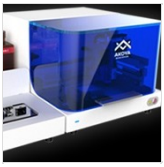

Dec 21, 2020

HuBMAP TMC-UF Validation of Custom conjugated Antibodies for CODEX

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1 Works for me dx.doi.org/10.17504/protocols.io.bkpzkvp6

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ABSTRACT

This protocol describes the methods for validating antibodies after custom conjugation with Akoya CODEX barcodes in FFPE tissues on coverslips. Included are the stepwise protocols for pre-staining, deparaffinization, antigen retrival, antibody staining, post-fixation, manual addition of CODEX Reporters and tissue mounting on microscope slides. The recommended single stain experimental design includes 3 tissues sections from the same block stained with the CODEX-tagged antibody alone (1), co-stained with a positive control(2), and co-satined with a negative control(3).

	Tissue 1	Tissue 2	Tissue 3
	CODEX-Stain Only	CODEX-stain with co-stain (Positive Control)	CODEX-stain with counter-stain (Negative Control)
DAPI Channel	Nuclear Stain	Nuclear Stain	Nuclear Stain
2nd Fluorescent Channel	CODEX-tagged Anitbody	CODEX-tagged Anitbody	CODEX-tagged Anitbody
3rd or 4th Fluorescent Channel	None	Control antibody targeting a different antigen expressed by the same phenotype or cell population	Control antibody targeting an antigen expressed by a different phenotype or cell population.

Antibody Validation Scheme Using 3 Tissues

The enitre process requires approximately 5.5 hours, including a 2 hour incubation. Except as noted, reagents and consumables should be prepared before begining the protocol.

ATTACHMENTS

one sheet protocol FFPE v1.pdf

DOI

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PROTOCOL CITATION

Marda Jorgensen, Jerelyn Nick 2020. HuBMAP TMC-UF Validation of Custom conjugated Antibodies for CODEX. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bkpzkvp6

KEYWORDS

Akoya Biosciences, CODEX, antibody staining, FFPE, deparaffinization, Antigen Retrival, Post-fixation, CODEX Barcoded Antibodies, Anitbody validation, validation of oligo conjugated antibodies

CREATED

Sep 02, 2020

LAST MODIFIED

Dec 21, 2020

PROTOCOL INTEGER ID

41433

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 2 hour incubation.
- 6-well plates can be washed and reused up to 5 times
- Protect fluorophores from light to prevent photobleaching
- To reduce background from non-spoecific binding , wait at least 48 hours after conjugation to test newly barcoded antibodies.

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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

Additional Materials for the Manual Addition of Repoerter and Tissue Mounting
DMSO
Parafilm
Lab tape
Microscope slides
Nail Ploish
Fluoromount -G
Nuclear Stain (DAPI)
Aluminum Foil or Box (to cover dyed tissues)
Amber eppendorf tubes (1.5 ml)
Assay Reagent
10X CODEX Buffer

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. Buffers containing DMSO should be collected and disposed of according to local regulations.

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Antibody Validation Scheme Using 3 Tissues

The enitre process requires approximately 5.5 hours, including a 2 hour incubation. Except as noted, reagents and consumables should be prepared before begining the protocol.

BEFORE STARTING

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

Tissue Pre-treatment 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.

4 °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. 25m

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 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.

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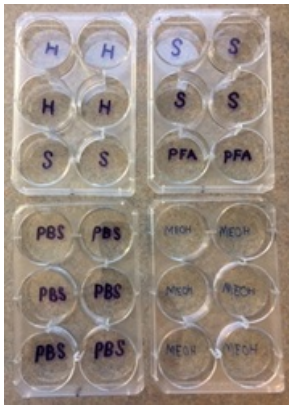

6-well plates for 2 tissues

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 top tray 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 After 20 minute incubation on warmer, place the sample coverslip(s) in the cover glass staining rack and wait 5 minutes to allow the tissue(s) to cool down. 5m

00:05:00

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Deparaffinization 50m

7 **Tissue Deparaffinization and Hydration** 50m

Start the deparafffinization and 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 **00:05:00** 5m

7.2 Immerse the staining rack in a second staining container of **Xylene** covered for **00:05:00** 5m

7.3 Immerse the staining rack in a staining container of **100% Ethanol** covered for **00:05:00**

7.4 Immerse the staining rack in a second staining container of **100% Ethanol** covered for **00:05:00** 5m

7.5 mmerse the staining rack in a staining container of **90% Ethanol** covered for **00:05:00** 5m

