

LAI High-Resolution Automated Colony Counting System – Mouse Lymphoma Assay: Performance Analysis

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Abstract

In the *in vitro* mutagenic assay using the L5178Y/TK^{+/-} mouse lymphoma cells, the relative abundance of small and large size colonies provides a profile that has been related to potential mutagenicity/carcinogenicity. As of 1995, the currently available automated colony counters did not accurately or repeatably measure the small colonies of less than 0.2mm (200 μ m) diameter. This report describes the development and test of an automated digital high resolution colony counter capable of rapid measurements of colony sizes of greater than or equal to 100 μ m.

Background

The agar based Mouse Lymphoma Assay requires the rapid and accurate assessment of small size colonies, down to 0.1mm (100 μ m) diameter. Due to the lack of a high precision colony counter, the assessment of small to large colony ratios was subject to variable error. Measurements were not easily replicated based on definitive cross-correlation with direct microscopic measurements. To improve the performance of the agar-based MLA assay we have developed a new digital high resolution digital instrument. The special requirements were to be able to quantitate cell size diameters down to 0.1mm, to accommodate colony review, and to provide a rapid, accurate and reproducible assay.

In the agar based mouse lymphoma assay, cell colonies are distributed in a 3-dimensional matrix. Since not all colonies exist in the same plane, the acquisition system must have a sufficient depth-of-field to view the entire scene depth. The relative abundance and size of colonies is not fixed in time due to colony growth.

Three classes of TFTr (Trifluorothymidine resistant) variants of L5178/TK^{+/-} mouse lymphoma cell colonies have been identified, l - large, s - small, and t - tiny. l and s colonies are identifiable with soft agar cloning. t mutants are generally below the resolution of current instruments and are slow growing. s and putative t mutants are thought to represent chromosomal mutations. l mutants are thought to represent less extensive mutations affecting the TK locus. The ability to distinguish and count small colonies (s and t mutants) is an important element of the L5178Y/TK^{+/-} mouse lymphoma assay.

Existing colony size distribution measurements incorporate size discriminators that quantitate colony size distribution. Colony size distribution is determined by calculating the difference between adjacent counts of 16 increasing size discriminator settings plotted as a histogram. Since the ARTEK only produces numerical results, it is not apparent whether or not this

instrument accounts for the uneven background related to individual dish variants.

LAI High Resolution Colony Counter for the Mouse Lymphoma Assay

The new high-resolution colony counter HRCC provides a very rapid (<24s/dish) automated count of colonies grown in agar media. Software provided with the HRCC compensates for lighting, gel and dish background patterns. Using this background corrector algorithm there is no need for manual dish-to-dish threshold setting.

The LAI High Resolution Colony Counter (HRCC) is based on a specially adapted high resolution quantitative-grade video camera (DAGE 81) modified for slow scan operation. High resolution digital images (2048x2048 pel, 4 Mbytes) are captured with a specially controlled A/D converter and digitizer. An effective pixel size of less than 40 μ m, based on the imaging of an 80mm dish, provides a 4 pixel foot print for a 100 μ m colony diameter. This footprint meets the Nyquist sampling criteria and allows the capture of images in which 100 μ m diameter colonies (ignoring the volume packing efficiency due to spherical shape 100 μ m corresponds to 125 20 μ m diameter cells) are acquired. The camera is fitted with a precision macro-lens and attached to an adjustable-height light stand. Background illumination is provided by a controlled intensity lightbox.

Specifically designed software provided with the HRCC, controls the capture and display of a single high resolution (2048 x 2048 pixel) image in 4 seconds. The image is automatically cropped to eliminate artifacts at the edge of the dish.

Each image is automatically background corrected to correct for the artifacts induced by the light, the dish and the agar gel. During the initial verification stage it was determined that the lighting background was also being induced by the agar media. To compensate for this, the LAI HRCC incorporates a rapid background corrector which results in a uniform background imaging field. Individual colonies are recognized as intensity departures from this flat corrected background. The discriminability of colonies from background therefore depends only on the digital noise of the instrument. The discrimination threshold can be set without reference to individual dishes. The ability to compensate “on-the-fly” for dish dependent background significantly improves the reliability and repeatability of the MLA system. Colonies are seen by the camera digitizer as distinct connected regions. Each colony is converted from area measurement to an effective diameter by the following equation.

$$d_{\text{eff}} = \frac{2}{\sqrt{\pi}} \sqrt{\text{number of pixels} \times \frac{\text{area}}{\text{pixel}}}$$

A pixel is a unit area measure of intensity. The area/pixel is determined by direct calibration of the image with a variable size colony standard. The software provides an immediate and direct presentation of the colony frequency as a function of cell diameter.

