

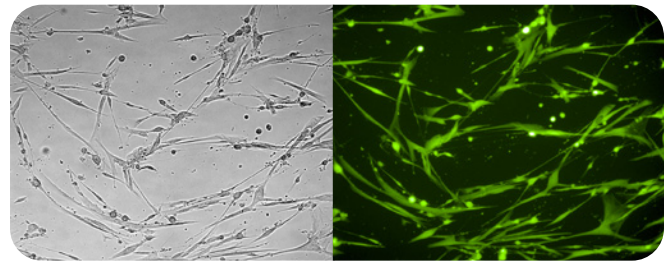
B cell selection and therapeutic antibody characterization using the Operetta high content imaging system

AIMM Therapeutics is a privately held research-based biotechnology company located in Amsterdam that uses proprietary state-of-the-art antibody technology platforms to develop therapeutic and prophylactic human monoclonal antibodies from immortalized and selected B cells. Research programs at AIMM target the prevention and treatment of diseases with a high unmet medical need, including cancer, inflammatory diseases, autoimmune diseases and infectious diseases.

Infection with the intestinal bacterium *Clostridium difficile* is the most common cause of healthcare-associated diarrhea that can develop in patients after hospitalization and treatment with antibiotics. The symptoms of *C. difficile* infection (CDI) vary from mild diarrhea to full-blown clinical expression with pseudomembranous colitis (PMC), a severe inflammation of the colon which can result in colonic perforation, septicemia and death¹. *C. difficile* is resistant to a wide range of antibiotics and, for this reason, new treatments for severe cases are desperately needed.

CDI is mediated by the production of toxins by the bacterium, with toxin A (ToxA) and toxin B (ToxB) responsible for the most common symptoms seen in the clinic¹. Treatment with toxin-binding agents such as antibodies is therefore a promising approach to reducing or inhibiting these clinical manifestations.

This case study describes the workflow that researchers at AIMM, including Dr. Mark Kwakkenbos and Dr. Pauline van Helden, are using to generate antibodies against *C. difficile* ToxB.



Therapeutic antibody development

Research at AIMM uses a proprietary technology called AIMSelect™ to transform peripheral blood memory cells from pathogen-exposed individuals into proliferative, cell surface B cell receptor positive, immunoglobulin-secreting cells, using genetic programming (Figure 1)². A recent example of such an experiment is described here.

The first step in the workflow to generate antibodies against *C. difficile* ToxB is the isolation of peripheral memory B cells from the blood of pathogen-exposed individuals using fluorescence-activated cell sorting (FACS). B cells are not typically suited for long term cultures as they undergo spontaneous terminal differentiation into plasma cells with arrested cell cycles. To circumvent this problem, researchers at AIMM have developed a genetic programming method to immortalize B cells.

To start immortalization, memory B cells are seeded onto a feeder cell layer, stimulated with cytokines, and transduced with a retrovirus which encodes the B cell lymphoma-6 (BCL-6) and B cell lymphoma-xL (BCL-xL) proteins, and GFP

as a transduction control. The BCL-xL protein promotes survival of activated B cells, whilst BCL-6 inhibits the differentiation of B cells into plasma cells. Together, these act to immortalize the B cells.

To identify B cell clones secreting ToxB specific antibodies, transduced B cells are diluted and seeded at cell densities of 10-50 cells per well into 96-well plates. After 18 days of cultivation, the supernatant is collected and tested for antibody binding to ToxB proteins in an ELISA assay performed on the EnVision® multilabel plate reader (primary screen). B cell cultures that produce ToxB binding antibodies are identified and sorted at 1 cell per well into 96-well plates using a cell sorter to obtain the ToxB specific clones from the mixture of cells.

