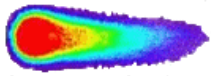
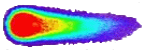


Helix3 Inc.

Technical Issues Influencing In Vivo Comet Results

Marie Z. Vasquez
Helix3 Inc.



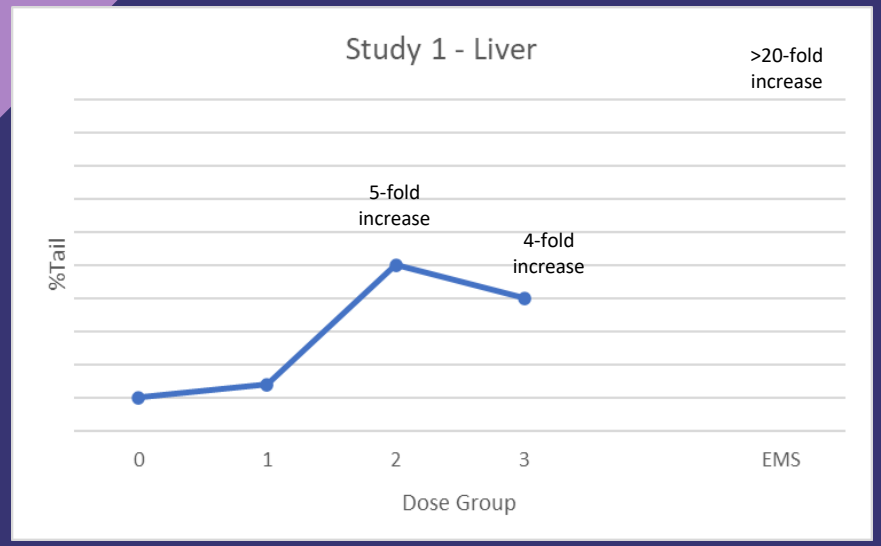
Helix3

Outline

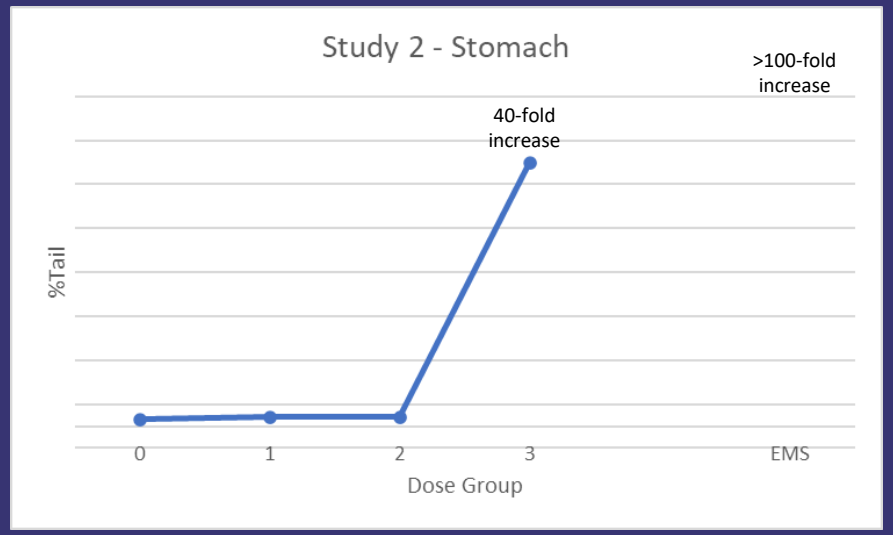
- Case studies with false positive results
- Possible technical concerns / issues
- Helix3 technical variation control methods
- Regulatory conclusions
- Points for Consideration
- Follow Up Questions

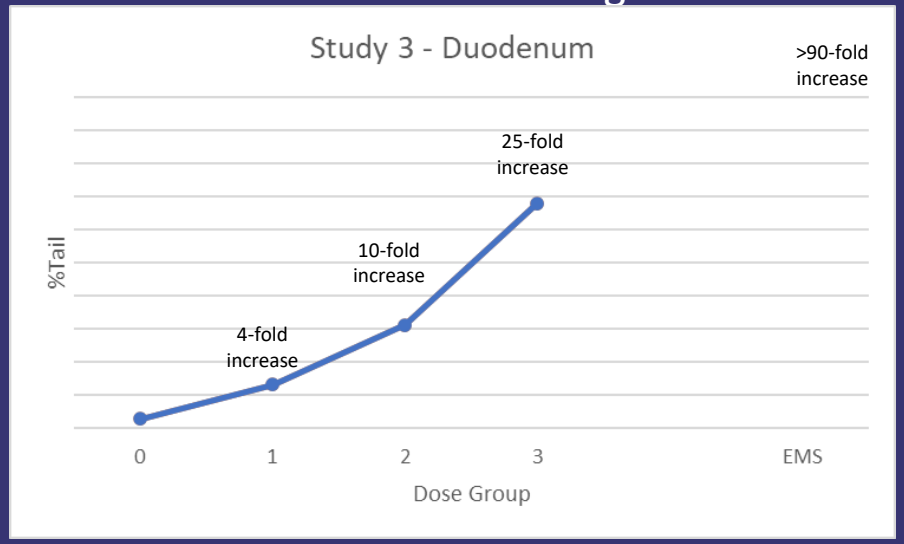
Helix3

Case Studies



- Statistically significant increases
- Dose-related response
- No histopath findings
- “Hedgehog” increase with dose
- Reproducible in repeat study
- Vehicle within HCD range

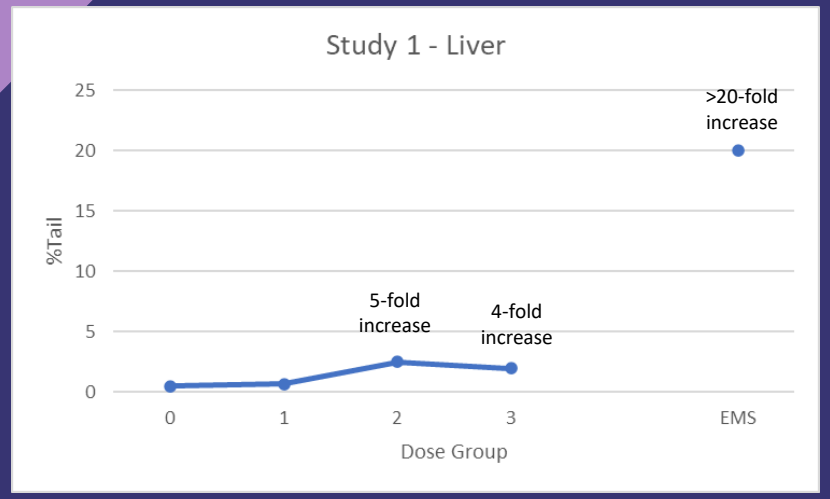


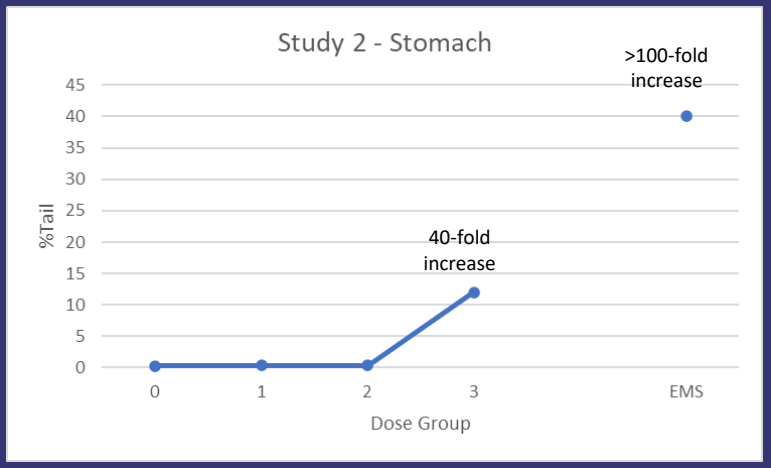


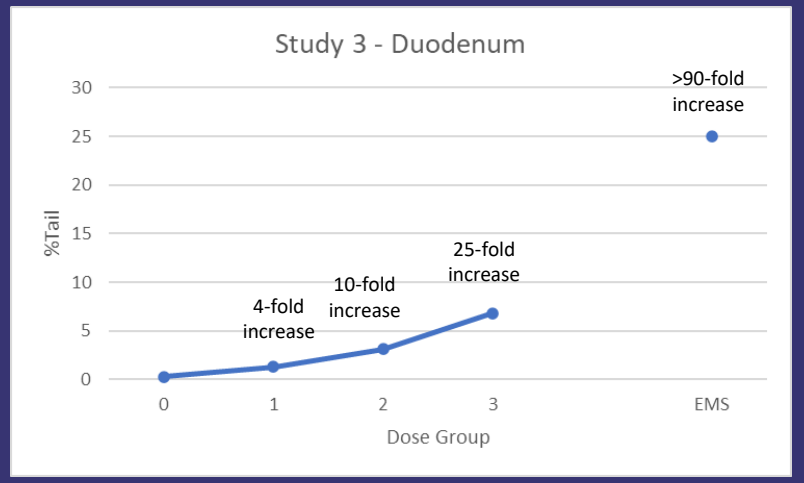
Helix3

Concerns

- Mean control %Tail values <1%
- Liver values = GI tract values
- HCD range with >100-fold difference between min and max values
- Overlapping negative and positive control HCD ranges
- TA “hedgehogs” ≥ EMS “hedgehogs”
- Extreme fold-increase detected in positive control

