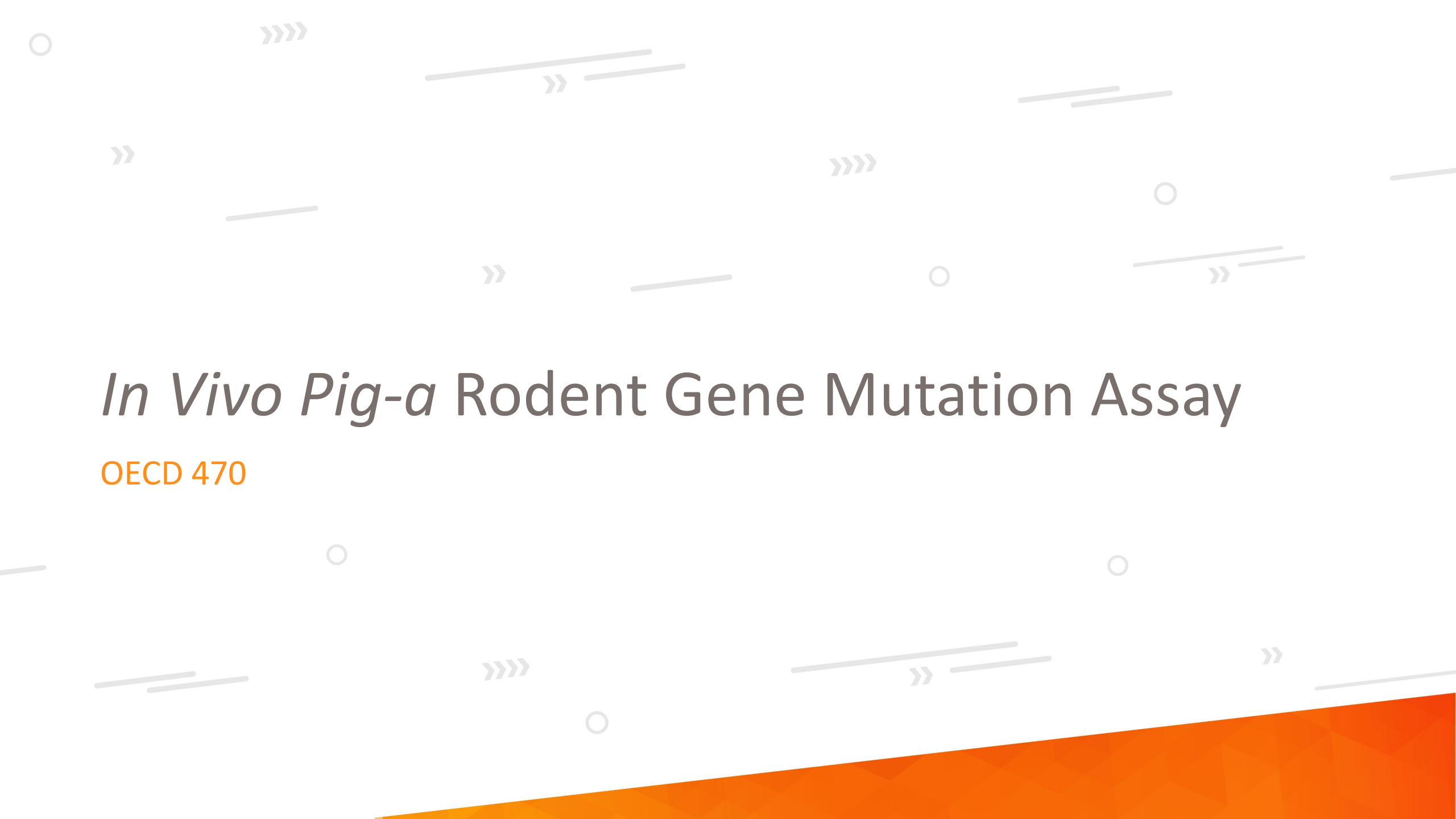


WHERE
insights LEAD TO
answers



In vivo Mutation Assays (*Pig-a* and Duplex Sequencing)

Christopher Sproul, PhD DABT

The background features a light gray field with various abstract elements: small circles, double chevrons (»), and several thin, parallel lines of varying lengths and orientations. These elements are scattered across the upper and middle portions of the slide. At the bottom, there is a large, solid orange shape that tapers from left to right, creating a sense of movement or a rising plane.

In Vivo Pig-a Rodent Gene Mutation Assay

OECD 470

Pig-a vs. TGR Assays

TGR Mutation Assay

- Advantages
 - > Can evaluate any organ or germ cells
 - site of metabolic action
 - first site of contact
 - fast and slow proliferating tissues
 - > Can store frozen tissue for subsequent analysis
 - > OECD test guideline (488)
- Disadvantages
 - > Requires use of expensive transgenic animals
 - > Restricted licensing resulting in long queues for starting studies
 - > Can't evaluate pre-dosing baseline MF (except for blood)
 - > Can't evaluate MF at multiple time points (except for blood)
 - > Laborious, lengthy analysis procedure involving manual scoring
 - > Can't assess mutation spectrum

Pig-a Gene Mutation Assay

- Advantages
 - > Any strain of rat or mouse
 - > Rapid, automated scoring by flow cytometry
 - > Can evaluate blood samples collected pre-dosing
 - to ensure no animal with an atypically high baseline mutation frequency (MF) is put on study
 - to evaluate sporadically elevated MFs following dosing (*i.e.*, jackpot mutation?)
 - > Can evaluate MF at multiple time points
 - > Can store frozen blood for subsequent analysis
 - > OECD test guideline (470)
- Disadvantages
 - > Requires bone marrow exposure
 - > Can't assess MF in other organs
 - > Can't assess mutation spectrum

Considerations for Use of the *Pig-a* Assay

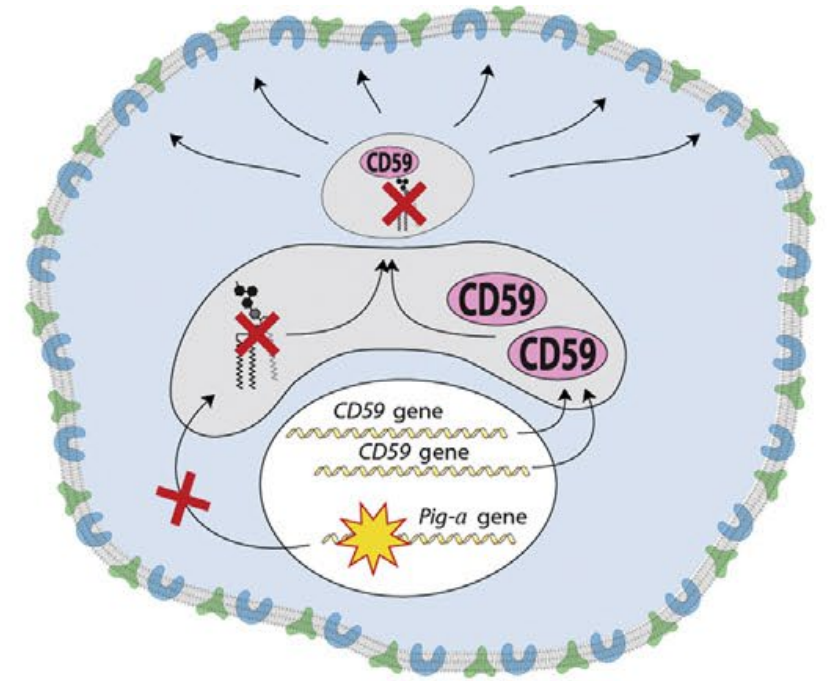
- If an *in vitro* positive result did not require metabolic activation (*i.e.*, -S9 or $\pm$ S9 conditions), one must be able to demonstrate exposure of bone marrow to the test article.
 - > Exposure can be ensured via intravenous dosing.
- If an *in vitro* positive result required metabolic activation (*i.e.*, +S9 condition), one must assume cause is a metabolite; unless the mutagenic metabolite is known and can be tested for in bone marrow/plasma/serum, the *Pig-a* assay will not be suitable.

Useful Tip:

- Tissues can be collected on *Pig-a* (or repeat dose toxicity) studies and stored for possible duplex sequencing to assess mutation frequency.

Pig-a Gene

- Phosphatidylinositol glycan, class A gene (*Pig-a*)
- The *Pig-a* gene product is essential to the synthesis of glycosylphosphatidylinositol (GPI) anchor proteins.
- GPI anchors attach proteins such as CD59 (rat) and CD24 (mouse) to the cell surface of red blood cells.
- *Pig-a* gene is located on the X-chromosome.
 - > Requires only a single hit to knock out function
 - > Can be used as a reporter gene



