APP NOTE
REV D | SEPTEMBER 2023

Discover Lead Molecules Against Difficult Targets in 1 Week

with Opto® Plasma B Discovery 4.0

APPLICATION SPOTLIGHT

- Access broad plasma cell diversity by screening up to 100,000 plasma cells
- Rapidly down-select lead candidates against difficult targets, by incorporating functional profiling into primary screens
- Sequence and re-express >1,000 functionally characterised antibodies for less than $100/molecule, in 1 week

INTRODUCTION

The number of antibody therapeutics approved for use in the United States and Europe has increased steadily since 1997, with 61 approvals between January 2014 and November 2020[1]. These therapies have been developed for a host of different diseases, ranging from autoimmune disorders to infectious diseases and cancer. Therapeutic antibodies act through multiple mechanisms, including blocking targeted molecular function, inducing apoptosis, or modulating signaling pathways. As the demand for antibody therapies grows, so does the need for technologies that can accelerate the discovery of functional therapeutic antibodies, also known as lead molecules. However, discovering novel lead molecules requires methods to screen and rapidly down-select a diverse panel of antibodies.

Antibody-producing B lymphocytes (plasma cells) are leveraged to identify lead molecules, because each plasma cell secretes a unique functional antibody. The diverse range of secreted antibodies in the plasma cell population is referred to as the plasma cell repertoire. Antibody discovery using traditional hybridoma methods requires the immortalisation of plasma cells by cell fusion, followed by a lengthy process of expansion and subcloning. This approach has been successful for finding lead molecules against simple targets but is limited when employed against difficult targets because the immortalisation step often results in the death of >90% of plasma cells, and therefore leads to significant loss of the plasma cell diversity that is necessary for identifying rare, functional antibodies such as those against membrane targets. Other commercialized technologies, such as plasma cell cloning using flow cytometry or droplet encapsulation, may access more plasma cell diversity because they enrich the plasma cell population, however, none of these approaches provide insights into antibody function. As a result, nonfunctional or irrelevant hits are moved down the drug development pipeline to costly sequencing, gene synthesis, and bacterial cloning steps before functional confirmation using well plate-based assays.

Membrane targets, such as GPCRs[2] and ion channels[3], are among the most widely exploited targets for therapeutics[4,5]. However, there has been limited success running functional screens against membrane targets due to their cryptic epitopes. Screening against recombinant antigens often does not produce viable drug candidates because the lead molecules lack the desired specificity or functional potency in *in vivo* models. For this class of targets cell-based assays are needed to allow screening against native antigens.

Finally, to increase the diversity of hit panels and shorten development timelines, methods that enable rapid sequencing and validation of thousands of antibodies in a single run are also needed. However, accurate sequencing of thousands of full-length antibody genes is prohibitively expensive and slow by conventional Sanger methods. Additionally, re-expressing antibodies using cDNA for validation requires expensive gene synthesis and time consuming bacterial cloning steps.

The Opto Plasma B Discovery 4.0 workflow (**FIGURE 1**) on the Beacon® optofluidic system addresses all these challenges. First, animals are immunized (**FIGURE 1A**) and plasma cell are harvested, enriched, and prepared for cloning using the Opto Plasma B Discovery Sample Prep kit (**FIGURE 1B**). Next, plasma cells are automatically cloned into NanoPen® chambers on a microfluidic OptoSelect® chip (**FIGURE 1C**) without the need for immortalisation. The small volume of NanoPen chambers enables secreted antibody concentrations to reach detectable levels in minutes. Hit panel generation and lead molecule down-selection are performed in a single workflow in just one day using multiple assays to screen for antigen specificity and function (**FIGURE 1D**). This drastically accelerates selection of lead molecules against difficult targets and eliminates the delays and expense incurred when irrelevant, non-functional hits are sequenced or cloned. Lastly, cell-based assays allow discovery of antibodies against even the most challenging membrane targets.

Once lead candidates have been functionally characterized, antibody sequences of lead molecules can be directly linked to their functional profiles to elucidate how genotypes contribute to phenotypic characteristics. Barcoded beads enable on-chip cDNA synthesis using the OptoSeq® Barcoded BCR kit (**FIGURE 1E**) and accurate sequencing of full-length, paired heavy/light chains (Hc/Lc) from >1,000 antibodies in a single run, on standard Illumina NGS sequencing platforms using the OptoSeq Barcoded BCR kit and Nextera XT DNA Library Preparation kit (**FIGURE 1G, I,** and **J**). PrimeSeq™ BCR software then allows these sequences to be linked to phenotypes characterized on-chip. Finally, the OptoSeq BCR Rapid Re-expression kit further reduces timelines by converting antibody sequences into lead molecules without gene synthesis and cloning (**FIGURE 1H**).

In this application note, we will demonstrate how the Opto Plasma B Discovery 4.0 workflow can be used to access broad plasma cell diversity, rapidly down-select lead candidates with functional profiling, and sequence and re-express >1,000 functionally characterized antibodies for less than $100/molecule, in 1 week.

