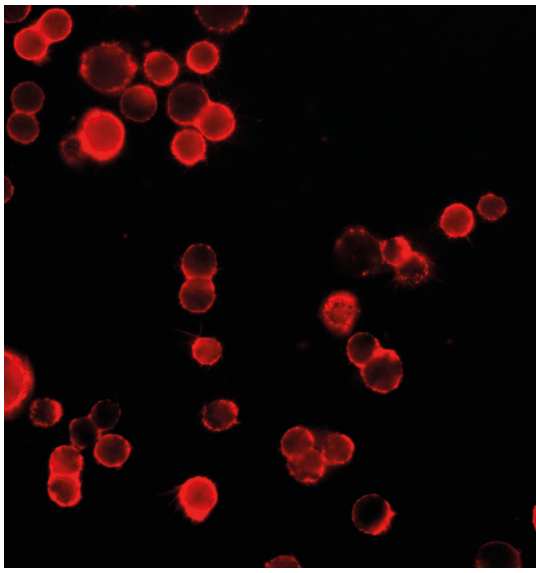
# Homogeneous Antibody-Binding Assay Using High-Throughput Microscopy

Schaefer W[1], Werdelmann B[1], Kollenda S[1], Sebens S[2], Geisen R[1], Hummel S[1] & Pirsch M[1]
[1]SYNENTEC GmbH, Elmshorn, Germany
[2]Institute for Experimental Cancer Research, CAU + UKSH Kiel, Germany

## ABSTRACT

**The production of high-affinity, antigen-specific antibodies is essential for various applications in research, diagnosis, and therapy. Typically, the antibody-producing cells are generated via hybridoma technology and subsequent single cell cloning. This requires a reliable method for selecting optimal clones. Various screening methods for antibodies in hybridoma supernatants exist, each with advantages and disadvantages. For example, enzyme-linked immunosorbent assay (ELISA) is common but requires extensive washing and can compromise antigen conformation. Flow cytometry is highly sensitive, but entails large sample volumes and complex data analysis, while fluorometric microvolume assay technology (FMAT) employs only a single laser. Here, we present a homogeneous antibody-binding assay, easy to perform in combination with our automated microscope NYONE® Scientific. This assay eliminates the need for washing steps and utilizes minimal sample volumes. NYONE® Scientific offers the versatility of different fluorescence channels for the detection of labeled cell detection and the ability to measure additional characteristics through the brightfield channel. We used an anti-HER2 antibody as a model antibody (ab). The HER2-expressing breast cancer cell line SK-BR-3 served as target cells, while HER2-negative MDA-MB-468 cells were used as a negative control. By optimizing the assay and the image settings, we achieved a detection limit of primary antibody up to 1 ng/mL with the Suspension Cell AB Binding (1F) application of YT-SOFTWARE®. Combined with our Single Cell Cloning application, this method enables both proof of monoclonality and screening of hybridoma supernatant with the same device and software.**



KEYWORDS: SINGLE CELL CLONING, CELL LINE DEVELOPMENT, NO-WASH, CELL-BASED, HOMOGENEOUS, ANTIBODY-BINDING ASSAY, MONOCLONAL ANTIBODY, HYBRIDOMA, HIGH-THROUGHPUT SCREENING (HTS)

## EASY IMAGING AND ANALYSIS OF NO-WASH ASSAYS

- Screening without plate washing
- Increased reproducibility and accuracy through simple assay design
- Fast imaging for high-throughput screening
- Detection of weak signal with high sensitivity up to 1 ng/mL
- Same device and software as for single cell cloning

## INTRODUCTION

Antibodies are glycoproteins that bind with high affinity and specificity to their target antigens and are widely used as research tools, diagnostics, and therapeutics [1]. The common method for producing antibodies is hybridoma technology, in which B lymphocytes producing the desired antibodies are fused with myeloma cells [2], [3]. The hybridoma cells are selected by single cell cloning to generate clones producing monoclonal antibodies (mAb) [4]. Identifying cells that produce antibodies with the desired properties of affinity, specificity, and functionality is a challenging task that requires efficient and reliable methods.

