Application Note
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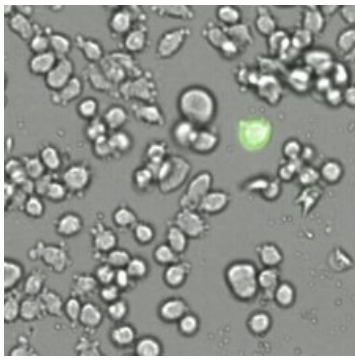
CRISPR/Cas9 Induced DHFR-Knockout in CHO Cells

Thies F[2], Christmann T[1], Werdelmann B[1], Hölscher M[1], Lüke J[1], Lückerath F[1], Mittmann K[2]

[1]SYNENTEC GmbH, [2]University of Applied Science Münster, Germany

Abstract

With the CRISPR/Cas9 system a new era of gene editing possibilities started in the year 2012. In comparison to other modern methods based on endonucleases like TALENS or ZNF, CRISPR/Cas9 provides a cheap and at the same time safe and precise method to specifically target a certain genetic sequence and edit it.
In this application note, we present a DHFR-knockout in CHO cells using the CRISPR/Cas9 method and the SYNENTEC Cell Imagers. So we used lipofection to transiently insert a Plasmid containing the Cas9, the gRNA against dhfr and a a tgfp as a selection marker for the transfection (fig. 1). After transfection, we used Limited Dilution to isolate single cells, which can proliferate to a monoclonal cell line of CRISPR treated cells using the GFP-fluorescence as a control (GFP-co-transfection).
To proof the DHFR knockout, we performed PCR, Western Blotting and a proliferation test using replica plating as well as an auxotrophy test with Methotrexate (MTX) as a DHFR inhibition in wild type CHO cells. For all necessary measurements we used the SYNENTEC's CELLAVISTA® and NYONE® Cell Imager and the associated image analysis YT®-software.



Keywords: CRISPR/Cas9, CHO cells, Transient Lipofection, Transfection, Fluorescence Microscopy, GFP, Methotrexate, DHFR-knockout, Monoclonal cell line, Cell line development, Limited Dilution, mAb

Introduction

The first discovery of a CRISPR locus happend in 1987 during a sequencing of *Escherichia coli* (Ishino et al. 1987). Since then the basic principles and functionalities of the CRISPR/Cas9 were revealed more and more until a detailed description and possible fields of application was published in 2012 and revolutionized modern gene editing (Sternberg et al. 2015).

Naturally occuring in bacteria or archaeal species as part of the immune system against viral intrusion and proliferation. A endonuclease protein named cas (CRISPR associated) cleaves viral DNA and transfers a fragment to the CRISPR–locus (Clustered Regularly Interspaced Short Palindromic Repeats) for incorporation. These fragments transcript for a precrRNA/crRNA, which guides another cas protein to DNA corresponding to the crRNA for cleaving (Mali et al. 2013).

Three different types of CRISPR/Cas systems have been identified in bacterias and archaea so far, with the type II system being the one best suitable for genome engineering purposes (Chira et al. 2017).

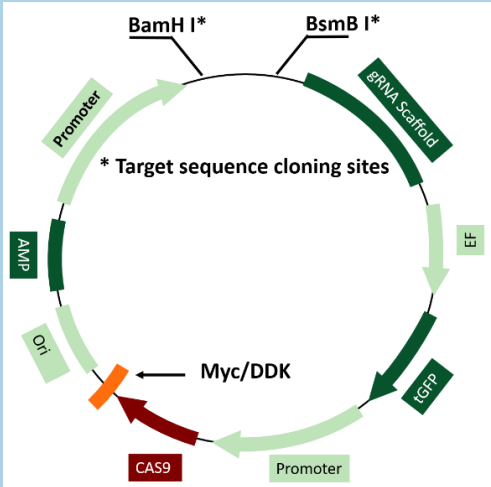


Fig. 1: Used plasmid for transfection. Plasmid contains gene for Cas9- and tGFP-protein as well as gRNA. Necessary components like ORI and promoters are presented but not named.

Type I and III systems use endonucleases which process pre-crRNA to crRNA, which is used further to form a Cas protein complex. This complex cleaves DNA complementary to the bounded crRNA.

Type II system on the other hand works slightly different, in a manner that another molecule called trans-activating crRNA (tracrRNA) is also necessary to cleave DNA. Furthermore a specific three base pair long sequence, called protospacer adjacent motif (PAM), is needed downstream to cleaving site for recognition (Jinek et al. 2012).

The combination of both crRNA and tracrRNA into a single molecule (gRNA), linked to a cas9 protein, allows a simple and versatile way of creating a programmable complex, that targets and cleaves DNA precisely.

Using either Non-Homologous End Joining (NHEJ) or Homology Directed Repair (HDR), the cell can repair the double strand break (Rodriguez 2016) (fig. 2). Due to the high chance of errors occurring during NHEJ, a gene knockdown or knockout can be artificially created. Similar to that a knockin can be realized by providing the cell a template DNA containing a desired sequence for HDR (Sternberg et al. 2015).

