Lights on, Knock out!

Using fluorescence and brightfield imaging to streamline gene editing in cells.

FrederikThies*[1], Tina Christmann[2], Ben Werdelmann[2], Martin Stöhr[3], Matthias Pirsch[3]

In 2012 the CRISPR/Cas9 system introduced a new era of gene editing possibilities. In comparison to other modern methods based on endonucleases like TALENS or ZNF, CRISPR/-Cas9 provides a cheap, safe and precise method to specifically target and edit a certain genetic sequence. In this poster, we present a Dhfr knockout in CHO-K1 cells using CRISPR/Cas9 method and SYNENTEC's Cell Imagers. We used lipofection to transiently insert a plasmid containing Cas9, gRNA against Dhfr and tGFP as a marker for the transfection. After transfection, we used Limited Dilution to isolate single cells, which can proliferate as monoclonal cell line of CRISPR treated cells using the GFP-fluorescence as a control (GFP-co-transfection). To prove Dhfr knockout, we performed PCR, Western Blotting and an auxotrophy test using replica plating as well as a proliferation test with Methotrexate (MTX) as DHFR inhibitor in wild type CHO cells. For all necessary measurements, we used SYNENTEC's CELLAVISTA® and NYONE® Cell Imager and the associated YT®-software. Based on the measurements performed, we also created a new CRISPR/Cas Cloning image processing algorithm for YT®-software to provide optimal image analysis for future CRISPR/Cas9 experiments. Chemiluminescence detection after Western Blotting showed no specific bands for DHFR protein for all samples. Since none of the samples showed a signal for DHFR but all showed signals for the house-keeping gene α-TUBULIN, we conclude zero or sub-detection level expression of Dhfr.

PCR results showed one transfected cell clone without a band for Dhfr, suggesting a successful knockout of Dhfr, although the primers targeted another exon of the Dhfr-gene as the gRNA and thus CRISPR/Cas9 system. This result was further confirmed in proliferation tests using replica plating, where the same cell clones showed decreased cell proliferation when seeded in cell medium without HT supplement, indicating a dhfr-knockdown rather than a complete knockout.

* corresponding author, [1]University of Applied Sciences Muenster, Stegerwaldstrasse 39, 48565 Steinfurt, Germany,
[2]SYNENTEC GmbH, Johann-Krane-Weg 42, 48149 Muenster, Germany, [3]SYNENTEC GmbH, Otto-Hahn-Strasse 9a, 25337 Elmshorn, Germany

