

# *In Vitro* and *Ex Vivo* Immuno-Oncology Drug Discovery



CrownBio's integrated Immuno-Oncology Platform allows end-to-end *in vitro* and *ex vivo* immunoprofiling, functional assay evaluation, and novel target validation

Discover how CrownBio's comprehensive Immuno-Oncology platform can advance your *in vitro* and *ex vivo* research today.

Immunotherapeutic evaluation faces many challenges from the earliest stages of drug development, with unique and diverse agents requiring specialized *in vitro* and *ex vivo* assays to determine functional, immuno-modulatory, and efficacy effects.

CrownBio's fully integrated *In Vitro* and *Ex Vivo* Immuno-Oncology Platform provides clients with all of the robust data needed to choose which drugs should be carried forward to *in vivo* evaluation.

Our one stop service for early stage immunotherapeutic assessment includes:

- Core *in vitro* immuno-oncology phenotyping and profiling, which allows full monitoring of the immune system and response to novel agents.
- A wide range of functional *in vitro* and *ex vivo* assays allowing clients to fully determine all aspects of how a novel agent works from MOA to immunomodulatory effects.
- Evaluation and validation of novel targets, demonstrating our commitment to furthering immuno-oncology research.

## Core Immunophenotyping and Profiling

### Functional Assays

Antigen Presentation &  
Immune System Activation

Immune Cell-Mediated  
Cancer Cell Killing

Regulation of  
Tumor Environment

## Novel Immuno-Oncology Targets

# In Vitro and Ex Vivo Immuno-Oncology Platform Key Facts

CrownBio's fully validated vast toolbox of *in vitro/ex vivo* assays covers a wide range of functions, across the innate and adaptive immune system, to provide clients with key next step decision making data.

Our integrated platform offering includes:

## Immunophenotyping and cell profiling including:

- Flow cytometry and IHC
- Immune cell separation
- Cell proliferation assays
- Cytokine profiling
- T cell receptor sequencing

## Identification of novel IO targets, including IDO and TDO assays

## Functional assays:

- Antigen presentation and immune system activation
  - » With T cell, macrophage, DC, NK cell, and tumor targets
- Regulation of tumor environment
  - » Microbiome sequencing
- Immune cell-mediated cancer cell killing
  - » ADCC, CDC, NK mediated, T cell killing

## In Vitro Immuno-Oncology Challenges

Immunotherapy represents the most promising cancer treatment approach since the first development of chemotherapies in the late 1940s<sup>(1)</sup>. However, developing novel immunotherapeutics still faces many challenges, from the early stages of *in vitro* drug development onwards.

Immune-targeted agents act against cancer via modulation of the host immune system, as opposed to conventional treatments which directly target tumor cells. This requires non-conventional immunology assays for the assessment of novel agent efficacy.

Specific classes of immunotherapeutic agents are also difficult to assess *in vitro* due to a lack of the heterogeneous components of immune cells found *in vivo* that are required for complete drug response e.g. evaluating biological agents.

In addition to assessment of antitumor response, there is also a need to assess inhibitory immune cell activity e.g. T<sub>reg</sub>, tumor associated-macrophages (TAMs) and others which can hinder efforts to mount cell mediated tumor killing. As a consequence, modulation of the immune system and its assessment captured as one time point may not provide a clear enough window into the net effect of the therapeutic approach.

CrownBio provides the wide range of validated *in vitro/ex vivo* assays that are required to move through:

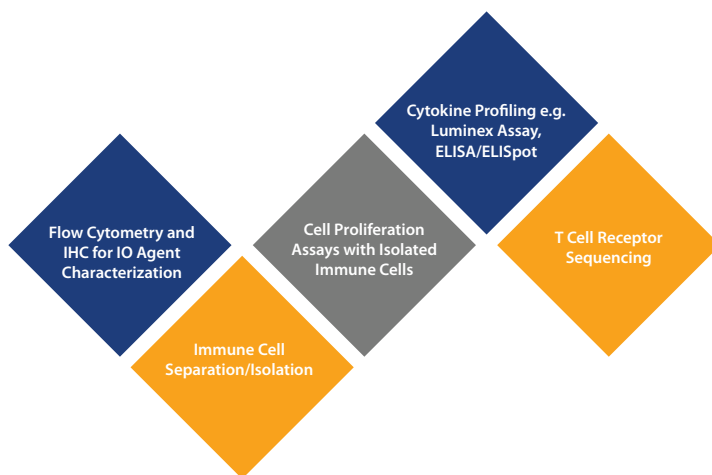
- Target expression profiling
- Determination of functional consequences and mechanism of action
- The understanding of the immunomodulatory effects of a novel agent

to provide robust data for moving agents to the next phase of immuno-oncology drug development.

