The *in-vivo* Comet Assay: Considerations for Validation of Optimal Study Design in a Regulatory Setting

Teresa Chirom, Julie Hayes, Lucinda Lillford
Gentronix (Part of Scantox Group), Alderley Park, United Kingdom

Introduction

The *in vivo* alkaline comet assay is a rapid and sensitive technique for detection of single and double strand DNA breaks, routinely used in the regulatory setting to assess genotoxicity in tissues exposed to a variety of test substances.

The Comet assay is employed as a follow up *in-vivo* test for test substances that have shown *in-vitro* liability, or as a second *in-vivo* test to provide weight of evidence.

To expand the Gentronix portfolio of *in-vivo* assays, validation of the Comet assay has commenced in Han Wistar rats in the following tissues:

- Liver - as the primary metabolic organ
- Stomach
- Duodenum

} site of contact tissues for orally administered test substances

Recently, the *in vivo* Comet assay has been the subject of criticism with respect to the reliability of the data it generates.

Sources of Variability

As part of the validation of the Comet assay for use in a regulatory setting, Gentronix has investigated the areas of variability that can occur within the assay procedures. Gentronix has endeavoured to adjust and optimise their methods accordingly to minimise these variability factors. This poster illustrates our findings for consideration.

1) Preparation of single cell suspension methods

Mechanical dissociation methods can produce DNA damage and lead to variability, for which the following tissue preparation methods were investigated.

- Liver – single cells released via gentle mincing with flat-tip forceps
- Stomach and duodenum – release of single cells via mincing and scraping methods

2) Electrophoresis

Variability can occur during the electrophoresis step:

- For a regulatory study, the total number of slides prepared per tissue type results in multiple electrophoresis runs
- The current within the tank may vary depending on the location of the slide in the tank
- Temperature of the buffer needs to be regulated

3) Scaling the methodology

As stated in OECD Test Guideline 489 '*slide preparation should be done as soon as possible (ideally within one hour) after single cell preparation*'.

This poses a challenge when scaling the methodology for a regulatory study in terms of the number of tissues to be processed.

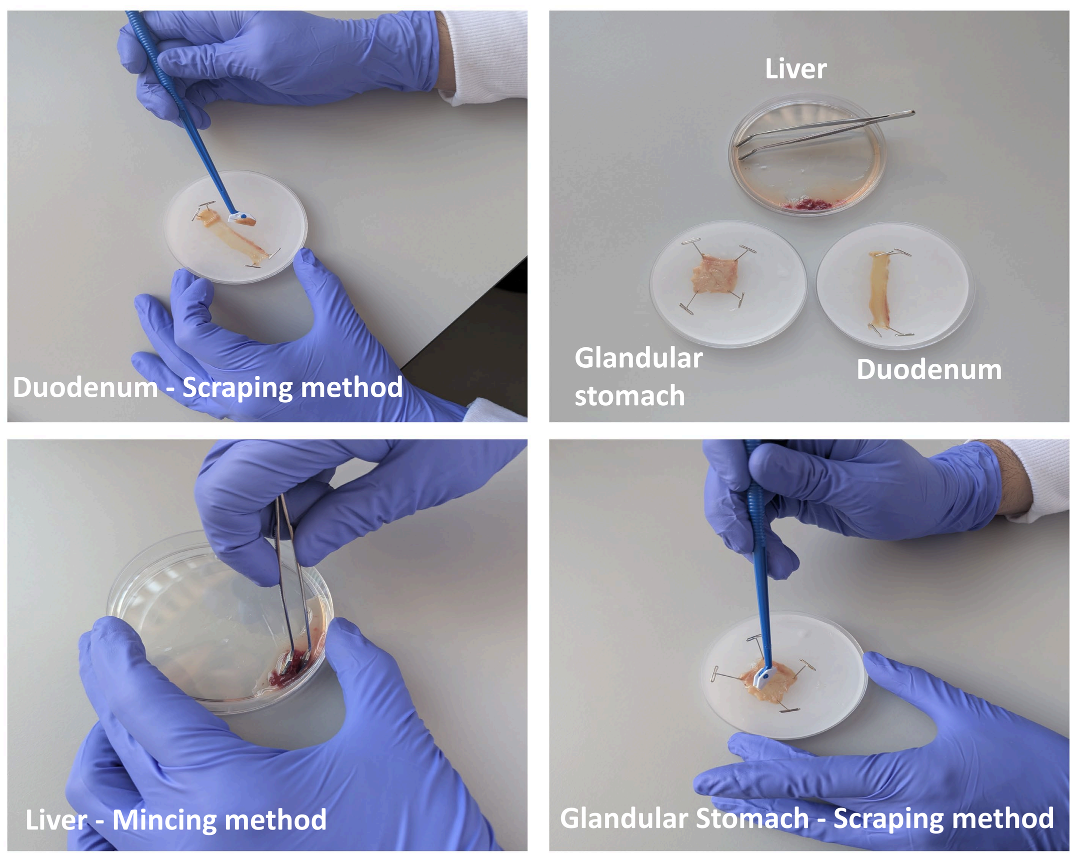
Reducing Variability in the *in vivo* Comet Assay

Tissue preparation

Good quality single cell suspensions were obtained via the gentle mincing method for liver and the scraping method for stomach and duodenum (see **Figure 1**).

