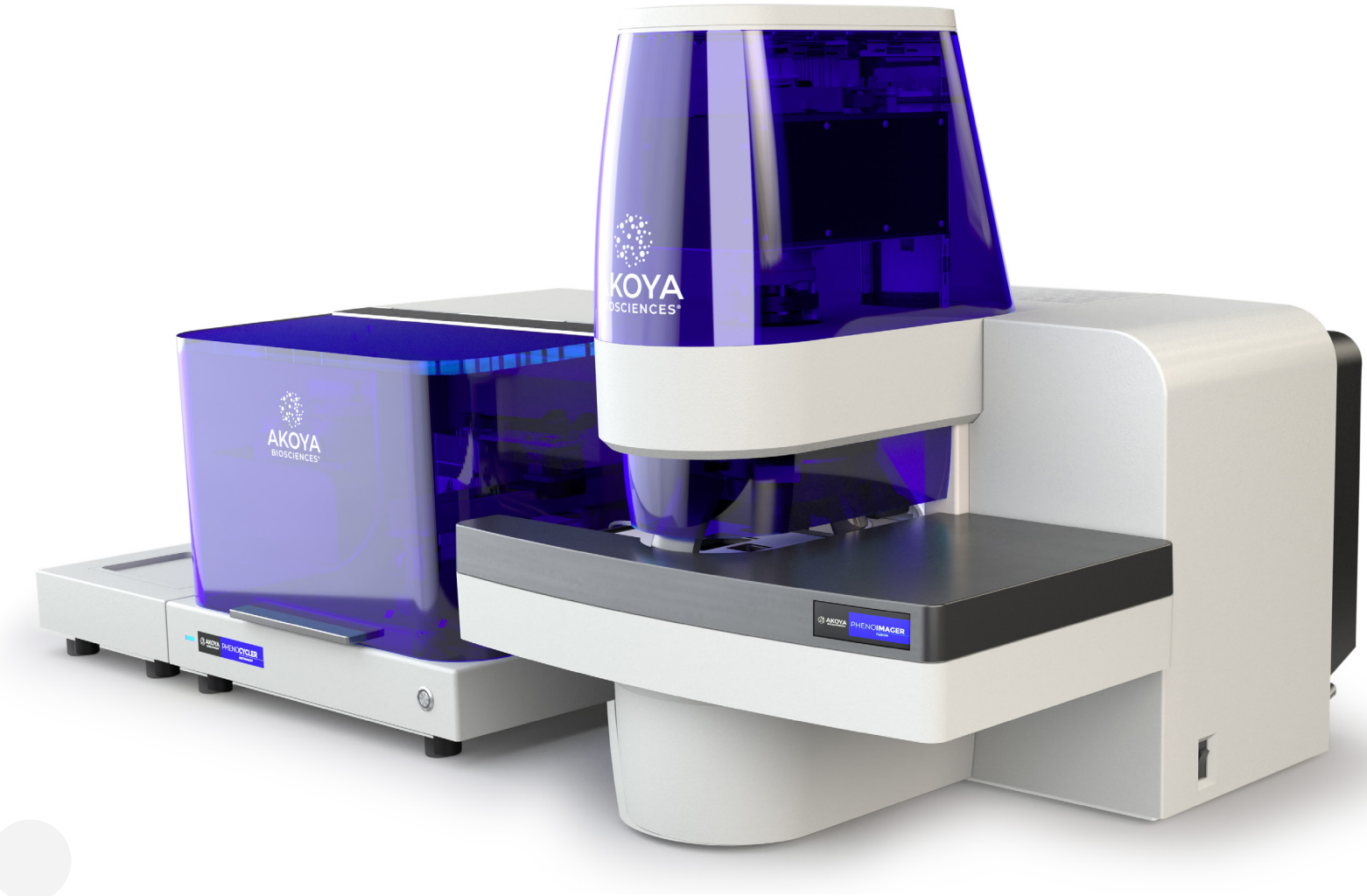
AKOYA BIOSCIENCES®
THE SPATIAL BIOLOGY COMPANY®

PhenoCycler-Fusion
USER GUIDE



PhenoImager Fusion SW version: 1.0.8 | Date: 032223

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CONTENTS

PREFACE

Welcome to the Akoya Biosciences' PhenoCycler-Fusion User's 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-Fusion instrument.

ASSOCIATED DOCUMENTS

- User's Manual (For use with PhenoCycler-Fusion)
- Opal Multiplex Assay Development Guide
- PhenoImager Fusion User Guide
- Phenochart Whole Slide Context Viewer for Annotation & the Review User's Manual
- inForm Advanced Image Analysis Software User's Manual

BOOK CONVENTIONS

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

	***DANGER: Warns the user of an imminently hazardous situation, which is 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 offers Field Service Engineers and internal specialists who are dedicated to supporting your hardware, software, and application development needs.

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.***

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.

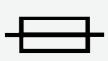
A급 기기 (업무용 정보통신기기)

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

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.

NOTE ***The figures below this table show the location of the labels on the instrument.***

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
 **NOTE**	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 (LABELING OF EQUIPMENT AND LOCATION)

DEFINITION OF SYMBOLS (CONTINUED)			
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.

PhenoCycler Nameplate

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 for technical support.*
	*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" on **page 13**.
- "Fuses" on **page 13**.
- "Cables and Adapters" on **page 13**.

POWER CORD SELECTION

Contact Akoya Biosciences Technical Support 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 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 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 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 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 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

Topics described in this chapter include:

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.

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.

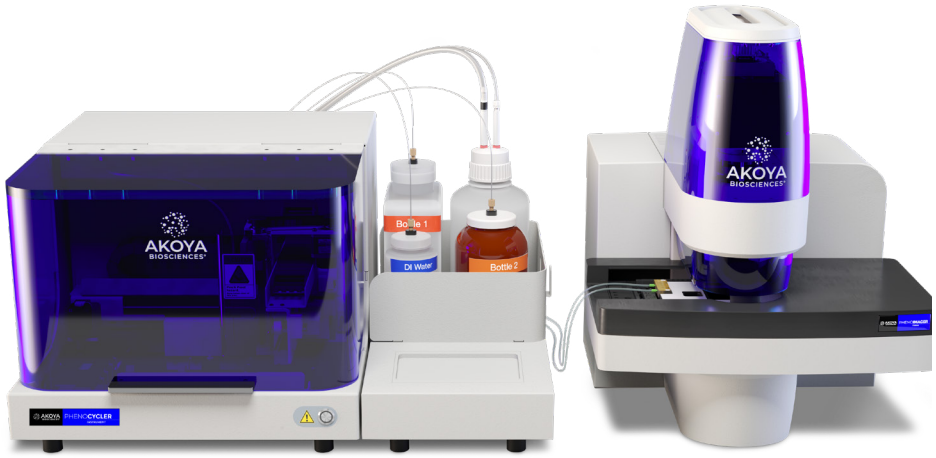


FIGURE 1-1: PhenoCycler-Fusion System

PHENOCYCLER TECHNOLOGY

Akoya Biosciences has designed and developed the PhenoCycler-Fusion system to accelerate the field of spatial biology. This integrated platform combines the strengths Akoya's automated, ultrahigh multiplex cycling platform, PhenoCycler, and its high-speed imaging platform, PhenoImager™ (formerly Phenoptics), into an end-to-end solution.

From building cell atlases to developing a novel spatial signatures novel spatial signature, the PhenoCycler-Fusion system has the power to match the scale of your studies today and tomorrow.

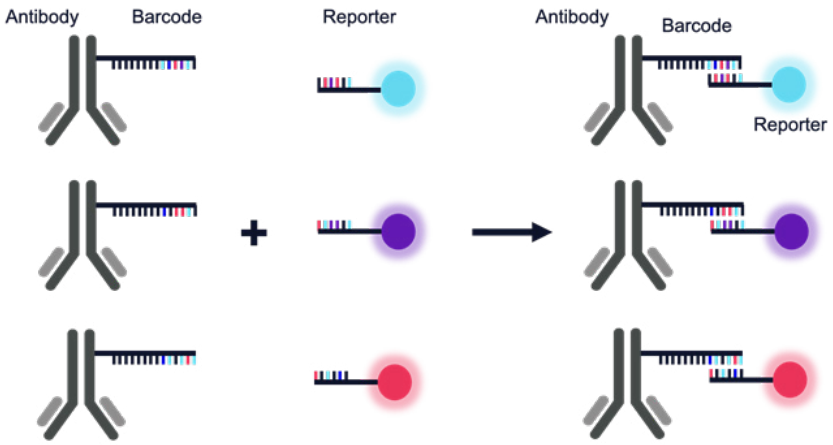


FIGURE 1-2: Reversible Hybridization Between the PhenoCycler Barcodes and Complementary PhenoCycler Reporters to Sequentially Reveal PhenoCycler Antibodies

PHENOCYCLER WORKFLOW NOMENCLATURE

FIGURE 1-3 shows the PhenoCycler barcoding system.

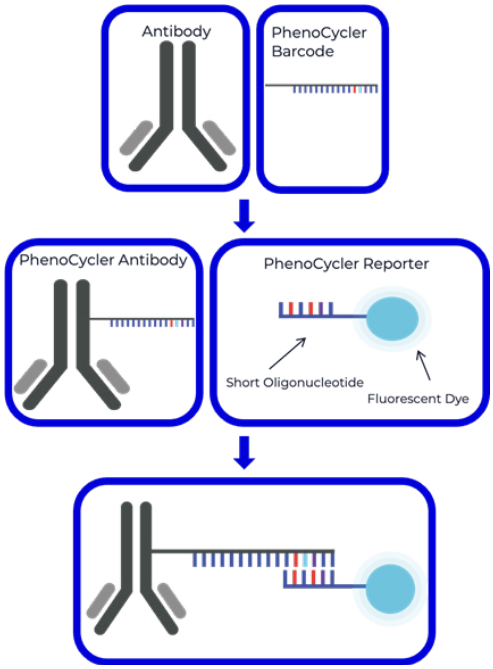


FIGURE 1-3: PhenoCycler Barcoding System

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. Reporter is comprised of a fluorescent dye and a short oligonucleotide.

TABLE 1-1 lists the PhenoCycler products.

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 Anoligonucleotide 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

TABLE 1-1: PhenoCycler Products

This next section gives a brief overview of the entire PhenoCycler-Fusion workflow. The subsequent chapters will focus on guiding the user through protocols related to the tissue preparation and antibody staining portions of the PhenoCycler-Fusion Solution.

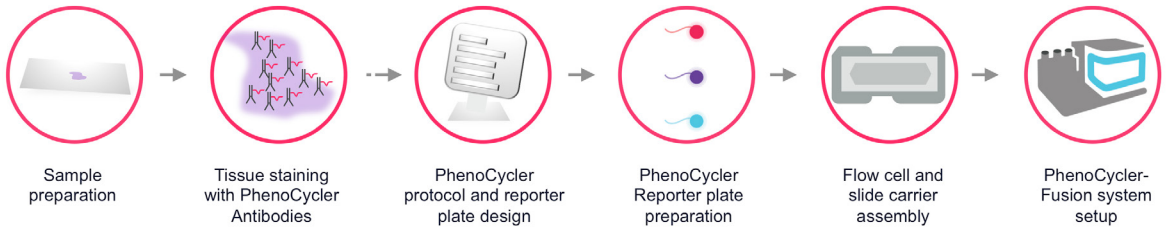


FIGURE 1-4: PhenoCycler Workflow

PHENOCYCLER INSTRUMENT

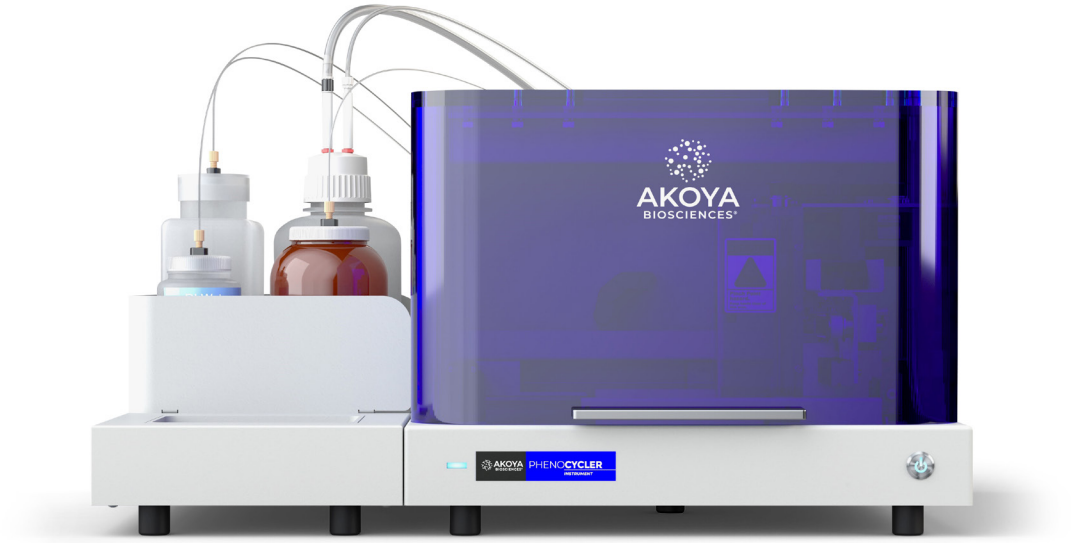


FIGURE 1-5: PhenoCycler Instrument

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

- A Robotic Cannula
- 4 Removable Reservoirs
- Buffer Tray for 4 Reservoirs
- One Holder for the 96-well reporter plate
- 4 Bottles on the side tray
- 1x PhenoCycler Buffer Bottle
- a Vacuum/Waste Bottle
- a DMSO Bottle
- a Water Bottle
- Flow Cell Assembly device
- Flow Cell Slide Carrier that 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. It is directed by the PhenoCycler-Fusion controller software, which also controls the image acquisition 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 Supplied

Sample Kit for PhenoCycler-Fusion (PN# 7000017)
This kit contains buffers and reagents to perform tissue stains with barcode-tagged PhenoCycler Antibodies, or custom-conjugated antibodies (**TABLE 1-2**).

Each kit contains enough reagents and flow cells for 10 tissue samples.

Contents	Storage	Related Protocols
Hydration Buffer	4°C	Tissue Staining
Staining Buffer		
Storage Buffer		
N Blocker		
J Blocker		
G Blocker	-20°C	
S Blocker		
Fixative Reagent		
Flow Cells	Ambient	Use of PhenoCycler-Fusion System

TABLE 1-2: Sample Kit for PhenoCycler-Fusion (PN# 7000017)

Antibody Conjugation Kit (PN# 7000009)
This kit contains the PhenoCycler reagents required for custom conjugation of non-inventoried antibodies with the PhenoCycler Barcodes to run a PhenoCycler experiment (TABLE 1-3).

Each kit contains enough reagents for 10 conjugations (antibodies not included).

Content	Storage	Related Protocols
Reduction Solution 1	-20°C	Antibody Conjugation
Filter Blocking Solution	4°C	
Reduction Solution 2		
Conjugation Solution		
Purification Solution		
Antibody Storage Solution		

TABLE 1-3: Antibody Conjugation Kit (PN# 7000009)

PhenoCycler à la Carte Items

TABLE 1-4 shows single order items used during Tissue Sectioning, Tissue Staining, Reporter Plate Preparation, and PhenoCycler runs.

Contents	PN#	Storage	Related Protocols
10X Buffer for PhenoCycler	7000001	RT	Tissue Staining Reporter Plate Preparation, Use of PhenoCycler-Fusion System
96-well plates for PhenoCycler	7000006		Use of PhenoCycler-Fusion System, Reporter Plate Preparation
96-well plate seals for PhenoCycler	7000007		
Assay Reagent for PhenoCycler	7000002	-20°C, and 4°C after the first thaw	Tissue Staining Reporter Plate Preparation Use of PhenoCycler-Fusion System
Nuclear Stain for PhenoCycler	7000003		

TABLE 1-4: PhenoCycler Reagents and Consumables

PhenoCycler Reagents

PhenoCycler reagents include PhenoCycler Antibodies, Reporters, and Barcodes (see TABLE 1-5). An updated list of available products can be found on our website: **akoyabio.com**.

