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QCSOP25-1: Fucose Detection in Antibodies

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We use this protocol and it's working

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Abstract

This protocol describes a **lectin-based ELISA** for detecting **core fucose residues** in antibody lots produced by the **Nonhuman Primate Reagent Resource (NHPRR)**. Using Biotinylated *Lens culinaris* agglutinin (LCA) and colorimetric detection, the assay distinguishes **fucosylated** from **afucosylated antibodies**, providing a **rapid and cost-effective** tool for **lot identification and quality control**.

Objective:

To establish a standardized procedure for detecting fucose residues in afucosylated monoclonal antibodies using a lectin-based ELISA, supporting NHPRR R&D and QC workflows such as glycoengineering, batch verification, and lot comparability.

Scope:

Applicable to laboratory personnel performing lectin-based ELISA assays for fucose detection in afucosylated monoclonal antibodies within NHPRR research and quality programs.

Background

Afucosylated monoclonal antibodies lack **core fucose** on Fc N-linked glycans, enhancing FcγRIIIa binding and antibody-dependent cellular cytotoxicity ADCC (Pereira et al., 2018). This lectin-based ELISA is a quick, qualitative method for confirming **the lot identity of NHPRR antibodies (e.g., anti-CD20)** before distribution.

Materials

NHPRR Specific Reagents:

- ☒ Reference afucosylated rhesus IgG1 (Anti-CD20-Af) [2B8R1F8] **Nonhuman Primate Reagent Resource (NHPRR) Catalog #CAT-00440**
- ☒ Reference rhesus IgG1 (Anti-CD20) [2B8R1] **Nonhuman Primate Reagent Resource (NHPRR) Catalog #CAT-00432**

Standard ELISA Materials and Reagents:

Material/Equipment	Purpose	Vendor	Catalog Number
Gibco™ PBS (10X), pH 7.2	Used in the preparation of the Washing Buffer	Gibco™	70-013-032
VWR® High-Performance [15 mL] Centrifuge Tubes with Flat or Plug Caps, PP	Used for the preparation/dilution of the test sample and other reagents	VWR or equivalent	89039-668
Gibco™ [1X] DPBS, calcium, magnesium, glucose, pyruvate	Diluent for preparation of the test sample and other reagents	Gibco™	14-287-080
VWR® [50 mL] Disposable Pipetting Reservoirs	For holding the test sample and other reagent solutions before transfer	VWR or equivalent	89094-684
Analytical Balance	For weighing BSA (part of the Blocking Buffer preparation)	of your choice	N/A
Biohazard Waste Bags	Collect used pipette tips and other waste from the experiment	of your choice	N/A
Bovine Serum Albumin (BSA)	Used in the preparation of the Blocking Buffer	Sigma-Aldrich	A9647-500G
Calculator	Calculate the dilution volumes	of your choice	N/A
Falcon® 96-well Polystyrene [Dilution] Microplates	Low-binding plates for preparation of the test sample and other reagents	Corning	353936
[Serological] Pipette, 10 mL and 5 mL, Graduated 1/10 mL, Sterile	Used for the preparation/dilution of the test sample and other reagents	Greiner Bio-One	606180 (5 ml) 607107 (10 mL)
INTEGRA VIAFLO 12-Channel 50-1250 µL Electronic Pipette	Used for the preparation, dilution, and transfer of the test sample and other reagents	INTEGRA	<i>Model: 12-channel VIAFLO</i>
ELISA Microplate Reader	Measure the OD values	Molecular Devices	<i>Model: SpectraMax ABS</i>
ELISA Plate Washer	Wash ELISA plates	BioTek	<i>Model #: 405LSRS</i>
Corning® 96-well Clear Flat Bottom Polystyrene High Bind Microplate, Nonsterile	High-binding plates for ELISA assay	Corning	9018
Sterile GRIPTIPS® INTEGRA 1250 µL Pipette Tips	Used for the preparation, dilution, and transfer of the test sample and other reagents	INTEGRA	6544 (non-filtered) 6545 (filtered)
Pipet-Lite™ XLS+ Manual 12-Channel Pipette, 20-200 µL	Used for transferring the test sample and other reagents from the dilution plate to the working plate	Rainin	17013810
Nalgene™ Rapid-Flow™ Sterile Filter Storage Bottles	Used for the preparation of the Blocking Buffer	Thermo Fisher Scientific	455-0250
Elisa Plate Reader Software	Analyze the OD values	Molecular Devices	<i>Model: SoftMax® Pro</i>
Plate Sealers	To cover ELISA plates for incubation	BioLegend	423601
PPE (Personal Protective Equipment)	For analyst protection: lab coat, nitrile gloves, and safety goggles	of your choice	N/A
Pipette Tips SR LTS 200µL F 960A/5	Used for transferring the test sample and other reagents from the dilution plate to the working plate	Rainin	17005859
Rainin Classic™ Manual Single-Channel Pipettes and Allocated Pipette Tips	Used for the preparation, dilution, and transfer of the test sample and other reagents	Rainin or equivalent	N/A
Timer	To keep track of incubation and developing time	of your choice	N/A
ELISA Stop Solution	Stops the enzymatic reaction	Invitrogen™	SS04
1-Step™ TMB ELISA Substrate Solutions	Acts as the substrate for the HRP enzyme	Thermo Fisher Scientific	34029

