APPLICATION NOTE

Rapid Selection of Antibodies Against Membrane Targets using Cell-Binding Assays and the Opto® Plasma B Discovery Workflow

APPLICATION SPOTLIGHT

- Hit generation and down-selection of lead antibody candidates against membrane targets using multiple assays in just 1 day
- Flexible assays that use transient or stably expressed cell lines and multiple cell types
- Confirmation of functional activity of lead candidates in plate-based assays

INTRODUCTION

Antibody therapeutic development is becoming faster and more streamlined as new tools have been developed. However, developing antibody drugs that target membrane proteins continues to challenge scientists due to the difficulties of screening against the native conformation of these proteins. Cell membrane proteins are difficult to extract and isolate, and recombinant membrane proteins tend to have low expression efficiency and can sometimes only be expressed as a truncated version of the full-length protein. In addition, resulting proteins are often misfolded, leading to selection of unviable drug candidates when these constructs are used as the target antigen for antibody screening. Cell-based assays that allow the full-length antigen to fold properly vastly improve the chances of selecting antibody lead candidates against these difficult targets. However, traditional cell-based assays extend development timelines due to the required cloning, re-expression and purification of antibodies for these assays as well as the difficulty of assay development and optimization.

Opto® Plasma B Discovery on the Beacon® optofluidic system accelerates the down-selection of lead candidates by enabling direct screening of plasma B cells using assays for both soluble antigens and cell membrane-bound antigens. Multiple assays can be run on the same plasma B cells, reducing lead candidate selection from 8–12 weeks to just 1 day (Figure 1). This early characterization translates to the identification of antibody therapeutic candidates more rapidly, reducing development timelines and downstream costs.

In this application note, we demonstrate rapid selection of antibodies that bind soluble antigen using bead binding assays followed by down-selection using membrane-bound antigen in cell-binding assays using transient or stable transfection methods. Antibody specificity was confirmed using plate-based assays after sequencing and re-expression, demonstrating that on-chip cell-based assays select antibodies that bind to the native conformation of cell membrane-bound antigens.

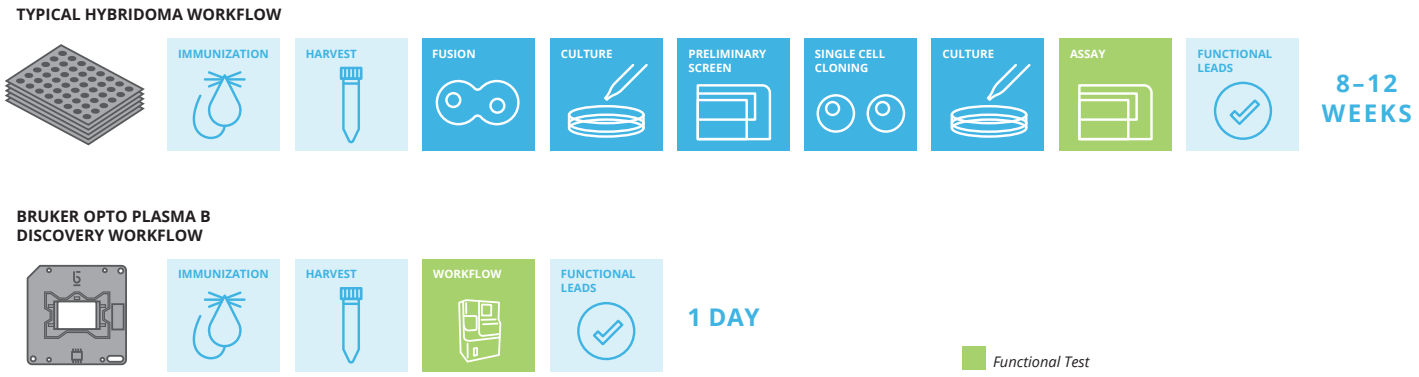


Figure 1. The Opto Plasma B Discovery workflow decreases screening and lead candidate selection from 8–12 weeks to 1 day. Antibodies can be functionally screened in a single day using both bead- and cell-based assays, enabling functional characterization during primary screening.