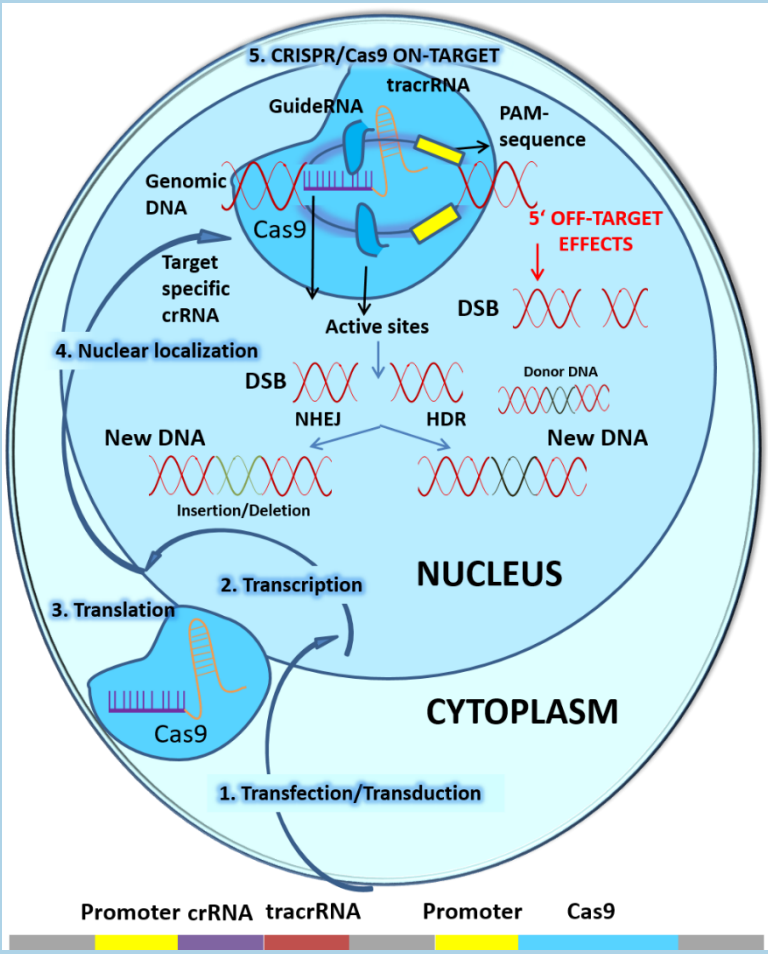


Fig. 2: Schematic presentation of gene editing using the CRISPR-system. Cell is transfected with a plasmid containing gene for Cas9- protein. Cas9 is transcripted and translated within the cell and cleaves genomic DNA according to guideRNA.

Gene editing can be applied in cell line development, by using a endonuclease to knockout the *dhfr* gene, creating a *dhfr* deficient cell line. Vectors designed for recombinant protein production contain an intact copy of the previous deleted *dhfr* gene close to the primary gene. Having such a cell line provides two major advantages for cell line development and production of recombinant proteins in cells. First, transfected cells can be selected by cultivating all cells in medium without Thymidine and Hypoxanthine. Only cells transfected with an intact *dhfr* gene can survive and proliferate within that medium (e.g. like well known CHO-DG44; ThermoFisher Scientific). This allows for an easy selection between transfected and non-transfected cells and a guarantee, that the primary gene is included in all cells (Urlaub et. Al. 1980).

The second advantage is, that the use of Methotrexate allows for a gene amplification of the primary gene. MTX acts as a competitive inhibitor for DHFR and a slowly increasing concentration of MTX exposed to the cell, forces it to increase gene expression of the *dhfr* gene to maintain proliferation. Since *dhfr* and primary gene are located close to each other, the higher expression rate also includes the desired protein (Christman et al. 1982).

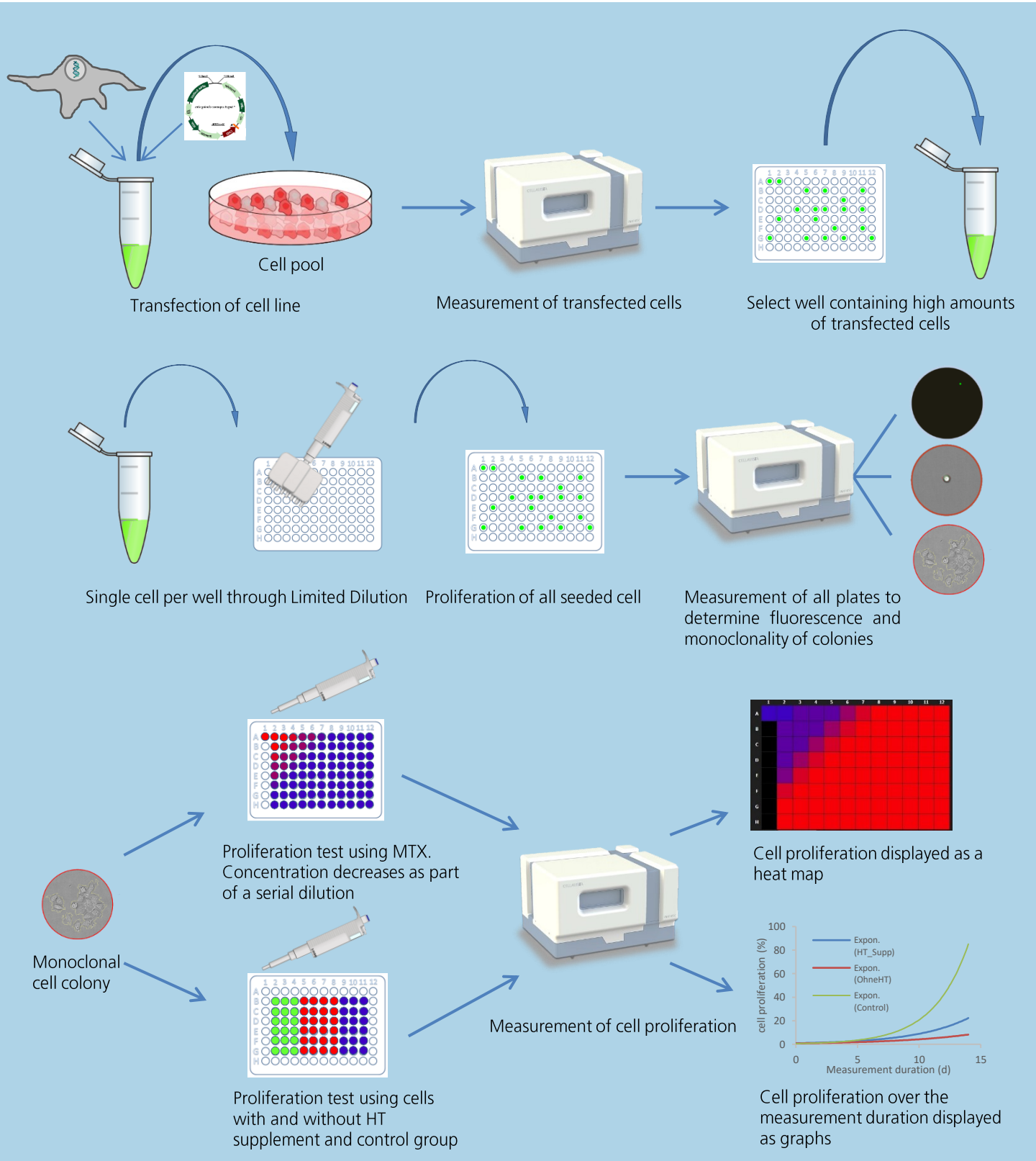


FIG. 3: SCHEMATIC PRESENTATION OF PROJECT'S WORKFLOW.
Each work-step is presented in graphical form to simply summarize each step performed. First step was a transfection of the cell line, followed by a single selection via Limited Dilution. Monoclonal colonies were used for a proliferation test, using Methotrexate as a natural DHFR inhibitor and a proliferation test, with and without external HT supplementation.

