

## Definiens Cellenger

# Comparison of Image Analysis Performance on Multiple Instrument Data

## Summary

This document investigates the comparability of output generated by image analysis of data collected on two different types of vendor instrumentation. Sources of variation were minimized and the data was shown to be highly comparable when analyzed with the same software system.

## Introduction

One of the most interesting questions currently being asked in the field of High Content Analysis of cell-based assays is the comparability and compatibility of image data (and results derived from such data using image analysis) collected and analyzed from different instrument and software platforms. This problem becomes particularly acute when an organization has more than one instrument platform available.

Different instruments have different design characteristics, strengths and weaknesses when applied to a particular problem. In some cases a certain feature of an instrument (such as confocality) is needed for a particular experiment, but often this is not the case. To what extent the results are determined by the choice of hardware is not yet clear.

While the physical characteristics of the imaging system are important sources of variation the effect of differing software systems can have an even greater impact on the results of an experiment. Even changing the parameters of the same algorithm can have a very big effect on data derived from the analysis.

With the advent of truly platform independent software tools for image analysis, we can begin to compare data from different instrument platforms and be able to draw meaningful conclusions by minimizing the sources of variation. In this application note we compare a data set of three plates imaged on hardware systems from two different vendors. The data was analyzed using Definiens Developer, Definiens Server and Definiens Cellenger.

## Description of the Experiment

The dataset used in this comparison consists of a 96 well plate containing several dose response experiments and control wells. The edge wells were not used in the experiment to minimize edge effects and one image was collected in each well on both imaging platforms.

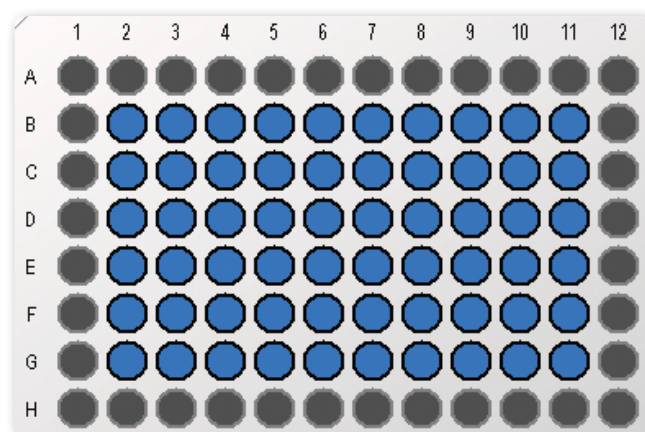


Figure 1 Experimental plate layout

## Description of Imaging Platforms

The two imaging platforms used to capture the data used in this comparison were the ImageXpress 5000A from Molecular Devices Corp [1] and the IN Cell Analyzer 3000 from GE Healthcare [2].

The ImageXpress is a non-confocal xenon arc lamp based system. The IN Cell 3000 is a laser based confocal instrument.

### KEY WORDS

Cell based assay –  
Image Analysis – HCA –  
Definiens Cellenger –  
Definiens Developer –  
Definiens Server –  
Platform Independence

The three major sources of variation in the image data are:

- Confocal versus non-confocal
- Image resolution
- Image bit depth

The confocal data from the IN Cell 3000 should have a better signal to noise ratio and this should help when trying to detect faint objects. The resolution of the ImageXpress is slightly better (20x objective give a resolution of 0.5 Microns/Pixel) than the IN Cell (0.582 Microns/Pixel). Finally the bit depth (data range) of the ImageXpress is 16-bits (65535 grey level range) as opposed to the 12-bit range of the IN Cell (4096 grey levels). The ImageXpress has a uniform illumination while the IN Cell has a shading artefact which has not been corrected. Other differences in the optical path such as excitation and emission filters may have an effect.

## Method

Comparing these datasets requires minimizing the sources of variation in the data. The key enabling technology which allows this comparison to be made is the Definiens platform independent cell-based analysis system Definiens Cellenger. By standardizing on one software platform the variations introduced by the image analysis part of the process can be minimized and managed. By contrast trying to compare data generated by two different (and often closed) algorithms is next to impossible as the sources of variation are not visible and can be huge. Standardizing on one software platform reduces the variation to that which is introduced by the imaging system and the parameters settings of the algorithms which account for these imaging variations. Given the differences between the two instruments used in this experiment described above although the same “algorithm” is used to analyze the data in each case, the exact settings of this algorithm must take into account the inherent differences between the image data. This presents a problem as to how we parameterize the algorithm in each case. As previously explained, derived data can be highly sensitive to the settings of an algorithm. The reason algorithms such as this have settings in the first place is to allow for experimental variations to be accommodated. To solve this problem we took overlapping geographic fields of view from the same well in each instrument set.

