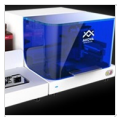

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CODEX Antibody Staining Protocol for FFPE tissues

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1 Works for me dx.doi.org/10.17504/protocols.io.bbsdina6

Human BioMolecular Atlas Program (HuBMAP) Method Development Community

ABSTRACT

This protocol describes the method for antibody staining of FFPE tissues on coverslips using CODEX Barcoded Antibodies. Included are the stepwise protocols for pre-staining, deparaffinization, antigen retrival, antibody staining and post-fixation. The enitre process requires approximately 6.5 hours, including a 3 hour incubation. Except as noted, reagents and consumables should be prepared before being the protocol. Stained tissues can be stored in CODEX Storage Buffer at 4º C for up to 2 weeks for use in CODEX multiplex imaging.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Akoya BiosciencesInc,CODEX User Maual REV A.0 2019

ATTACHMENTS

one sheet protocol FFPE v1.pdf

GUIDELINES

- The coverslips used to mount the tissue must be poly-l-Lysine coated.
- Take care to protect tissues from drying during transfer steps
- Always pipette at the corner of the coverslip and allow the liquid to flow over the tissue to minimize damage.
- Coverslips are fragile; use the reccommmended bent-tip forceps to handle the coverslips
- The hummidty chamber should be placed on a solid, vibration free table or bench top to maintain surface tension during the 3 hour incubation.
- 6-well plates can be washed and reused up to 5 times

MATERIALS TEXT

CODEX® Staining Kit (*Product Number:*7000008):
Stored at 4 C:
Hydration Buffer
Staining Buffer
Storage Buffer
N Blocker
G Blocker
Nuclear Stain
CODEX® Barcoded Antibodies
CODEX® Reporters
Stored at -20 C:
J Blocker
S Blocker
Fixative Reagent
Assay Reagent

Consumables & Glassware:
6-well plates Thermo14075
14 ml Tubes Thermo 339651
Screw cap vials/tubes 1.5 ml Fisherbrand02-681-372; 5.0 ml Fisherbrand 02-681-130
50 ml beaker (3) Fisherbrand 100-50

Equipment & Tools

Heating Plate that can be set to 55°C
Instant Pot IP-Duo 6 quart Model IPDuo60 V3
Eppendorf mini spin plus centrifuge for 1.5 ml tubes
Coverglass staining rack- 5 place ThermoFisher 116
Bent tip tweezers- Fine Science tools Dumont 1151-33 5/45C (recommended; no substitutions)
Humidity Chamber (homemade) empty ART 1.0 ml pipette tip box with lid

Solvents and Buffers

Xylene Fisher X3P-1GAL
Ethanol 200 proof Decon Lab 2701
Methanol Acros 61009-0040
16% formaldehyde Solution (methanol free) Thermo 28906
DPBS 1X Gibco 14190-44
Nuclease-Free water (not DEPC treated) Ambicon AM9938
Citrate Buffer pH 6.0 100X Abcam ab64236

SAFETY WARNINGS

Use of a fume hood is recommended for working with flammable solvents. Methanol should be kept in a refrigerator approved for storage of flammable liquids. Xylene, ethanol, PFA and methanol must be disposed of in the appropriate hazardous waste containers.

BEFORE STARTING

Prepare reagents, except as noted, and consumables before beginning protocol.

Tissue Pre-treatment ent 30m

1 Turn on the heating plate and set it at 55°C.

55 °C

2 Once the heating plate has reached 55°C, retrieve the FFPE samples on poly-l-Lysine treated coverslip(s) from 4°C storage.

55 °C

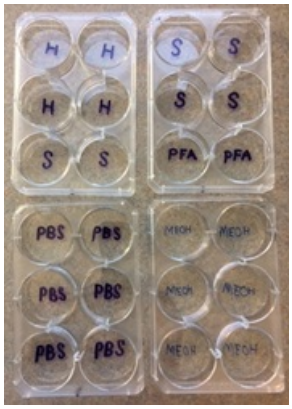
3 Using bent tip forceps, place the sample coverslip(s) on the hot plate with the tissue facing up.
Incubate 20-25 minutes until wax thoroughly melts.

55 °C

00:20:00 - **00:25:00**

4

NOTE:**[While wax is melting from tissue on coverslips, label and fill 6 well plates. Add 5.0 ml of reagent per well. Each coverslip requires two wells of Hydration Buffer (H), three of Staining Buffer (S) and three of DPBS. Allow reagents to warm to room temperature. One well each of PFA and ice cold methanol will be filled later in the protocol. Store the empty methanol plate at 4C.



