TECHNICAL NOTE

AlphaLISA Assay for Measurement of Core Fucosylated Glycoproteins Using LCA Lectin

AlphaLISA #26

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AlphaLISA® Technology

This AlphaLISA immunodetection assay measures the level of core fucosylated glycoprotein.

Lens culinaris agglutinin (LCA) AlphaLISA Acceptor Beads

- AL140C: 250 µg, 500 assay points*
- AL140M: 5 mg, 10,000 assay points*
- AL140R: 25 mg, 50,000 assay points*

*0.5 µg/assay point

Model System

IgG and lactoferrin were used as model glycoproteins to show the application of AlphaLISA to detect core fucosylated glycoproteins.

AlphaLISA Assays

The AlphaLISA technology allows performing no-wash homogeneous proximity immunoassays using Alpha Donor and AlphaLISA Acceptor Beads. In this technical note, we present the optimization of assays for the detection of glycoproteins. Detection of the glycoproteins was performed by the addition of Streptavidin (SA) Alpha Donor beads, biotinylated anti-glycoprotein antibody and AlphaLISA Acceptor beads conjugated to LCA lectin. LCA lectin was extensively characterized and shown to detect biantennary core α1,6-fucosylated oligosaccharides by Tateno *et al.* Glycobiology vol. 19 no. 5 pp. 527–536, 2009. Upon laser irradiation of the beads-target complexes at 680 nm, short-lived singlet oxygen molecules produced by the Donor beads can reach the Acceptor beads in proximity to generate an amplified chemiluminescent signal at 615 nm. The intensity of light emission is proportional to the level of fucosylated glycoprotein detected.

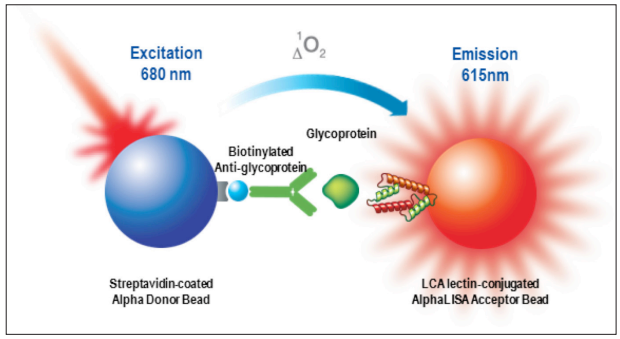

Figure 1. Schematic representation of the AlphaLISA detection of a fucosylated glycoprotein.

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Development of a Core Fucosylated Glycoprotein Assay

Reagents needed for this assay:

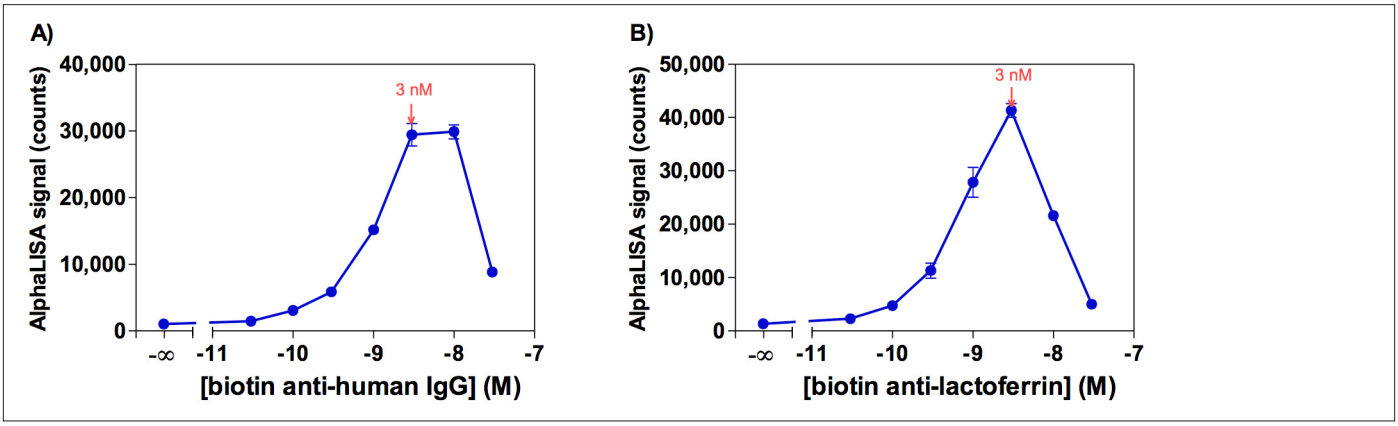
Lens culinaris agglutinin AlphaLISA Acceptor beads	PerkinElmer # AL140
Alpha Streptavidin Donor beads	PerkinElmer # 6760002
Immunoassay buffer, 10X, 10 mL	PerkinElmer # AL000C
White opaque OptiPlate™-384	PerkinElmer # 6007290
TopSeal™-A film	PerkinElmer # 6050195
Mouse anti-human lactoferrin antibody*	AbD Serotec # MCA2764
Goat $(Fab')_2$ anti-human IgG-Fc fragment*	Bethyl Laboratories # A80-248A
Lactoferrin from human milk	Sigma # L0520
Human IgG4 protein from myeloma plasma	Fitzgerald # 31-AI20
ChromaLink Biotin (DMF Soluble)	Solulink # B-1001

*Antibodies were biotinylated with Chromalink Biotin, using a standard biotinylation procedure.