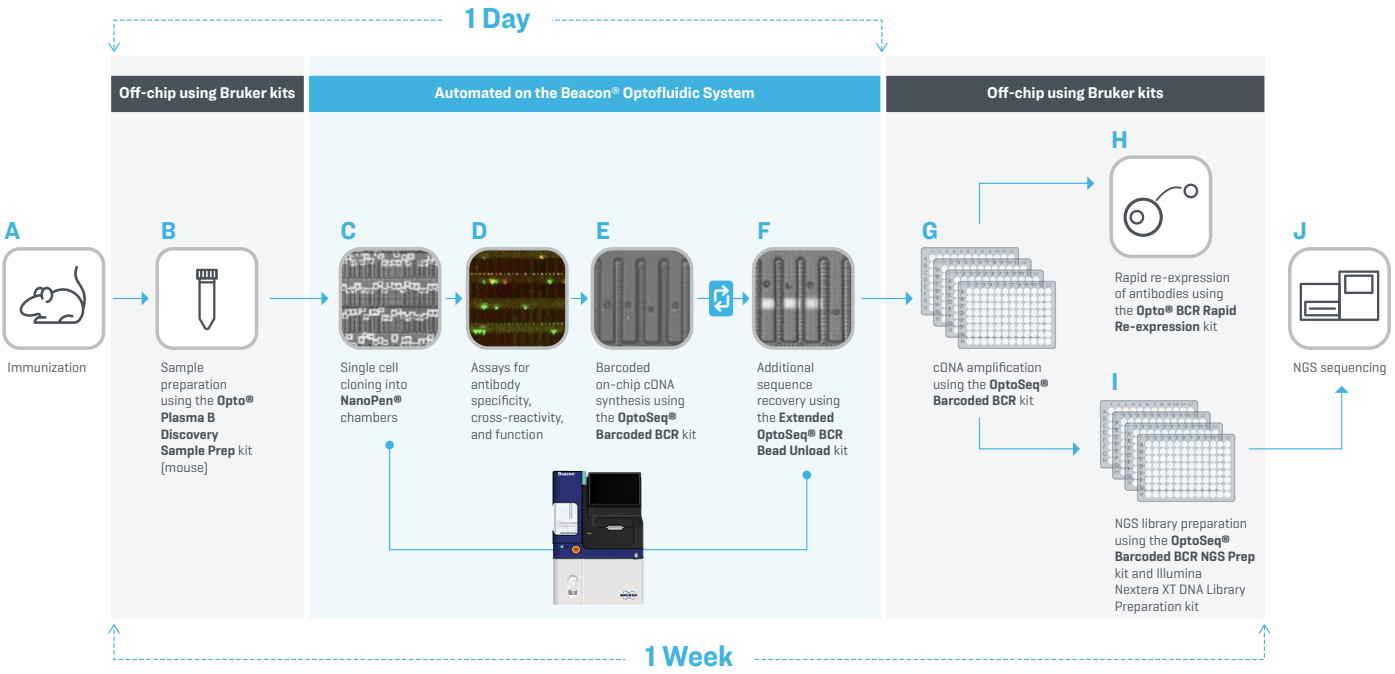


FIGURE 1: The Opto Plasma B Discovery 4.0 workflow. The Opto Plasma B Discovery 4.0 workflow enables screening of broad B cell diversity, rapid down-selection of lead molecules with functional profiling in 1 day, followed by high-throughput sequencing and re-expression of lead molecules which can be completed in 1 week.

MATERIALS AND METHODS

CELL PREPARATION AND ASSAY REAGENTS

For the standard Opto Plasma B Discovery 4.0 workflows, we harvested bone marrow and spleen of wild-type mice immunized with a model antigen. Plasma cells were enriched by magnetic-activated cell sorting (MACS) using the CD138+ Plasma Cell Isolation Kit, Mouse kit (Miltenyi Biotec), using our Opto Plasma B Discovery Sample Prep kit (mouse) and Plasma B Cell Isolation for Mouse Tissues protocol (MAN-000048). The Opto Plasma B Discovery Sample Prep kit (mouse) includes proprietary media that has been demonstrated to maintain plasma cell viability over a 2-day period. This allows a portion of the plasma cell sample to be cultured overnight, using standard tissue culture practices, which doubles the screening throughput.

For the PD-L1 experiments, we isolated primary plasma cells from the bone marrow and spleen of Balb/c mice immunized with Fc-fused PD-L1 extracellular domain (huPD-L1 ECD-FC) using a CD138+ plasma cell isolation kit (Miltenyi Biotech). We then prepared PD-1-AF488 by labeling a recombinant PD-1-Fc fusion protein (ChemPartner) using an AF488 labeling kit (Thermo Fisher Scientific). We prepared recombinant PD-L1 beads by coupling biotinylated PD-L1 (ChemPartner) to streptavidin polystyrene particles (Spherotech Inc.). Finally, CHO-K1 cells were engineered to over-express human PD-L1 (ChemPartner).

To identify anti-TIM-3 antibodies, 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 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).

PLASMA CELL CLONING

Using the Opto Plasma B Discovery 4.0 workflow, tens of thousands of single mouse plasma cells were automatically cloned into NanoPen chambers on up to four OptoSelect™ 20k chips. To increase the number of plasma cells screened, plasma cells were cultured overnight using proprietary media in the Opto Plasma B Discovery Sample Prep kit (mouse).

ASSAYS

Following loading, plasma cells were screened for antibody specificity. These assays were performed using Assay Beads (Bruker), conjugated to a target antigen using our Biotinylated Soluble Antigen Beads Preparation protocol (MAN-000024), and fluorescently-labeled secondary antibodies.