Material & Methods

1. Transfection:

Material:

- Chinese hamster ovary cells (K1); suspension adapted
- Panserin H5000 with 0.05 % Insulin
- XtremeGene™ HP DNA transfection reagent
- Gibco™ HT-supplement (100x)
- Plasmids containing gene for Cas9-protein, tGFP-protein, gRNA and gRNA scaffold
- Phosphate buffered saline (PBS^{--}, w/o Ca^{2+} and Mg^{2+})
- Trypan Blue 0.02 % stock
- Transparent 96-well half area plate (e.g. Corning™ cat. # 3695)
- Black 96-well plate (e.g. Greiner µClear® cat. # 655096)
- CELLAVISTA® Imaging System
- SYNENTEC's YT®-software

Methods:

For cell counting and viability determination a sample of CHO-K1 SuPa with a final dilution of 1:40 was set up with PBS^{--} and Trypan Blue. The sample was measured with the CELLAVISTA® and the **Trypan Blue** wizard.
The amount of XtremeGene™ HP DNA transfection reagent and cells per well was calculated according to the manufacturers instruction using the results from **Trypan Blue** image processing.
As part of the plate preparation cell solution was diluted and a volume of 100 µL containing 1E+04 cells and 1 µL of HT Supplement was filled in each well. Based on previous testing 2 µg of plasmid were incubated with a transfection reagent in a ratio of 1:1.5. To each well 20 µL of the incubated solution was added. The plate was stored 10 minutes at room temperature for better results.

Regular measurements with **Suspension cell count 1F** wizard of YT®-software were performed over a period of 10 days to monitor transfection efficiency.

This wizard consist of 2 channels:
- one for cell detection in brightfield
- one for fluorescence (in this case the transiently expressed tGFP (tab. 1))

Transfection efficiency was analyzed using the result property 1F/BF [%] generated by image processing.

Tab. 1: Optical settings for **Suspension Cell Count 1F** wizard

Channel	Brightfield	Fluorescence
Objective	10x (default)	
LED	LED_Brightfield (default)	LED_Blue (default)
Emission Filter	Emi_Green (default)	Emi_Green (default)
Intensity	100 %	100 %
Exposure Time	23 ms	300 ms
Gain	0 %	10 %

2. Limited Dilution:

Material:

- CHO-SuPa cells
- Transfected CHO-SuPa cells
- Panserin H5000 with 0.05 % Insulin and 1 % Penicillin/Streptomycin
- Gibco™ HT-supplement (100x)
- Multichannel pipettor
- Black 96-well plate (e.g. Greiner µClear® cat. # 655096)
- CELLAVISTA® 3.1 Imaging System and NYONE® Imaging System
- SYNENTEC's YT®-software

Methods:

For cell seeding with Limited Dilution the selected cell solution was transferred to a reaction tube and the cell density was determined with **Suspension Cell Count** wizard. According to the calculated dilution factor media was mixed with HT-supplement in a ratio of 1:100 and with the cell suspension and was distributed into sample carrier with approximately 0.5 cells per well with a multichannel pipettor (final volume 0.2 mL per well).

Plates were measured daily within a time period of 8 days using the brightfield and fluorescence channels for transfected cells and the brightfield channel for non-transfected cells. For image processing the YT®-software wizards **Suspension Cell Count 1F** and **Suspension Cell Count** were used (tab. 2 and tab. 3).

The **Single Cell Cloning** operator was used after 8 days to analyze monoclonality of grown colonies.

TAB. 2: OPTICAL SETTINGS FOR **SUSPENSION CELL COUNT 1F** WIZARD

Channel	Brightfield	Fluorescence
Objective	10x (default)	
LED	LED_Brightfield (default)	LED_Blue (default)
Emission Filter	Emi_Green (default)	Emi_Green (default)
Intensity	100 %	100 %
Exposure Time	17 ms	100 ms
Gain	0 %	40 %

TAB. 3: OPTICAL SETTINGS FOR **SUSPENSION CELL COUNT** WIZARD

Channel	Brightfield
Objective	10x (default)
LED	LED_Brightfield (default)
Emission Filter	Emi_Green (default)
Intensity	100 %
Exposure Time	3 ms
Gain	0 %

3. Methotrexate-Test:

Material:

- CHO-SuPa cells
- Panserin H5000 with 0.05 % Insulin and 1 % Penicillin/Streptomycin
- Phosphate buffered saline (PBS^{--}, w/o Ca^{2+} and Mg^{2+})
- DMSO
- Methotrexate lyophilized
- Trypan Blue 0.02 % stock
- Transparent 96-well half area plate (e.g. Corning™ cat. # 3695)
- Transparent 96-well plate (e.g Nunc™ MicroWell™ cat. # 167008)
- CELLAVISTA® Imaging System
- SYNENTEC's YT®-software

Methods:

For cell density and viability acquisition a Trypan Blue measurement (see 1. Transfection) was performed.
Prior to measurement the lyophilized MTX was resuspended in DMSO to create a 2 mM solution and then diluted in medium with a ratio of 1:1000 for a final concentration of 2 µM.
For plate preparation each well of a Nunclon 96 plate was prefilled with 100 µL medium (leave A1 empty). 200 µL of the MTX solution was added to well A1, and after that stepwise diluted as part of a serial dilution by the factor 2.
Finally cells were diluted to get 1000 cells per 100 µL and that volume was pipetted in each well, increasing the MTX final dilution factor to 3.
For a period of 7 days the plate was measured using brightfield channel and analyzed with **Cell Confluence** wizard (tab. 4).

