Automating the Proof of Monoclonality using SYBOT-800® and CELLAVISTA®

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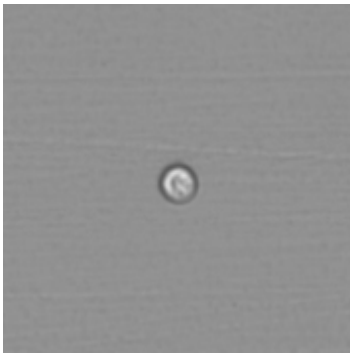
Abstract

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 on how to automatically seed cells using cytena's single-cell printer™ and conduct the proof of monoclonality using the high resolution optics of CELLAVISTA® in conjunction with SYNENTEC's proprietary image processing (YT-software®). We outline a possible image-based process qualification comparing different seeding methods taking advantage of the high-throughput imaging systems and plate handling system SYBOT-800® (the previous model of the new SYBOT-1000®; refer Fig. 11) 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, 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, crop) 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 deposition. This can be conducted regardless of the means that are used to isolate single cells.

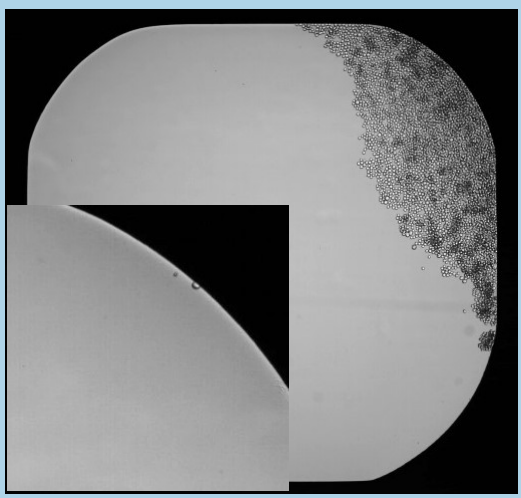


Fig. 1: Well-lit, full well image from CELLAVISTA®
The image contains one single colony imaged at a high resolution, using one single image in a 384 well plate. In the image crop from seeding day the colony's single cell progenitor is depicted.

Today's cell line development is technology-driven, new findings in vector design and construction, codon optimization, 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 state-of-the-art processes involve the generation of stable or transient genetically modified cell lines by transfection resulting in transfected pools of cells that express the product of interest. The pools usually are quite heterogeneous regarding the expression rate and the quality of the expressed product. 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 cell lines, 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 speeds up cell line generation significantly by making another round of single cell cloning irrelevant. 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].

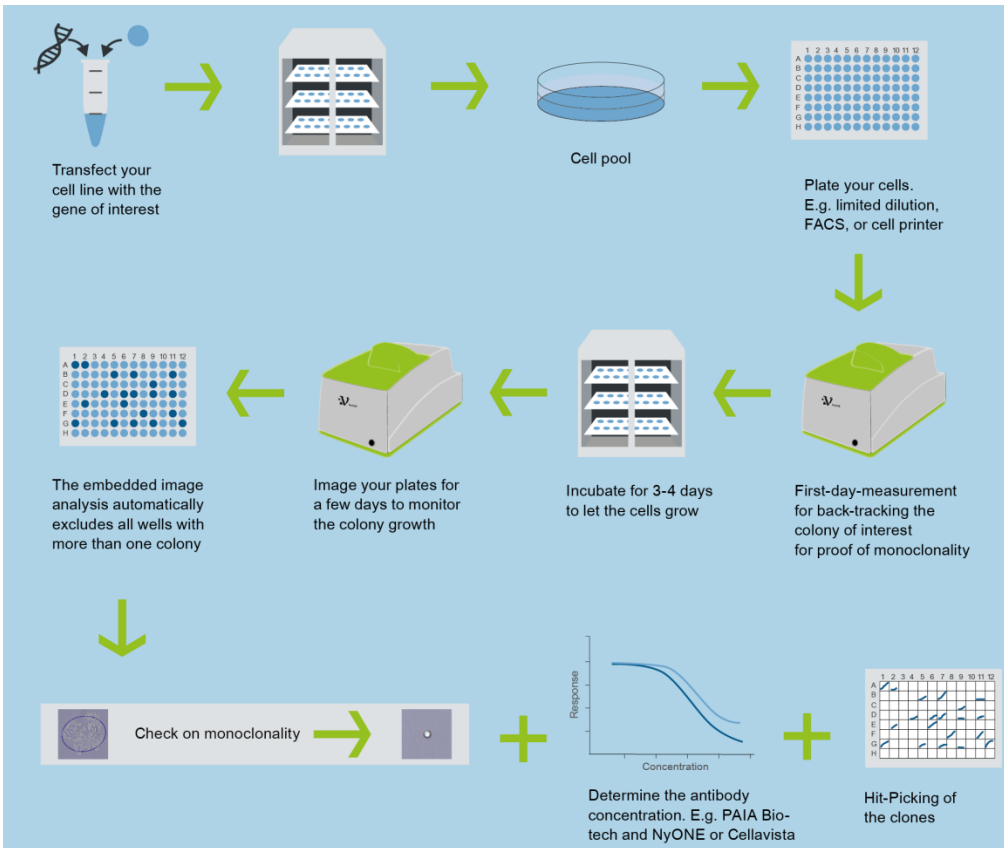


FIG. 2: PROCESS SCHEME IN CELL LINE DEVELOPMENT
After transfection and culturing of transfected pools, the cells can be plated by several means to achieve single cell status. SYNENTEC's imagers can be used for whole well imaging on seeding day. The growth of the cells is monitored over time by imaging 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.

