RayBio® Human Glycosylation Antibody Array L-3

For the simultaneous detection of the glycosylation protein profile of 500 human proteins in serum, plasma, cell culture supernatants, cell/tissue lysates or other body fluids.

User Manual (Revised Nov. 4th, 2020)

Human Glycosylation Array L-3
Cat# GAH-GCM-L-3-4 (4 Sample Kit)
Cat# GAH-GCM-L-3-8 (8 Sample Kit)

Please read manual carefully before starting experiment

RayBiotech
Empowering your proteomics

Your Provider of Excellent Protein Array Systems and Services

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I. Introduction

Glycosylation is the addition of sugar molecules onto cellular proteins, lipids, and polysaccharides that results from a co- and post-translational modification. Recent appreciation of glycosylation changes on the cell surface have been linked to numerous cellular diseases, most notably in several cancers. Understanding the changes that occur in the cellular process of molecule glycosylation can unveil new biomarkers of disease or pave the way for new drug targets.

Recent technological advances by RayBiotech have enabled the largest commercially available glycosylation antibody array to date. With the Human Glycosylation Array L-3, researchers can now efficiently obtain glycosylation profiles in their samples. This assay allows the simultaneous detection of glycosylation profiles of 500 human proteins, including, but not limited to, cytokines, chemokines, adipokines, growth factors, angiogenic factors, proteases, soluble receptors, soluble adhesion molecules in cell culture supernatants, serum, plasma and other samples types. Using a set of 5 lectins that recognize a broad array of glycosylation moieties on the surface of proteins, this assay allows researchers to identify glycosylation changes on the surface of these proteins directly.

How it Works

Capture antibodies for the 500 proteins are printed onto glass slides and the glycans on these capture antibodies are removed. The glass slide arrays come pre-blocked and are ready to be incubated with samples. After incubation with samples and washing to remove unbound proteins, five unique biotin-labeled lectins are incubated with the array. These lectins bind their respective glycan moiteies on the captured proteins present on the glass surface. Streptavidin-conjugated fluorescent dye (Cy3 equivalent) is then added to the array which recognizes the biotin attached to any bound lectin molecule. Finally, the glass slide is dried and laser fluorescence scanning is used to visualize the signals. These signals are then compared to the array map to identify glycosylated proteins present in the samples.

II. Materials Provided

A. Storage Recommendations

Upon receipt, the kit should be stored at -20°C until needed. Use within 6 months from the date of shipment is recommended. After initial use, remaining reagents should be stored at 4°C and may be stored for up to 3 months. Unused glass slides should be kept at -20 °C and repeated freeze-thaw cycles should be avoided (slides may be stored for 6 months).

ITEM	Description	4 Sample Kit (GAH-GCM-L-3-4)	8 Sample Kit (GAH-GCM-L-3-8)
A	Human Glycosylation Array L-3 Glass Slide*	1 pre-coated slide	2 pre-coated slides
B	Dilution Buffer	1 bottle (8 mL)	2 bottles (8 mL/ea)
D	20X Wash Buffer 1 Concentrate	1 vial (30 ml)	2 vials (30 ml/ea)
E	20X Wash Buffer 2 Concentrate	1 vial (30 ml)	2 vials (30 ml/ea)
F	Cy3-Conjugated Streptavidin**	1 vial	2 vials
G	Biotin-labeled Lectin Mixture***	2 vials	4 vials
H	Adhesive Plastic Strips	1	2
M	30 mL Centrifuge Tube	1 tube	1 tube

*Each slide contains 4 identical subarrays
**HiLyte Plus™ 555
*** See page 13 for lectin mixture composition

B. Additional Materials Required

- ddH_2O
- Small plastic or glass containers
- Orbital shaker or oscillating rocker
- 1 ml tube
- Pipettors, pipette tips and other common lab consumables
- Laser scanner for fluorescence detection (list available online)
- Aluminum foil

III. Overview and General Considerations

A. Preparation and Storage of Samples

1) Preparation of Cell Culture Supernatants

1. Seed cells at a density of $1x10^6$ cells in 100 mm tissue culture dishes.*
2. Culture cells in complete culture medium for ~24–48 hours. **
3. Replenish with serum-free or low-serum medium such as 0.2% FCS/FBS serum, and then incubate cells again for ~48 hours. ** If high serum medium such as 10% FCS/FBS is used, high background can occur on glass slide arrays due to serum interactions with the slide surface.
4. To collect supernatants, centrifuge at 1,000 g for 10 min and store as ≤1 ml aliquots at -80°C until needed.
5. Measure the total wet weight of cultured cells in the pellet and/or culture dish. You may then normalize between arrays by dividing fluorescent signals by total cell mass (i.e., express results as the relative amount of protein expressed/mg total cell mass). Or you can normalize between arrays by determining cell lysate concentration using a total protein assay (BCA Protein Assay Kit, Pierce, Prod #: 23227).

