Improved Single Cell Clone Analysis with the Automated SynenTec Cellavista® System and Eppendorf Cell Culture Plates

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Abstract
Recombinant cell lines stably expressing target proteins are an important tool in drug screening today. Single cell cloning is one critical step in stable cell line development. To ensure the monoclonal character of the population, a reliable microscopic identification of single cells is essential. Manual observation of single cell clones can be time-consuming, unreliable, and difficult to document. Automated cell analyzers like the SynenTec Cellavista System are convenient to use, allow a high throughput, and reliable tracking and documentation from single cell status to colony formation.

Besides instrumentation also the consumable can influence the analysis: evaporation and meniscus effects can result in disturbing shadows and optical interferences limiting the area of observation. Single cell clones may stay undetected or be falsely analyzed. Here we show that the automated Cellavista System together with the Eppendorf Cell Culture Plates are a perfect combination for successful single cell clone analysis.

The automated Cellavista System is a fast and flexible image-based platform with brightfield and multiple fluorescence capabilities delivering excellent results for a broad range of cellular applications.

The Eppendorf 96-Well Cell Culture Plate has excellent optical properties and an outer moat that can be filled with liquid to insulate specifically the edge wells to minimize evaporation. In addition the complete inter-well space can be filled with liquid.
Introduction

Transfection is the procedure of introducing foreign genetic material into a host cell with the aim to produce a genetically modified cell. Transient transfection of cells allows gene expression for a limited time span usually with a peak expression between 24-96 hours post transfection. Nucleic acid is not integrated into the genome and is lost during subsequent cell divisions. Transient transfection is a valuable tool for the study of gene function and expression in fundamental and medical research. Stable transfected cell lines arisen from one single cell clone allow continuous gene expression by a genetically homogeneous population. Introduced genetic material is integrated into the host genome and is passed to subsequent cell generations by using additional selection markers. In 1986 the first therapeutic protein produced in CHO cells (human tissue plasminogen activator, tPA/Activase) obtained international market approval. It was the start of using mammalian cells as “production factories” for therapeutic protein products in medicine. The advantage of using mammalian expression systems compared to e.g. bacterial cells is the allowance of post-translational processing and modification of proteins. By this, quality and efficacy of a therapeutic protein can be improved. Nowadays approximately 60–70 % of all recombinant protein pharmaceuticals are produced in mammalian cells [1]. CHO cells are still one of the most popular cell lines used for the production of therapeutic proteins for several reasons: They can be easily adapted to suspension culture for large scale production and can grow in chemically defined serum-free media ensuring an improved reproducibility between different production batches. Besides that cells derived from mouse myeloma (NS0), baby hamster kidney (BHK), human embryonic kidney (HEK-293) and human retina (PerC6) have gained regulatory approval for recombinant protein production [2].

Today specialized biotechnology companies offer services from vector design and stable cell line generation to targeted assay development [3]. The process of stable cell line generation is a time consuming and labor intensive process (figure 1). Initially the cells are transfected with a recombinant gene expression vector containing the gene of interest (GOI) and an adequate selection marker. After having selected the positive transfected cells this polyclonal culture can be further processed to isolate monoclones using different single cell cloning techniques such as limited dilution, fluorescence activated cell sorting (FACS) or automated clone picking.

Independent from the method it is critical to verify that the resulting colony derives from a single cell. Manual microscopic observation of single cell clones and colony formation can be time-consuming, unreliable and difficult to document. Automated cell analyzers like the SynenTec Cellavista System can monitor cell growth of colonies from single cell status by repeated measurements of the same microplate over a span of typically two to three weeks. The fast growing colonies can be selected and monoclonality ensured by tracking colony growth back to the first image. The system is able to follow the growth of the clones and can completely document this process by the imaging software and advanced data management in a high throughput manner.

In this application note we show the optimal image quality of the Cellavista System, which allows distinguishing clearly single cells from e.g. doublets. We also analyze the influence of the consumable in single cell cloning. A uniform well illumination without interfering shadows at the well edges is important to detect cell colonies growing at the well periphery. We will show that the Eppendorf Cell Culture Plate facilitates excellent well illumination by its reduced edge- and meniscus effect.

