RayBio® Label-based (L-Series) Rat Antibody Array L-90 Membrane Kit

Patent Pending Technology
User Manual (Revised May 24, 2013)

For the simultaneous detection of the relative expression of 90 (L-90) rat proteins in cell culture supernatants.

Cat# AAR-BLM-1-2 (2 Sample Kit)
Cat# AAR-BLM-1-4 (4 Sample Kit)

Please read manual carefully
before starting experiment

RayBiotech, Inc.
the protein array pioneer company

Your Provider for Excellent Protein Array Systems and Services

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RayBiotech, Inc

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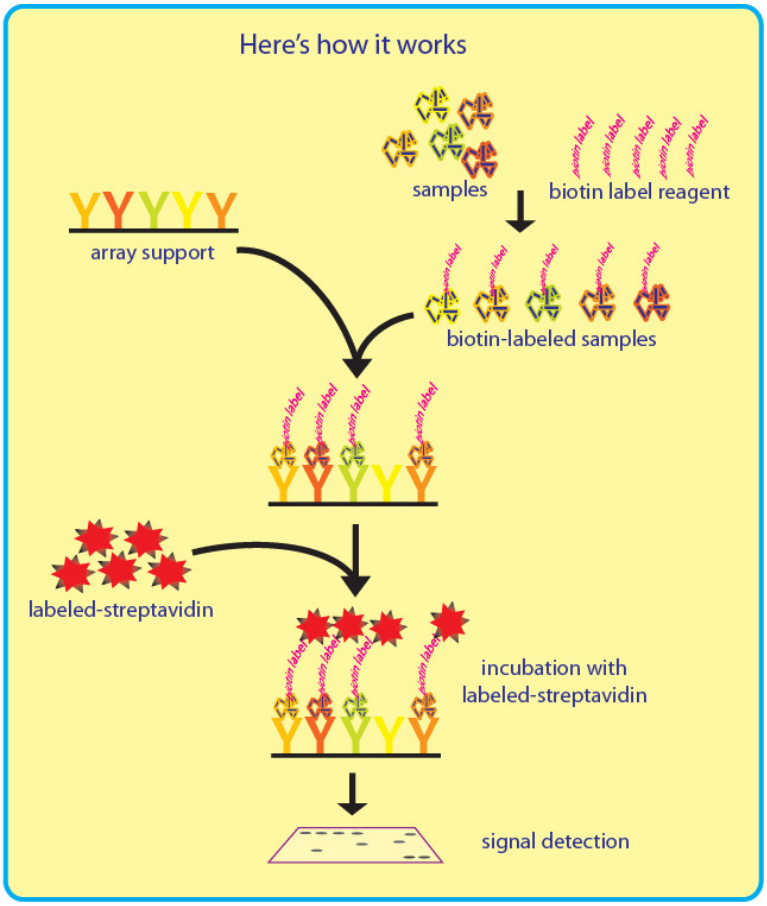
I. Introduction

Recent technological advances by RayBiotech have enabled the largest commercially available antibody array to date. With the RayBio® L-Series Rat Antibody Array 90, researchers can now obtain a broad, panoramic view of cytokine expression. The expression levels of 90 rat proteins can be simultaneously detected, including cytokines, chemokines, adipokine, growth factors, angiogenic factors, proteases, soluble receptors, soluble adhesion molecules and other proteins in cell culture supernatants.

The first step in using the Rat L-90 is to biotinylate the primary amine of the proteins in the sample. The membrane arrays are then blocked, similar to a Western blot, and the biotin-labeled sample is added onto the membrane array which is pre-printed with capture antibodies and incubated to allow for interaction of target proteins. After incubation with HRP-Conjugated Streptavidin, the signals can be visualized by chemiluminescence.



II. Materials Provided

A. Storage Recommendations

Upon receipt, Box 1 should be stored at -20 °C and Box 2 should be stored at 4 °C. The kit must be used within 6 months from the date of shipment. After initial use, Blocking Buffer, Stop Solution, HRP-Conjugated Streptavidin, Detection Buffers C and D should be stored at 4 °C to avoid repeated freeze-thaw cycles (may be stored for up to 3 months, Labeling Reagent, Item B should be fresh preparation before use). The Array Membrane should be kept at -

20 °C and avoid repeated freeze-thaw cycles (may be stored for up to 6 months).

