RayBio® Human Glycosylation Antibody Array 493

User Manual (Revised Dec 9, 2019)

For the simultaneous detection of the glycosylation protein profile of 493 human proteins in serum, plasma, cell culture supernatants, cell/tissue lysates or other body fluids.

Human Glycosylation Array 493
Cat# GAH-GCM-493-4 (4 Sample Kit)
Cat# GAH-GCM-493-8 (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 493, researchers can now efficiently obtain glycosylation profiles in their samples. This assay allows the simultaneous detection of glycosylation profiles of 493 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 493 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 moieties 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-493-4)	8 Sample Kit (GAH-GCM-493-8)
A	Human Glycosylation Array 493 Glass Slide*	1 GCM-493 slide	2 GCM-493 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 2 identical subarrays

** 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.

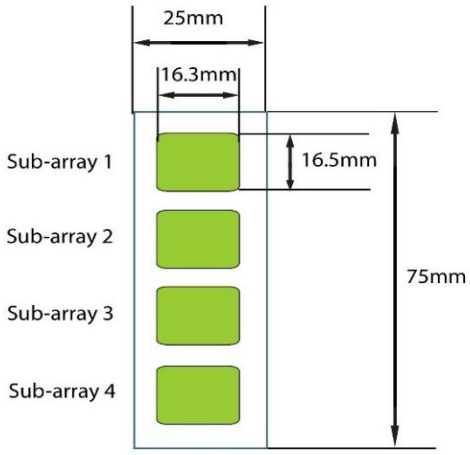
4. Transfer supernatant to a clean tube and store at -80°C.

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 493 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).

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.

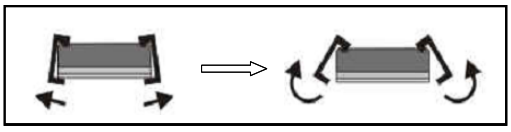
Note: Avoid exposure to light in Steps 13-20 by covering the Glass Slide Assembly with aluminum foil or incubate in dark room.

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

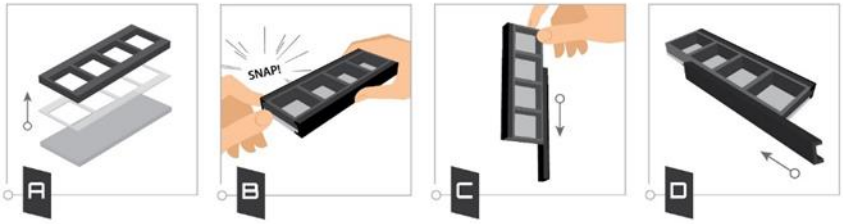
19. Allow glass chip to dry in a laminar flow hood protected from light for 20 minutes or until slide is completely dry. Place chip under an aluminum foil tent to protect it from light. Make sure the slides are absolutely dry before scanning or storage.

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: Please protect glass slides from temperatures above RT and store them in the dark protected from light. 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

A. RayBio® Human Glycosylation Antibody Array 493 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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34	496	496	497	497	498	498	499	499	500	500	501	501	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	P-3c	P-3c	P-2c	P-2c	P-1c	P-1c