Please refer to “PhenoCycler Nomenclature” on page 2-3 for information on the design and structure of PhenoCycler reagents.

Content	Storage	Related Protocols
PhenoCycler Antibodies	4°C	Tissue Staining
PhenoCycler Barcodes	-20°C	Antibody Conjugation
PhenoCycler Reporters	-20°C and 4°C after the first thaw	Reporter Plate Preparation, Use of PhenoCycler-Fusion System Validation of Custom-Conjugated Antibodies.

TABLE 1-5: Storage of PhenoCycler Reagents

Materials Not Supplied

The materials listed in Table 1-6 are not supplied, but are required for the entire PhenoCycler workflow.

Type	Item	Vendor	PN#	Section
Consumables	Coplin jars	VWR	513200	Tissue Staining
	Glass microscope slides (1in x 3in)	Leica Slide White Apex Superior Adhesive or Fisherbrand Superfrost Plus	3800080 or 12-550-15	Tissue sectioning, tissue staining, tissue storage
	Staining tray	VWR	M918-2	Tissue Staining
	Microscope Slide Box	Fisher Scientific	03-448-1	Tissue Sectioning, Tissue Staining, Tissue Storage
	Buffer reservoirs - 4 Required. No substitutions.	Beckman Coulter	BK372790	Use of PhenoCycler-Fusion System (1 set included with instrument)
	Buffer reservoir tray - Required. No substitutions.	Beckman Coulter	BK372795	Use of PhenoCycler-Fusion System (1 set included with instrument)
	1 mL, 1.5 mL, 2 mLtubes	Customer choice	--	Tissue StainingUse of PhenoCycler-Fusion System
	Amber 1.5 mL tubes	Customer choice	--	PhenoCycler Reporter PreparationUse of PhenoCycler-Fusion System
	Serological Pipet	Customer choice	--	Tissue Sectioning Tissue StainingUse of PhenoCycler-Fusion System
	5, 15, 50 mL conical tubes	Customer choice	--	Tissue Staining
	Disposable Filter Units	Nalgene™ Rapid-Flow™ (Recommended)	156-4020	Use of PhenoCycler-Fusion System
	Compressed Air Duster	Customer choice	--	Use of PhenoCycler-Fusion System
	Kimwipes	Customer choice	--	Use of PhenoCycler-Fusion System
	Parafilm	Customer choice	--	Tissue staining

TABLE 1-6: Materials Required for the Entire PhenoCycler Workflow

Materials Not Supplied (Continued)

Type	Item	Vendor	PN#	Section
Biologics/ Reagents	16% Paraformaldehyde	Electron Microscopy Sciences (Recommended)	15710	Tissue Staining
	1X PBS	Life Technologies	14190144	Antibody Conjugation Tissue Staining
	Nuclease-Free Water	Thermo Fisher Scientific	AM9938	Reporter Plate Preparation
	Fluoromount-G™ (optional)	Thermo Fisher Scientific	00-4958-02	Validation of Custom- Conjugated Antibodies
	ddH_2O or Milli-Q® H2O	Customer choice	--	Use Of PhenoCycler-Fusion System Antibody Conjugation
	Methanol	Sigma-Aldrich	34860-1L-R	Tissue Staining
	DMSO - ACS reagent,≥99.9%	Sigma-Aldrich	472301-4L	Validation of Custom- Conjugated Antibodies Used PhenoCycler-Fusion System
Instrumentation	UPS (Recommended)	APC Back-UPS Pro 1500	BR1500G	Use of PhenoCycler-Fusion System
	Vacuum Pump	Customer choice	---	Validation of Custom- Conjugated Antibodies
	Fume Hood (Highly Recommended)	Customer choice	---	Tissue Staining Waste Collection

TABLE 1-7: Materials Required for entire PhenoCycler-Fusion Workflow

The materials listed in TABLE 1-8 are not supplied, but are required for for Fresh-Frozen tissue sections.

Type	Item	Vendor	PN#	Section
Consumables	Drierite Adsorbents	Fisher Scientific	23-116582	Tissue Staining
Solvents	Acetone	Sigma-Aldrich	650501-1L	Tissue Staining
Instrumentation	Cryostat	Customer choice	---	Tissue Sectioning

TABLE 1-8: Materials Required for for Fresh-Frozen Tissue Sections

The materials listed in TABLE 1-9 are NOT supplied, but are required for Formalin-Fixed-Paraffin-Embedded (FFPE) Tissue Sections.

Type	Item	Vendor	PN#	Section
Materials required for Formalin-Fixed-Paraffin-Embedded (FFPE) Tissue Sections				
Additional Materials	Aluminum Foil	Customer choice		Antigen Retrieval
	10 Solvent-resistant Containers with lids	EZ-Quick Slide Staining Set, IHC World	IW-2510	Tissue Staining
	Slide staining rack	EZ-Quick Slide Staining Set, IHC World	IW-2512	Tissue Staining
Solvents	10x AR6 OR 10x AR9 Buffer	Akoya Biosciences	PN#AR6001KT or PN#AR9001KT	Antigen Retrieval
	Ethanol or Reagent Alcohol	Sigma Aldrich	79317-16GA-PB	Tissue Staining
	Histo-Choice Clearing 1x	VWR	H103-4L	Tissue Staining
Equipment	Microtome	Customer choice		Tissue Sectioning
	Instant Pot	Instant Pot		Antigen Retrieval

Table 1-9: Materials Required for Conjugation

The materials listed in TABLE 1-10 are required for Conjugation.

Materials Required for Custom Conjugation				
Consumables	50kDa MWCO filter - No size substitutions (25kDa and 100kDA result in failure)	EMD Millipore	UFC505096	Antibody Conjugation
	Screw-top 1.7 mL or 2 mL tubes	Customer choice	---	Antibody Conjugation
	Parafilm	Customer choice	---	Conjugation Verification
Biologics/Regents	Purified antibodies	Customer choice	---	Antibody Conjugation
	NuPAGE™ LDSSample Buffer (14X)	Thermo Fisher Scientific	NP0008	Conjugation Verification
	NuPAGE™ Sample Reducing Agent (10X)	Thermo Fisher Scientific	NP0009	Conjugation Verification
	NuPAGE™ 4-12% Bis-Tris Protein Gels	Thermo Fisher Scientific	NP0321BOX	Conjugation Verification
	Novex™ Sharp Pre- Stained Protein Standard 3.5-260 kDa	Thermo Fisher Scientific	LC5800	Conjugation Verification
	Novex™ SimplyBlue™ SafeStain	Thermo Fisher Scientific	LC6065	Conjugation Verification
	NuPAGE™ MOPSSDS Running Buffer (20X)	Thermo Fisher Scientific	NP0001	Conjugation Verification

Type	Item	Vendor	PN#	Section
Instrumentation	Centrifuge	Customer choice		Antibody Conjugation
	XCell SureLock™ Mini-Cell Electrophoresis System	Customer choice		Conjugation Verification
	95°C dry bath	Customer choice		Conjugation Verification
	Nanodrop	Customer choice		Antibody Conjugation
	Shaker (optional)	Customer choice		Conjugation Verification
	Microwave (optional)	Customer choice		Conjugation Verification

TABLE 1-10: Materials Required for Conjugation

PHENOCYCLER-FUSION SOFTWARE SUITE

PhenoCycler-Fusion makes use of a software suite containing four different programs – PhenoCycler-Fusion Acquisition Software, the PhenoCycler Experiment Designer App, Phenochart Visualization software, and inForm software for analysis.

PhenoCycler-Fusion will write whole slide scans in QPTIFF format, which contains all image layers and metadata. Whole slide image files written from PhenoCycler-Fusion are viewable in Phenochart software.

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

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

PHENOCYCLER-FUSION EXPERIMENT OVERVIEW

In a PhenoCycler-Fusion experiment, a tissue section on a slide is stained manually using a panel of PhenoCycler Antibodies simultaneously. After staining is complete, a flow cell is attached on top of the slide, placed into the flow cell slide carrier which then is 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. The runs are comprised of multiple cycles: in each cycle, the reporters reveal up to three markers of interest (and DAPI) simultaneously, the tissue is imaged in each spectrally distinct fluorescence channels, 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."

FIGURES 1-6 and 1-7 illustrate the procedures for PhenoCycler Antibody staining and PhenoCycler multicycle runs.

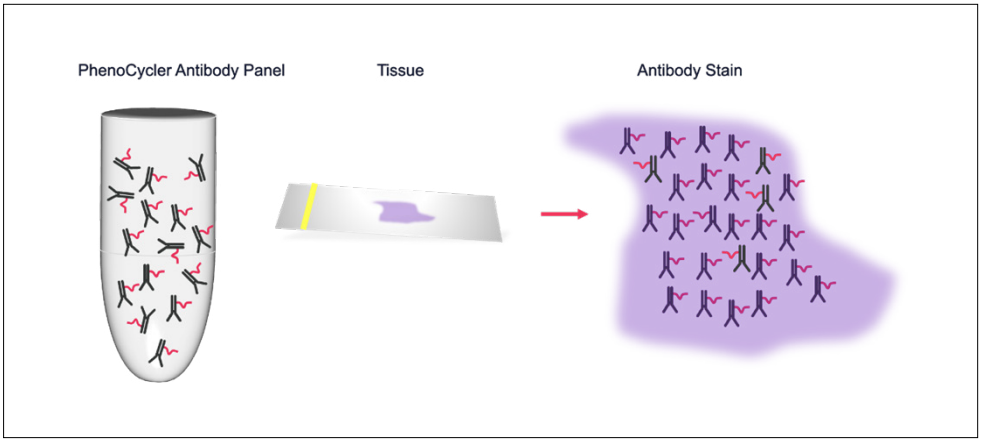


FIGURE 1-6: Single Staining Step: A Panel of PhenoCycler Antibodies is Used to Stain a Tissue Section in a Single Step

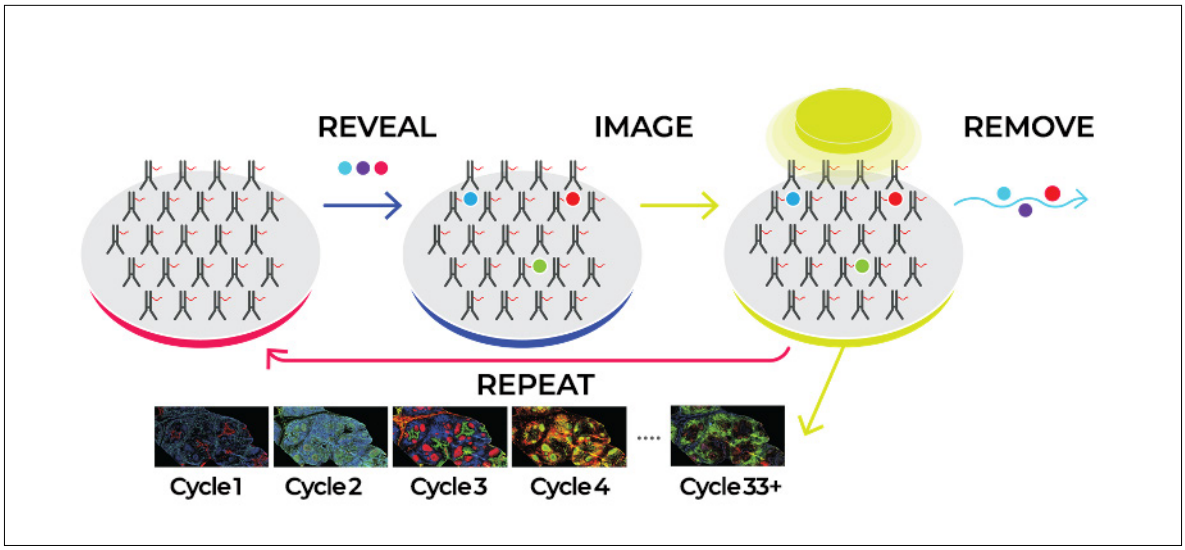


FIGURE 1-7: PhenoCycler-Fusion Runs (Reveal-Image-Remove-Repeat)

The PhenoCyclerWorkflow: 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 which 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 and are, at the end of the run, available as whole slide scans in QPTIFF format, which contains all 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 then be used in external applications.

PHENOCYCLER-FUSION PROCEDURE OVERVIEW

1. Adhere tissue sections to recommended standard 1"x3" positively-charged glass microscope slides. (Refer to **Chapter 2**)
2. Design a PhenoCycler Antibody panel. Antibody panels are customizable and can include both commercially available inventoried PhenoCycler Antibodies, as well as custom-conjugated antibodies. For instructions on how to conjugate PhenoCycler Barcodes to antibodies of interest, please 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. (Refer to **Chapter 3**).
4. Using the Experiment Designer app, create, design the experiment protocol and reporter well plate layout (refer to **Chapter 4**).

   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 up to three spectrally distinct Reporters. Each group of Reporters, in addition to DAPI, comprises one cycle and are placed into separate wells of a 96-well plate that will be dispensed onto the tissue during separate PhenoCycler cycles. (Refer to **Chapter 5**).

5. Prior to a PhenoCycler-Fusion run, attach flow cell to the antibody-stained tissue slide, assemble the flow cell slide carrier with a blank flow cell, connect the carrier to the PhenoCycler instrument lines, launch the Fusion controller software, and follow the prompts to prepare and prime the PhenoCycler instrument. Refer to **Chapters 3 and 6** in the PhenoImager Fusion User Guide.
6. After preparing and priming the instrument, load the flow cell attached to stained tissue section into the flow cell slide carrier and placed it onto the stage.
7. Initiate the PhenoCycler multicycle run.

After the PhenoCycler multicycle run is complete, remove the tissue, replace with a blank flow cell into the slide carrier, and clean the PhenoCycler Instrument.

USER GUIDE OVERVIEW

FIGURE 1-8 shows an illustrated overview of the PhenoCycler procedure with references to the chapters where more information can be found for each step.

	Chapter or Appendix	Title
	2	Sample Preparation
	3	Tissue staining
	4	Experiment Protocol and Reporter Plate design using the PhenoCycler Experiment Designer Software
	5	PhenoCycler Reporter Plate preparation
	A	Specifications
	B	Custom Conjugation

CHAPTER 2

Sample Preparation

Topics described in this chapter include:

INTRODUCTION

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, fresh-frozen or FFPE tissues are sectioned and directly adhered to standard sized (1" x 3") positively-charged glass microscope slides. Using non-standard microscope slides and/or tissue preparation techniques that deviate from this protocol is not recommended.

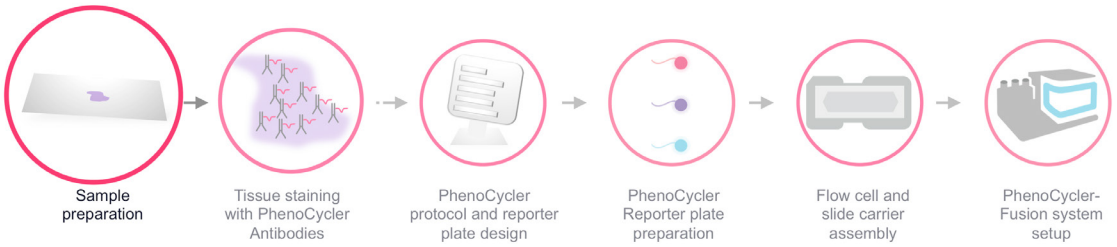


FIGURE 2-1: PhenoCycler Workflow - Sample Preparation

FRESH-FROZEN TISSUE SECTIONING

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 manual are specific to the PhenoCycler workflow, and they 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 as 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 Included

Type	Item	Notes
Tissue	Fresh-frozen tissue block of interest	
Consumables	Dry ice	
	Compressed/ canned air duster	
Laboratory equipment	1" x 3" positively charged glass microscope slides	as prepared in Recommended: Leica Apex Adhesive Slide size 1" x 3" x .04" (Catalog #3800080) or Fisherbrand Superfrost Plus Microscope Slides (Catalog 12-550-15) Please 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. Recommended slide dimensions: 24-25 mm width; 74-75mm length
	Microscope slide storage box	
	Polystyrene container	for Dry ice
	Blade for tissue sectioning	we recommend 63069-LP Low Profile Microtome Feather® Blade by Electron Microscopy Sciences
	Cryostat for tissue sectioning	

TABLE 2-1: List of Tissue, Consumables and Laboratory Equipment Required for Fresh-Frozen Tissue Sectioning

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 TISSUES - 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 the - 80°C freezer 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 10 µm as this will affect the autofocusing capabilities of the microscope. Avoid folds and tears in the tissue, as these artifacts will affect image quality and data analysis.***

7. Gently place the tissue section in the center of the slide within the imageable area as shown in grey patterned rectangle below in FIGURE 2-2.

