APPLICATION NOTE

Discover Lead Molecules Against Difficult Membrane Targets Inaccessible by Hybridoma Methods

APPLICATION SPOTLIGHT

- Discover up to 10-fold more hits against soluble and cell membrane antigens in days not weeks
- Generate diverse lead panels by sampling >50-fold greater sequence diversity
- Increase campaign yield and efficiency to reduce costs by 20-fold

INTRODUCTION

Acceleration of lead molecule discovery is a significant opportunity for improving drug development, which is currently plagued by clinical trials that are costly, time-consuming and subject to high failure rates. Lead molecule discovery necessitates the generation of large, diverse hit panels and rapid methods to down-select lead molecules from these panels. This is especially true for difficult membrane targets for which lead molecules often need to bind cryptic epitopes or trigger complex molecular functions and are, as a result, rare.

Antibody discovery using the traditional hybridoma approach requires the immortalization of B cells by cell fusion, followed by a lengthy process of expansion, subcloning, and screening. This approach has been successful for finding monoclonal antibodies against simple targets but is limited when employed against difficult targets because the immortalization step often results in the death of >90% of B cells, and therefore leads to significant loss of B cell diversity.

The Opto™ Plasma B Discovery workflow on the Beacon® optofluidic system maximizes diversity of antibodies through direct functional profiling of plasma B cells and accelerates the down-selection of lead candidates through rapid, high-throughput sequencing and re-expression of all discovered antibodies. Using this workflow, functional lead molecules can be delivered in just 1 week (**Figure 1A** and **B**).

In this application note, we demonstrate how the Opto Plasma B Discovery workflow increases hit recovery for a difficult membrane-bound target and provides access to more than 50-fold greater sequence diversity than a hybridoma workflow. Lead molecules were selected using on-chip assays to identify antibodies that bound to both soluble and cell membrane forms of a target antigen (**Figure 1C**).

MATERIALS AND METHODS

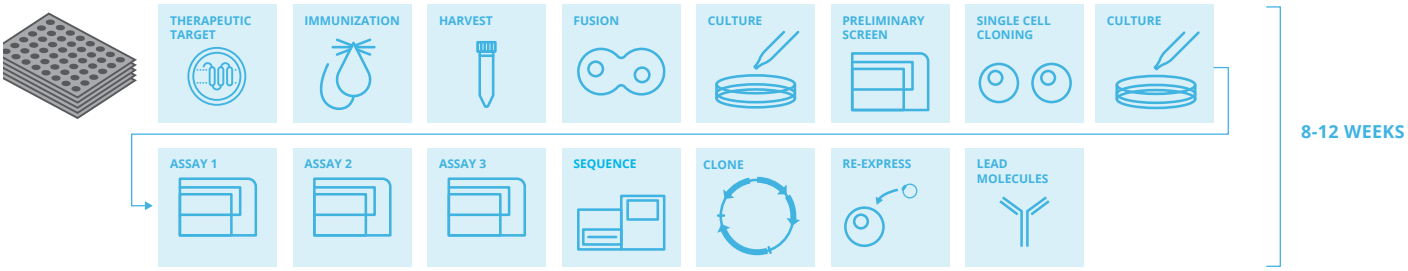
Mouse immunization and Plasma B cell purification

16 BALB/c mice were immunized with recombinant TIM-3 (Acro Biosystems) by a third-party contract provider using a rapid immunization protocol. Sera titers were measured for all mice and the top 12 mice were split into 2 cohorts of 6 mice each for screening using the hybridoma and Opto Plasma B Discovery workflows, respectively. The