Gene editing workflow Dhfr knock

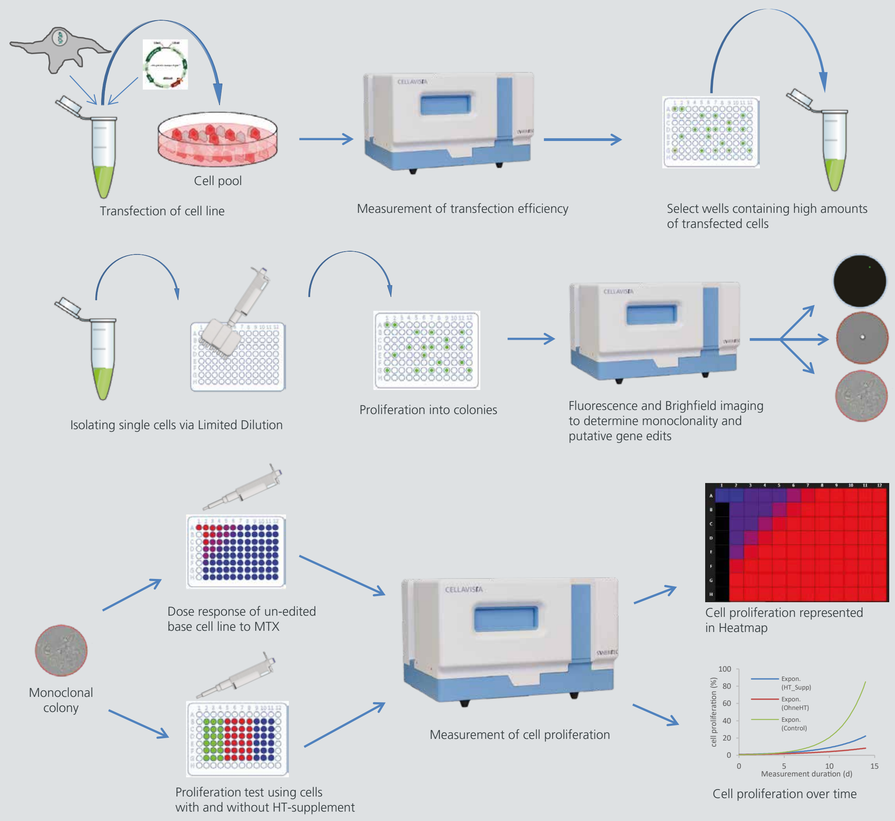


Fig. 1 Schematic illustration of gene editing workflow. The gene-editing workflow using SYNENTEC's imaging systems results in monoclonal cell lines containing the desired knock-out to a high degree of probability due to confirmation using fluorescent reporter genes on the same plasmid as the editing genes. The workflow was exemplified using dhfr as a model for biotechnologically relevant knock-outs. The knock-out was phenotypically confirmed using minimal medium without HT-supplement, all measurements were carried out using SYNENTEC's imaging systems NYONE and CELLAVISTA.

CRISPR/Cas Cloning image processing

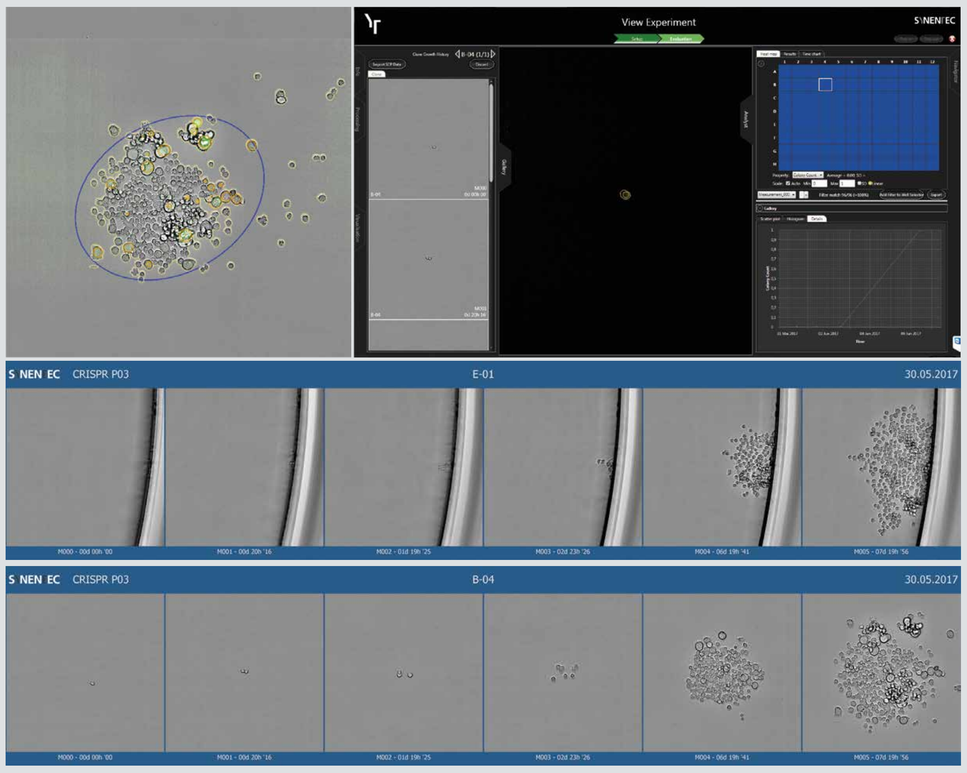
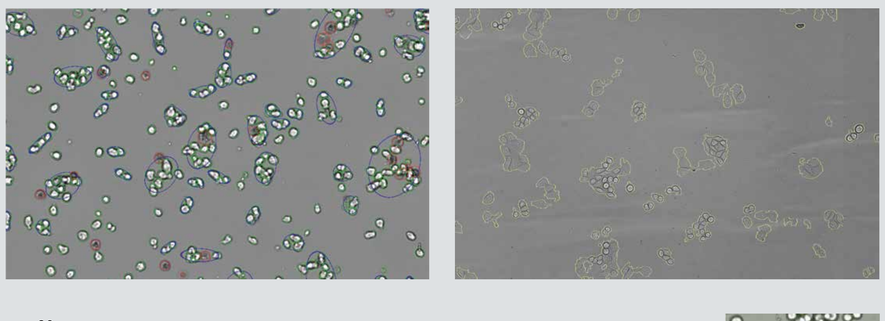
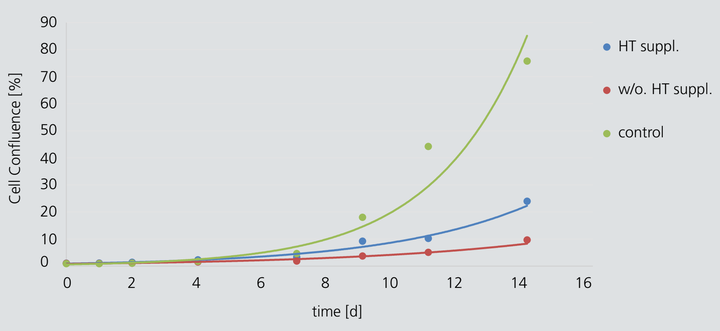