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

B. RayBio® Human Glycosylation Antibody Array 493 Target List

Number	Name	Number	Name	Number	Name	Number	Name	Number	Name
1	Positive-1a	61	ApoE3	121	Ceruloplasmin	181	EpCAM	241	GPR-39
2	Positive-2a	62	ApoD	122	CFHR2	182	EphA1	242	GPX1
3	Positive-3a	63	ApoM	123	Chemerin	183	EphA2	243	GPX3
4	Blank	64	ApoH	124	CHI3L1	184	EphA3	244	Pancreastatin
5	11b-HSD1	65	APP	125	Chromogranin A	185	EphA4	245	GRP
6	2B4	66	ASPH	126	Chymase	186	EphA5	246	GRP75
7	4-1BB	67	Attractin	127	cIAP-2	187	EphA6	247	GRP78
8	ABL1	68	B3GNT1	128	Ck beta 8-1	188	EphA7	248	GSR
9	ACE	69	BAF57	129	CK-MB	189	EphA8	249	GST
10	ACE-2	70	BAFF	130	Claudin-3	190	EphB1	250	HADHA
11	ACK1	71	BAI-1	131	Claudin-4	191	EphB2	251	HAI-1
12	ACPP	72	BCAM	132	CLEC3B	192	EphB3	252	HAI-2
13	ACTH	73	Beta 2M	133	Clusterin	193	EphB4	253	hCG alpha
14	ADAM-9	74	Beta Defensin 4	134	CNDP1	194	EphB6	254	hCGb
15	Neurokinin-A	75	Beta IG-H3	135	Factor XIII A	195	ERRa	255	Hck
16	ADAMTS-1	76	Biglycan	136	Factor XIII B	196	Erythropoietin R	256	HE4
17	ADAMTS-L2	77	BLAME	137	COCO	197	ESAM	257	Hemopexin
18	ADAMTS-4	78	BMP-9	138	C2	198	EV15L	258	Hepcidin
19	ADAMTS-5	79	BMX	139	C3a	199	EXTL2	259	HSP32
20	ADAMTS-10	80	BNIP2	140	C5/C5a	200	FABP1	260	HOXA10
21	ADAMTS-13	81	Btk	141	C7	201	FABP2	261	Haptoglobin
22	ADAMTS-15	82	ApoC1	142	C8B	202	FABP4	262	HSP10
23	ADAMTS-17	83	CA 9	143	C9	203	FAK	263	HSP20
24	ADAMTS-18	84	CA 15-3	144	Complement factor H	204	FAP	264	HSP27
25	ADAMTS-19	85	CA 19-9	145	Contactin-1	205	Fc RIIB/C	265	HSP40
26	Adipsin	86	CA 125	146	Contactin-2	206	Fen 1	266	HSP60
27	Afamin	87	Cadherin-13	147	Corticosteroid-binding globulin	207	FER	267	HSP70
28	AFP	88	Calbindin	148	COX-2	208	Ferritin	268	HSP90
29	ALBUMIN	89	Calbindin D	149	C-peptide	209	Fetuin A	269	HSPA8
30	IL-36RN	90	Calcitonin	150	Creatinine	210	Fetuin B	270	HTRA2
31	Aldolase A	91	Calreticulin	151	CRP	211	FGFR1	271	IBSP
32	Aldolase B	92	Calsyntenin-1	152	CRTAM	212	FGFR1 alpha	272	IGF2BP1
33	Aldolase C	93	CPN2	153	CSH1	213	FGFR2	273	IGFBP-5
34	ALK	94	CART	154	gamma-Thrombin	214	Fibrinogen	274	IL-23p19
35	Alpha Lactalbumin	95	Caspase-3	155	CutA	215	Fibrinopeptide A	275	IL-33
36	Alpha 1 AG	96	Caspase-8	156	cTnT	216	Fibronectin	276	IL-34
37	A1BG	97	Cathepsin B	157	Cyclin D1	217	Ficolin-3	277	INSRR
38	A1M	98	Cathepsin D	158	Cystatin A	218	FIH	278	Integrin alpha V
39	A2M	99	Cathepsin L	159	Cystatin B	219	FOLR1	279	CD61
40	TPM1	100	Cathepsin S	160	Cystatin C	220	FOXN3	280	Itk
41	ALPP	101	CBP	161	Cytochrome C	221	FoxO1	281	ITM2B
42	pro-MMP13	102	CCK	162	Cytokeratin 8	222	FoxP3	282	Kallikrein 2
43	AMICA	103	CD23	163	Cytokeratin 18	223	FRK	283	ApoC3
44	AMPKa1	104	CD24	164	Cytokeratin 19	224	FSH	284	Kallikrein 5
45	Amylin	105	CD36	165	DBI	225	Furin	285	Kallikrein 6
46	ANGPTL3	106	CD38	166	DCBLD2	226	Fyn	286	Positive-1b
47	ANGPTL4	107	CD44	167	D-Dimer	227	GADD45A	287	Positive-2b
48	Annexin A7	108	CD45	168	DEFA1/3	228	Galectin-1	288	Positive-3b
49	APC	109	CD46	169	Defensin	229	Galectin-3BP	289	neg
50	APCS	110	CD47	170	Desmin	230	Galectin-7	290	Kallikrein 7
51	Apelin	111	CD55	171	DLL1	231	Gas1	291	Kallikrein 8
52	Apex1	112	CD59	172	DLL4	232	Gastrin	292	Kallikrein 10
53	APN	113	CD71	173	DMP-1	233	GATA-3	293	Kallikrein 11
54	ApoA1	114	CD74	174	DPPIV	234	GATA-4	294	Kallikrein 14
55	ApoA2	115	CD90	175	BNP	235	Gelsolin	295	KCC3
56	ApoA4	116	CD97	176	E-Cadherin	236	Ghrelin	296	KCTD10
57	ApoB	117	CD 79 alpha	177	Endorphin Beta	237	GLP-1	297	KIF3B
58	ApoC2	118	CD200	178	Endothelin Receptor A	238	GPI	298	KLF4
59	ApoB100	119	CEA	179	Enolase 2	239	GPBB	299	LAG-3
60	ApoE	120	CEACAM-1	180	ENPP2	240	GMNN	300	pro-Glucagon