TAB. 4: OPTICAL SETTINGS **FOR CELL CONFLUENCE** WIZARD

Channel	Brightfield
Objective	10x (default)
LED	LED_Brightfield (default)
Emission Filter	Emi_Green (default)
Intensity	100 %
Exposure Time	16 ms
Gain	10 %

4. Proliferation test:

Material:

- CHO-SuPa cells
- Transfected CHO-SuPa cells
- Gibco® CD CHO-medium with 1 % P/S and 3 mM stable Glutamin
- Gibco™ HT-supplement (100x)
- Phosphate buffered saline (PBS^{--}, w/o Ca^{2+} and Mg^{2+})
- Trypan Blue 0.02 % stock
- Transparent 96-well half area plate (e.g. Corning™ cat. # 3695)
- Transparent 96-well plate (e.g Nunc™ MicroWell™ cat. # 167008)
- CELLAVISTA® Imaging System
- SYNENTEC's YT®-software

Methods:

As before, a sample of CHO-K1 SuPa with a final dilution of 1:40, was set up with PBS^{--} and Trypan Blue. Sample was then measured with the CELLAVISTA® Imaging System and the **Trypan Blue** image analysis algorithm.
For testing, a Nunclon plates were prepared with 100 µL medium per well, adding HT supplement to the first four columns of each plate in a ratio of 1:100.
Cell solution containing non-transfected cells was added to the last four columns of each plate with a cell count of 1000 cells per well. In the same way the rest of the plates was filled with transfected cells.
A daily measurement using **Cell Confluence** application of the YT®-software over a period of 14 days was performed. For this only the brightfield channel for cell detection was necessary (tab. 5).

Tab. 5: Optical settings for **Cell Confluence** wizard (Plate B4)

Channel	Brightfield
Objective	10x (default)
LED	LED_Brightfield (default)
Emission Filter	Emi_Green (default)
Intensity	100 %
Exposure Time	8 ms
Gain	10 %

Results & Discussion

The results of each experiment are presented in the order experiments were conducted. First experiment was the transfection of the CHO-SuPa cells using a vector containing *cas9* and *tgfp*. After that, cells were seeded via Limited Dilution to receive monoclonal colonies. Finally two tests were performed on printed cells, a proliferation test using Methotrexate (MTX) as an inhibitor and second a proliferation test using replica plating.
It is possible to process the taken images during measurement or afterwards by switching to the evaluation mode of SYNENTEC's YT®-software. To inspect the experiment data later, open the YT®-software, choose evaluation (not measurement), open your experiment with *View Experiment* and press the *Evaluation* button in the YT®-software main window.

Transfection:

Previous to cell seeding for transfection, we measured viability and cell density. For this purpose the SYNENTEC's Viability Assay was carried out (see TechNote TN-B004-XV-17).
The analysis of the CHO-SuPa cell sample with the **Trypan Blue** wizard (well number n=4) shows an average viability of 86.78 % (±0.45 %) and an average VCD (viable cell density) of 1.32E+06 (±0.06E+06) cells/mL (fig. 4).

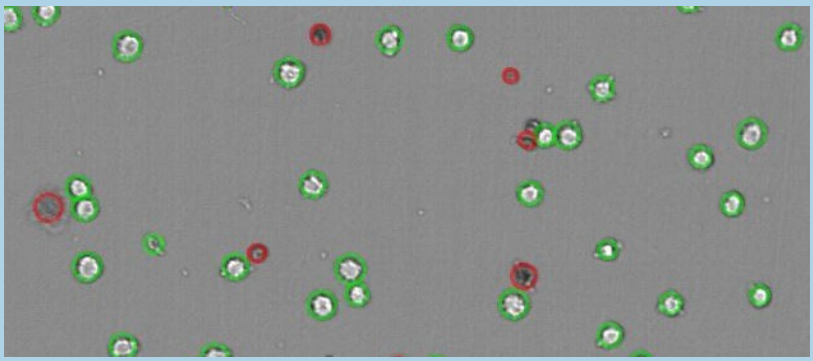


Fig. 4: SYNENTEC's **Trypan Blue** image analysis.
Brightfield image of Trypan Blue stained CHO-SuPa cells. SYNENTEC's image analysis automatically marks viable cells (green), dead cells (red) and Aggregates (blue).

Therefore the cell solution was diluted by the factor 132 and the 96-well full area plate for transfection was set up with 100 µL per well.

1. Transfection efficiency

After transfection, measurements with **Suspension Cell Count 1F** wizard of the YT®-software were carried out over a period of 10 days. To quantify the transfection efficiency, the amount of fluorescent cells in comparison to the total cell count (BF AND Fluo Area)/BF Area [%] of the result table or the heat map) was chosen.

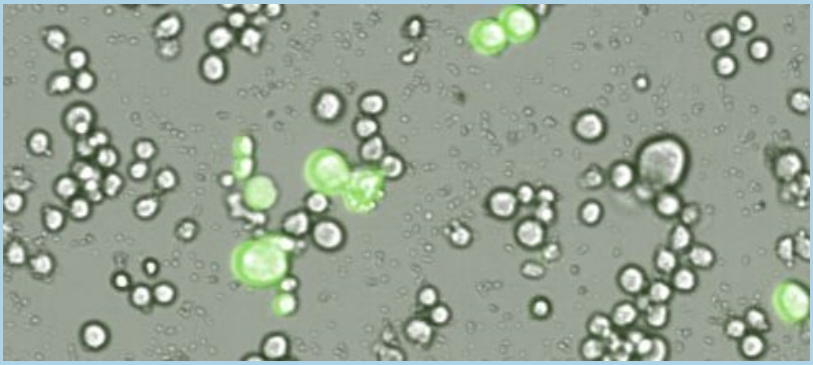


Fig. 5: SYNENTEC's CELLAVISTA® measurement using brightfield and fluorescence channel.
Brightfield image of transfected CHO-SuPa cells overlapping with fluorescence image.

Transfected Cells were measured with the CELLAVISTA® Cell Imager (fig. 5). Based on this measurement the **Suspension Cell Count 1F** image analysis calculated an average transfection efficiency of 10.74 % (±1.17 %) with n=3.

