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Evaluation of the efficacy of the in *vitro micronucleus method* using a commercial kit and comparison with the microscopy method

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ABSTRACT
In vitro *micronucleus testing* is an important tool in the investigation of the genotoxic potential of products. Performing the test by the optical microscopy method requires training and attention from the technician reading the slides and can be an extremely slow process. The technique of micronucleus analysis by flow cytometry emerges as a highly complex assay that makes it possible to increase the number of analyses and reduce the time spent on experiments. Some commercial kits are sold to perform this test, however, in some cases, they can be very costly for the laboratory. This work demonstrated that the technique of micronuclei in vitro by microscopy and cytometry are equivalent and that it is also possible to optimize the use of commercial reagents to reduce costs.

Keywords: Micronucleus, In vitro test, Cytometry.

1 INTRODUCTION

Product safety analysis is essential for the overall evaluation of medicines, cosmetics, food additives, insecticides and other consumer agents to ensure that they will not harm human health or that of those who have contact with them. An important test routinely performed and recommended by the OECD to analyze the safety of products is the investigation of their genotoxic potential (Türkez et al., 2017).

Products considered genotoxic are those that, when in contact with a cell, interact with genetic material, producing changes in its structure or function. When these alterations occur and are transmitted to daughter cells, they are called mutations. Mutations are hereditary and these changes in the structural content of DNA, whether in a somatic cell or a germ cell, can cause a variety of diseases to the affected individual, including cancer (Buick et al., 2020; Türkez et al., 2017).

One of the tests routinely used to evaluate genotoxic potential is the in *vitro* micronucleus assay (MNvit), which detects the presence of micronuclei (MN) in the cytoplasm of interphase cells. Micronuclei can originate from fragmented acentric chromosomes, or entire chromosomes that have been unable to migrate to the poles during one of the phases of cell division, anaphase. MNvit is an *in vitro* method that provides a comprehensive basis for investigating the potential for chromosomal

damage. It is able to detect both clastogenic (which break chromosomes) and aneugenic (which induce aneuploidy or abnormal chromosomal segregation) effects in cells that have undergone cell division during or after exposure to the test chemical (Buick et al., 2020).

The presence of micronuclei in human cells is an indicator of damage to genetic material and may be related to the development of reproductive problems and diseases such as cancer. Bhatia & Kumar, 2013 demonstrated that the presence of micronuclei in cells from people exposed to mutagens is associated with an increased risk of developing lung, head and neck, and bladder cancers. In addition, it reported that the presence of micronuclei can affect the reproductive capacity and overall health of individuals. Fenech, 2011

In mammalian cells it is particularly relevant to evaluate the potential of chemical and physical agents to affect human health. Studies show that these *in vitro* tests are useful for identifying genotoxic and carcinogenic agents or proving the biocompatibility of materials. One of the studies already carried out analyzed the genotoxicity of dental implant extracts of (Bryce et al., 2008; Witt et al., 2008) *Porous NiTi* (pNiTi) produced by *Metal Injection Molding* (MIM). Using the *in vitro* micronucleus test, the researchers demonstrated cellular compatibility of the product with the cell lines L-929 (fibroblast) and V79-4 Another study analyzed (N W N A et al., 2023). *black cohosh* extract (ECB), a dietary supplement widely used to relieve symptoms of gynecological diseases in women, which has not yet had its toxicity well characterized. Tests have shown that ECB induces chromosomal damage, causing micronuclei in blood cells when grown at normal levels of folic acid, raising concerns about its safety (Smith- Roe et al., 2018).

In vitro *micronucleus testing* (MNVit) can be performed by both conventional microscopy and flow cytometry. The conventional microscopy technique is the most widely used for the detection of micronuclei, but it requires technical skills and careful analysis of the results. Flow cytometry, on the other hand, is an automated and objective technique that allows the detection and analysis of a large number of cells in a short period of time (Lenzi et al., 2017, 2018; Witt et al., 2008; Zhou et al., 2013).

In this context, the main objective of this study was to compare the results obtained between the techniques of analysis of MNVit by microscopy and by flow cytometry. By providing a comparative analysis of techniques, this study provides valuable information for informed decision-making on the choice of the most appropriate methodologies to evaluate the genotoxic potential of products.