Material/Equipment	Purpose	Vendor	Catalog Number
TWEEN® 20	Used in the preparation of the Washing Buffer	Sigma Aldrich	P6585-100ML
Fisherbrand™ Hexagonal Polystyrene Weighing Dishes	Used for weighing BSA during Blocking Buffer preparation	Fisher Scientific	02-202-103

Assay-Specific ELISA Reagents:

Product Name	Vendor	Catalog Number	Lot	Purpose	RRID
Streptavidin-HRP	Thermo Fisher Scientific	N100	-	Signal Amplifier	-
Biotinylated LCA	Vector Laboratories	B-1045	-	Detection Reagent	-
Reference afucosylated rhesus IgG1 (Anti-CD20-Af) [2B8R1F8]	NHPRR	CAT-00440	Test Lot	Coating: Example Test Antibody	AB_3719830
Reference afucosylated rhesus IgG1 (Anti-CD20-Af) [2B8R1F8]	NHPRR	CAT-00440	Reference Lot	Coating: Reference Antibody	AB_3719830
Reference rhesus IgG1 (Anti-CD20) [2B8R1]	NHPRR	CAT-00432	-	Coating: Positive Control	AB_3695603

Protocol materials

- ☒ Biotinylated LCA **Vector Laboratories Catalog #B-1045**
- ☒ HRP-Conjugated Streptavidin **Thermo Fisher Scientific Catalog #N100**
- ☒ Reference afucosylated rhesus IgG1 (Anti-CD20-Af) [2B8R1F8] **Nonhuman Primate Reagent Resource (NHPRR) Catalog #CAT-00440**
- ☒ Reference rhesus IgG1 (Anti-CD20) [2B8R1] **Nonhuman Primate Reagent Resource (NHPRR) Catalog #CAT-00432**



Troubleshooting

Problem

Positive Control Shows Low or No Signal:

Solution

This issue may result from the degradation of the Biotinylated LCA or Streptavidin-HRP, incorrect coating concentration or dilution, as well as insufficient signal development. To address this, confirm that the Biotinylated LCA and Streptavidin-HRP have been stored properly and are within their expiration dates. Additionally, verify that the antibody coating concentrations and volumes match the protocol specifications. Then, repeat the assay using freshly prepared dilutions of Biotinylated LCA and Streptavidin-HRP, and if available, use a different lot or a new vial from the same lot, provided it is still within its expiry date. Use a freshly opened TMB Substrate, and ensure that incubation times strictly follow the protocol. If the signal remains low, consider extending the TMB Substrate incubation period up to 30 minutes while visually monitoring color development throughout the process.

Problem

Blank Wells Show High Signal:

Solution

The issue may result from incomplete washing, insufficient blocking, or buffer contamination. To address this, confirm that all wash steps are performed using the correct volume, Washing Buffer, and cycle count. Repeat the assay with fresh Blocking Buffer, ensuring full plate coverage during the blocking step. Use new pipette tips for all reagent transfers, and make sure reagent reservoirs are clean or replaced.

Problem

Reference Antibody Shows Unexpected High Signal:

Solution

The issue may result from cross-contamination, preparation errors, or problems with the detection reagent, Biotinylated LCA, and/or the signal amplifier, Streptavidin-HRP. To address this, confirm that the reference antibody is correctly diluted and handled from the stock. Then, repeat the assay with a new high-binding ELISA plate and freshly prepared reference antibody solution. Additionally, try using either a new lot or a fresh vial of the same lot (within expiry) for Biotinylated LCA and Streptavidin-HRP. Finally, inspect the plate reader calibration and check for OD saturation if unusually high readings occur.

Problem

All Wells Show Low Signal:

Solution

The issue may result from inactive Streptavidin-HRP or TMB Substrate, incorrect incubation temperature, or systemic pipetting errors. To address this, verify reagent activity by testing Streptavidin-HRP and TMB Substrate on a known positive sample. Additionally, confirm that all incubation steps were performed at room temperature or according to SOP specifications. Next, inspect the performance of the plate reader. Finally, repeat the assay using a new high-binding ELISA plate with newly prepared signal amplification reagents from different lots or fresh aliquots.

Problem

High Variability Between Duplicates:

Solution

The issue may be caused by inconsistent pipetting or incomplete mixing of reagents. To address this, use calibrated pipettes and maintain a consistent technique; a multichannel pipette is recommended for uniform loading of the samples and reagents. Additionally, ensure that all samples and reagents are thoroughly mixed before application.

Before start

Ensure that all materials listed in the **"Materials"** section are available before starting the assay, including a biohazard waste bag for discarding used pipette tips and other waste generated during the procedure.