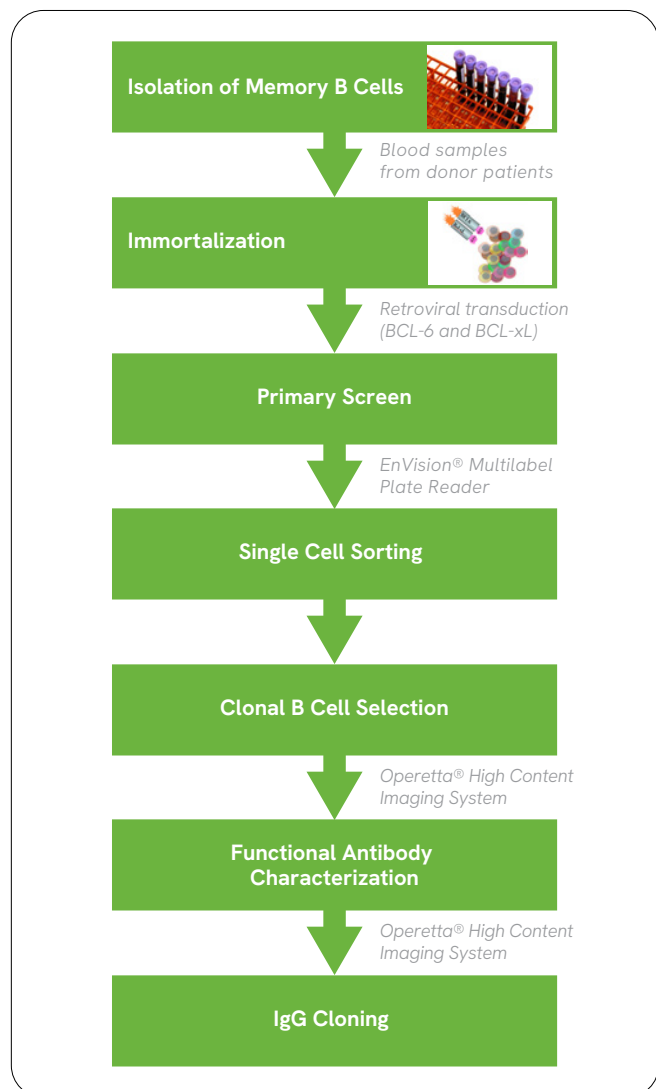


Figure 1: AIMSelect™ workflow of ToxB therapeutic antibody development. Full details on this method can be found in Kwakkenbos et al. (2010).



- Dr. Mark Kwakkenbos

“Before we used the Operetta system we analyzed plates with a conventional microscope, which resulted in the investigator getting many headaches! Now, the Operetta allows us to automate imaging and to achieve higher speed and throughput. The Operetta keeps the cells undisturbed and is a very sensitive instrument as we are able to analyze as few as five cells. This would not work with other assays.”

Clonal B cell selection assay

After 14 days of culture the growth of the clonal B cells is checked using a B cell detection assay. Wells containing GFP-positive B cells are identified using the Operetta® high-content analysis system, coupled to a plate::handler workstation, and the Columbus™ image data storage and analysis system.

Images are acquired on the Operetta system, in the GFP channel, using the 2X objective in widefield mode (Figure 2). The images are then imported into the Columbus system (superseded by Signals Image Artist) for analysis and The Find Cells building block is applied. The number of detected cells is used as the readout parameter.

Wells containing GFP positive cells are considered as hit wells as they contain transduced B cells of which the antibodies in the supernatant can be tested for ToxB binding. Wells that do not contain B cells do not have to be screened, greatly reducing the amount of work, time and reagents needed for further screening.

After an additional four days of culture, the supernatants of the positive hit wells from the clonal B cell selection assay are collected using the JANUS® automated workstation and tested for ToxB binding in an ELISA.