For recombinant PD-L1 bead binding assays, we imported PD-L1 coated beads, in suspension with a fluorescently labeled anti-mouse secondary antibody (AF568). Secreted antibodies diffused from the NanoPen chambers into the channel where binding of the secreted antibody was detected optically as in-channel "blooms" in the TRED imaging channel. Blooms observed adjacent to the center of the NanoPen indicated positive PD-L1 binding.

Cell-based functional assays can be performed either in the chip channel or in the pens themselves using reporter cell lines that homogeneously express antigens of interest on the cell surface. For the in-pen PD-L1 cell binding assays, plasma cells were co-incubated with CHO-K1-PD-L1 cells for 1 hour to allow for secreted antibodies to saturate the receptors. A fluorescently labeled anti-mouse secondary antibody (AF568) was then perfused through the chip and allowed to diffuse into the NanoPen chambers. Anti-PD-L1 cell-binding antibodies were identified by locating pens with fluorescent CHO-K1- PD-L1 cells when imaged on the Beacon system using a TRED filter cube. After completing the in-pen cell binding assay, we perfused a fluorescently labeled, soluble PD-1-Fc fusion protein (AF488) through the chip. PD-1 binding to the reporter cells was detected in the FITC imaging channel. NanoPen chambers containing CHO-K1-PD-L1 cells that were positive in both the TRED and FITC channels confirmed the presence of secreted antibodies that have PD-L1 binding, but no blocking activity. NanoPen chambers containing CHO-K1-PD-L1 cells that were positive in TRED but negative in FITC contained secreted antibodies that had both PD-L1 binding and PD-1/PD-L1 blocking activity.

For the TIM-3 experiments, we conjugated recombinant TIM-3 antigen to AF647 using Alexa Fluor 647 NHS Ester (Thermo Fisher Scientific) following the manufacturer's instructions. RPMI 8226 (ATCC), a human cell line with high endogenous TIM-3 expression levels, 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). A multiplexed assay mixture of anti-mouse IgG coated beads (Spherotech), secondary anti-mouse IgG-AF488 (Jackson ImmunoResearch), and AF647-labeled recombinant TIM-3 was used for in-channel bead binding assays. 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.

SEQUENCE RECOVERY

Antigen-specific antibody sequences were recovered using the OptoSeq Barcoded BCR kit. Automated cell lysis and reverse transcription were performed on-chip to generate stable cDNA on mRNA-capture beads with dual optical and genetic barcodes, to enable bulk recovery of up to 1,152 beads per 96-well plate, using the OptoSeq Barcoded BCR kit. Amplification of cDNA, also using the OptoSeq Barcoded BCR kit, is performed after bulk recovery. Additional beads were recovered using the Extended OptoSeq Bead Unload kit.

NGS libraries were generated using the Next-Generation Sequencing of OptoSeq Barcoded BCR Exports for the Beacon Optofluidic system protocol (MAN-08137), the OptoSeq Barcoded BCR kit and the Nextera XT DNA Library Preparation kit, allowing accurate sequencing of the full-length variable region of paired heavy/light chain antibody genes on standard Illumina sequencing platforms. Bioinformatics software, PrimeSeq BCR, was then used to link the sequences to phenotypic data for all recovered antibody sequences.

RAPID RE-EXPRESSION

Antibodies were re-expressed, for further downstream characterization, using the Rapid Re-Expression of Antibodies from Opto Plasma B Discovery protocol (MAN-08148) and the Opto BCR Rapid Re-expression Kit.

RESULTS AND DISCUSSIONS

ACCESSING BROAD B CELL DIVERSITY

Plasma cells are loaded onto OptoSelect 20k chips that contain more than 20,000 NanoPen chambers, each less than 1 nL in volume. This enables screening of up to 50,000 plasma cells in a single automated 4-chip workflow [**FIGURE 2A**]. To double the number of plasma cells screened, a second 4-chip workflow can be performed on the same plasma cell sample. To achieve this a portion of the plasma cell sample is cultured overnight, in proprietary plasma media, and screened the next day. Through this process, over 98,000 single plasma cells were screened over 2 days, enabling access to a broad range of antibody diversity [**FIGURE 2A**].

Following loading, plasma cells can be screened using multiple assays for antibody specificity, cross-reactivity, and function against soluble or cell membrane antigens. More than 6,000 antigen specific hits were identified from the two 4-chip workflows [**FIGURE 2A**]. The antigen hit rate remained constant over several hours, enabling multiple sequential assays to be run [**FIGURE 2B**].

FUNCTIONAL PROFILING DURING PRIMARY SCREENING ALLOWS EARLY DOWN-SELECTION OF LEAD CANDIDATES

Assays can be performed either in the channel, [in-channel assays], or within the pens [in-pen assays] using beads, coated with capture molecules, or reporter cells with homogeneously expressed antigens of interest [**FIGURE 3**]. For in-channel assays, beads or reporter cells suspended in media that contains secondary antibody are imported onto the OptoSelect chip. As secreted antibodies diffuse out of pens they are captured at the bead surface and fluorescence increases as the secondary antibody binds [**FIGURE 3A**]. For in-pen assays, beads or reporter cells are co-penned with plasma cells, and secreted antibodies then interact with the beads or reporter cells. For a binding hit, the secreted antibody binds to the surface of beads or reporter cells and fluorescence increases as the secondary antibody binds [**FIGURE 3B**]. For blocking hits the reporter cells will show little to no fluorescence, as the secreted antibody prevents binding of a fluorescently tagged ligand to the surface of the bead or reporter cell [**FIGURE 3C**].

