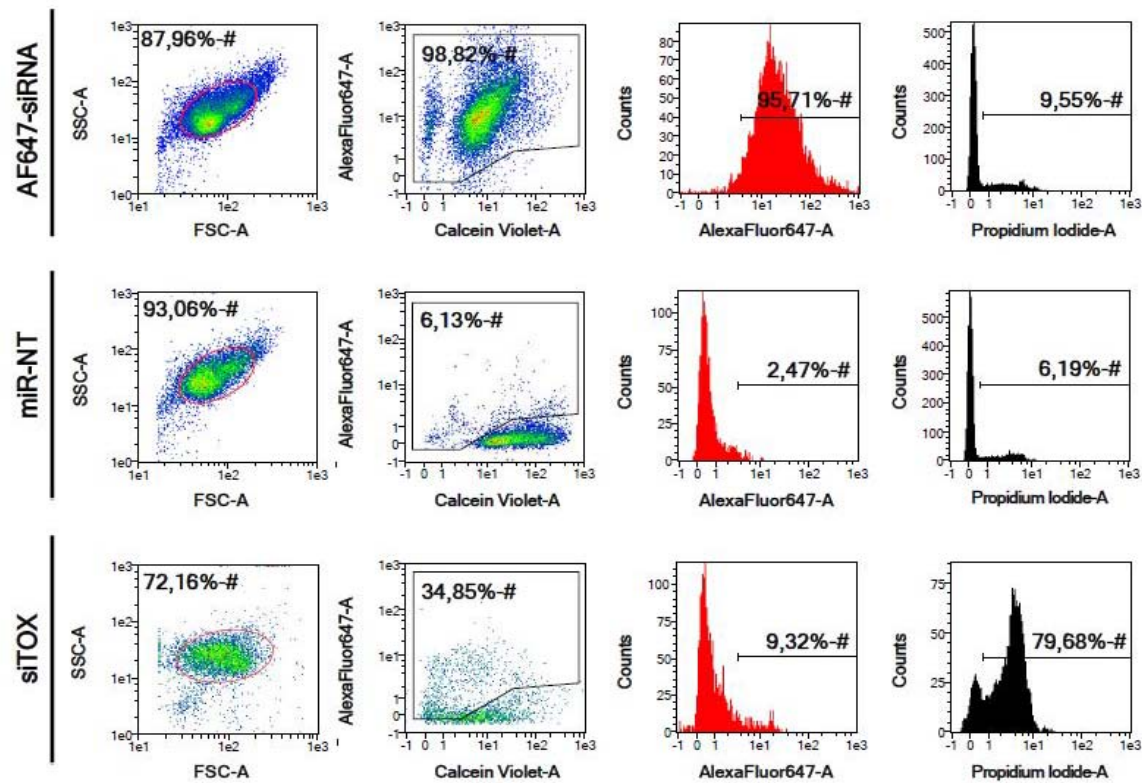


Figure 3 Determination of transfection efficiency of CHO-mAb3 cells after transient transfection with the Alexa Fluor®647 labeled non-target-siRNA (AF647-siRNA), non-labeled miR-NT and the cell death inducing control siRNA (siTOX). Cells were transfected using Screen Fect® A plus and analyzed for Calcein Violet positive (viable), Alexa Fluor®647 positive and propidium iodide positive cells (necrotic) at 48 h post transfection via flow cytometry.

function in a rather cell line specific manner, miR-30c-5p and miR-557 increased recombinant protein production in all cell lines tested (Figure 4B).

Taken together, all tested miRNA mimics were able to induce a significant increase in volumetric or specific productivity in several CHO cell lines (Figure 5), with each cell line expressing a different antibody or antibody-derived product. Although CHO-mAb1 cells were found to be least amenable to the optimized transfection process compared to the other cell lines, we could observe an increased cellular productivity upon transfection but at lower significance levels. Transfection of CHO-mAb2 and -mAb4 cells, however, showed the greatest significance in product titer increasing effects among all tested CHO cell lines (Figure 5). Notably, miR-30c-5p and miR-557 generally exhibited a very good performance in all CHO cell lines tested.

