NanoBiT Homogeneous Immunoassay: A Simple, Sensitive, and Rapid Method for Analyte Detection using Luminescent Signal

Nidhi Nath, Hicham Zegzouti, Martha O'Brien, Dan Lazar, Becky Godat, Rod Flemming, and Marjeta Urh

Promega Corporation, 2800 Woods Hollow Rd, Madison, WI 53711

1. Introduction

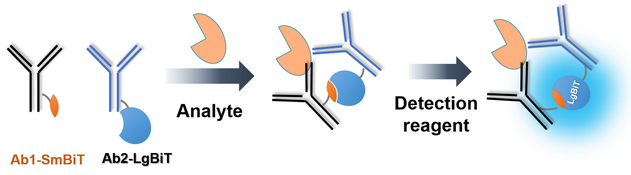
ELISAs are routinely used for protein detection, and although sensitive, these assays are time-consuming and involve multi-step processes. Several improvements in immunoassay technology have been made such as use of microfluidics, colored magnetic beads, energy transfer, automation, and so forth but they require expensive instrumentation and considerable expertise to implement.

We describe a novel NanoLuc Binary Technology (NanoBiT), which is a luminescent-based structural complementation reporter designed for biomolecular interaction studies. NanoBiT is used to develop a novel homogeneous immunoassay method (NanoBiT immunoassay), which is sensitive, rapid, and simple. NanoBiT immunoassays require wash steps and need only a simple luminometer for detection. We present several case studies, including detection of cell membrane protein receptors, cytokines, and therapeutic antibodies to demonstrate the specificity, sensitivity, and robustness of this assay format.

2. NanoBiT Homogeneous Assay

NanoLuc® Binary Technology (NanoBiT): The NanoBiT® system is composed of two small subunits, Large BiT (LgBiT; 18kDa) and Small BiT (SmBiT; 11 amino acid peptide), that have been optimized for stability and minimal self-association (weak affinity).

NanoBiT Homogeneous Immunoassay: Two antibodies (Ab1 & Ab2) are chemically labeled with SmBiT and LgBiT. In the presence of an analyte the two antibodies come in close-proximity and allow SmBiT and LgBiT to form an active enzyme and generate a bright luminescence signal proportional to analyte concentration.



Detection reagent: Nano-Glo Reagent for Immunoassay

3. Simple, Add-Mix-Read Format

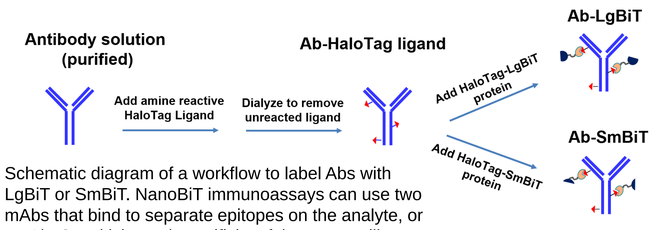
Step 1:
Add 20µl of sample

Step 2:
20µl of Ab1-LgBiT
+
20ul of Ab2-SmBiT

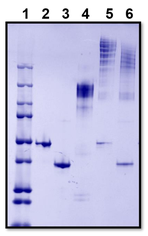
Incubate 5-30min

Step 3:
Add 20µl Nano-Glo Immunoassay detection reagent and read luminescence

4. Simple Method for Labelling Antibodies with SmBiT and LgBiT



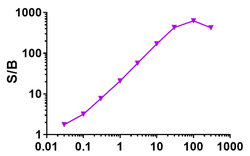
Schematic diagram of a workflow to label Abs with LgBiT or SmBiT. NanoBiT immunoassays can use two mAbs that bind to separate epitopes on the analyte, or a pAb. Sensitivity and specificity of the assay will depend on the antibodies.



Non-denaturing SDS-PAGE for anti-EGFR Ab labeling. Disappearance of 150kDa band for full-length Ab and appearance of high molecular weight bands indicate efficient labeling.

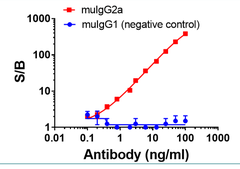
Lane	Protein
1	MW marker
2	HT-LgBiT
3	HT-SmBiT
4	Anti-EGFR pAb
5	anti-EGFR-LgBiT
6	anti-EGFR-SmBiT

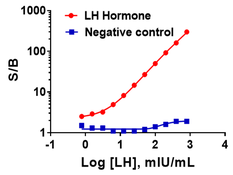
5. NanoBiT Direct Immunoassay



EGFR Calibration Curve
- Analyte: Human EGFR
- MW: 76kDa
- Detection Abs: anti-EGFR pAb labeled with LgBiT and SmBiT

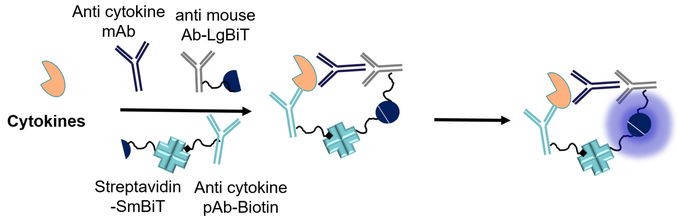
Mouse IgG2a calibration curve
- Analyte: Mouse IgG
- MW: 150kDa
- Detection Abs:
  - Anti-mouse pAb IgG-LgBiT
  - Anti-mouse mAb IgG2a-SmBiT