NOTE ***Make sure that the tissue section is placed in the center of the slide, as shown in FIGURE 2-2. Print this page out and use the true-to-size figure below as a guide. Tissue under the adhesive portion of the flow cell will affect bonding and 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.***

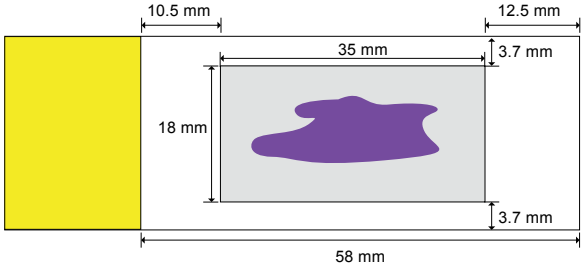


FIGURE 2-2: Fresh Frozen Tissue Placement Guide

9. Adhere the tissue section to the slide by placing a gloved finger underneath 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 OCT (Optimal Cutting Temperature 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.***

10. Place the mounted slide in a single slot of a microscope slide box.

11. Repeat **Steps 6-9** for each tissue section.

12. Once complete, cover the slide storage box with the lid.

13. Place the box of mounted slides on dry ice for transport to a -80°C freezer, as show in FIGURE 2-3.

FIGURE 2-3: Mounted Tissue Stored in Slide Storage Box

STOPPING POINT ***If prepared and stored properly, samples can be stored at*** **-80°C for up to 6 months.** ***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 our website, "Guidelines: Tissue processing – Best practices" at akoyabio.com.***

FFPE TISSUE SECTIONING

FFPE tissue sections are mounted onto slides. Preparation and storage of tissue sections are critical for sample integrity. The instructions outlined in this manual 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 at, "Guidelines: Tissue processing – Best practices" at: **akoyabio.com**.

GUIDELINES

Tissues

- FFPE Tissues sectioned onto slides can be stored at **4°C for up to 6 months**.
- It is critical not to exceed a thickness of 10 µm as 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 Included

Type	Item	Notes
Tissue	FFPE Tissue Block	
Consumables	Compressed/ canned air duster	
	1" x 3" positively charged glass microscope slides	As prepared in "Preparing Slides" Recommended: Leica Apex Adhesive Slide size 1" x 3" x .04" (Catalog #3800080) or Fisherbrand Superfrost Plus Microscope Slides (Catalog 12-550-15))
Laboratory equipment	Microtome for tissue sectioning	
	Blade for tissue sectioning	We recommend using 63069-LP Low Profile Microtome Feather® Blade by Electron Microscopy Sciences
	40°C water bath	
	An angled slide holder	For laying and drying the slides after adhering tissue sections (recommended, but optional)
	Slide storage box	

TABLE 2-2: List of Tissue, Consumables and Laboratory Equipment Required for FFPE Tissue Sectioning

PREPARE MICROTOME

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

FFPE TISSUES - 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 10 µm 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 towards the tissue. Doing so, the tissue will lay on the slide as it is moved out of the water bath.

NOTE ***Make sure that the tissue section is placed in the center of the slide, as shown in FIGURE 2-4. Print this page out and use the true-to-size figure below as a guide. Tissue under the adhesive portion of the flow cell will affect bonding and 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.***

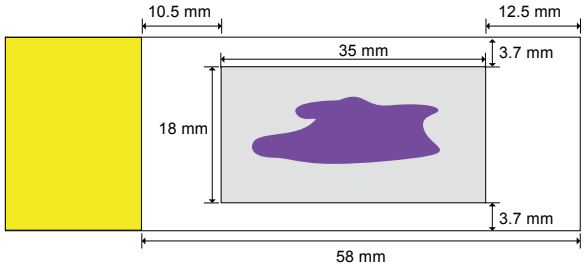


FIGURE 2-4: FFPE Tissue Placement Guide

9. Put the slide on a clean surface (with the tissue facing up) or on the angled slide holder and let it **dry overnight at RT**, as shown in 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. Place the mounted slide in a single slot of the slide storage box.

STOPPING POINT ***If stored properly, samples can be stored at 4°C for up to six months. Store box in a secure location, kept upright as to minimize movement of slides.***

CHAPTER 3

Tissue Staining

Topics described in this chapter include:

Fresh-Frozen Tissue Staining

FFPE Tissue Staining

INTRODUCTION

This chapter describes the process of staining tissue sections with PhenoCycler Antibodies.

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 fresh-frozen tissue sections and 7 hours for FFPE tissues. This time includes a 3-hour incubation step. Do not exceed or shorten this incubation time.

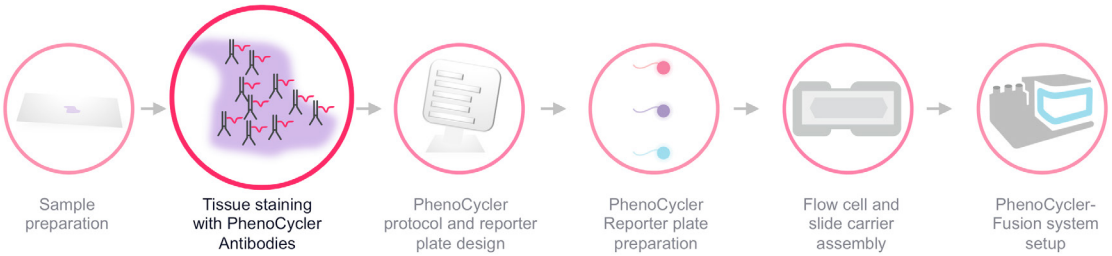


FIGURE 3-1: PhenoCycler Workflow – Tissue Staining

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. (Ab-BXxxx) See akoyabio.com for an up-to-date list of available PhenoCycler Antibodies.

Sample Slide Handling

- It is imperative to avoid tissue drying by minimizing tissue exposure to air. Tissue drying is detrimental to staining quality.
- To avoid damaging the tissue, avoid dispensing liquid directly onto the tissue. Always pipette solutions onto the corner of the slide and allow the liquid to flow over the tissue.
- It is good practice to stain more than one 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, and a piece of 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. Incubation times have been optimized for fixing or staining the tissue, ensuring the tissue does not dry. Do not exceed or shorten these recommended incubation times.***

Safety

- Acetone, PFA, and methanol are toxic chemicals and should be handled following 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 barrier. This may cause issues during PhenoCycler-Fusion run, as fluids may not be able to pass over the tissue properly.***

FRESH-FROZEN TISSUE PRE-STAINING

This section describes the preparation of fresh-frozen tissues for staining with PhenoCycler Antibodies. When working with freshly conjugated antibodies (as described in Appendix B **"Custom Conjugation"**), we recommend waiting at least two 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 Included in Kit Provided by Akoya Biosciences

Content	Storage
Akoya Hydration Buffer	4°C
Akoya Staining Buffer	
N Blocker	
J Blocker	
G Blocker	-20°C
S Blocker	
Flow Cells	Ambient

TABLE 3-1: Sample Kit for PhenoCycler-Fusion (PN# 7000017)

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.

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

TABLE 3-2: Antibodies (Materials Not Included in the Kit)

Follow these guidelines for materials listed in **TABLE 3-2**

- Obtain these materials immediately before use in the **"Pre-Staining Procedure"** on page 42 and place on ice.

Storage Buffer and Fixative Reagent will be used in **"Fresh-Frozen Tissue Post-Staining"** on page 47.

Materials Not Included in Kit

Type	Item	Notes
Lab Solvents	Acetone	Dispense right before use (~40 mL per Coplin jar)
	Cold methanol	Stored at 4°C. Dispense right before use (~40 mL per Coplin jar).
Lab Chemicals/Buffers	16% Paraformaldehyde (PFA)	Recommended: PFA from Electron Microscopy Sciences, PN# 15710.
Plastic Consumables/Tools	Coplin Jars	
	Slide staining box	VWR Slide Staining Tray, PN#M918-2
	Parafilm	Used to cover solutions directly applied to tissue sample slides
	Scissors	
	Drierite Absorbent Beads	
	1.5 mL Eppendorf Tubes	
	50 mL Conical Tube	
	Ice Bucket	
Laboratory Equipment	Fume Hood	Use of a fume hood is highly recommended for steps involving the use of acetone and PFA.

TABLE 3-3: List of Chemicals, Lab Supplies, and Equipment Required for the Tissue Staining

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, **Step 3: Tissue Retrieval,** 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 (**TABLE 3-4**).

- Retrieve PhenoCycler antibodies from 4°C and place on ice. **CAUTION:** If antibodies have just been custom-conjugated as 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 antibody dilutions document.
- For custom-conjugated antibodies, the volume of antibody solution used to stain any tissue needs to be determined by titration. The recommended starting dilution factors are: for FF- 1:250; for FFPE - 1:50.

- If you are staining several samples at the same time, you should make a stock solution accordingly.

To determine the volume of Blocking Buffer (**TABLE 3-7**) per sample, 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 ul)] – [Total Antibody Volume] = Blocking Buffer Volume

# 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

TABLE 3-4: Volume of Blocking Buffer (µL)

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 is 1:250 for FF.***

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

PRE-STAINING PROCEDURE

To pre-stain Fresh-Frozen samples:

1. Coplin Jar Configuration for Fresh-Frozen 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 Coplin jars ahead of time.

   a. Fill designated Coplin jars with 40mL of Hydration Buffer (2 jars) and Staining Buffer (1 jar).

2. Wait until **Step 5** to prepare and dispense 40 mL of Pre-Staining Fixing Solution.

3. Tissue Retrieval

   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 freezer.

   c. Place the slides in the box directly on top of the Drierite beads with the tissue facing up, as shown in FIGURE 3-2. Close the lid and **wait for 5 minutes**.

FIGURE 3-2: Fresh-Frozen Tissue Sample in Box with Drierite Beads

4. Acetone Incubation
   Remove the sample slide(s) from the Drierite beads, and place in the Coplin jar containing acetone.

5. Incubate for **10 min** in Acetone.

6. Tissue Drying

   a. Remove the sample slide(s) from the acetone.

   b. Place the sample slide(s) in the slide staining box with tissue facing up, as shown in FIGURE 3-3.

   c. Let the sample slide(s) sit in the box for **up to 2 min.**

FIGURE 3-3: FF Tissue Sample Drying in Slide Staining Box

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

Tissue Hydration

d. 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).

e. **Incubate for 2 min at RT**.

f. Place each sample slide(s) into the second Coplin jar containing 40 mL of Hydration Buffer.

g. **Incubate for another 2 min at RT** for a total of 2 washes.

h. Prepare the Pre-Staining Fixing Solution as described in **Step 6** during incubation.

7. Fix Tissue

a. Prepare the Pre-Staining Fixing Solution in a conical tube (see **TABLE 3-5**).

Pre-Staining Fixing Solution	1 jar (up to 5 samples)	2 jars (6-10 samples)
16% PFA [mL]	4	8
Hydration Buffer [mL]	36	72
Total Volume [mL]	40	80

TABLE 3-5: Pre-Staining Fixing Solution

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%.Add Pre-Staining Fixing Solution to a clean Coplin jar.***

Add the sample slide(s) to the Coplin jar containing Pre-Staining Fixing Solution.

b. **Incubate for 10 min at RT**.

8. 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 the bottom of the slides.

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

9. 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 mins** in the Staining Buffer.

c. Prepare Antibody Cocktail (see **"Fresh-Frozen Tissue Staining"** on page 3-12) during equilibration.

NOTE ***Sample slides can stay in the Staining Buffer for a maximum time of 30 min 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 (fresh-frozen 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 examples in TABLE 3-6 to verify how to achieve the correct dilution factor.

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 less than 1 µL. If the volume pipetted will be less than 1 µL, werecommend making a stock solution first.		

TABLE 3-6: Dilution Factor Examples

- 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 3-7.

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

TABLE 3-7: Blocking Buffer Components

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

3. Label one 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 roughly the size and shape of the non-label portion of the sample slide (roughly 1x2 in).
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, as shown in FIGURE 3-4. Ensure that the liquid covers the entire tissue.

CRITICAL ***Be careful not to pipette the solution directly on 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, **as shown in** FIGURE 3-5.
7. Repeat **Steps 1-6** above for each sample slide.

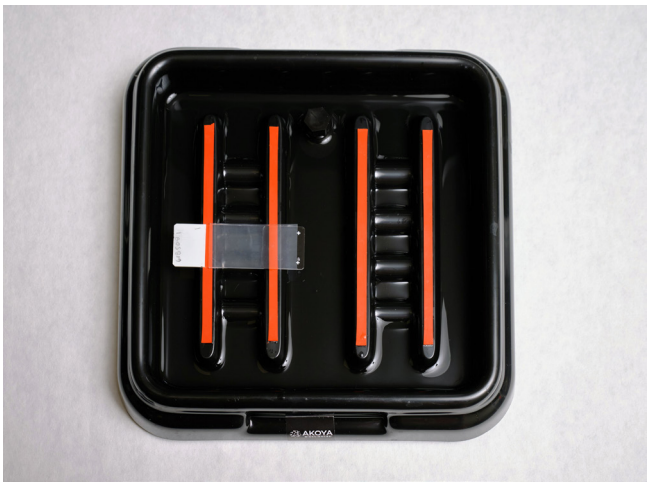


FIGURE 3-5: Placing Parafilm on Slide After Dispensing Antibody Cocktail

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 ***The slide staining box must be placed on a stable surface 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 ***It is critical to prepare all reagents and consumables ahead of time to prevent degradation of the sample(s).***

Pre-Experiment Preparation

Materials Included in the Kit

TABLE 3-8: Staining Buffer and Storage Buffer

Item	Kit	Storage
Staining Buffer	Sample Kit for PhenoCycler-Fusion (PN 7000017)	4°C
Storage Buffer		

TABLE 3-8: Materials that Should Be Obtained Now

TABLE 3-9: Fixative reagent

Item	Kit	Storage
Fixative Reagent 1 tube for every 5 tissues (Single-Use)	Sample Kit for PhenoCycler-Fusion (PN 7000017)	-20°C

TABLE 3-9: Materials that Should Be Thawed Immediately Before Use in Step 6

CRITICAL ***Do not thaw Fixative Reagent before Step 5. Cut necessary tubes off strip without thawing entire strip. Fixative reagent cannot be frozen and reused after initial thawing.***

Materials Not Included in the Kit

Type	Item	Notes
Solvents	Refrigerated Methanol,40mL per Coplin jar	Keep at 4°C until use in Step 4.
Chemicals/ Buffers	16% Paraformaldehyde(PFA)	Recommended: 16% PFA from Electron Microscopy Sciences,PN# 15710.
	1X PBS	
Plastic Consumables/ Tools	Slide staining box	VWR Slide Staining Tray, PN#M918-2
	Parafilm	
	1.5 mL Eppendorf Tubes	
	50 mL Conical Tube	

TABLE 3-10: List of Chemicals and Lab Supplies Required for the Post-Staining Process

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 40mL of Staining Buffer and fill 3 Coplin jars with 40mL 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 40mL 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.

FRESH-FROZEN TISSUE POST-STAINING PROCEDURE

To post-stain fresh-frozen tissue:

1. **Wash 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 to 3 times to ensure the removal of the Antibody Cocktail from both sides of the slide(s).

   c. Incubate for 2 min.

   d. Place the sample slide(s) in the second Coplin jar containing Staining Buffer.

   e. Incubate for another 2 mins for a total of 2 washes.

2. **Fix Tissue**

   a. Prepare the Post-Staining Fixing Solution (**see TABLE 3-11**).

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

TABLE 3-11: Post-Staining Fixing Solution

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

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 min at RT**.