Various methods are available to screen secreted mAb in hybridoma supernatants for binding of the antibody to the target antigen, e.g., enzyme-linked immunosorbent assay (ELISA), flow cytometry, and fluorometric microvolume assay technology (FMAT). ELISA is a widely used method that involves coating the target antigen on a solid surface and detecting the bound antibody with a secondary antibody conjugated to an enzyme [5]. However, ELISA has several limitations, such as the need for multiple washing steps, the possibility of denaturing the antigen during coating, the difficulty in detecting antibodies that recognize native conformational epitopes on cell surface antigens and clones that express high levels of a low-affinity antibody can give an equivalent signal to clones that express low levels of a high-affinity antibody [1], [4]. Flow cytometry is a laser-based method that can measure antibody binding to cell surface antigens using fluorescently labeled antibodies and cells. However, flow cytometry requires expensive equipment, large sample volumes, and complex data analysis. Another technique using a laser scanner and measuring the fluorescence signal of a secondary antibody conjugated to a fluorophore binding to the primary antibody on the cell surface is FMAT. FMAT provides a homogeneous assay without washing steps and can detect antibody binding at low concentrations (5 ng/mL) and antigen densities [4].

SYNENTEC's widefield imagers NYONE® or CELLAVISTA® are already well known and accepted for the proof of monoclonality in the antibody production process [6]-[9]. However, screening of high-yield clones is often performed using one of the previously mentioned methods on other instruments, which is both time-consuming and resource-intensive. Therefore, our aim in this study was to develop a homogeneous antibody-binding assay for fast screening of hybridoma supernatants using NYONE® Scientific and YT-SOFTWARE®. This assay for use with the CELLAVISTA® or NYONE® is designed to combine the single cell quantification capabilities of flow cytometry screening with the no-wash setup, low sample volume, sensitivity, and specificity of FMAT assays. In addition, it offers higher throughput capacity and the ability to use different fluorophores, as NYONE® and CELLAVISTA® have up to six different excitation sources.

## MATERIAL

### Cell Culture and Treatment

- SK-BR-3 (cell line from breast cancer that overexpresses HER2)
- MDA-MB-468 (cell line from breast cancer, HER2 negative)
- Accutase (e.g., PAN Biotech)
- RPMI 1640 medium (e.g., PAN Biotech) supplemented with 10 % (v/v) FCS, 1 % (v/v) L-glutamine, 1 % (v/v) sodium pyruvate
- Trypan Blue 0.02 % (e.g., Gibco)

### Plate

- 384-well plates, black with transparent bottom (e.g. Falcon)

### Staining

- Rat Anti-HER2 antibody [ICR12] (abcam, ab11710)
- Goat Anti-Rat IgG H&L Alexa Fluor 647 (abcam, ab150159)
- Staining Buffer, PBS with 10 % BSA, 10 % NaAC, 0.5 M EDTA

### Imaging and Analysis

- SYNENTEC's imaging device (here NYONE® Scientific)
- SYNENTEC's YT-SOFTWARE®

## METHODS

### Cell culture and cell counting

We routinely cultured the cancer cells in RPMI 1640 medium containing FCS (see above) using standardized cell culture conditions (37 °C, 5 % $CO_2$, humidified atmosphere). Before

**TAB. 1:** EXCITATION AND EMISSION SETTINGS

| Channel | Excitation LED [nm] | Emission Filter [nm] |
|---|---|---|
| Brightfield | Brightfield | Green (530/43) |
| Alexa647 | Red (632/22) | Far Red (685/40) |

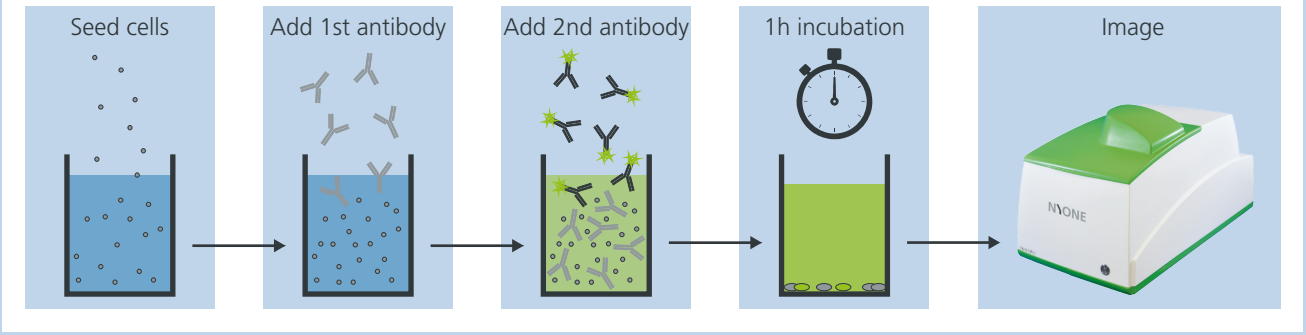



**FIG. 1.** WORKFLOW

No-wash assay for the detection of antibodies starts with seeding of 10 µL of cell suspension per well in a black 384-well plate with transparent bottom. 5 µL of primary (1st) and fluorescently labeled secondary antibody (2nd) were added in sequence. After an incubation of 1 hour at 37 °C, the plate was imaged using NYONE® Scientific and analyzed with **Suspension Cell AB Binding (1F)** applicaton of YT-SOFTWARE®.

seeding cells for experiments, we detached them from their culture vessels using Accutase, centrifuged and resuspended them in RPMI 1640 medium, and counted them using SYNENTEC's **Trypan Blue** application.