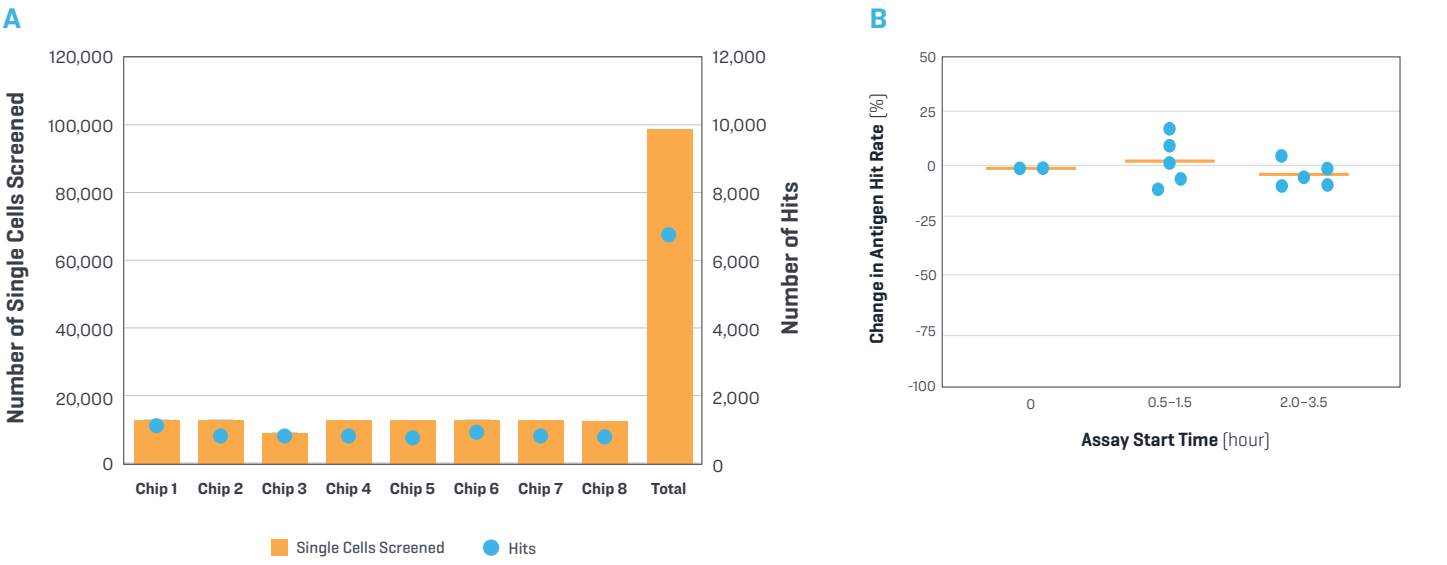
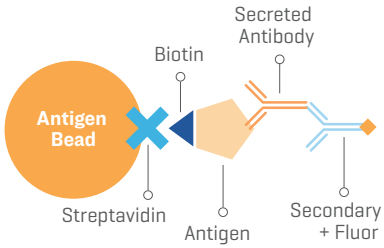


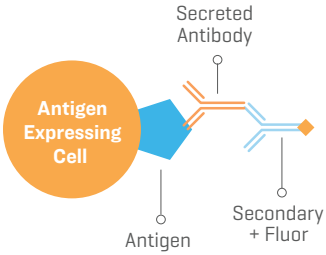
FIGURE 2: Screen up to 100,000 single plasma cells and perform multiple assays. A. The Opto Plasma B Discovery workflow enables screening of up to 100,000 single plasma cells in a two 4-chip Beacon workflows using OptoSelect 20k chips. Additionally, over 6,000 antigen positive hits were identified. **B.** The antigen hit rate remains constant over several hours, which means multiple assays, such as antibody specificity, cross-reactivity and functional assays, can be performed without loss of activity and on the same cell.

To avoid wasting valuable time and resources recombinantly expressing non-functional antibodies, a series of assays can be performed to down-select lead candidates. This decreases the burden associated with downstream processing and ultimately means that only the best possible candidates are pursued. In a PD-L1 case study, we used an in-channel antigen binding bead assay followed by in-pen reporter cell binding and blocking assays to identify rare PD-1/PD-L1 ligand receptor blocking antibodies [FIGURE 3D].