Hormone calibration curve
- Analyte: Hormone
- MW: 26kDa
- Detection Abs:
  - mAb1-LgBiT
  - mAb2-SmBiT

6. NanoBiT Indirect Immunoassays for Detection of Cytokines



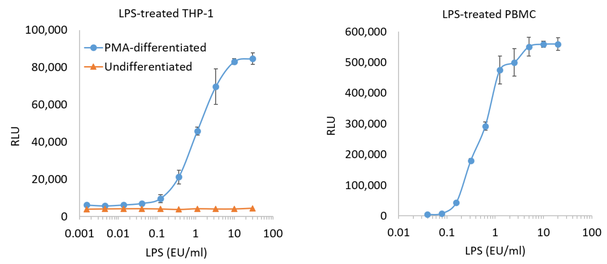
Schematic diagram of detection of variety of cytokines using indirect immunoassays. Sensitivity and specificity of the assay will depend on the antibodies.

1. Paired antibodies against cytokines available from R&D Systems were used.
2. Paired antibodies are:
   a. Biotinylated pAb
   b. MAb
3. Streptavidin was labeled with SmBiT.
4. Anti-mouse Ab was labeled with LgBiT.
5. Antibodies and Streptavidin were mixed at 1.0µg/ml and added to the substrate.
6. After 30-60min incubation detection reagent was added and luminescence signal is read.

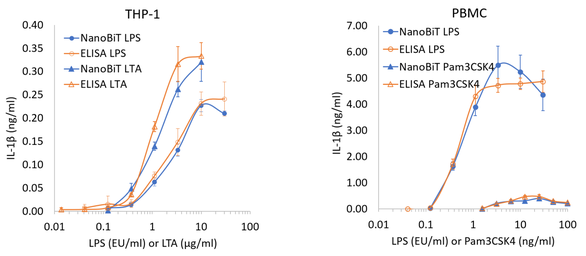
Cytokine	LOD (pg/mL)
VEGF	10-20
TNFa	20-40
IL-10	300-350
IL-6	10-20
IFN-γ	10-20
IL-2	10-20

LOD: Limit of detection

7. NanoBiT Direct Immunoassay for Detection of Released IL-1β from Cells

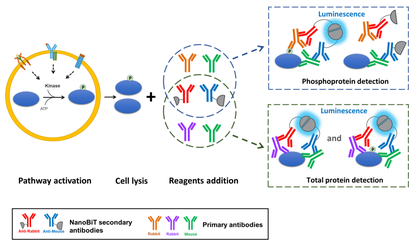


THP-1 cells (ATCC) in RPMI-1640 + 10% FBS were plated (5 x 10^4/well in 100µl) in 96-well plates and differentiated with 20nM phorbol myristate acetate for 2-3 days. The medium was replaced before treatment. PBMCs (BioIVT) were pooled from 4 donors and frozen. Upon thawing, cells were resuspended in RPMI-1640 + 10% FBS, plated (7.5 x 10^4/well in 100µl) in 96-well plates and treated. A titration of LPS was added to both cell types (5hr treatment for THP-1 cells and overnight treatment for PBMCs). After treatment, IL-1β release was monitored following the direct format.



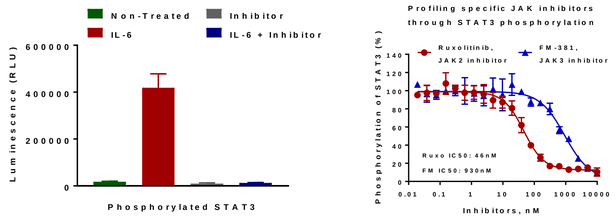
IL-1β released from THP-1 cells and PBMCs was measured comparing the NanoBiT® IL-1β immunoassay and the QuantiGlo® IL-1β ELISA (R&D Systems). The NanoBiT immunoassay detects the same amount of IL-1β as the QuantiGlo ELISA.

8. NanoBiT Cell-Based Kinase Immunoassay



1. Activate pathway
2. Lyse Cells
3. Add antibody mix
4. Incubate 90 minutes
5. Add Nano-Glo reagent
6. Read Luminescence

Monitoring IL6-Mediated Phosphorylation of STAT3 in A431 Cells



NanoBiT STAT3 immunoassay reveal the predicted biology of JAK/STAT pathway after IL6 treatment: Activation of STAT3 **phosphorylation** (pY705) and its **inhibition** with specific JAK inhibitors.

9. Conclusions

Current immunoassay methods like ELISAs have multiple time-consuming steps (e.g., washing and protein/antibody immobilization). NanoBiT immunoassays are solution-based and may minimize artifacts introduced by immobilization.

1. Assays are homogeneous (add-and-read) and require no washing.
2. Luminescence based detection provides wide dynamic range and large assay window
3. Assays are quick (5-30min) and requires low sample volume (5-20µl).
4. Use of 96/384 well white plates will enable flexible throughput and automation capabilities.

Corresponding author: nidhi.nath@promega.com