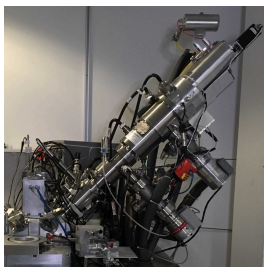
May 14, 2022 Version 5

MIBI staining V.5



DOI
dx.doi.org/10.17504/protocols.io.dm6gprk2dvzp/v5

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DOI: https://dx.doi.org/10.17504/protocols.io.dm6gprk2dvzp/v5

Protocol Citation: Marc Bosse, Sean Bendall, Mike Angelo 2022. MIBI staining. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.dm6gprk2dvzp/v5**Version created by **Marc Bosse**

Protocol status: Working
We use this protocol and it's working

Created: May 14, 2022

Last Modified: February 01, 2024

Protocol Integer ID: 62555

Keywords: staining procedure, standard ffpe tissue, multiplex ion beam imaging time, mibi, multiplex ion beam imaging time of flight instrument, mibi-tof, glutaraldehyde fixation, immunohistochemistry, art of immunohistochemistry, final washes prior tissue dehydration, prior tissue dehydration

Abstract

This protocol is the standard FFPE tissue staining procedure recommended for Multiplex Ion Beam Imaging Time of Flight instrument (MIBI_TOF) developed in the Sean C. Bendall and Michael R. Angelo labs. The protocol has been successfully used for MIBI and is the result of extensive optimization experiments. It is inspired from state-of-the art of immunohistochemistry staining procedures but differs in some very important steps, namely, glutaraldehyde fixation and final washes prior tissue dehydration. Failure to follow exactly all steps described in this procedure may result in inconsistencies in output data after MIBI_TOF acquisition.

Guidelines

Staining tissue sections is fairly straightforward but there are few things to be cognizant of, when preparing samples:

- Always try to limit mechanical damage to the sample surface. This can occur when moving the samples with forceps, particularly when the mounting substrates are small.
- Once the samples have been rehydrated, they cannot dry out until the end of the protocol.
- Be careful at all times to not touch the tissue, in order to not leave any residue.
- Once the samples have been stained, fixed, and dehydrated, they have an indefinite shelf life and can be imaged at any time but need to be stored properly such as in a vacuum chamber or in a sealed vacuum bag
- MIBI_TOF observes the basic principles of Mass spectrometry. Any contaminant ions present in water or in the air can potentially compromise the integrity of the sample.
- Therefore to prevent potential contamination, it is important to **always use single use lab ware containers** to make the solutions. The protocol has been validated using the level of precision of graduated Nalgene bottles and 50 mL tubes.
- **The use of washed beakers or graduated cylinders is not considered as good a practice, due possible introduction of contaminants (exemples of sources: barium from lab ware soap or calcium from air dried lab ware)**

Materials

	A	B	C
	Products	**Provider**	**Catalogue No.**
	Alcohol ethyl ETHANOL 200 PROOF	Gold Shield	412811
	Alcohol ethyl ETHANOL 190 PROOF	Gold Shield	412602
	TBS IHC Wash Buffer with Tween 20	Cell Marque	935B-09
	PBS IHC Wash Buffer with Tween 20	Cell Marque	934B-09
	Target Retrieval Solution, pH 9	Agilent (Dako)	S236784-2
	UltraPure water	Invitrogen	10977-015
	Avidin/Biotin Blocking Kit	Biolegend	927301
	Hydrogen peroxide	Sigma	216763-100ML
	Gelatin (cold water fish skin)	Sigma-Aldrich	G7765-250
	Xylene HISTOLOGICAL GRADE	Sigma-Aldrich	534056-500
	Glutaraldehyde 8% Aqueous Solution EM Grade	EMS	16020
	Bovine Albumin (BSA), heat shock treated	Fisher	BP1600-100
	Centrifugal filters (0.1µm)	Millipore	UFC30VV00
	ImmEdge hybrophobic barrier pen	Vector lab	H-4000
	MIBI slides	IonPath	567001
	Levamisole	Vector Labs	SP-5000
	Horse serum	Vector Labs	S-2000
	VectaMount Permanent Mounting Medium	Vector Labs	H-5000
	Equipments	Provider	Cat No.
	Thermo Scientific™ Lab Vision™ PT Module	Thermo Fisher Scientific	A80400012
	Leica ST4020 Small Linear Stainer	Leica	14050946425

