Optimization Guidelines

Normalization

Seahorse XF Imaging and Normalization System

XFe24 Hoechst33342 Staining Optimization Guidelines

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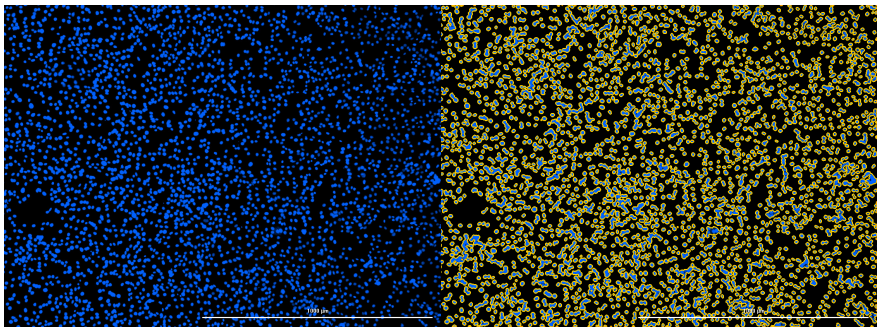
Please refer to the XF Imaging and Cell Counting User Manual before reading this.

The purpose of this assay procedure is to optimize the Hoechst dye concentration for use with the XF Imaging and Normalization system for one cell line of interest. The protocol guides the user through a Hoechst dye and cell density titration for fluorescence imaging and cell counting. The results should allow the user to determine the optimal concentration of Hoechst dye for their cells. It is imperative that this procedure be followed closely for best results and accurate cell counting.

In order to identify what works best, see the examples and recommendations below to help identify and troubleshoot staining efficacy. Note that the images shown below were captured using the XFe96 platform, but that the same phenomenon and troubleshooting practices can be applied to the XFe24.

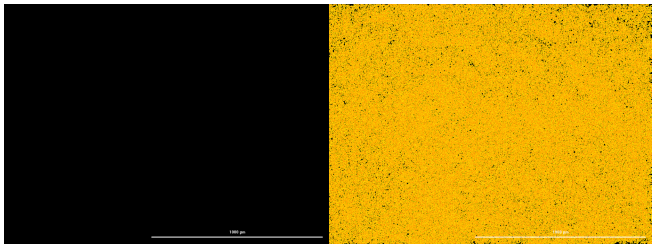
1. Good, uniform stain:

   This exemplified a “good” sample – the brightness of the objects are uniform across the entire well and the mask shows that the software can identify all of the individual objects.



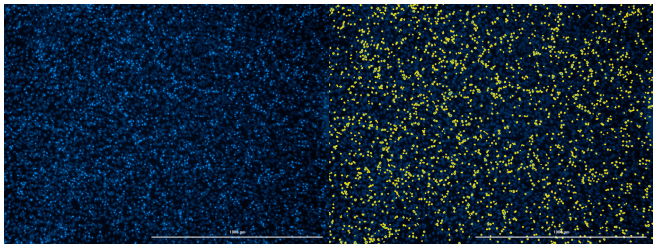
2. No dye/background wells

   If a background well with no cells is imaged or the dye fails to inject, there will be no objects visible. However, the cell count displayed may seem extraordinarily high. This is because the software bases the detection threshold on the difference between the stained objects and the background. What is shown in the image below, on the right is that the software is picking up a lot of background noise and identifying it as objects. If this well is defined as a background well in Wave, it will automatically overwrite to 0. If not, the user will have to delete this cell count data from the data set.



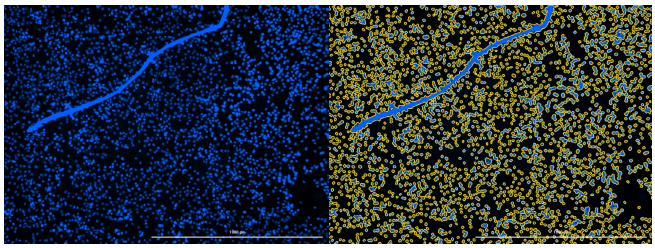
3. Non-uniformity in staining/understained:

   In this case, we can tell that many of the objects in the image have not been detected by the cell counting software. This is because of the difference in brightness across the sample. To mitigate this, some suggestions include additional incubation time for the stain or increasing the concentration of the dye in the assay.