Always store antibodies at 2–8 °C, both before and immediately after use. Do not reuse pipette tips, as this may cause cross-contamination and lead to inaccurate assay results. *Pipette tips may only be reused when transferring solutions from the dilution plate to the working plate in sub-steps 6.10–6.15, or when dispensing the same reagent at the same concentration from a reagent reservoir onto the ELISA working or dilution plate.

Definitions and Acronyms

1 Definitions:

Positive Control: Reference rhesus IgG1 (**Anti-CD20**) [2B8R1], NHPRR, **CAT-00432**, RRID: AB_3695603)

A positive control is a sample known to produce a positive result, confirming that the assay is functioning correctly under current run conditions (Abcam, 2024). This particular ELISA is designed to detect fucose. So, the positive control is an antibody sample known to be fucosylated, meaning it retains its core fucose and has not undergone the post-translational removal of this sugar.

Note

If the positive control fails to generate the expected signal, the assay may be invalid, and the run should be repeated (BosterBio, n.d.).

Reference Antibody: (Reference **afucosylated** rhesus IgG1 (**Anti-CD20-Af**) [2B8R1F8], NHPRR, **CAT-00440**, **Reference Lot**, RRID: AB_3719830)

A reference antibody is a sample or product that serves as a benchmark for expected assay response and is used to compare with test samples. In this fucose ELISA, the reference antibody should be included on each plate to confirm the expected response of an afucosylated antibody. It serves as both a visual and analytical control.

Note

If a test sample yields a significantly higher signal than the Reference Lot, additional testing (e.g., glycan mapping) may be required. Conversely, if the signal is lower, this suggests a lower fucose content and no further action is needed (BDP, 2017; Sluis et al., 2014; WHO, 2004).

Detection Reagent:

Biotinylated *Lens culinaris* agglutinin (LCA) that binds specifically to **fucose residues**.

A detection reagent binds specifically to the analyte or a relevant epitope, enabling signal generation (*ELISA Guide; Part 1: Introduction to ELISA, Formats and Signal Amplification*, 2023; NCBI, 2020).

Blank:

A **buffer-only well** that is used to establish the background signal and verify the absence of non-specific binding. Any unexpected signal indicates contamination or assay error.

Note

If a blank well exhibits an unexpected signal, this may indicate contamination or non-specific binding, and the assay should be considered invalid (Jackson ImmunoResearch, n.d.).

Signal Amplifier:

Streptavidin-HRP, which binds to the Biotinylated LCA and catalyzes color development with **TMB Substrate**, amplifying the detection signal (*ELISA Guide; Part 1: Introduction to ELISA, Formats and Signal Amplification*, 2023). A signal amplifier is a molecule or complex that enhances the detection signal generated by analyte-reagent binding.

Nonhuman Primate (NHP) Immunoglobulin G (IgG):

Immunoglobulin G (IgG) is a monomeric antibody primarily found in serum that plays a crucial role in the humoral immune response. It consists of two heavy chains and two light chains, each containing variable and constant regions. There are four subclasses of IgG (IgG1, IgG2, IgG3, and IgG4). Although the Nonhuman Primate IgG is structurally and functionally similar to Human IgG, the subclasses and their Fc interactions were shown to have interspecies variation (Crowley & Ackerman, 2019).

Antibody-Dependent Cellular Cytotoxicity (ADCC):

Antibody-dependent cellular cytotoxicity (ADCC) is an immune response in which antibodies bind to antigens on the surface of target cells and interact with immune cells,

such as natural killer (NK) cells and macrophages, through Fc receptor interaction, leading to the destruction or lysis of the target cells (ScienceDirect, n.d.).

CD20:

The CD20 antigen is a non-glycosylated phosphoprotein that is expressed by both normal and malignant B cells. It functions as a receptor for B-cell activation and differentiation (Leicabiosystems.com, n.d.).

FcγRIIIa:

This is a low-affinity receptor for Immunoglobulin G (IgG), found on natural killer (NK) cells, macrophages, and other effector cells. This recognizes and binds to the Fc region of the IgG antibodies, triggering their effector function, such as antibody-dependent cellular cytotoxicity (ADCC) (Bruhns et al., 2009; Marshall et al., 2017).

Afucosylation:

This is a post-translational modification in which the core fucose is removed from the N-linked glycans in the Fc region of Immunoglobulin G (IgG) antibodies. In monoclonal antibodies (mAbs), this post-translational modification increases binding affinity to FcγRIIIa receptors, thereby enhancing antibody-dependent cellular cytotoxicity (ADCC). (Pereira et al., 2018; Golay et al., 2022).

Dilution Plate:

A **low-binding microplate** that is used to prepare dilutions of samples, controls, or standards before transfer to the working plate.

Working Plate:

The specific **ELISA plate** used for coating, blocking, detection, and reading at **450 nm**.

Blocking Buffer:

A blocking buffer is applied after the coating step to cover the unoccupied binding sites on the plate surface, minimizing background noise. It also serves as a diluent for detection and secondary antibodies, reducing non-specific binding (Excedr, 2023).

