Promega

TECHNICAL MANUAL

Anti-HiBiT Monoclonal Antibody, XFD Fluorophore Conjugates

Instructions for Use of Products
N7221 and N7231

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Anti-HiBiT Monoclonal Antibody, XFD Fluorophore Conjugates

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Email Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

The High BiT (HiBiT) protein tag is an 11-amino-acid peptide that binds with high affinity to Large BiT (LgBiT) in NanoLuc® Binary Technology (NanoBiT) to reconstitute NanoBiT® Luciferase, a bright, luminescent enzyme (1,2). Proteins tagged with HiBiT can be easily quantified by homogeneous luminescent assays in multiple formats. Because of its small size and sensitive detection, HiBiT makes an ideal tag for insertion by genome-editing techniques like CRISPR-Cas9, resulting in endogenous expression of HiBiT-tagged proteins (3). The HiBiT tag can also be added to proteins using available HiBiT cloning vectors or directly to existing protein expression constructs by PCR-based or gene synthesis methods. The sequence and rights to synthesize the HiBiT tag can be obtained by reviewing and accepting the Terms and Conditions of Use at: **www.promega.com/HiBiT-Synthesis**

Anti-HiBiT Monoclonal Antibody is a potent and specific mouse monoclonal antibody (mAb) that binds to the HiBiT tag, enabling applications such as immunoblotting, immunofluorescence, immunoprecipitation and fluorescence-activated cell sorting (FACS). The antibody binds to HiBiT with high affinity, with a K_D in solution of approximately 6pM. Because of this high affinity and low background binding, many HiBiT-tagged proteins can be detected at endogenous expression levels.

Anti-HiBiT Monoclonal Antibody, XFD Fluorophore Conjugates[a] (Cat.# N7221, N7231), are fluorescently labeled derivatives of the Anti-HiBiT Monoclonal Antibody. These conjugates include XFD488 and XFD647, which are structurally identical to Alexa Fluor® dyes at the same wavelength. The fluorophore-conjugated antibodies are suitable for use in standard antibody-based detection protocols. This Technical Manual includes example protocols and representative data for FACS.

Table 1. Anti-HiBiT Monoclonal Antibody, XFD Fluorophore Conjugate, Specifications.

Concentration	0.5µg/µl
Storage Buffer	Phosphate-buffered saline (PBS, pH 7.3) with 50% glycerol
Conjugate	Anti-HiBiT Monoclonal Antibody XFD488 (Cat.# N7231) or Anti-HiBiT Monoclonal Antibody XFD647 (Cat.# N7221)
Clone Number	30E5
Host Species	Mouse
Antibody Form	Purified immunoglobulin
Isotype	IgG2c with kappa light chain
Purification Method	Protein A resin
Immunogen	Synthetic HiBiT peptide

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Anti-HiBiT Monoclonal Antibody XFD488	**100µg**	**N7231**
Anti-HiBiT Monoclonal Antibody XFD647	**100µg**	**N7221**

Storage Conditions: Store the antibody at –30°C to –10°C protected from light. Anti-HiBiT Monoclonal Antibody conjugates are provided as a sterile-filtered solution in PBS with 50% glycerol without sodium azide. The antibody is shipped ambiently. Upon receipt, centrifuge briefly to collect any solution present on the tube sides or cap. Antibody will remain a liquid at storage temperature.

3. Fluorescence-Activated Cell Sorting (FACS) with Live Cells

3.A. General Considerations

The HiBiT peptide tag can be fused to plasma membrane proteins via an extracellular terminus or by insertion into a surface loop. In these cases, antibody staining of living cells can be used to quantify cell-surface expression or to isolate clonal cell lines expressing the HiBiT-tagged protein. FACS analysis of cells expressing intracellular HiBiT will require fixation, as discussed in Section 4.

Materials to Be Supplied by User

- phosphate-buffered saline (e.g., DPBS, GIBCO® Cat.# 14190)
- FACS buffer (DPBS + 1% FBS or 1% BSA)
- fetal bovine serum (FBS)
- bovine serum albumin (BSA; e.g., Sigma, Cat.# A7979)
- VERSENE® (Thermo Fisher Scientific Cat.# 15040066)
- flow cytometer (e.g., BD LSRFortessa™ X-20 Cell Analyzer or BD Accuri™ C6 Plus Flow Cytometer)
- polystyrene centrifuge tube with filter cap (e.g., Corning® Falcon® Cat.# 352235)

3.B. Example Protocol

Include parental cells lacking expression of a HiBiT fusion protein as a negative control.

