# Validation of Immunofluorescent Anti-Kinetochore (CREST) Staining in Rat Bone Marrow as part of the in vivo Micronucleus assay

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## BACKGROUND

The mammalian erythrocyte micronucleus test is a widely accepted *in vivo* assay used to evaluate the genotoxic liability of chemicals through the detection of micronuclei in hematopoietic stem cells within the bone marrow.

The presence of elevated levels of micronuclei is indicative of chromosomal damage that may have arisen from two primary mechanisms, clastogenic or aneugenic modes of action. However, the standard test conditions of this assay cannot identify the mechanism of action of a chemical, leading to the development of anti-kinetochore (CREST) staining. This labelling procedure utilises anti-kinetochore antibodies discovered in the serum of patients with an autoimmune condition called scleroderma pigmentosum (CREST syndrome).

## TECHNICAL LIMITATIONS TO THE ASSAY

The application of CREST antibodies allows for specific binding to kinetochores; small protein structures located at the centromeres of chromosomes. In conjunction with a fluorochrome-conjugated anti-human IgG antibody and fluorescence microscopy, the presence of kinetochore proteins can be visualised, allowing the quantification and subsequent identification of aneugenic compounds.

Kinetochore labelling in rat models is necessary, as the centromeric region is not highly conserved, rendering Fluorescent *in situ* hybridisation (FISH) ineffective. However, the fluorescent signal produced through kinetochore labelling is considerably smaller, making it much more challenging to detect.

## MODIFICATIONS TO METHODOLGY

In this validation experiment, anti-kinetochore staining was applied to rat bone marrow preparations from animals administered with the reference aneugen Vinblastine Sulphate.

Several procedural aspects were modified to enhance the precision of micronucleus evaluation and to effectively discern whether micronuclei primarily arose through aneugenic or clastogenic mechanisms, thereby elucidating the predominant mode of action.

Gentronix Ltd now offer immunofluorescent kinetochore labelling as an additional investigative tool alongside the *in vivo* micronucleus test in the rat for regulatory studies.

## MATERIALS AND METHODS

**Table 1**

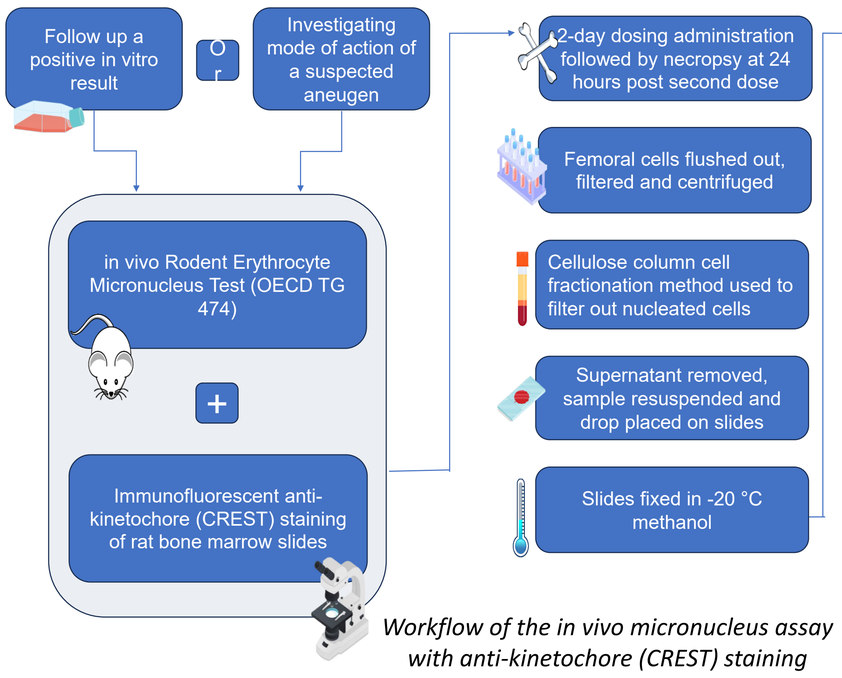
| Treatment | Dose route | Number and sex/ group | Dose level (mg/kg/day) | Dose volume | Formulation concentration mg/mL |
|---|---|---|---|---|---|
| Sterile Saline | IP | 5/M | 0 | 5 | 0 |
| Vinblastine sulphate (VIN) | IP | 5/M | 0.125 | 5 | 0.025 |
| | | 5/M | 0.25 | 5 | 0.05 |
| | | 5/M | 0.5 | 5 | 0.1 |
| Cyclophosphamide monohydrate | Oral | 5/M | 20 | 10 | 2.0 |

IP: intraperitoneal injection

9–10-week-old male Han Wistar rats were used. Femoral bone marrow harvested, and bone marrow smears were prepared using the cellulose column cell fractionation:

- Bone marrow cell suspension diluted with Hanks' Balanced Salt Solution (HBSS).
- Cell suspension layered onto cellulose columns (1:1 of Type 50 and α-cellulose).
- Once eluted, 0.5 mL of HBSS added to each column.
- Eluant centrifuged for 5 minutes at 200 x *g.*
- The supernatant was discarded, the sample vortexed and each pellet resuspended in Fetal Bovine Serum (FBS).
- Centrifuged for 5 minutes at 200 x g.
- Supernatant removed, pellet resuspended, and sample placed on slides. Slides then underwent antibody labelling, as detailed in workflow below.

