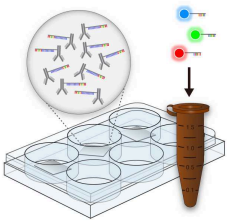
May 11, 2022

CODEX® Multiplexed Imaging | Tissue Staining and Reporter Plate Preparation



DOI
dx.doi.org/10.17504/protocols.io.n92ldzro9v5b/v1

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Vanderbilt Diabetes Res...

Islet and Pancreas Analysis Core
Vanderbilt Diabetes Research Center

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.n92ldzro9v5b/v1

Protocol Citation: Diane Saunders, Conrad Reihsmann, Marcela Brissova, Alvin C. Powers 2022. CODEX® Multiplexed Imaging | Tissue Staining and Reporter Plate Preparation . **protocols.io https://dx.doi.org/10.17504/protocols.io.n92ldzro9v5b/v1**

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

Created: April 08, 2022

Last Modified: May 11, 2022

Protocol Integer ID: 60459

Keywords: multiplexed imaging of the human pancrea, performing multiplexed imaging, use by the vanderbilt diabetes research center islet, vanderbilt diabetes research center islet, pancreas analysis, reporter plate preparation, human pancrea, pancrea

Disclaimer

This protocol is adapted from the **CODEX User Manual, revision C** (Akoya Biosciences, Dec. 2020). For a quick reference and overview, see **FF Tissue Staining and Reporter Plate Preparation**. Although the protocol is designed for flash frozen (unfixed) tissue, we have validated that it also works for lightly PFA-fixed tissue.

Abstract

This protocol describes the staining and pre-imaging preparation currently in use by the Vanderbilt Diabetes Research Center **Islet & Pancreas Analysis (IPA) Core** and Powers/Brissova Research Group prior to performing multiplexed imaging of the human pancreas using the CO-Detection by indEXing (**CODEX®**) platform (now **PhenoCycler™**; Akoya Biosciences). See also **CODEX® Multiplexed Imaging | Modality overview.**

Materials

CODEX® Supplies:

- 10X CODEX Buffer **Akoya Biosciences Catalog #**7000001
- Assay Reagent **Akoya Biosciences Catalog #**7000002
- Nuclear Stain **Akoya Biosciences Catalog #**7000003
- 96 well plate **Akoya Biosciences Catalog #**7000006
- 96 well Plate Foil Seals **Akoya Biosciences Catalog #**7000007
- CODEX Staining kit **Akoya Biosciences Catalog #**7000008

  Contains: Hydration Buffer, Staining Buffer, Storage Buffer, N/J/G/S Blockers, Fixative reagent

- Primary antibodies with CODEX® oligonucleotide barcodes
- CODEX® barcoded reporters

Additional Reagents and Consumables:

- 1X Dulbecco's Phosphate Buffered Saline (DPBS) **Thermo Fisher Scientific Catalog #**14190094
- 16% Paraformaldehyde **Fisher Scientific Catalog #**15710
- Bent-tip tweezers **Fine Science Tools Catalog #**11251-33
- Acetone **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**650501-1L
- Methanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**34860-1L-R
- Drierite™ indicating absorbents **Fisher Scientific Catalog #**23-116582
- Amber 1.5-mL tubes **VWR International (Avantor) Catalog #**89000-030
- Nuclease free water
- 6 well plates - non-tissue culture treated

Additional Supplies:

- 15-mL glass beakers

Protocol materials

- Bent-tip tweezers **Fine Science Tools** **Catalog #**11251-33
- Assay Reagent **Akoya Biosciences** **Catalog #**7000002
- Methanol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**34860-1L-R
- Drierite™ indicating absorbents **Fisher Scientific** **Catalog #**23-116582
- Nuclear Stain **Akoya Biosciences** **Catalog #**7000003
- 96 well plate **Akoya Biosciences** **Catalog #**7000006
- 16% Paraformaldehyde **Fisher Scientific** **Catalog #**15710
- 10X CODEX Buffer **Akoya Biosciences** **Catalog #**7000001
- Amber 1.5-mL tubes **VWR International (Avantor)** **Catalog #**89000-030
- 96 well Plate Foil Seals **Akoya Biosciences** **Catalog #**7000007
- CODEX Staining kit **Akoya Biosciences** **Catalog #**7000008
- 1X Dulbecco's Phosphate Buffered Saline (DPBS) **Thermo Fisher Scientific** **Catalog #**14190094
- Acetone **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**650501-1L
- Drierite™ indicating absorbents **Fisher Scientific** **Catalog #**23-116582
- Nuclear Stain **Akoya Biosciences** **Catalog #**7000003
- 96 well plate **Akoya Biosciences** **Catalog #**7000006
- 96 well Plate Foil Seals **Akoya Biosciences** **Catalog #**7000007
- Assay Reagent **Akoya Biosciences** **Catalog #**7000002

Troubleshooting

Tissue Preparation

1 **Prepare pre-staining reagents (per individual coverslip):**

- 15-mL beaker with 10 mL acetone

> Note
>
> After acetone fixation, all incubations and washes in this portion of the protocol are done in 6-well plates using approximately 3 mL of reagent per well (one well per coverslip). If using an alternative container, this volume must be sufficient to submerge the coverslip.