**The density of cells per dish used is dependent on the cell type. More or less cells may be required.*

***Optimal culture time may vary and will depend on the cell line, treatment conditions and other factors.*

2) Extracting Protein from Cells

1. Centrifuge Cells:

    a. Adherent Cells:

        i. Remove supernatant from cell culture and wash cells gently twice with cold 1X PBS taking care not to disturb cell layer.

        ii. Add enough cold 1X PBS to cover cell layer and use cell scraper to detach cells. Proceed to 1b. Cells in Suspension.

    b. Cells in Suspension: Pellet the cells by centrifugation using a microcentrifuge at 1500 rpm for 10 min.

2. Make sure to remove any remaining PBS before adding cell lysis buffer. Solubilize the cells at $2x10^7$ cells/ml lysis buffer.

Note: We recommend RayBio® 2X Cell Lysis Buffer (Cat# AA-LYS) or another lysis buffer suitable for immunoprecipitations. We also recommend supplementing the buffer with a protease inhibitor cocktail prior to homogenization.

3. Pipette up and down to resuspend cells and rock the lysates gently at 2–8 °C for 30 minutes. Transfer extracts to microfuge tubes and centrifuge at 13,000 rpm for 10 min at 2-8 °C.

Note: If the lysates appear to be cloudy, transfer the lysates to a clean tube, centrifuge again at 13,000 rpm for 20 minutes at 2-8°C. If the lysates are still not clear, store them at -20°C for 20 minutes. Remove from the freezer and immediately centrifuge at 13,000 rpm for 20 minutes at 2-8°C.

4. Transfer lysates to a clean tube. Determine cell lysate concentrations using a total protein assay (BCA Protein Assay Kit, Pierce, Prod# 23227). Aliquot the lysates and store at -80°C.

3) Extracting Protein from Crude Tissue

1. Transfer approximately 100 mg crude tissue into a tube with 1 ml lysis buffer.
2. Homogenize the tissue according to homogenizer manufacturer instructions.
3. Transfer extracts to microcentrifuge tubes and centrifuge for 20 min at 13,000 rpm (4°C).

Note: If the supernatant appears to be cloudy, transfer the supernatants to a clean tube, centrifuge again at 13,000 rpm for 20 minutes at 2-8°C. If the supernatant is still not clear, store the lysate at -20°C for 20 minutes. Remove from the freezer, immediately centrifuge at 13,000 rpm for 20 minutes at 2-8°C.

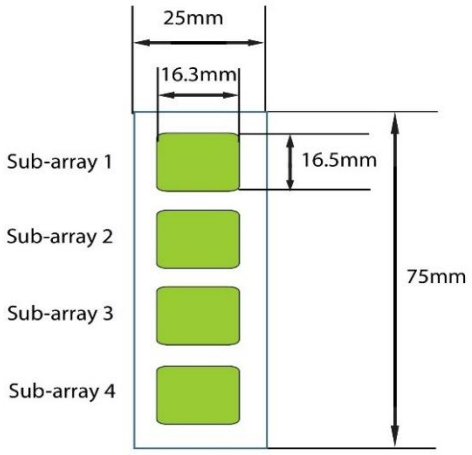
4. Transfer supernatant to a clean tube and store at -80°C.

B. Handling the Glass Slides

- The microarray slides are delicate. Please do not touch the array surface with pipette tips, forceps or your fingers. Hold the slides by the edges only.
- Handle the slides with powder-free gloves and in a clean environment.
- Do not remove the glass slide from the chamber assembly until step 14 on page 10, and take great care not to break the glass slide when doing so.
- Remove reagents/sample by gently applying suction with a pipette to corners of each chamber. Do not touch the printed area of the array, only the sides as seen in image below.

