A Novel Peptide Tag Enables Simple and Sensitive Bioluminescent Quantification of Tagged Proteins

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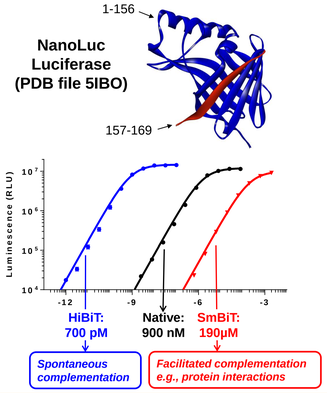
1. Introduction

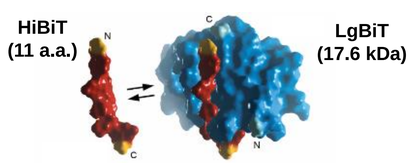
Dysregulation of protein expression is a key mechanism of tumorigenesis. The most commonly used approach to monitor changes in expression is to perform SDS-PAGE, followed by immunoblotting, a labor-intensive process that requires high-quality antibodies to detect proteins at endogenous levels of expression. We have developed a novel peptide tag utilizing NanoLuc® Binary Technology (NanoBiT), a binary complementation system based on NanoLuc® luciferase. The tag, designated High BiT (HiBiT), is only 11 amino acids in length, which minimizes potential interference with protein function. The amount of HiBiT-tagged protein is measured using a lytic detection reagent containing Large BiT (LgBiT), which binds with high affinity to HiBiT (K_D~1 nM) to reconstitute a bright, luminescent enzyme. HiBiT-tagged proteins can be quantified in cell lysates over 7 orders of magnitude of linear dynamic range with a limit of detection of less than 10^{-19} moles (3 fg of 30 kDa protein). The simple add-mix-read assay protocol can be completed in minutes, providing an assay that is compatible with high-throughput applications. The sensitivity of the assay allows quantification at endogenous levels of expression, and the small tag size is ideal for CRISPR-mediated genome editing. HiBiT-tagged proteins separated by SDS-PAGE can be detected on blots at sub-picogram levels with a detection reagent containing LgBiT. By eliminating the multiple steps of blocking, binding, and washing of traditional blotting techniques, the protocol takes minutes instead of hours. Additionally, the cell surface expression, internalization, or secretion of HiBiT-tagged proteins can be measured in minutes using a non-lytic detection reagent containing cell-impermeable LgBiT and furimazine. HiBiT represents a next-generation protein tag that allows simple quantification of proteins of interest in their cellular context and following SDS-PAGE.

2. NanoBiT™ Technology Overview



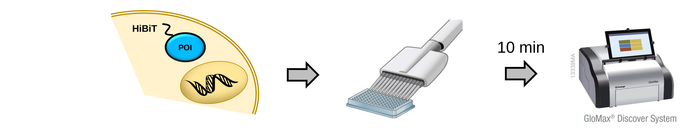
- Numerous split sites were identified with a low affinity for self-association
- Site between 156/157 selected for development
- 1-156 fragment evolved for enhanced structural stability to give Large BiT (LgBiT)
- Numerous peptides screened with LgBiT
- Highest affinity peptide, PEP86, was selected as High BiT (HiBiT)
- Lowest affinity peptide, PEP114, was selected as Small BiT (SmBiT), which is used for protein:protein interaction assays

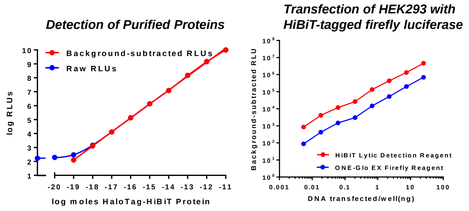
3. HiBiT Technology Overview



- High-affinity interaction between HiBiT and LgBiT (K_D = 700 pM) drives rapid binding of purified LgBiT in the reagent to HiBiT in the sample
- Interaction generates a bioluminescent enzyme that gives a bright, extended glow-type signal in the presence of furimazine substrate (>10-fold brighter than firefly luciferase)
- HiBiT can be fused to the N- or C-terminus of proteins, or even placed in accessible internal locations
- Enables lytic, live-cell, and blotting applications

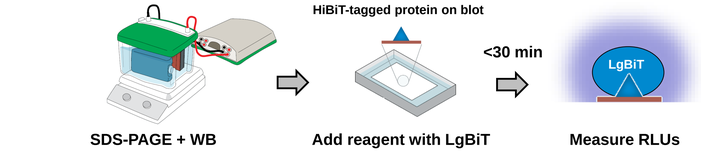
4. Simple, Sensitive, and Rapid Protein Quantification with Large Dynamic Range

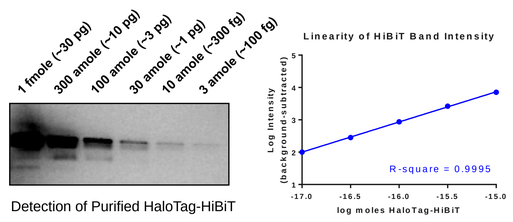




- Homogeneous assay format with measurement 10 minutes after reagent addition and signal stability for hours
- Much brighter than firefly luciferase for the sensitivity to measure endogenous levels of proteins
- Protein quantification in solution over 7 logs of linearity (<10 fg to >100 ng of a 30 kDa protein)