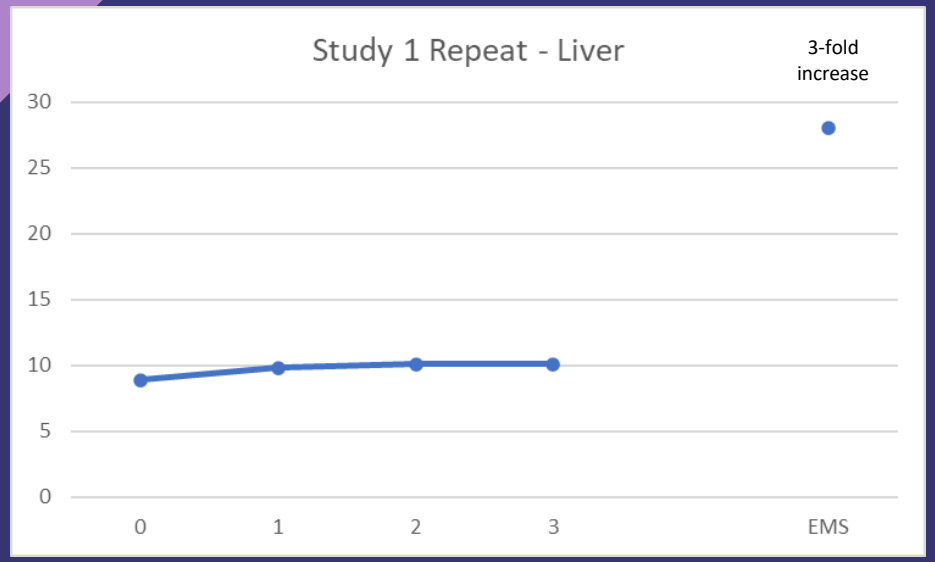




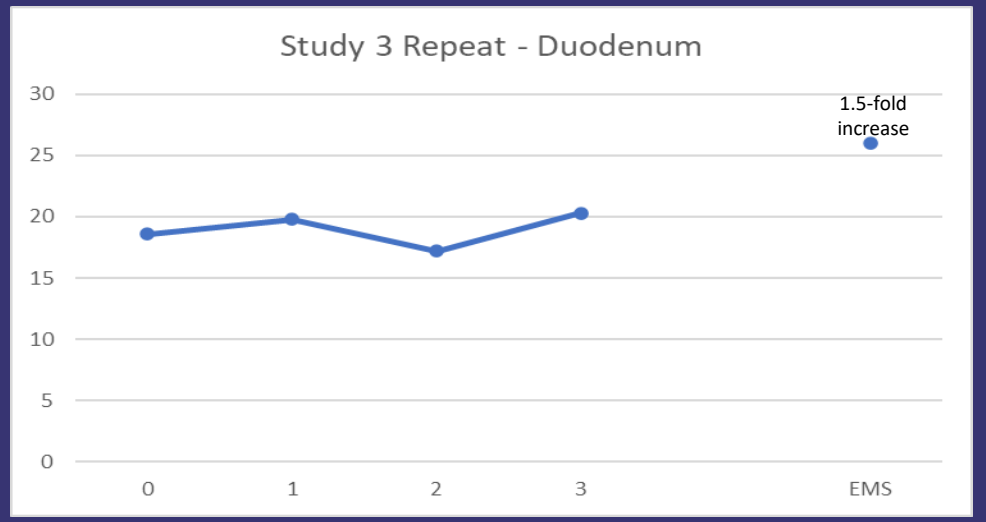


Helix3

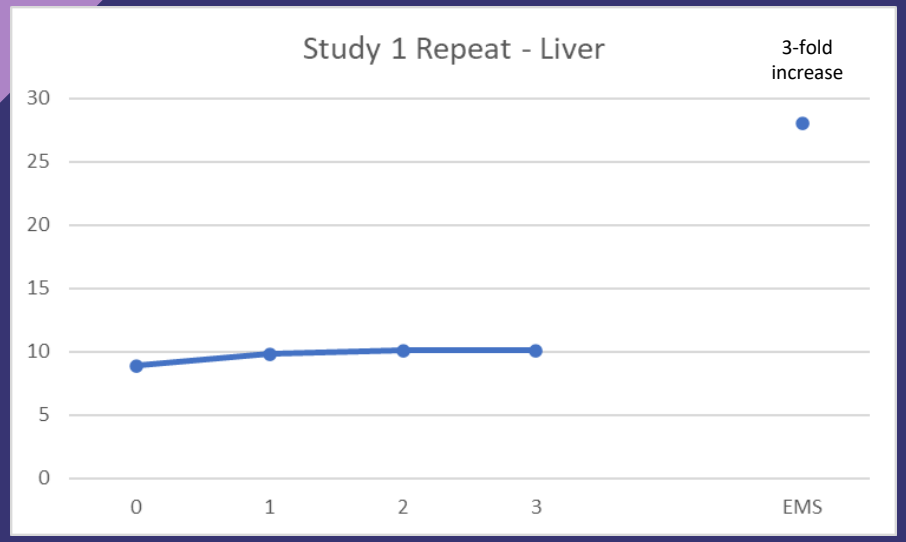
Repeat Studies at Helix3



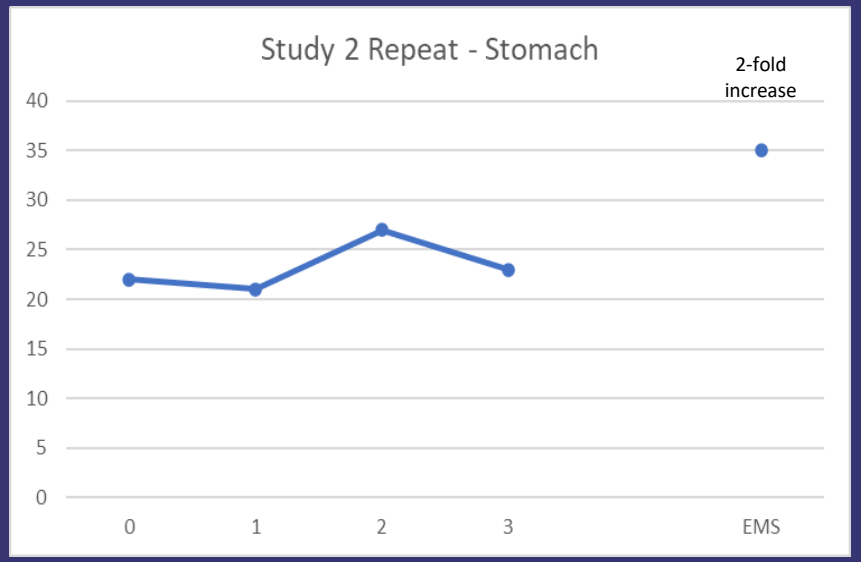
- No increase at any dose
- No dose-related response
- No histopath findings
- No “ghost” increase
- Vehicle within HCD range
- ≥ Plasma concentrations

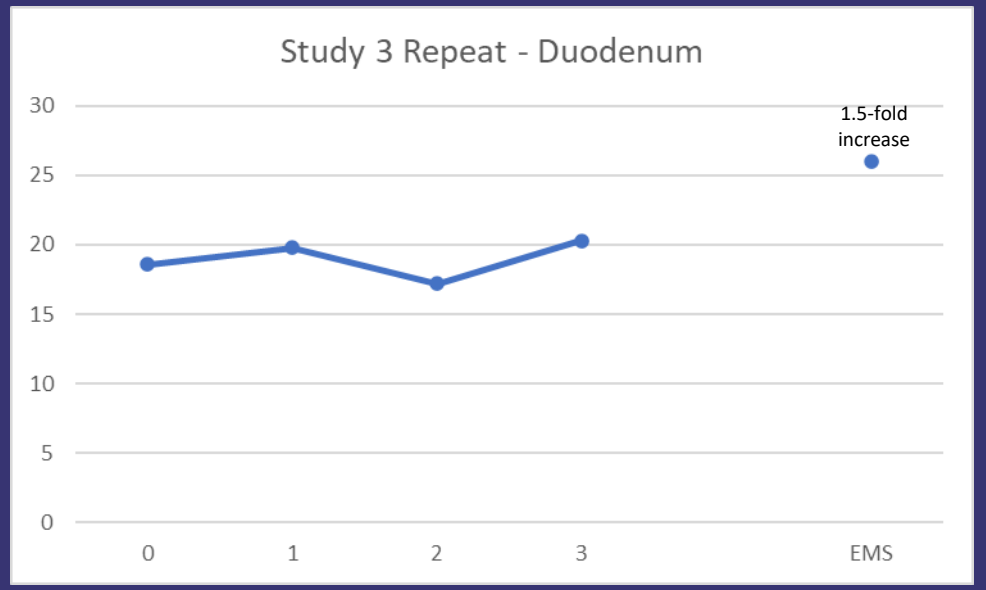


Helix3 Differences

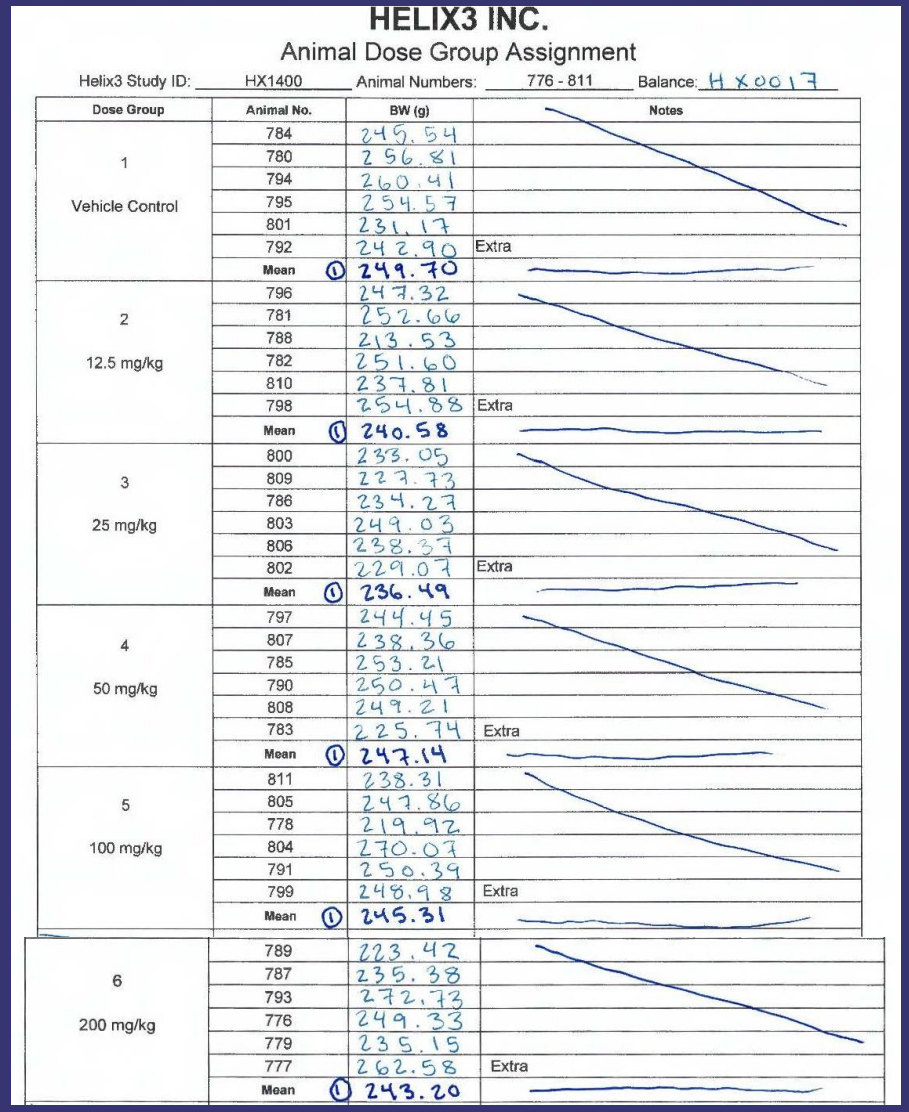


- Mean control %Tail values >5%
- Liver values < GI tract values
- HCD range with ≤3-fold difference between min and max values for all tissues (n=18)
- No overlap between negative and positive control HCD ranges





Sample Coding



HELIX3 INC.