Monoclonal Antibody (mAb):

An antibody preparation derived from a single B-cell clone, recognizing one specific epitope on an antigen and therefore having a single binding specificity. In this SOP, the monoclonal antibody Test Article (TA-mAb) is a mouse anti-rhesus IgA antibody evaluated for its binding to recombinant monomeric and dimeric rhesus IgA.

Acronyms:

LCA - *Lens culinaris* agglutinin

1X DPBS – 1X Dulbecco's Phosphate-Buffered Saline (pH 7); used to dilute coating reagents

10X DPBS – 10X Dulbecco's Phosphate-Buffered Saline (pH 7.4); used to prepare Washing Buffer

TMB – 3,3',5,5'-Tetramethylbenzidine; a chromogenic substrate oxidized by HRP

OD – Optical Density; the absorbance value read at 450 nm

AU - Absorption Units

BSA – Bovine Serum Albumin; used in Blocking Buffer to prevent nonspecific binding

HRP – Horseradish Peroxidase; conjugated to streptavidin for signal amplification

LC-MS - Liquid Chromatography - Mass Spectroscopy

UPLC/UHPLC - Ultra High Performance Liquid Chromatography

ADCC: Antibody-Dependent Cellular Cytotoxicity

Positive Control Schematic:

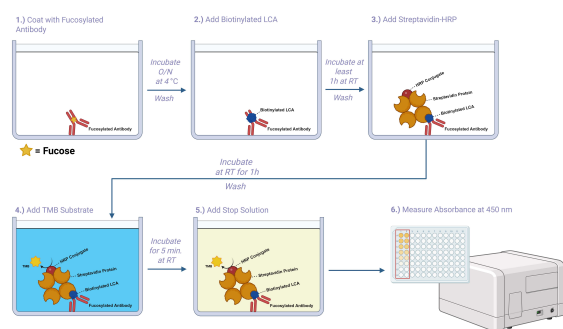


Figure 1: Workflow Overview of Fucose Detection ELISA

Description:

The first step of this fucose detection process involves coating the plate with an antibody. In this particular schematic, the plate is coated with a fucosylated antibody, which means it contains fucose in the N-linked glycans of its Fc region, near the hinge. This is represented by the yellow star in the first step of the schematic. After the fucosylated antibody is incubated overnight and the plate is washed, Biotinylated LCA is added and binds to the fucose. Once the Biotinylated LCA has incubated for 1 hour at RT and the plate is washed, Streptavidin-HRP is added, which is a tetrameric protein known to bind to biotin. Since Biotinylated LCA contains biotin, Streptavidin-HRP binds to it. After 1 hour of Streptavidin-HRP incubation and plate washing, the TMB Substrate is added. The HRP conjugated to Streptavidin catalyzes the oxidation of TMB, causing the well solution to turn blue. After 5 minutes, the Stop Solution is added to stop the reaction, causing the solution to turn yellow. This allows the ELISA plate reader to measure the absorbance of each well at 450 nm.

Concerning coating with an afucosylated antibody, when the Biotinylated LCA is added, it does not bind the coated antibody near its hinge region. If Biotinylated LCA cannot bind, then, during the washing step, it will be washed away. If no Biotinylated LCA is in the wells, then no biotin will be present to be bound to by Streptavidin-HRP. So, following the addition of Streptavidin-HRP, it is washed away as well. Subsequently, in the absence of Streptavidin-HRP, there is no HRP available to catalyze the oxidation of the TMB Substrate, which is responsible for the color change. Essentially, the ELISA wells coated with an afucosylated antibody would appear clear throughout the entire ELISA process.

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<https://BioRender.com/bdwtm78>

Washing Buffer Preparation (1X DPBS + 0.05% Tween-20)

- 2 Take a 20 L clean container.
- 2.1 Add 18 L of deionized (DI) water.
- 2.2 Add 2 L of 10X PBS and mix gently.
- 2.3 Then, add 50 mL of Tween-20 to the above mixture.
- 2.4 Mix the above contents until fully homogeneous.
- 2.5 Label the container with reagent name, concentration, date of preparation, and initials.
- 2.6 If using a transparent container, protect from light by wrapping with aluminium foil.



- 2.7 If an opaque container is being used, then there is no need to wrap the container with aluminium foil.
- 2.8 The above preparation can be scaled up or down as needed.

Blocking Buffer Preparation (3% BSA in 1XDPBS + 0.05% Tween-20)

- 3 It is ideal to prepare this buffer on the day of use, which is usually **Day 2**.
- 3.1 Take a graduated cylinder and measure  150 mL of **Washing Buffer (1X DPBS + 0.05% Tween-20)**.
- 3.2 Weigh  4.5 g of BSA (Bovine Serum Albumin).
- 3.3 Add the above contents to a  200 mL sterile bottle or container.
- 3.4 Mix thoroughly until BSA is completely dissolved. 
- 3.5 Label this container as "Blocking Buffer," and note the operator's initials and date on the label.
- 3.6 **Calculation:**
 $4.5 \text{ g (BSA)} \div 150 \text{ mL (Washing Buffer)} = 0.03$
 $0.03 \cdot 100 = 3\%$
- 3.7 The above preparation can be scaled up or down based on the need.