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 prior to using the process for projects that will be subjected to the BLA.

Well Bottom Quality in 384 Well Plates

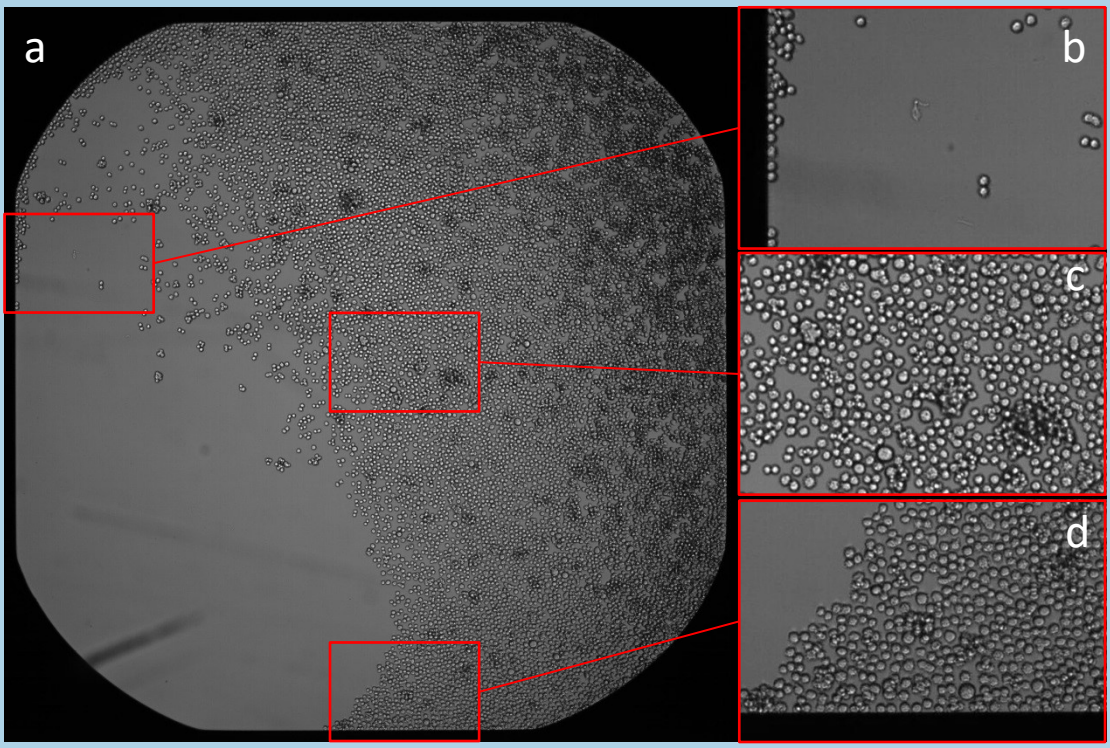


Fig. 3 Example of different lens effect across well bottom
The lens effect can be used to clearly differentiate cells from debris (b, c). The differences in height of the plate bottom can cause the lens effect to be less pronounced towards the edge of the well. The CELLAVISTA® is resolving the cells very clearly (a, 4x lens, one focus per well, Corning 3895BC). Due to very good optical quality of the imager and the plate, the single cell identification is less relying on a lens effect (d).

Fig. 3 illustrates the effects of using good quality plates (a) in 4x magnification. In the middle of the well close to the focused area, the cells exhibit a prominent lens effect that clearly helps in single cell identification (c). Towards the edge of the well the lens effect is less pronounced in the cells (b), but doublets are clearly resolved, even at the well edge. In the lower part of the well, the lens effect is not present at all, only very little differences in cell brightness are visible, but the combination of a high resolution imager and a very good quality plate make doublet identification easily possible, even if the lens effect is less pronounced.

Additionally the edge of the well has no "radius", so it is noticeable that single cells can not settle above the focal plane of the imager, which in turn shows that this plate-imager combination can be used for proving monoclonality. It is thus very unlikely that one single cell could not be detected due to optical issues resulting in a ghost well, or false clonal well.

The plates used for the production of clonal cell lines still have to be thoroughly qualified, as there are critical properties of different plate types, that can make the plate useless for proving monoclonality in BLA contexts.

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. 4).