MATERIALS AND METHODS

Mouse immunization and plasma B cell purification

Mouse immunizations were carried out at the Josman Facility (Josman, LLC, Napa, CA). Balb/c mice received initial immunizations subcutaneously with Complete Freund's Adjuvant (CFA) followed by boost injections with Incomplete Freund's Adjuvant (IFA) every two weeks for eight weeks. Different cohorts of mice were immunized with recombinant antigens CD40 (ACROBiosystems) or CD137 (ACROBiosystems). The mice received final boosts without adjuvant 3–5 days before harvesting of the organs. Plasma B cells were then purified using a CD138+ plasma cell isolation kit (Miltenyi Biotec).

Recombinant assay reagent preparation

We conjugated recombinant antigens CD40 and CD137 to AF647 using Alexa Fluor 647 NHS Ester (Thermo Fisher Scientific) following the manufacturer's instructions.

Reporter cell line generation: Transient transfection

We generated reporter cells by transiently transfecting an expression vector with a gene of interest (CD40) into a HEK293T cell line (ATCC) or using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific). A commercial transfection protocol was adapted (Thermo Fisher Scientific) to increase transfection efficiency of the expression vector.

Reporter cell line generation: Stable transfection

We generated reporter cells by stably transfecting a K562 cell line with a CD137 expression vector. The CD137 expression vector contained a puromycin resistant selection marker that rendered successfully-transfected cells resistant to puromycin. After 3 days of culture we incubated the cells with a lethal concentration of puromycin for 3 weeks. We then analyzed surviving cells for CD137 expression using a BD FACSMelody and bulk sorted for high CD137 expressing cells.

Antibody screening assay: Bead-based assay

We first loaded single mouse plasma B cells into individual NanoPen® chambers on OptoSelect® 11k chips using Bruker's OEP® technology. We then loaded a multiplexed assay mixture of anti-mouse IgG coated beads (Spherotech), AF488-labeled secondary antibody (Jackson ImmunoResearch), and either AF647-labeled recombinant CD137 or CD40. Secreted antibodies diffused from the NanoPen chambers into the channel where they bound the beads and secondary antibodies, forming fluorescent halos ("blooms") in the channels adjacent to the pens containing antigen-specific plasma B cells. We imaged the OptoSelect chips using a FITC fluorescent filter cube to detect IgG secretion and a Cy5 filter cube to detect antigen specificity. Blooms

observed only in the FITC channel indicated secretion of IgG antibodies that were not specific to the target antigen (CD137 or CD40). Blooms detected in both the FITC and CY5 channels indicate positive IgG secretion and antigen specificity.

Antibody screening assay: Cell-binding assay

After performing the bead-based assays, we flushed the bead assay mixture out of the chip and loaded a cell-binding assay mixture consisting of AF488-labeled secondary and CD137-expressing K562 cells or CD40-expressing HEK293T cells. We detected secreted antibodies capable of binding the membrane-bound antigens presented on the transfected reporter cells as blooms in the FITC channel. Cell-based assays were scored by human verification.

Sequence recovery and functional confirmation

We recovered sequences for antibodies specific to both soluble (bead-based assay) and membrane-bound (cell-binding assay) antigens using either single cell or OptoSeq® BCR exports into 96-well PCR plates. We amplified and recovered antibody heavy and light chain sequences using components of the Opto® Plasma B Discovery cDNA Synthesis Kit (Bruker) and the Opto® Plasma B Discovery Sanger Prep Kit, Mouse (Bruker). We then cloned recovered sequences into expression constructs, and screened the re-expressed antibody supernatants for soluble antigen specificity using plate-based ELISA measurements.

We used flow cytometry to confirm off-chip cell-binding of re-expressed antibodies to the same cell lines that were used for the on-chip cell-binding assays. We plated CD137-transfected K562 or parental K562 cells in a 96-well plate and stained with a viability stain. After viability staining, we incubated the cells with re-expressed antibody supernatants collected from transfected HEK293T cells for 30 minutes at 4°C. After the incubation, we washed, resuspended, and incubated them in Alexa Fluor 488 anti-mouse IgG secondary antibody (Jackson ImmunoResearch) for 30 minutes at 4°C. We then washed and resuspended the cells in FACS buffer and analyzed them for cell associated fluorescence using a BD FACSCelesta. We used a similar approach to confirm cell-binding of re-expressed anti-CD40 antibodies using HEK cells transiently expressing CD40.