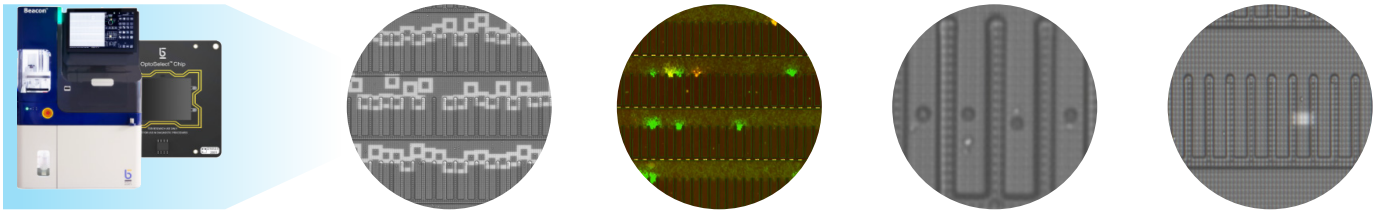
A

BERKELEY LIGHTS OPTO PLASMA B DISCOVERY WORKFLOW

TYPICAL HYBRIDOMA METHOD



B



BEACON SYSTEM AND OPTOSELECT CHIP

STEP 1: SINGLE-CELL CLONING

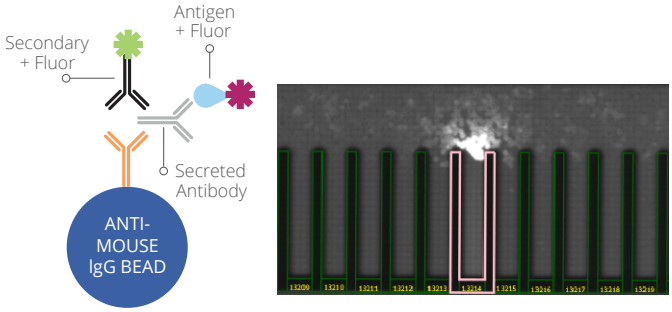
STEP 2: FUNCTIONAL PROFILING

STEP 3: cDNA SYNTHESIS

STEP 4: SEQUENCE RECOVERY

C

SOLUBLE ANTIGEN BEAD-BINDING ASSAY



MEMBRANE-BOUND ANTIGEN CELL-BINDING ASSAY

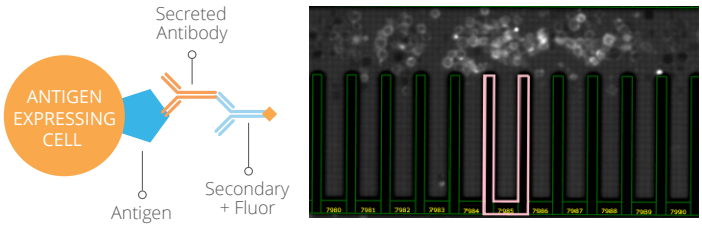


Figure 1. The Opto Plasma B Discovery workflow enables rapid functional profiling of plasma B cells, sequencing, and re-expression in just 1 week. A. The Opto Plasma B Discovery workflow drastically shortens timelines and the number of manual steps required compared to a typical hybridoma workflow. **B.** The Opto Plasma B Discovery workflow integrates single cell cloning, assays, cDNA synthesis and sequence recovery into a single, automated workflow. **C.** Bead- and cell-binding assays can be used to discover antibodies specific to the soluble and membrane-bound forms of a target antigen.

LEARN MORE AT

berkeleylights.com/antibody-discovery/

mice received final boosts without adjuvant either 3 or 5 days before harvesting of the organs for the hybridoma and Opto Plasma B Discovery workflows, respectively. For the Opto Plasma B Discovery workflows, plasma B cells were purified using mouse CD138 microbeads (Miltenyi Biotec).

Soluble antigen assay reagent preparation
We conjugated recombinant TIM-3 antigen to AF647 using Alexa Fluor 647 NHS Ester (Thermo Fisher Scientific) following the manufacturer's instructions.

Reporter Cell Line
RPMI 8226 (ATCC) is a human B lymphocyte-derived cell line with high endogenous TIM-3 expression levels and was used as a reporter cell line. Cells were stained with a PE mouse anti-human TIM-3 antibody (Biolegend) to characterize surface expression via FACS (data not shown).

