# Analysis Guidelines for the Incucyte® ATP Analysis Software Module

The Incucyte® ATP Analysis Software Module is used to acquire, quantify and visualize real-time measurements of cytoplasmic ATP levels utilizing the Incucyte® CytoATP Lentivirus Kit. The assay is compatible with the Incucyte® SX5 Live-Cell Analysis System configured with an Incucyte® SX5 Metabolism Optical Module, enabling image-based measurement of cytoplasmic ATP within your choice of cells and treatments.

This guideline covers the following topics for defining ATP Live-Cell Analysis Parameters:

- Acquiring images using the ATP Analysis Software
- Creating a plate map identifying CytoATP and Non-binding Indicator wells
- Defining ATP Analysis Parameters
- Defining metrics and visualizing ATP Ratio

The following procedures are for example purposes only and are designed to provide a frame of reference for defining the ATP Analysis Parameters within the Analysis Guided Interface.

For new Incucyte® users, we recommend that you review the Incucyte® Systems User Manual. It will be helpful to have experience scheduling and acquiring scans, viewing images, performing image analysis, and visualizing results prior to reviewing this technical note.

## Acquiring images using the ATP Analysis Software Module

This module enables acquisition of phase and fluorescence images in flasks, multi-well plates or dishes. Please see the Incucyte® Systems User Manual, Section 1 for a detailed description of how to log in to the Incucyte® and launch the Acquisition Window. Follow instructions for scheduling a scan as described in the User's Manual until the "Scan Type" Window is displayed.

1. In the Scan Type Window, select ATP.

2. In the Scan Settings Window, all three channels will be selected (Phase, 485X | 578M, and 535X | 578M). Phase is optional but recommended for monitoring changes in morphology.

    a. 485X | 578M; changes in signal intensity represent changes in cytoplasmic ATP.

    b. 535X | 578M (direct ex/em of mKOk); signal represents total protein expression. The fluorescence segmentation mask is generated using the 535X | 578M channel.

3. 10X and 20X objectives are supported. Generally, 10X is recommended for adherent cell types and 20X for non-adherent cell types. 20X will generate higher quality images but will slightly increase scan time.

4. Proceed through the remaining windows referencing the Incucyte® Systems User Manual as needed.

## Creating a plate map identifying CytoATP and Non-binding Indicator wells

The following section will guide you through creating an ATP plate map to designate corresponding CytoATP and Non-binding Lentivirus wells to enable Corrected ATP Ratio images and metrics.

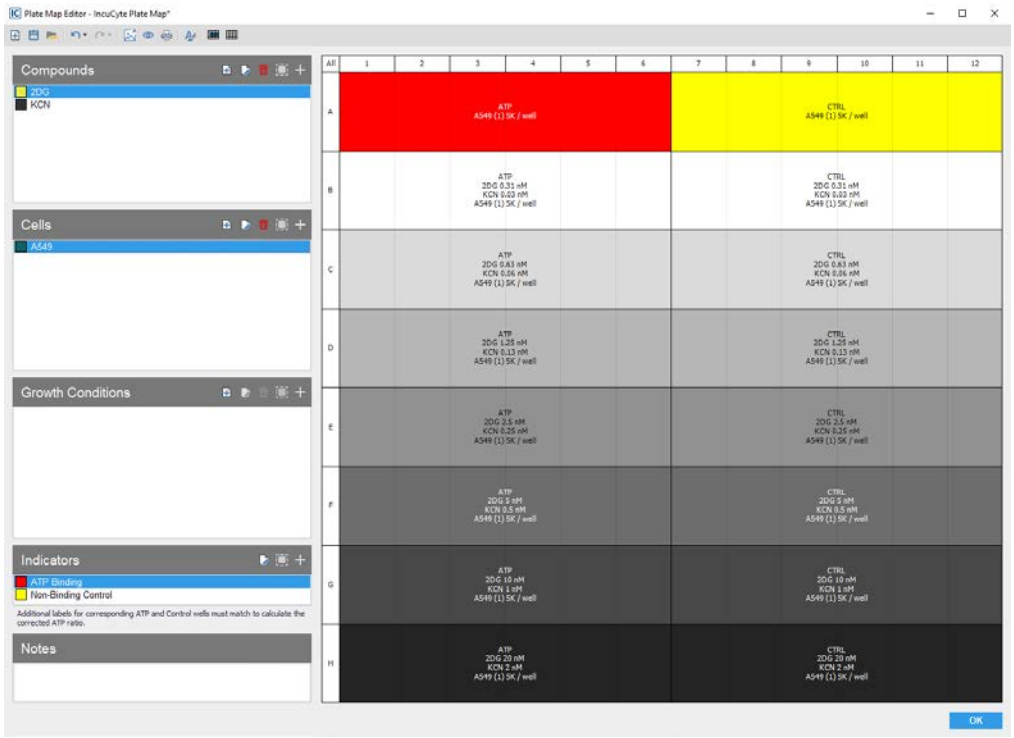
1. Open the ATP Plate Map Editor during ATP scan setup or launch from a vessel following acquisition of images using ATP scan type.