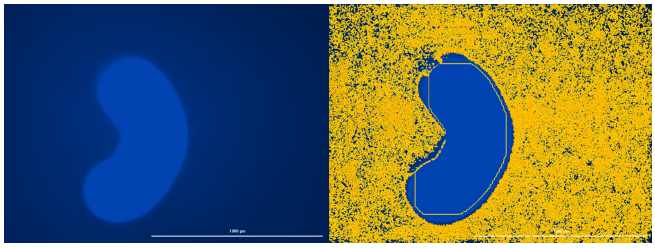
4. Debris on bottom surface of plate:

   It appears as if a fiber or some other debris is stuck on the bottom surface of the plate. This appears as one large object in the image. Because the software uses object area to calculate total cell count, the count for this image may be inaccurate. It is necessary to wipe the bottom surface of every plate with an ethanol/isopropyl (5% in water) soaked wipe to remove any debris.



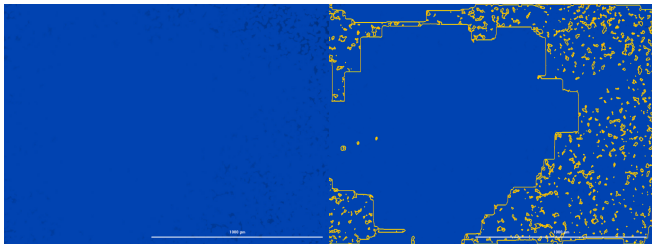
5. Debris in well (affects focus):

   It appears as though there was an object in this well that affected the focus. In this case, none of the cells are identified. It is recommended that all assay media is filtered prior to use.



6. Overstaining/overexposure (this example is particularly severe)

   In the example below, the well appears to be flooded with Hoechst Dye. The image is completely overexposed, so the edges of the objects cannot be seen.



The XF Imaging and Normalization solution is intended to be used with single nucleated, adherent cell lines grown in a monolayer. This is not intended for cells grown in suspension, 3D samples, multi-nucleated cells, or primary immune cells.

Please consult the following resources to provide an initial starting point for cell density values specific to your needs.

1. Cell Reference and/or XF publication data base: a searchable data base by cell type - http://www.agilent.com/ cell-reference-database/ and http://www.agilent.com/ publications-database/.
2. Assay Guides and Template Library: pre-made XF assay templates for many cell types with cell density and FCCP concentration values - http://www.agilent.com/en-us/ support/cell-analysis-(seahorse)/seahorse-assay-guidestemplates.

While suggested values may be found in the resources above, it is encouraged to still perform both cell density and FCCP titration analyses to ensure optimal cellular function under the assay conditions used.

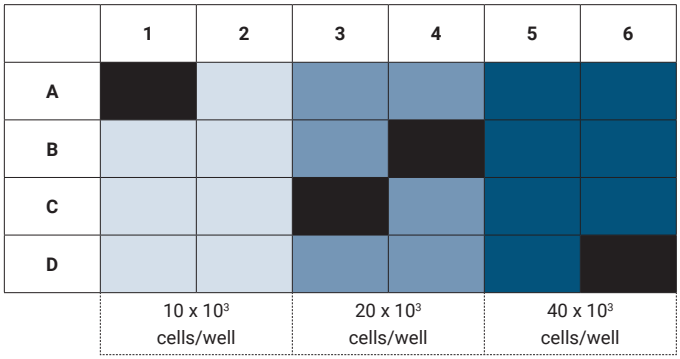
All of these resources are in the Seahorse XF Assay Learning Center

For information and recommendations on Normalizing your XF Data, see our Tech Note: XF Assay Normalization - Technical Overview

Method

This method is for simultaneously testing three different cell densities and four different Hoechst dye concentrations using an XF24 cell culture plate and XFe24 cartridge with an XFe24 Analyzer and a BioTek Cytation Instrument.