Antibody screening assay: Bead-based assay
We first loaded single mouse plasma B cells into individual NanoPen™ chambers on OptoSelect™ 11k chips using Berkeley Lights' OEP™ technology. We then loaded a multiplexed assay mixture of anti-mouse IgG coated beads (Spherotech), secondary anti-mouse IgG-AF488 (Jackson ImmunoResearch), and AF647-labeled recombinant TIM-3. 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 FITC and Cy5 filter cubes to detect antibody secretion and specificity to the soluble antigen, respectively.

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 secondary anti-mouse IgG-AF647 (Jackson ImmunoResearch) and TIM-3-expressing RPMI 8226 cells. Secreted antibodies capable of binding the membrane-bound antigen presented on the reporter cells were identified by fluorescent blooms in the Cy5 channel. Cell-based assays were scored by human verification.

Sequence recovery and functional confirmation
We recovered sequences for antibodies specific to both soluble and membrane-bound antigens using OptoSeq™ BCR exports into 96-well PCR plates. We amplified antibody heavy and light chain genes and performed NGS sequencing on the Illumina MiSeq platform. We then used the Opto™ BCR Rapid Re-expression kit to rapidly clone recovered sequences into expression constructs and screen the re-expressed antibody supernatants for confirmation of 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 RPMI 8226 cells in a 96-well plate and stained with a viability stain (Zombie NIR, BioLegend). 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 cells with 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.

Hybridoma Generation
The spleen and lymph nodes from 6 BALB/c mice immunized with TIM-3 were fused to a mouse myeloma cell line. After HAT selection and library expansion, the number of viable hybridoma cells in the library population was counted to estimate the number of successful fusion events. Single cell hybridomas were then sorted by SSC/FSC and viability on a BD FACS Aria3 using Propidium Iodide to gate out dead cells. Hybridoma supernatants were used to perform ELISA measurements against the soluble TIM-3 antigen and for FACS measurements using the TIM-3 expressing RPMI 8226 cell line.

RESULTS AND DISCUSSION

More hits in less time

Membrane proteins are considered difficult targets due to small extracellular regions, high sequence homology, and poor cell surface expression when recombinantly expressed. For this experiment, we immunized mice with a representative challenging target, human T-cell immunoglobulin and mucin domain 3 (TIM-3), and harvested B cells for parallel discovery efforts using traditional hybridoma and Opto Plasma B Discovery workflows. For each workflow we screened for antigen specificity using both a soluble form of TIM-3 (soluble antigen) and a TIM-3 expressing cell line (cell membrane antigen).

For the hybridoma workflow, fusions from 6 mice were pooled and single-cell sorted into sixty 384-well plates. After culturing the plates for 3 weeks, the supernatants from each well were analyzed by ELISA and flow cytometry to identify antibodies specific to both soluble and cell membranes forms of TIM-3.

For the Opto Plasma B Discovery workflows, we screened more than 100,000 plasma B cells from the spleen and lymph nodes of 5 mice. Antibodies specific to both soluble and cell membrane TIM-3 were identified using a combination of bead-based and cell-based assays performed in less than 4 hours. As a result, the Opto Plasma B Discovery workflow yielded a hit panel several weeks earlier than the hybridoma workflow.

The Opto Plasma B Discovery workflow discovered nearly 8 times as many hits as the hybridoma workflow against the soluble TIM-3 antigen (677 vs. 88), and 9 times as many antibodies specific to cell membrane TIM-3 (505 vs. 57, Figure 2). The Opto Plasma B Discovery workflow yielded 327 antibodies that cross-reacted with both soluble and cell membrane forms of TIM-3 whereas hybridoma workflows yielded just 57 cross-reactive antibodies. This ability to discover more hits increases the probability of identifying successful lead molecules.

Access to greater sequence diversity

The diversity of a hit panel can be significantly compromised by the discovery of multiple antibodies with identical sequences. To assess the true diversity of antibodies discovered by the Opto Plasma B Discovery and hybridoma workflows, we performed next-generation sequencing on the antibodies discovered by both workflows.