Limited Dilution:

1. Monoclonality of cell lines

To prove monoclonality of cell colonies seeded via the Limited Dilution, the **Single Cell Cloning** image analysis tool of the YT®-software was used to track cell colonies and create a clone gallery to show the cell colony developing over the measurement period.

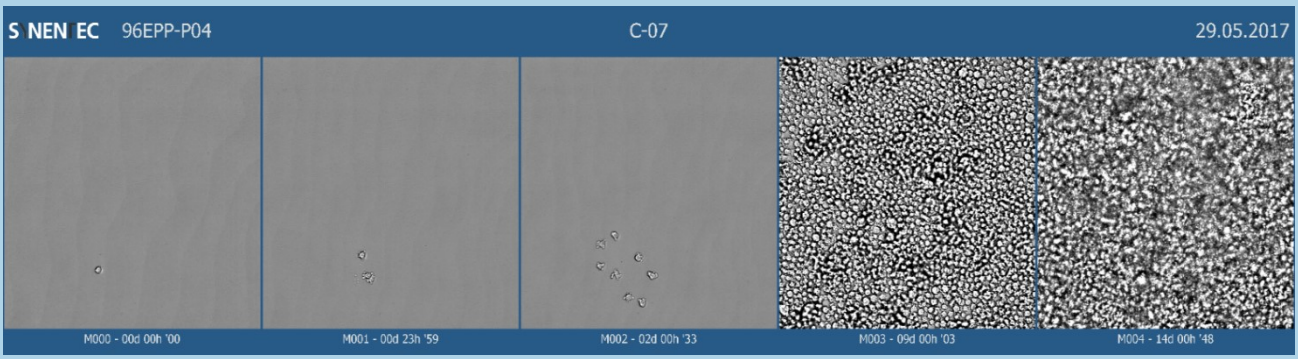


FIG. 6: SYNENTEC'S AUTOMATICALLY GENERATED CLONE GALLERY USING **SINGLE CELL CLONING** IMAGE ANALYSIS.
Brightfield images of CHO-SuPa cells over the measurement period. SYNENTEC's image analysis automatically marks visible colonies and tracks them back based on colony location.

Each seeded single cell was tracked using the SYNENTEC **Single Cell Cloning** image analysis. After the measurement period of 8 days the colonies were counted and single colonies were checked for monoclonality. As an additional function a clone gallery was created to showcase the cell growth from the single cell to the monoclonal colony (fig. 6). Multiple cells proliferated into a monoclonal colony after printing but only the best growing was selected for further cultivating and testing.

2. Transient GFP-Expression of transfected cells

To proof whether a printed cell was previously transfected or not, cell measurements were analyzed using the **Suspension Cell Count 1F** wizard to detect wells containing a fluorescent cell.
Cells after Single Cell Printing were measured and analyzed with brightfield and fluorescent channel. **Suspension Cell Count 1F** image analysis marks the cell as a fluorescent one based on the fluorescence being stronger than the background, so it can be assumed the cell is one of the previous transfected cells (fig. 7).

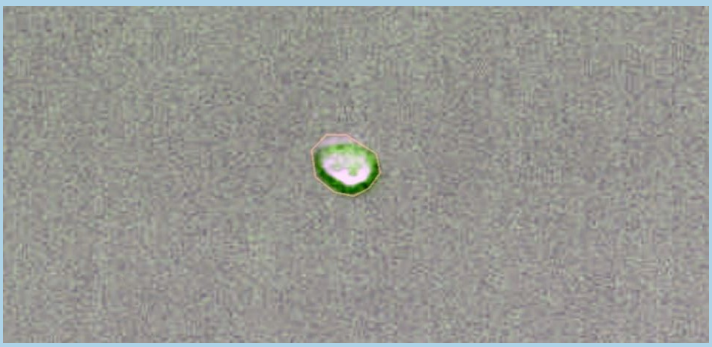


FIG. 7: SYNENTEC'S **SUSPENSION CELL COUNT 1F** IMAGE ANALYSIS.
Brightfield and fluorescence image overlay of transfected and printed CHO-SuPa cells, SYNENTEC's image analysis automatically tags visible cells and marks fluorescence.

Methotrexate-Test:

1. Trypan Blue viability determination

To seed a nearly equal and known number of cells per well for the proliferation test using MTX, a viability measurement using **Trypan Blue** and the according experiment wizard of the YT®-software was performed (fig. 8). The measurement resulted in an average viability of 86.95 % (±0.47 %) and an average VCD (viable cell density) of 1.54E+05 (±0.04E+05) cells/mL cell solution. Based on the VCD the dilution factor 15.4 was calculated to get a concentration of 1000 cells per 100 µL.

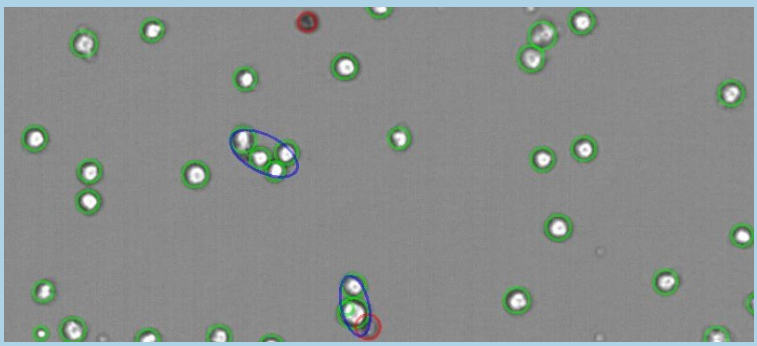


FIG. 8: SYNENTEC'S **TRYPAN BLUE** IMAGE ANALYSIS.
Brightfield image of Trypan Blue stained CHO-SuPa cells. SYNENTEC's image analysis automatically marks viable cells (green), dead cells (red) and Aggregates (blue).

2. Seeding control

After viability- and cell counting the plate was set up as described before. To check whether the number of cells per well is sufficient, the 96-well plate was analyzed using **Suspension Cell Count** wizard after seeding.

The analysis of the plate showed an average living cell count of 3394 (±634) cells/well. Since the plate was measured directly after seeding without prior centrifugation cells sedimented during measurement. Based on this the cell count between well A1 (first well to measure) and H12 (last well to measure) differs in some amount and the average cell count and the according standard deviation are influenced (fig. 9).

