

Automated Micronucleus Assay System Micronucleus Scoring Validation Test: Accuracy, Repeatability

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Abstract

This report summarizes the results of analyzing a set of 18 slides using the LAI Automated Micronucleus Assay System. This report details the accuracy, repeatability and variance observed when scoring the same slides multiple times using the automated system. This document also illustrates the new quantitative data that is available by using the automated system versus a manual method of analysis.

LAI Automated Micronucleus Assay System Description

The LAI Automated Micronucleus Assay System features automatic discrimination and scoring of micronucleated PCEs and NCEs in Giemsa stained bone marrow or peripheral blood preparations.



The LAI Automated Micronucleus Assay System is based on color discrimination of PCEs and NCEs. The system accommodates the existence of non-target (non PCE or NCE) cells in the slide preparations and isolates and ignores these non-target cells in analysis. Micronucleated cells found automatically by the system may be automatically relocated under the microscope eyepieces and reviewed by the user. Reports are available *immediately* after scoring of the experiment is complete. This eliminates transcription errors and the time necessary for statistical analysis completion.

Results of pharmaceutical laboratory use of the LAI Micronucleus Assay System show a 75% reduction in man-hours required for scoring of a Micronucleus Assay for a single compound. Manual interaction is only required to load the slides onto a 15-slide capacity stage and for editing

the potentially micronucleated cells after the slides have been automatically scored by the system. This reduction in time and man-power requirements significantly boosts the throughput of compound testing. Throughput increases of 2X without requiring more personnel to prepare animals has been realized.

Based on individual laboratory GLP validation, there is no statistical difference between manual scoring and the Automated Micronucleus Assay System. At the same time, however, the per dose variance is significantly reduced in the automated system. The quantitative repeatability in discriminating erythrocytes by scoring the same experiment/slide multiple times using the automated micronucleus system has also been demonstrated.

Test Setup

Several parameters must be set to identify cells and micronuclei according to the laboratory's individual protocol. These parameters include the number of PCEs to be analyzed, the area of the slide to be scored, and the definition of size, shape and color of cells and micronuclei to be scored. (Note that these parameters are normally set up only once in the laboratory according to the laboratory's protocol and staining methods. This parameter set is then used for all subsequent studies that follow the same protocol.) The setup of these parameters for the analysis of the slides is described below. The system was set up by LAI personnel to score 1000 PCEs per slide.

Area of slides to be scored

A region of scoring was set up to be within the smear, as shown in Figure 1a. Figure 1b shows the scanning pattern used for searching for cells within the region of interest. The system automatically searches for cells in a horizontal scanning pattern starting in the center of the region of scoring. The system scans horizontally right across the slide, then moves above the center line and scans horizontally left across the slide, and then moves below the center line and scans horizontally to the right side of the slide. This process continues until the target number of PCEs are found or until the entire region has been searched. With all of the slides, 1000 PCEs were found within the region established.

Figure 1(a) illustrates the region of scoring established for the Huntington Research Slides. This region is set up prior to scoring all slides and encompasses the area of the smear.

Figure 1(b) illustrates the scanning pattern used by the automated system. The system horizontally scans a slide in a spiral pattern, starting at the center of the region of analysis.

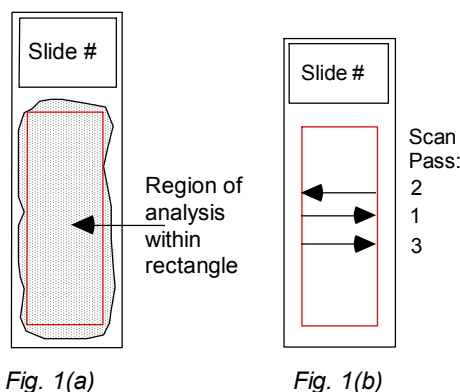


Fig. 1(a)

Fig. 1(b)

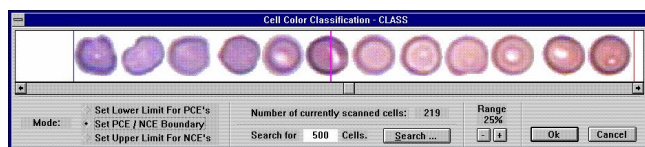
Size, shape, and color of cells to be scored

The system was set to score cells with an area ranging from 6.87 microns² to 41.97 microns². This setting was based on viewing a representative set of cells. (A graphical interface is set up in the system to view various cells and set the minimum and maximum cell sizes based on the reviewed cells.)

A criterion was set within the system that the cells to be scored must conform to a near-circular shape. This criterion is based on a parameter that utilizes the relationship between the area and circumference of a circle to identify round or oval objects.

