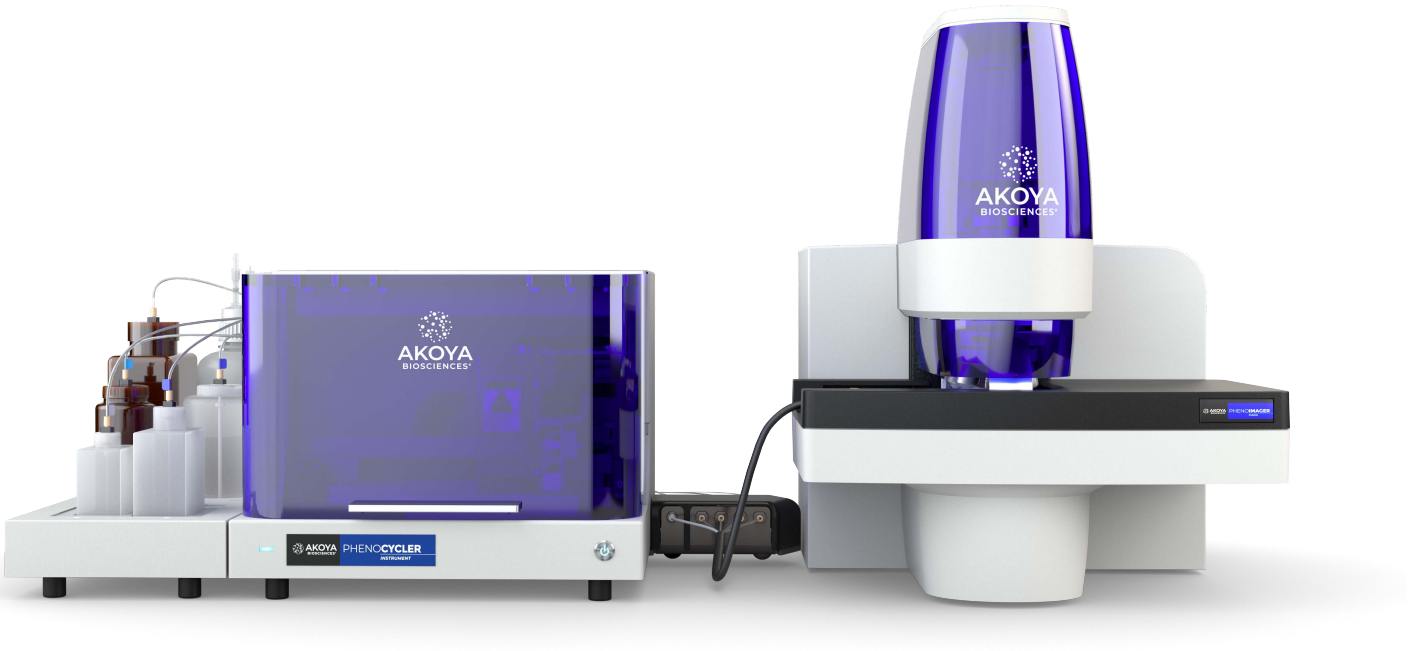
AKOYA BIOSCIENCES®
THE SPATIAL BIOLOGY COMPANY®

PhenoCycler-Fusion
USER GUIDE



PD-000011 Rev L

PhenoImager Fusion SW version 2.1.0 | Date: 08.15.2023

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CONTENTS

PREFACE

Welcome to the Akoya Biosciences PhenoCycler®-Fusion User Guide.

INTENDED AUDIENCE FOR THIS DOCUMENT

This document is intended for laboratory personnel who have received instruction in:

- General safety practices for laboratories.
- Specific safety practices for the PhenoCycler™ and PhenoImager Fusion instruments.

ASSOCIATED DOCUMENTS

- Opal® Multiplex Assay Development Guide
- PhenoImager™ Fusion 2.0 User Guide
- PhenoImager™ Fusion User Guide
- Phenochart™ Whole Slide Context Viewer for Annotation & the Review User Guide
- inForm® Advanced Image Analysis Software User Guide

GUIDE CONVENTIONS

This guide contains the following information highlights and cross-references:

	***DANGER: Warns the user of an imminently hazardous situation, which if not avoided, will result in death or serious injury.***
	***Warning: Warns the user regarding actions that may result in physical damage to the system or personal injury.***
	***Caution: Cautions the user regarding actions that may result in operational issues or data loss.***
NOTE >	***NOTE: Identifies important points, helpful hints, special circumstances, or alternative methods.***
CRITICAL >	***CRITICAL: Identifies areas where the user should pay close attention to the instructions to avoid possible physical damage to the system or personal injury.***

PRODUCT SERVICE AND CUSTOMER SUPPORT PLANS

Akoya offers a full range of services to ensure your success. From our original factory warranty to a comprehensive line of customer support plans, Akoya provides Field Service Engineers and internal specialists who are dedicated to supporting your hardware, software, and application development needs. Contact Akoya Technical Support at support@akoyabio.com.

Our programs can include such useful services as:

- Preventive maintenance.
- Diagnostic servicing performed on-site by Akoya Field Service Engineers or remotely via Technical Support.
- Validation performed on-site by Akoya Field Service Engineers.
- Extended use of the Akoya Technical Support Center.
- Software updates.
- Parts, labor, and travel expense coverage.
- Other customized services upon request.

CE

CE	*This device complies with all CE rules and requirements.*

NOTE > *Changes or modifications to this equipment not expressly approved by the party responsible for compliance could void the user's authority to operate the equipment.*

A급 기기 (업무용 정보통신기기)

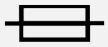
이 기기는 업무용으로 전자파적합등록을 한 기기이오니 판매자 또는 사용자는 이 점을 주의하시기 바라며, 만약 잘못판매 또는 구입하였을 때에는 가정용으로 교환하시기 바랍니다.

KOREAN CERTIFICATIONS (KC)

This device complies with MSIP (Ministry of Science, ICT, and Future Planning) EMC Registration requirements. This instrument is registered as a Class A instrument for business use only. Product sellers and users should notice that this equipment is not for household use.

DEFINITION OF SYMBOLS (LABELING OF EQUIPMENT AND LOCATION)

This section details the meaning, intent, and location of the labels (containing symbols) that appear on the PhenoCycler-Fusion system and/or in the user documentation.

DEFINITION OF SYMBOLS			
Symbol	Description	Symbol	Description
	This symbol indicates danger. It indicates an imminently hazardous situation, which, if not avoided, will result in death or serious injury.		Lifting Hazard. May result in injury. (ISO 3864)
	This symbol warns the user regarding actions that may result in physical damage to the system or personal injury.		Risk of Explosion. (ISO 3864)
	This symbol indicates caution. Cautions the user regarding actions that may result in operational issues or data loss.		Bright Lights.
	This symbol is a Note. A note is a cautionary statement, an operating tip, or maintenance suggestion. Ignoring the note may result in instrument damage if not followed.		Protective ground symbol. (IEC 60417-5019)
	This symbol represents hazardous voltage. Hazardous voltage causes risk of electric shock, per IEC 60417-6042.		Ground Symbol. (IEC 60417-5017)
	Crush Hazard. Risk of body parts, hair, jewelry, or clothing getting caught in a moving part. (ISO 3864)		Fuse. (IEC 60417-5016)
	Risk of Fire. (ISO 3864)		Alternating Current. (IEC 60417-5032)

DEFINITION OF SYMBOLS			
Symbol	**Description**	**Symbol**	**Description**
	On (Power)		Signifies unit has passed safety tests for grounding, power line transmissions, and current leakage (screen cap)
	Off (Power)		Input
	CE Compliance Mark		Output
	Serial Number		WEEE Symbol
	Date of Manufacture	**Equipment labels are color coded:**	YELLOW: Caution, risk of danger RED: Stop BLUE: Mandatory action GREEN: Safe condition or information

LOCATION OF INSTRUMENT LABELS

The images below illustrate the nameplate for the PhenoCycler-Fusion (top) and its location with respect to the assembled instrument (bottom).

PhenoCycler nameplate.

Assembled PhenoCycler-Fusion instrument.

SAFETY CONSIDERATIONS

Safety information for the PhenoCycler-Fusion system is included in this guide. Read and review all safety information before operating the PhenoCycler-Fusion instrument.

REQUIRED TRAINING

Ensure that all personnel involved with the operation of the instrument have:

- Received instruction in general safety practices for laboratories.
- Received instruction in specific safety practices for the instrument.

	*Warning: Use this product only in the manner described in this manual. If the equipment is used in a manner not specified by the manufacturer, the protection provided by the equipment may be impaired.*

DANGER, WARNINGS, AND CAUTION SIGNS

Observe the following dangers, warnings, and cautions when using the PhenoCycler-Fusion system.

General Hazards

None.

Electrical Safety

The PhenoCycler-Fusion is powered by a 100-120VAC/200-240VAC, 50-60Hz (±10%) input power supply.

	*The wall outlet or the power cable connector on the left side of the instrument should be accessible after the system's installation. This enables trained service personnel to safely disconnect power from the system during servicing.* *Warning: Do not operate the system in an environment with explosive or flammable gases.*
	*Warning: Do not remove instrument covers. There are no user serviceable parts inside. The covers are intended to be removed only by qualified Akoya Biosciences service personnel. They are not intended to be removed during operation or for maintenance by users. Contact Akoya Biosciences Technical Support at support@akoyabio.com.*
	*Warning: Turn off the electrical power to the PhenoCycler-Fusion system by shutting down the system before cleaning any part of the instrument where electrical or fiber optic cables connect.*
	*Warning: Do not spray cleaning solutions directly onto the monitor screen. Sprays and liquids that come into contact with the screen may result in damage to the system or electric shock. Always spray the cleaning solution onto a cloth and then wipe the screen with the cloth.*
	*Warning: The PhenoCycler instrument has moving parts. Do no attempt to open the door when the instrument is running.*

Additional electrical safety information can be found in the following sections:

- Power Cord Selection
- Fuses
- Cables and Adapters

POWER CORD SELECTION

Contact Akoya Biosciences Technical Support at support@akoyabio.com to order replacement power cords.

	*Warning: Use only the power supply cord set provided with the PhenoCycler-Fusion system. If the correct cord for the location was not provided, contact Akoya Biosciences Technical Support at support@akoyabio.com for a replacement. Do not use power supply cords with inadequate ratings.*
	*Warning: Use only a properly grounded power outlet when connecting the system to power.*
	*Warning: The appliance inlet is a disconnecting device. Place the device or equipment where disconnecting the device is always accessible.*

FUSES

Contact Akoya Biosciences Technical Support at support@akoyabio.com to order replacement fuses.

	*Warning: The fuses in this instrument are only replaceable by trained Akoya Biosciences personnel.*

CABLES AND ADAPTERS

Some cables and adapters supplied with the system have proprietary specifications.

	*Warning: Do not connect components supplied by Akoya Biosciences using unqualified cables or adapters. Contact Akoya Biosciences Technical Support at support@akoyabio.com to order replacement cables and adapters.*

MECHANICAL SAFETY

	*Warning: Do not connect components supplied by Akoya Biosciences using unqualified cables or adapters. Contact Akoya Biosciences Technical Support at support@akoyabio.com to order replacement cables and adapters.*

WEIGHT WARNING

	*Warning: LIFTING HAZARD. The PhenoCycler-Fusion instrument weighs ~120 lbs. (~54 kg). Do not move the PhenoCycler-Fusion instrument. Installing, servicing, and moving the PhenoCycler-Fusion instrument should be performed only by qualified Akoya Biosciences service personnel. Contact Akoya Biosciences Technical Support at support@akoyabio.com if help is required.*

BRIGHT LIGHT

	*Warning: BRIGHT LIGHT HAZARD. The interior of the PhenoCycler-Fusion system includes a Class 2 LED Light. Do not look into the bright light to avoid an eye injury.* *Caution: Do not stare directly into the beam.*

CHAPTER 1

PhenoCycler-Fusion Solution Overview

PHENOCYCLER-FUSION SOLUTION

The PhenoCycler®-Fusion system is the fastest spatial biology solution that enables ultrahigh-plex spatial phenotyping of whole slides at single-cell resolution by integrating automated fluidics and iterative imaging. (See Figure 1.1.)

This integrated platform combines the strengths of Akoya's automated, ultrahigh multiplex cycling platform, PhenoCycler™, and its high-speed imaging platform, PhenoImager®, into an end-to-end workflow that includes instrumentation, reagents and consumables, and acquisition and analysis software.

FIGURE 1.1. The PhenoCycler-Fusion system.

PHENOCYCLER TECHNOLOGY

Akoya Biosciences has designed and developed the PhenoCycler-Fusion system to accelerate the field of spatial biology. (See Figure 1.2.) From building cell atlases to developing a novel spatial signature, the PhenoCycler-Fusion system has the power to match the scale of your studies today and tomorrow.

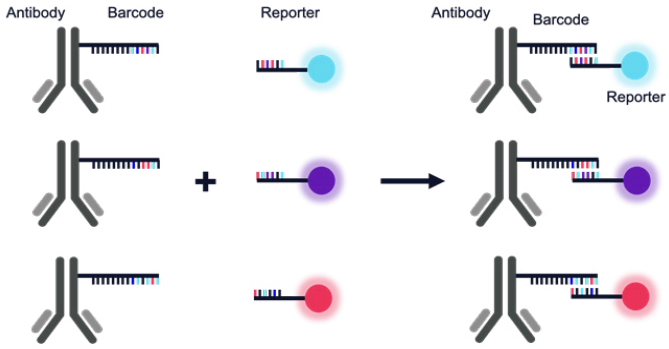


FIGURE 1.2. Reversible hybridization between the PhenoCycler Barcodes and complementary PhenoCycler Reporters to sequentially reveal PhenoCycler Antibodies.

PHENOCYCLER WORKFLOW NOMENCLATURE

The PhenoCycler Barcoding System is illustrated in Figure 1.3.

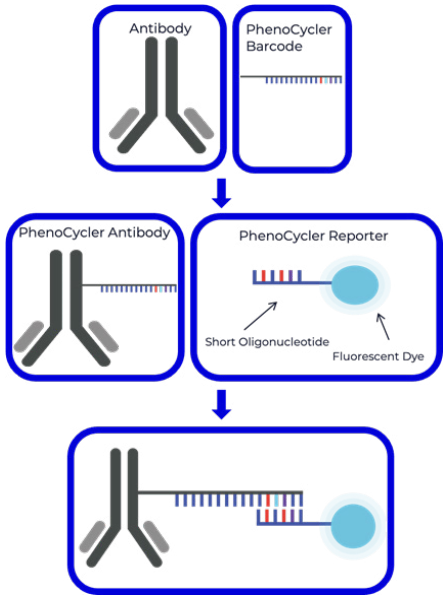


FIGURE 1.3. PhenoCycler barcoding system.

The multiplexing capability of PhenoCycler technology is based on the proprietary PhenoCycler Barcoding System. Each PhenoCycler Antibody is conjugated to a unique oligonucleotide sequence, referred to as the PhenoCycler Barcode. Each PhenoCycler Barcode, in turn, is complementary to a unique PhenoCycler Reporter. The reporter is comprised of a fluorescent dye and a short oligonucleotide.

PhenoCycler product nomenclature is defined in Table 1.1.

TABLE 1.1. PhenoCycler product nomenclature

Product	Definition	Naming Structure	Abbreviation	Example
PhenoCycler Antibody	Successfully validated antibody conjugated to a PhenoCycler Barcode	Antibody—Barcode	Ab–BXxxx	CD4–BX018
PhenoCycler Barcode	PhenoCycler Barcode oligonucleotide that can be custom conjugated to antibodies of interest	Barcode	BXxxx	BX001
PhenoCycler Reporter	Fluorophore conjugated to an oligonucleotide that can hybridize with a specific complementary PhenoCycler Barcode	Fluorophore—Reporter	Dye–RXxxx	AF750–RX003

The following section provides a brief overview of the entire PhenoCycler-Fusion workflow. (See Figure 1.4.) Subsequent chapters will focus on guiding you through protocols related to the tissue preparation and antibody staining portions of the PhenoCycler-Fusion solution.

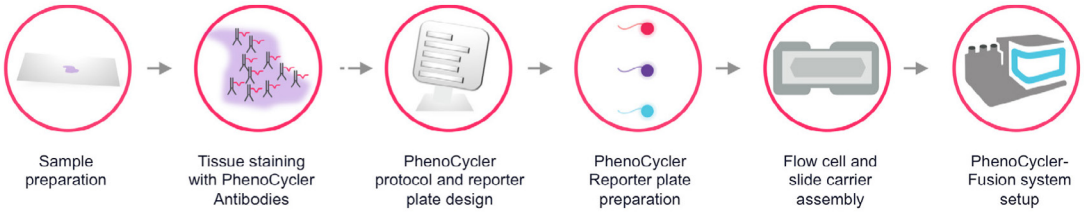


FIGURE 1.4. PhenoCycler workflow.

PHENOCYCLER INSTRUMENT

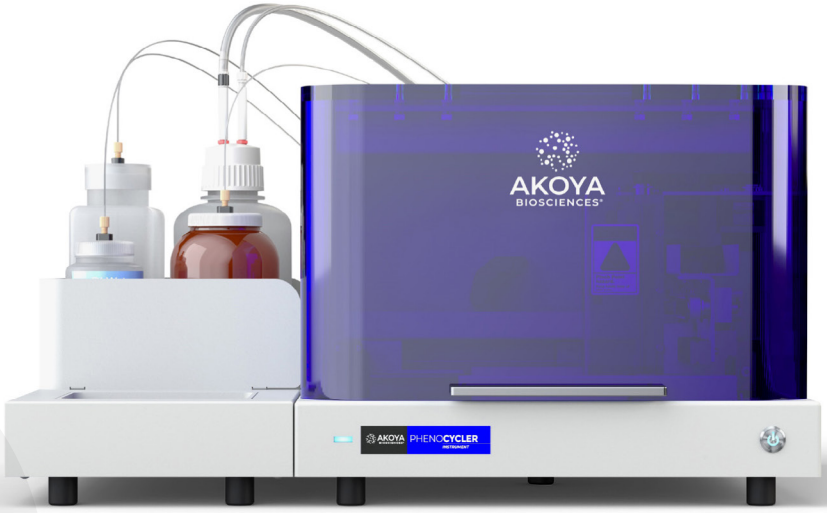


FIGURE 1.5. PhenoCycler instrument.

The PhenoCycler Instrument performs all fluidic operations required for a PhenoCycler-Fusion run. It is equipped with:

- Robotic Cannula.
- 2 Removable Reservoirs.
- 1 Holder for the 96-well Reporter Plate.
- 6 Bottles on the Side Tray.
- 1X PhenoCycler Buffer Bottle.
- Vacuum/Waste Bottle.
- High DMSO Bottle.
- Low DMSO Bottle.
- ACD 1X Wash Buffer Bottle.
- Water Bottle.
- Flow Cell Assembly Device.
- Flow Cell Slide Carrier (integrates with the PhenoCycler-Fusion and is placed on the PhenoCycler-Fusion stage).

The PhenoCycler instrument automates the gentle washes and incubations of the tissue sample with reagent mixtures during a PhenoCycler-Fusion run. The controller software directs the PhenoCycler-Fusion controls the image acquisition with every cycle. Fluidics and imaging of the tissue sample are conducted sequentially every cycle during a PhenoCycler run.

REAGENTS AND CONSUMABLES FOR PHENOCYCLER-FUSION WORKFLOW

Materials Provided

Sample Kit for PhenoCycler-Fusion

The Sample Kit for PhenoCycler-Fusion (P/N 7000017) contains buffers and reagents to perform tissue stains with barcode-tagged PhenoCycler Antibodies or custom-conjugated antibodies. (See Table 1.2.)

TABLE 1.2. Sample Kit for PhenoCycler-Fusion provides reagents and flow cells for 10 tissue samples

Item	Storage	Related Protocols	P/N
Hydration Buffer	4°C	Tissue Staining	7000017
Staining Buffer			
Storage Buffer			
N Blocker			
J Blocker			
G Blocker	-20°C		
S Blocker			
Fixative Reagent			
Flow Cells	RT*	Use of PhenoCycler-Fusion System	
Flow Cell 10 pk	RT	Use of PhenoCycler-Fusion System	240205
Flow Cell 2 pk	RT	Use of PhenoCycler-Fusion System	240204

*Room temperature (RT).

NOTE > ***Unless otherwise stated, the buffers are 1X and do not require dilution.***

Antibody Conjugation Kit

The Antibody Conjugation Kit (P/N 7000009) contains the PhenoCycler reagents required for custom conjugation of non-inventoried antibodies with the PhenoCycler Barcodes to run a PhenoCycler experiment. (See Table 1.3.)

TABLE 1.3. Antibody Conjugation Kit provides reagents for 10 conjugations (antibodies not included)

Item	Storage	Related Protocols	P/N
Reduction Solution 1	-20°C	Antibody Conjugation	7000009
Filter Blocking Solution	4°C		
Reduction Solution 2			
Conjugation Solution			
Purification Solution			
Antibody Storage Solution			

PhenoCycler à la Carte Items

Table 1.4 lists single-order items used during tissue sectioning, tissue staining, Reporter Plate preparation, and PhenoCycler runs.

