# Novel Bioluminescent Tools for the Assessment of ADC Fc Function and Bystander Killing

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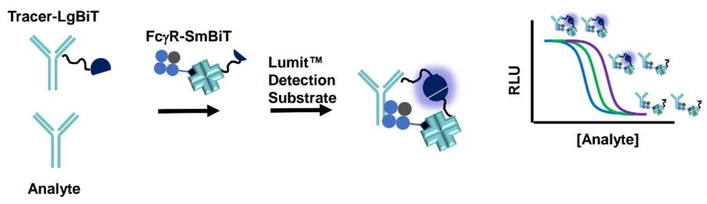
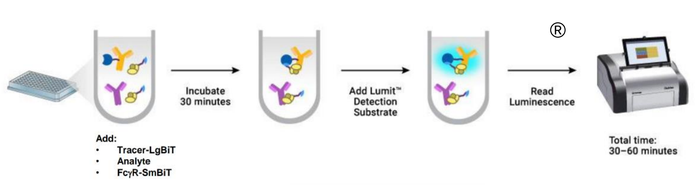
## 1. Introduction

Antibody Drug Conjugates (ADC) are complex drugs with multiple mechanisms of action (MoA), which may include direct cytotoxicity following internalization by antigen-expressing cells, killing of bystander cells by cleaved payload, blockade of antigen function, and induction of immune-mediated cytotoxicity (such as ADCC and ADCP) by the antibody Fc region. Complete characterization and potency testing of ADC requires a matrix of assays that reflect each MoA. To streamline the characterization and potency testing of ADC, we have developed a suite of bioluminescent tools for measurement different aspects of ADC function.

## 2. Lumit™ FcR Binding Assays




**Assay Principle:**

- LgBiT and SmBiT have low affinity, and negligible association in solution
- Proximity-induced complementation by Tracer-LgBiT binding to FcR-SmBiT produces bright luminescent NanoBiT® enzyme
- Analyte (antibody or ADC) competes for FcR binding, resulting in loss of signal

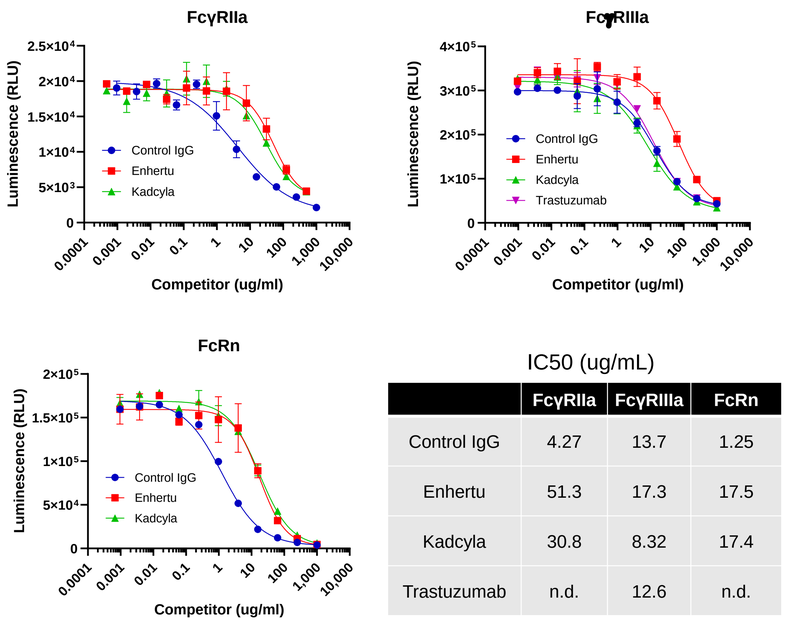
**Workflow:**

- Incubate Tracer-LgBiT, FcR-SmBiT, and Analyte in assay plate for 30 min.
- Add Lumit™ Detection Substrate
- Read luminescence

**Benefits:**

- Solution-based, no immobilization
- Homogeneous, no-wash protocol
- >60 min. workflow
- Wide dynamic range
- Small sample volume required