Figure 1: Simplified procedure of the development of a stable transfected cell line.
1. Transfection of host cell line with expression vector containing the gene of interest (GOI) and a selection marker gene.
2. Selection of positive cells.
3. Subsequent single cell cloning to generate a monoclonal culture derived from one cell.
4. Further clone evaluation (e.g. quality of recombinant protein) and expansion of selected clone prior to cell banking.
Materials and Methods

Single Cell Seeding Procedure
The inter-well space and the outer moat of the Eppendorf Cell Culture Plates, 96-Well, TC treated (n=5) were filled with sterile, distilled water to minimize evaporation of medium over time (outer moat 6 mL, inner area 10 mL). Plates were kept in the incubator until cell seeding. Competitor plates (n=5), which do not offer the option of filling the moat as well as non-filled Eppendorf Cell Culture Plates were placed in the incubator at the same time prior to seeding. Adherent CHO-GH3 cells (Log-Phase, 60 % confluence) were prepared and seeded in a concentration of 0.5 cells per well in 200 µL medium using a multichannel pipette. Two wells per plate were filled with a higher cell number to adjust illumination and focus. To avoid drifting of cells to the well edges the plate were set at room temperature for 30 min prior transferring it to the incubator.

Analysis

Edge effect & tracking of colony growth
Measurement of plates was started after all cells attached to the well bottom (~ 1-2 hours). Images (n=4) were taken with the Cellavista System on day 0 and day 1 followed by measurements every 2-3 days until day 10-14 post seeding: One autofocus per well at each measurement and one whole plate scan (not all data shown). In order to prevent loss of cells no change of medium was performed.

Meniscus effect
To analyze the meniscus effect Eppendorf 96-Well Cell Culture Plates, TC treated as well as high quality plates of a competitor were seeded with different volumes of medium from 1 – 6 µL/mm². Volumes were adapted to volume/surface area to comprise variances in growth areas of plates. In a second experiment cells were seeded using 3.31 µL/mm² and stained with trypan blue. All plates were analyzed using the Cellavista System.

Results and Discussion

Edge effect
During long incubation periods evaporation in 96-well plates is a critical factor. Especially in the edge wells of the plate liquid loss becomes apparent, because these wells are not completely surrounded by neighboring wells. This effect is also called edge effect. With the Eppendorf Cell Culture Plates evaporation can be reduced to a minimum by filling the outer moat and the inter-well space. The detailed process is described in the Eppendorf Application Note 326 [4]. To monitor the influence of the edge effect on image quality during long-term incubation, filled (moat + inter-well space) and non-filled Eppendorf Cell Culture Plates were incubated after single cell seeding for 14 days and especially the edge wells were monitored at different time points. Figure 2B shows that filling the moat and the inter-well space of the Eppendorf plate leads to clear well illumination and brightness throughout the whole length of incubation. In comparison to that, the non-filled Eppendorf plate (figure 2A) shows a slight decrease of illumination uniformity over time, which is most severe on day 14 of incubation.

Here interfering shadows at the well edges are visible. Colony growth in the edge wells of the plate can be detected clearly throughout the whole incubation time in the filled Eppendorf plate (figure 2B). The visible decrease in illumination to the well edge in the non-filled Eppendorf plates makes it slightly more difficult to detect the colony at later time points of measurement (figure 2B). As the evaporation from the edge wells in non-filled plates is higher than in the filled plates [4], a direct relation between evaporation and shadow formation is assumed. Evaporation leads to a loss of liquid and reduction of the filling height. As the filling height of the liquid influences also the meniscus formation of the liquid we further analyze the influence of different filling heights on the meniscus effect in the last experiment (Meniscus Effect, see page 6). The evaporation from the edge wells can be easily prevented in the Eppendorf Cell Culture Plates by simply filling the outer moat e.g. with distilled water [4].
A: Without filling the moat a slight decrease of brightness and illumination uniformity can be seen with increased length of incubation time. Cells growing in the well edge are partly in the shadow. (Upper panel: 4x objective, brightfield; lower panel: image detail)

B: Filling of the outer moat minimizes evaporation and obtains optimal illumination of the well also after 14 days of incubation. The complete colony is clearly visible even in the well edge. (Upper panel: 4x objective, brightfield; lower panel: image detail)