	A	B	C
	Digital incubators, INCU-Line®, IL 10 and IL 23	VWR	390-0384
	Bel-Art™ SP Scienceware™ Lab Companion Cabinet Style Vacuum Desiccators, Clear	**Fisher Scientific**	**08-648-109**
	Oribital shaker	Boekel	270200
	Moist chamber	Ted Pella	21051

Protocol materials

MIBI slides **IonPath Catalog #**567001

Troubleshooting

Safety warnings

All organic solvents should be manipulated under a chemical hood.

Before start

Verify the stocks of all reagents and place an order or prepare solutions, if some reagents are running low.

Slide for MIBI

1 FFPE or frozen sections should be deposited on special conductive slides for MIBI
It is recommended to use freshly cut tissue sections. Otherwise tissue section slides should be stored properly using different state of the art methods (vacuum chamber, nitrogen chamber or vacuum sealed bags)
MIBI slides **IonPath Catalog #**567001

Slide baking and PT module preparation

2 Bake the sections at 70 °C for 00:20:00 in a dry incubator

Optional : 01:00:00 ; Overnight

Note

Note: Some tissues or section size may need longer baking time.

Recommended to bake at least 1 hour for brain tissue or TMA. This can be extended to 16 h (overnight).

2.1 Last 10 min place the slide (s) vertically with the label side up to allow drip down the paraffin

3 **Prepare Target retrieval solution**
2.5 mL of target retrieval solution 10x (3-in-1), DAKO
in 22.5 mL of ultrapure (type 1, >18 MOhms) water

	Total volume (mL)	Volume (mL) Target retrieval	Volume (mL) H2O
	25	2.5	22.5
	50	5	45
	100	10	90

4 Put in the containers with the diluted target retrieval solution in the PT Module

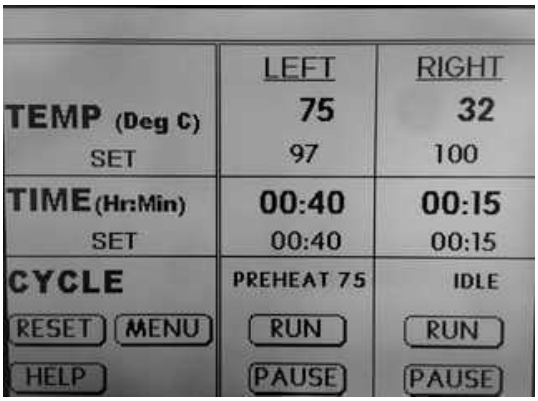
Equipment

Thermo ScientificTM Lab VisionTM PT Module	NAME
Programmed Water bath	TYPE
Thermofisher	BRAND
A80400012	SKU
https://www.thermofisher.com/order/catalog/product/A80400012#/A80400012	LINK

PNG

5 **PT Module Preheat**

Press RUN on digital screen and check for PREHEAT 75 on display



Slide deparaffination

6 **Linear Stainer**

Pour out reagent containers and fill with fresh reagents:

Xylene x 3, 100% Ethanol x 2, 95% Ethanol x 2, 80% Ethanol, 70% Ethanol, ddH2O x 2, exit stainless steel tank = ultrapure (type 1, >18 MOhms) water

Note

IMPORTANT: **Use fresh xylene for every deparaffination.**

6.1 Prior loading the slides on the Linear Stainer, immerse the slides in a container with fresh xylene. Make sure that the xylene solution covers all the paraffin. Incubate for a minimum of 00:05:00 5m

Note

The MIBI staining protocol was originally design with the Target retrieval Solution pH 9, (3in1) 10x, S2375. This product is now discontinued. **Deparaffination is a critical step for successful staining. Longer deparaffination time might be needed for some tissues.**