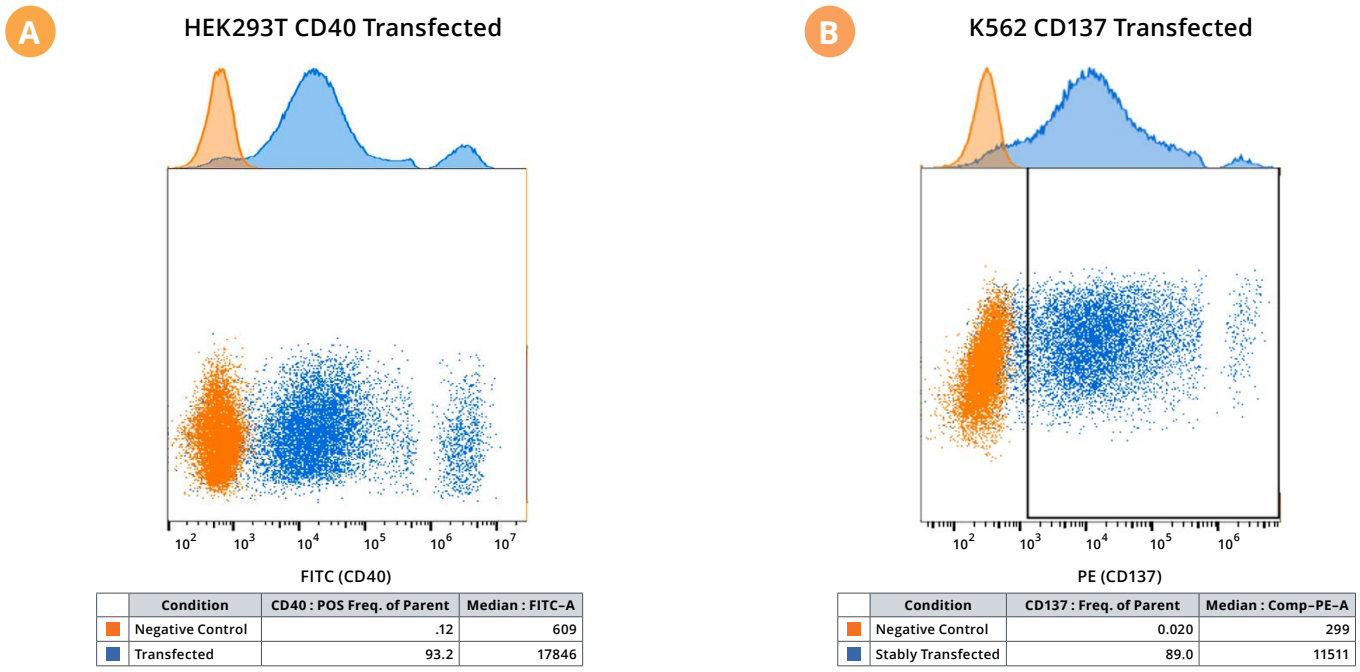
RESULTS AND DISCUSSION

Generation of stable and transient cell lines expressing membrane-bound antigens

We expressed two model membrane antigens, CD40 and CD137, in transient HEK293T and stable K562 cell lines, respectively. We confirmed that the transiently transfected HEK293T cells were efficiently expressing CD40 protein by staining the cells with FITC-anti-human CD40 antibody for analysis with flow cytometry. 93.2% of the transfected HEK293T cells had surface-expressed CD40 with a median fluorescent intensity (MFI) nearly 2 orders of magnitude greater than the parental negative control HEK293T cells (Figure 2A). The same method was used to confirm that K562 cells were stably expressing CD137 after initial transfection. 89.0% of cells stably expressed CD137 with an MFI nearly 2 orders of magnitude greater than the parental negative control (Figure 2B).