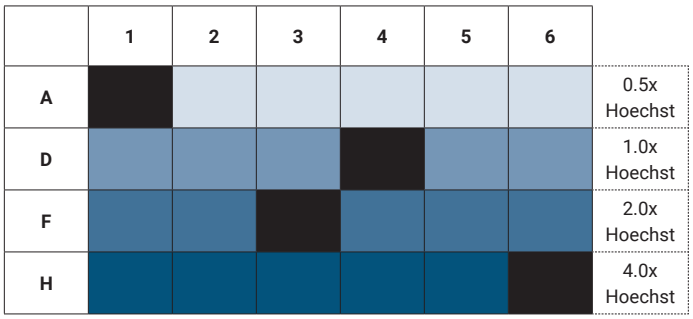
1. In preparation of installation/training, prepare a cell plate for use.
   a. Choose three cell densities to test. Either cover the range found in the Cell Reference database, or seed the recommended cells/well value (1x) plus 0.5x cells/well and 2x cells/well. For example, 10×10^3, 20×10^3, and 40×10^3 cells/well in each of the groups shown in the diagram below. See plating procedure. Do not seed wells A1, B4, C3, or D6 (these wells are used for background correction).
   CRITICAL: Stained cells must be in B3 and C4 for the auto-exposure settings to work properly.



   b. Hydrate an XFe24 cartridge the day prior to the XF Assay. Follow this link for detailed instructions.

2. Day of: make sure systems are set up, drives/image databases are correct, and instruments/barcode wand are operating. See the XF Imaging and Normalization User Manual for more information.
3. Prepare XF Assay Medium and warm to 37 °C. Ensure that the pH is 7.4 ±0.1 at 37 °C. Click here for detailed instructions.
4. Retrieve the cell culture plate from the CO_2 incubator.
5. View the cells under the microscope to confirm cell health, morphology and seeding uniformity.
6. Wash cells with prepared Seahorse XF Assay Medium. Final well volume is 500 µL. Click here for detailed procedure.
7. Transport the plate to the Cytation Instrument. Wipe the bottom of the wells with ethanol/isopropyl to clear them of any debris. In the XF Imaging and Cell Counting application, scan the plate barcode with the handheld barcode wand and begin brightfield imaging (see XF Imaging and Cell Counting user guide for more information). Leave the plate in the Cytation for one hour prior to the assay – there is a timer within the software that will alert the user when the plate has completed outgassing.
8. Design an assay protocol in Wave. For this initial study, only Hoechst dye will be injected. This will only generate basal OCR and ECAR data. See the Wave protocol design section on page 6 of this document.
9. Prepare a titration of Hoechst dye injections. See the layout below – use 4 different concentrations. It is recommended to start at a concentration of 20 µM (for 1.0x concentration). If the concentrations suggested do not result in uniform brightness with low background, adjust the stain concentrations as necessary.

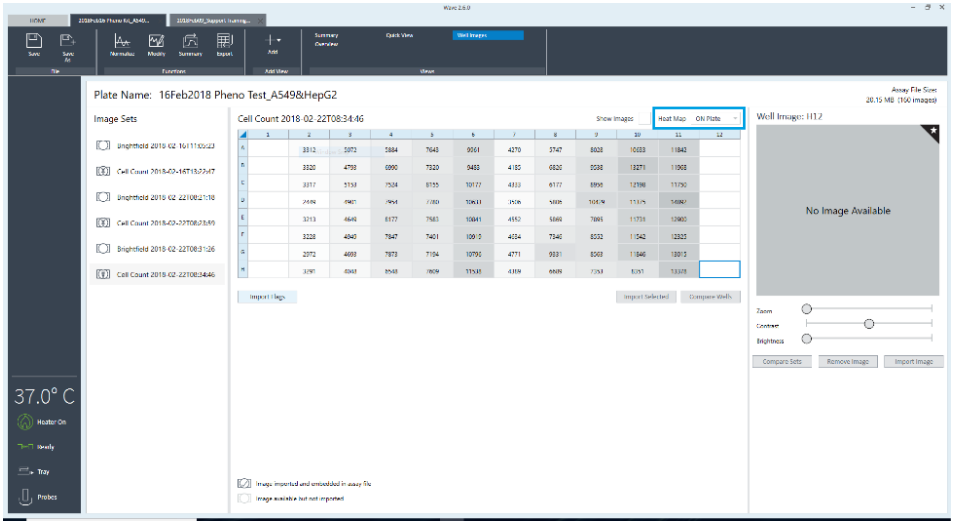
Use the table below to prepare enough of each injection solution for 1 XF24 plate:



Concentration	Volume XF Assay Medium	Volume Hoechst Dye (20 mM Solution)	Total Volume
10 µM (0.5x)	1,999 µL	1 µL	2.0 mL
20 µM (1.0x)	1,998 µL	2 µL	2.0 mL
40 µM (2.0x)	1,996 µL	4 µL	2.0 mL
80 µM (4.0x)	1,992 µL	8 µL	2.0 mL

10. Load the sensory cartridge using the format shown below:
    a. Add 55 µL to all port A's of the sensor cartridge. Be sure to add to every well, even background. See cartridge loading guidelines here.
11. Run Assay: when calibration is complete and outgassing has finished, retrieve the plate from the Cytation to load on the Analyzer. Note the estimated assay completion time after loading the cell plate.
12. As soon as the assay has completed, retrieve the cell plate from the instrument and run the fluorescent imaging procedure in Imaging & Cell Counting.
    a. During the first few experiments staining samples for your XF Assays, it is recommended to stand by while the imaging completes – the fluorescent protocol should take no longer than 10 minutes to complete. Once the images are available to review, observe examples from each group with and without the detection mask, and determine if further optimization is necessary.
    b. If staining does not appear to produce a bright enough signal and cell count is compromised, the first recommendation is to wait an additional 30 minutes, and image the plate again to visualize.
    c. If staining is still not bright enough, try adjusting the Hoechst dye concentrations.

13. After fluorescence imaging has completed, and data is ready to review, navigate to the Wave assay results.
14. Click on the Normalization tab and click the **Import** button, and then **Apply**.
15. Click the **Add** button, and open the "Well Image View" tab. Here, import the image sets and use the Heat Map option (on plate) to show the cell counts across the plate for review of counting accuracy.



Analysis and troubleshooting:

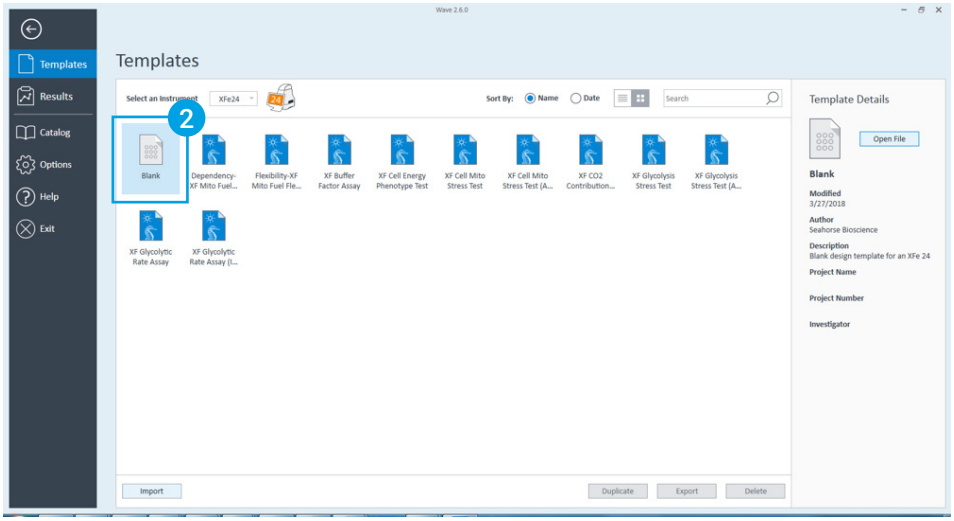
1. Using the images and cell counts generated, select the concentration that best represents the original seeding density and shows the most consistency across the concentration group. Note that the counts shown in Cell Imaging and Wave are different – importing into Wave extrapolates the cell counts to represent the entire area of the well bottom.
2. Use the image examples shown on pages 1 and 2 of this document to determine the accuracy of object detection and to troubleshoot staining and imaging.
3. If the brightness of the objects is not uniform within the wells, the detection and cell counts will not be accurate. Timing after the Hoechst Dye injection and dye concentration should be adjusted for each cell type. Note that adding stain to different reagents (i.e. with XF Assay Kit reagents) can affect the uniformity and timing of staining and imaging the cells. For example, if Hoechst dye is combined with Antimycin A and Rotenone in an XF Cell Mito Stress Test Kit, most samples are very uniform and brightly stained.