2. Using the pre-populated options in the Indicators box, designate wells containing cells expressing the ATP Binding (CytoATP) or Non-binding Control Indicator See Figure1

3. Designate Compounds, Cells, and Growth Conditions as desired, ensuring that corresponding CytoATP and Non-binding Control wells are denoted identically across each treatment group. If this is not done properly, Corrected ATP Ratio images and metrics will not be enabled (only Uncorrected Ratio Analysis will be available).

*The standard plate map editor does not contain the Indicators option that is necessary for linking corresponding CytoATP and Non-binding Indicator wells to generate Corrected ATP Ratio (recommended).*

*Figure 1. Example plate map generated with the ATP plate map editor. Note the Indicators box, which is present only when the editor is launched during ATP scan setup or from a vessel scanned in ATP acquisition mode.*



## Defining ATP Analysis Parameters

1. Launch analysis of vessel and select "Create New Analysis Definition". Proceed through the wizard to select ATP analysis.

2. **Image Channel Selection:** 485X | 578M and 535X | 578M are required for ATP analysis. Phase is selected by default and is optional for generating standard phase metrics (e.g. confluence).

3. **Image Set Selection:** Select a representative set of images from both CytoATP and Non-binding Control wells, incorporating images with and without treatment (if applicable, include treatment-induced morphological changes over the course of the experiment).

4. **Analysis Definition:** The ATP segmentation mask is generated using 535X | 578M (direct mKOk Ex/Em) images. The segmentation mask and Top Hat radius for background subtraction is then automatically applied to 485X | 578M, enabling calculation of the ATP Ratio image.

    a. It is recommended to first preview the image set using the default analysis parameters.

    b. Evaluate the ATP Mask and refine using the Analysis Definition parameters. See Figure 2 and Table 1.
        i. Turning the phase channel on and off can be helpful for determining fluorescence intensity.
        ii. If fluorescence signal is dim, turning off the auto scale and decreasing the max/min range can help with visualization.
        iii. The Top Hat radius should be chosen to reflect the size of fluorescent objects but contain sufficient background to reliably estimate background fluorescence; 60-100 µm is a useful starting point.
        iv. Optimize threshold (535X | 578M) to be sufficiently high to avoid spillover of mask outside the boundary of bright cells while still capturing most of fluorescent cell area of dimmer cells.
        v. Recommended: set a minimum area filter (e.g. 300 $\mu m^2$) to avoid masking cellular debris.
        vi. If drug treatments induce substantial balling up of cells associated with cell death, a maximum intensity filter in conjunction with Edge Split can be useful for filtering out dead/dying cells.

    c. Once you have previewed all the images within the wizard and are satisfied with the parameters, complete the Launch wizard analysis to select Scan Times and Wells to be analyzed and assign an analysis definition name.

*The best way to begin setting up the Analysis Definition is to use the preset values. Therefore, do not change the Segmentation, Cleanup, or Filters at this time.*

*Figure 2. Generating the ATP Mask. The ATP Mask is generated based on fluorescent objects in 535X | 578M via analysis parameters defined by the user. The same segmentation mask and top hat radius is automatically applied to 485X | 578M.*