Box 1 (store at -20 °C):

ITEM	DESCRIPTION	Cat#: AAR-BLM-1-2	Cat#: AAR-BLM-1-4
B	Labeling Reagent	1 vial	2 vials
D	Stop Solution	1 vial (50 ul)	
E	RayBio® L-Series Rat 90 Antibody Array Membranes	2 membranes L-90	4 membranes L-90
F	Blocking Buffer	1 vial (30 ml)	2 vials (30 ml/ea)
I	500X HRP-Conjugated Streptavidin Concentrate	1 vial (100 ul)	2 vials (100 ul/ea)
M	Detection Buffer C	1 vial (10 ml)	1 vial (10 ml)
N	Detection Buffer D	1 vial (10 ml)	1 vial (10 ml)
Other Kit Components:		Plastic Sheets	

Box 2 (store at 4 °C):

ITEM	DESCRIPTION	Cat#: AAR-BLM-1-2	Cat#: AAR-BLM-1-4
A	Dialysis Vials	2 vials	4 vials
G	20X Wash Buffer 1 Concentrate	1 vial (30 ml)	1 vial (30 ml)
H	20X Wash Buffer 2 Concentrate	1 vial (30 ml)	1 vial (30 ml)
J	Spin Columns	2 columns	4 columns
N/A	Plastic Incubation Trays (w/lid)	1 tray	
M	Floating Dialysis Rack	1 rack	

B. Additional Materials Required

- 1X PBS, pH=8.0
- Shaker
- 2~5 ml tube
- 50 ml conical collection tubes
- Distilled water
- Kodak X-Omat™ AR film (REF 165 1454) and film processor or Chemiluminescence imaging system
- large beaker
- stir plate
- Eppendorf tube

III. Overview and General Considerations

A. Handling Array Membranes

- Always use forceps to handle membranes and grip the membranes by the edges only.
- Never allow membranes to dry during the experiment.
- Avoid touching membranes with hands or any sharp tools.

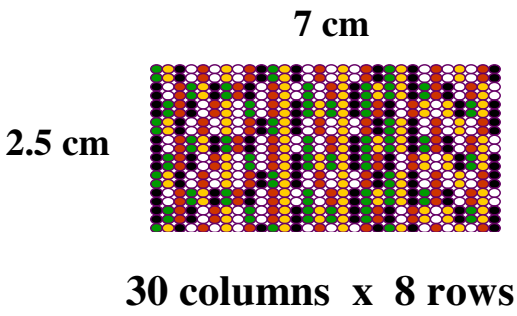
B. Incubation

- Completely cover membranes with sample or buffer during incubation and cover Plastic Incubation Tray with lid to avoid drying.
- Avoid foaming during incubation steps.
- Perform all incubation and wash steps under gentle rotation.

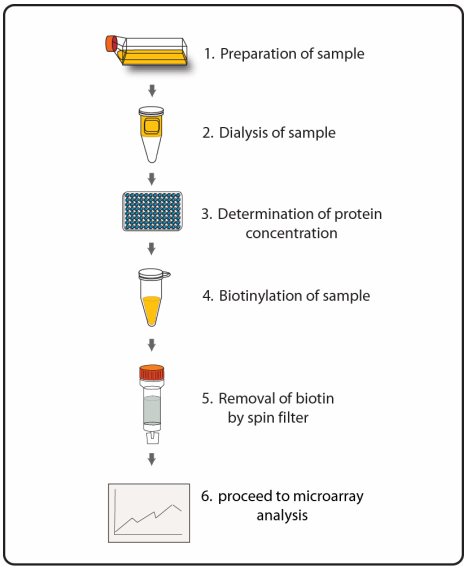
- Several incubation steps such as step 3 in page 10 (sample incubation) or step 7 in page 11 (HRP-Conjugated Streptavidin incubation) may be done at 4 °C for overnight.