Wave Assay Protocol

Follow the steps below to set up the protocol in Wave software. The example shown uses cells (A549) plated at 10,000, 20,000, and 40,000 cells/well in an XF24 well plate and Hoechst Dye injection solution at concentrations of 10, 20, 40, and 80 µM (port concentration).

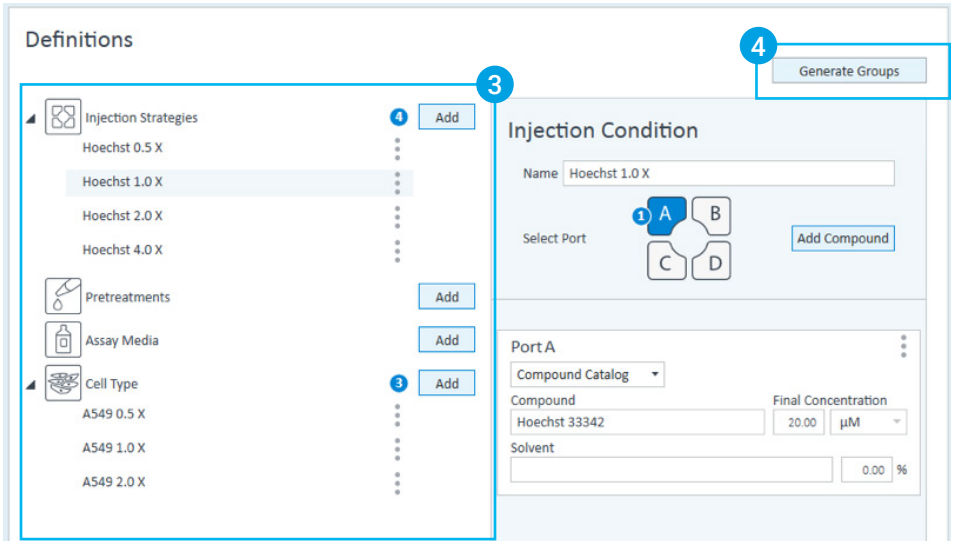
1. Open Wave software.

