

miRNAscope[™] RED LS combined with Immunohistochemistry: Integrated Co-Detection Workflow (ICW) on Leica Bond RX

Introduction

This Technical Note provides guidelines for performing automated chromogenic co-detection of miRNA/ ASO/ siRNA/short RNAs and protein on the Leica BOND RX System. The Integrated Co-Detection Workflow (ICW) combines ACD's miRNAscope LS Reagent Kit RED (Cat. No. 324600) with fully automated DAB or semi-automated Green immunohistochemistry (IHC). In addition to ACD's Red ISH assays, you will need the Leica BOND Polymer Refine Red Kit for ISH detection and the Leica BOND Polymer Refine Kit for immunohistochemistry. Before starting the procedure, create protocols for miRNA-Protein Co-Detection Part A and miRNA-Protein Co-Detection Part B on the BOND RX controller with the help of your ACD FAS. For every chemical, read the Safety Data Sheet (SDS) and follow handling instructions. Wear appropriate protective eyewear, clothing, and gloves. For the latest service and support information, go to **www.acdbio.com/support.**



Workflow







Chromogen Combinations for ICW

For optimal results using ISH – IHC chromogen combinations, see the following table:

ACD ISH Assay	ISH	IHC	Reagents for ISH Detection	IHC Detection System/Reagents
	Chromoge	Chromogen		
	n			
miRNAscope	Red	Green	miRNAscope LS Reagent Kit – RED;	Co-Detection Antibody Diluent
			Leica BOND Refine Red Detection	Leica BOND Refine Detection Kit;
			Kit	RNAscope 2.5 LS Green Accessory Pack
miRNAscope	Red	DAB	miRNAscope LS Reagent Kit – RED;	Co-Detection Antibody Diluent
			Leica BOND Refine Red Detection	Leica BOND Refine Detection Kit
			Kit	

Materials Required

ACD LS Chromogenic ISH Detection Kits

miRNAscope LS Reagent Kit- RED

The miRNAscope LS Reagent Kit - RED (Cat. No. 324600) provides reagents to stain ~60 standard slides on Leica Biosystems' BOND RX System. The miRNAscope LS Probes are available separately. The reagents are Ready-To-Use (RTU) and are stored as indicated in the following table:

For Research Use Only. Not for use in diagnostic proceduresTN 324600/ Rev A/ Effective Date 10/01/2021



	RNAscope 2.5 LS Reagent K	it - RED (Cat. No. 322150)	
\checkmark	Reagent	Quantity	Storage
	RNAscope 2.5 LS Hydrogen Peroxide	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Protease III	21 mL x 1 bottle	2–8°C
	miRNAscope LS AMP 1	21 mL x 1 bottle	2–8°C
	miRNAscope LS AMP 2	21 mL x 1 bottle	2–8°C
	miRNAscope LS AMP 3	21 mL x 1 bottle	2–8°C
	miRNAscope LS AMP 4 – RED	21 mL x 1 bottle	2–8°C
	miRNAscope LS AMP 5 – RED	21 mL x 1 bottle	2–8°C
	miRNAscope LS AMP 6 – RED	21 mL x 1 bottle	2–8°C
	RNAscope 2.5 LS Rinse	29 mL x 2 bottles	2–8°C

RNAscope 2.5 LS Green Accessory Pack (Optional)

For green IHC staining, we recommend the RNAscope 2.5 LS Green Accessory Pack (Cat. No. 322550). Following co-detection, this accessory pack provides reagents to stain ~60 standard slides offline on Leica Biosystems' BOND RX System. The reagents are Ready-To-Use (RTU) and are stored as indicated in the following table:

RNAscope	2.5 LS Green Accessory Pack (Cat. No. 322550)	
Reagent	Quantity	Storage
RNAscope 2.5 LS Duplex Green A	12 mL x 1 bottle	2–8°C
RNAscope 2.5 LS Duplex Green B	240 μL x 1 tube	2–8°C
RNAscope 50X Wash Buffer	60 mL x 1 bottle	Room temp (20–25°C)

Additional Reagents

Additional Reagents for Co-Detection					
Reagent	Source / Ordering Info	Quantity	Storage		
Co-Detection Antibody Diluent	ACD / Cat No. 323160	120 mL x 1 bottle	2–8°C		
RNAscope LS Protease IV	ACD / Cat No. 322140 (5 mL)	21 mL	2–8°C		
Primary Antibody Concentrate	User	As needed	Per manufacturer's recommendation		
10% Neutral Buffered Formalin	User	5 – 10 mL	Per manufacturer's recommendation		

Required Materials from Leica BOND RX

The Integrated Co-Detection Workflow (ICW) requires specific materials and equipment available *only* from Leica Biosystems.

Component	Cat. No.	Storage
BOND 30 mL Open Containers	OP309700	Room temp (20–25°C)
BOND 7mL Open Containers	OP79193	Room temp (20–25°C)
BOND Universal Covertiles 100 pack	S21.2001	Room temp (20–25°C)
BOND Polymer Refine Detection (DAB) and Hematoxylin *	DS9800	2–8°C
 BOND Epitope Retrieval Solution 1-1L (RTU)	AR9961	2–8°C

For Research Use Only. Not for use in diagnostic procedures

in diagnostic procedures TN 324600/ Rev A/ Effective Date 10/01/2021 LS Chromogenic RNA-Protein Co-Detection



 BOND Epitope Retrieval Solution 2-1L (RTU)	AR9640	2–8°C
BOND Dewax Solution – 1L (RTU)	AR9222	2–8°C
BOND Wash Solution 10X Concentrate – 1L	AR9590	2–8°C
BOND Aspirating Probe Cleaning System	CS9100	2–8°C
BOND Mixing Stations	S21.1971	Room temp (20–25°C)
 BOND Polymer Refine Red Detection and Hematoxylin*	CS9390	2–8°C

*Do not substitute with any other chromogen kit.

Run the Assay

Part 1: Add and Register Reagents for Co-Detection

The Integrated Co-Detection Workflow requires the addition of new reagents in the software, including Co-Detection Antibody and 10% NBF. To ensure optimal ISH detection, perform IHC with a concentrated primary antibody diluted in Co-Detection Antibody Diluent. Using Ready-to-Use (RTU) primary antibody in this workflow could result in suboptimal RNA detection. You must place the antibody in a Leica Open Container and register the antibody as an ancillary reagent on the Leica BOND RX instrument. To ensure optimal IHC detection in this workflow, crosslink with 10% NBF. Place the NBF reagent in a 7mL Open Container registered as an ancillary reagent, and keep the container closed when not on the Leica BOND RX instrument.

Add new co-detection reagents

- 1. Select the **Reagent Setup** icon at the top of the screen.
- 2. To add 10% NBF as a new co-detection reagent, do the following steps:

Slide setup	Protocol setup	Reagent setup	Slide history	Search	Help	Log out +		Lei
Mrs. Potato Head	Reage	nt setup						
	Add	Open De	Panels					
Slinky Dog	*ACD Amp	Na 0 1	me		Abb. name *ACDAmp1	Type Ancillary	Supplier Advanced Cell Diagnostics	Pref.
	*ACD Amp	2			*ACDAmp2	Ancillary	Advanced Cell Diagnostics	~
	*ACD Amp	3			*ACDAmp3	Ancillary	Advanced Cell Diagnostics	~
	*ACD Amp	o 4			*ACDAmp4	Ancillary	Advanced Cell Diagnostics	~
	*ACD Amp	5 Brown			*ACDAmp5Br	Ancillary	Advanced Cell Diagnostics	~
	*ACD Amp	5 Red			*ACDAmp5Red	Ancillary	Advanced Cell Diagnostics	~
	*ACD Amp	6 Brown			*ACDAmp6Br	Ancillary	Advanced Cell Diagnostics	~
	*ACD Amp	6 Red			*ACDAmp6Red	Ancillary	Advanced Cell Diagnostics	~
	*ACD Blue	•			*ACDBlue	Ancillary	Advanced Cell Diagnostics	~
	*ACD Dup	lex AMP 1			*ACD_Du_AMP1	Ancillary	Advanced Cell Diagnostics	~
	*ACD Dup	lex AMP 10			*ACD_Du_AMP10	Ancillary	Advanced Cell Diagnostics	~
	*ACD Dup	lex AMP 2			*ACD_Du_AMP2	Ancillary	Advanced Cell Diagnostics	*
	Package type	:	Rea	gent type:		Supplier:	Preferred status:	
	All reagents		- AI	1		▼ All	 Preferred 	

- 3. Select Add.
- 4. Enter the name **10% NBF** in the Name text box.
- 5. Enter **NBF** in the Abbreviated name text box.
- 6. Select **Ancillary** in the Type drop-down menu.

Note: You may leave the Supplier text box empty.

For Research Use Only. Not for use in diagnostic procedures

TN 324600/ Rev A/ Effective Date 10/01/2021



7. Select **Preferred** and **Hazardous**, then **Save**.

IMPORTANT!

For waste disposal, follow local guidelines.

