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813.1 Multiplexed Immunofluorescence Phenocycler-Fusion® Imaging of FFPE Lung Sections V.2

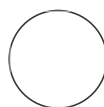
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ABSTRACT

This protocol describes multiplexed immunofluorescent (MxF) staining and imaging of FFPE lung tissue sections utilizing the Phenocycler-Fusion platform (Akoya Biosciences). The approach is based on the CODEX multiplexed immunofluorescence staining technology developed by Garry Nolan and colleagues (1). The protocol is closely aligned with the attached Phenocycler-Fusion User Guide provided by Akoya (2).

The protocol includes A) FFPE lung tissue section preparation, B) Labeling of lung tissue sections with antibody-barcode conjugates, C) Barcode-Reporter plate and experiment design, D) Multiplexed imaging and analysis, E) Reference for H&E stain to follow MxF and F) Reference to custom antibody conjugation. Thus, the protocol is designed to provide information regarding specific reagents (i.e., antibodies), conditions (i.e., dilutions), and procedures used in multiplexed immunofluorescent staining of lung tissue.

ATTACHMENTS

[PhenoCycler-Fusion User Guide.pdf](#)

GUIDELINES

An overview of Multiplexed Imaging using barcode conjugated antibodies can be found in the attached Phenocycler-Fusion User Guide. Onsite training in staining procedures, experimental setup and design, as well as image acquisition is provided by Field Application Scientists from Akoya Biosciences.

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We use this protocol and it's working

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MATERIALS

Key equipment, reagents, buffers, and supplies can be found in the attached Phenocycler-Fusion User Guide (Akoya Biosciences).

1. Surgipath Apex Superior Adhesive Slides; P/N: 3800086 Orange (Leica® Biosystems, Richmond, IL)
2. AR9 buffer; PN#AR9001KT, (Akoya Biosciences, Marlborough, MA)
3. Paraformaldehyde (20% solution); P/N: 15713-S EM Grade (Electron Microscopy Sciences, Hatfield, PA)
4. dPBS; 17-512Q (Lonza, Walkersville, MD).
5. Sample Kit for PhenoCycler-Fusion; P/N:7000017 (Akoya Biosciences)
Contains Hydration, Staining, and Storage buffers, blocking buffers N, Gv2, J, and S, and final fixative reagent.
6. 10X Buffer Kit for PhenoCycler-Fusion; P/N: 7000019 (Akoya Biosciences)
Contains 10x Buffer for Phenocycler and Buffer Additive
7. Assay Reagent; P/N: 7000002 (Akoya Biosciences)
8. DMSO, ACS Reagent Grade ($\geq 99.9\%$); 472301-1L Sigma-Aldrich.
9. Flow Cells (10 pack) Akoya Biosciences

SAFETY WARNINGS



Procedures involving xylenes and paraformaldehyde should be performed in a chemical fume hood. Nitrile gloves are permeable to xylenes therefore change gloves promptly upon contact with xylenes.

Use heat resistant gloves to handle Coplin jars after antigen retrieval.

ETHICS STATEMENT

The protocol does not utilize laboratory animals.

BEFORE START INSTRUCTIONS

The Phenocycler Fusion system must be setup and calibrated by an installation engineer. Akoya Biosciences will provide a list of materials required for training.



Lung section preparation

3h

1 Bake Sections to promote tissue adherence to the slide.

Since stained slides can be stored in storage buffer for a maximum 5 days without diminution of staining signal intensity, and Phenocycler-Fusion imaging typically ranges from 16-24h, staining more than 5 slides at a time is not recommended.



1.1 FFPE Lung sections are prepared as described in dx.doi.org/10.17504/protocols.io.kxygxejwdv8j/v2 and mounted on Leica® Surgipath Apex Superior Adhesive Slides.