The LAI HRCC program has been developed to minimize operator interaction. Various user-specified protocols can be invoked which prompt the user for the proper dish and which automatically builds an annotated experiment data-base. At the user's option, images can be saved for each dish. These images can also be size-compacted by retaining only the range of intensities spanning the colonies. Individual uncompacted images are 4 Mbytes, compacted images typically require only 100 Kbytes storage.

The program provides for the automated operation of custom protocols. Data is automatically recorded in an Excel© report format which is easily customized.

The colony-frequency distributions are automatically determined on-the-fly and recorded with total colony counts, and with pertinent acquisition parameters.

The special features of the LAI HRCC-MLA demonstrated during this effort are summarized below.

FEATURES OF THE LAI HRCC-MLA

- Rapid Automated Operation
- Compensates for Dish/Agar/Lighting Background
- Colony Sizing > 100µm
- Integrated Database and Report Generation
- Frequency Distribution and % Small Colony Automatically Captured
- Digital Record of Dish Images, as Required

Methods For Proving Colony Size Mensuration

Three separate techniques were proposed for defining system performance 1) comparison with direct microscopic measurement, 2) cross-correlation with the ARTEK 880 and 3) measurement of a colony standard which mimics the colony sizes expected in the MLA.

Sensitivity-Microscopic Comparison

Sensitivity is defined as the efficiency of a system to detect colonies of a given size. Ninety-five percent (95%) efficiency was defined as the lower limit of colony detection.

To quantify the lower limit of colony detection, seven day growth negative control mutant plates were evaluated. The seven day growth time point was chosen to enhance the number of small colonies present. Specific colonies were first located and measured using an inverted microscope with a 3.5X objective. The mutant plate was subsequently analyzed by the LAI HRCC-MLA. Six replicated measurements were performed and the data from each analysis was then compared to the microscopic evaluation data.

Microscopic evaluation consisted of sequential location of each colony and measurement of the colony diameter. A clear plastic lid was used to cover the plate while searching for colonies. A reticle present within the microscope eyepiece was used to measure colony diameter. The unit gradation of the reticle measured 28.57 microns. The plate was searched in a concentric pattern beginning with the outside edge and progressing in a spiral pattern towards the center. After a colony was located, it was centered on the measuring reticle and the diameter was documented. A permanent ink marker was then used to identify the colony's location by marking the clear plastic lid. Marking the lid provided a method that negated double sampling.

Negative control plates were analyzed six times on the LAI HRCC-MLA system. A list of colonies detected and their diameter resulted from each analyses. The results were compared by sorting the colonies detected in each analysis by diameter in descending order. Colonies were then grouped across analyses and the system measurements were compared to the microscopic measures.

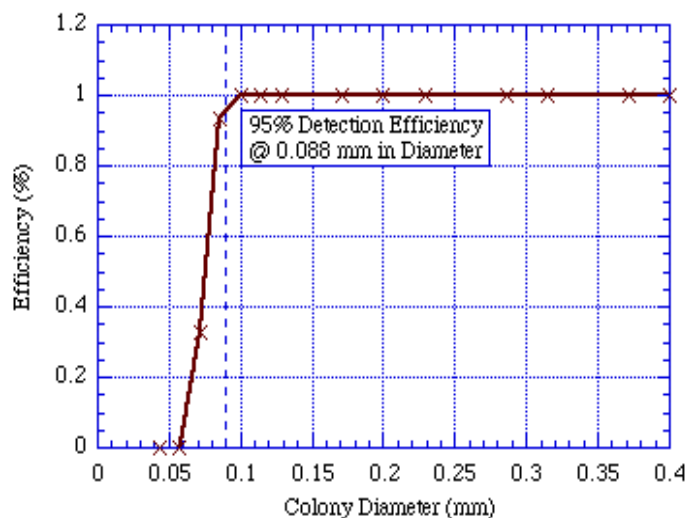


Figure 1 Colony Detection Efficiency

A plot of colony efficiency identifying the 95% detection limit is shown in Figure 1. The colony detection limit of the LAI HRCC-MLA system is determined to be 0.09mm in diameter. Note that colonies with a diameter smaller than 0.09mm did not exhibit the 95% detection limit. A colony with a measured diameter of 0.07 was only found 2 out of 6 trials. Three colonies below 0.06mm were not detected by the LAI HRCC-MLA system.