1. Prepare FACS buffer by combining DPBS with 1% (v/v) FBS. Alternatively, 1% (w/v) BSA can be used instead of FBS if serum-mediated receptor internalization is a concern.

Harvest and Count Cells

2. Dislodge adherent cells with VERSENE® or similar mild dissociation solution. Do not use trypsin to dislodge cells due to proteolytic cleavage of the HiBiT sequence. Gently scrape adherent cells from the surface of the flask, if needed.
3. Centrifuge at 300 × *g* for 5 minutes and aspirate supernatant.
4. Gently resuspend cell pellet in FACS buffer to 10^6 cells/ml.
5. Add 1ml of cell suspension per tube.
6. Centrifuge at 300 × *g* for 5 minutes and wash once in 1ml of FACS buffer.

Add Antibody

7. Dilute the Anti-HiBiT Monoclonal Antibody, XFD Fluorophore Conjugate, to 1µg/ml (500X dilution of stock) in FACS buffer.
8. Gently resuspend cell pellet from Step 6 by adding 50µl of primary antibody solution per tube.
9. Incubate tubes on ice for 30 minutes with periodic mixing. Protect samples from light for the remainder of protocol (e.g., cover with foil when samples are not in use). Keep live cell samples at 4°C and use ice-cold reagents to reduce receptor internalization.

Wash Cells

10. Add 1ml of FACS buffer per tube.
11. Centrifuge at 300 × *g* for 5 minutes and aspirate supernatant.
12. Gently resuspend pellets using 1ml of FACS buffer per tube.
13. Centrifuge at 300 × *g* for 5 minutes and aspirate supernatant.
14. Gently resuspend pellets using 500µl of FACS buffer per tube.
15. Filter cells into a 5ml polystyrene tube with filter cap.
16. Analyze by flow cytometry using FITC filters (or similar) for Anti-HiBiT Monoclonal Antibody XFD488 and APC filters (or similar) for Anti-HiBiT Monoclonal Antibody XFD647.

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3.C. Example Data

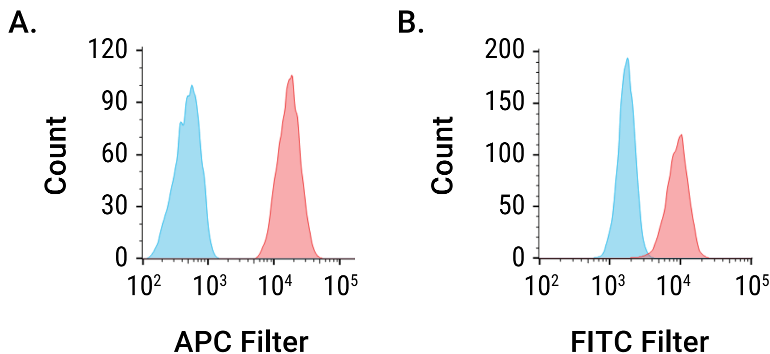

Figure 1. Live-cell FACS with HeLa and HeLa/HiBiT-EGFR cells. CRISPR-Cas9 was used to create a heterozygous knock-in of HiBiT at the extracellular N-terminus of EGFR in HeLa cells. A clonal HiBiT-EGFR cell line and the parental HeLa cells were stained using the protocol in Section 3.B, with BSA replacing FBS in the FACS buffer to prevent receptor internalization. HeLa parental cells (blue) and HeLa/HiBiT-EGFR cells (red) were stained with 1µg/ml of Anti-HiBiT Monoclonal Antibody XFD647 (**Panel A**) or Anti-HiBiT Monoclonal Antibody XFD488 (**Panel B**). Flow cytometry was performed on a BD Accuri™ C6 Plus Flow Cytometer using APC (**Panel A**) or FITC (**Panel B**) filters.