1.2 Heat Slide(s) in oven at  60 °C  Overnight .

Labeling of lung tissue sections with antibody-barcode conjug... 2h 15m

2 Deparaffination 15m

Paraffin wax is removed from lung sections by incubation in xylene (3X)

- Xylene #1  00:05:00
- Xylene #2  00:05:00
- Xylene #3  00:05:00

3 Rehydration 40m

The tissue sections are rehydrated through descending ethanol series followed by molecular biology grade distilled water (2X).

- 100% Ethanol #1  00:05:00
- 100% Ethanol #2  00:05:00
- 90% Ethanol  00:05:00
- 70% Ethanol  00:05:00
- 50% Ethanol  00:05:00
- 30% Ethanol  00:05:00
- ddH2O #1  00:05:00
- ddH2O #1  00:05:00

4 High pH Antigen Retrieval


Heat-Induced Epitope Retrieval (HIER) reverses protein crosslinking in FFPE tissue

4.1 Dilute AR9 buffer (Akoya Biosciences) 1/10 in ddH2O and fill plastic Coplin Jar to 90-95% volume. Add slides containing tissue sections to be stained. Cover entire Coplin Jar with aluminum foil. **(Do not Cap)**

4.2 Place Coplin Jar in an InstantPot® pressure cooker containing ddH2O to 1/3-1/2 depth of Coplin Jar.

4.3 Heat on high pressure setting  00:20:00

20m

4.4 After releasing pressure per pressure cooker directions, remove Coplin Jar, partially unwrap foil without uncovering slides and allow slides to cool for a minimum of  01:00:00 . Attempting to rinse/wash without allowing slides to cool may reduce tissue adherence to glass slide.







1h


5 Prepare Blocking Buffer

Prepare Blocking Buffer no earlier than 1 h before staining (i.e. while slide are cooling in AR9 buffer and/or incubating in Staining buffer, see below) and keep on ice.

5.1 Table 1. Blocking Buffer Component Table

Component	2 Slides	5 slides	N Slides
Staining Buffer	 362 µL	 905 µL	 181 µL x N
N Blocker	 9.5 µL	 23.75 µL	 4.75 µL x N
G Blocker	 9.5 µL	 23.75 µL	 4.75 µL x N
J Blocker	 9.5 µL	 23.75 µL	 4.75 µL x N
S Blocker	 9.5 µL	 23.75 µL	 4.75 µL x N
Total Volume	 400 µL	 1000 µL	 200 µL x N

5.2 Prepare Antibody Cocktail

In a 1.5 ml microfuge tube, mix volume of blocking buffer =  200 µL x N slides to be stained **minus total volume of antibodies (µl/slide x N)** to be used in experiment (See Table below). The Blocking buffer volume must be **≥ 60% of total antibody cocktail volume** for effective blocking. If needed, reduce staining buffer volume to achieve 60% blocking buffer volume in Ab solution.

Example antibody panel (final column = microliter of antibody per single slide):

Antibody	Vendor	Cat#	Barcode	Dilution	µl/200µl/1slide
SMA	Akoya	4450049	BX013	1:200	1

Antibody	Vendor	Cat#	Barcode	Dilution	μl/200μl/1slide
PanCK	Akoya	4450020	BX019	1:200	1
MPO	Akoya	4250083	BX098	1:200	1
Ki67	Akoya	4250019	BX047	1:200	1
Keratin5	Akoya	4450090	BX101	1:200	1
HLADR	Akoya	4550118	BX033	1:200	1
FOXP3	Akoya	4550071	BX031	1:200	1
ColIV	Akoya	4550122	BX042	1:200	1
CD8	Akoya	4250012	BX026	1:200	1
CD68	Akoya	4550113	BX015	1:200	1
CD45	Akoya	4550121	BX021	1:200	1
CD4	Akoya	4550112	BX003	1:200	1
CD3e	Akoya	4550119	BX045	1:200	1
CD31	Akoya	4450017	BX001	1:200	1
CD20	Akoya	4450018	BX007	1:200	1
CD163	Akoya	4250079	BX069	1:200	1
CD14	Akoya	4450047	BX037	1:200	1
CD11c	Akoya	4550114	BX024	1:200	1
E-Cadherin	Akoya	4250021	BX014	1:200	1
TPSAB1*	Abcam	ab2378	BX041	1:1000	0.2
SFTPC*	Invitrogen	PA5-71842	BX020	1:500	0.4
SCGB1A1*	R&D System	MAB4218	BX043	1:400	0.5
β-III-Tubulin*	R&D Systems	MAB1195	BX055	1:400	0.5
SCEL*	Abcepta	Abcepta	BX052	1:100	2
RAGE*	Abcam	ab228861	BX028	1:100	2
LYVE1*	R&D Systems	AF2089	BX025	1:100	2
COL1A1*	Abcam	ab88147	BX054	1:100	2
CD298*	Abcam	ab167390	BX005	1:100	2
CD1c*	Novus	ab156708	BX016	1:50	4
SCGB3A2*	Abcam	ab240255	BX002	1:400	0.5