TABLE 1.4. PhenoCycler reagents and consumables

Item	Storage	Related Protocols	P/N
10X Buffer Kit for PhenoCycler-Fusion	RT	Tissue Staining Reporter PlatePreparation Use of PhenoCycler-Fusion System	7000019
96-well plates for PhenoCycler	RT	Use of PhenoCycler-Fusion System Reporter Plate Preparation	7000006
96-well plate seals for PhenoCycler			7000007
Assay Reagent for PhenoCycler	-20°C, then 4°C after first thaw	Tissue Staining Reporter Plate Preparation Use of PhenoCycler-Fusion System	7000002
Nuclear Stain for PhenoCycler			7000003

PhenoCycler Reagents

PhenoCycler reagents include PhenoCycler Antibodies, Reporters, and Barcodes. (See Table 1.5.) An updated list of available products can be found on the Akoya website at http://akoyabio.com.

For information on the design and structure of PhenoCycler reagents, see the **PhenoCycler Workflow Nomenclature** section.

TABLE 1.5. Storage of PhenoCycler reagents

Product	Storage	Related Protocols
PhenoCycler Antibodies	4°C	Tissue Staining
PhenoCycler Barcodes	-20°C	Antibody Conjugation
PhenoCycler Reporters	-20°C, then 4°C after the first thaw	Reporter Plate Preparation Use of PhenoCycler-Fusion System

Materials Not Provided

The materials required for the entire PhenoCycler workflow but NOT provided are listed in Table 1.6.

TABLE 1.6. Materials required (but NOT provided) for the entire PhenoCycler Workflow

Type	Item	Vendor	Section/s	P/N
Consumables	Coplin jars	VWR	Tissue Staining	513200
	Glass microscope slides, positively charged (1 inch x 3 inch)	Leica® Slide White Apex Superior Adhesive or Fisherbrand™ Superfrost Plus	Tissue sectioning, Tissue Staining, Tissue storage	3800080 or 12-550-15
	Staining tray	VWR	Tissue Staining	M918-2
	Microscope slide box	Fisher Scientific	Tissue Sectioning, Tissue Staining, Tissue Storage	03-448-1
	Buffer reservoirs—4 required; no substitutions	Beckman Coulter	Use of PhenoCycler-Fusion System (1 set included with instrument)	BK372790
	1 mL, 1.5 mL, 2 mL tubes	Customer Choice	Tissue Staining, Use of PhenoCycler-Fusion System	MLS
	Amber 1.5 mL tubes	Customer Choice	PhenoCycler Reporter Preparation, Use of PhenoCycler-Fusion System	MLS
	Serological pipet	Customer Choice	Tissue Sectioning, Tissue Staining, Use of PhenoCycler-Fusion System	MLS
	5, 15, 50 mL conical tubes	Customer Choice	Tissue Staining	MLS
	Disposable filter units	Nalgene™ Rapid-Flow™ (Recommended)	Use of PhenoCycler-Fusion System	156-4020
	Compressed air duster	Customer Choice	Use of PhenoCycler-Fusion System	MLS
	Kimwipes®	Customer Choice	Use of PhenoCycler-Fusion System	MLS
	Parafilm	Customer Choice	Tissue Staining	MLS

TABLE 1.6. Materials required (but NOT provided) for the entire PhenoCycler-Fusion Workflow

Type	Item	Vendor	Section/s	P/N
Biologics/ Reagents	16% PFA*	Electron Microscopy Sciences (recommended)	Tissue Staining	15710
	1X PBS	Thermo Fisher Scientific	Antibody Conjugation, Tissue Staining	14190144
	ddH_2O	Customer Choice	Use Of PhenoCycler-Fusion System, Antibody Conjugation	MLS**
	Methanol	Sigma-Aldrich	Tissue Staining	34860-1L-R
	DMSO—ACS reagent, ≥99.9%	Sigma-Aldrich	Use of PhenoCycler-FusionSystem	472301-4L
Instrumentation	UPS (recommended)	APC Back-UPS Pro 1500	Use of PhenoCycler-Fusion System	BR1500G
	Fume Hood (highly recommended)	Customer Choice	Tissue Staining, Waste Collection	MLS
	Magnetic stir plate and bar	Customer Choice	Preparing the 1X PhenoCycler Fusion Buffer	MLS

*Paraformaldehyde (PFA).

**Major laboratory supplier (MLS).

NOTE > ***Do not use the extended frost/extended label versions of the slides. These slides may interfere with the proper adhesion of the flow cell and/or result in flow cell breakage.***

NOTE > ***Recommended slide dimensions: 24–25 mm width x 74–75 mm length.***

The materials required for fresh frozen (FF) tissue sections but NOT provided are listed in Table 1.7.

TABLE 1.7. Materials required (but NOT provided) for FF tissue sections

Type	Item	Vendor	Section	P/N
Consumables	Drierite® Adsorbents	Fisher Scientific	Tissue Staining	23-116582
Solvents	Acetone	Sigma-Aldrich	Tissue Staining	650501-1L
Instrumentation	Cryostat	Customer Choice	Tissue Sectioning	MLS

The materials required for formalin-fixed, paraffin-embedded (FFPE) tissue sections but NOT provided are listed in Table 1.8.

TABLE 1.8. Materials required (but NOT provided) for FFPE tissue sections

Type	Item	Vendor	Section	P/N
Additional Materials	Aluminum foil	Customer Choice	Antigen Retrieval	MLS
	Solvent-resistant containers with lids (Qty 10)	EZ-Quick Slide Staining Set, IHC World	Tissue Staining	IW-2510
	Slide staining rack	EZ-Quick Slide Staining Set, IHC World	Tissue Staining	IW-2512
Solvents	10X AR6 or 10X AR9 Buffer	Akoya Biosciences	Antigen Retrieval	AR6001KT or AR9001KT
	Ethanol or Reagent Alcohol	Sigma-Aldrich	Tissue Staining	79317-16GA-PB
	Histo-Choice® Clearing 1X	VWR	Tissue Staining	H103-4L
Equipment	Microtome	Customer Choice	Tissue Sectioning	MLS
	Instant Pot	Instant Pot	Antigen Retrieval	MLS

The materials required for conjugation are listed in Table 1.9.

TABLE 1.9. Materials required for conjugation

Type	Item	Vendor	Section	P/N
Consumables	50kDa MWCO filter—no size substitutions (25kDa and 100kDa result in failure)	EMD Millipore	Antibody Conjugation	UFC505096
	Screw-top 1.7 mL or 2 mL tubes	Customer Choice	Antibody Conjugation	MLS
	Parafilm	Customer Choice	Conjugation Verification	MLS
Biologics/ Reagents	Purified antibodies	Customer Choice	Antibody Conjugation	MLS
	NuPAGE™ LDS Sample Buffer (14X)	Thermo Fisher Scientific	Conjugation Verification	NP0008
	NuPAGE Sample Reducing Agent (10X)	Thermo Fisher Scientific	Conjugation Verification	NP0009
	NuPAGE 4–12% Bis-Tris Protein Gels	Thermo Fisher Scientific	Conjugation Verification	NP0321BOX
	Novex™ Sharp Pre-Stained Protein Standard 3.5–260 kDa	Thermo Fisher Scientific	Conjugation Verification	LC5800
	Novex SimplyBlue™ SafeStain	Thermo Fisher Scientific	Conjugation Verification	LC6065
	NuPAGE MOPS SDS Running Buffer (20X)	Thermo Fisher Scientific	Conjugation Verification	NP0001

Type	Item	Vendor	Section	P/N
Instrumentation	Centrifuge	Customer Choice	Antibody Conjugation	MLS
	XCell SureLock™ MiniCell Electrophoresis System	Customer Choice	Conjugation Verification	MLS
	95°C dry bath	Customer Choice	Conjugation Verification	MLS
	Nanodrop	Customer Choice	Antibody Conjugation	MLS
	Shaker (optional)	Customer Choice	Conjugation Verification	MLS
	Microwave (optional)	Customer Choice	Conjugation Verification	MLS

PHENOCYCLER-FUSION SOFTWARE SUITE

PhenoCycler-Fusion uses a software suite containing 4 different programs:

- PhenoCycler-Fusion Acquisition Software.
- PhenoCycler Experiment Designer App.
- Phenochart Visualization Software.
- inForm Software for Analysis.

The PhenoCycler-Fusion software suite has a unique architecture designed for modular use. You can use part of it (e.g., for data acquisition and processing) and use other commercial or custom-written software for image analysis or single-cell data analysis.

PhenoCycler-Fusion writes whole slide scans in QPTIFF format that contains all image layers and metadata. Whole slide image files written from PhenoCycler-Fusion are viewable in the Phenochart software.

Users can segment cells in Akoya's inForm software. These cell segmentation tables can be used in external applications.

PHENOCYCLER-FUSION EXPERIMENT OVERVIEW

In a PhenoCycler-Fusion experiment, a tissue section on a slide is manually stained using a panel of PhenoCycler Antibodies simultaneously. After staining is complete, a flow cell is attached on top of the slide and placed into the Flow Cell Slide Carrier. The carrier is then placed into the Fusion stage and a PhenoCycler-Fusion run is performed on the stained tissue section using the PhenoCycler-Fusion system.

A PhenoCycler run is fully automated and executed by the controller software. PhenoCycler Reporters are dispensed onto the tissue by the PhenoCycler instrument and revealed via fluorescence microscopy using the PhenoCycler-Fusion. Runs are comprised of multiple cycles. In each cycle, the reporters reveal up to 3 markers of interest (and DAPI) simultaneously. The tissue is imaged in each spectrally distinct fluorescence channel, and then the reporters are removed from the tissue by a gentle isothermal wash. The repetition of these cycles using different reporters allows for the visualization of a full PhenoCycler antibody panel in a single experiment and on the same tissue area.

The PhenoCycler workflow uses a proprietary chemistry that preserves both tissue morphology and the antibody staining throughout the experiment. Users can purchase PhenoCycler inventoried antibodies or customize their panel by conjugating purified antibodies to PhenoCycler Barcodes. (See **Appendix B: Custom Conjugation**.)

The procedures for PhenoCycler Antibody staining and PhenoCycler multicycle runs are illustrated in Figures 1.6 and 1.7.

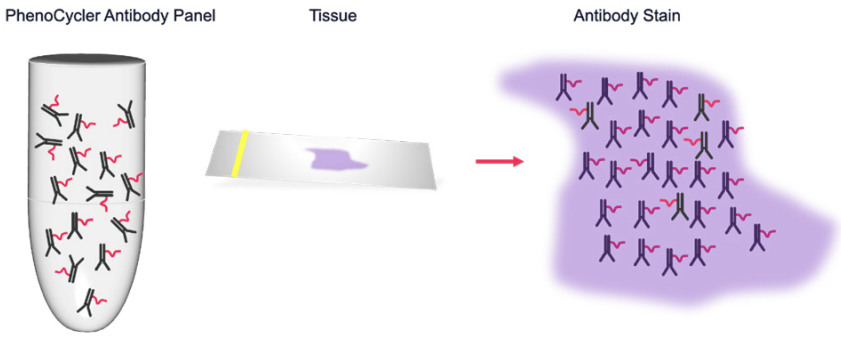


FIGURE 1.6. Single staining step. A panel of PhenoCycler Antibodies is used to stain a tissue section in a single step.

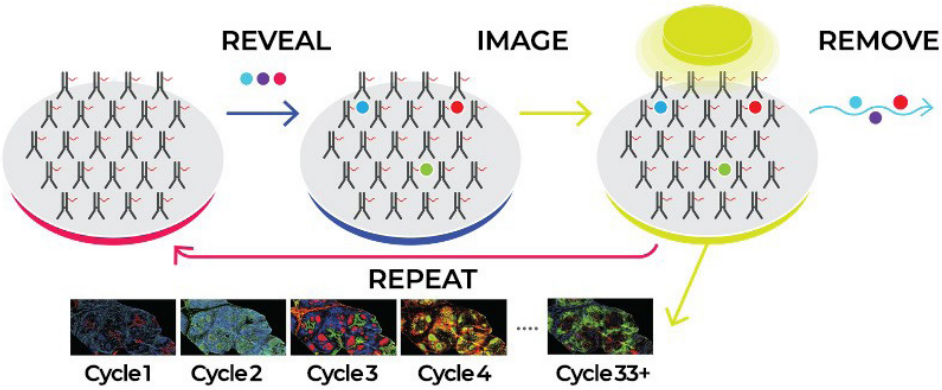


FIGURE 1.7. PhenoCycler-Fusion runs. The cyclical process of Reveal—Image—Remove—Repeat.

In the PhenoCycler workflow, the PhenoCycler-Fusion system performs consecutive cycles of dispensing reporters, acquiring images, and removing reporters:

- **Reveal:** Up to 3 PhenoCycler Reporters are dispensed onto the antibody-stained tissue slide by the PhenoCycler instrument and allowed to incubate. PhenoCycler Reporters hybridize to complementary barcodes conjugated to target antibodies.
- **Image:** The tissue is scanned by the PhenoImager Fusion microscope that is integrated to the PhenoCycler instrument.
- **Remove:** A gentle isothermal wash is performed to remove the reporters.

During a PhenoCycler-Fusion multicycle experiment, acquired images are processed in parallel. At the end of the run, images are available as whole slide scans in a QPTIFF format that contains the image layers and metadata. Whole-slide image files written from PhenoCycler-Fusion are viewable in Akoya's Phenochart software.

Cell segmentation can then be performed using Akoya's inForm software. The cell segmentation tables can be used in external applications.

PHENOCYCLER-FUSION PROCEDURE OVERVIEW

1. Adhere tissue sections to recommended standard 1 inch x 3 inch positively charged glass microscope slides. (See **Chapter 2: Sample Preparation**.)
2. Design a PhenoCycler Antibody panel. Antibody panels are customizable and can include both commercially available inventoried PhenoCycler Antibodies and custom-conjugated antibodies. Instructions on conjugating PhenoCycler Barcodes to antibodies of interest are included at the end of this guide. (See **Appendix B: Custom Conjugation**.) When designing the panel, verify that each antibody is conjugated to a unique barcode; barcodes may not be duplicated within a panel.
3. Stain the tissue with the PhenoCycler antibody panel. (See **Chapter 3: Tissue Staining**.)
4. Using the Experiment Designer app, create and design the experiment protocol and reporter well plate layout. (See **Chapter 4: Experiment Protocol and Reporter Plate Design**.)
5. Prepare the PhenoCycler Reporter plate using the layout prepared in Step 4. Reporters complementary to the barcodes used in the antibody panel are organized into groups of <3 spectrally distinct reporters. Each group of reporters, in addition to DAPI, comprises 1 cycle and is placed into separate wells of a 96-well plate to be dispensed onto the tissue during separate PhenoCycler cycles. (See **Chapter 5: Preparing PhenoCycler Reporter Plate**.)
6. Prior to a PhenoCycler-Fusion run, attach a Flow Cell to the antibody-stained tissue slide. Assemble the Flow Cell Slide Carrier with a blank Flow Cell and connect the carrier to the PhenoCycler instrument lines. Launch the Fusion controller software and follow the prompts to prepare and prime the PhenoCycler instrument. (See PhenoImager Fusion User Guide, Chapters 3 and 6.)
7. After preparing and priming the instrument, load the Flow Cell that is attached to the stained tissue section into the Flow Cell Slide Carrier and place it onto the stage.
8. Initiate the PhenoCycler multicycle run.
9. After the PhenoCycler multicycle run is complete, remove the tissue and replace it with a blank Flow Cell in the Slide Carrier.
10. Clean the PhenoCycler instrument.

USER GUIDE OVERVIEW

An illustrated overview of the entire PhenoCycler procedure with references to the chapters where more information can be found for each step is provided below. (See Table 1.10.)

TABLE 1.10. User guide overview

Step	Title	Reference
	Sample Preparation	Chapter 2
	Tissue Staining	Chapter 3
	Experiment Protocol and Reporter Plate Design Using the PhenoCycler Experiment Designer Software	Chapter 4
	PhenoCycler Reporter Plate Preparation	Chapter 5
	Specifications	Appendix A
	Custom Conjugation	Appendix B

CHAPTER 2

Sample Preparation

Step	Stopping Point	Page
FF Tissue Sectioning Guidelines	Can be stored at -80°C for up to 6 months.	26
FFPE Tissue Sectioning	Can be stored at 4°C for up to 6 months.	29

This chapter outlines the techniques for preparation and storage of tissue samples for PhenoCycler-Fusion experiments. These steps must be completed prior to starting the PhenoCycler-Fusion workflow. (See Figure 2.1.)

For PhenoCycler-Fusion experiments, FF or FFPE tissues are sectioned and directly adhered to standard sized (1 inch x 3 inch) positively charged glass microscope slides. Use of non-standard microscope slides and/or tissue preparation techniques that deviate from this protocol is not recommended.

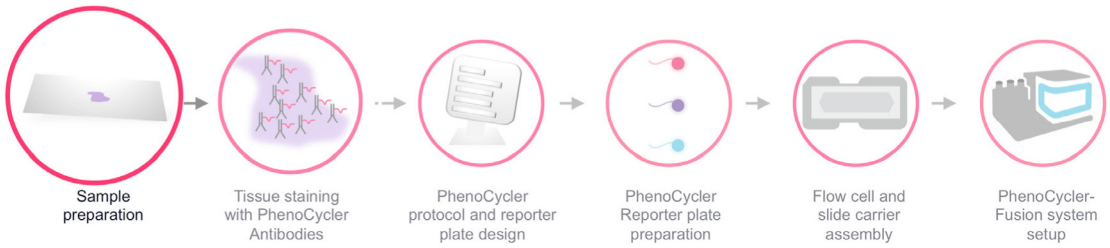


FIGURE 2.1. PhenoCycler workflow, sample preparation.

FRESH FROZEN TISSUE SECTIONING GUIDELINES

Fresh frozen tissue sections are mounted directly onto slides. Appropriate preparation and storage of tissue sections are critical to ensure sample integrity. The instructions provided in this guide are specific to the PhenoCycler workflow and are not intended to be a comprehensive guide for tissue processing.

GUIDELINES

Tissue Sections

- Tissue sections adhered directly onto slides can be stored at -80°C for up to 6 months before staining.
- Tissue thickness must not exceed 10 µm since this can affect the autofocusing capabilities of the microscope.
- For best results, tissue sections should be completely adhered to the slide without folds or tears.
- To ensure that tissue sections are not damaged, it is critical that the tissue slides are not stacked on top of one another.

PRE-EXPERIMENT PREPARATION

Materials Not Provided

TABLE 2.1. List of tissue, consumables, and laboratory equipment required (but NOT provided) for FF tissue sectioning

Type	Item	Vendor	P/N
Tissue	FF tissue block of interest	--	--
Consumables	Dry ice	Customer Choice	MLS
	Compressed/canned air duster	Customer Choice	MLS
Equipment	Glass microscope slides, positively charged (1 inch x 3 inch)*	Leica Slide White, Apex Superior Adhesive or Fisherbrand, Superfrost Plus	3800080 or 12-550-15
	Microscope slide box	Fisher Scientific	03-448-1
	Polystyrene container	Customer Choice	MLS
	Blade for tissue sectioning	Electron Microscopy Sciences Low Profile Microtome Feather® Blade	63069-LP
	Cryostat for tissue sectioning	Customer Choice	MLS

*Recommended slide dimensions: 24–25 mm width x 74–75 mm length.

NOTE > ***Do not use the extended frost/extended label versions of the slides. These slides may interfere with the proper adhesion of the Flow Cell and/or result in Flow Cell breakage.***

PREPARE CRYOSTAT CHAMBER

Standard cryostats with temperature control are recommended for tissue sectioning. Most tissues are sectioned in temperatures ranging from -15°C to -25°C. The exact temperature is unique to each tissue type and should be determined according to standard sectioning procedures.

FRESH FROZEN TISSUE SECTIONING PROCEDURE

1. Set the cryostat chamber to tissue-specific temperature range.
2. Place the slide storage box in the cryostat chamber to equilibrate to the cryostat temperature.
3. Once the cryostat reaches the programmed temperature, transfer the tissue from -80°C storage to the cryostat using a container filled with dry ice.
4. Use compressed air to remove dust and lint from the slides before use.
5. Place the prepared slides in the cryostat chamber to equilibrate temperature for 20–30 seconds.
6. Section the tissue at a thickness of 5–10 µm.

CRITICAL > ***Do not exceed a 10 µm thickness when sectioning tissue. The autofocusing capabilities of the microscope will be adversely affected.***

CRITICAL > ***Avoid folds and tears in the tissue; these artifacts will negatively affect image quality and data analysis.***

7. Gently place the tissue section in the center of the slide within the imageable area. (See Figure 2.2., gray area.)

NOTE > ***Ensure the tissue section is placed in the center of the slide as illustrated in Figure 2.2 (gray area). Print this page and use the true-to-size figure below as a guide. Tissue placed under the adhesive portion of the Flow Cell will affect bonding and will likely fail. This could result in a leaking Flow Cell and hence loss of the tissue slide. Additionally, ensure the tissue is placed on the positively charged side of the slide to ensure proper adhesion.***

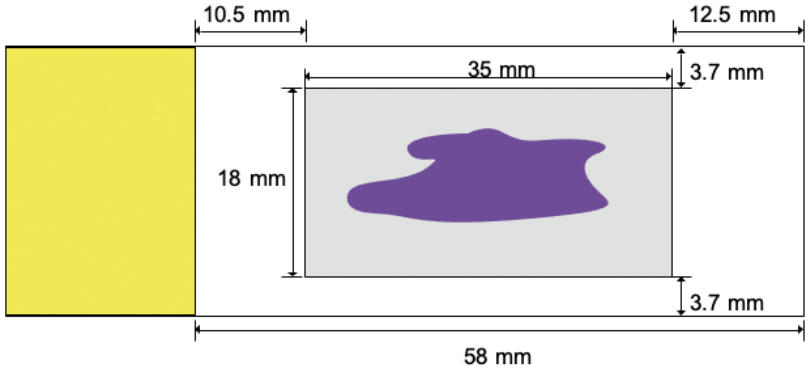


FIGURE 2.2. Fresh frozen tissue placement guide.

