Considerations to the Proof of Monoclonality using NYONE® and CELLAVISTA®

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Application Note
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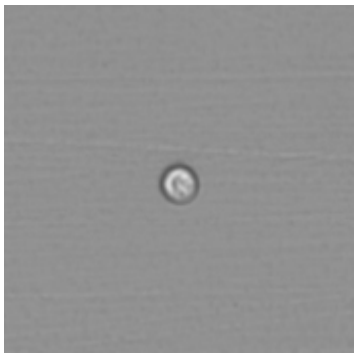


Abstract

The production of cell lines for recombinant products is regulated in part by the ICH Q5D[1] and the EMEA/CHMP/BWP/157653/-2007[2] These guidelines state that "For recombinant products, the cell substrate is the transfected cell containing the desired sequences, which has been cloned from a single cell progenitor."[1] and "The cell substrate to be used for the production of the monoclonal antibodies should be a stable and continuous monoclonal cell line...[2]". Here we provide an overview of how to conduct the proof of monoclonality using the high resolution optics of CELLAVISTA® and NYONE® in conjunction with SYNENTEC®'s proprietary image processing (YT-software®). We outline possible image based process qualifications for Single Cell Cloning taking advantage of the high-throughput imaging systems engineered by SYNENTEC®. Furthermore we exemplify and elucidate possible ways to generate supporting data for regulatory approval of clonal cell lines for biopharmaceutical production.

KEYWORDS: CELL LINE DEVELOPMENT, PROCESS QUALIFICATION, REGULATORY APPROVAL, FDA, MONOCLONALITY

Introduction

The use of monoclonal cell lines for biopharmaceutical production is not only a regulatory necessity, it also contributes to product quality and consistency in production processes as well as safety and efficacy. Historically two rounds of limited dilution and statistical procedures were used to ensure monoclonality of cell lines[3]. There are several drawbacks to using a statistical approach that is proven to not account for critical attributes of cells within that process ("sticky cells")[4]. The use of high-throughput microtiter plate (MTP) based imaging technologies enables the generation of indisputable images displaying one complete well (fig. 1) containing only one cell. These images can be used to prove the monoclonality of cell lines derived from imaged wells. SYNENTEC®'s imagers NYONE® and CELLAVISTA® possess bespoke features that enable scientists to conduct imaging in high-throughput, matching current processes and timelines in CLD with one round of single cell cloning.

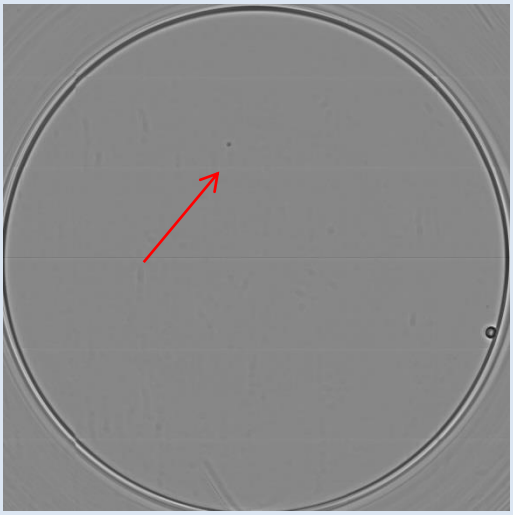


Fig. 1: Well lit, full well image taken with NYONE®
The image contains one single cell imaged at a high resolution, without stitching effects (e.g. pixelloss and visible borders).

Modern cell line development is technology driven, new findings in vector design and construction, codon optimization and host cell engineering and transfection as well as screening technologies enable modern processes involving protein expression to be more efficient than ever before[5].

Some modern processes still involve the generation of stable or transient genetically modified cell lines by transfection resulting in transfected pools of cells that express the protein of interest. The pools usually are quite heterogenous regarding the expression rate and the quality of the expressed protein. The undesired diversity is dealt with by using monoclonal cell lines derived from one single cell progenitor. The pools are deposited at one cell per well in microtiter plates and expanded after reaching a certain confluence as a monoclonal colony (fig. 2).

The single cell deposition can be facilitated by several means e.g. limited dilution, single cell printing or FACS. After single cell deposition and expansion of monoclonal colonies, these are subjected to several rounds of screening involving screens for production, stability, quality and other characteristics. Prior to the first passaging of putatively monoclonal colonies, the colonies can be checked to assure monoclonality[6].

An image based workflow for the proof of monoclonality speeds up cell line generation significantly by making another round of single cell cloning by limited dilution redundant. Using non-image based approaches that statistically exhibit enough confidence in probability of monoclonality are difficult to demonstrate[4].

Even though a high probability of monoclonality can be calculated[7], the presence of one colony in a well can not be used as an estimate for monoclonality as it does not account for adhesion of cells to each other[8]. Each method used for the assurance of monoclonality has it's drawbacks. Considering imaging in plate based assays the focal plane of the imager and the plate quality are specific critical parameters that need thorough analysis and evaluation.