Antibody	Vendor	Cat#	Barcode	Dilution	µl/200µl/1slide
TP63*	Abcam	ab214790	BX006	1:100	2
MUC5AC*	Abcam	ab212636	BX040	1:100	2
PROX1*	R&D Systems	AF2727	BX050	1:200	1
CXCL4*	Peprotech	500-P05	BX004	1:200	1
					41.1 µlAb/200µl blocking buffer / 1 slide


Note: Whenever possible barcodes and reporters were assigned to specific antibodies based on predicted antigen abundance and relative channel sensitivity in accordance with the PhenoCycler-Fusion User Guide (Akoya Biosciences).

*Denotes custom-conjugated antibody. Refer to custom-conjugation section at the end of the protocol.

6 Incubate with Ab + Blocking Buffer Cocktail Solution





6.1 Wash slides in Coplin Jars containing dd H2O

2m

- dd H2O #1 - 3 dips
- dd H2O #2 - immersion  00:02:00

6.2 Immerse Slides in sequential Coplin jars containing the following buffers from Akoya Biosciences

54m

- Hydration buffer #1  00:02:00
- Hydration buffer #2  00:02:00
- Staining buffer  00:20:00 -  00:30:00 max.

6.3 Carefully dry slide around tissue with a Kimwipe™.

Then pipette 190 µL Ab cocktail solution onto slide to cover tissue section.
Avoiding pipetting directly onto tissue.

6.4 Incubate slides, covered in a lightly humidified chamber, for

 03:00:00  Room temperature

3h

7 Post Stain Wash and Multiple Fixations

Tissue slides are briefly washed in staining buffer followed by sequential fixation with paraformaldehyde, ice-cold methanol, and final fixation solution.

7.1 Incubate in sequential Coplin Jars containing:

19m

- **Staining Buffer #1.** ⌚ 00:02:00
- **Staining Buffer #2.** ⌚ 00:02:00
- 1.6% **paraformaldehyde** (Diluted in Storage buffer, Akoya Biosciences from 20% stock).
⌚ 00:10:00
- Rinse slides sequentially in 3 Coplin Jars containing 1 x **PBS** (3 dips each)
- pre-chilled, 🌡️ -20 °C, **methanol on ice** ⌚ 00:05:00
- Rinse slides sequentially in 3 Coplin Jars containing 1 x **PBS** (3 dips each)

7.2 Prepare Final Fix 2% dilution

20m

20 µl aliquot of **Final Fix** (Akoya Biosciences) diluted in 1 ml PBS

Carefully dry slide around tissue with a Kimwipe™

Then pipette 190 µL of **2% Final Fix solution** onto slide to cover tissue section while avoiding pipeting directly onto tissue.

Incubate ⌚ 00:20:00 .

7.3 Rinse slides sequentially in 3 Coplin Jars (3 dips each) PBS

8 Photobleaching and Storage

8.1 Slides may be stored for up to 5 days in a Coplin Jar containing **Storage buffer** 🌡️ 4 °C .

8.2 In order to reduce autofluorescence from the tissue, on the day prior to imaging, lay the slide to be imaged flat in a 100 cm² dish containing **Storage Buffer** (Akoya Biosciences).