## In Vitro Immuno-Oncology Phenotyping and Profiling

CrownBio's core *in vitro* immuno-oncology phenotyping and profiling platform provides a range of comprehensive assays required to fully monitor the immune system and response to novel agents, shown in **Figure 1**.

**Figure 1: CrownBio's In Vitro Immuno-Oncology Phenotyping and Profiling Platform**



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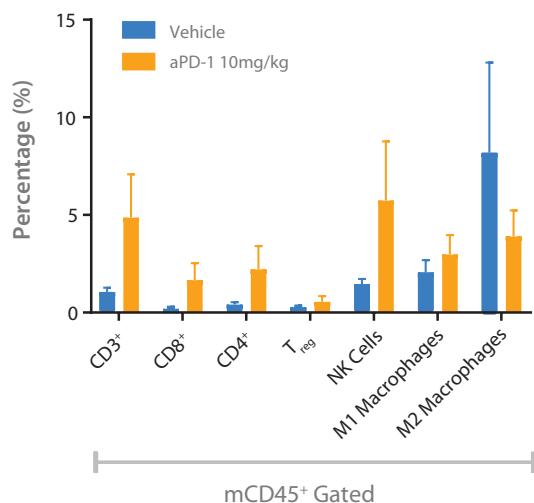
## Flow Cytometry and Immunohistochemistry

Flow cytometry is available for a range of sample types including small animal specimens (evaluating surface and intracellular proteins from spleen, lymph node, tumor, and blood cells) and cell lines (evaluating surface and intracellular proteins). We have a validated panel of markers for both human and murine cell analysis to monitor the effects of immunotherapeutics on immune cell populations including:

- B cells
- T cells (total, helper, cytotoxic, T<sub>reg</sub>)
- Dendritic cells
- Natural killer (NK) cells
- Macrophages
- Monocytes
- Neutrophils
- MDSC
- Checkpoint antigens

Example data for immunophenotyping of murine EMT6 breast cancer tumors is shown in **Figure 2**. A range of our markers are also validated for immunohistochemistry analysis e.g. human and/or murine CD3, CD4, CD8, PD-1, and PD-L1.

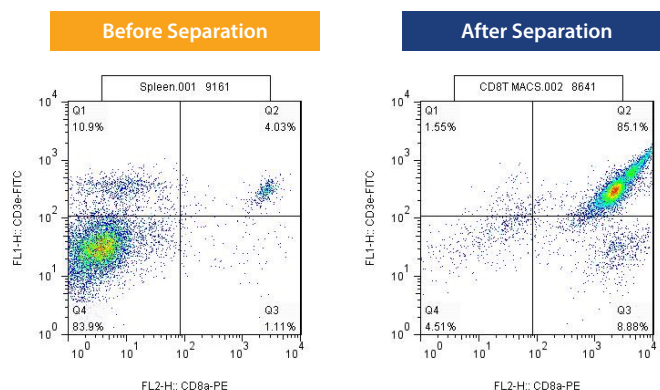
**Figure 2: EMT6 Tumor Immunophenotyping following Anti-PD-1 Treatment**



## Immune Cell Separation

CrownBio provides immune cell separation delivering subset enrichment for downstream applications. We are able to isolate immune cells through magnetic bead separation; example separation and enrichment of murine CD8<sup>+</sup> T cells shown in **Figure 3**.

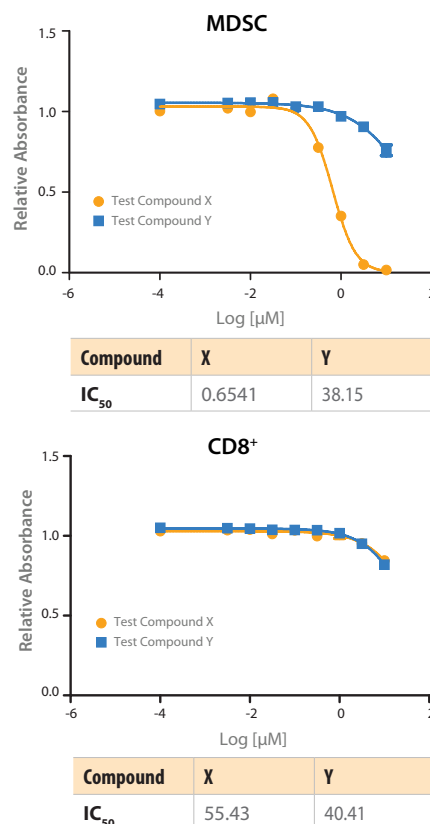
**Figure 3: CD8<sup>+</sup> Murine Immune Cell Separation and Subset Enrichment**



## Cell Proliferation Assays with Isolated Immune Cells

CrownBio provides *in vitro* CellTiter-Glo® assays on isolated immune cells to evaluate how novel immunotherapeutics affect cell proliferation in specific populations such as MDSC and CD8<sup>+</sup> T cells, and assess a compounds cytotoxic potential on subsets of immune cells (**Figure 4**).