Day 1 - Preparation of Positive Control, Reference Antibody, and Test Antibody

- 4 The antibodies will be coated onto the working plate.
- 4.1 Each antibody is diluted to  1.25 µg/mL and run in duplicate; each well on the working plate (two columns per antibody) receives approximately  0.125 µg of antibody.
- 4.2 Calculate the volumes using $C_1V_1 = C_2V_2$ (see **Table 1**).
- C_1 = initial stock concentration
 V_1 = volume of stock solution required
 C_2 = final desired concentration of the diluted reagent
 V_2 = final total volume of diluted solution
- 4.3 **Example Calculations:**
- (Positive Control)**
-  Reference rhesus IgG1 (Anti-CD20) [2B8R1] Nonhuman Primate Reagent Resource (NHPRR) Catalog #CAT-00432
- For this **positive control** antibody with an initial concentration of  10.2 mg/mL :
- (10,200 µg/mL) (V_1) = (1.25 µg/mL) (8 mL)
 - (10,200 µg/mL) (V_1) = 10
 - V_1 = 0.000980 mL or **0.980 µL**

(Reference Antibody)

 Reference afucosylated rhesus IgG1 (Anti-CD20-Af) [2B8R1F8] **Nonhuman Primate**
Reagent Resource (NHPRR) Catalog #CAT-00440

For this **Reference Lot antibody** with an initial concentration of **11.6 mg/mL** :

- (11,600 µg/mL) (V_1) = (1.25 µg/mL) (8 mL)
- (11,600 µg/mL) (V_1) = 10
- V_1 = 0.000862 mL or **0.862 µL**

(Example Test Antibody)

 Reference afucosylated rhesus IgG1 (Anti-CD20-Af) [2B8R1F8] **Nonhuman Primate**
Reagent Resource (NHPRR) Catalog #CAT-00440

For this **Test Lot antibody** with an initial concentration of **10.5 mg/mL** :

- (10,500 µg/mL) (V_1) = (1.25 µg/mL) (8 mL)
- (10,500 µg/mL) (V_1) = 10
- V_1 = 0.000952 mL or **0.952 µL**

Antibody	Lot	[C1] Initial Concentration (mg/mL)	[C2] Final Concentration (µg/mL)	1X DPBS Volume to Take Out (µL)	[V1] Antibody Volume to be Added (µL)
Positive Control: Reference rhesus IgG1 (Anti-CD20) [2B8R1]	-	10.20	1.25	0.980	0.980
Reference Antibody : Reference afucosylated rhesus IgG1 (Anti-CD20-Af) [2B8R1F8]	Reference	11.60	1.25	0.862	0.862
Test Antibody : Reference afucosylated rhesus IgG1 (Anti-CD20-Af) [2B8R1F8]	Test	10.50	1.25	0.952	0.952

Table 1: The volumes in this table correspond to the amount of antibody required to prepare 8 mL of a 1.25 µg/mL working solution (prepared in excess).

- 4.4 Prepare three labeled **15 mL** sterile centrifuge tubes and add **8 mL** of 1X DPBS to each, using a 10 mL serological pipette.
- 4.5 For each tube, remove the calculated volume of 1X DPBS and replace it with the corresponding amount of antibody (refer to **Table 1**).
- 4.6 Ensure each centrifuge tube is labeled with the correct lot information, operator's initials, and the date of preparation.
- 4.7 Gently mix each tube by inverting several times.



Note

In this protocol example, the **Test Lot** and **Reference Lot** are both the NHPRR product, CAT-00440, but they come from different lots. The **Test Lot** was used to demonstrate how to coat with different test antibodies. The **Reference Lot**, on the other hand, will serve as a reference antibody for future assays. The remaining wells may be used for other test antibodies as needed; it is recommended to run all test antibodies in duplicate, as outlined in this SOP.

Day 1 - Plate Coating

1d

5 Pour antibody solutions into separate labeled reagent reservoirs.

5.1 Using a multichannel pipette, dispense $100\ \mu\text{L}$ of antibody solution at 1 mg $1.25\ \mu\text{g}/\text{mL}$ into each corresponding well (each well gets $0.125\ \mu\text{g}$ of antibody). Utilize **Figure 2** as a guide.