Mutation in
Pig-a gene



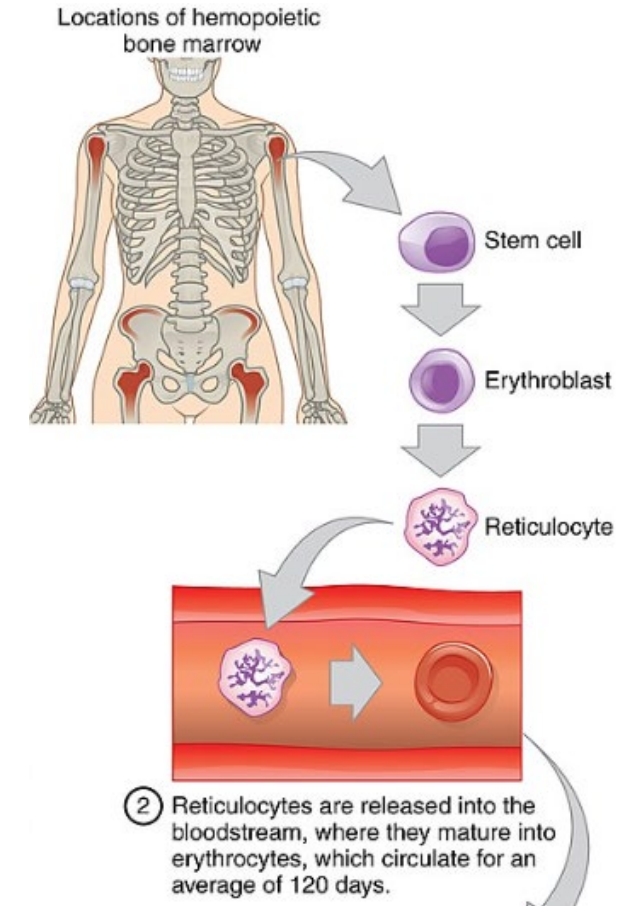
Loss of functional
GPI anchors



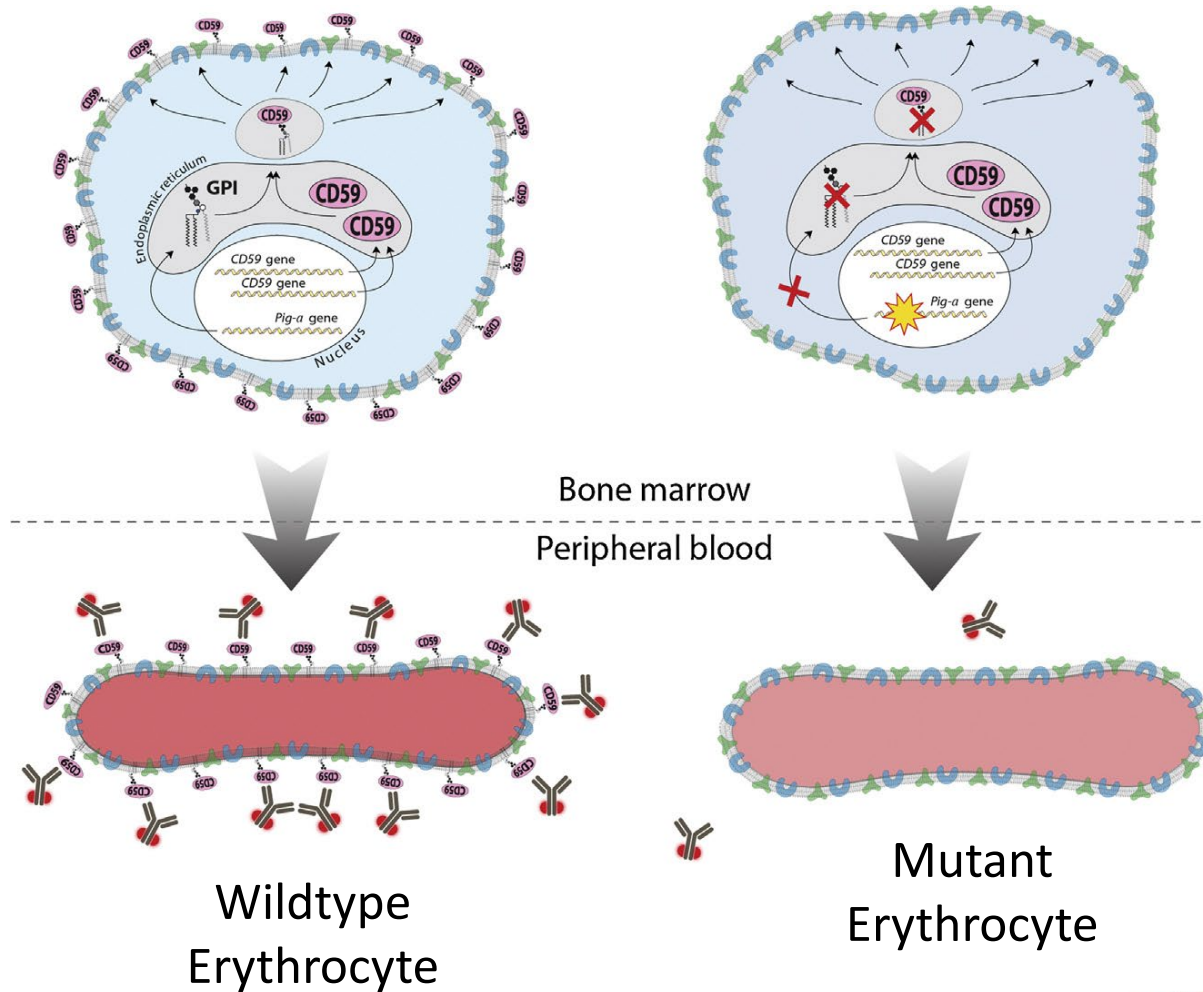
Lack of cell surface
markers

Erythropoiesis and Mutagenesis in the *Pig-a* Assay

- Need damage/alteration to DNA structure or sequence (*Pig-a* gene) that results in loss of expression/function of the target protein (responsible for attaching GPI anchors to cell surface markers such as CD59)
- That alteration to the DNA needs to be heritable and replication needs to take place so that subsequent daughter cells acquire the mutation
- The target of the *Pig-a* assay are dividing progenitor cells in the bone marrow that still have a nucleus and are dividing/differentiating into reticulocytes and erythrocytes; 15-28 days to manifest as peripheral RET and RBC mutants.

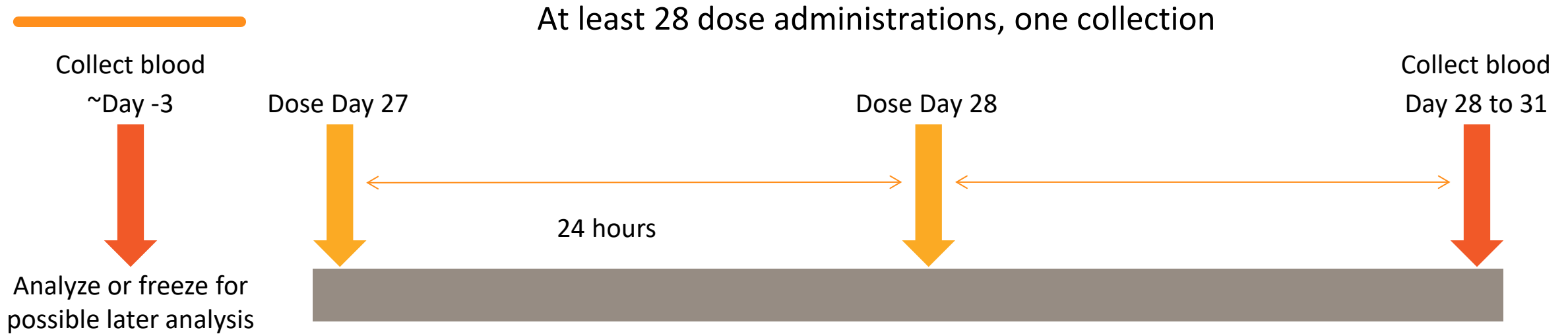


Pig-a Gene Mutation Assay



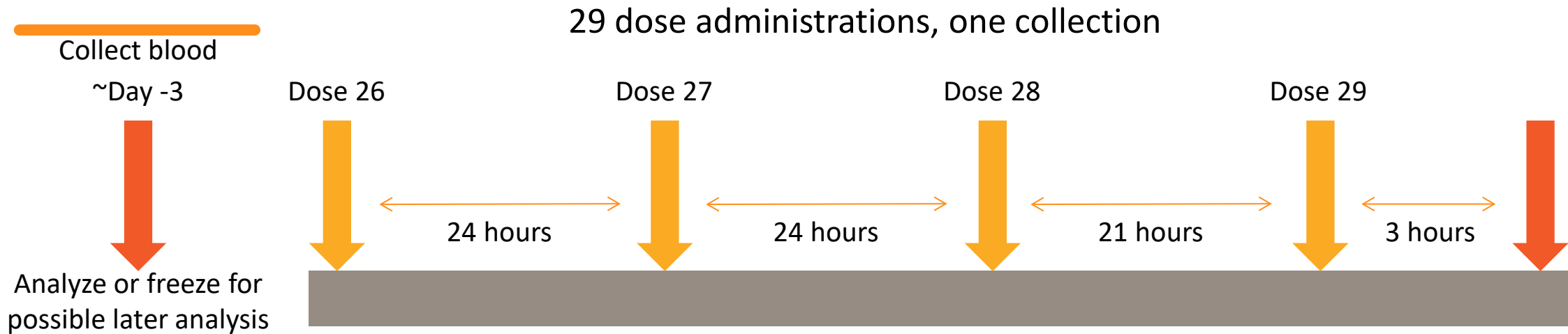
- Assay for loss of:
- CD59 (rat)
 - CD24 (mouse)

Pig-a Gene Mutation Assay Design



- ENU positive control dosed on days 1-3
- At necropsy, blood collected, stabilized with EDTA, and frozen at -80°C ; blood (plasma) collected for potential bioanalytical evaluation (exposure confirmation).
- Blood cells thawed, washed, labeled with antibodies, and analyzed on a flow cytometer.
- RET and total red blood cells evaluated for *Pig-a* mutant frequency (genotoxicity); concurrent measurement of RET frequency (cytotoxicity).

Combined *Pig-a*/MN/Comet Assay Design



- ENU on days 1-3, EMS on days 27-29
- At necropsy:
 - > blood collected, stabilized with EDTA, and frozen at -80°C for the *Pig-a* assay, AND
 - > blood collected, stabilized with EDTA or heparin, fixed and frozen at -80°C for the MN assay, AND/OR
 - > bone marrow harvested and slides prepared for the MN assay (potential bone marrow for CREST), AND/OR
 - > tissue(s) harvested and frozen for the comet assay and possible histopathology (cytotoxicity/exposure confirmation)
 - > blood (plasma) collected for potential bioanalytical evaluation (exposure confirmation)

Pig-a Gene Mutation Assay Design

Any strain of rat or mouse

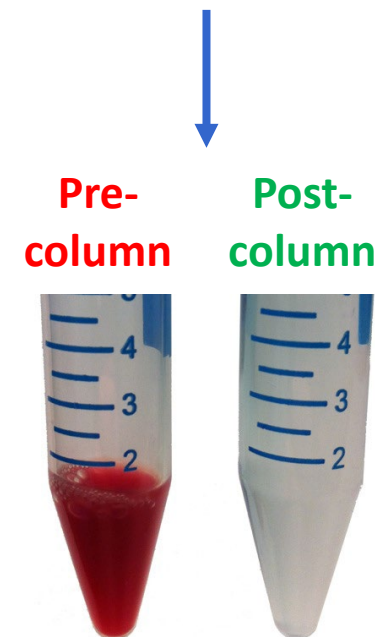
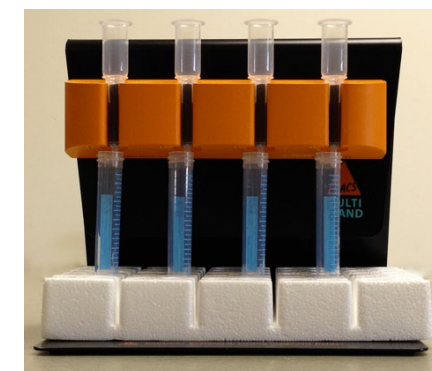
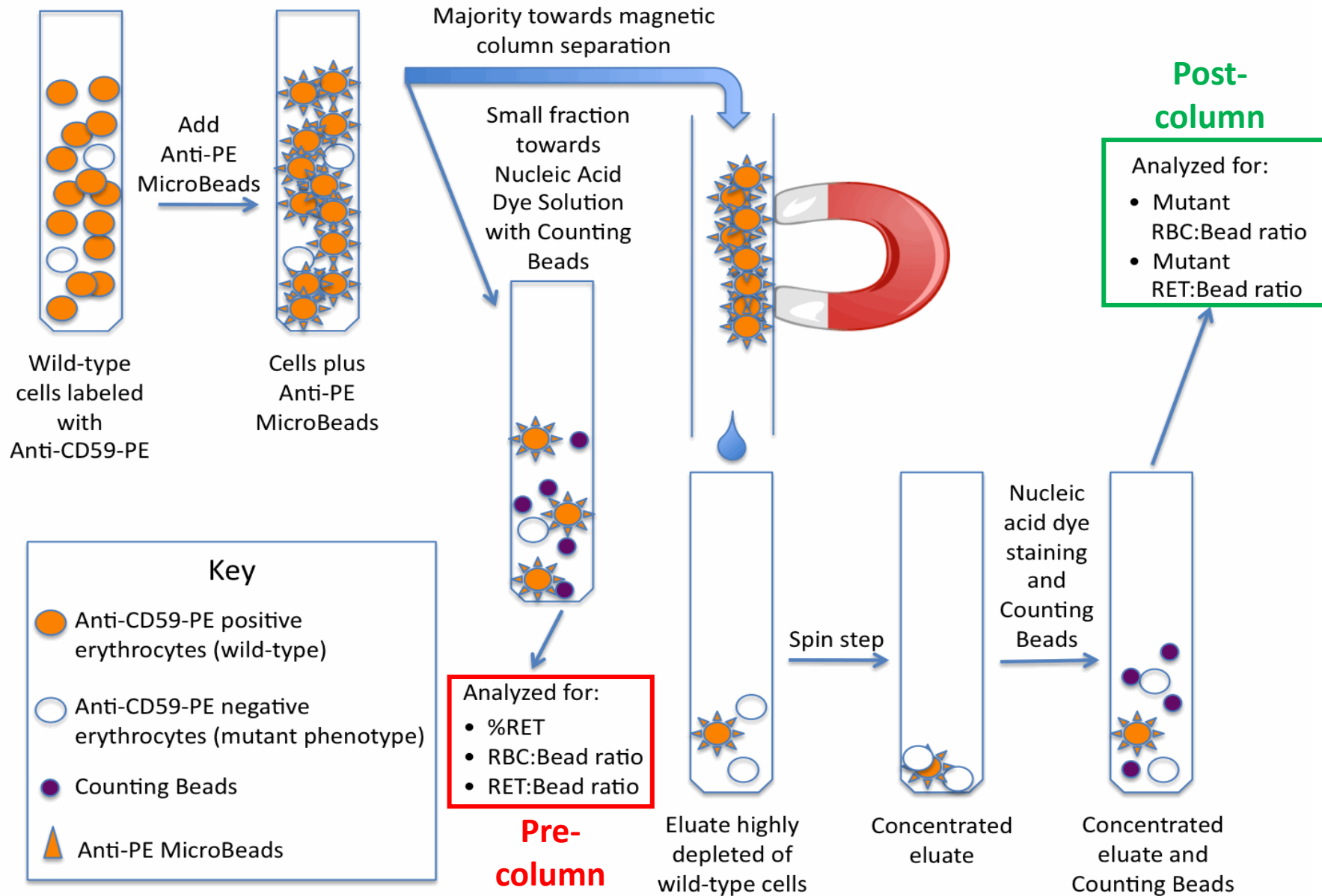
Vehicle control and three test article dose levels – 6 animals/group