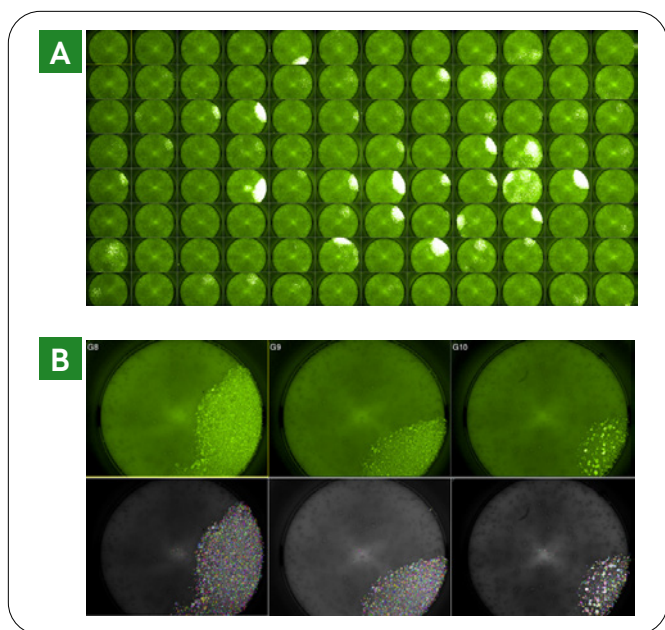


Figure 2: Clonal B cell selection assay. A) Plate overview of a 96-well plate in the GFP channel. The hit wells contain a GFP-positive B cell colony. Images were acquired using the Operetta high-content analysis system equipped with a 2X objective in widefield mode. B) Magnified view of three wells containing B cell colonies. The upper row shows the GFP channel, the lower row shows the cell detection using the *Find Cells* building block in the Columbus system.

Toxin neutralization assay

In the next step, a functional antibody characterization assay is performed to identify which antibody is the most effective, i.e. offers the highest protection against the toxic effects of *C. difficile* ToxB.

Human lung fibroblast (IMR-90) cells are seeded at a nearly confluent level (10,000 cells per well) into CellCarrier 96-well black-walled microplates (Revvity, Cat-No. 6005558) and incubated overnight at 37 °C and 5% CO₂. On day two, the *C. difficile* ToxB, derived from a bacterial culture (50,000 x diluted ~pg/mL range), is mixed with the medium alone (negative control), diluted antibody (positive control) or B cell supernatant at a 1:1 ratio (vol/vol). The mixtures are pre-incubated for 60 min and then used to replace the cell culture medium of the IMR-90 cells. Cells are incubated for 24 h in the presence of the toxin/antibody mixture.

The following day, cells are stained with the live cell stain Calcein AM for 1 h at a final concentration of 1 μM and imaged on the Operetta system in widefield mode using the 10X objective (Figure 3).

