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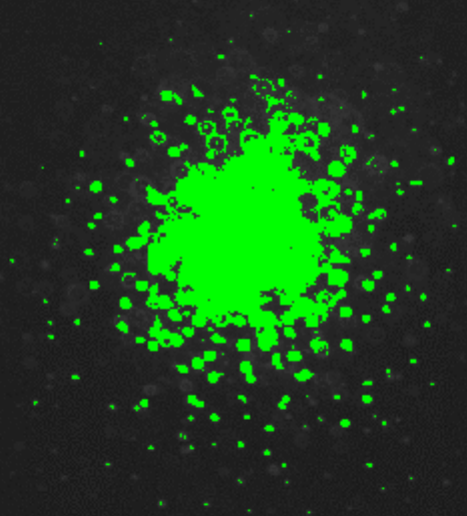


Cell Selection and Retrieval

Antibody Discovery

Automated Drug Discovery Workflows to Identify the Best Producing Clones With Highest Productivity

- Documented image based monoclonality proof
- 5 channel fluorescence for productivity measurements
- Nanowell technology to assess and verify high numbers of clones before transferring the top producers
- Highly viable hybridoma transfer from semi-solid media such as methylcellulose and Matrigel®

Antibody Discovery

Antibodies are the ideal therapeutic biomolecule due to their ability to bind to a variety of targets with high affinity and specificity. Innovations in hybridoma and phage display technologies have allowed drug developers to exploit the natural diversity in antibodies, leading to the wide use of monoclonal antibodies (mAbs) in treating cancers, chronic inflammation, and infectious diseases. Whilst single B-cell screening has significantly evolved and is the main source for obtaining antigen-specific mAb sequences, hybridoma screening using semi-solid media still represents an important technology.

This application guide will focus on both methods within the antibody discovery workflow, and how the CellCelector Flex can help simplify progress.

Advantages of the CellCelector Flex in Antibody Discovery

- Screen up to 5 million B-cells or hybridomas in a single plate using Nanowell technology – in less than 8 hours
- Antigen specific identification and isolation of single B cells
- Use of plate coating, beads or reporter cells to capture the antibody of interest
- Multiplex screening of up to 14 antigens in a single run
- High-throughput interaction studies of multiple cell types in parallel

CellCelector B-Cell Screening Workflow

Combining the CellCelector Flex with Nanowell technology enables the implementation of a high-throughput cell isolation, imaging and secretion assay workflow to enable the processing of millions of B-cells within hours. Target-specific secreting cells can be selected from thousands of potential candidates before being transferred into PCR tubes for further downstream analysis.