**Figure 4: Test Compound Effects on MDSC and CD8<sup>+</sup> T Cells**



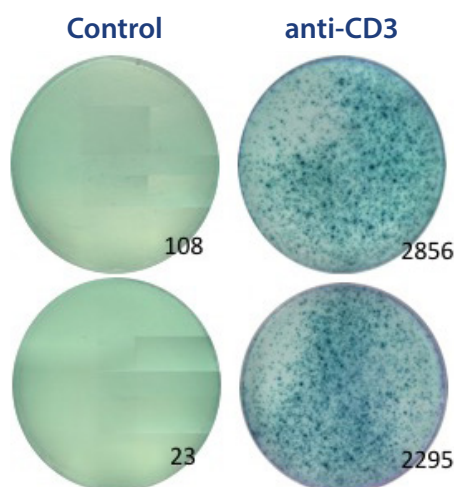
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## Cytokine Profiling

We provide multiple platforms for quantifying human and murine cytokines and chemokines, and the frequency of cytokine-producing cells:

- ELISA: for evaluating proinflammatory cytokine panels, commonly from serum or cell supernatant
- ELISPOT: for visualization of the secretory molecules of individual stimulated or responding cells, providing qualitative (cytokine/chemokine specific) and quantitative (number of responding cells) detection and analysis. Example ELISPOT data for increased IFN- $\gamma$  production following *in vitro* T cell stimulation using anti-CD3 antibody is shown in **Figure 5**.
- Luminex™ assays including utilizing the Cytokine Mouse Magnetic 20-Plex Panel to simultaneously quantify 20 murine cytokines, chemokines, and growth factors in serum, plasma, and tissue culture supernatant.

**Figure 5: ELISPOT IFN- $\gamma$  Release *In Vitro* Assay Following T Cell Stimulation**



## T Cell Receptor Sequencing

T cell receptors undergo dynamic rearrangements that allow the adaptive immune system to recognize and respond to a vast range of antigens. High throughput immunosequencing identifies millions of T cell receptors in a single sample enabling quantification of T cell diversity at an unprecedented level<sup>(2)</sup>. T cell receptor sequencing can be used to evaluate T cell differences and clonality in response to immunotherapeutic treatment<sup>(2)</sup>, as well as in the development of biomarkers and diagnostic tools<sup>(3)</sup>.

CrownBio provides advanced analysis of T cell receptor sequencing and T cell clonality, based on the understanding of the enumeration of T cells (using FACS) and the clonal expansion of T cell subsets (through sequencing)<sup>(2)</sup>.

## Functional Assays: Antigen Presentation and Immune System Activation

CrownBio offers a variety of assays for assessing the immunogenicity of experimental proteins and peptides *in vitro* and *ex vivo*, and to investigate the mechanism of action of novel cancer immunotherapy agents (**Figure 6**). Our assays are customized to target immune cells, including T and NK cells, macrophages, and dendritic cells, as well as tumor cells.

**Figure 6: CrownBio's Antigen Presentation and Immune System Activation Functional Assay Platform**

Antigen Presentation and Immune System Activation			
T Cell Targets and Activation	Macrophage and Dendritic Cell Targets	NK Cell Targets	Tumor Targets
<ul style="list-style-type: none"><li>• T cell proliferation and survival including MLR</li><li>• Recalled T cell function</li><li>• T cell migration</li><li>• Target expression profiling</li><li>• Cytokine profile modulation</li></ul>	<ul style="list-style-type: none"><li>• Target expression profiling</li><li>• Modulation of M1/M2/MDSC like differentiation</li><li>• IDO modulation</li><li>• DC migration</li></ul>	<ul style="list-style-type: none"><li>• Target expression of NK cells</li><li>• Modulation of activation and killing</li></ul>	<ul style="list-style-type: none"><li>• Target expression profiling</li><li>• IDO/TDO modulation</li><li>• Effects on antigen presentation/killing assays</li><li>• Checkpoint ligand expression modulation</li><li>• Tumor derived immune modulating factors modulation</li><li>• Direct cytotoxicity</li></ul>

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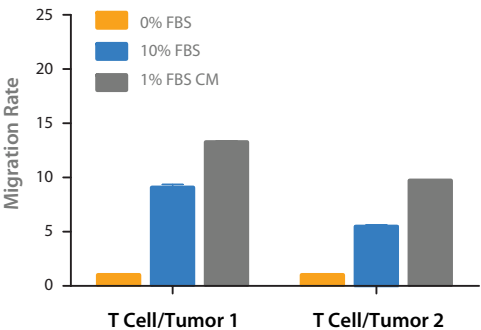


## T Cell Targets and Activation

We provide a range of assays to assess T cell activation, function, and activity, and to evaluate how this is modulated *in vitro* by test immunotherapeutic agents. Individual assay types are discussed below, and summarized in **Figure 6**.

