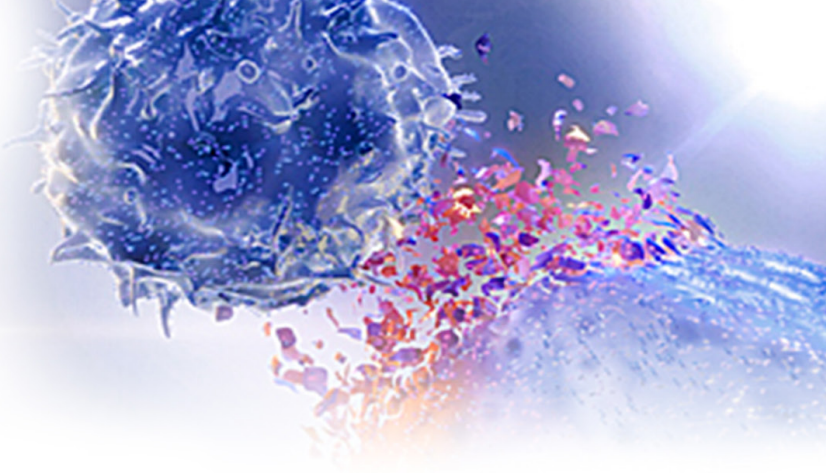

ABZENA

CASE STUDY

IMPLEMENTING ROBUST & REPRODUCIBLE KILR READY-TO-USE BIOASSAYS FROM SCREENING TO LOT RELEASE: APPLICATIONS FOR MEASURING DIRECT CYTOTOXIC CELL DEATH

ABSTRACT

Therapeutic regulators require assay data on the impact of Fc effector-mediated function of antibodies during their development as therapeutics. The antibodies are required to demonstrate immune cell-mediated target cell killing via Antibody Dependent Cell-Mediated Cytotoxicity (ADCC) and/ or Antibody Dependent Cellular Phagocytosis (ADCP) rather than measuring a surrogate endpoint that exhibits antibody engagement of antigen on target cells. The Eurofins DiscoverX® KILR® cytotoxicity assay platform specifically measures antibody-mediated killing of antigen-expressing target cells in co-culture with effector cells. This platform has been developed into a ready-to-use (RTU) bioassay format for several tumor models, including Raji and Daudi, for use in screening and lot-release applications. The KILR assay platform is robust and flexible and can be used for analyzing multiple mechanism-of-actions (MOAs), including ADCC, ADCP, complement-dependent cytotoxicity (CDC), and T-cell redirection. Abzena®, a leading partner research organization that offers integrated discovery, development, and cGMP manufacturing services for biologics and bioconjugates, independently evaluated the bioassay format for KILR Raji target cell model in ADCC and ADCP assays to offer clients a robust platform for ranking and characterization studies. This case study demonstrates the ability of these bioassays to provide a quantitative readout for ADCC, ADCP, and CDC applications that produce large assay windows and excellent reproducibility (key aspects that help accelerate potency testing to QC lot release) when used with KILR CD16 Effector Cells.

Robust and MOA-Reflective Platform

The KILR platform provides a robust and sensitive solution to measure ADCC, ADCP, and CDC as a simpler and quicker alternative to traditional readouts, providing larger assay windows and lower variability than other assay formats

Fc-MEDIATED EFFECTOR FUNCTIONS FOR MEASURING DIVERSE CELL CYTOTOXICITY MECHANISMS: A CASE STUDY FOR ADCC, ADCP & CDC APPLICATIONS

Assessment of Fc-mediated effector functions is key in the development of biotherapeutics. Fc-mediated effector functions play a role in the MOA of antibodies and can have an impact on product safety and efficacy. Among the most common subclasses of therapeutic antibodies, human IgG1 can potently activate these mechanisms, whereas human IgG4 elicits much lower to no activity depending on modifications. Depending on these wanted/unwanted activities, Fc-mediated effector functions can be assessed at various stages of the drug discovery & development processes, such as part of the screening cascade, characterization, safety evaluation, or potency testing for lot release.