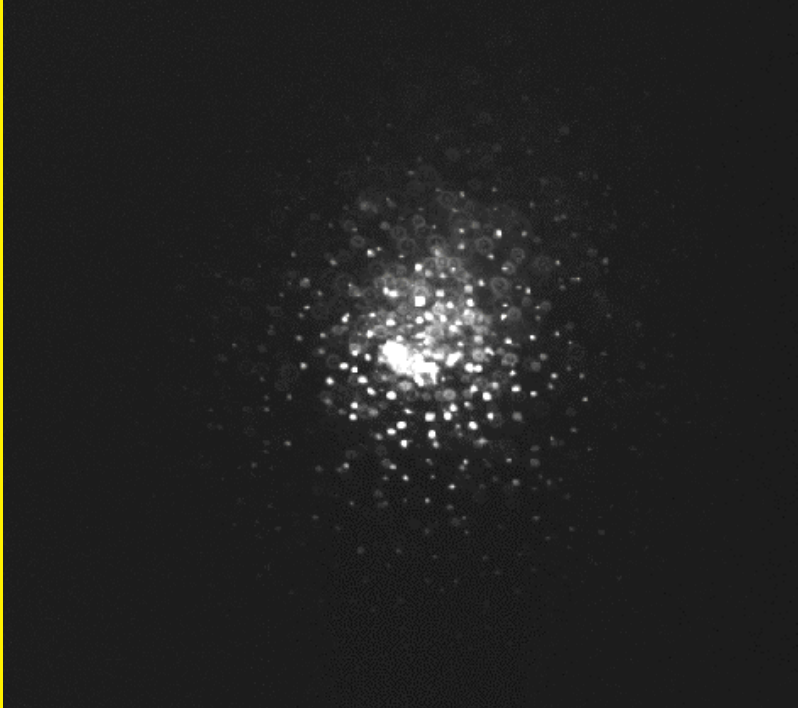
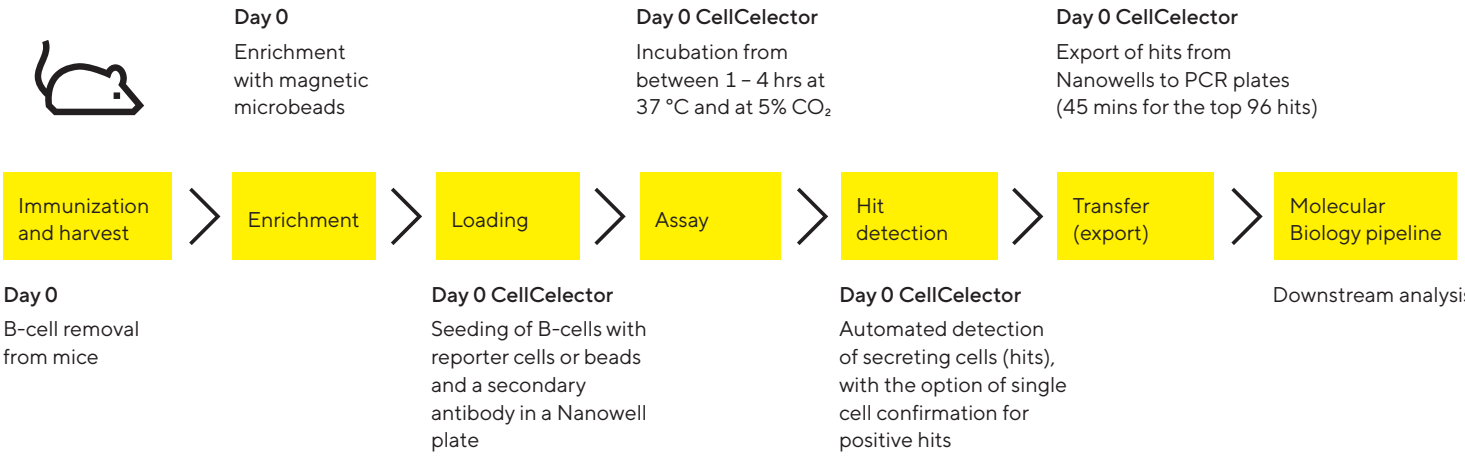


Figure 1: *Antibody Discovery workflow.*



Note: B-cells are harvested from mice spleen, lymph nodes or spinal fluid and enriched using magnetic microbeads. Nanowell plates are prepared with between 20,000 – 50,000 B-cells added per macrowell (more can be used if monoclonality is not important). Different bead types and sizes, or antigen-expressing reporter cells labelled with cell tracked dyes can be used. A dye-conjugated secondary antibody (15 µg/mL) should be added to each macrowell, with the subsequent assay being incubated for between 1 – 4 hours at 37 °C. Optimally secreting cells are then identified on the CellCelector and subsequently ranked before the top 96 hits are transferred into PCR tubes for downstream analysis.

CellCelector Advantages in B-Cell Screening

Nanowell Technology
Nanowell plates feature up to hundreds of thousands of Nanowells at the bottom of each macrowell. Despite local separation, cells are covered by the same medium allowing uninhibited cellular crosstalk (Fig. 2).

Further information on Nanowell Technology can be found within the "**CellCelector Flex Capillaries, Tips and Consumables Brochure**".