Positive control group not required (but recommended by Inotiv)

Blood collected into K₂EDTA tubes at interim or terminal timepoints

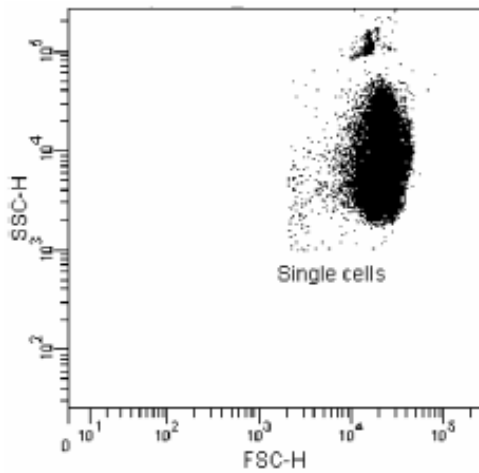
Analysis using flow cytometry and MutaFlow^{PLUS} assay kit (Litron Laboratories).

- Mutation frequency can be assessed across multiple time points.
- Blood transferred to freezing solution (Litron Laboratories) and stored at -80°C for subsequent analysis
- Option #1: analysis pre-dosing to screen out animals with pre-existing “jackpot” mutations
- Option #2: collect and freeze blood pre-dosing for possible later analysis to help interpret results

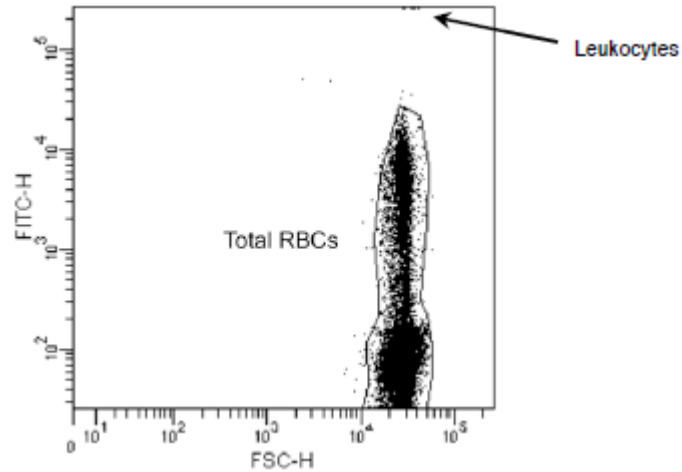


Flow Cytometry Set-Up Gating

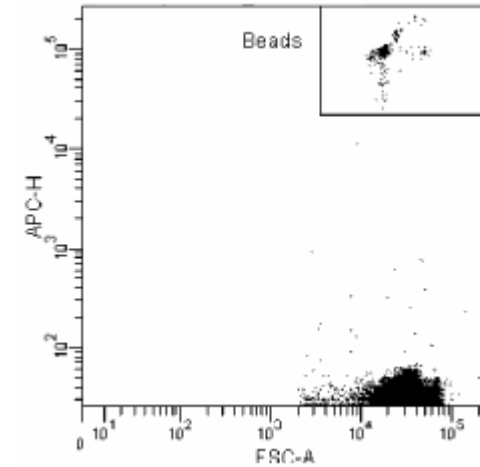
BD FACSCalibur:



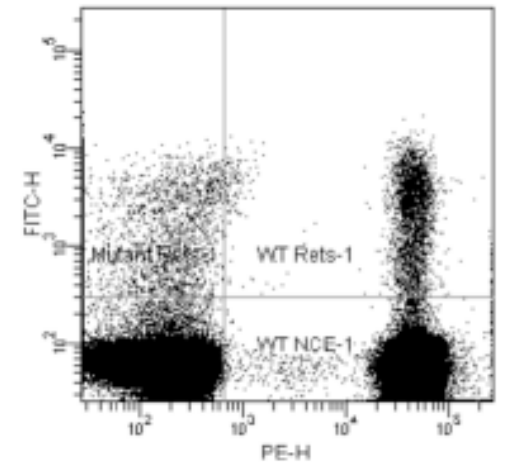
Single cell gate



Total red blood cells gate



Bead gate

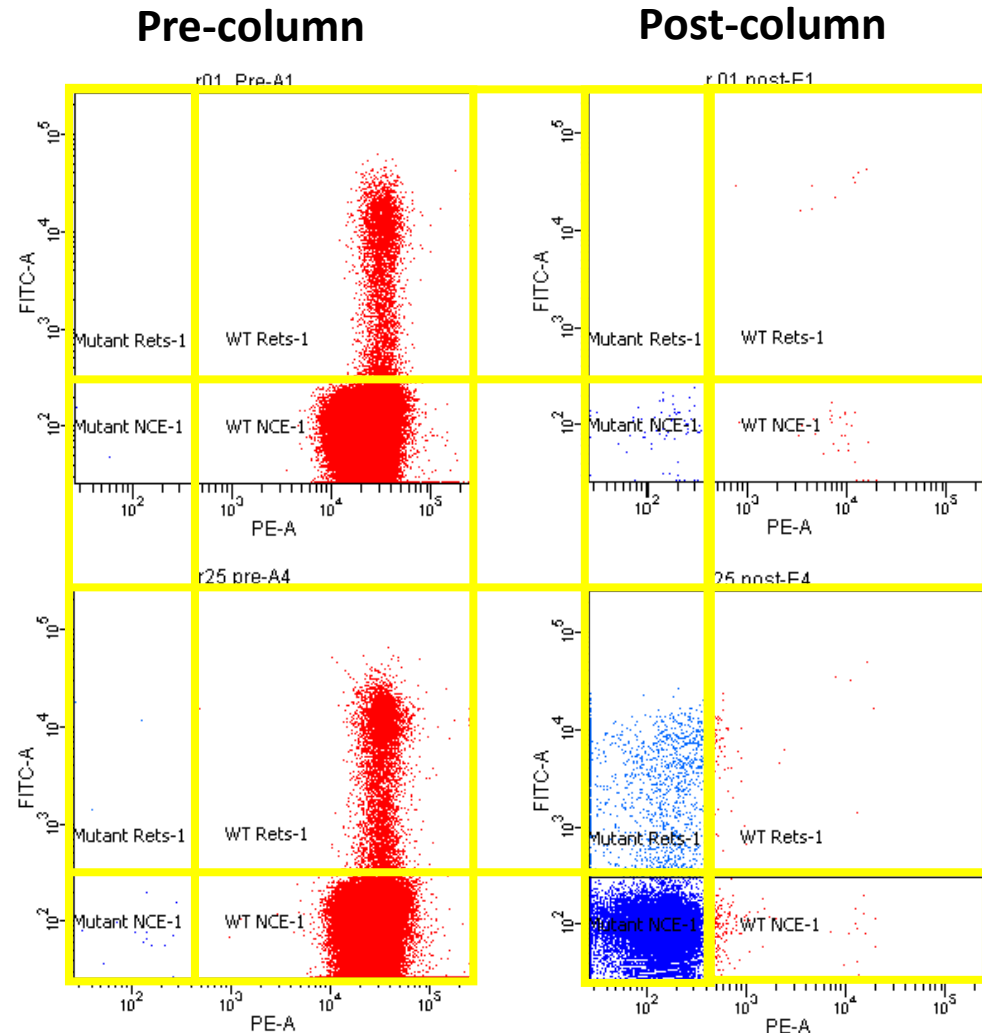


Internal Calibration Control
(mutant mimic)

Flow Cytometry Plots

BD FACSCanto:

Vehicle



N-Ethyl-*N*-nitrosourea

RETs = reticulocytes (young erythrocytes)
NCE = normochromatic erythrocytes
(mature erythrocytes)
WT = Wild type

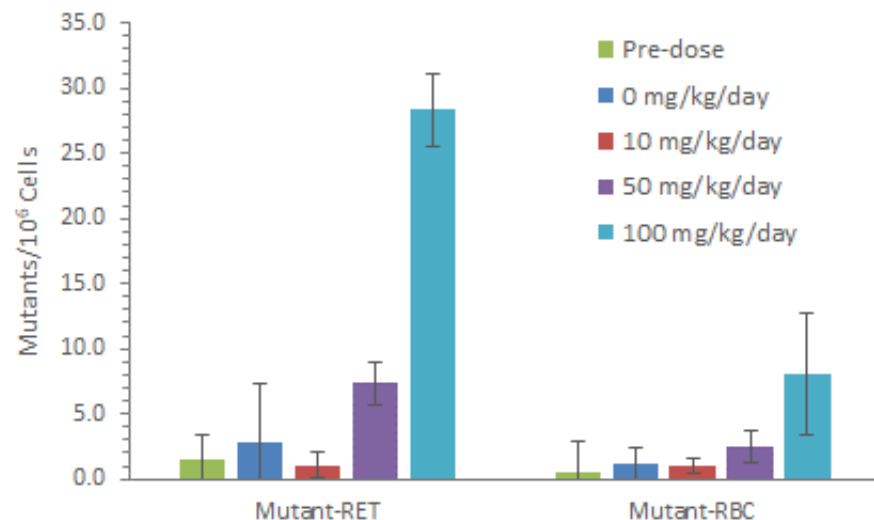
Total red blood cells = RETs + NCE

- Minimum of $1-3 \times 10^6$ RET and total red blood cells evaluated

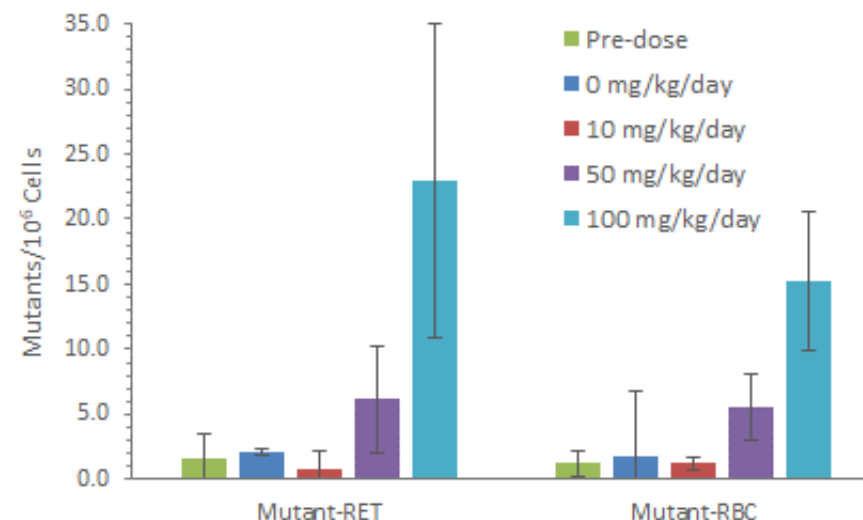
Pig-a Mutant Frequency Dose Response to Benzo[a]pyrene (B[a]P)