### Cell seeding

We seeded 10 µL of cell suspension into 384-well plates (700 cells/well). We let the cells settle to the bottom without centrifugation during antibody staining.

### Antibody staining

We prepared a 4 x staining solution by diluting the antibody in buffer. Directly after cell seeding, we added 5 µL of the primary antibody solution to each well and 5 µL of the secondary antibody solution. After an incubation for 1 hours at 37 °C, imaging was performed without further washing steps (Fig. 1).

### Imaging and processing

Imaging was performed using the 10 x objective of NYONE® Scientific capturing brightfield and fluorescence channel (Tab. 1). For the image analysis we used the **Suspension Cell AB Binding (1F)** application of YT-SOFTWARE®.

### Data evaluation

We exported the data of the processed experiments from YT-SOFTWARE® and subsequently analyzed them with the statistics software GraphPad Prism.

## RESULTS AND DISCUSSION

### Establishment of the Assay

The present study aimed to demonstrate the easy use of NYONE® Scientific and the **Suspension Cell AB Binding (1F)** application of YT-SOFTWARE® for an antibody-binding assay. To do this, we used an anti-HER2 antibody as a model antibody and two breast cancer cell lines with different HER2 expression levels [10]. SK-BR-3 cells express high levels of HER2 while MDA-MB-468 cells are HER2-negative and were used as a negative control [11]. Without washing in between, we added cells, different concentrations of anti-HER2 antibody, and a secondary antibody to a 384-well plate to facilitate high-throughput screening. Here, we used an Alexa647-coupled secondary antibody, as autofluorescence and fluorescence background is usually lower with longer wavelengths compared to shorter ones.

The images acquired by NYONE® Scientific were analyzed using the **Suspension Cell AB Binding (1F)** application. This application detects the suspension cells in brightfield and analyzes the background-corrected average fluorescence intensity within these

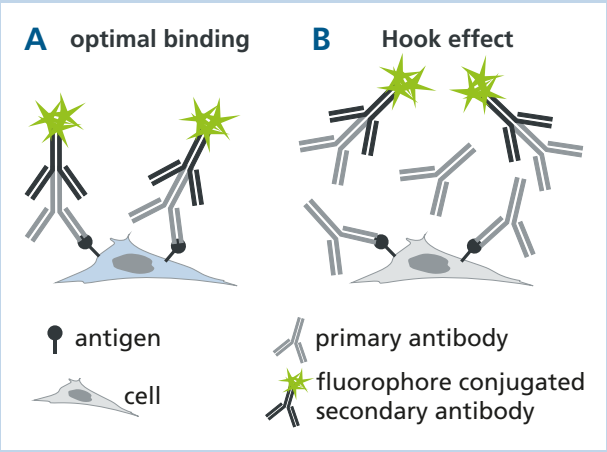



**FIG. 2.** HOOK EFFECT

The 1st antibody (primary, light grey) binds specifically to the antigen (dark grey) on the cell, and the 2nd antibody (secondary, middle grey with green mark) binds to the 1st antibody. A) Balancing primary and secondary antibodies results in an optimal binding and fluorescent signal. B) An excess of primary antibodies results in secondary antibodies binding to the unbound primary antibodies in solution rather than to the antigen-bound antibodies. This process, known as the Hook effect, leads to a lack of fluorescent labelling of the cells (based on Lee et al. [4]).

cells. A defined threshold is set for calculating the percentage of positive cells.

We titrated the primary antibody from 0.0002 ng (0.01 ng/mL) to 500 ng (25 µg/mL) and added a consistent concentration of secondary antibody (0.1 µg/mL) to each well. A phenomen occuring with high primary antibody concencentrations is the well-known 'Hook effect'. In this case, the excess primary antibodies bind the labeled antibodies in solution, leading to false negative results (Fig. 2) [4]. Thus, the background-corrected average fluorescence intensity (Avg Fluo CH1 Intensity BC) of the stained SK-BR-3 cells increased corresponding to the amount of primary antibody up to a certain amount (1.33 ng) and then began to decrease (Fig. 3 A).