This was done by identifying an object in both images and using this to calculate the relative position of the image boundaries. A simple “registration” using one ground control point (GCP) is all that is required here. Although the images only partially overlap the same geographic area in the well, they are well aligned (i.e. there is no rotational variation) and the images are not distorted in any other non-linear way relative to each other. The resulting “registered” images are different sizes however due to the different resolution of the two imaging systems.

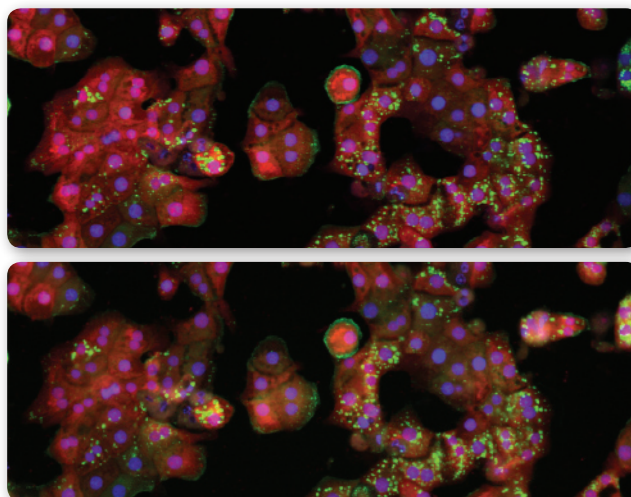


Figure 2 Overlapping FOV IN Cell (top) ImageXpress (bottom)

Unfortunately the offset between the two instruments is not constant from well to well so any automated procedure would need to identify GCP's in each pair of images. To complicate matters further, each second image collected on the IN Cell 3000 has reflectional symmetry in the x-axis as compared to the ImageXpress because of the line scanning nature of the instrument.

For the purposes of this experiment however we need only register one pair of images. This allows for a direct comparison of the image analysis algorithm with a view to determining the settings which produces the minimum variation in derived data. Once these settings have been determined we can analyze the complete dataset and compare the important results which are relevant to the desired experiment. We should also be in a position to make estimates of the experimental error introduced by the measurement system (consisting of both instrument and software).

## Nuclei Detection

Because Nuclei are fairly large, well separated (in most cases) and very bright by comparison to the background they make a good candidate for analysis comparison. Nuclei can also be used to improve the cell detection (although multiple nuclei can belong to the same cell). Because of the high signal to background inherent in the nuclei channel (of both instrument's images) the results should be less variable with respect to the parameter settings of the algorithm than the case where S/N and S/B are low. For the purposes of this experiment the Cellenger Nuclei Detection (DAPI/Hoechst) module is used as the DNA stain in this case is DAPI.



Figure 3 NucleiDetection Action (DAPI)

Before we begin to configure the *Nuclei Detection Action* we need to account for two of the three major differences between the images; the resolution and the bit depth. These are configured in the *General Settings* of the solution (figure 4).

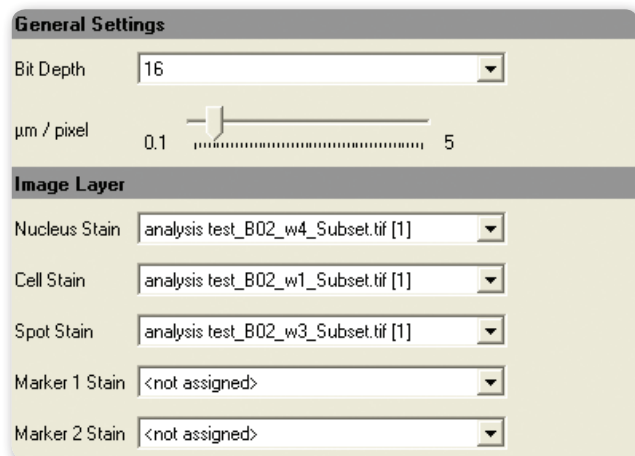


Figure 4 General Settings

Starting with the default settings the *Nuclei Detection Action* parameters were set to try and achieve the best possible nuclei detection and separation in each image.