For the Opto Plasma B Discovery workflow, plasma B cells were lysed and mRNA was captured on beads for on-chip cDNA synthesis, enabling efficient recovery of paired heavy/light chain sequences from 553 of 693 (79.8%) recovered beads (Figure 3A). This panel of antibodies contained >50-fold greater sequence diversity than the hybridoma method. 50.5% (279/553) of recovered heavy chain CDR3 sequences from the Opto Plasma B Discovery workflows were unique, as compared with only 11.9% (8/67) of the antibodies sequenced from the hybridoma workflow. The panel of antibodies discovered by the Opto Plasma B Discovery workflow spanned a wide range of heavy chain V gene families, including the 8 V-gene families from which antibodies were discovered via the hybridoma workflow (Figure 3B). Therefore, the

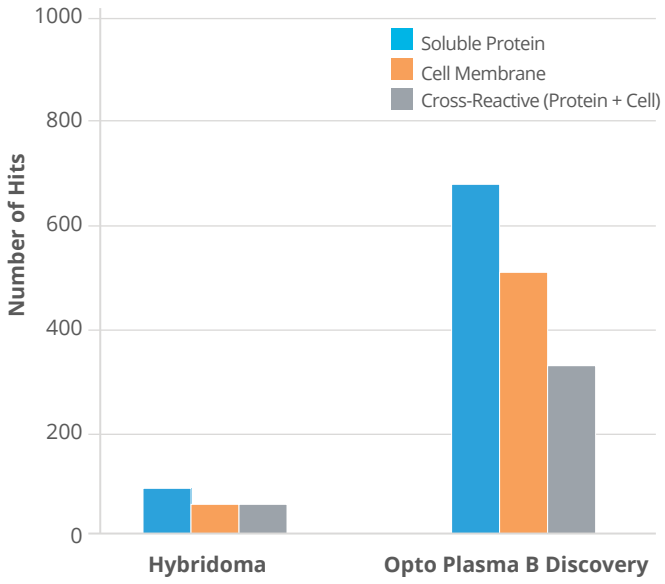


Figure 2. The Opto Plasma B Discovery workflow delivers more hits against both soluble and membrane forms of the TIM-3 antigen as compared with a traditional hybridoma workflow. The Opto Plasma B Discovery workflow discovered nearly 8 times as many hits as the hybridoma workflow against the soluble TIM-3 antigen, 9 times as many antibodies specific to cell membrane TIM-3, and nearly 6 times as many antibodies that cross-reacted with both soluble and cell membrane forms of TIM-3.