## 3. Use of ADCs in Lumit™ FcR Binding Assays

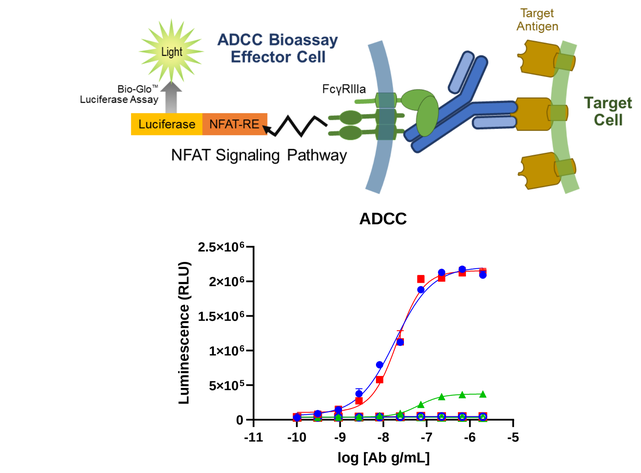


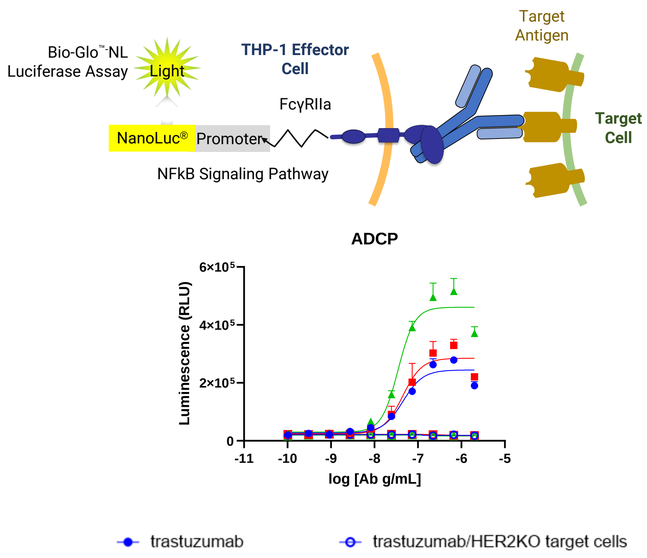

IC50 (ug/mL)

| | FcγRIIa | FcγRIIIa | FcRn |
|---|---|---|---|
| Control IgG | 4.27 | 13.7 | 1.25 |
| Enhertu | 51.3 | 17.3 | 17.5 |
| Kadcyla | 30.8 | 8.32 | 17.4 |
| Trastuzumab | n.d. | 12.6 | n.d. |

ADCs were tested in Lumit™ FcR Binding Assays in parallel with control IgG or unconjugated trastuzumab. Enhertu and Kadcyla showed reduced binding to FcγRIIa and FcRn relative to control IgG. Only Enhertu showed reduced binding to FcγRIIIa.

## 4. Measurement of ADCC and ADCP Activity with Cell-Based Reporter Bioassays



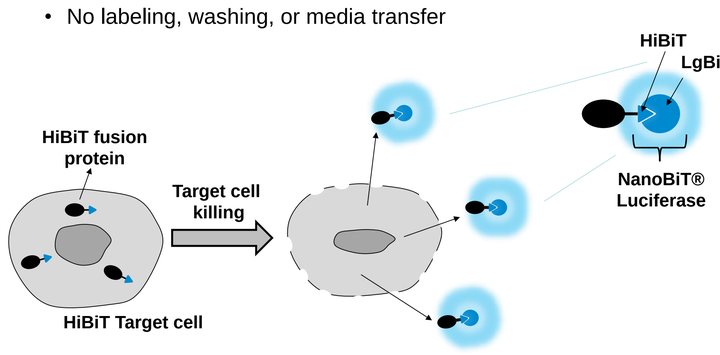




ADCC or ADCP effector cells were combined with SKOV3 target cells, or SKOV3/HER2KO target cells, as indicated, and serial dilutions of trastuzumab or ADCs were added. After 6 hours incubation, detection reagents were added and luminescence was read on a Glomax® Discover luminometer. Luminescent signal was fully dependent on antigen expression by the target cell. Trastuzumab and Kadcyla performed similarly in the assays, while Enhertu had lower ADCC activity and elevated ADCP activity relative to the unconjugated antibody.

## 5. Bystander Killing Assay

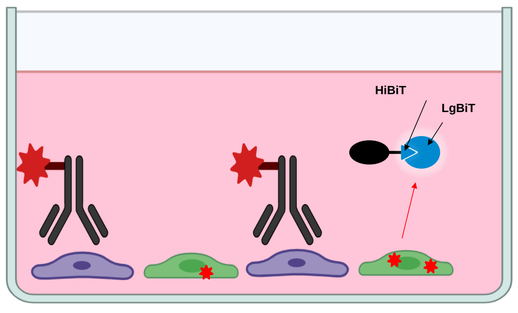
**HiBiT Target Cell Killing:**

- Engineered target cells express intracellular HiBiT reporter
- HiBiT is released from lysed cells
- Extracellular HiBiT binds LgBiT with high affinity, forming a bright luminescent enzyme
- Luminescent signal is proportional to cell death.
- No labeling, washing, or media transfer

