Litron Laboratories

In Vivo Micronucleus

Measure DNA Damage in Red Blood Cells

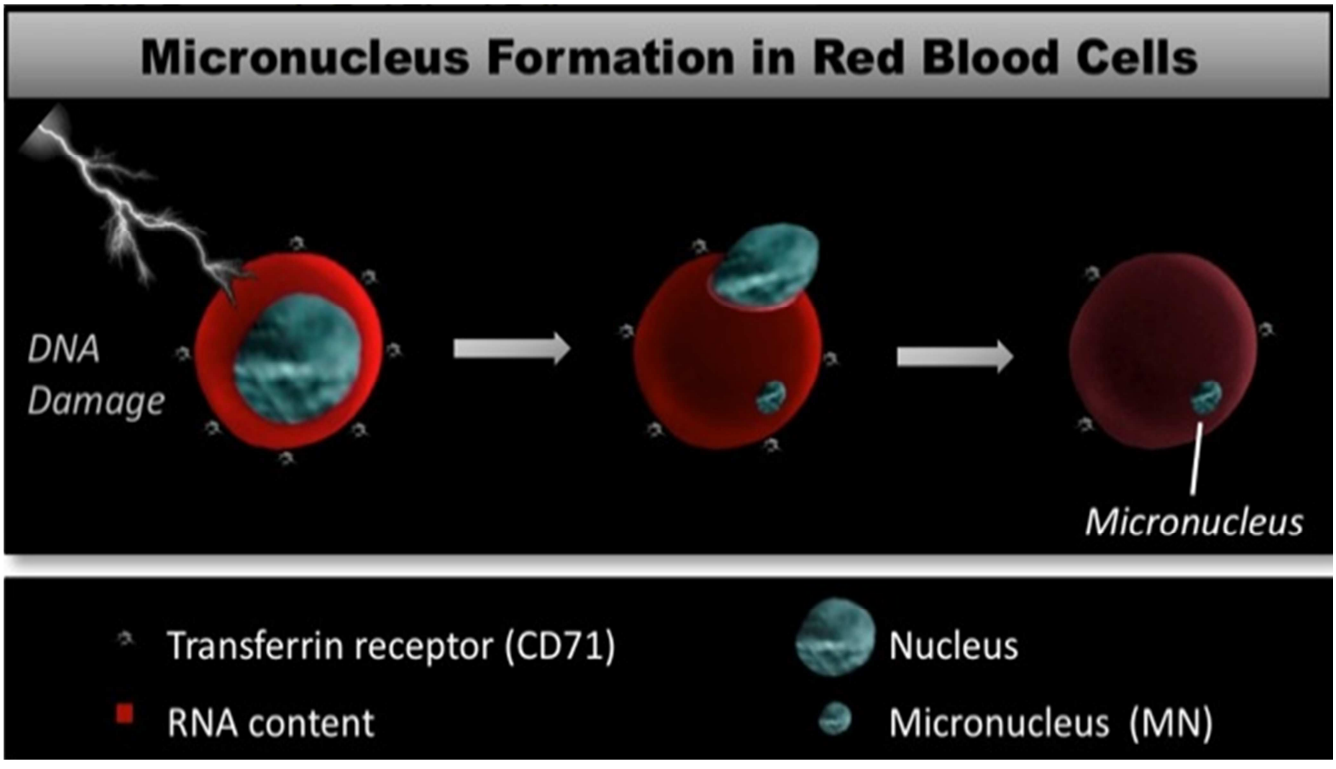
This test detects damage to the chromosomes, or spindle apparatus, of cells. After exposure to a test substance, precursor cells divide. It is upon division that this type of damage can result in the formation of a smaller 'micronucleus', apart from the main nucleus.

At this point, red blood cells expel their nuclei. When stained, micronuclei (i.e., pieces of DNA) are easily seen in a population of cells with no nuclear DNA.

MicroFlow® Kits

Litron's MicroFlow kits use flow cytometry to analyze micronuclei *in vivo*.

Anti-CD71 antibodies distinguish mature and immature RBCs. Micronuclei are easily identified with a DNA stain. Biological standards supplied with the kits allow reproducible results: day-to-day and lab-to-lab



Benefits

- Conforms to New ICH Guidelines (20,000 RETs scored per sample)
- Provides analysis of entire study in one day
- Supplies Mode of Action information
  (aneugens versus clastogens)
- Uses biological standards to get reproducible results, across days and between labs

Unlimited Technical Support

Get answers to your questions, help with validation studies, or send example plots for troubleshooting. We're always here to assist you.