IV. Protocol

Layout of L-90 Array Membrane



Assay Diagram



A. Preparation of Samples

1). Seed cells at a density of $1x10^6$ cells in 100 mm tissue culture dishes.*
2) Culture in complete culture medium for ~24–48 hours.**
3) Replenish with serum-free or low-serum medium, such as 0.2% FCS/FBS, and then re-incubate cells for ~48 hours***
4) Collect the cell culture supernatant and centrifuge at 1,000 g for 10 minutes and store in ≤1 ml aliquots at -80 °C until needed.
5) Measure the total wet weight of the cultured cells in the pellet and/or culture dish. 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). Normalization can also be done between arrays by determining the total protein concentration using a total protein assay (RayBiotech recommends the Pierce BCA Protein Assay Kit, cat# 23227).

Note: * The density of cells per dish used is dependent on the cell type. More or less cells may be required but should be determined empirically.

** Optimal culture time may be different and depends on cell lines, treatment conditions, and other factors.

***Bovine serum proteins produce detectable signals on the RayBio® L-Series membrane arrays at concentrations as low as 0.2%. When testing serum-containing media, it is

recommended test an uncultured media blank sample for comparison with sample results.

B. Dialysis of Sample

Note: Samples must be dialyzed prior to biotin-labeling (Steps 5–7).

1. Prepare dialysis buffer (1X PBS) by dissolving 0.6 g KCl, 24 g NaCl, 0.6 g KH_2PO_4 and 3.45 g Na_2HPO_4 in 2500 ml de-ionized or distilled water. Adjust to a pH of 8.0 with 1M NaOH and adjust final volume to 3000 ml with de-ionized or distilled water.
2. Load each sample into a separate Dialysis Vials (Item A), 2.5-3.0 ml of sample per vial for dialyzing. Carefully place all Dialysis Vials into the Floating Rack.

3. Place the Floating Rack into ≥500 ml dialysis buffer in a large beaker. Place beaker on a stir plate and dialyze for at least 3 hours at 4 °C, occasionally gently stirring the dialysis buffer. Then exchange the dialysis buffer with fresh buffer and repeat dialysis for at least 3 hours at 4 °C. Transfer dialyzed samples into a clean eppendorf tube. Centrifuge dialyzed samples for 5 minutes at 10,000 rpm to remove any particulates or precipitates and then transfer and combine each sample into one clean eppendorf tube. Mix well by gently pipetting.

Note: The sample volume may change during dialysis.

Note: Dialysis procedure may proceed overnight.

C. Biotin-labeling of Sample

Avoid contamination with any solution containing amines (i.e., Tris, glycine) as well as azides during the biotinylation process.

4. Immediately before use, prepare 1X Labeling Reagent by briefly centrifuging down the Labeling Reagent vial (Item B) and add 100 µl 1X PBS (pH=8.0) into the vial. Pipette up and down or vortex briefly to dissolve the powder.
5. Add an appropriate amount* of 1X Labeling Reagent into the tube containing the sample and immediately mix the reaction solution. Incubate the reaction solution at room temperature for 30 minutes with gentle shaking. Gently tap the tube to mix the reaction solution every 5 minutes.

* Use 7.2 µl of 1X Labeling Reagent for labeling 1 mg of total protein in samples. For example, if sample's total protein concentration is 0.5 mg/ml you need to add 10.8 µl 1X Labeling Reagent to 3 ml dialyzed sample.

Note: The total protein concentration needs to be determined if the sample volume changes after dialysis or if the total protein concentration was determined before the dialysis step.

6. Add 5 µl Stop Solution (Item D) into the reaction solution and

then use the Spin Column (Item J) to remove any unbound biotin.

a). Twist off the bottom closure of the Spin Column and loosen the cap (but keep the cap on). Place the Spin Column into a 50 ml conical collection tube.

b). Centrifuge the Spin Column at 1,000 g for 3 minutes to remove storage solution.