7.6 Immerse the staining rack in a staining container of **70% Ethanol** covered for **00:05:00** 5m

7.7 Immerse the staining rack in a staining container of **50% Ethanol** covered for **00:05:00** 5m

7.8 Immerse the staining rack in a staining container of **30% Ethanol** covered for **00:05:00**

7.9 Immerse the staining rack in a staining container of **ddH2O** covered for **00:05:00** 5m

7.10 Immerse the staining rack in a second staining container of **ddH2O** covered for **00:05:00** 5m

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Antigen Retrival 45m

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 Pouro **1200 mL** f DI water into the Instant Pot chamber and carefully place the sealed beaker in the chamber.

11 Close the Instant Pot lid.Set pressure to **HIGH**, timer for **00:20:00** (turn off the keep warm setting). Press PRESSURE COOK to start.

12 After the cycle ends (about 40-45 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 briefly.
Step 12 about . **00:45:00** total.

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

13 Place staining rack in a 50 ml beaker containing **40 mL** of **ddH2O** for a few seconds to cool. 3s
Room temperature **Room temperature**

14 Transfer staining rack to a second beakerr containing **40 mL** **ddH2O**, emerse for **00:10:00** 10m
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

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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 in water (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. 5s

00:00:05

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

00:00:05

18 Move coverslip to the well containing **5 mL** I**Staining Buffer**. Incubate for 20-30 minutes. 20m

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

Room temperature

Do not exceed **00:30:00** in **Staining Buffer**

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 (1:200 dilution) . Titer may require adjustment. Newly conjugate antibodies are typically tested at 1, 2 or 4 ul per tissue (dilution of 1:200, 1:100, 1:50, respectively)

If standard fluorescent antibodies are being used as co- and counter-stains thay should be added here in the predetermined dilution.

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

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

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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 Staining 2h

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 2 hours. Do not disturb the chamber during incubation.

190 µl **02:00:00** **Room temperature**

Post-Staining 1h 30m

21

NOTE: **At 1.5 hours of incubation: Prepare Methanol and PFA

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).

5 mL **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

5 mL **1.6 % volume** **Room temperature**

22 2m
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 **5 mL** **Staining Buffer** well #1 of the second cluster dish containing (see STEP 4). Incubate 2 minutes.

00:02:00 **Room temperature**

23 2m
Transfer coverslip to **Staining Buffer** well #2 of the second cluster dish. Incubation 2 minutes.

00:02:00 **Room temperature**

24 10m
Transfercoverslip to **PFA** well. Incubate 10 minutes.

00:10:00 **Room temperature**

25 20s
Transfer coverslip to **PBS** well #1. Immerge coverslip **00:00:20**

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26 Transfer coverslip to **PBS** well #2. Immerge coverslip **00:00:20** 20s

27 Transfer cover slip to **PBS** well #3. Immerge coverslip **00:00:20** . 20s

28 Transfer coverslip to well containing **Ice cold methanol**. Incubate for 5 minutes on ice. 5m

On ice

00:05:00

29 Prepare Final Fix Solution 1m

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 µl** of **Final Fix** to **1 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. 20s

Tissues in methanol tend to dry out quickly

31 Immediately transfer coverslip to **PBS** well #2. 20s

32 Immediately transfer coverslip to **PBS** well #3 20s

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. 20m

190 µl **00:20:00** **Room temperature**

34 Reuse **PBS** wells: Transfer coverslip to PBS well #1. 20s

35 Immediately transfer coverslip to **PBS** well #2. 20s

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36 Immediately transfer cover slip to **PBS** well #3 20s

37 Place each coverslip tissue side up in labeled well of 6-well plate containing **5 mL** ml **Storage Buffe**r. Wrap edges of 6-well plate with parafilm and store at 4ºC.Or proceed to manual addition of CODEX reporter.

4 °C

Stained tissue can be stored for up to 2 weeks.

