**CODEX Antibody Staining Protocol for FFPE tissues**

In 1 collection

**Marda Jorgensen¹, Jerelyn Nick¹**

¹University of Florida

**Human BioMolecular Atlas Program (HuBMAP) Method Development Community**

Tech. support email: Jeff.spraggins@vanderbilt.edu

**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 retrieval, antibody staining and post-fixation. The entire 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 Manual REV A.0 2019

**ATTACHMENTS**

one sheet protocol FFPE

v1.pdf

**DOI**

dx.doi.org/10.17504/protocols.io.bbsdina6

**PROTOCOL CITATION**

Marda Jorgensen, Jerelyn Nick 2020. CODEX Antibody Staining Protocol for FFPE tissues. protocols.io
https://dx.doi.org/10.17504/protocols.io.bbsdina6

**MANUSCRIPT CITATION** please remember to cite the following publication along with this protocol

Akoya BiosciencesInc, CODEX User Manual REV A.0 2019

**COLLECTIONS**

2020 Featured Protocols

**KEYWORDS**

Akoya Biosciences, CODEX, antibody staining, FFPE, deparaffinization, Antigen Retrival, Post-fixation, CODEX Barcoded Antibodies

**LICENSE**

This is an open access protocol distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**CREATED**

Jan 24, 2020
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 recommended bent-tip forceps to handle the coverslips.
- The humidity 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.
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

1. **Turn on the heating plate and set it at 55°C.**
   - $55 \, ^\circ \text{C}$

2. **Once the heating plate has reached 55°C, retrieve the FFPE samples on poly-l-Lysine treated coverslip(s) from 4°C storage.**
   - $55 \, ^\circ \text{C}$

---

Citation: Marda Jorgensen, Jerelyn Nick (02/27/2020). CODEX Antibody Staining Protocol for FFPE tissues. [https://dx.doi.org/10.17504/protocols.io.bbsdina6](https://dx.doi.org/10.17504/protocols.io.bbsdina6)

This is an open access protocol distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
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 4°C.

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.

Citation: Marda Jorgensen, Jerelyn Nick (02/27/2020). CODEX Antibody Staining Protocol for FFPE tissues. https://dx.doi.org/10.17504/protocols.io.bbsdina6

This is an open access protocol distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
Place the sample coverslip(s) in the cover glass staining rack and wait 5 minutes to allow the tissue(s) to cool down.

Deparaffinization

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.

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.

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 Immerse 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.

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

¢ Room temperature

14 Transfer staining rack to a second beaker of 40 ml ddH2O, emerse for 10 minutes.

¢ 00:10:00

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/cover slips you are staining.]

¢ Room temperature

<table>
<thead>
<tr>
<th>CODEX BB</th>
<th>2 samples</th>
<th>3 samples</th>
<th>4 samples</th>
<th>6 samples</th>
<th>10 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining buffer</td>
<td>362 ul</td>
<td>543 ul</td>
<td>724 ul</td>
<td>1086 ul</td>
<td>1810 ul</td>
</tr>
<tr>
<td>N Blocker</td>
<td>9.5 ul</td>
<td>14.25 ul</td>
<td>19 ul</td>
<td>28.5 ul</td>
<td>47.5 ul</td>
</tr>
<tr>
<td>G Blocker</td>
<td>9.5 ul</td>
<td>14.25 ul</td>
<td>19 ul</td>
<td>28.5 ul</td>
<td>47.5 ul</td>
</tr>
<tr>
<td>J Blocker</td>
<td>9.5 ul</td>
<td>14.25 ul</td>
<td>19 ul</td>
<td>28.5 ul</td>
<td>47.5 ul</td>
</tr>
<tr>
<td>S Blocker</td>
<td>9.5 ul</td>
<td>14.25 ul</td>
<td>19 ul</td>
<td>28.5 ul</td>
<td>47.5 ul</td>
</tr>
<tr>
<td>Total</td>
<td>400 ul</td>
<td>600 ul</td>
<td>800 ul</td>
<td>1200 ul</td>
<td>2000 ul</td>
</tr>
</tbody>
</table>

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

Citation: Marda Jorgensen, Jerelyn Nick (02/27/2020). CODEX Antibody Staining Protocol for FFPE tissues. https://dx.doi.org/10.17504/protocols.io.bbsdina6

This is an open access protocol distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
Move coverslip to the well containing 5 ml Staining Buffer. Incubate for 20-30 minutes.

\[\text{Room temperature}\]

\[\text{00:20:00} \rightarrow \text{00:30:00}\]

**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

\[\text{_______} \#\text{Primaries @ 1ul per tissue X ______# Tissues=} \text{_______ul total Primary volume}\]

Final antibody cocktail will be:

\[\text{Antibody Cocktail}
\]

\[\text{_______ul volume Blocking Buffer (STEP 15)}\]

\[-_______ul total primary volume (calculated above)\]

\[=_______ul adjusted volume of Blocking Buffer\]

\[+_______ul total Primary @ 1ul per tissue\]

\[=_______ul Final volume of Primary cocktail (will be equivalent to initial volume of Blocking Buffer)\]

Antibody Staining

\[3h\]

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.

\[\text{190 µl Room temperature}\]

\[\text{03:00:00}\]

**NOTE:** At 2.5 hours of incubation:

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

\[\text{On ice}\]

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

\[\text{Room temperature}\]

Post-Staining

\[1h 30m\]

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.

Citation: Marda Jorgensen, Jerelyn Nick (02/27/2020). CODEX Antibody Staining Protocol for FFPE tissues. https://dx.doi.org/10.17504/protocols.io.bbsdina6

This is an open access protocol distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
Transfer coverslip to staining buffer well #2 of the second cluster dish. Incubation 2 minutes.

Transfer coverslip to PFA well. Incubate 10 minutes.

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

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

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

Transfer coverslip to well containing ice cold methanol. Incubate for 5 minutes on ice.

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.

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

Immediately transfer coverslip to PBS well #2.

Immediately transfer coverslip to PBS well #3.

Using bent tip forceps, carefully transfer coverslips one at a time to the cleaned humidity chamber. Immediately add
190 μl 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 4°C. 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.