Migration assays via the transwell system are performed by CrownBio, to evaluate T cell migration under stimulus from conditioned medium (CM). Cultured syngeneic tumor cells and the CM are collected, and used to stimulate T cells isolated from splenocytes derived from animals injected with the same syngeneic tumor cells. Migration transwell assay data is shown in **Figure 7** for two example tumors.

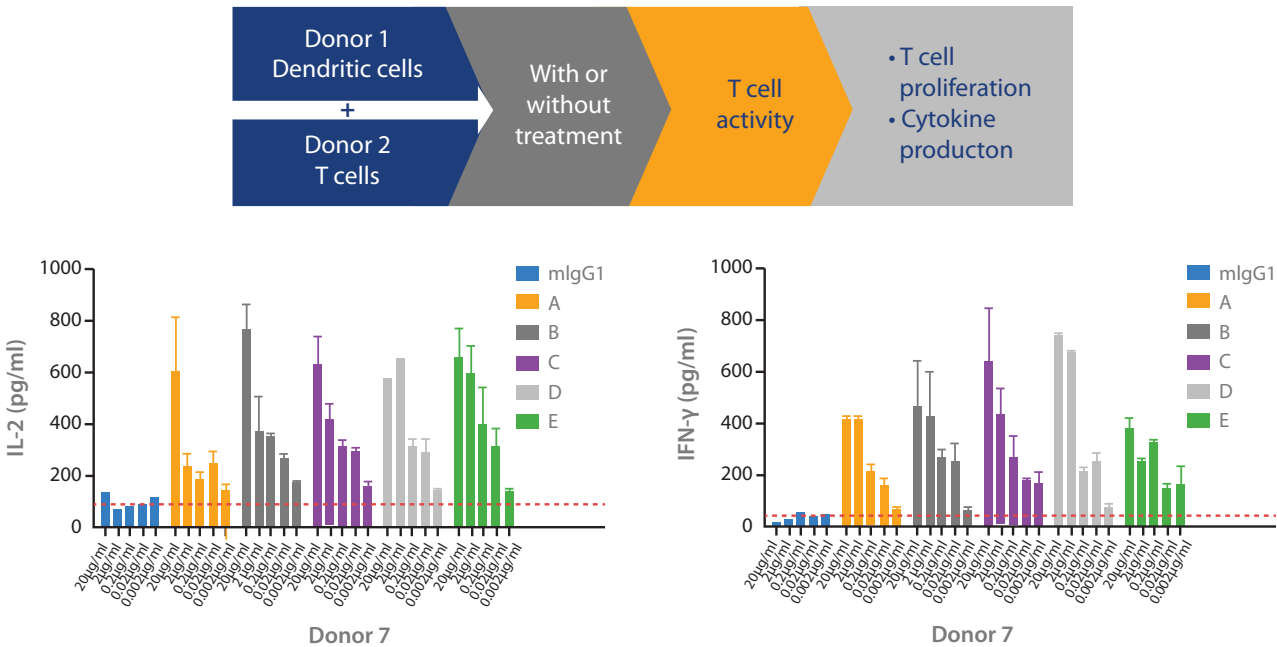
**Figure 7: T Cell Migration Under Stimulation from Conditioned Media, via Transwell Migration Assay**



T cell proliferation assays are the gold standard of measuring T cell activation following *in vitro* stimulation, and we offer both colorimetric (MTT) and dye based (CFSE) approaches. CrownBio also provides the Mixed Lymphocyte Reaction (MLR) functional assay, which allows the rapid identification of agents that modulate T cell activation, through the measurement of T cell proliferation and cytokine production. We use two separate donors to provide stimulator allogeneic dendritic cells and responder T cells, which are combined with or without antibody treatment to assess T cell response (assay principle and example data for test antibody effect on cytokine production is shown in **Figure 8**).

