Bioluminescent Assays for Antibody-Drug Conjugate Development: Internalization and Bystander Killing Functions

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1. Introduction

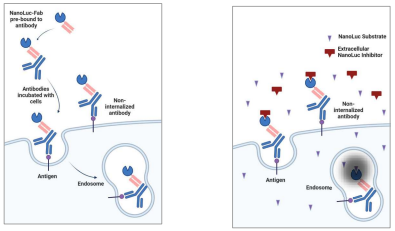
Antibody-drug conjugates (ADCs) combine targeted delivery with potent cytotoxic payloads, utilizing multiple mechanisms of action (MoA). These include direct cytotoxicity following internalization of the ADC by antigen-expressing cells, killing of bystander cells through cleaved payloads, antigen function blockade, and immune-mediated cytotoxicity (e.g., ADCC and ADCP) driven by the antibody Fc region. To address the need to capture MoA of ADCs, we developed bioluminescent tools to streamline ADC evaluation, focusing on antibody internalization and bystander killing. Studying bystander killing is particularly important in immunotherapy strategies for cancer, as it allows ADCs to target heterogenous tumors where not all cells express the antigen.

The internalization assay employs luciferase-labeled Fab fragments with a quenching mechanism to measure intracellular antibody localization. The assay was validated using HER2-expressing SKOV3 cells with anti-HER2 monoclonal antibodies as model systems. It shows strong time- and antigen-dependent luminescence, achieving over 100-fold signal increases compared to controls, enabling quantitative ADC screening. Additional assays include FcR binding assays and cell-based reporter bioassays for ADCC and ADCP activities. The HiBiT technology is a luminescent tagging system that enables precise and sensitive detection of protein interactions and cellular events, making it a powerful tool for evaluating Target Cell Killing (TCK) by effectors and cytotoxic payloads carried by antibodies. To assess payload efficacy, a HiBiT enabled bystander killing assay was developed, distinguishing ADCs with varying payload properties.

Taken together, the internalization assay and bystander killing assay based on bioluminescent platforms, offer a high-throughput solution for evaluating ADC functionality. With exceptional sensitivity, broad dynamic range, and efficient workflows, these tools enable precise optimization of therapeutic efficacy, delivering significant value for preclinical ADC development.

2. NanoLuc® Internalization Assay Principle

Antibody Internalization **Internalization Measurement**



Assay Principle:

1. Test antibodies are pre-incubated with a Nanoluc® -labeled Fab fragment against human Fc. Complexes are then added to cells and incubated to allow internalization.
2. At desired time points, media is exchanged to remove free antibody, and a non-lytic detection reagent is added.
3. Extracellular NanoLuc® Inhibitor in the detection reagent quenches signal from non-internalized antibody, while the membrane permeable substrate diffuses into endosomes.
4. Digitonin lysis allows the inhibitor to quench intracellular NanoLuc®. The drop in signal quantifies internalized antibody, since any residual signal from extracellular antibody is normalized away.

3. Sensitive Quantification of Internalized Antibody

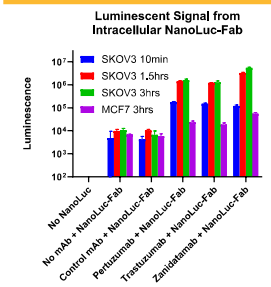


Figure 2. Anti-HER2 mAbs generate time-dependent increase in signal. Primary antibodies were pre-incubated with NanoLuc-Fab and incubated with SKOV3 (HER2+) or MCF7 (HER2-) cells for various amounts of time before performing the NanoLuc Antibody Internalization Assay. The three anti-HER2 antibodies (Pertuzumab, Trastuzumab, and Zanidatamab) show time-dependent increases in levels of internalized antibody, with low signals for the control cell line not overexpressing HER2.

4. ADCs Exhibit Near Identical Internalization to Parent Antibody

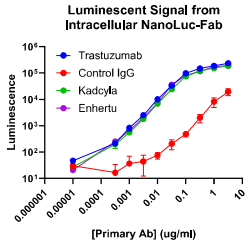
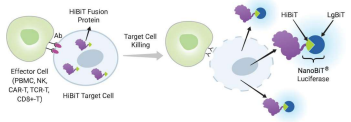


Figure 3. Titrations of ADCs Complexed with NanoLuc Fab. Kadcyla®, Enhertu®, Trastuzumab®, or control IgG were incubated with NanoLuc®-Fab at a 2:1 ratio for 15min, serially diluted into media, and added to SKOV3 cells. After 3hr incubation, cells were washed once before adding assay reagent.
After the first luminescence measurement, cells were permeabilized with digitonin, allowing the extracellular NanoLuc® inhibitor to inhibit internalized NanoLuc®-Fab. The difference between the two luminescence measurements is plotted as the signal from intracellular NanoLuc®

5. Bystander Killing Assay Principle

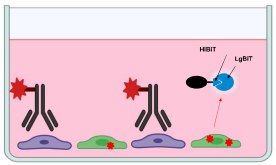


HiBiT Target Cell Killing:

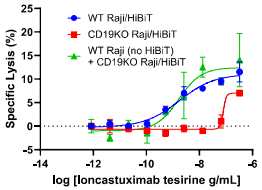
- Engineered target cells express intracellular HiBiT reporter
- Upon cell lysis HiBiT released and binds LgBiT with high affinity, forming luciferase
- Luminescent signal is proportional to cell death.
- No labeling, washing, or media transfer

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

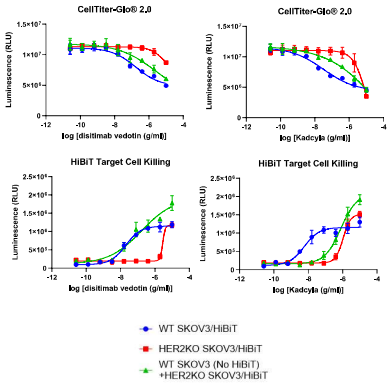


6. Detection of Bystander Killing by Loncastuximab Tesirine



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 → direct lysis of Raji/HiBiT cells (EC50 1.7 ng/mL). Red→ off-target lysis of CD19KO Raji/HiBiT cells at high conc (EC50 78 ng/mL).
Green→ Raji cells not expressing HiBiT 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

We have developed Bioluminescent Assays for characterizing ADCs:

Luminescent cell-based internalization assay for ADCs

- simple quantitative screening of both binding and internalization of unlabeled ADC
- Internalization of antibodies can be sensitively quantified by pre-binding an anti-Fc Fab chemically labeled with NanoLuc® luciferase

Bystander Killing Assay Using HiBiT Target Cell Killing (TCK) Cell lines

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

ADCC and ADCP Reporter Bioassays & Lumit™ FcR Binding Assays *(not shown here)*

- Cell-based assay directly measures Fc effector functions
- Binding Assays to measure interaction between ADC and Fc receptors, incl. FcRn
- Suitable for QC/Lot Release testing

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