Reporter Plate and Experiment Design

- 9 Reporter plate design and Phenocycler-Fusion run protocols are developed using the PhenoCycler Experiment Designer Software (Akoya Biosciences).

10 Prepare 1x Buffer Phenocycler Buffer and Reporter Stock Solution

Reporter Stock Solution is prepared according to guidelines in the attached Phenocycler-Fusion User Guide (Akoya Biosciences®)

- 10.1 Prepare 1 L of 1 x Phenocycler Buffer with Buffer additive, sufficient for 10 imaging runs. This is used in the side-car Phenocycler Buffer bottle, in the reporter stock solution and in the mounting of the flow cell. Do not filter. Store at room temperature for up to 2 weeks. Combine in clean glass container, mix well, avoid bubbles:

- 800 mL ddH₂O
- 100 mL 10x Buffer for Phenocycler (Akoya Biosciences)
- 100 mL Buffer Additive for Phenocycler Fusion (Akoya Biosciences)

10.2 Prepare Reporter Stock Solution

Combine, mix gently by inversion:

- 1 x Phenocycler Buffer with Buffer additive 270 μ L X N
- Assay Reagent 25 μ L X N
- Nuclear Stain 5 μ L X N

Total volume should equal 300 μ L x N = **number of cycles plus two blank cycles** (# reporter plate wells) to be done in the imaging run.

11 Prepare Reporter Solutions for each cycle in an opaque or foil wrapped tube.

Keep Reporters on ice.

Centrifuge Reporter stock tubes briefly prior to removing aliquot.

Protect the fluorescent dye-containing Reporters from photobleaching.

Avoid introducing bubbles into the solutions.

Important to keep the reporter plate dust-free.

An imaging cycle = 4 channel detection including nuclear stain and 3 reporters, depending on filter set chosen, for example one each of ATTO550, Cy5/AF647, AF750. A multi-cycle experiment composes an imaging run.

11.1 Label an opaque microtainer for each well, each well = one imaging cycle of the imaging run. To each tube add 5 µl of each predetermined reporters complementing the antibody barcodes to be detected in that cycle, and add the Reporter Stock Solution to make total volume 250 µl per microtainer.

11.2 Pipette 245 µl of each reporter mix into a well of a light-opaque 96 well microtiter plate (Akoya Biosciences) according to the Experimental Design developed in the experimental design app.

Also, pipette 245 µl reporter stock solution (blanks) into two wells of row H to create 2 blank wells.

Seal the solution-containing wells with Akoya foil seals. The plate may be stored @ 4°C for up to 14 days in accordance with the attached PhenoCycler-Fusion User Guide (Akoya Biosciences).

Multiplexed Imaging and Analysis



12 Image Acquisition via Phenocycler-Fusion® (i.e., CODEX V2)


12.1 Use the Experiment Designer app, located on the PhenoCycler-Fusion Acquisition PC desktop, to define the design of the Reporter plate and imaging parameters to be used in the experiment. Follow details provided in the attached PhenoCycler-Fusion User Guide.

12.2 If necessary, warm reporter plate to  Room temperature

12.3 After photobleaching in storage buffer, wash slides in PBS (250 ml; Coplin Jar)

10m

 Room temperature  00:10:00

12.4 After the wash, dry the bottom of the slide and around the edges of the tissue with a kimwipe. Attach a flow cell using the flow cell assembly device (Akoya Biosciences). Center and press the flow cell onto the slide for  00:00:30

30s

12.5 Cure the flow cell adhesive by incubating the slide in 1X phenocycler buffer (Akoya biosciences)

10m

 00:10:00  Room temperature

- 12.6** Fill respective Reagent reservoirs on the Phenocycler side car with DMSO, 1X phenocycler buffer, and ddH₂O, and place a blank flow cell in the flow cell carrier attached to the fluidics tubing.
- 12.7** Start an imaging run by turning on the Phenocycler fluidics system and the Phenoimager, followed by launching the Fusion software. Select Start experiment and follow the prompts.
- 12.8** Images were acquired utilizing the 20X (0.5 μ M/pixel) objective and Fusion 1.0.8 software.
- 12.9** Image processing is automated via the Fusion 1.0.8 software and completed at the end of the experiment run.