Between the SK-BR-3 and the MDA-MB-468 cells, there was already a detectable difference in intensity at a low primary antibody amount with a detection limit nearby 0.01 ng (< 1 ng/mL) (Fig. 3 B). Looking at the raw pictures of the wells with low or high antibody concentrations, our eyes can hardly detect the cells (Fig. 3 C). However, the software is still able to detect the signal and distinguish it from background.

To obtain meaningful results in the antibody-binding assay, various parameters must be considered. Of course, the concentration of the primary and secondary antibodies is essential. The cell number is also important and should neither be too high nor too low. Further, the total volume in the well and the medium or buffer used are important, as these affect the fluorescence of the background. Also, a high-quality microtiter plate suitable for fluorescence

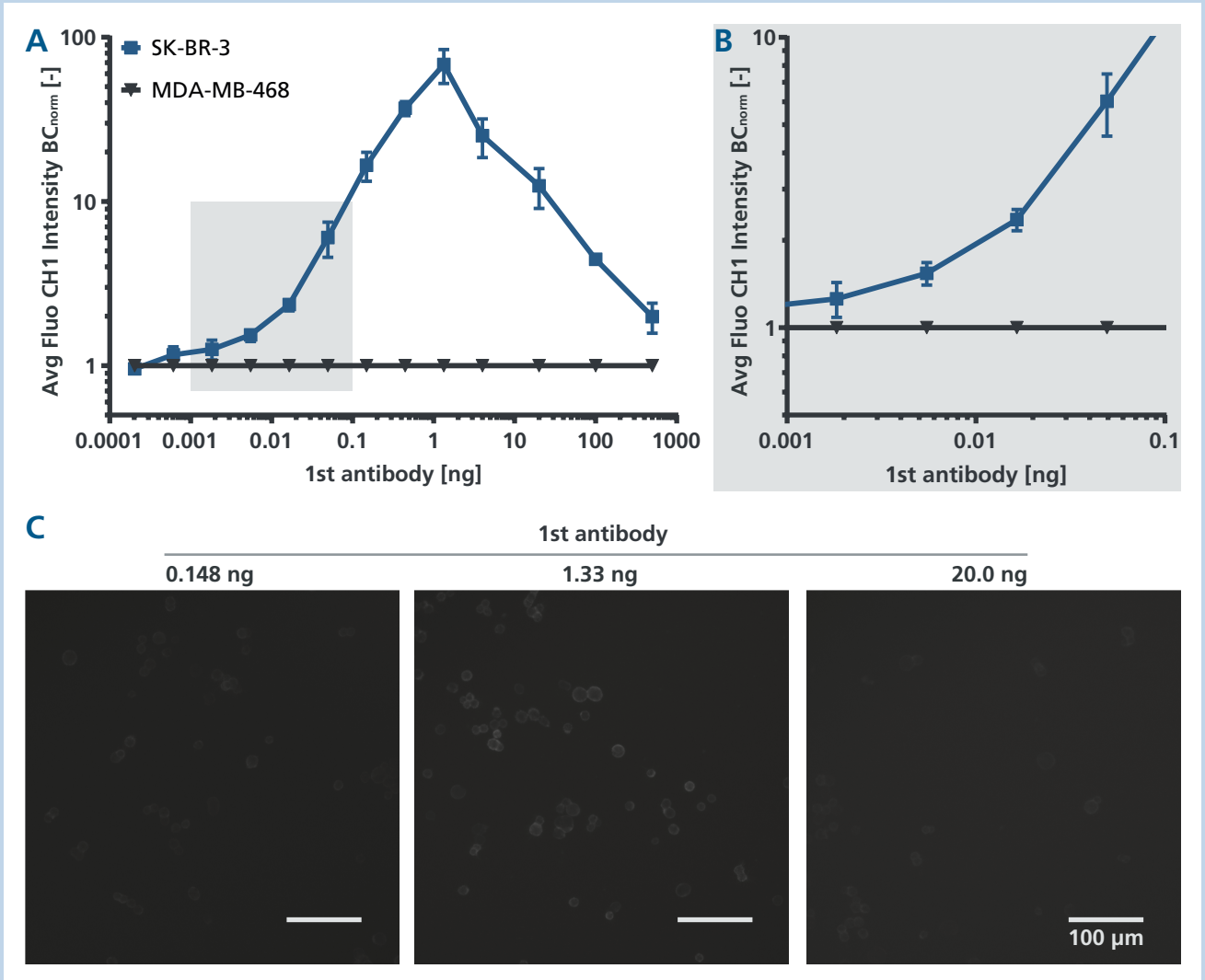



**FIG. 3.** PRIMARY ANTIBODY TITRATION

We seeded HER2-expressing SK-BR-3 and HER2-negative MDA-MB-468 cells in a 384-well plate and added different amounts of primary antibody (1st) as well as a consistent amount of fluorescently labeled secondary antibody (0.1 µg/mL). A) Using the primary antibody amount from 0.2 ng (0.01 ng/mL) to 500 ng (25 µg/mL), the intensity of stained SK-BR-3 cells was analyzed with the **Suspension Cell AB Binding (1F)** application. B) A difference between the SK-BR-3 and MDA-MB-468 signal was already detectable at the lower range, demonstrating the sensitivity in detecting low antibody concentrations (technical triplicates, 16 bit, 120 ms). C) Images of the fluorescence signal of the three representative primary antibody sets illustrating how weak the signal is (16 bit, 120 ms). Scale bar: 250 µm.

