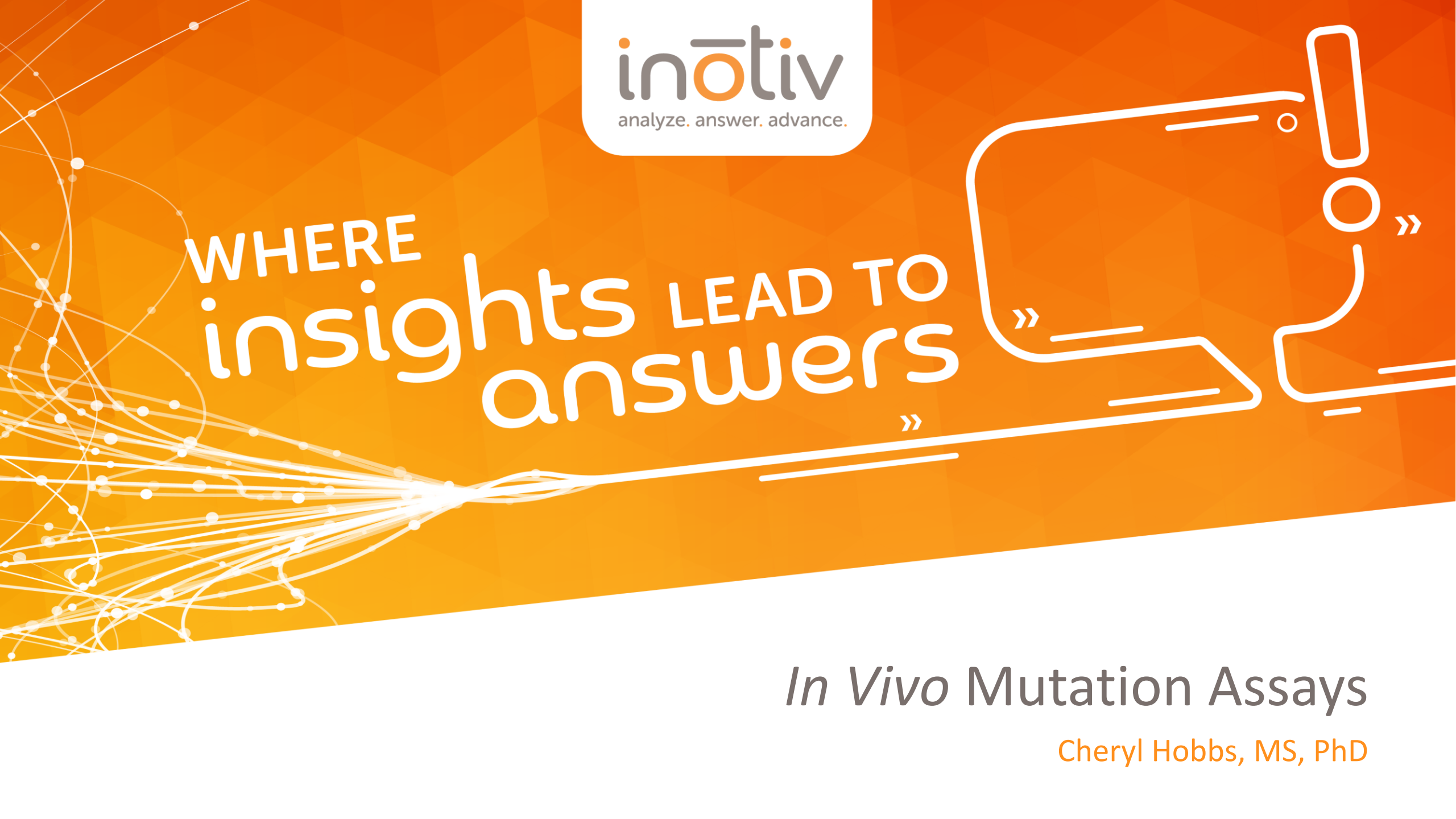
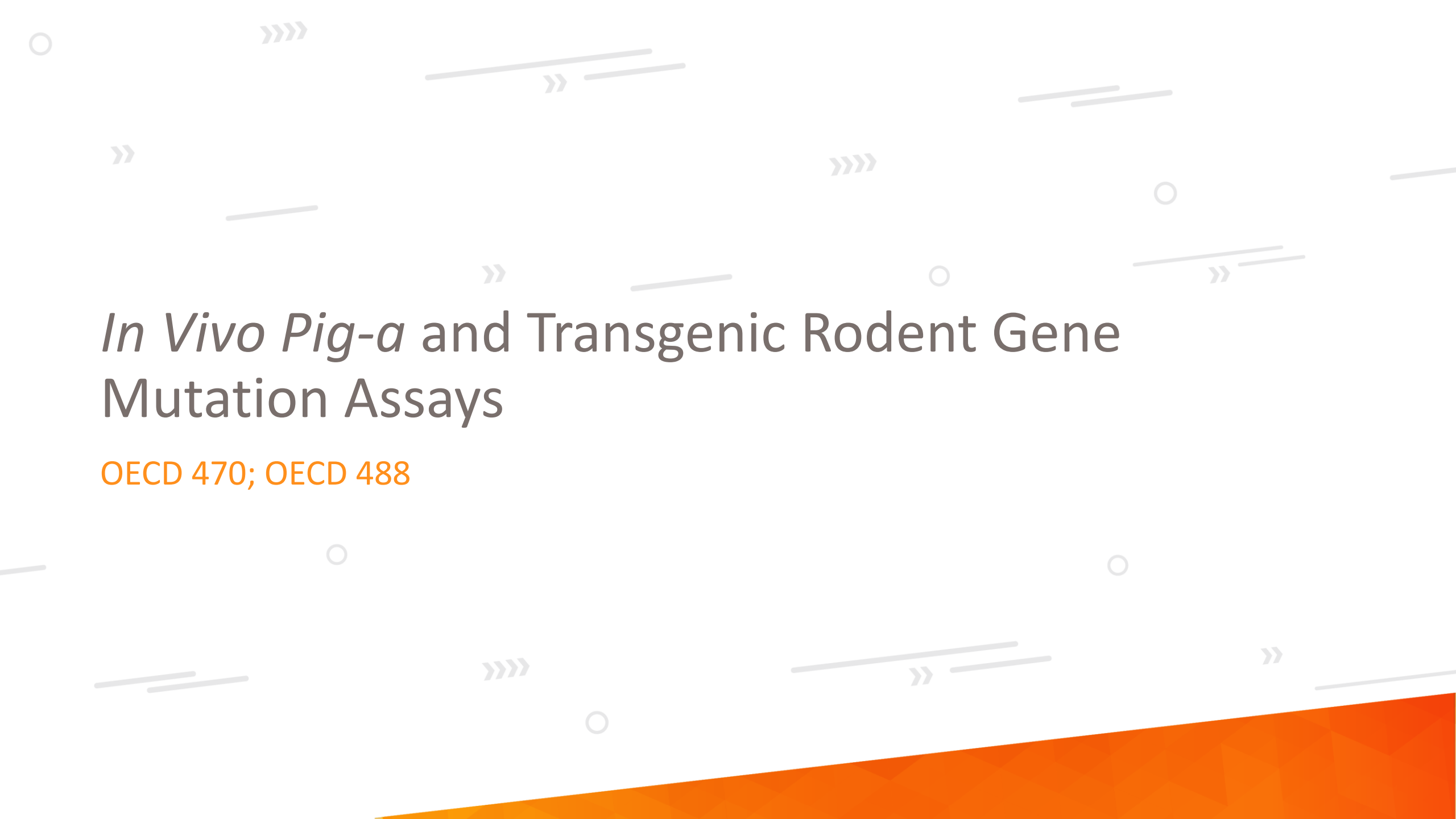




WHERE
insights LEAD TO
answers

In Vivo Mutation Assays

Cheryl Hobbs, MS, PhD

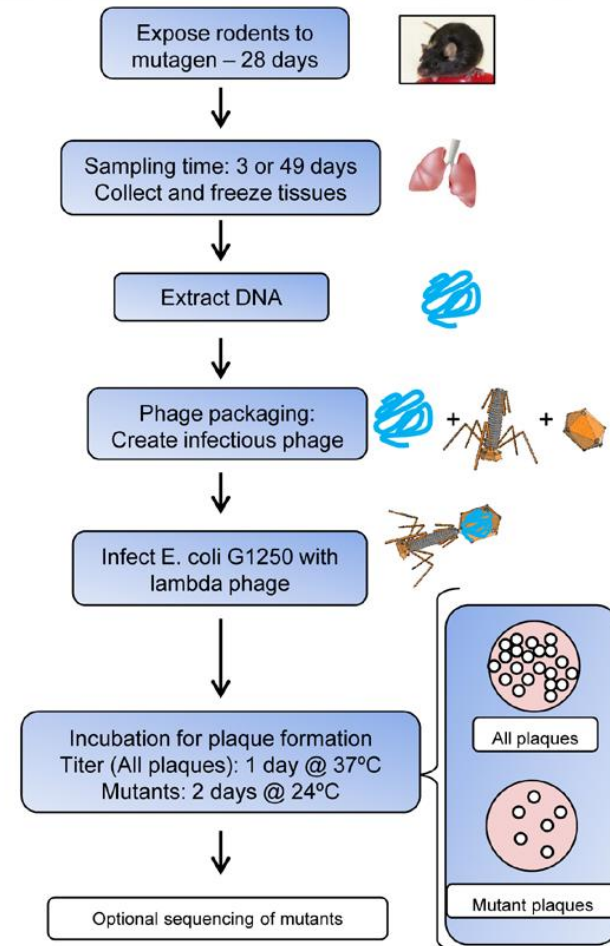


In Vivo Pig-a and Transgenic Rodent Gene Mutation Assays

OECD 470; OECD 488

Transgenic Rodent (TGR) Models for Mutation Assays

- Commonly used transgenic models
 - > Muta™ Mouse (*lacZ E. coli* gene; *cII* lambda gene)
 - > Big Blue® mouse and rat (*lacI E. coli* gene; *cII* lambda gene)
 - > *lacZ* plasmid mouse (*lacZ E. coli* gene)
 - > *gpt* delta mouse and rat (*gpt E. coli* gene; *red/gam* lambda genes)
 - > multiple copies of transgenes integrated into lambda phage or plasmid shuttle vectors
- *in vitro* processing of genomic DNA isolated from tissue(s) of interest (*i.e.*, packaging of lambda vectors, or ligation and electroporation of plasmids to recover the shuttle vector)
- scoring based on reporter gene phenotype in a bacterial host deficient for the reporter gene under non-selective or selective conditions