7 Insert slides into slide carriers

Place the slide carrier into first xylene container

8 Press on **Menu**

Check for Processing time = 30 sec, Lift bar = 976, Number of dips = 3

Continue to press **Menu** until the screen displays **Start at: __**

Set Start position corresponding to the first slide carrier position

Exemple: If the first slide carrier is at position 4, use Plus (**+**) or Minus (**-)** button to increase or decrease to get **Start at: 04**

8.1 Then press **Enter**

8.2 **Synchronize** when the PT module temperature has reached **75˚C**

then Press **Run** on the Linear Stainer

8.3 Allow the rehydration process and wait until the slides have reached the stainless steel tank and stop

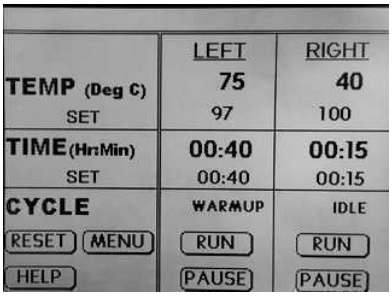
9 Bring the stainless steel tank with the slides in close to the PT module

Antigen Retrieval

10 Open the PT Module and insert the slides in the warm **Target retrieval solution** container

Discard water immediately from the stainless steel tank

11 **Press RUN** again and check for first **WARMUP** then **HEAT** on display, once the temperature has reached 97˚C



12 Verify stock of 1x PBS wash buffer and prepare accordingly if running low

	Reagents	Qty for 1000 mL
	PBS IHC Wash Buffer with Tween 20 (mL)	50
	Bovine Albumin (BSA), heat shock treated (g)	1
	Ultrapure (type 1) water (mL)	949

13 Allow to run for 40 min at 97 °C and then cool down for approximately 50 min and reach 65 °C

14 When the alarm sounds Stop the PT module

15 Take out the slides and let cool down at room temperature for at least 00:05:00

16 Prepare two coplin jars filled with MIBI 1x PBS wash buffer

17 Transfer the slides in the **first** MIBI 1x PBS wash buffer and use orbital shaker set for 5 min, 70 rpm

18 Transfer the slides to the **second** 1x PBS wash buffer and use orbital shaker set for 5 min, 70 rpm

Hydrophobic barrier pen

19 Make sure to dry with a folded-tissue paper the slide, leaving a square of wet surface surrounding of the tissue section

Note: Do not let AIR DRY the tissue section, this will result high background and false positive staining

20 Draw a square following the outside edges of the wet square with an hydrophobic barrier pen (ImmEdge pen)

Optionnal: blocking endegenous biotin

21 If a biotinylated antibody or a probe is used, it is recommended to block endogenous biotin

Place the slides in the moist chamber

Add drops of Avidin solution (Avidin/Biotin blocking kit, Biolegend) sufficient to cover the sample and incubate for 00:10:00 at Room temperature

21.1 Wash in coplin jar with MIBI 1x PBS wash buffer and use orbital shaker set for 5 min, 70 rpm

21.2 Add drops of Biotin solution sufficient to cover the sample and incubate for 00:10:00 at Room temperature

21.3 Wash in coplin jar with MIBI 1x PBS wash buffer and use orbital shaker set for 5 min, 70 rpm

21.4 Next day, use anti-biotin metal-labeled antibody (clone 1D4-C5) in ***Stain 2*** panel

Blocking

22 Add 100 µL of Blocking Buffer for 18 mm^2

For blocking solution preparation refer to **MIBI and IHC solutions protocols**

	Estimated Surface area (mm)	10×10	15×15	18×18	20×20	20x 45
	Volume (µL)	50	70	100	150	350

23 Place the slides in a moist chamber at Room temperature and incubate 00:20:00 to 01:00:00

Multiplex Antibody mix

24 Prepare antibody mix based on the putative multiplex antibody panel

Make sure that all the antibodies are ready to use **BEFORE** starting to build the panel

It is highly recommended to prepare all the antibodies, ready to use, a day before the panel is built