2 MATERIALS AND METHODS

2.1 V79 CELL CULTURE ESTABLISHMENT

Aliquots containing 3x105 V79 cells were cultured with DMEM medium (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum in 75cm2 culture bottles. At the moment when the cells reached 80% confluency, they were trypsinized and plated in 6-well plates (1.5x10; ^{5} cells per well) for the microscopy assay and in 96-well plates (1x104 cells per well) for the flow cytometry assay. The plates were then incubated for 24h ± 0.5h in a CO2 greenhouse with an atmosphere of 5% ± 0.5% CO2, humidity of 90% ± 10% and temperature of 37oC ± 1oC.

2.2 CELL TREATMENT

The cell treatment protocol was the same for both forms of evaluation (by microscopy and cytometry), differing only in the amount of reagents, which was calculated proportionally to the area of the wells.

After 24 hours of incubation, the culture medium was replaced by the treatments, namely positive control, negative control and vehicle control. The positive control consisted of a 0.75µg/mL colchicine solution in DMEM medium + 10% Fetal Bovine Serum (FBS), the negative control only half DMEM + 10% FBS, and the vehicle control by 0.5% DMSO diluted in DMEM medium + 10% SFB. In each well of the plate, the respective treatments were added in triplicates, being 100uL for the 96-well plates and 2mL for the 6-well plates.

After the addition of the treatments, the plates were incubated for 3 h in a CO2 greenhouse with an atmosphere of 5% ± 0.5% CO2, humidity of 90% ± 10% and temperature of 37oC ± 1oC.

3 MICROSCOPY ANALYSIS PROTOCOL

3.1 EXPOSURE OF CELLS TO CYTOCHALASIN B

After the time of exposure to the treatments, the plates were removed from the greenhouse and analyzed under an inverted microscope to evaluate the appearance of the cultures for the presence of precipitation, morphology and cell death.

After the evaluation, they were treated with cytochalasin B (Sigma, code C6762), a mycotoxin that acts by blocking the formation of the contractile ring of actin and myosin, necessary for the division of the cytoplasm and the formation of daughter cells, causing the cells to stop dividing in the cytokinesis phase, remaining with two or more nuclei in the same cytoplasm Click or tap here to enter the text. (Wessells et al., 1971).

For this, the culture medium was removed and all wells with cells were washed with phosphate buffer (PBS) for 2 times. Then, the DMEM culture medium containing cytochalasin B at the final

concentration of 3 µg/mL prewarmed to a temperature of 37 °C was added. The plate was homogenized gently with circular motions to avoid air bubbles.

The cultures were incubated at 37 °C and 5 % CO2 for 21 hours, which corresponds to approximately 1.5 times the cell cycle time of the V79 cells, and then submitted to fixation treatment.

3.2 CELL FIXATION

The culture medium from each well was aspirated and transferred to 15mL tubes. Then, the cells were trypsinized and added to the corresponding tube. The cultures were then centrifuged for 5 minutes at 1000 rpm. The supernatant was discarded and the cells were resuspended in 3 mL of sterile 0.075 M potassium chloride (KCl) previously cooled. After 3 minutes in KCl, 500 µL of the cell fixative (methanol + acetic acid, in a 3:1 ratio) were added. Subsequently, the cultures were centrifuged again for 5 minutes at 800 rpm. The supernatant was carefully removed and the pellet resuspended in 5 mL of fixative. They were centrifuged once again and resuspended again in 5 mL of fixative.

3.3 ASSEMBLING THE BLADES

The fixed cells were homogenized and 3 drops were added to the central areas of the previously identified slides. After this process, they were placed at an angle in an exhaust hood for 12 hours at room temperature for complete drying. After this period they were stained with panopticon dye.

3.4 SLIDE READING

The slides were analyzed under a Nikon optical microscope with a 10x eyepiece and a 40x objective. Whenever necessary, a 100x objective (with immersion oil) was used to confirm the presence of micronuclei. The multiplication rate was performed by reading the slides to evaluate the Replication Index. We counted 500 cells per slide and recorded the number of mononucleated, binucleated and multinucleated cells. Approximately 500 binucleate cells were analyzed for the presence of micronuclei in each slide.

3.5 STATISTICAL ANALYSIS

The analyses were performed using the GraphPad Prism software.