Animal Dose Group Assignment

Helix3 Study ID: HX1400 Animal Numbers: 776 - 811 Balance: HX0017

Dose Group	Animal No.	BW (g)	Notes
1 Vehicle Control	784	245.54	
	780	256.81	
	794	260.41	
	795	254.57	
	801	231.17	
	792	242.90	Extra
	Mean	① 249.70	
2 12.5 mg/kg	796	247.32	
	781	252.66	
	788	213.53	
	782	251.60	
	810	237.81	
	798	254.88	Extra
	Mean	① 240.58	
3 25 mg/kg	800	233.05	
	809	227.73	
	786	234.27	
	803	249.03	
	806	238.37	
	802	229.07	Extra
	Mean	① 236.49	
4 50 mg/kg	797	244.45	
	807	238.36	
	785	253.21	
	790	250.47	
	808	249.21	
	783	225.74	Extra
	Mean	① 247.14	
5 100 mg/kg	811	238.31	
	805	247.86	
	778	219.92	
	804	270.07	
	791	250.39	
	799	248.98	Extra
	Mean	① 245.31	
6 200 mg/kg	789	223.42	
	787	235.38	
	793	272.73	
	776	249.33	
	779	235.15	
	777	262.58	Extra
	Mean	① 243.20	

Helix3

- Random animal and sample codes are used throughout study
- Avoids procedural bias

Other Labs

- Obvious animal and sample codes (e.g., 00-99=control, 100-199=group 1, 200-299= group 2, etc.) are used
- Potential procedural bias

Dosing / Sample Collections

HELIX3 INC.
Animal Dosing

Helix3 Study ID: HX1400

Animal No	Dose	Pre-Dose Ob(s)	BW (g)	Dose Volume* (mL)	Time Dosed	Post Dose Ob(s)^ Ob(s)	Post Dose Ob(s)^ Time
784	1	1	257.67	2.6	1:00	1	1:15
780	1	1	275.22	2.8	1:05	1	1:20
789	6	1	242.66	2.4	1:10	1	1:25
787	6	1	256.33	2.6	1:15	1	1:30
811	5	1	260.43	2.6	1:20	1	1:35
805	5	1	267.00	2.7	1:25	1	1:40
797	4	1	264.30	2.6	1:30	1	1:45
807	4	1	259.35	2.6	1:35	1	1:51
800	3	1	247.94	2.5	1:40	1	1:56
809	3	1	246.15	2.5	1:45	1	2:00
796	2	1	273.97	2.7	1:50	1	2:05
781	2	1	273.67	2.7	1:55	1	2:10
794	1	1	281.96	2.8	2:00	1	2:15
795	1	1	271.14	2.7	2:05	1	2:20
793	6	1	294.40	2.9	2:10	1	2:25
776	6	1	271.26	2.7	2:15	1	2:30
778	5	1	240.10	2.4	2:20	1	2:35
804	5	1	293.45	2.9	2:25	1	2:40
785	4	1	278.20	2.7	2:30	1	2:45
790	4	1	274.79	2.7	2:35	1	2:50
786	3	1	248.09	2.5	2:40	1	2:55
803	3	1	267.50	2.7	2:45	1	3:00
788	2	1	231.56	2.3	2:50	1	3:05
782	2	1	270.36	2.7	2:55	1	3:10
801	1	1	250.63	2.5	3:00	1	3:15
Initials/Date	KD 16 Mar 22				KO 16 Mar 22	KO 16 Mar 22	

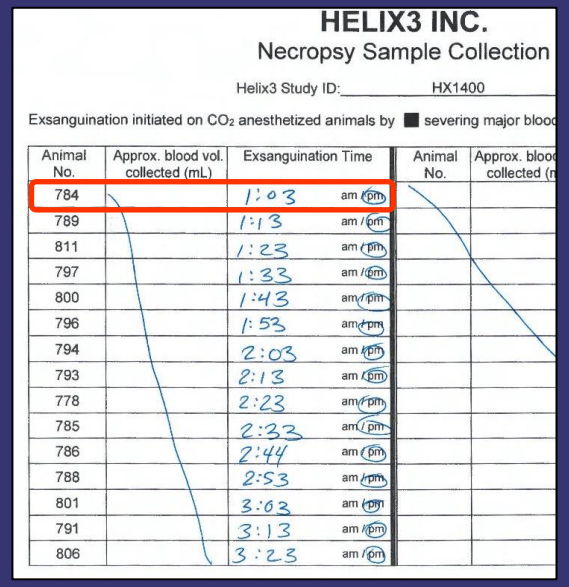
*Target dose volumes are entered before dosing and based on individual BW. Doses are administered in this volume except where otherwise noted
^See Clin Ob Key (Helix3 Form 7093)

HELIX3 INC.
Animal Dosing

Helix3 Study ID: HX1400

Animal No	Dose	Pre-Dose Ob(s)	BW (g)	Dose Volume* (mL)	Time Dosed	Post Dose Ob(s)^ Ob(s)	Post Dose Ob(s)^ Time
784	1	1	252.52	2.5	9:00	1	9:15
780	1	1	288.01	2.9	9:06	1	9:20
789	6	1	230.96	2.3	9:10	1	9:25
787	6	1	237.29	2.4	9:15	1	9:30
811	5	1	259.98	2.6	9:20	1	9:35
805	5	1	263.75	2.6	9:25	1	9:40
797	4	1	270.13	2.7	9:30	1	9:45
807	4	1	268.19	2.7	9:35	1	9:50
800	3	1	257.44	2.6	9:40	1	9:56
809	3	1	256.62	2.6	9:45	1	10:00
796	2	1	286.35	2.9	9:50	1	10:06
781	2	1	283.98	2.8	9:56	1	10:10
794	1	1	294.52	2.9	10:00	1	10:15
795	1	1	282.88	2.8	10:05	1	10:20
793	6	1	276.90	2.8	10:10	1	10:25
776	6	1	266.05	2.7	10:15	1	10:30
778	5	1	237.43	2.4	10:20	1	10:35
804	5	1	296.24	3.0	10:25	1	10:40
785	4	1	275.40	2.8	10:30	1	10:50
790	4	1	276.50	2.8	10:35	1	10:50
786	3	1	259.87	2.6	10:40	1	10:56
803	3	1	279.87	2.8	10:45	1	11:00
788	2	1	241.89	2.4	10:50	1	11:05
782	2	1	283.46	2.8	10:55	1	11:10
801	1	1	264.94	2.6	11:00	1	11:16
Initials/Date	KO 17 Mar 22				KO 17 Mar 22	KO 17 Mar 22	

*Target dose volumes are entered before dosing and based on individual BW. Doses are administered in this volume except where otherwise noted
^See Clin Ob Key (Helix3 Form 7093)

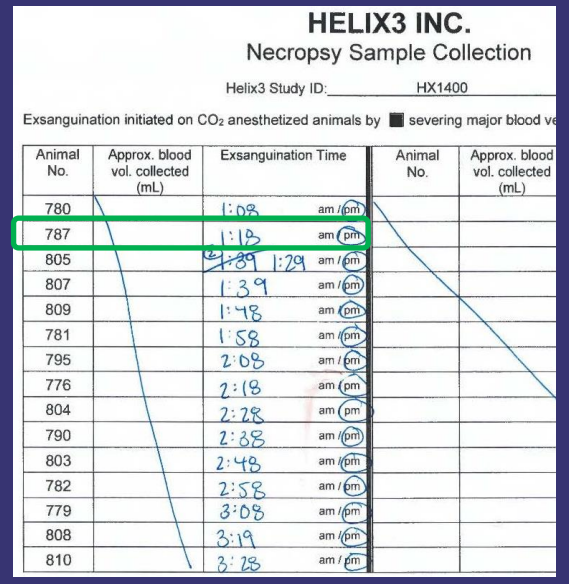


HELIX3 INC.
Necropsy Sample Collection

Helix3 Study ID: HX1400

Exsanguination initiated on CO_2 anesthetized animals by ■ severing major blood

Animal No.	Approx. blood vol. collected (mL)	Exsanguination Time	Animal No.	Approx. blood collected (mL)
784		1:03 am / pm		
789		1:13 am / pm		
811		1:23 am / pm		
797		1:33 am / pm		
800		1:43 am / pm		
796		1:53 am / pm		
794		2:03 am / pm		
793		2:13 am / pm		
778		2:23 am / pm		
785		2:33 am / pm		
786		2:44 am / pm		
788		2:53 am / pm		
801		3:03 am / pm		
791		3:13 am / pm		
806		3:23 am / pm		



HELIX3 INC.
Necropsy Sample Collection

Helix3 Study ID: HX1400

Exsanguination initiated on CO_2 anesthetized animals by ■ severing major blood ve