ADCC APPLICATION AND ASSAY

ADCC is part of the host immune defense where an effector cell of the immune system is directed to lyse target cells that have been opsonized by antibodies. The Fc portion of the target-bound antibody binds to FcγRIII receptors expressed on effector cells (mainly natural killer (NK) cells). Once the binding occurs, the effector cells release cytokines and cytotoxic granules, leading to direct target cell lysis[1]. The KILR® Raji ADCC Bioassay Kit measures target cell death using the gain-of-signal function based on the Eurofins DiscoverX® enzyme fragment complementation (EFC) technology. This technology employs two fragments (a small enzyme donor (ED) and a large enzyme acceptor (EA)) of a split β-galactosidase (β-gal) enzyme that produces a sensitive luminescence readout with excellent signal-to-background (S/B) ratios and large assay windows. See Figure 1 for the assay principle.

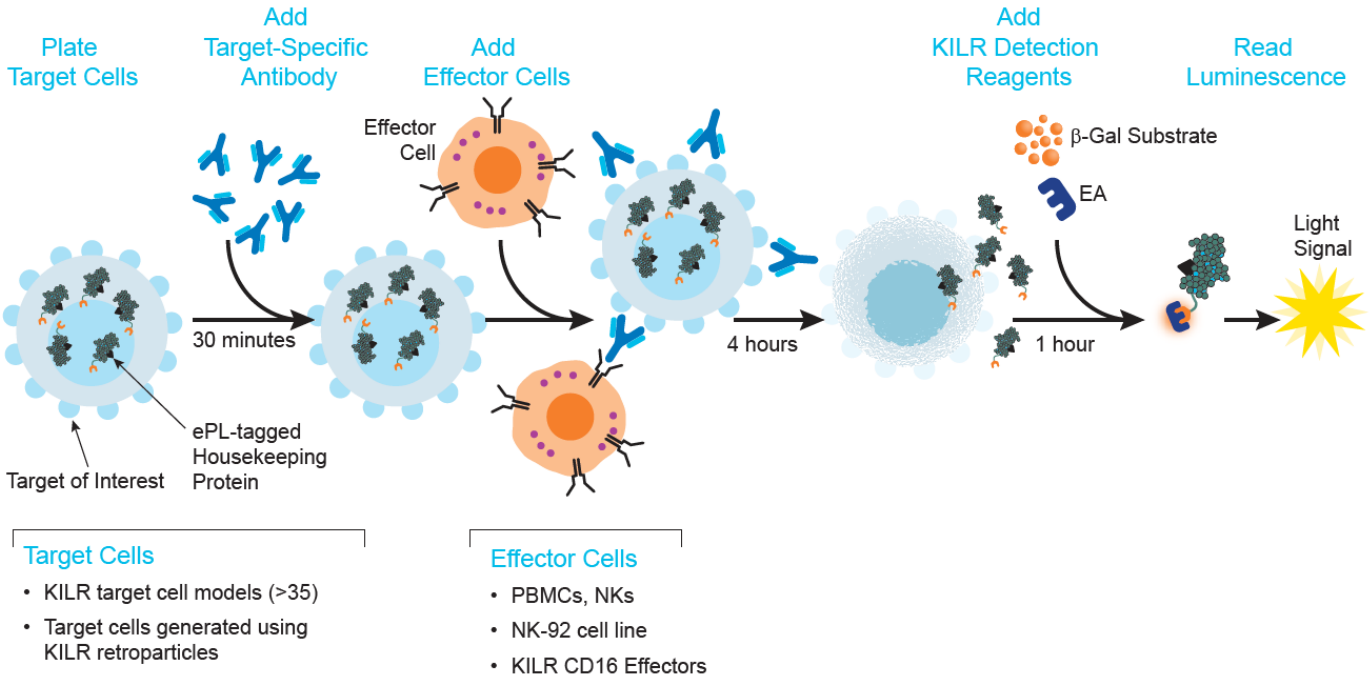

Figure 1. KILR ADCC Assay Principle.