## WORKFLOW AND REFINEMENTS




*Workflow of the in vivo micronucleus assay with anti-kinetochore (CREST) staining*

- Slides underwent pre-treatment in a solution containing 0.025% Sodium Dodecyl Sulfate (SDS) and 0.1% Triton X-100 to enhance penetration of antibodies into erythrocytes.
- The primary antibody was diluted with 0.25% Bovine Serum Albumin (BSA) serving as a blocking agent to minimise non-specific antibody binding and mitigate background staining.
- Throughout all washing steps in the protocol, Phosphate Buffered Saline (PBS) containing 0.1 % Tween-20 (PBST) was used to reduce non-specific antibody binding.
- As the slides were wet-mounted CoverGrip™ slide sealant was used to prevent seepage of the Vectashield®.
- The presence of fluorescent centromeric-positive micronuclei with FITC excitation was examined under an Olympus BX43 microscope using a triple band pass filter.

## RESULTS

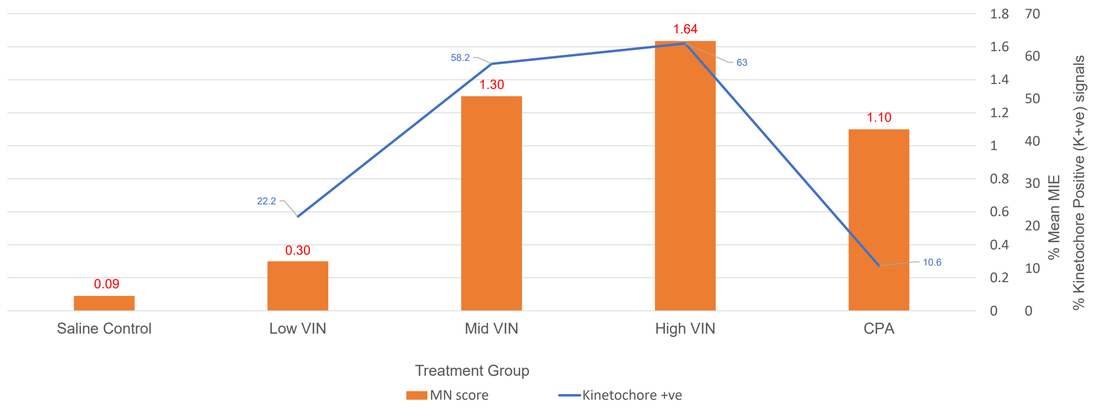
- Following IP administration of VIN, results showed a dose-dependent increase in centromeric-positive micronuclei (K+ve), with 22.2%, 58.2% and 63% K+ve at 0.125 mg/kg, 0.25 mg/kg and 0.5 mg/kg respectively.
- The clastogenic control, cyclophosphamide at 20 mg/kg, yielded 10.6% K+ve micronuclei (89.4% K-ve). No data was obtained from the vehicle control animals due to insufficient levels of micronucleated erythrocytes.

**Table 2**

| | Micronucleus Data | | Kinetochore Data | |
|---|---|---|---|---|
| | Mean % MIE | St dev | Mean %K+ve | St dev |
| Saline Control | 0.09 | 0.02 | ND | ND |
| VIN 0.125 | 0.30 | 0.10 | 22.2 | 9.73 |
| VIN 0.25 | 1.30 | 0.38 | 58.2 | 6.14 |
| VIN 0.5 | 1.64 | 0.45 | 63 | 7.91 |
| CPA 20 | 1.10 | 0.10 | 10.6 | 7.8 |

ND = No Data

**Figure 1**




## CONCLUSIONS

Refinements to the methodology have facilitated the optimisation of kinetochore labelling by significantly reducing, though not totally eliminating issues related to non-specific binding and suboptimal antibody penetration into micronucleated erythrocytes. This enhanced method can be applied to elucidate the predominant mode of action of a compound, with results demonstrating the detection of centromeric-positive micronuclei up to 63% for reference aneugen, vinblastine sulphate. There is currently no published literature on the acceptance criteria for aneugenic compounds therefore the number of kinetochore-positive micronuclei required to classify a compound as aneugenic remains undefined. Gentronix Ltd now offer immunofluorescent kinetochore labelling as an additional investigative tool alongside the *in vivo* micronucleus test in the rat for regulatory studies.

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