🜈 BOND - (bondpowe	ruser) - Windows Internet Explorer						_ 🗆 🗵
Slide setup	Protocol setup	Slide history Search	Help Add reag	Log out		×	Leica BIOSYSTEMS
Mrs. Potato Head	Reagent setu Setup In Add Open	Name: Abbreviated name: Type: Supplier:	10% NBF NBF Ancillary				
Slinky Dog	*ACD Amp 1	Available bulks:		Compatit	ble bulks:		Pref.
	*ACD Amp 2		>>	*BWash *DI			~
	*ACD Amp 3		<<				~
	*ACD Amp 4						~
	*ACD Amp 5 Brown	✓ Preferred ✓ Haz	ardous				~
	*ACD Amp 5 Red						4
	*ACD Amp 6 Brown		Save	Cancel			~
	*ACD Amp 6 Red		Асратрокец	Anumary	Auvanced Cell Diagnostics		~
	*ACD Blue		*ACDBlue	Ancillary	Advanced Cell Diagnostics		4
	*ACD Duplex AMP 1		*ACD_Du_AMP1	Ancillary	Advanced Cell Diagnostics		4
	*ACD Duplex AMP 10		*ACD_Du_AMP10	Ancillary	Advanced Cell Diagnostics		4
	*ACD Duplex AMP 2		*ACD_Du_AMP2	Ancillary	Advanced Cell Diagnostics		4
	Package type: All reagents	Reagent type:		Supplier:	· · · · · · · · · · · · · · · · · · ·	Preferred status: Preferred	•

- 8. To create a generic Co-Detection antibody reagent, do the following steps:
- 9. Select Add.
- 10. Enter **Co-Detection Antibody 1** in the Name text box.
- 11. Enter **CoD Ab1** in the Abbreviated name text box.

🜔 BOND - (bondpov	veruser) - Windows Internet Exp	lorer				
Slide setup	Protocol setup Reagent s	etup Slide history	Search Hel	p Log out ∠∩		Jeica
				Add reagent	×	BIOSVSTEMS
Mrs. Potato Head	Reagent setu	Name:	Co-Detection An	tibody 1		
	Setup Im	Abbreviated name:	CoD Ab1			
	Add	Туре:	Ancillary	*		
		Supplier:				Pref
Slinky Dog	Probe 14	Available bulks:		Compatible bulks	s:	
	Probe 15			>> *DI		4
	Probe 2			<<		4
G	Probe 3					
	Probe 4	 Preferred 	Hazardous			
100	Probe 5					4
	Probe 6		Sa	Cancel		
	Probe 7	_	Prb7	Prohe RNA		
100 12	Probe 8		Prb8	Prohe RNA		
	Probe 9		Prb9	Probe RNA		
	Antibody 7		Ab7	Primary antibody		
8	10% NBE		NBE	Ancillary		
				Once it a	D. (
	All reagents	All	r type.	All	Prefe	rred

For Research Use Only. Not for use in diagnostic procedures

TN 324600/ Rev A/ Effective Date 10/01/2021



12. Select Ancillary in the Type drop-down menu.

Note: You may leave the Supplier text box empty.

- 13. Select **Preferred**, then **Save**.
- 14. Register additional Co-Detection antibody reagents as needed.

IMPORTANT! For Co-Detection antibodies, you must select **Ancillary** as the reagent type. Reagents registered as **Antibody** are not compatible with this protocol. To avoid confusion, include "Co-Detection" in the name (for example, Co-Detection CD3).

- 15. To register a probe for use with the ICW workflow, do the following steps:
- 16. Select Add.
- 17. Enter a probe name under **Name** and enter an abbreviated name.
- 18. Select Ancillary as Reagent Type.
- 19. Select **Preferred** and **Hazardous**, then **Save**.
- 20. Register additional probes as needed.

IMPORTANT! For probes, you must select **Ancillary** as the reagent type. Make sure that the name of the probe is unique and does not match the name of any existing reagent registered as a Probe.

BOND - (bondpo	weruser) - Windows Internet Explorer									- 21
Slide setup	Protocol setup	Slide history	Search	Help	Log out ¢					Leica
Mrs. Potato Head	Reagent setup	Panels	P							
	Add Open De	slete			Add	reagent		*		
8:04 AM		Name		Name:	miRNA probe 1				Supplier	Pref.
Slinky Dog	2.5 TBP-dapB			Abbreviated name:	miP1					4
9:38 AM	ACD Duplex Amp 1			Туре:	Ancillary	-				4
9:47 AM	ACD Duplex Amp 10			Supplier:	ACD					~
	ACD Duplex Amp 2			Available bulks:		Compatible	e bulks:			~
	ACD Duplex Amp 3					*BWash *DI				~
	ACD Duplex Amp 4					<<				4
	ACD Duplex Amp 5			_						~
	ACD Duplex Amp 6			 Preferred 	 Hazardous 					~
	ACD Duplex Amp 7							_		~
	ACD Duplex Amp 8				Save	Cancel				~
	ACD Duplex Amp 9				UpixA9	Ancillary	ACD			4
	ACD Duplex Wash				DplxWsh	Ancillary	ACD			1
	ACD miRNA ISH Red probe 1				miR P1	Probe RNA	ACD			4
	ACD miRNA ISH Red probe 2	2			miR P2	Probe RNA	ACD			4
	ACD Multiplex Amp 2				Mux Amp2	Ancillary	ACD			~
	ACD Multiplex Amp 3				Mux Amp3	Ancillary				4
	ACD Multiplex Amp1				Mux Amp1	Ancillary	ACD			4
	ACD Multiplex HRP Blocker				HRPblock	Ancillary				*
	ACD Multiplex HRP-C1				HRP-C1	Ancillary	ACD			4
	Package type:		Re	agent type:		Supplier:			Preferred status:	
			•			All			Preferred	*



Part 2: Create Co-Detection Software Protocols

This section provides instructions for creating two staining protocols for ICW. Use the protocols together in a sequential dual stain procedure on the Leica BOND RX System. Part A applies primary antibody followed by crosslinking, RNAscope Pretreatment, and ISH staining. Part B applies and detects secondary antibody. If you choose Green IHC, perform the chromogen and counterstaining steps offline. If you choose Brown IHC, these steps are fully automated.

Create Part A: primary antibody and ISH detection

- 1. The figures and table from the following procedure display the steps for the Leica miRNAscope protocol. In the Protocol setup screen, select Staining under the Protocol group menu.
- 2. Highlight the *ACD 2.5 Red Rev B protocol. Select Copy.
- 3. Change the protocol name for your first probe to **ACD miRNA-Protein Co-Detection Part A** in the Name text box, **miP-CoDA** in the Abbreviated name text box, and **ACD Red miRNA-Protein Co-Detection Part A** in the Description text box.
- 4. For Staining method, select **First**.
- 5. From the reagent drop-down menu select the appropriate miRNA probe on steps 1, 2, and 3.
- 6. For step 3, change the probe hybridization temperature to 37°C.

me:	ACD miRNA-Protein Co-Dete	ACD miRNA-Protein Co-Detection Part A				
breviated name:	miP-CoDA					
scription:	ACD Red miRNA-Protein Co					
ining method:	Single 🗹 First	Second				Preferr
BOND RX				-	Import protocol	Protocol type: ISH detecti
eferred detection sy	ystem: Bond Polymer Re	fine Red Detection				
Step N° Wasł	n Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type
1	miRNA probe 1	ACD	~		0:00	150 μL
2	miRNA probe 1	ACD	~		0:00	150 µL
3	miRNA probe 1	ACD		37	120:00	150 µL
15	miRNAscope Amp 1	ACD		42	1:00	150 µL
16	miRNAscope Amp 1	ACD		42	30:00	150 μL
25	*LS Rinse	Advanced Cell Diagnostics	~		5:00	150 µL
26	*LS Rinse	Advanced Cell Diagnostics	~		5:00	150 μL
31	miRNAscope Amp 2	ACD		42	<mark>1:00</mark>	150 µL
้วา		ACD		۲٨	45.00	4501
Show wash step	DS				Insert wash	Insert reagent Delete st

- 7. From the appropriate drop-down menus for the Amp steps, change the reagents *ACD Amp 1, *ACD Amp 2,..., *ACD Amp 6 to miRNAscope Amp 1, miRNAscope Amp 2, ..., miRNAscope Amp 6.
- 8. If using 5.2 software, select the appropriate tab for your instrument (BOND RXm or BOND RX).

For Research Use Only. Not for use in diagnostic procedures TN 324600/ Rev A/ Effective Date 10/01/2021 LS Chromogenic RNA-Protein Co-Detection

×



New protocol properties

ame:	ACD miRNA-Protein Co-Dete	ection Part A					
breviated name:	miP-CoDA						
escription:	ACD Red miRNA-Protein Co	-Detection Part A					
aining method:	Single 🗹 First	Second				~	Preferre
BOND RX					Import protocol	Protocol type: 19	H detection
eferred detection syste	em: Bond Polymer Re	fine Red Detection					
Step N° Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type	
1	miRNA probe 1	ACD	1		0:00	150 µL	Í
2	miRNA probe 1	ACD	~		0:00	150 µL	
3	miRNA probe 1	ACD		37	120:00	150 µL	
15	miRNAscope Amp 1	ACD		42	1:00	150 µL	
16	miRNAscope Amp 1	ACD		42	30:00	150 µL	
25	*LS Rinse	Advanced Cell Diagnostics	~		5:00	150 µL	
26	*LS Rinse	Advanced Cell Diagnostics	~		5:00	150 μL	
31	miRNAscope Amp 2	ACD		42	1:00	150 µL	
აე		A0D		40	45-00	4501	
Show wash steps					Insert wash	Insert reagent	Delete ster

9. Select **Show wash** steps, and add steps 1–35 from the following table before the probe step. Once additional steps have been added, verify that probe application miRNA probe 1 begins at step 36.