In vitro cell lysis induced by ADCC is typically measured by quantifying isotope, fluorophore, or enzyme release. These readouts can be challenging depending on the choice of target cells and often result in low S/B ratios. In contrast, the KILR Raji Bioassay Kit offers a convenient and robust MOA-reflective alternative and measures direct target cell death. The KILR Raji ADCC Bioassay utilizes KILR Raji target cells that stably express an ED-tagged reporter protein that are opsonized with a therapeutic antibody targeting the relevant antigen endogenously expressed on the target cells. This is co-incubated with immune effector cells (e.g., KILR CD16 Effector Cells) (Figure 1). When the opsonized target Raji cells are used in a cytotoxicity assay, effector-mediated killing results in the loss of the cell membrane integrity, thus releasing the tagged reporter protein into the media. The reporter protein is detected by adding KILR detection reagents containing the EA fragment of the β-gal enzyme. The ED and EA fragments complement through protein-protein interactions. This leads to the formation of the active β-gal enzyme that hydrolyzes a substrate resulting in a robust chemiluminescent signal that is detected on any benchtop luminescence reader.

Most ADCC assays utilize primary human effector cells, such as peripheral blood mononuclear cells (PBMCs) or NK cells that show large donor-to-donor variability. This variability presents a challenge especially for release assays, where consistency and reproducibility are essential for assay qualification and validation. As a solution to this problem, KILR CD16 Effector Cells offer an excellent alternative by yielding reproducibly high target cell lysis. KILR CD16 Effector Cells are $CD8^+$ T-cells isolated from a single healthy donor and engineered to express the V158 variant of CD16. Our highly controlled manufacturing process ensures reproducible production of these effector cells between lots, resulting in much lower variation than is seen between lots of primary cells from the same donor or between donors. These cells also cause very low background killing for most cell lines, ensuring larger response windows relative to primary PBMCs or NK cells.

KILR RTU bioassay Raji cells can also be used to evaluate other effector-mediated functions of clinical antibodies as well, such as ADCP and CDC.

ADCP APPLICATION AND ASSAY

ADCP is part of the host immune defense where macrophage effector cells are directed to phagocytose target cells that have been opsonized by antibodies. The Fc portion of target-bound antibodies binds to FcγRIIa (the predominant receptor for ADCP, but FcγRI and FcγRIIIa are also involved) expressed on macrophages and other phagocytic cells. Once the binding occurs, the signaling initiated in the effector cells leads to the phagocytosis of the target cells[2]. *In vitro* phagocytosis is most often measured by labeling effector cells and target cells with two different fluorescent probes, followed by the identification of double-positive events either by flow cytometry or imaging, or using a pH-sensitive readout. While these methods may provide a means of visualizing phagocytosis, obtaining high-throughput quantitative datasets can be challenging. KILR® bioassays offer a possible solution to the challenges stated above.

In the KILR ADCP assay (see Figure 2), M0 or M1 macrophages are co-incubated with antibody opsonized KILR Raji target cells expressing the KILR reporter protein for 24 hours, followed by cell lysis. Cells phagocytosed by macrophages in the presence of an antibody drug leads to a dose-dependent decrease in EFC signal that is then converted to % killing (% ADCP) by normalization to vehicle-treated target cells.