Expected result

- A. Folder with slide/sample name containing:
- The respective Ab-Reporter (.xpd) file generated by the Phenocycler Experiment designer.
 - Akoya whole slide scan .qptiff (~8-12 GB for a 30-36 marker panel; ~1 cm² lung section)
- B. The following .temp file contents:
- CombineInputs
 - Coverslip Mask
 - qptiff raw files: 8-12 GB for each cycle (30-36 marker panel; 1 cm² section)
 - qptiff.intermediate: 8-12 GB for each cycle (30-36 marker panel; 1 cm² section)
 - FocusMap
 - FocusTable
 - Label
 - MarkerList
 - Overview BF
 - Overview FL
 - SampleMask

- 12.10** Checking the Sample mask, BF overview, and FL overview, by dragging and dropping files into ImageJ after the first cycle is recommended. If major issues are observed, the run may be aborted to

preserve reporters.

Do not attempt to open raw or intermediate cycle.qptiff during the imaging run.

- 12.11** Rapid review of the resultant image.qptiffs is performed utilizing PhenoChart 1.2.0 software. If necessary, exposure time (ms) is adjusted in the Phenocycler Experiment Designer to obtain readily detectable, specific marker signals that are below saturation. Note that after viewing qptiffs in PhenoChart an_annotations.xml.lock file will appear in the image folder.
- 12.12** After the run return the tissue slide with attached flow cell to storage buffer . If necessary, slides can be reimaged with a new set of reporters up to 5 days post staining without loss of signal.

13 Record Imaging Run MetaData

- 13.1** Record Slide ID, staining and imaging date in the Metadata Excel file located in the Fusion folder on the Phenocycler PC.
- 13.2** Likewise record the dates for biweekly Brightfield reference calibration and maintenance washes as well as monthly fluorescent and phenocycler reference calibration.
- 13.3** The "source storage time" = length of time (Days) between slide staining date and imaging date.
- 13.4** The image acquisition time = length of time between time stamp on the MarkerList or CombineInputs files (**START**) and the time stamp on the Akoya whole slide scan file .qptiff (**FINISH**).
- 13.5** The time_since_acquisition_instrument_calibration_value is the time interval (days) between the phenocycler reference calibration date and the imaging run date.

Data backup and storage

14 Upload files from Phenocycler-Fusion runs to the URMC local Bluehive-Archive/archive/lungmap_lab codex folder.

14.1 Maintain directory structure for each run by creating a new folder labeled with the slide ID containing a scan# subfolder, and a temp subfolder within the scan folder.

14.2 Include the following files in the upload:

Slide folder:

Scan Folder:

- A. (.xpd) file (Phenocycler Experiment designer)
- B. Akoya whole slide scan .qptiff
- C. The ome.tiff generated via bfconvert (See step 17)

Temp folder:

- i. CombineInputs
- ii. Coverslip Mask
- iii. qptiff raw files for each cycle
- iv. qptiff.intermediate files for each cycle
- v. FocusMap
- vi. FocusTable
- vii. Label
- viii. MarkerList
- ix. Overview BF
- x. Overview FL
- xi. SampleMask