The color of the cells to be scored was initially set based on a set of 1000 cells that met the size and shape criteria specified above.

The cells found were ranked by color (e.g. blue to pink). Using this ranked display of cells, a discrimination line was set between the cell types (non-target cells, PCEs, NCEs) based on their color. A discrimination line was set between the non-target cells and PCEs so that non-target cells would be eliminated from analysis. A discrimination line was set between PCEs and NCEs so that anything below the discrimination line was considered a PCE and anything above the discrimination line was considered an NCE.



Color Classification Dialog

Typically, this color discrimination setting is defined using a negative control slide. The negative control slides normally produce a bimodal distribution, with one

histogram peak representing PCEs and the second histogram peak representing NCEs. Viewing both the histogram as well as the color ranking of the cells allows the user to more easily set the color discrimination line between PCEs and NCEs, which typically falls in the "valley" between the PCE peak and NCE peak of the histogram. (Because we did not know the group assignments of the blinded slides, we viewed histograms and color rankings from 3 slides to determine the PCE / NCE discriminations.)

Size, shape, and location of micronuclei to be scored

The system was set up to identify micronuclei that met the following criteria: 1) the micronucleus must be in the same plane as the cells, 2) the micronucleus must be round or oval, 3) the micronucleus must be contained within the cell membrane (i.e. do not include extruding micronuclei in the MPCE count), and 4) the micronucleus must be less than 2.38 microns². We allowed the system to search for micronuclei in both PCEs and NCEs.

Scoring & Review of Potential Micronucleated Cells

The slides were scored overnight by the system and LAI personnel reviewed the potential micronucleated cells during normal working hours. Each slide was scored and reviewed at least one time. Results of scoring are discussed in the next section.

As the system automatically scores a slide, it saves a "postage stamp" picture of each potential micronucleated cell. The system will score a cell as micronucleated if it meets the color, size, and shape criterion established. After a slide has been scored, the user must review the potential micronuclei found and verify that the cell is truly micronucleated. Figure 2 shows an example of the postage stamp images of potential micronuclei that were found for Slide 223. The red boxes indicate cells that were identified as true micronucleated cells.

When reviewing and verifying the micronuclei that were automatically detected for the slides, we followed a conservative policy of "if in doubt, leave it out.". If we had any questions as to whether the potential micronucleus found was truly a micronucleus, we removed it from the MPCE count.

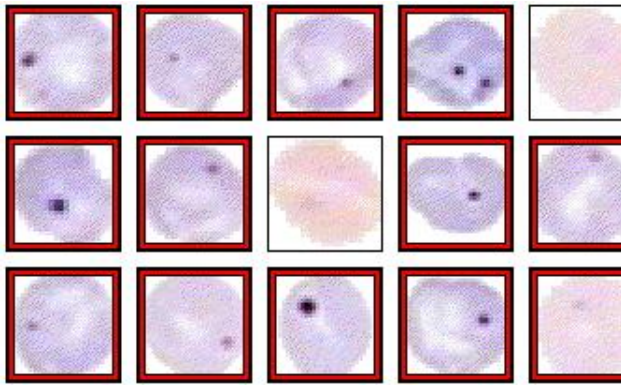


Figure 2 illustrates the postage stamp images saved of potential micronuclei found on slide 223.

Results

Table 1 shows the results that were obtained from using the LAI Automated Micronucleus Assay System to score the slides. Slides were "blinded" when provided to LAI; therefore, LAI had no indication as to the expected results of individual slides.

Slide	% PCEs	MPCEs / 1000
201	50.3	1
202	45.8	1
203	63.1	4
204	47.5	2
205	47.6	3
206	56.3	2
207	57.5	0
208	48.1	1
209	53.8	6
210	50.8	3
221/241	27.2	18
222/242	25.5	13
223/243	58.8	45
224/244	35.8	36
225/245	32.1	22
226/246	51.7	34
228/248	64.1	27
230/250	38.9	35

Table 1: Scoring Results

As described in the previous methodology, the system automatically searched for cells and micronucleated PCEs according to the objective criterion established. When reviewing potential micronucleated cells found by the automated system, we noticed some cells that were questionable as to whether the object detected as a micronucleus was a true micronucleus or an artifact. However, following our conservative "if in doubt, leave it out" policy, we did not count the questionable cells as truly micronucleated.

Minimal Variance of Repeated Scoring Using the Automated System

To demonstrate the quantitative repeatability of the system, we scored a subset of the slides five times. The results shown in Table 1 for the first repetition of scoring each slide are very consistent with the results obtained when scoring the an individual slide multiple times. Because of the quantitative parameters used in scoring the slide (providing objectivity), the variance in results when scoring a slide multiple times is minimized.