6-well plates for 2 tissues

5

NOTE: **[While wax is melting from tissue on coverslips, create Humidity Chamber from an empty pipette tip box with lid. Wet a paper towel and place it at the bottom of the pipette box. Fill the pipette box with enough ddH2O at the bottom to fully cover the paper towel (ca. 1-2 cm deep). Rinse and dry the tray for holding pipette tips before placing it back in the box. Label different positions in the tray if working with multiple sample coverslips.Cover with the lid.

Hummidity Chamber

6 Place the sample coverslip(s) in the cover glass staining rack and wait 5 minutes to allow the tissue(s) to cool down.
00:05:00

Deparaffinizationion 1h

7 **Tissue Deparaffinization and Hydration**
Start the rehydration process by placing the coverglass staining rack in the following solvent series. Each incubation step lasts for 5 minutes. Make sure the coverslip(s) are completely covered by the liquid and move the rack gently at start of each new step to make sure the liquid in the space between coverslips is exchanged. Close the containers with lids during incubation.
100 ml each **00:05:00 each step**

Note: It is highly recommended that you perform this procedure in a fume hood; organic solvents are toxic and highly volatile.

7.1 Immerse the staining rack in a staining container of Xylene covered for 5 minutes. 5m

7.2 Immerse the staining rack in a second staining container of Xylene covered for 5 minutes.

7.3 Immerse the staining rack in a staining container of 100% Ethanol covered for 5 minutes.

7.4 Immerse the staining rack in a second staining container of 100% Ethanol covered for 5 minutes.

7.5 Immerse the staining rack in a staining container of 95% Ethanol covered for 5 minutes

7.6 mmerse the staining rack in a staining container of 90% Ethanol covered for 5 minutes.

7.7 Immerse the staining rack in a staining container of 70% Ethanol covered for 5 minutes.

7.8 Immerse the staining rack in a staining container of 50% Ethanol covered for 5 minutes.

7.9 Immerse the staining rack in a staining container of 30% Ethanol covered for 5 minutes.

7.10 Immerse the staining rack in a staining container of ddH2O covered for 5 minutes.

7.11 Immerse the staining rack in a second staining container of ddH2O covered for 5 minutes.

Antigen Retrival rival 40m

8 In a 50 ml Pyrex Beaker, for each rack of slides, prepare 40 ml of 1x citrate buffer . Dilute 100x citrate buffer pH6.0 to 1X citrate buffer in ddH2O.

9 Immerse the staining rack(s) in the beaker(s) containing the 1x citrate buffer and wrap tightly with aluminum foil to ensure the best sealing possible. Seal well around the rim and spout of the beaker to prevent water vapor from entering the beaker.

10 Pour 1200 ml of DI water into the Instant Pot chamber and carefully place the sealed baker in the chamber.

11 Close the Instant Pot lid.Set pressure to HIGH, timer for 20 minutes (turn off the keep warm setting). Press PRESSURE COOK to start.

12 After the cycle ends (about 35-40 min), slowly release the pressure cooker vent. Using a hot mit, and lifting from beaker rim, carefully take the rack out from the pressure cooker. Allow to cool on the bench until no longer hot to the touch.
00:40:00

Caution: beaker will be HOT when removed form pressure cooker!

13 Place staining rack in a 40 ml beaker of ddH2O for a few seconds.
Room temperature **Room temperature**

14 Transfer staining rack to a second beakerr of 40 ml ddH2O, emerse for 10 minutes.
00:10:00 **Room temperature**

15

NOTE: **[During STEP 14, make CODEX Blocking Buffer by adding Blockers N, G, J, and S to staining buffer according to the number of samples/coverslips you are staining.]

Room temperature

CODEX BB	2 samples	3 samples	4 samples	6 samples	10 samples
Staining buffer	362 ul	543	724	1086	1810
N Blocker	9.5 ul	14.25	19	28.5	47.5
G Blocker	9.5 ul	14.25	19	28.5	47.5
J Blocker	9.5 ul	14.25	19	28.5	47.5
S Blocker	9.5 ul	14.25	19	28.5	47.5
Total	400 ul	600	800	1200	2000

CODEX BLOCKING BUFFER by number of tissue samples

Tissue Washing 30m

16 After 10 min incubation (STEP 14) carefully pick up each cover slip with bent tip forceps and place in prepared well #1 containing 5 ml of Hydration buffer. Incubate 5 seconds.