Initially, the percentages of micronuclei found in each group were tabulated and analyzed by T-test, and the analysis was unpaired and with Gaussian distribution. Subsequently, all groups were analyzed by One-way ANOVA, without matching and with multiple comparisons of the means of each group.

3.6 MICROFLOW KIT PROTOCOL – CYTOMETRY ANALYSIS

After 3 h of incubation, the treatments were removed and each well was added DMEM medium with 10% SFB, 100µL in 96-well plates and 2mL in 6-well plates. The plates were then incubated for 21 h in the greenhouse of CO2 until they are removed from the greenhouse and submitted to the Microflow kit protocol (Commercial kit *In Vitro MicroFlow*® (BD, Cod 562354). For the 96-well plates, the reagents were reduced to 1/5 of the manufacturer's recommendation, which indicates the use of 12-well plates.

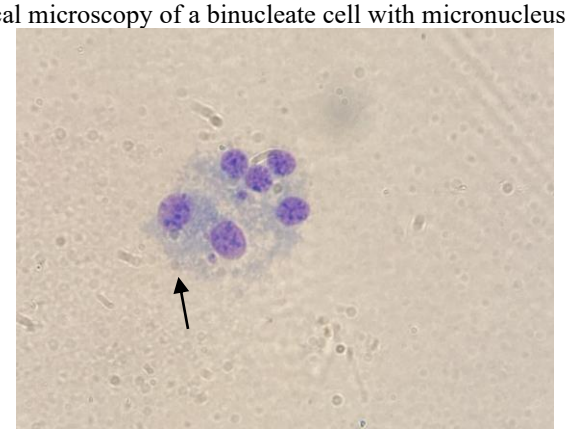
According to the specifications of the manufacturer of the kit used the reagent *Nucleic Acid Dye A* (ethidium monoazide or EMA), crosses the compromised exterior of the membrane of apoptotic and necrotic cells. After that, the cells are washed and the cytoplasmic membranes are digested with detergent to release nuclei and micronuclei. During the lysis step, the reagent *Nucleic Acid Dye B* (SYTOX Green) is introduced and marks all chromatin. In this way, it is possible to achieve differential staining of healthy chromatin versus that of dead or apoptotic cells.

After the completion of the protocol steps, the samples were acquired using the Beckson Dickson (BD) flow cytometer model FACSVia.

4 RESULTS

In order to prove the efficacy of the in vitro *micronucleus technique* performed in cytometry, this study compared the results of flow cytometry assays with light microscopy analyses (Figure 1). For this, treatments were performed with positive controls (colchicine), vehicle control (DMSO) and negative control (culture medium only). The treatments were performed in the same way in the experiments for analysis by microscopy and cytometry, differing only in the amount of reagents, which was proportional to the size of the culture plates and the number of cells used per well.

Figure 1: Image obtained by optical microscopy of a binucleate cell with micronucleus formation indicated by the arrow.



Fonte: Carla Lujan

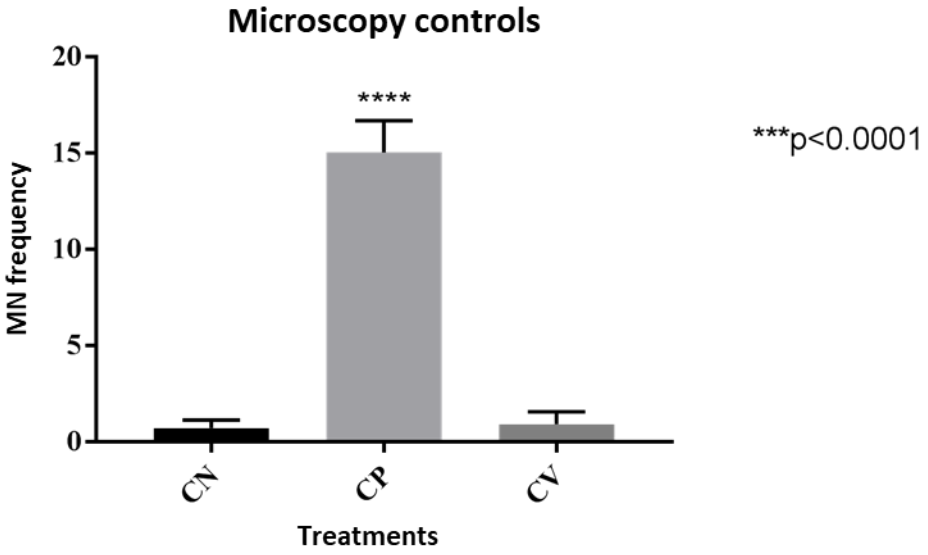
Data from 3 different experiments were obtained for the analysis of each type of micronucleus evaluation (microscopy and cytometry) and the percentage of micronuclei found in the negative, positive and vehicle control samples evaluated by optical microscopy are shown in Table 1.