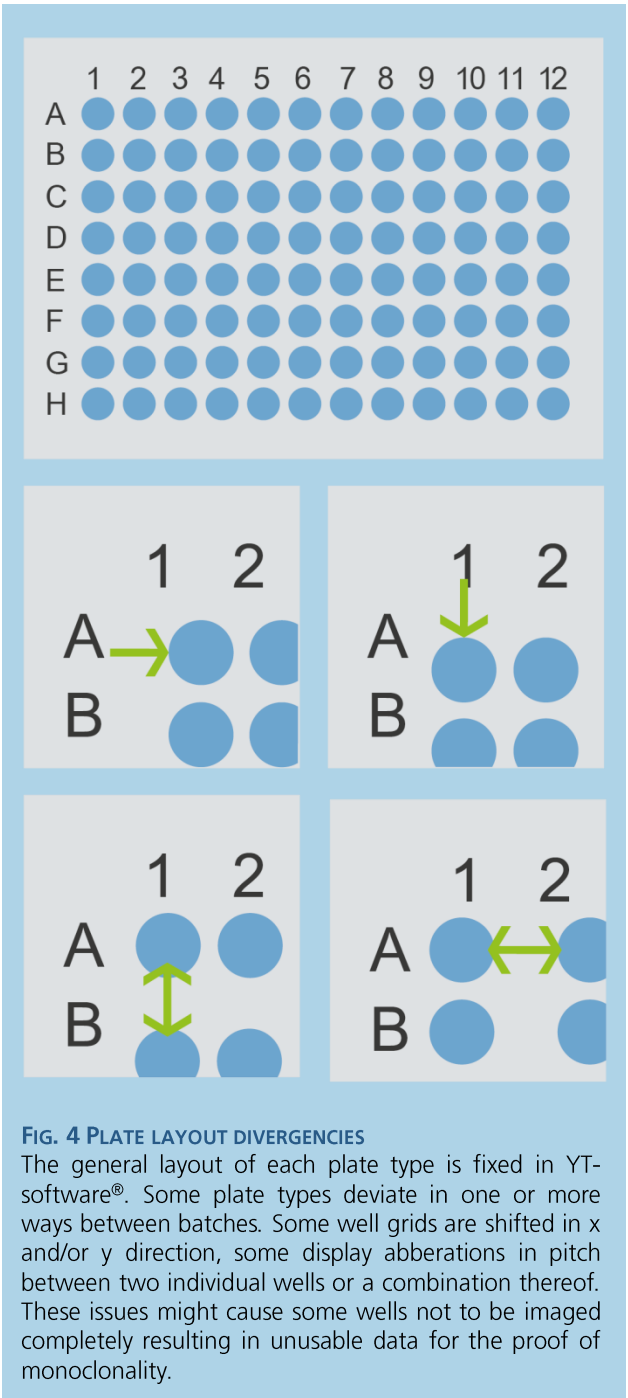


Fig. 4 Plate layout divergencies
The general layout of each plate type is fixed in YT-software®. Some plate types deviate in one or more ways between batches. Some well grids are shifted in x and/or y direction, some display abberations in pitch between two individual wells or a combination thereof. 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 be 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 assessed 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 consistency

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

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 can 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.