SD rats dosed with B[a]P by oral gavage for 10 days (5 days per week for 2 weeks)
Blood sampled on day 14 or day 28

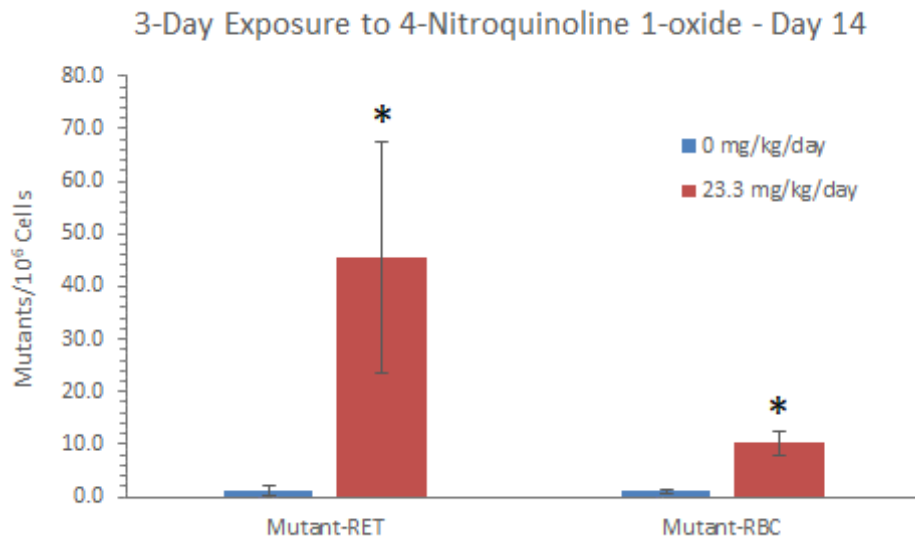
10-Day Exposure to Benzo[a]pyrene - Day 14



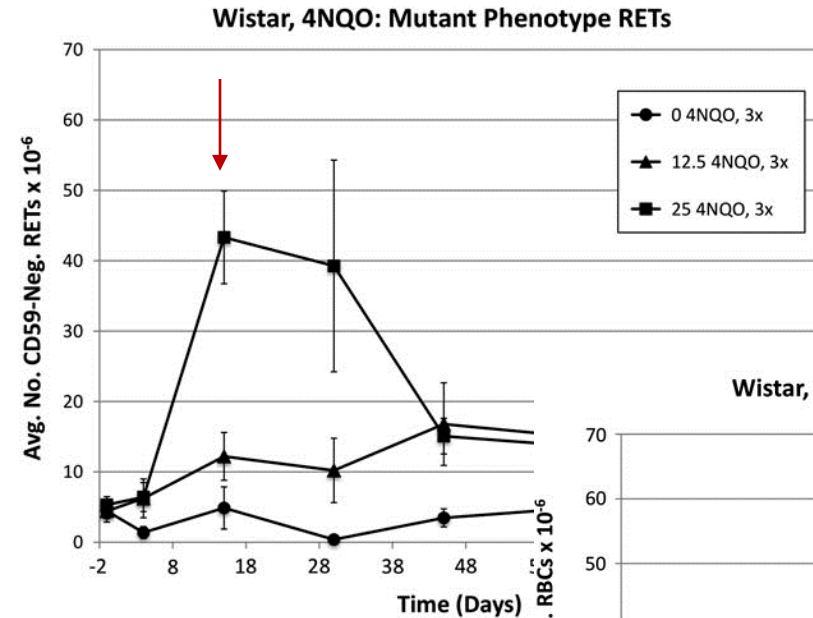
10-Day Exposure to Benzo[a]pyrene - Day 28



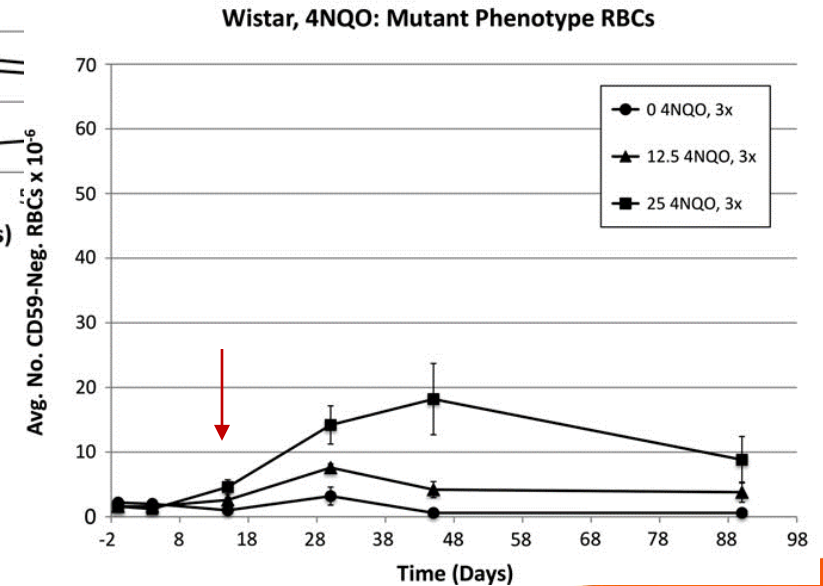
Pig-a Mutant Frequencies in Response to 4-nitroquinoline 1-oxide (4NQO): Comparison with Published Results



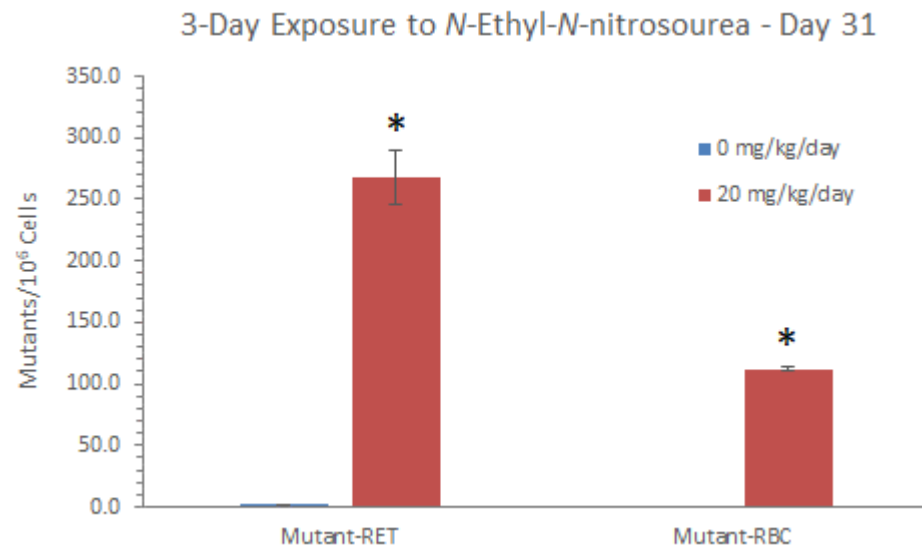
SD rats dosed with 4NQO by oral gavage for 3 days
Sampled on Day 14



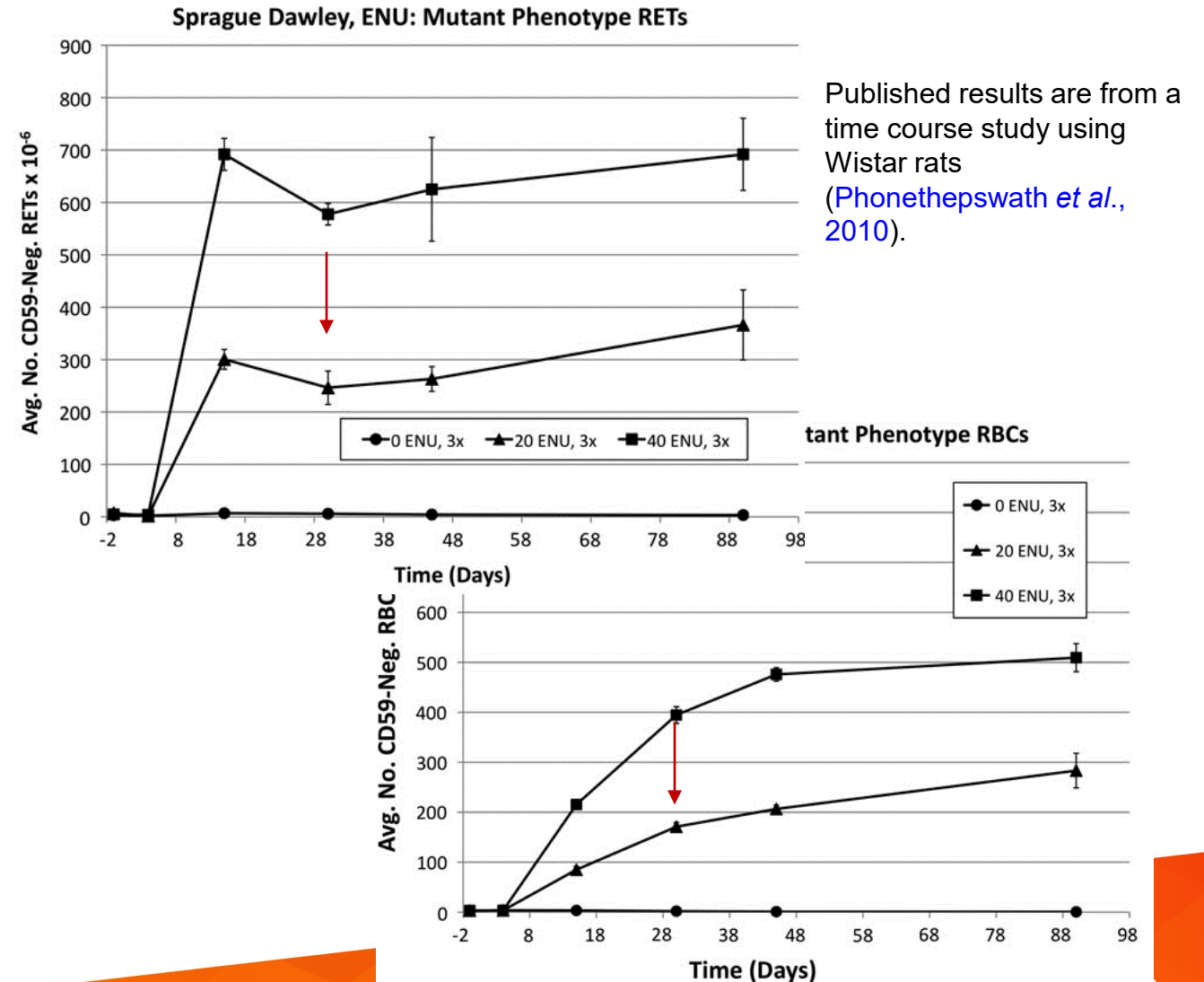
Published results are from a time course study using Wistar rats ([Phonethepswath et al., 2010](#)).