8. Adhere the tissue section to the slide by placing a gloved finger under the slide for 1-2 seconds.

CRITICAL > ***Do not keep your finger on the slide for longer than the minimum time necessary to melt the Optimal Cutting Temperature (OCT) compound.***

NOTE > ***The directed heat transfer should melt the OCT, thereby ensuring tissue adherence. Chemical fixation of the tissue will take place during the staining protocol.***

9. Place the mounted slide in a single slot of a microscope slide box.
10. Repeat Steps 6–10 for each tissue section.
11. Once complete, cover the slide storage box with the lid.
12. Place the box of mounted slides on dry ice for transport to -80°C storage. (See Figure 2.3.)

FIGURE 2.3. Mounted tissue stored in slide storage box.

STOPPING POINT >

Samples can be stored at -80°C for up to 6 months if prepared and stored properly. Limit exposure to changes in temperature and keep the storage box upright and secure to minimize slide movement.

NOTE > ***Tissue processing and sectioning are critical steps and need to be performed by trained users. Resources for tissue processing best practice procedures can be found on the Akoya website at Tissue Processing–Best Practices.***

FORMALIN-FIXED, PARAFFIN-EMBEDDED TISSUE SECTIONING

Formalin-fixed, paraffin-embedded tissue sections are mounted onto slides. Preparation and storage of tissue sections are critical for sample integrity. The instructions outlined in this guide are specific to the PhenoCycler workflow and are not intended to be a comprehensive guide on tissue processing. Further guidance on tissue processing for FFPE samples can be found on the Akoya website at Tissue Processing–Best Practices.

GUIDELINES

Tissues

- FFPE Tissues sectioned onto slides can be stored at 4°C for up to 12 months.
- Do not to exceed a thickness of 10 µm when sectioning tissue since this may disrupt the autofocusing capabilities of the microscope.
- For best results, the tissue should be completely adhered to the slide with minimal tears or folds.
- To ensure that tissue sections are not damaged, it is critical that the tissue slides are stored properly and not stacked on top of one another.

PRE-EXPERIMENT PREPARATION

Materials Not Provided

TABLE 2.2. Tissue, consumables, and laboratory equipment required (but NOT provided) for FFPE tissue sectioning

Type	Item	Vendor	P/N
Tissue	FFPE tissue block	--	--
Consumables	Compressed/canned air duster	Customer Choice	MLS
	Glass microscope slides, positively charged (1 inch x 3 inch)	Leica Slide White Apex Superior Adhesive or Fisherbrand Superfrost Plus	3800080 or 12-550-15
Equipment	Microtome for tissue sectioning	Customer Choice	MLS
	Blade for tissue sectioning	Electron Microscopy Sciences Low Profile Microtome Feather® Blade	63069-LP
	40°C water bath	Customer Choice	MLS
	Angled slide holder* (recommended but optional)	Customer Choice	MLS
	Microscope slide box	Fisher Scientific	03-448-1

*For storing and drying the slides after adhering tissue.

PREPARE MICROTOME

Prepare the Microtome for use at RT following the standard operating procedures of the instrument.

FFPE TISSUE SECTIONING PROCEDURE

1. Prepare a water bath at 40°C and place it next to the microtome.
2. Prepare a clean, dry surface for placing the coated slides next to the microtome.
3. Use compressed air to remove dust and lint from the slides prior to use.
4. Place the slides next to the microtome.
5. Insert a new blade for sectioning each new block or as paraffin accumulates on the blade.
6. Section the tissue at a thickness of 5–10 µm.

CRITICAL > ***Do not exceed a thickness of 10 µm when sectioning tissue as this can disrupt the autofocusing capabilities of the microscope. Avoid folds and tears as these artifacts will affect image quality and data analysis.***

7. Place the sectioned tissue in the water bath for a few seconds to allow the tissue to flatten out.
8. Once the tissue is completely flat and devoid of folds or wrinkles, quickly place a slide in the water bath and gently move it toward the tissue. This technique helps the tissue lay on the slide as it is removed from the water bath.

NOTE > ***Make sure the tissue section is placed in the center of the slide. (See Figure 2.4.) Print this page and use the true-to-size figure below as a guide. Tissue placed under the adhesive portion of the Flow Cell will affect bonding and will likely fail. This could result in a leaking Flow Cell and hence loss of the tissue slide. Additionally, ensure the tissue is placed on the positively charged side of the slide to ensure proper adhesion.***

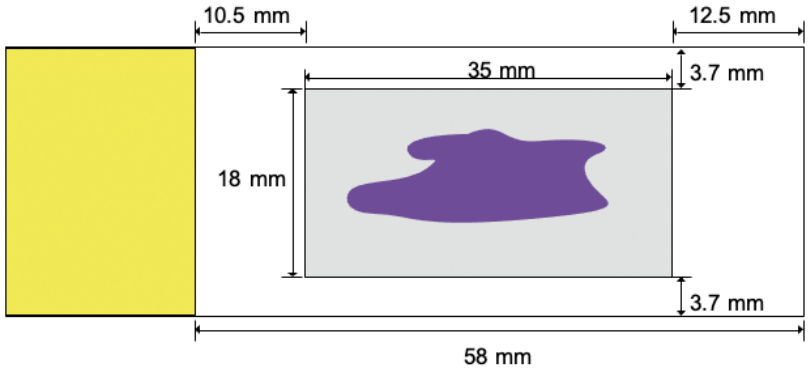


FIGURE 2.4. Approximate accessible imaging area (in gray) for PhenoCycler Flow Cell Carrier. The sample is illustrated in the center of the imaging area (in purple). The smaller square on the left (in yellow) indicates the slide label..

9. With the tissue facing up, place the slide on a clean surface or on the angled slide holder and let it dry overnight at RT. (See Figure 2.5.)

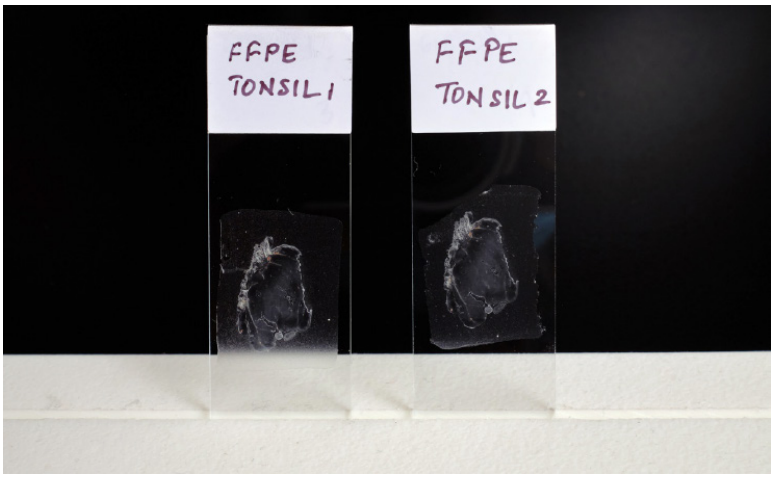


FIGURE 2.5. FFPE tissue drying overnight on angled slide holder.

10. Repeat Steps 6–9 for each tissue section.

11. When the sections are dry, place each tissue slide in a single slot of the storage box and cover the storage box with the lid.

STOPPING POINT >

Samples can be stored at 4°C for up to 6 months if stored properly. Store slide storage box in a secure location and kept upright to minimize movement of slides.

CHAPTER 3

Tissue Staining

FF Tissue Staining

Duration	Step	Time	Stopping Point
5.5 hours	Fresh Frozen Tissue Pre-Staining: Adhere Tissue to Slide	1.5 hours	--
	Fresh Frozen Tissue Staining: Stain Tissue with panel of PhenoCycler Antibodies	3 hours	--
	Fresh Frozen Tissue Post-Staining: Wash tissue to remove unbound antibodies from tissue and adhere bound antibodies to tissue section	50 minutes	--
	Store the stained samples in storage buffer in Coplin jar at 4°C	--	Store up to 5 days

FFPE Tissue Staining

Duration	Step	Time	Stopping Point
7 hours	FFPE Tissue Pre-Staining: Deparaffinization and antigen retrieval	2.5 hours	--
	FFPE Tissue Staining: Stain Tissue with panel of PhenoCycler Antibodies	3 hours	--
	FFPE Tissue Post-Staining: Wash tissue to remove unbound antibodies from tissue and adhere bound antibodies to tissue section	50 minutes	--
	Store the stained samples in storage buffer in Coplin jar at 4°C	--	Store up to 5 days

Tissues must be mounted on slides prior to tissue staining. Buffers and reagents required to perform tissue staining are provided in the Sample Kit for PhenoCycler-Fusion. PhenoCycler Antibodies are purchased separately.

Tissues are stained with the entire antibody panel at once. This can be entirely made of commercial PhenoCycler Antibodies and custom-conjugated PhenoCycler Antibodies or a combination of both. It is critical that each PhenoCycler Barcode is used only once in the antibody panel. Tissues are stained with an Antibody Cocktail Solution comprised of Blocking Buffer and the panel of PhenoCycler Antibodies.

The entire staining process will take approximately 5.5 hours for FF tissue sections and 7 hours for FFPE tissues. This time includes a 3-hour incubation step. Do not exceed or shorten this incubation time.

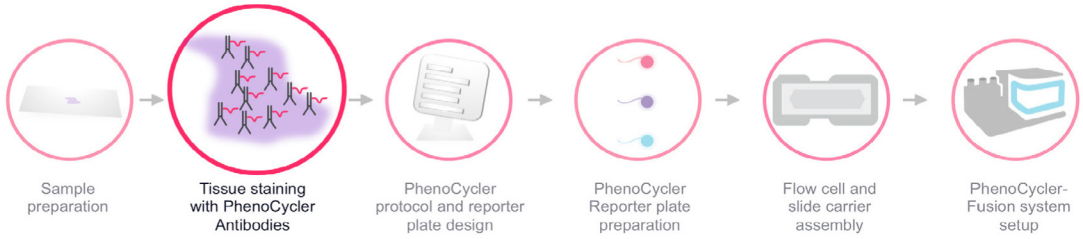


FIGURE 3.1. PhenoCycler workflow—tissue staining.

GUIDELINES

Terminology

- In the protocol, the term “sample slide/s” refers to tissue sections mounted onto the glass microscope slides.
- The PhenoCycler Antibody is a successfully validated antibody conjugated to a PhenoCycler Barcode (nomenclature: Ab-BXxxx). See the Akoya Biosciences website for an up-to-date list of available antibodies at PhenoCycler Antibodies.

Sample Slide Handling

- Avoid tissue drying by minimizing tissue exposure to air. Tissue drying is detrimental to staining quality.
- Avoid dispensing liquid directly onto the tissue to avoid damaging it. Always pipette solutions onto the corner of the slide and allow the liquid to flow over the tissue.
- Best practice is to stain >1 sample slide with the same antibody panel as a backup.

Slide Staining Box Use

- During the 3-hour antibody incubation step, the sample slide is placed in a slide staining box covered with the Antibody Cocktail solution. Parafilm is added on top to ensure the solution evenly covers the sample. The slide staining box should be placed on a stable surface with minimal shaking or vibration. The box is closed with a lid to prevent tissues from drying out.
- When transferring samples between Coplin jars and the slide staining box, you can gently remove any excess liquid on the slide by dabbing the edge gently with a paper towel or Kimwipe.

CRITICAL > ***In some steps, liquids are dispensed onto sample slide/s inside the slide staining box. Always rinse the slide staining box with distilled water between uses to minimize cross-contamination.***

CRITICAL > ***Incubation times have been optimized for fixing or staining the tissue to ensure the tissue does not dry. Do not exceed or shorten these recommended incubation times.***

Safety

Acetone, paraformaldehyde (PFA), and methanol are toxic chemicals and should be handled following Material Safety Data Sheet (MSDS) guidelines. Dispose of each in the designated hazardous waste immediately after use.

Coplin Jars

Coplin jars can be reused after rinsing with ddH_2O.

NOTE > ***Do not reuse jars without washing.***

CRITICAL > ***Do not use PAP pens during staining to create hydrophobic barriers. This may cause issues during the PhenoCycler-Fusion run since fluids may not be able to pass over the tissue properly.***

FRESH FROZEN TISSUE PRE-STAINING

This section describes the preparation of FF tissues for staining with PhenoCycler Antibodies. When working with freshly conjugated antibodies (described in Appendix B: Custom Conjugation), we recommend waiting at least 2 days before using the antibodies for tissue staining. Otherwise, high levels of nuclear background may be observed.

CRITICAL > ***Allow the Hydration, Staining, and Storage Buffers to equilibrate to RT before using. Prepare all buffers and consumables ahead of time to prevent sample degradation.***

PRE-EXPERIMENT PREPARATION

Materials Provided

The following items are provided in the Sample Kit for PhenoCycler-Fusion.

TABLE 3.1. Sample Kit for PhenoCycler-Fusion

Item	Storage	P/N
Hydration Buffer	4°C	7000017
Staining Buffer		
N Blocker		
J Blocker		
G Blocker	-20°C	
S Blocker		
Flow Cells	RT	

Follow these guidelines for materials listed in Table 3.1:

- Obtain these materials now.
- Keep Blockers in an ice bucket.
- Allow Hydration and Staining Buffers to equilibrate to RT.

Materials Not Provided

The following items are required (but NOT provided) in the Sample Kit for PhenoCycler-Fusion.

TABLE 3.2. Antibodies

Item	Storage
PhenoCycler Antibodies	4°C
Custom-Conjugated Antibodies	4°C

Follow these guidelines for materials listed in Table 3.2.

- Obtain these materials immediately before use in the section on Pre-Staining Procedure and place on ice.
- Storage Buffer and Fixative Reagent will be used in the section on Fresh Frozen Tissue Post-Staining.

Materials Not Provided

The following items are required (but NOT provided) for tissue staining.

TABLE 3.3. List of chemicals, lab supplies, and equipment required for the tissue staining

Type	Item	Vendor	P/N
Solvents	Acetone, dispense right before use. (~40 mL per Coplin jar)	Sigma-Aldrich	650501-1L
	Refrigerated methanol (40 mL per Coplin jar); keep at 4°C until use.	Sigma-Aldrich	34860-1L-R
Chemicals/Buffers	16% PFA	Electron Microscopy Sciences	15710
Plastic Consumables/Tools	Coplin jars	VWR	513200
	Slide staining box	VWR	M918-2
	Parafilm	Customer Choice	MLS
	Scissors	Customer Choice	MLS
	Drierite™ absorbent beads	Fisher Scientific	23-116582
	1.5 mL Eppendorf tubes	Customer Choice	MLS
	50 mL conical tube	Customer Choice	MLS
	Ice bucket	Customer Choice	MLS
Laboratory Equipment	Fume hood	Customer Choice	MLS

Prepare Slide Staining Box

- Fill the tray with ddH_2O until the bottom layer is covered.

Prepare Drierite Absorbent Beads

- Locate an empty pipette tip box with a lid or similar container.
- Immediately prior to obtaining samples in the Pre-Staining Procedure section (Tissue Retrieval, Step 3), fill the bottom with Drierite absorbent beads (approximately 1–2 cm deep).
- Cover with lid.

Preparing the Antibody Cocktail Solution

When preparing the Antibody Cocktail Solution, make sure to factor in the number of antibodies and volume per antibody. The total volume of antibodies will be subtracted to determine the Antibody Stock Solution used per sample slide. (See Table 3.4.)

- Retrieve PhenoCycler antibodies from 4°C and place on ice. **CAUTION**: If antibodies have just been custom-conjugated (described in Appendix B: Custom Conjugation), allow at least 2 days before using them for tissue staining or high nuclear background staining may be observed.
- The total volume of the staining solution made for each slide is 200 µL, of which 190 µL will be applied to each sample slide.
- If the dilution factor for an antibody is 1:200, the amount of antibody used per sample slide will be 1 µL.
- For commercial PhenoCycler Antibodies, recommended dilution factors are reported in the PhenoCycler Antibody Dilutions document.
- For custom-conjugated antibodies, the volume of antibody solution used to stain any tissue needs to be determined by titration. Recommended starting dilution factors are FF at 1:250 and FFPE at 1:50.
- If you are staining several samples at the same time, you should make a stock solution accordingly.

To calculate the volume of Blocking Buffer per sample (see Table 3.7), determine the Total Volume of Antibodies (depends on the total number of PhenoCycler Antibodies) and subtract it from Total Volume Per Tissue of the Antibody Cocktail (200 µL). (See Table 3.4.)

[Total Volume Per Tissue (200 µL)] – [Total Antibody Volume] = Blocking Buffer Volume

TABLE 3.4. Volume of Blocking Buffer (µL)

# of Antibodies	Volume of Each Antibody (µL)	Total Volume of Antibodies (µL)	Volume of Blocking Buffer (µL)
24	1	24	176
8	1 or 2	10	190
8	2	16	184

NOTE > ***For custom-conjugated antibodies, the volume of antibody solution used to stain any tissue needs to be determined by titration. The recommended starting antibody dilution factor for FF is 1:250.***

Example 1

If 24 PhenoCycler Antibodies are used to stain a single tissue with 1 µL of each antibody to be added for a total of 24 µL of antibodies, then 176 µL of Blocking Buffer should be used.

200 µL – 24 µL = 176 µL

Example 2

If 8 PhenoCycler Antibodies are used to stain a single tissue with 1 µL of each antibody to be added for 6 of them and 2 µL each for 2 of them for a total of 10 µL of antibodies, then 190 µL of Blocking Buffer should be used.

200 µL – 10 µL = 190 µL

Example 3

If 8 PhenoCycler Antibodies are used to stain a single tissue with 2 µL of each antibody to be added for a total of 16 µL of antibodies, then 184 µL of Blocking Buffer should be used.

200 µL – 16 µL = 184 µL

PRE-STAINING PROCEDURE

To pre-stain FF samples:

1. Set up Coplin jars for FF samples.

During the following steps, the sample slide/s will be incubated in various PhenoCycler reagents in Coplin jars and the slide staining box. For efficient tissue staining, prepare and label 4 Coplin jars as follows:

   a. 40 mL Hydration Buffer.
   b. 40 mL Hydration Buffer.
   c. 40 mL Staining Buffer.
   d. 40 mL Pre-Staining Fixing Solution.

NOTE > ***Wait until Step 6 to prepare and dispense the Pre-Staining Fixing Solution.***

2. Retrieve the tissue:
   a. Prepare 40 mL of acetone in a Coplin jar.
   b. With a prepared box of 1-2 cm Drierite beads in hand, obtain sample slide/s from -80°C storage.
   c. Place the slides in the box directly on top of the Drierite beads with the tissue facing up. (See Figure 3.2.) Close the lid and wait 5 minutes.

FIGURE 3.2. FF tissue sample in box with Drierite beads.

3. Incubate in acetone:
   a. Remove the sample slides from the Drierite beads and place in the Coplin jar containing acetone.
   b. Incubate for 10 minutes in acetone.
4. Dry the tissue:
   a. Remove the sample slide/s from the acetone.
   b. Place the sample slide/s in the slide staining box with tissue facing up. (See Figure 3.3.)
   c. Let the sample slide/s sit in the box for up to 2 minutes.

FIGURE 3.3. FF tissue sample drying in slide staining box.

NOTE > ***Immediately dispose of acetone in the proper waste container.***

5. Hydrate the tissue:
   a. Lift and immerse each sample slide/s into the first Coplin jar of Hydration Buffer 2–3 times to ensure removal of acetone from the top and bottom of the slide/s.
   b. Incubate for 2 minutes at RT. (Save Coplin jar for use in step 7.a.)
   c. Place each sample slide/s into the second Coplin jar containing 40 mL of Hydration Buffer.
   d. Incubate for another 2 minutes at RT for a total of 2 washes. (Save Coplin jar for use in step 7.c.)
   e. Prepare the Pre-Staining Fixing Solution as described in Step 6 during incubation.
6. Fix the tissue:
   a. Prepare the Pre-Staining Fixing Solution in a Coplin jar. (See Table 3.5.)

TABLE 3.5. Pre-staining fixing solution

Pre-Staining Fixing Solution (mL)	1 Jar (≤5 samples)	2 Jars (6-10 samples)
16% PFA	4	8
Hydration Buffer	36	72
Total Volume	40	80

NOTE > ***The Pre-Staining Fixing Solution is 1 part 16% PFA solution in 9 parts of Hydration Buffer at 1:9 (v/v) for a final concentration of 1.6% PFA. Stock PFA must be 16%.***

   b. Add Pre-Staining Fixing Solution to a clean Coplin jar.
   c. Add the sample slide/s to the Coplin jar containing Pre-Staining Fixing Solution.
   d. Incubate for 10 minutes at RT.
7. Wash the tissue:
   a. Remove the sample slide/s from the Pre-Staining Fixing Solution and place them in Coplin jar containing Hydration Buffer used from the tissue hydration steps.
   b. Lift and immerse the sample slide 2–3 times to make sure that the Pre-staining Fixing Solution is completely removed from both the top and bottom of the slides.

c. Quickly move sample slide/s to the second Coplin jar containing Hydration Buffer used in the tissue hydration steps.