Animal No.	Approx. blood vol. collected (mL)	Exsanguination Time	Animal No.	Approx. blood vol. collected (mL)
780		1:08 am / pm		
787		1:18 am / pm		
805		~~1:29~~ 1:29 am / pm		
807		1:39 am / pm		
809		1:48 am / pm		
781		1:58 am / pm		
795		2:08 am / pm		
776		2:18 am / pm		
804		2:28 am / pm		
790		2:38 am / pm		
803		2:48 am / pm		
782		2:58 am / pm		
779		3:08 am / pm		
808		3:19 am / pm		
810		3:28 am / pm		

Helix3

- Dose groups rotated
- Timing consistent between animals
- Samples collected and processed in <10 min after exsanguination

Other Labs

- Dosing and processing conducted in order by dose
- Less control of timing

Dosing / Sample Collections

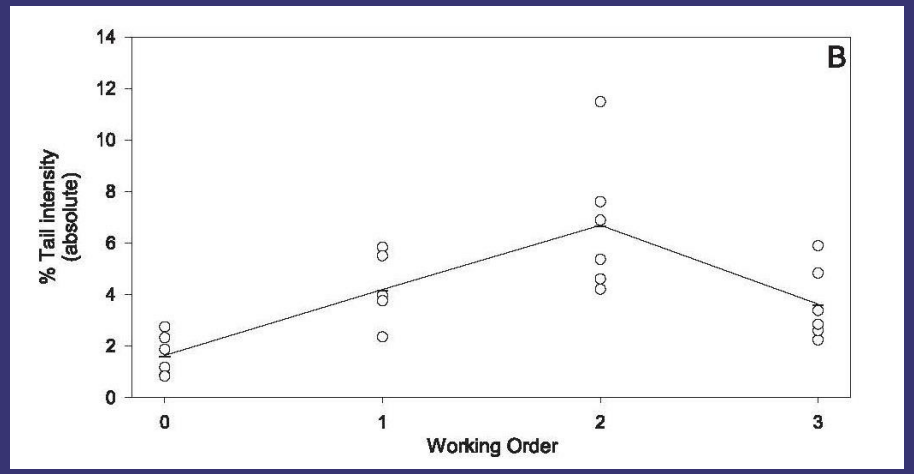
Mutagenesis Advance Access published March 9, 2011
Mutagenesis pp. 1–2, 2011 doi:10.1093/mutage/ger007

LETTER TO THE EDITOR

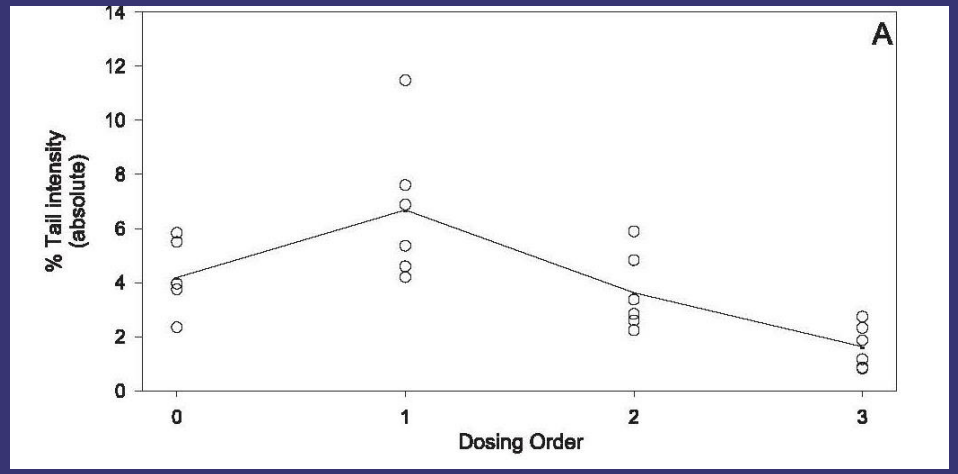
Possibility of methodical bias in analysis of comet assay studies
RE: DNA damage detected by the alkaline comet assay in the liver of mice after oral administration of tetrachloroethylene. (*Mutagenesis*, 25, 133–138, 2010)

Melanie Struwe*, Andreas Zeller, Thomas Singer and Elmar Gocke

Dosing / Processing Order: High dose, Control, Low dose, Mid dose

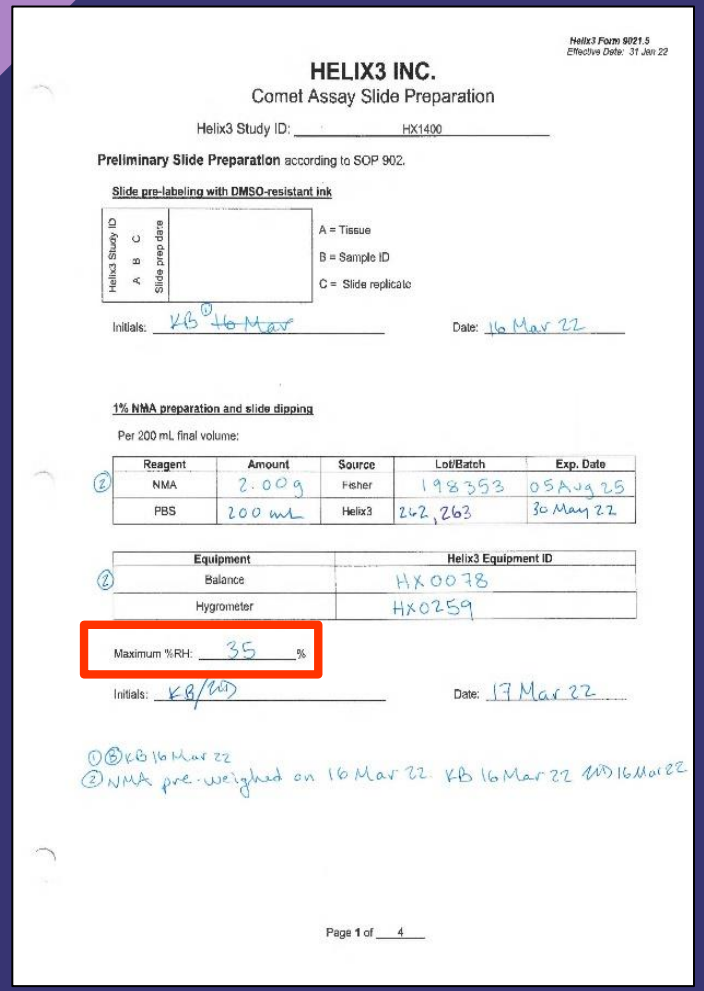


Plotted by order of processing
Dose response



Plotted by dose
No dose response

Slide Preparations



Helix3 Form 9021.5
Effective Date: 31 Jan 22

HELIX3 INC.
Comet Assay Slide Preparation

Helix3 Study ID: HX1400

Preliminary Slide Preparation according to SOP 902.

Slide pre-labeling with DMSO-resistant ink

Helix3 Study ID | A | B | C | Slide prep date

A = Tissue
B = Sample ID
C = Slide replicate

Initials: KB ① 16 Mar Date: 16 Mar 22

1% NMA preparation and slide dipping

Per 200 mL final volume:

	Reagent	Amount	Source	Lot/Batch	Exp. Date
②	NMA	2.00 g	Fisher	198353	05 Aug 25
	PBS	200 mL	Helix3	262, 263	30 May 22

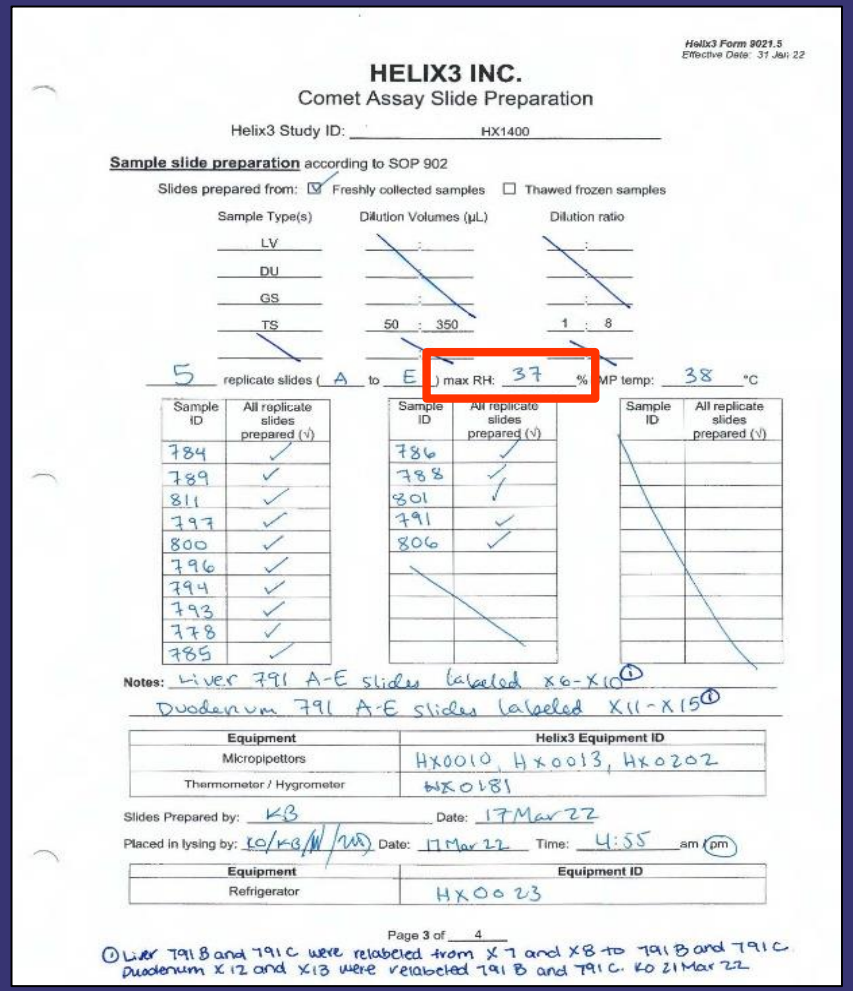
	Equipment	Helix3 Equipment ID
②	Balance	HX0078
	Hygrometer	HX0259