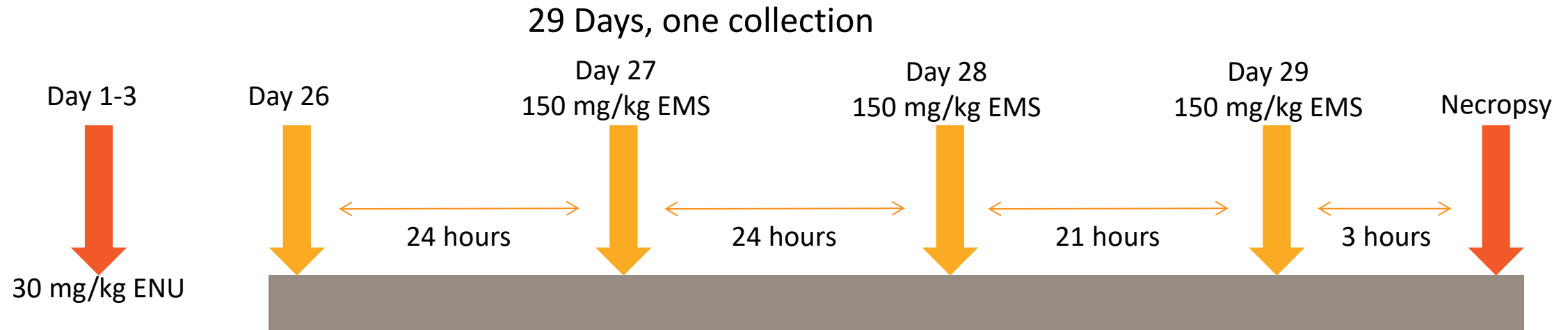
Pig-a Mutant Frequencies in Response to *N*-ethyl-*N*-nitrosourea (ENU): Comparison with Published Results



SD rats dosed with ENU by oral gavage for 3 days
Sampled on Day 31



Combined *Pig-a*/MN/Comet Assay Design: Historical Control Studies in Support of an EFSA submission for Oral Exposure to Styrene

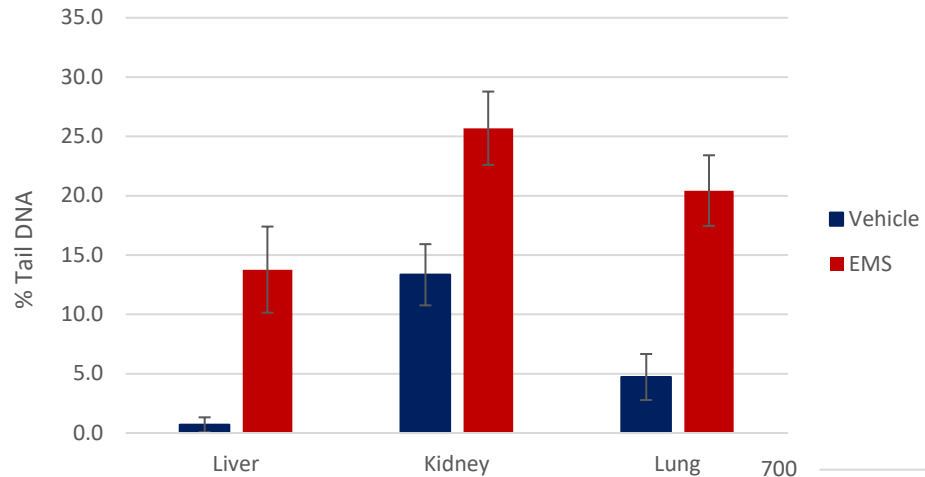


- At necropsy:
 - > blood collected, stabilized with EDTA, and frozen at -80°C for the *Pig-a* assay, AND
 - > blood collected, stabilized with EDTA or heparin, fixed and frozen at -80°C for the MN assay, AND
 - > tissues harvested for the comet assay

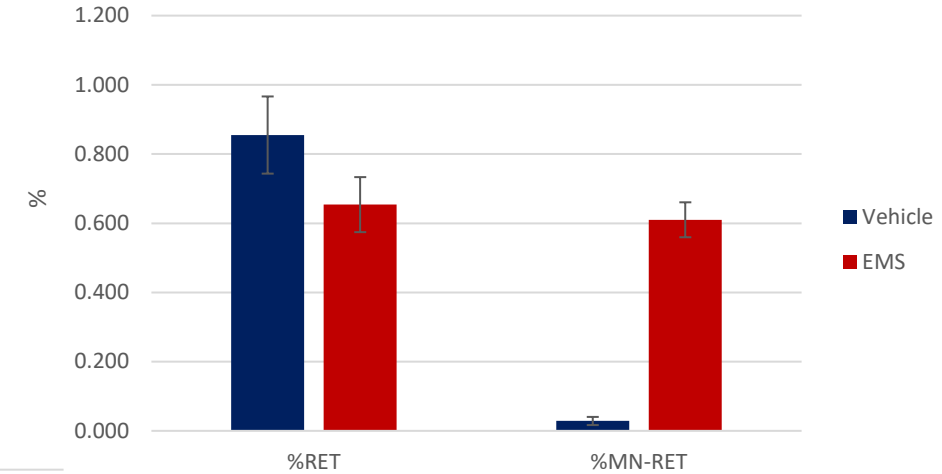
See: Gollapudi BB. Investigations on the genotoxic potential of styrene in Fischer 344 rats using multiple endpoints. *Environ Mol Mutagen.* 2024

Combined *Pig-a*/MN/Comet Assay Design: Fisher F344 Rats

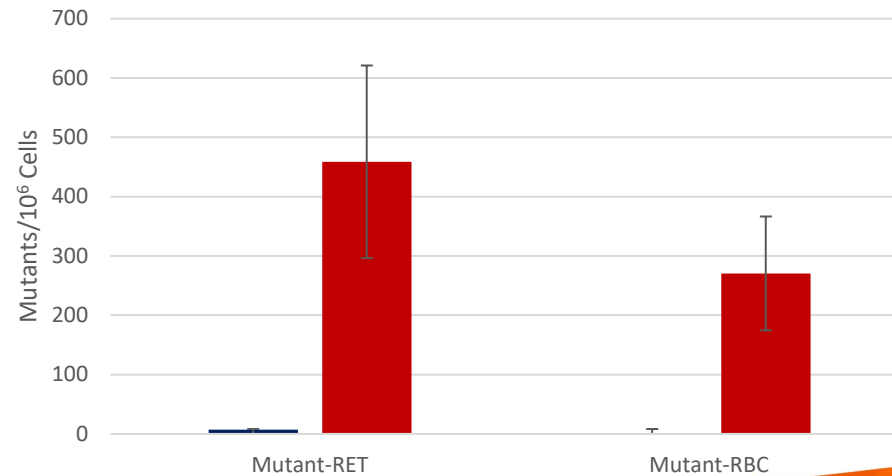
Comet Results



MN Results

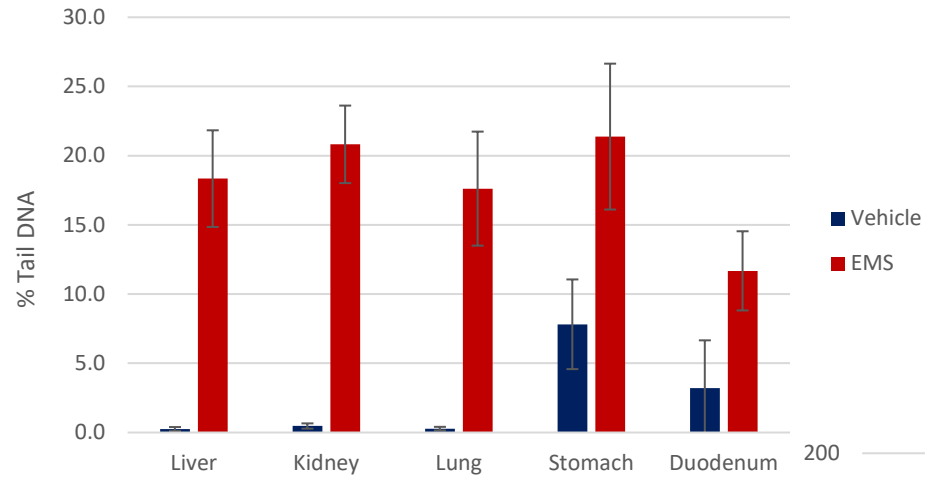


Pig-a Results

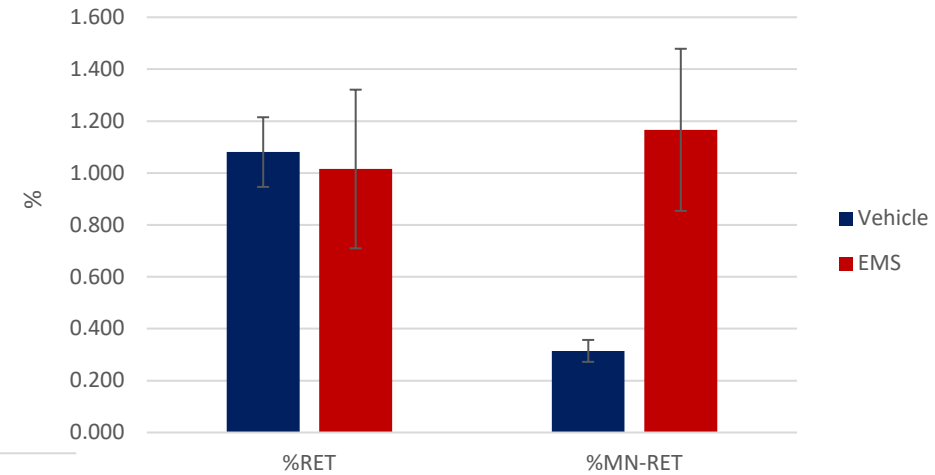


Combined *Pig-a*/MN/Comet Assay Design: B6C3F1 Mice

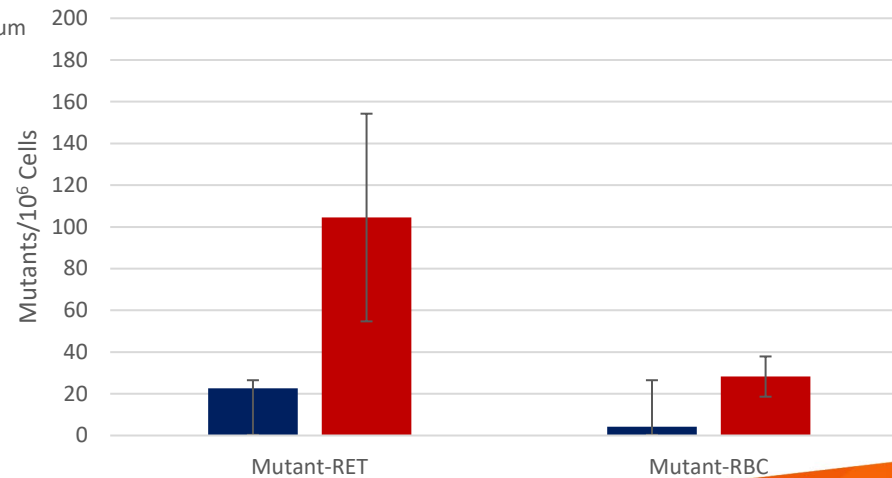
Comet Results



MN Results



Pig-a Results



Considerations and Acceptance Criteria for a Valid *Pig-a* Assay

- Highest dose up to the MTD, MFD, or limit dose of 1000 mg/kg/day (≥ 14 days dosing) with subsequent doses typically @ 2–4-fold intervals
 - > MTD may be defined as dose that causes a statistical decrease in %RET, but should not go below 20% of vehicle
 - > When MTD is less certain, adding a 4th dose group can be a contingency against loss of animals in the high dose group to help ensure 3 usable dose groups
 - > Doses should span max toxicity to little no toxicity, so adding a 4th dose group may be useful if this range is large
- At least 3 dose groups and vehicle, at least 6 animals per group (we use 7 to ensure 6 surviving animals per group)
- At least 1×10^6 RET and RBC scored per animal for *Pig-a* mutant frequency
- Negative control data should not exceed the upper bound of the negative HCD
- If positive control used, it should cause a statistical increase in mutant frequency (RET and RBC) and be “compatible” with HCD

