

CHAPTER 4

Experiment Protocol and Reporter Plate Design

Topic	Page
Launching the Experiment Designer	58
Creating a New Experiment Design	59
Editing the Experiment Design Layout	64
Adding a Custom-Conjugated Marker to the Experiment Design	65
Loading an Existing Well Plate	68

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

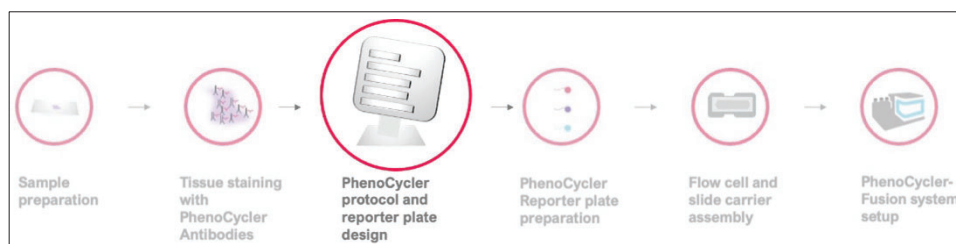


Figure 4.1. PhenoCycler Workflow – PhenoCycler Protocol and Reporter Plate Design.

LAUNCHING THE EXPERIMENT DESIGNER

Launch the Experiment Designer by double-clicking on the Experiment Designer app located on the PhenoCycler-Fusion Acquisition PC desktop. The main screen appears as shown in Figure 4.2. Select either Protein or RNA Assay experiments.

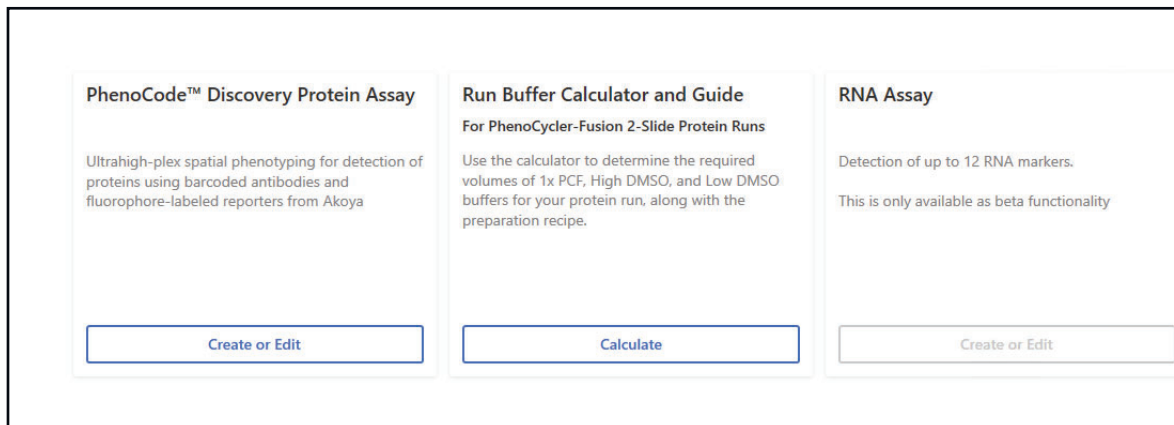


Figure 4.2. Main screen on Experiment Designer.

The Run Buffer Calculator and Guide is a tool for determining the volumes needed to prepare buffers for the experiment. Refer to the Phenolmager Fusion User Guide, Chapter 6: Buffer Preparation.

USING THE APPLICATION

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

The main screen provides 4 buttons on the left side:

- Home
- New
- Load
- Save



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

CREATING A NEW EXPERIMENT DESIGN

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

To create a new experiment design:

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

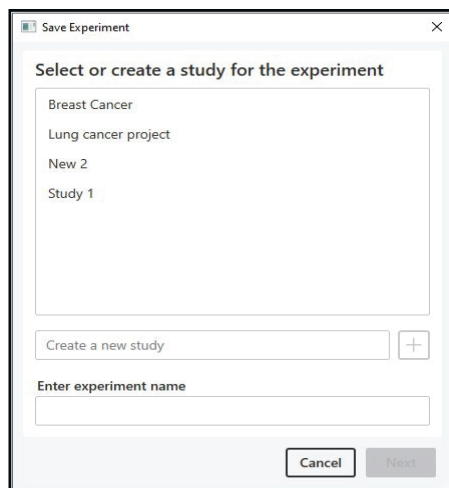


Figure 4.4. Save Experiment screen.