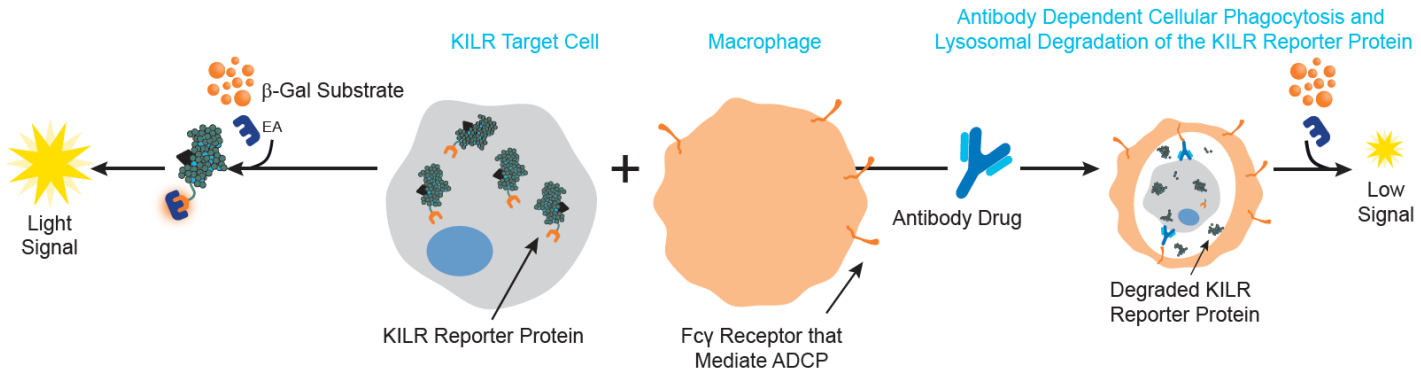

Figure 2. KILR ADCP Assay Principle.

CDC APPLICATION AND ASSAY

CDC is the mechanism by which antibody-coated target cells recruit and activate complement cascade components, leading to the formation of a Membrane Attack Complex (MAC) on the cell surface and subsequent cell lysis[3]. In the CDC assay (see Figure 3), target cells (e.g. KILR Raji cells) expressing the target antigen and KILR reporter protein are incubated with a titration of target-specific antibodies in human serum containing active complement. This leads to the production of MAC on the surface of the cells. Like the ADCC assay, complement-mediated target cell killing results in the loss of the cell membrane integrity, thus releasing the tagged reporter protein into the media. The reporter protein is detected by the addition of KILR detection reagents containing the EA fragment of the β-gal enzyme. The ED and EA fragments complement through protein-protein interactions leading to the formation of the active β-gal enzyme. The active enyme hydrolyzes a substrate leading to an increase in luminescence signal corresponding to cell death that can be read on any benchtop luminescence reader. This assay performs well and can be used as an indicator of cell lysis mediated by CDC, as shown in Figure 6 for Rituximab-mediated CDC of KILR Raji cells.

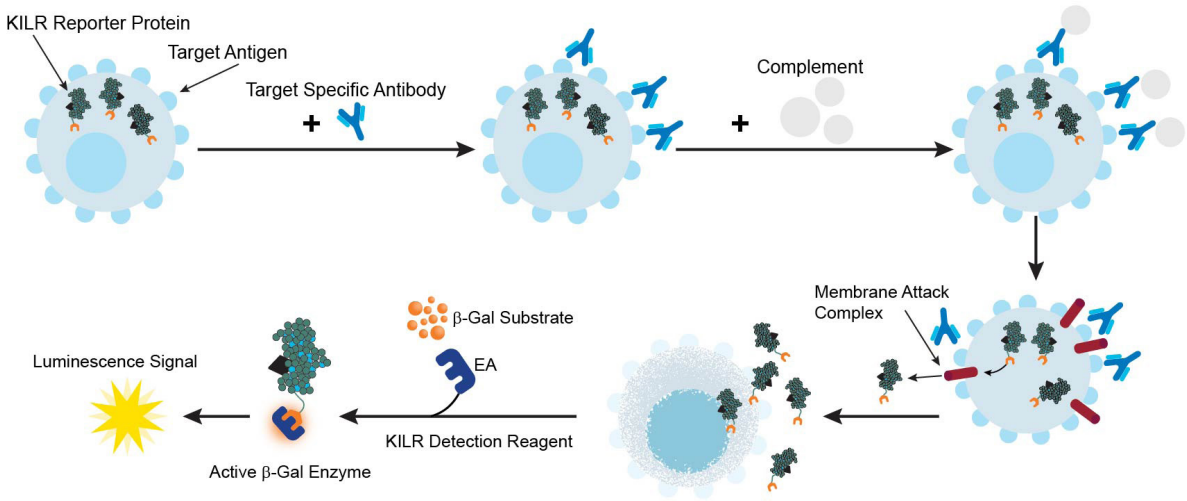

Figure 3. KILR® CDC Assay Principle.