Note: The resin should appear compacted after centrifugation.

c). Add 5 ml 1X PBS (pH=8.0) into the Spin Column and centrifuge at 1,000 x g for 3 minutes to remove the 1X PBS. Repeat an additional 2 times to wash the Spin Column.

d). Place the Spin Column in a new 50 ml conical collection tube and slowly load 3.5 ml of sample to the center of the compact resin bed.

Note: The maximal sample volume is 4 ml for each Spin Column. Do not load over 4 ml of sample into a Spin Column.

e). Centrifuge the Spin Column at 1,000 x g for 3 minutes. The sample should filter through the resin and deposit into the 50 ml conical collection tube. Store at -80 ^{0}C until needed. Discard the Spin Column after use.

D. Blocking and Incubation

7. Place each membrane printed side up into a Plastic Incubation Tray (provided). 1 membrane per tray.

   Note: The printed membrane will have a "-" mark in the upper left corner of the membrane.

8. Add 2.5 ml of Blocking Buffer (Item F) to each membrane and cover with the lid. Incubate at room temperature with gentle shaking for 1 hour.

9. Aspirate Blocking Buffer from each tray. Add 2.5 ml of diluted* or undiluted sample onto each membrane and cover with the lid. Incubate at room temperature with gentle shaking for 2 hours.

   Note: 1). It is recommended to use 2.5 ml of 5-fold diluted biotin-labeled cell culture supernatant. Dilute sample using Blocking Buffer.

   Note: 2). The concentration of sample used depends on the abundance of proteins. The samples can be concentrated if the overall signals are too weak. If the overall signals are too strong, the sample can be diluted further.

   Note: 3). Incubation may be done at room temperature with gentle shaking for 2 hours or overnight at 4°C.

10. Dilute 20X Wash Buffer 1 with deionized or distilled water to prepare the 1X Wash Buffer 1. Aspirate the samples from each tray and then wash by adding 3 ml of 1X Wash Buffer I at room temperature with gentle shaking (5 min per wash). Repeat the wash 2 more times for a total of 3 washes.

11. Aspirate the 1X Wash Buffer 1 from each tray. Dilute 20X Wash Buffer 2 with deionized or distilled water to prepare the 1X Wash Buffer 2. Wash 3 times with 3 ml of 1X Wash Buffer 2 at room temperature with gentle shaking.

12. Aspirate the 1X Wash Buffer 2 from each tray. Dilute the 500X HRP-Conjugated Streptavidin with Blocking Buffer to prepare the 1X HRP-Conjugated Streptavidin. Add 2.5 ml of 1X HRP-Conjugated Streptavidin to each membrane.

Note: Ensure that the vial containing the 500X HRP-Conjugated Streptavidin is mixed well before use, as precipitation can form during storage.

13. Incubate at room temperature with gentle shaking for 2 hours.

Note: incubation may be done at 4 ^{0}C for overnight.

14. Wash as directed in steps 10 and 11.

E. Detection

* Do not let the membrane dry out during detection. The

detection process must be completed within 40 minutes without stopping.

15. For detection of 2 membranes, add 2.5 ml of Detection Buffer C and 2.5 ml of Detection buffer D into a tube and mix both solutions. Drain off excess wash buffer. Place membrane antibody side up ("-" symbol is marked in the top left corner of each membrane) on a clean plastic plate or its cover (provided in the kit). Pipette 2 ml of the mixed Detection Buffers on to each membrane and incubate at room temperature for 2 minutes with gentle shaking. Ensure that the detection mixture is evenly covering the membrane without any air bubbles.

16. Gently place the membrane with forceps (antibody side up) on a plastic sheet (provided) and cover the membrane with another plastic sheet. Gently smooth out any air bubbles. Avoid using pressure on the membrane. Work as quickly as possible.

17. The signal can be detected directly from the membrane using a chemiluminescence imaging system or by exposing the array to x-ray film (we recommend using Kodak X-Omat™ AR film) with subsequent development. Expose the membranes for 40 seconds. Then re-expose the film according to the intensity of signals. If the signals are too strong (background too high), reduce exposure time (eg, 5–30 seconds). If the signals are too weak, increase exposure time (eg, 5–20 min or overnight). Or re-incubate membranes overnight with 1X HRP-Conjugated Streptavidin, and repeat detection on the second day.