C. Layout of Human Glycosylation Array L-3 Glass Slide

Four identical sub-arrays on one slide



4 printed sub-arrays per glass chip

D. Incubations and Washes

- During incubations and washes, cover incubation chamber with a Plastic Adhesive Strip (Item H) to prevent evaporation, particularly those incubations lasting 2 hours or longer.
- During incubation and wash steps avoid foaming and be sure to remove all bubbles from the sub-array surface.
- Perform all incubation and wash steps with a gentle rocking motion (~0.5 to 1 cycle/sec).
- Wash steps in Wash Buffer II and all incubation steps may be performed overnight at 4°C, with gentle rocking.
- Avoid cross-contamination of samples to neighboring wells. To remove Wash Buffers and other reagents from chamber wells, you may invert the Glass Slide Assembly to decant, and aspirate the remaining liquid.

- Unlike most Cy3 fluors, the HiLyte Plus™ 555 used in this kit is very stable at RT and resistant to photobleaching on the hybridized glass slides. However, please protect glass slides from strong direct light and temperatures above RT.

IV. Protocol

A. Drying of the Glass Slide

1. Remove the package containing the assembled glass slide (Item A) from the freezer. Place unopened package on the bench top for approx. 15 min, and allow the assembled glass slide to equilibrate to room temperature (RT).

2. Open package, and take the assembled glass slide out of the sleeve (do not disassemble the glass slide from the chamber assembly). Place glass slide assembly in laminar flow hood or similar clean environment for 1-2 hours at RT.

Note: Protect the slide from dust or other contaminants.

B. Incubations

Note: Glass slide should be completely dry before continuing with this procedure.

3. Immediately prior to sample incubation, spin samples for 5 min at 10,000 rpm to remove any particulates or precipitates. Dilute samples with 1X Dilution Buffer (Item B). Recommended dilution of samples with Blocking Buffer is 2-10 fold for serum/plasma and cell culture supernatants or body fluid, 100-500ug/ml (total protein concentration) for cell/tissue lysate.

Note: Optimal sample dilution factor will depend on the abundance of target proteins. If the background or antigen-specific antibody signals are too strong, the sample can be diluted further in subsequent experiments. If the signal is too weak, more concentrated samples can be used.

4. Add 400 µl of diluted samples into appropriate wells. Remove any bubbles on array surfaces. Incubate arrays with gentle rocking for 2 hours at RT or overnight at 4°C.

Note: Avoid sample flow through into neighboring wells.

5. Dilute 20X Wash Buffer I Concentrate (Item D) 20-fold with ddH_2O. Decant the samples from each well, and wash 3 times with 800 µl of 1X Wash Buffer I at RT with gentle rocking for 5 min per wash.

6. Obtain a clean container (e.g., pipette tip box or slide-staining jar), place the assembled glass slide into the container with enough volume of 1X Wash Buffer I to completely cover the entire assembly, and remove any bubbles in wells. Wash 2 times at RT with gentle rocking for 10 min per wash.

7. Dilute 20X Wash Buffer II Concentrate (Item E) 20-fold with ddH_2O. Decant the Wash Buffer I from Step 6. Place the assembled glass slide into the container with enough volume of 1X Wash Buffer II to completely cover the entire assembly, and remove any bubbles in wells. Wash 2 times at RT with gentle rocking for 5 min per wash.

8. Prepare Biotin-labeled Lectin Mixture (Item G): Add 1 mL 1x Dilution Buffer directly into the tube Item G. Pipette up and down gently to mix and then pipette 400 µL into each well on the array slide.

9. Incubate slide at RT with gentle rocking for 45-50 minutes.

10. Repeat washing steps from Step 5 to Step 7.

11. Prepare 1X Cy3-Conjugated Streptavidin:

    a. Briefly spin down tube containing the Cy3-Conjugated Streptavidin (Item F) immediately before use.

    b. Add 1000 µl of Dilution Buffer (Item B) into the tube to prepare a concentrated Cy3-Conjugated Streptavidin stock solution. Pipette up and down to mix gently (do not store the stock solution for later use).

    c. To prepare 1X Cy3-Conjugated Streptavidin, add 400 µl of the concentrated Cy3-Conjugated Streptavidin stock solution into a tube with 1600 µl of Dilution Buffer (Item B). Mix gently.

12. Carefully remove Assembled Glass Slide from container. Remove all of Wash Buffer II from the wells. Add 400 µl of 1X Cy3-Conjugated Streptavidin to each sub-array. Cover the incubation chamber with the plastic adhesive strips.

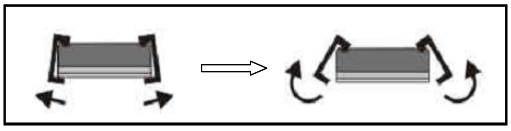
Note: Avoid exposure to light in Steps 13-20 by covering the Glass Slide Assembly with aluminum foil or incubate in dark room.