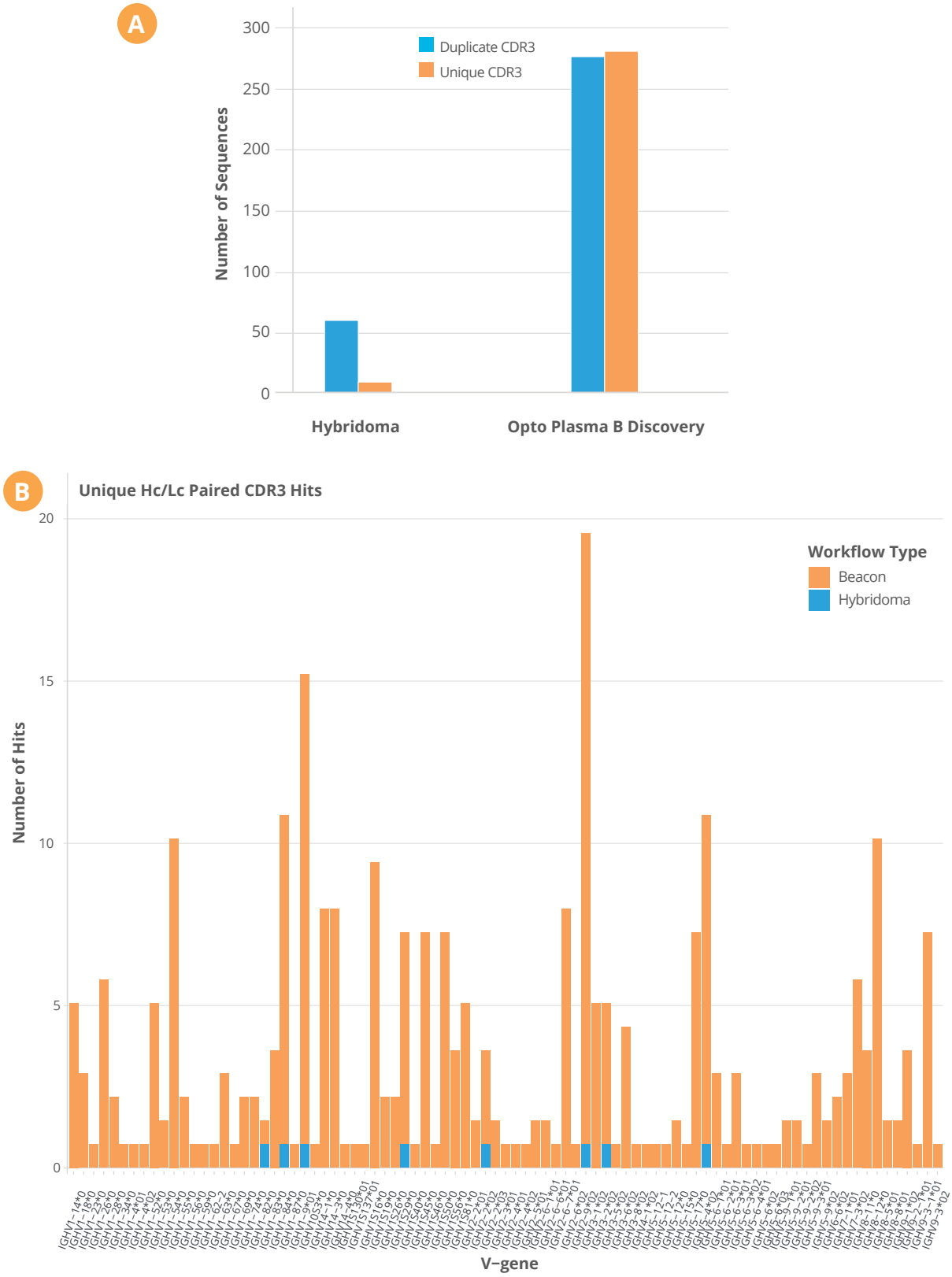


Figure 3. The Opto Plasma B Discovery workflow yielded a larger, more sequence-diverse panel of antibodies than the hybridoma workflow. **A.** Only 8 out of 67 antibodies (11.9%) discovered by the hybridoma workflow contained unique heavy chain CDR3 sequences as compared with 279 of 553 antibodies (50.5%) discovered by the Opto Plasma B Discovery workflow. **B.** The Opto Plasma B Discovery workflow discovered antibodies spanning a wide range of V gene families including all families from which antibodies were discovered via the hybridoma workflow.

Opto Plasma B Discovery workflow yielded both a larger and more sequence-diverse panel of TIM-3-specific antibodies as compared with the hybridoma workflow.

Increased efficiency reduces campaign costs

We rapidly re-expressed antibodies using the Opto BCR Rapid Re-expression kit, which enables amplified cDNA to be directly cloned into expression constructs without laborious bacterial cloning or costly gene synthesis. We confirmed that 106 out of 150 re-expressed antibodies (70.7%) exhibited specificity against both soluble and membrane forms of the TIM-3 antigen as measured by ELISA and FACS, respectively. Thus, the Opto Plasma B Discovery workflow yielded >10-fold more functionally-confirmed, unique lead molecules than the hybridoma workflow (106 vs. 8, Figure 4A). This increased workflow efficiency resulted in a >20-fold reduction in the cost per unique lead molecule (Figure 4B).

CONCLUSION

Discovery of antibodies against difficult targets requires a "more shots on goal" approach in order to yield lead molecules with a desired functional profile. However, traditional hybridoma methods are slow and often do not yield the necessary volume of diversity that is required when tackling difficult targets.