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

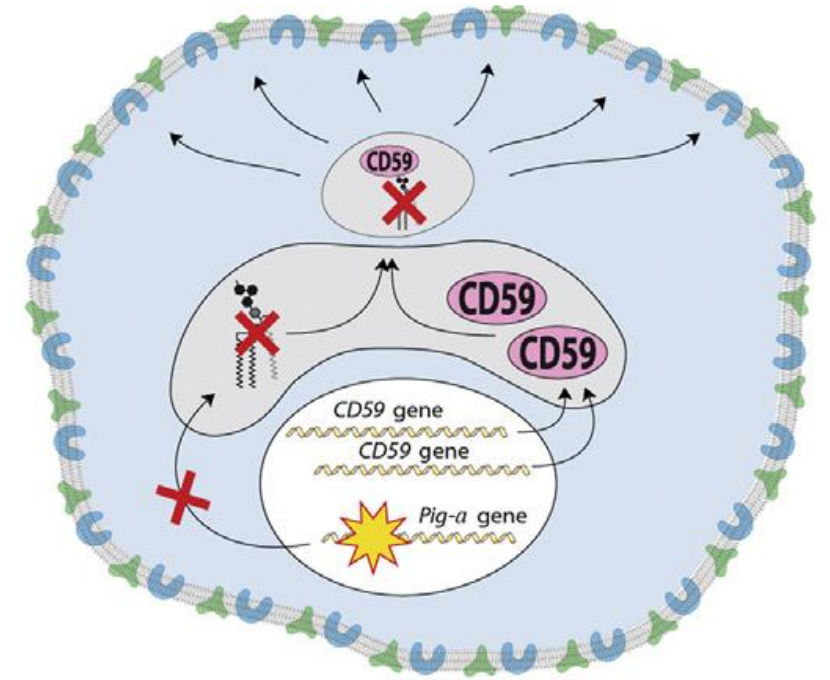
- If an *in vitro* positive result did not require metabolic activation (*i.e.*, -S9 or $\pm$ S9 conditions), one must be able to demonstrate exposure of bone marrow to the test article.
 - > Exposure can be ensured via intravenous dosing.
- If an *in vitro* positive result required metabolic activation (*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

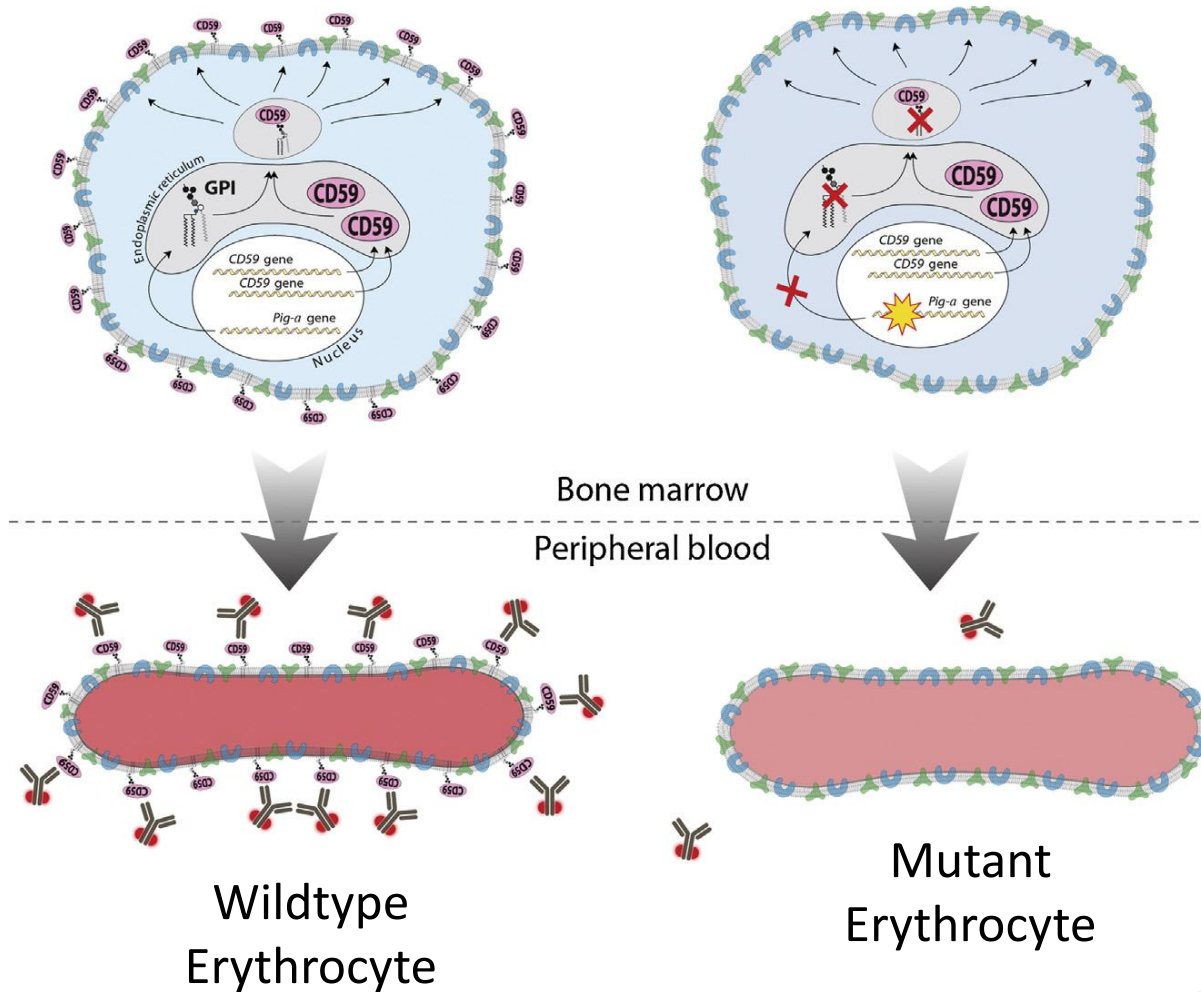


Loss of functional
GPI anchors



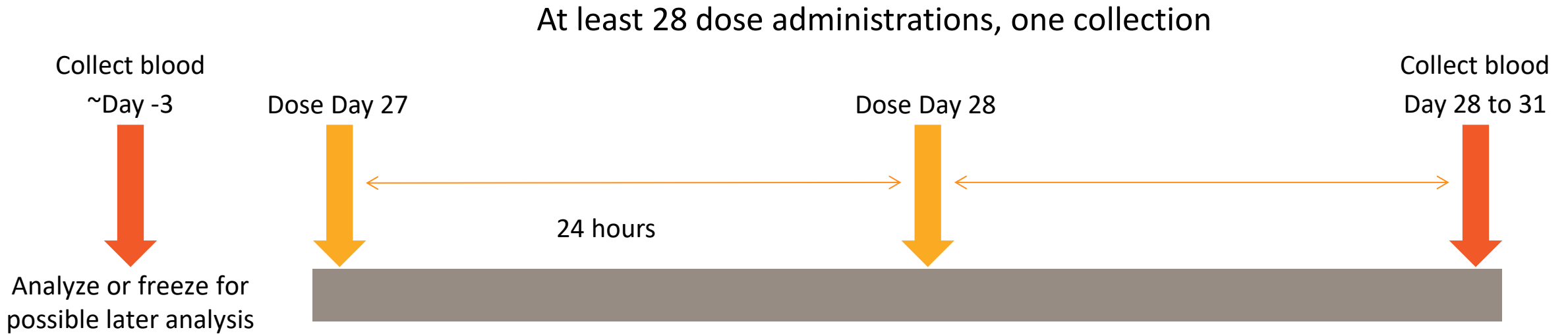
Lack of cell surface
markers

Pig-a Gene Mutation Assay



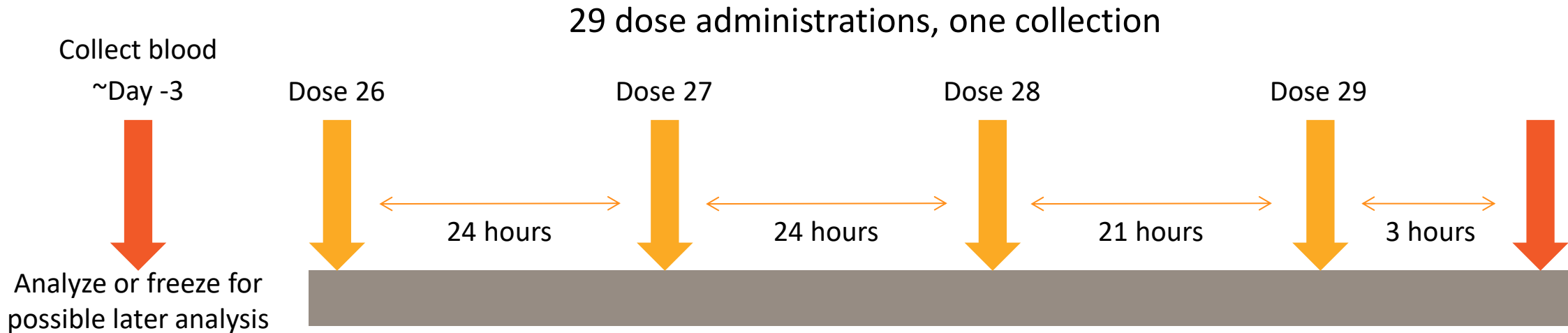
- Assay for loss of:
- CD59 (rat)
 - CD24 (mouse)