8. Equilibrate tissue in Staining Buffer:

a. Move sample slide/s to the Coplin jar containing Staining Buffer.

b. Equilibrate sample slide/s by incubating for 20-30 minutes in the Staining Buffer.

c. Prepare Antibody Cocktail during equilibration. (See below Fresh Frozen Tissue Staining.)

NOTE > ***Sample slides can stay in the Staining Buffer for a maximum time of 30 minutes prior to antibody staining.***

FRESH FROZEN TISSUE STAINING

Understanding Antibody Dilution

Each PhenoCycler Antibody is optimized to a specific dilution factor to offer the best staining performance in control tissues. In some cases, antibodies may have to be re-titrated to optimize for specific tissues of interest. We recommend starting with the dilution factor indicated on the antibody dilution document. Consider the dilution factors indicated for the species (human or mouse) and tissue type (FF or FFPE) being tested. The total volume of the staining solution per tissue sample is the sum of the volume of each antibody and the Blocking Buffer, equaling 200 µL.

Refer to the dilution factor examples in Table 3.6 to verify how to achieve the correct dilution factor. (See Table 3.6.)

TABLE 3.6. Dilution factor examples

Dilution Factor	1:200	1:500
Antibody Volume per sample slide (µL)	1.0	0.4*
Total Volume of Antibody Cocktail per sample slide (µL)	200.0	200.0

*We do not recommend pipetting <1 µL. If the volume pipetted will be <1 µL, we recommend making a stock solution first.

- If the dilution factor of the antibody of interest is 1:200, 1 µL of antibody is required in the total volume of 200 µL of Antibody Cocktail.
- If the dilution factor of the antibody of interest is 1:500, 0.4 µL of antibody is required in the total volume of 200 µL of Antibody Cocktail.

PREPARING THE ANTIBODY COCKTAIL SOLUTION

1. Remove selected antibodies from 4°C and keep on ice until use. Spin down the tubes to collect any liquid from caps.

2. Prepare a stock solution of Blocking Buffer to be used for the Antibody Cocktail/s. (See Table 3.7.)

TABLE 3.7. Blocking Buffer components

PhenoCycler Reagent (µL)	2 Samples	4 Samples	6 Samples	8 Samples	10 Samples
Staining Buffer	362	724	1086	1448	1810
N Blocker	9.5	19	28.5	38	47.5
G Blocker	9.5	19	28.5	38	47.5
J Blocker	9.5	19	28.5	38	47.5
S Blocker	9.5	19	28.5	38	47.5
Total Volume	400	800	1200	1600	2000

CRITICAL > ***Prepare Blocking Buffer just before staining--no earlier than 1 hour before. Keep on ice until ready to use.***

3. Label 1 tube for each unique Antibody Cocktail Solution.
4. Add Blocking Buffer to each of the tubes designated for Antibody Cocktail Solution/s. The volume of Blocking Buffer to be prepared for each sample slide can vary depending on the number and volume of antibodies used. The final volume of the Antibody Cocktail is a total of 200 µL per tissue. Refer to the antibody datasheet for the recommended dilution factor.

CRITICAL > ***The volume of Blocking Buffer should always be greater than 60% of the total Antibody Cocktail solution. Otherwise, sufficient blocking may not occur. If the Blocking Buffer must be <60% of the total Antibody Cocktail to accommodate more antibodies, decrease the volume of the Staining Buffer. Do not adjust the volumes of blocking components.***

5. Add the appropriate volume of each PhenoCycler Antibody to the Antibody Cocktail Solution.
6. Pipette to mix or vortex gently.

TISSUE STAINING

CRITICAL > ***Each sample slide should be removed from the Coplin jar containing Staining Buffer and stained one at a time to avoid drying of the tissue.***

1. Cut a rectangular piece of parafilm roughly the size and shape of the non-label portion of the sample slide (approximately 1 inch x 2 inch).
2. Draw up 190 µL of the Antibody Cocktail into a pipette. Set aside.
3. Remove sample slide from the Coplin jar containing Staining Buffer and use a Kimwipe to carefully and gently absorb excess buffer.
4. Place sample slide on the tray of the slide staining box.
5. Quickly dispense the 190 µL of the Antibody Cocktail to the top corner of the sample slide. (See Figure 3.4.) Ensure that the liquid covers the entire tissue.

CRITICAL > ***Be careful not to pipette the solution directly onto the tissue and minimize bubbles.***

FIGURE 3.4. Dispensing Antibody Cocktail onto FF sample slide.

6. Gently place the parafilm on top of the Antibody Cocktail Solution. (See Figure 3.5.)
7. Repeat Steps 1-6 above for each sample slide.

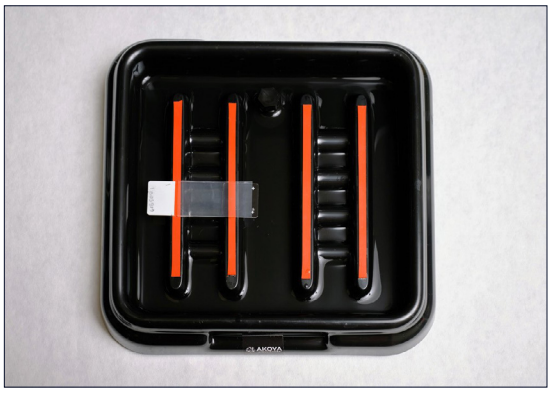


FIGURE 3.5. Placing parafilm on slide after dispensing Antibody Cocktail.

8. Place the lid on the slide staining box.
9. Incubate for 3 hours at RT.

After 3 hours, proceed immediately to Fresh Frozen Tissue Post-Staining. It Is critical to prepare the initial solutions from Fresh Frozen Tissue Post-Staining before the end of this 3-hour incubation.

CRITICAL > ***Place the slide staining box on a stable surface that is free of vibrations.***

FRESH FROZEN TISSUE POST-STAINING

The following steps are performed to remove unbound antibodies and fix the bound antibodies to tissues.

CRITICAL > ***Prepare all reagents and consumables ahead of time to prevent degradation of sample/s.***

Pre-Experiment Preparation

Materials Provided

TABLE 3.8. Staining Buffer and Storage Buffer (obtain these materials now)

Item	Storage	P/N
Staining Buffer	4°C	7000017
Storage Buffer		

NOTE > ***These items are components in the Sample Kit for PhenoCycler-Fusion.***

TABLE 3.9. Fixative Reagent

Item	Storage	P/N
Fixative Reagent (1 tube for every 5 tissues [Single-Use])	-20°C	7000017

NOTE > ***This item is a component in the Sample Kit for PhenoCycler-Fusion.***

CRITICAL > ***Do not thaw Fixative Reagent before Step 6.c. Cut the necessary tubes off of strip to avoid thawing the entire strip. Fixative reagent cannot be frozen and reused after initial thawing.***

Materials Not Provided

TABLE 3.10. List of chemicals and lab supplies required (but NOT provided) for the post-staining process

Type	Item	Vendor	P/N
Solvents	Refrigerated methanol, 40 mL per Coplin jar. Keep at 4°C until use in Step 4.	Sigma-Aldrich	34860-1L-R
Chemicals/ Buffers	16% PFA	Electron Microscopy Sciences	15710
	1X PBS	Thermo Fisher Scientific	14190144
Plastic Consumables/ Tools	Slide staining box	VWR	M918-2
	Parafilm	Customer Choice	MLS
	1.5 mL Eppendorf tubes	Customer Choice	MLS
	50 mL conical tube	Customer Choice	MLS

COPLIN JAR CONFIGURATION FOR FRESH FROZEN SAMPLES

In this section, the sample slide/s will be transferred from the solvents to PhenoCycler buffers in Coplin jars. Subsequently, they will be transferred to the slide staining box and finally, to a Coplin jar containing Storage Buffer for storage at 4°C. Prepare and label Coplin jars ahead of time.

- For every 5 samples, fill 2 Coplin jars with 40 mL of Staining Buffer and fill 3 Coplin jars with 40 mL of 1X PBS: these Coplin jars will be used 3 times in Steps 3, 5, and 7.
- Fill Coplin jars designated for Post-Staining Fixing Solution, methanol, and Storage Buffer with 40 mL of the corresponding solution immediately before use.
- The Coplin jar containing Storage Buffer will be used for tissue storage. Each sample slide should be labeled directly on the slide label to differentiate them.

FRESH FROZEN TISSUE POST-STAINING PROCEDURE

To post-stain FF tissue:

1. Wash the tissue:
   a. Following the 3-hour antibody incubation, gently remove the parafilm and place the sample slide/s in the first Coplin jar containing Staining Buffer.
   b. Lift and immerse the sample slide/s 2–3 times to ensure the removal of the Antibody Cocktail from both sides of the slide/s.
   c. Incubate for 2 minutes.
   d. Place the sample slide/s in the second Coplin jar containing Staining Buffer.
   e. Incubate for an additional 2 minutes for a total of 2 washes.
2. Fix the tissue:
   a. Prepare the Post-Staining Fixing Solution. (See Table 3.11.)

TABLE 3.11. Post-staining fixing solution

Post-Staining Fixing Solution (mL)	1 Coplin Jar (<5 samples)	2 Coplin Jars (6-10 samples)
16% PFA	4	8
Storage Buffer	36	72
Total Volume	40	80

NOTE > ***The Post-Staining Fixing Solution is 1 part 16% PFA solution in 9 parts Storage Buffer at a 1:9 (v/v).***

b. Add 40 mL of Post-Staining Fixing Solution to each Coplin jar.

c. Place the sample slide/s in the Coplin jar containing Post-Staining Fixing Solution.

d. Incubate for 10 minutes at RT.

NOTE > ***During the 10-minute incubation, prepare the ice-cold methanol in the Coplin jar for Step 4.***

3. Wash the tissue:

a. Prepare 3 Coplin jars of 1X PBS per 5 sample slides.

b. Remove each sample slide/s from the Coplin jar containing Post-Staining Fixing Solution.

c. Place the sample slide/s in the first Coplin jar containing 1X PBS. Lift and immerse each sample slide 2–3 times to ensure the Fixing Solution is removed from the top and bottom of each slide.

d. Immediately move the sample slide/s to the second Coplin jar containing 1X PBS. Lift and immerse the sample slide 2–3 times.

e. Immediately move the sample slide/s to the third Coplin jar containing 1X PBS for a total of 3 washes. Lift and immerse each sample slide 2–3 times.

4. Incubate in ice-cold methanol:

a. Retrieve ice-cold methanol from the refrigerator at 4°C.

b. Pour approximately 40 mL of methanol into the Coplin jar.

c. Remove each sample slide from the third Coplin jar containing 1X PBS and place them in the Coplin jar containing ice-cold methanol.

d. Incubate at 4°C for 5 minutes.

5. Wash the tissue:

a. Quickly transfer the sample slide/s from methanol to the first corresponding 1X PBS Coplin jar.

CRITICAL > ***Methanol dries tissue faster than other buffers. Move quickly to prevent sample degradation.***

b. Ensure the slide is fully immersed in PBS. Lift and immerse the sample slide 2–3 times to ensure methanol is removed from the bottom and top of the slide.

c. Transfer the sample slide/s to the second 1X PBS Coplin jar. Lift and immerse the sample slide 2–3 times.

d. Transfer the sample slide/s to the third 1X PBS Coplin jar for a total of 3 washes. Lift and immerse each sample slide 2–3 times.

6. Fix the tissue:

a. Rinse and dry the slide staining box if not already done.

b. Add 1 mL of 1X PBS to an Eppendorf tube for every 5 samples that are being prepared.

c. Retrieve 1 aliquot of Fixative Reagent tube from -20°C storage (1 tube for every 5 samples). Each tube should contain 20 µL of reagent. Cut each tube selected for use from the tube strip.

CRITICAL > ***Do not thaw the entire strip. Do not remove Fixative Reagent ahead of time. Let it melt quickly between gloved fingers. Each tube is for single use; do not re-freeze.***

d. Briefly spin down the Fixative Reagent to collect any liquid from the cap.

e. Prepare the Final Fixative Solution by diluting the 20 µL of the PhenoCycler Fixative Reagent in 1 mL of 1X PBS. (See Table 3.12.)

TABLE 3.12. Final fixative solution

Final Fixative Solution (µL)	≤5 Samples	6-10 Samples
1X PBS	1000	2000
Fixative Reagent	20	40

f. Mix thoroughly by vortexing the solution.

g. Cut a rectangular piece of parafilm to the approximate size of the non-label portion of the sample slide (approximately 1 inch x 2 inches).

h. Draw up 200 µL of the Final Fixative Solution with a pipette. Set aside.

i. Remove the sample slide/s from the Coplin jar and place each on the tray of the slide staining box.

j. Add 200 µL of Final Fixative Solution to the top corner of each sample slide. Cover the entire section with reagent. Do not pipette the solution directly onto the tissue.

k. Carefully place the parafilm over the fixative solution.

l. Repeat Steps g-k for all sample slides.

m. Place lid on the slide staining box and incubate for 20 minutes.

7. Wash the tissue:

a. Remove the sample slide/s from the slide staining box, gently remove the parafilm, and place the slide/s in the first Coplin jar containing 1X PBS. Lift and immerse the sample slide 2–3 times to ensure Fixative Solution is removed.

b. Move sample slide/s to the second Coplin jar containing 1X PBS. Lift and immerse each sample slide 2–3 times.

c. Move sample slide/s to the third Coplin jar containing 1X PBS for a total of 3 washes. Lift and immerse each sample slide 2–3 times.

8. Store the tissue:

a. Label a new Coplin jar and pipet 40 mL of Storage Buffer for every 5 sample slides.

b. Place the sample slide/s in the Coplin jar ensuring the tissue faces the same direction for all slides.

c. Cap the Coplin jar and seal around the edges with parafilm (optional) for storage.

STOPPING POINT >

Tissues can now be used directly to run a PhenoCycler-Fusion Experiment. For best results, store at 4°C for ≤5 days.

FFPE TISSUE PRE-STAINING

This section describes the preparation of FFPE tissues for staining with PhenoCycler Antibodies. When working with freshly conjugated antibodies (described in Appendix B: Custom Conjugation), we recommend waiting at least 2 days before using the antibodies for tissue staining. Otherwise, high levels of nuclear background may be observed.

CRITICAL > ***Allow the Hydration, Staining, and Storage Buffers to equilibrate to RT before using. Prepare all buffers ahead of time to prevent sample degradation.***

PRE-EXPERIMENT PREPARATION

Obtain the Sample Kit components listed in Table 3.13 now.

Materials Provided

TABLE 3.13. Sample Kit components

Item*	Storage	P/N
Hydration Buffer	4°C	7000017
Staining Buffer		
N Blocker		
J Blocker		
G Blocker	-20°C	
S Blocker		
Flow Cells	RT	

*These items are components in the Sample Kit for PhenoCycler-Fusion.

Follow these guidelines for materials listed in Table 3.13.

- Keep Blockers in an ice bucket.
- Allow Hydration and Staining Buffers to equilibrate to RT.

Materials Not Provided

TABLE 3.14. Antibodies

Item	Storage
PhenoCycler Antibodies	4°C
Custom-Conjugated PhenoCycler Antibodies	

Follow these guidelines for materials listed in Table 3.14.

- Obtain these materials immediately before use in the **Preparing the Antibody Cocktail Solution** step and place on ice.
- Storage Buffer and Fixative Reagent will be used in the **FFPE Tissue Post-Staining** section.

Materials Not Provided

TABLE 3.15. Chemicals, lab supplies, and equipment required (but NOT provided) for tissue staining

	Item	Vendor	P/N
Solvents	Ethanol or Reagent Alcohol	Sigma-Aldrich	79317-16GA-PB
	1X HistoChoice Clearing Agent	VWR	H103-4L
Chemicals/Buffers	10X AR6 or 10x AR9 Buffer*	Akoya Biosciences	AR6001KT or AR9001KT
	16% PFA	Electron Microscopy Sciences	15710
Plastic Consumables/ Tools	Coplin jars	Customer Choice	MLS
	Slide staining box	VWR	M918-2
	1.5 mL Eppendorf tubes	Customer Choice	MLS
	50 mL conical tube	Customer Choice	MLS
	Parafilm (to cover solutions directly applied to tissue sample slides)	Customer Choice	MLS
	Solvent-resistant containers with lids (Qty 10)	IHC World	IW-2510, EZ-Quick Slide Staining Set
	Slide staining rack	IHC World	IW-2512, EZ-Quick Slide Staining Rack
	Aluminum foil	Customer Choice	MLS
Laboratory Equipment	Pressure cooker	Customer Choice	MLS
	Fume hood	Customer Choice	MLS

*Some clones may require antigen retrieval in AR6 instead of AR9.

Prepare Slide Staining Box

- Fill the tray with ddH_2O until the bottom is covered.

Determine Antibodies to Constitute the Antibody Cocktail for FFPE Samples

- If antibodies have just been custom-conjugated (described in Appendix B: Custom Conjugation) wait >2 days before using them for tissue staining since high nuclear background staining may be observed.

PREPARING THE ANTIBODY COCKTAIL SOLUTION

When preparing the Antibody Cocktail Solution, make sure to factor in the number of antibodies and volume per antibody. The total volume of antibodies will be subtracted to determine the Antibody Stock Solution used per sample slide.

- The total volume of the Antibody Cocktail made for each slide is 200 µL, of which 190 µL will be applied to each sample slide.
- If the antibody dilution factor is 1:200, the amount of antibody used per sample slide will be 1 µL.
- For commercial PhenoCycler Antibodies, recommended dilution factors are reported in the antibody dilutions document.

- When staining several samples simultaneously, make a stock solution accordingly.
- For custom-conjugated antibodies, the volume of antibody solution used to stain any tissue needs to be determined by titration. Recommended starting dilution factors are FF at 1:250 and FFPE at 1:50.
- To determine the volume per sample of the Blocking Buffer (see Table 3.18), first determine the Total Volume of Antibodies (depends on the total number of PhenoCycler Antibodies) and subtract it from Total Volume Per Tissue of the Antibody Cocktail (200 µL). (See Table 3.16.)

Blocking Buffer Volume = [Total Volume Per Tissue (200 µL)] – [Total Antibody Volume]

TABLE 3.16. Volume of Blocking Buffer (µL)

# of Antibodies	Volume of Each Antibody (µL)	Total Volume of Antibodies (µL)	Volume of Blocking Buffer (µL)
24	1	24	176
8	1 or 2	10	190
8	2	16	184

NOTE > ***For custom-conjugated antibodies, the volume of antibody solution used to stain any tissue needs to be determined by titration. The recommended starting antibody dilution factor for titration for FFPE is 1:50.***

Example 1

If 24 PhenoCycler Antibodies are used to stain a single tissue, with 1 µL of each antibody to be added for a total of 24 µL of antibodies, 176 µL of Blocking Buffer should be used.

200 µL – 24 µL = 176 µL

Example 2

If 8 PhenoCycler Antibodies are used to stain a single tissue, with 1 µL of each antibody to be added for 6 of them and 2 µL each for 2 of them (for a total of 10 µL of antibodies), 190 µL of Blocking Buffer should be used.

200 µL – 10 µL = 190 µL

Example 3

If 8 PhenoCycler Antibodies are used to stain a single tissue, with 2 µL of each antibody to be added for a total of 16 µL of antibodies, 184 µL of Blocking Buffer should be used.

200 µL – 16 µL = 184 µL

PREPARE SOLVENTS FOR TISSUE DEPARAFFINIZATION AND HYDRATION

NOTE > ***Organic solvents can be used for up to 2 weeks before they should be changed. Monitor volumes since alcohol will evaporate over time. Dispose of used solvents in dedicated waste containers following institutional protocols.***

CRITICAL > ***It is highly recommended to perform this procedure under a fume hood since organic solvents are highly volatile.***

1. Depending on the container used for the incubation of tissues in the solvent series, determine the volume required to ensure that the slide staining rack is fully submerged in the liquid.
2. Prepare 10 containers with the required volume of the following solvents:
   - HistoChoice Clearing Agent
   - HistoChoice Clearing Agent
   - 100% Ethanol/Reagent Alcohol

- 100% Ethanol/Reagent Alcohol
- 90% Ethanol/Reagent Alcohol
- 70% Ethanol/Reagent Alcohol
- 50% Ethanol/Reagent Alcohol
- 30% Ethanol/Reagent Alcohol
- ddH_2O
- ddH_2O

TISSUE PRE-TREATMENT AND ANTIBODY STAINING

FFPE tissues must undergo deparaffinization, rehydration, and antigen retrieval before antibody labeling. In this protocol, Akoya-recommended procedures for FFPE tissue pre-treatment are provided.

Pre-Staining Procedure

To pre-stain FFPE tissue:

1. Pre-treat the tissue:
   a. Place the sample slide/s in the slide staining rack.
   b. Bake sample slide/s in an incubator at 65°C for 1-3 hours to melt paraffin.
   c. Depending on tissue compostion and sectioning, some samples can have portions partially lift off the slide. To improve tissue adherance to the slide, increase the baking time.
2. Tissue deparaffinization and hydration:

Start the deparaffinization process by placing the sample slide/s in the rack in the following solvent series. Ensure that the sample slide/s are completely submerged in the solvents. Move the rack/s gently to make sure the liquid in the space between the slides is exchanged. Seal the container with a lid during incubation to reduce solvent evaporation.