Step No.	Reagent	Step Type	Incubation Time	Temperature
1	*Bond Wash Solution	Wash	0 MIN	Ambient
2	*Bond Wash Solution	Wash	0 MIN	Ambient
3	*Bond Wash Solution	Wash	0 MIN	Ambient
4	Co-Detection Antibody 1	Reagent	15 MIN	Ambient
5	*Bond Wash Solution	Wash	0 MIN	Ambient
6	*Bond Wash Solution	Wash	0 MIN	Ambient
7	*Bond Wash Solution	Wash	0 MIN	Ambient
8	*Bond Wash Solution	Wash	0 MIN	Ambient
9	*Bond Wash Solution	Wash	0 MIN	Ambient
10	10% NBF	Reagent	30 MIN	Ambient
11	*Bond Wash Solution	Wash	0 MIN	Ambient
12	*Bond Wash Solution	Wash	0 MIN	Ambient
13	*Bond Wash Solution	Wash	0 MIN	Ambient
14	*Bond Wash Solution	Wash	2 MIN	Ambient
15	*Bond Wash Solution	Wash	2 MIN	Ambient
16	*Bond Wash Solution	Wash	0 MIN	Ambient
17	*ACD Enzyme	Reagent	0 MIN	40°C

For Research Use Only. Not for use in diagnostic procedures

TN 324600/ Rev A/ Effective Date 10/01/2021



18	*ACD Enzyme	Reagent	30 MIN	40°C
19	*Bond Wash Solution	Wash	0 MIN	Ambient
20	*Bond Wash Solution	Wash	0 MIN	Ambient
21	*Bond Wash Solution	Wash	0 MIN	Ambient
22	*Open 0 Haz	Reagent	10 MIN	Ambient
23	*Bond Wash Solution	Wash	0 MIN	Ambient
24	*Bond Wash Solution	Wash	0 MIN	Ambient
25	*Bond Wash Solution	Wash	0 MIN	Ambient
26	*Bond Wash Solution	Wash	0 MIN	Ambient
27	*Bond Wash Solution	Wash	0 MIN	Ambient
28	*Bond Wash Solution	Wash	0 MIN	Ambient
29	*Bond Wash Solution	Wash	0 MIN	Ambient
30	*Bond Wash Solution	Wash	0 MIN	Ambient
31	*Bond Wash Solution	Wash	0 MIN	Ambient
32	*Bond Wash Solution	Wash	0 MIN	Ambient
33	*Bond Wash Solution	Wash	0 MIN	Ambient
34	*Bond Wash Solution	Wash	0 MIN	Ambient
35	*Bond Wash Solution	Wash	0 MIN	Ambient
36	miRNA probe 1	Reagent	0 MIN	Ambient
37	miRNA probe 1	Reagent	0 MIN	Ambient
38	miRNA probe 1	Reagent	120 MIN	37°C

IMPORTANT! Ensure the temperature is set correctly. For heated steps, you must deselect **Ambient** before inputting the heated temperature.

Note: For steps 17–18, select *ACD Enzyme, which is the appropriate protease for the miRNAscope assay.

Note: Add steps 1–35 at the start of the protocol. After adding these steps, previous protocol steps should begin at step 36.

10. Delete two ***Bond Wash Solution 1 MIN** steps directly before the ***LS Rinse** steps that precede ***Mixed Red Refine**. For the miRNAscope assay, these are steps 120–121.

OTE

Name:		ACD miRNA-Protein Co	-Detection Part A			
Abbrevia	ited name:	miP-CoDA				
Descript	ion:	ACD Red miRNA-Protei	in Co-Detection Part A			
Staining	method:	Single 🖌 First	Second			 Preferre
BOND F	x				Import protocol	Protocol type: ISH detection
Preferred det	ection syste	m: Bond Polymer Ref	ine Red Detection			
Step N°	Wash	Reagent	Supplier	Ambient Temper	ature Inc. (min)	Dispense type
119	~	*Bond Wash Solution	Leica Microsystems	1	1:00	150 µL
120	~	*Bond Wash Solution	Leica Microsystems	×	1:00	150 µL
121	~	*Bond Wash Solution	Leica Microsystems	~	1:00	150 µL
122		*LS Rinse	Advanced Cell Diagnostics	4	5:00	150 µL
123		*LS Rinse	Advanced Cell Diagnostics	4	5:00	150 µL
		*Mixed Red Refine	Leica Microsystems	4	1:00	150 µL
124		*Mixed Red Refine	Leica Microsystems	~	10:00	150 µL
124					0.00	150 ul
124 125 126	~	*Deionized Water		~	0.00	loo pe

11. The Wash step after *Mixed Red Refine is the final step of the Part A protocol. For the miRNAscope assay, delete all steps following step124 *Deionized Water.

lame:		ACD miRNA-Protein Co-Detecti	on Part A				
Abbreviated	name:	miP-CoDA					
Description:		ACD Red miRNA-Protein Co-De	etection Part A				
Staining met	hod:	Single 📝 First	Second				Preferred
BOND	RX				Import protocol	Protocol type:	SH detection
Preferred de	tection syste	m: Bond Polymer Refine	e Red Detection				
Step N°	Wash	Reagent	Supplier	Ambient Tempera	ture Inc. (min)	Dispense type	
117	~	*Bond Wash Solution	Leica Microsystems	~	1:00	150 µL	
118	~	*Bond Wash Solution	Leica Microsystems	~	1:00	150 µL	
119	1	*Bond Wash Solution	Leica Microsystems	~	1:00	150 µL	
120		*LS Rinse	Advanced Cell Diagnostics	~	5:00	150 µL	
121		*LS Rinse	Advanced Cell Diagnostics	~	5:00	150 µL	
122		*Mixed Red Refine	Leica Microsystems	~	1:00	150 µL	
123		*Mixed Red Refine	Leica Microsystems	~	10:00	150 µL	
124	~	*Deionized Water	Leica Microsystems	~	0:00	150 µL	Ţ
		*BaseScope LSx Rinse					D. Law .
Show	wash steps	*Bond Wash Solution			Insert wash	Insert reagent	Delete step
		*Deionized Water	-				
		*GeoMx Buffer W					

12. Change the Wash step after *Mixed Red Refine from *Deionized Water to *Bond Wash Solution. 13. Select Save.

For Research Use Only. Not for use in diagnostic procedures TN 324600/ Rev A/ Effective Date 10/01/2021 LS Chromogenic RNA-Protein Co-Detection

~



New protocol properties

Name:	ACD miRNA-Protein Co-Dete	ction Part A			
Abbreviated name:	miP-CoDA				
Description:	ACD Red miRNA-Protein Co-	Detection Part A			
Staining method:	Single 🔽 First	Second			Preferred
BOND RX				Import protocol	Protocol type: ISH detection
Preferred detection s	ystem: Bond Polymer Ref	ine Red Detection			
Step N° Wasi	n Reagent	Supplier	Ambient Temperatu	e Inc. (min)	Dispense type
117 🖌	*Bond Wash Solution	Leica Microsystems	~	1:00	150 μL
118 🖌	*Bond Wash Solution	Leica Microsystems	~	1:00	150 µL
119 🖌	*Bond Wash Solution	Leica Microsystems	~	1:00	150 μL
120	*LS Rinse	Advanced Cell Diagnostics	~	5:00	150 μL
121	*LS Rinse	Advanced Cell Diagnostics	~	5:00	150 µL
122	*Mixed Red Refine	Leica Microsystems	~	1:00	150 µL
123	*Mixed Red Refine	Leica Microsystems	~	10:00	150 µL
124 🖌	*Bond Wash Solution	Leica Microsystems	~	0:00	150 µL
Show wash step	os			Insert wash	I Insert reagent Delete step
		Save	Cancel		

14. Select Yes to proceed.

Note: Additional Part A protocols must be created for each new probe and primary antibody combination.

15. To create a protocol for each additional probe and primary antibody, follow these steps:

a. Highlight the ACD miRNA-Protein Co-Detection Part A protocol. Select Copy.

New protocol properties

- b. Change the protocol name by adding your antibody and probe name (for example, ACD miRNA-Protein Co-Detection Part A CD3 Hs-miR-21) in the Name text box. Change the Abbreviated name text and Description text box accordingly.
- c. Under Staining Method, select First.