Pig-a Gene Mutation Assay Design



- 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



- 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, 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

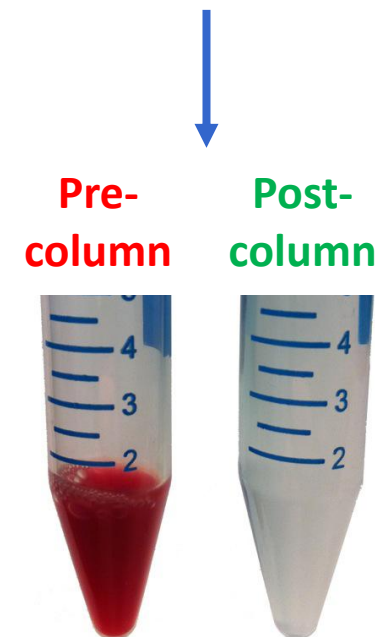
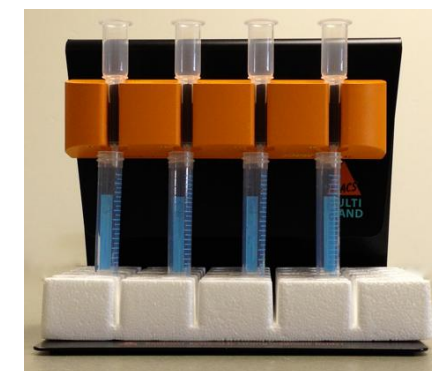
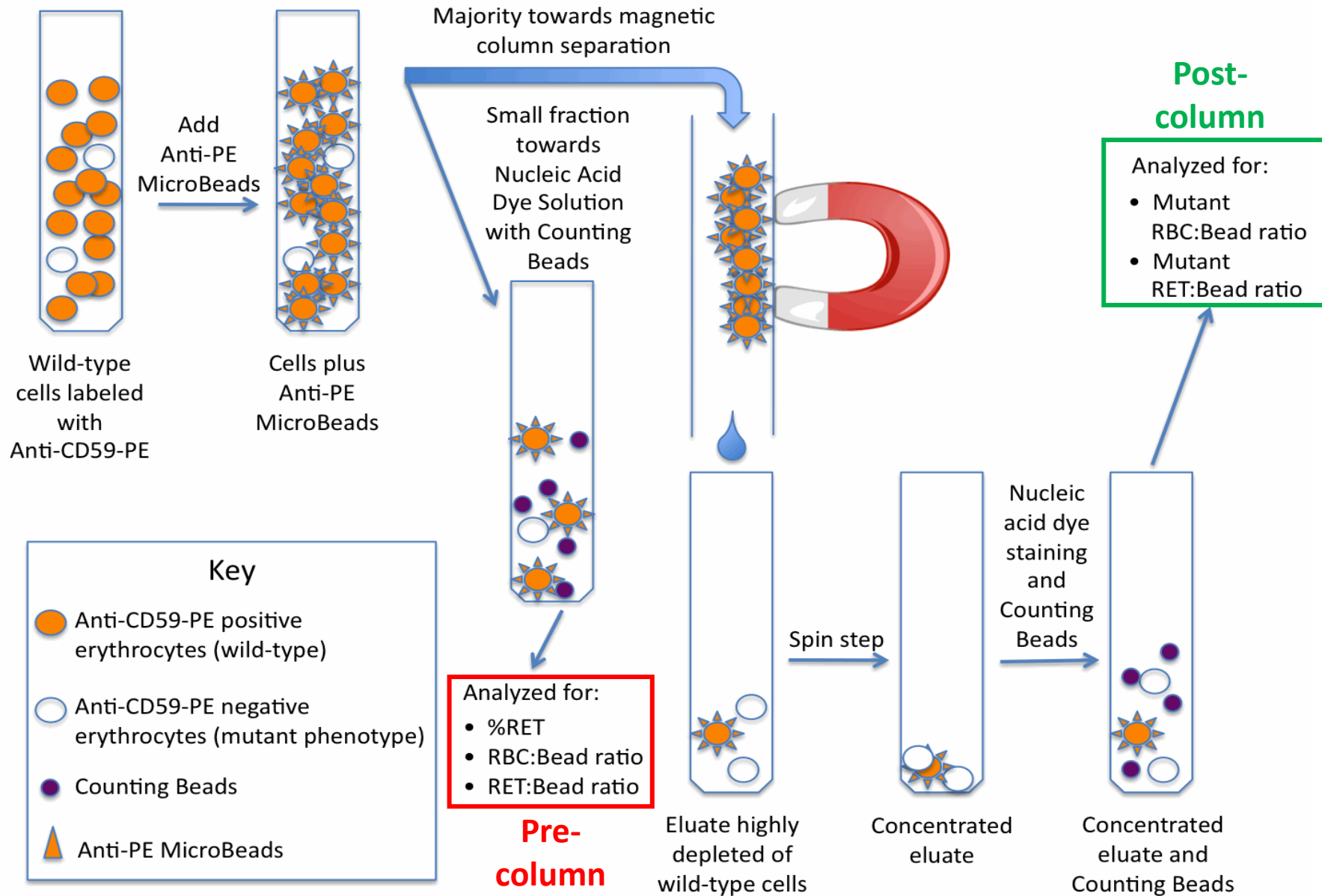
Vehicle control and three test article dose levels – 6 animals/group

Positive control group not required (but recommended by Inotiv)

Blood collected into K₂EDTA tubes at interim or terminal timepoints

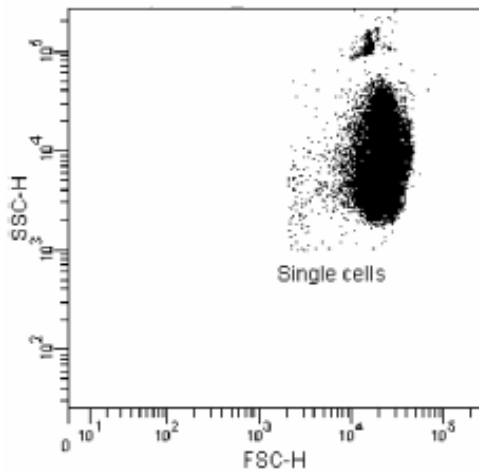
Analysis using flow cytometry and MutaFlow^{PLUS} assay kit (Litron Laboratories).

- Mutation frequency can be assessed across multiple time points.
- Allows for collection of samples at in-life lab and shipment to another lab for analysis
- 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

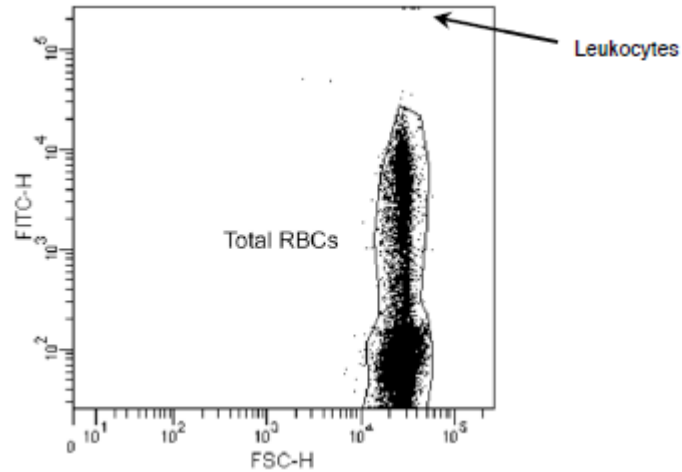


Flow Cytometry Set-Up Gating

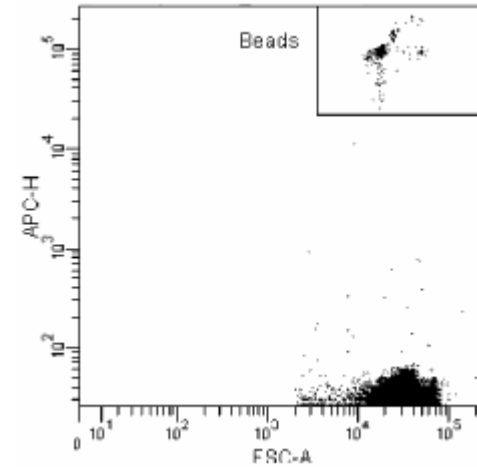
BD FACSCalibur:



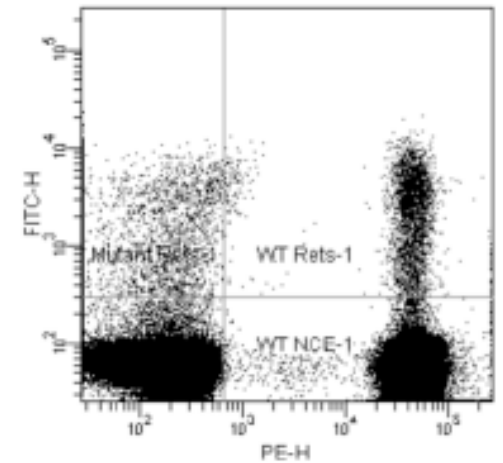
Single cell gate



Total red blood cells gate



Bead gate

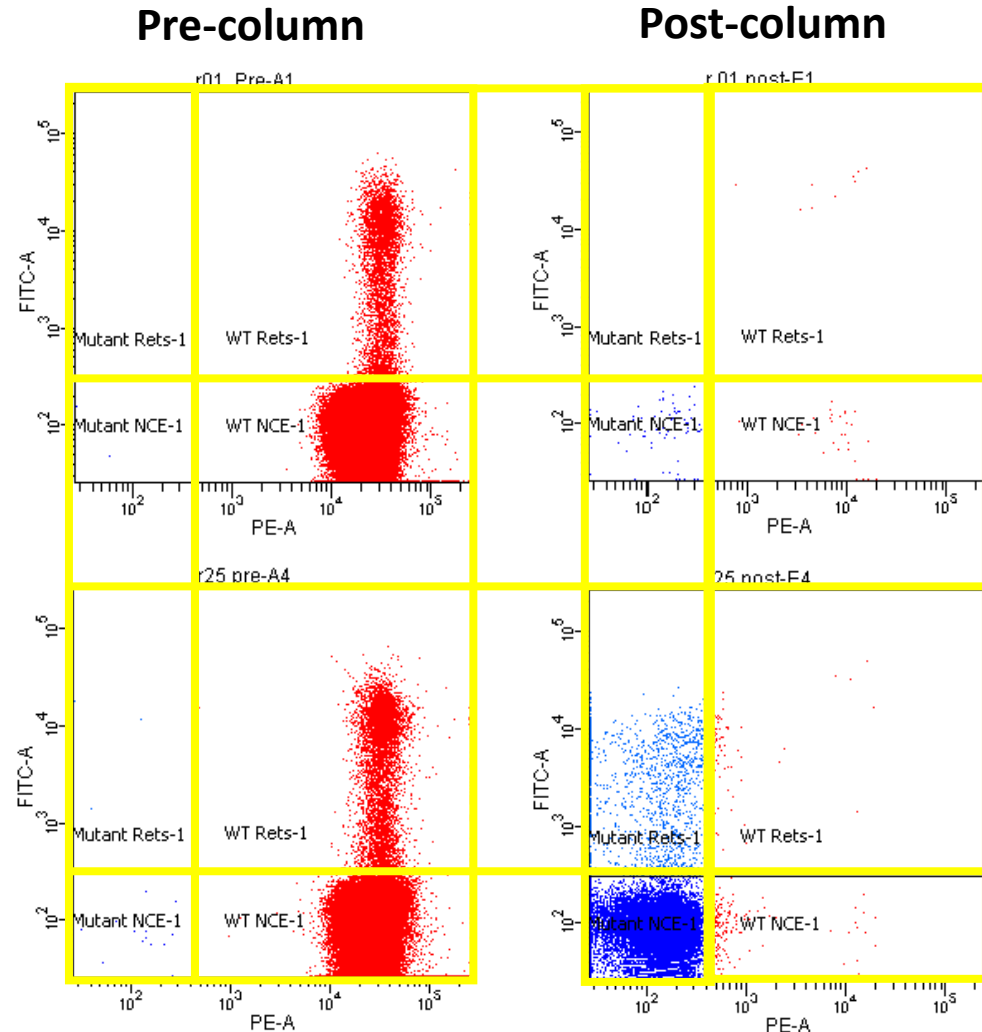


Internal Calibration Control
(mutant mimic)

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

The background is white with various light gray geometric elements scattered across it, including circles, double chevrons (»»), and parallel lines. An orange geometric pattern, consisting of overlapping triangles, is located in the bottom right corner.

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

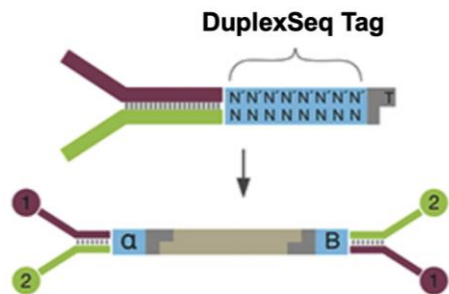
ecNGS Technologies for Mutagenesis

- 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.)

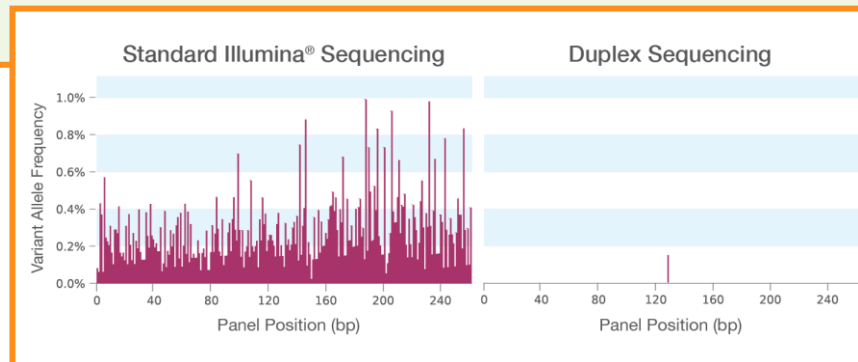
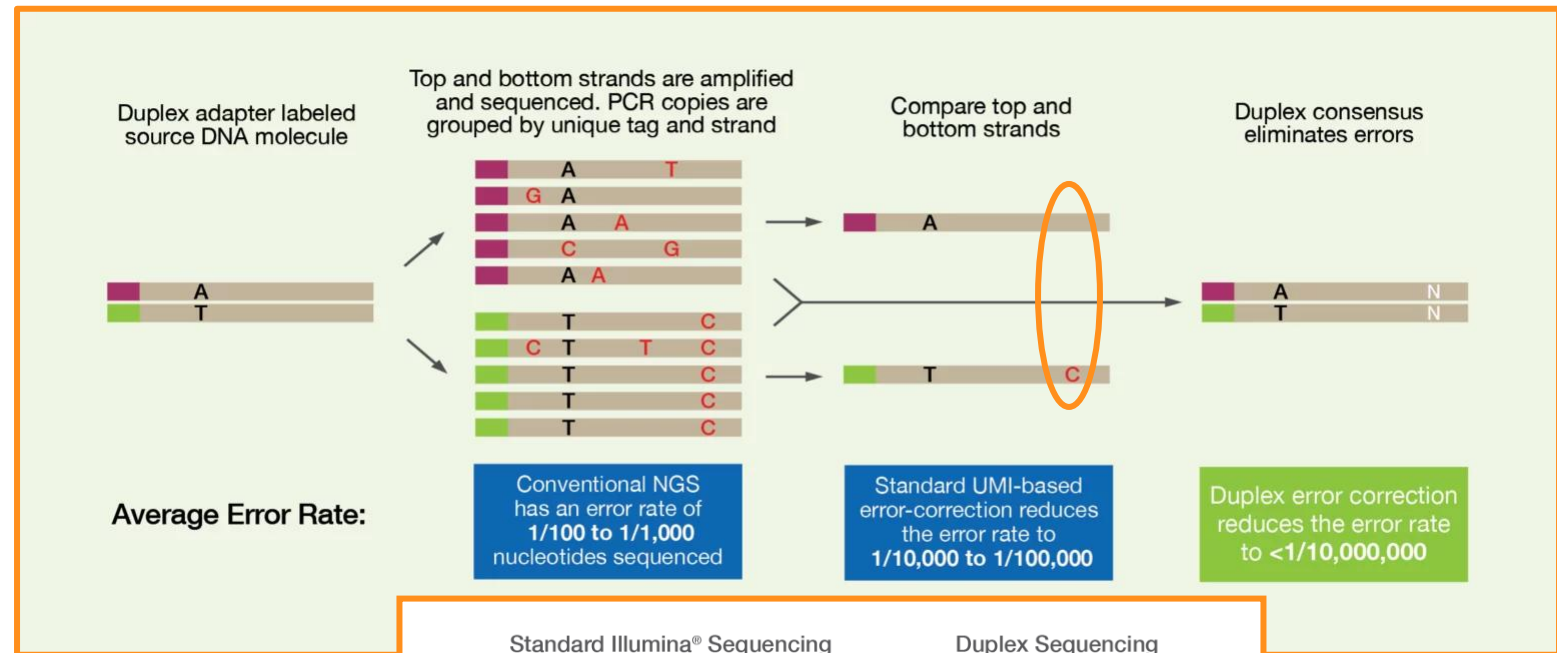
Duplex Sequencing (DuplexSeq™)

- 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) 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