00:00:05

17 Move cover slip to Hydration buffer well #2. Incubate 5 seconds.

00:00:05

18 Move coverslip to the well containing 5 ml Staining Buffer. Incubate for 20-30 minutes.

00:20:00 - **00:30:00**

Room temperature

19

NOTE: **[During STEP 18, prepare Antibody Cocktail by adding the primary barcoded antibodies to the Codex Blocking Buffer prepared in STEP 15.] Akoya inventoried antibodies are applied at 1ul per tissue stained. Titer may require adjustment.

Subtract the total volume of antibodies from the volume of CODEX blocking buffer prepared

_____ #Primaries @ 1ul per tissue X _____# Tissues= _____ul total Primary volume

Final antibody cocktail will be:Antibody Cocktail
_____ ul volume Blocking Buffer (STEP 15)
-_____ ul total primary volume (calculted above)
=_____ul adjusted volume of Blocking Buffer
+_____ul total Primary @ 1ul per tissue
=_____ul Final volume of Primary cocktail (will be equivalent to initial volume of Blocking Buffer)

Antibody Stainingng 3h

20 At the completion of STEP 18 add antibody cocktail to the coverslips.Working one tissue at a time, remove coverslip from staining buffer with bent tip forceps, tip to drain, and place on humidity chamber. Immediately add 190 ul of antibody cocktail to a corner of the coverslip. Avoid pipetting directly over tissue. Avoid bubbles. Repeat for each coverslip. Cover the humidity chamber with lid and incubate at room temperature for 3 hours. Do not disturb the chamber during incubation.

190 µl **03:00:00** **Room temperature**

Post-Staining 1h 30m

21

NOTE: **At 2.5 hours of incubation:

Add 5.0 ml ice cold methanol to the prelabled 6-well plate, one well is needed for each coverslip. Set plate on ice (See STEP 4).

On ice

Prepare the PFA solution. To 9 mls of storage buffer add 1.0 ml of 16% PFA. For each coverslip, place 5.0 ml into the well of a labeled 6-wellplate. see STEP 4 **Room temperature**

22 After antibody incubation is completed:
Using bent tip forceps, carefully lift the coverslip out of the humidity chamber, tip to drain antibody solution and place in staining buffer well #1 of the second cluster dish (see STEP 4). Incubate 2 minutes.

00:02:00 **Room temperature**

23 Transfer coverslip to staining buffer well #2 of the second cluster dish. Incubation 2 minutes.

00:02:00 **Room temperature**

24 Transfercoverslip to PFA well. Incubate 10 minutes.
00:10:00 Room temperature

25 Transfer coverslip to PBS well #1. Immerge coverslip 2-3 times.

26 Transfer coverslip to PBS well #2. Immerge coverslip 2-3 times.

27 Transfer cover slip to PBS well #3. Immerge coverslip 2-3 times.

28 Transfer coverslip to well containing Ice cold methanol. Incubate for 5 minutes on ice.
On ice
00:05:00

29

NOTE: **[Wash/dry the staining chamber and prepare Final Fix solution, near the end of STEP 28. Rapidly thaw CODEX Final Fix Reagent and spin down briefly.Add 20 ul of Final Fix to 1.0 ml of PBS. Mix thoroughly.]
Room temperature

30 Reuse the filled PBS wells: Quickly transfer coverslip to PBS well #1. Avoid drying of the coverslip.

31 Immediately transfer coverslip to PBS well #2.

32 Immediately transfer coverslip to PBS well #3

Final Fix 20m

33 Using bent tip forceps, carefully transfer coverslips one at a time to the cleaned humidity chamber. Immediately add 190 ul of the final fix solution to the corner of the coverslip. Repeat with each coverslip. Incubate for 20 minutes.
190 µl 00:20:00 Room temperature

34 Reuse PBS wells: Transfer coverslip to PBS well #1.

35 Immediately transfer coverslip to PBS well #2.

36 Immediately transfer cover slip to PBS well #3

37 Place each coverslip tissue side up in a bottom labeled, well containing 5.0 ml Storage Buffer. Wrap edges of 6-well plate with parafilm and store at 4ºC. Record date on the cluster dish, stained tissue is stable for 2 weeks.
4 °C

Stained tissue can be stored for up to 2 weeks.

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