Single Cell Secretion Assays
Single cell secretion assays are performed on individual cells within in the Nanowell arrays and allow for the rapid identification of secreting cells of interest. The CellCelector B-cell workflow supports a number of cellular assays, including assays with plate capture coatings, bead-based assays and antigen-expressing reporter cell assays (Fig. 3).

Multiplex Screening
Six LEDs combined with an excitation filter generate light with the desired excitation wavelength at 10 different intensity levels. Secretion assays can be set-up using fluorescence excitation | emission measurements in the blue, green, red, far-red and near infra-red channels.

Figure 2: *Schematic view of cell growth in a normal well (A and B), compared to that of a Nanowell plate (C and D).*

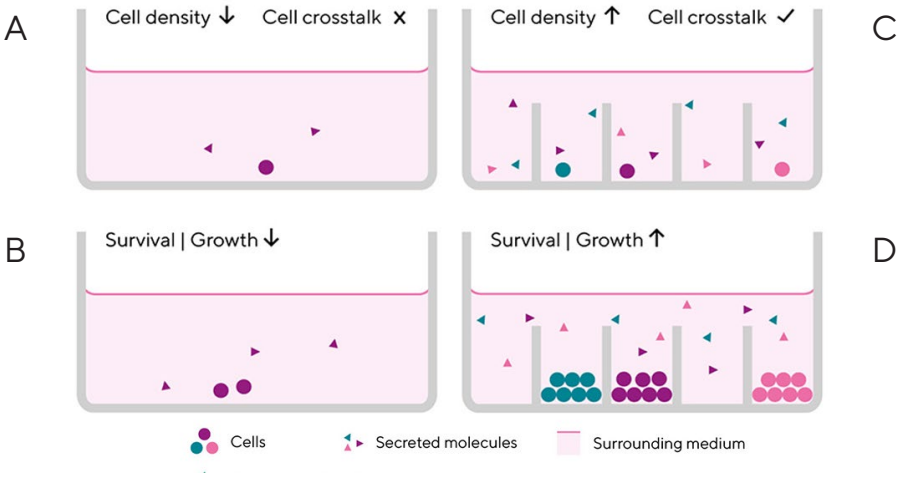
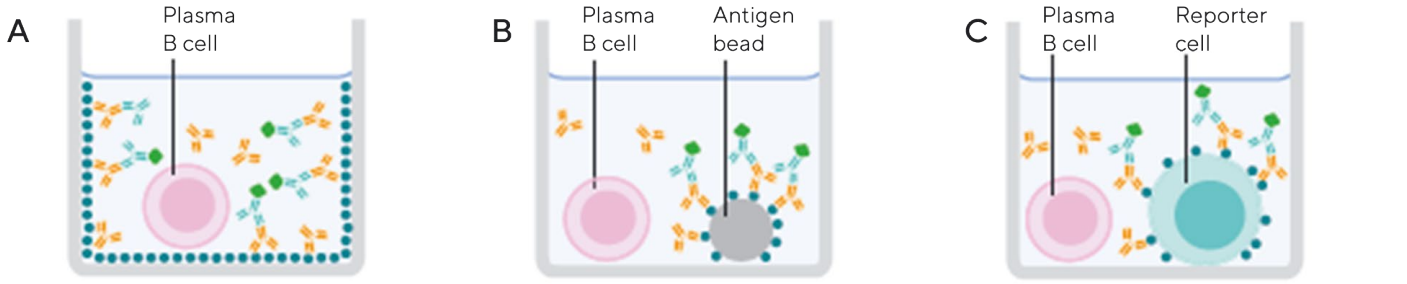


Figure 3: *Schematic of the three main cellular assays. (A) plate capture coating, (B) bead-based assay, and (C) antigen-expressing reporter cell assay.*



Assay with plate capture coating

Bead-based assay

Antigen-expressing reporter cell assay

Single B-Cell Bead and Reporter Cell Protocols

B-Cell Screening Nanowell Plates

The CellCelector 6-well H100-100 360K Nanowell plate contains 60,000 Nanowells per macrowell, featuring noncytotoxic low attachment properties to help prevent cell attachment, and can therefore be used for all type of cells independently of their adhesion properties or their optimization for suspension conditions. If necessary, the surface can be also blocked with BSA to improve cell repellent properties and avoid non-specific protein absorption.