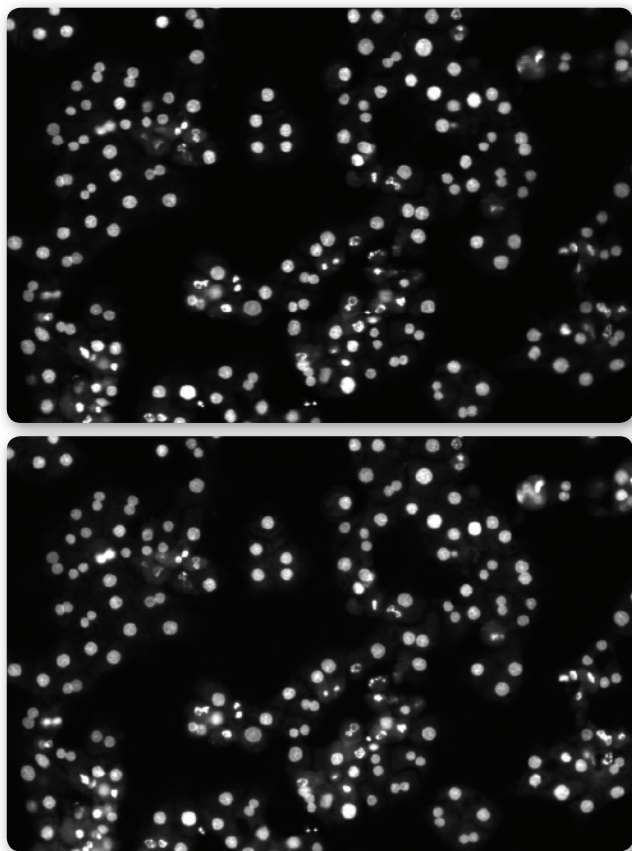


Figure 5 Nuclei Channel IN Cell (top) IX (bottom)

There are six parameters which can be used to configure this *Action* (figure 6):

- Ignore objects at image border
- Image contains clusters of nuclei
- Nuclei have irregular shapes
- Low contrast
- Nuclei have variable size
- Characteristic background

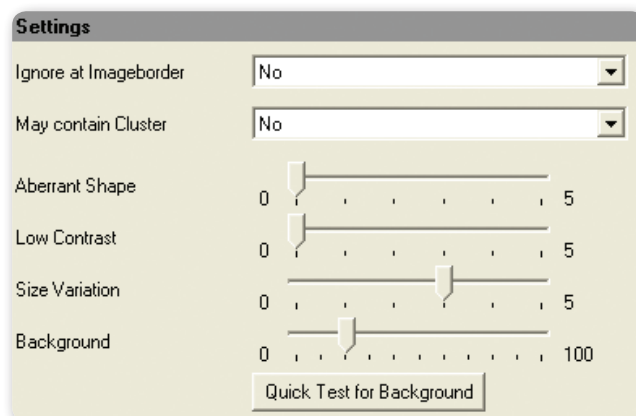


Figure 6 Nuclei Detection Action (DAPI) Settings

Most of these we would not require to differ between the two images as they refer to invariant properties when looking at the same field of view. We wish nuclei at the border of the image to be included (in both cases). Since we are looking at the same cells again, in both cases there are not significant clusters of nuclei which need to be separated. Even though there are a number of dead cells in this image, there are not significantly irregular shaped nuclei present which would require special shape processing. Again this is invariant between the images. In both cases contrast is high (we have already taken account of the different bit depth of the images). Because we measure the size of an object in microns, not pixels we have already taken account of variation in size of the same objects between images by setting the correct resolution in the general settings. There is however a reasonable size range of nuclei in this image so we set the value higher in both cases.

The final parameter in this *Action* is the threshold setting for the background. While not a hard and fast threshold it gives the algorithm an idea of what brightness range the objects of interest lie in. While we have taken account of the differing available brightness range between the images in the bit depth settings, we have not accounted for possible differences in the relative levels of signal and background. This difference could occur as a result of a number of different things including the power of incident light, the sensitivity of the camera and differing non-linear camera responses.

If we look at the pixel value range of each image as a percentage of the total available range we get the values in table 1.