Selection of antibodies against membrane-bound antigens

We loaded single plasma B cells from mice immunized with either recombinant CD40 or CD137 into OptoSelect 11k chips and performed a soluble antigen bead-binding assay to identify antibodies specific to soluble CD40 (Figure 3A, middle) or CD137 (Figure 3A, right). The assay mixture was then flushed from the chip. Next, either transiently transfected HEK293T cells expressing CD40 or stably transfected K562 cells expressing CD137 were loaded onto the chip to perform a cell-binding assay using membrane-bound antigen (Figure 3B). Using these model systems, we demonstrated that the Opto Plasma B Discovery workflow can screen against diverse membrane antigens using both transient and stable transfection methods in multiple cell types.



	Condition	CD40 : POS Freq. of Parent	Median : FITC-A
	Negative Control	.12	609
	Transfected	93.2	17846

	Condition	CD137 : Freq. of Parent	Median : Comp-PE-A
	Negative Control	0.020	299
	Stably Transfected	89.0	11511

Figure 2. Confirmed expression of CD40 and CD137 membrane-bound antigens in transiently transfected HEK293T and stably transfected K562 cells. A. Histogram of CD40 transiently transfected HEK293T (blue) and parental HEK293T cells (orange) stained with FITC anti-human CD40 antibodies confirms high transfection efficiency (>93%). **B.** Histogram of CD137 stably transfected K562 (blue) and parental K562 cells (orange) stained with PE anti-human CD137 antibodies confirms high transfection efficiency (89%).

Down-selection of antibody lead candidates using cell-based assays

By performing cell-binding assays after bead-binding assays, we were able to drastically down-select lead candidates in a single day. Of the 8,853 single plasma B cells loaded from CD40-immunized mice, 616 (7.0%) were positive for IgG secretion, 206 (2.3%) were positive for recombinant CD40 binding, and 172 (2.0%) were positive in the HEK293T reporter cell-binding assay (Figure 4, blue bars). Of the 13,753 single plasma B cells loaded from CD137-immunized mice, 139 (1.0%) were positive for IgG secretion, 17 (0.1%) were positive for recombinant CD40 binding, and 4 (0.03%) were positive in the K562 reporter cell-binding assay (Figure 4, orange bars). Hit rates for the CD137 model system were lower due to a freeze/thaw cycle performed on the plasma B cells prior to screening.

The rapid down-selection of antibodies using both soluble and cell-binding antigens accelerates lead candidate selection by removing the need to recover, sequence, clone, re-express, purify and characterize antibodies that only bind the soluble form and not the native conformation of cell membrane antigens.

Confirmation of soluble and cell-based antigen binding by ELISA and flow cytometry

Sequences from 16 anti-CD137 antibodies and 50 anti-CD40 antibodies were recovered using either cell or OptoSeq® BCR exports, followed by antibody heavy/light chain sequencing, re-expression, and further characterization. Single paired heavy and light chain BCR sequences were directly cloned and transfected into HEK293T cells for re-expression. After three days of culture, supernatant from the HEK cells was isolated and binding to soluble antigens was confirmed using an ELISA assay. 15 anti-CD137 antibodies (94%) and 41 anti-CD40 antibodies (82%) demonstrated confirmed antigen binding by ELISA (data not shown). Binding of antibodies to membrane-bound targets was assessed by