For stomach and duodenum, the top mucosal layer was scraped off to expose the epithelial layer, which was then carefully scraped with the aid of a cell scraper.

Figure 1

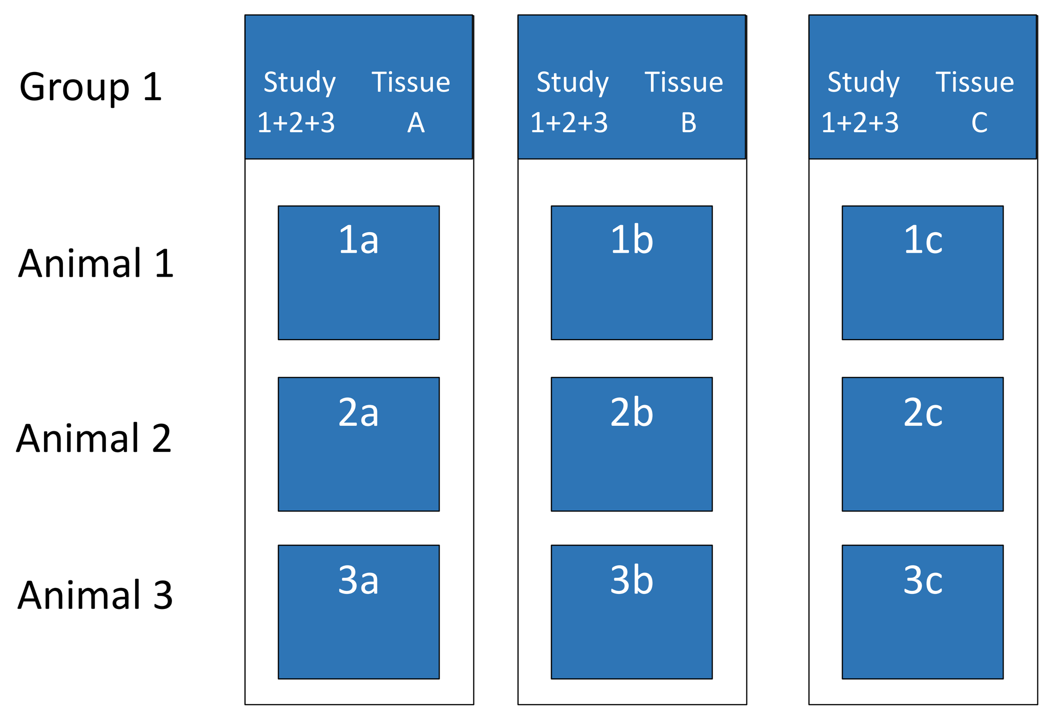


Slide preparation

To minimise any tank location variability, 3 gels/slide were prepared from 3 animals/group as illustrated in **Figure 2.** All slides were placed in a single large electrophoresis tank using a randomised block design.

Adopting this design on a standard regulatory, OECD 489 compliant study, means only one electrophoresis run is required for each tissue, thus preventing tank to tank variation.

Figure 2



Temperature and voltage regulation

To consistently regulate the temperature of the buffer and ensure that several electrophoresis tanks can be run concurrently, a large fridge with the capacity to house multiple tanks for the duration of the unwinding and electrophoresis run was installed (see **Figure 3**).

The buffer volume in each tank was also optimised to run at a consistent voltage of 0.7 V/cm.

Figure 3


Large fridge to house multiple tanks

Preliminary Data Obtained Using the Comet Assay IV™ Image Analysis System

Table 1

in-vitro Cell Line	Treatment (µg/mL)	Median % Tail Intensity
Mouse lymphoma L5178Y	DMSO	4.94
	MMS 5	7.31
	MMS 10	15.22
	MMS 20	32.70

Table 2

Rat Tissue	Control Animal	Median % Tail Intensity
Liver	1	1.51
	2	2.57
	3	0.38
Stomach	1	4.91
	2	8.78
	3	6.77
Duodenum	1	0.14
	2	6.73
	3	3.73