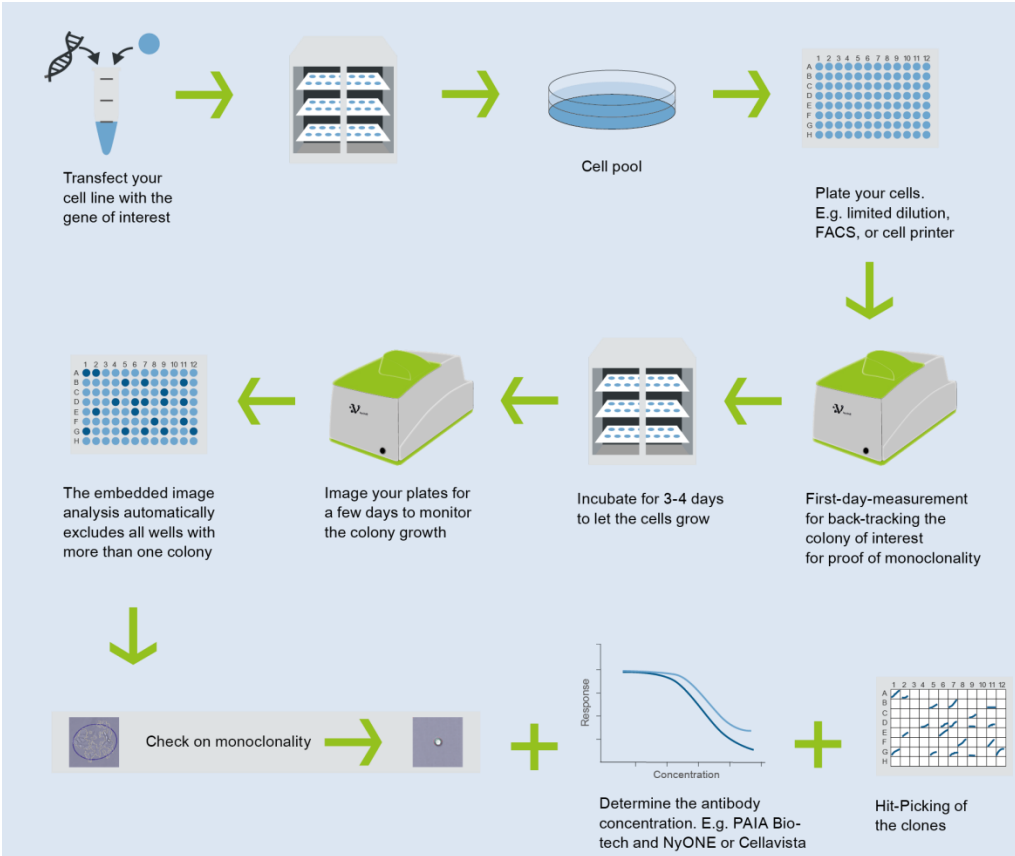


Fig. 2: Process scheme in cell line development
After transfection and culturing of transfectant pools, the cells can be plated by several means to achieve single cell status. SYNENTEC®'s imagers can be used for whole well imaging on seeding day. The growth of the cells is monitored over time by imaging and wells containing one colony can easily be identified using image processing. Prior to the next expansion step, it is possible to back-track colonies to seeding day in order to confirm the monoclonality of each colony prior to hit-picking.

Well Edge Quality

The well edge, especially the transition from well bottom to well wall can cause severe issues for single cell imaging. This radius or slope, depending on it's size might enable single cells to settle above the focal plane of the imager, resulting in poor single cell visibility.

A prominent radius at the well edge can result in unclear imaging that cannot be used for proof of single cell status (fig. 3, bottom right) A prominent radius at the well edge can result in unclear imaging that cannot be used for proof of single cell status (fig. 3, bottom right) as the cell is not depicted clearly. The compliance of the well edge quality to single cell imaging standards has to be assessed prior to process qualification. The well edge is one critical parameter that has to be considered when selecting a plate. Each plate type has a different appearance in that regard and has to be qualified as part of the plate quality, but is not the only critical attribute of general plate quality.