Here we have demonstrated that the Opto Plasma B Discovery workflow can be used to rapidly generate larger, more diverse hit panels than a traditional hybridoma approach against a model difficult target. Antibodies discovered by the Opto Plasma B Discovery workflow exhibited substantial sequence diversity and contained multiple candidates in all V gene families containing antibodies discovered from the hybridoma workflow. Finally, the Opto Plasma B Discovery workflow yielded >10-fold more functionally-confirmed, unique lead molecules than the hybridoma method. This allows the Opto Plasma B Discovery workflow to provide the speed, diversity, and efficiency required to rapidly advance lead molecules against difficult targets to the clinic.

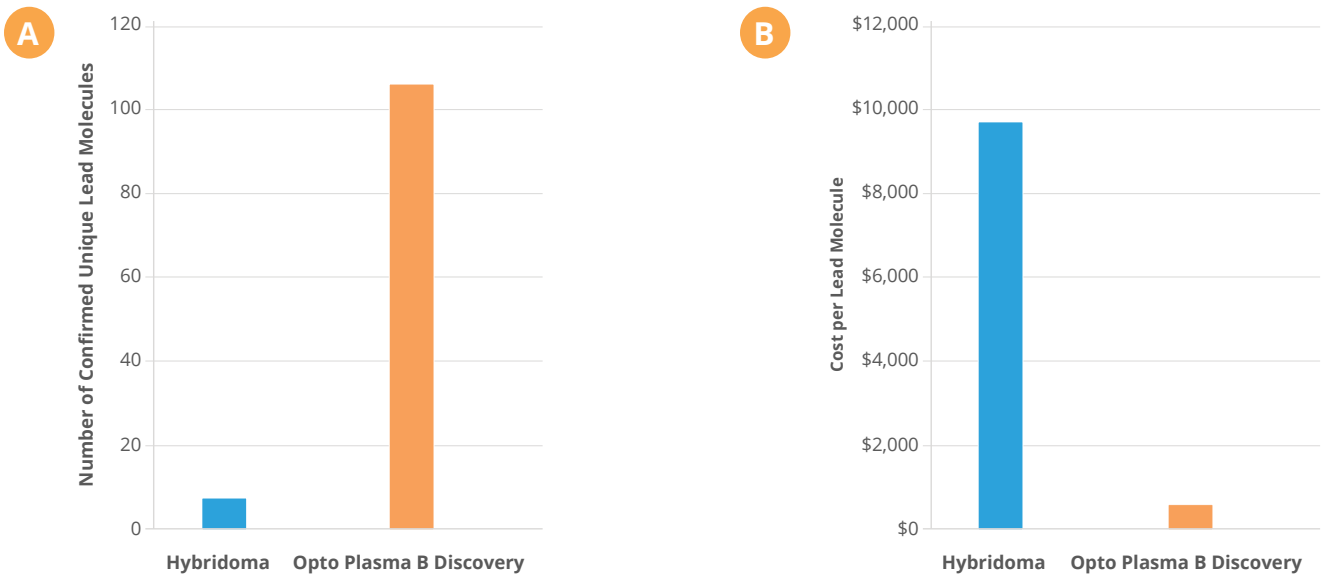


Figure 4. The Opto Plasma B Discovery workflow yielded more functionally-confirmed, unique lead molecules than the hybridoma workflow, resulting in a large reduction in the overall cost per molecule. A. The Opto Plasma B Discovery workflow yielded 106 unique lead molecules versus the 8 molecules yielded by the hybridoma workflow. **B.** The Opto Plasma B Discovery workflow resulted in a >20-fold reduction in the cost per unique lead molecule.

FOR MORE INFORMATION, VISIT

berkeleylights.com/antibody-discovery/

BERKELEY LIGHTS®

5858 Horton Street | Suite 320 | Emeryville, CA 94608

info@berkeleylights.com
+1-510-858-2855
berkeleylights.com

0150 Rev A