Table 1: Percentage of micronuclei by control groups in each microscopy experiment.

	Negative Control	**Vehicle Control**	**Positive Control**
Experiment 1	0,36%	0,33%	16,60%
Experiment 2	0,6%	0,8%	15,2%
Experiment 3	1,16%	1,6%	13,3%

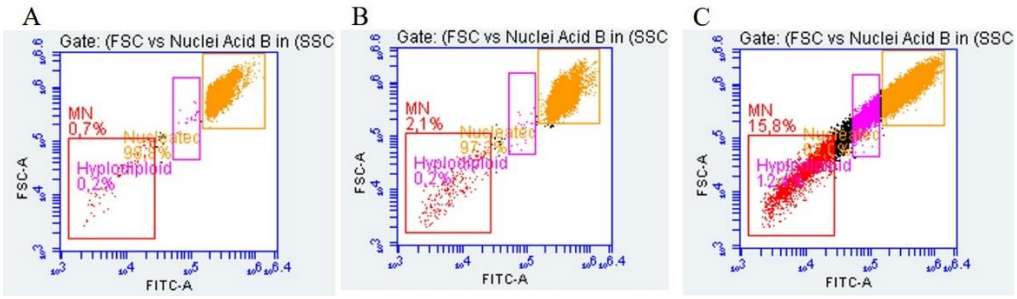
When analyzing the data from the vehicle controls and negative control, the differences in the means were not statistically significant. The opposite is observed when comparing the first two with the Positive Control, which presented statistically significant differences, with a p-value lower than 0.0001 (Figure 2).

Figure 2: Statistical analysis of the micronucleus frequency of the Negative Control (NC), Positive Control (CP) and Vehicle Control (NC) groups in the NM experiments by microscopy. The mean of the positive control showed a significant difference (P<0.001) in relation to the other groups.



For the micronucleus experiments *In fashion* In vitro cytometry, data from 3 different experiments obtained from the acquisition of samples treated with the MicroFlow kit were also used in the cytometer (BD FACSVia). EMA-staining and SYTOX-positive cells are analyzed for micronucleus quantification. The gates were designed according to the recommendations of the kit, and the micronuclei consist of the group of events that present fluorescence in the SYTOX channel with values between 1 and 10% of those found in the gate of the nucleated cells. The graphs obtained in the analyses are shown in Figure 3.

Figure 3: Graphs obtained in the BD Research software. By analyzing the SYTOX signal (FITC-A) by size (FSC-A) it is possible to quantify the Micronuclei (MN – in red), the hypodiploid cells (in purple) and the nucleated cells (in orange). Chart A shows the negative control samples; B the vehicle control and C the positive control.



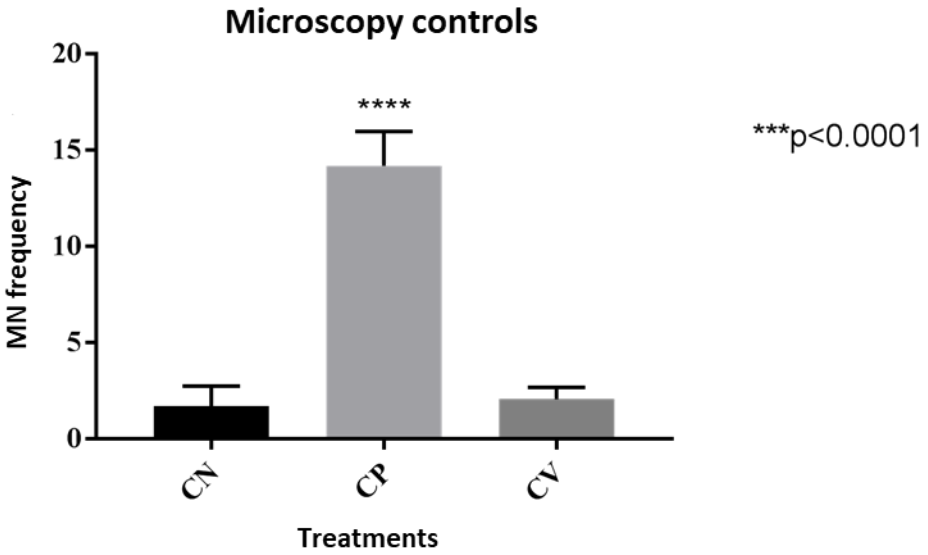
The percentage of micronuclei found in the negative, positive and vehicle control samples are shown in Table 2.