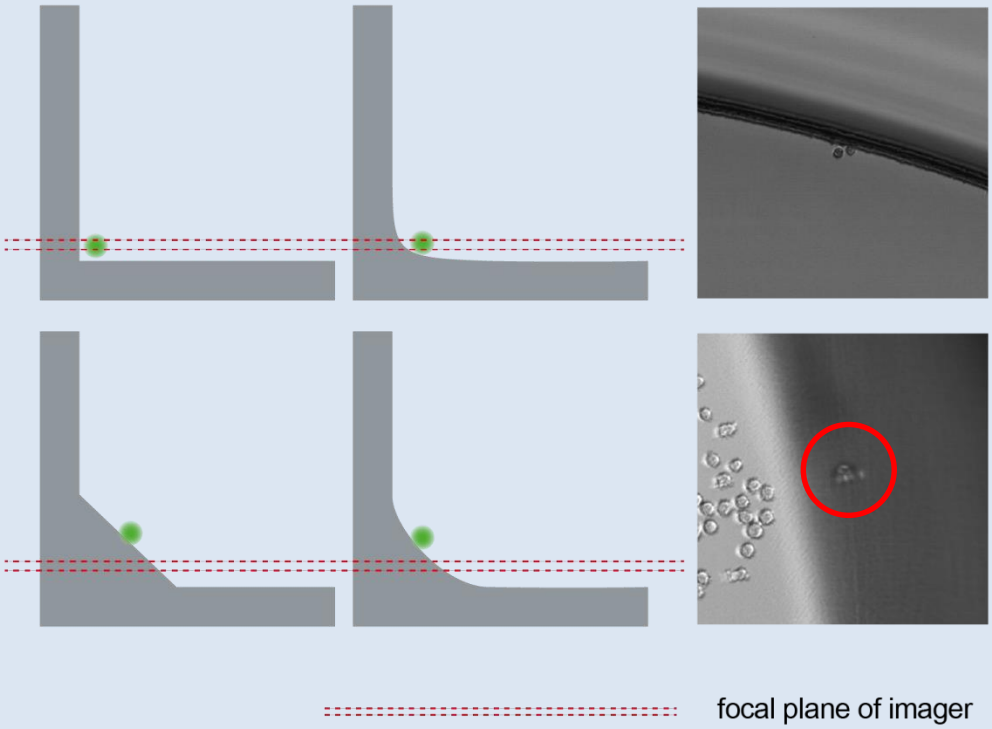


Fig. 3 Well edge attributes in single cell cloning applications
Seeded cells often settle at the very edge of the well. The well edge radius from different plate types varies in suitability for single cell imaging. (Top, Eppendorf 96 well TC Treated 0030730119) Small Radius in curved and edged variants. A small radius cannot be a reason for ghost wells as the imager's full resolution is used and the cells have no chance settling above the focal plane of the imager. (Bottom) A bigger radius enables cells settling above the focal plane of the imager resulting in ambiguous images or even ghost wells.

A small radius at the well edge ensures that the imager's highest resolution and sharpness is used. If a high quality plate with a good quality well edge is used, a decision on single cell status can be made effortlessly (fig. 3, top right).

Another critical plate attribute is the well bottom quality. Scratches, bottom thickness, residual debris and other impurities in the well and well bottom have massive impact on the optical performance of the plate. These parameters can vary on a batch to batch basis, so this should be assessed prior to plate selection. The most critical attribute of plates is the amount of difference in plate bottom dimensions across wells and also across plates.

Well Bottom Quality

The plates used for imaging have to have a flat surface due to the focal plane of imaging systems, 7.444 µm @ 10x magnification[9]. Nonetheless, there are certain tolerances in manufacturing. Some deviations in plate bottom dimensions are within these tolerances specified by the plate manufacturer (not available publicly).

The most critical attribute is the plate bottom thickness. This needs to stay consistent across the plates to achieve good autofocusing and imaging while keeping the throughput as high as possible. While the recommended setting for high throughput single cell cloning is one focus per well, sufficient focusing performance must not be impeded by high deviations in the plate bottom thickness. Please refer to the Operating Guide of the imager to check on the autofocusing system. Considering one focus per well in high throughput applications, the flatness of each well can cause issues if it is not consistent. Some plate types exhibit critical height deviations from the middle towards the outer parts of the well or even across the complete well (fig. 4).

The focus offset enables the cells to be imaged exhibiting the lens effect of the cytoplasm (fig. 4, top). This lens effect is used to distinguish cells. If the well bottom deviates in height from the center of the well the lens effect will not be exhibited properly (fig. 4). Debris or media precipitates do not show this lens effect, which enables the user to distinguish clearly between artifacts and single cells in a well. To achieve a good lens effect with poor quality plates, the focusing mode would have to be changed to more frequent focusing, which impedes throughput.

Using good quality plates with very low differences in bottom height is key to having good image quality and still using high throughput capabilities of SYNENTEC®'s imagers.

