Miltenyi Biotec

MACSQuant® Tyto® and cellenONE® X1

Development of monoclonal cell lines

Time- and cost-efficient production of high-expressing and fast-expanding monoclonal cell lines using the MACSQuant® Tyto® Cell Sorter in combination with the cellenONE® X1 single-cell deposition unit

François Monjaret[1], Gianluigi Atzeni[1], Jens Gaiser[2], Bastian Ackermann[2], and Guilhem Tourniaire[1]

[1]Cellenion SASU, Lyon, France
[2]Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Background

Monoclonal cell lines – procedures and challenges

The cost- and time-efficient generation of high-expressing and fast-expanding monoclonal cell lines for the production of biologicals such as antibodies has become more and more important, especially in the context of innovative immunotherapies for cancer.

Creating a monoclonal cell line expressing a certain protein involves three steps. 1) Modifying suitable host cells by transfection to induce protein expression. This results in the generation of a mixed population of cells that do or do not express the desired protein. Moreover, expression levels can vary greatly within the population. 2) Selecting the cell subpopulation that has undergone the transformation of interest. 3) Cloning this subpopulation to select the best-performing clone. This process can be laborious and costly.

Cloning by manual limiting dilution

Currently, the most commonly used method for cloning cells to generate a cell line is manual limiting dilution of the transfected cells into multi-well plates and subsequent screening for high-expressing and fast-expanding colonies derived from single cells. This method however is highly inefficient and time and cost consuming as most wells will not contain only a single cell. If the protein expressed by the cell line will be used for therapeutic or diagnostic purposes, the cloning process should be performed at least twice in order to guarantee monoclonality, as recommended by the WHO Expert Committee on Biological Standardization.[1] This further complicates the process.

Isolation of high-expressing cells by flow sorting

To isolate cells expressing high levels of the desired protein by fluorescence-activated cell sorting, the cells are often genetically modified to co-express the protein of interest and GFP. While cloning by manual dilution is considered to be gentle to cells, traditional flow sorting typically involves high shear and decompression forces acting on the cells. Usually, mechanical stress substantially affects the viability of sorted cells which, in turn, reduces the chance of isolating healthy single cells capable of forming monoclonal cell lines. Additionally, conventional droplet sorters require highly trained operators who constantly monitor and optimize parameters during sorting.

Effective and gentle automated sorting of high-expressing cells

Compared to conventional droplet sorters, sorting on Miltenyi Biotec's MACSQuant® Tyto® is much gentler to cells, typically resulting in viabilities >95%. As the MACSQuant Tyto operates fully automatically, it can be handled by any lab professional after only a short training period. Therefore, the MACSQuant Tyto is the ideal solution for effective and efficient sorting of high-expressing cells prior to cloning.

Efficient automated cloning of high-expressing cells

The cellenONE® X1 system from Cellenion allows for fully automated cell cloning. The instrument enables efficient deposition of single cells into various different plate formats such as 96-well and 384-well microplates. Typically, close to 100% of the wells contain only a single cell. Additionally, cells do not get harmed due to the gentle deposition mechanism, resulting in high cloning efficiencies. The combination of MACSQuant Tyto and cellenONE X1 is a time- and cost-efficient solution for establishing high-expressing, fast-expanding monoclonal cell lines.

Materials and methods

Cell line

A mixed cell suspension consisting of 75% wild type (wt) HEK293T and 25% stably transduced GFP⁺ HEK293T cells (expression rate 90%) was prepared for cloning experiments. HEK293T cells is a commonly used cell line for the manufacture of biotherapeutics.[2]

Cell culture

Cells were cultured under standard conditions (37 °C, 5% CO_2) in DMEM with high glucose, 10% FBS, pen/strep. Before cloning, cells were split every 3 days at 1:5 dilutions in T75 flasks. On the day of cloning, cells were resuspended using trypsin-EDTA (0.05%, phenol red) before suspending them in MACSQuant® Tyto® Running Buffer at the desired concentration. After cloning, 96-well microplates were incubated for 12 days.