RayBio® Human Glycosylation Antibody Array L-493 Target List ...continued

Number	Name	Number	Name	Number	Name	Number	Name
301	Layilin	361	Pappalysin-1	421	S100A4	481	TRPC6
302	LDL R	362	Pancreatic Polypeptide	422	S100A6	482	TRPM7
303	Legumain	363	Presenilin 1	423	S100A8	483	Trypsin 1
304	LH	364	PARK7	424	S-100b	484	TSH
305	LIMPII	365	Visfatin	425	SART1	485	TSLP
306	LIN41	366	P-Cadherin	426	SART3	486	TXK
307	Livin	367	PCAF	427	SCG3	487	Uromodulin
308	LOX-1	368	PD-1	428	Selenoprotein P	488	TFF1
309	LPS	369	PTH	429	SEMA3A	489	VDUP-1
310	LRG1	370	Troponin C	430	Serotonin	490	VEGF R1
311	LTF	371	PDX-1	431	Serpin A1	491	VGF
312	LTK	372	PEDF	432	Serpin A12	492	VIP Receptor 2
313	Lumican	373	PEPSINOGEN I	433	Serpin A3	493	Vitamin D Receptor
314	Lyn	374	PEPSINOGEN II	434	Serpin A4	494	Vitamin D-BP
315	LYRIC	375	Vasopressin	435	Serpin A5	495	Vitamin K-dependent protein S
316	LYVE-1	376	PGRP-S	436	Serpin A8	496	Vitronectin
317	LZTS1	377	PI 16	437	Serpin A9	497	VWF
318	Mammaglobin A	378	PI 3Kinase p85 beta	438	Serpin B5	498	Wilms Tumor 1
319	Marapsin	379	PIM2	439	Serpin D1	499	XIAP
320	MATK	380	PKM2	440	Serpin I1	500	ZAG
321	MBL	381	Plasminogen	441	SERPING1	501	ZAP70
322	MBL-2	382	Podocalyxin	442	SERTAD2	502	Neg
323	Mer	383	POMC	443	SHBG	503	Neg
324	Mesothelin	384	PON1	444	SMAC	504	Neg
325	MICB	385	PON2	445	SNCG	505	Neg
326	Midkine	386	PPARg2	446	SSTR5	506	Neg
327	MINA	387	PPP2R5C	447	Somatotropin	507	Neg
328	FABP3	388	NR3C3	448	SOST	508	Positive-3c
329	MSHa	389	INSL3	449	SOX17	509	Positive-2c
330	MTUS1	390	Pro-BDNF	450	SOX2	510	Positive-1c
331	Myoglobin	391	Procalcitonin	451	SPARCL1		
332	NAIP	392	Pro-Cathepsin B	452	SPINK1		
333	Nanog	393	Thrombin	453	SRMS		
334	NELL2	394	Prohibitin	454	SSEA-1		
335	NEP	395	ProSAAS	455	SSEA-4		
336	Galanin	396	Prostasin	456	SSTR2		
337	Nesfatin	397	PSP	457	Survivin		
338	Nestin	398	Pro-MMP-7	458	SYK		
339	NET1	399	Pro-MMP-9	459	Syndecan-1		
340	Netrin G2	400	Protein p65	460	Syndecan-3		
341	Netrin-4	401	PSA-Free	461	TACE		
342	Neuropeptide Y	402	PSA-total	462	TAF4		
343	NF1	403	PTHLP	463	Tyk2		
344	NM23-H1/H2	404	PTN	464	Tec		
345	Presenilin 2	405	PTPRD	465	TFF3		
346	Notch-1	406	PYK2	466	Thrombomodulin		
347	NPTX1	407	PYY	467	Thymidine Kinase-1		
348	NPTXR	408	Ras	468	Thyroglobulin		
349	Progesterone	409	RBP4	469	TIM-1		
350	Ntn1	410	RECK	470	TNK1		
351	OCT3/4	411	RELM alpha	471	TOPORS		
352	Omentin	412	Resistin	472	TPA		
353	Osteocalcin	413	RET	473	TRA-1-60		
354	Osteopontin	414	RIP1	474	TRA-1-81		
355	OX40	415	ROCK1	475	Transferrin		
356	p21	416	ROCK2	476	Trappin-2		
357	p27	417	ROR1	477	TRKB		
358	p53	418	ROR2	478	TROPONIN I		
359	PAI-1	419	ROS	479	TYRO10		
360	PAK7	420	RYK	480	TRPC1		