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

3. **Wash 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. **Ice-cold Methanol Incubation**

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

b. Pour ~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 mins.**

5. **Wash 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 Tissue**

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

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

c. Retrieve one aliquot of Fixative Reagent tube from storage in -20°C freezer (one tube for every 5 samples). Each tube should contain 20 uL 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**).

Final Fixative Solution	1-5 Samples	6-10 Samples
1x PBS	1000 µL	2000 µL
Fixative Reagent	20 µL	40 µL

TABLE 3-12: Final Fixative Solution

f. Mix thoroughly by vortexing the solution.

g. Cut a rectangular piece of parafilm roughly the size of the non-label portion of the sample slide (roughly 1x2in).

h. Draw up 200 µL of the Final Fixative Solution with a micro-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 min**.

7. **Wash 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 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 Tissue**

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

b. Place the sample slide(s) in the Coplin jar keeping with the tissue facing 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 no longer than 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 (as described in Appendix B: "Custom Conjugation") we recommend waiting at least two 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

Material Included in Kit

Content	Kit	Storage
Hydration Buffer	Sample Kit for PhenoCycler-Fusion (PN 7000017)	4°C
Staining Buffer		
N Blocker		
J Blocker		
G Blocker		-20°C
S Blocker		
Flow Cells		Ambient

Table 3-13: Sample Kit Components

Follow these guidelines for materials listed in **TABLE 3-13**.

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

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

TABLE 3-14: Antibodies (Materials Not included in the Kit)

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 **"FFPE Tissue Post-Staining" on page 59**.

Materials Not Included in Kit

Type	Item	Notes
Solvents	Ethanol or Reagent Alcohol	Sigma Aldrich, PN# 79317-16GA-PB
	1x HistoChoice Clearing Agent	VWR, PN# H103-4L
Chemicals/Buffers	10x AR6 OR 10x AR9 Buffer	Akoya Biosciences, PN#AR6001KT OR PN#AR9001KT
	16% paraformaldehyde (PFA)	Recommended: PFA from Electron Microscopy Sciences, PN# 15710
Plastic Consumables/ Tools	Coplin jars	
	Slide staining box	VWR Slide Staining Tray, PN#M918-2
	1.5 mL Eppendorf Tubes	
	50 mL Conical Tube	
	Parafilm	Used to cover solutions directly applied to tissue sample slides
	10 solvent-resistant containers with lids	Recommended: EZ-Quick Slide Staining Set, PN# IW-2510, IHC World
	Slide staining rack	Recommended: EZ-Quick Slide Staining Rack, PN# IW-2512, I HC World
	Aluminum Foil	
Required Laboratory Equipment	Pressure Cooker	Instant pot
	Fume Hood	Use of a fume hood is highly recommended for steps involving PFA
**Some clones may require antigen retrieval in AR6 instead of AR9.*		

TABLE 3-15: Chemicals, Lab Supplies and Equipment Required for the Tissue Staining

Prepare Slide Staining Box

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

Determine Antibodies to Constitute the Antibody Cocktail for FFPE Samples

- If antibodies have just been custom-conjugated as described in Appendix B **"Antibody Conjugation,"** wait at least 2 days before using them for tissue staining or 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 which 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 antibody dilutions document.
- If you are staining several samples at the same time, you should 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. The recommended starting dilution factors are: for FF- 1:250; for FFPE - 1:50.
- To determine the volume of Blocking Buffer (TABLE 3-18) per sample, 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**.

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

# 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

TABLE 3-16: Volume of Blocking Buffer (µL)

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 is 1:50 for FFPE.***

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 thema 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 two weeks before they should be changed. Monitor volumes as 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 as organic solvents are highly volatile.***

a. Depending on the container used for the incubation of tissues in the solvent series, determine the volume required to make sure that the slide staining rack is fully submerged in the liquid.

b. Prepare containers containing 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. TISSUE PRE-TREATMENT

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. 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 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, as organic solvents are toxic and highly volatile.***

a. Immerse the staining rack in the container containing the following reagents for 5 min each:

1. HistoChoice Clearing Agent
2. HistoChoice Clearing Agent
3. 100% Ethanol/Reagent Alcohol
4. 100% Ethanol/Reagent Alcohol

5. 90% Ethanol/Reagent Alcohol
6. 70% Ethanol/Reagent Alcohol
7. 50% Ethanol/Reagent Alcohol
8. 30% Ethanol/Reagent Alcohol
9. ddH_2O
10. ddH_2O

3. ANTIGEN RETRIEVAL

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

1. Dilute the stock 10x AR9 solution to 1x with ddH_2O.
2. If staining less than 5 sample slides, fill a Coplin jar with 50mL of 1x AR9. If staining 5-24 sample slides, fill a EZ Quick slide staining vessel with 250mL of 1x AR9.

ALTERNATIVE BUFFER *(optional)* ***Some clones may require antigen retrieval in AR6 instead of AR9. In this case, AR6 from Akoya Biosciences (PN#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.

3. Immerse slides in the vessel. Ensure there is enough 1x AR9 to fully cover the sample slide, as shown in FIGURE 3-6.

FIGURE 3-6: Slide Staining Rack Immersed in Vessel with 1x AR9 Buffer

4. Cover the vessel with aluminum foil, as shown in FIGURE 3-7.

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

5. Fill the Instant Pot pressure cooker with ~1L of ddH_2O.
6. Place staining vessel with slides into the pressure cooker.

FIGURE 3-7: Vessel with Slides in Pressure Cooker with 1L of ddH_2O

7. Secure the lid.

8. Set the pressure cooker to the high-pressure protocol and **let the samples cook for 20 min.**

9. After the incubation in the Pressure cooker, release the pressure and carefully remove the vessel from the pressure cooker and **equilibrate the samples to RT for at least 30 min**.

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

10. Remove the samples from the cooled 1x AR9 solution and quickly immerse it 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 TISSUE

11. 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.

12. Incubate for 2 min.

13. Place each sample slide(s) into the second Coplin jar of Hydration Buffer.

14. Incubate for another 2 mins for a total of 2 washes.

5. EQUILIBRATE TISSUE IN STAINING BUFFER

15. Move sample slide(s) to the Coplin jar containing Staining Buffer.

16. Equilibrate sample slide(s) by incubating for 20-30 mins in the Staining Buffer.

17. Prepare Antibody Cocktail (for "FFPE Tissue Staining") during equilibration.

NOTE ***Sample slides can stay in the Staining Buffer for a maximum time of 30 min 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 on the antibody dilution document. Consider the dilution factors indicated for the species (human or mouse) and tissue type (fresh-frozen 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 examples in TABLE 3-17 to verify how to achieve the correct dilution factor.

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 less than 1 µL. If the volume pipetted will be less than 1 µL, we recommend making a stock solution first.*		

TABLE 3-17: Dilution Factor Examples

- 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**.

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

TABLE 3-18: Blocking Buffer Components

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

Label one tube for each unique Antibody Cocktail Solution.

3. 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.***

4. Add the appropriate volume of each PhenoCycler Antibody to the Antibody Cocktail Solution.

5. 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.***

6. Cut a rectangular piece of parafilm roughly the size and shape of the non-label portion of the sample slide (roughly 1x2 in).

7. Draw up 190 µL of the Antibody Cocktail into a pipette. Set aside.

8. Remove sample slide from the Coplin jar containing Staining Buffer and use a Kimwipe to carefully and gently absorb excess buffer.

9. Place sample slide on the tray of the slide staining box.

10. Quickly dispense the 190 µL of the Antibody Cocktail to the top corner of the sample slide, as shown in FIGURE 3-8. Ensure that the liquid covers the entire tissue.

CRITICAL ***Be careful not to pipette the solution directly on the tissue, and minimize bubbles.***

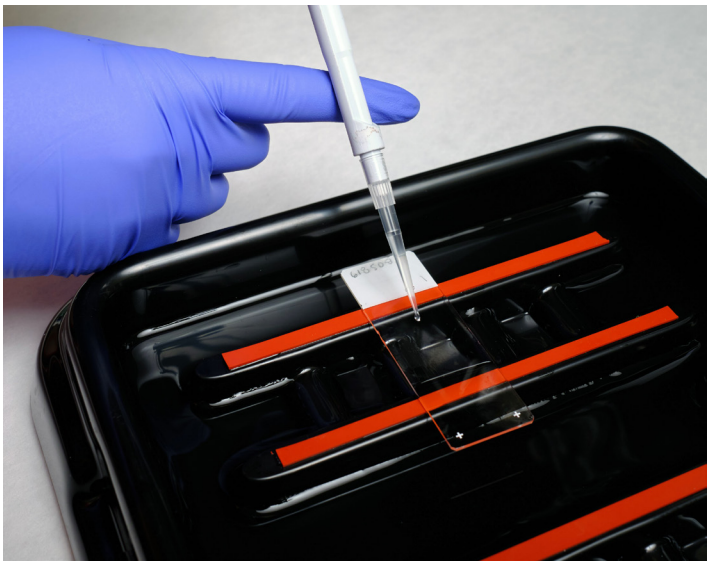


FIGURE 3-8: Dispensing Antibody Cocktail onto FFPE Sample Slide

11. Gently place the parafilm on top of the Antibody Cocktail solution, as shown in FIGURE 3-9.

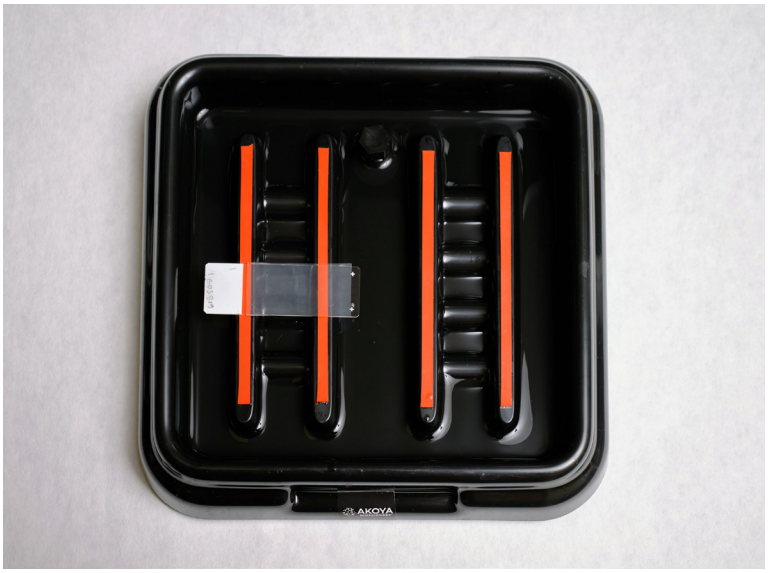


FIGURE 3-9: Placing Parafilm on Slide After Dispensing Antibody Cocktail

12. Repeat **Steps 1-6** above for each sample slide.
13. Place the lid on the slide staining box.
14. **Incubate for 3 hours at RT.**

After 3 hours, proceed immediately to **"FFPE Tissue Post-Staining"**. It Is critical to prepare the initial solutions from "FFPE Tissue Post-Staining" before the end of this 3-hour Incubation.

CRITICAL ***The slide staining box must be placed on a stable surface 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

Materials Included in the Kit

Item	Kit	Storage Location
Staining Buffer	Sample Kit for PhenoCycler-Fusion (PN 7000017)	4°C
Storage Buffer		

TABLE 3-19: Materials That Should Be Obtained Now

TABLE 3-20: Fixative reagent

Item	Kit	Storage Location
Fixative Reagent1 tube for every 5 tissues (Single-Use)	Sample Kit for PhenoCycler-Fusion	-20°C

TABLE 3-20: Materials That Should Be Thawed Immediately Before Use in Step 6

CRITICAL ***Do not thaw Fixative Reagent before Step 5. Cut necessary tubes off strip without thawing entire strip. Fixative reagent cannot be frozen and reused after initial thawing.***

Materials Not Included in the Kit

Type	Item	Notes
Solvents	Refrigerated Methanol, 40mL per Coplin jar	Keep at 4°C until use in Step 4.
Chemicals/ Buffers	16% Paraformaldehyde (PFA)	Recommended: 16% PFA from Electron MicroscopySciences, PN# 15710.
	1X PBS	
Plastic Consumables/ Tools	Coplin Jars	
	Parafilm	
	1.5 mL Eppendorf Tubes	
	50 mL Conical Tubes	
	Serological Pipets	

TABLE 3-22: Post-Staining Fixing Solution

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 40mL of Staining Buffer and fill 3 Coplin jars with 40mL of 1x PBS.
- Fill Coplin jars designated for Post-Staining Fixing Solution, Methanol and Storage Buffer with 40mL 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:

6. **Wash 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 to 3 times to ensure the removal of the Antibody Cocktail from both sides of the slide(s).

   b. Incubate for 2 min.

   c. Place the sample slide(s) in the second Coplin jar containing Staining Buffer.

   d. Incubate for another 2 mins for a total of 2 washes.

7. **Fix Tissue**

Prepare the Post-Staining Fixing Solution (TABLE 3-22).

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

TABLE 3-22: Post-Staining Fixing Solution

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

   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 min at RT.**

NOTE ***During the 10 min incubation, prepare the ice-cold methanol Coplin jar for Step 3.***

8. **Wash 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.

9. **Ice-cold Methanol Incubation**

   a. Retrieve methanol from the refrigerator (4°C).

   b. Pour ~ 40mL 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 mins.**

10. **Wash 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.

11. Fix Tissue

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

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

c. Retrieve one aliquot of PhenoCycler Fixative Reagent tube from storage in -20°C freezer (one tube for every 5 samples). Each tube should contain 20 uL 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 **18**).

Final Fixative Solution	1-5 Samples	6-10 Samples
1x PBS	1000 µL	2000 µL
Fixative Reagent	20 µL	40 µL

TABLE 3-18: Final Fixative Solution

f. Mix thoroughly or vortex the solution.

g. Cut a rectangular piece of parafilm roughly the size of the glass microscope slide (roughly 1x2 in).

h. Draw up 200 µL of the Final Fixative Solution with a micro-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 min**.

12. Wash Tissue

n. 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.

o. Move each sample slide(s) to the second Coplin jar containing 1x PBS. Lift and immerse each sample slide 2-3 times.

p. 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.

13. Store Tissue

q. Label a new Coplin and pipette 40mL of Storage Buffer for every five sample slides.

r. Place the sample slide(s) in the Coplin jar keeping with the tissue facing the same direction for all slides.

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

t. Tissues can now be used directly to run a PhenoCycler-Fusion Experiment.

STOPPING POINT ***For best results, store at 4°C for no longer than 5 days.***

CHAPTER 4

Experiment Protocol and Reporter Plate Design

Using the PhenoCycler Experiment Designer Software

Topics in this chapter include:

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 eg: inventoried antibodies, and one of the two 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 two dedicated wells in H row for blank cycles.
- A saved experiment design (.xpd file) can then be accessed and loaded into the PhenoCycler-Fusion app. This is discussed in the PhenoImager Fusion user guide, **Chapter 6: PhenoCycler-Fusion Workflow**.

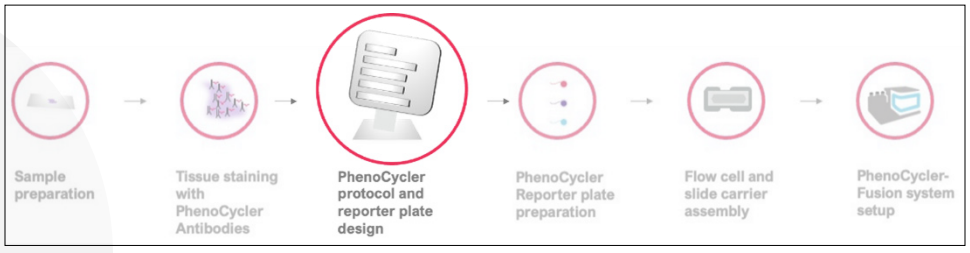


FIGURE 4-1: PhenoCycler Workflow – PhenoCycler Protocol and Reporter Plate Design

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.