Figure 2: Well brightness and illumination uniformity. Outer well 6A of the Eppendorf 96-Well Cell Culture Plate was measured on indicated time points using the Cellavista System.
Tracking colony growth

Figure 3 shows extracted pictures from the colony growth from the day of single cell seeding (day 0) up to day 14 post seeding. It is clearly visible that the colony in figure 3A originates from a single cell (=monoclonal) and can be used for further processes whereas the colony in figure 3B originates from two cells (=polyclonal) and has to be discarded as monoclonality of the culture is not given. The optimal image quality of the Cellavista System allows distinguishing single cells clearly from doublet cells. The system facilitates tracking of culture development of a whole plate experiment throughout the incubation period (in this experiment 14 days) and allows reliable documentation.

Figure 3: Tracking colony growth. The optimal image quality of the Cellavista System allows distinguishing single cells from doublet cells. (Cellavista, 4x objective, brightfield).
Meniscus Effect
A common problem when imaging 96- and 384-well formats is the meniscus formation of the liquid. The curvature of the liquid causes a refraction of the light with the result of uneven illumination of the sample which may lead to a distorted image like looking through a porthole. Due to the special production technique, the Eppendorf Cell Culture Plates have a minimized liquid meniscus [5]. The filling height of the liquid influences the curvature of the meniscus. To analyze the meniscus effect, different liquid filling heights were measured in the Eppendorf Cell Culture Plate, 96-Well TC-treated and an analogous high quality plate from a competitor brand. The measurement was done with the Cellavista Analyzing System with a 2 x objective to allow visualizing the whole well. Figure 4 shows that the Eppendorf plate allows an excellent illumination of the whole well-area also at reduced filling heights. Only with very small volumes (1 µL/mm²) a disturbing meniscus effect is visible resulting in shadow formation at the well edge. In comparison to that the competitor plate already shows disturbing shadows and uneven illumination when using higher filling levels. A constant decrease in illumination with lower filling levels is visible.

Figure 4: Comparison of meniscus effect in Eppendorf Cell Culture Plates, 96-Well, TC-treated and competitor plates. Volumes were adapted to volume/surface area to comprise variances in growth areas sizes). The lower the volume the more critical is the dark shadow formation (Cellavista, 2x objective, brightfield-adjustment: BF1=8 ms, BF2=12 ms, BF3=20 ms)
In figure 5 trypan blue stained cells were analyzed in Eppendorf plates and comparable plates from a competitor using the Cellavista Imaging System. An interfering shadow can be observed in the competitor plate at the edge of the well whereas in the Eppendorf plate the shadow is reduced to a minimum.

![Figure 5: Comparison of meniscus effect with trypan blue stained cells.](image)

**Figure 5:** Comparison of meniscus effect with trypan blue stained cells. No shadow at the edge is visible in the Eppendorf Cell Culture Plate. Volumes were adapted to volume/surface area (Cellavista, 2x objective, brightfield)

**Conclusion**

An efficient microscopic analysis is crucial in single cell cloning to verify monoclonality of cultures. Single cell clones that have previously been generated by limited dilution, fluorescence activated cell sorting (FACS) or automated clone picking can be easily identified and monitored with the automated SynenTec Cellavista System. The Cellavista can monitor cell growth of colonies from single cell status by repeated measurements of the same microplate over a span of typically two to three weeks. By this, documentation of colony, development is made easy. The optimal image quality of the Cellavista Imaging System allows distinguishing clearly single cells from doublets. By this a reliable selection of monoclonal colonies is possible.

Eppendorf Cell Culture Plates have an optimized microscopic performance: The excellent bottom planarity and well design and the reduced meniscus enable a uniform illumination without disturbing shadows at the edges.

Due to the possibility to reduce evaporation in the plate to a minimum, Eppendorf Cell Culture Plates enable an improved microscopic performance also in long-term incubation experiments. Single cell cloning using SynenTec’s automated analyzing system Cellavista together with the Eppendorf Cell Culture Plates is best suited for successful single cell clone analysis.

**Acknowledgement**

Experiments were performed by SynenTec GmbH, Johann-Krane-Weg 42, 48149 Münster, Germany. Information on the Cellavista System can be found on [www.synentec.com](http://www.synentec.com).
Literature


Ordering Information

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