KILR BIOASSAYS ARE ROBUST AND REPRODUCIBLE IN MEASURING DIRECT CYTOTOXICITY

Abzena® independently evaluated the RTU bioassay format for KILR Raji target cell model in ADCC, ADCP, and CDC assays to offer clients a robust platform for ranking and characterization studies. For the ADCC assay, assay performance was evaluated using engineered KILR CD16 Effector Cells relative to primary human PBMCs from two healthy donors. As seen in Figure 4, the KILR Raji bioassay showed excellent reproducibility in two independent ADCC assays with KILR CD16 Effector Cells. Further, it was demonstrated that assay window (E_{Max}) and data quality were much improved using the KILR CD16 Effector Cells relative to primary human PBMCs.

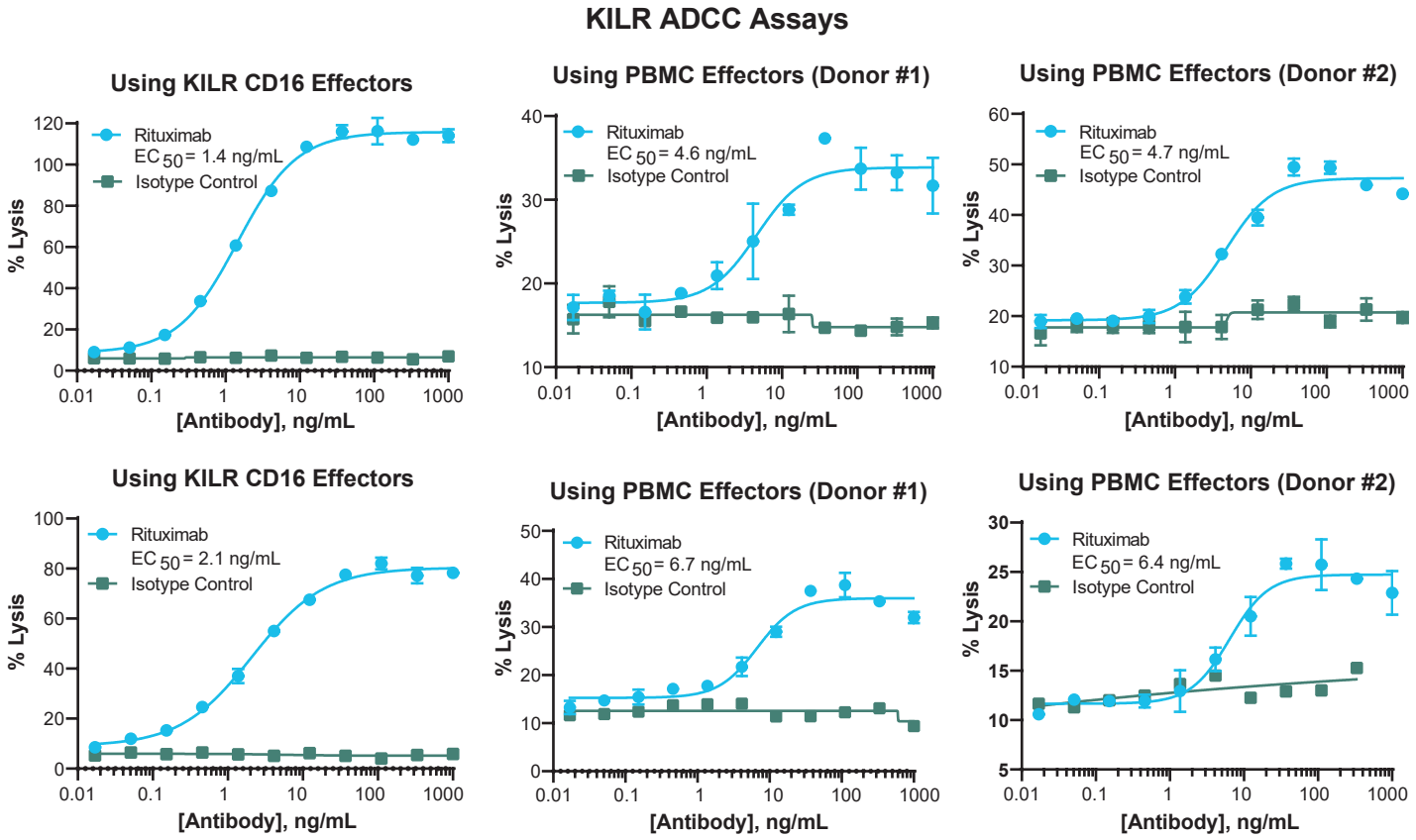

Figure 4. Robust ADCC Bioassay Performance. Experiments performed by Abzena with varying amounts of Rituximab (antibody drug) to study the percent lysis of target tumor cells (KILR CD16 Effectors and PBMC cells from 2 healthy donors). The KILR ADCC Bioassay Kit shows sensitive and robust results with large assay windows (indication of % lysis), especially using the KILR CD16 Effector Cells.