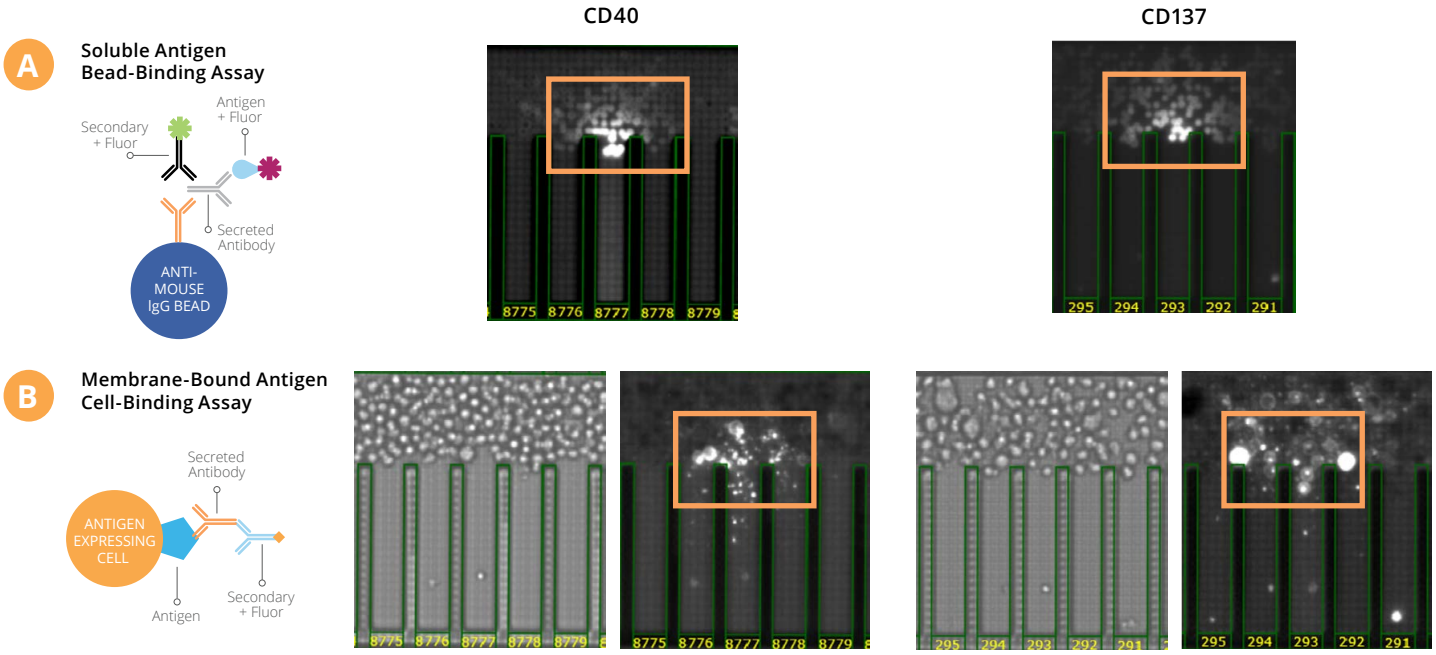


Figure 3. The Opto Plasma B Discovery workflow enables screening of plasma B cells with both bead-based and cell-based assays in just 1 day. A. The soluble antigen bead-binding assay performed in-channel identifies plasma B cells secreting antibodies that bind recombinantly-expressed soluble antigen. **B.** The cell-binding assay, performed in-channel after the bead-binding assay, down-selects plasma B cells secreting antibodies that bind cell membrane-bound antigens, such as CD40 (middle) or CD137 (right) antigens expressed in transiently (HEK293T) or stably transfected (K562) target cell lines, respectively.

flow cytometry (representative data found in Figure 5). Of the re-expressed antibodies that scored positive for cell-binding in the on-chip cell-binding assays, 100% of anti-CD137 (4 of 4) and 62% of anti-CD40 antibodies (23 of 37) demonstrated binding to the membrane-bound antigens in off-chip flow cytometry measurements. None of the re-expressed antibodies that scored negative in the on-chip cell-binding assay bound to the membrane-bound antigens as measured by flow cytometry (data not shown).

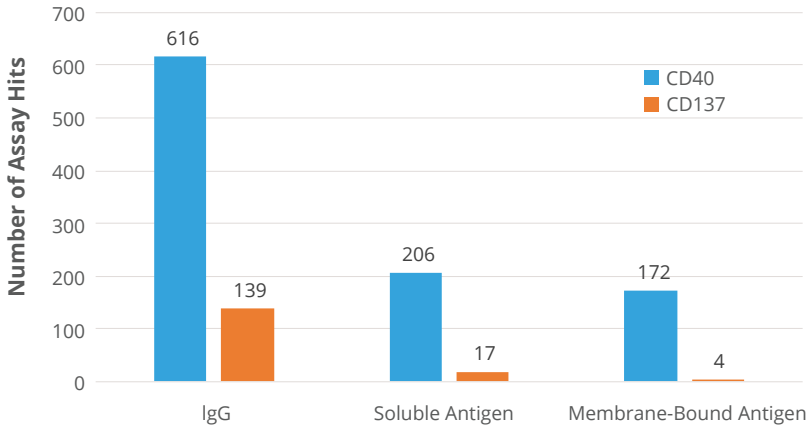


Figure 4. The Opto Plasma B Discovery workflow enables down-selection of lead candidates by screening against both soluble and membrane-bound antigens. Plasma B cells from mice immunized with either CD40 or CD137 were screened using bead-based in-channel assays to identify antibodies that bound the soluble form of these antigens. Antibodies that bound the native, membrane-bound conformation of cell membrane antigens were selected by screening with either HEK293T cells transiently expressing CD40 or K562 cells stably expressing CD137.