4. Fluorescence-Activated Cell Sorting with Fixed Cells

4.A. General Considerations

To quantify expression of intracellular HiBiT-tagged proteins, HiBiT assays like the Nano-Glo® HiBiT Lytic Detection System (Cat.# N3030) or live-cell assays involving expression of LgBiT inside the cell are generally preferred, as they provide increased sensitivity. However, FACS analysis of fixed, permeabilized cells that have been stained with Anti-HiBiT Monoclonal Antibody, XFD Fluorophore Conjugates, can be used to measure the protein expression distribution in a cell population or to correlate those expression levels to another protein.

Materials to Be Supplied by User

- Cyto-Fast™ Fix/Perm Buffer Set (BioLegend Cat.# 426803)
- phosphate-buffered saline (DPBS, GIBCO® Cat.# 14190)
- FACS buffer (DPBS + 1% FBS or 1% BSA)
- fetal bovine serum (FBS)
- bovine serum albumin (BSA; e.g., Sigma Cat.# A7979)
- flow cytometer (e.g., BD LSRFortessa™ X-20 Cell Analyzer or BD Accuri™ C6 Plus Flow Cytometer)
- polystyrene centrifuge tube with filter cap (e.g., Corning® Falcon® Cat.# 352235)

4.B. Example Protocol

The following example uses the Cyto-Fast™ Fix/Perm Buffer Set. Alternative reagents or protocols for fixation or permeabilization are also expected to work. Include parental cells lacking expression of a HiBiT fusion protein in your experiment as a negative control.

1. Trypsinize adherent cells and resuspend in six volumes of growth medium.

   Note: If cells are expressing an extracellular HiBiT tag, do not use trypsin. Instead use a gentler cell dissociation reagent like VERSENE® solution.

2. Pellet 10^6 cells and wash once in 1ml of DPBS.
3. Resuspend the cell pellet in 100µl of DPBS.
4. Add 150µl of Cyto-Fast™ Fix/Perm Buffer and mix.
5. Incubate for 20 minutes at room temperature.
6. Dilute the Cyto-Fast™ Perm Wash solution (a component of Cyto-Fast™ Fix/Perm Buffer Set) 10X using deionized water.
7. Add 1ml of 1X Cyto-Fast™ Perm Wash solution to sample.
8. Centrifuge at 300 × *g* for 5 minutes and aspirate supernatant.
9. Repeat Steps 7 and 8.

10. Dilute Anti-HiBiT Monoclonal Antibody, XFD Fluorophore Conjugate, to 1µg/ml (500X dilution of stock) using 1X Cyto-Fast™ Perm Wash Solution. Resuspend cell pellets by adding 100µl of the respective primary antibody solution per tube. Protect sample from light for the remainder of protocol (e.g., cover with foil when sample is not in use).
11. Incubate for 30 minutes at room temperature.
12. Add 1ml of Cyto-Fast™ Perm Wash solution to sample and mix.
13. Centrifuge at 300 × *g* for 5 minutes and aspirate supernatant.
14. Resuspend cell pellet by adding 1ml of FACS buffer (DPBS + 1% FBS or 1% BSA) per tube and mixing.
15. Centrifuge at 300 × *g* for 5 minutes and aspirate supernatant.
16. Resuspend cell pellet by adding 500µl of FACS buffer and mixing.
17. Filter cells into a 5ml polystyrene tube with filter cap (Corning® Falcon® Cat.# 352235).
18. Analyze by flow cytometry using FITC filters (or similar) for Anti-HiBiT Monoclonal Antibody XFD488 and APC filters (or similar) for Anti-HiBiT Monoclonal Antibody XFD647.

4.C. Example Data

Figure 2 shows the results of FACS analysis with CRISPR-modified cells. CRISPR-Cas9 was used to insert HiBiT at the C terminus of the endogenous locus of JAK2 and EGFR in HEL 92.1.7 and HeLa cells, respectively.