Overview of cloning methods

Three cloning methods were compared:
1) cells from the mixed HEK293T cell suspension were cloned manually using a multichannel pipette to dispense cells into 96-well plates.
2) In parallel, the mixed HEK293T cell suspension was cloned into 96-well microplates using the cellenONE® X1 instrument, and
3) the MACSQuant Tyto was utilized to enrich cells showing high GFP expression levels. The enriched high-expressing GFP⁺ fraction was subsequently used for cloning by the cellenONE X1 instrument into 96-well plates. Cloned cells were then expanded for 12 days. Cloning efficiency was evaluated using a microscope. GFP expression levels of each viable clone were determined by flow cytometry using the MACSQuant Analyzer 10. The same mixed cell suspension (25% GFP⁺) was used to compare the three cloning methods. For an overview see figure 1.

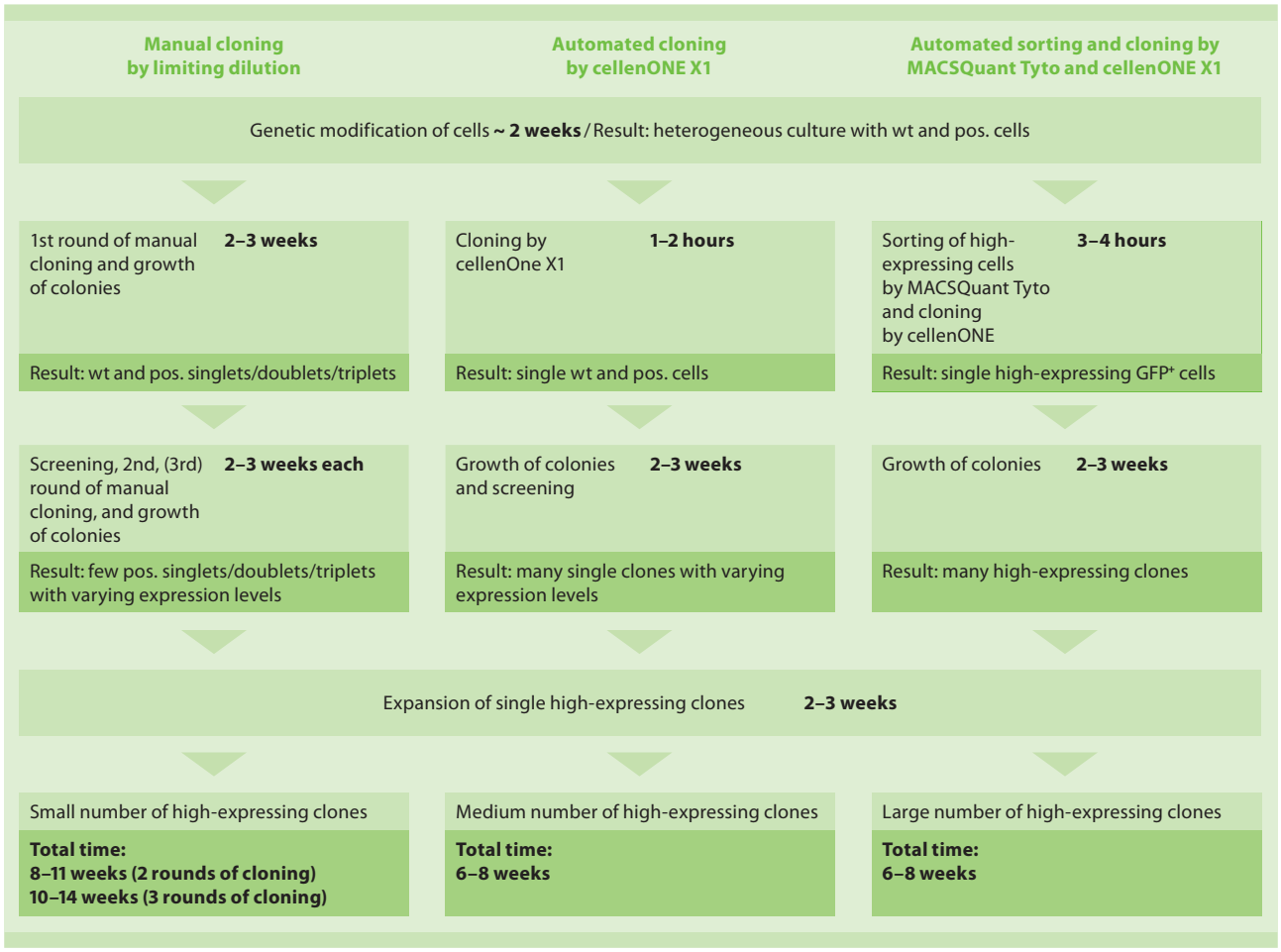

Figure 1: Scheme of the three different cloning methods investigated.