13. Incubate with Cy3-Conjugated Streptavidin at RT for 2 hours with gentle rocking.

***Note: Incubation may be done overnight at 4°C* with gentle rocking.**

14. Decant the solution and disassemble the glass slide from the incubation frame and chamber. Disassemble the device by pushing clips outward from the side, as shown below. Carefully remove the glass slide from the gasket.

Note: Be careful not to touch the printed surface of the glass slide, which is on the same side as the barcode.



15. Gently place the glass slide into the 30 ml Centrifuge Tube (Item I). Add enough 1X Wash Buffer I to cover the entire glass slide. Wash with gentle rocking for 10 min. Remove Wash Buffer I. Repeat 2 times for a total of 3 washes.

16. Repeat step 15 with 1X Wash Buffer II. Repeat one time with Wash Buffer II, for a total of two washes for 5 min per wash.

17. Finally, wash the glass slide with 30 ml of ddH_2O for 5 min with gentle rocking. Remove glass slide and decant water from Centrifuge Tube.

18. Remove water droplets from slide by applying suction gently with a pipette tip.

Note: Be careful not to touch the array portions of the slide with your pipette tip, only touch the sides of the slide.

C. Fluorescence Detection

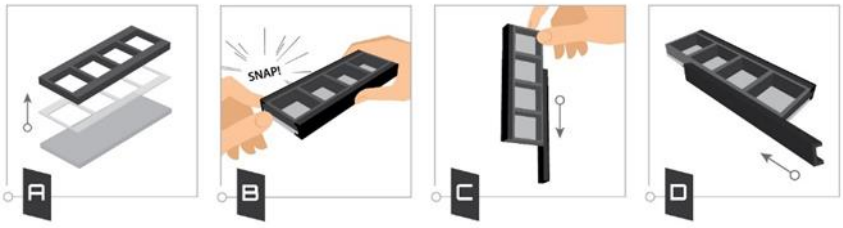
19. Allow glass chip to dry in a laminar flow hood protected from light for 20 minutes or until slide is completely dry. Place chip under an aluminum foil tent to protect it from light. Make sure the slides are absolutely dry before scanning or storage.

20. You may proceed immediately to scanning, or you may store the slide at -20 °C in the Centrifuge Tube provided or at RT and to scan at a later time.

Note: ***<u>Please protect glass slides from temperatures above RT and store them in the dark protected from light</u>. Do not expose glass slide to strong light, such as sunlight or a UV lamp.***

Note: ***If you need to repeat any of the incubations after finishing the experiment, you must first re-assemble the glass slide into the incubation chamber by following step as shown in the figures below. To avoid breaking the printed glass slide, you may first want to practice assembling the device with a blank glass slide.***

1. *Apply slide to incubation chamber barcode facing upward as in image A (below).*
2. *Gently snap one edge of a snap-on side as shown in image B.*
3. *Gently press other of side against lab bench and push in lengthwise direction (image C).*
4. *Repeat with the other side (image D)*



V. Antibody Array Map

RayBio® Human Glycosylation Antibody Array L-3 Map

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	P 1a	P 1a	P 2a	P 2a	P 3a	P 3a	neg	neg	5	5	6	6	7	7	8	8	9	9	10	10	11	11	12	12	13	13	14	14	15	15
2	16	16	17	17	18	18	19	19	20	20	21	21	22	22	23	23	24	24	25	25	26	26	27	27	28	28	29	29	30	30
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35	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	positi	posit	posit	posit	posit	positi

Biotin-Labeled Lectin Mixture

	Lectin Name	Sugar specificity
1	Concanavalin A	αMan, αGlc
2	Dolichos Biflorus Agglutinin	αGalNAc
3	Peanut Agglutinin	Galβ3GalNAc
4	Ulex Europaeus Lectin 1	αFuc
5	Wheat Germ Agglutinin	GlcNAc