Name:	ACD miRNA-Protein Co-Dete	ection Part A-CD3-Hs-miR21						
Abbreviated name:	miPCoDA2							
Description:	ACD Red miRNA-Protein Co	Detection Part A-CD3-Hs-miR21						
Staining method:	Single 📝 First	Second			Preferred			
BOND RX					Import protocol	Protocol type: ISH detectio		
Preferred detection sy	ystem: Bond Polymer Re	fine Red Detection						
Step N° Wash	n Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type		
4	Co-Detection Antibody 1		~		15:00	150 μL		
10	10% NBF		1		30:00	150 µL		
17	*ACD Enzyme	Advanced Cell Diagnostics		40	0:00	150 µL		
18	*ACD Enzyme	Advanced Cell Diagnostics		40	30:00	150 µL		
22	*Open 0 Haz	User	~		10:00	150 µL		
36	miRNA probe 1	ACD	~		0:00	150 µL		
37	miRNA probe 1	ACD	~		0:00	150 µL		
38	miRNA probe 1	ACD		37	120:00	150 µL		
EN				40	4-00	450		
Show wash step	DS				Insert wash	Insert reagent Delete step		

For Research Use Only. Not for use in diagnostic procedures

TN 324600/ Rev A/ Effective Date 10/01/2021



- d. If using 5.2 software, select the appropriate tab for your instrument (BOND RXm or BOND RX).
- e. Select ***Co-Detection Antibody 1**. Change the Reagent to your registered ancillary antibody (for example, **Co-Detection CD3**).

ame:	ACD miRNA-Protein Co-Dete	ection Part A-CD3-Hs-miR21					
bbreviated name:	miPCoDA2						
escription:	ACD Red miRNA-Protein Co	-Detection Part A-CD3-Hs-miR21					
taining method:	Single 🖌 First	Second					 Preferre
BOND RX					Import protocol	Protocol type:	ISH detection
Preferred detection sys	stem: Bond Polymer Re	fine Red Detection 🔹					
Step N° Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type	
4	Co-Detection CD3		4		15:00	150 µL	
10	10% NBF		1		30:00	150 µL	
17	*ACD Enzyme	Advanced Cell Diagnostics		40	0:00	150 µL	
18	*ACD Enzyme	Advanced Cell Diagnostics		40	30:00	150 µL	
22	*Open 0 Haz	User	~		10:00	150 µL	
36	miRNA probe 1	ACD	1		0:00	150 µL	
37	miRNA probe 1	ACD	~		0:00	150 µL	
38	miRNA probe 1	ACD		37	120:00	150 µL	
		٨٥٥		17	4-00	4601	
EN						A AND AND AND AND AND AND AND AND AND AN	

f. Select **miRNA probe 1**. Change the Reagent to your registered ancillary probe (for example, **Hs-miR-21**).

			ropordoo			
Name:	ACD miRNA-Protein Co-De	ection Part A-CD3-Hs-miR21				
Abbreviated name:	miPCoDA2					
Description:	ACD Red miRNA-Protein Co	o-Detection Part A-CD3-Hs-miR21				
Staining method:	Single 🗹 First	Second				V Preferre
BOND RX				h	mport protocol	Protocol type: ISH detection
Preferred detection s	ystem: Bond Polymer Re	efine Red Detection				
Step N° Wasl	n Reagent	Supplier	Ambient 1	Temperature	Inc. (min)	Dispense type
4	Co-Detection CD3		~		15:00	150 µL
10	10% NBF		~		30:00	150 μL
17	*ACD Enzyme	Advanced Cell Diagnostics		40	0:00	150 μL
18	*ACD Enzyme	Advanced Cell Diagnostics		40	30:00	150 μL
22	*Open 0 Haz	User	~		10:00	150 μL
36	Hs-miR21	ACD	*		0:00	150 μL
37	Hs-miR21	ACD	~		0:00	150 μL
38	Hs-miR21	ACD		37	120:00	150 µL
EN				40	4.00	4601
Show wash step	DS				Insert was	n Insert reagent Delete ste

For Research Use Only. Not for use in diagnostic procedures

in diagnostic procedures TN 324600/ Rev A/ Effective Date 10/01/2021 LS Chromogenic RNA-Protein Co-Detection



Create Part B: semi-automated green or fully automated brown IHC detection

1. On the Protocol setup screen, select IHC Staining under the Protocol type menu.

BOND - (bondpov	weruser) - Windows Internet Explorer							
Slide setup	Protocol setup	Slide history	Search H	elp Log out				Leica
Mrs. Potato Head	Protocol setup					Сору Ор	en Delete	Report
	Protocol nar	ne	Protocol type	Desc	iption	Modified by	Mod. date	Pref.
	*AccuCyte CTC IF Protocol		IHC staining	AccuCyte CTC IF protocol		Leica	2/21/2020	~
	*AccuCyte CTC IHC Protocol		IHC staining	AccuCyte CTC IHC protoc	l	Leica	2/21/2020	~
Slinky Dog	*IF Protocol		IHC staining	IF protocol		Leica	2/21/2020	~
3 5 4 7 8 3 3 6 9 2 5 6 9 8 7 3 5 8 3 5 4 7 8 1 3 6 9	*IHC Open Dispense Template		IHC staining	IHC template with Open Ar	cillary and Chromogen	Leica	2/21/2020	~
	*IHC Protocol F		IHC staining	Bond Polymer Refine IHC	protocol	Leica	2/21/2020	~
	*IHC Protocol F RX 37M		IHC staining	IHC Protocol F with market	step at 37C	Leica	2/21/2020	~
	*IHC Protocol F RX 40M		IHC staining	IHC Protocol F with market	step at 40C	Leica	2/21/2020	~
	*IHC Protocol J		IHC staining	Bond Polymer Refine Red	IHC protocol	Leica	2/21/2020	~
	*IHC Protocol J RX 37M		IHC staining	IHC Protocol J with marker	step at 37C	Leica	2/21/2020	~
	*IHC Protocol J RX 40M		IHC staining	IHC Protocol J with marker	step at 40C	Leica	2/21/2020	~
	*IHC Protocol K		IHC staining	ChromoPlex 1 Dual IHC pr	otocol	Leica	2/21/2020	~
	*IHC Protocol K - 50 Test		IHC staining	ChromoPlex 1 Dual IHC pr	otocol	Leica	2/21/2020	~
	*IHC Protocol N		IHC staining	IHC Protocol using 1/60 mi	xed chromogen	Leica	3/10/2020	~
	*IHC Protocol S		IHC staining	IHC HRP FLEX Protocol w	ith Blue Chromogen	Leica	3/10/2020	~
	*IHC Protocol T		IHC staining	IHC HRP FLEX Protocol w	ith Green Chromogen	Leica	3/10/2020	~
	Protocol group:	Protocol type:		taining status:	Protocol origin:	Pref	erred status:	
	Staining	- IHC staining		All	All	▼ Pr	eferred	

C BOND - (bondpo	weruser) - Windows								
Slide setup	Protocol setup	Reagent setup	Slide history	Search	Help L	.og out + 🕽			Leica
Mrs. Potato Head	Protoc	ol setup					Сору	Open Delete	Report
		Protocol na	ame	Protocol ty	/pe	Description	Modified b	y Mod. date	Pref.
	*AccuCyte	e CTC IF Protocol		IHC stair	ning AccuCyte C1	C IF protocol	Leica	2/21/2020	 ✓
	*AccuCyte	e CTC IHC Protocol		IHC stair	ning AccuCyte CT	C IHC protocol	Leica	2/21/2020	~
Slinky Dog	*IF Protoc	ol		IHC stair	ning IF protocol		Leica	2/21/2020	~
	*IHC Ope	n Dispense Template		IHC stair	ning IHC template	with Open Ancillary and Chrome	ogen Leica	2/21/2020	~
	*IHC Prot	ocol F		IHC stair	ning Bond Polyme	er Refine IHC protocol	Leica	2/21/2020	~
	*IHC Prot	ocol F RX 37M		IHC stair	ning IHC Protocol	F with marker step at 37C	Leica	2/21/2020	~
	*IHC Prot	ocol F RX 40M		IHC stair	ning IHC Protocol	F with marker step at 40C	Leica	2/21/2020	~
	*IHC Prot	locol J		IHC stair	ning Bond Polyme	er Refine Red IHC protocol	Leica	2/21/2020	~
	*IHC Prot	ocol J RX 37M		IHC stair	ning IHC Protocol	J with marker step at 37C	Leica	2/21/2020	~
	*IHC Prot	ocol J RX 40M		IHC stair	ning IHC Protocol	J with marker step at 40C	Leica	2/21/2020	~
	*IHC Prot	ocol K		IHC stair	ning ChromoPlex	1 Dual IHC protocol	Leica	2/21/2020	~
	*IHC Prot	ocol K - 50 Test		IHC stair	ning ChromoPlex	1 Dual IHC protocol	Leica	2/21/2020	~
	*IHC Prot	ocol N		IHC stair	ning IHC Protocol	using 1/60 mixed chromogen	Leica	3/10/2020	~
	*IHC Prot	ocol S		IHC stair	ning IHC HRP FLI	EX Protocol with Blue Chromoge	n Leica	3/10/2020	~
	*IHC Prot	locol T		IHC stair	ning IHC HRP FLI	EX Protocol with Green Chromog	gen Leica	3/10/2020	v .
	Protocol grou	ıp:	Protocol type:		Staining status:	Protocol or	igin:	Preferred status:	
	Staining		 IHC staining 		All	✓ All	•	Preferred	•

- 2. To create an IHC protocol, highlight ***IHC Protocol F** in the protocol set up page and select **Copy**.
- 3. Change the protocol name to **ACDmiRNA-Protein Co-Detection Part B** in the Name text box and **miP-CoD B** in the Abbreviated name text box.
- 4. For Staining Method, select Second.