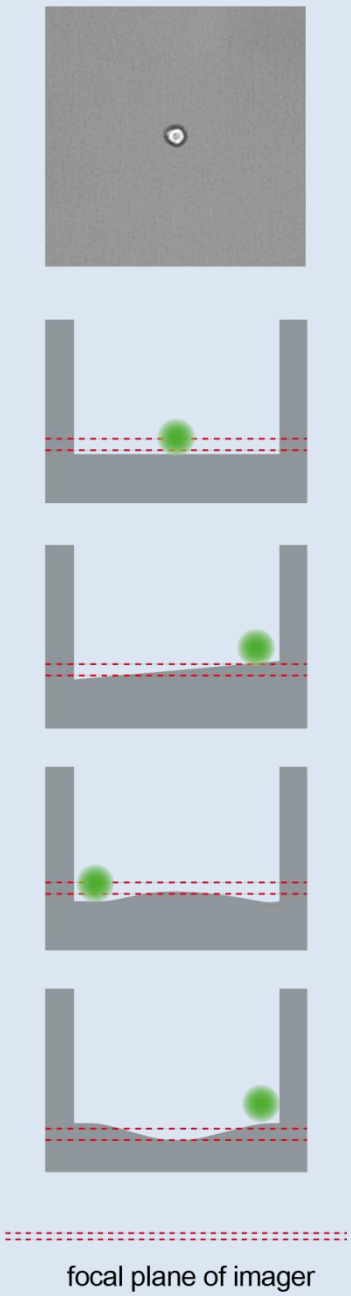


Fig. 4 Schematic Well Bottom Attributes in Single Cell Cloning Applications
Using a microscopic imager for the proof of monoclonality requires the plate bottom to be flat. Depending on the quality of the plate, there are some plate types that exhibit different deviations within the specifications of the plate bottom. The well bottom deviations can be in form of a slope across the well or in height differences towards the edges. All of these can cause the cells' lens effect to change and thus decreasing the cells visibility, if one focus per well is used.

Well Bottom Quality

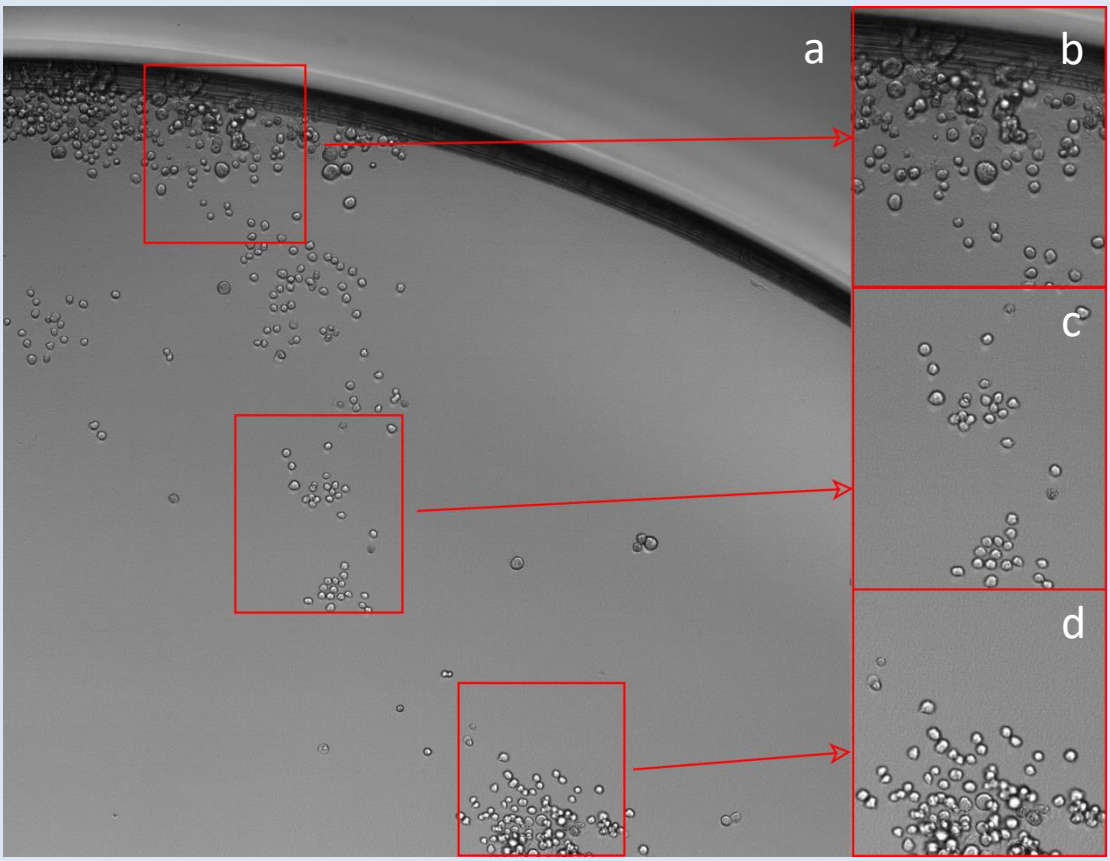


Fig. 5 Example of different Lens Effect acrosss Well Bottom
The lens effect is used to clearly differentiate cells from debris (a, c, d). The differences in height of the plate bottom cause the lens effect to be less obvious towards the edge of the well. The radius at the well edge causes cells to reside above the imager's focal plane, resulting in unclear images (b).

Fig. 5 illustrates the effects of uneven well bottoms (a). In the middle of the well close to the fosused area the cells exhibit a prominent lens effect that clearly differentiates them from debris (d). Towards the edge of the well the lens effect is less prominent in the cells (c). At the edge of the well the cells do not exhibit a lens effect which makes the differentiation from cell shaped debris a challenging task. At the very edge of the well it is noticeable that some cells have settled above the focal plane of the imager, which in turn shows that this plate should not be used for single cell cloning as one single cell possibly could not be detected resulting in a ghost well, or false clonal wells.