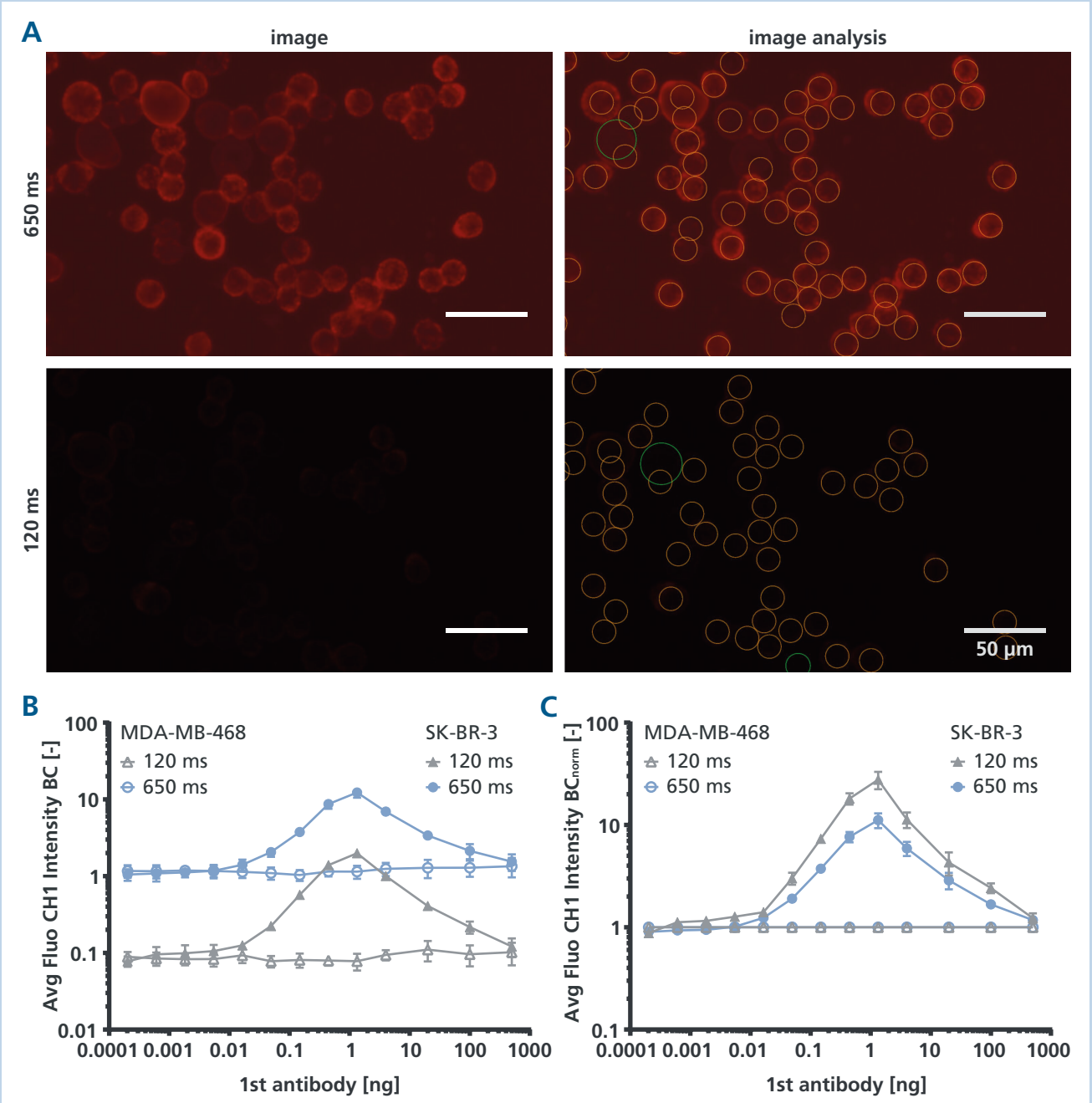



**FIG. 4.** EXPOSURE TIME

HER2-overexpressing SK-BR-3 and HER2-negative MDA-MB-468 cells were seeded in a black 384-well plate and different amounts of primary antibody (1st ab) as well as a consistent amount of secondary antibody (0.1 µg/mL) were added. The stained cells were imaged with NYONE® Scientific with an exposure time of 120 ms and 650 ms. A) The images show that although the raw images differ greatly, the image analysis with the **Suspension Cell AB Binding (1F)** application detects the fluorescence of the cells similarly. Unstained cells are encircled in green and stained cell are encircled in orange (8-bit, 1 µg/mL secondary antibody, 1.33 ng primary antibody). B) The background-corrected average fluorescence intensity (Avg Fluo CH1 Intensity BC) of 650 ms exposure time was 10 times higher than the values of 120 ms exposure time. C) The normalized intensities indicate a better signal-to-noise ratio for the shorter exposure time (technical triplicates, 8-bit). Scale bar: 50 µm.

imaging should be selected. All of these parameters, as well as the optimal imaging settings, need to be established for the individual conditions of the antibody-binding assay. Here, we describe the influence of some of these parameters in our settings (data from other optimizations not shown).

**Exposure time**

We analyzed two different exposure times for imaging the stained cells with NYONE® Scientific. The longer exposure time (650 ms) is according to the Operating Guide for YT-SOFTWARE®. The guide recommends an average gray value of 130 for 8-bit images

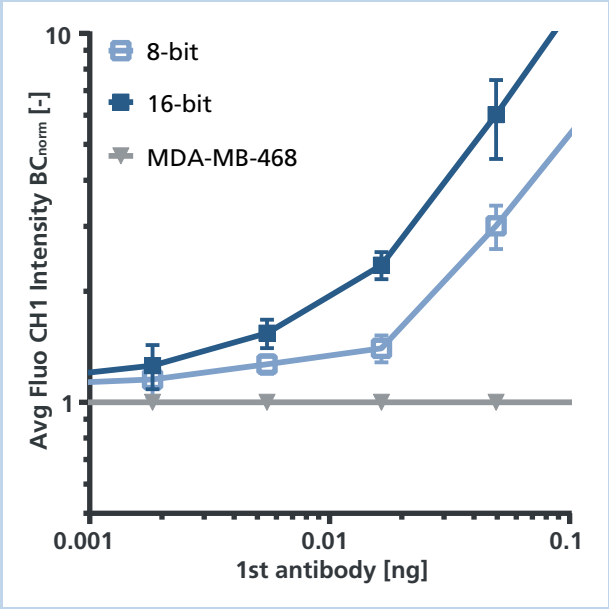