Multiplexed Imaging and Analysis

15 Image Analysis and Segmentation

15.1 Analysis of processed image.qptiff files is performed utilizing QuPath (See Reference 3).

- 15.2** Cell segmentation based on (DAPI) stained nuclei is performed utilizing the respective StarDist extension (See Reference 4) in QuPath.
- 15.3** Cell detection measurements (e.g. X,Y coordinates, fluorescent intensity data for all channels) are saved as a text file, opened in Excel as a tab delimited file, and resaved as a CSV (comma delimited) file.
- 15.4** Cell segmentation masks are exported from QuPath running the following ExpCell script within Script Editor:

```

import qupath.lib.images.servers.LabeledImageServer
import qupath.lib.roi.RectangleROI
import qupath.lib.scripting.QP

def imageData = getCurrentImageData()
def project = getProject()
def server = imageData.getServer()

// Define output path (relative to project)
def name =
GeneralTools.getNameWithoutExtension(imageData.getServer().getMetadata(
).getName())
def pathOutput = buildFilePath(PROJECT_BASE_DIR, 'export')
mkdirs(pathOutput)
// Define output resolution
double requestedPixelSize =
server.getPixelCalibration().getAveragedPixelSize()
// Convert to downsample
double downsample = requestedPixelSize /
imageData.getServer().getPixelCalibration().getAveragedPixelSize()
println('Downsample factor : '+downsample)
// Create an ImageServer where the pixels are derived from annotations
def labelServer = new LabeledImageServer.Builder(imageData)
    .backgroundLabel(0, ColorTools.BLACK) // Specify background label
(usually 0 or 255)
    .downsample(downsample) // Choose server resolution; this should
match the resolution at which tiles are exported
    .useCells()
    .useInstanceLabels() //Export as unique label
    .multichannelOutput(false) // If true, each label refers to the
channel of a multichannel binary image (required for multiclass
probability)
    .build()

annotation = getSelectedObject()

//Export the mask
def region = RegionRequest.createInstance(labelServer.getPath(),
downsample, annotation.getROI())
def outputPath = buildFilePath(pathOutput, 'labels_mask.ome.tiff')
writeImageRegion(labelServer, region, outputPath)

resetSelection()

```

15.5 Vascular smooth muscle and airway epithelial features are annotated and classified by thresholding the ACTA2 and PanCK signals utilizing the Pixel classifier in QuPath.

15.6 Annotation masks are exported from QuPath running the following ExpCell script within Script Editor:

```
def imageData = getCurrentImageData()

// Define output path (relative to project)
def outputDir = buildFilePath(PROJECT_BASE_DIR, 'export')
mkdirs(outputDir)
def name =
GeneralTools.getNameWithoutExtension(imageData.getServer().getMetadata(
).getName())
def path = buildFilePath(outputDir, name + "-labels.png")

// Define how much to downsample during export (may be required for
large images)
double downsample = 8

// Create an ImageServer where the pixels are derived from annotations
def labelServer = new LabeledImageServer.Builder(imageData)
    .backgroundLabel(0, ColorTools.WHITE) // Specify background label
(usually 0 or 255)
    .downsample(downsample) // Choose server resolution; this should
match the resolution at which tiles are exported
    // Choose output labels (the order matters!)
    .addLabel('Airway', 1)
    .addLabel('Vasculature', 2)
    .addLabel('Other', 3)
    .multichannelOutput(false) // If true, each label refers to the
channel of a multichannel binary image (required for multiclass
probability)
    .build()

// Write the image
writeImage(labelServer, path)
```

15.7 Object Data is exported as a GeoJSON file.

Conversion of .qptiff files to ome.tiff for upload to Omero util...

- 16 Download Command Line Tools from <https://www.openmicroscopy.org/bio-formats/downloads/>
- 17 Execute the following in command prompt:
 - 17.1 C:\>Set BF_MAX_MEM=10240M
 - 17.2 C:\>bfconvert -no-sas -series 0 -compression LZW -pyramid-resolutions 5 -pyramid-scale 2 INPUT.qptiff OUTPUT_ome.tiff
 - 17.3 Upload the resulting ome.tiff file to the CODEX folder in Omero and the respective tissue donor subfolder (e.g. D265) utilizing the Omero importer.