CRITICAL > ***It is highly recommended to perform this procedure under a fume hood since organic solvents are toxic and highly volatile.***

a. Immerse the staining rack in each of the 10 containers with the following reagents for 5 minutes each:
   - HistoChoice Clearing Agent
   - HistoChoice Clearing Agent
   - 100% Ethanol/Reagent Alcohol
   - 100% Ethanol/Reagent Alcohol
   - 90% Ethanol/Reagent Alcohol
   - 70% Ethanol/Reagent Alcohol
   - 50% Ethanol/Reagent Alcohol
   - 30% Ethanol/Reagent Alcohol
   - ddH_2O
   - ddH_2O

3. Antigen Retrieval:

NOTE > ***If staining less than 5 sample slides, fill a Coplin jar with 50 mL of 1X AR9. If staining 5-24 sample slides, fill an EZ Quick slide staining vessel with 250 mL of 1X AR9.***

a. Dilute the stock 10X AR9 solution to 1X with ddH_2O.

b. If staining less than 5 sample slides, fill a Coplin jar with 50 mL of 1X AR9. If staining 5-24 sample slides, fill an EZ Quick slide staining vessel with 250 mL of 1X AR9.

***ALTERNATIVE BUFFER** (optional) > Some clones may require antigen retrieval in AR6 instead of AR9. In this case, AR6 from Akoya Biosciences (P/N AR6001KT) is recommended. The recommended AR6 antigen retrieval conditions include a 20-minute incubation at high pressure in a pressure cooker.*

Most commercial PhenoCycler Antibodies work with both AR6 and AR9 retrieval methods. If an antibody requires one or the other retrieval methods specifically, it is critical to ensure that the rest of the panel is compatible with the antigen retrieval method selected.

c. Immerse slides in the vessel, ensuring sufficient 1X AR9 to fully cover the sample slide. (See Figure 3.6.)

FIGURE 3.6. Slide Staining Rack immersed in vessel with 1X AR9 Buffer.

d. Cover the vessel with aluminum foil. (See Figure 3.7.)

***NOTE** > Wrapping with aluminum foil will prevent the vapor from the pressure cooker from entering the beaker.*

e. Fill the Instant Pot pressure cooker with approximately 1 L of ddH_2O.

f. Place staining vessel with slides into the pressure cooker.

FIGURE 3.7. Vessel with slides in pressure cooker containing 1 L of ddH_2O.

g. Secure the lid.

h. Set the pressure cooker to the high-pressure protocol and cook the samples for 20 minutes.

i. After the incubation in the pressure cooker, release the pressure and carefully remove the vessel from the pressure cooker. Equilibrate the samples to RT for at least 30 minutes.

CRITICAL > ***Failure to equilibrate samples at RT for at least 30 minutes will result in tissue detachment from the slide.***

   j. Remove the samples from the cooled 1X AR9 solution and quickly immerse them in a Coplin jar filled with ddH_2O. Place the samples in a second Coplin jar filled with ddH_2O and incubate for 2 minutes.

4. Wash the tissue:
   a. Remove the sample slide/s from the water container and place them in the Coplin jar containing Hydration Buffer. Lift and immerse the sample slide 2–3 times.
   b. Incubate for 2 minutes.
   c. Place each sample slide/s into the second Coplin jar of Hydration Buffer.
   d. Incubate for another 2 minutes for a total of 2 washes.
5. Equilibrate the tissue in Staining Buffer:
   a. Move sample slide/s to the Coplin jar containing Staining Buffer.
   b. Equilibrate sample slide/s by incubating for 20–30 minutes in the Staining Buffer.
   c. Proceed to preparing the Antibody Cocktail for FFPE Tissue Staining during equilibration.

NOTE > ***Sample slides can stay in the Staining Buffer for a maximum time of 30 minutes prior to antibody staining.***

FFPE TISSUE STAINING

Understanding Antibody Dilution

Each PhenoCycler Antibody is optimized to a specific dilution factor to offer the best staining performance in control tissues. In some cases, antibodies may have to be re-titrated to optimize for specific tissues of interest. We recommend starting with the dilution factor indicated in PhenoCycler Antibody Dilutions. Consider the dilution factors indicated for the species (human or mouse) and tissue type (FF or FFPE) being tested. The total volume of the staining solution per tissue sample is the sum of the volume of each antibody and the Blocking Buffer, equaling 200 µL.

Refer to the dilution factor examples to verify how to achieve the correct dilution factor. (See Table 3.17.)

TABLE 3.17. Dilution Factor examples

Dilution Factor	1:200	1:500
Antibody volume per sample slide (µL)	1.0	0.4*
Total volume of Antibody Cocktail per sample slide (µL)	200.0	200.0

*We do not recommend pipetting <1 µL. If the volume pipetted will be <1 µL, we recommend making a stock solution first.

- If the dilution factor of the antibody of interest is 1:200, 1 µL of antibody is required in the total volume of 200 µL of Antibody Cocktail.
- If the dilution factor of the antibody of interest is 1:500, 0.4 µL of antibody is required in the total volume of 200 µL of Antibody Cocktail.

Preparing the Antibody Cocktail Solution

To prepare the Antibody Cocktail solution:

1. Remove selected antibodies from 4°C and keep them on ice until use. Spin down the tubes to collect any liquid from caps.
2. Prepare a stock solution of Blocking Buffer to be used for the Antibody Cocktail/s. (See Table 3.18.)

TABLE 3.18. Blocking Buffer components

PhenoCycler Reagent (µL)	2 Samples	4 Samples	6 Samples	8 Samples	10 Samples
Staining Buffer	362	724	1086	1448	1810
N Blocker	9.5	19	28.5	38	47.5
G Blocker	9.5	19	28.5	38	47.5
J Blocker	9.5	19	28.5	38	47.5
S Blocker	9.5	19	28.5	38	47.5
Total Volume	**400**	**800**	**1200**	**1600**	**2000**

CRITICAL > ***Prepare Blocking Buffer just before staining—no earlier than 1 hour before. Keep on ice until ready to use.***

3. Label 1 tube for each unique Antibody Cocktail Solution.
4. Add Blocking Buffer to each of the tubes designated for Antibody Cocktail Solution/s. The volume of Blocking Buffer to be prepared for each sample slide can vary depending on the number and volume of antibodies used. The final volume of the Antibody Cocktail is a total of 200 µL per tissue. Refer to the antibody datasheet for the recommended dilution factor.

CRITICAL > ***The volume of Blocking Buffer should always be greater than 60% of the total Antibody Cocktail solution. Otherwise, sufficient blocking may not occur. If the Blocking Buffer must be less than 60% of the total Antibody Cocktail to accommodate more antibodies, adjust the volume of the Staining Buffer down. Do not adjust the volumes of blocking components.***

5. Add the appropriate volume of each PhenoCycler Antibody to the Antibody Cocktail Solution.
6. Pipette to mix or vortex gently.

TISSUE STAINING

CRITICAL > ***Each sample slide should be removed from the Coplin jar containing Staining Buffer and stained one at a time to avoid drying of the tissue.***

1. Cut a rectangular piece of parafilm to the approximate size and shape of the non-label portion of the sample slide (approximately 1 inch x 2 inches).
2. Draw up 190 µL of the Antibody Cocktail into a pipette. Set aside.
3. Remove sample slide from the Coplin jar containing Staining Buffer and use a Kimwipe to carefully and gently absorb excess buffer.
4. Place sample slide on the tray of the slide staining box.
5. Quickly dispense the 190 µL of the Antibody Cocktail to the top corner of the sample slide. (See Figure 3.8.) Ensure that the liquid covers the entire tissue.

CRITICAL > ***Be careful not to pipette the solution directly onto the tissue to minimize bubbles.***

FIGURE 3.8. Dispensing Antibody Cocktail onto FFPE sample slide.

6. Gently place the parafilm on top of the Antibody Cocktail solution. (See Figure 3.9.)

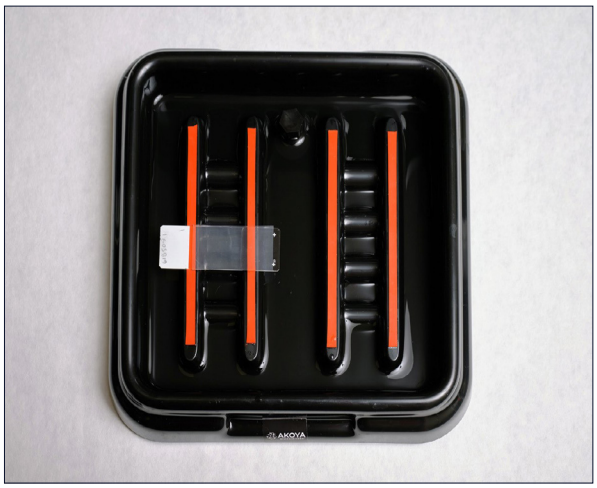


FIGURE 3.9. Parafilm placed on slide after dispensing Antibody Cocktail.

7. Repeat Steps 1–6 above for each sample slide.
8. Place the lid on the slide staining box.
9. Incubate for 3 hours at RT.

After 3 hours, proceed immediately to FFPE Tissue Post-Staining in the following section. It Is critical to prepare the initial solutions from FFPE Tissue Post-Staining before the end of this 3-hour incubation.

CRITICAL > ***Place the slide staining box on a stable surface that is free of vibrations.***

FFPE TISSUE POST-STAINING

The following steps are performed to remove unbound antibodies and fix the bound antibodies to tissues.

CRITICAL > ***It is critical to prepare all reagents and consumables ahead of time to prevent degradation of the sample/s.***

Pre-Experiment Preparation

TABLE 3.19. Materials provided (obtain now)

Item	Storage	P/N
Staining Buffer	4°C	7000017
Storage Buffer		

NOTE > ***These items are components in the Sample Kit for PhenoCycler-Fusion.***

TABLE 3.20. Fixative reagent (materials to thaw immediately before use in Step 6)

Item	Storage	P/N
Fixative Reagent (1 tube for every 5 tissues) Single-Use	-20°C	7000017

NOTE > ***This item is a component in the Sample Kit for PhenoCycler-Fusion.***

CRITICAL > ***Do not thaw Fixative Reagent before Step 6. Cut necessary tubes off strip without thawing the entire strip. Fixative reagent cannot be frozen and reused after initial thawing.***

Materials Not Provided

TABLE 3.21. Post-Staining Fixing Solution

Type	Item	Vendor	P/N
Solvents	Refrigerated methanol, 40 mL per Coplin jar. Keep at 4°C until use in Step 4.	Sigma-Aldrich	34860-1L-R
Chemicals/ Buffers	16% PFA	Electron Microscopy Sciences	15710
	1X PBS	Thermo Fisher Scientific	14190144
Plastic Consumables/ Tools	Coplin jars	VWR	513200
	Parafilm	Customer Choice	MLS
	1.5 mL Eppendorf tubes	Customer Choice	MLS
	50 mL conical tubes	Customer Choice	MLS
	Serological pipets	Customer Choice	MLS

COPLIN JAR CONFIGURATION FOR FFPE SAMPLES

In this section, the sample slide/s will be transferred from the solvents to PhenoCycler buffers into Coplin jars. Subsequently, they will be transferred to the slide staining box and finally, to a Coplin jar containing Storage Buffer for storage at 4°C. Prepare and label Coplin jars ahead of time. (See Figure 3.7.)

- For every 5 samples, fill 2 Coplin jars with 40 mL of Staining Buffer and fill 3 Coplin jars with 40 mL of 1X PBS.
- Fill Coplin jars designated for Post-Staining Fixing Solution, Methanol, and Storage Buffer with 40 mL of the corresponding solution immediately before use.
- The Coplin jar containing Storage Buffer will be used for tissue storage. Each sample slide should be labeled directly on the slide label to differentiate.

FFPE TISSUE POST-STAINING PROCEDURE

To post-stain FFPE tissue:

1. Wash the tissue:
   a. Following the 3-hour antibody incubation, gently remove the parafilm and place the sample slide/s in the first Coplin jar containing Staining Buffer. Lift and immerse the sample slide/s 2–3 times to ensure the removal of the Antibody Cocktail from both sides of the slide/s.
   b. Incubate for 2 minutes.
   c. Place the sample slide/s in the second Coplin jar containing Staining Buffer.
   d. Incubate for another 2 minutes for a total of 2 washes.

Prepare the Post-Staining Fixing Solution

TABLE 3.22. Post-Staining Fixing Solution

Post-Staining Fixing Solution (mL)	1 Coplin jar (1–5 samples)	2 Coplin jars (6–10 samples)
16% PFA	4	8
Storage Buffer	36	72
Total Volume	40	80

NOTE > ***The Post-Staining Fixing Solution is 1 part 16% PFA solution in 9 parts Storage Buffer at a 1:9 (v/v).***

2. Fix the tissue:
   a. For each Coplin jar, add 40 mL of Post-Staining Fixing Solution.
   b. Place the sample slide/s in the Coplin jar containing Post-Staining Fixing Solution.
   c. Incubate for 10 minutes at RT.

NOTE > ***During the 10-minute incubation, prepare the ice-cold methanol Coplin jar for Step 4.***

3. Wash the tissue:
   a. Prepare 3 Coplin jars of 1X PBS per 5 sample slides.
   b. Remove each sample slide/s from the Coplin jar containing Post-Staining Fixing Solution.
   c. Place the sample slide/s in the first Coplin jar containing 1X PBS. Lift and immerse each sample slide 2–3 times to ensure the Fixing Solution is removed from the top and bottom of each slide.
   d. Immediately move the sample slide/s to the second Coplin jar containing 1X PBS. Lift and immerse the sample slide 2–3 times.

e. Immediately move the sample slide/s to the third Coplin jar containing 1X PBS for a total of 3 washes. Lift and immerse each sample slide 2–3 times.

4. Incubate in ice-cold methanol:

a. Retrieve methanol from 4°C storage.

b. Pour approximately 40 mL of methanol into the Coplin jar.

c. Remove each sample slide from the third Coplin jar containing 1X PBS and place them in the Coplin jar containing ice-cold methanol.

d. Incubate at 4°C for 5 minutes.

5. Wash the tissue:

a. Quickly transfer the sample slide/s from methanol to the first corresponding 1X PBS Coplin jar.

CRITICAL > ***Methanol dries tissue faster than other buffers. Move quickly to prevent sample degradation.***

b. Ensure the slide is fully immersed in PBS. Lift and immerse the sample slide 2–3 times to ensure methanol is removed from the bottom of the slide as well as the top.

c. Transfer the sample slide/s to the second 1X PBS Coplin jar. Lift and immerse the sample slide 2–3 times.

d. Transfer the sample slide/s to the third 1X PBS Coplin jar for a total of 3 washes. Lift and immerse each sample slide 2–3 times.

6. Fix the tissue:

a. Rinse and dry the slide staining box if not already done.

b. Add 1 mL of 1X PBS to an Eppendorf tube for every 5 samples that are being prepared.

c. Retrieve 1 aliquot of PhenoCycler Fixative Reagent tube from -20°C storage (1 tube for every 5 samples). Each tube should contain 20 µL of reagent. Cut each tube selected for use from the tube strip.

CRITICAL > ***DO NOT THAW THE ENTIRE STRIP. Do not remove Fixative Reagent ahead of time. Let it melt quickly between gloved fingers. Each tube is for single use; do not re-freeze.***

d. Briefly spin down the Fixative Reagent to collect any liquid from the cap.

e. Prepare the Final Fixative Solution by diluting the 20 µL of the PhenoCycler Fixative Reagent in 1 mL of 1X PBS. (See Table 3.23.)

TABLE 3.23. Final Fixative Solution

Final Fixative Solution	1–5 Samples	6–10 Samples
1X PBS	1000 µL	2000 µL
Fixative Reagent	20 µL	40 µL

f. Mix thoroughly or vortex the solution.

g. Cut a rectangular piece of parafilm roughly the size of the glass microscope slide (approximately 1 inch x 2 inches).

h. Draw up 200 µL of the Final Fixative Solution with a pipette. Set aside.

i. Remove the sample slide/s from the Coplin jar and place each on the tray of the slide staining box.

j. Add 200 µL of Final Fixative Solution to the top corner of each sample slide. Cover the entire section with reagent. Do not pipette the solution directly onto the tissue.

k. Carefully place the parafilm over the fixative solution.

l. Repeat Steps g–k above for all sample slides.

m. Place lid on the slide staining box and incubate for 20 minutes.

7. Wash the tissue:
   a. Remove the sample slide/s from the slide staining box, gently remove the parafilm, and place in the first Coplin jar containing 1X PBS. Lift and immerse the sample slide 2–3 times to ensure Fixative Solution is removed.
   b. Move each sample slide/s to the second Coplin jar containing 1X PBS. Lift and immerse each sample slide 2–3 times.
   c. Move each sample slide/s to the third Coplin jar containing 1X PBS for a total of 3 washes. Lift and immerse each sample slides 2–3 times.
8. Store the tissue:
   a. Label a new Coplin and pipet 40 mL of Storage Buffer for every 5 sample slides.
   b. Place the sample slide/s in the Coplin jar keeping the tissue facing the same direction for all slides.
   c. Cap the Coplin jar and seal around the edges with parafilm (optional) for storage.
   d. Tissues can now be used directly to run a PhenoCycler-Fusion Experiment.

STOPPING POINT >

For best results, store at 4°C for ≤5 days.

CHAPTER 4

Experiment Protocol and Reporter Plate Design

USING THE PHENOCYCLER EXPERIMENT DESIGNER SOFTWARE

This chapter describes how to design the PhenoCycler-Fusion run protocol using the PhenoCycler Experiment Designer Software. The Experiment Designer allows you to define the run panel, organize the cycles, assign exposure times, and create the reporter plate layout in advance. The following is a summary of the key steps used to create experiment protocols using this application:

- Load and update the PhenoCycler inventory to make sure it is up to date.
- Create a new experiment design or edit or select a previously saved protocol from the study folder using the load button.
- When creating a new design, enter the tissue type, panel source, e.g., inventoried antibodies, and 1 of the 2 filter set combinations depending on the reporter dyes used in a given experiment.
- Within the Panel Content window, select the markers to be used. Using Table Mode, custom-conjugated antibodies can be added to the experiment run panel.
- Identify the starting well and the blank wells. Note: Every experiment must have 2 dedicated wells in row H for blank cycles.
- A saved experiment design (.xpd file) can then be accessed and loaded into the PhenoCycler-Fusion app. See the PhenoImager Fusion User Guide, Chapter 6: PhenoCycler-Fusion Workflow.

FIGURE 4.1. PhenoCycler Workflow—PhenoCycler Protocol and Reporter Plate Design.

LAUNCHING THE EXPERIMENT DESIGNER

To launch the Experiment Designer:

Double-click on the Experiment Designer app located on the PhenoCycler-Fusion Acquisition PC desktop to open the Experiment Designer. The main screen appears as shown in Figure 4.2.

Select Select PhenoCode Discovery Protein Assay to run PhenoCycler Protein experiments. RNA detection assay is only available as beta functionality.

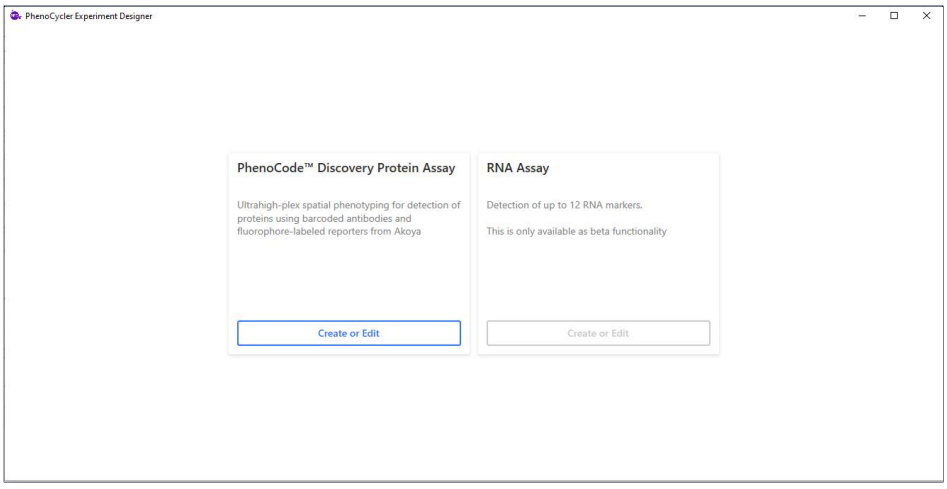


FIGURE 4.2. Main screen on Experiment Designer.

USING THE APPLICATION

The buttons on the main screen of the Experiment Designer are shown in Figure 4.3. This section describes how to use these 4 buttons to perform various tasks.

The main screen provides 4 buttons on the left side:

- Home.
- New.
- Load.
- Save.

The Grid menu button is on the far right. Each button is described in this chapter.

FIGURE 4.3. Buttons on the main screen of the Experiment Designer.

CREATING A NEW EXPERIMENT DESIGN

You can either create a new experiment design or load an existing design. Follow this procedure to create a new design. To load a saved experiment design, see the Load an Existing Well Plate section.

To create a new experiment design:

1. Select the New button on the main screen. (See Figure 4.3.) The Save Experiment screen displays. (See Figure 4.4.)

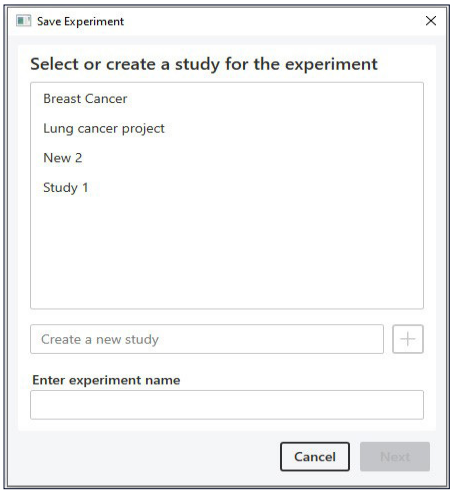


FIGURE 4.4. Save Experiment screen.