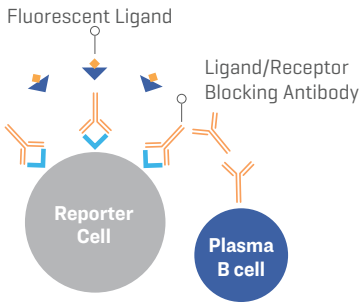
A **In-channel bead binding assay**



B **In-channel cell binding assay**



C **In-pen receptor/ligand blocking assay**



D **Down-selection using functional assays**

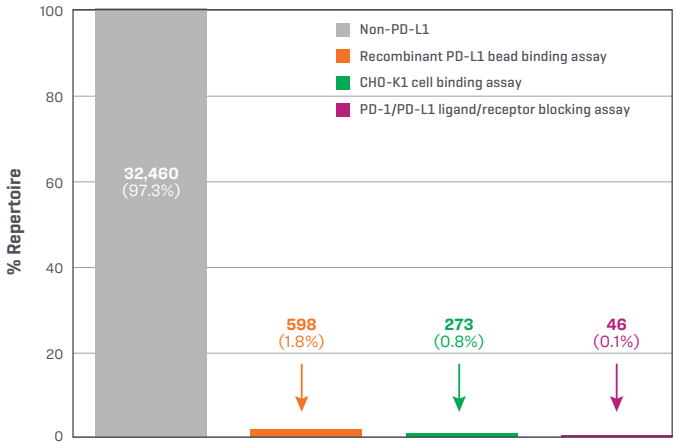
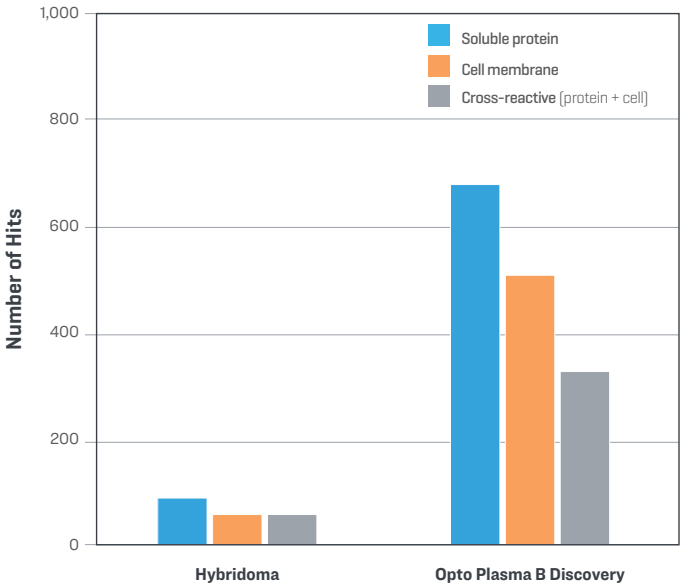


FIGURE 3: The Opto Plasma B Discovery workflow enables hit identification and lead candidate down-selection using multiple assays in just one day. A. In-channel bead binding assay. The highlighted pen, which the fluorescent bloom is centered on, contains the plasma cell that is secreting an antigen binding antibody. Intensity is due to the fluorophore on the secondary antibody. B. In-channel cell binding assay. The cells adjacent to the highlighted pen are binding secreted antibody and a fluorescently tagged secondary antibody. Intensity is due to the fluorophore on the secondary antibody. C. In-pen bead or cell blocking assay. Reporter cells, within the highlighted pen, were incubated with antibody secreting plasma cells. A fluorescently tagged ligand was introduced that is capable of binding to receptors, on reporter cells, that were not blocked by secreted antibody. Therefore, cells with low intensity indicate a blocking event. Intensity is due to the fluorescently tagged ligand. D. In a PD-L1 case study, fewer than 2% of screened plasma cells secreted antibodies that bound recombinantly expressed PD-L1 presented on beads. Fewer than 1% of plasma cells screened bound to PD-L1 presented at the cell surface. Only 0.1% of plasma cells screened blocked PD-1 binding to PD-L1 expressed on the cell surface.

SCREEN AGAINST MEMBRANE TARGETS USING CELL-BASED ASSAYS FOR FUNCTIONAL PROFILING



As mentioned previously, there has been limited success running functional screens against difficult membrane targets, such as GPCRs and ion channels, because lead molecules often need to bind cryptic epitopes or trigger complex molecular functions. To demonstrate the utility of functional screens on the Beacon system, cell binding assays using the Opto Plasma B Discovery workflow were performed alongside a traditional hybridoma workflow to identify antibodies that target challenging membrane protein targets. The Opto Plasma B Discovery workflow yielded 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 4**). 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 against even difficult targets.

FIGURE 4: 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.

ACCESS GREATER SEQUENCE DIVERSITY BY RECOVERING THOUSANDS OF ANTIBODY SEQUENCES

Once functionally characterized leads have been identified, the OptoSeq Barcoded BCR method enables rapid, efficient sequencing of thousands of antibodies in a single run. Antibody sequences are recovered in bulk by using a mixture of beads containing 12 distinct optical and genetic barcodes (**FIGURE 5A**). A single barcoded bead is loaded into each NanoPen chamber containing an antigen-specific plasma cell and cells are lysed to capture mRNA. After on-chip cDNA synthesis, the optical barcodes of every bead are decoded by fluorescent imaging (**FIGURE 5A**). Up to 1,152 beads can then be recovered into a single 96-well plate by bulk recovery of up to 12 beads with distinct barcodes into each well. From just two 4-chip workflows, nearly 12,000 beads can be recovered with high (>90%) efficiency by using the Extended OptoSeq Bead Unload kit (**FIGURE 5B**).