Custom antibody conjugation

- 18 Custom Antibody Conjugation is performed as described dx.doi.org/10.17504/protocols.io.3fugjnw.
 - 18.1 For antibodies containing sodium azide (0.05-0.1%) or trehalose (5%), buffer exchange was performed utilizing Zeba Spin Desalting columns 7K MWCO (89890, 2ml, Thermoscientific) equilibrated in PBS in accordance with the manufacturer's recommendations.
 - 18.2 Success of Antibody-Barcode chemical conjugation is determined by resolving unconjugated and conjugated Ab's on BioRAD™s MiniProtean TGX Gel 4-15% Bis-Tris Protein Gels in accordance with Guidelines in the Phenocycler-Fusion User Guide (Akoya Biosciences®, Malborough, MA).

Expected result

Barcode conjugated Ig heavy and light chains exhibit an Electrophoretic Mobility Shift (EMS) relative to the corresponding unconjugated Ab.

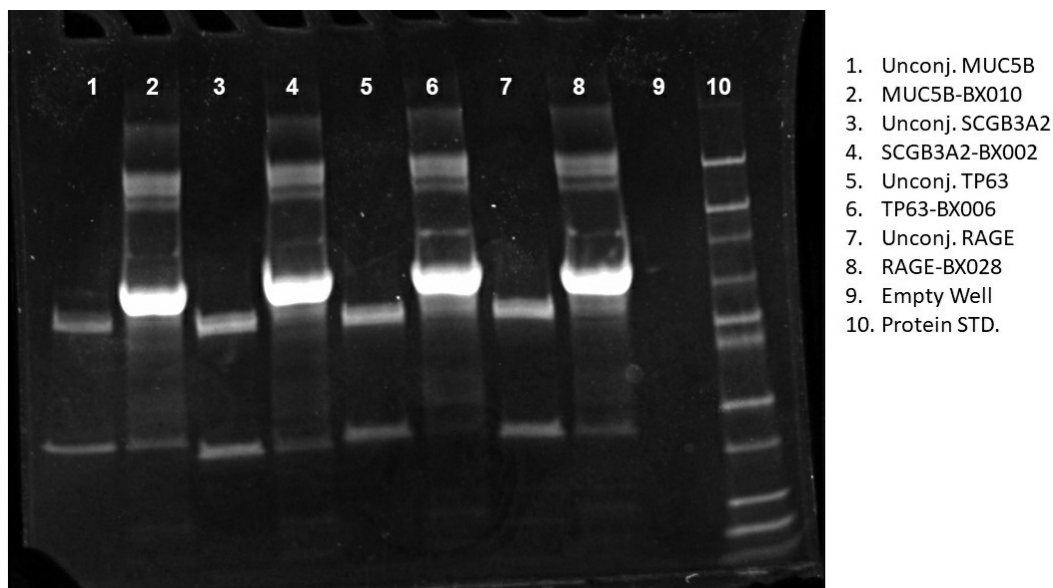


Figure 1. A representative SDS-PAGE Gel providing quality assurance of Ab barcode conjugation.

H&E staining Post Phenocycler-Fusion

10m

19 It is useful to stain the tissue sections with hematoxylin & eosin following the multiplexed fluorescence imaging in order to match histology with antibody staining. We have stained lung sections with an aqueous H&E stain as described dx.doi.org/10.17504/protocols.io.kqdg397yeg25/v1 but this resulted in an overall rather grey stain and so we have opted instead to remove the flow cell by soaking in xylene prior to H&E staining of lung sections as described in Step 19.1.

19.1 After all MxF imaging is completed for the slides, remove the flow cell covered slides from storage buffer (Akoya Biosciences), wipe dry with Kimwipe and, utilizing a vacuum-suction apparatus similar to what is described in dx.doi.org/10.17504/protocols.io.kqdg397yeg25/v1, remove buffer from the flow cell.

- Incubate slides in a Coplin Jar containing Xylene for up to **04:00:00** in order to weaken or dissolve the flow cell adhesive.
- Carefully pry flow cell off the slide with razor blade or preferably a thin spatula.
- Proceed with H&E staining according to the standard protocol described here



[dx.doi.org/10.17504/protocols.io.36wgqjnj3vk5/v4](https://doi.org/10.17504/protocols.io.36wgqjnj3vk5/v4) beginning at Step 5.