Instrument	% of available intensity range used
IN Cell 3000	95
ImageXpress	66

**Table 1** 12-bit IN Cell data uses a far greater percentage of the available data range than the 16-bit ImageXpress.

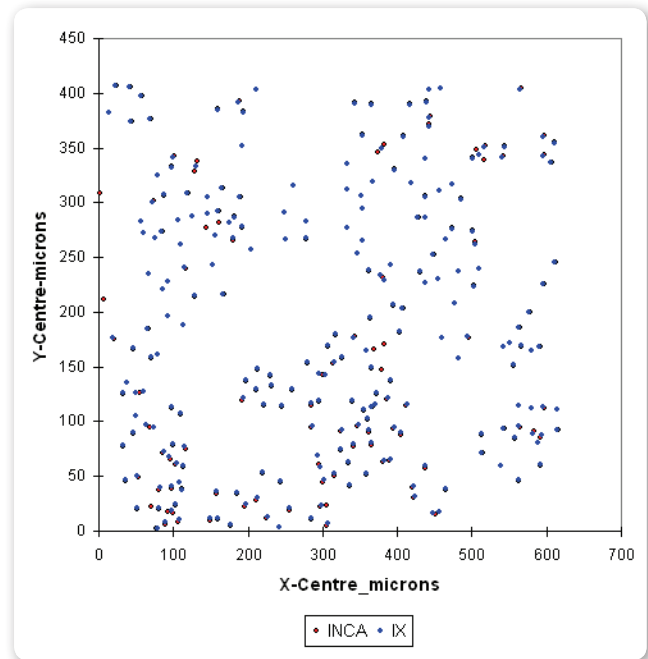
We can see from table 1 that the IN Cell is using a much larger proportion of its available intensity range than is the ImageXpress. It appears however that the relative levels of signal and background are similar. In this case we can stick to the same value for this setting in both versions of the algorithm.

For nuclei detection, only the bit depth and resolution differ between the algorithm used to analyse the images in each case.

## Nuclei Detection Results

When the two versions of the *Nuclei Detection Action* are run on the corresponding overlapping field of view we get a very similar (but not exact) count for the number of detected Nuclei; 241 Nuclei detected in the ImageXpress image and 243 in the IN Cell image. We cannot assume however that there is 100% overlap between all the detected nuclei in each image. In order to determine the rate of exactly overlapping detected nuclei we calculate the coordinates of each object (in microns) from the origin of the overlapping FOV in each image. We then calculate the distance between the centre of mass of each object in the ImageXpress image to the centre of mass of every object in the IN Cell image. If this distance is less than 3 microns (approximately  $\frac{1}{4}$  of a nuclear diameter) and we get a unique match then we can assume with a fairly high degree of confidence that the same object has been detected accurately in both images.

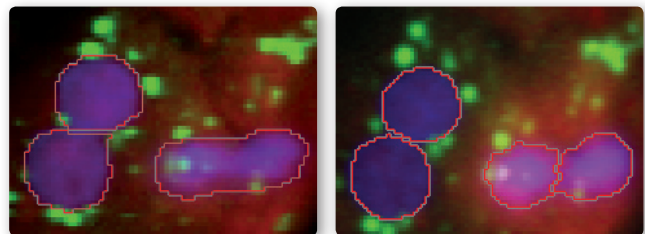
When we apply this post processing to the output of the *Nuclei Detection actions* we get 91% of the objects detected in both images with a high degree of confidence.



**Figure 7** Plot of location of detected nuclei in each image. IX points overlay INCA points completely in the majority of cases.

The next question is why do 9 % of the objects detected in the ImageXpress data not match exactly with objects in the IN Cell data.

On close examination the majority of failed matches occur because overlapping adjacent nuclei have been split in the ImageXpress data and not split in the IN Cell data (figure 8).



**Figure 8** Split nuclei ImageXpress (left) and not in IN Cell (right)

This can be explained by the higher spatial resolution (more pixels representing the boundary between the nuclei) and higher intensity resolution (more grey levels to represent the subtle gradient between the nuclei) in the ImageXpress data as compared to the IN Cell.

Other failures happen because object are found in one image and not in the other; mainly small and dim objects which are detected in the IN Cell image and not in the ImageXpress image. This could be accounted for by the lower S/N provided by the confocal nature of the IN Cell instrument. In a very small number of cases false positives are detected in the IN Cell image. It is possible however that these failure modes could be reduced further by changing the parameters of the *Nuclei Detection Action*.