2. Select the name of an available study from the Select or create a study for the experiment list. (See Figure 4.5.) When opening the application for the first time, this list will appear blank.

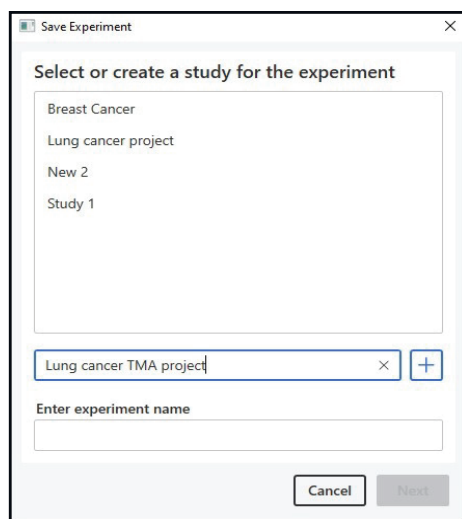


Figure 4.5. Save Experiment screen—selecting study.

3. To add a new study, enter the name in the Create a New Study field and select the + button.
4. With the appropriate study selected, enter the name of the experiment in the Enter Experiment Name field and select Next. (See Figure 4.6.)

Figure 4.6. Save Experiment screen–Enter Experiment Name.

5. The Experiment Creator screen displays (see Figure 4.7.). Experiment Creator allows you to define the Staining Information, Panel Content, Starting Well, the (2) Blank Wells, and the Exposure Times for each channel.

Run Parameters

Tissue Type

Human FF

Panels Used

Inventoried PhenoCycler Antibodies

Filters

DAPI-ATTO550-CY5-AF750

Scan Resolution

0.5um/pixel

Panel Content - Inventoried Antibodies

ATTO550 (0/12)

☐ CD2 - BX002
☐ CD104 - BX005
☐ PD-1 - BX014
☐ CD278 - BX017
☐ CD45RO - BX017
☐ Podoplanin - BX023
☐ HLA-DR - BX026
☐ CD31 - BX032
☐ CD34 - BX035
☐ CD69 - BX041
☐ Ki67 - BX047
☐ Podoplanin - BX121

CY5/AF647 (0/6)

☐ CD19 - BX003
☐ CD3 - BX015
☐ CD45RO - BX017
☐ CD4 - BX021
☐ CD11c - BX027
☐ CD49f - BX033

AF750 (0/7)

☐ CD45 - BX001
☐ CD8 - BX004
☐ CD38 - BX007
☐ CD138 - BX010
☐ CD21 - BX013
☐ Pan-Cytokeratin - BX019
☐ CD90 - BX022

Clear All

Well Plate Layout

Starting Well

A1

Blank Cycles

Blank #1

H1

Blank #2

H2

Exposures

DAPI

1

ms

ATTO550

150

ms

CY5

150

ms

AF750

150

ms

Custom-conjugated antibodies can be added in a well content editor on the next view

Cancel

Create

Figure 4.7. Experiment Designer screen.