Increase in Assay Sensitivity: ARTEK System Comparison

A seven day growth (+) control mutant plate was cross-analyzed on the LAI HRCC-MLA system and the ARTEK counter. The mutant plate was analyzed ten times consecutively on both the ARTEK 880 and LAI HRCC-MLA systems. A calibration curve supplied for the ARTEK 880 size settings was used to compare the colony size frequency distributions. The derived colony diameter calibration curve (Figure 2) was used for the ARTEK 880 to translate the size units of the ARTEK to physical units (colony diameter). The mean and standard deviation of the measurements for each colony diameter was

calculated. This data was best fit with an exponential relationship and the curve was used to compare the frequency distribution data across systems.

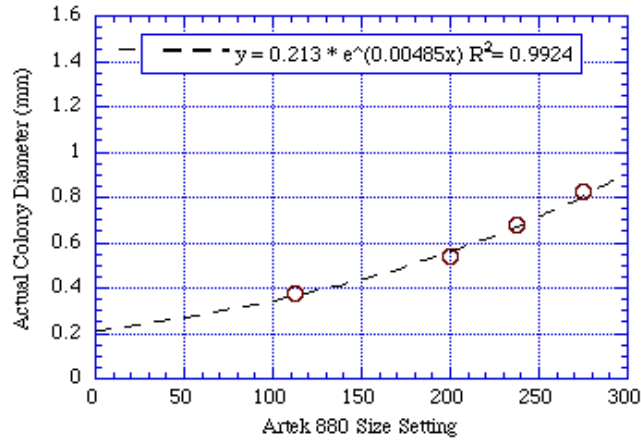


Figure 2 Artek 880 Colony Size Calibration

The LAI MLA program provides a frequency distribution for each analysis. Each mutant plate was analyzed ten times.

The ARTEK 880 creates a colony size distribution by calculating the difference between colony counts of adjacent size settings. Ten samples were taken at each size setting.

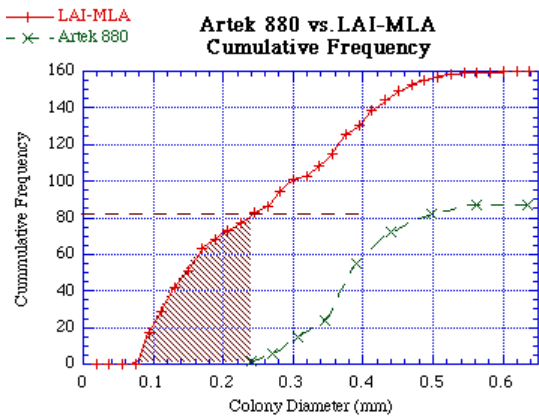


Figure 3

Cumulative frequency histograms were derived from the average of the frequency data, to account for differences in colony diameter bin sizes. The comparison of the cumulative frequency distribution from both systems is shown in Figure 3. The graph demonstrates an increase of approximately 77 additional small colonies detected by the LAI HRCC-MLA system. These colonies were predominantly in the small size classes below 0.24mm.

Table 1 summarizes the results based on the mean data for both instruments.

	Total No. Colonies	No. of Colonies < 0.24m	No. of Colonies > 0.24m
HRCC- MLA	160	77	83
ARTEK	87	0	87

Table 1

Accuracy Reference Transparency Standard

The accuracy of the system was determined by sampling a distributed number of quasi-circular dots of a known diameter and comparing the sampled diameter to its true diameter. The dot pattern was superimposed on a dish size transparency. The transparency comprised of 1041 black dots (colony surrogates) on a clear film that is roughly the dimensions of an 80mm agar plate as illustrated in Figure 4. The dots diameters are distributed as shown in Table 2.

# of Dots	Diameter (mm)
16	0.125
25	0.175
900	0.625
80	0.9275
20	1.5

Table 2

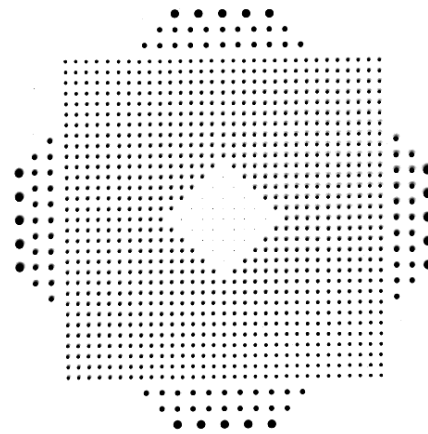


Figure 4

Note that in the center square region there are an additional 16 dots which are not visible in this reproduction.