18. Save membranes at –20 °C to –80 °C for future reference.

V. Antibody Array Map

RayBio® L-Series Human Antibody Array 90 Maps – if needed, larger versions of these maps can obtained by contacting technical support at 770-729-2992 or techsupport@raybiotech.com.

RayBio® Biotin Label-based Mouse Antibody Array I Map – Larger versions of this can obtained by contacting technical support at 770-729-2992.

	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-2a	P-3a	Blank	Neg	Neg	Blank	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	Blank	Blank	Blank	Blank
2	P-1a	P-2a	P-3a	Blank	Neg	Neg	Blank	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	Blank	Blank	Blank	Blank
3	Blank	Blank	Blank	Blank	Blank	Blank	Blank	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	Blank	Neg	Neg	Neg
4	Blank	Blank	Blank	Blank	Blank	Blank	Blank	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	Blank	Neg	Neg	Neg
5	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	Blank	Blank	Blank	Blank
6	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	Blank	Blank	Blank	Blank
7	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	Blank	P-3b	P-2b	P-1b
8	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	Blank	P-3b	P-2b	P-1b

RayBio® L-Series Rat Antibody Array 90 (L-90) List

Number	Name		Number	Name		Number	Name		Number	Name
1	P-1a		31	Blank		61	IL-1 beta		91	Neuropilin-2
2	P-2a		32	Blank		62	IL-1 R6/IL-1 R rp2		92	NGFR
3	P-3a		33	Blank		63	IL-2		93	Orexin A
4	Blank		34	Blank		64	IL-3		94	Osteopontin/SPP1
5	Neg		35	Blank		65	IL-4		95	PDGF-AA
6	Neg		36	Blank		66	IL-5		96	Prolactin R
7	Blank		37	Blank		67	IL-6		97	RAGE
8	Activin A		38	EG-VEGF/PK1		68	IL-10		98	RALT/MIG-6
9	ACTH		39	E-Selectin		69	IL-12/IL-23 p40		99	RELM beta
10	ADFP		40	FADD		70	IL-13		100	Resistin
11	Adiponectin/Acrp30		41	Fas Ligand/TNFSF6		71	Integrin alpha M beta 2		101	TAL1A
12	AMPK alpha 1		42	Fas/TNFRSF6		72	Inuslin		102	TGF-beta1
13	B7-1/CD80		43	FGF-BP		73	IP-10		103	TGF-beta2
14	BDNF		44	Follostatin-like -1(FSL1)		74	Leptin (OB)		104	TGF-beta3
15	beta-Catenin		45	Fractalkine		75	LIX		105	Thrombospondin
16	basic-FGF		46	GFR alpha-1		76	L-Selectin/CD62L		106	TIE-2
17	beta-NGF		47	GFR alpha-2		77	MCP-1		107	TIMP-1
18	CCR4		48	GM-CSF		78	MDC		108	TIMP-2
19	CD106		49	Growth Hormone		79	MIF		109	TIMP-3
20	CINC-2 alpha/beta		50	Growth Hormone R		80	MIP-1 alpha		110	TLR4
21	CINC-3		51	Hepassocin		81	MIP-2		111	TNF-alpha
22	CNTF		52	ICAM-1/CD54		82	MIP-3 alpha		112	TRAIL
23	CNTF R alpha		53	ICK		83	MMP-13		113	TROY
24	CSK		54	Insulin Degrading Enzyme		84	MMP-2		114	Ubiquitin
25	CXCR4		55	IFN-gamma		85	MMP-8		115	VEGF
26	EGFR		56	IL-1 alpha		86	MuSK		116	VEGF-C
27	Blank		57	Blank		87	Blank		117	Blank
28	Blank		58	Neg		88	Blank		118	P-3b
29	Blank		59	Neg		89	Blank		119	P-2b
30	Blank		60	Neg		90	Blank		120	P-1b