For Research Use Only. Not for use in diagnostic procedures TN 324600/ Rev A/ Effective Date 10/01/2021



6.

×

5. If using 5.2 software, select the appropriate tab for your instrument (BOND RXm or BOND RX).

ime:	ACD MIRNA-Protein Co-Det	lection Part B				
breviated name:	miP-CoDB					
scription:	Bond Polymer Refine IHC pro	otocol				
aining method:	Single First	 Second 				✓ Preferre
BOND RX				Import	protocol	Protocol type: IHC staining
eferred detection su	stem Bond Polymer Ref	fine Detection				
Step N° Wash	Reagent	Supplier	Ambient Temperatu	ure Inc. (min)	Dispen	se type
1	*Peroxide Block	Leica Microsystems	~	5:00	150 µL	
5	*MARKER	Leica Microsystems	~	15:00	150 µL	
9	*Post Primary	Leica Microsystems	4	8:00	150 µL	
13	*Polymer	Leica Microsystems	4	8:00	150 µL	
17	*Mixed DAB Refine	Leica Microsystems	~	0:00	150 μL	
18	*Mixed DAB Refine	Leica Microsystems	~	10:00	150 µL	
22	*Hematoxylin	Leica Microsystems	1	5:00	150 µL	
Show wash step ect Show	° wash steps.	Save	Cancel	INS		
Show wash step ect Show Name:	s wash steps.	Save New proto tection Part B	Cancel	105		
Show wash step ect Show Name: Abbreviated name:	s wash steps. ACD miRNA-Protein Co-Det miP-CoDB	Save New proto tection Part B	Cancel	115		
Show wash step ect Show Name: Abbreviated name: Description:	s wash steps. ACD miRNA-Protein Co-Def miP-CoDB Bond Polymer Refine IHC pr	Save New proto tection Part B	Cancel col properties			
Show wash step ect Show Name: Abbreviated name: Description: Staining method:	s wash steps. ACD miRNA-Protein Co-Del miP-CoDB Bond Polymer Refine IHC pr Single First	Save New proto tection Part B rotocol Second	Cancel col properties			✓ Preferred
Show wash step ect Show Name: Abbreviated name: Description: Staining method: BOND RX	s wash steps. ACD miRNA-Protein Co-Det miP-CoDB Bond Polymer Refine IHC pr Single First	Save New proto tection Part B rotocol Second	Cancel col properties	Import pr	otocol F	Preferred
Show wash step ect Show Name: Abbreviated name: Description: Staining method: BOND RX Preferred detection	s wash steps. ACD miRNA-Protein Co-Del miP-CoDB Bond Polymer Refine IHC pr Single First system: Bond Polymer Ref	Save New proto tection Part B rotocol Second	Cancel col properties	Import pr	otocol F	Preferred Protocol type: IHC staining
Show wash step ect Show Name: Abbreviated name: Description: Staining method: BOND RX Preferred detection Step N° Wa	s Wash steps. ACD miRNA-Protein Co-Det miP-CoDB Bond Polymer Refine IHC pr Single First system: Bond Polymer Re sh Reagent	Save New proto tection Part B rotocol Second afine Detection	Cancel Col properties	Import pr	otocol F Dispense ty	Preferred Protocol type: IHC staining
Show wash step ect Show Name: Abbreviated name: Description: Staining method: BOND RX Preferred detection Step N° Wa 1	s wash steps. ACD miRNA-Protein Co-Def miP-CoDB Bond Polymer Refine IHC pr Single First system: Bond Polymer Re sh Reagent *Peroxide Block	Save New proto tection Part B rotocol second Efine Detection Supplier Leica Microsystems	Cancel Col properties	Import pr e Inc. (min) 5:00	otocol F Dispense ty 150 μL	Preferred Protocol type: IHC staining
Show wash step ect Show Name: Abbreviated name: Description: Staining method: BOND RX Preferred detection Step N [®] Wa 1 2	s Wash steps. ACD miRNA-Protein Co-Del miP-CoDB Bond Polymer Refine IHC pr Single First System: Bond Polymer Ref sh Reagent *Peroxide Block *Bond Wash Solution	Save New proto tection Part B rotocol fine Detection Leica Microsystems Leica Microsystems Leica Microsystems	Cancel Col properties	E Inc. (min) 5:00 0:00	Dispense ty 150 µL	Preferred Protocol type: IHC staining
Show wash step ect Show Name: Abbreviated name: Description: Staining method: BOND RX Preferred detection Step N° Wa 1 2 2 3	s Wash steps. ACD miRNA-Protein Co-Det miP-CoDB Bond Polymer Refine IHC pr Single First system: Bond Polymer Res sh Reagent *Peroxide Block *Bond Wash Solution *Bond Wash Solution	Save New proto tection Part B rotocol Second efine Detection Leica Microsystems Leica Microsystems Leica Microsystems Leica Microsystems Leica Microsystems	Cancel Col properties	Import pr E Inc. (min) 5:00 0:00	Dispense ty 150 µL 150 µL Open	Preferred Protocol type: IHC staining
Show wash step ect Show Name: Abbreviated name: Description: Staining method: BOND RX Preferred detection Step N° Wa 1 2 2 3 4	s wash steps. ACD miRNA-Protein Co-Def miP-CoDB Bond Polymer Refine IHC pr Single First system: Bond Polymer Res sh Reagent *Peroxide Block *Bond Wash Solution *Bond Wash Solution Bond Wash Solution	Save New proto tection Part B rotocol fine Detection Supplier Leica Microsystems Leica Microsystems Leica Microsystems Leica Microsystems Leica Microsystems Leica Microsystems	Cancel Col properties	Import pr 	оtocol F Dispense ty 150 µL 150 µL 0pen 150 µL	Preferred Protocol type: IHC staining
Show wash step ect Show Name: Abbreviated name: Description: Staining method: BOND RX Preferred detection Step N° Wa 1 2 3 4 4 5	s ACD miRNA-Protein Co-Def miP-CoDB Bond Polymer Refine IHC pr Single First system: Bond Polymer Re sh Reagent *Peroxide Block *Bond Wash Solution	Save New proto tection Part B rotocol afine Detection Leica Microsystems Leica Microsys	Cancel Col properties	Import pr Inc. (min) 5:00 0:00 0:00 0:00 15:00	Dispense ty 150 µL 150 µL 150 µL 150 µL 150 µL	Preferred Protocol type: IHC staining
Show wash step ect Show Name: Abbreviated name: Description: Staining method: BOND RX Preferred detection Step N [®] Wa 1 2 3 4 4 5 6	s ACD miRNA-Protein Co-Del miP-CoDB Bond Polymer Refine IHC pr Single First System: Bond Polymer Ref sh Reagent *Peroxide Block *Bond Wash Solution	Save New proto tection Part B rotocol fine Detection Supplier Leica Microsystems	Cancel Col properties	Import pr 5:00 0:00 0:00 0:00 0:00 0:00 0:00 0:00 0:00 0:00 0:00	Dispense ty 150 µL 150 µL 0pen 150 µL 150 µL 150 µL	Preferred Protocol type: IHC staining
Show wash step ect Show Name: Abbreviated name: Description: Staining method: BOND RX Preferred detection Step N° Was 1 2 V 3 V 4 3 V 4 5 6 V 7 V	s ACD miRNA-Protein Co-Det miP-CoDB Bond Polymer Refine IHC pr Single First system: Bond Polymer Ref sh Reagent *Peroxide Block *Bond Wash Solution	Save New proto tection Part B rotocol Second Supplier Leica Microsystems	Cancel col properties Ambient Temperature ·	Import pr 5:00 0:00 0:00 0:00 0:00 0:00 0:00 0:00 0:00 0:00	Dispense ty 150 µL 150 µL	Preferred Protocol type: IHC staining
Show wash step A step Show Name: Abbreviated name: Description: Staining method: BOND RX Preferred detection Step N° Wa 1 2 3 4 4 5 6 7 8 8	s ACD miRNA-Protein Co-Def miP-CoDB Bond Polymer Refine IHC pr Single First system: Bond Polymer Re sh Reagent *Peroxide Block *Bond Wash Solution	Save New proto tection Part B rotocol Second Supplier Leica Microsystems	Cancel	Import pr 5:00 0:00 0:00 0:00 0:00 0:00 0:00 0:00 0:00 0:00 0:00 0:00 0:00 0:00	Dispense ty 150 µL 150 µL 150 µL 150 µL 150 µL 150 µL 150 µL	Preferred Protocol type: IHC staining

7. Set up semi-automated green or fully automated brown IHC detection by choosing one of the following procedures:



a. For semi-automated green IHC detection:

i. Modify the protocol according to the following table, and verify that the final protocol is 11 steps.

ii. (Optional) Change the incubation time for *Post Primary and *Polymer to 16 MIN.