The plates used for the production of clonal cell lines thus have to be thoroughly qualified.

To achieve a good lens effect with poor quality plates, the focusing mode would have to be changed to more frequent focusing with the focusing options (each image, each well, pattern) in YT-software® which can be used to account for difficult plate types.
But, using good quality plates with very low differences in bottom height is key to having good image quality and still using high throughput capabilities of SYNENTEC®'s imagers.

Plate Layout Consistency

The plate layout for each plate type used, once defined in the plate setup wizard, is a fixed parameter in each experiment conducted (please refer to the Operating Guide of the imager). Particularly in high throughput applications and most notably in robotic systems users assume the plate layout to be consistent between batches of the same plate type. Sometimes the plate layout changes between batches e.g. A1 is slightly shifted in x and or y direction. On some occasions we have noticed pitch variances between wells, that alter the distance between individual wells (fig. 6).

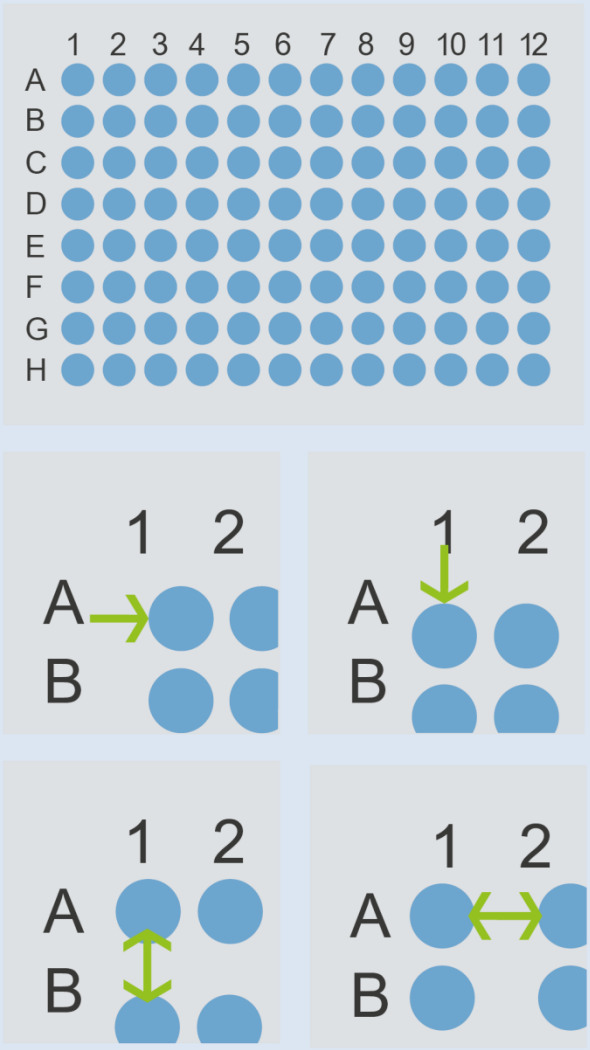


Fig. 6 Plate Layout divergencies
The general layout of each plate type is fixed in YT-software®. Some plate types deviate in one or more ways between batches. Some well grids are shifted in x and/ or y direction, some display abberations in pitch between two individual wells or a combination thereof. These issues might cause some wells not to be imaged completely resulting in unusable data for the proof of monoclonality.

All these discrepancies can result in wells that are not completely imaged, ultimately leading to unusable data for the proof of mono-clonality. In this case the user would not able to provide a full well image for the clone selected or even the complete run of single cell cloning.

Avoiding such issues involves testing several batches of each plate type to be used for Single Cell Cloning. This should be conducted prior to process qualification and is of high importance as processes are set to be used for several years. If plate quality wasn't assesed properly or the quality declines over time, we suggest testing each batch of new plates for consistency and quality regarding plate layout and other factors like scratches and debris.

Plate attributes to evaluate:

- Well edge quality
- Well bottom flatness
- Well bottom thickness
- Well bottom clarity
- Plate layout constistency

After suitable plate type selection, process qualification can be planned. In the following chapter a possible workflow is described.

Material & Methods

Material:

- mAB-CHO (mAb expressing chinese hamster ovary cells)
- Chemically defined media
- 96-well plates (transparent)
- Cell counting device e.g. NYONE®
- SYNENTEC®'s imaging systems (here: CELLAVISTA®
- SYNENTEC®'s YT-Software®

Methods:

mAb-CHO cells were counted using an automated cell counter and subsequently diluted into serum free chemically defined media to a final concentration of 5 cells/mL and 0.5 cells/mL. After seeding the cells at the given concentrations dispensing 200 µL per well, 12 plates were centrifuged for 5 minutes at 300 x g. Four plates were left standing for at least 30 minutes after seeding to demonstrate ghost wells as a negative control. The plates were imaged on a CELLAVISTA® using the *Single Cell Cloning* Wizard in YT-software®.