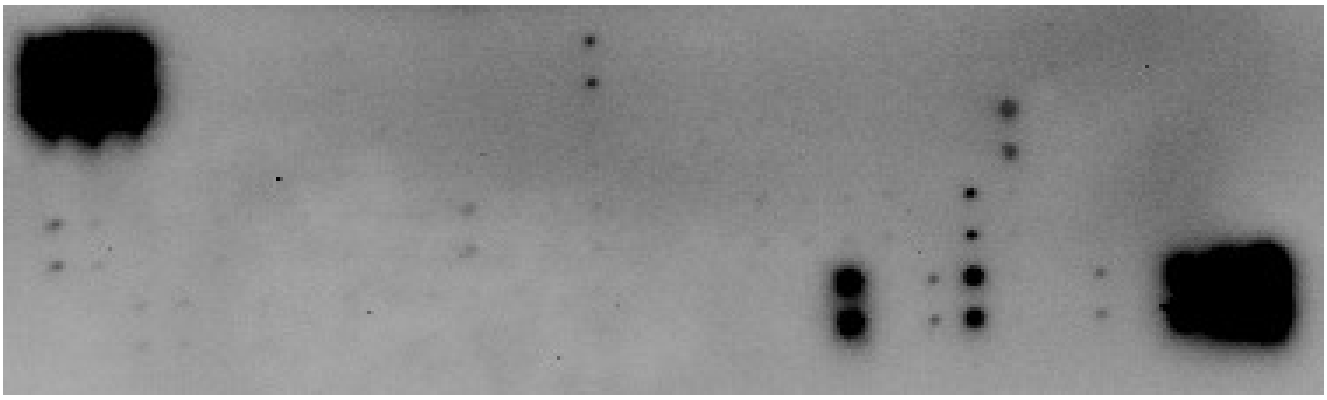
VI. Interpretation of Results

The following images show the RayBio® L-Series Rat Antibody Array 90 captured using a chemiluminescence imaging system (UVP BioImaging Systems). To obtain optimal results, it is suggested to try several different exposure times until the best one is determined. Then, by comparing the signal intensities, relative expression levels of the target proteins can be made. The intensities of signals can be quantified by densitometry. Anti-HRP (P-1a, P-2a, P-3a) and anti-streptavidin (P-1b, P-2b, P-3b) will produce positive control signals, which can be used to identify the orientation and help normalize the results from different arrays being compared.

Antibody affinity to its target varies significantly between antibodies. The intensity detected on the array with each antibody depends on this affinity; therefore, signal intensity comparison can be performed only within the same antibody/antigen system and not between different antibodies.

The RayBio® Analysis Tool is a program specifically designed for analysis of RayBio® L-Series Rat Antibody Antibody Arrays. This tool will not only assist in compiling and organizing your data, but also reduces your calculations to a "copy and paste." Call RayBiotech, Inc. at 770-729-2992 for ordering information.

L-90 image



VII. Troubleshooting Guide

Problem	Cause	Recommendation
Weak signal or no signal	1. Taking too much time for detection.	1. The whole detection process must be completed in 30 min.
	2. Film developer does not work properly.	2. Fix film developer.
	3. Did not mix HRP-streptavidin well before use.	3. Mix tube containing HRP-Conjugate Streptavidin well before use since precipitates may form during storage.
	4. Sample is too dilute.	4. Increase sample concentration
	5. Other.	1.Check if there were any contamination with any solution containing amines in biotin-labeling step
		2. Slightly increase HRP concentrations.
		3. Work as quickly as possible after mix Detection Buffer C and D
		4. Expose film for overnight to detect weak signal.
Uneven signal	1. Bubbles formed during incubation.	1. Remove bubbles during incubation.
	2. Membranes were not completely covered by solution.	2. Completely cover membranes with solution.
High background	1. Exposure time is too long.	1. Decrease exposure time.
	2. Membranes dry out during experiment.	2. Completely cover membranes with solution during experiment. Cover tray w/ lid
	3. Sample is too concentrated.	3. Dilute sample.

VIII. Reference List

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Note:

Note:

Note:

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