Manual cloning

To obtain an optimal cloning efficiency, an average seeding density of 0.25 cells/well was chosen. This density results in a minimal number of wells containing multiple cells (i.e. doublets, triplets etc.), and still affords a reasonable number of single clones (singlets) for further processing. Cloning was achieved by stepwise dilution of the initial cell suspension to 2.5 cells/mL and adding 100 µL of this dilution into 96-well plates pre-filled with 100 µL culture medium per well.

Cloning by cellenONE® X1

Cells were suspended in MACSQuant Tyto Running Buffer at 10^5 cells/mL, and 10 µL of the cell suspension was taken up into a PDC 90 piezo dispense capillary for cloning. For isolation of single cells onto microscope slides, an array of 10×10 positions was programmed, with a center-to-center distance of 500 µm.

For isolation on a 96-well plate, the cellenONE® X1 instrument was programmed to dispense a single cell in each well of the microplate. The following cellenONE X1 parameters were used during single cell isolation:

Detection parameters: LGV = 25; HGV = 255; Area min. = 80; Area max. = 10,000. Printing parameters: Area min. = 250; Area max. = 700; Circularity max. = 1.2; Elongation max. = 2.0. Environmental control: No humidity control; microplate holder temperature = 4 °C.

GFP⁺ cell sorting by MACSQuant® Tyto®

For sorting on the MACSQuant® Tyto®, cells were resuspended in MACSQuant Tyto Running Buffer at $1×10^6$ cells/mL. Cells were transferred into a primed MACSQuant Tyto Cartridge using a 10-mL syringe and a Pre-Separation Filter (20 µm) attached to the input chamber. Sorting was performed at 4 °C and the sort gate was set on the high-expressing GFP⁺ cells (fig. 2). The sort was stopped after 20 min when approx. 170,000 cells were successfully sorted – enough cells for further processing by the cellenONE X1.

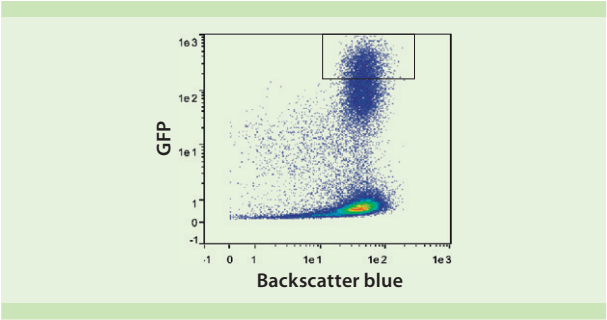

Figure 2: Flow cytometry analysis of a mixed cell suspension consisting of wt and stably transduced GFP⁺ HEK293T cells.
The sort gate to enrich high-expressing GFP⁺ cells is shown.

Colony evaluation

After 12 days of culture, each well of the microplates was inspected under an inverted microscope. Colony-containing wells were recorded and the number of colonies per well was determined.

Flow cytometry analysis of GFP expression by MACSQuant® Analyzer 10

Culture medium was removed from each colony-containing well. Accutase (25 µL) was added for 5 minutes at RT and deactivated with 25 µL PBS (Ca^{2+} and Mg^{2+} free). Wells were thoroughly flushed to suspend individual cells. Cells were then transferred to a round-bottom 96-well plate before processing on the MACSQuant® Analyzer 10.

Results

Flow cytometry analysis of the mixed starting population of wt and GFP⁺ HEK293T cells

To determine cell viability and percentage of GFP⁺ cells, the mixed cell population was analyzed using the MACSQuant Analyzer 10. The sample used for the analysis shown in figure 3 consisted of 99% viable cells and about 25% GFP⁺ cells with heterogeneous expression levels. GFP⁺ cells were divided into three subsets: low-, medium-, and high-expressing cells, according to fluorescence intensities.