A graph of the sampled versus the true diameter of the 1,041 dots of the reference standard transparency is shown in Figure 5.

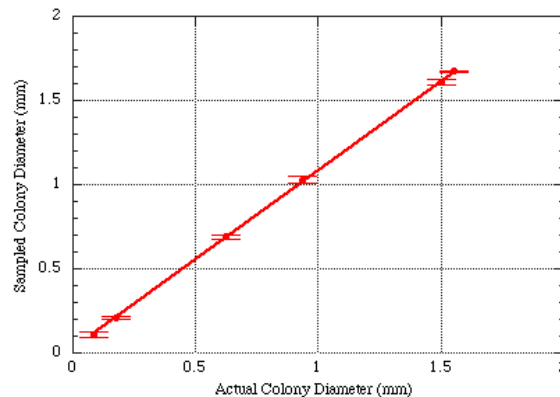


Figure 5 Colony Sampling Accuracy

Another aspect of accuracy is the reliability of a system to detect a colony. To demonstrate the system reliability, the reference standard transparency was analyzed ten times. The true or false

spot detected are presented in Table 3. Notice the true dots were found consistently on each attempt. Also, the majority of the false positives were consistently identified as small dirt particles.

Trial	1	2	3	4	5	6	7	8	9	10
Total	1047	1047	1045	1049	1047	1046	1049	1047	1047	1046
TP	1041	1041	1041	1041	1041	1041	1041	1041	1041	1041
FP	6	6	4	8	4	5	8	6	6	5

Total True Dots 1041

Table 3

Precision

Precision is defined as a system’s ability to repeatably measure the same size of a given colony. The system precision is measured as the sampled area variance of a colony with respect to the size of the colony and the number of times sampled.

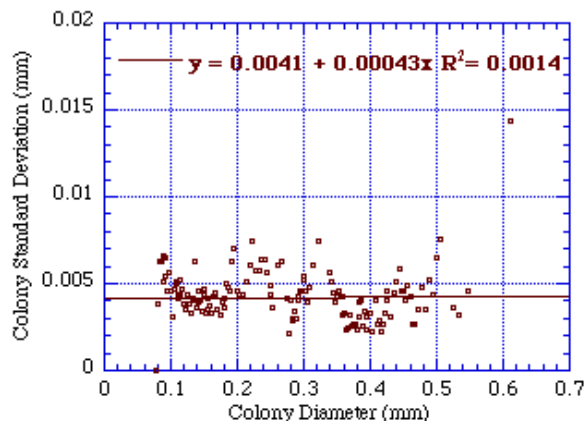


Figure 6 Colony Diameter Sampling Precision

Sampling Variance

Ten repetitions of the measurements from a (+) control mutant plate were made. Each analysis resulted in a diameter value for each colony detected. Colonies were grouped by sorting each analysis from the largest to the smallest colony and then grouping colonies starting with the largest in each analysis and progressively getting smaller. The mean and standard deviation was calculated for each group. The standard deviation is plotted as a function of average diameter in Figure 6.

Conclusions

The LAI HRCC-MLA system is capable of identifying, counting and measuring the diameter of mouse lymphoma cell colonies greater than and equal to 100 microns in diameter. Identification of colonies 100 micron in diameter and greater has been demonstrated by microscopic comparison. An increase in counting efficiency for colonies was demonstrated for the LAI HRCC-MLA at diameters below 0.24mm.

Small Colony Size Measurement:

The resolution provided by the LAI MLA HRCC permits reliable measurement of colonies with deff < 0.1mm. Comparison with concomitantly identified small colonies by microscopy indicates a lower limit cutoff of approximately 90µm .

Cross-comparison experiments with early gel-dish mouse lymphoma colonies showed that the LAI MLA HRCC provides a more sensitive measure of the small colony to large colony ratio since it was able to capture colonies in the range of 0.1 to 0.3mm.

Repeatability/Threshold Sensitivity

The repeatability of the LAI HRCC-MLA was determined by a series of measurements at various lighting and threshold levels using a transparent colony standard.

References

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