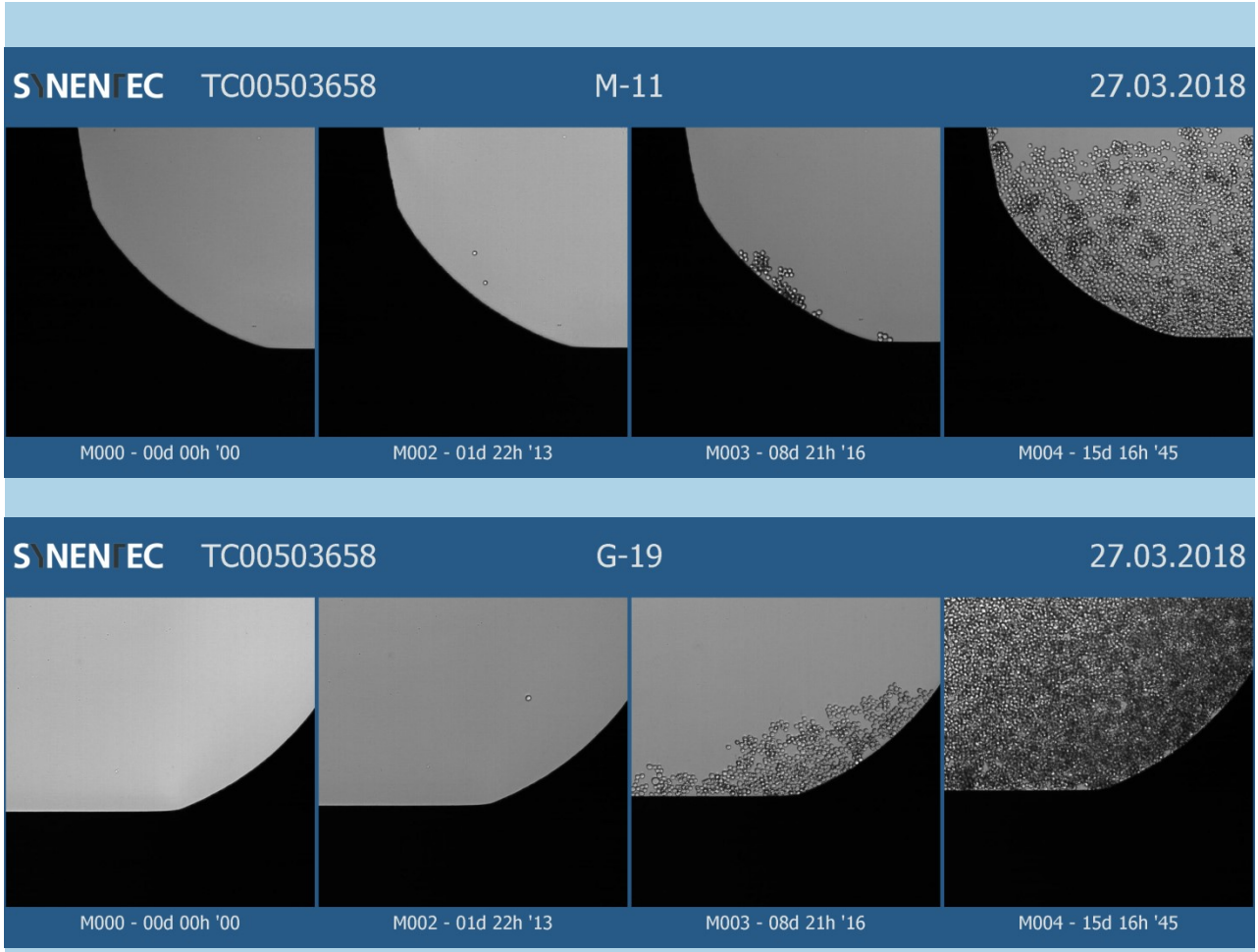


FIG 5: EXAMPLE GALLERIES
The clone gallery can be exported for each well and will be displayed as a filmstrip including all measurements and images in a comprehensive overview. M-11 is a non-clonal well containing one colony originating from two cells. G-19 is a monoclonal well with a clear and unambiguous single cell image. The exported gallery is used solely for illustration and quick identification of single cell status, it neither eliminates the need to check the whole well prior to master cell banking of lead production cell lines, nor it is replacing a full well image in the cell line generation report.

The galleries depicted in Fig. 5 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.

SYBOT-800®

SYBOT-800® is SYNENTEC's automated plate loading system, that automates imaging for a batch of up to 20 lidded plates. SYBOT-800® is reliably and quickly grabbing plates from the source rack, puts them into the Imager and removes them after imaging into the target rack. SYBOT-800® takes 1 minute to remove the imaged plate and feed a new plate to the device, thus enabling walk-away times up to 2h requiring no user intervention.

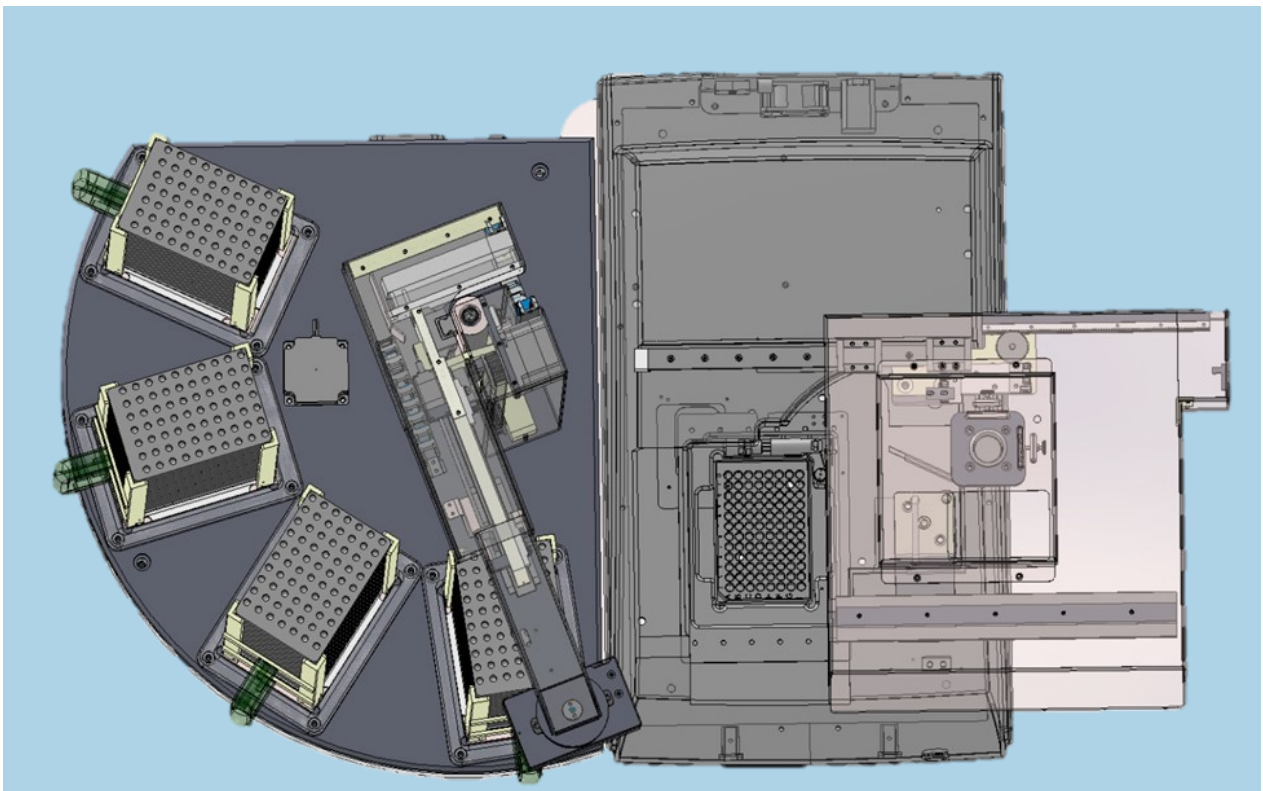


FIG 6: SYBOT-800® WITH AUTOMATED NYONE®
SYBOT-800® is SYNENTEC's plate handling solution that was developed to reliably automate plate loading into SYNENTEC's imaging systems NYONE ® and CELLAVISTA ®. SYBOT-800 ® is completely integrated in YT-Software which allows users to use all SYNENTEC products in a comprehensive manner, e.g. increasing throughput using Batch Processing and automatic exporting, resulting in a tailored workflow meeting demands in regards to throughput, consistency and data handling.