**Figure 8: Effect of Test mAbs on Human IL-2 and IFN- $\gamma$  Production, Assayed by MLR**

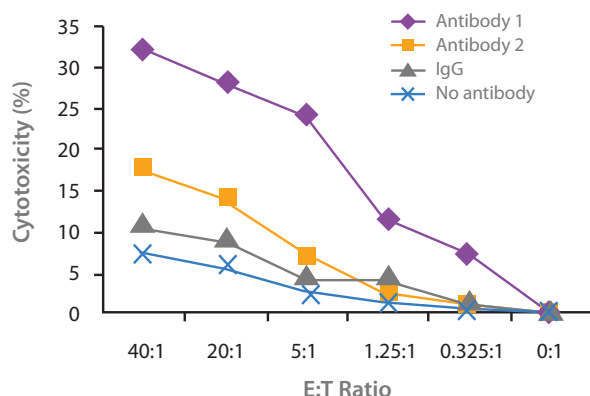
Assay principle and example MLR data with allogeneic DC cells.



# In Vitro and Ex Vivo Immuno-Oncology Platform Application Note

How novel agents affect cytotoxic T lymphocyte (CTL) activity can be evaluated by the recalled T cell function assay. Tumor-bearing mice are treated with novel antibodies which stimulate the immune system, causing tumor rejection. Splenocytes are then cultured, and T cells isolated which are used to assess tumor cell killing *in vitro* (Figure 9).

**Figure 9: CTL Activity Analysis for Novel Immunotherapeutics Evaluated via Recalled T Cell Function Assay**



## Further Cellular and Tumor Targets

CrownBio also provides a range of functional assays for other targets (summarized in Figure 6):

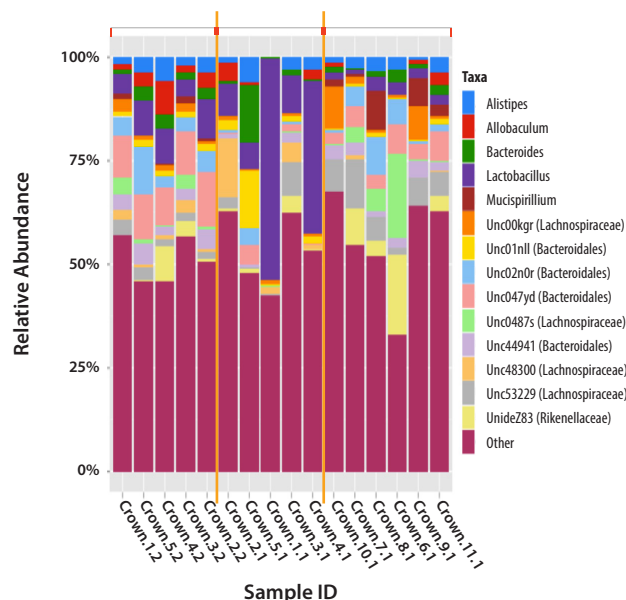
- Macrophage and dendritic cells (including the transwell migration assay discussed above for dendritic cells)
- NK cells
- Tumor

## Functional Assays: Regulation of Tumor Environment

Microbiota play an important role in determining an organism's response to anticancer treatment, even in tumors far from the gastrointestinal tract, possibly because of their proinflammatory properties which activate the immune system. For example, commensal *Bifidobacterium* has been shown to promote antitumor immunity and facilitate anti-PD-L1 efficacy against melanoma xenografts<sup>(4)</sup>.

In order to gain insights into the complex interaction between the microbiome and cancer therapy, CrownBio provides fecal collection and microbiome profiling (16S rRNA sequencing) to compare and evaluate changes in gut microbiomes across our syngeneic models (example data shown in Figure 10). A wide range of standard of care and immunotherapeutic agents have been trialed on our syngeneic models, allowing correlation of microbiome analysis data with response to therapy.

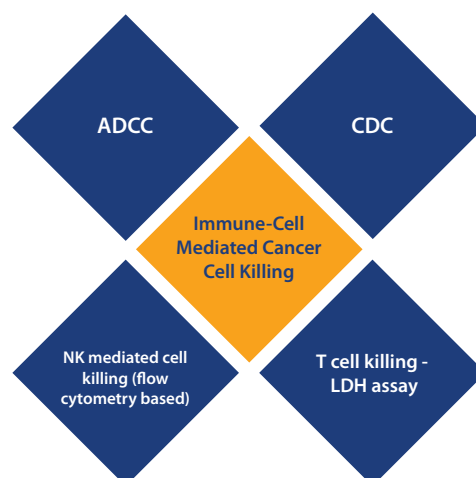
**Figure 10: Comparison of Gut Microbes Across Syngeneic Models**



## Functional Assays: Immune Cell-Mediated Cancer Cell Killing

Enhancement of effector cell cancer killing functions can be achieved by appropriately priming the immune system with targeted antibodies, and the use of agents such as monoclonal antibodies (mAbs) in oncology has been a successful and important strategy for treating cancer patients<sup>(5)</sup>. At CrownBio we provide a variety of *in vitro* immune cell mediated cancer cell killing strategies, with multiple endpoint readouts including flow cytometry and LDH assay, to evaluate the cytotoxic effects of novel agents (Figure 11).