Table 4.1. Run Parameter Definitions

Parameter Name	Description
Tissue Type	Select the tissue-type: - Human FFPE - Human FF - Mouse FF
Panel Used	Select the source of antibodies used in the run, e.g., Inventoried PhenoCycler Antibodies. Currently, the primary source is the ready-to-use inventoried antibodies provided by Akoya. Users can include custom-conjugated antibodies in the panel. To add detection of the custom-conjugated antibodies during a run, add each custom-conjugated marker of interest into the desired cycles in the next window. NOTE The ready-to-use markers available differ based on the filter set selected.
Filters	Select the filter set based on the reporter combination of choice. Filter set options include: - DAPI-ATTO550-CY5-AF750 (recommended for FFPE) - DAPI-ATTO550-CY5-AF488 (recommended for FF)
Scan Resolution	0.5 µm/pixel

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

Table 4.2. Parameters to define markers

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



Figure 4.8. Experiment Designer screen – Panel Content.

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

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

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

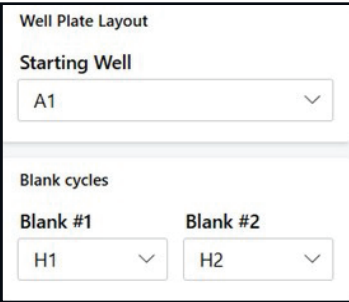


Figure 4.9. Experiment Designer Screen – Well Plate Layout.

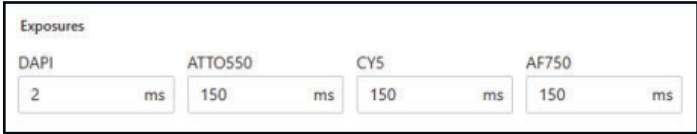


Figure 4.10. Setting Exposures (ms) for the 4 parameters.

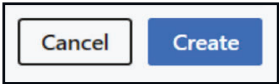


Figure 4.11. Click Create to continue or Cancel to return to the main screen.

8. Selecting Create displays the cycle layout/table mode. In this layout, the designer app sorts the marker detection into the minimum number of required cycles (See Fig. 4.12.). Each cycle consists of detection in DAPI channel plus up to three channels. The filter set selected in the main window—either ATTO550, Cy5, or AF750 (or AF488)—determines the three marker channels available.

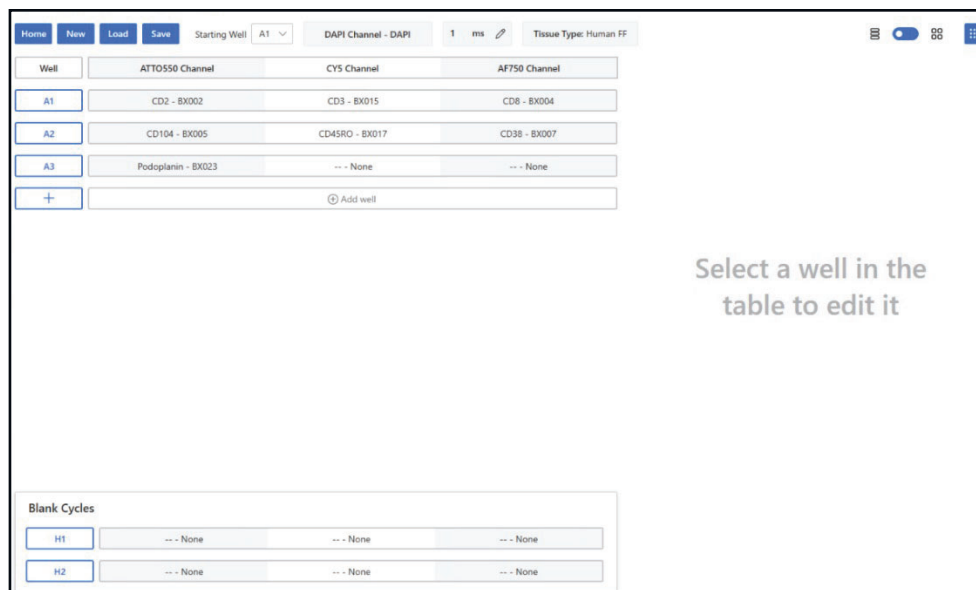


Figure 4.12. Cycle or Table Mode Layout.