**FIG. 5.** 8-BIT AND 16-BIT IMAGE ANALYSIS

Image analysis of a 16-bit image allows better signal separation than 8-bit images using the **Suspension Cell AB Binding (1F)** application due to the higher count of gray values. When using the identical concentrations of the secondary antibody, the normalized background-corrected average fluorescence intensity (Avg Fluo CH1 Intensity BC) is higher in the low 1st (primary) antibody concentration range when using 16-bit images than with the 8-bit images. This provides an even lower detection limit when using 16-bit images (technical triplicates, 120 ms exposure time).

and 33,000 for 16-bit images for medium intensity fluorescent objects. This exposure time makes the cells clearly visible (1.33 ng (66.7 ng/mL) primary and 2 ng (0.1 µg/mL) secondary antibody) (Fig. 4 A). In contrast, for the low exposure time (120 ms), the gray value exhibits a much lower magnitude than these values (8-bit ~30, 16-bit ~7000), making the cells scarcely visible. However, the background signal also experiences a considerable decrease. The background-corrected average fluorescence intensity (Avg Fluo CH1 Intensity BC) at 650 ms exposure time was higher than the intensity at 120 ms exposure time (Fig. 4 B). When normalizing the results to the HER2-negative MDA-MB-468, the reduced background value improved the signal-to-noise ratio (Fig. 4 C). This ratio enables the **Suspension Cell AB Binding (1F)** application to better detect fluorescence objects in images with low exposure time. Furthermore, lower exposure times leads to a shorter measurement duration, under six instead of nearly ten minutes per 384-well plate, and enables higher throughput.