- 2 wells **Hydration Buffer**
- 1 well **Pre-staining Fixation Solution** (1.6% PFA in Hydration Buffer)
- 1 well **Staining Buffer**

2 Remove coverslip from freezer and place face up in container with layer of Drierite™ indicating absorbents **Fisher Scientific Catalog #**23-116582 for 00:02:00 to dry.

3 Place coverslip into an individual 10-mL beaker containing **acetone**, making sure entire tissue area is submerged. Incubate for 00:10:00 .

4 Carefully remove coverslip from beaker and place back into Drierite container for another 00:02:00 .

5 Transfer coverslip to 6-well plate and perform **two** washes in 3 mL **Hydration Buffer**, incubating for 00:02:00 each time. If staining more than one sample at a time, make sure coverslip order/orientation in 6-well plates is kept consistent so you don't lose track of samples.

6 From second well of Hydration Buffer, transfer coverslip to well containing 3 mL **Pre-Staining Fixation Solution** ([M] 1.6 % (v/v) PFA in Hydration Buffer) and incubate for 00:10:00 .

7 Wash coverslip **twice** in 3 mL **Hydration Buffer** to remove fixative. No incubation is required, and aliquots from step 5 can be reused.

8 Incubate coverslip in 3 mL **Staining Buffer** for 00:20:00 . Place 6-well plate onto a fixed-angle rocker for the incubation period.

Primary Antibody Stain

9 Prepare **CODEX Blocking Buffer** for primary antibody cocktail:

- Staining Buffer - [M] 91 % (v/v)
- N/G/J/S blockers - [M] 2.4 % (v/v) each

	A	B
	Total number of samples	1
	Total volume (µl)	210
	Staining Buffer (µl)	190
	N blocker (µl)	5
	G blocker (µl)	5
	J blocker (µl)	5
	S blocker (µl)	5

Table 1: Blocking Buffer. Copy and paste all cells above into an Excel sheet, then enter value into cell B1. The volumes for each reagent will automatically be returned in cells B2-B7.

10 Calculate required amounts for each primary antibody (based on optimized dilution) as well as the total antibody volume. Remove the total volume from the **Blocking Buffer**, then add primary antibodies. Vortex gently.

ⓘ Preconjugated antibody dilutions: **PhenoCycler Antibody Dilutions**

11 Place coverslip face up in humidity chamber (can use an empty pipet tip box with damp paper towel in the bottom; coverslips rest on the removable insert). Carefully pipette 200 µL of **antibody cocktail solution** on top, making sure solution pools to cover the entire tissue area.

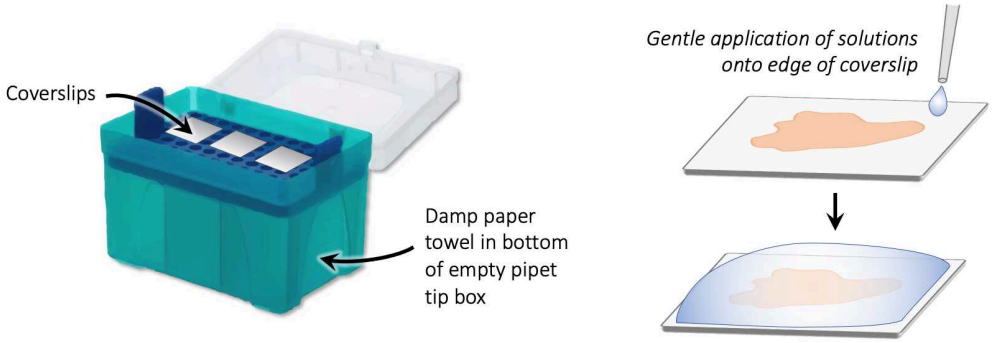

Figure 1. Schematic of a humidity chamber made from an empty pipet tip box with coverslips arranged on top of the insert (left). Right panel shows how solution should be pipetted to pool and cover the tissue for incubation.

12 Incubate at Room temperature for 03:00:00 .

Post-Stain Procedure

13 **Prepare post-staining reagents (per individual coverslip):**

> Note
>
> All incubations and washes in this portion of the protocol, with the exception of Final Fixative, are done in 6-well plates using approximately 3 mL of reagent per well (one well per coverslip). If using an alternative container, this volume must be sufficient to submerge the coverslip.

- 1 well **Post-staining Fixation Solution** (1.6% PFA in **Staining Buffer)**
- 2 wells **Staining Buffer**
- 1 well **methanol** On ice
- 3 wells 1X **DPBS**

14 Transfer coverslip from humidity chamber to 6-well plate and perform **two** washes in 3 mL **Staining Buffer**, incubating for 00:02:00 each time.

15 From second well of Staining Buffer, transfer coverslip to well containing 3 mL **Post-Staining Fixation Solution** ([M] 1.6 % (v/v) PFA in Staining Buffer) and incubate for 00:10:00 .