RayBio® Human Glycosylation Antibody Array L-3 List

number	name	number	name	number	name	number	name	number	name
1	Pos 1a	61	alpha Tubulin	121	beta 1 Spectrin	181	Cathepsin X/Z/P	241	Collagen I a1
2	Pos 2a	62	AlphaA Crystallin/CRYAA	122	beta B1 Crystallin/CRYBB1	182	CBS	242	Collagen III
3	Pos 3a	63	ALS	123	beta -I Tubulin	183	CCDC126	243	Collagen IVa6
4	Neg	64	Als2	124	beta III Tubulin/CUBB3	184	CCDC25	244	Collagen IX
5	14-3-3 beta	65	ALS+B62:B1212CR1	125	BID	185	CCT3	245	Collagen V
6	14-3-3 epsilon	66	Aminoacylase	126	BIN2	186	CD109	246	Collagen VI
7	14-3-3 eta	67	Androgen Receptor	127	BIRC6	187	CD133	247	Collagen X
8	14-3-3 gamma	68	ANGPTL6	128	BLMH	188	CD155	248	Collagen XV alpha 1
9	14-3-3 sigma	69	ANGPTL8	129	BLVRB	189	CD157	249	COMP
10	14-3-3 theta	70	ANK	130	BMP-1	190	CD16	250	Complement Factor B
11	14-3-3 zeta	71	Ankrd26	131	BPGM	191	CD21	251	Contactin-3
12	53BP1	72	Annexin A1	132	BPIFB1	192	CD32	252	COPS8
13	67LR	73	Annexin A2	133	BPIL1	193	CD35	253	Corneodesmosin
14	ABAT	74	Annexin A6	134	BRCA 2	194	CD39L4	254	Coronin 3
15	ABCF1	75	Annexin V	135	BRD2	195	CD41	255	Cortactin
16	ABI3BP	76	ANP	136	Brevican	196	CD42b	256	COTL1
17	ACAA1	77	Antithrombin III	137	Brg1	197	CD48	257	CPE
18	ACAA2	78	APA	138	BRSK1	198	CD5L	258	CPEB3
19	ACACA	79	APLP-1	139	BTD	199	CD9	259	CPM
20	Acetyl-CoA acetyltransferase/ACAA	80	APM2	140	BTF3	200	CD98	260	CPN1
21	ACLP	81	Apo (a)	141	C 1q	201	CDA	261	CPNE3
22	ACLY	82	APOA1BP	142	C 1q S	202	CDC5L	262	CPS1
23	Aconitase 1	83	Apolipoprotein F	143	C1QB	203	CDK2	263	Creatine Kinase MM/CKMM
24	ACTBL2	84	Apolipoprotein L 1	144	C1qR1	204	CEACAM-8/CD66b	264	CRF21
25	ACTC1	85	Apolipoprotein L 2	145	C1RL	205	CECR1	265	CRHBP
26	Actinin alpha 1	86	ARFBP1	146	C1s	206	CENPF	266	CrkL
27	ADAMDEC1	87	ARFGEF3	147	C3orf75	207	CEP57	267	CRMP2
28	ADAS	88	Argininosuccinate Lyase/ASL	148	C4.4A	208	CES1	268	CRTAC1
29	ADH1B	89	ArgRS	149	C4BPA	209	CETP	269	CS
30	ADH1C	90	ARP19	150	C5b-9	210	Cezanne	270	Ctip2
31	ADH4	91	Arp2	151	C6 -N-t	211	CFHR 1	271	Cux2
32	ADH5	92	ARP2/3	152	C8G	212	CFHR4	272	Cyclophilin A
33	ADM	93	Arp3	153	C9orf40	213	CFHR5	273	Cyclophilin B
34	Advillin-N-t	94	ARPC2	154	CA1	214	CFI	274	Cystatin D
35	AFG3L2	95	ARPC3	155	CA150	215	CFL1	275	Cystatin E/M
36	AGA	96	ART3	156	CA2	216	CFVII	276	Cystatin S
37	Aggrecan	97	ARTS1	157	CA3	217	CHC17	277	Cystatin SN
38	AGXT	98	ARX	158	CACNB4	218	Chitobiase	278	Cysteine-rich Protein 1
39	AHNAK	99	ASH2L	159	CAD	219	Chitotriosidase	279	CYTL1
40	Ahsp	100	ASGR2	160	Cadherin 22	220	CHORDC1	280	Cytochrome b5
41	AIF	101	ASK1	161	Cadherin-6	221	CHREBP	281	Cytochrome c
42	AK2	102	Aspartate Aminotransferase /AST	162	Caldesmon/CALD1	222	Chromogranin B	282	Cytokeratin 1
43	AKAP9	103	Aspartyl Aminopeptidase/DNPEP	163	CALML5	223	Chromogranin C	283	Cytokeratin 10
44	AKR1B1	104	ASXL1	164	Calmodulin	224	CIP29	284	Cytokeratin 13
45	AKR1C3	105	ATBF1/ZFHX3	165	Calpain 1	225	CKB	285	Cytokeratin 14
46	AKR7A2	106	ATP5A	166	Calpain S1	226	CLIC1	286	Pos 1b
47	ALAD	107	ATP5O	167	Calpastatin	227	CLIC4	287	Pos 2b
48	Alanine Transaminase/ALT	108	ATPB	168	Calretinin	228	CLIP170-N-t	288	Pos 3b
49	Alcohol Dehydrogenase/ADH	109	B3GNT2	169	Calumenin	229	CL-P1	289	Neg
50	Aldehyde Oxidase 1/AOX1	110	B4GalT1	170	CAP1	230	CLPS	290	Cytokeratin 15
51	ALDH16A1	111	B7-H2	171	CapG	231	CLTA	291	Cytokeratin 16
52	ALDH1A1	112	B7-H3	172	CAPZA1	232	CNN2	292	Cytokeratin 17
53	ALDH9A1	113	BAD	173	Carboxypeptidase B2/CPB2	233	CNOT1	293	Cytokeratin 20
54	ALKP	114	Band 3	174	CARHSP1	234	CO4A2	294	Cytokeratin 3
55	ALP	115	BASP1	175	Caspase-14	235	COG4	295	Cytokeratin 4
56	alpha 1,2 Mannosidase IA	116	Bassoon	176	Catalase	236	COL19A1	296	Cytokeratin 5
57	alpha Actinin 4	117	BAZ2B	177	Cathelicidin	237	COL4A3	297	Cytokeratin 9
58	Alpha Fodrin	118	BCHE	178	Cathepsin A	238	Col6A2	298	D4 GDI
59	alpha Glucosidase II	119	Bcl-w	179	Cathepsin G	239	COL9A3	299	DAK
60	alpha -Synuclein	120	BCOR	180	Cathepsin H	240	COLEC10	300	DAN