Automation, even in small footprint formats like SYBOT-800® are becoming more popular in all fields of modern biotechnology. SYBOT-800® was designed to be extremely robust and reliable, while maintaining an exceptional precision and speed, due to the delicacy of some cells or cell lines. An option to incorporate an automated incubation system is not available yet.

CYTENA SCP™

The patented single-cell isolation technology, developed by cytena, enables fully automated isolation of single cells into standard into 96- and 384-microwell plates with assurance of monoclonality by images of the single-cell dispensation. The instrument uses an inkjet-like principle featuring a disposable, dispensing cartridge. The cell sample is pipetted into the cartridge and an actuator is used to eject droplets out of it. An optical sensor allows for determination of cell number in each droplet. A fast shutter mechanism sorts the droplets containing exactly one single-cell into the substrate. Unwanted droplets are deflected into waste. cytena's disposable, cartridge takes up to 80 µL of cell sample. The single-packed, sterilized cartridges can simply be loaded by hand-pipetting. Its silicon micro-fluidic chip generates free-flying picoliter droplets acting as transport vessels for the cells. One cartridge can fill many well-plates with single cells. After use, the cartridge is disposed. This innovative approach prevents cross-contamination and saves extensive cleaning steps.

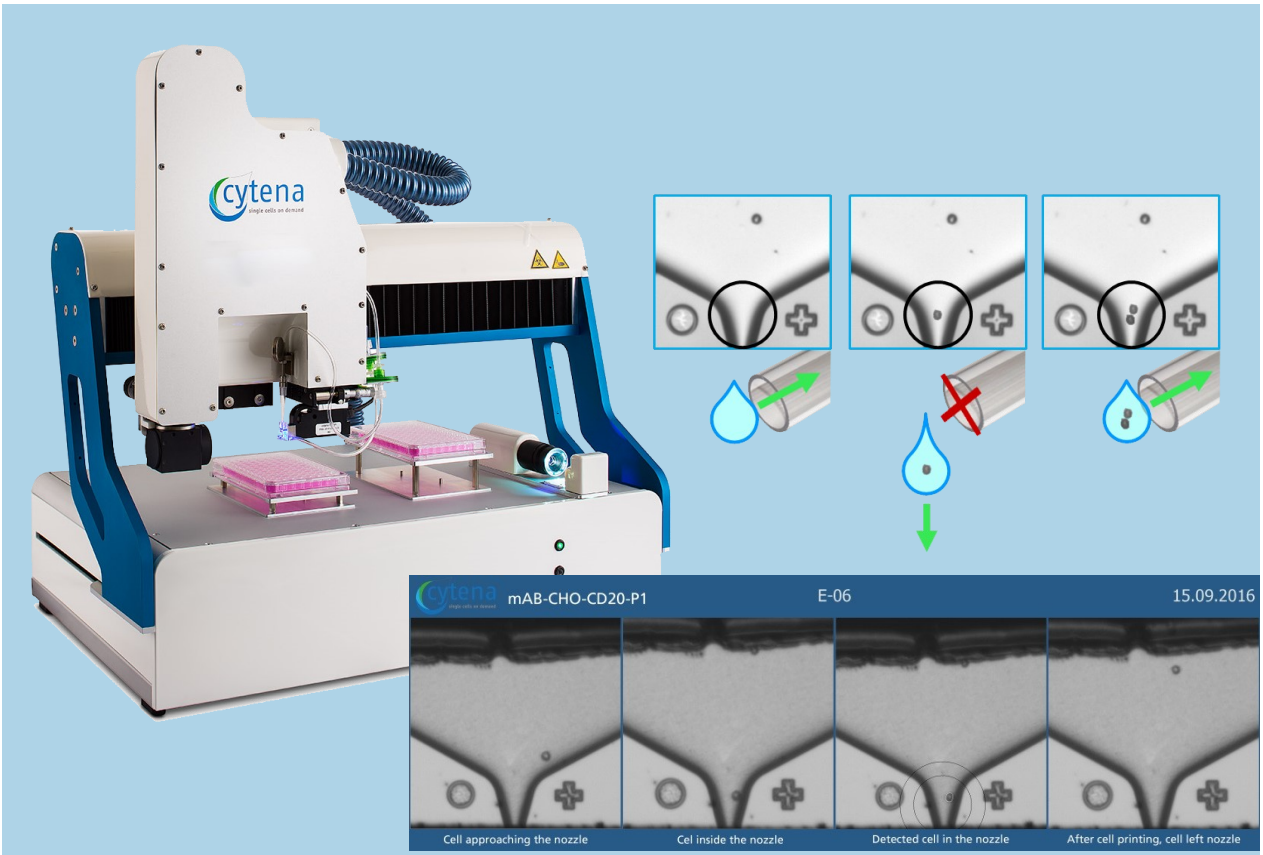


FIG 7: CYTENA SINGLE-CELL PRINTER™
cytena's single-cell printer™ (scp™) with details of the dispensing system. A cell suspension is pipetted into the cartridge and reaches the dispenser chip via an inlet. A piezo-driven actuator deflects a membrane on the back of the dosage chamber and a droplet is formed. The properties of cells at the nozzle are captured by the camera system within a region of interest before droplet formation. Only droplets containing a single cell are delivered to the substrate. A shutter system removes unwanted droplets containing no or more than one cell. The images of single-cell dispensing provide additional assurance of monoclonality and can be imported into SYNENTEC's YT-software®.These can also be exported into film strips according to the clone gallery.

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 analyzed, 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 detection of wells without a corresponding single cell seeding day image (p'), namely „ghost wells". The one-sided upper confidence interval on said percentage at 95 % confidence level ($\alpha = 0.05$) is used to deduct the probability of monoclonality in order to show the cloning method's compliance to defined acceptance levels (e.g. 95 % probability of monoclonality) or not. The use of this method for process qualification is in line with "cloning ghost well rate"[10] as it is based on ghost wells per plated wells and empty wells.