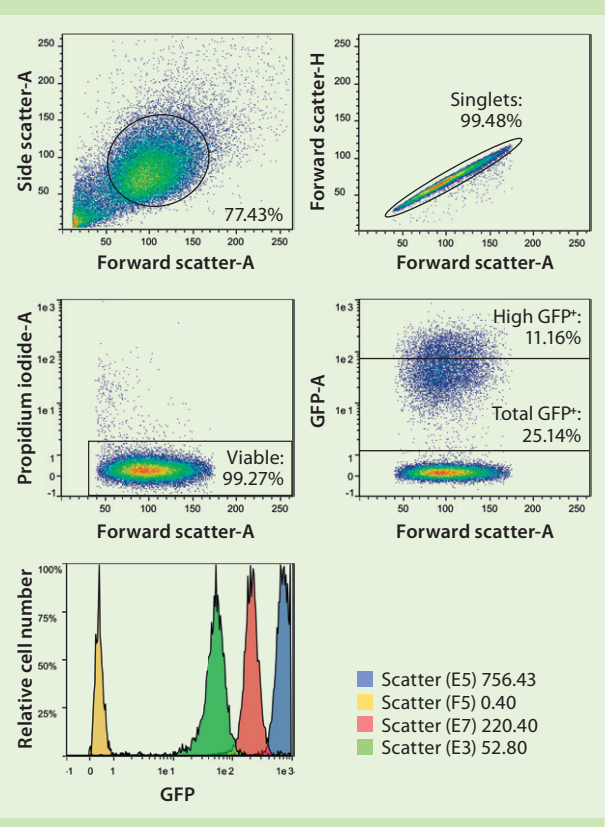

Figure 3: Flow cytometry analysis of a mixed cell suspension consisting of wt and stably transduced GFP⁺ HEK293T cells.
Viability was determined by dead cell exclusion based on propidium iodide fluorescence. Histograms show GFP⁻ cells (yellow) and three populations with low (green), medium (red), and high (blue) GFP expression levels.

Manual cloning

After the first round of manual cloning, only 13% of the wells contained viable single colonies. According to Poisson's law, however, 25% of the wells should have contained single colonies. As expected, a considerable percentage of wells (5%) contained more than one colony per well. Due to the presence of multiple colonies per well, monoclonal cell line development would require two or more rounds of cloning which drastically lengthens the procedure.
A proportion of 25% of the colonies expressed GFP, which was in accordance with 25% GFP-expressing cells contained in the starting cell suspension prior to cloning.

Cloning using the cellenONE® X1 instrument

Prior to cloning, the precision of the cellenONE® X1 instrument in isolating single cells was verified by depositing single cell–containing droplets as an array of 10×10 positions onto a microscope slide. Microscopic inspection confirmed an outstanding single-cell isolation rate as every position contained exactly one cell (fig. 4).

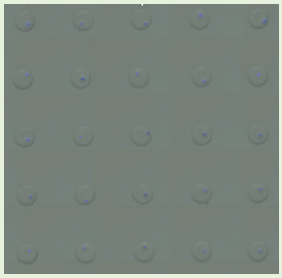

Figure 4: Microscopic evaluation of single-cell isolation by the cellenONE X1 instrument. The image was acquired at 20× magnification; bright field and DAPI images were merged. The image shows a subsection (5×5) of a 10×10 array.

Subsequently the mixed cell suspension consisting of wt and stably transduced GFP⁺ HEK293T cells was used for cloning into 96-well microplates by the cellenONE X1 instrument.
On average, 66% of the wells contained viable single colonies, and importantly, no wells contained multiple colonies. The recovered colonies contained 27% GFP-expressing cells, which corresponded approximately to the expression levels measured by the MACSQuant Analyzer 10 in the starting cell suspension prior to cloning.