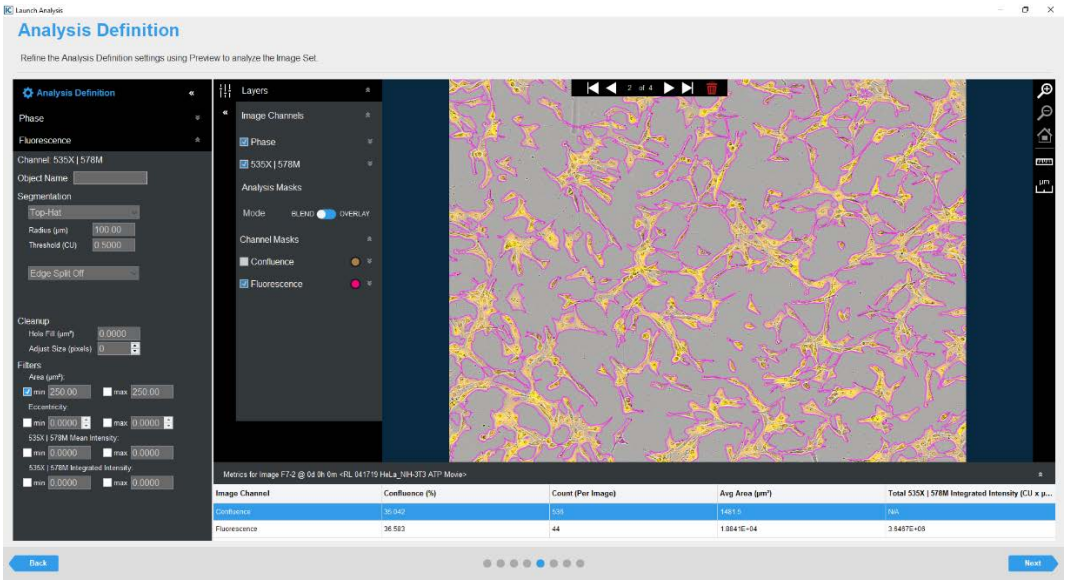


*Table 1: ATP Analysis Definition Options*

| Option | Description |
|---|---|
| Object Name | Allows user to define the metric with a custom name. |
| Segmentation | Top-Hat segmentation subtracts background fluorescence from object fluorescence and segments fluorescent objects within image. |
| Radius | A disk of this radius size is used to identify fluorescent objects from background. The radius should be set such that it is slightly larger than fluorescent objects, containing sufficient background to reliably estimate background fluorescence. |
| Threshold | Objects that are brighter than the set threshold level are detected in the background-subtracted image and masked as objects. |
| Edge Split | Determines sensitivity for detection of object edges. This tool is turned off as a default. When turned on, slide to the right to increase edge detection sensitivity. |
| Cleanup | Cleanup parameters are used to adjust ATP mask closer or further from relative to fluorescent objects |
| Hole Fill | Removes any holes in the segmentation mask that are smaller than the defined value |
| Adjust Size | Grows or shrinks the mask around fluorescent objects on a pixel basis |
| Filters | Allows user to further exclude or include masked objects |
| Area | Defines a range of sizes (in $\mu m^2$) for objects and eliminates objects that fall outside this range. Useful for eliminating cellular debris. |
| Eccentricity | Defines a range of roundness for the object and eliminates objects that fall outside this range. Eccentricity ranges from 0 to 1 with a perfect circle having a value of 0. |
| 535X \| 578M Mean Intensity | Defines a range of total mean intensity for objects in 535X \| 578M and eliminates objects that fall outside this range. |
| 535X \| 578M Integrated Intensity | Defines a range of total integrated intensity for objects in 535X \| 578M and eliminates objects that fall outside this range. |

## Defining metrics and visualizing ATP Ratio

The following section will guide you through defining the ATP metrics and visualizing the ATP ratio to measure intracellular ATP.

1. After the vessel images have been analyzed, the Corrected Ratio (recommended) and Uncorrected Ratio can be quantified and visualized. See Table 2 for definition of metrics.

    a. Corrected ATP Ratio images and metrics will be displayed as unavailable if CytoATP and Non-binding Control wells have not been designated correctly in the Plate Map Editor.

*Table 2: ATP Ratio Metric Definitions*

| Option | Description |
|---|---|
| ATP Metrics | |
| Corrected Ratio | Defined as ((Uncorrected Ratio$_{CytoATP}$/Mean Uncorrected Ratio$_{Non\text{-}binding\ Control}$) -1) for any given treatment for which corresponding CytoATP and Non-binding Control wells have been designated via the plate map editor. Provides correction for any artifacts that may affect the ratio and sets a 0 point (i.e. the limit of detection = Non-binding Control ATP ratio). |
| Uncorrected Ratio | Represents direct ratio output of 485X \| 578M and 535X \| 578M fluorescent images |

2. The ATP Ratio Image is displayed as a default when opening an analysis. The image is color coded to denote ATP Ratio values for pixels included in segmentation mask generated using the Analysis Definition.

    a. Ratio Image Autoscale Max and Min values are denoted at the top and bottom of the legend in the bottom right corner of the screen. See Figure 3

    b. If Autoscale is set to well, "Max" and "Min" will be displayed to reflect variable values for each well.