EDITING THE EXPERIMENT DESIGN LAYOUT

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

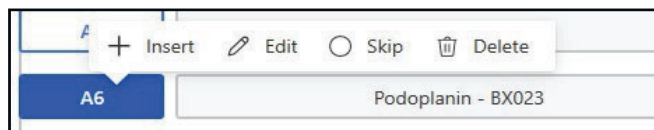


Figure 4.13. Edit Layout options within Table Mode.

To edit the contents within a cycle/well:

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

Well A7 ☐ Skip well

ATTO550 Channel

Marker/Barcode: CD8 - BX026

Exposure: 150 ms

Clone: C8/144B

CY5 Channel

Marker/Barcode: CD3e - BX045

Exposure: 150 ms

Clone: EP449E

AF750 Channel

Marker/Barcode: -- None

Exposure: 150 ms

Clone: None

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

ADDING A CUSTOM-CONJUGATED MARKER TO THE EXPERIMENT DESIGN

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

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

AF750 Channel

Marker/Barcode: -- None

Exposure: 150 ms

Clone: None

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

The Add Additional Item window will display.

2. Select Custom Conjugations from the Item Source drop-down. (See Figure 4.16.)

CRITICAL

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

The screenshot shows a dialog box titled "Add Additional Item". Inside, under the "CY5 Channel" section, there are four input fields: "Item Source" (a dropdown menu showing "Custom Conjugations"), "Marker" (a text box with "ABC"), "Barcode" (a dropdown menu with "BX054"), and "Clone" (a text box with "123"). There is also a "Reporter" field showing "RX054". At the bottom right, there are "Cancel" and "OK" buttons.

Figure 4.16. Edit content options within a cycle/well – Add Additional Item.

The screenshot shows a configuration screen for "Well A7". At the top right, there is a "Skip well" checkbox. Below this, there are three channel configurations:

- ATTO550 Channel:** Marker/Barcode is "HLA-DR - BX026", Exposure is "100 ms", and Clone is "L243".
- CY5 Channel:** Marker/Barcode is "ABC - BX054", Exposure is "100 ms", and Clone is "123".
- AF488 Channel:** Marker/Barcode is "CD90 - BX022", Exposure is "100 ms", and Clone is "5E10".

 Each channel's Marker/Barcode field has a dropdown arrow and a "+" button. At the bottom right, there are "Cancel" and "Update" buttons.

Figure 4.17. Assign barcodes and exposure time to channels.