Cloning using MACSQuant® Tyto® and cellenONE X1

For sorting GFP-positive cells on the MACSQuant® Tyto®, only cells expressing high levels of GFP were selected as the target fraction, which corresponded to 11% of total viable cells. After 20 minutes 170,000 cells were successfully sorted. At the end of the sorting process, an aliquot of the positive fraction was incubated with ethidium homodimer (EthD-1) for assessment of cell viability by flow cytometry on the MACSQuant Analyzer 10. GFP-expressing cells in the sorted fraction were enriched to 98% purity and showed 98% viability, demonstrating the effectiveness and gentleness of the MACSQuant Tyto System.

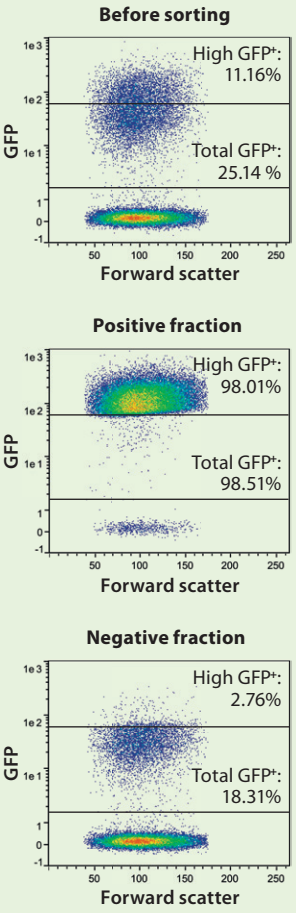

Figure 5: Sorting of high-expressing GFP⁺ cells by the MACSQuant Tyto. Only high-expressing cells were sorted, as indicated by the gate in the upper plot. Dot plots for positive and negative fractions after cell sorting illustrate the high sorting effectiveness.

After cloning cells from the positive fraction using the cellenONE® X1 instrument and subsequently incubating the single cells to obtain colonies, on average 56% of the wells contained viable single colonies. An example is shown in figure 6. No wells contained multiple colonies, which confirmed the aforementioned cloning results. More than 75% of the colonies showed high GFP expression levels (figs. 7 and 8).

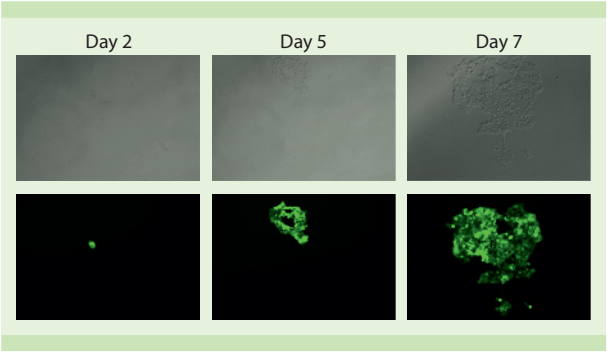

Figure 6: Microscopy images of a GFP-expressing colony growing from a single cell. The images were taken at 20× magnification in a well of a 96-well plate at the indicated time points after enrichment with MACSQuant Tyto and cloning with cellenONE X1. Brightfield images (top) and GFP fluorescence (bottom) are shown.

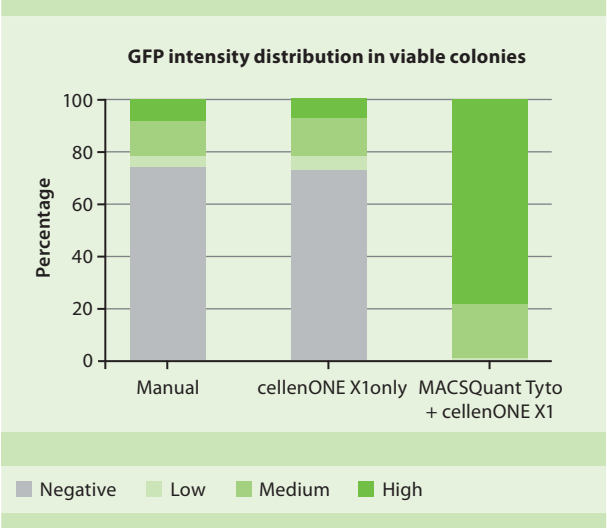

Figure 7: Diagram representing the percentage of GFP-negative colonies as well as GFP-positive colonies with low, medium, and high expression levels. Cells were either cloned manually or by cellenONE X1 only or by MACSQuant Tyto in combination with cellenONE X1.