Required Materials

- H100-100 360K Nanowell Plate (Product No. CC0080)
- CellCelector 50 µm glass capillary (Product No. CC0007)
- Antigen-coated beads (for antigen-bead assay only)
- Fluorescently labelled secondary antibody
- Centrifuge with microplate adapter
- Absolute ethanol
- BSA 2% (optional)

Single B-Cell Screening

Step 1: Nanowell Priming

1. Add 3 mL of absolute ethanol in each well by holding pipette tip against well wall, close to bottom
2. Immediately centrifuge the plate for 2 min at 700 x G to remove any residual air microbubbles formed in the Nanowells
3. Remove 1 mL of ethanol, leaving 2 mL
4. Add 3 mL of 1 x PBS and then remove 3 mL
5. Repeat washing with PBS 3 - 4 more times

Step 2: Nanowell Preparation

1. (Optional) Rinse the plate 3-5 times with 2% BSA, always leaving 2 mL in the well. Incubate (block) with the BSA for 1 hour at RT
2. Following the rinsing instructions above, wash with supplemented culture media 3 times. Leave 2 mL of media

Step 3: Cell Preparation and Seeding

1. Per well to be seeded, prepare 1.0 mL of cell suspension, following the instructions in Table 1
2. Per well to be seeded, add 1.0 mL of prepared cell suspension by dispensing dropwise in a 'Z' shape across the well
3. (Optional) Allow cells to settle for 5 - 10 minutes at RT
4. Centrifuge for 5 minutes at 300 x G
5. Observe seeded wells using the CellCelector or a standard cell culture microscope (optional)
6. Incubate the plate for 1 hr - 3 hrs at 37 °C and 5% CO_2 (the optimal incubation time and the temperature depends on the assay and cell origin)

Table 1: *B-cell assay parmeters for antigen coated bead and reporter cell-based assays.*

Harvested and Enriched Plasma B - cells | mL	
▪ 20,000 - 50,000 per well. More cells can be used if monoclonality is not important ▪ Fresh B-cells provide higher detection signal compared to frozen ones, which can also have a large percentage of dead cells, thus decreasing hit rate	
Antigen Coated Bead Assays:	**Reporter Cell-Based Assays:**
▪ Different types and sizes of bead can be used. Avoid using beads <1 µm. Adjust the quantity of the beads to cover rather than a large part of the Nanowell bottom to guarantee earlier detection ▪ For larger beads (≥5 µm) use 1.5M - 2M per well (at least 20 beads on average per Nanowell)	▪ Use 300,000 - 500,000 antigen - expressing reporter cells per well labelled with a cell tracking dye
Dye - Conjugated Secondary Antibody	
▪ Concentration should be 15 µg | mL before adding to the well ▪ As 1 mL of cell suspension with mixed secondary antibody is to be added to 2 mL of media already present in the well, the final concentration will be 5 µg | mL	

B-Cell Secretion Assay with Antigen Plate Coating Protocol

Step 1: Nanowell Priming

1. Place the Nanowell plate horizontally and open the lid
2. Dispense 3 mL of PBS in each well to be seeded
3. Centrifuge the plate for 5 min at room temperature (RT) at the highest speed allowed by the centrifuge and the rotor. After centrfiugation, the bottom of the well should look transparent and free of any pattern. Repeat centrifugation if this is not the case
4. Remove 1 mL, leaving 2 mL