2. Select the name of an available study from the Select or create a study for the experiment list. When opening the application for the first time, this list will appear blank.

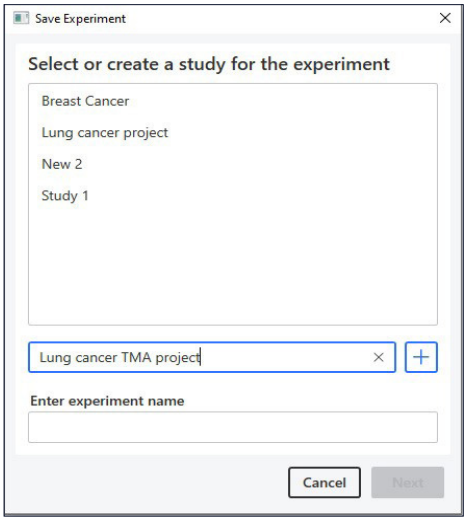


FIGURE 4.5. Save Experiment screen—selecting study.

3. To add a new study, enter the name in the Create a New Study field and select the **+** button.
4. With the appropriate study selected, enter the name of the experiment in the Enter Experiment Name field and select Next.
5. The Experiment Creator screen displays. (See Figure 4.7.) Experiment Creator allows you to define the Staining Information, Panel Content, Starting Well, the (2) Blank Wells, and the Exposure Times for each channel.

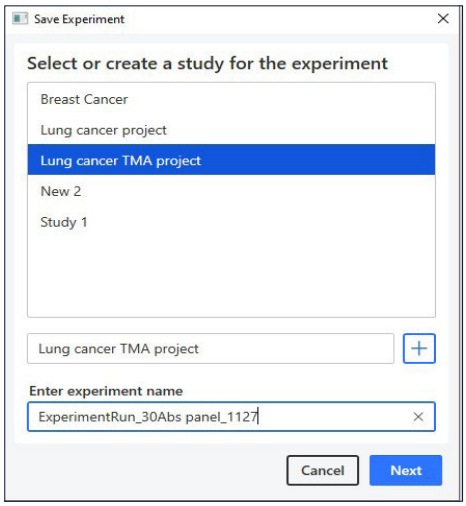


FIGURE 4.6. Save Experiment screen—Enter Experiment Name.

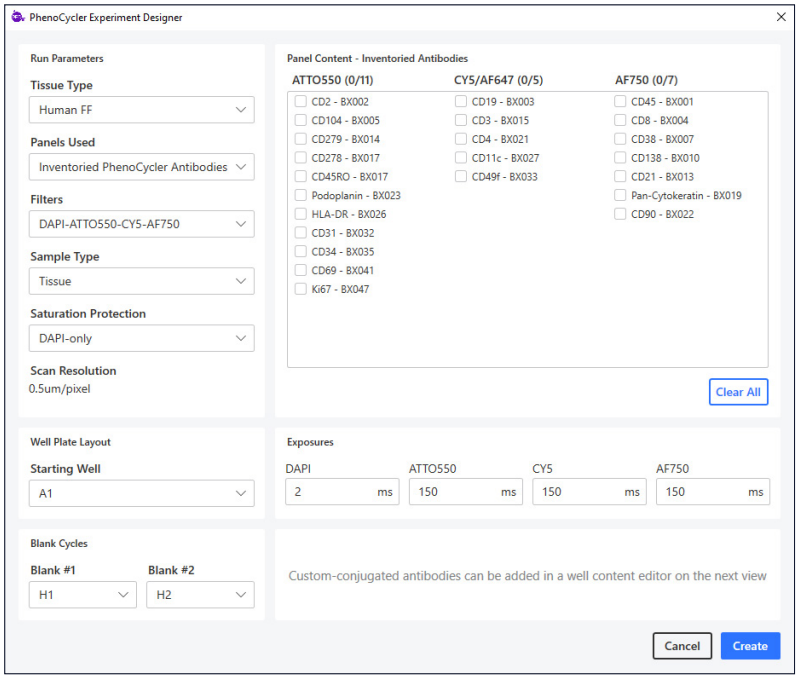


FIGURE 4.7. Experiment Designer screen.

TABLE 4.1. Run Parameter Definitions

Parameter Name	Description
Tissue Type	Select the tissue-type: Human FFPE, Human FF, or Mouse FF.
Panel Used	Select the source of antibodies used in the run, e.g., Inventoried PhenoCycler Antibodies. Currently, the primary source is the ready-to-use inventoried antibodies provided by Akoya. Users can include custom-conjugated antibodies in the panel. To add detection of the custom-conjugated antibodies during a run, add each custom-conjugated marker of interest into the desired cycles in the next window. **NOTE >** ***The ready-to-use markers available differ based on the filter set selected.***
Filters	Select the filter set based on the reporter combination of choice. Filter set options include: DAPI-ATTO550-Cy5-AF750 (recommended for FFPE) or DAPI-ATTO550-Cy5-AF488 (recommended for FF).
Sample Type	PhenoCycler-Fusion has a specialized algorithm for finding whole tissue samples vs finding TMA samples. If your samples are TMAs, select a Sample Type of TMA.
Saturation Protection	Saturation Protection helps prevent overexposure when acquiring fluorescence imagery. If this option is selected and saturated pixels are detected in your imagery, the instrument lowers the exposure time for the entire scan to reduce the number of saturated pixels. The recommended option for PhenoCycler experiments is Only DAPI. **NOTE >** ***Using Saturation Protection does not increase scan times noticeably, i.e., PhenoCycler-Fusion can record each image using 2 camera settings within 1 readout.*** Consider disabling Saturation Protection if your sample has bright debris that will saturate at much lower exposure times than your true sample. This could cause the exposure times to be reduced too low for proper visualization of the sample.
Scan Resolution	0.5 µm/pixel

6. Select the markers to be used in the Panel Content area. (See Table 4.2.)

TABLE 4.2. Parameters to define markers

Parameter Name	Description
Panel Content	Select the markers to be used by clicking in the checkboxes. The available antibodies will change based on the tissue type and filter set selected. The markers are grouped by reporter dye options for easy filtering: • ATTO550 • Cy5/AF647 ***NOTE >*** ***The experiment may have reporters with either Cy5 or AF647 or include both dye options. Both can be imaged within the Cy5 channel with the same exposure times.*** • AF488 or AF750 Each PhenoCycler Antibody will have a PhenoCycler Barcode (BX###) that is complementary to a specific PhenoCycler Reporter (RX###). For example, PhenoCycler Barcode BX001 corresponds to PhenoCycler Reporter RX001. For all custom-conjugated antibodies, conjugations must be performed prior to this step. (See **Appendix B: Custom Conjugation**.) ✔Ensure that each antibody within a run has a unique barcode that is not shared with any other antibody in the multicycle run. ✔Assign each antibody to a cycle number, ensuring that a given dye/channel is used only once in each cycle. This process is critical to guarantee the true signal of the resulting fluorescence images concerning a specific biomarker and will determine the final number of necessary cycles. Each cycle can have 1 or 2 Reporters instead of 3, if necessary.

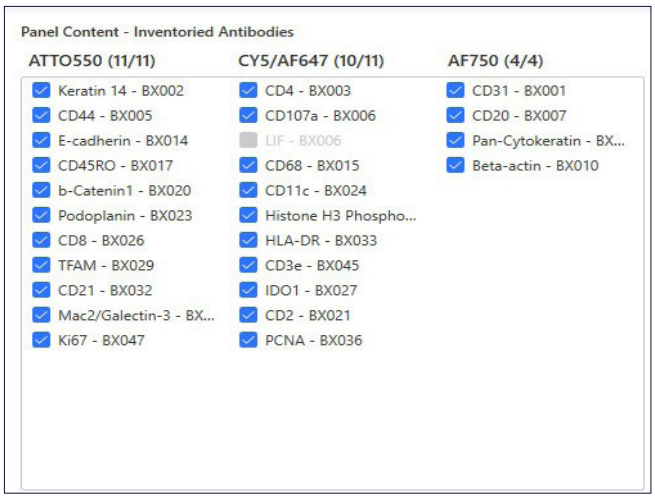


FIGURE 4.8. Experiment Designer screen—Panel Content.

7. Identify the starting well and the blank cycle wells in the Well Plate Layout and Blank Cycles areas. (See Table 4.3.)

TABLE 4.3. Parameters to define Well Plate Layout and Blank Cycles

Parameter Name	Description
Starting Well	Choose the starting well from the drop-down box. Choices range from A1–A12 through G1-G12.
Blank Cycles	Blank images are recorded in 3 fluorescence channels at the beginning and at the end of a PhenoCycler multicycle run. Blank cycles are used for subtracting background signal. The Nuclear Stain (DAPI) will be deposited during the blank cycles and used for auto-focusing. Row H of the Reporter Plate is dedicated for blank cycles. The user must add aliquots of the Reporter Stock Solution (with Nuclear Stain but without Reporters) to 2 wells in row H for every Phenocycler experiment. Choose the blank cycle wells (Blank #1 and Blank #2) from the drop-down box. Choices range from H1–H12 for each, but each well in row H can only be used once for each blank cycle in a given experiment, i.e., both blank cycles cannot be H2.

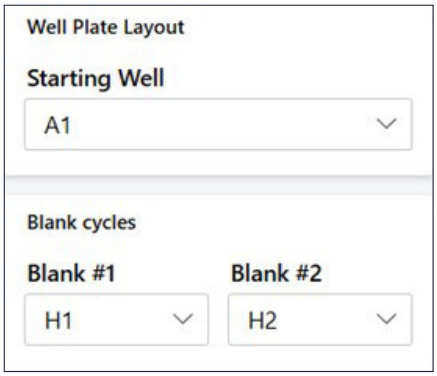


FIGURE 4.9. Experiment Designer Screen—Well Plate Layout.

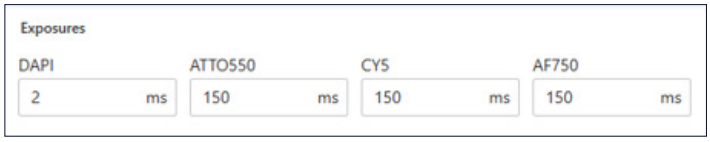


FIGURE 4.10. Setting Exposures settings (ms) for the 4 parameters.

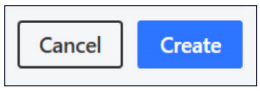


FIGURE 4.11. Click Create to continue or Cancel to return to the main screen.

8. Selecting Create displays the cycle layout/table mode. In this layout, the designer app sorts the marker detection into the minimum number of required cycles. Each cycle consists of detection in DAPI channel plus up to three channels. The 3 marker channels available depend on the filter set selected in the main window, either ATTO550, Cy5, or AF750 (or AF488).

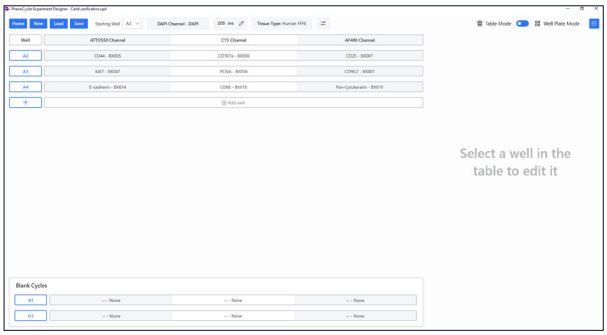


FIGURE 4.12. Cycle or Table Mode Layout.

EDITING THE EXPERIMENT DESIGN LAYOUT

To edit the cycle layout, click on the button correspondingto the well (e.g., see Figure 4.13, A6) and use the Insert, Skip, or Delete options to add, skip, or remove cycles.

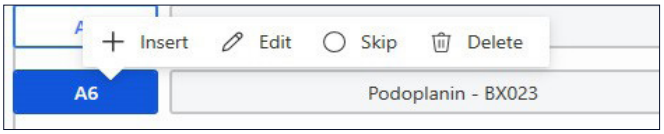


FIGURE 4.13. Edit Layout options within Table Mode.

To edit the contents within a cycle/well:

1. Select the well/cycle you want to edit and select Edit. The contents of that well displays on the right side of the screen. (See Figure 4.14.)
2. Alter the exposure times for particular markers within a cycle by editing the exposure times for their respective individual channels.
3. For inventoried antibodies, use the Marker/Barcode drop-down box to select the marker/barcode combination to be used. To add an additional item, select Add Additional Item from the drop-down box and specify the details for that item.
4. Repeat Steps 1 and 2 as needed to edit the layout of the wells.
5. When finished, select Update to save your layout or select Cancel.

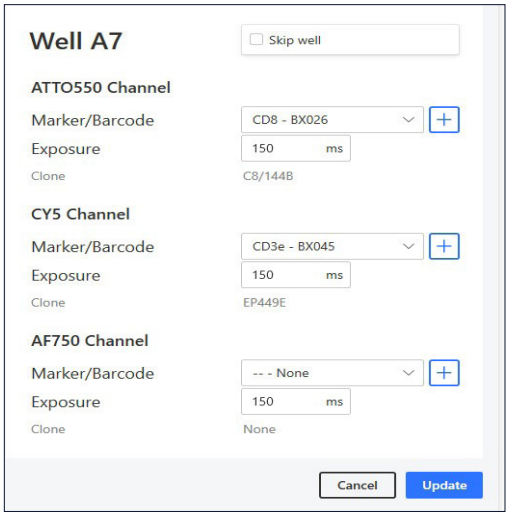


FIGURE 4.14. Edit content options within a cycle/well.

ADDING A CUSTOM-CONJUGATED MARKER TO THE EXPERIMENT DESIGN

To add a custom-conjugated antibody to an empty channel within a cycle:

1. Select the appropriate channel within a cycle/well and select the **+** button next to the Marker/Barcode field. (See Figure 4.15.)

FIGURE 4.15. Select appropriate channel to add custom-conjugated antibody.

The Add Additional Item window will display.

2. Select Custom Conjugations from the Item Source drop-down.

CRITICAL > ***Enter the appropriate marker and clone names, select the proper barcode, and then select OK. It is critical to ensure that a unique barcode is selected for each new marker.***

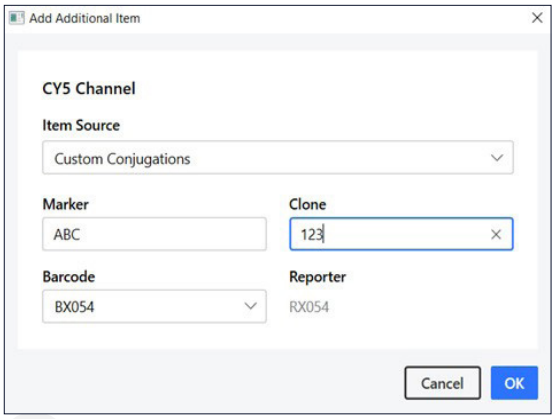


FIGURE 4.16. Edit content options within a cycle/well—Add Additional Item.

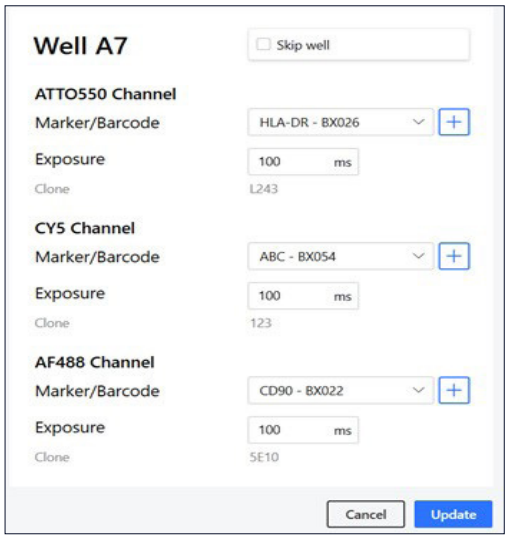


FIGURE 4.17. Assign barcodes and exposure time to channels.

3. Edit all the channels within the cycle/well appropriately.
4. Select Update to update the experiment design.
5. Repeat Steps 1–4 to add all required custom-conjugated antibodies to the experiment.

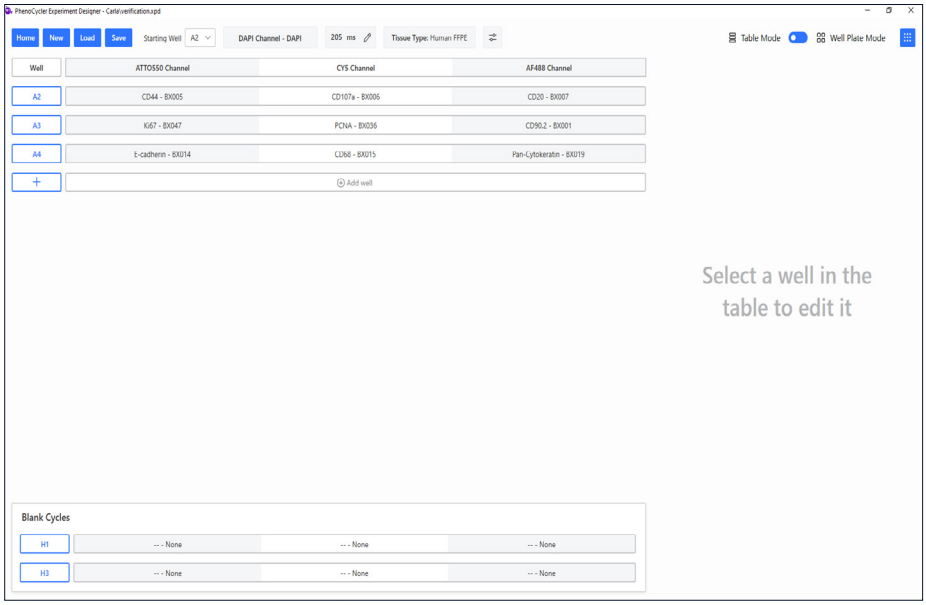


FIGURE 4.18. Experiment Designer Screen—Table Mode Window (List View).

6. The updated cycle layout will display all selected markers, including the custom-conjugated antibodies.
7. Select Well Plate Mode to view the well plate layout using the toggle button. The layout displays the reporters (RX-xxx) corresponding to each well. This layout will be used in **Chapter 5: Preparing PhenoCycler Reporter Plate** to prepare the reporter plate.

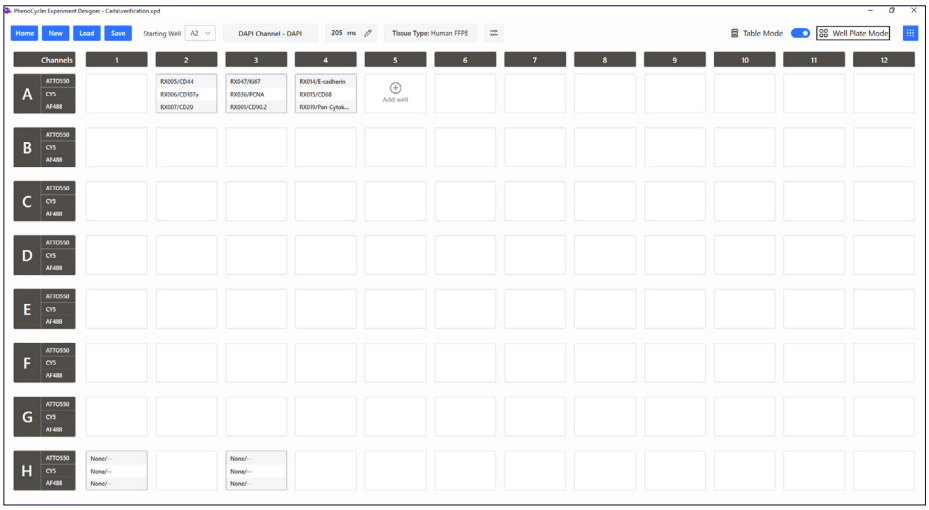


FIGURE 4.19. Experiment Designer Screen—Well Plate Mode to use in reporter plate preparation.

Critical: If using custom feature, ensure all Marker names in experiment file are unique, or final qptiff generation will be corrupted.

LOADING AN EXISTING WELL PLATE

To load an existing well plate:

1. Select the Load button on the Main Screen. (See Figure 4.20.)

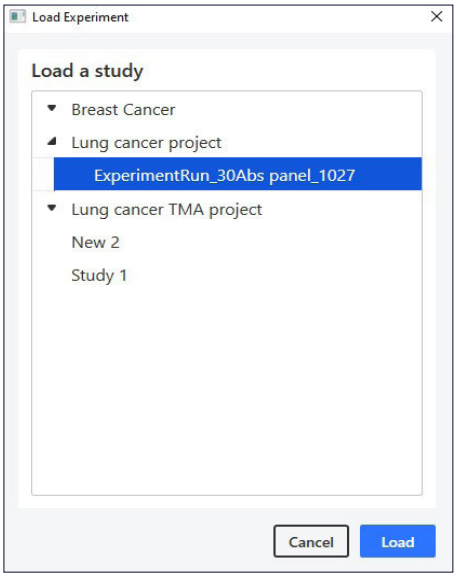


FIGURE 4.20. Load a Study window.

2. Select the desired saved protocol in the available Study folders.
3. Click Load.

SAVING THE NEW OR MODIFIED WELL PLATE

Select Save on the main screen. (See Figure 4.21.)

