Focal Points

Application Note FP-147

UVP, LLC Upland, CA | (800) 452-6788 | (909) 946-3197 | info@uvp.com
Ultra-Violet Products Ltd. Cambridge UK | +44(0)1223-420022 | uvp@uvp.co.uk
Web Site: uvp.com

Radial diffusion assay and analysis for proteases and other hydrolytic enzymes using the ColonyDoc-It® Imaging Station

A fast and convenient way to analyze crude and purified samples of proteases, amylases, and cellulases.

The UVP ColonyDoc-It Imaging Station has a number of features that enable radial diffusion or cup based enzyme and inhibition zone analysis. This includes a range of light sources and lighting modes and specialized software functions for imaging, numbering, quantitating single zones as well as arrays, and exporting to Excel. In addition, the high-resolution camera is capable of detecting very small features with great accuracy.

Radial diffusion based assays are versatile and have many applications. The example described in this note is for analyzing proteolytic enzymes (Gallagher et al. , 1986).

- Briefly, the sample is placed in a small well or on a small filter paper square on an agar or agarose plate. As the sample diffuses into the surrounding gel from the loading zone, any proteolytic enzymes in the sample hydrolyzes the substrate dispersed in the agarose.
- Once the gel is stained for the remaining substrate, the size of the cleared zones indicate the level of enzymatic activity. Purified and crude mixed types of hydrolytic enzymes such as amylase, cellulose, and proteases are readily detected and accurately quantitated in samples ranging from cellular homogenates to feed stocks (e.g., Dingle et al, 1953, Gallagher et al., 1986, Walsh et al., 1995; 2005). This is similar to inhibition zone assays in which a bacterial lawn is grown on the agar and antibiotics are tested for their ability to lyse bacteria by creating cleared zones around the point of sample application (UVP Focal Point-145).
- Evaluating the cleared zones for area, diameter, or perimeter is performed automatically with ColonyDoc-It software, yielding an accurate measure of the amount or concentration of the enzyme. In the case of bacterial zone inhibition, the zone diameter gives an indication of the effectiveness of the antibiotic.

ColonyDoc-It Imaging Station

Materials and Methods

Preparation of the agarose plate and the plant cell extracts containing the proteolytic enzymes is described in Gallagher et al (1985).

- Briefly, a 1% agaorse gel containing 200 ug/ml boiled casein was heated to 90C to melt the agarose, cooled to 70C, and then injected into an electrophoresis gel mold lined with GEL-FIX^{tm} (Serva) plastic sheet to adhere the agarose and giving 125 x 125 x 0.75 mm gel.
- Once solidified, the agarose gel plate was removed and 3mm wells were punched in the gel, using a template placed underneath the agarose plate.
- 2.5 ul aliquots of sample were added to each well and the plate left for 24 hours in a water-saturated chamber.
- The plate was fixed and stained with Coomassie Blue R-250 to visualize the cleared zones where the protein was hydrolyzed by the enzyme.
- The intact casein representing the background is stained blue.

- Once stained, the radial diffusion plate is placed in the ColonyDoc-It Imaging Station and imaged using white light transilluminaton.

Analysis of a radial diffusion plates is straightforward. The ColonyDoc-It Imaging Station and software simplifies image capture and, once a preset template is saved, automatically detects, numbers, and then perform analysis on each zone. Templates and macros are saved and allow standard settings to be used for various assays with the click of a mouse. A typical series of analysis steps are given below.

1. Place zone plate in the ColonyDoc-It Imaging Station and capture image using white light transillumination.
2. Use manual analysis to set up the template and perform the analysis (Figure 1). Select the Zone analysis box.

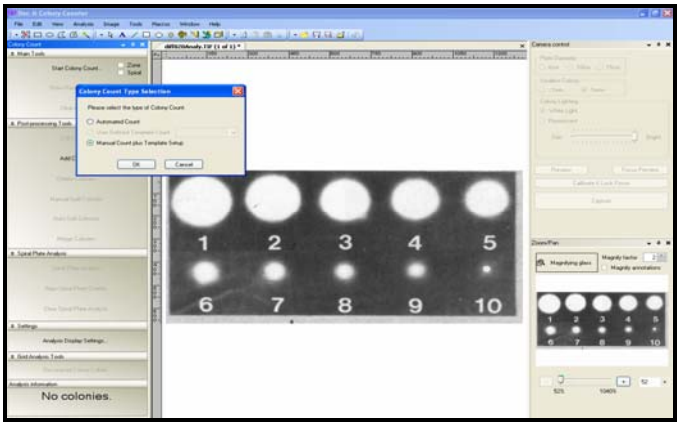

Figure 1

3. Using the area of interest (AOI) tools, apply an AOI enclosing the assay zones on the plate. Depending on the shape of the plate and variety of shapes can be used. In this case, a rectangular AOI is used to analyze the 10 zones. Set the class and points by placing mouse cursor in the cleared zone and performing a left click. This sets the intensity/color level that represents the cleared zone. Save and name the template for future use. Opening the template will automatically populate the memorized settings for that specific template (Figure 2).