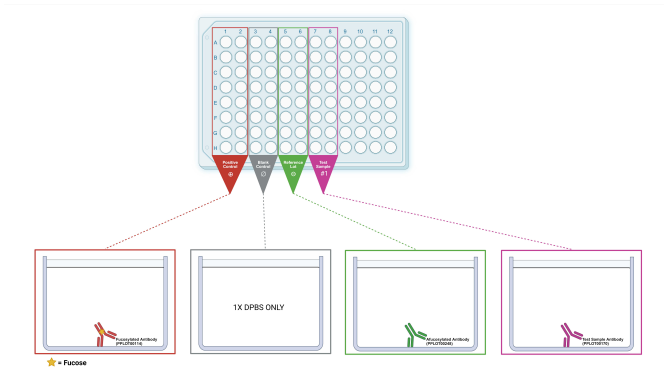


Figure 2: Fucose Detection Assay Coating Schematic

Columns 1 and 2: Positive Control - (Reference rhesus IgG1 (Anti-CD20) [2B8R1], NHPRR, CAT-00432, RRID: AB_3695603)

Columns 3 and 4: Blank - (1X DPBS only)

Columns 5 and 6: Reference Antibody - (Reference afucosylated rhesus IgG1 (Anti-CD20-Af) [2B8R1F8], NHPRR, CAT-00440, Reference Lot, RRID: AB_3719830)

Columns 7 and 8: Test Antibody - (Reference afucosylated rhesus IgG1 (Anti-CD20-Af) [2B8R1F8], NHPRR, CAT-00440, Test Lot, RRID: AB_3719830)

Columns 9 and 12: These wells can be used for additional test antibodies, 1X DPBS, or can be left empty.

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<https://BioRender.com/eena8wa>

5.2 Make sure to add $100\ \mu\text{L}$ of 1X DPBS into each Blank well (refer to **Figure 2**).

5.3 Seal the working plate and incubate Overnight at $4\ ^\circ\text{C}$.



Day 2 - Blocking

1h

6 Retrieve the working plate that was coated the previous day.

6.1 Wash the plate three times with the Washing Buffer using an ELISA plate washer.



- 6.2 Add  275 μL of Blocking Buffer to each well using a multichannel pipette.
- 6.3 Seal the plate and incubate for  01:00:00 at  Room temperature or overnight at  4 $^{\circ}\text{C}$.

1h



Note

Potential pause point: After blocking the ELISA working plate with Blocking Buffer, the plate may be stored at 4 $^{\circ}\text{C}$ overnight, and the assay can be continued the following day.

Day 2 - Biotinylated LCA Preparation

- 7 The following sub-steps can be performed while the working plate is set aside for blocking. Before proceeding, refer to the guidelines to understand the difference between a "Working Plate" and a "Dilution Plate".
- 7.1 The  Biotinylated LCA Vector Laboratories Catalog #B-1045 needs to be diluted to  10 $\mu\text{g/mL}$ in a  15 mL sterile centrifuge tube using $C_1V_1 = C_2V_2$ (refer to **Table 2**).
- 7.2 **Example Calculation:**
- (5,000 $\mu\text{g/mL}$) (V_1) = (10.00 $\mu\text{g/mL}$) (4 mL)
 - (5,000 $\mu\text{g/mL}$) (V_1) = 40
 - $V_1 = 0.008$ mL or **8.00 μL**

	Name	[C1] Initial Concentration (mg/mL)	[C2] Final Concentration ($\mu\text{g/mL}$)	Blocking Buffer Volume to take Out (μL)	[V1] Antibody Volume to be Added (μL)	[V2]
	Biotinylated LCA	5.00	10.00	8.00	8.00	

Table 2: Volumes stated correspond to the amount of Biotinylated LCA required to prepare 4 mL of a 10.00 $\mu\text{g/mL}$ working solution.

- 7.3 Take a  15 mL sterile centrifuge tube and add  4 mL of Blocking Buffer to it, using a 5 mL serological pipette.
- 7.4 Pipette out  8.00 μL of Blocking Buffer and replace it with the same volume of Biotinylated LCA (refer to **Table 2**).
- 7.5 Gently mix the solution by inverting the tube several times (do not vortex).
- 7.6 Obtain a low-binding dilution plate.
- 7.7 Add  300 μL of the prepared  10 $\mu\text{g/mL}$ Biotinylated LCA solution to each well in Row A of the dilution plate.
- 7.8 Take a reagent reservoir and add  20-25 mL of Blocking Buffer to it.
- 7.9 Using a multichannel pipette, add  200 μL of Blocking Buffer to all wells of the dilution plate, except for Row A.



Note

If using an electronic Integra pipette, then use the "repeat dispense" setting.

- 7.10 Follow the steps below to prepare serial 1:3 dilutions in the dilution plate, as shown in **Figure 3**.
- 7.11 Using a multichannel pipette, transfer  100 μL from Row A to Row B and mix thoroughly by pipetting up and down at least 5–6 times.
- 7.12 Transfer  100 μL from Row B to Row C and mix thoroughly again.
- 7.13 Continue this process down through Row G.
- 7.14 After mixing in Row G, discard the  100 μL remaining in the pipette tips to maintain a consistent volume across all wells.
- 7.15 Row H should contain only Blocking Buffer (no Biotinylated LCA).

Note

If the above steps are being done using an electronic Integra pipette, the "serial dilutions" setting can be used.