Bone Marrow or Blood

- Mouse
- Rat
- Other species available
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Advantages of Peripheral Blood

- Easily integrates into existing general toxicology studies
- Integration reduces the number of animals in your studies
- Allows each subject to be sampled multiple times
- Requires very low volumes of blood

Specifications

In Vivo MicroFlow Kits

Analyze Samples Yourself (PLUS Kits)	**Send Samples to Litron for Analysis** (BASIC Kits)

Available in Two Sizes

- 60 Samples (Standard Kit)
- 15 Samples (Trial Kit)

Available in Two Formats

- Blood
- Bone Marrow

Shipping Conditions of the Kit

- Ambient
- Ice packs (Biological Standards)

Storage Conditions of Kit Components

- Freezer (-80 °C)
- Freezer (-20 °C)
- Refrigerator (4 °C)
- Ambient

What's Included in the Kit

- **Anticoagulant/Diluent** (blood only)
  Dilutes blood and prevents clotting
- **Strainer Cap Tubes** (bone marrow only)
  Prevents flow cytometer clogs
- **Long-Term Storage Solution (LTSS)**
  Stabilizes fixed samples
- **Buffer Solution**
  To wash fixed cells for transfer into LTSS and for staining; used to prepare solutions

Available in Two Sizes

- 60 Samples (Standard Kit)
- 15 Samples (Trial Kit)

Available in Three Formats

- Fixed Blood
- Fixed Bone Marrow
- Whole Blood

Shipping Conditions of the Kit

- Ambient

Storage Conditions of Kit Components

- Freezer (-20 °C)
- Refrigerator (4 °C)
- Ambient

What's Included in the Kit
(Fixed Blood or Bone Marrow Kits)

- **Anticoagulant/Diluent** (blood only)
  Dilutes blood and prevents clotting
- **Long-Term Storage Solution (LTSS)**
  Stabilizes fixed samples
- **Buffer Solution**
  To wash fixed cells for transfer into LTSS
- **Cryovials and Storage Box**
  For storing and shipping samples
- **Vacuum-Insulated Shipping Container**

- **RNase Solution**
  Removes RNA
- **Anti-CD71 Antibody**
  Labels young red blood cells
- **Anti-Platelet Antibody**
  Labels platelets
- **DNA Stain**
  Stains DNA (e.g., micronuclei)
- **Biological Standards**
  To calibrate flow cytometer

Additional Materials Required

- Flow cytometer capable of 488 nm excitation
- Fixative (methanol)
- Freezer (-80 °C), freezer (-20 °C), and refrigerator (4 °C)
- Centrifuge and other general lab supplies
- FBS, cellulose, and columns for bone marrow

Archive Kits

For more flexibility, MicroFlow PLUS Archive Kits allow you to collect, fix, and store samples, then analyze only if necessary.

For sample shipment

- **Ice Packs**
  For sample shipment

What's Included in the Kit
(Whole Blood Kits)

- **K_2EDTA Vacutainer Tubes**
  For blood collection
- **Exakt-Pak Shipping Container**
  For blood shipment
- **Cold Packs**
  For blood shipment

Additional Materials Required

- Fixative (methanol)
- Freezer (-80 °C), freezer (-20 °C), and refrigerator (4 °C)
- Centrifuge and other general lab supplies
- FBS, cellulose, and columns for bone marrow

Archive Kits

For more flexibility, MicroFlow BASIC Archive Kits allow you to collect, fix, and store samples, then analyze only if necessary.

New ICH S2(R1) Guideline

Signed November 9, 2011

The new S2(R1) ICH Guideline, "Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use" was signed on November 9, 2011. Many of the important changes in this update were designed to: 1) reduce the number of irrelevant positive results for *in vitro* mammalian assays, and 2) follow the 3Rs for genotoxicity testing (Replacement, Refinement and Reduction).

What does this regulation mean for *in vivo* micronucleus assays?

ICH states, "Systems for automated analysis (image analysis and flow cytometry) can be used if appropriately validated…"

ICH considers assays validated if they meet the criteria as stated by the International Workshop on Genotoxicity Testing (IWGT) in **this article**. These criteria are:

- Provide % MN-RET, % MN-NCE and % RET.
- Detects both fragments and whole chromosomes.
- Score consistently within and between experiments.
- Understand how known artifacts behave in the system.