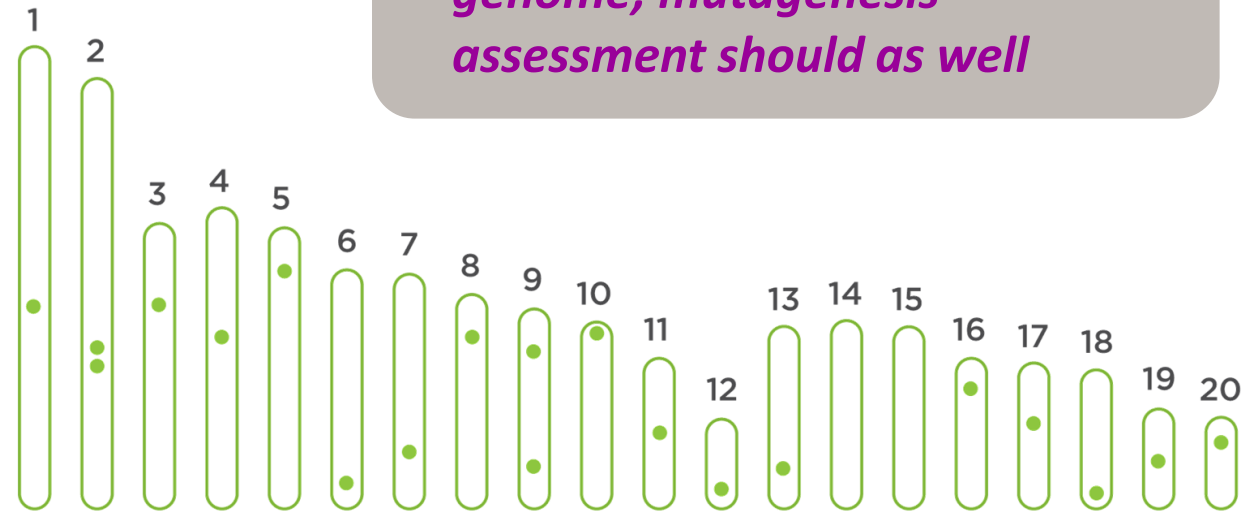
Genomic dsDNA is fragmented into ~300 bp duplexes and both strands are tagged with unique molecular identifiers and non-complementary adaptors for strand asymmetry.



DuplexSeq™ Mutagenesis Panels

- The 48 kb of targets comprise twenty 2.4 kb regions spread randomly throughout the genome.
- The proportion of %GC, genic/non-genic, coding/non-coding and trinucleotide composition is representative of the whole genome.
- Selected sites have no known role in cancer and are unlikely to be significantly influenced by positive or negative selection.

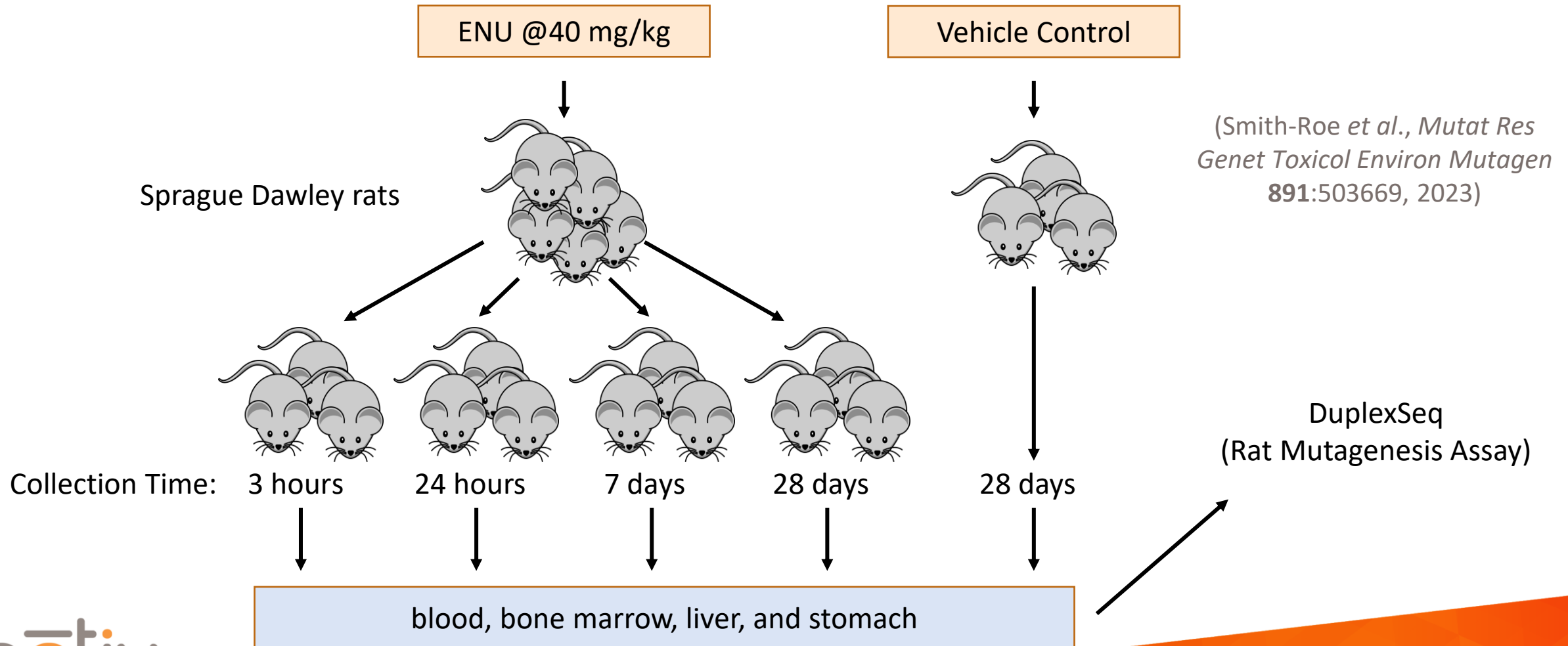
Mutagens touch the entire genome; mutagenesis assessment should as well



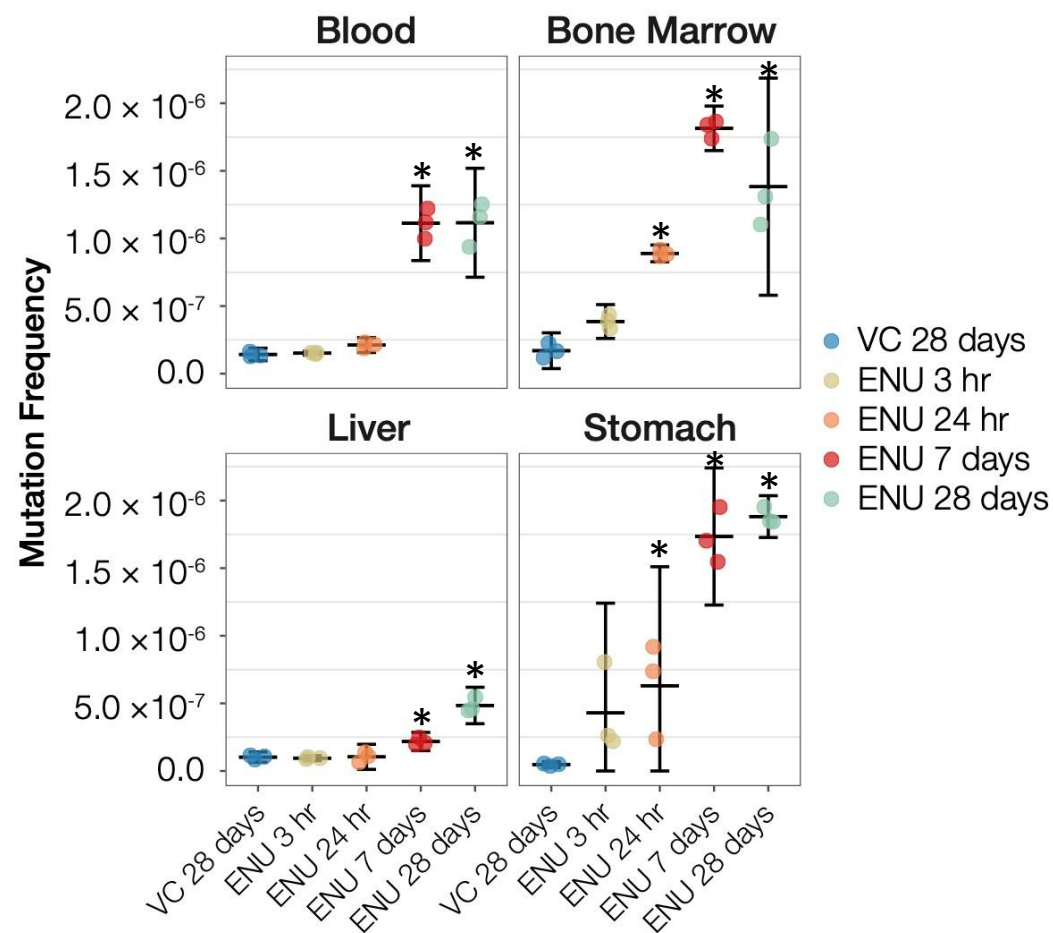
Locations of genomic targets in the DuplexSeq Rat Mutagenesis panel. Mouse and human panels are similarly constructed.