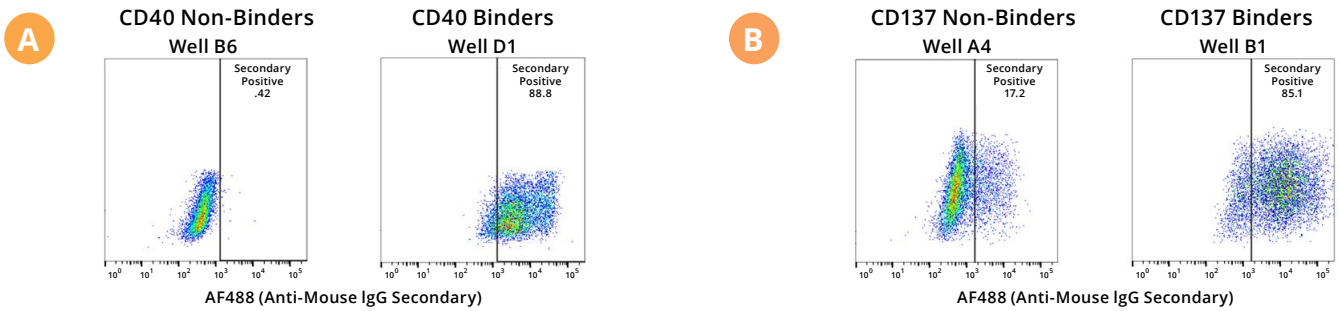


Figure 5. Confirmation of cell-surface binding of anti-CD40 and CD-137 antibodies. A. HEK293T cells transiently expressing CD40 and **B.** K562 cells stably expressing CD137 were incubated with supernatants collected from HEK293T cells transfected to re-express antibodies selected from Opto Plasma B Discovery workflows. After the incubation, the cells were stained with a fluorescent secondary antibody and analyzed using flow cytometry. Antibodies that bound the cell-membrane antigens demonstrated fluorescence 1–2 orders of magnitude above the non-binding antibodies. Non-specific binding to the secondary antibody to the parental K562 cell line resulted in fluorescence from a small fraction of cells (~17%) incubated with CD137 non-binding antibodies with fluorescence greater than the threshold. This figure shows representative data for both antibodies that bind and don't bind the membrane-bound antigens.

CONCLUSION

Antibody therapeutics against complex membrane proteins hold great promise due to the many different physiological processes they regulate. However, a fast and simple method to screen and identify candidates has remained elusive due to the challenges related to screening antibodies against full-length, native cell membrane proteins.

The Opto Plasma B Discovery workflow overcomes this hurdle by enabling cell-binding assays to be run in conjunction with bead-based binding assays to downselect antibodies against cell membrane antigens in just 1 day, compared to 8–12 weeks using traditional methods. The early down-selection of antibodies that bind cell membrane antigens can reduce the time and resources traditionally wasted characterizing non-functional hits in well plates.

Here we have shown that the Opto Plasma B Discovery workflow can screen plasma B cells with both soluble protein based bead-binding assays and membrane-bound protein cell-binding assays. We performed cell-binding assays using different transfection methods (stable and transient), cell types (HEK293T and K562), and antigens (CD40 and CD137), demonstrating the flexibility of the assays that can be performed using this workflow. We confirmed that antibodies that bound soluble and membrane-bound antigens in on-chip assays demonstrated equivalent specificity when re-expressed and measured using off-chip ELISA and flow cytometry assays. This demonstrates that the Opto Plasma B Discovery workflow simplifies and dramatically reduces the time needed to down-select potential lead candidates, even for difficult membrane proteins.

FOR MORE INFORMATION, VISIT

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