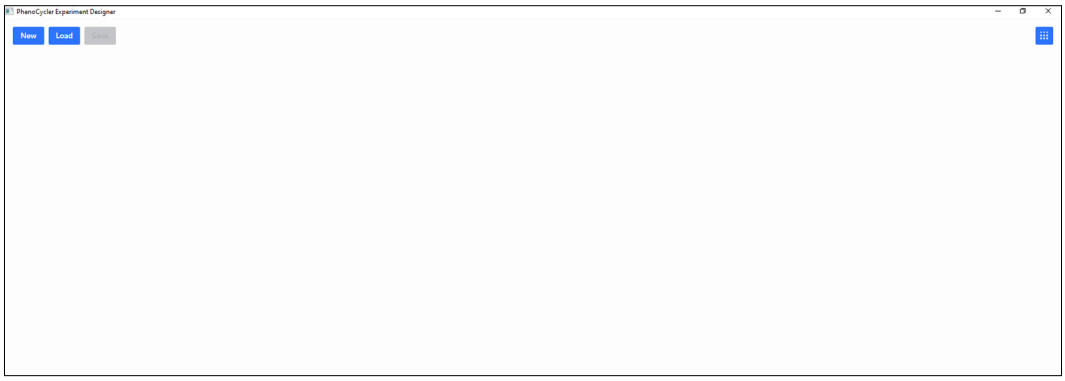


FIGURE 4-2: Main Screen on Experiment Designer

The main screen provides three buttons on the left side:

- New
- Load
- Save

The Grid menu button is on the far right. Each of these will be described in this chapter.

USING THE APPLICATION

FIGURE 4-3 shows the buttons on the main screen of the Experiment Designer. This section describes how to use these three buttons to perform various tasks.

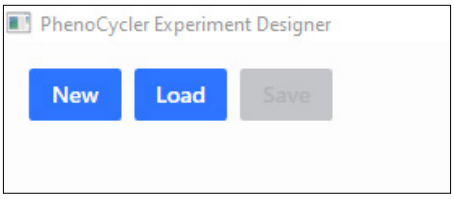


FIGURE 4-3: Buttons on the Main Screen of the Experiment Designer

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, reference 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).

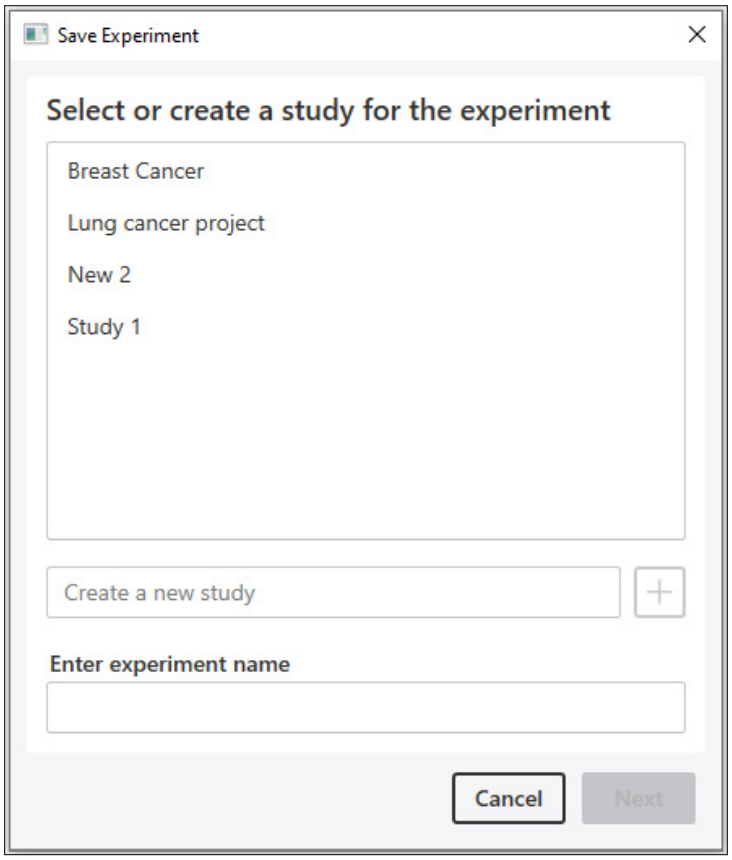


FIGURE 4-4: Save Experiment Screen

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

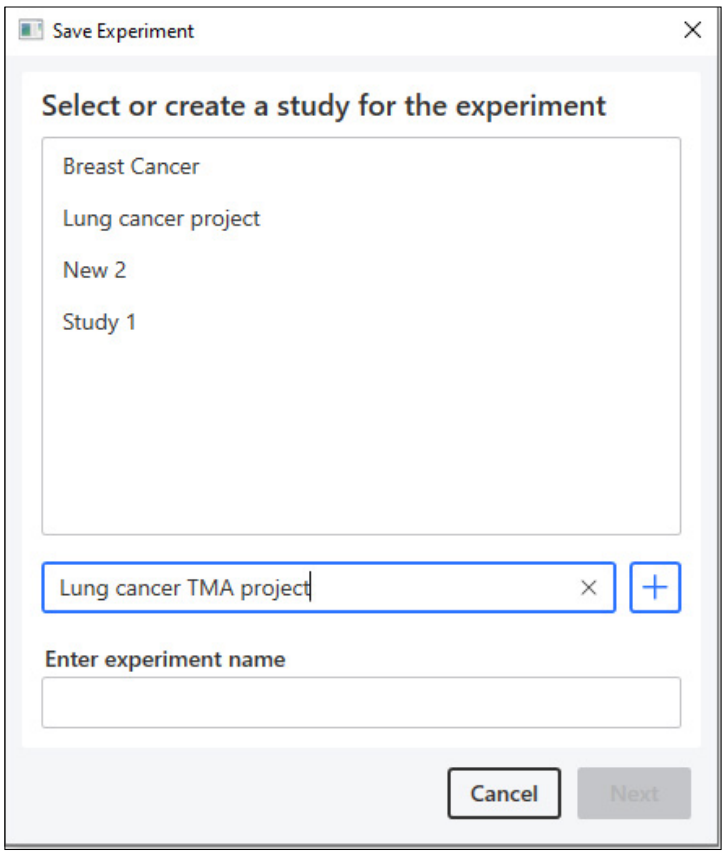


FIGURE 4-5: Save Experiment Screen – Selecting Study

3. To add a new study, enter the name in the **Create a new study** field and hit the "+" button.
4. With the appropriate study selected, enter the name of the experiment in the **Enter experiment name** field and hit "Next".
5. On selecting Next, Experiment Creator screen displays (see FIGURE 4-7). Define the Staining Information, Panel Content, Starting well, the two blank wells, and exposure times for each channel on this screen.

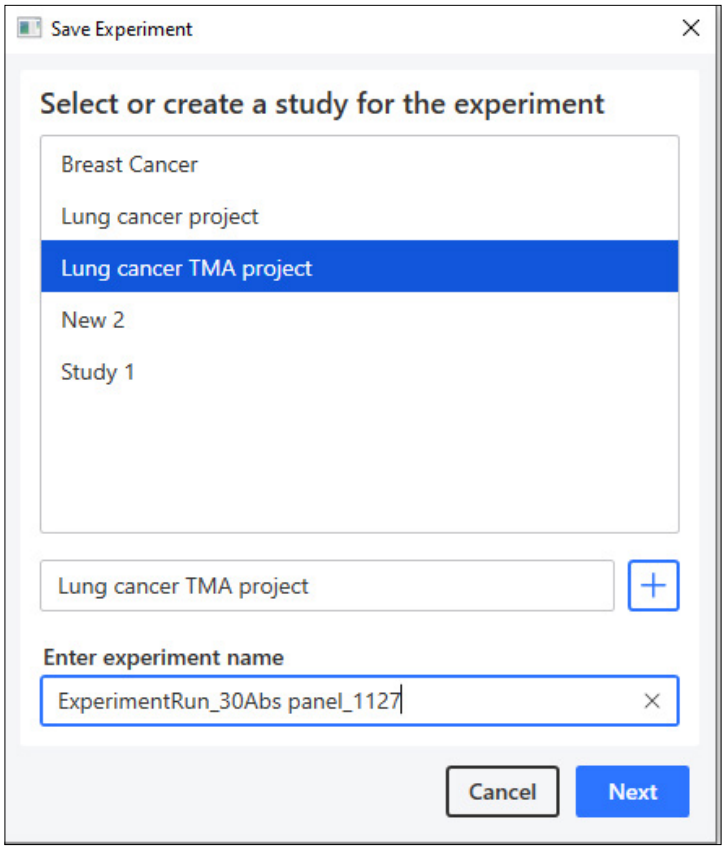


FIGURE 4-6: Save Experiment Screen – Enter Experiment Name

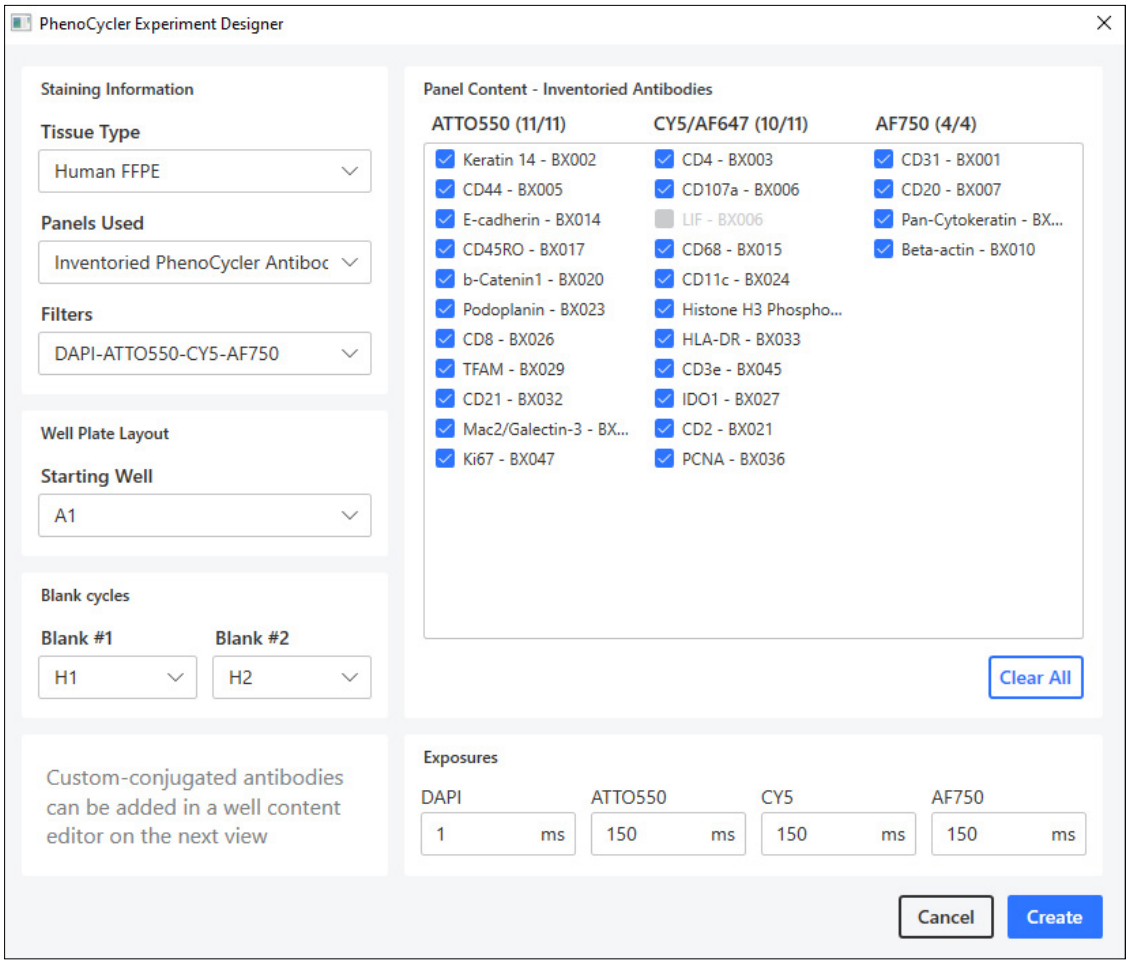


FIGURE 4-7: Experiment Designer Screen

Parameter Name	Description
Tissue Type	Select the tissue-type Human FFPE, Human FF, or Mouse FF
Panel source	Select the source of antibodies used in the run (eg: Inventoried PhenoCycler Antibodies). At present, 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 are different based on the filter set selected
Filters	Select the filter set based on the reporter combination of choice: Filter set options are: DAPI-ATTO550-CY5-AF750 (recommended for FFPE) OR DAPI-ATTO550-CY5-AF488 (recommended for FF)

TABLE 4-1: Parameters to Define Staining Information

6. Select the markers to be used in the Panel Content area as described in **TABLE 4-2**).

Parameter Name	Description
Panel Content	Select the markers to be used by clicking on the checkboxes.The Antibodies available 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: your 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). ✔ Ensure that each antibody within a run has a unique barcode, 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 one or two Reporters instead of three, if necessary.

TABLE 4-2: Parameters to Define Markers

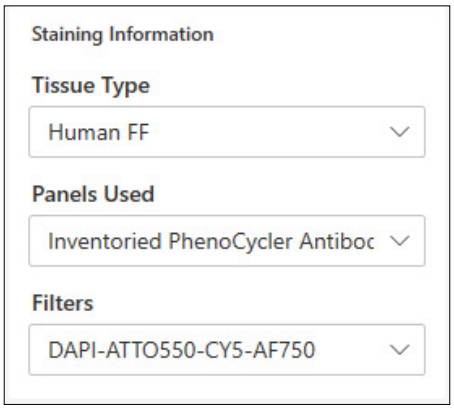


FIGURE 4-8: Experiment Designer Screen - Staining Information

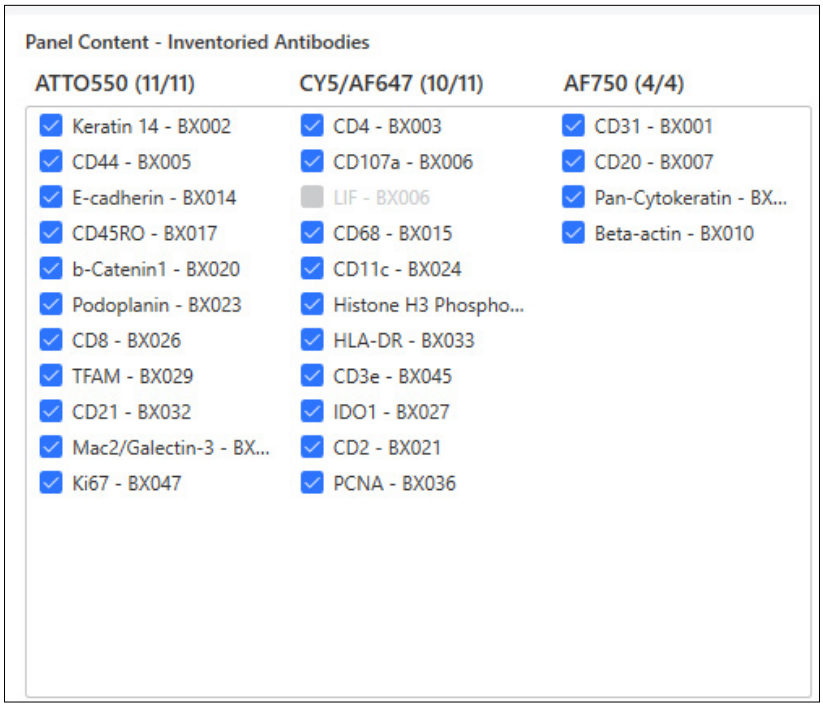


FIGURE 4-9: Experiment Designer Screen – Panel Content

7. Identify the starting well and the blank cycle wells in the Well Plate Layout area (as described in **TABLE 4-3**).

Parameter Name	Description
Starting Well	Choose the starting well from the dropdown box. Choicesrange from A1-A12, B1-B12, 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 any Reporters) to two wells in row H for every Phenocycler experiment. Choose the blank cycle wells (Blank #1 and Blank #2) from the dropdown box. Choices range from H1-H12 for each, but each well in the H row can only be used once for each blank cycle in a given experiment. Eg: both blank cycles cannot be H2.