Maximum %RH: 35 %

Initials: KB/MD Date: 17 Mar 22

① KB 16 Mar 22
② NMA pre-weighed on 16 Mar 22. KB 16 Mar 22 MD 16 Mar 22

Page 1 of 4



Helix3 Form 9021.5
Effective Date: 31 Jan 22

HELIX3 INC.
Comet Assay Slide Preparation

Helix3 Study ID: HX1400

Sample slide preparation according to SOP 902

Slides prepared from: ☑ Freshly collected samples ☐ Thawed frozen samples

Sample Type(s)	Dilution Volumes (µL)	Dilution ratio
LV	:	:
DU	:	:
GS	:	:
TS	50 : 350	1 : 8

5 replicate slides (A to E) max RH: 37 % MP temp: 38 °C

Sample ID	All replicate slides prepared (√)
784	✓
789	✓
811	✓
797	✓
800	✓
796	✓
794	✓
793	✓
778	✓
785	✓

Sample ID	All replicate slides prepared (√)
786	✓
788	✓
801	✓
791	✓
806	✓

Sample ID	All replicate slides prepared (√)

Notes: Liver 791 A-E slides labeled X6-X10 ①
Duodenum 791 A-E slides labeled X11-X15 ①

Equipment	Helix3 Equipment ID
Micropipettors	HX0010, HX0013, HX0202
Thermometer / Hygrometer	HX0181

Slides Prepared by: KB Date: 17 Mar 22

Placed in lysing by: KO/KB/MD/MA Date: 17 Mar 22 Time: 4:55 am (pm)

Equipment	Equipment ID
Refrigerator	HX0023

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① Liver 791B and 791C were relabeled from X7 and X8 to 791B and 791C
Duodenum X12 and X13 were relabeled 791B and 791C. KO 21 Mar 22

Helix3

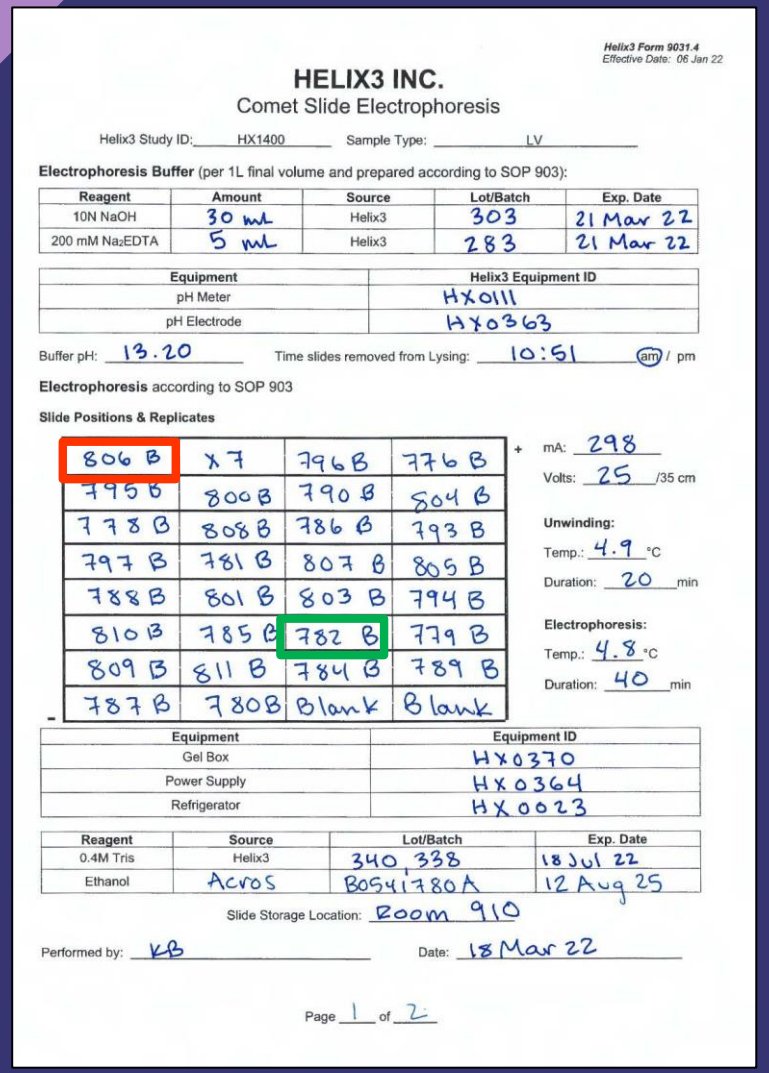
- Relative Humidity is maintained at 30-50%
- Higher RH (≥60%, Summer) decreases LMP concentration and DNA migration levels*
- Lower RH (≤20%, Winter) increases LMP concentration and DNA migration levels*

Other Labs

- No record or control of RH
- Evidence of seasonal influence on results

*Vasquez, M.Z. (2010) Combining the in vivo comet and micronucleus assays: a practical approach to genotoxicity testing and data interpretation. *Mutagenesis*, 25,187–199
Vasquez, M.Z. and Frötschl, R. (2016) The In Vivo Comet Assay Test. In: Proudlock R, editor. Genetic Toxicology Testing: A Laboratory Manual. Academic Press. pp. 345–382

Slide Preparations



Helix3 Form 9031.4
Effective Date: 06 Jan 22

HELIX3 INC.
Comet Slide Electrophoresis

Helix3 Study ID: HX1400 Sample Type: LV

Electrophoresis Buffer (per 1L final volume and prepared according to SOP 903):

Reagent	Amount	Source	Lot/Batch	Exp. Date
10N NaOH	30 mL	Helix3	303	21 Mar 22
200 mM Na_2EDTA	5 mL	Helix3	283	21 Mar 22

Equipment	Helix3 Equipment ID
pH Meter	HX0111
pH Electrode	HX0363

Buffer pH: 13.20 Time slides removed from Lysing: 10:51 (am) / pm

Electrophoresis according to SOP 903

Slide Positions & Replicates

806 B	X7	796 B	776 B
795 B	800 B	790 B	804 B
778 B	808 B	786 B	793 B
797 B	781 B	807 B	805 B
788 B	801 B	803 B	794 B
810 B	785 B	782 B	779 B
809 B	811 B	784 B	789 B
787 B	780 B	Blank	Blank

mA: 298
Volts: 25 /35 cm

Unwinding:
Temp.: 4.9 °C
Duration: 20 min

Electrophoresis:
Temp.: 4.8 °C
Duration: 40 min

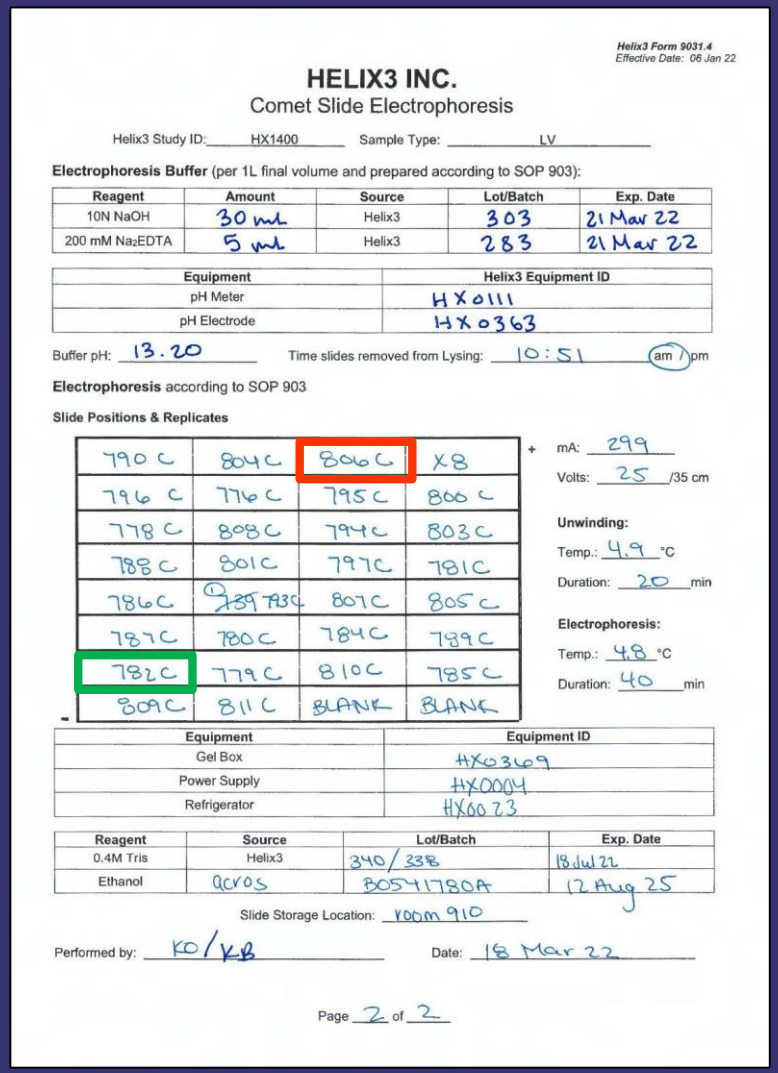
Equipment	Equipment ID
Gel Box	HX0370
Power Supply	HX0364
Refrigerator	HX0023

Reagent	Source	Lot/Batch	Exp. Date
0.4M Tris	Helix3	340, 338	18 Jul 22
Ethanol	Acros	B0541780A	12 Aug 25

Slide Storage Location: Room 910

Performed by: KB Date: 18 Mar 22

Page 1 of 2



Helix3 Form 9031.4
Effective Date: 06 Jan 22

HELIX3 INC.
Comet Slide Electrophoresis

Helix3 Study ID: HX1400 Sample Type: LV

Electrophoresis Buffer (per 1L final volume and prepared according to SOP 903):

Reagent	Amount	Source	Lot/Batch	Exp. Date
10N NaOH	30 mL	Helix3	303	21 Mar 22
200 mM Na_2EDTA	5 mL	Helix3	283	21 Mar 22