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

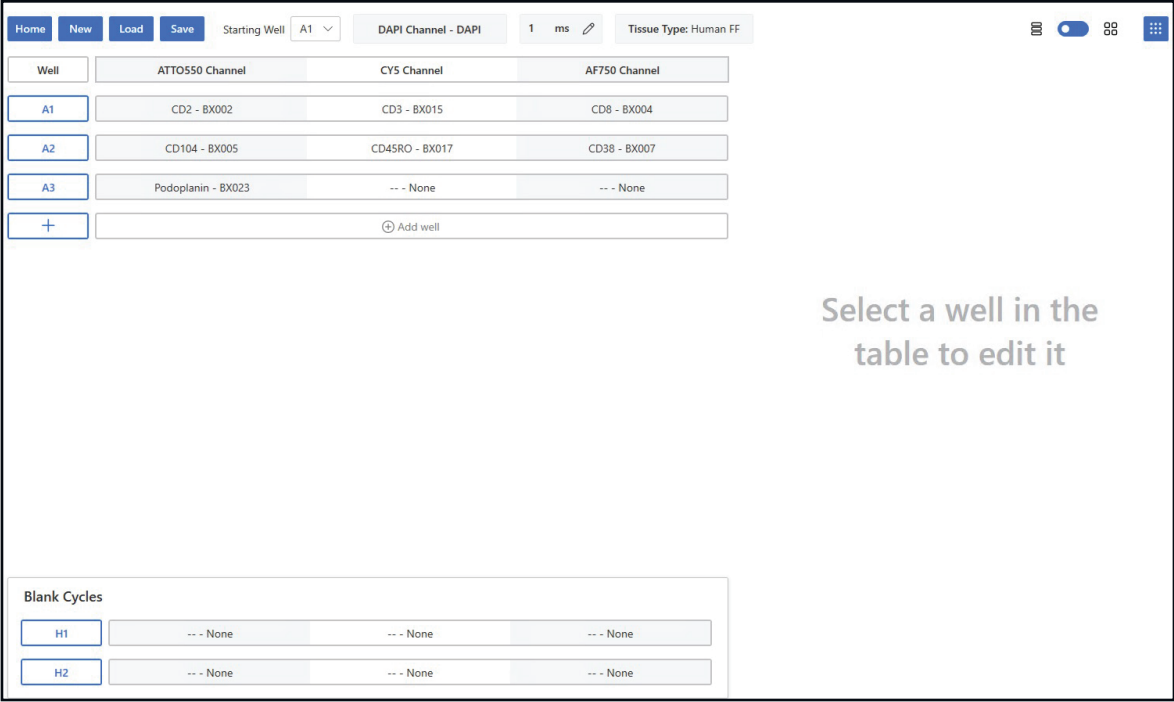


Figure 4.18. Experiment Designer Screen – Table Mode Window (List View).

6. The updated cycle layout will display all selected markers, including the custom-conjugated antibodies.
7. Select Well Plate Mode to view the well plate layout using the toggle button. The layout displays the reporters (RX-xxx) corresponding to each well (See Figure 4.19.). This layout will be used in [Chapter 5: Preparing PhenoCycler Reporter Plate](#) to prepare the reporter plate.

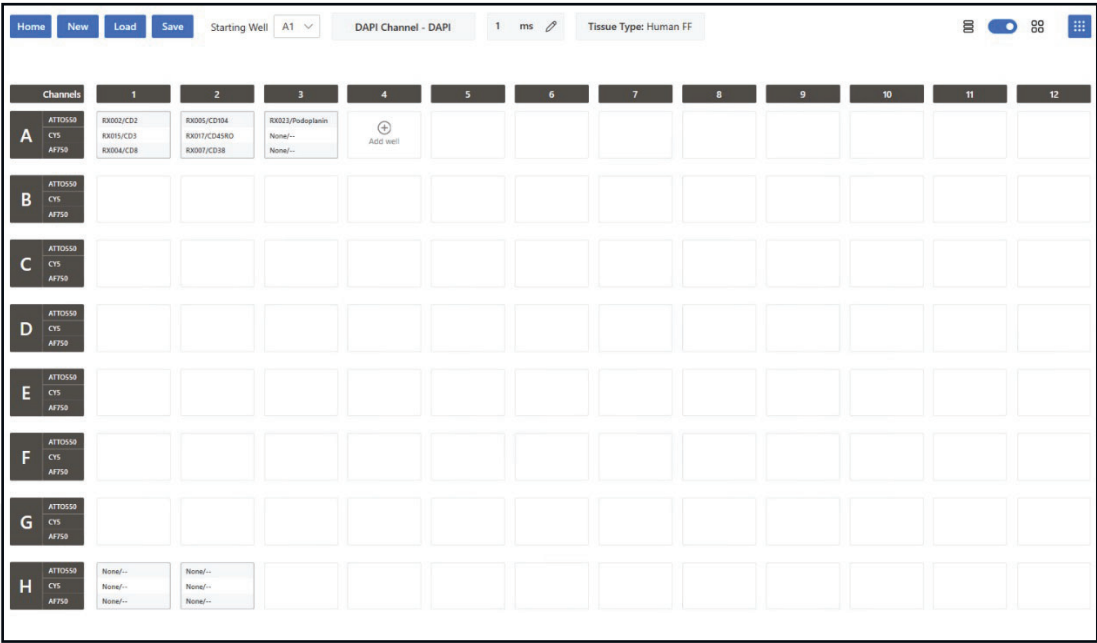


Figure 4.19. Experiment Designer Screen – Well Plate Mode to use in reporter plate preparation.