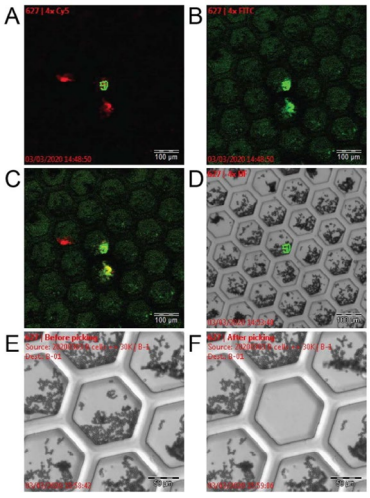
Alternative to Steps 2 - 4

- Pre-wet the Nanowells by dispensing 3 mL of absolute ethanol in each well to be seeded by holding pipette tip against the well wall, close to the bottom. Slowly disperse as ethanol spreads via capillary action until the entire surface is covered
- Ensure that the rotor is properly balanced prior to centrifugation, and centrifuge the plate for 2 min at 700 x G to remove any residual air microbubbles formed in the Nanowells
- Remove 1 mL of ethanol, leaving 2 mL
- Add 3 mL of 1 x PBS and then remove 3 mL
- Repeat washing with PBS 3 -4 times

Step 2: Cell Preparation and Seeding

1. Add 1.0 mL of antigen at 50 to 100 µg | mL and incubate overnight
2. Rinse the plate 3 - 5 times with 2% BSA, always leaving 2 mL in the well. Incubate (block) with BSA for 1 hour at RT
3. Wash with media 3 times, leaving 2 mL of media
4. Prepare 1.0 mL of single cell suspension per well to be seeded, mixing:
    a) No more than 50,000 secreting cells | mL in fresh media. More cells can be used if monoclonality is not important
    b) 5 µg | mL of dye - conjugated secondary antibody
5. Add 1.0 mL of prepared cell suspension by dispensing in a 'Z' shape across the well
6. (Optional) Allow cells to settle for 5 - 10 minutes at RT
7. Centrifuge for 5 minutes at 300 x G
8. Observe seeded wells using the CellCelector
9. Allow plate to incubate for 1 hr or 2 hrs at 37 °C (the optimal incubation time is cell type dependent)
10. Scan the plate on the CellCelector at 10 x for positive reactivity (fluorescent channel corresponding to the second antibody fluorophore) and pick cells from positive wells

Figure 4: *Single B-cell antigen coated bead assay example.*



Note: Two million CD138+ antibody secreting cells (ASCs) were obtained, and 60,000 cells loaded into one well of a six-macrowell Nanowell plate to achieve an average occupancy of one cell per Nanowell. EGFR was used as the model antigen. A mixture of anti-human IgG Fc specific capture beads, and anti-human IgG conjugated AF647, biotinylated EGFR and AF488 conjugated streptavidin incubated with the cells. A total of 52,000 Nanowells were scanned in brightfield and fluorescence to identify Nanowells containing B-cells that secrete antibodies (AF647+) and are positive for binding to EGFR (AF488+). Images are centered and a green outline auto-assigned to the well and B-cell to be picked. (A) Image of fluorescence specific 647 nm emission (RED); it is observed two neighboring wells contain ASCs. (B) Image of fluorescence specific 488 nm emission (GREEN); it is observed one neighboring well contains an ASC specific to EGFR. (C) Overlay of images from both 647 and 488 nm channels and (D) brightfield image used to pick an EGFR+ ASC. Brightfield images (E) before and (F) after an EGFR+ ASC is picked from Nanowell. Brightfield images demonstrate all contents within the Nanowell are picked.