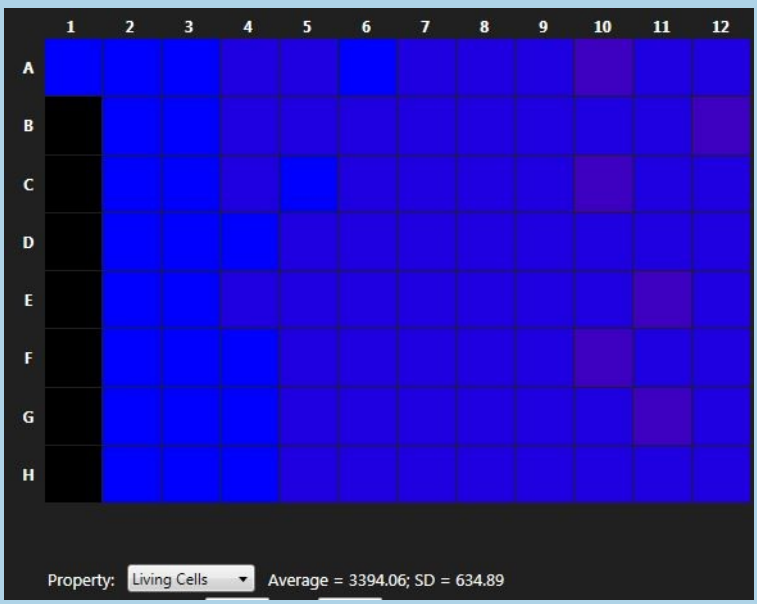


FIG. 9: HEAT MAP CREATED BY SYNENTEC'S **CELL CONFLUENCE** IMAGE ANALYSIS OF MTX – INHIBITION TESTING MEASUREMENTS.
Heat map showing last measurement's Cell Confluence for each single well in comparison to the other wells.

As described in Material and Method part (see 3. Methotrexate test), the growth of cells treated with different concentrations of Methotrexate was measured over a period of 1 week to investigate the effect of MTX on cell proliferation. The highest MTX concentration was prepared in well A1 with 2 µM. With each step the concentration was diluted 1:3 to the lowest amount of MTX in well H12 with 5.16 pM.

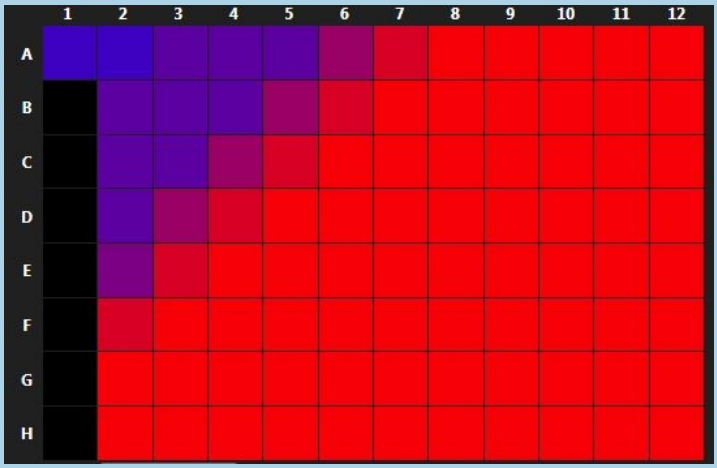


FIG. 10: HEAT MAP CREATED BY SYNENTEC'S **CELL CONFLUENCE** IMAGE ANALYSIS OF MTX – INHIBITION TESTING MEASUREMENTS.
Heat map showing last measurement's Cell Confluence for each single well in comparison to the other wells. High amounts of cell density are colored red and low amounts blue.

The cell confluence of all wells is presented as a heat map (fig. 10). The cell confluence has its lowest value in Well A1 and rises inverse proportional to the sinking MTX concentration, as part of the serial dilution, in the direction of well H12, with a maximum of 99.54 in well D8. This shows the inhibitory effect of the MTX in cells and the effect of a non-working DHFR.

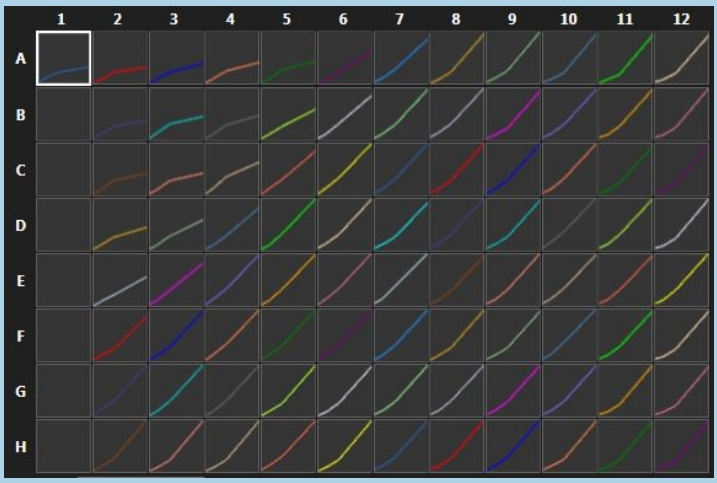


FIG. 11: CELL PROLIFERATION GRAPHS CREATED SYNENTEC'S **CELL CONFLUENCE** IMAGE ANALYSIS.
Graphical display of the timely progression of cell proliferation for each well. Each well can be selected individually for better conspicuity and more information.

The analyzed **Cell Confluence** over the entire measurement period for each well can also be presented as graphs over the measurement period (fig. 11). As shown before in the heat map, the cell growth correlates with MTX concentration. It is furthermore observable, that the starting concentration of 2 µM MTX is nearly enough to inhibit the cell growth completely.

Proliferation test:

1. Trypan Blue viability determination

Similar to the proliferation test using MTX as an inhibitor, a known number of cells were seeded equally in all wells prior to testing. For this viability measurement was performed (n=4) and evaluated via YT®-software (fig. 12).
The image analysis calculated an average viability of 82.71 % (±1.93 %) for transfected cells and an average VCD (viable cell density) of 2.98E+06 (±0.15E+06) cells/mL cell solution. For printed wild type CHO-SuPa cells an average viability of 83.78 % (±2.82 %) and an average VCD (viable cell density) of 2.28E+06 (±0.5E+05) cells/mL cell solution was measured.