In Vivo MicroFlow kits meet all of these criteria.

IWGT also stated that "A number of different flow cytometric-based micronucleus assays have been developed, but at the present time the validation data are most extensive for the flow cytometric method using anti-CD71 fluorescent staining especially in terms of inter-laboratory collaborative data."

The "anti-CD71" method referred to here is the MicroFlow method.

ICH also states "…each laboratory should determine the appropriate minimum sample size to ensure that scoring error is maintained below the level of animal-to-animal variation."

Using MicroFlow kits, 20,000 cells are routinely analyzed per sample. This is considered sufficiently large to ensure scoring error is below the level of animal-to-animal variation.

Can I integrate into other toxicity studies?

Yes. ICH states, "In case of repeated administrations, attempts should be made to incorporate the genotoxicity endpoints into toxicity studies…".

The MicroFlow method, requiring very low volumes of blood, makes it easy to integrate into existing studies.

Can I use rat peripheral blood?

Yes*.* ICH states, "Rat blood can be used for micronucleus analysis provided methods are used to ensure analysis of the newly formed reticulocytes…and the sample size is sufficiently large to provide appropriate statistical sensitivity…".

The guideline also indicates that rat peripheral blood can be used for MN assessment as long as MN are measured in the immature erythrocytes (polychromatic erythrocytes/reticulocytes).

MicroFlow kits are available for rat blood and can discriminate between mature and immature erythrocytes using the anti-CD71 antibody.

Can I use in vitro micronucleus as a part of the standard battery?

Yes. *ICH states, "Several in vitro mammalian cell systems are widely used and can be considered sufficiently validated: The in vitro metaphase chromosome aberration assay, the in vitro micronucleus assay and the mouse lymphoma L5178Y cell Tk (thymidine kinase) gene mutation assay (MLA). These three assays are currently considered equally appropriate and therefore interchangeable for measurement of chromosomal damage..."*

MicroFlow *In Vitro* Kits use flow cytometry to analyze hundreds of samples in just a few hours. This method supports both attachment and suspension cell lines. **Contact us** for more information on how *In Vitro* MicroFlow kits can support your regulatory submissions.

Regulatory Information

In Vivo MicroFlow Kits

Are these data accepted by regulatory agencies?

Yes. Data obtained using the MicroFlow method have been evaluated and accepted by the regulatory authorities in the US and EU. These results meet OECD test guideline 474 and comply with the new ICH S2(R1) guideline. See at right.

Does this method meet OECD test guideline 474 for micronucleus assays?

Yes. The guideline states “Systems for automated analysis (image analysis and cell suspensions flow cytometry) are acceptable alternatives to manual evaluation if appropriately justified and validated.”

Assays are considered validated if they meet the criteria as determined by the International Workshop on Genotoxicity Testing (IWGT) in **this article**. To meet these criteria, a method must:

- Provide % MN-RET, % MN-NCE and % RET.
- Identify fragments and whole chromosomes.
- Score consistently within and between experiments.
- Understand how known artifacts behave in the system.

MicroFlow kits meet all of these criteria.

Are MicroFlow BASIC analyses, performed at Litron, GLP-compliant?

Yes. Litron routinely performs these analyses and complies with FDA GLP regulations and the OECD Principles of GLP.

Does this method comply with the new ICH S2(R1) guideline?

Yes. The guideline states “Systems for automated analysis (image analysis and flow cytometry) can be used if appropriately validated…” Assays are considered validated if they meet the criteria as determined by the International Workshop on Genotoxicity Testing (IWGT) in **this article**. See the criteria, at left, under "Does this method meet OECD test guideline 474 for micronucleus assay?"

The guideline states “…each laboratory should determine the appropriate minimum sample size to ensure that scoring error is maintained below the level of animal-to-animal variation." And “Rat blood can be used for micronucleus analysis provided:

- Methods are used to ensure analysis of the newly formed reticulocytes…
- And the sample size is sufficiently large to provide appropriate statistical sensitivity…”

MicroFlow kits meet all the criteria of IWGT. They restrict analysis to the newest red blood cells (newly formed reticulocytes). In addition, 20,000 cells can be routinely analyzed per sample that is sufficiently large to be below the level of animal-to-animal variation, as well as providing the appropriate statistical sensitivity

In Depth

In Vivo MicroFlow Kits

Introduction to the *In Vivo* Micronucleus Assay

The *in vivo* micronucleus test is widely used in rodents to evaluate compounds for aneugenic and clastogenic potential. In the past, micronuclei (MN) were scored in peripheral blood or bone marrow through microscopic inspection of erythrocytes (also known as red blood cells or RBCs). While it is often simple to identify RBCs that contain MN using microscopy, the scarcity of these events makes scoring tedious and time-consuming. For this reason, thousands of cells must be evaluated to achieve a scoring error that is less than the level of inter-animal variation **[Kissling et al., 2007]**.