The *Single Cell Cloning* application is used to image whole wells in high resolution (10x-lens, 1.1 µm/px, Tab. 1) for the proof of monoclonality. Subsequent imaging runs with the same settings were repeated for each plate (within the same experiment) to use SYNENTEC®'s image processing in order to identify wells with one colony on d7 or d14. Single colonies were checked by eye for a single cell image on day 0. The monoclonality check was conducted using the Clone Gallery feature in YT-Software®. The center of each colony is cropped and extracted from the images of each measurement and displayed in the gallery thus enabling quick confirmation of monoclonality status and discarding of non-monoclonal wells. The wells were categorized into three categories: monoclonal well, polyclonal well and ghost well. Monoclonal wells were passaged into 1.5 mL shaking cultures in 24 well plates to screen for mAb-production (data not shown).

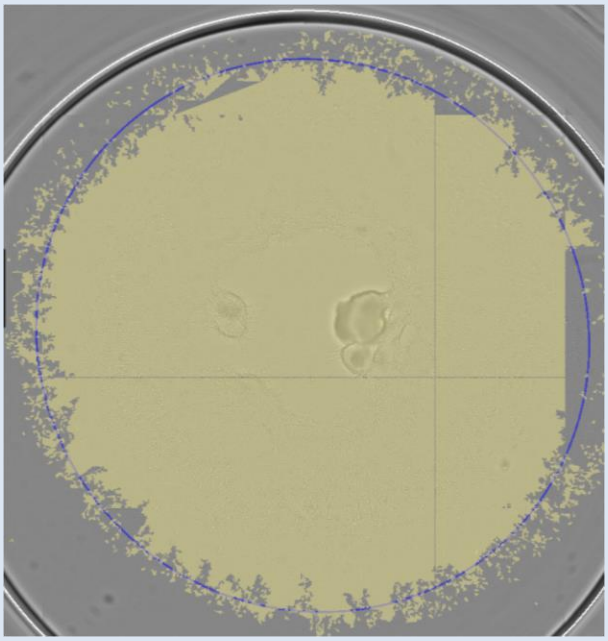


Fig 7: Detected colony in *Single Cell Cloning* Wizard
The yellow area is the cell area (fill polygons checked) whereas the blue line marks the single colony. The results are accessible in YT-Software® as Cell Confluence 70 % and Colony Count 1.

Tab. 1: Optical settings for *Single Cell Cloning* application

Channel	Brightfield
Objective	10x (default)
LED	LED_Brightfield (default)
Emission Filter	Emi_Green (default)
Intensity	26 %
Exposure Time	5 ms
Gain	10 %

Clone Gallery

After successful colony detection the clone galleries are created and used to categorize each colonized well. The categorization is used to enable the calculation of probability of monoclonality, which needs to be incorporated into the supporting data of IND and BLA submissions. Incomplete or lacking data will lead to either rejection of application or to the need of extensive production process quality monitoring and controls throughout the product's lifecycle.