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

LOADING AN EXISTING WELL PLATE

To load an existing well plate:

1. Select the Load button on the Main screen.
2. Select the desired saved protocol in the available Study folders. (See Figure 4.20.)

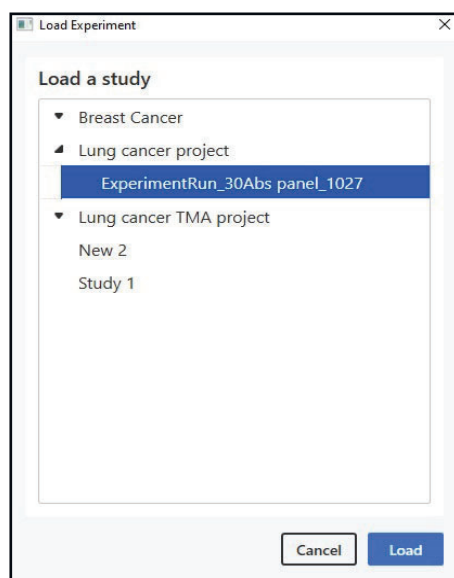


Figure 4.20. Load a Study window.

3. Click Load.

SAVING THE NEW OR MODIFIED WELL PLATE

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

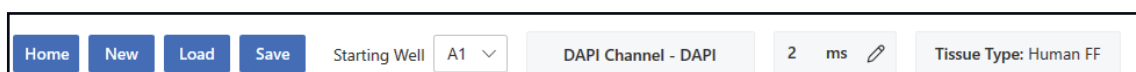


Figure 4.21. Experiment Designer – Main screen – Save.

Using Options from the Grid Menu

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

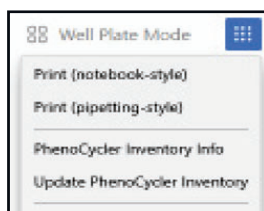


Figure 4.22. Drop-down options available from the grid menu.

Table 4.4. Options available from grid menu

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

Study: Steel_WPA_Test
Experiment: Test
Starting well: A1
Tissue type: Human FF
Report date: 10/17/2023 11:16:56 AM

Well	DAPI Channel	ATTO550 Channel	CY5 Channel	AF750 Channel
A1	DAPI	CD2 - BX002	CD3 - BX015	CD8 - BX004
A2	DAPI	CD104 - BX005	CD45RO - BX017	CD38 - BX007
A3	DAPI	Podoplanin - BX023	-- None	-- None

Blank cycles

Well	DAPI Channel	ATTO550 Channel	CY5 Channel	AF750 Channel
H1	DAPI	-- None	-- None	-- None
H2	DAPI	-- None	-- None	-- None

Page 1 of 1

Printer: Microsoft Print to PDF

Copies: 1

Paper format: NorthAmericaLetter

Font size: 14

Page orientation: Portrait

Margins: Narrow

[Print using system print dialog](#)

Cancel Print

Figure 4.23. Print preview – notebook style.

Study
Experiment
Starting well
Block type
Report date

Steel WPA test
test
A1
Plasma PP
10/17/2022 11:10:02 AM

	1	2	3	4	5	6	7	8	9	10	11	12
DA1/1	NoneDA1/1	NoneDA1/1	NoneDA1/1									
A1110580	930502C022	930505C02194	9305237Pab...									
CYS	930515C023	930517C046	None...									
A17750	930504C038	930507C038	None...									
DA1/1												
A110580												
CYS												
A17750												
DA1/1												
A110580												
CYS												
A17750												
DA1/1												
A110580												
CYS												
A17750												
DA1/1												
A110580												
CYS												
A17750												
DA1/1	NoneDA1/1	NoneDA1/1										
A110580	None...	None...										
CYS	None...	None...										
A17750	None...	None...										

Printer
Microsoft Print to PDF

Copies
1

Paper format
NorthAmericaLetter

Font size
10

Page orientation
Landscape

Margins
Narrow

[Print using system print dialog](#)

Cancel Print

Figure 4.24. Print preview – pipetting style.