**Figure 11: CrownBio's Immune-Cell Mediated Cancer Cell Killing Platform**





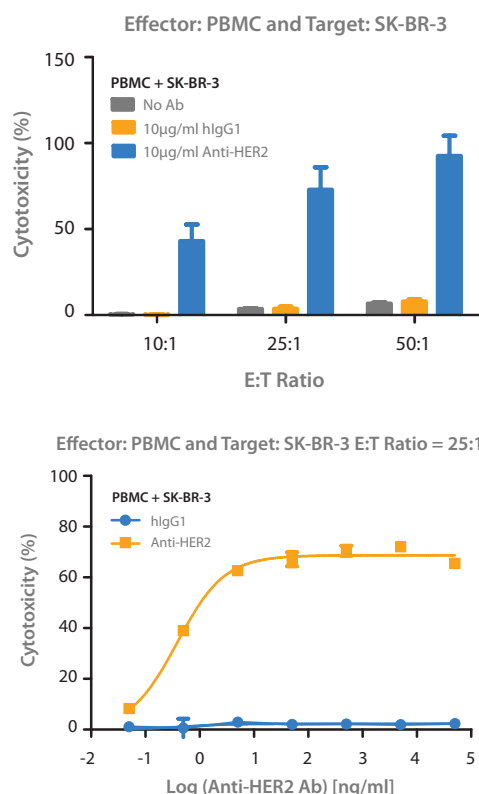
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## Antibody Dependent Cell-Mediated Cytotoxicity with PBMC Effector Cells

CrownBio has established an ADCC platform to provide a high quality assay for determining the cytotoxic activity of selected antibodies against specified target cell lines, using effector PBMC.

**Figure 12** exemplifies our ADCC assay showing the cytotoxicity of an anti-HER2 antibody against the human breast cancer cell line SK-BR-3, which overexpresses the HER2/c-erb-2 gene product<sup>(6)</sup>.

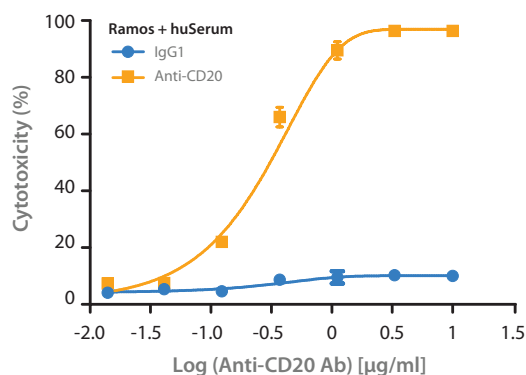
**Figure 12: ADCC Evaluation of Cytotoxicity of Anti-HER2 Antibody Against SK-BR-3 Cell Line**



## Complement Dependent Cytotoxicity

CrownBio's CDC assay measures the lysis of target tumor cells following the addition of novel test mAbs, based on the antibody triggering complement cascade activation, which results in membrane attack complexes being inserted on the tumor causing cell death. Our CDC assays are fully optimized for complement concentration used in the serum, with optimal concentrations used to evaluate cytotoxicity of novel mAbs. Example CDC data is shown in **Figure 13** for the Ramos Burkitt's lymphoma cell line treated with an anti-CD20 antibody.

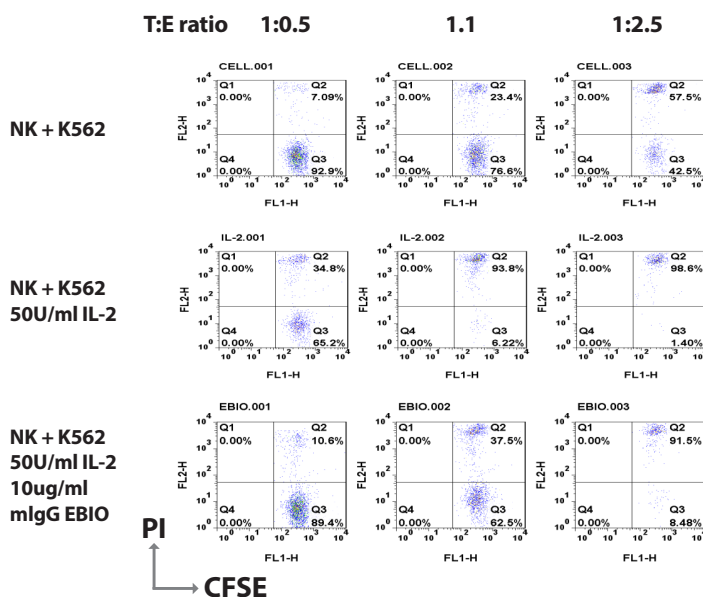
**Figure 13: CDC Evaluation of Cytotoxicity of Anti-CD20 Antibody Against Ramos Cell Line**