Matacho et al. (2022). Antibody Therapeutics, Volume 5, Issue 1, January 2022, Pages 11–17

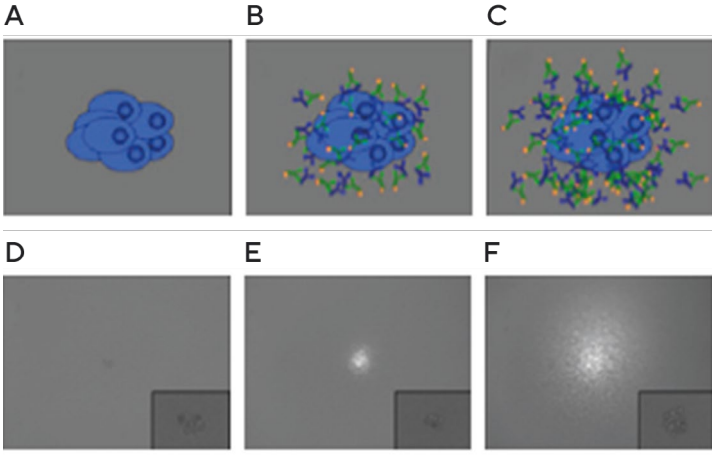
CellCelector Advantages in Hybridoma Screening

Hybridoma Secretion Assays
Secreted antibodies are made visible by fluorescence-labelled secondary antibodies, appearing as a halo around the producing hybridoma. CellCelector software compares the size of the cell colony with the diameter of the halo to calculate the level of antibody production, allowing the ranking of hybridomas based on antibody production across multiple plates (Fig. 5).

Example data of secreted protein from a stable CHO transfected cell line grown on semi-solid media can be found in figure 6.

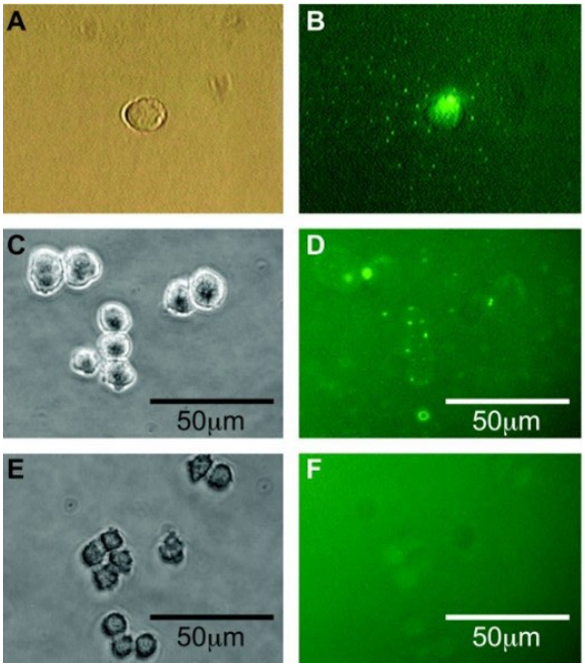
Beads and Reagents
Sartorius iQue Qbeadsare® a family of reagents that capture specific proteins on distinct bead types for the multiplexed quantitation of cytokines, adhesion molecules, enzymes, growth factors receptors. Qbeads® can be used to perform CellCelector single cell secretion assays with confidence.

Figure 5: *Hybridoma secretion assays.*



Note: A - C: chematic view; D - F; overview of brightfield and fluorescence illumination. Colony producing no antibodies (A and D), colony producing medium amounts of antibodies (B and E); colony producing high amounts of antibodies (C and F).

Figure 6: *Detection of secreted IGF-E5 from stable CHO transfectants by fluorescent labelling in semi-solid media.*



Note: A, C and E: transmitted light images. B, D and F: green fluorescence at 510 nm from anti-His Tag/FITC antibody present in semi-solid medium; fluorescent dots formed by reaction of anti-His Tag/FITC with secreted IGF-E5 bearing His Tag moiety. A and B: longer exposure from single cell showing a cloud of fluorescent dots. C and D: positive CHO-IGF-E5 colony: signal is weak, but sufficient for detection of high producing cells. E and F: negative control; CHO cells secreting an unrelated product do not react with anti-His Tag antibody.

Caron et al. (2009). Fluorescent labelling in semi-solid medium for selection of mammalian cells secreting high-levels of recombinant proteins.

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