Equipment	Helix3 Equipment ID
pH Meter	HX0111
pH Electrode	HX0363

Buffer pH: 13.20 Time slides removed from Lysing: 10:51 (am) / pm

Electrophoresis according to SOP 903

Slide Positions & Replicates

790 C	804 C	806 C	X8
796 C	776 C	795 C	800 C
778 C	808 C	794 C	803 C
788 C	801 C	797 C	781 C
786 C	785 783	807 C	805 C
787 C	780 C	784 C	789 C
782 C	779 C	810 C	785 C
809 C	811 C	BLANK	BLANK

mA: 299
Volts: 25 /35 cm

Unwinding:
Temp.: 4.9 °C
Duration: 20 min

Electrophoresis:
Temp.: 4.8 °C
Duration: 40 min

Equipment	Equipment ID
Gel Box	HX0369
Power Supply	HX0004
Refrigerator	HX0023

Reagent	Source	Lot/Batch	Exp. Date
0.4M Tris	Helix3	340/338	18 Jul 22
Ethanol	Acros	B0541780A	12 Aug 25

Slide Storage Location: room 910

Performed by: KO/KB Date: 18 Mar 22

Page 2 of 2

Helix3

- Slides randomized with samples balanced across gel boxes
- Constant Voltage
- Starting current (300±10 mA) ensures consistency between same size gel boxes with slight volume variations*
- Current changes during run to prevent damage to power supply as conductivity of buffer changes*

*Vasquez, M.Z. and Frötschl, R. (2016) The In Vivo Comet Assay Test. In: Proudlock R, editor. Genetic Toxicology Testing: A Laboratory Manual. Academic Press. pp. 345–382

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Scoring

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- Andor (a.k.a. Kinetic) KometGLP
- “Ghosts” determined and excluded by image analysis system during scoring

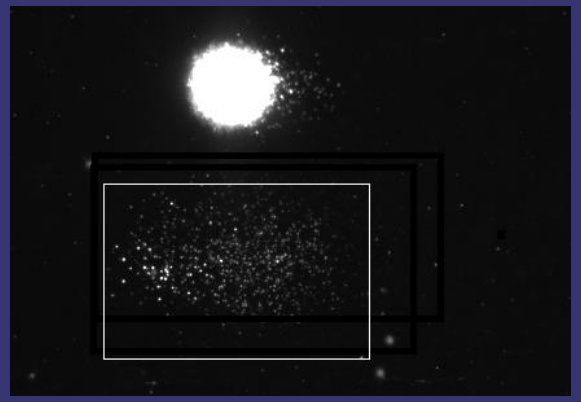
Other Lab

- Instem (a.k.a. Perceptives) Comet IV
- “Hedgehogs” determined manually by technician before image analysis scoring and subjectively excluded during scoring

Helix3

Hedgehog Definitions

Helix3



No discernible or accurate detection of head

Image from: Vasquez, M.Z. and Frötschl, R. (2016) The In Vivo Comet Assay Test. In: Proudlock R, editor. Genetic Toxicology Testing: A Laboratory Manual. Academic Press. pp. 345–382

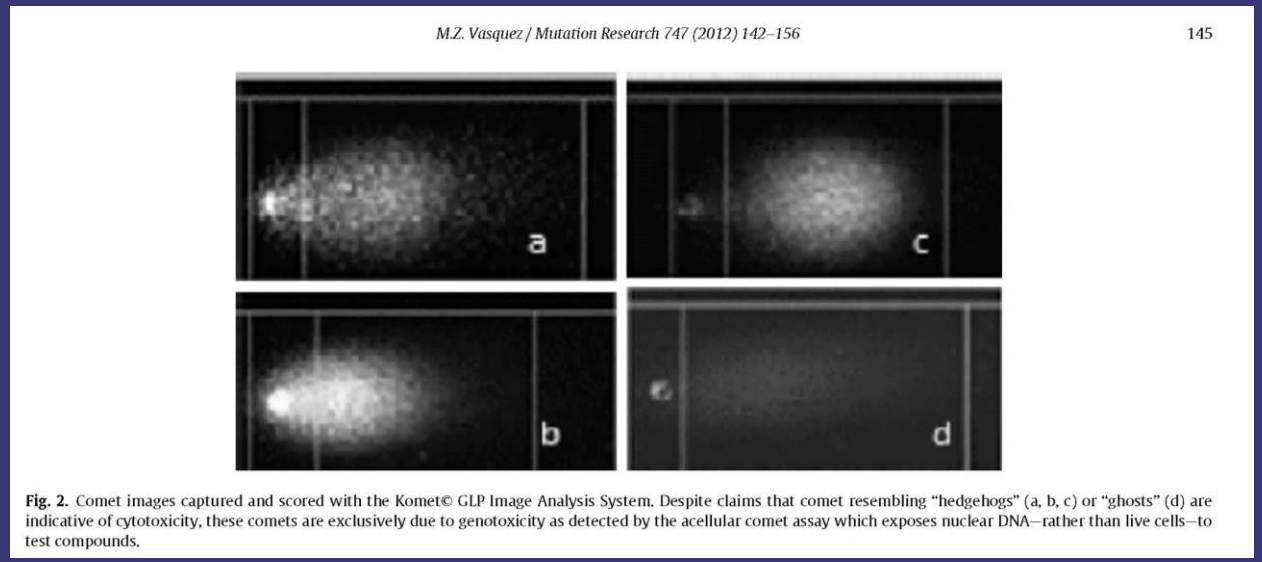


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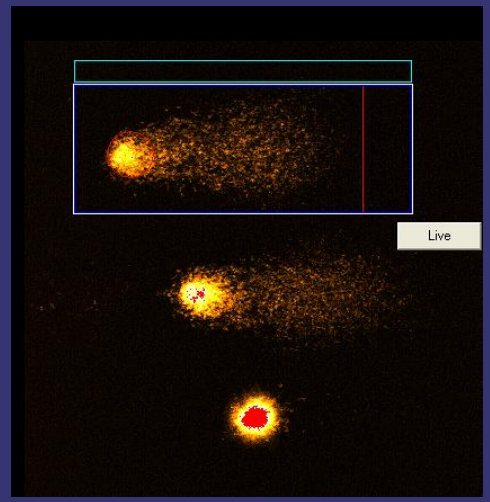
Fig. 2. Comet images captured and scored with the Komet© GLP Image Analysis System. Despite claims that comet resembling "hedgehogs" (a, b, c) or "ghosts" (d) are indicative of cytotoxicity, these comets are exclusively due to genotoxicity as detected by the acellular comet assay which exposes nuclear DNA—rather than live cells—to test compounds.

Other Lab



"high" tail migration

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Measurements

Duodenum

1	Head Len	Tail Length	Head Inte	Tail Intens	ail Mome	Total Area	Mean Gre	Total Inter	Width	Year
19	25.2353	12.05271	99.88028	0.11972	0.011559	411.5448	29.9445	86869	22.22218	2021
20	30.5084	11.67606	100	0	0	440.6268	24.6803	76657	22.22218	2021
21	27.4952	9.039532	100	0	0	372.3906	26.648	69951	21.84554	2021
22	12.4293	5.27306	99.4741	0.525896	0.02521	89.79933	9.913112	6275	10.92277	2021
23	26.7419	14.31259	95.40721	4.592795	0.349435	496.3789	26.17891	91600	25.23536	2021
24	26.7419	13.18265	99.89124	0.108755	0.013015	445.3082	28.41383	89191	23.35212	2021
25	26.7419	12.05271	99.74424	0.255764	0.002272	451.2665	26.42628	84062	24.85871	2021
26	25.2353	11.29941	100	0	0	421.1915	29.16773	86599	24.10542	2021
27	24.4820	15.06589	96.6732	3.326796	0.312834	422.752	22.41309	66791	21.84554	2021
28	27.4952	11.29941	100	0	0	386.8606	20.31243	55392	21.84554	2021
29	5.64970	6.779649	83.1648	16.8352	0.691601	155.0564	8.233303	8999	19.9623	2021
30	32.0150	17.32577	96.17217	3.827832	0.387861	858.1298	10.82724	65494	37.66472	2021
31	24.4820	12.42936	99.04419	0.955808	0.081052	393.3863	9.319149	25842	22.97548	2021
32	22.9754	11.29941	97.00938	2.990621	0.246077	338.2016	7.111158	16953	20.71559	2021
33	27.4952	11.29941	100	0	0	459.2108	13.13006	42502	25.98865	2021
34	25.9886	12.05271	95.69283	4.307174	0.261394	441.1942	12.5865	39144	24.48207	2021
35	22.2221	11.67606	98.46723	1.532766	0.139869	332.8108	13.93265	32686	20.33895	2021
36	28.2485	11.67606	100	0	0	413.8146	8.995201	26239	23.35212	2021
37	24.4820	11.67606	99.63335	0.366646	0.036847	287.8402	8.871858	18001	16.57248	2021
38	25.2353	10.92277	99.94233	0.057667	0.000521	337.3504	7.292262	17341	20.71559	2021
39	21.4688	10.92277	97.33173	2.668273	0.189021	316.7803	14.87013	33205	21.09224	2021
40	27.4952	11.29941	100	0	0	439.0663	11.04588	34187	24.10542	2021
41	28.2485	8.662885	100	0	0	371.1138	10.85054	28385	22.97548	2021
42	25.2353	9.792826	100	0	0	348.9832	9.304065	22888	22.22218	2021
43	23.7287	9.416179	100	0	0	295.7845	9.759712	20349	19.9623	2021
44	13.9359	6.026355	99.67312	0.326877	0.007524	124.2721	9.429224	8260	13.93595	2021
45	13.1826	5.649707	100	0	0	94.19709	6.349398	4216	10.92277	2021
46	22.9754	8.662885	100	0	0	258.1908	9.771978	17785	18.07906	2021
47	21.4688	11.29941	96.6749	3.325099	0.258405	332.1015	8.645878	20240	21.46889	2021
48	25.2353	12.05271	97.9217	2.078299	0.11233	401.4725	14.62191	41380	22.59883	2021
49	10.1694	4.143119	100	0	0	53.62425	7.15873	2706	8.286238	2021
50	25.2353	11.29941	99.9509	0.049102	0.003408	410.5518	14.77816	42768	24.10542	2021
51	23.7287	10.54612	100	0	0	320.6106	17.19469	38860	19.58565	2021

Other Lab

- ~ 30% cells in vehicle control group with 0% Tail
- Measurable tail length when 0% Tail
- Extremely low values despite actively dividing and heterogeneous cell population in tissue
- GI tract %Tail ≤ Liver %Tail
- Caused by camera settings/sensitivity, and/or selective scoring?