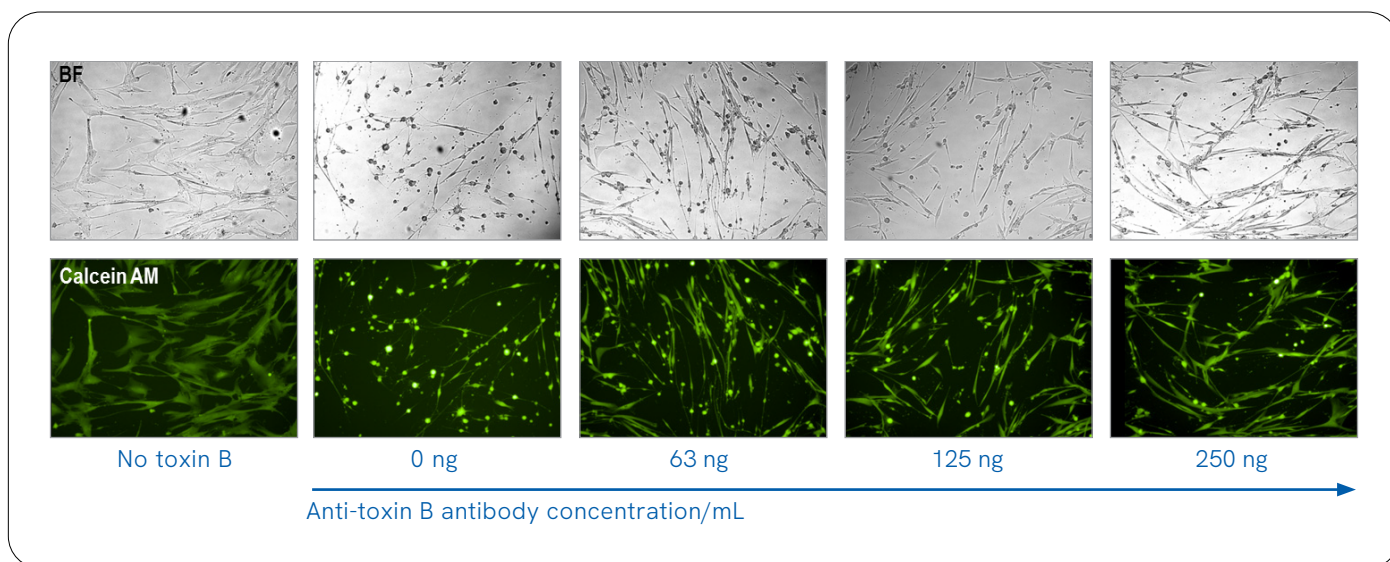


Figure 3: Toxin neutralization assay for functional characterization of B cell supernatants. As a proof of principle, human lung fibroblast (IMR-90) cells were incubated with *C. difficile* ToxB mixed with various concentrations of a positive control antibody. Without ToxB, the cells show a healthy, elongated phenotype. Incubation with the toxin alone (0 ng antibody) leads to cell rounding, and eventually promotes cell death. The toxic effect is less pronounced however if a toxin/antibody mixture is applied, proving that the antibody neutralizes the toxin and protects against the toxic mechanisms in a dose-dependent manner. BF = Brightfield.

The images are then transferred to the Columbus system for analysis. Incubation with the toxin leads to cell rounding; therefore the toxicity of the toxin/antibody mixture is scored in Columbus by using the cell roundness of the IMR-90 cells as a readout parameter. The Columbus analysis sequence first uses a *Find Cells* building block for cell detection on the Calcein AM channel, followed by a *Calculate Morphology Properties* building block to calculate the cell roundness. A *Select Population* building block is then used to classify all cells with a roundness value greater than 0.85 as dying cells. The percentage of dying cells is then used as the final readout parameter to score the neutralizing potential of the antibody.

Following the toxin neutralization assay, those B cells which express functional antibodies are then expanded and the encoding DNA is isolated and cloned into an expression vector for stable expression of the antibody at higher yields in mammalian cells.

Conclusion

This case study illustrates how Revvity's high content imaging solutions are enabling therapeutic antibody development. Two steps in the AIMSelect™ ToxB workflow (Figure 1), the clonal B cell selection and the functional antibody characterization, rely on high content screening (HCS) using the Operetta high-content analysis system. The combination of the Operetta system with the Columbus image data storage and analysis system extends the image analysis capabilities and allows measurements to be performed on the Operetta system while analyzing the data in parallel on the Columbus system. In addition, the coupling of the

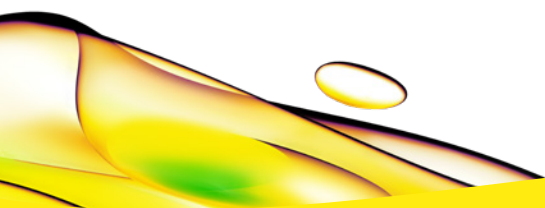
Operetta system to a plate::handler workstation allows the group to run plates both day and night, further increasing their throughput. The EnVision multilabel plate reader and the JANUS automated workstation are essential parts of the workflow that contribute to the throughput of the process and reduce the variability of liquid handling steps. All of these instruments and software products complement one another and have allowed researchers at AIMM to create a workflow for developing therapeutic antibodies that could significantly improve the treatment of diseases with high unmet medical need.

Acknowledgements

Many thanks to Dr. Mark Kwakkenbos and Dr. Pauline van Helden from AIMM Therapeutics, Amsterdam, for providing the data for this case study. Thanks also to Dr. Karin Boettcher (Revvity) and Dr. Sarah Piper (Revvity) for writing the manuscript.

References

1. Rupnik et al. (2009) *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. *Nature Reviews Microbiology*, 7 (7): 526–536.
2. Kwakkenbos et al. (2010) Generation of stable monoclonal antibody-producing B cell receptor-positive human memory B cells by genetic programming. *Nature Medicine*, 16 (1): 123–128.



revvity