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

Eq. 1: Wilson centered probability of „ghost wells"
n = sum of plated 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 plated 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"

The probability of (non-)imaging a cell in the seeding process is to be multiplied by the probability of dispensing two cells on error and is also calculated using Wilson's method. This is due to the fact that these two low probabillity events have to coincide to result in a false-monoclonal cell line[11] and is the probability of monoclonality.

Material & Methods

Material:

- Transfected cell pools for product types (bispecific antibody, FC fusion protein, 2xIgG1, IgG4
- Proprietary chemically defined media
- Black 384-well plates (Corning 3985BC)
- Cell counting device e.g. NYONE®
- SYNENTEC's imaging systems (here: CELLAVISTA®
- SYNENTEC's plate handling system SYBOT-800®
- Cytena scp™ & BD FACSAria™ Fusion

Methods:

Difficult to clone mAb-CHO cells were counted using an automated cell counter and subsequently diluted into sterile PBS or media to an appropriate concentration for the single cell isolation devices. After seeding the cells using Single Cell Printing and Fluorescence Activated Cell Sorting (FACS) plates were centrifuged for 5 minutes at 1126 x g (Evaluation of this parameter not shown, but please refer to [8]). All plates were imaged on a CELLAVISTA® using *Single Cell Cloning* wizard in YT-software® , while 50 % of all plates (30 of 60) where automatically fed into the imager using SYBOT-800®.
The *Single Cell Cloning* application is used to image whole wells in high resolution (4x-lens, 2 µm/px, Tab. 1) before and after single cell isolation for the proof of monoclonality. The imaging of "empty" wells is due to very rare plate related imaging difficulties and well bottom artefacts. 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. Seeding efficiency was scored by eye in all wells on seeding day and day one. The wells were categorized into monoclonal well, polyclonal well, empty well and ghost well categories.

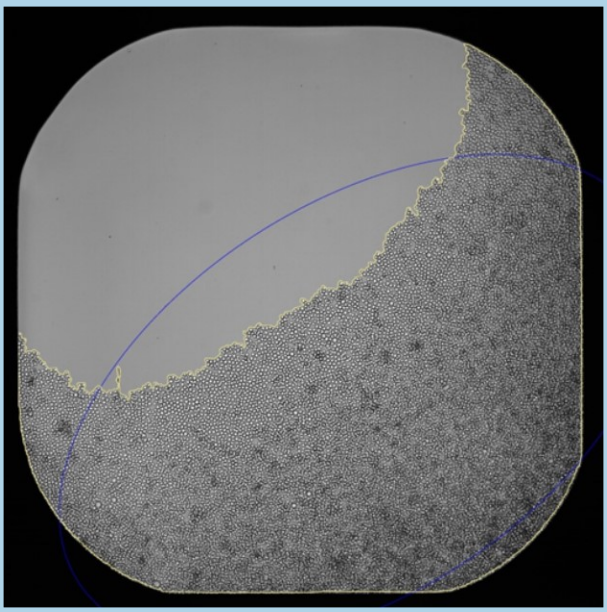


Fig 9: Detected colony in Single Cell Cloning wizard
The yellow area is the cell area (fill polygons un-checked) whereas the blue line marks the single colony. The results are accessible in YT-software® as *Cell Confluence* 42 % and *Colony Count* 1.