Interpretation of the *Pig-a* Assay

- Assuming a valid test, the test article is clearly negative if all the following are met:
 - > No dose level shows a statistical increase in mutant RET / RBC frequency compared to the concurrent negative control group
 - > There is no dose related trend for increased mutant RET / RBC frequency when evaluated by an appropriate trend test
 - > No dose group has mutant RET / RBC frequencies that exceed the upper bound of the control limit for the laboratory's HCD
 - > There is evidence of exposure to the bone marrow:
 - Direct systemic bioanalysis of test article or related metabolite (dose related levels implied)
 - Previous PK/ADME data from an independent study using the same dosing route and species
 - Indirect evidence (may be less compelling): Clinical signs related to the central nervous system (e.g., change of spontaneous activity, ataxia, sedation, staggering), that usually only occur if the test article is systemically bioavailable.

Interpretation of the *Pig-a* Assay

- Assuming a valid test, the test article is clearly positive if all the following are met:
 - > At least one dose level shows a statistical increase in mutant RET / RBC frequency compared to the concurrent negative control group
 - > There is a dose related trend for increased mutant RET / RBC frequency when evaluated by an appropriate trend test
 - > At least one dose level shows mutant RET / RBC frequencies exceed the upper bound of the control limit for the laboratory's HCD
 - > There is no need to demonstrate bone marrow exposure if all other criteria for a positive test are clearly met

Interpretation of the *Pig-a* Assay

- What if some criteria are met for a clearly positive test, but not all?
- Additional investigation and/or a weight of evidence approach and sound scientific judgment should be applied in an effort to interpret the results as positive or negative
 - > Is the increase in mutant frequency dose related (e.g. is only a low or middle dose “positive”)
 - > What is the magnitude of the response (e.g. a statistical increase that is only 20% elevated may not be relevant)
 - > Is the increase in mutant populations consistent in both RET and RBC (e.g. an increase in only one population is suspect)?
 - > What is the number of individual animals in each group that exceed HCD and is that dose related?
 - > Criteria a (pairwise comparisons) and b (trend test) hold more weight than c (comparison with HCD)
 - > Are there outliers driving response
 - > Can the data be put in context of other studies (Ames etc...)?
- There is no requirement for a positive/negative result, the data can be equivocal. Equivocal studies may prompt a repeat experiment using different experimental conditions or change in test system .

The background features a light gray field with scattered abstract elements: thin horizontal lines, double chevrons (»), and small open circles. An orange geometric pattern, composed of various triangles, forms a diagonal band across the bottom right corner.

Error Corrected Next Generation Sequencing (ecNGS) Applied to Measuring Gene Mutation

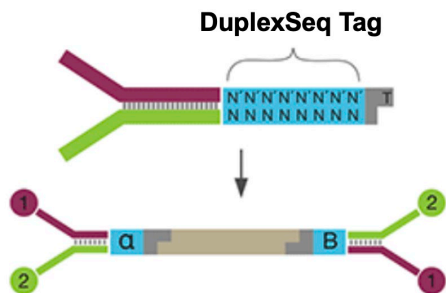
ecNGS Technologies for Mutagenesis

- Duplex Sequencing (DuplexSeqTM; TwinStrand Biosciences)
- Concatenating Original Duplex for Error Correction (CODEC; Broad Institute)
- Single molecule real-time, high fidelity sequencing (HiFi; PacBio)
- Single Molecule Mutation Sequencing (SMM-SeqTM; Mutagentech)
- Hypothesis alignment with weak overlap sequencing (Hawk-SeqTM; Kao Corporation)
- Nanorate sequencing (Nanoseq; Sanger Institute)
- Bottleneck sequencing (BotSeqS; Johns Hopkins U.)

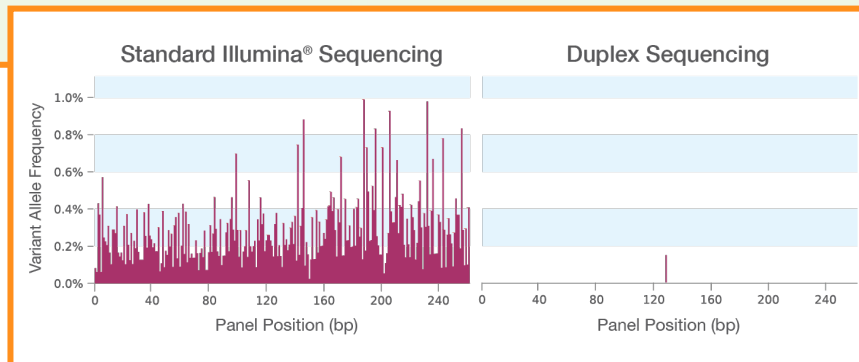
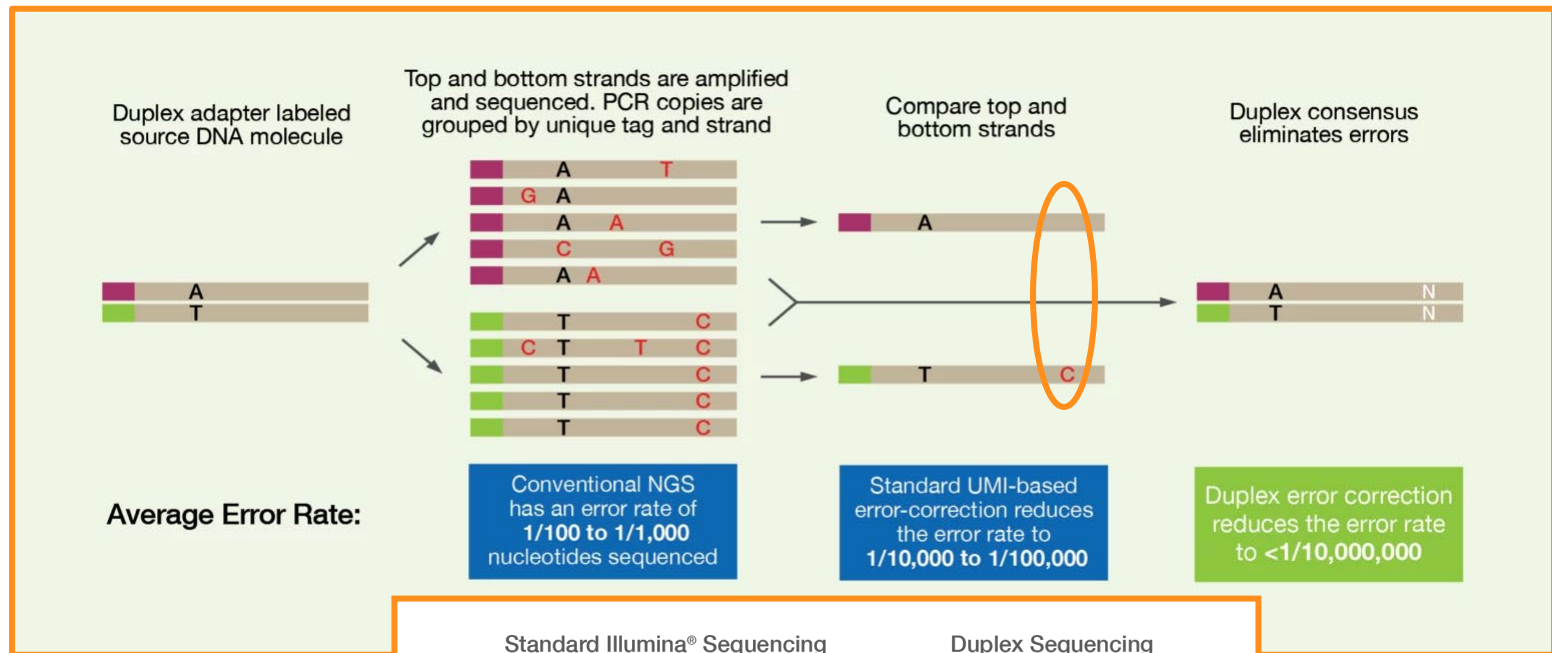
Duplex Sequencing (DuplexSeq™)

- Ultra-accurate, error-corrected next generation sequencing (ecNGS) strategy
- Permits direct evaluation of mutation frequency (MF) and spectrum (e.g., base substitution profile) in any tissue.
 - > Note that TGR and *Pig-a* assays measure MF via phenotypic reporter systems.
- DuplexSeq can be integrated into standalone genetox assays and repeat dose toxicity studies.
- DuplexSeq™ Mutagenesis Assay kits for rat, mouse, and human samples (TwinStrand Bioscience now Scantox) have demonstrated superb interlaboratory reproducibility.
- Due to long queues for TGR assays, DuplexSeq has rapidly gained popularity, especially for evaluation of nitrosamine impurities.