Similarly, the KILR® Raji bioassay cells were utilized to evaluate ADCP mediated by Rituximab, using macrophages isolated from two different healthy donors. As shown in Figure 5, comparable E_{Max} (% lysis) and EC_{50} values with robust assay windows were obtained with the two different donors using this simple homogeneous ADCP assay format.

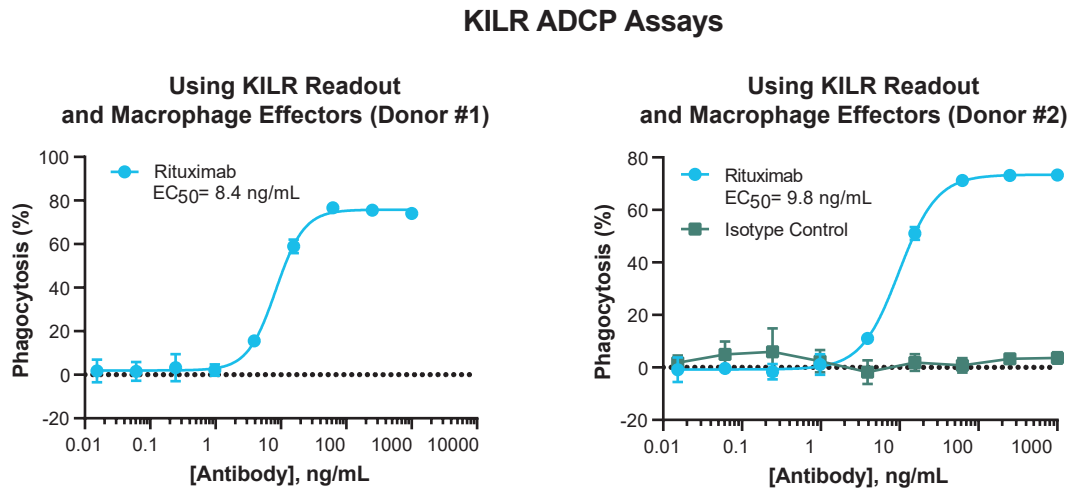

Figure 5. ADCP activity was assessed with KILR Bioassay Cells. The KILR assay platform exhibits highly sensitive and robust assay performance when assessing phagocytic activity.

Finally, the KILR Raji bioassay was also utilized to evaluate CDC mediated by Rituximab. As shown in Figure 6, the bioassay delivered a good assay window and expected EC_{50} with consistent performance. The KILR Raji bioassay can be used as an indicator of cell lysis mediated by CDC and may have advantages in case of challenging targets, where CDC is difficult to quantify without a target-specific luminescence method. The ability of a single platform, such as KILR to interrogate three different Fc-effectors, offers significant advantages in gaining a comprehensive understanding of the cytotoxicity profile of the therapeutic antibody.