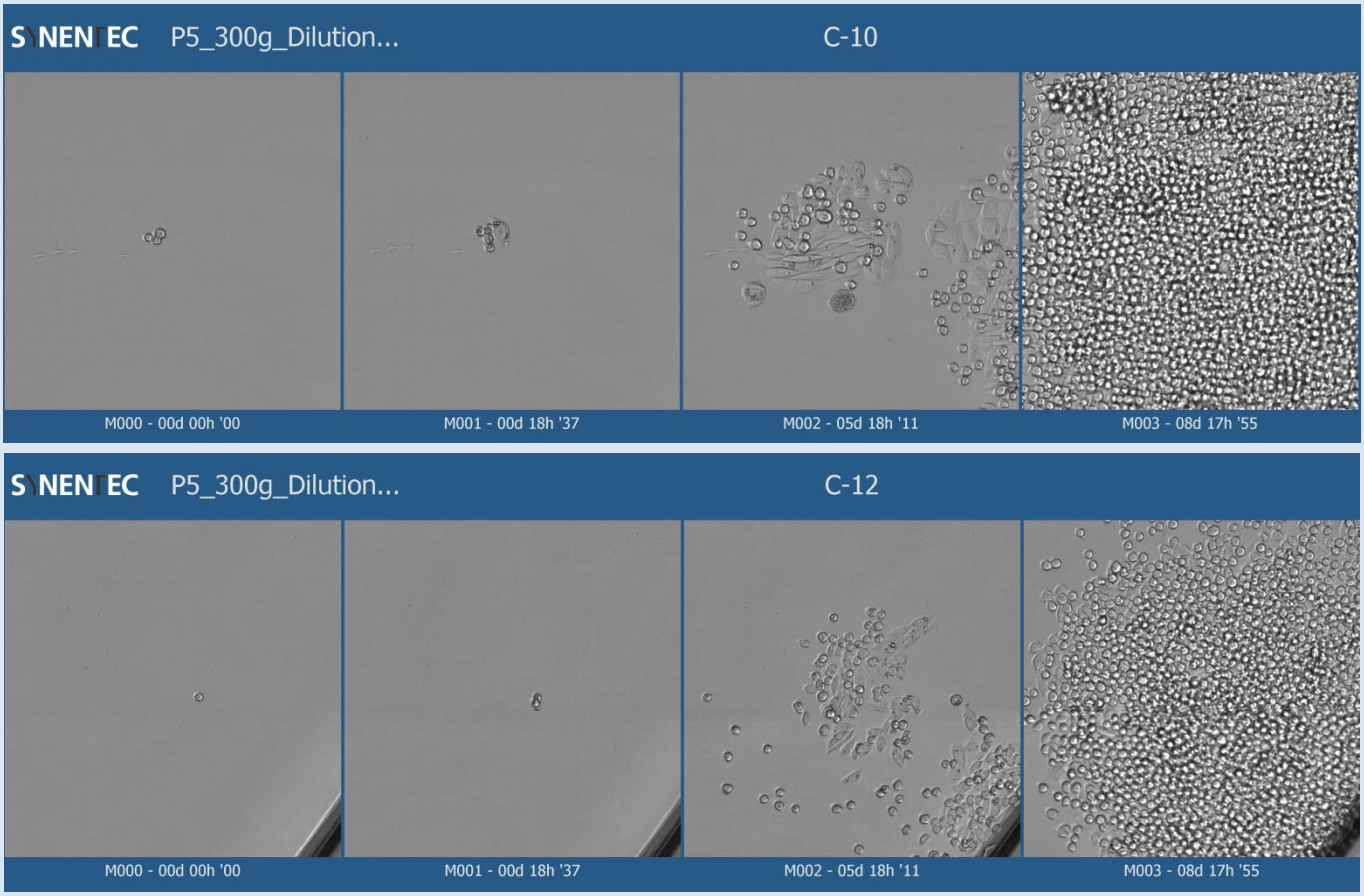


Fig 8: Example galleries
The clone gallery can be exported for each well and will be displayed as a filmstrip including all measurements and images in a comprehensive overview. C-10 is a non-clonal well containing one colony originating from a cell triplet. C-12 is a monoclonal well with a clear and unambiguous single cell image. The exported gallery is used solely for illustration and quick identification of single cell status, it does not eliminate the need to check the whole well prior to master cell banking of lead production cell lines.

The galleries depicted in fig. 8 do not replace the need to check clonality status of to be banked cell lines by two independent users to confirm monoclonality individually per well.

This prevents user bias and results in true monoclonal cell lines that ensure consistent quality and safety throughout the derived products life cycles.

Probability of Monoclonality

The probability of monoclonality (p_m) is one very important tool in describing processes resulting in the generation of monoclonal cell lines used for production of therapeutic agents. Several ways of calculating such probabilities exist, historically using Poisson Distributions and their implications (not accounting for „sticky cells“[3][4]). Here the use of confidence intervals estimates and accounts for uncertainty present in the empirical dataset analized, and is thus a reliable way of proving conformity to certain regulations[7][8] and self-set quality standards in cell line development.

In this case, Wilson's method is used to construct confidence intervals (w^+) on the measured percentage of colonies without a corresponding seeding day image ($\hat{p}$), namely „ghost wells“. It also accounts for experimental variablilty (s'), thus resulting in less than 100 % probability of monoclonality despite the dataset suggesting otherwise. The one-sided upper confidence interval on said percentage at 95 % confidence level ($\alpha = 0.05$) is used to deduct the probability of monoclonality (p_m=1-w^+) in order to show the cloning method's compliance to defined acceptance levels (e.g. 95 % probability of monoclonality) or not.

$$p' = \frac{\hat{p} + \frac{z_{1-\alpha}^2}{2n}}{1 + \frac{z_{1-\alpha}^2}{n}}$$

Eq. 1: Wilson centered probability of **„ghost wells“**
n = sum of colonized wells,
1-α = target confidence level
$\hat{p}$ = observed proportion of ghost wells
$z_{1-\alpha}$ = 1-α^{th} percentile of standard normal distribution

$$s' = \frac{\sqrt{\frac{\hat{p}(1-\hat{p})}{n} + \frac{z_{1-\alpha}^2}{4n^2}}}{1 + \frac{z_{1-\alpha}^2}{n}}$$

Eq. 2: Wilson centered standard deviation of **„ghost wells“**
n = sum of colonized wells,
1-α = target confidence level
$\hat{p}$ = observed proportion of ghost wells
$z_{1-\alpha}$ = 1-α^{th} percentile of standard normal distribution

$$w^+ = p' + s'$$

Eq. 3: Upper boundary of one sided confidence interval
p' = Wilson centered probability of „ghost wells“
S' = Wilson centered standard deviation of „ghost wells“

Results

During the conducted trial, using 12 96-well microtiter plates colony outgrowth measured with YT-Software® was 6 % for a seeding density of 0.1 cell/well, and 44 % for 1 cell/well respectively (tab. 2). Single cell status for each colony was checked by eye in the seeding day images and wells were categorized into the categories „monoclonal", „polyclonal" and „ghost". No ghost wells were present when plates were centrifuged at 300 x g for 5 minutes. In 4 of the non-centrifuged plates 8 colonies did not have a single cell depicted on seeding day.

