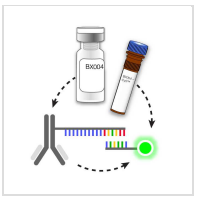

May 10, 2022

CODEX® Multiplexed Imaging | Antibody Conjugation and Validation

Diane Saunders[1], Conrad Reihsmann[2], Marcela Brissova[1], Alvin C. Powers[1]

[1]Vanderbilt University, Vanderbilt University Medical Center; [2]Vanderbilt University Medical Center

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Vanderbilt Diabetes Research Center

Islet and Pancreas Analysis Core
Vanderbilt Diabetes Research Center

This protocol is adapted from the CODEX User Manual, revision C (Akoya Biosciences, Dec. 2020).

This protocol describes the antibody conjugation and validation processes for the CODEX® (now PhenoCycler™) system by Akoya Biosciences. For the comprehensive multiplexed imaging workflow currently in use at the Vanderbilt Diabetes Research Center, please see **CODEX® Multiplexed Imaging | Modality overview**.

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protocol ,

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In steps of
CODEX® Multiplexed Imaging | Modality Overview

For Conjugation:

- 50kDa MWCO filter **Emd**
- **Millipore Catalog #UFC505096** Step 2
- CODEX® Conjugation Kit **Akoya**
- **Biosciences Catalog #7000009** Step 2

Contains: Filter Blocking Solution, Reduction Master Mix, Conjugation Solution, Purification Solution, Antibody Storage Solution

- CODEX® oligonucleotide barcodes
- Primary antibodies (50 µg each)

For Validation:

- 10X CODEX Buffer **Akoya**
- **Biosciences Catalog #7000001**
- Assay Reagent **Akoya**
- **Biosciences Catalog #7000002**
- Nuclear Stain **Akoya**
- **Biosciences Catalog #7000003**
- Dimethyl sulfoxide ≥99.9% **Sigma**
- **Aldrich Catalog #472301-6X1L**
- Invitrogen™ SlowFade™ Gold Antifade Mountant **Fisher Scientific Catalog #S36936** Step 18

- CODEX® barcoded reporters
- See CODEX® Multiplexed Imaging | Tissue Staining and Reporter Plate Preparation for complete list

Equipment:

- Benchtop microcentrifuge
- Nanodrop

:

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Primary Antibody Screening

1 Establish the efficacy of the primary antibody using an indirect immunofluorescence protocol (e.g., steps 1-20 of Immunofluorescent Staining of Mouse Pancreas).

> Primary antibodies used for conjugation must be **free of additives** (e.g., BSA, glycerol), so keep this in mind when selecting antibodies to screen. Some manufacturers offer alternate formulations of listed clones or a "carrier-free" format. In our experience, a low concentration of sodium azide is permissible.

ⓘ See also: Antibody Screening and Custom-conjugation - Tips and guidelines (Akoya Biosciences).

Custom Antibody Conjugations

2 **Gather reagents:**

- CODEX® Conjugation Kit **Akoya Biosciences Catalog #7000009**
- 50kDa MWCO filter **Emd Millipore Catalog #UFC505096**
- Oligonucleotide barcodes (Akoya Biosciences)
- 50 µg each primary antibody to be conjugated (vendor of choice)

ⓘ For an overview of the conjugation workflow, see Conjugating CODEX® tags on antibodies of choice (Akoya Biosciences).

3 Label one tube and and one filter per antibody. Block each filter with **500 µL Filter Blocking Solution**, then centrifuge **12.000 x g, 00:02:00** . Discard the flow-through.

4 Measure actual concentration using a NanoDrop™ or comparable system and calculate the volume for **50 µg** .

Prepare each antibody in a minimum volume of **100 µL** (dilute with 1X PBS if needed). Centrifuge **12.000 x g, 00:08:00** and discard the flow-through.

5 Reduce antibodies by applying **260 µL Reduction Master Mix** to each filter/tube unit and incubating for **00:30:00** at room temperature. Centrifuge **12.000 x g, 00:08:00** and discard the flow-through.

6 **Prepare oligonucleotide barcodes:**

Add **10 µL** of nuclease-free water to each barcode vial to dissolve, then add **210 µL**

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Conjugation Solution. Pipet up and down gently to dissolve all material.

Add suspended barcode to the top of the corresponding filter unit. Close lid and briefly vortex, then incubate for **00:02:00** at room temperature. Centrifuge **12.000 x g, 00:08:00** and discard the flow-through.

7 Wash by adding **450 µL** **Purification Solution** to the top of each filter unit. Centrifuge **12.000 x g, 00:08:00** and discard the flow-through.

8 **go to step #7** and repeat x2 for a total of 3 washes/spins.

9 Label a new collection tube and remove the lid. Add **100 µL** of **Antibody Storage Solution** to each filter unit. Place the new tube face-down on top of the filter unit. Invert the whole apparatus for collection into the new tube (some liquid will come off the filter immediately). Centrifuge **3.000 x g, 00:02:00** and discard the flow-through.

10 Transfer conjugated antibody (~ **120 µL**) to a screw-top storage tube. Label as follows:

AB - BX###
Conjugation date
AB lot number

Antibodies should be stored at **4 °C** .

Conjugated Antibody Screening

11 Follow the staining protocol CODEX® Multiplexed Imaging | Tissue Staining and Reporter Plate Preparation through step 20.

12 Prepare **Screening Buffer** and then transfer **3 mL** aliquots into 6-well plates.

A	B
Total number of samples	4
Total volume (mL)	40
ddH2O (mL)	28
DMSO (mL)	8
10X CODEX buffer (mL)	4

Table 1: Screening 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 B3-B5.

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13 Move coverslips through 3 washes (= 3 aliquots Screening Buffer per coverslip).

14 Prepare **Reporter Stock Solution (RSS)**:

A	B
Total number of samples	4
Total volume (µl)	400
1X CODEX buffer (µl)	379
Assay reagent (µl)	20
Nuclear stain (µl)	1

Table 2: Reporter Stock Solution. Copy and paste all cells above into an Excel sheet, then enter value into cell B1 (up to 12). The volumes for each reagent will automatically be returned in cells B3-B5.

15 Make up a reporter mix for each coverslip in a light-protected microcentrifuge tube:

100 µL Reporter Stock Solution (RSS)

2.5 µL each CODEX® barcoded reporter (RX); up to 3 RX (one of each fluorophore) per coverslip

16 Affix a piece of parafilm onto the bench using lab tape. Use a Sharpie to label spaces for each coverslip, then pipet reporter mixes into the corresponding spaces. Using bent-tip tweezers, place each coverslip **face-down** onto the pooled drop of reporter mix. Cover with a box top or other light-protective structure and incubate for **00:05:00** .

17 **go to step #13** to complete 3 washes in screening buffer (can reuse same aliquots from step 13).

18 Mount coverslips face-down onto microscope slides for imaging. For this step, either **10 µL** of 1X CODEX buffer or Invitrogen™ SlowFade™ Gold Antifade Mountant **Fisher Scientific Catalog #S36936** can be used.

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