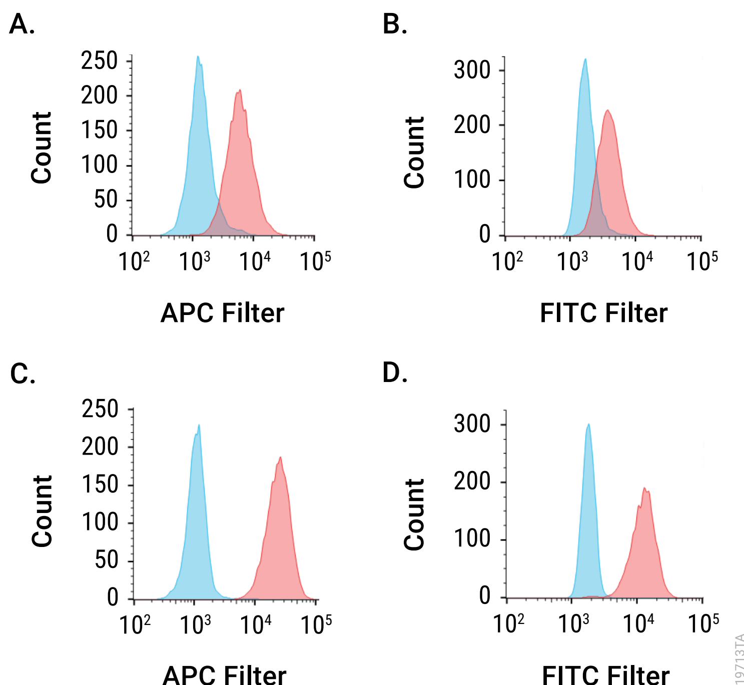

Figure 2. FACS with fixed CRISPR-modified cells. Parental cells and CRISPR-modified clones expressing HiBiT-tagged target proteins at endogenous levels. Samples were prepared using the protocol in Section 4.B with 1µg/ml of Anti-HiBiT Monoclonal Antibody, XFD Fluorophore Conjugate. Parental HEL 92.1.7 cells and CRISPR-modified JAK2-HiBiT cells were stained with Anti-HiBiT Monoclonal Antibody XFD647 (**Panel A**) or Anti-HiBiT Monoclonal Antibody XFD488 (**Panel B**). Parental HeLa cells and CRISPR-modified EGFR-HiBiT cells were stained with Anti-HiBiT Monoclonal Antibody XFD647 **(Panel C**) or Anti-HiBiT Monoclonal Antibody XFD488 (**Panel D**). Flow cytometry was performed on a BD Accuri™ C6 Plus Flow Cytometer using FITC or APC filters.

5. Antibody Binding Affinity and Competition with LgBiT

Anti-HiBiT Monoclonal Antibody binds with high affinity to form a stable complex with HiBiT peptide or HiBiT-tagged proteins. Antibody binding largely blocks productive interaction of HiBiT with the LgBiT subunit. Therefore, the presence of Anti-HiBiT Monoclonal Antibody in a sample can affect quantitation of HiBiT in bioluminescent assays in which LgBiT is a component of the reagent (e.g., the Nano-Glo® HiBiT Lytic Detection System). Similarly, the presence of LgBiT subunit in a sample (e.g., a LgBiT expressing cell line) may decrease the extent of antibody binding or slow its rate of association with HiBiT, due to competition. However, because of its ~100-fold higher affinity, the antibody may often be able to outcompete LgBiT for binding to HiBiT.

6. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. Email: **techserv@promega.com**

Symptoms	Causes and Comments
The fluorescence signal from the HiBiT-expressing cells is weak	The expression level of the HiBiT-tagged protein may be too low to give a fluorescence signal significantly over background, even if the HiBiT luminescent signal is well over background in orthologous assays.
	Optimize the concentration of primary antibody by testing higher concentrations.
	Make sure the flow cytometer settings (laser, alignment, PMT) are correct for the fluorophore being used.
	Trypsinization of adherent cells could destroy surface antigens. When HiBiT is expressed on the surface, use a gentle cell dissociation method like VERSENE® solution.
	For fixed cells, ensure that the permeabilization conditions are appropriate for the subcellular localization of the protein.
	Cell-surface target proteins may internalize during staining. Keep samples at 4°C and use ice-cold reagents to reduce internalization.
The fluorescence signal or background is too high	Optimize the concentrations of primary antibody by testing lower concentrations.
	Cell debris can cause high background. Gate to exclude low-scatter debris from your analysis.
	Adjust the gain and offset values on the flow cytometer. Perform additional washes to remove unbound antibody.

7. References

1. Dixon, A.S. *et al.* (2016) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol.* **11**, 400–8.
2. Hall, M.P. *et al.* (2012) Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. *ACS Chem. Biol.* **7**, 1848–57.
3. Schwinn, M.K. *et al.* (2018). CRISPR-mediated tagging of endogenous proteins with a luminescent peptide. *ACS Chem. Biol.* **13**, 467–74.

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