We repeated the scoring of individual slides five times for 9 of the slides provided; these 9 slides were selected randomly from the complete set of 18 slides. For each repetition of scoring, the automatic scanning began in the center of the region of analysis. Variance in the repeated scoring results from the fact that although the same general region is scored, the system does not score exactly the same fields of view during each repetition.

Figure 3 shows the positive results of scoring Slide 221/241 five times. Figure 3a shows the distribution of the color of erythrocytes scored by the automated system. The left hand side of the graph (erythrocyte discrimination color value of 0.875) shows the number of the "bluest" PCEs. Cell color moves from blueness to pinkness while moving to the right hand side of the graph. Note that the color discrimination line representing the cell color cutoff between PCEs and NCEs is shown at a erythrocyte color discrimination value of 1.025.

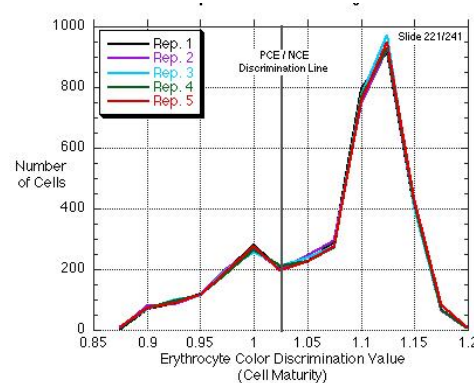


Fig. 3(a)

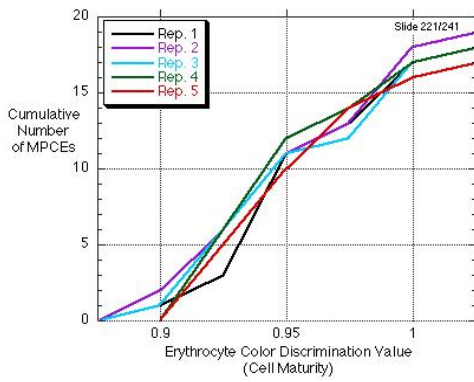


Fig. 3(b)

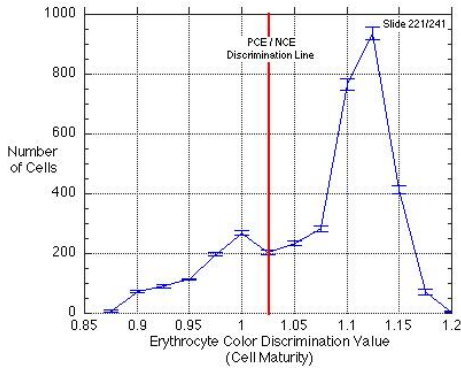


Fig. 3(c)

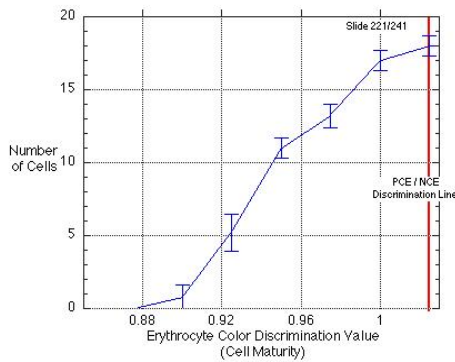


Fig. 3(d)

Figure 3 illustrates five repetitions of the scoring of Slide 221/245. Graphs (a) and (c) show the number of cells found per cell color bin, while (b) and (d) show the number of micronuclei found per PCE color bin. Graphs (a) and (b) show the results of each individual repetition. Graphs (c) and (d) show the average and standard deviation of the scoring results over the five repetitions.

The color distributions of cells found in each scoring repetition overlap tightly. Figure 3c illustrates the average and standard deviation of the number of cells found in each color bin over the five repetitions. The coefficient of variation of number of cells found in each color bin ranges

from 0.2% to 1.3%, which falls well within an acceptable level of error.

Figure 3b shows the number of micronuclei found in each PCE color bin over each repetition of scoring the slide. The erythrocyte color discrimination value axis has been reduced to values between 0.875 and 1.025 to show only micronucleated PCEs. The left hand side of the graph represents the "bluest" cells and the right hand side shows the maximum color value of PCEs, which is 1.025. Any cell with a larger color value would be considered an NCE.

The distribution of the number of micronuclei found in each repetition also match well. Figure 3d illustrates the average number of micronuclei found in each color bin, as well as the standard deviation of the number of micronuclei found in each color bin, over the five repetitions. The coefficient of variation of the number of micronuclei found in each PCE color bin ranges from 0% to 14.3%. (The coefficient of variation is at its highest values for slides where the sample size of micronuclei found is small.)