2. Click "Blank" template, and Open File to begin designing a new assay protocol:

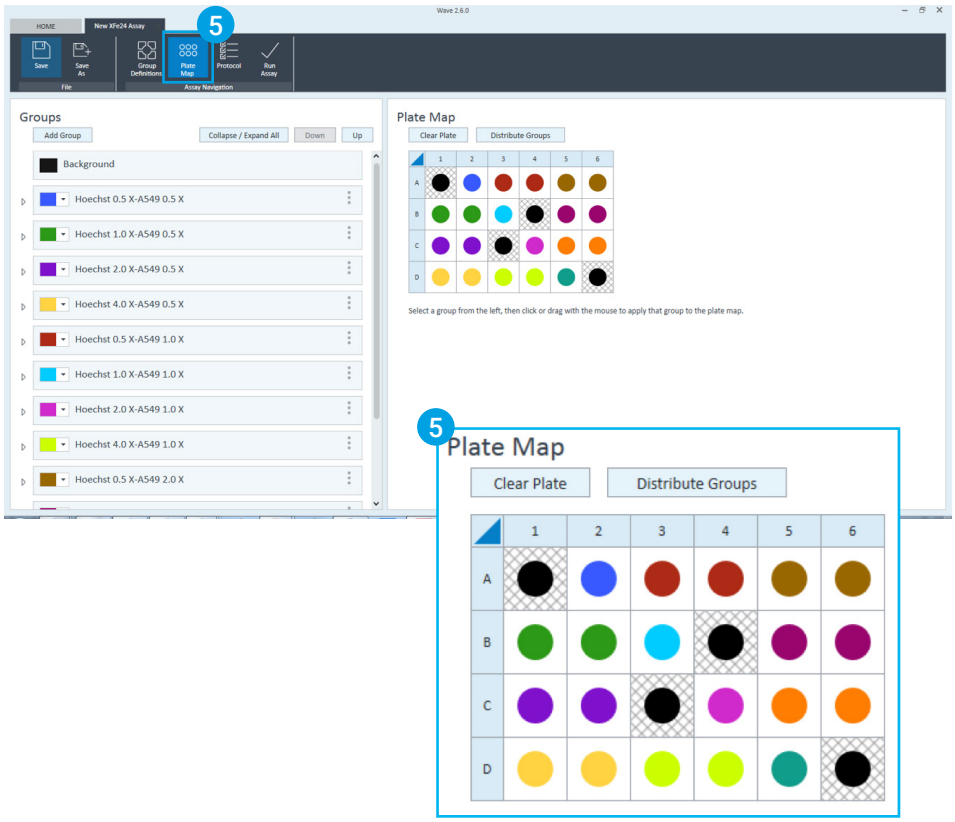


3. In the Group Definitions tab, add Injection Strategy and Cell Type information as appropriate for your assay conditions; see example below. Add more or less detail as you deem necessary.

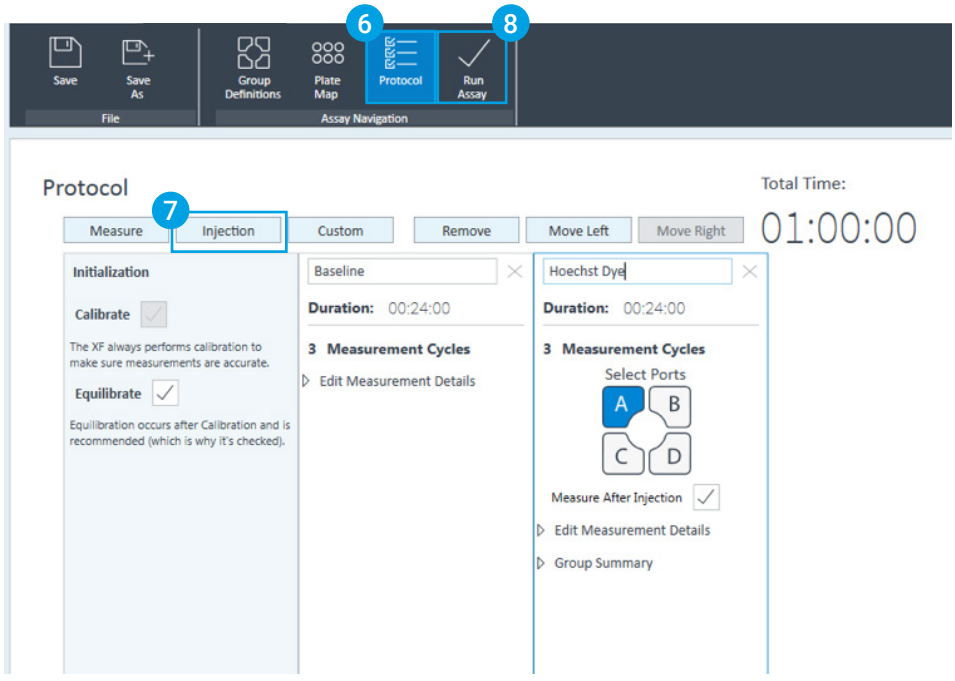
4. Once all 4 injection strategies are defined and 3 cell groups are defined, click the **Generate Groups** button. This will create 12 groups for the plate.



5. Click the “Plate Map” tab. Add the groups as shown in the following images. You can click and drag on the plate map to form a group in Wave.



6. Click on the “Protocol” tab. Ensure that the “Equilibrate” option is checked.
7. Click **Injection**. This should automatically create a command for injecting Port A and include three additional measurement cycles following the injection. The total assay time will be approximately 1 hour.
8. Click the “Run Assay” tab. Add all necessary information and begin the assay when the cartridge is ready to be calibrated.



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