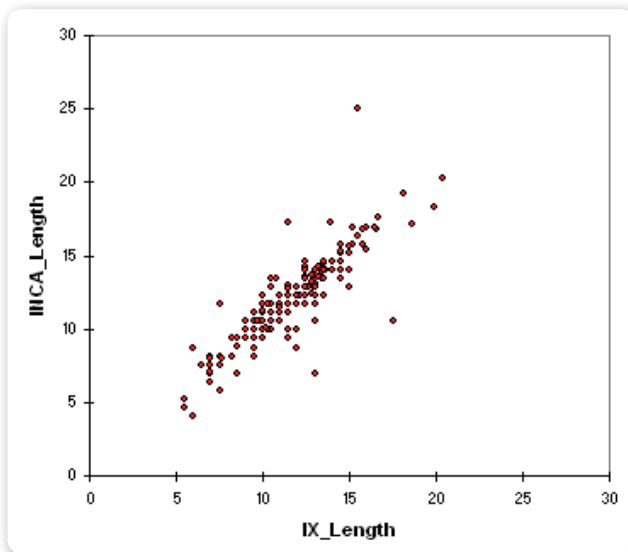
However the co-detection of nuclei is remarkably robust overall to changes in the parameters of one or both versions of the configured algorithm (table 2).

Background IX	Background IN Cell	% distance match
20.5	20.5	91%
17.5	20.5	92%
17.9	17.9	92%
20.5	17.9	90%
20.5	30.5	91%

**Table 2** Comparison of % matches for different parameter values in each version of the Nuclei Detection Action

Now that the non-matching objects can be accounted for and removed we are left with an exact list of matching nuclei detected in both images. It is now possible to do a direct object-by-object comparison of their properties.

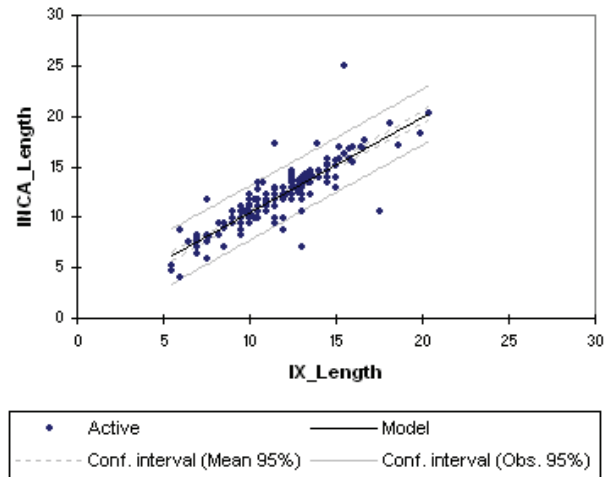
Comparing the length of nuclei in microns as measured by the analysis on both sets of data yields the following results. Plotting the length data from each object on a scatterplot shows the data is highly correlated (figure 9).



**Figure 9** Nuclei length measurements for matching objects. The data is highly correlated (0.89).

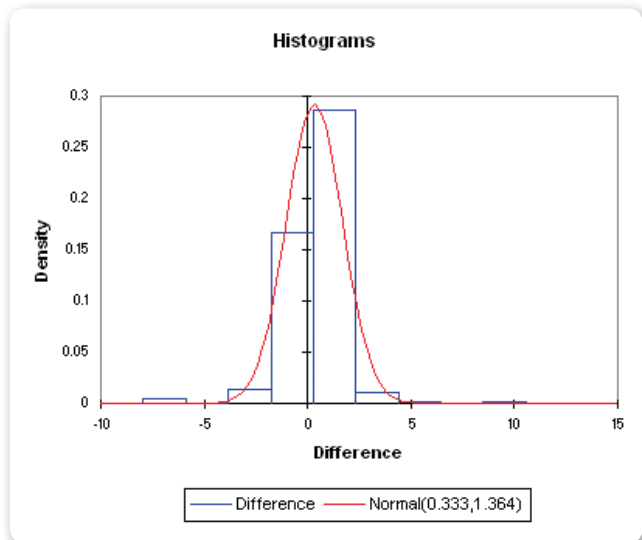
The correlation coefficient (0.89) conforms this. For the data to be comparable however requires that the slope of the correlation to be close to 1 and the intercept to be at the origin. Figure 10 shows the linear regression of this data from which the model is calculated.