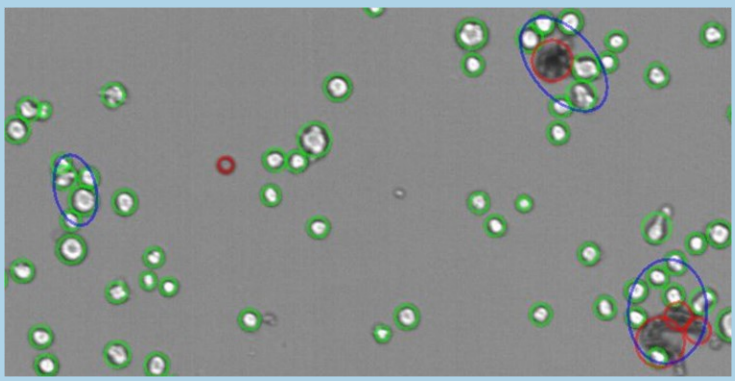


FIG. 12: SYNENTEC'S **TRYPAN BLUE** IMAGE ANALYSIS.
Brightfield image of Trypan Blue stained CHO-SuPa cells. SYNENTEC's image analysis automatically marks viable cells (green), dead cells (red) and Aggregates (blue).

2. Seeding Control

A measurement using **Suspension Cell Count** wizard was performed directly after seeding to control the correct number of cells per well and homogenous distribution of cells within the wells. A heat map showing the amount of living cells for each well was created (fig. 13). With the exception of three wells (F2, H3, H9), which contain over 1500 cells each, all wells have nearly the same amount of cells with an average number of 797 (±182) cells. Since the MTX test is based on cell proliferation and not on the total amount of cells, a plate seeded with a slightly lower or higher amount of cells as the desired 1000 cells per well is acceptable, as long as the amount is nearly equal in all wells. So the plate prepared and measured is suitable for the MTX test. Although for later observation of cell confluence for single measurements the different cell amount could influence the result to some degree.

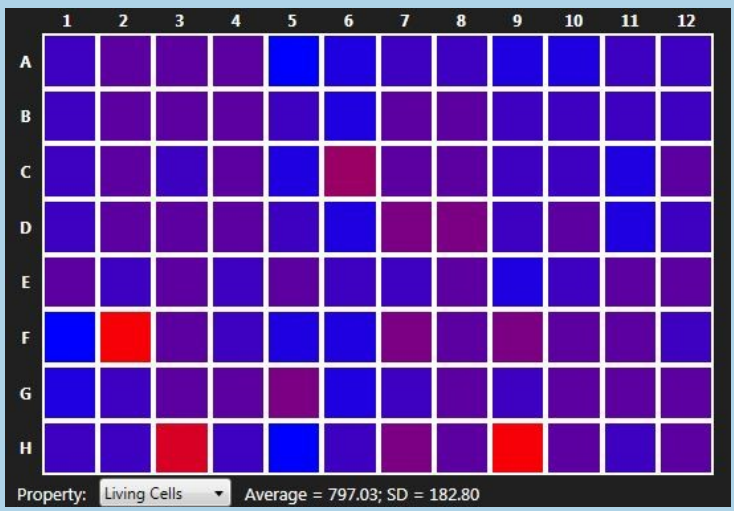


FIG. 13: SYNENTEC'S **TRYPAN BLUE** IMAGE ANALYSIS.
Brightfield image of Trypan Blue stained CHO-SuPa cells. SYNENTEC's image analysis automatically marks viable cells (green), dead cells (red) and Aggregates (blue).

3. Cell Proliferation

The plate was measured over a time period of 14 days with regular measurements. Based on these measurements and using the **Cell Confluence** image analysis, the cell proliferation of each group was measured and proliferation rates where calculated.

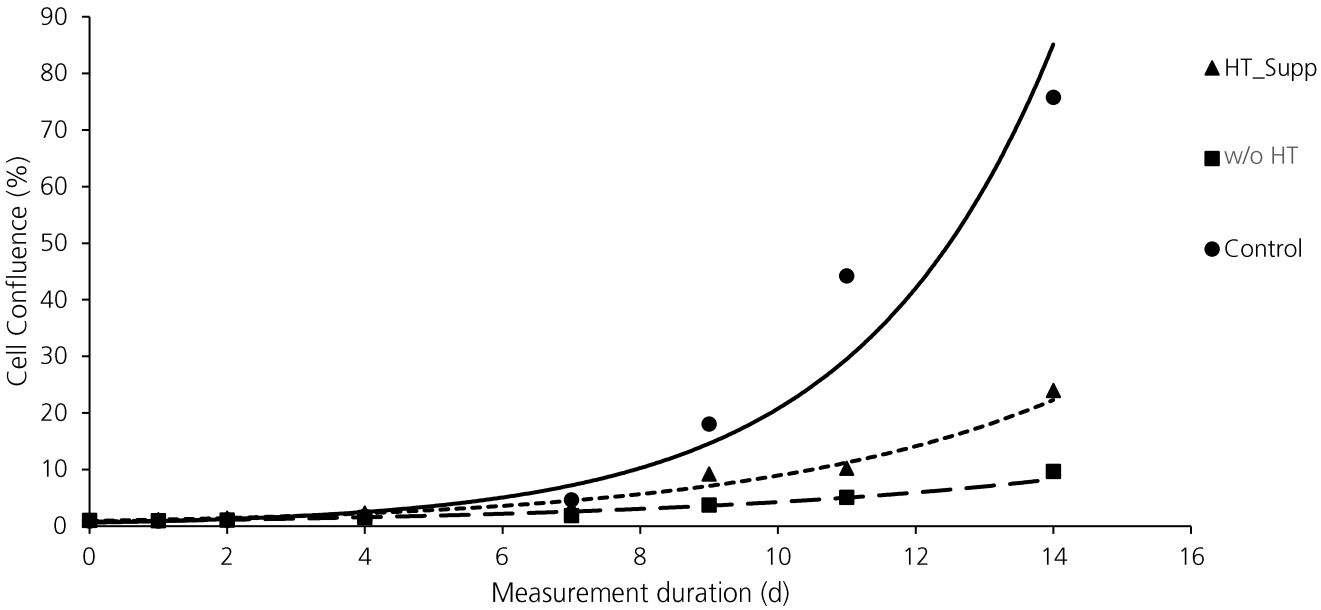


FIG. 14: CELL CONFLUENCE OVER THE MEASUREMENT PERIOD FOR EACH OF THE TESTED SCENARIOS.
Average Cell Confluence of measured plate (HT supplemented transfected cells (blue), transfected cells without (w/o) HT (red) and non-transfected cells as control (green) and corresponding regression curves (same colors)).