Standard Protocol

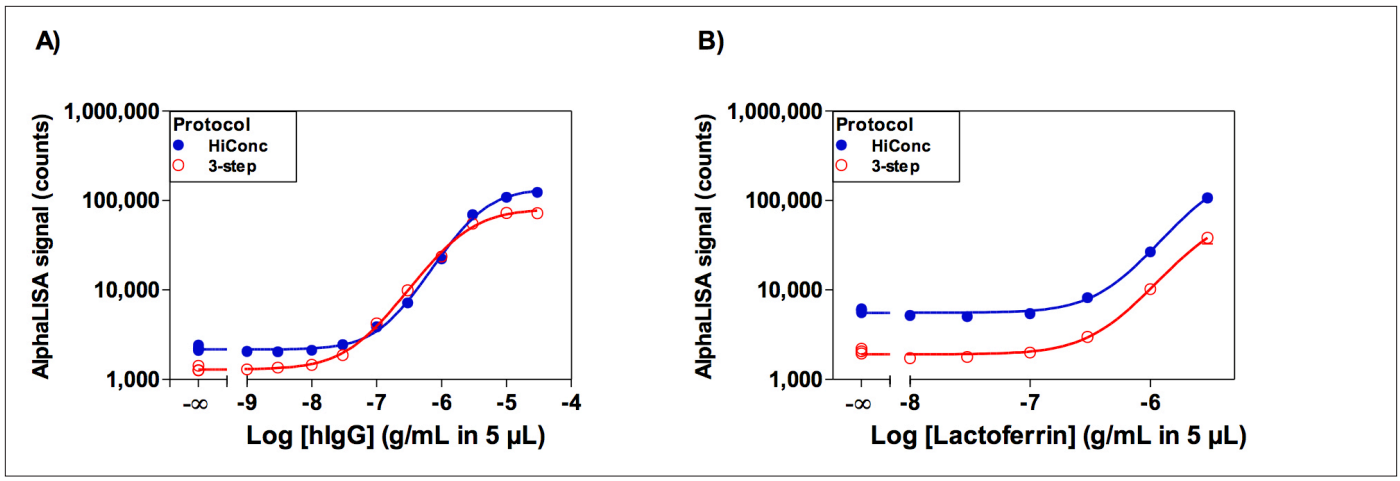
- Prepare the glycoprotein dilutions to test in 1X Immunoassay buffer.
- Prepare a 10X mix of *Lens culinaris* agglutinin AlphaLISA Acceptor Beads and biotinylated anti-human lactoferrin antibody or biotinylated anti-human IgG antibody at 100 µg/mL and 30 nM, respectively, in 1X Immunoassay buffer. Final concentrations are, respectively, 10 µg/mL and 3 nM in 50 µL total assay volume.
- Add to the wells of a white OptiPlate-384:
  - 5 µL of glycoprotein dilutions
  - 5 µL of 10X Acceptor beads/biotinylated antibody mix.
- Cover with TopSeal-A film and incubate 60 min at RT.
- Prepare 1.25X Streptavidin Donor beads at 50 µg/mL in 1X Immunoassay buffer, in subdued light (final concentration of 40 µg/mL in 50 µL total assay volume).
- Add 40 µL of Donor beads in subdued light.
- Cover with TopSeal-A film and incubate in the dark for 30 min at RT.
- Read signal in Alpha mode with the EnVision® or EnSpire® readers.