**Regression of INCA\_Length by IX\_Length (R<sup>2</sup>=0.763)**



**Figure 10** Linear Regression of IN Cell Nuc. Length by IX Nuc. Length. Model parameters: Intercept=0.925, Slope=0.949.

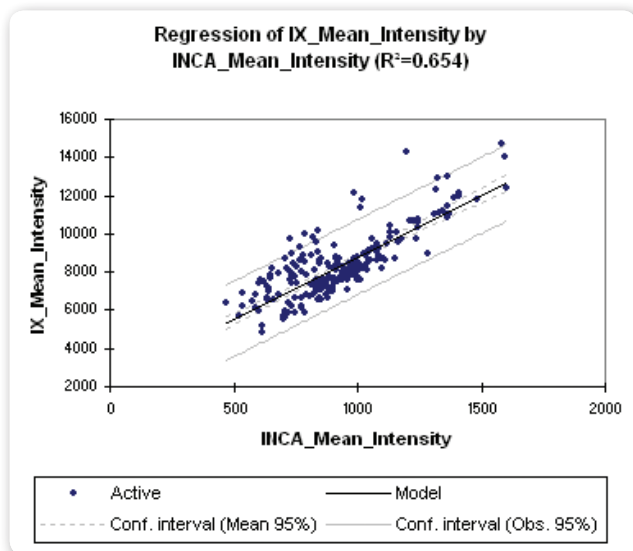
The slope of this model has a value (0.949) very close to 1 which suggests that the length increases at the same rate in both datasets. The intercept however is not close to zero (0.925) which suggests the IN Cell data results in slightly higher values (constant offset) for the length than does the ImageXpress. Investigating this a little further, figure 11 shows the distribution of the difference between the length values (INCA\_Length – IX\_Length).



**Figure 11** Difference between INCA Length and IX Length. Positive mean value indicates INCA longer on average.

If the data were perfectly comparable then the resulting mean and sigma would be zero. An offset from zero would indicate a systematic bias in the length calculation of one dataset relative to the other. The mean value of 0.333 in this case however is less than the limit of resolution of both instruments (0.582 and 0.5 microns). The standard deviation gives us an estimate of the error inherent in the measurement systems.

We can repeat this process with the mean intensity measurement pair calculated from each nucleus object (Figure 12).



**Figure 12** Linear Regression of INCA Mean Nuc. Intensity and IX Mean Nuc. Intensity. Model Parameters: Intercept=2304 Slope=6.471. Correlation=0.73.

This data also shows a high degree of correlation (0.73), but not as close as the morphology parameters. This is to be expected given the large difference in the intensity scales and also because of the stochastic nature of photon emission (shot noise in the detector), detector read noise and other noise in the detector. These differences also manifest themselves in the parameters of the linear regression model, however the measurements are clearly comparable.

## Conclusions

When the major sources of variation between differing image systems are accounted for and the same image analysis system is used to analyse the data then the results obtained can be shown to be highly comparable. This means that assays can be developed on one imaging platform with the corresponding image analysis and transferred directly to a different imaging platform without having to repeat the image analysis part of the experiment.

The degree of comparability between datasets will depend directly on the relative quality of the image data being compared, for example large differences in the spatial or spectral resolution, signal to noise or signal to background will increase the error between the measurements being made. When data from two instruments with similar characteristics are compared with the same image analysis system, estimates for the error inherent in the complete measurement process (imaging plus analysis) can be made.

## Acknowledgements

Image data courtesy of Advanced Science and Technology Labs of AstraZeneca, Charnwood, UK.

## References

1. [http://www.moleculardevices.com/pages/instruments/imagexpress\\_5000a.html](http://www.moleculardevices.com/pages/instruments/imagexpress_5000a.html)
2. [http://www5.amershambiosciences.com/aptrix/upp00919.nsf/Content/drugscr\\_applications~drugscr\\_applic\\_technol~drugscr\\_incell~DrugScr+IN+Cell+Analyzer+3000](http://www5.amershambiosciences.com/aptrix/upp00919.nsf/Content/drugscr_applications~drugscr_applic_technol~drugscr_incell~DrugScr+IN+Cell+Analyzer+3000)

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