Step No.	Reagent	Step Type	Incubation Time	Temperature
1	*Peroxide Block	Reagent	5 MIN	Ambient
2	*Bond Wash Solution	Wash	0 MIN	Ambient
3	*Bond Wash Solution	Wash	0 MIN	Ambient
4	*Bond Wash Solution	Wash	0 MIN	Ambient
5	*Post Primary	Reagent	8 MIN /16 MIN	Ambient
6	*Bond Wash Solution	Wash	2 MIN	Ambient
7	*Bond Wash Solution	Wash	2 MIN	Ambient
8	*Bond Wash Solution	Wash	2 MIN	Ambient
9	*Polymer	Reagent	8 MIN /16 MIN	Ambient
10	*Bond Wash Solution	Wash	2 MIN	Ambient
11	*Bond Wash Solution	Wash	2 MIN	Ambient

Note: Green chromogen and counterstaining are performed offline.

iii. Select Save.

b. For fully automated brown IHC detection:

i. Modify the protocol according to the following table, and verify that the protocol is 21 steps.

Step No.	Reagent	Step Type	Incubation Time	Temperature
1	*Peroxide Block	Reagent	5 MIN	Ambient
2	*Bond Wash Solution	Wash	0 MIN	Ambient
3	*Bond Wash Solution	Wash	0 MIN	Ambient
4	*Bond Wash Solution	Wash	0 MIN	Ambient
5	*Post Primary	Reagent	8 MIN / 16 MIN	Ambient
6	*Bond Wash Solution	Wash	2 MIN	Ambient
7	*Bond Wash Solution	Wash	2 MIN	Ambient
8	*Bond Wash Solution	Wash	2 MIN	Ambient
9	*Polymer	Reagent	8 MIN / 16 MIN	Ambient
10	*Bond Wash Solution	Wash	2 MIN	Ambient
11	*Bond Wash Solution	Wash	2 MIN	Ambient
12	*Deionized Water	Wash	0 MIN	Ambient
13	*Mixed Refine DAB	Wash	0 MIN	Ambient
14	*Mixed Refine DAB	Wash	10 MIN	Ambient
15	*Deionized Water	Wash	0 MIN	Ambient
16	*Deionized Water	Wash	0 MIN	Ambient
17	*Deionized Water	Wash	0 MIN	Ambient
18	*Hematoxylin	Reagent	5 MIN	Ambient
19	*Deionized Water	Wash	0 MIN	Ambient
20	*Bond Wash Solution	Wash	0 MIN	Ambient

For Research Use Only. Not for use in diagnostic procedures

TN 324600/ Rev A/ Effective Date 10/01/2021

ACD	TI	ECHNICAL NOTE			
	21	*Deionized Water	Wash	0 MIN	Ambient
-	ii. Sele	ect Save.			

Create an HIER protocol for use with Co-Detection protocols

IMPORTANT! We recommend using an extended heat-induced epitope retrieval (HIER) incubation for optimal RNA and protein co-detection. Before proceeding to slide setup, refer to **Appendix A** for instructions on how to create an **ACD HIER 30 min with ER2 (95)** protocol.

Part 3: Set up a Study for Co-Detection

Build a study

1. Select the **Slide setup** icon at the top of thescreen.



- 2. Select Add study and enter a name in the Study ID field (keep the Dispense volume at 150 μ L as shown.
- 3. For FFPE tissues, select ***Bake and Dewax** as the Preparation protocol (leave blank for other tissue types).



4. Select OK.

Add a slide for ICW in FFPE samples

Note: To set up slides for fresh frozen samples, refer to **Appendix B**. To set up slides for fixed frozen samples, refer to **Appendix C**.

- 1. Select Add slide.
- 2. Enter the probe name and primary antibody under the Comments field.
- 3. Select **Sequential DS** from the Staining mode drop down menu.

Study ID:	Hs-miR21/CD3					
test	Tissue type:	Dispense volume:				
Researcher:	Test tissue	100 µL				
Slide ID:	Negative tissue	🐼 150 uL				
Study N°:	Positive tissue					
8 64 - 4	Staining mode:					
study comments:	Single	Routine				
Date created: 3/2/2020 1-01-39 PM	Single	1				
5/2/2020 1.01.30 F M	Sequential DS					
	Parallel DS IHC ISH					
	Marker:		-			
	Protocols					
	Preparation:	*Bake and Dewax	-			

4. Under the First tab, select **ISH**

For Research Use Only. Not for use in diagnostic procedures

TN 324600/ Rev A/ Effective Date 10/01/2021



- 5. Select Mock Probe as Marker.
- 6. Under Protocols:
- For each probe, select a protocol from the Staining drop down menu. Make sure that each probe and primary antibody combination is associated with a different Part A protocol (for example, ACD miRNA-Protein Co-Detection Part A – Hs-miR-21 CD3).
- 8. For HIER protocol, select ACD HIER 30 min with ER2 (95) as the HIER protocol.
- 9. Select *--- for Enzyme.

Note: The Part A protocol already includes a protease step. Additional enzyme pretreatment can negatively impact IHC detection.

- 10. Select ***DEFAULT*** for Probe Application and Probe Removal.
- 11. Select *--- for Denaturation and ACD 1 min Hybridization for Hybridization.

	Hs-miR21/ CD3	
est	Tissue type:	Dispense volume:
Researcher:	Tast tissue	100 vi
	 Test tissue 	100 μL
lide ID:	Negative tissu	e 🍼 150 µL
tudy N°:	Positive tissue	3
) Studu annunanta	Staining mode:	
study comments:	Sequential DS	✓ Routine
Date created: 3/2/2020 1:01:39 PM	First	Second
	Process:	UHC 🕑 ISH
	Marker:	Mock Probe (ACD)
	Protocols	
	Staining:	ACD miRNA-Protein Co-Detection Part A-CD3-Hs-miR21
	Preparation:	*Bake and Dewax
	HIER:	ACD HIER 30 min with ER2 (95)
	-	
	Enzyme:	Tabaa
	Probe Application:	*DEFAULT*
	Enzyme: Probe Application: Denaturation:	*DEFAULT*
	Enzyme: Probe Application: Denaturation: Hybridization:	*DEFAULT* * ACD 1 Min Hybridization *

- 12. Under the Second tab, select IHC.
- 13. Select *Negative as Marker.
- 14. Under Protocols:
- 15. Select ACD miRNA-Protein Co-Detection Part B from the Staining drop down menu.
- 16. For HIER, select *---.
- 17. For Enzyme, select *---.
- 18. Select Add Slide.



Study ID:	Hs-miR21/ CD3	
Deserved and the second s	Tissue type:	Dispense volume:
	Test tissue	100 μL
Slide ID:	Negative tissue	e 🕜 150 μL
Study N°: 8	Positive tissue	
Study comments:	Staining mode:	
Date created:	Sequential DS	Routine
3/2/2020 1:01:39 PM	First	Second
	Process:	IHC SIN
	Marker:	*Negative
	Protocols	
	Staining:	ACD miRNA-Protein Co-Detection Part B
	HIER:	
	Enzyme:	* v

19. Repeat steps 1–18 for each slide.

Complete the study

- 1. After adding slides to the study, select **Close** to return to the Slide setup screen.
- 2. Select **Print labels** to print barcodes and attach to theslides.
- 3. Place slides into the Leica BOND Rx Slide Staining Assemblies (SSAs) and carefully place Leica Covertiles on each slide.
- 4. Place the SSA in the Leica BOND RX, and press the button to load the tray onto the machine.

Note: If performing the miRNAscope assay, you can run up to three SSAs simultaneously using ICW.

5. Once the slides have been scanned, select the **PLAY** (triangular) button on the screen located under the start tray to start the run. Alternatively, right-click on the scanned label images and select **Delayed Start** to start the run at a future time.

Note: If using Delayed Start, set the staining procedure to begin within six hours of loading reagents.

Part 4: Detect green IHC staining off the instrument

Note: If you are performing Brown IHC detection, it is already included in the automated protocol. Proceed to **Dry and Mount the Samples**.

Prepare reagents and equipment

• Before the run completes, remove the Green A and Green B reagents from the refrigerator and warm to ambient temperature.

 IMPORTANT!
 View the wash step video at www.acdbio.com/technical-support/learn-more

 before proceeding.
 View the wash step video at www.acdbio.com/technical-support/learn-more



1. As soon as the run is complete, press the button on the front of the instrument and unload the slides immediately.

IMPORTANT! Do not let sections dry out between incubation steps. Work quickly and make sure the sections are hydrated at all times.

- 2. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Agitate slides by moving the slide rack up and down in the staining dish.
- 3. Repeat Step 2 with fresh 1X WashBuffer.
- 4. Briefly spin down the contents of the Green B tube to be sure content is at the bottom of the tube before opening the cap.
- 5. Prepare 200 µL of GREEN working solution per slide using a 1:50 ratio of Green B to Green A. Mix well.

IMPORTANT! Use the GREEN solution within **5 MIN**. Do not expose to direct sunlight or UV light.