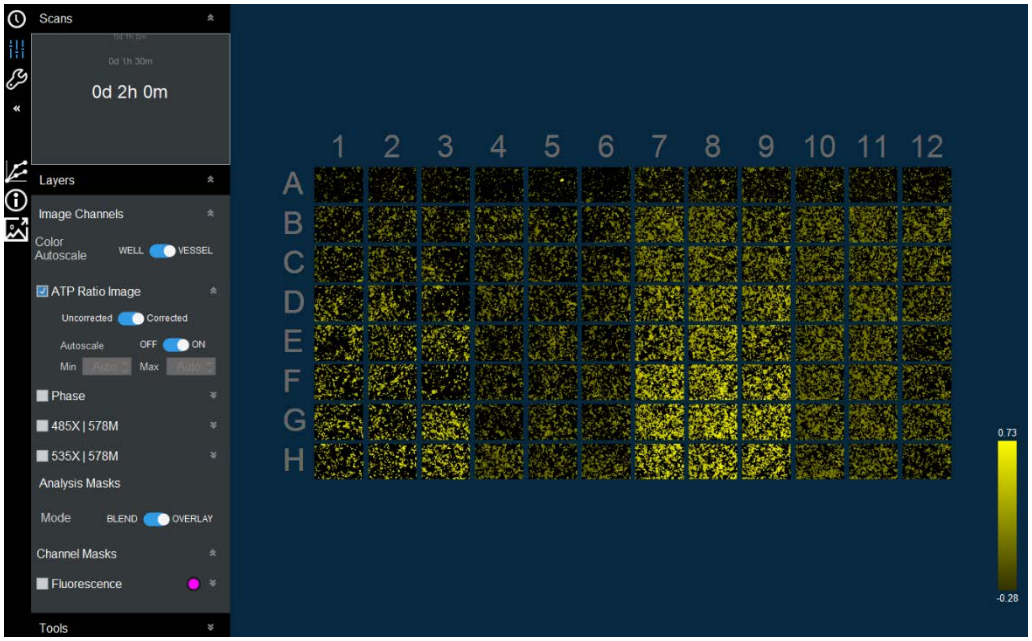
3. Options for toggling between Uncorrected and Corrected ATP Ratio images can be found in the dropdown menu.

    a. Note that ratio image appearance will only change between Uncorrected and Corrected if treatments have an effect on Non-binding Control ATP Ratio values. The max and min values on the legend will always change when toggling between the two options.

4. Additional visualization options include Phase, 485X | 578M, 535X | 578M, and ATP Analysis mask (segmentation mask).

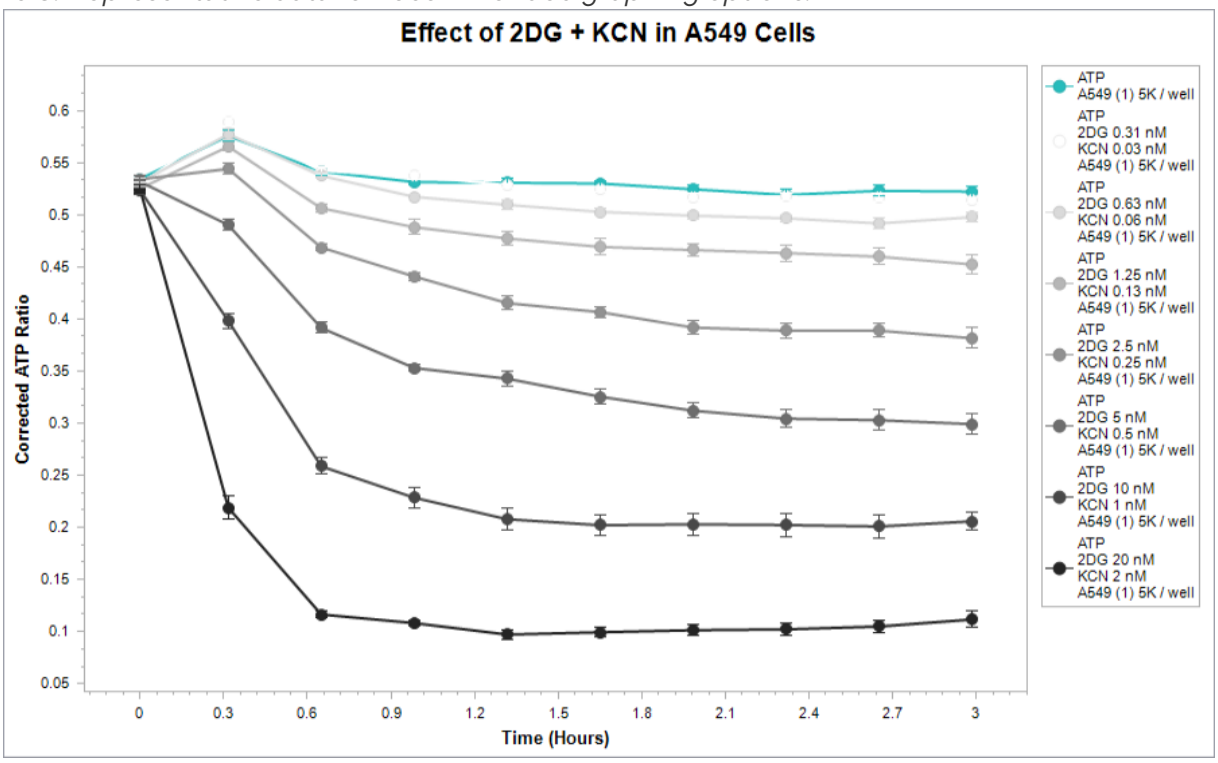
    a. Visualization of 485X | 578M and/or 535X | 578M can be useful for investigating causes of unexpected ATP ratio results (e.g. misalignment between two fluorescent images, auto fluorescence of debris)