### 8-bit versus 16-bit images

In the Scientific line of SYNENTEC's devices, the camera can capture 8-bit and 16-bit images. An 8-bit image corresponds to 256 shades of gray per pixel, while images with 16-bit displays 65,536 gray values. The higher bit depth considerably increases the dynamic range of 16-bit images, enabling signals to be displayed in much greater detail and therefore better recognized than in 8-bit images. In comparison to the results of 8-bit images, the detected fluorescence intensities are almost twice as high for the 16-bit images (Fig. 5). This enables the detection of lower antibody concentrations up to 0.016 ng (1 ng/mL). Although 8-bit imaging is sufficient for a wide range of primary antibody concentrations.

### Secondary antibody concentration

For the best result of the antibody-binding assay, we tested five different concentrations of secondary antibody with a consistent amount of primary antibody (1.33 ng). We added these to SK-BR-3 and MDA-MB-468 cells and captured them with 120 ms exposure time with NYONE® in 8-bit and 16-bit images. The background-corrected average fluorescence intensity (Avg Fluo CH1 Intensity BC) normalized to the signal of the MDA-MB-468 cells illustrates the influence of the secondary antibody on the signal to noise ratio as well as the better resolution with 16-bit images (Fig. 6 A). This result is also reflected in the 16-bit images (Fig. 6 B). Although the cells at low secondary antibody concentrations are barely visible in the images, image analysis using the **Suspension Cell AB Binding (1F)** application reveals that at low antibody concentrations, more cells are marked with an orange circle as positive than at the high antibody concentration. In this case, more cells are marked with a green circle as unstained, although they are visually clearer. Nonetheless, the signal-to-noise ratio is crucial for the analysis and not the visibility for the human eye.

## CONCLUSION

The antibody-binding assay using NYONE® Scientific is a simple, fast, and reliable method for screening and validating antibodies against target molecules. The assay uses cells or beads that express or carry the target molecule, as well as negative controls that lack the target molecule, to measure the specificity and affinity of antibodies. The assay does not require any washing steps, only the addition of the antibody solutions e.g., by using a liquid handler. For the assay, only micro volumes of antibody and cell/bead suspensions are needed, reducing the cost and consumption of reagents or hybridoma supernatants. The assay is performed in a 384-well plate format, enabling high-throughput screening of antibody candidates. To achieve the best results, the assay must be optimized individually by adjusting various parameters, such as the concentration of the primary and secondary antibodies, the number of cells or beads per well, the incubation time, and the buffer or media composition. These parameters affect the signal-to-noise ratio as well as the detection limit and can lead to the Hook effect. The optimal conditions for each parameter depend on the characteristics of the antibodies, the target molecules, and the media.