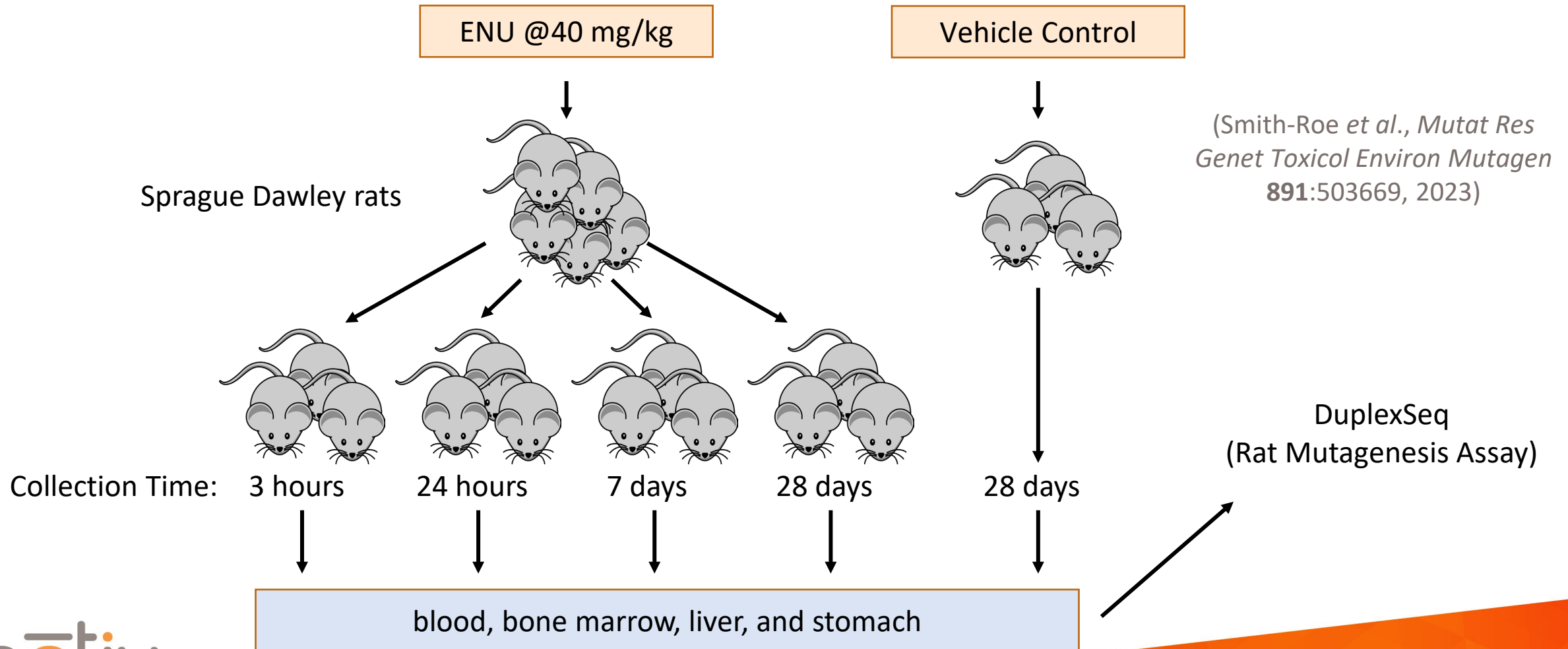
DuplexSeq™ Technology



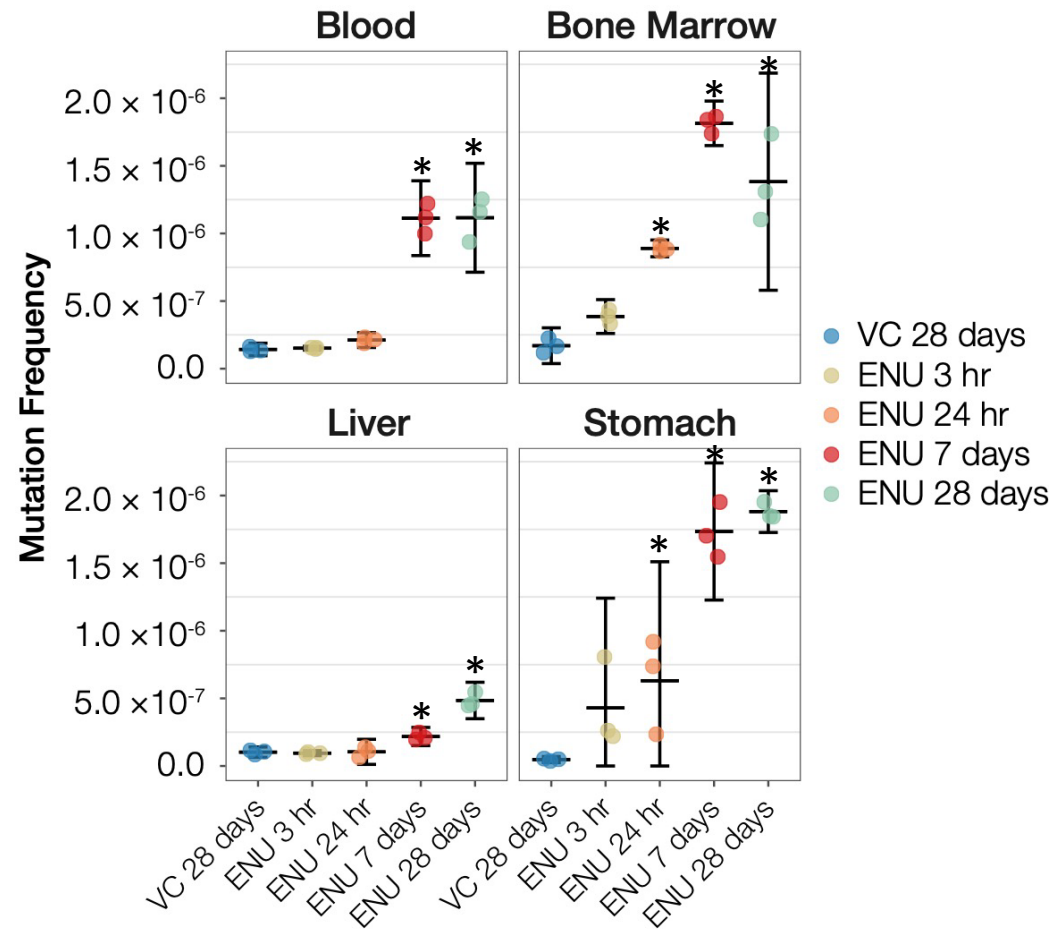
Genomic dsDNA is fragmented into ~300 bp duplexes and both strands are tagged with unique molecular identifiers and non-complementary adaptors for strand asymmetry.



Proof of Concept Study – *N*-Ethyl-*N*-Nitrosourea



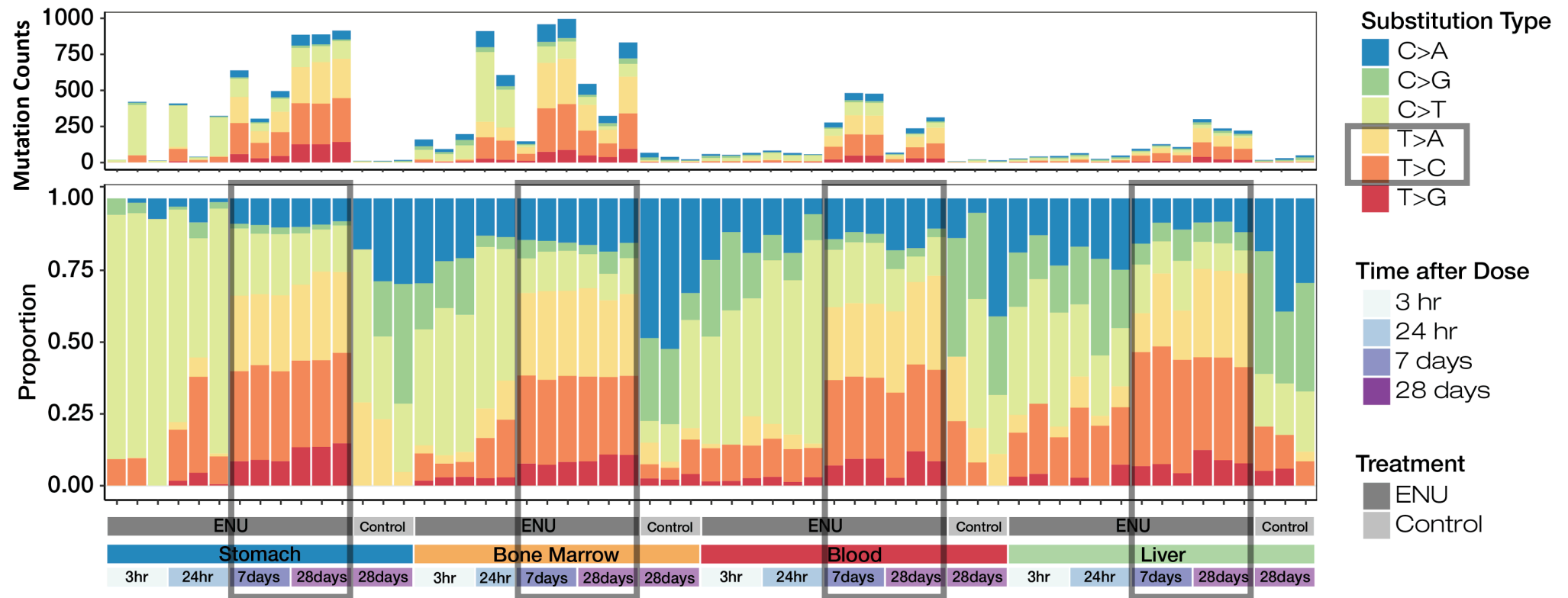
Mutation Frequency Over Time



- Significant increase in mutation frequency as early as 24 hours in fast proliferating bone marrow and stomach.
- Significant increase in mutation frequency in all tissues by 7 days.
- In stomach, bone marrow, and blood, not much difference in mutation frequency between 7 and 28 days.
- Small, but significant, increases in mutation frequency in slow proliferating liver.

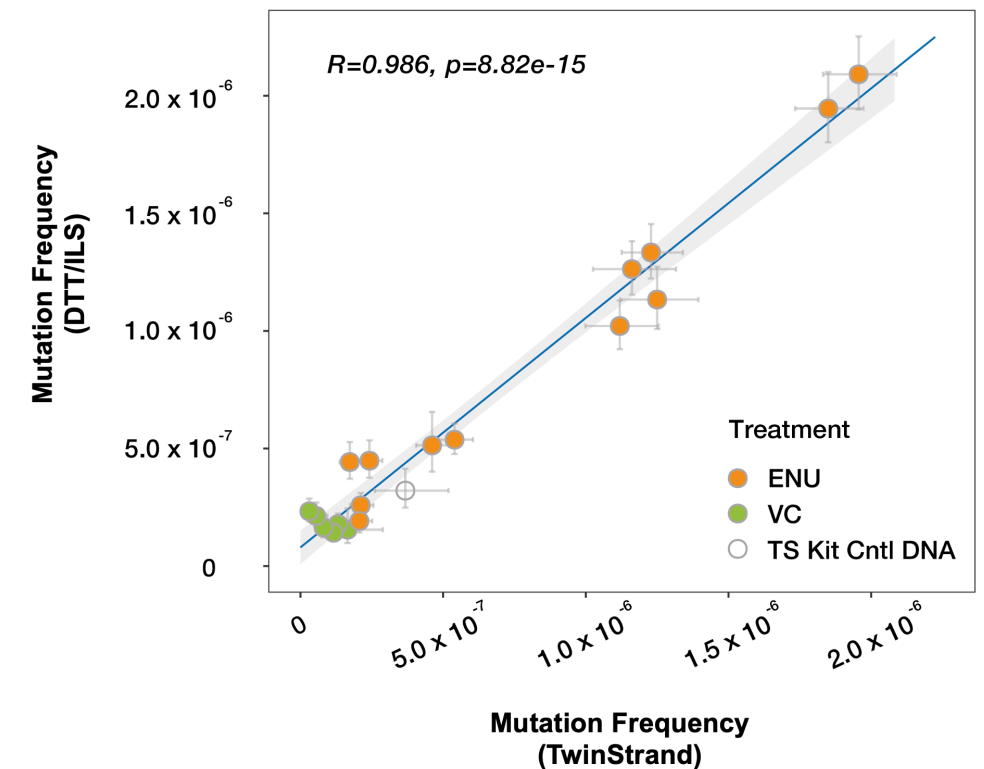
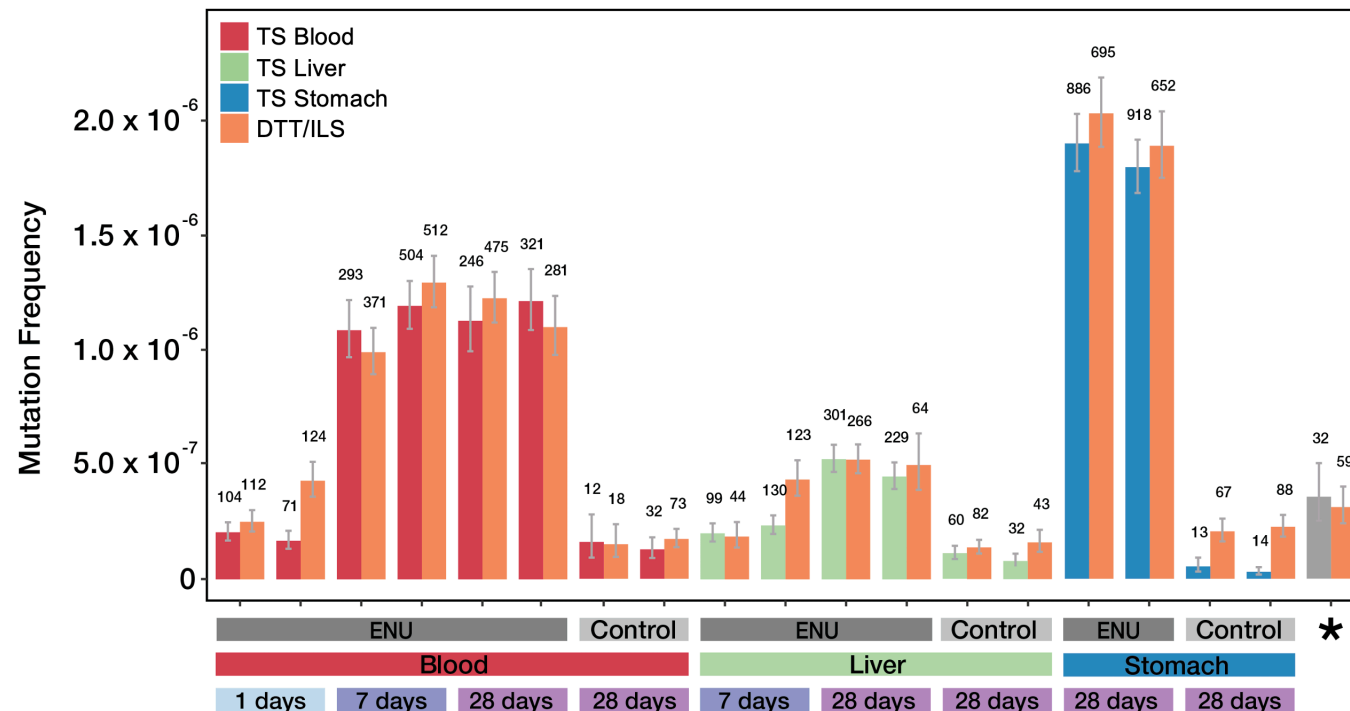
Simple Mutation Spectra