Based on this data, probabilities of monoclonality were calculated using a standard spreadsheet program. Sample preparation without the incorporation of a centrifugation step at 300 x g, 5 min resulted in a probability of monoclonality of 91.71 % which is not compliant to the minimum of 95 % probability. When plates were centrifuged prior to imaging on CELLAVISTA® the probability of monoclonality was 99.11 % and cell lines derived from that process (tab. 3) are acceptable to be used in the production of therapeutic agents for human use, such as therapeutic proteins or cell and gene therapies. This shows the compliance of SYNENTEC®'s imagers to FDA, EMEA and ICH standards.

Tab. 2: Results of monoclonality study
Displayed are the outgrowth rates (wells containing colonies) for different seeding densities as well as the results of well categorization into clonal, non-clonal and ghost wells. No ghost wells were detectable when plates were centrifuged, 8 ghost wells were present when plates were not centrifuged.

300 x g	0.1 cell / well							Outgrowth [%]
Well category	P1	P2	P3	P4	P5	P6	Total	
Monolonal	5	6	4	2	4	4	25	5.90
Polyclonal	2	2	1	1	1	2	9	
Ghost	0	0	0	0	0	0	0	
300 x g	1 cell / well							Outgrowth [%]
Well category	P1	P2	P3	P4	P5	P6	Total	
Monolonal	12	8	8	18	9	11	66	45.83
Polyclonal	30	48	32	30	27	31	198	
Ghost	0	0	0	0	0	0	0	
0 x g	1 cell / well							Outgrowth [%]
Well category	P1	P2	P3	P4			Total	
Monolonal	16	11	13	17			57	43.49
Polyclonal	25	25	33	19			102	
Ghost	2	1	1	4			8	

Tab. 3: Overview of probabilities of monoclonality deducted from results of monoclonality study
The table summarizes the probabilities of monoclonality calculated from the results of the well categorization using Wilson's method for the generation of 95 % confidence intervals for the expected percentage of non-imaged cells on seeding day. The probability of monoclonality for non-centrifuged plates was 91.71 % whereas the centrifugation resulted in 99.11 % probability of monoclonality.

x g	poly-Clonal	monoclonal	Ghost Wells	Colonies	Wilson Centr. Prob. (Ghost well)	Wilson St.Dv. (Ghost well)	Upper Bound. Of CI (Ghost well)	exp. % Missed cells at 95 % CI	Probability of Monoclonality [%]
0	102	57	8	167	0,05507	0,0278	0,083	8,29	91.71
300	207	91	0	298	0,00447	0,0045	0,009	0,89	99.11

Conclusion & Outlook

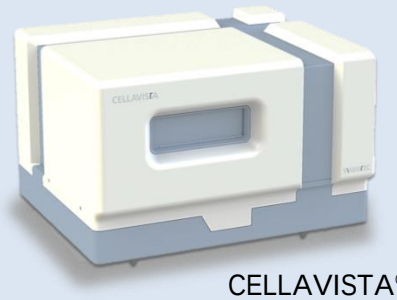
The experiments performed using SYNENTEC®'s imagers are a very potent tool to qualify and conduct single cell processes for the production of monoclonal cell lines and also to monitor user and process performance on a routine basis. Seeding methods like fluorescent activated cell sorting, limited dilution, cell printing or other methods can also be qualified in the presented approach in a cell line development setting monitoring the complete process involving seeding performance, cloning efficiencies and ultimately the probability of monoclonality. These trials can be included as supporting data in BLA and IND applications to display the method's capabilities for the proof of monoclonality in modern cell line development.

SYNENTEC®'s imagers CELLAVISTA® and NYONE® have proven to be valuable tools in cell culture labs in different areas of biology and biotechnology and are capable of being used in the production of monoclonal cell lines.

Other high throughput applications in cell line development

- Confluence Screenings
- Transfection Efficiency
- Trypan Blue Viability
- PAIA protein titer measurements
- PAIA glycosylation measurements
- Fluorescent Activated Single Cell Cloning (FASCC)

All applications mentioned in this AppNote are measurable and evaluatable with all our devices and are implemented in the YT-software®.



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DISCLAIMER

This article provides tips and suggestions for creating a monoclonal cell line.
However, it does not claim to provide a complete, FDA, EMEA, ICH compliant guide and does not supersede discussions with the agencies.

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