(Adapted from TwinStrand Biosciences Literature)

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



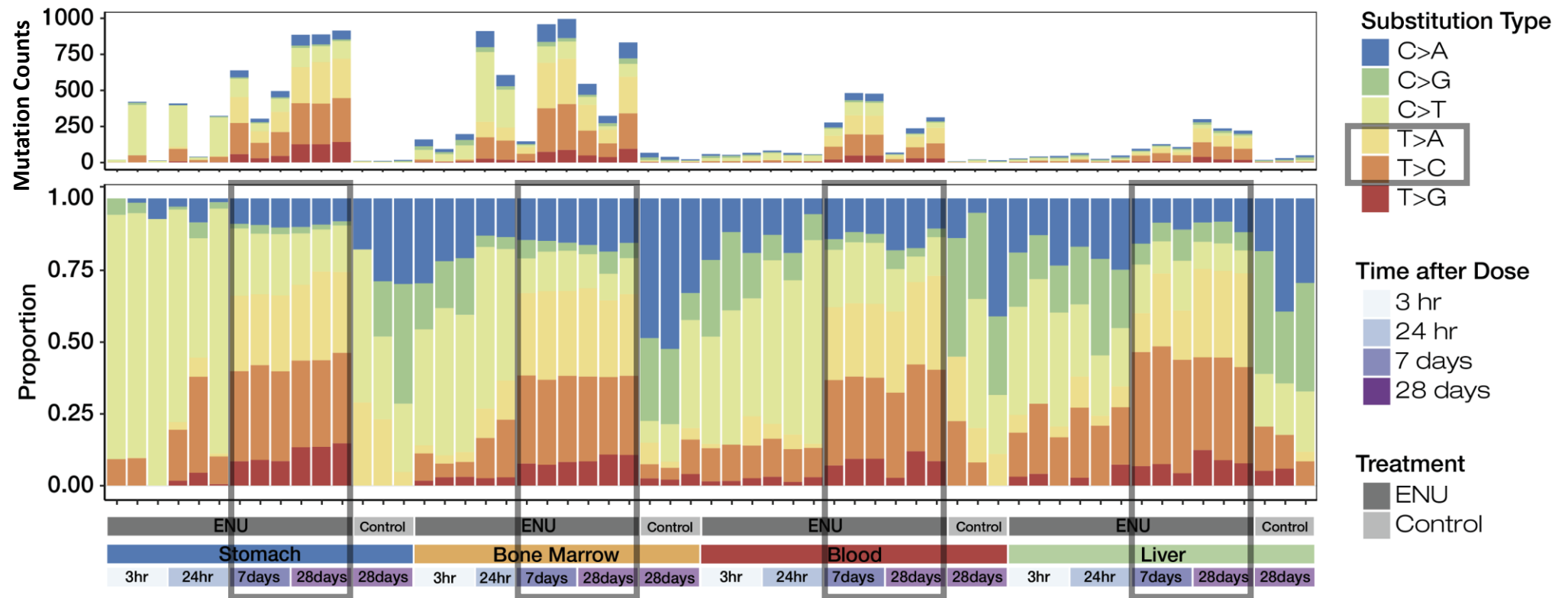
Mutation Frequency Over Time



- 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.

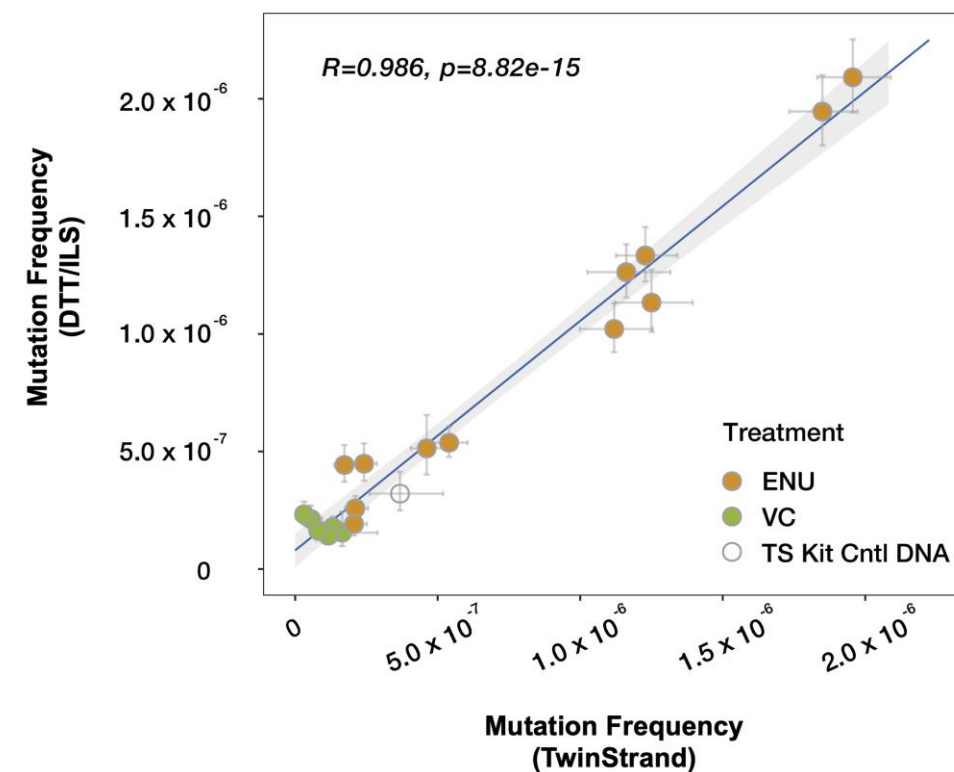
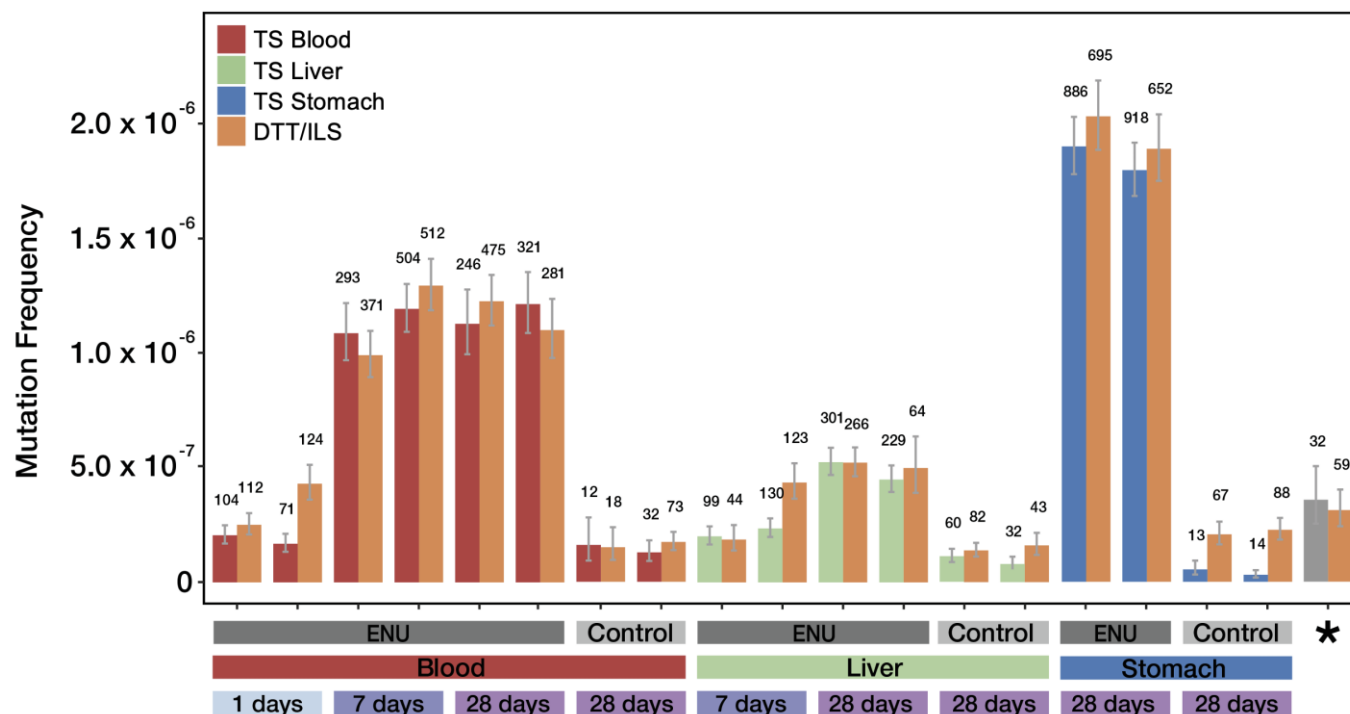
Simple Mutation Spectra

ENU mutational signature evolves over time:



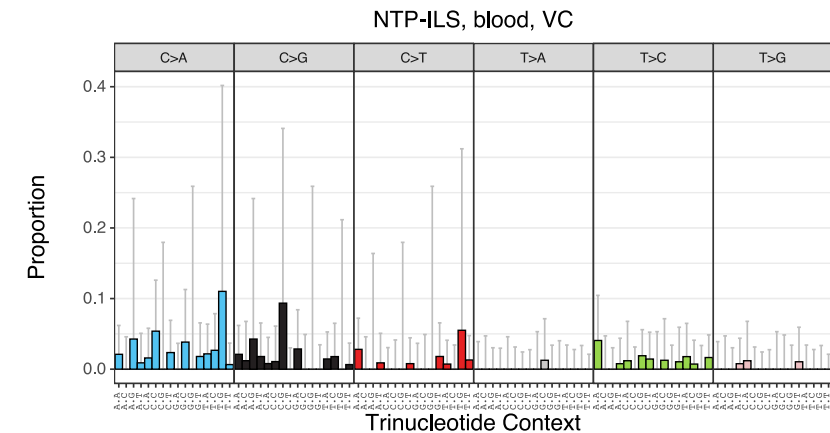
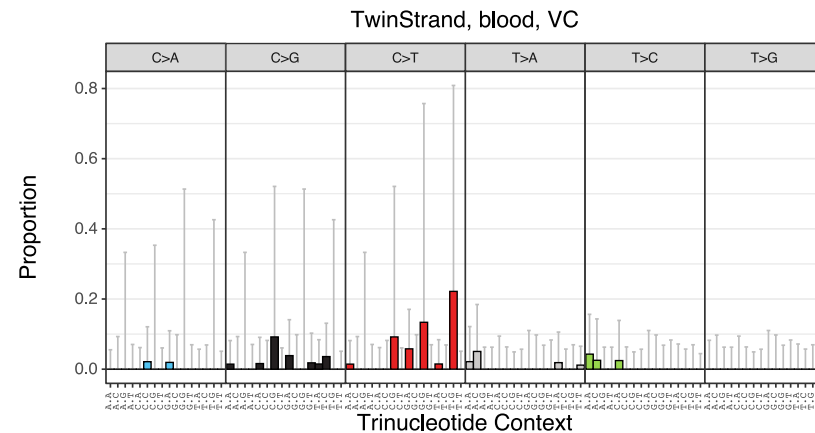
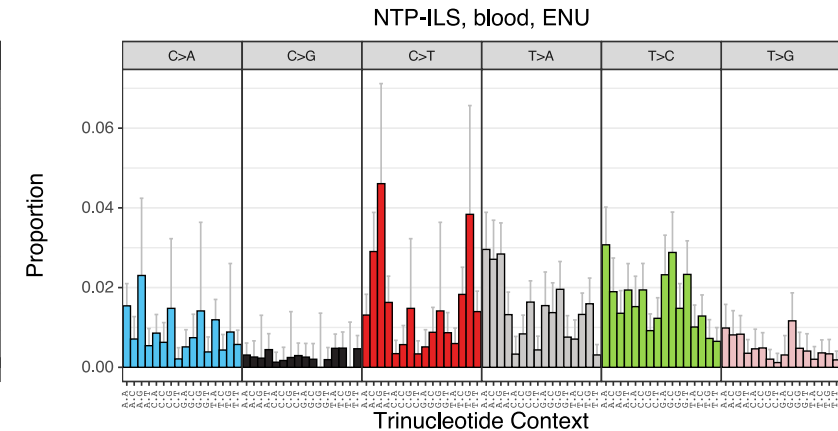
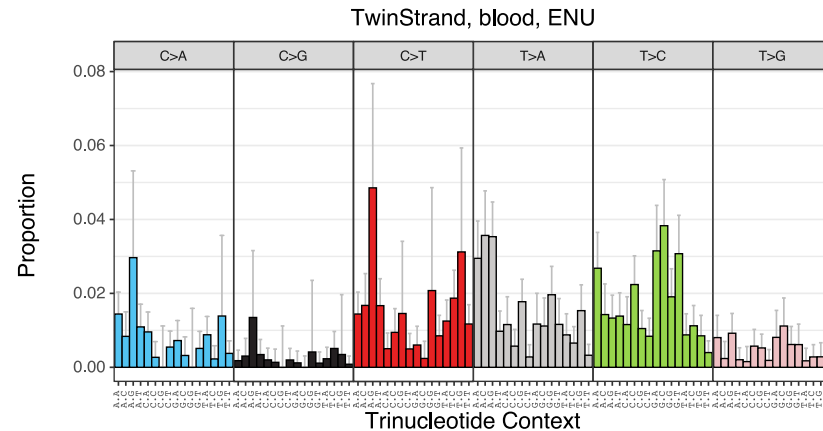
Interlaboratory Reproducibility: Mutation Frequency

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



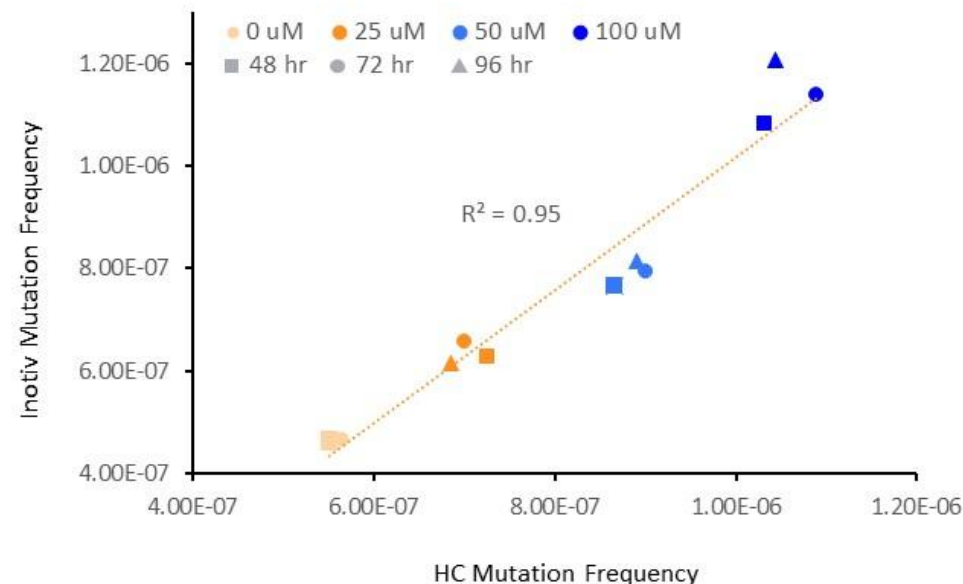
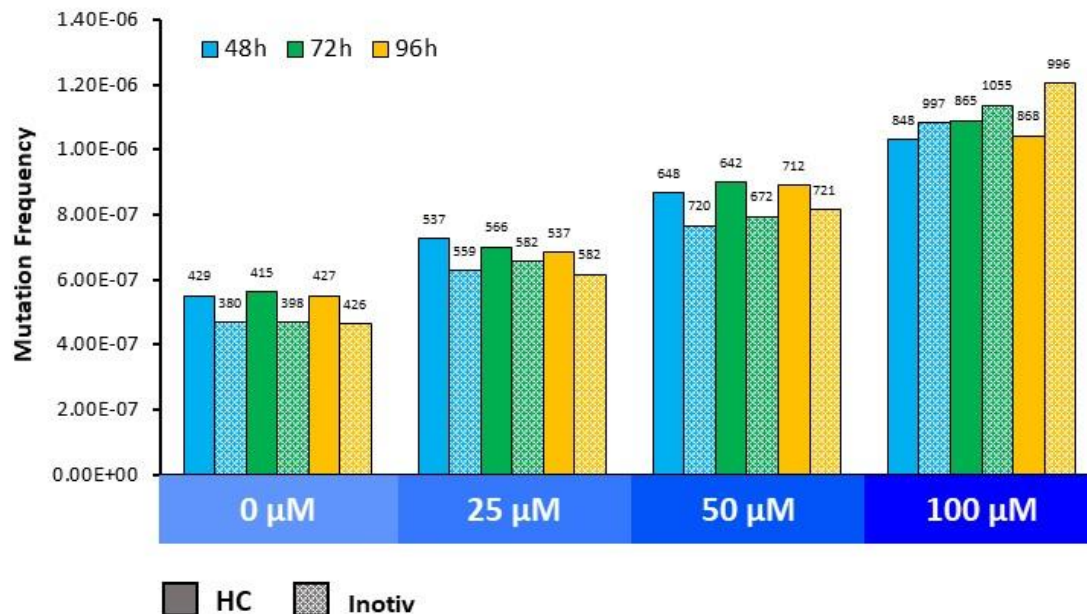
Interlaboratory Reproducibility: Trinucleotide Spectra

Good interlaboratory concordance in trinucleotide spectra:



Interlaboratory Reproducibility

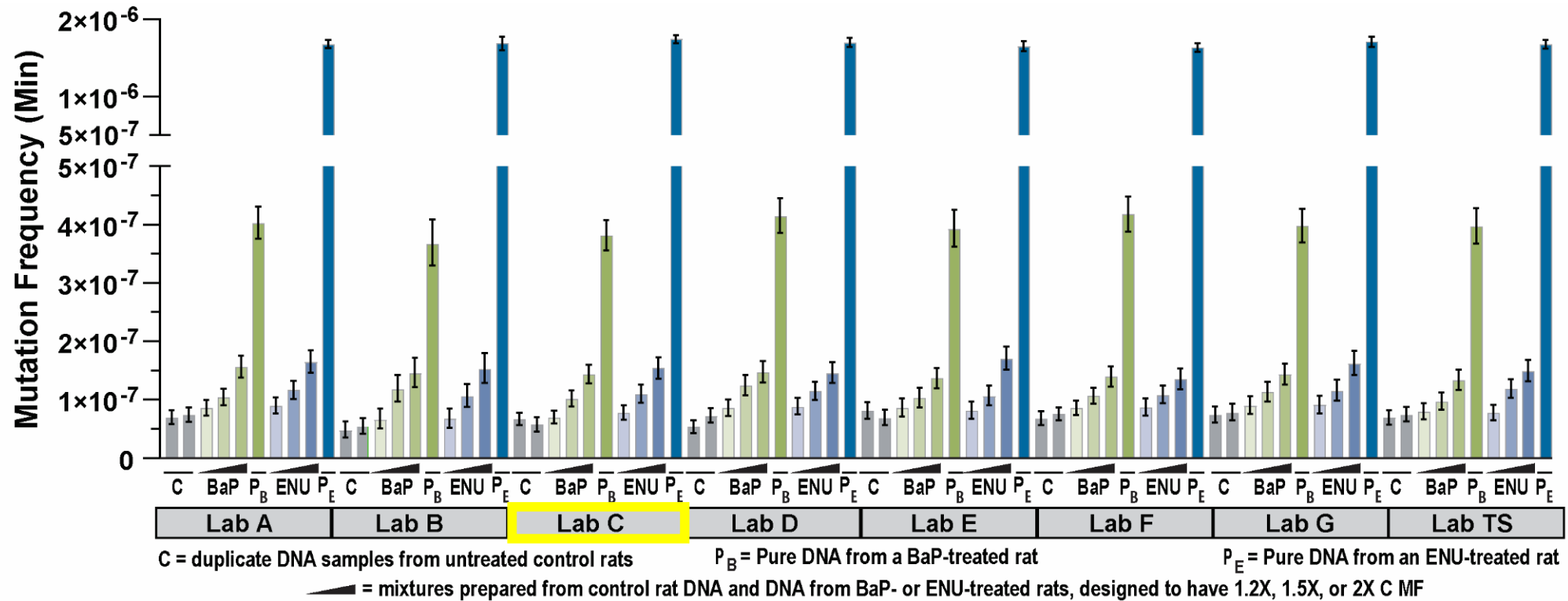
Comparison of mutation frequency for experiments in human TK6 cells conducted independently by Inotiv RTP and Health Canada:



(Cho *et al.*, *Mutat Res Genet Toxicol Environ Mutagen* **891**:503649, 2023)

Superior Interlaboratory Reproducibility

HESI-GTTC “Reconstruction Experiment”



TGR vs. DuplexSeq vs. Comet for Assessing Nitrosamine Potency

Mutation Research - Genetic Toxicology and Environmental Mutagenesis 891 (2023) 503685



Contents lists available at ScienceDirect

Mutation Research - Genetic Toxicology
and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox

Comparison of the transgenic rodent mutation assay, error corrected next generation duplex sequencing, and the alkaline comet assay to detect dose-related mutations following exposure to *N*-nitrosodiethylamine

Joel P. Bercu^{a,*}, Shaofei Zhang^{b,*}, Zhanna Sobol^c, Patricia A. Escobar^c, Phu Van^d,
Maik Schuler^b

^a Gilead Sciences, Inc., Nonclinical Safety and Pathobiology (NSP), Foster City, CA 94404, USA

^b Pfizer Research, Development, and Medical, Groton, CT, USA

^c Merck & Co., Inc., Rahway, NJ, USA

^d TwinStrand Biosciences, Inc., Seattle, WA, USA

“Therefore, DS and comet assays showed good utility for hazard identification and dose-response analysis of a representative *N*-nitrosamine comparable to the TGR gene mutation assay.”

Received: 9 April 2024 | Revised: 11 June 2024 | Accepted: 14 June 2024

DOI: 10.1002/em.22615

RESEARCH ARTICLE

Environmental and
Molecular Mutagenesis



WILEY

Assessing the genotoxicity of *N*-nitrosodiethylamine with
three in vivo endpoints in male Big Blue[®] transgenic and
wild-type C57BL/6N mice

Shaofei Zhang¹ | Stephanie L. Coffing¹ | William C. Gunther¹ |
Michael L. Homiski¹ | Richard A. Spellman¹ | Phu Van² | Maik Schuler¹

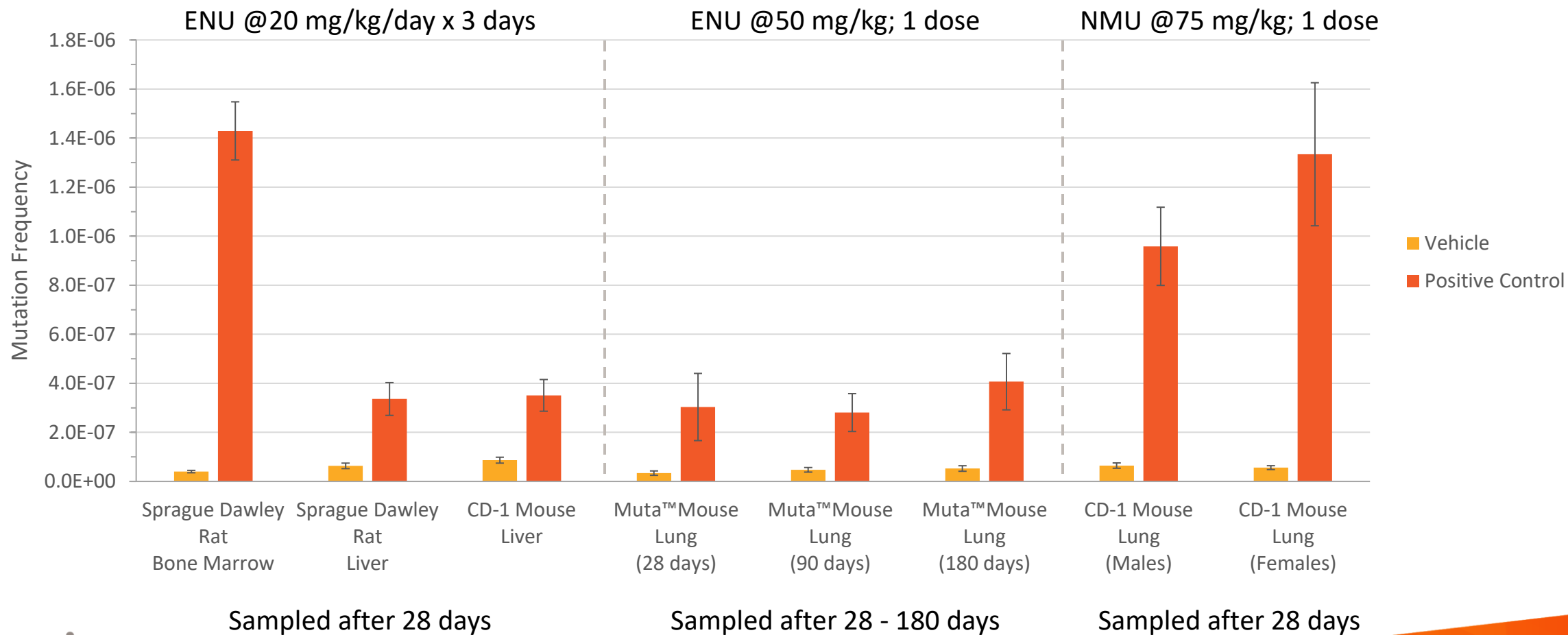
“The sensitivities of three in vivo assays, if compared by the lowest dose of NDEA that can induce a statistically significant response relative to the vehicle control, were duplex sequencing (0.1 mg/kg/day) > Big Blue[®] (1 mg/kg/day) > comet (3 mg/kg/day).”

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

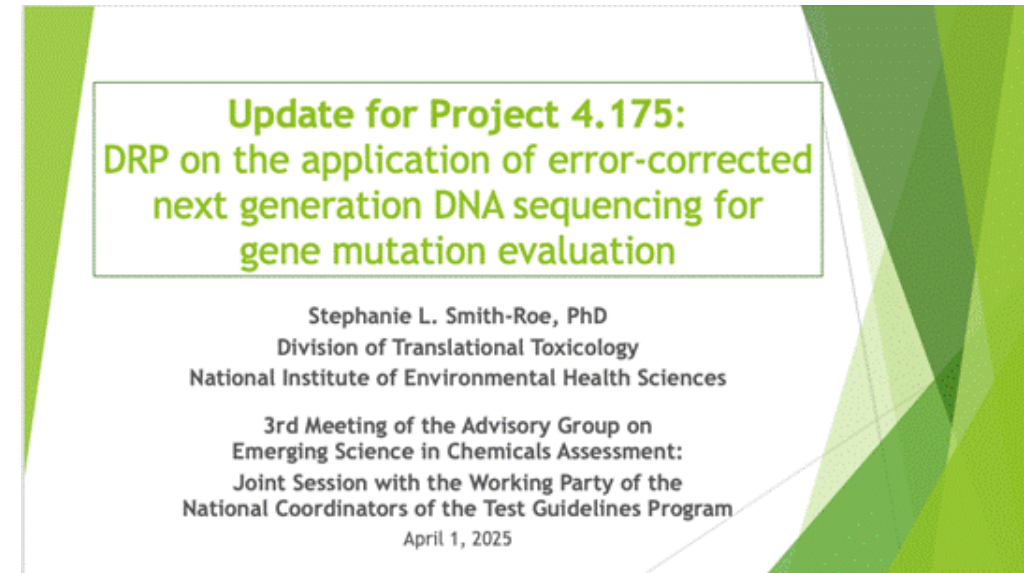


ecNGS Emergence in the Regulatory Landscape

- Expert working groups are actively driving advancement of the technology.
 - > HESI Genetic Toxicology Technical Committee (GTTC)
 - “Reconstruction Experiment” demonstrating interlaboratory reproducibility and sensitivity of duplex sequencing – revised manuscript submitted
 - Developing a strategy to fill data gaps (e.g., more chemicals; validating alternative technologies to DuplexSeq)
 - Developing a plan for establishing consensus protocols for *in vitro* studies
 - > HESI Emerging Systems Toxicology for the Assessment of Risk (eSTAR) Committee
 - Exploring use of ecNGS and cancer driver genes to identify non-genotoxic tumorigenic agents
 - Pilot study completed comparing study designs to detect clonal expansion
 - > IWGT – submission of guidance manuscript by June 2025
- Requests for DuplexSeq data from two regulatory agencies!

ecNGS Emergence in the Regulatory Landscape

- April 2024 – SPSF proposal approved to write a Detailed Review Paper & Retrospective Performance Assessment
- April 1, 2025 – OECD update on status of ecNGS field
 - > OECD WNT
 - > Advisory Group on Emerging Science in Chemicals Assessment (ESCA)
- Future plan – submit new SPSF proposal aimed at amending relevant gene mutation test guidelines to include ecNGS approaches.
 - > Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays (TG 488)
 - > In Vitro Mammalian Cell Gene Mutation Test Using Thymidine Kinase Gene (TG 490)
 - > Bacterial Reverse Mutation Test (TG 471)



Remove requirement for
transgenic animals!

Explore our Genetic Toxicology Services