16 Wash coverslip **three times** in 3 mL **DPBS** to remove fixative. No incubation is required.

17 Incubate coverslip in 3 mL cold **methanol** On ice for 00:05:00 .

18 Wash coverslip **three times** in 3 mL **DPBS** to remove methanol. No incubation is required, and aliquots from step 16 can be reused.

19 Prepare **Final Fixative** ([M] 2 % (v/v) Fixative Reagent in DPBS).

⚠ *Thaw Fixative Reagent right before use and discard any unused volume. One tube provides adequate volume to fix up to 5 samples.*

20 Place coverslip face-up in humidity chamber and carefully pipette 200 µL of **Final Fixative** onto coverslip, making sure it covers the entire tissue area. Incubate at Room temperature for 00:20:00 . 20m

21 Wash coverslip **three times** in 3 mL **DPBS** to remove fixative. No incubation is required, and aliquots from steps 16/18 can be reused.

22 Transfer coverslip into 3 mL of **Storage Buffer** and label 6-well plate. Add a piece of parafilm before placing lid to ensure solution does not evaporate. Store at 4 °C for up to 2 weeks. Label the top of the plate with Sharpie or tape to keep track of sample(s).

Preparation of Reporter Plate

23 Gather supplies and reagents:

- Nuclear Stain **Akoya Biosciences Catalog #**7000003
- Assay Reagent **Akoya Biosciences Catalog #**7000002
- 96 well plate **Akoya Biosciences Catalog #**7000006
- 96 well Plate Foil Seals **Akoya Biosciences Catalog #**7000007

Prepare **Reporter Stock Solution:**

- Nuclease-free water - [M] 81.33 % (v/v)
- 10X CODEX Buffer - [M] 10.00 % (v/v)
- Assay Reagent - [M] 8.33 % (v/v)

- CODEX Nuclear Stain - [M] 0.33 % (v/v)

	A	B
	Total number of samples	3
	Reporter cycles per sample	10
	Total volume (mL)	9
	Nuclease-free water (mL)	7.32
	10X CODEX buffer (µl)	900
	Assay Reagent (µl)	750
	CODEX Nuclear Stain (µl)	30

Table 2: Reporter Stock Solution. Copy and paste all cells above into an Excel sheet, then enter values into cells B1-B2. The volumes for each reagent will automatically be returned in cells B3-B7.

⚠ *Ensure you prepare adequate Reporter Stock Solution to account for Blank cycles (no reporters); formulas in Table 2 reflect this.*

24 Label one **1.5 mL light-blocking microcentrifuge tube** for each reporter cycle and aliquot Reporter Stock Solution (250 µL per sample). You should have Reporter Stock Solution remaining (for Blank cycles) after aliquoting.

25 Prepare reporter mixes by adding appropriate reporters to microcentrifuge tubes (5 µL per sample for each reporter):

	A	B
	Total number of samples	3
	Total volume Reporter Stock Solution (µl)	750
	Reporter for channel 1 (µl)	15
	Reporter for channel 2 (µl)	15
	Reporter for channel 3 (µl)	15

Table 3: Reporter mixes (per cycle). Copy and paste all cells above into an Excel sheet, then enter value into cell B1. The volumes for each reagent will automatically be returned in cells B2-B5.

⚠ *Make sure to vortex reporter tubes briefly before adding to reporter mix.*

26 Mix contents of tubes by gently pipetting up and down with pipette or by brief vortex. Spin tubes using benchtop microcentrifuge to collect solution at bottom of tubes.

27 Add 250 µL of reporter mix to each corresponding well in a light-protected 96-well plate, making sure to leave blank wells before and after reporter mixes. Fill these wells (corresponding to Blank cycles) with 250 µL **Reporter Stock Solution** (no reporters in solution).

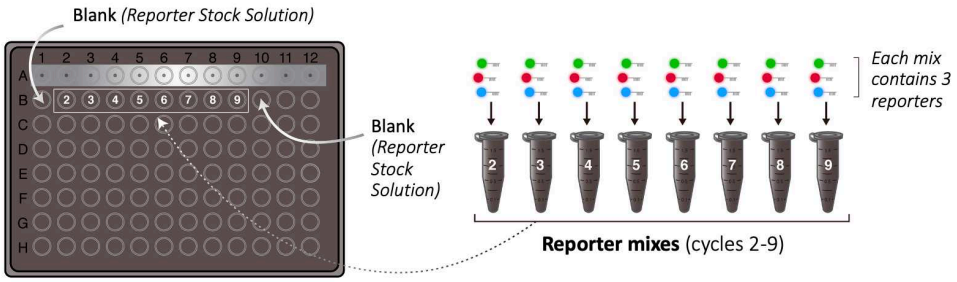

Figure 2. Schematic of 96-well Reporter Plate. In this example a total of 24 primary antibodies will be visualized in eight imaging cycles, with additional cycles before (#1) and after (#10) serving as "Blank" cycles with DAPI only. After reporter mixes are made up in tubes, each mix (labeled by its cycle number, #2-9) is transferred to its respective well (B2-B9). Reporter Stock Solution is added to wells B1 and B10 for Blank cycles.

28 Seal plate with **foil plate seal** and store at 4 °C until imaging.