TABLE 4-3: Parameters to Define Well Plate Layout

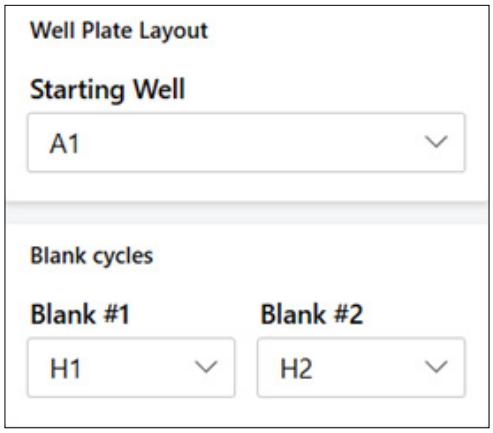


FIGURE 4-10: Experiment Designer Screen – Well Plate Layout

Select Create to continue or Cancel to return to the main screen.

8. When you select Create, the cycle layout/table mode displays.
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 three marker channels available depend on the filter set selected in the main window - ATTO550, Cy5, AF750 (OR AF488).

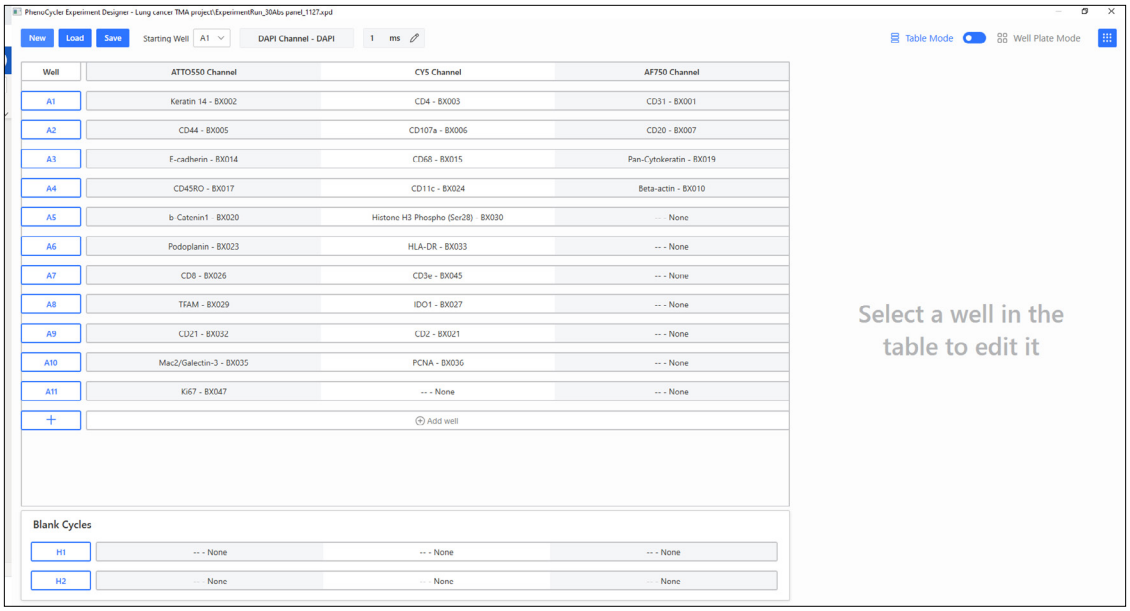


FIGURE 4-11: Cycle or Table Mode Layout

EDITING THE EXPERIMENT DESIGN LAYOUT

To edit the cycle layout, click on the well button (eg here A7) and use the

Insert, Skip, Delete options to add, skip or remove cycles.

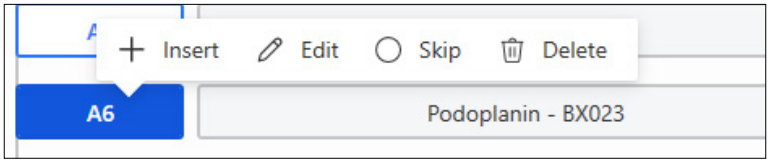


FIGURE 4-12: Edit Layout Options Within the Table Mode

To edit the contents within a cycle/well:

1. Select the well/cycle you want to edit. Select **Edit.** The contents of that well displays on the right side of the screen.
2. To alter the exposure times for particular markers within a cycle, edit the exposure times for individual channels appropriately.
3. For inventoried antibodies, use the **Marker/Barcode** dropdown box to select the marker/barcode combination to be used. If you want to add an additional item, select Add additional item from the dropdown 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 you are done, select **Update** to save your layout or select **Cancel**.

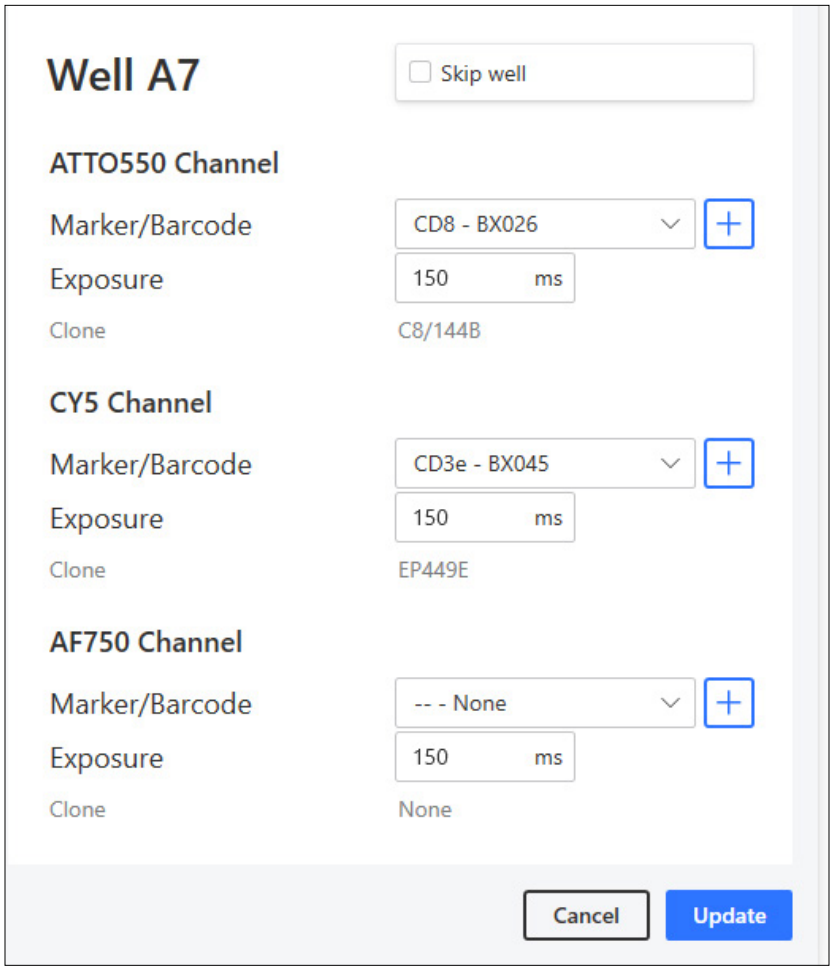


FIGURE 4-13: Edit Content Options within a Cycle/Well

ADDING A CUSTOM-CONJUGATED MARKER TO THE EXPERIMENT DESIGN

1. To add a custom-conjugated Ab to an empty channel within a cycle, select the appropriate channel within a cycle/well and hit "+" button next to the Marker/Barcode field.

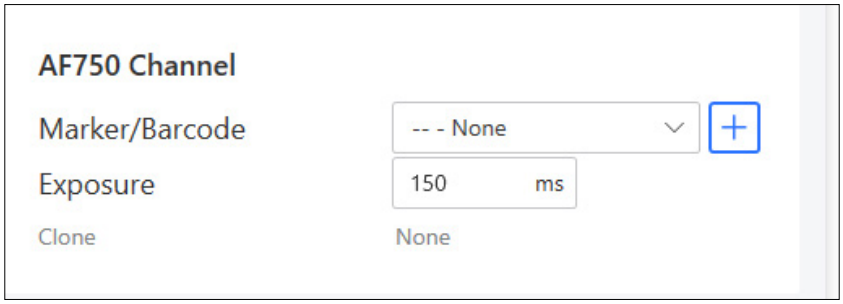


FIGURE 4-14 : Select Appropriate Channel to Add Custom-Conjugated Antibody

2. A**dd Additional item** window will display.
3. Within the window, select **"Custom Conjugations"** from the **Item Source** dropdown.

CRITICAL ***Enter the appropriate marker and clone names, and select the proper barcode, and press*** **OK. It is critical to make sure a unique barcode is selected for each new marker.**

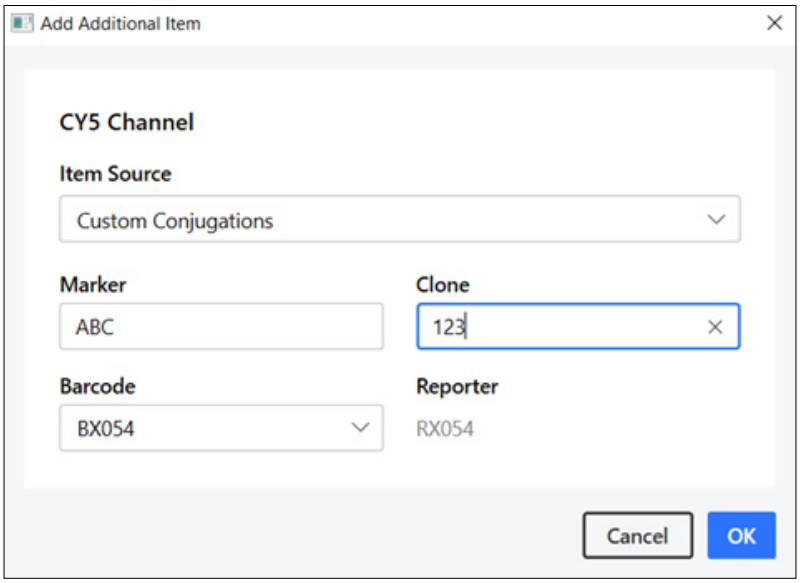


FIGURE 4-15: Edit Content Options within a Cycle/Well – Add Additional Item

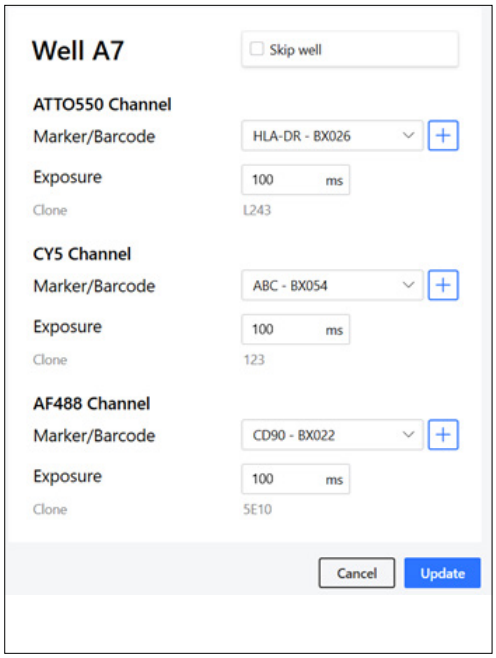


FIGURE 4-16

4. Edit all the channels within the cycle/well appropriately and then select “Update”.

Edit content options within a cycle/well – Press Update button to update the experiment design.

Repeat **Steps 1-5** to add all required custom conjugated antibodies to the experiment.

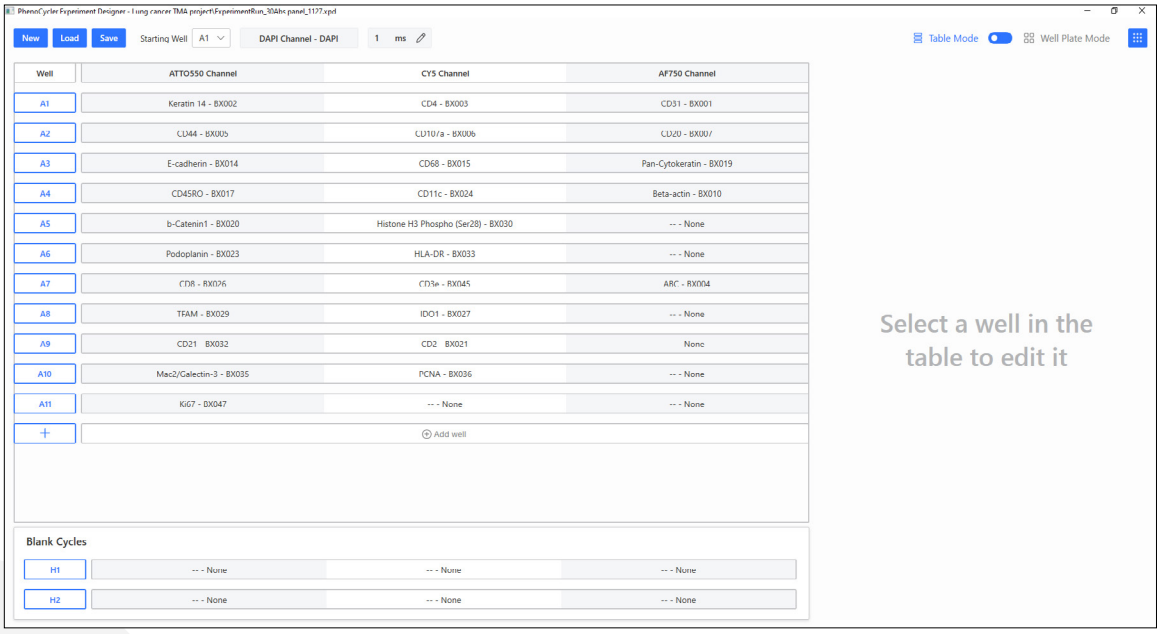


FIGURE 4-17: Experiment Designer Screen – Table Mode Window (List View)

5. The updated cycle layout will display all selected markers, including the custom-conjugated Abs.

6. Using the toggle button, you can select **the Well Plate Mode** to view the well plate layout. Please note that this layout instead displays the reporters (RX-xxx) corresponding to each well . And this layout will be used in **Chapter 5** to prepare the reporter plate.