- 6. Take each slide one at a time from the Tissue-Tek[®] Slide Rack and tap and/or flick to remove the excess liquid.
- 7. Pipette ~200 µL GREEN solution onto each tissue section. Ensure sections are covered.
- 8. Incubate the slides for 15–30 MIN at RT to achieve the desired level of chromogen intensity.
- 9. To remove the GREEN working solution from the slides, tilt each slide one at a time over a waste container and tap the corner on the edge of the container. Immediately insert the slide into a Tissue-Tek Slide Rack submerged in a Tissue-Tek Staining Dish filled with distilled water.
- 10. Quickly rinse the slides with fresh distilled water for less than 30 seconds.

IMPORTANT! Proceed quickly to the next step. GREEN substrate may fade if stored in water for too long.

Counterstain the slides

1. Move the Tissue-TekSlide Rack into the staining dish containing 50% Hematoxylin I staining solution for 30 SEC at **RT**. Tissue should look purple.

IMPORTANT! Proceed quickly to the next step. GREEN substrate can fade if in Hematoxylin for longer than 30 seconds.

- 2. Immediately transfer the slide rack into a staining dish filled with tap water. Do not let the slides remain in the water for more than 30 seconds.
- 3. Repeat Step 2 once or twice.

Dry and mount the samples

1. Remove the slide rack from the staining dish and dry slides in a 60°C dry oven for 30 MIN.

IMPORTANT! GREEN substrate is alcohol sensitive. Do not dehydrate the slides in alcohol.

- 2. Cool the slides for **5 MIN** at **RT**.
- 3. Briefly dip one slide into fresh pure xylene and immediately place 1–2 drops of VectaMount[®] Mounting Medium on the slide before the xylene dries.
- 4. Carefully place a 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
- 5. Repeat steps 2 and 3 for eachslide.
- 6. Air dry the slides for **5 MIN**.

Appendix A. Creating a new HIER Protocol

For optimal RNA and protein detection, we recommend using an extended heat-induced epitope retrieval (HIER) step. Follow the steps to edit the Epitope retrieval procedure in the software.

Create a prestaining protocol

1. Open the Leica BOND software and click on the Protocol Setup icon on the home screen.

🜔 BOND - (bondpov	reruser) - Window	s Internet Explorer								_1	IX
Slide setup	Protocol setup	Reagent setup	Slide history	Search	Help	Log out				•	t and
BOND - (bondpow	reruser) - Windows	Internet Explorer						_		_0	12
Slide setup	Protocol setup	Reagent setup	Slide history	Search	Help	Log out				Leica	. 1
ð	1	ปั	Ś	Q	0	•U				BIGSYSTEMS	- 1
											_
Mrs. Potato Head	Protoco	ol setup						Copy O	pen Delete	Report	
		Protocol na	ame 🔺	Pro	tocol type		Description	Modified by	Mod. date	Pref.	e l
	*AccuCyte	e CTC HIER ER2 8 r	nins	Heat	pretreatment	AccuCyte CTC H	IIER ER2 8 mins	Leica	2/21/2020	~	1
	*ACD HIE	R 15 min with ER2 (95)	Heat	pretreatment	ACD RNAscope	heat pretreatment	Leica	2/21/2020	~	11
Slinky Dog	"HIER 10	min with ER1		Heat	pretreatment	10 min Heat Ret	rieval using ER1	Leica	2/21/2020	~	
[] 7:13 AM	"HIER 10	min with ER2		Heat	pretreatment	10 min Heat Ret	rieval using ER2	Leica	2/21/2020	~	
	"HIER 20	min with ER1		Heat	pretreatment	20 min Heat Ret	rieval using ER1	Leica	2/21/2020	~	
	"HIER 20	min with ER2		Heat	pretreatment	20 min Heat Ret	rieval using ER2	Leica	2/21/2020	~	
	"HIER 25	min with ER1 (97)		Heat	pretreatment	25 min Heat Ret	rieval using ER1	Leica	2/21/2020	~	
	"HIER 30	min with ER1		Heat	pretreatment	30 min Heat Ret	rieval using ER1	Leica	2/21/2020	~	
	"HIER 30	min with ER2		Heat	pretreatment	30 min Heat Ret	rieval using ER2	Leica	2/21/2020	~	
	"HIER 40	min with ER1		Heat	pretreatment	40 min Heat Ret	rieval using ER1	Leica	2/21/2020	~	
	*HIER 40	min with ER2		Heatp	pretreatment	40 min Heat Ret	rieval using ER2	Leica	2/21/2020	~	
	"HIER 5 m	nin with ER1		Heat	pretreatment	5 min Heat Retri	eval using ER1	Leica	2/21/2020	~	
	*RNAscop	pe 2.5 LSx Target Re	trieval (88)	Heat	pretreatment	RNAscope 2.5 L	Sx heat retrieval 88C	Leica	2/21/2020	~	
	*RNAscop	pe 2.5 LSx Target Re	trieval (95)	Heat	pretreatment	RNAscope 2.5 L	Sx heat pretreatment 95C	Leica	2/21/2020	~	
	*ViewRNA	A HIER 10 min, ER1	(95)	Heat	pretreatment	10 min heat pret	reatment, ER1 @ 95C for	Leica	2/21/2020	~	
	A.Con ONI	UICD 10 min. CD2	(00)	Lant.	rotrootmont	10 min hant area	rootmont ED2 @ 000 for	Loise	2/24/2020		•
	Protocol grou	ip:	Protocol type:		Staining :	status:	Protocol origin:	Pre	eferred status:	_	- 1
	Prestaining		 Heat pretreat 	atment	·		▼ All	•	Preferred	•	- 1
											5
											4
							*Dew	ax *DI *BWash *∖	Alcohol "Blk/Mast *B	IKWast *HazW	ast

- 2. Under the Protocol group menu, select **Prestaining**.
- 3. Under Protocol type menu, select Heat Pretreatment.
- 4. Highlight the *ACD HIER 15min with ER2 (95) protocol. Select Copy.
- 5. Rename the protocol as ACD HIER 30min with ER2 (95). Rename the abbreviated name as

Abbreviated name:	ACDHet30	mont					
Description.	ACD Reviscope near preveau	ingrit.					 Preferre
BOND RX					Import protocol	Protocol type:	Heat pretreatme
							
Step N* Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type	~
1	"Bond ER Solution 2	Leica Microsystems	~		0:00	150 µL	
2	*Bond ER Solution 2	Leica Microsystems	~		0:00	150 µL	
3	"Bond ER Solution 2	Leica Microsystems		95	15:00	Intermediate	
4	*Bond ER Solution 2	Leica Microsystems	~		0:00	150 µL	
Snow wasn steps	5						

ACDHet30.



- 6. Highlight the third ***BOND ER Solution 2** step. Change the incubation time to **30 MIN**.
- 7. Select Save.

Appendix B. LS Chromogenic ICW using Fresh Frozen Samples

Part 1. Prepare Fresh Frozen Samples

Prepare reagents and equipment

- 1. Remove tissue and trim to fit into cryomolds.
- 2. Freeze on dry ice or liquid Nitrogen within **5 MIN** of harvest.
- 3. Embed frozen tissue in cryo-embedding medium and freeze blocks.
- 4. Store the frozen block in an air-tight container at -80°C.

Note: Embedded tissue may be stored for at least three months

- 5. Equilibrate block to **–20°C** in a cryostat **~1 HR**.
- 6. Cut 10-20 µm sections and mount ONLY onto SuperFrost® Plus slides.

Abbreviated name:	ACDHet30	(00)					
Description:	ACD RNAscope heat pretrea	atment					✓ Preferre
BOND RX					Import protocol	Protocol typ	e: Heat pretreatme
							Ĩ
Step N* Wash	Reagent	Supplier	Ambient	Temperature	Inc. (min)	Dispense type	
1	*Bond ER Solution 2	Leica Microsystems	~		0:00	150 µL	
2	*Bond ER Solution 2	Leica Microsystems	~		0:00	150 µL	
3	*Bond ER Solution 2	Leica Microsystems		95	30:00	Intermediate	
4	"Bond ER Solution 2	Leica Microsystems	~		0:00	150 µL	
Show wash steps							

- 7. Dry slides for **1 HR** at **-20°C**.
- 8. Store in air-tight slide boxes at **-80°C** until use.

Note: Use sectioned tissue within three months.

Prepare the slides

- 1. Add fresh 10% NBF or 4% PFA to a Tissue Tek Staining Dish.
- 2. Remove slides from **-80°C** and place in a Tissue Tek Slide Rack.
- 3. *Immediately* immerse slides in staining dish containing 10% NBF or 4% PFA. Fix for **90 MIN** at **ROOM TEMPERATURE (RT)**.
- 4. Prepare four Tissue Tek Staining Dishes with about 200mL of fresh 50% EtOH, 70% EtOH, and 100% EtOH (2x).
- 5. Remove slides from the fixative and immediately place in 50% EtOH for **5 MIN** at **RT**.
- 6. Place the slides in 70% EtOH for **5 MIN** at **RT**.
- 7. Place the slides in 100% EtOH for **5 MIN** at **RT**.
- 8. Repeat with fresh 100% EtOH for **5 MIN** at **RT**.
- 9. Remove slides from 100% EtOH and allow to air dry for **5 MIN** at **RT** on absorbent paper.