VI. Interpretation of Results:

A. Explanation of Controls Spots

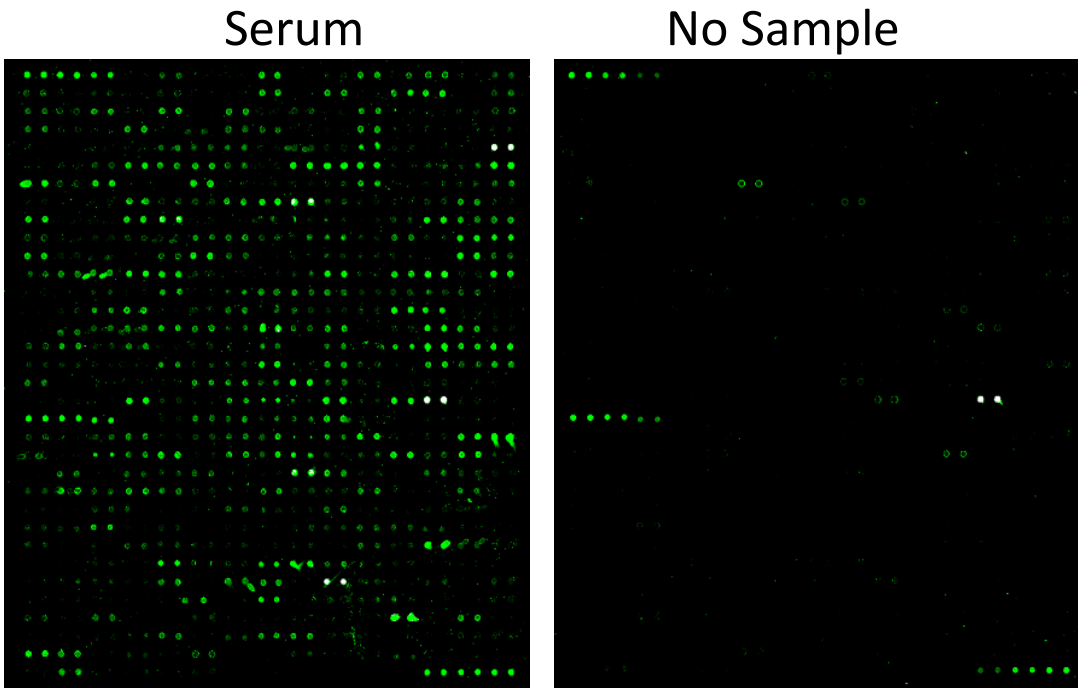
1) **Positive Control spots (POS1, POS2, POS3)** are standardized amounts of biotinylated IgGs printed directly onto the array. All other variables being equal, the Positive Control intensities will be the same for each sub-array. This allows for normalization based upon the relative fluorescence signal 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 493 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 493



RayBio Human Glycosylation Antibody Array 493. 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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