Experiment 1: Biotinylated Antibody Titration Curves



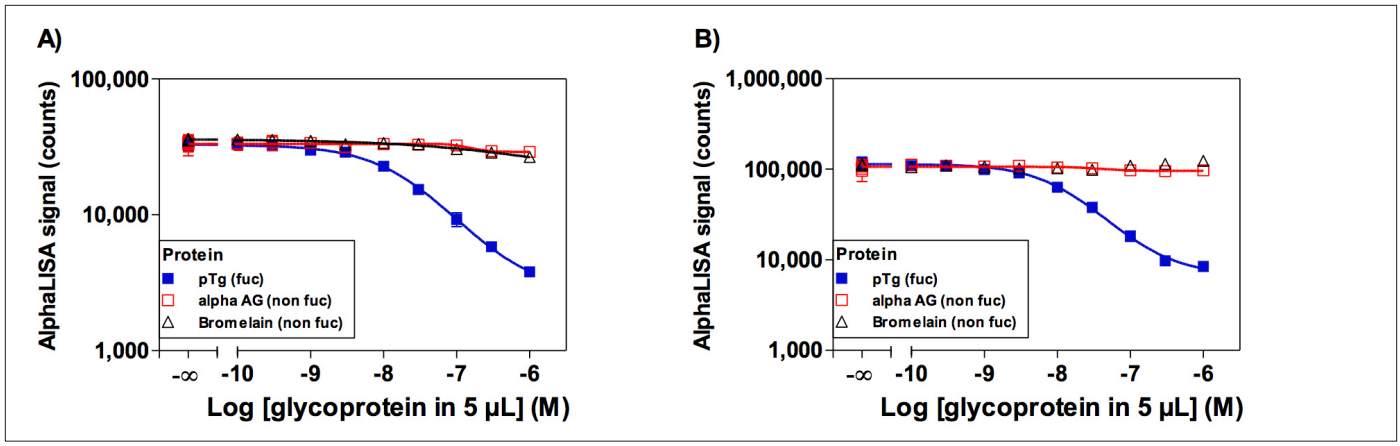
Biotinylated antibody titration curves were performed by incubating 5 µL of the glycoprotein (3 µg/mL) (A: IgG, B: Lactoferrin) with 10 µL of biotinylated antibody (0.03 to 30 nM)for 30 minutes, at RT. Then, an addition of 10 µL of a 5X solution (50 µg/mL) of Acceptor beads was followed by a 60-minute incubation, at RT. Finally, 25 µL of Streptavidin Donor beads were added and signal was read after 30 min. A final concentration of 3 nM biotinylated antibody was selected for all subsequent experiments.

Experiment 2: Assay Protocol Comparison



Two assay protocols were compared: a two-step High Concentration (HiConc) protocol (see Standard Assay Protocol) and a 3-step protocol. The 3-step protocol consisted in adding 5 µL of each serial dilution of the glycoprotein (A: IgG, B: Lactoferrin) in triplicate and then 10 µL of a 5X solution of LCA Acceptor beads (50 µg/mL for a final concentration of 10 µg/mL) followed by 30-minute incubation at RT. Then, 10 µL of a 5X solution of biotinylated antibody (15 nM for a final concentration of 3 nM) were added, followed by a 60-minute incubation at RT. Finally, 25 µL of a 2X solution of Streptavidin Donor beads (80 µg/mL for a final concentration of 40 µg/mL) were added and signal was read after 30 min. The High Concentration protocol was selected for all subsequent experiments. A 2-fold higher background signal was obtained in the lactoferrin detection assay (B) compared to the IgG assay (A), which was likely due to the binding of the LCA Acceptor Beads to core fucose on the anti-lactoferrin antibody molecule. The IgG was captured by an $F(ab')_2$, which does not contain core fucose.

Experiment 3: Specificity – Competition With Glycoproteins And Monosaccharides



In order to verify the specificity of the assay for core α1-6-fucose, three different glycoproteins (one known to be fucosylated and two without core α1-6-fucose) (Skrikrishna *et al.* JBC vol. 272 no. 41 pp.25743-25752, 1997) were used to compete with the human IgG and lactoferrin for the interaction with the LCA on the Acceptor beads. Indeed, the following proteins were tested: porcine thyroglobulin (pTg) (contains biantennary core-substituted Fucα1,6GlcNAc), Human α1-acid glycoprotein (Alpha AG) (complex bi-, tri-, and tetraantennary glycans, with outer Fucα1,3GlcNAcβ and no core substitution) and pineapple stem bromelain (Oligomannose glycan, with core Fucα1,3GlcNAcβ and Xylβ1,2Manβ). For the assay, 5 µL of the glycoprotein (3 µg/mL) (A: IgG, B: Lactoferrin) was added, followed by 5 µL of each serial dilution of the "competitor glycoproteins" in triplicate (concentrations from 0.1 nM to 1 µM) and 5 µL of a 10X mix of biotinylated antibody (30 nM) and LCA Acceptor beads (100 µg/mL) (final concentrations of biotinylated antibody and Acceptor beads are 3 nM and 10 µg/mL, respectively). The reactions were then incubated for 60 minutes, at RT. Finally, 35 µL of a 1.43X solution of Streptavidin Donor beads (57.1 µg/mL for a final concentration of 40 µg/mL) were added and signal was read after 30 min. The assays were shown to be specific, recognizing the glycoprotein containing core α1-6-fucoses (pTg) and not the two glycoproteins that do not have core α1-6-fucoses (α1-acid glycoprotein and pineapple stem bromelain). In a separate experiment, the possible competition by glucose and fucose were also tested using the same protocol. Fucose showed no interference up to 30 mM and glucose showed 50% inhibition at 10 mM (data not shown). Certain biological matrices may contain mM amounts of glucose; therefore, the possible interference by glucose should be taken into consideration when analyzing samples.

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