RayBio® Human Glycosylation Antibody Array L-3 List ...continued

number	name	number	name	number	name	number	name
301	DARS2	361	ERAB	421	GCLC	481	Histone H2B K
302	DCI	362	ERAP2	422	GCSH	482	Histone H3.3
303	DCXR	363	ERp29	423	GDA	483	Histone H4
304	DDAH1	364	ERp57	424	GDF7	484	HLA-C
305	DDT	365	ERp72	425	GDI1	485	HMGB1
306	DDX3Y	366	ESD	426	GDI2	486	HMGB2
307	DEFA6	367	ESR1	427	Gephyrin	487	HMGB3
308	DEP-1	368	ETL	428	GFAP	488	HMGN2
309	Der p2	369	EVC2	429	GHRF	489	HN1
310	Dermcidin	370	Ezrin	430	GIP	490	HNF-3 alpha /FoxA1
311	Desmocollin 1	371	F11	431	GLIPR2	491	hnRNP A1
312	Desmocollin-2	372	FABP5	432	GLRX1	492	hnRNP A2B1
313	Desmocollin-3	373	Factor IX	433	G6PD	493	hnRNP C1 + C2
314	Desmoglein-1	374	Factor V	434	Glucosidase 2 subunit beta/PRKCSH	494	hnRNP G
315	Desmoglein-2	375	Factor XII	435	GLUD1	495	hnRNP L
316	Desmoplakin	376	Factor XIII	436	Glutamyl hydrolase gamma /CGH	496	hnRNP M1-M4
317	Desmuslin	377	FAM20C	437	glutathione S transferase Omega 1/GSTO1	497	hnRNP U
318	Destrin	378	FAM3C	438	Glutathione Synthetase/GSS	498	Hornerin
319	DGK	379	Fascin	439	Glycerol 3 Phosphate Dehydrogenase	499	Hoxb3
320	DISC 1	380	FASN	440	Glycoprotein V	500	HOXD11
321	DMGDH	381	fast skeletal Myosin	441	Glyoxalase II	501	HP1BP3
322	DMRN9	382	FASTKD5	442	GM2A	502	HPD
323	Dopamine beta Hydroxylase/DBH	383	FBP 38	443	GMF beta	503	HPR
324	DOT1L	384	FBP2	444	GNB1	504	HPRT
325	DPEP2	385	FBPase 1	445	GNPTG	505	HRG
326	DPP3	386	FCGBP	446	GOLPH2	506	HRSP12
327	DPPI	387	FDPS	447	GOLPH4	507	HSC70
328	DRIL1	388	FH	448	GOT2	508	HSP47
329	DSCAM	389	Fibrillin 1	449	GPCR GPR116	509	Neg
330	DSPG3	390	Fibrinogen gamma chain/FGG	450	GPLD1	510	Neg
331	Dystroglycan	391	Fibrinogen-like 2	451	Grainyhead-like protein 1 homolog/GRHL1	511	Neg
332	E1 Ubiquitin Activating Enzyme/UBA1	392	Fibrinopeptide B	452	Granzyme M	512	Neg
333	ECHS1	393	Fibulin 3	453	GRHPR	513	Neg
334	ECM-1	394	Ficolin-2	454	GRP	514	Neg
335	EEF1G	395	Filamin A	455	GSTM1	515	Neg
336	EEF2	396	Filamin B	456	GSTP1	516	Neg
337	EFEMP2	397	Filamin C	457	Guanylin	517	Neg