The results of scoring individual slides five times are also displayed graphically in Figure 4. Figure 4a shows the average and standard deviation of % PCEs value for each slide that was scored five times. Figure 4b shows the corresponding average and standard deviation of the MPCEs / 1000 PCEs for each slide that was scored five times.

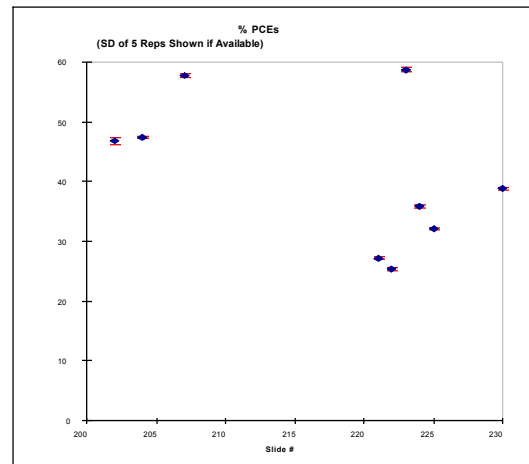


Fig. 4(a)

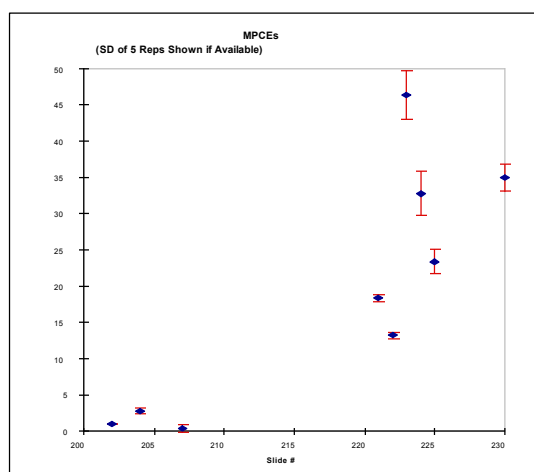


Fig. 4(b)

Figure 4 illustrates the results of scoring individual slides five times. The average and standard deviation of the % PCEs value and MPCEs/1000 PCEs are shown for each slide that was scored multiple times. Note the small variance between repetitions as reflected by the small standard deviation.

Increased Quantitative Data Available When Using the Automated System

In addition to the benefits of increased compound testing throughput and increased objectivity and quantitative repeatability of scoring, the LAI Automated Micronucleus Assay system also provides the ability to view quantitative data that is not available when using manual methods of scoring.

The LAI Automated MN System displays distributions of cells found by color for each slide. This provides information related to cytotoxicity and cell maturity rates beyond that obtained by a single PCE / NCE ratio value.

For example, negative control compounds typically portray a bimodal histogram. Figure 5a illustrates the cell color distribution found on Slide 202, which is bimodal. Notice the large peak on the left, indicating PCEs, and the large peak on the right, indicating NCEs. There is a "valley" between these peaks indicating the presence of transitional cells. Figure 5b illustrates the cell color distribution for Slide 221/241, which is not a bimodal distribution. The "valley" between PCEs and NCEs has been filled in.

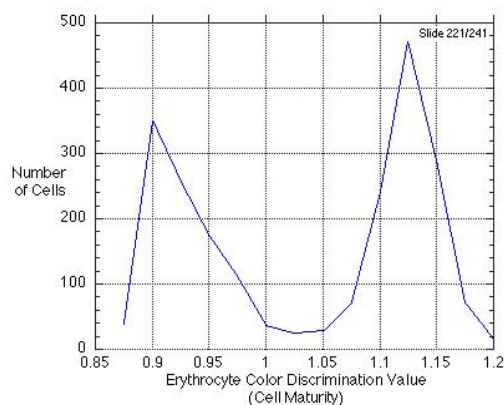


Fig. 5(a)

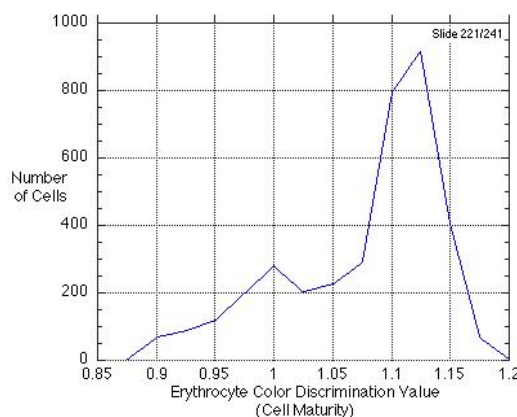


Fig. (b)

Figure 5 illustrates the color distribution of PCEs and NCEs scored on two slides. (a) Slide 202 shows a bimodal distribution, which is typical of negative control compounds; (b) Slide 221 shows a non-bimodal distribution. There are more transitional cells seen on this slide.