To compare the cell proliferation of each of the three groups within the cell proliferation test, the average cell confluence for each measurement was calculated and plottet over the measurement's time period. A exponentionell regression curve was added for each data series (fig. 14). After the 14 day measurement duration the control group showed the highest cell confluence (84.56 % (±6.98 %); n=11), followed by HT supplemented cells (20.37 % (±9.12 %); n=11). The non-supplemented cells showed the lowest cell confluence (5.25 % (±2.15 %); n=13). Since all cells were seeded with nearly the same amount the cell confluence is directly linked to the cell proliferation.

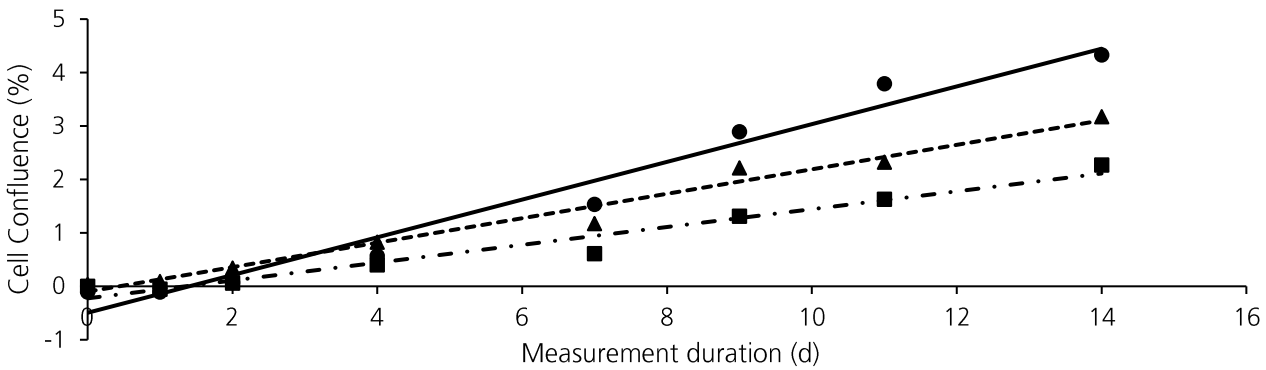


FIG. 15: LOGARITHMIZED CELL CONFLUENCE OVER THE MEASUREMENT PERIOD FOR EACH OF THE TESTED SCENARIOS.
Previous measurement data were logarithmized and plotted again, to determine ratio between the different groups (HT supplemented transfected cells (blue), transfected cells without HT (red) and non-transfected cells as control (green).

For cell proliferation calculation, the measurement data was logarithmized and the according gradient determined. Each gradient was put into ratio with the lowest amount to better compare cell proliferation.
This calculation showed, that the control group proliferates 111 % and the HT supplement cell group 37 % faster than the cell group without HT supplement. Based on this, it can be assumed, that the transfection successfully reduced DHFR activity or expression. Also an overall reduction in cell proliferation due to stress put on cell through the transfection, was determined (fig. 15).

Conclusion & Outlook

The experiments showed that a DHFR-knockout in CHO-K1 cells using the CRISPR/Cas9 method was performed successfully. Furthermore each step of the experiment, from the transfection to the proliferation tests, could be monitored and measured with the SYNENTEC CELLAVISTA® and NYONE® Cell Imager and the YT®-software. As part of the experiment a transfection protocol using the XtremeGene™ HP transfection reagent was successfully established with an average transfection efficiency of around 10 %.

Limited Dilution represents a simple and standardized method to separate cells and works reliably in combination with high resolution image based analysis of the seeded wells. Because the monoclonality is also documented via camera, monoclonality can be tracked back at each time with the stored images and the generated clone gallery.

Using Limited Dilution a monoclonal DHFR-knockout and wild type CHO-cell line have been established and could be used for further testing. Two of these tests were proliferation tests using Methotrexate as an DFHR inhibitor in established wild type CHO-SuPa cells and HT supplemented replica plating with transfected cells with wild type CHO-SuPa cells as control groups. The MTX proliferation test showed, that the cell proliferation is reduced according to a rising MTX concentration and that an inactive DHFR leads to reduced cell growth and potential cell death. The maximum MTX concentration of 2 µM almost inhibited the cell proliferation completely.

Finally the replica plate proliferation test, using HT supplemented and HT free medium, showed, that the cell growth without HT added to the medium was lower than the HT supplemented cells, so it can be assumed, that the transfection altered the DHFR gene to be less effective.

Possible future improvements could be:

- Changing plasmids target sides to reduce *dhfr* expression further or completely
- Switching GFP as a selection marker with an antibiotic resistance could facilitate cell selection after transfection. The **Cell Confluence** Wizard can be used to accurately measure cell proliferation of transfected cells
- Increasing transfection efficiency through transfection protocol improvement

  (e.g. test other transfection reagent, change reagent, cell and plasmid amount and ratio)
- Reducing cell stress by improving transfection protocol
- Checking other cell culture mediums for better cell proliferation and viability
- Using **Single Cell Cloning** image analysis, the viability and proliferation rate of cells can be measured to test, whether other automated sorting methods (flow cytometry) reduces or increases cell stress manual Limited Dilution

This applications are measurable and evaluatable with all our devices and are implemented in the YT®-software.

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SYNENTEC GmbH
Otto-Hahn-Str. 9A
25337 Elmshorn/Germany
Phone. +49 (0) 4121 46311-0
Email. appsupport@synentec.com

www.synentec.com