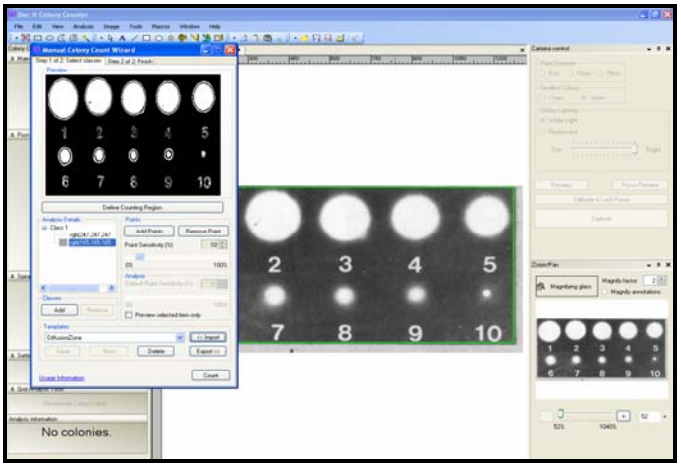

Figure 2

4. Run the analysis by selecting count. Note the zones are automatically numbered sequentially from left to right, then top to bottom. This enables arrays of data to be readily analyzed (Figure3).

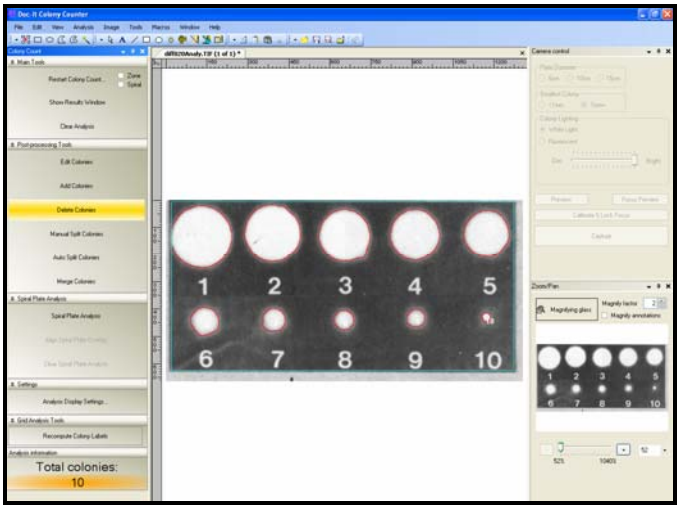

Figure 3

5. (Optional) Adjust the Circularity and Smallest Colony slidebars, until all area of interest appear in the main image window.
6. (Optional) Save the template for repeating with similar plates of the same growth medium and bacteria.
7. (Optional) Delete defects or incorrectly identified areas.
8. (Optional) Click “Recompute Colony Labels” to number the colonies from left to right, top to down.
9. Open data table to inspect the zone by zone data. The parameters include area, perimeter, and average diameter for each zone (figure 4). Note the data units are in pixels. Alternatively, the image can be calibrated in a variety of measurement units such as mm or um using the calibration tools of the software.

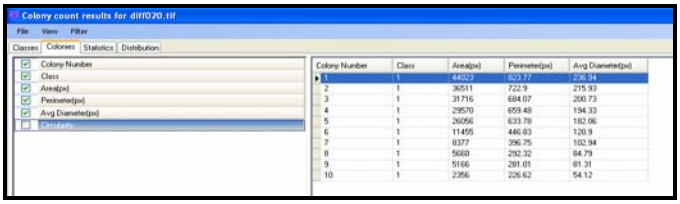

Figure 4

10. Export to Excel if further analysis is needed.

Graph showing the analysis of the endopeptidase trypsin using the radial diffusion assay is shown in figure 5.

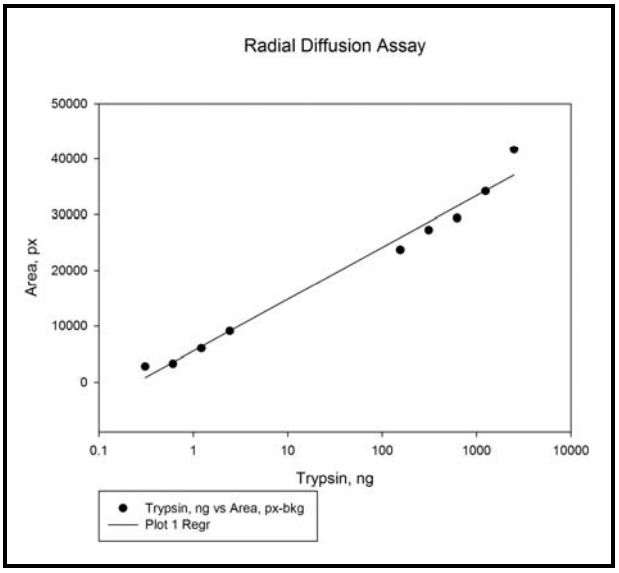

Figure 5

References

1. Gallagher, S.R.; Carroll, E.J.; Leonard, R.T. A Sensitive Diffusion Plate Assay for Screening Inhibitors of Protease Activity in Plant Cell Fractions. *Plant Physiol.* **1986,** *81,* 869-874.

2. J. Dingle; W. W. Reid, ; G. L. Solomons, The enzymic degradation of pectin and other polysaccharides. II - Application of the Cup-plate assay to the estimation of enzymes. *J. Sci. Food Agric.* **1953,** *4,* 149-155.

3. Walsh, G.; Murphy, R.A.; Killeen, G.F.; Power, R.F. Quantification of supplemental enzymes in animal feedingstuffs by radial enzyme diffusion. *Appl. Microbiol. Biotechnol.* **2005,** *67,* 70-74.

4. Walsh, G.A.; Murphy, R.A.; Killeen, G.F.; Headon, D.R.; Power, R.F. Technical note: detection and quantification of supplemental fungal beta-glucanase activity in animal feed. *J. Anim. Sci.* **1995,** *73,* 1074-1076.

5. Walsh, G.A.; Power, R.F.; Headon, D.R. Enzymes in the animal-feed industry. *Trends Biotechnol.* **1993,** *11,* 424-430.