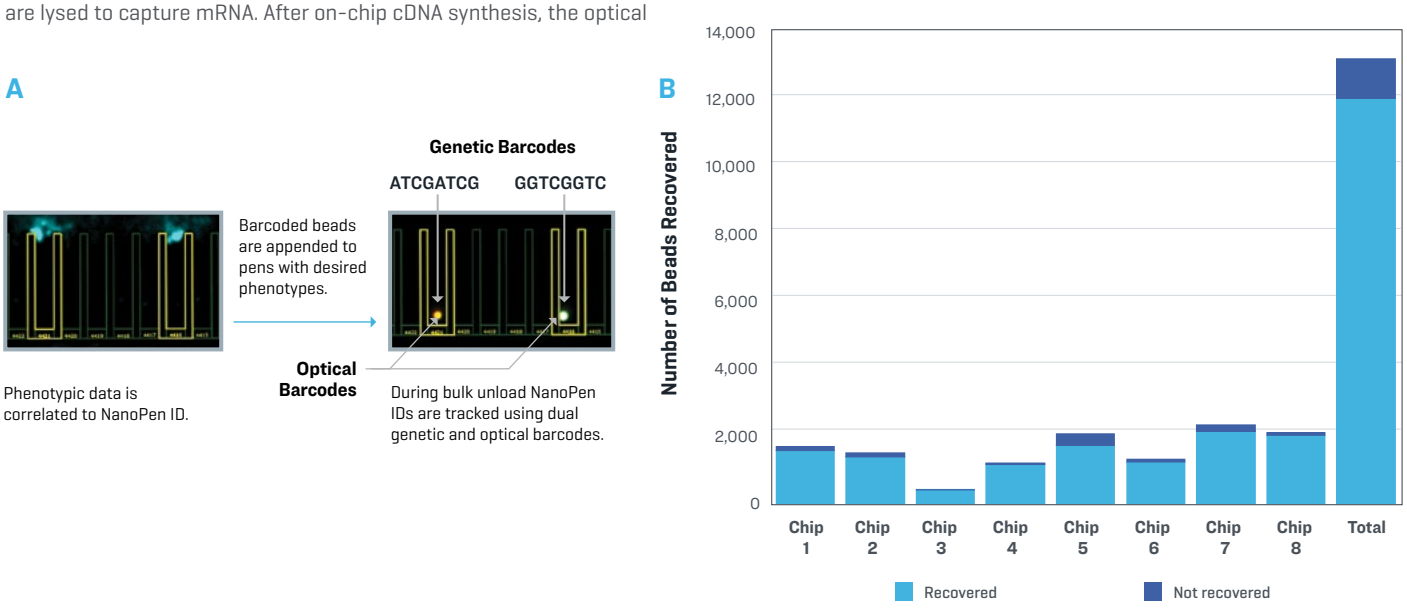


FIGURE 5: OptoSeq Barcoded BCR enables linkage of sequence to function of thousands of antibodies. A. Dual genetic and optical barcodes enable bulk bead recovery while preserving the linkage of sequence to function. Phenotypic data is correlated to NanoPen identifiers, barcoded beads are then appended to pens with desired phenotypes and this allows NanoPens data to be tracked to sequences. **B.** Using the Extended OptoSeq Bead Unload kit almost 12,000 beads can be recovered from two 4-chip Opto Plasma B Discovery workflows with high efficiency (>90%).

OptoSeq Barcoded BCR then enables rapid, accurate sequencing of paired heavy/light chain antibody genes by DNA fragmentation, NGS sequencing, and bioinformatics analysis (**FIGURE 6A** and **FIGURE 6B**). Bioinformatic assembly of full-length antibody variable regions from short reads (150 x 150) enables accurate sequencing of thousands of antibodies from a single run on standard Illumina sequencing platforms (**FIGURE 7A**). From a single 96-well plate, paired heavy/light chain sequences from over 500 antibodies were recovered (**FIGURE 7B**).