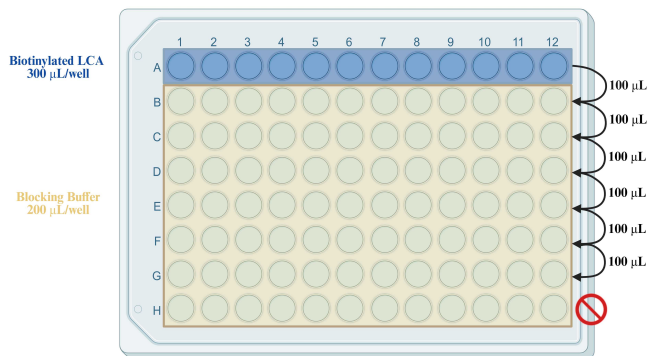


Figure 3: Schematic Representation of 1:3 Vertical Serial Dilution

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<https://BioRender.com/771n54a>

Day 2 - Transfer to ELISA Working Plate

1h

- 8 Remove the seal from the working plate that has been incubating with the Blocking Buffer for 1 hour.
- 8.1 Wash the plate three times with the Washing Buffer using an ELISA plate washer. 
- 8.2 Using a manual multichannel pipette, starting from Row H (the lowest LCA concentration), transfer  100 μL from each well of the dilution plate to the

corresponding wells on the working plate; proceed upward toward Row A (highest Concentration).

8.3 Pipette tips do not need to be changed between rows since the transfer proceeds from lower to higher concentrations.

8.4 After transferring, seal the working plate and incubate for 01:00:00 at Room temperature . 1h

Day 2 - Streptavidin-HRP / Signal Amplifier Preparation

1h

9 Dilute 8 HRP-Conjugated Streptavidin Thermo Fisher Scientific Catalog #N100 to 0.125 µg/mL in a 15 mL sterile centrifuge tube using $C_1V_1 = C_2V_2$ (see Table 3).

9.1 Example Calculation:

- $(1,250\text{ }\mu\text{g/mL}) (V_1) = (0.125\text{ }\mu\text{g/mL}) (13\text{ mL})$
- $(1,250\text{ }\mu\text{g/mL}) (V_1) = 1.625$
- $V_1 = 0.0013\text{ mL}$ or **1.3 µL**

Name	[C1] Initial Concentration (mg/mL)	[C2] Final Concentration (µg/mL)	Blocking Buffer Volume to take Out	[V1] Antibody Volume to be Added (µL)	[V2] Final Volume (mL)
HRP-Conjugated Streptavidin	1.25	0.125	1.30	1.30	13.00

Table 3: Volumes stated correspond to the amount of Streptavidin-HRP required to prepare 13 mL of a 0.125 µg/mL working solution.

9.2 Add 13 mL of Blocking Buffer into a 15 mL sterile centrifuge tube, using a 10 mL serological pipette.

9.3 Pipette out 1.3 µL of Blocking Buffer and replace it with the same volume of Streptavidin-HRP (refer to Table 3).

9.4 Gently mix the solution by inverting the tube several times. 

9.5 Pour the diluted Streptavidin-HRP working solution into a reagent reservoir.

9.6 Remove the seal from the working plate that has been incubating with the Biotinylated LCA for 1 hour.

9.7 Wash the plate three times using the Washing Buffer. 

9.8 Using a multichannel pipette, add 100 µL of Streptavidin-HRP to all wells of the working plate; each well gets 0.0125 µg of Streptavidin-HRP.

9.9 Seal the plate and incubate for 01:00:00 at Room temperature . 1h

Day 2 - Detection and Reading

5m

10 Remove the seal from the working plate that has been incubating with the Streptavidin-HRP for 1 hour.



- 10.1 Using a multichannel pipette, add  100 μ L of TMB Substrate to all wells of the working plate and incubate for  00:05:00 .
- 10.2 Stop the reaction by adding  100 μ L ELISA Stop Solution to each well.
- 10.3 Read the absorbance at **450 nm** within 10 minutes using a calibrated plate reader.

5m



Data Analysis Using GraphPad Prism v10.3.0

- 11 Export OD450 absorbance data from the Spectra Max ABS plate reader into Microsoft Excel (refer to **Attachment #3**).
- 11.1 Organize the data by sample type following the plate map layout: Positive Control, Blank, Reference Antibody, Test Antibody 1, etc., as shown in **Attachment #2**.
- 11.2 Open GraphPad Prism and start new project:
- 11.3 Select "**X = numbers**" for the X-Axis values.
- 11.4 Set "Y" to include 2 replicates (side-by-side).
- 12 Set up the data tables:
- 12.1 Label the X-axis as "Biotinylated LCA (μ g/mL)."
- 12.2 Input the titrated concentrations of Biotinylated LCA in the allotted column.
- 12.3 Create a separate column for each sample group: Positive Control, Blank, Reference Antibody, Test Antibody 1, Test Antibody 2, etc., as shown in **Attachment #1**.
- 12.4 Enter the OD450 absorbance values into the appropriate rows for each group (refer to **Attachment #1**).
- 13 Generate the graph:
- 13.1 Click the graph tab and select the graph type labeled "**Points and Connecting Line with Error Bars**."
- 14 Apply curve fitting:
- 14.1 Go to "**Analyze**" and select "**Nonlinear Regression**."
- 14.2 Then, select "**Sigmoidal, 4PL, X is log (concentration)**."
- 15 Format the X-axis:
- 15.1 Right-click on the X-axis and select "**Format Axis**."