Table 2: Percentage of Micronuclei by Control Groups in Each Cytometry Experiment

	Negative Control	**Vehicle Control**	**Positive Control**
Experiment 1	1,34%	2,51%	16,22%
Experiment 2	0,89%	1,33%	12,87%
Experiment 3	2,87%	2,31%	13,49%

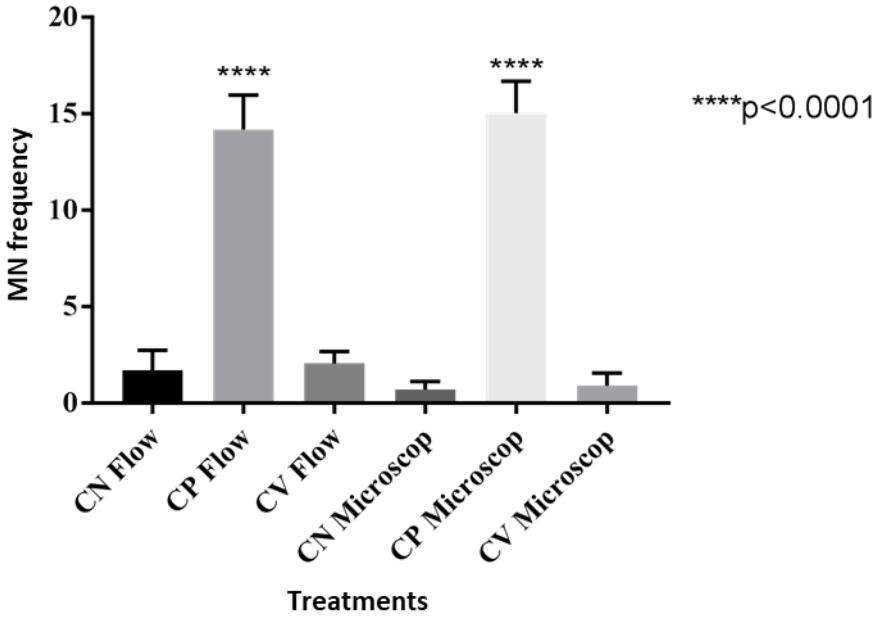
In the cytometry tests, as well as in the microscopy experiments, the statistical analyses of the data from the vehicle and negative controls showed no statistical difference. The opposite is observed when comparing the first two with the Positive Control (Figure 4).

Figure 4: Statistical analyses of the micronucleus frequency of the Negative Control (NC), Positive Control (CP) and Vehicle Control (NC) groups in the NM experiments by cytometry. The mean of the positive control showed a significant difference (P<0.001) in relation to the other groups.



When we performed the comparative statistical analyses of the cytometry and microscopy approaches, we found a consistency in the observed patterns. The analysis of the negative controls in both techniques did not show significant differences, as well as in relation to the vehicle controls. However, significant differences were observed when comparing the positive controls with the negative controls and the vehicle controls. It is worth noting that no significant differences were identified between the positive controls obtained through microscopy and cytometry analyses. Even with the different approaches used for the analysis of the micronuclei, the results indicate no statistically significant difference between the negative controls and the vehicle controls ($p>0.001$). Relevant differences appeared exclusively between the positive controls and the other groups ($p<0.001$), and such differences were not evidenced between the positive controls of the different groups ($p>0.001$).

Figure 5: Statistical analyses of the micronucleus frequency of the Negative Control (NC), Positive Control (CP) and Vehicle Control (NC) groups in the NM experiments by cytometry (Flow) and microscopy (Microscop). The means of the positive controls showed a significant difference ($P<0.001$) in relation to the other groups.