Figure 4

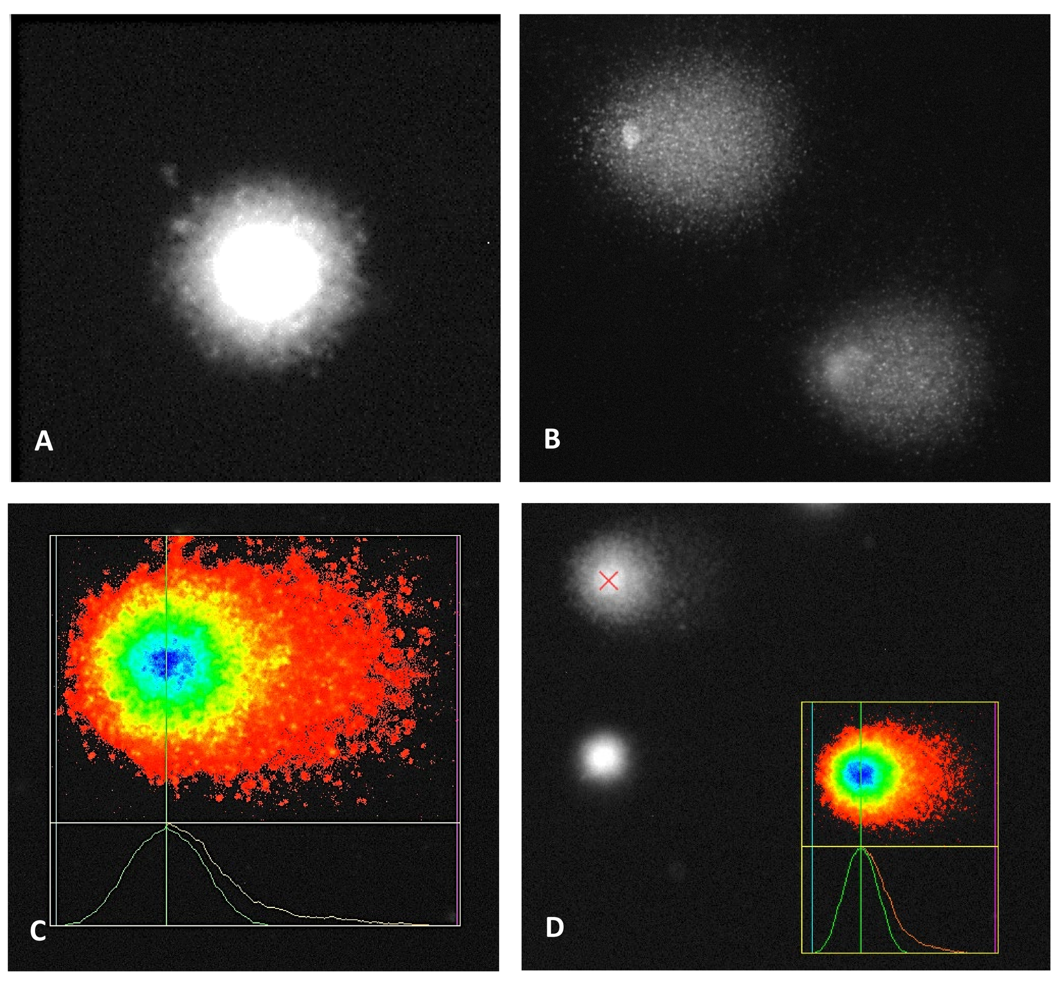


Table 1

Preliminary *in-vitro* comet data obtained from mouse lymphoma L5178Y cells dosed with MMS for 3 hours. 50 cells were scored for each concentration. A total of 7.41% hedgehogs were observed at 20 µg/mL MMS.

Table 2

Preliminary *in-vivo* data obtained from an untreated control group of Han Wistar rats. For each animal 150 cells were scored.

Figure 4

A. Liver cell
B. Hedgehogs observed on a duodenum slide
C. Liver cell with % Tail Intensity of 23.5%, dosed with 20 mg/kg CPA
D. Cells observed on a liver slide, cell with % Tail Intensity of 21.91%, dosed with 20 mg/kg CPA

CONCLUSIONS

Numerous considerations were taken into account for the first stage of the comet assay validation to refine and optimise the various steps involved in conducting the methods. As a 3R's initiative, Gentronix will perform further work to integrate the Comet assay into the established GLP-compliant *in-vivo* bone-marrow micronucleus assay running at Gentronix, thereby greatly reducing animal use. In addition, an enhanced randomisation approach will be adopted in the *in-vivo* experimental design to aid with reducing assay variability and any subsequent impact on data. Finally, a laboratory historical control database will be generated, which forms an essential component for aiding with data contextualization in regulatory studies.

Gentronix Limited | Alderley Park | Cheshire | United Kingdom info@gentronix.co.uk