Manual Addition of CODEX Reporter

38 Prepare **35 mL** **Screening Buffer** in a glass beaker. For 3 coverslips use:

Screening Buffer
3.5 ml 10X CODEX Buffer
24.5 ml ddH2O
7.0 ml DMSO

Screening Buffer should be prepared fresh for each experiment.
This process is exothermic. Allow to **equillibatrate for 20 minute** prior to use

39 Add **5 mL** Screening Buffer to 6-well plate. 3 wells per tissue. Also, add **5 mL** 1X CODEX Buffer to 6-well plate, 1 well per coverslip. **Room temperature**

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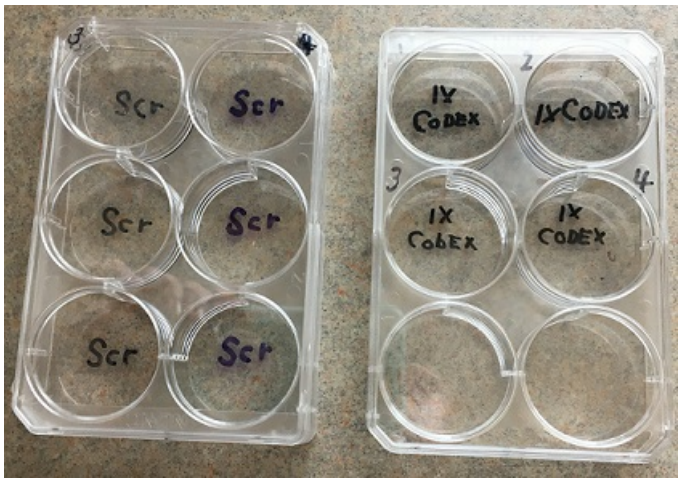

6-well plates

40 Place coverslip in well #1 **Screening Buffer** 10s

40.1 Move cover slip to well #2 **Screening Buffer** 10s

40.2 Move cover slip to Well #3 **Screening Buffer** 10s

Coversplips can be left in Screening Buffer for MAX of **00:15:00**

41 Prepare **1X CODEX Buffer**. For 3 coverslips prepare at least **20 mL**

Nuclease Free Water **18 mL**

10x CODEX Buffer **2 mL**

42 Prepare **Reporter Stock Solution** for 3 sample coverslips

1X CODEX Buffer **284.5 µl**

Assay Reagent **15 µl**

Nuclear Stain **1 µl**

43 Prepare **Reporter Master Mix.** Thaw reporters, if frozen, and spin briefly. Label 3 *amber* eppendorf tube for each experiment. Add Reporter Stock Solution according to the number of CODEX tagged antibodies/Reporters per sample coverslip. add 2.5 ul of each Reporter. Reporter Master Mix final violume **100 µl** .

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1 coverslip + 1 reporter = **97.5 µl** Reporter Stock solution + **2.5 µl** Reporter

1 coverslip + 2 reporters= **95 µl** Reporter Stock solution + **2.5 µl** EACH of 2 Reporters

On ice Reporter Master Mix

Use amber colored eppendorf tubes to protect fluorescent reporter stock solutions from light.

Incubate Sample coverslip with CODEX Reporters 5m

44 Tape a square of parafilm to the benchtop. Carefully pipette **95 µl** of each Reporter master Mix onto the paraflim

Reporter Master Mix Droplets

45 Remove the sample cover slip from the screening buffer
INVERT the coverslip onto the corresponding droplet of Reporter master Mix

Place coverslip down by gently placing one edge first and then slowly lowering the coverslip on top of the liquid.

Coverslip Inverted for Reporter Incubation

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46 Cover samples with aluminum foil or a small box to prevent light exposure. Incubate **Room temperature** **00:05:00** . 5m
Change gloves.

Protect reporters from light to prevent photobleaching

47 **Invert** coverslip so that tisssue is facing up. Place in well #1 **Screening Buffer**. 10s

48 Move coverslips to well #2 **Screening Buffer** 10s

49 Mover coverslip to well # 3 **Screening Buffer** 10s

50 Move coverslip to Well containing 1X CODEX Buffer

Mount Tissue 7m

51 Label 3 microscope slides, one for each coverslip.

52 Cut the end off of a 200 ul pipette tip. Add **15 µl** of Fluoromount-G to each slide. Remove coverslip from 1X CODEX buffer and INVERT onto mountant.

Take care not to trap bubbles under coverslip. If bubbles form, remove tissue coverslip immerse in 1X CODEX buffer and repeat mounting procedure.

53 Blot coverslip edges on kimwipe to remove excess liquid. cover edges with nail polish to seal. Allow 5-7 minutes to dry in the dark. 7m

54 After sealant has dried, remove buffer salts from top of the sample coverslip with a wet Kimwipe.

55 Image with the fluorescent microscope associated with the CODEX Instrument using the same settings used for

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CODEX. For example, all 4 channels, 20X magnification with the same acquisition times.

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