DISCUSSION

CHO cell engineering is of utmost importance to constantly improve cellular performances and product qualities in biopharmaceutical production processes [5]. This is especially true for the ever increasing number of sophisticated and therefore difficult-to-express proteins. Facing this challenge, cell line engineers will be compelled to develop novel state-of-the-art strategies.

We aimed to set-up and optimize a high-throughput cultivation and transfection process for industrial CHO cell lines

to enable screening of miRNA mimics. At first, we evaluated two different small-scale high-throughput cell cultivation formats and subsequently established a workflow for an efficient and functional delivery of small regulatory RNAs into proprietary CHO production cell lines. Results of batch cultivation processes suggest that Duetz® 96-deepwell plates appear to be highly comparable to the standard shake flasks and 48-well FlowerPlates® in terms of growth behavior rendering them suitable for high-throughput transfection procedures. Since the tested CHO cell line also showed very good cultivation performance in 48-well FlowerPlates®, this format is an appropriate alternative in high-throughput applications thereby enabling more extensive analytics due to increased culture volumes.

To study the delivery of small ribonucleic acids into CHO cell lines, a fluorescently-labeled non-targeting siRNA (siRNA-AlexaFluor®647) was transfected into four different antibody producing CHO cell lines using several different transfection reagents. Albeit all cells used in this study were CHO cells, transfection efficiency varied substantially between the tested transfection reagents. It is known that cell culture media can greatly influence nucleic acid transfection due to media ingredients that interfere with the transfection reagents [7]. Given that different CHO cell lines are closely related but nevertheless profoundly different [17], uptake of small RNAs may vary due to heterogeneity in cell surface composition which directly affects transfection efficiency [18]. Therefore, the aim was to identify a non-viral transfection reagent capable of delivering