## NK Mediated Cell Killing Flow Cytometry Based Assay

CrownBio has developed an NK mediated cell killing assay using flow cytometry analysis for cytotoxicity. Our assay is based on target cell labeling with CFSE and subsequent DNA-labeling with PI for identification of target cells with compromised cell membranes, which provides a measure of cytotoxicity of novel agents. Our assay has been validated with K562 leukemia cells, which are sensitive to NK-cell mediated lysis which is stimulated by IL-2 addition, and which can be blocked by concurrent addition of mlgG (**Figure 14**).

**Figure 14: NK Mediated Cell Killing of K562 Cells Stimulated by IL-2**



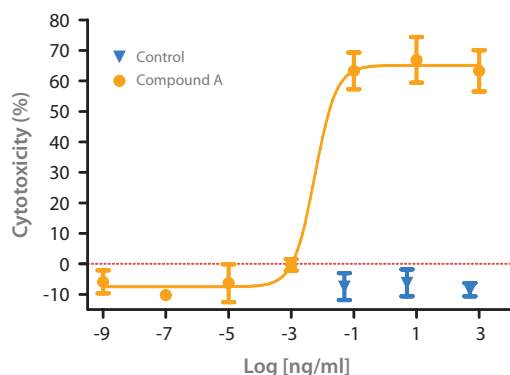
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## T Cell Killing LDH Assay

T cell mediated cell death can be indirectly measured through the release of lactate dehydrogenase (LDH) by cells enduring membrane damage. We have validated our T cell killing LDH assay to evaluate both single agent and combination regimens, combining T cells isolated from human PBMCs with target tumor cells (example data shown in **Figure 15** for the SU-DHL-4 B cell lymphoma treated with a test agent).

**Figure 15: T Cell Killing LDH Assay: SU-DHL-4 Cell Line**

T cells and tumor cells plated at E:T of 10:1.



## Novel Immuno-Oncology Targets: IDO and TDO

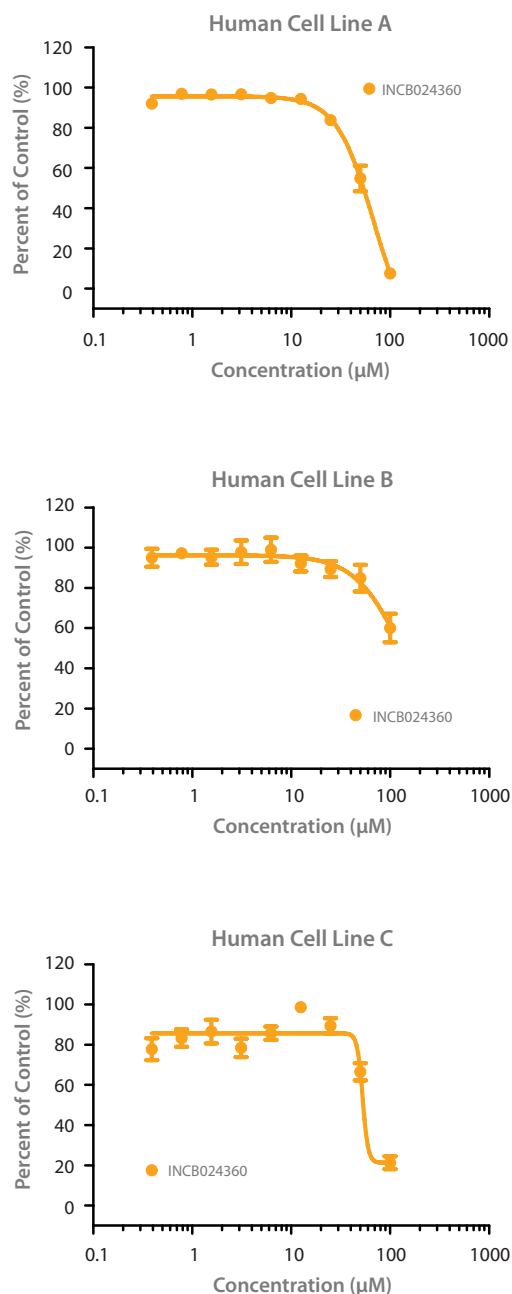
Immuno-oncology is a fast paced field, and the identification and validation of novel targets is vital to new agent development. At CrownBio we are constantly enhancing our *in vitro* immuno-oncology platforms, and now provide a variety of assays to evaluate IDO and TDO modulation, as well as IDO inhibitor effects on human and murine tumor cell proliferation *in vitro*.