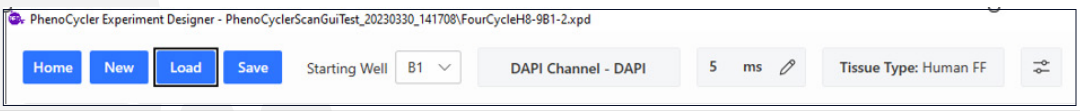


FIGURE 4.21. Experiment Designer—Main screen—Save.

USING OPTIONS FROM THE GRID MENU

Each option illustrated in Figure 4.22 is described in Table 4.4.

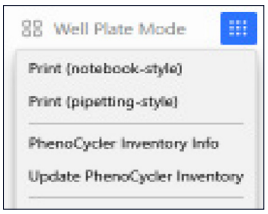


FIGURE 4.22. Drop-down options available from the grid menu.

TABLE 4.4. Options available from grid menu

Menu Option	Description
Print (notebook-style)	Prints the cycle design information in a condensed format for your lab notebook. (See Figure 4.23.)
Print (pipetting-style)	Prints the cycles organized in a 96-well plate format. This view is useful for preparing the Reporter Plate. (See Figure 4.24.)
PhenoCycler Inventory Info	Displays the publication date of your PhenoCycler Inventory so you can determine if your information is up to date.
Update PhenoCycler Inventory	Update your PhenoCycler Inventory from the web. You will need to access the web to locate and identify the up-to-date PhenoCycler Inventory.
Exit	Closes the Grid menu.

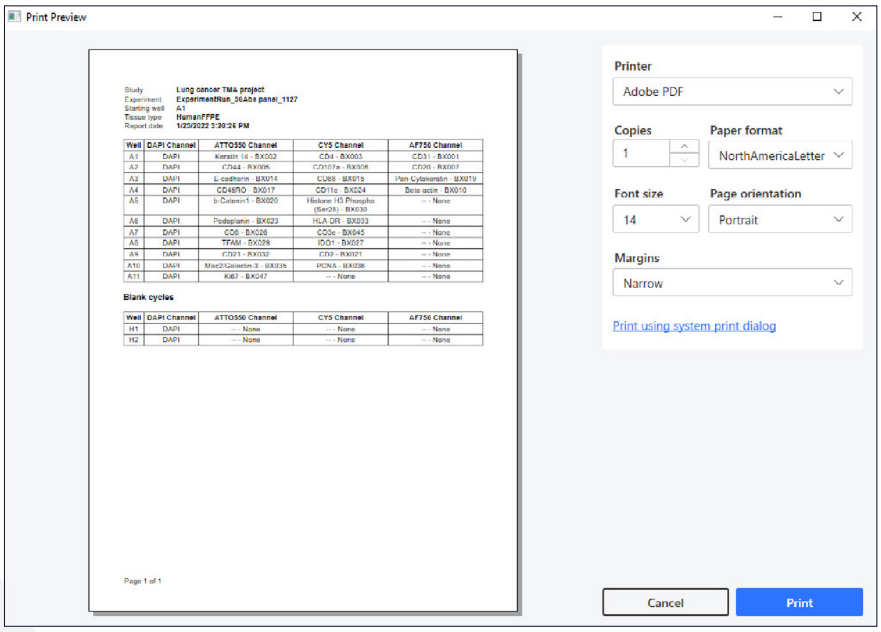


FIGURE 4.23. Print preview—notebook style.

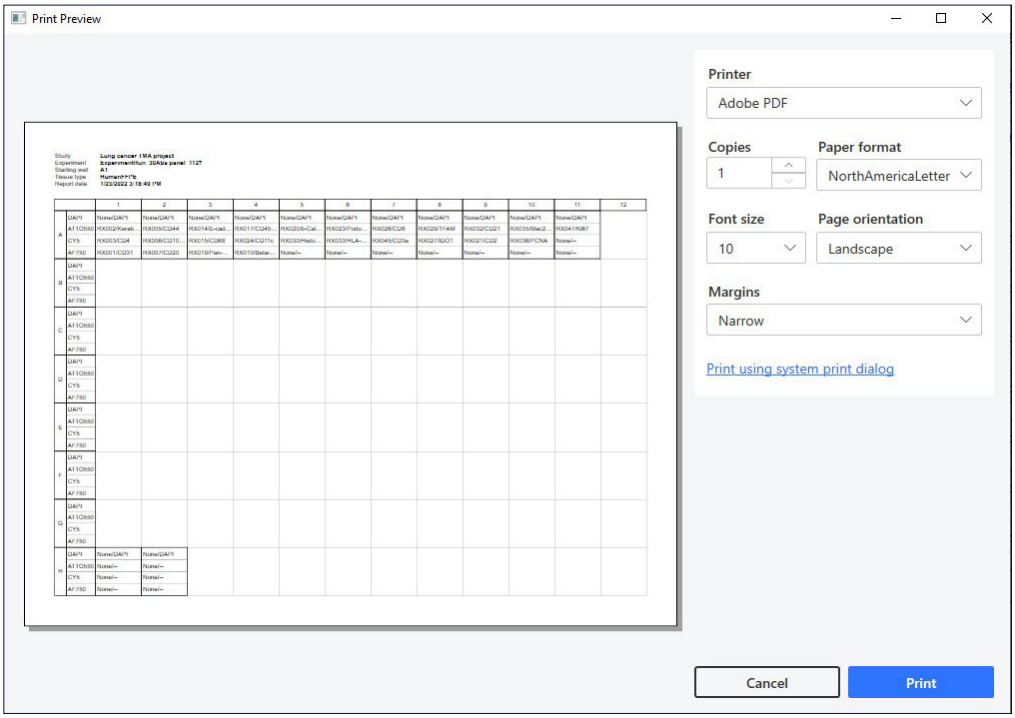


FIGURE 4.24. Print preview—pipetting style.

CHAPTER 5

Preparing PhenoCycler Reporter Plate

This chapter describes how to prepare and organize the PhenoCycler Reporters revealed in a PhenoCycler-Fusion multicycle run. A Reporter comprises a fluorescent dye conjugated to a PhenoCycler oligonucleotide sequence complementary to one specific antibody barcode. As part of Reporter plate preparation, unique Reporters are grouped together in mixtures of up to 3 spectrally distinct dyes, along with a nuclear stain. Each of these mixtures is called a Reporter Master Mix and is pipetted into a single well of a 96-well plate. Each well corresponds to 1 experiment cycle.

Timeline to Prepare PhenoCycler Reporters

Duration	Step	Time	Stopping Point
Variable	Prepare Reporter Stock Solution: Combine 10XBuffer for PhenoCycler, Assay Reagent, NuclearStain, and H_2O to create the Reporter Stock Solution.	10 minutes	--
	Prepare Reporter Master Mix for each cycle: Combine Reporter Stock Solution with predetermined Reporters to create a Reporter Master Mix for each cycle.	30 minutes–1 hour for 10 cycles (estimated)	--
	Create a 96-well reporter plate: Add the Reporter Master Mix contiguously by row to a 96-well plate, seal with foil seal, and store at 4°C.	Variable	Store up to 2 weeks

During each cycle, the Reporter Master Mix is drawn from 1 well on the 96-well plate and applied to the sample prior to image acquisition. The cycle ends with the removal of the Reporters from the tissue sample.

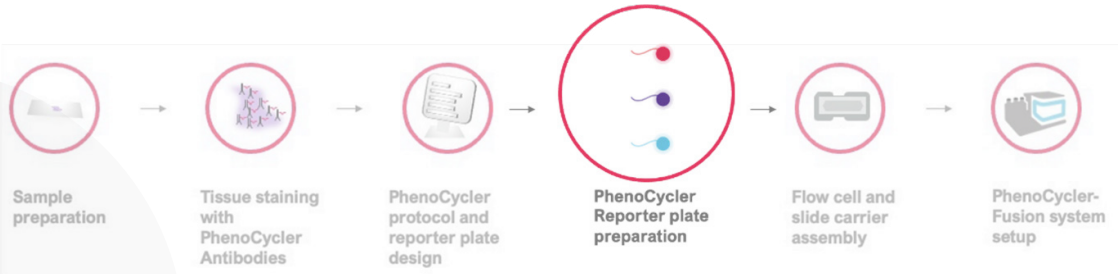


FIGURE 5.1. PhenoCycler Workflow—PhenoCycler Reporter Plate preparation.

CONFIGURING CYCLES FOR A PHENOCYCLER-FUSION EXPERIMENT

A PhenoCycler-Fusion run requires the preparation of a Reporter Master Mix for every cycle. The reporter plate can be designed using the Experiment Designer app. (See Chapter 4: Experiment Protocol and Reporter Plate Design.) Each Reporter Master Mix will be placed in a separate well of a 96-well plate.

CRITICAL > ***Make sure that Reporters in the same cycle are paired to unique fluorescent dyes. If 2 reporters are paired with the same dye in a cycle, they will be revealed at the same time in 1 fluorescence channel, making it impossible to distinguish the signal coming from the 2 corresponding biomarkers.***

NOTE > ***Plan which reporters are to be revealed in each cycle ahead of time using the Experiment Designer App. (See Chapter 4: Experiment Protocol and Reporter Plate Design.)***

CRITICAL > ***It is important that the complete multicycle experiment is designed in Experiment Designer before performing the antibody staining and preparing the 96-well reporter plate. The Reporter Plate can be prepared UP TO 2 WEEKS ahead of the experimental run and must be stored with a seal at 4°C until it is time to start the run. The plate set-up will reflect the cycle order for the PhenoCycler-Fusion multicycle run.***

CONFIGURING REPORTER PLATE

Once all PhenoCycler Reporters are assigned to a cycle number using the Experiment Designer App, cycles can be associated with specific wells following an order from left-to-right (1–12) and from top-to-bottom (Row A–Row H), as shown in Figure 5.2. During the PhenoCycler run, in each cycle, the instrument withdraws the Reporter Master Mix from 1 well of the 96-well plate.

NOTE > ***The PhenoCycler Experiment Designer allows skipping some wells and starting from a well other than A1, if necessary. If a well has been contaminated, the user can simply skip that well in the plate design using the Experiment Designer app.***

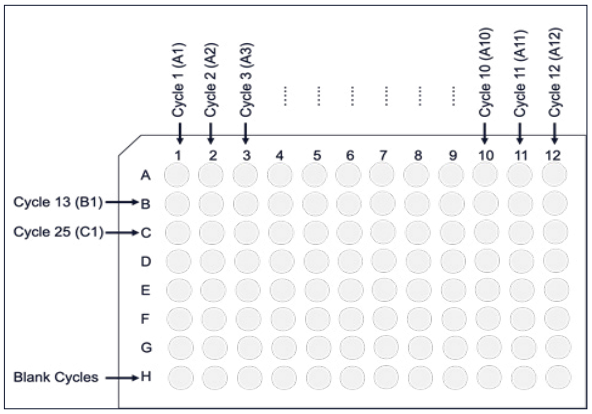


FIGURE 5.2. Configuration of 96-well plate for PhenoCycler Reporters.

An example of a 96-well plate configuration as shown in Figure 5.2. Well A1 contains the solution for cycle 1; well A2 contains the solution for cycle 2, etc. A multicycle PhenoCycler run can start from any well on the plate, and wells can be skipped if this information is included in the PhenoCycler Experiment Designer before starting the experiment. For information regarding the PhenoCycler Experiment Designer, see Chapter 4: Experiment Protocol and Reporter Plate Design.

BLANK CYCLES

Row H of the Reporter Plate is dedicated for blank cycles. The user must add aliquots of the Reporter Stock Solution (with Nuclear Stain but without Reporters) to 2 unused wells in row H for every PhenoCycler experiment.

PREPARING PHENOCYCLER REPORTER PLATE

Guidelines

Akoya 96-Well Plates for PhenoCycler (P/N 7000006) must be used for PhenoCycler Reporter plate preparation. Alternative plates may not have the proper dimensions required to run a PhenoCycler-Fusion experiment.

Akoya Plate Seals for PhenoCycler (P/N 7000007) must be used for sealing the prepared plates. Alternative seals may stick to the instrument during the aspiration steps.

Reporter plates can be created up to 2 weeks in advance. Prepared Reporter plates should be sealed and stored at 4°C.

PRE-EXPERIMENTATION

Akoya Consumables

TABLE 5.1. Materials provided

Item	Storage	Use At	P/N
96-Well Plates for PhenoCycler	RT	RT	7000006
Plate Seals for PhenoCycler			7000007
10X Buffer Kit for PhenoCycler-Fusion			7000019
Assay Reagent for PhenoCycler	-20°C, and 4°C after the first thaw	Place on ice	7000002
Nuclear Stain for PhenoCycler			7000003
PhenoCycler Reporters			--

TABLE 5.2. Materials not provided

Item	Vendor	P/N
Nuclease-free water	Thermo Fisher Scientific	AM9938
Opaque 1.5 mL tubes	Customer Choice	MLS
15 mL tube covered with foil	Customer Choice	MLS
Bucket of ice	Customer Choice	MLS

PREPARE 1X PHENOCYCLER-FUSION BUFFER

Prepare 1 L of 1X PhenoCycler-Fusion Buffer using components from 10X Buffer kit for Phenocycler-Fusion (P/N 7000019):

1. In a clean glass beaker (or similar container) add 800 mL ddH_2O.
2. Pipet 100 mL 10X Buffer for PhenoCycler.
3. Pipet 100 mL Buffer Additive for PhenoCycler-Fusion.
4. Mix by pipetting up and down with pipet aid or by using magnetic stir bar.

NOTE > ***The 1X PhenoCycler-Fusion Buffer was previously referred to as "1X Buffer for PhenoCycler with Buffer Additive."***

NOTE > ***To avoid bubble formation, do not mix by shaking. If mixing in a screw top bottle, avoid inverting to prevent leakage. If spills occur, clean with 70% ethanol.***

NOTE > ***The above 1x solution should be stored at RT and is stable for 2 weeks.***

Use

This 1X solution is used:

- To fill the Buffer bottle in the PhenoCycler sidecar. (See Figure 6.1.)
- For the reporter stock solution in the 96-well plate. (See Chapter 5: Preparing PhenoCycler Reporter Plate.)
- For the incubation step after mounting the Flow Cell.

PHENOCYCLER REPORTER PLATE

To prepare the PhenoCycler Reporter Plate:

1. Prepare the Reporter Stock Solution:
   a. Prepare the Reporter Stock Solution based on the total number of cycles (including 2 blank cycles) for the experiment in an opaque 1.5 mL tube or a 15 mL tube covered with foil.

CRITICAL > ***For PhenoCycler 2.0, see Table 5.3. For PhenoCycler 1.0, see Table 5.4.***

TABLE 5.3. Prepare reporter stock solution based on the number of cycles for the experiment (PhenoCycler 2.0)

PhenoCycler 2.0	Cycles/Wells			
Reporter Stock Solution (µL)	5	10	15	20
1X PhenoCycler-Fusion Buffer	1325	2650	3975	5300
Assay Reagent	125	250	375	500
Nuclear Stain	50	100	150	200
Total Volume	1500	3000	4500	6000

TABLE 5.4. Prepare reporter stock solution based on the number of cycles for the experiment (PhenoCycler 1.0)

PhenoCycler 1.0	Cycles/Wells			
Reporter Stock Solution (µL)	5	10	15	20
1X PhenoCycler-Fusion Buffer	1350	2700	4050	5400
Assay Reagent	125	250	375	500
Nuclear Stain	25	50	75	100
Total Volume	1500	3000	4500	6000

b. After adding all reagents, mix by gently pipetting the Reporter Stock Solution up and down at least 5x to ensure solution is thoroughly mixed.

NOTE > ***Nuclear Stain may precipitate initially upon addition. Gently mix the solution to ensure homogeneity.***

CRITICAL > ***Prevent the formation of bubbles. Do not shake or vortex the solution vigorously.***

2. Prepare the blank cycles.
Pipette 245 µL of Reporter Stock Solution into 2 wells within row H (e.g., H1 and H2) as designated in the well plate layout using the designer app. (See Chapter 4: Experiment Protocol and Reporter Plate Design.)

3. Prepare the Reporter Master Mix for each cycle:

a. For each cycle, label an opaque 1.5 mL tube with the associated cycle number or well number (e.g., A1).

b. Add the Reporter Stock Solution to each opaque tube. The volume of Stock Solution will vary depending on whether 1, 2, or 3 Reporters will be revealed. (See Table 5.5.)

TABLE 5.5. Volume of the reporter stock solution based on the number of reporters to be revealed

Reporter Stock Solution, Volume (µL)			
3 Reporters per Cycle	2 Reporters per Cycle	1 Reporters per Cycle	Blank Cycle
235	240	245	250

Place Reporters in an ice bucket before use:

c. Briefly spin tubes down using a benchtop centrifuge.

d. Add 5 µL of each Reporter to its corresponding opaque tube. (See Table 5.6.)

TABLE 5.6. Total volume of all reporters per opaque tube based on the number of reporters per opaque tube

Number of Reporters Per Opaque Tube	Total Volume of All Reporters Per Opaque Tube (µL)
1	5
2	10
3	15

e. Mix the contents of the tube by gently pipetting up and down or gently inverting the tube. Minimize the number of bubbles generated during this process. Do not shake or vortex the solution vigorously.

f. Repeat Steps a–e for every cycle.

4. Create the Reporter 96-well plate:

a. Obtain the 96-well plate once all tubes have been prepared.

b. Pipette 245 µL of Reporter Master Mix from each tube into its corresponding well on the 96-well plate.

CRITICAL > ***Use caution when pipetting into the plate. Do not touch, drip, or pipette into wells other than the corresponding designated one. Any cross-contamination will alter the staining profile. Do not use wells that have been contaminated. In the Experiment Design app, use the skip wells feature to skip the contaminated wells. Prevent the formation of bubbles while pipetting.***

CRITICAL > ***Keep the filled wells in the dark to protect fluorescent dyes from photobleaching.***

c. Remove the adhesive layer from a foil plate seal.

d. Cover the entire plate and do not move or tear the foil once it has adhered to the plate. (See Figure 5.3.)

e. To ensure optimal sealing, carefully press down on top of each filled well.

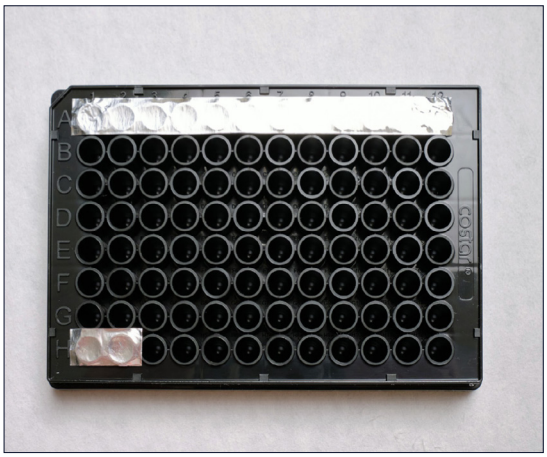


FIGURE 5.3. Filled and sealed 96-well plate.

CRITICAL > ***Take caution if reusing a 96-well plate for an additional PhenoCycler run. It is best to cover the unused wells with the foil seal in order to keep them dust-free for the next run. Do not disturb the foil seal that is covering the used wells to avoid contamination. Do not cover wells to be pierced by the PhenoCycler instrument with more than 1 layer of foil seal.***

STOPPING POINT >

PhenoCycler 96-well Reporter Plate can be used directly to run a PhenoCycler-Fusion Experiment or stored at 4°C for up to 2 weeks.

Once the tissue and reporter plate are prepared, see the PhenoImager Fusion User Guide for Flow Cell Assembly and instruction on setting up runs.

APPENDIX A

Specifications

PERFORMANCE SPECIFICATIONS

TABLE A.1.

Imageable Tissue Thickness	≤10 µm
Maximum Fluidics Capacity	Up to 40 cycles
Imageable Area	18 mm x 34 mm

More information can be found on the PhenoCycler support page at http://akoyabio.com.

ENVIRONMENTAL CONDITIONS

TABLE A.2.

Operating Temperature	20°C–24°C
Humidity	20%–80%, noncondensing
Input Voltage	100–240VAC ~ 2A 50/60 Hz

INSTRUMENT SPECIFICATIONS

TABLE A.3.

General Overview	
Dimensions (W X D X H inches)	PhenoCycler: 28 X 22 X 14.5; Fusion: 25 X 20 X 26
Weight* (lbs. / kg)	PhenoCycler: ~67 lbs. / 30.3 kg; Fusion:~120 lbs. / 54.4 kg
Tissue Format	Whole-slide, Tissue Microarray, and Tissue Sections
Speed (1.5 cm X 1.5 cm)	Fluorescence: 25 min
Resolution	Up to 0.25 µm/pixel (40X)
Automation	Version 1.0: 1 slide, Version 2.0: 2 slides
Multiplexing Capability	Supports up to 100+ biomarkers depending on barcode compatibility
File format	Akoya Biosciences whole slide scan image (qptiff), color images (JPEG, BMP, PNG)
Platform Electrical	
Input Voltage	PhenoCycler: 100–120V, 50/60Hz (±10%) Fusion: 100–240V, 50/60Hz (±10%)
Input Current	2A, 50/60Hz
Backup UPS (Optional, but recommended)	APC Smart-UPS 1500VA
Operating Environment	
Temperature	+20°C to +26°C (68°F to 80°F)
Humidity	30%–60%, No condensation

*Measurements are approximations and could vary slightly for the final shipments.

APPENDIX B

Custom Conjugation

This chapter outlines how to custom-conjugate third-party, non-inventoried, purified antibodies to PhenoCycler Barcodes. The conjugation allows converting clones of interest into antibodies tagged with PhenoCycler Barcodes that can then be used in PhenoCycler multicycle experiments.