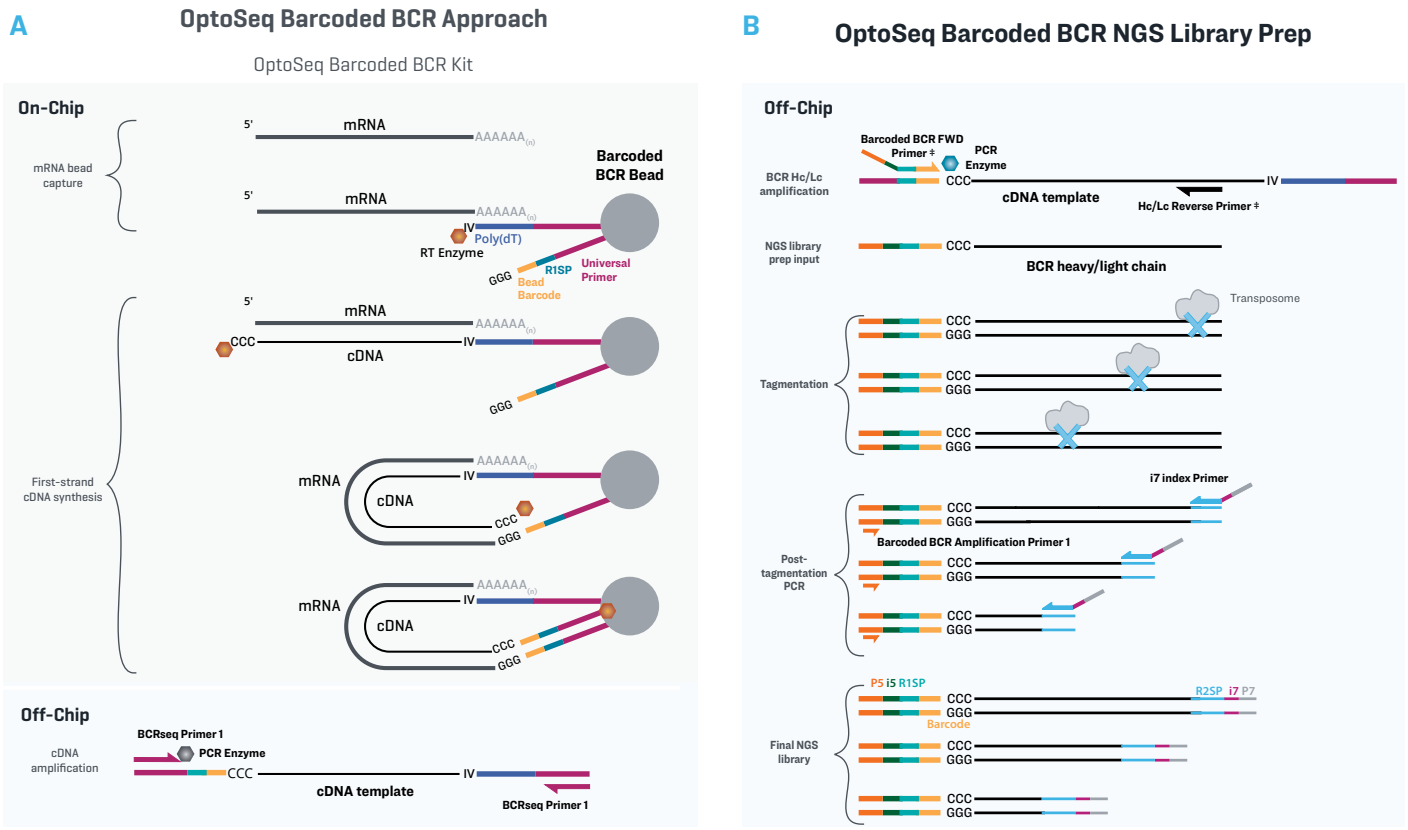


FIGURE 6: OptoSeq Barcoded BCR enables accurate sequencing of full-length, paired heavy/light chains (Hc/Lc) from >1,000 antibodies from a single run on standard Illumina NGS sequencing platforms. **A.** The OptoSeq Barcoded BCR kit is used for on-chip cell lysis, on-chip cDNA synthesis and off-chip cDNA amplification from recovered beads. **B.** The OptoSeq Barcoded BCR NGS Prep kit and Nextera XT DNA Library Prep kit use amplified cDNA to prepare NGS libraries for 150x150 sequencing compatible with standard Illumina sequencing platforms.

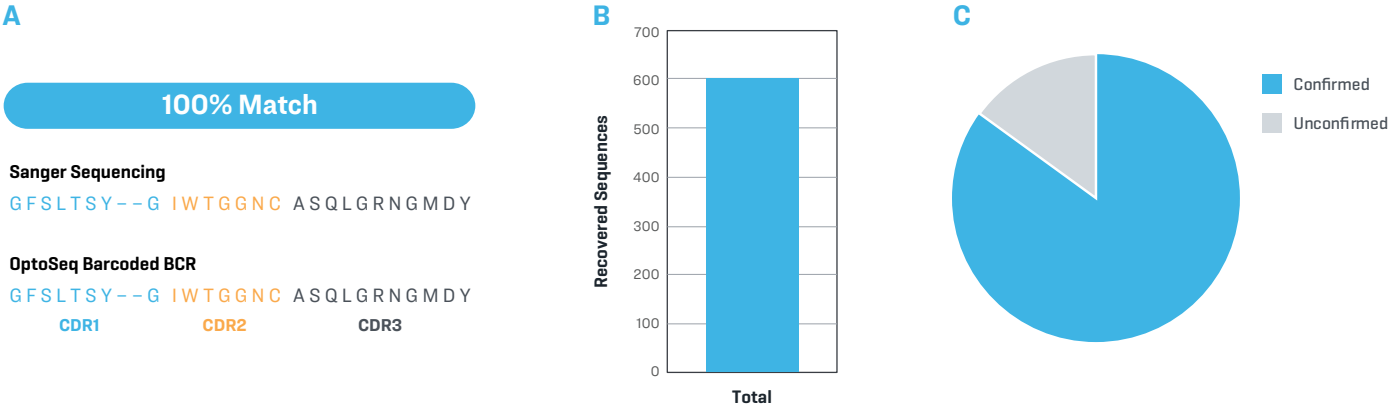


FIGURE 7: OptoSeq Barcoded BCR enables accurate sequencing and re-expression of >1,000 antibodies in just 1 week. **A.** Accurate, full length variable region sequences are obtained using a combination of fragmentation, NGS sequencing, and bioinformatics analysis. The ability to reconstruct full-length antibody genes from short reads enables rapid sequencing of hundreds of antibodies from a single run on standard Illumina sequencing platforms. **B.** Paired heavy/light chain sequences from over 500 antibodies were recovered from a single 96-well plate with high efficiency (73%). **C.** Over 80% of re-expressed antibodies (n = 271) demonstrated confirmed function in plate-based ELISA assays.

After sequencing the exports, the PrimeSeq BCR pipeline is used to demultiplex the bulk exports and link the full-length variable domain antibody sequences to each NanoPen chamber and the phenotypic assay data associated with that pen [**FIGURE 8**]. Additionally, PrimeSeq BCR's visualization features, including a dendrogram and a multiple sequence alignment, allow users to easily choose sequences to investigate based on sequence similarity.