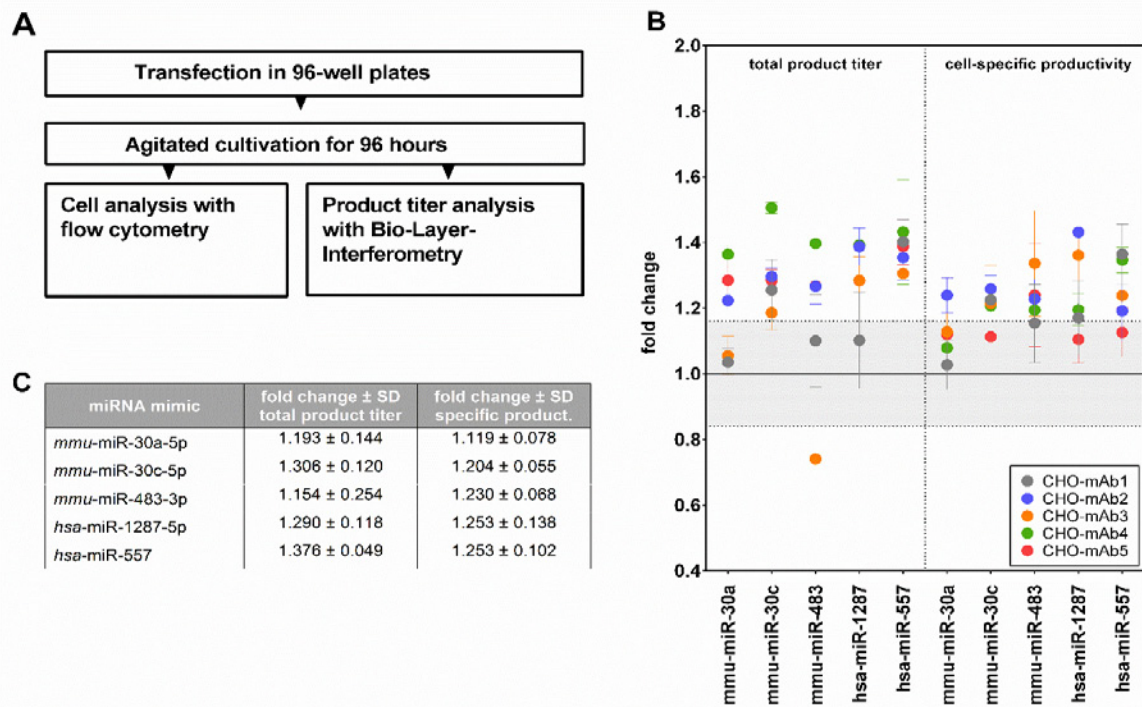


Figure 4 Transient small-scale transfection of potential pro-productive miRNA mimics in proprietary CHO cell lines. (A) Schematic overview of experimental workflow. (B) Dot-plot of miRNA mimics' performance with regard to the effect on total product titer and specific productivity. For each miRNA, the fold change in total product titer and specific productivity normalized to the mean value of the respective on-plate control is plotted. Grey background indicates the variance region (20 % from zero effect). (C) Performance of the miRNAs merged over all tested CHO cell lines.

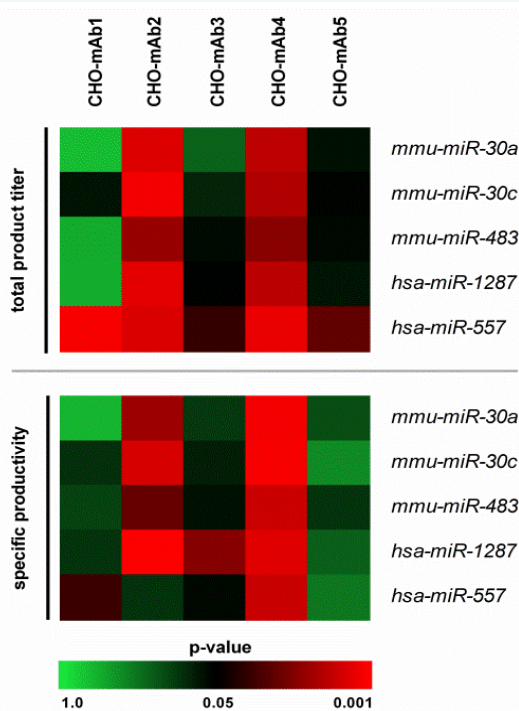


Figure 5 Heat-map plot representing the P-value of volumetric antibody productivity and specific productivity enhancement of the respective miRNA mimics. Highly significant values are shown in red. P-Values lower than 0.05 (black) were considered as significant.

miRNA mimics into several different CHO cell lines at high efficiency and low cytotoxicity. Data obtained from fluorescence microscopy and flow cytometry showed that ScreenFect® *Aplus* was the only transfection reagent fulfilling these prerequisites in a reproducible manner for all cell lines tested. In an optimized transfection procedure, we were able to evaluate potential pro-productive miRNAs known from the literature which significantly increased the productivity of standard and difficult-to-express monoclonal antibodies in various BI-HEX® CHO cell lines and thus were confirmed as cell line and product independent pro-productive miRNAs. Especially, miR-30c-5p and miR-557 were able to induce a significant increase in volumetric antibody yield in all tested CHO cell lines.

In recent years, discoveries in genetic engineering such as precise genome editing, overexpression of beneficial genes or exploitation of non-coding RNAs had remarkable impact on CHO cell line development [5]. Current state-of-the-art industrial cell line generation processes are capable of generating clonal cell lines exceeding volumetric productivities of 5 g/L. However, efforts are focused on the improved production of difficult-to-express proteins that actually fail to achieve product titers in the multigram per liter range. Especially the removal of intracellular bottlenecks which prevent CHO cells from generating sufficient amounts of product can be addressed by next-generation cell engineering approaches such as microRNAs. This is required predominantly for novel biopharmaceutical formats such as bi- and multi specific antibodies, antibody derived fragments or conjugates as well as other heterologous proteins. As the

genomic knock-out of a gene-of-interest that is suspected to be counterproductive for the cell can partly result in unexpected (negative) phenotypic changes, introducing a miRNA in order to down-regulate a multitude of different genes concomitantly represents a smart approach to improve CHO cell factories in the future.

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