338	EFTUD2	398	FKBP12	458	GULP1/CED-6	518	Neg
339	EHD1	399	FKBP25	459	H6PD	519	Neg
340	EHD3	400	FKBP51	460	HABP2	520	Neg
341	EIF3S2	401	FLG2	461	HBZ	521	Neg
342	eIF4A1-N-t	402	FOLR3	462	HCFC1	522	Neg
343	eIF5A	403	Frizzled 8	463	HDGF	523	Pos 1c
344	ELAVL1	404	FRY	464	HEG1	524	Pos 2c
345	EMILIN1	405	FSH	465	Hemoglobin	525	Pos 3c
346	EMSY	406	FTL	466	Hemoglobin A1c	526	
347	EN2	407	FUCA1	467	Hemoglobin subunit beta/HBB	527	
348	Endorepellin	408	FUCA2	468	Hemoglobin subunit delta/HBD	528	
349	ENO1	409	Fumarylacetoacetate hydrolase/FAH	469	Hemoglobin subunit gamma 2/HBG2	529	
350	ENO1 + ENO2 + ENO3	410	G0/G1switch 2	470	HEXB	530	
351	ENSA	411	G3BP	471	HGFA	531	
352	Envoplakin	412	GALNT2	472	hGH	532	
353	Eosinophil derived neurotoxin/EDN	413	gamma Catenin	473	hHR23b	533	
354	EPB41	414	GAPDH	474	HIBADH	534	
355	EPCR	415	GARNL1	475	HINT1	535	
356	Ephrin B1	416	GART	476	HIP1R	536	
357	Ephrin B2	417	Gastrokine 1	477	Histone H1.2	537	
358	EPHX2	418	GATM - C-terminal	478	Histone H1.3	538	
359	EPPK1	419	GBE1	479	Histone H2A	539	
360	Eps 15	420	GCDFP 15	480	Histone H2A.Z	540	

VI. Interpretation of Results:

A. Explanation of Controls Spots

1) **Positive Control spots (POS1, POS2, POS3)** are standardized amounts of biotinylated IgGs printed directly onto the array. All other variables being equal, the Positive Control intensities will be the same for each sub-array. This allows for normalization based upon the relative fluorescence signal responses to a known control, much as "housekeeping" genes or proteins are used to normalize results in PCR or Western blots, respectively.

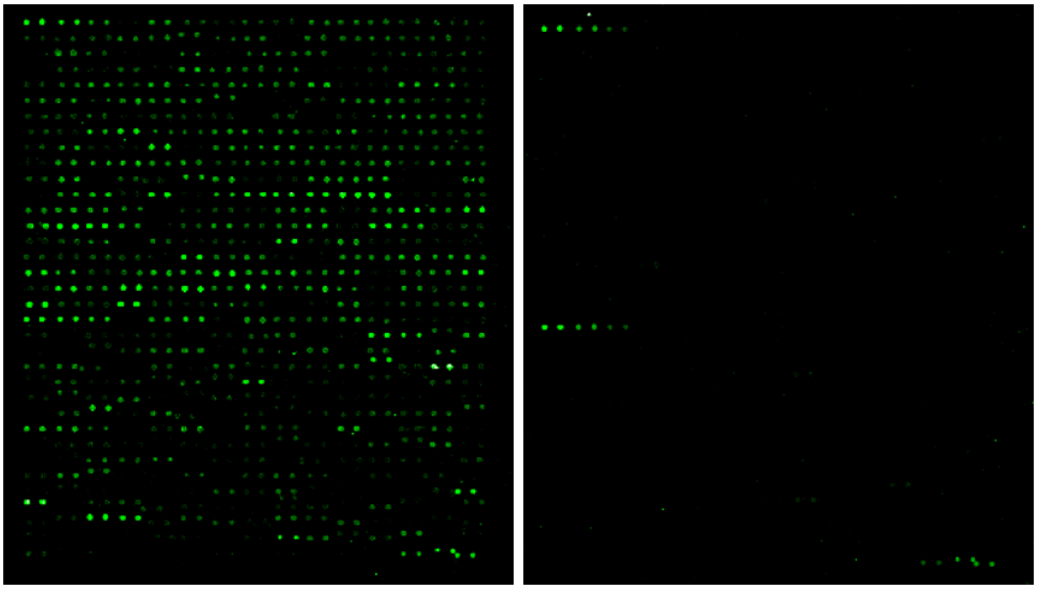
2) **Negative Control (NEG) spots** contain a protein-containing buffer (used to dilute antibodies printed on the array). Their signal intensities represent non-specific binding of the Biotin-conjugated lectin mixture and/or the Cy3-Conjugated Streptavidin. Negative control signal intensities are usually very close to background signals in each sub-array.