Fig. 2 The newly developed "CRISPR/Cas Cloning" image processing uses CELLAVISTA's and NYONE's high resolution imaging capabilities to image single cells, detect outgrown colonies in brightfield and facilitates a check for low fluorescent signals on single cell and colony level. In that way both monoclonality and gene editing status can be evaluated based on co-expression of fluorescent proteins and Cas9 from the transfected plasmid. Thus putative monoclonal knock-out cell lines are identified automatically in an early stage of cell line development using SYNENTEC's Clone Gallery.

Phenotypical proof of genome editing





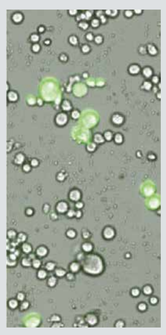


Fig. 3 Transfection efficiency was measured using SYNENTEC's Confluence(1F) wizard to detect GFP fluorescence after successful transfection, the efficiency was 10% (bottom right). In order to prove a gene knockout using CRISPR/Cas9 we successfully performed several assays, e.g. PCR and Western-Blot analysis (data not shown). To show deficiencies in nucleic acid synthesis from Dhfr knockout, we used HT withdrawal to show the cell line's auxotrophy regarding Hypoxanthine and Thymidine. Equal numbers of cells per well (1000) were seeded with and without HT supplement. CHO-K1 host cell line was used as a control. Before seeding, cell densities and viablilties were measured using SYNENTEC's TrypanBlue assay, resulting in an average viability of 83% and cell densities of 3 x 10^6 and 2.2 x 10^6 cells/mL for transfected cells and wildtype respectively (top left). The cells were cultivated over 14 days and were subjected to daily imaging. The imaging was performed in brightfield using SYNENTEC's Confluence wizard (top right), the data generated was analysed and displayed in a spreadsheet program. Plotting the confluence values over time shows the transfected cell's incapability to exhibit comparable doubling times to the control group, regardless of HT supplementation. However the edited cells in non-supplemented media show a 37% lower doubling time compared to the supplemented media, indicating a successful knockout of dhfr.

Conclusion and Outlook

In this poster we presented a streamlined gene-editing process that is compatible to SYNENTEC's imaging systems CELLAVISTA and NYONE using an image processing pipeline that has been developed in this study. CRISPR/Cas Cloning image processing enables the usage of SYNENTEC's Clone Gallery in conjunction with highly sensitive fluorescence imaging, resulting in monoclonal cell lines that are checked for the presence of a fluorescence reporter as a proxy for successful gene editing.
The knockout of dhfr is shown in the proliferation test using HT withdrawal and further analysed via PCR and Western-Blot analysis using specific probes for DHFR, indicating an incomplete knockout.
The reduced doubling time in the HT-supplemented cells indicates a high stress level in the cells after transfection, thus the "edited" cell lines in further experiments have to be transfected using an optimised protocol to avoid stress effects on the cells. During the optimisation of transfection, the low transfection efficiency (10%) has to be improved as well.

To validate the assay pipeline, we are planning to target other genes of interest, like fut8 to improve glykosylation patterns or glutamine synthase as an additional selection marker.

This work was performed by Frederik Thies during his master thesis at the University of Applied Sciences Muenster in collaboration with SYNENTEC.

Fachhochschule Münster University of Applied Sciences