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Measurements

Duodenum

Slide	Repli	Cell_Area	Head_DNA	Tail_DNA	Length_to_HDiam	TExtMoment	Olive_TM	Tail_Length	Comet_Mode	Co
721	B	1412.57	89.82	10.18	2.63	4.21	1.45	41.37	332.56	
721	B	1252.15	87.25	12.75	2.04	3.67	1.13	28.79	148.84	
721	B	1344.43	96.66	3.34	2.21	1.11	0.61	33.26	118.83	
721	B	1369.72	74.2	25.8	3.47	10.57	2.29	40.96	244.55	
721	B	881.73	80.18	19.82	1.79	3.54	1.28	17.84	37.31	
721	B	1599.92	88.73	11.27	2.73	4.48	1.78	39.75	205.21	
721	B	2397.41	80.85	19.15	2.45	8.77	4.34	45.83	128.97	
721	B	701.44	98.93	1.07	0.83	0.06	0.12	6.08	69.76	
721	B	684.69	74.49	25.51	1.06	2.07	1.23	8.11	193.45	
721	B	739.7	95.98	4.02	0.9	0.26	0.27	6.49	230.76	
721	B	814.9	85.15	14.85	0.91	1.14	1	7.71	279.84	
721	B	641.18	97.15	2.85	0.83	0.16	0.22	5.68	57.59	
721	B	1117.51	94.43	5.57	1.66	1.31	0.66	23.52	248.61	
721	B	6674.37	16.57	83.43	6.31	94.4	29.71	113.15	201.56	
721	B	1221.45	97.52	2.48	1.87	0.71	0.45	28.79	203.19	
721	B	901.6	86.28	13.72	1.52	2.39	0.98	17.44	133.43	
721	B	664.66	92.93	7.07	0.88	0.46	0.4	6.49	234.82	
721	B	527.56	90.01	9.99	0.77	0.45	0.57	4.46	358.11	
721	B	553.67	81.91	18.09	1	1.32	0.97	7.3	31.63	
721	B	659.24	85.85	14.15	0.84	0.86	0.86	6.08	360.95	
721	B	586.51	82.79	17.21	1	1.26	0.98	7.3	224.28	
721	B	567.62	95.08	4.92	1.05	0.42	0.28	8.52	81.52	
721	B	618.03	87.01	12.99	1.18	1.21	0.81	9.33	113.56	
721	B	1201.09	71.96	28.04	2.95	11.15	2.53	39.75	77.46	
721	B	4008	52.61	47.39	3.44	29.4	14.18	62.05	232.39	
721	B	723.77	82.9	17.1	1.42	2.43	1.09	14.19	286.73	
721	B	1594.33	94.85	5.15	2.74	2.34	1.31	45.42	100.58	
721	B	556.95	97.72	2.28	0.75	0.09	0.19	4.06	217.38	
721	B	2565.55	60.06	39.94	2.96	19.12	6.48	47.86	133.02	
721	B	1288.44	97.62	2.38	1.53	0.64	0.46	26.77	77.87	
721	B	2422.86	80.53	19.47	3	10.26	4.16	52.72	129.37	
721	B	617.54	81.82	18.18	1.32	2.06	1.11	11.36	354.46	
721	C	5657.18	26.39	73.61	5.57	66.58	17.86	90.44	98.96	
721	C	865.8	93.94	6.06	1.09	0.64	0.47	10.54	122.07	
721	C	1922.89	70.61	29.39	3.32	14.78	4.49	50.29	215.35	
721	C	1216.03	80.17	19.83	1.71	4.67	1.69	23.52	142.76	
721	C	4289.93	49.91	50.09	4.38	33.11	13.58	66.11	166.28	
721	C	1806.47	67.76	32.24	2.13	11.11	3	34.47	103.42	
721	C	1034.59	94.76	5.24	1.42	0.98	0.51	18.66	103.82	
721	C	785.67	78.48	21.52	1.45	3.14	1.33	14.6	250.23	
721	C	767.12	80.72	19.28	1.13	1.95	1.14	10.14	307.42	
721	C	1633.74	88.28	11.72	2	3.71	1.67	31.63	262.4	
721	C	726.4	93.84	6.16	0.89	0.42	0.47	6.89	38.93	
721	C	3063.72	58.1	41.9	3.31	22.94	8.32	54.75	227.52	
721	C	1280.72	80.8	19.2	2.17	6	2.03	31.23	105.45	
721	C	899.3	81.76	18.24	1.17	2.07	1.19	11.36	63.27	
721	C	1452.14	55.49	44.51	3.06	16.61	3.67	37.31	40.56	

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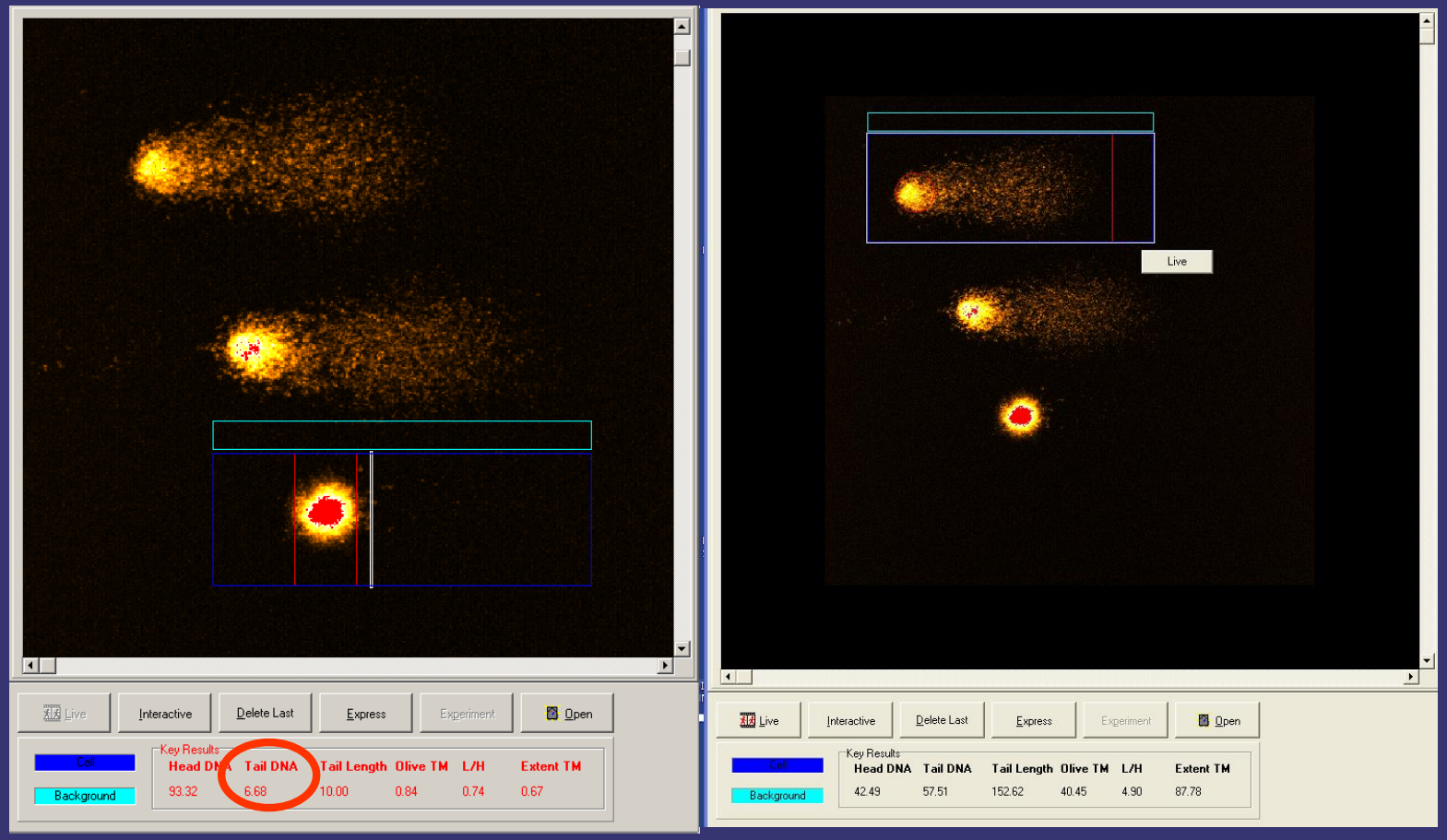
- Zero cells in vehicle control group with 0% Tail
- ≤0.5% of total study comets (n=13500) scored = 0% Tail
- Tail measured from edge of head giving zero tail length when 0% Tail
- GI Tract %Tail > Liver %Tail

Slide	Replicate	Cell_Area	Head_DNA	Tail_DNA	TLength_to_HDiam	TExtMoment	Olive_TM	Tail_Length	Cor
756	C	2165.57	80.2	19.8	2.56	8.59	3.58	43.4	
756	C	1011.93	98.56	1.44	1.31	0.25	0.21	17.03	
756	C	916.21	97.21	2.79	0.87	0.21	0.31	7.71	
756	C	714.74	99.24	0.76	0.88	0.05	0.08	6.49	
756	C	1568.06	91.85	8.15	2.17	2.64	1.35	32.44	
756	C	746.43	99.53	0.47	0.76	0.03	0.07	5.68	
756	C	5189.88	36.43	63.57	5.53	49.24	20.29	77.46	
756	C	1212.58	68.02	31.98	2.36	8.69	2.31	27.17	
756	C	906.03	72.96	27.04	1.67	4.61	1.74	17.03	
756	C	919.82	87.86	12.14	1.65	2.27	1.05	18.66	
756	C	841.5	99.7	0.3	0.74	0.02	0.04	5.27	
756	C	2351.27	75.89	24.11	4.72	13.2	6.62	54.75	
756	C	897.49	95.8	4.2	1.57	0.77	0.42	18.25	
756	C	714.25	99.6	0.4	0.7	0.02	0.05	4.06	
756	C	636.09	100	0	-1.62	0	0	0	
756	C	1306.34	91.18	8.82	2.28	2.93	1.03	33.26	
756	C	792.41	83.35	16.65	1.1	1.62	1.03	9.73	