Tab. 1: Optical settings for Single Cell Cloning application

Channel	Brightfield
Objective	4x
LED	LED_Brightfield (default)
Emission Filter	Emi_Green (default)
Intensity	100 %
Exposure Time	7 ms
Gain	1 %

Results

In the trial conducted, using 60 384 microtiter plates (Corning 3985BC) colony outgrowth per plate measured with YT-software® was 18.0 % for Single Cell Printing and 3.6 % for FACS The colony outgrowth per clone is nearly identical (Tab. 2). Single cell status for each well was checked by eye in the seeding day images and wells were categorized into the categories "empty",„monoclonal", „polyclonal" and „ghost". In total 2334 wells did not have a single cell depicted on seeding day, 5 of them where Ghost wells. Based on this data, probabilities of monoclonality were calculated using a standard spreadsheet program. Plates seeded prior to imaging on CELLAVISTA® using the scp™ had 517 clonal colonies and 3 ghost wells resulting in a probability of monoclonality of 99.89 %. Plates seeded with FACS had 104 colonies and 2 ghost wells with a probability of monoclonality of 99.99 %. Cell lines, regardless of the seeding method, derived from the scp™ 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. Although Single Cell Printing is extremely efficient in Single Cell Deposition (98.6% vs. 19.6% FACS) and generally results in higher cloning efficiency (18.0% vs. 3.6% FACS, e.g. colonies per plate) the monoclonal outgrowth per clone is comparable (18.2% vs. 18.6% FACS).

Tab. 2: Results of seeding method comparison
Displayed are the single cell efficiencies and outgrowth rates per clone (wells containing colonies) for different seeding methods.

Method	Imaged Wells	Single Cell Efficiency		Clonal outgrowth (15% confluence + confirmed clonality)		Cloning Efficiency
		No.	%	No.	%	%
scp™	2880	2841	98.6	517.0	18.2	18.0
FACS	2850	504	19.6	104.0	18.6	3.6

FACS had less non-clonal wells (Tab.2) which results in a slightly higher Probability of Monoclonality of FACS deposition and imaging. Considering the amount of cloning plates needed to result in a sufficient amount of clones to be passed on to the next screening stage, the scp™ is the more efficient device for single cell isolation. The nearly exact rates for clonal outgrowth per single cell show a great potential for process optimization using SYNENTEC's imagers and approaches like DoE or other multivariate analysis. No effect on ghost well rate, cloning efficiency or clonal outgrowth was measureable in plates imaged using SYBOT-800®.

Tab. 3: Probabilities of monoclonality deducted from results of seeding method comparison
The table summarizes the probabilities of monoclonality calculated from the results of the well categorization and cell deposition accuracy monitoring using Wilson's method for the generation of 95 % confidence intervals for the expected percentage of non-imaged cells and the likelyhood of depositing more than one cell per droplet. The probability of monoclonality for scp™ was 99.89 % whereas FACS deposition resulted in 99.99 % probability of monoclonality.

Method	Empty Wells	Non-Clonal	Clonal Wells	Ghost Wells	Colonies	PM
scp™	31	8	2841	3	517	99.89
FACS	2288	2	504	2	104	99.99