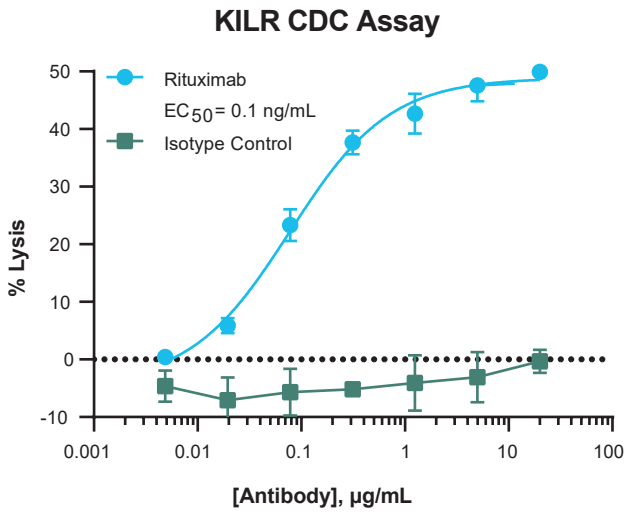

Figure 6. CDC activity was assessed with KILR Bioassay Cells. As shown with ADCC and ADCP, the KILR assays performs well in a CDC assay showing sensitive and robust results with large assay windows (indication of % lysis).

CONCLUSION

Therapeutic antibodies cause cytotoxic effects on tumor cells through a variety of mechanisms. Most of the mechanisms require engagement with the host immune system components via ADCC, ADCP, and CDC. For the therapeutic modalities that utilize Fc-effector mechanisms, the regulatory agencies have been routinely recommending the use of cell-based assays that measure immune effectors-mediated direct cell-death rather than a surrogate endpoint for antibody engagement of antigen on target cells.

Existing ADCC assays suffer from drawbacks such as measuring cell death that is not specific to target cells, thereby relying on a predictive approach to determining the MOA rather than reflective of the true MOA. Other limitations stem from the use of dye-based assays, donor-to-donor variability of effector cells, or low throughput. To address the existing challenges with traditional cytotoxicity assays and the increasing requirement of regulators for evaluation of Fc effector mechanism during development of antibody based therapeutics, Eurofins DiscoverX® offers the KILR® cytotoxicity assay platform. The KILR platform offers a simple, dye-free, and non-radioactive assay format to directly measure ADCC, ADCP, and CDC, as well as other immune-cell mediated killing MOAs not covered here.

Abzena® independently evaluated the bioassay format for the KILR Raji target cell model in ADCC, ADCP, and CDC assays to offer clients a robust platform for ranking and characterization studies. For ADCC, the KILR Raji bioassay showed excellent reproducibility in two independent ADCC assays with KILR CD16 Effector Cells as well as primary PBMCs. Further, it was demonstrated that assay window (E_{Max}) and data quality were much improved using the KILR CD16 Effector Cells relative to primary human PBMCs. ADCP mediated by Rituximab, using macrophages isolated from two different healthy donors was also assessed. It was demonstrated that comparable E_{Max} (% phagocytosis) and EC_{50} values with robust assay windows were obtained with the two different donors using this simple homogeneous ADCP assay format. Finally, the KILR Raji bioassay were utilized to evaluate CDC. It was determined that the KILR platform is an excellent option to asses this mode of action as well, allowing all three MOAs to be evaluated with the same target cells.

Taken together, we have demonstrated that the KILR platform provides a robust and sensitive solution to measure ADCC, ADCP, and CDC. These robust and MOA reflective assays offer a means of measuring target cell lysis and phagocytosis by primary effector cells, and are a simpler and quicker alternative to traditional readouts, providing larger assay windows and lower variability than other assay formats. The RTU bioassay kits for the KILR target cells simplifies the use and implementation of these cytotoxicity assays.

To learn more about Eurofins DiscoverX's KILR ADCC Bioassay Kits, visit discoverx.com/KILR-Bioassays.

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