Introduction to MicroFlow® Kits

In Vivo MicroFlow, a flow cytometric technique, allows users to analyze enough cells to sufficiently reduce scoring error, even in species with low spontaneous frequencies. This system also has the following benefits:

- Results in a fraction of the time compared to microscopy
- High potential for integration into other toxicology studies
- Reduce inter-lab and inter-experiment variability using biological standards for instrument calibration
- Able to be measured across species
- Validated method (rodent only)
- Accepted by regulatory agencies (rodent only)

The MicroFlow method involves fixed blood or bone marrow specimens being incubated with anti-CD71-FITC, RNase, and anti-CD61-PE. Anti-CD71 differentiates reticulocytes (RETs) from mature RBCs. RNase, in conjunction with propidium iodide, is used to distinguish those RBCs with MN from those without, based on DNA content. Anti-CD61 prevents platelets from interfering with MN scoring. Four populations of erythrocytes can be identified: mature and immature erythrocytes, with and without MN. See next column.

In Vivo MicroFlow kits address a chief technical hurdle associated with reliable MN scoring. The use of fixed malaria-infected blood as biological standards provides consistent configuration of instrument settings, which is not present in other systems **[Tometsko et al., 1993; Dertinger et al., 2000]**. Malaria parasites mimic MN within RBCs, and the use of these biological standards controls intra- and inter-laboratory variability. This consistent configuration of instrument settings has been demonstrated in several international validation studies **[Torous et al., 2005; Dertinger et al., 2006]**.

Benefits of MicroFlow

RBCs containing MN are not actively eliminated from the circulation of mice by the spleen. The same cannot be said for most species of toxicological interest **[MacGregor et al., 1980]**. The MicroFlow method overcomes this challenge by restricting analysis to the youngest RBCs. For instance, several species who have spleens that filter MN-RBCs have shown significantly elevated MN-RET frequencies in subjects who have been exposed to genotoxic agents **[Abramsson-Zetterberg et al., 2000; MacGregor et al., 2006; Harper et al., 2007; Dertinger et al., 2007; Hotchkiss et al., 2008]**. This method allows reduction of animal usage, since it facilitates integration of a genetox endpoint into key toxicology studies, the majority of which are conducted with species other than mice.

Unpublished data has also demonstrated that scoring many thousands of RETs per rat blood sample could be used to establish a No Observable Effects Level (NOEL) for the alkylating agent methyl methanesulfonate. Additionally, other data show that clastogens can be distinguished from aneugens by the fluorescence intensity of their MN. Using this flow cytometric method allows researchers to simultaneously determine a chemical's Mode of Action along with its genotoxicity.

Human Analysis

In Vivo MicroFlow Kits

Measure DNA damage in red blood cells

This test detects damage to the chromosomes, or spindle apparatus, of cells. After exposure to a test substance, precursor cells divide. It is upon division that this type of damage can result in the formation of a smaller 'micronucleus', apart from the main nucleus.

At this point, red blood cells expel their main nuclei. When stained, micronuclei (which contain DNA) are easily seen in a population of cells with no nuclear DNA. These 'micronuclei' are also referred to as Howell-Jolly Bodies (HJB).

MicroFlow®Kits

Litron's MicroFlow kits use flow cytometry to analyze micronuclei in human blood samples. This method is based on Litron's rodent micronucleus kits.

Anti-CD71 antibodies distinguish between mature and immature RBCs. Micronuclei are easily identified with a DNA stain.

Disclaimer
The methods for human blood analyses by MicroFlow are for **Research Use Only (RUO)** and have not been approved, cleared, validated or intended for clinical diagnostic use or to serve as a basis for individual patient management.

Examine trends or changes in populations

- Environmental exposure
- Workplace safety
- Clinical research
- Post-market surveillance
- Other epidemiological studies

I want to measure micronuclei in human blood. Can you help me?

Yes. Blood samples can be shipped to us for flow cytometric analysis. For information on how to send samples for this method, contact us.

Can I measure micronuclei at my own facility?

Currently, this method is only offered as a service at Litron.