4.1 MICROFLOW® IN VITRO *COMMERCIAL KIT OPTIMIZATION* (BD, COD 562354)

The amount of reagents used for the treatment of the cells that were analyzed by cytometry was reduced by 1/5 compared to the value recommended by the manufacturer for 12-well plates. This ratio was chosen to maintain a minimum volume of reagents per well and to maximize the number of analyses with the same kit. The results showed that this reduction did not interfere with the labeling and identification of micronuclei.

5 DISCUSSION

One of the main advantages of micronucleus testing *in vitro* It is the possibility of performing accurate and detailed evaluations using cells grown in the laboratory. This allows for standardization of the test, minimizing variability between assays. In addition, the use of *in vitro* It allows the selection of different cell types, representing specific tissues and organs, which increases the correspondence with the human organism Added to this is the use of (Lenzi et al., 2017, 2018). *in vitro* It reduces the number of experimental animals by aligning with ethical principles and regulatory guidelines aimed at reducing the use of animals in scientific research. (Jennings, 2015)

The main objective of this study was to provide data to support the use of the micronucleus detection technique *in vitro* by cytometry in V79 cells of known genotoxic and non-genotoxic agents.

Our study demonstrated the equivalence of the MNVit method when analyzed by microscopy and cytometry. Even reducing the number of reagents and cells indicated by the kit manufacturer, we can prove that by performing the test in cytometry it is possible to analyze a larger number of cells (10 thousand events in cytometry versus 500 cells in microscopy), in a few minutes, while the complete test performed by a technician in microscopy can take weeks. depending on the skill of the reader.

Several studies corroborate our data by demonstrating that the efficacy of the in vitro micronucleus test is similar when performed by microscopy or cytometry. Zhou et al., 2013, demonstrated that primary murine bone marrow and blood cells exposed to genotoxic and non-genotoxic chemicals were able to generate a good match between the two methods of analysis. The authors also emphasized that cytometry is a faster and more objective method than manual microscopy. This conclusion has been reached in other studies using different mammalian cells associated with different compounds: human lymphocytes treated with compounds known to be clastogenic and aneugenic; in hamster epithelial cells (CHO-K1) treated with 21 chemical compounds; in peripheral blood of mice. (Lenzi et al., 2018) (Shi et al., 2010) (Witt et al., 2008)

Recent studies have created and validated in some laboratory's software capable of automating analyses. This program can quantify the micronuclei of cells previously labeled with antibodies and specific probes submitted to analysis in a specific flow cytometer, capable of recording images of the cells. Such analyses are based on photographs, just like in the microscopy method, but with the automation of the process, the need for a trained operator in the analyses is eliminated and the difference in readings between operators is zeroed. Another study compared the MicroFlow technique with another semi-automated evaluation technique, Metafer, to evaluate the formation of micronuclei (MN) in human lymphoblastoid cells exposed to different chemical compounds. MicroFlow has been shown to be comparable to the Metafer approach in terms of sensitivity, but with some non-significant differences in micronucleus quantification. (Rodrigues et al., 2021; Wills et al., 2021) (Verma et al., 2017)

The development of automated cytometry-based methods to calculate micronucleus frequencies in cells is a major technological advance when compared to manual slide-based methods. Unlike microscopy, the cytometry method sorts cells in suspension. Individually, the cells flow through a focused laser beam, and the signals of scattering and fluorescence of the light are detected by the cytometer. This automation provides very large numbers of micronucleated cells, improving the power of the assay. (Lenzi et al., 2018; Ueoka et al., 2023; Zhou et al., 2013)

Another important aspect is the speed and efficiency of micronucleus testing *in vitro*. Compared to tests *in vivo*, the essay *in vitro* It requires significantly less time to deliver results, increases production capacity, and can save resources by eliminating labor time spent on manual reading, making it a valuable tool in screening studies (Lenzi et al., 2018; Ueoka et al., 2023; Zhou et al., 2013)

6 FINAL THOUGHTS

Performing the micronucleus test *in vitro* The flow cytometry technique demonstrated equivalence of the data found in the microscopy. Allied to this technique, the use of the Commercial Kit *In Vitro MicroFlow*® (BD, Cod 562354) with the reduction of the amount of reagents proved to be effective, thus allowing the analysis of more samples in less time and with cost optimization for the laboratory.

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