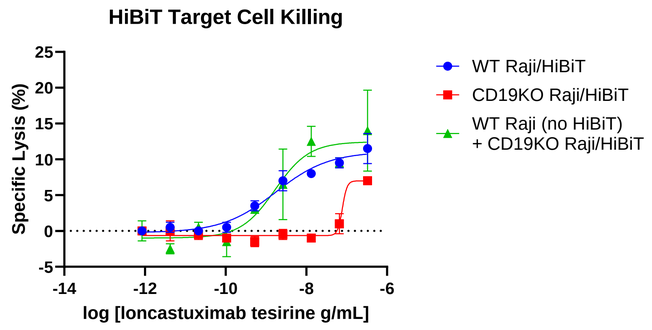



**Bystander Killing Assay Principle**:

- ADC binds **wild type** cell and payload is released
- Payload kills **HiBiT-expressing reporter** cell
- Luminescence is specific to bystander killing

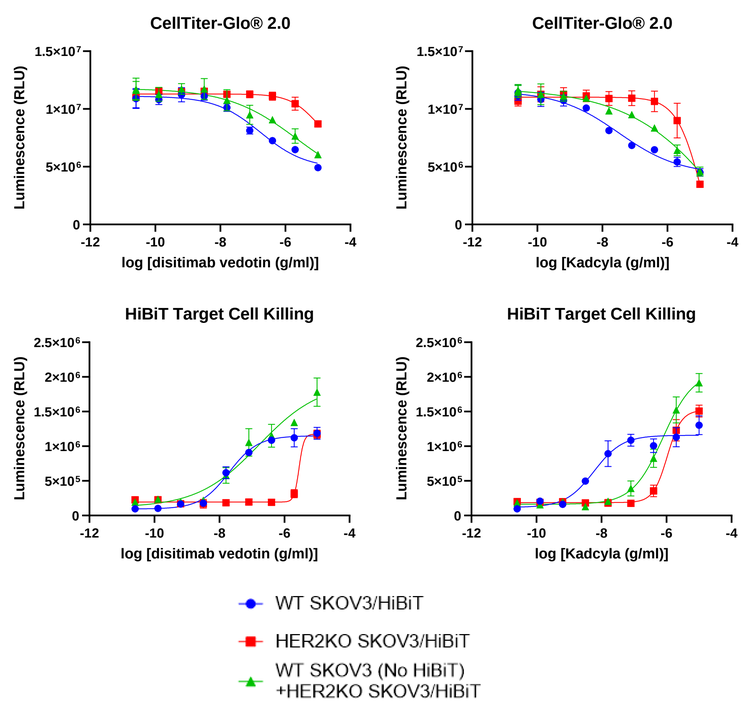



## 6. Detection of Bystander Killing by Loncastuximab Tesirine




Raji cells were incubated for 96 hours with a serial dilution of the anti-CD19 ADC loncastuximab tesirine, then extracellular HiBiT was detected with Bio-Glo-NB™ TCK detection reagent. Blue indicates direct lysis of Raji/HiBiT cells (EC50 1.7 ng/mL). Red indicates off-target lysis of CD19KO Raji/HiBiT cells at high concentrations (EC50 78 ng/mL). When Raji cells not expressing HiBiT are mixed at an 8:1 ratio with CD19KO Raji/HiBiT cells (Green), bystander killing is observed (EC50 1.7ng/mL).

## 7. Bystander Killing Assay can Distinguish Between ADC With and Without Bystander Killing Activity




SKOV3 (WT, HiBiT, or HER2KO/HiBiT) cells were incubated for 96 hours with serial dilutions of the anti-HER2 ADCs disitimab vedotin and Kadcyla (ado-trastuzumab emtansine.) In parallel plates, total cell death was detected with CellTiter-Glo® 2.0 (upper row) and death of HiBiT-expressing cells was detected with Bio-Glo-NB™ TCK (lower row).

CellTiter-Glo® 2.0 was unable to distinguish between ADC with (disitimab vedotin) and without (Kadcyla) bystander killing properties.

Bystander killing can be specifically detected with Bio-Glo-NB™ TCK. Direct lysis of SKOV3/HiBiT cells is indicated in blue. Red indicates off-target lysis of HER2KO SKOV3/HiBiT cells at high concentrations of drugs. When SKOV3 cells not expressing HiBiT are mixed at a 10:1 ratio with HERKO SKOV3/HiBiT cells (Green), the curve shifts to the left for disitimab vedotin, but not Kadcyla, reflecting differential bystander killing activity of the two drugs

## 8. Conclusions

**Lumit™ FcR Binding Assays**

- Rapidly measure interaction between ADC and Fc receptors, including FcRn
- Homogeneous add-mix-read workflow with no washing
- Amenable to 384 well assay plates

**ADCC and ADCP Reporter Bioassays**

- Cell-based assay directly measures Fc effector functions
- Suitable for QC/Lot Release testing

**Bystander Killing Assay**

- Measures killing of antigen-negative bystander cells in mixed culture
- Distinguishes ADC with and without bystander killing activity
- No washing, labeling, or media transfer



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