24.1 Evaluate the total volume of multiplex antibody mix by counting the number of slides and the surface area per slide

Refer to the chart for the volume of antibody to apply according to the estimated surface area

	Estimated Surface area (mm)	10×10	15×15	18×18	20×20	20x 45
	Volume (µL)	50	70	100	150	350

24.2 Build an antibody mix table information to make the antibody panel as follow:

Conjugation ID, Target name, Channel, Antibody concentration, Titer, Volume

Exemple:

	A	B	C	D	E	F
	ID	**Target**	**Channel**	**Concentration µg/mL**	**Titer (µg/mL)**	**Volume (µL)**
	1565	CD45	169	50	0.25	2.5
	1516	CD8	158	50	0.5	5
	...	...	...	...	...	...

	A	B	C	D	E	F
					Total	500
					Antibody mix	7.5
					Antibody diluent (NHS 3%)	492.5

For **Antibody Diluent (NHS 3%)** solution preparation refer to **MIBI and IHC solutions protocols**

25 Add 400 µL of antibody diluent (NHS 3%) to a Centrifugal 0.1 µm filter unit (Millipore, UFC30VV00)

25.1 10000 rcf, Room temperature, 00:01:00 1m

25.2 Discard flow through

26 Add antibody mix to the filter unit

26.1 10000 rcf, Room temperature, 00:01:00 1m

Stain 1 (Overnight)

27 Remove the blocking solution by tapping the slide on a side

Immediately add the filtered multiplex antibody mix

28 Place the moist chamber at 4˚C Overnight , preferably in a place with low disturbance (e.g. a designated area in a cold room)

Wash buffer

29 Prepare two Coplin jars filled with 1x PBS wash buffer

29.1 Transfer the slides into the **first** Coplin jar and use orbital shaker set for 5 min, 70 rpm

29.2 Transfer the slides into the **second** Coplin jar and use orbital shaker set for 5 min, 70 rpm

Stain 2 (1h)

30 Add adequate volume of the selected sub-panel of antibody mix

Refer to the chart for the volume of antibody to apply

	Estimated Surface area (mm)	10×10	15×15	18×18	20×20	20x 45
	Volume (µL)	50	70	100	150	350
	Estimated # of drops	1	2	3	4	8-9

31 Place sample in a sealed humidity chamber, transfer to 4^{O}C refrigerator, and incubate 01:00:00

Wash buffer

32 After 1h incubation

than go to step 33

Prepare solutions

33 **Prepare fresh glutaraldehyde fixing solution**

Glutaraldehyde fixing solution

1. Add 30 mL of 1x PBS low barium in a 50 mL tube
2. Break the glass glutaraldehyde 8% (amber vial)
3. Add the content of the glutaraldehyde (10 mL) by inverting it and tapping the bottom of the vial in the 50 mL tube
4. Transfer the content in a linear stainer container

34 **Set the linear stainer containers**

Fill containers with the following solution and order

Glutaraldehyde x 1, PBS low barium x 1, TRIS 100 mm pH 8.5 × 3, ddH2O x 2, 70% Ethanol x1, 80% Ethanol x1, 95% Ethanol x 2, 100% Ethanol x 2, exit stainless steel tank = empty

Glutaraldehyde fixation

35 Mount the slides on the linear slide holder

Fix for 00:05:00

36 Rinse briefly with 1x PBS low barium

Dehydration and Storage

37 Press on **Menu**

Check for Processing time = 30 sec, Lift bar = 976, Number of dips = 3

Continue to press **Menu** until the screen displays **Start at: __**

Set Start position corresponding to the first slide carrier position

Exemple: If the first slide carrier is at position 3, use Plus (**+**) or Minus (**-)** button to increase or decrease to get **Start at: 03**

37.1 Then press **Enter**

37.2 Press **Run** on the Linear Stainer

37.3 Allow the dehydration process and wait until the slides reached the **empty stainless steel tank** and stop

37.4 Store the slides immediately under vacuum until MIBI acquisition

Alternatively, the stained slides can be stored in a vacuum sealed bag for longterm storage pre and post MIBI acquisition