NOTE > ***Please refer to Akoya's PhenoCycler Screened Antibody List for clones that have been successfully conjugated and have shown positive and specific staining patterns in relevant tissue samples. These purified clones are commercially available from other antibody vendors and require conjugation by the end user.***

NOTE > ***PhenoCycler Antibodies purchased from Akoya are already tagged with PhenoCycler Barcodes. Therefore, conjugation is not necessary.***

NOTE > ***Custom conjugation should be performed at least 2 days before use.***

TABLE B.1. Experiment Overview

Duration	Step	Time	Stopping Point
4 hours, 10 minutes	Reduce purified antibody: Perform partial reduction reaction to reveal reactive thiol groups.	1 hour (includes 30-minute incubation)	--
	Conjugate antibody with PhenoCycler Barcode: Combine partially reduced antibody with PhenoCycler Barcode and incubate.	2.5 hour incubation (includes 2-hour incubation)	--
	Purify PhenoCycler custom-conjugated antibody: Filter PhenoCycler custom-conjugated antibody solution to remove unconjugated components.	40 minutes (includes 4 x 8 minute spin downs)	--
	Store PhenoCycler custom-conjugated antibody: Add PhenoCycler Antibody Storage Solution to sample and store at 4°C.	--	Store up to 1 year

The success of the conjugation can be verified via gel electrophoresis, which is used as quality control. Please note that this step requires additional equipment and materials not provided in the Antibody Conjugation Kit. (See Verifying the Success of Conjugation.)

CONJUGATING ANTIBODIES

GUIDELINES FOR ASSIGNING PHENOCYCLER BARCODES TO ANTIBODIES

- **Identify and validate unconjugated antibody clone.** Prior to conjugation with PhenoCycler Barcodes, it is critical to identify the best-suited antibody clone and verify positive staining using the unconjugated/purified antibody clone in the tissue of interest. Please refer to Akoya's Screened Antibody List for clones that have been successfully conjugated and have shown positive staining patterns in relevant tissue samples. Optional: At this point, you may also consider assessing the specificity of the purified antibody clone. This can be done by staining with the antibody clone and a positive and negative counterstain when possible.
- **Assess quantities of reagents.** The Antibody Conjugation Kit (P/N 7000009) contains reagents sufficient for 10 conjugation reactions, each using 50 µg of purified antibody per reaction.
- **Purchase PhenoCycler Barcodes and PhenoCycler Reporters.** Each Barcode corresponds to a specific Reporter and, consequently, to a well-defined and spectrally distinct fluorescence channel. Each unique barcode can only be used for a single antibody in any PhenoCycler panel.
- **Consider antigen abundance and fluorescence channel sensitivity when selecting barcodes for conjugation.** Less abundant antigens produce lower-intensity signals and perform better if conjugated to PhenoCycler Barcodes assigned to fluorescence channels with low autofluorescence. The corresponding reporter dyes are Cy5/AF647 and ATTO550 for FF tissues and Cy5/AF647 for FFPE. For antibodies targeting highly expressed antigens, we recommend using PhenoCycler Barcodes corresponding to AF488 for FF tissues and ATTO550 and AF750 for FFPE tissues. These channels are recommended for highly expressed antigens due to the possibility of high autofluorescence (AF488 for FF and ATTO550 for FFPE) and due to varying camera quantum efficiency (AF750). For FFPE samples, we recommend conjugating antibodies to Barcodes corresponding to AF750 only after having performed a preliminary conjugation on a different channel, for example, Cy5/AF647. This additional step is recommended because camera sensitivities tend to decrease approaching the Near Infrared Region (NIR), and only after ensuring a strong signal in a different channel should the marker be expected to work well for AF750.

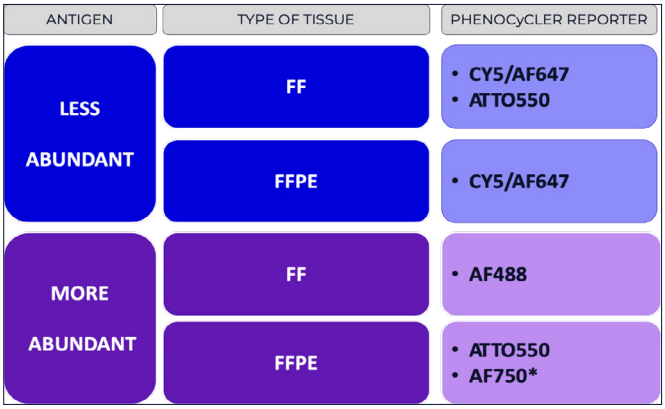


FIGURE B.1. Use cases for PhenoCycler Reporters.

*After Preliminary Screening.

*Since AF750 dye is in the Near IR region, low camera sensitivity may cause conjugations to appear as a failure although another channel will show successful conjugation. We suggest doing preliminary screening on a different channel to confirm successful conjugation of a particular antibody clone and then conjugating more abundant antigens/stronger antibody signal intensity to this channel.

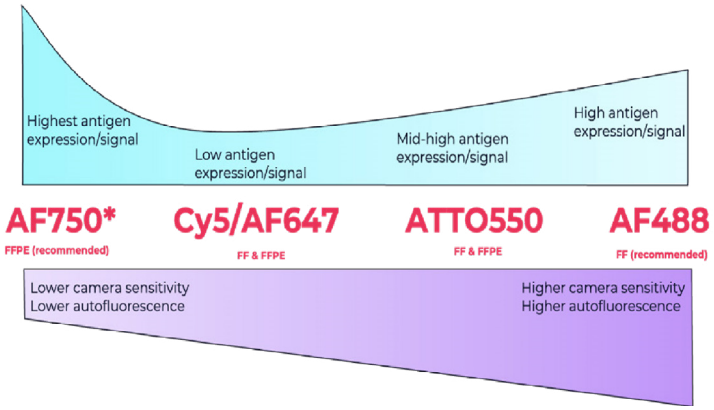


FIGURE B.2. Use cases for PhenoCycler Reporters.

USING PURIFIED ANTIBODIES

Purchase pre-purified antibodies. When selecting clones for conjugation to PhenoCycler Barcodes, we recommend purchasing purified antibodies in PBS or a similar buffer that is free of carrier proteins and other chemicals. Otherwise, purification is necessary. Many vendors may also allow you to purchase a "custom formulation" free of carrier proteins if requested.

Purify before conjugation if necessary. If purified clones are not commercially available, a purification process must be performed before conjugation. Carriers including BSA, gelatin, and glycerol must be removed prior to conjugation. The presence of sodium azide does not interfere with conjugation and does not need to be removed.

Quantify antibodies accurately. Be sure to measure the concentration of commercial antibodies using a NanoDrop or similar instrument. This is important for calculating the amount of antibody to use for conjugation.

NOTE > ***The success of custom conjugation is highly dependent on the ratios of antibody to barcode. The concentrations labeled on the antibody tubes are oftentimes inaccurate.***

ANTIBODY FILTRATION

The purified antibody is added to the top of a 50 kDa Molecular Weight Cut-Off (MWCO) filter.

Centrifugation steps are performed, resulting in concentrated antibody solution in the filter unit and flowthrough in the bottom of the tube. (See Figure B.3.) Flow-through solution should be discarded after each step as instructed. A 50 kDa MWCO filter must be used. Use of filters other than 50 kDa MWCO will likely result in poor purification, poor conjugation, and/or loss of tagged antibody.

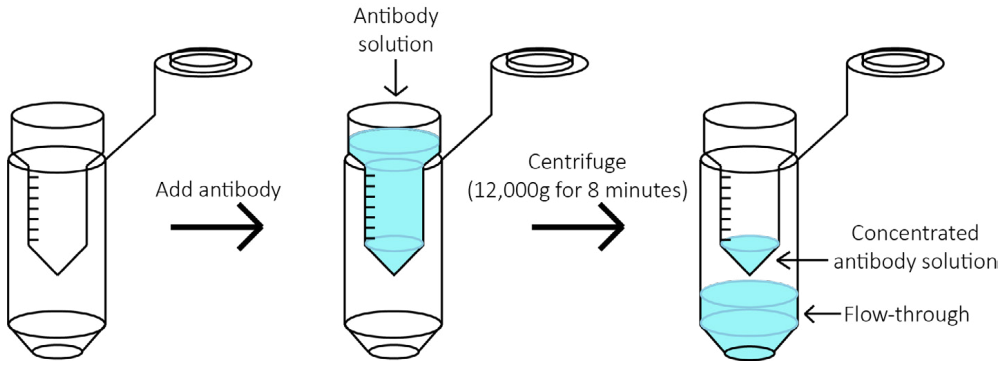


FIGURE B.3. Antibody filtration.

PRE-EXPERIMENT PREPARATION

Materials Provided

TABLE B.2. Material included in the Antibody Conjugation Kit (P/N 7000009)

Antibody Conjugation Kit	Storage	Notes
Retrieve before beginning the experiment:		
Reduction Solution 1	-20°C	Single use tubes. Do NOT reuse after thawing once. Each tube has enough reagent for up to 3 conjugations. Discard any remaining reagent.
Reduction Solution 2	4°C	
Filter Blocking Solution	4°C	
Retrieve after 30-minute incubation in Step 5:		
Conjugation Solution	4°C	

Materials Not Provided

TABLE B.3. Antibody Conjugation Kit

Type	Item
Biologics	Purified antibody/s
Consumables	50 kDa MWCO filter
	1.5 mL screw-top sterile tube/s
	Nuclease-free molecular biology-grade water
	PBS
	0.2 mL PCR tubes (For Quality Check, see **Verifying the Success of Conjugation**.)
	Bucket of ice for antibodies
Instrumentation	Centrifuge for 1.5 mL tubes
	NanoDrop™ spectrophotometer
	Vortex (optional)

CONJUGATION PROCEDURE

To conjugate antibodies:

1. Assign a PhenoCycler Barcode to each antibody that will be conjugated.
2. Label a 50 kDa MWCO filter for each antibody to be conjugated.
3. Block non-specific binding of antibody to MWCO filter:
   a. Add 500 µL of Filter Blocking Solution to the top of each 50 kDa MWCO filter.
   b. Spin down at 12,000g for 2 minutes.
   c. Remove all liquid on the top of the column, as well as the flow-through solution. Use a micropipette if desired.
4. Measure and calculate the protein concentration:
   a. Set up a NanoDrop spectrophotometer or similar instrument for absorbance readings. Use pre-set IgG settings.
   b. Calculate the volume of solution that corresponds to 50 µg of antibody.
5. Concentrate the purified antibody solution:

Add the volume corresponding to 50 µg of the antibody volume calculated in Step 4b to each 50 kDa MWCO filter. If the volume is less than 100 µL, adjust the volume to 100 µL by adding 1X PBS.

   a. Spin down tubes at 12,000 g for 8 minutes.
   b. During the centrifugation, prepare Antibody Reduction Master Mix as described in Step 6.c. Discard the flow-through.
6. Initiate the antibody reduction:
   a. Prepare the Antibody Reduction Master Mix based on the number of antibodies to be conjugated. (See Table B.4.) One tube of Reduction Solution can be used for up to 3 conjugation reactions.

TABLE B.4. Number of antibodies to be conjugated

Number of Conjugations	1	2	3	4	5	6	7	8
Reduction Solution 1 (µL)	6.6	13.2	19.8	26.4	33	39.6	46.2	52.8
Reduction Solution 2 (µL)	275	550	825	1100	1375	1650	1925	2200
Total (µL)	281.6	563.2	844.8	1126.4	1378	1689.6	1971.2	2252.8

CRITICAL > ***Thawed aliquots of Reduction Solution 1 should NOT be reused.***

b. Add 260 µL of the Antibody Reduction Master Mix to the top of each filter unit.

c. Briefly vortex solution in filter units for 2-3 seconds to mix.

d. Incubate the tube at RT for 30 minutes.

CRITICAL INCUBATION ***Incubate for 30-minute. It is critical NOT to exceed 30 minutes.***

7. Buffer exchange of the Antibody Solution:

a. After the 30-minute incubation has completed, spin down the tubes at 12,000 g for 8 minutes.

b. Discard the flow-through solution.

c. Add 450 µL of Conjugation Solution to the top of each column.

d. Spin down at 12,000g for 8 minutes.

e. During centrifugation, prepare PhenoCycler Barcode Solution in Step 8.

8. Prepare PhenoCycler Barcode Solution:

NOTE > ***Each Barcode is used once for every 50 µg of antibody.***

CRITICAL INCUBATION ***After retrieving barcodes, do not spend more time than necessary on protocol steps since barcode integrity can start to degrade. We recommend conjugating no more than 6 barcodes per experiment.***

a. Add 10 µL of Nuclease-free molecular biology-grade water to each lyophilized barcode container.

b. Add 210 µL of Conjugation Solution to each suspended barcode.

c. Pipette up and down to dissolve all the lyophilized material.

d. Mix by gentle pipetting. Set aside.

9. Set up Antibody Conjugation Reaction:

a. After the spin has completed in Step 7.d., discard the flow-through.

b. Add the respective PhenoCycler Barcode Solution created in Step 7 to the top of each filter.

c. Close the lid and vortex the solution for 2–3 seconds to mix.

d. Incubate the antibody conjugation reaction for 2 hours at RT.

INCUBATE > ***Incubate for 2 hours at RT.***

10. Purify the PhenoCycler Antibody conjugates:

a. After the 2-hour incubation, remove a 5 µL aliquot of the purified solution into a new 0.2 mL PCR tube for validation of conjugation. (See Verifying the Success of Conjugation.)

NOTE > ***Do not skip Step 10a. Once a conjugated antibody is placed in Antibody Storage Solution, a gel cannot be run on the sample to verify conjugation.***

b. Spin down the remainder of the solution at 12,000g for 8 minutes.

c. Discard the flow-through solution.

d. Add 450 µL of Purification Solution to the top of each column.

e. Spin down at 12,000g for 8 minutes.

f. Repeat Steps c–e 2 more times for a total of 3 purifications.

g. After the third centrifugation, discard the flow-through solution.

h. The filter will contain the conjugated antibody solution.

11. Collect the PhenoCycler Antibody Solution:

a. For each antibody, label a new outer tube that can hold filter units with the corresponding antibody name.

b. If desired, cut the lid off of the tube to minimize damage during centrifugation.

c. Add 100 µL of Antibody Storage Solution to each filter unit.

d. After it is labeled, place the new empty tube upside-down on top of the filter.

e. Invert the filter unit for collection into the new collection tube.

f. Spin solution down at 3,000g for 2 minutes. The final volume in the tube should be about 120 µL.

STOPPING POINT >

Transfer the solution to a sterile screw-top tube for storage at 4°C for up to 1 year.

After antibody conjugation, do not use these antibodies for tissue staining for at least 2 days. If used for staining sooner, you may observe high levels of background nuclear staining.

VERIFYING THE SUCCESS OF CONJUGATION

Protein gel electrophoresis can be performed to verify the success of the antibody conjugation reaction. Please note that this procedure only assesses the success of the chemical reaction used for barcode-antibody conjugation.

Heavy chains of conjugated antibodies will show higher molecular weights than their unconjugated counterparts. This comparison can be done by loading a protein gel using the following components:

- 5 µL of each conjugated antibody from Step 10.a.
- 1 µg (usually corresponding to 2 µL) of unconjugated antibody to be used as control.
- A protein ladder to be used as a molecular weight standard.

PRE-EXPERIMENTATION PREPARATION

Materials Not Provided

Use the reagents and protein gel of choice. In this example, we used the items in Table B.5.

The materials listed in Table B.5 are required, but not supplied, for verifying conjugation.

TABLE B.5. Materials required (but NOT supplied) for verifying conjugation

Type	Item	Vendor	P/N
Reagents and Protein Gel	NuPAGE™LDS Sample Buffer (4X)	Thermo Fisher Scientific	NP0008
	NuPAGE Sample Reducing Agent (10X)	Thermo Fisher Scientific	NP0009
	NuPAGE4–12% Bis-Tris Protein Gels	Thermo Fisher Scientific	NP0321BOX
	Novex™ Sharp Pre-Stained Protein Standard—3.5–260 kDa	Thermo Fisher Scientific	LC5800
	XCell SureLock™ Mini-Cell Electrophoresis System	Thermo Fisher Scientific	EI001 and related
	NuPAGE MOPS SDS Running Buffer (20X)	Thermo Fisher Scientific	NP0001
	Novex SimplyBlue™ SafeStain	Thermo Fisher Scientific	LC6065
	ddH_2O	Customer Choice	--
	Nuclease-free water	Customer Choice	--
Instrumentation	95°C dry bath	Customer Choice	--
	Shaker	Customer Choice	--
	Microwave	Customer Choice	--

Procedure

To validate custom-conjugated antibodies (with gel electrophoresis):

1. Prepare the sample:
   a. Dilute each of the conjugated antibodies and the unconjugated antibody control to a final volume of 13 µL with nuclease-free water.
   b. Add 5 µL of NuPAGE LDS Sample Buffer (4X) (NP0008) or an analogous product to each sample tube.
   c. Add 2 µL of NuPAGE Sample Reducing Agent (10X) (NP0009) or an analogous reducing agent to each sample tube.
   d. Denature all samples at 95°C in a dry bath for 10 minutes.
2. Set up the gel:
   a. Prepare enough volume of buffer for running the gel. In this example, we prepared 800 mL of 1X NuPAGE MOPS SDS Running Buffer by diluting 40 mL of NuPAGE MOPS SDS Running Buffer (20X) in 760 mL of ddH_2O.
   b. Prepare the gel and place it in the tank following manufacturer instructions.

c. Pour the buffer in the gel tank making sure that the liquid fully covers the gel.

d. Load 1 well with the protein standard to determine molecular weight.

e. Load a second well with 20 µL of the denatured control unconjugated antibody solution.

f. Load each of the remaining wells with 20 µL of the denatured conjugated antibody solution.

g. Run the gel at 200 V for 30–40 minutes until completion.

h. Turn off the current when the protein standard appears at the end of the gel.

3. Visualize the gel:

a. Remove the gel from the plastic cassette.

b. Gently transfer the gel into a microwavable container filled with ddH_2O.

c. Microwave the gel until the first bubbles form.

d. Stain the gel with Novex SimplyBlue SafeStain (LC6065) or an analogous product according to manufacturer's instructions.

e. Microwave the gel again until the first bubbles form.

f. Place the gel in the shaker for 10 minutes.

g. Wash the gel with ddH_2O and leave it on the shaker until bands are visible. (See Figure B.4.) More microwaving steps can be added to accelerate this process or it can be left overnight on a shaker.

NOTE > ***It is important to change the water.***

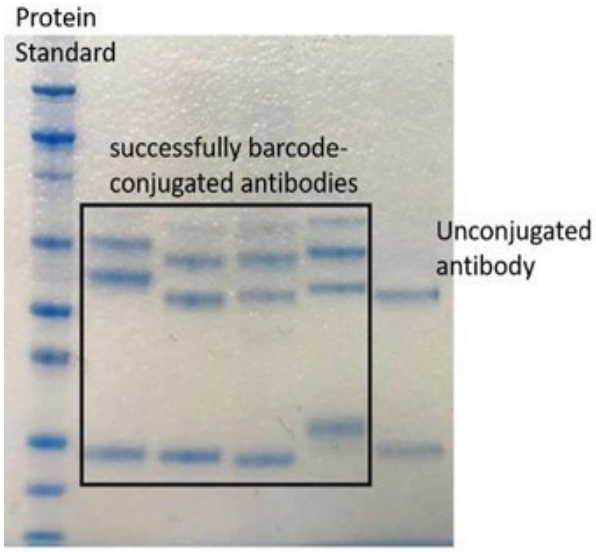


FIGURE B.4. Gel visualization.

The following describes the gel visualization (from left-to-right):

- First column shows the protein standard.
- Columns 2–5 (in this example) show the band of successfully barcode-conjugated antibodies.
- The last column shows the heavy and light chain bands from a control unconjugated antibody.

NOTE > ***Microwaving steps are optional. They are used to accelerate the gel readout.***

NOTE > ***Wait at least 2 days before using newly conjugated antibodies for tissue staining. Otherwise, you may experience high levels of background nuclear staining.***

APPENDIX C

PhenoCycler Fusion EULA

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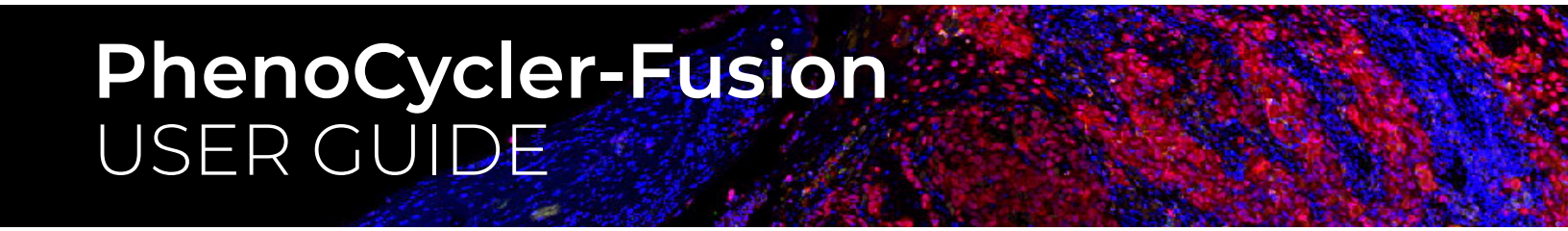

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