B. Typical Results

The following figure shows the RayBio® Human Glycosylation Antibody Array L-3 probed with a conditioned media sample. The images were captured using an Axon GenePix laser scanner. The strong signals in row 20 and the upper left and lower right corners of each array are Positive Controls, which can be used to identify the orientation and help normalize the results between arrays.

RayBio® Human Glycosylation Antibody Array L-3

Conditioned medium No Sample



RayBio Human Glycosylation Antibody Array L-3. Probed with biotinylated lectins: (b- Con A; DBA, PNA, UEA I, WGA)

If scanned using optimal settings, 3 distinct signal intensities will be seen: POS1>POS2>POS3. If all of these signals are of similar intensity, try increasing or decreasing laser power and/or signal gain settings.

C. Background Subtraction:

Once you have obtained the fluorescence intensity data, you should subtract the background and normalize to the Positive Control signals before proceeding to analysis.

Most laser fluorescence scanner software has an option to automatically measure the local background around each spot. For best results, we recommend comparing signal intensities representing the MEDIAN background signals minus local background. If your resulting fluorescence signal intensity reports do not include these values (e.g., a column labeled as "MED532-B532"), you may need to subtract the background manually or change the default settings on your scanner's data report menu.

D. Normalization of Array Data:

To normalize signal intensity data, one sub-array is defined as "reference" to which the other arrays are normalized. This choice is arbitrary. For example, in our Analysis Tool Software (described below), the array represented by data entered in the left-most column each worksheet is the default "reference array."

You can calculate the normalized values as follows:

X(Ny) = X(y) * P1/P(y)

Where:
P1 = mean signal intensity of POS spots on reference array
P(y) = mean signal intensity of POS spots on Array "y"
X(y) = mean signal intensity for spot "X" on Array "y"
X(Ny) = normalized signal intensity for spot "X" on Array "y"

The RayBio® Analysis Tool software is available for use with data obtained using RayBio® Arrays. You can copy and paste your signal intensity data (with and without background) into the Analysis Tool, and it will automatically normalize signal intensities to the Positive Controls.

To order the Analysis Tool, please contact us toll-free at +1-888-494-8555 or info@raybiotech.com for more information.

E. Threshold of significant difference in expression:

After subtracting background signals and normalization to Positive Controls, comparison of signal intensities between and among array images can be used to determine relative differences in expression levels of each protein between samples or groups.

Any ≥1.5-fold increase or ≤0.65-fold decrease in signal intensity for a single analyte between samples or groups may be considered a measurable and significant difference in expression, provided that both sets of signals are well above background (Mean background + 2 standard deviations, accuracy ≈ 95%).

VII. Troubleshooting Guide

Problem	Cause	Recommendation
Weak signal	Inadequate detection	Check laser power and PMT parameters
	Inadequate reagent volumes or improper dilution	Check pipettors and ensure correct preparation
	Short incubation times	Ensure sufficient incubation time and change sample incubation step to overnight
	Too low protein concentration in sample	Decrease dilution Or concentrate sample
	Improper storage of kit	Store kit at suggested temperature
High background	Sample is too concentrated	Use more diluted sample
	Excess of streptavidin	Make sure to use the correct amount of streptavidin
	Inadequate detection	Check laser power and PMT parameters
	Inadequate wash	Increase the volume of wash buffer and incubation time
Uneven signal	Bubbles formed during incubation	Avoid bubble formation during incubation
	Arrays are not completely covered by reagent	Completely cover arrays with solution

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