5. Sensitive Quantification of HiBiT-Tagged Proteins on Blots with a Simple Protocol

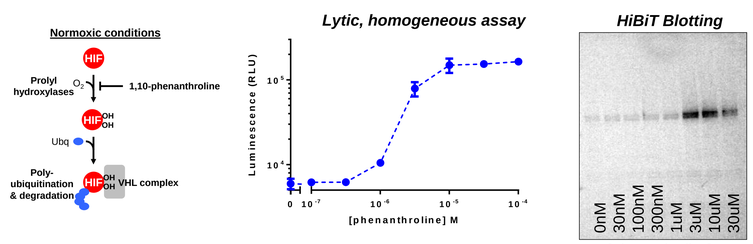




Detection of Purified HaloTag-HiBiT

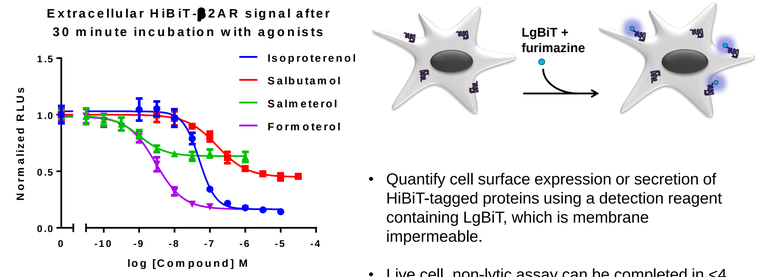
- Selectivity of NanoBiT™ complementation means no blocking, washing, or antibody addition steps are required, allowing quantification of HiBiT-tagged proteins on a blot in <30 minutes.
- Dynamic range of >6 logs (100 fg to >100 ng of a 30 kDa protein, with different exposure times)

6. Quantify Changes in Stability or Degradation of Proteins at Endogenous Levels



- Quantify changes in expression of HiBiT-tagged proteins at endogenous levels using lytic or blotting assays
- Regulated degradation of Hif1a is inhibited by 1,10-phenanthroline, a hypoxia mimetic (left panel).
- Transiently transfected HEK293 cells were treated with phenanthroline for 4 hours with Hif1a-HiBiT accumulation measured using lytic (middle panel) or blotting (right panel) applications

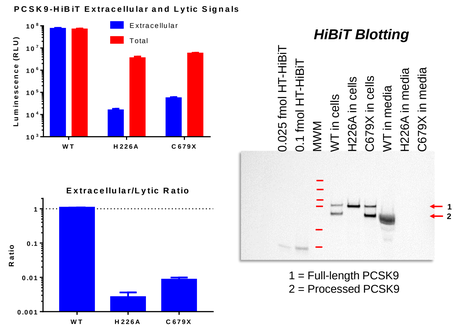
7. Measure Receptor Internalization in Minutes with a Live-Cell Non-Lytic Assay for Extracellular HiBiT



Agonist	EC50	Percentage Receptor Remaining on Surface
Isoproterenol	50.9 nM	16%
Salbutamol	161 nM	45%
Salmeterol	1.04 nM	63%
Formoterol	2.92 nM	16%

- Quantify cell surface expression or secretion of HiBiT-tagged proteins using a detection reagent containing LgBiT, which is membrane impermeable.
- Live cell, non-lytic assay can be completed in <4 minutes to measure rapidly changing biology at a particular time.
- Determine both ligand potency and efficacy for receptor internalization (left).

8. Monitor PCSK9 Processing & Secretion with HiBiT Extracellular, Lytic, and Blotting Assays



- PCSK9 undergoes auto-proteolytic processing in the ER and subsequent trafficking and secretion
- The H226A mutation blocks auto-proteolytic processing and secretion, while the C679X mutation blocks only secretion.
- HEK293 cells were transfected with HiBiT-tagged PCSK9 variants. After 24hrs, surface and total protein levels were measured by sequential addition of the HiBiT Extracellular Reagent and digitonin (left panels). Cell lysates and media were also analyzed by HiBiT blotting after SDS-PAGE (middle).

9. Conclusions

HiBiT is a small, directly detected peptide tag with the sensitivity to quantitatively analyze proteins at endogenous levels using simple workflows.

- **Small**
  - 11 amino acid size reduces any potential impact on fusion partner function
  - Small size greatly facilitates CRISPR/Cas9 workflow for genomic knock-ins
  - Perfect for insertion into viral genomes for infection/replication studies
- **Simple**
  - Homogeneous, add-and-read assay protocol
  - Measurement of surface-expressed or total HiBiT tag in 4-10 minutes
  - Blotting protocol with no blocking or washing steps
- **Quantitative**
  - >7-log linear dynamic range
  - Monitor regulated changes in protein stability or degradation
  - Precisely measure changes in the surface expression of receptors or other membrane proteins
- **Study Endogenous Biology**
  - Sub-attomole sensitivity enables measurement of proteins at endogenous levels
  - Use CRISPR/Cas9 to easily add HiBiT tag to endogenous locus (see Poster 4517)
  - With transient transfection, express at much lower levels for more physiologically relevant results