ENU mutational signature evolves over time:



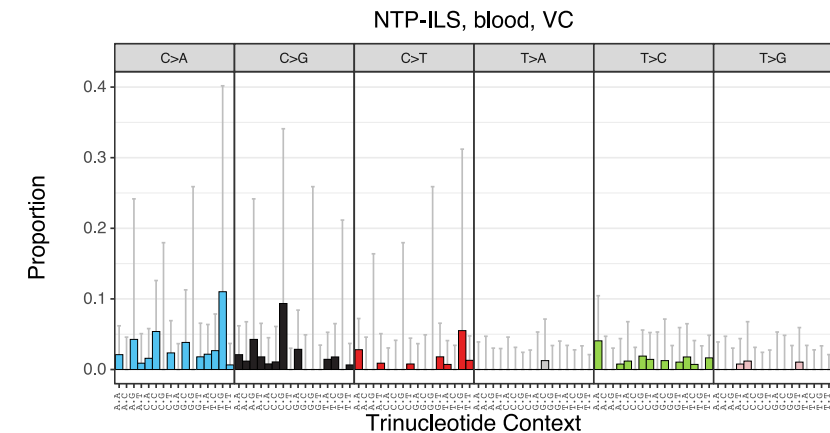
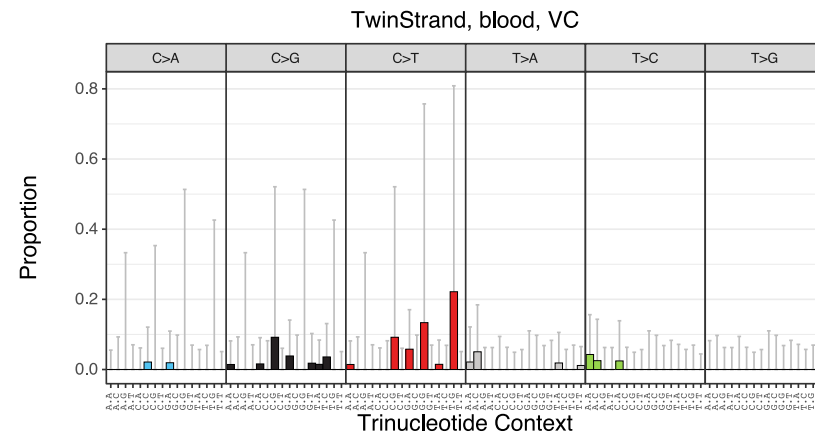
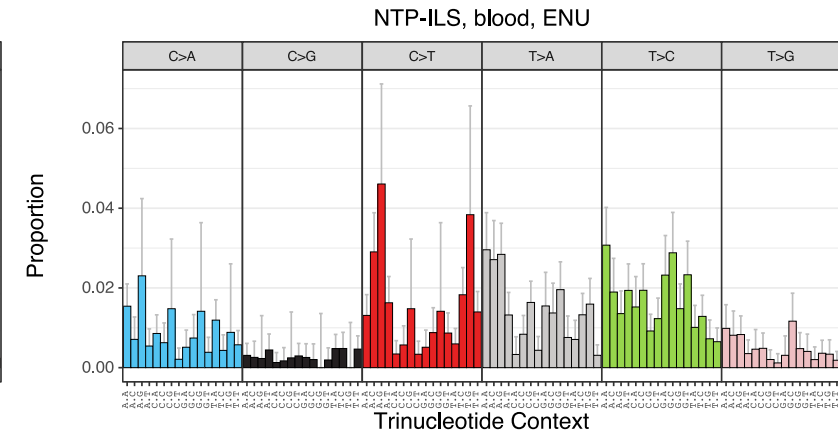
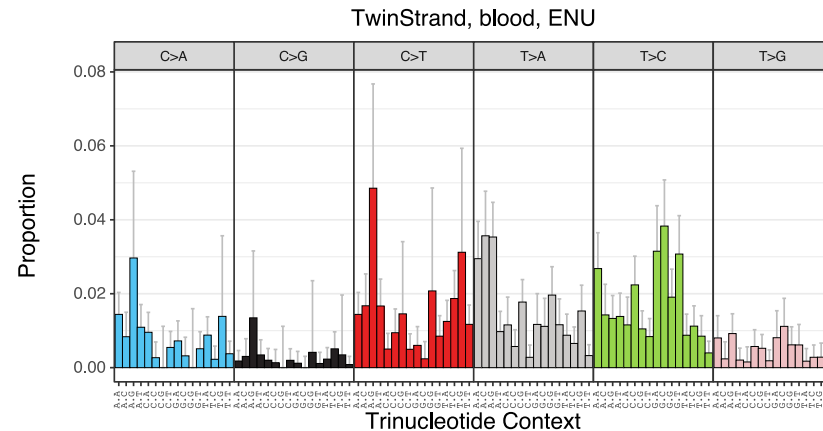
Interlaboratory Reproducibility: Mutation Frequency

Near perfect correlation in mutation frequencies measured by TwinStrand and ILS (now Inotiv):



Interlaboratory Reproducibility: Trinucleotide Spectra

Good interlaboratory concordance in trinucleotide spectra:

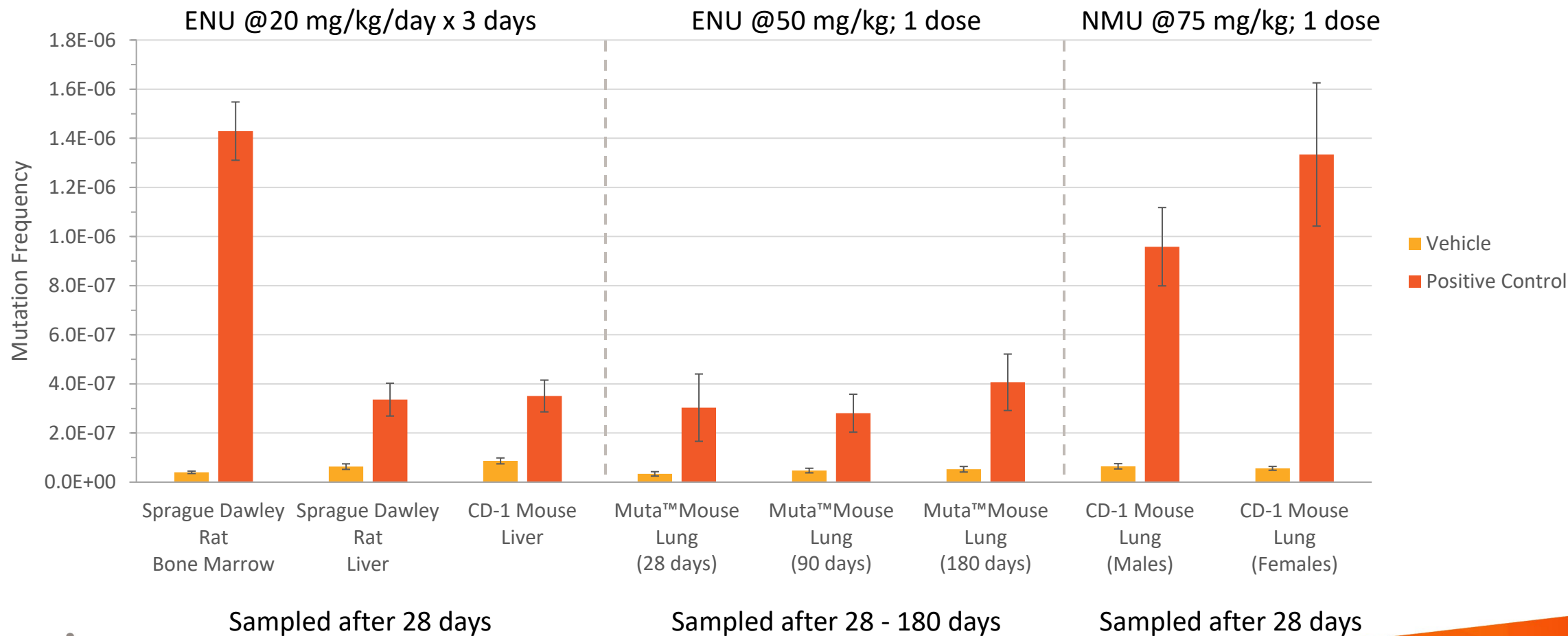


Inotiv's DuplexSeq Experience Related to Nitrosamines

Nitrosamine Studies						
Chemical	Species/strain	Organ	Expectation	TGR*	<i>Pig-a</i> /MN	DuplexSeq
NDSRI #1	CD-1 mouse	Liver	Negative	-	-	Negative
NDSRI #2	Sprague Dawley rat	Bone marrow	Metabolite that doesn't reach bone marrow	-	Neg./Neg.	Negative
		Liver		-	-	Positive
NA #3	Muta™ Mouse	Liver	Positive	Positive (repeat dosing) Positive (single dose)	-	Positive (repeat dosing) Positive (single dose)

*Conducted elsewhere

Examples of Inotiv's DuplexSeq Data – Control Samples



Thank you!



Questions?