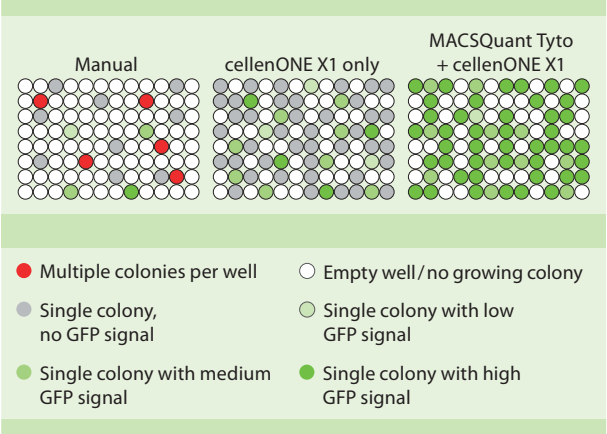

Figure 8: Scheme representing viable colonies and their respective GFP expression levels for each tested condition on 96-well microplates. Left: manual cloning (representative of five 96-well plates), middle: cellenONE X1 cloning (representative of three 96-well plates), right: MACSQuant Tyto enrichment and cellenONE X1 cloning (representative of three 96-well plates).

Conclusion

In this study, a HEK293T cell population showing heterogeneous expression of GFP was used to model variable levels of recombinant protein expression as typically observed after transfection. To demonstrate the power of combining MACSQuant® Tyto® and cellenONE® X1, high-expressing GFP$^+$ HEK cells were sorted out of the heterogeneous starting population and subsequently dispensed into single clones to establish monoclonal cell lines.

- Using the combination of MACSQuant Tyto for GFP$^+$ cell enrichment and cellenONE X1 for cloning, it was possible to isolate cells with high expression levels and obtain large numbers of viable clonal colonies.
- The cloning process was highly efficient as most wells of a microplate contained high-expressing GFP$^+$ colonies.
- A large number of clonal colonies was obtained in a shorter period of time compared to manual cloning.
- The process enables drastic cost savings due to high efficiency.
- The fully automated and effortless cell sorting procedure avoids supervision by highly trained operators, in contrast to traditional droplet sorters.

Manual cloning	Cloning by MACSQuant Tyto and cellenONE X1	Benefits
Multiple cloning steps required. Total time to high-expressing clonal colonies: 8–14 weeks	No need for multiple cloning steps. Total time to high-expressing clonal colonies: 6–8 weeks	• Faster establishment of monoclonal cell lines (minimum 25% time saving)
Small number of high-expressing clones	Only high-expressing clones	• Efficient production of high-expressing cell lines
Small number of clonal colonies	Large number of clonal colonies	• More cell lines available
Large amounts of consumables and solutions required	Smaller amounts of consumable and solutions suffice	• Lower expenditure
Manual process	High level of automation	• Hassle-free procedure • Saves operator time • No need for highly trained personnel

Table 1: Features of manual cloning and cloning by MACSQuant Tyto and cellenONE X1. Combined use of the automated instruments affords compelling benefits compared to the manual process.

References

1. WHO Expert Committee on Biological Standardization, Sixty-first report (2013) WHO Technical Report Series No. 978: 110ff.
2. Dumont, J. *et al.* (2016) Crit. Rev. Biotechnol. 36: 1110–1122.

Product	Order no.
Flow sorting*	
MACSQuant® Tyto®	130-103-931
MACSQuant Tyto Cartridges	130-106-088
MACSQuant Tyto Running Buffer	130-107-206
Pre-Separation Filter (20 µm)	130-101-812
Flow cytometry*	
MACSQuant Analyzer 10	130-096-343
Cell cloning**	
cellenONE® X1	F00C
cellenONE PDC90 (Piezo dispense capillary)	P-2050-C

For further information on products and how to order visit
*www.miltenyibiotec.com
**www.cellenion.com/technology/cellenone/

Miltenyi Biotec

Miltenyi Biotec provides products and services worldwide. Visit **www.miltenyibiotec.com/local** to find your nearest Miltenyi Biotec contact.