*Figure 3. ATP Ratio Image enables a quick look at experimental results within vessel view.*



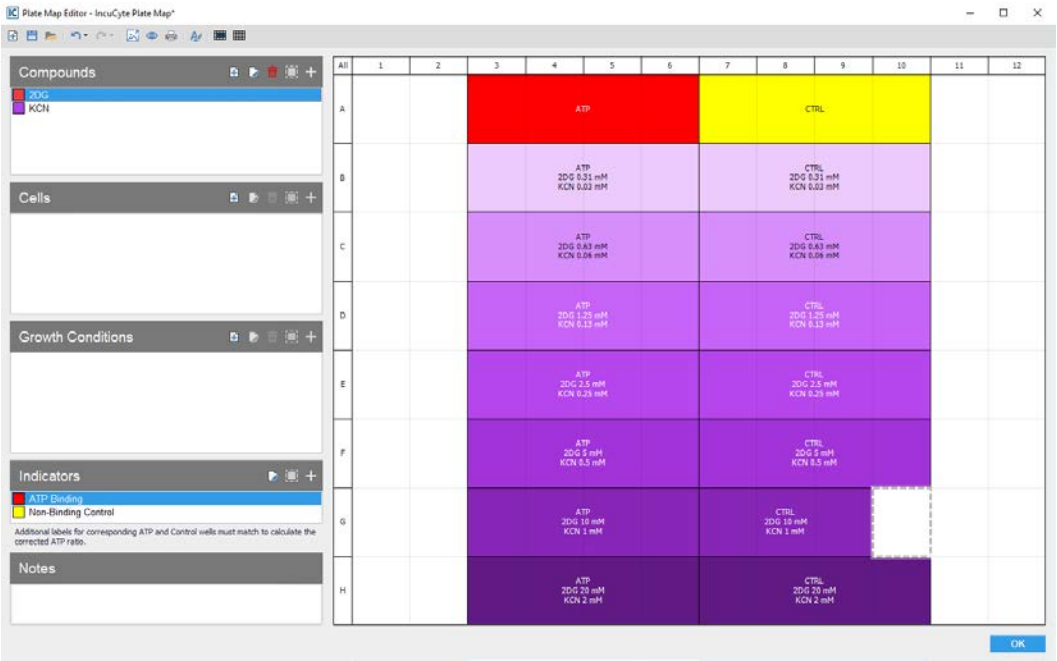

5. Corrected Ratio and Uncorrected Ratio are provided as default metrics (485X | 578M and 535X | 578M data may be plotted by creating a User Defined metric). Metrics, time points, wells, and microplate or graph options are described in more detail in the Incucyte® Systems User Manual.
    a. Selecting the Corrected ATP Ratio and plotting only the wells containing the CytoATP-expressing cells is recommended (See Figures 4 and 5). The Non-binding Control wells will be automatically included in the correction analysis for the corresponding CytoATP wells whether or not they are selected (See more in Step 7 below).

*Figure 5. Representative data for recommended graphing options.*




6. CytoATP outliers may be excluded by deselecting from the "Select Wells" section when graphing.
7. Deselecting Non-binding Control wells from the "Select Wells" section will exclude wells from being plotted but will NOT exclude them from inclusion in the Corrected Ratio metric calculation.
    a. If excluding Non-binding Control outlier wells is desired, they must be cleared from the group via the ATP plate map editor (See Figure 6). Corrected metrics will be calculated based on the mean of the remaining Non-binding Control wells for that treatment group.

*Figure 6. Example of exclusion of well G10 from Corrected ATP Ratio calculations. The Corrected Ratio for wells G3-6 would be calculated using the mean of wells G7-9.*




Visualization of Analysis Results is described Incucyte® Live-Cell Analysis Systems User Manual.

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