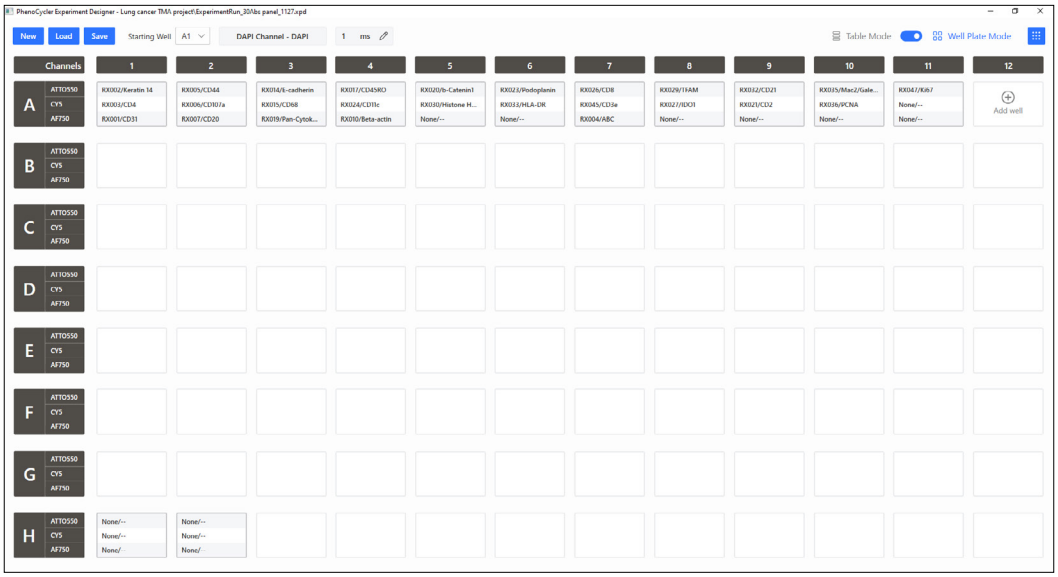


FIGURE 4-18: Experiment Designer Screen – Well Plate Mode (to Use in Reporter Plate Preparation)

LOADING AN EXISTING WELL PLATE

To load an existing well plate:

1. Select the **Load** button on the Main Screen (see FIGURE 4-19).

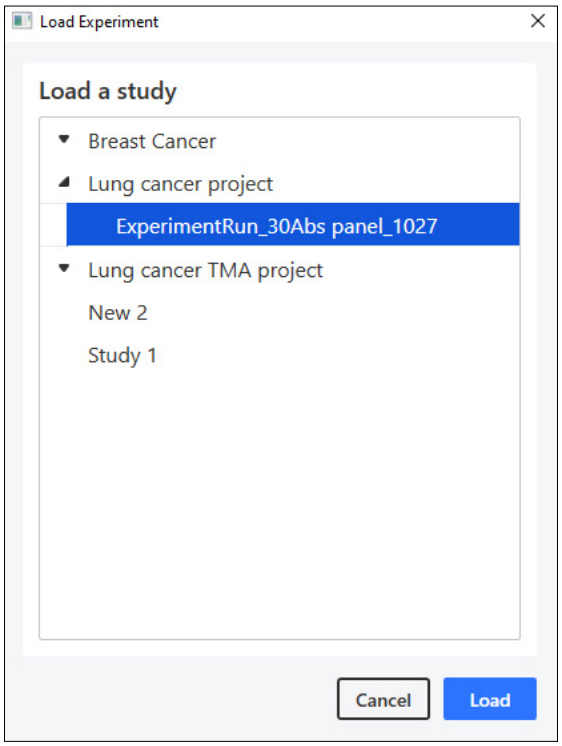


FIGURE 4-19: Load a Study Window

2. From the available Study folders, select the desired saved protocol from the appropriate folder and press **"Load."**

SAVING THE NEW OR MODIFIED WELL PLATE

To save a new or modified well plate:

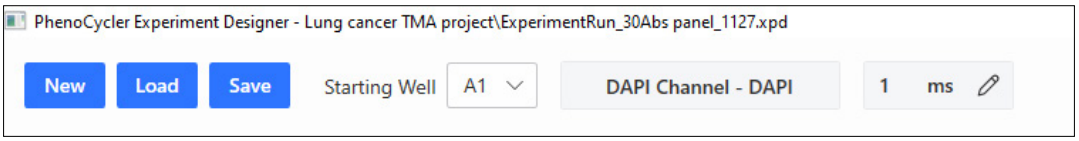


FIGURE 4-20: Experiment Designer – Main Screen – Save

Select the **Save** button on the Main Screen (see **FIGURE 4-20**).

Using options from the grid menu

FIGURE 4-21: Shows the Options Available from the Grid Menu

Each option is described in TABLE 4-4

Menu Option	Description
Print (notebook-style)	Prints the cycle design information in a condensed format for your lab notebook. FIGURE 4-22
Print (pipetting-style)	Prints the cycles organized in a 96 well-plate format. This view is useful for preparing the reporter plate. FIGURE 4-23
PhenoCycler Inventory Info	Shows the publication date of your PhenoCycler Inventory so you can determine if your information is up to date.
Update PhenoCycler Inventory	Allows you to 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.

TABLE 4-4: Options Available from Grid Menu

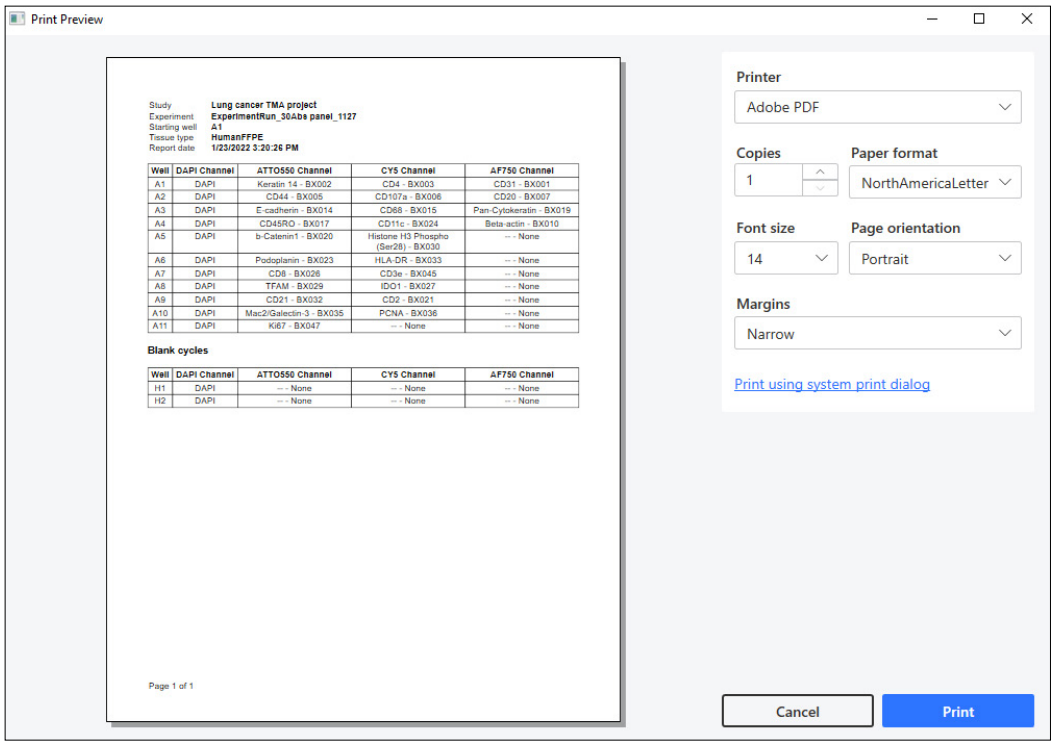


FIGURE 4-22

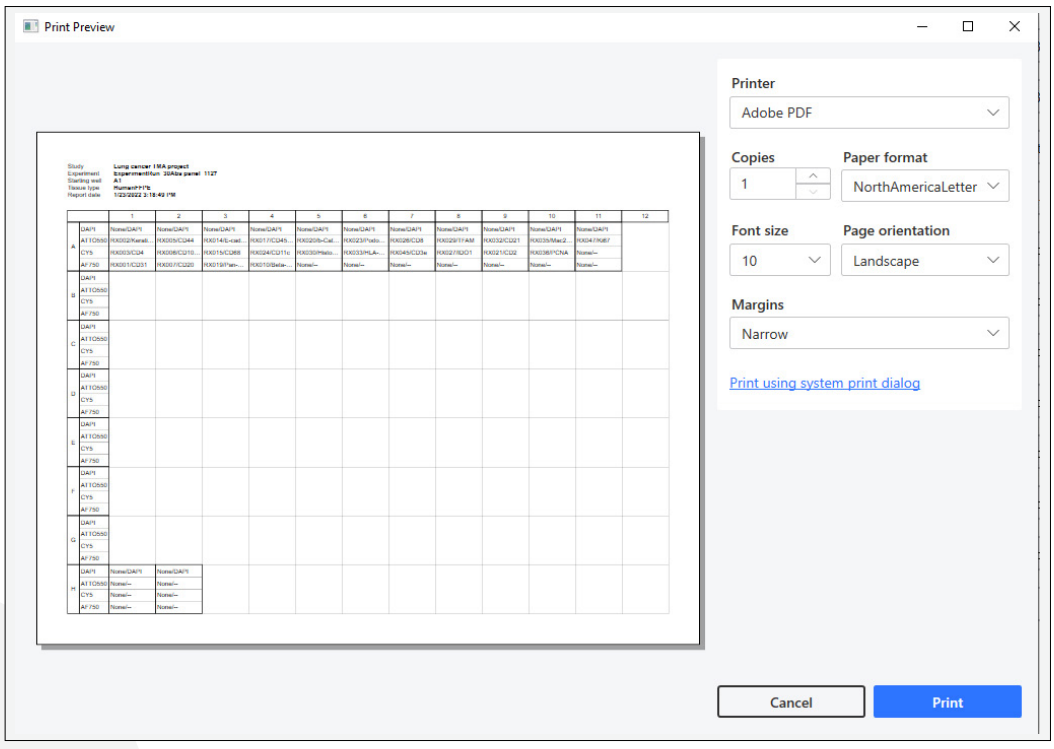


FIGURE 4-23

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 three spectrally distinct dyes, along with a nuclear stain. Each of these mixtures is called a Reporter Master Mix and is pipetted in one well of a 96-well plate. Each well corresponds to one experiment cycle.

Timeline to Prepare PhenoCycler Reporters

Duration	Step	Time	Stopping Point	Page
Variable	Prepare Reporter Stock Solution: Combine 10X Buffer for PhenoCycler, Assay Reagent, Nuclear Stain, and H_2O to create the Reporter Stock Solution.	10 m		77
	Prepare Reporter Master Mix for each cycle: Combine Reporter Stock Solution with predetermined Reporters to create a Reporter Master Mix for each cycle.	30 min-1 hr for 10 cycles (estimated)		78
	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	80

During each cycle, the Reporter Master Mix is drawn from one 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.

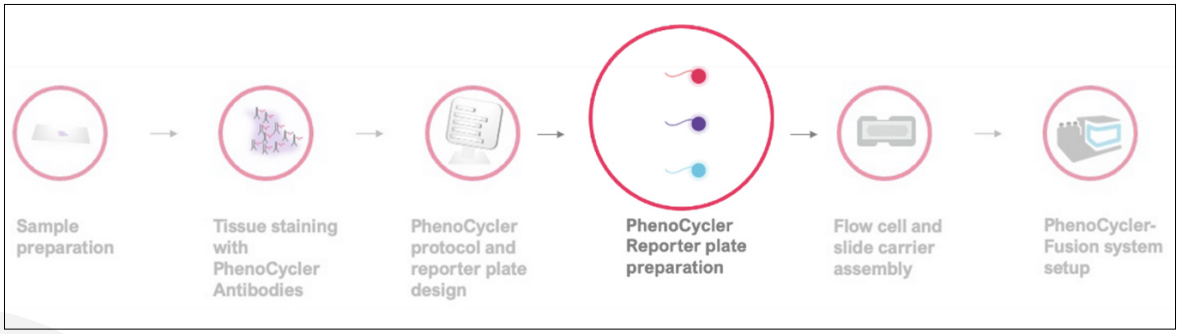


FIGURE 5-1: PhenoCycler Workflow - PhenoCycler Reporter Plate Preparation

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 (**Chapter 4**) 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 two reporters are paired with the same dye in a cycle, they will be revealed at the same time in one fluorescence channel, making it impossible to distinguish the signal coming from the two corresponding biomarkers.***

NOTE ***Plan which reporters are to be revealed in each cycle ahead of time using the Experiment Designer App. Refer to "Chapter 4: Using the Experiment Designer."***

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 5-3. During the PhenoCycler run, in each cycle, the instrument withdraws the Reporter Master Mix from one well of the 96-well plate.

Note that 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.

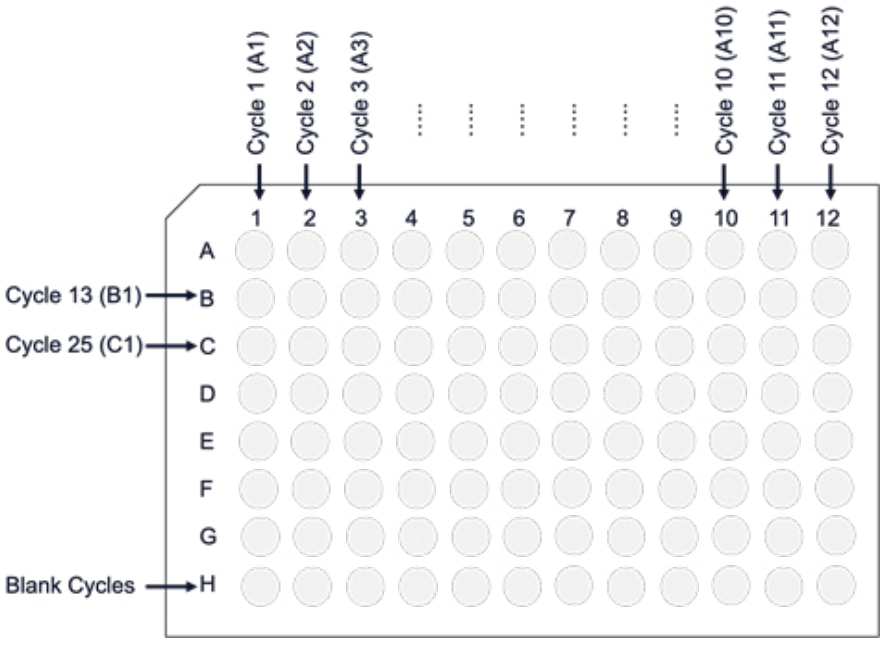


FIGURE 5-2: 96-Well Plate Configuration for PhenoCycler Reporters

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, and so on. A multicycle PhenoCycler run can start from any well on the plate, and wells can be skipped, given this information is included in the PhenoCycler Experiment Designer before starting the experiment. Refer to **Chapter 4** for information regarding the PhenoCycler Experiment Designer.

Add print out view from the Experiment Designer app.

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 any Reporters) to two unused wells in row H for every PhenoCycler experiment.

PREPARING PHENOCYCLER REPORTER PLATE

Guidelines

Akoya 96-Well Plates for PhenoCycler (PN# 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 (PN #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

Lists the materials available for purchase from Akoya.

Item	Storage Location	Use At
96-Well Plates for Phenocycler (PN# 7000006)	RT	RT
PlateSeals for Phenocycler (PN# 7000007)		
10x Buffer for Phenocycler (PN# 7000001) Buffer Additive for PhenoCycler-Fusion (PN#240257. To order, contact your Field Application Scientist)		
Assay Reagent for Phenocycler (PN# 7000002)	-20°C, and 4°C after the first thaw	Place on Ice
Nuclear Stain for Phenocycler (PN# 7000003)		
Phenocycler Reporters		

TABLE 5-1: Materials Available for Purchase from Akoya

Materials Not available for purchase from Akoya

Item
Nuclease-free water
Opaque 1.5 mL tubes
A 15 mL tube covered with foil
A bucket of ice

TABLE 5-2: Materials Not Available for Purchase from Akoya

PREPARE 1X BUFFER FOR PHENOCYCLER

Prepare 1L of 1x PhenoCycler Buffer with Buffer Additive

1. In a clean glass beaker (or similar container) add 800mL ddH2O.
2. Pipet 100mL 10x Buffer for PhenoCycler.
3. Pipet 100mL Buffer Additive for PhenoCycler-Fusion and rise pipet with the mixture from above to ensure complete transfer
4. Mix by pipetting up and down with pipet aid or by using magnetic stir bar.

NOTE ***Do not shake to mix to avoid bubble formation. If using a bottle to mix, do not invert a lid-screwed bottle to mix to avoid leakage. If any spills occur, clean with 70% ethanol.***

NOTE ***Do not filter. Previous user manual states to do this. The above 1x solution should be stored at room temperature and is stable for 2 weeks.***

This 1x solution is used:

- to fill the Buffer bottle in the PhenoCycler sidecar
- for the reporter stock solution in the 96 well plate (Chapter 5, PhenoCycler-Fusion User Guide)
- for incubation step after mounting the flow cell

PREPARING THE PHENOCYCLER REPORTER PLATE

To prepare the PhenoCycler Reporter Plate:

1. Preparing the Reporter Stock Solution

   a. Prepare the Reporter Stock Solution based on the total number of cycles (including two blank cycles) for the experiment in an opaque 1.5 mL tube or a 15 mL tube covered with foil (see TABLE 5-3).

Report Stock Solution	Cycles/Wells			
	5	10	15	20
1x Buffer for PhenoCycler with Buffer Additive (µL)	1350	2700	4050	5400
Assay Reagent (µL)	125	250	375	500
Nuclear Stain (µL)	25	50	75	100
Total (µL)	1500	3000	4500	6000

TABLE 5-3: Prepare Reporter Stock Solution Based on the Number of Cycles for the Experiment

   b. After adding all reagents, mix by gently inverting the Reporter Stock Solution tube a few times.