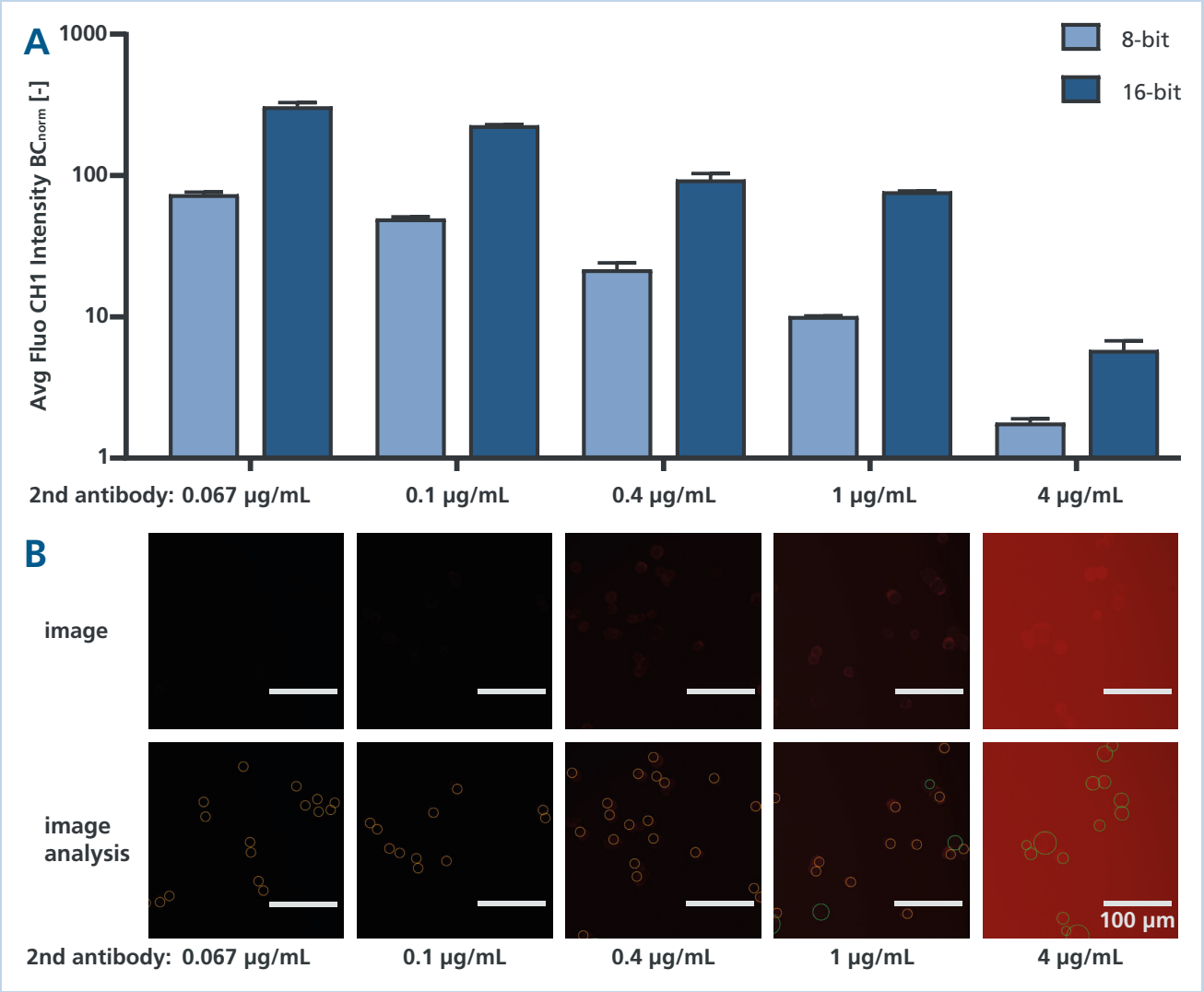



**FIG. 6.** SECONDARY ANTIBODY CONCENTRATION

To analyze the influence of the secondary antibody, we used five different concentrations with a consistent amount of primary antibody (1.33 ng) for the staining of SK-BR-3 and MDA-MB-468 cells. We imaged with an exposure time of 120 ms with NYONE® Scientific for 8-bit and 16-bit images and analyzed the cells with **Suspension Cell AB Binding (1F)** application of YT-SOFTWARE®. A) The background-corrected average fluorescence intensity (Avg Fluo CH1 Intensity BC) of the different secondary antibody concentrations illustrate the better signal to noise ratio at lower concentrations due to the increase in background signal with increasing concentration. This is even higher for 16-bit images. B) Images of stained SK-BR-3 cells with different concentrations of secondary antibody and the corresponding image analysis illustrate the discrepancy between visualization and detection. At low concentrations, all cells are marked as stained with an orange circle, and at higher concentrations, the clearly visible cells are still marked as unstained with a green circle (16-bit images, 120 ms exposure time). Scale bar: 100 µm.

Imaging this assay with NYONE® Scientific and analysis with YT-SOFTWARE® are efficient and fast. Imaging can be performed in a single image per well at 10 x magnification, eliminating the need to scan the entire well by quantifying the fluorescence intensity of each individual cell rather than the total fluorescence of the cell sample. Higher sensitivity is achieved with 16-bit images than with 8-bit images of NYONE® Scientific. With the optimal exposure time, background noise can be minimized, signal separation increased, and duration reduced. Thus, a full 384-well plate can be scanned in under 6 minutes, enabling the screening of more than 10,000 samples per day. The antibody-binding assay using NYONE® Scientific and **Suspension Cell AB Binding (1F)** application YT-SOFTWARE® is therefore a time-saving, easy-to-perform and fast-to-analyze method for antibody screening and validation. The same device and software can be used for monoclonality validation of single cell cloning and for screening of antibodies in hybridoma supernatants to identify high-quality antibodies for various applications.

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**THIS APPLICATION IS COMPATIBLE WITH ALL OUR DEVICES AND IS IMPLEMENTED IN YT-SOFTWARE®. AN AUTOMATED LENS CHANGER IS NOT MANDATORY.**


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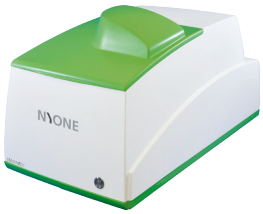


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**SYNENTEC Gmbh**
**Otto-Hahn-Str. 9A**
**25337 Elmshorn/Germany**
**Phone. +49 (0) 4121 46311-0**
**Email. appsupport@synentec.com**

**www.synentec.com**