Tryptophan (Trp or L-Trp) is an essential amino acid with a variety of biological functions, the catabolism of which is a central pathway maintaining the immunosuppressive microenvironment in many types of cancers<sup>(7)</sup>. Cancer cells overexpress the activated forms of indoleamine 2,3-dioxygenases (IDO1 and IDO2) and tryptophan 2,3-dioxygenase (TDO) enzymes that catalyze the first and rate limiting step of the kynurenine (Kyn) Trp degradation pathway. This results in the depletion of Trp and subsequent inhibition of T cell response, creating an immunotolerant environment where cancer cells can survive, grow, invade, and metastasize<sup>(8)</sup>.

Our range of IDO and TDO assays include:

- IDO expression modulation via IFN- $\gamma$ , in both human and murine cell lines
- IDO modulation via tryptophan catabolism assays (for macrophage and dendritic cells, as well as tumor targets)
- TDO modulation via tryptophan catabolism assays (for tumor targets)
- Cell proliferation assays with IDO1 inhibitors, in both human and murine cell lines (**Figure 16**).

**Figure 16: Cell Proliferation Assays of Human and Cancer Cell Lines with IDO1 Inhibitor**





# In Vitro and Ex Vivo Immuno-Oncology Platform Application Note

## Summary

As immunotherapy continues to grow and further influence cancer standard of care treatments, enhanced early stage drug development is required to fully characterize potential new agents and their immunomodulation and efficacy, and to provide robust information on which drugs should be carried forward to *in vivo* evaluation.

CrownBio has validated an *In Vitro* and *Ex Vivo* Immuno-Oncology Research Platform for preclinical drug development, covering a range of functions, and providing key decision making data.

Our core *in vitro* immuno-oncology phenotyping and profiling platform provides a comprehensive range of assays for the full monitoring of the immune system and response to test immunotherapeutics. This includes flow cytometry and IHC for immuno-oncology agent characterization, as well as cytokine profiling, cell proliferation assays, and immune cell purification.

We also offer a full *in vitro/ex vivo* functional assay platform – built around antigen presentation and immune system activation (with assays customized to target immune cells, including T cells, natural killer cells, macrophages, and dendritic cells, as well as tumor cells), regulation of the tumor environment via microbiome analysis, and immune-cell mediated cancer cell killing including ADCC, CDC, and multiple assay readouts.

CrownBio also has a firm commitment to furthering immuno-oncology research, including the validation and identification of novel targets. We now provide a variety of assays to evaluate IDO and TDO modulation, as well as IDO inhibitor effects on human and murine cancer cell proliferation *in vitro*.

## References

<sup>1</sup>Cancer Research Institute Website. Cancer Immunotherapy <http://www.cancerresearch.org/cancer-immunotherapy> Accessed 16 June 2016.

<sup>2</sup>Adaptive Biotechnologies website. immunoSeq overview <http://www.adaptivebiotech.com/immunoseq> Accessed 08 September 2016.

<sup>3</sup>Woodsworth DJ, Castellarin M, Holt RA. Sequence analysis of T-cell repertoires in health and disease. *Genome Medicine* 2013;5(10): 98.

<sup>4</sup>Sivan A, Corrales L, Hubert N *et al.* Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy. *Science* 2015;350(6264): 1084-1089.

<sup>5</sup>Yamashita M, Kitano S, Aikawa H *et al.* A novel method for evaluating antibody-dependent cell-mediated cytotoxicity by flowcytometry using cryopreserved human peripheral blood mononuclear cells. *Scientific Reports* 2016;6: 19772.

<sup>6</sup>ATCC website. SK-BR-3 [SKBR3] (ATCC® HTB-30™) Characteristics [https://www.lgcstandards-atcc.org/Products/All/HTB-30.aspx?geo\\_country=gb#characteristics](https://www.lgcstandards-atcc.org/Products/All/HTB-30.aspx?geo_country=gb#characteristics) Accessed 09 September 2016.

<sup>7</sup>Platten M, von Knebel Doeberitz N, Oezen I *et al.* Cancer Immunotherapy by Targeting IDO1/TDO and Their Downstream Effectors. *Frontiers in Immunology* 2014;5: 673.



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