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

2. Preparing blank cycles:

Pipette 245 uL of Reporter Stock Solution into two wells within row H (I.e., H1 and H2) as designated in the well plate layout using the designer app (Refer to **Chapter 4**).

3. Preparing 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 (for example, "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. (**TABLE 5-4**).

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

TABLE 5-4: Volume of the Reporter Stock Solution Based on the Number of Reporters to be Revealed

c. Put Reporters in an ice bucket before use.

d. Briefly spin tubes down using a benchtop centrifuge.

e. Add 5 µL of each Reporter to its corresponding opaque tube (**see TABLE 5-5**).

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

Table 5-5: Total Volume of All Reporters Per Opaque Tube Based on the Number of Reporters Per Opaque Tube

f. Mix the contents of the tube by gently pipetting up and down or gently inverting the tube.

NOTE Minimize the number of bubbles generated during this process. Do not shake or vortex the solution vigorously.

g. Repeat **Steps a-f** above for every cycle.

4. Creating the Reporter 96-Well Plate:

a. Once all tubes have been prepared, obtain the 96-well plate.

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, as shown in FIGURE 5-3.

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

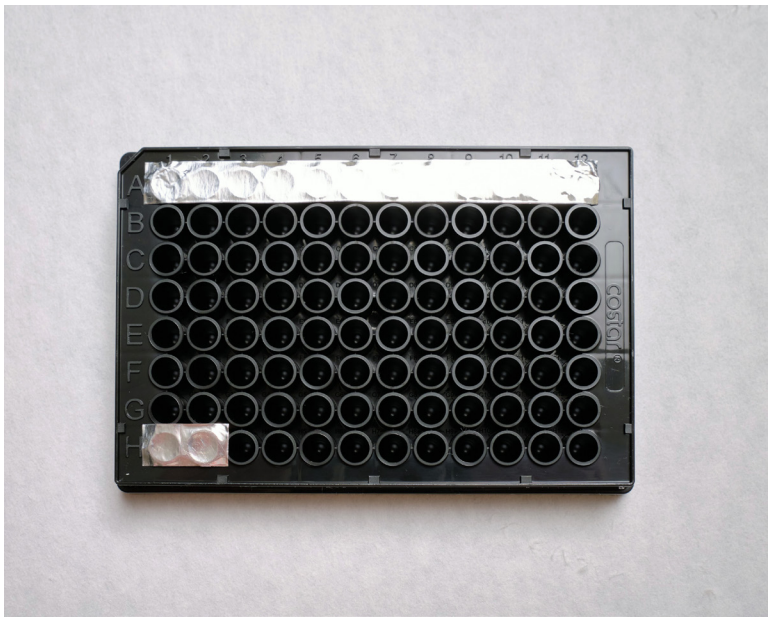


FIGURE 5-3: Filled and Sealed 96-Well Plate

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 two weeks.**

APPENDIX A

Specifications

Topics described in this chapter include:

PERFORMANCE SPECIFICATIONS

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 our PhenoCycler support page (see akoyabio.com).	

TABLE A-1: Performance Specifications

ENVIRONMENTAL CONDITIONS

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

TABLE A-2: Environmental Conditions

INSTRUMENT SPECIFICATIONS

General Overview	
Dimensions (W X D X H in)	PhenoCycler: 28”X 22”X 14.5”; Fusion: 25”X 20”X 26”
Weight*	PhenoCycler: ~67 lbs. / 30.3 kg ; Fusion :~120 lbs. / 54.4 Kg
Tissue Format	Whole-slide, Tissue Microarray and Tissue Sections
Speed (1.5cmX1.5cm)	Fluorescence: 25 min
Resolution	Up to 0.25 µm/pixel (40X)
Throughput	1 slide
Multiplexing Capability	Supports up to 100+ biomarkers depending on barcode compatibility
Image Analysis Software	inForm®: Intuitive learn-by-example interface that automatically segment and quantitate, tissue structures, cells and sub-cellular signatures
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***	

TABLE A-3: Instrument Specifications

APPENDIX B

Custom Conjugation

Topics described in this chapter include:

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.***

Duration	Step	Time	Stopping Point	Page
4h 10m	Reduce purified antibody: Perform partial reduction reaction to reveal reactive thiol groups	1 h (includes 30 min incubation)		
	Conjugate antibody with PhenoCycler barcode: Combine partially reduced antibody with PhenoCycler barcode and incubate	2.5 h incubation (includes 2 hour incubation)		
	Purify PhenoCycler custom-conjugated antibody: Filter PhenoCycler custom-conjugated antibody solution to remove unconjugated components	40 m (includes 4x8min spin downs)		
	Store PhenoCycler custom-conjugated antibody: Add PhenoCycler Antibody Storage Solution to sample and store at 4°C.		Store up to 1 year	

TABLE B-1: Experiment Overview

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 included in the Antibody Conjugation Kit. For details see"Verifying Conjugation."

CONJUGATING ANTIBODIES

GUIDELINES

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 (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 fresh-frozen tissues and Cy5/AF647 for FFPE. For antibodies targeting highly expressed antigens, we recommend using PhenoCycler Barcodes corresponding to AF488 for fresh-frozen 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 fresh-frozen 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 extra 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.

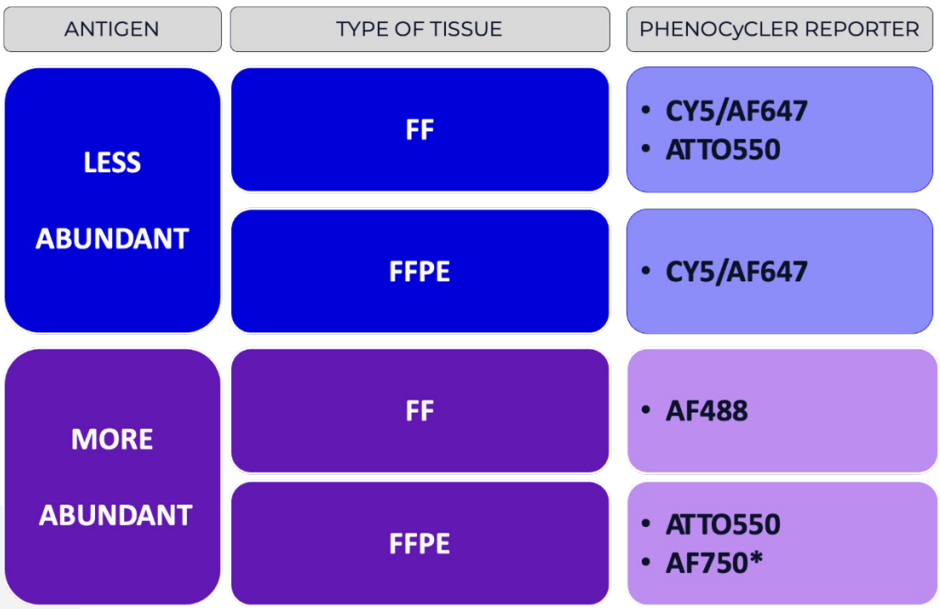


FIGURE B-1 Use Cases for PhenoCycler Reporters

* 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. As such, 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.

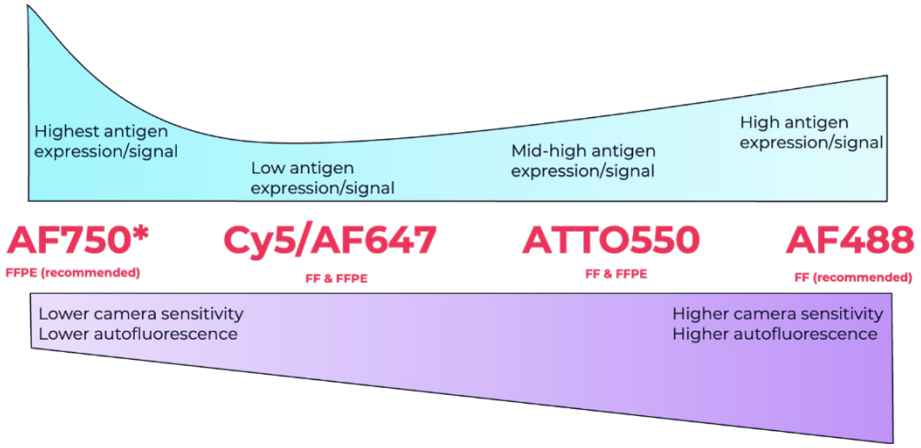


FIGURE B-2 Use Cases for PhenoCycler Reporters

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, 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 like BSA, Gelatin, Glycerol, etc. 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. Often, the concentrations labeled on the antibody tubes are not accurate.***

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 flow-through in the bottom of the tube (see FIGURE 4-4). 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.

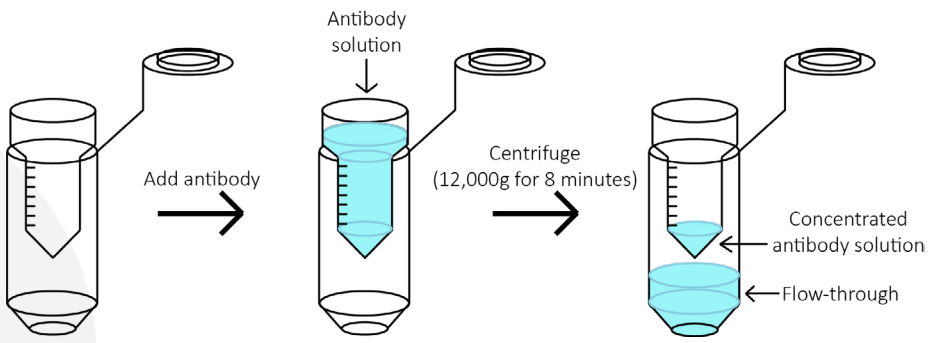


FIGURE B-3: Antibody Filtration

PRE-EXPERIMENT PREPARATION

Materials Included in the Kit

TABLE B-2 lists the materials included in the Antibody Conjugation Kit.

Antibody Conjugation Kit	Stored at	Notes
Retrieve before beginning the experiment		
Reduction Solution 1	-20°C	Single use tubes. Do NOT re-use after thawing once. Each tube has enough reagent for up to 3 conjugations. Any remaining reagent should be discarded.
Reduction Solution 2	4°C	
Filter Blocking Solution	4°C	
Retrieve after 30-min incubation in Step 5		
Conjugation Solution	4°C	

TABLE B-2 Material Included in the Antibody Conjugation Kit (7000009)

Materials Not Included in the Kit

TABLE B-3 lists material not included in the Conjugation Kit for PhenoCycler

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 “Validating Custom-Conjugated Antibodies” on page B-2)
	Bucket of ice for antibodies
Instrumentation	Centrifuge for 1.5 mL tubes
	NanoDrop™ spectrophotometer
	Vortex (Optional)

TABLE B-3: Material Not Included in the Antibody Conjugation Kit

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 min.
    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 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 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 min.
    b. During the centrifugation, prepare Antibody Reduction Master Mix as described in **Step 6**..
    c. Discard flow-through.

6. Initiate Antibody Reduction
    a. Prepare the Antibody Reduction Master Mix based on the number of antibodies to be conjugated (see TABLE 4-3). One tube of Reduction Solution 1 can be used for up to 3 conjugation reactions.

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

TABLE B-4: Number of Antibodies to be Conjugated

CRITICAL ***Note: 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 min.

CRITICAL INCUBATION ***30-minute incubation. It is critical NOT to exceed 30 min.***

7. Buffer Exchange of the Antibody Solution
    a. After the 30-minute incubation has completed, spin down the tubes at 12,000g for 8 min.
    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 min.
    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 as barcode integrity can start to degrade. We recommend conjugating no more than six Barcodes at a time for this reason.***

   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 ***2-hour incubation at RT.***

10. Purify 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 Conjugation" section**).

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 min.

   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 min.

   f. Repeat **Steps c – e** above two more times for a total of three purifications.

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

   h. The filter will contain the conjugated antibody solution.

11. Collect 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 min. 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 10a.**
- 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 Included in the Kit

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 not supplied, but are required for verifying conjugation.

Type	Item	Vendor	Catalog #
Reagents and Protein Gel	NuPAGE™ LDS Sample Buffer (4X)	Thermo Fisher Scientific	NP0008
	NuPAGE™ Sample Reducing Agent (10X)	Thermo Fisher Scientific	(NP0009
	NuPAGE™ 4-12% 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		
	Nuclease-free Water		
Instrumentation	95°C dry bath		
	Shaker		
	Microwave		

TABLE B-5: Materials Required for Verifying Conjugation

PROCEDURE

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

1. Sample Preparation

   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 min.

2. Gel Setup

   a. Prepare enough volume of buffer for running the gel. In our 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 one 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 min until completion.

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

3. Gel Visualization

   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 min.

   g. Wash the gel with ddH_2O and leave it on the shaker until bands are visible (see FIGURE B-5). More microwaving steps can be added to accelerate this process or it can be left overnight on a shaker. Additionally, it is important to change the water.

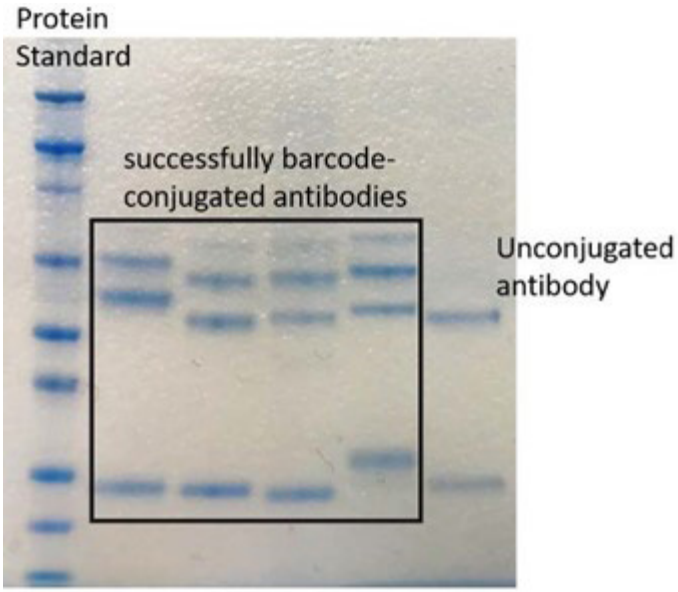


FIGURE B-5: Gel Visualization

The following describes the gel visualization. From left to right:

- First column shows the protein standard.
- Columns 2-5 (in this example) shows 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

Legal Terms of Use

The following is an agreement (the "Agreement") between you and Akoya Biosciences, Inc., 100 Campus Drive, 6th Floor, Marlborough, MA 01752 ("Akoya") for software known as Research Use Only PhenoImager-Fusion and PhenoCycler-Fusion, including any updates, upgrades or enhancement provided by Akoya and any and all accompanying documentation (collectively, the "Software").

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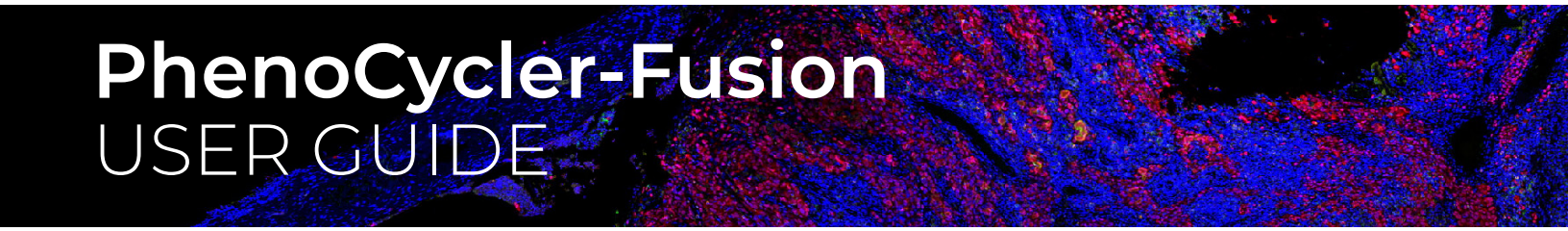

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