Helix3

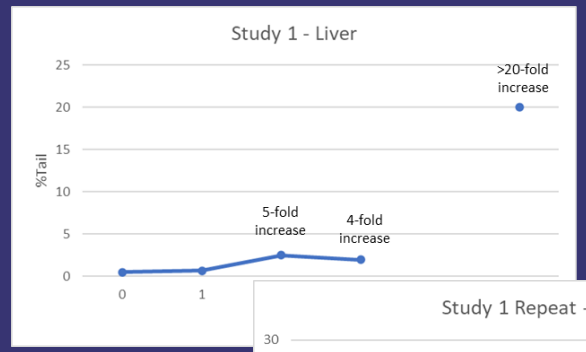
Normal Cell Population Heterogeneity

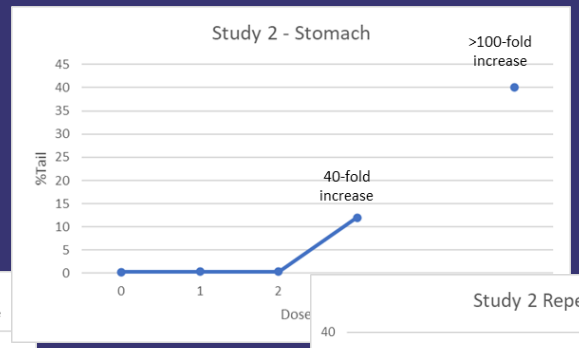


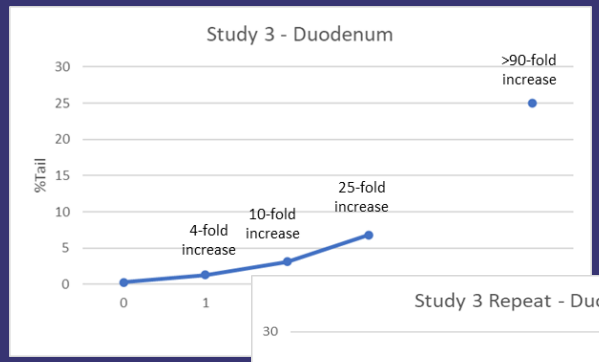
- Untreated whole blood leukocytes (non-dividing cell population)
- ~7% Tail even in low migration cells
- %Tail range from 6.68-57.51 in same cell population

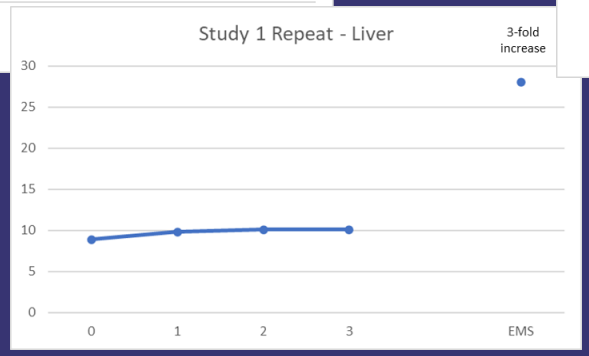
Statistical Analysis

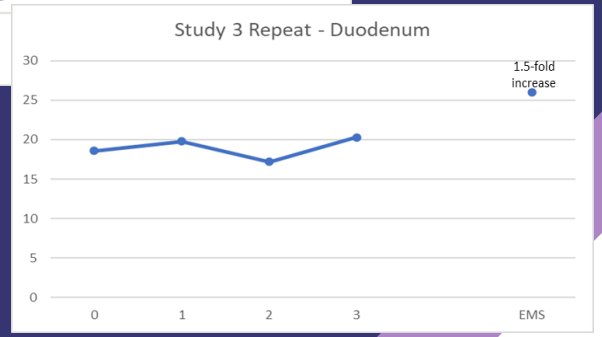
- Animal means of slide medians; Group mean of means (mean of medians) presented*
- Helix3 replicated the same statistical method of original lab (e.g., log transformations, specific tests).
- *Helix3 results the same for mean of medians vs. mean of means

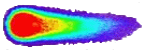












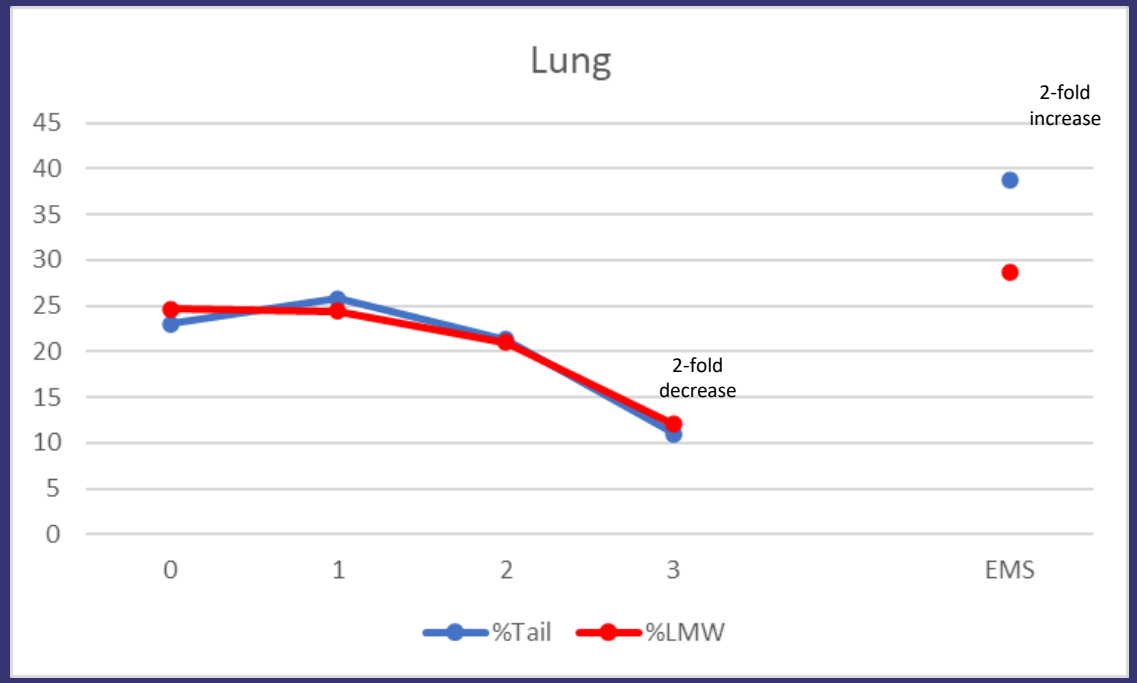
Regulatory Decisions

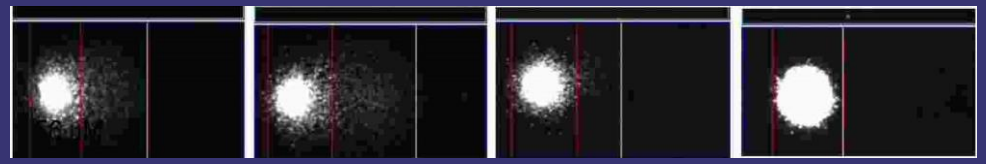
In all three case studies:

- The concerns regarding the quality of the previous study data and its potential susceptibility to statistical artifacts appeared to be confirmed by the negative results generated when technical variations were more carefully controlled
- Regulators accepted the Helix3 negative results over the previous positive results
- The FDA specifically inspected the Helix3 studies and reported no findings
- The test compounds were progressed to clinical trials with at least one progressing to Phase III

Helix3

Case Study 4





Cisplatin Dose Response

Image from: Vasquez, M.Z. and Frötschl, R. (2016) The In Vivo Comet Assay Test. In: Proudlock R, editor. Genetic Toxicology Testing: A Laboratory Manual. Academic Press. pp. 345–382

- Ames negative; Chrom Ab positive compound
- Inhalation study in rats
- Standard processing and electrophoresis
- Statistically significant and dose related decrease in %Tail and %LMW
- Unusually high image intensities noted in high dose slides
- Persistent trend with PK slide treatment
- Evidence of significant hyperplasia and metaplasia in lung
- **Conclusion: Positive for DNA-DNA crosslink induction in lung similar to ames negative monocrotaline pyrrole (Wagner et al. 1993)**

Helix3

Conclusions

1. Methodical bias can increase the risk of false positive results
2. To minimize methodical bias:
    a. Animals and samples/slides should be coded throughout processing
    b. Doses should be administered and samples processed in a rotating and balanced manner (vs in order by dose group)
    c. Dosing and sample collection timing should be maintained consistent between animals
    d. Environmental conditions (%RH) during slide preparations should be recorded and maintained consistent

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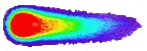
Conclusions

3. “Hedgehogs” or “ghosts”

    a. Are just cells with high levels of DNA damage and NOT indicative of cytotoxicity (Vasquez 2010, 2012, 2016; Hartmann et al. 2007; Collins et al. 2008; Morley et al., 2006; Meintieres et al., 2003)

    b. May only be indicative of Image analysis (IA) system/setting sensitivity

    c. Should be determined by IA system exclusions during scoring to minimize subjective cell exclusions and possible scoring bias



Helix3

Conclusions

4. Emphasis on HCD should be modified to:

   a. Eliminate HCD requirement for proof of proficiency with every tissue, species, strain, etc. to comply with 3Rs

   b. Eliminate use of HCD to qualify results and instead rely on concurrent control data to demonstrate sensitivity and interpret results

   c. Use HCD to identify possible poor control over technical variations (e.g., 100-fold difference between min and max values for similar vehicles/study designs)

   d. Use HCD to determine when comet may be inappropriate for evaluating a certain tissue with a specific vehicle and route(e.g., high background level in GI tract tissue caused by corrosive vehicle administered orally)

Helix3

Additional Concerns

1. Background levels close to zero (0-2%) with low variability (±0.01 to 0.1 SD) likely increase the risk of statistical artifacts and the false positives while eliminating the ability to detect crosslinks

2. ≥20-fold increases in the positive control biases scoring and/or misrepresents the sensitivity of the assay

   Note: Other labs reported "hedgehog" increases in affected TA doses, but rarely in the positive control. Helix3 reported no "ghost" increases.

3. Directives/ attempts to decrease background levels close to zero and/or <u>below</u> a specified %Tail value for every tissue, vehicle, and experimental design create technical bias, statistical artifacts, and unreliable results