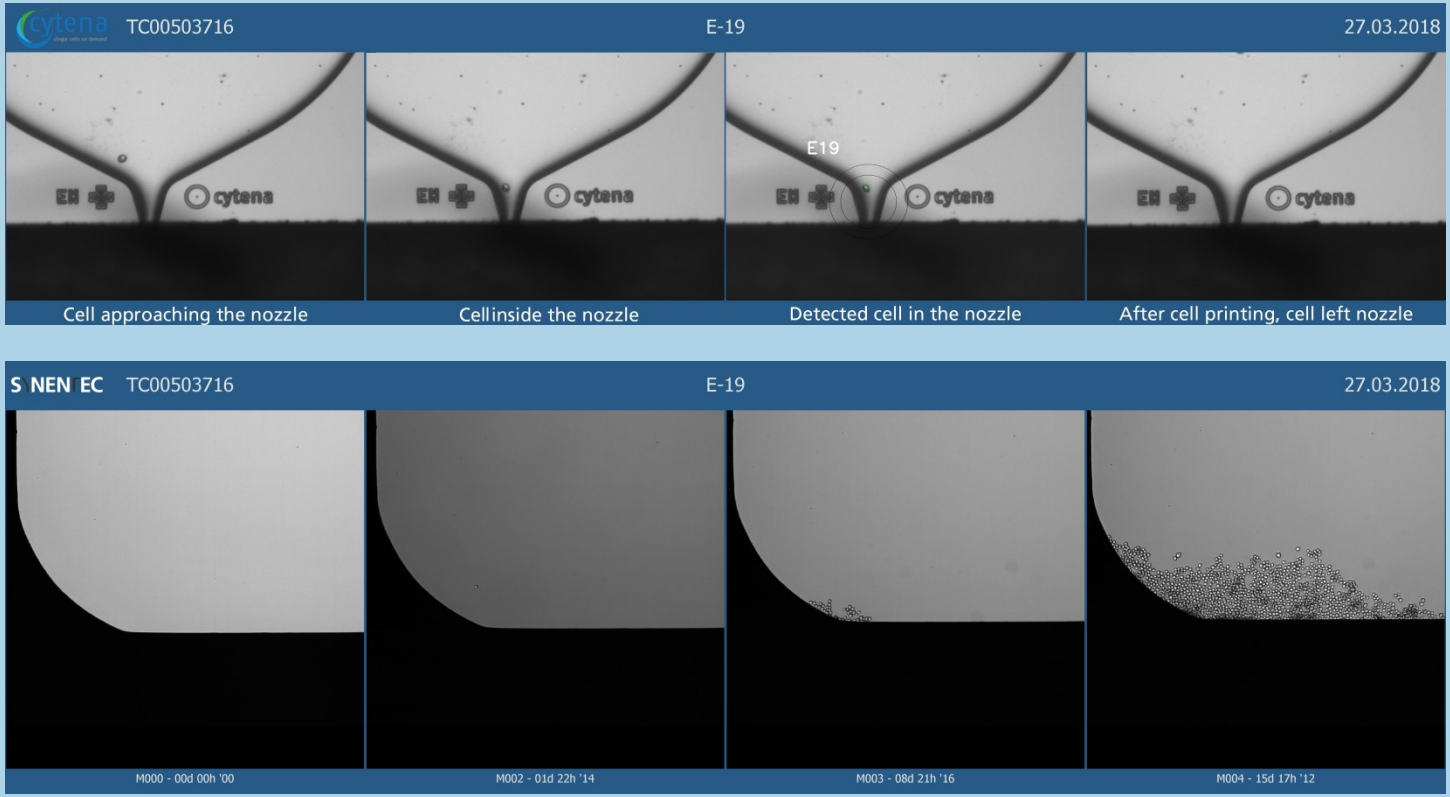


FIG. 10: EXAMPLE GALLERIES GENERATED IN YT-SOFTWARE
YT-Software enables users to not only conduct monoclonality checks for seeding and imaging rapidly in one software, it also allows the export of galleries in a comprehensive "film-strip" format for illustration purposes in presentations or even cell line generation reports.

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 be and were qualified and compared 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 potentially be included as supporting data in BLA and IND applications to display the method's capabilities for the proof of monoclonality in data and evidence driven cell line development. The use of automation, here SYBOT-800® (the previous model of the new SYBOT-1000®; refer Fig. 11), in combination with well-known and sufficiently qualified processes and operations enables the same quality of work compared to highly skilled scientists doing routine tasks which are highly repetitive and thus very ineffective.

Increasing walk away times by automating basic tasks is one mayor benefit of automation. The use of SYBOT-800® thus reduces the need to performing routine and repetitive tasks like loading plates in devices every 3-7 minutes without. SYNENTEC's imagers CELLAVISTA® and NYONE® as well as the plate handling solution SYBOT-800® 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

- Distinguishable cell studies[10]
- Confluence Screenings
- Transfection Efficiency
- Trypan Blue Viability
- PAIA Protein Titer Measurements
- PAIA Glycosylation Measurements
- Fluorescent Activated Single Cell Cloning (FASCC)

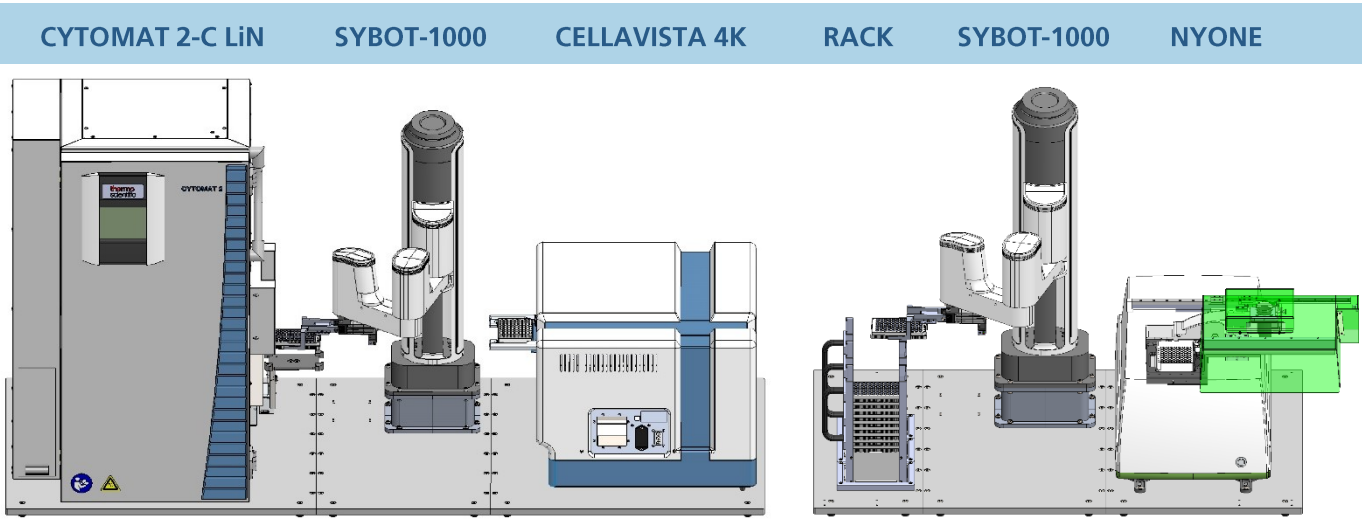


Fig. 11: Combinability depending on Throughput, Budged and Footprint
From simple plate loading with SYBOT-1000 at ambient temperature or long-term imaging combining SYBOT-1000 with CYTOMATTM 2 C-LiN to high end integrations with external collaborators into cutting edge automation enclosures, SYNENTEC has a solution for your automation project.

SYBOT-1000, the follow-up model of the SYBOT-800

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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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