Note: Slides may be stored in 100% EtOH at **-20°C** for up to one week.

For Research Use Only. Not for use in diagnostic procedures TN 324600/ Rev A/ Effective Date 10/01/2021



Setup a Protocol

- 1. Refer to **Part 1: Add and Register Reagents for Co-Detection** on page 4 to add and register new reagents.
- 2. Refer to **Part 2: Create Co-Detection Software Protocols** on page 7 to create co-detection software protocols. If needed, modify the Protease step in the **Part A** protocol according to the following table:

Assay	Protease Reagent	Step No.	Registration Name	Step Type	Incubation Time	Temperature
miRNAscope LS Red	Protease IV	17	*Enzyme 1	Reagent	0 MIN	Ambient
	Protease IV	18	*Enzyme 1	Reagent	30 MIN	Ambient

+ The table lists the standard Protease conditions for miRNAScope LS. We recommend using standard conditions unless your tissue type requires additional time and temperature optimization. If stronger protease treatment is needed, add an additional **30 MIN** Enzyme reagent step directly following Step 18.

Setup a study

- 1. Go to the **Slide setup** screen. Select **Add Study** and add study information.
- 2. For Preparation, select *- --.

IMPORTANT! The ***Frozen Slide Delay** preparation selection is not recommended for fresh frozen samples, as it could result in non-uniform staining.

- 3. Select Add slide.
- 4. Enter the probe name and primary antibody under the Comments field.
- 5. Select **Sequential DS** from the Staining mode drop downmenu.
- 6. Under the First tab, select **ISH**. Select **Mock Probe** as Marker.
- 7. Under Protocols:
- 8. Select a protocol from the Staining drop down menu for each probe. Make sure that each probe and primary antibody combination is associated with a different Part A protocol (for example, **ACD miRNA-Protein Co-Detection Part A CD3 Hs-miR-21**).
 - a. For HIER and Enzyme, select *---.
- 9. The Part A protocol already includes a protease step. Additional Enzyme pretreatment can negatively impact IHC detection. Select ***DEFAULT*** for Probe Application and Probe Removal.
- 10. Select *--- for Denaturation and ACD 1 min Hybridization for Hybridization.
- 11. Under the Second tab, select **IHC**. Select ***Negative** as Marker.
- 12. Under Protocols:
- 13. Select ACD miRNA-Protein Co-Detection Part B from the Staining drop down menu.
 - a. For HIER and Enzyme, select *---.
- 14. Select Add Slide.
- 15. Repeat steps 1–14 for each additional slide.

Complete the study

- 1. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
- 2. Select **Print labels** to print barcodes to attach to the slides.
- 3. Place slides into the Leica BOND RX Slide Staining Assemblies (SSAs) and carefully place Leica Covertiles on each slide.
- 4. Place the SSA in the Leica BOND RX, and press the button to load the tray onto the machine.

Note:If performing the miRNAscope assay, you can run up to three SSAs simultaneously using ICW.For Research Use Only. Not for use in diagnostic proceduresTN 324600/ Rev A/ Effective Date 10/01/2021LS Charmenenic BNIA Bristin Co. Detection



1. Once the slides have been scanned, select the **PLAY** (triangular) button on the screen located under the start tray to start the run.

Appendix C. LS Chromogenic ICW using Fixed Frozen Samples

Part 1. Prepare Fixed Frozen Samples

Post fixation

- 1. Remove slides from **-80°C** and place in a Tissue Tek Slide Rack.
- 2. Bake slides for **30–60 MIN** at **60°C**.
- 3. Immerse slides in staining dish containing 10% NBF or 4% PFA. Fix for 15 MIN at 4°C.

Note: Formalin that has been stored for more than six months, exposed to air for more than one week, or used repeatedly can result in suboptimal post-fixation.

- 4. Prepare four Tissue Tek[®] Staining Dishes with about 200mL of fresh 50% EtOH, 70% EtOH, and 100% EtOH (2x).
- 5. After 15min post-fixation, remove slides from the fixative and *immediately* place in 50% EtOH for **5 MIN** at **RT**.
- 6. Place the slides in 70% EtOH for **5 MIN** at **RT**.
- 7. Place the slides in 100% EtOH for **5 MIN** at **RT**.
- 8. Repeat with fresh 100% EtOH for **5 MIN** at **RT**.
- 9. Remove slides from 100% EtOH and allow to air dry for **5 MIN** at **RT** on absorbent paper.

Note: You can store slides in 100% EtOH at **-20°C** for up to one week.

Part 2. Run the Assay

IMPORTANT! This procedure uses the heat-induced epitope retrieval (HIER) protocol ACD HIER 5 min with ER2 (95). Before continuing, make sure you have this protocol on your instrument. For an example of how to create a new HIER protocol, see Appendix A.

Setup a protocol

- 1. Refer to Part 1: Add and Register Reagents for Co-Detection on page 4 to add and register new reagents.
- Refer to Part 2: Create Co-Detection Software Protocols on page 7 to create co-detection software protocols.

Note: For fixed frozen samples, changes to protease conditions are unnecessary.

Setup a study

- 1. Go to the Slide setup screen. Select **Add Study** and add study information.
- 2. For Preparation, select *- --.

IMPORTANT! The ***Frozen Slide Delay** preparation selection is not recommended for fixed frozen samples, as it can result in non-uniform staining.

- 3. Select Add slide.
- 4. Enter the probe name and primary antibody under the Comments field.
- 5. Select Sequential DS from the Staining mode drop downmenu.
- 6. Under the First tab, select **ISH**. Select **Mock Probe** as Marker.
- 7. Under Protocols:

For Research Use Only. Not for use in diagnostic procedures TN 324600/ Rev A/ Effective Date 10/01/2021 LS Chromogenic RNA-Protein Co-Detection



- Select a protocol from the Staining drop down menu for each probe. Make sure that each probe and primary antibody combination is associated with a different Part A protocol (for example, ACD miRNA-Protein Co-Detection Part A – CD3 Hs-miR-21.
- 9. For HIER protocol, select ACD HIER 5 min with ER2 (95).
- 10. For Enzyme, Select *- --.
- 11. The Part A protocol already includes a protease step. Additional Enzyme pretreatment can negatively impact IHC detection. Select ***DEFAULT*** for Probe Application and Probe Removal.
- 12. Select *--- for Denaturation and **ACD 1 min Hybridization** for Hybridization.
- 13. Under the Second tab, select IHC, and select *Negative for Marker.
- 14. Under Protocols:
- 15. Select ACD miRNA-Protein Co-Detection Part B from the Staining drop down menu.
- 16. For HIER and Enzyme, select *---.
- 17. Select Add Slide.
- 18. Repeat steps 3–17 for each additional slide.

Complete the study

- 1. After adding all the slides to the study, select **Close** to return to the Slide setup screen.
- 2. Select **Print labels** to print barcodes to attach to theslides.
- 3. Place slides into the Leica BOND RX Slide Staining Assemblies (SSAs), and carefully place Leica Covertiles on each slide.
- 4. Place the SSA in the Leica BOND RX, and press the button to load the tray onto the machine.

Note: If performing the miRNAscope assay, you can run up to three SSAs simultaneously using ICW.

5. Once the slides have been scanned, select the **PLAY** (triangular) button on the screen located under the start tray to start the run.

Appendix D. ICW Troubleshooting Guide

You may need to use a higher primary antibody concentration for the ICW workflow than you would normally use for IHC alone. To optimize protein detection, we recommend optimizing the antibody concentration.

The crosslinking and pretreatment conditions in this Tech Note provide optimal miRNA and protein detection across most tissue samples. If further optimization is required for a specific sample or target of interest, adjust the following parameters:

Reagent	Incubation Temperature	Recommended Incubation Time	Optimization Range
HIER	95°C	30 MIN	15–30 MIN at 88-95°C
Primary Antibody	Ambient	15 MIN	15–60 MIN
10% NBF	Ambient	30 MIN	15–60 MIN
Protease	40°C*	30 MIN	15–30 MIN†
Post Primary	Ambient	8 MIN	8–16 MIN
Polymer	Ambient	8 MIN	8–16 MIN

*For fresh frozen samples, we recommend incubating at ambient temperature. Higher temperatures can compromise RNA quality.

+Some samples may require stronger protease treatment. For these samples, add an additional Enzyme reagent step directly following step 18.

For Research Use Only. Not for use in diagnostic procedures TN 324600/ Rev A/ Effective Date 10/01/2021

For Research Use Only. Not for use in diagnostic procedures.

Advanced Cell Diagnostics, Inc. and/or its affiliate(s) warrant their products as set forth in the ACD General Terms and Conditions of Sale found on the ACD website. Advanced Cell Diagnostics, Inc. reserves the right to change its products and services at any time to incorporate technological developments. This document is subject to change without notice. Although this document has been prepared with every precaution to ensure accuracy, Advanced Cell Diagnostics, Inc. assumes no liability for any errors, omissions, or for any damages resulting from the use of this information.

© 2021 Advanced Cell Diagnostics. All rights reserved. RNAscope is a registered trademark of Advanced Cell Diagnostics, Inc. All other trademarks belong to their respective owners.