15.2 Set the scale to **Log**.

15.3 Set **Minimum = 0.001** and **Maximum = 100**.

16 Format the Y-axis:

16.1 Right-click on the Y-axis and select "**Format Axis**."

16.2 Set **Minimum = 0** and **Maximum = 5**.

16.3 Label the Y-axis as "**Absorbance (450 nm)**."

17 **Assay Results:** Positive Control, Blank, Reference Antibody, and Test Antibody

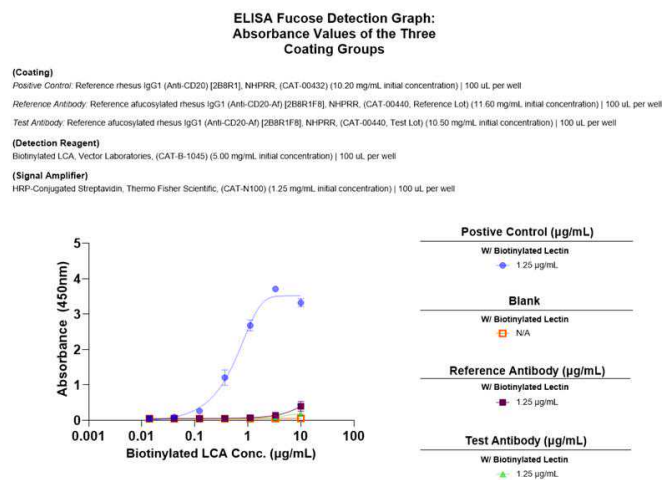


Figure 4: Example ELISA Signal and Validity Checks

Graph Legend: The blue line with circles represents the positive control, where wells were consistently coated with 1.25 µg/mL of this reagent. The orange line with outlined squares represents the blank wells, which were coated with buffer only. The purple line with filled-in boxes relates to the reference antibody, and the green line with triangles represents the test antibody, both coated at a constant 1.25 µg/mL. The X-axis shows the titrated concentration of Biotinylated LCA, which served as the detection reagent, while the Y-axis shows the resulting absorbance values. To read the graph, locate the desired LCA concentration on the X-axis, move up to the corresponding data point, and then across to the Y-axis to determine the absorbance value.

Attachment 1: GraphPad Prism File

ELISA Fucose Detection Graph_Abs...

Attachment 2: Plate Map PDF

Plate Map for Fucose Detection Pro...

Attachment 3: Raw Absorbance Data Excel File

Fucose Detection Protocol Assay R...

Acceptance Criteria

**18 Positive Control:**

☒ Reference rhesus IgG1 (Anti-CD20) [2B8R1] Nonhuman Primate Reagent Resource (NHPRR) Catalog #CAT-00432

The optical density (OD) should increase with increasing concentrations of Biotinylated LCA and **plateau** within the range of **3.0 to 4.0 AU** ($\pm 20\%$).

19 Reference Antibody:

☒ Reference afucosylated rhesus IgG1 (Anti-CD20-Af) [2B8R1F8] Nonhuman Primate Reagent Resource (NHPRR) Catalog #CAT-00440

The afucosylated reference antibody should produce flat or near-baseline binding curves, indicating minimal to no binding of Biotinylated LCA, as expected for afucosylated antibodies. The OD values of ≤ 0.6 AU ($\pm 20\%$) are considered acceptable for the reference signal.

20 Test Sample:

☒ Reference afucosylated rhesus IgG1 (Anti-CD20-Af) [2B8R1F8] Nonhuman Primate Reagent Resource (NHPRR) Catalog #CAT-00440

The test sample should not exhibit an OD value substantially higher than that of the afucosylated reference antibody. As an operational guideline, values exceeding approximately twice the reference signal (**i.e., $> 2 \times 0.6$ AU**) may indicate the presence of fucose. If such a criterion is not met, the sample should be subjected to further investigation using glycan mapping.

21 Blank Wells:

No visible color development should be observed, and the OD value, representing any background signal, should be ≤ 0.10 AU ($\pm 20\%$).

22 If the assay does not meet acceptance criteria, refer to the "Troubleshooting" section of the protocol for possible causes and corrective actions.

Note

*The above acceptance criteria were established based on NHPRR qualification data for this assay. A **$\pm 20\%$ variance** has been included to account for potential differences between laboratories and instruments. Additionally, the specified OD values may change if the protocol's concentrations, Volume and dilutions are proportionally scaled up or down.*

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