RAPIDLY RE-EXPRESS >1,000 LEAD MOLECULES WITHOUT GENE SYNTHESIS AND CLONING

Production of lead molecules for downstream characterization typically includes costly gene synthesis and laborious bacterial cloning. The Opto BCR Rapid Re-expression kit enables re-expression of antibodies in 1 week to confirm function in plate-based assays [**FIGURE 9**], without the need for gene synthesis and bacterial cloning. Amplified cDNA is cloned into expression constructs in one step to enable generation of >1,000 antibodies for functional confirmation. Over 80% of re-expressed antibodies demonstrate confirmed function in plate-based ELISA assays [**FIGURE 7C**]. This decreases timelines associated with traditional sequence validation methods, and costs less than $100/molecule.

FIGURE 8: The PrimeSeq BCR software enables visualization of antibody sequence similarity and links sequences to antibody phenotype. After sequencing the exports, the PrimeSeq BCR pipeline is used to demultiplex the bulk exports and link the full length variable domain antibody sequences to each NanoPen chamber and the associated assay data. The CDR1, CDR2, and CDR3 regions are highlighted in blue. Various point mutations, between related sequences, are shown in bolded white font.

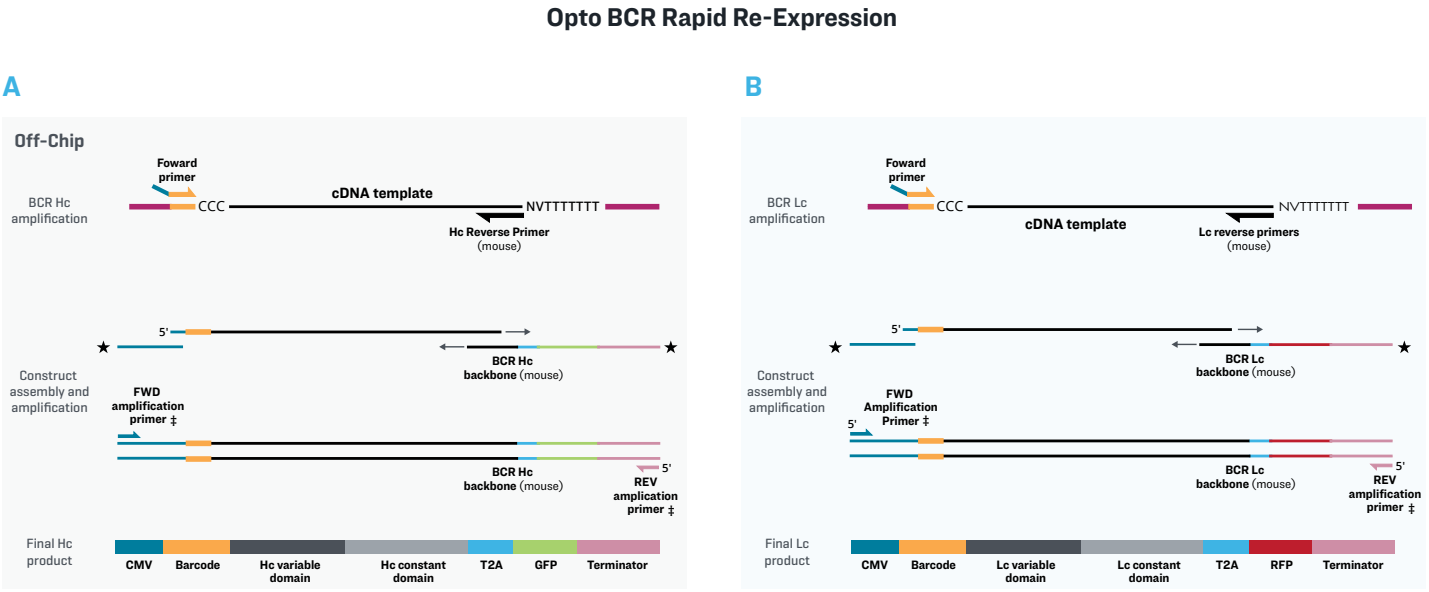


FIGURE 9: The Opto BCR Rapid Re-expression kit converts antibody sequences into molecules in 1 week for plate-based confirmation assays. cDNA captured from the OptoSeq Barcoded BCR kit is amplified and directly cloned into expression constructs for rapid re-expression of mouse IgG2α antibodies. The CMV region of both BCR Hc Backbone and BCR Lc Backbone are connected to the respective Constant Domain, T2A, GFP, and Terminator region as a single molecule [ ]. The FWD and REV Amplification Primers (‡) are pooled in the BCR Construct Amplification Primers. **A.** Heavy chain constructs. **B.** Light chain constructs.

CONCLUSION

Antibody discovery necessitates screening a diverse panel of molecules, using a variety of methods to rapidly down-select molecules from these panels. With hybridoma and plasma cell cloning technologies, functional characterization is performed after sequencing and as a result nonfunctional or irrelevant hits are moved down the drug development pipeline wasting valuable time and resources. To circumvent these challenges, functional profiling of a broad diversity of plasma cells must be performed ahead of sequencing, which can easily be accomplished using the Opto Plasma B Discovery 4.0 workflow on the Beacon system.

We have shown that the Opto Plasma B Discovery 4.0 workflow enables recovery of 1,000s of hits by screening up to 100,000 plasma cells, down-selecting lead candidates by functional profiling, and sequencing and re-expressing >1,000 functionally characterized antibodies. By integrating functional profiling of broad plasma cell diversity with high-throughput sequencing and rapid re-expression, the ability to map genotype to functional characteristics is enabled at the earliest possible time using live individual plasma cells. This workflow accelerates the discovery of lead molecules against difficult targets by allowing you to go from plasma cell to functionally characterized protein in 1 week.

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