Validation of an *In Vivo Pig-a* Gene Mutation Assay for Use in Regulatory Toxicology Studies

J. Godin-Ethier[1], F. Leroux[1], N. Wang[1], S. Thébaud[1], F. Merah[1], S. D. Dertinger[2] and A. Nelson[1].

([1]) ITR Laboratories Canada Inc., Montreal, Canada; ([2]) Litron Laboratories, Rochester, NY

Abstract

Integration of *in vivo* genotoxicity testing into standard toxicology studies presents multiple advantages as it reduces animal use and costs, accelerates data generation, and provides concurrent data that are useful for interpreting results. The *in vivo Pig-a* assay is a mammalian gene mutation assay which utilizes peripheral blood. In this study we evaluated the performance of the *in vivo Pig-a* gene mutation assay (MutaFlow® kit, Litron Laboratories) in rats and conducted an assay validation to characterize it for subsequent use in GLP toxicology studies.

Male Sprague-Dawley rats were treated daily for three consecutive days with 0, 20 or 40 mg/kg *N*-ethyl-*N*-nitrosourea via oral gavage. Blood was collected on multiple occasions from day 15 up to day 71. Blood samples were analyzed for *Pig-a* mutation using the rat MutaFlow® kit. Frequencies of reticulocytes (RET), mutant-phenotype RET (RET^{CD59-}) and mutant-phenotype erythrocytes (RBC^{CD59-}) were determined. Intra-assay and inter-assay variability, ruggedness, robustness and blood storage stability were assessed. Persistence of mutant-phenotype cells was also evaluated. Over the course of the study mutant cell frequencies remained low in negative control samples. RET^{CD59-} and RBC^{CD59-} accumulated in a dose-dependent manner and remained detectable 71 days after dosing. The assay demonstrated acceptable intra-run and inter-run variability with coefficients of variation of less than 4.8% and 20.6% respectively. The method was shown to be independent of the analyst performing the assay and unaffected by small changes in assay conditions. Finally, comparable results were obtained from freshly collected samples and samples refrigerated for up to 4 days.

Although technically challenging, the rat *Pig-a* gene mutation assay using a high throughput automated method was shown reliable. The different validation parameters evaluated during the conduct of this study yielded acceptable results.

Introduction

The integration of *in vivo* genotoxicity testing into repeat dose toxicology studies in safety assessment offers multiple advantages:

- it reduces animal use;
- it is cost effective;
- it allows rapid data generation;
- it can be combined with other endpoints.

The *in vivo Pig-a* gene mutation assay utilizes peripheral blood and thus has a high integration potential. It could be used as a follow-up to positive responses in *in vitro* gene mutation assays, as required by regulatory safety guidelines[1].

The *Pig-a* assay principles:

- *Pig-a* is a X-linked gene.
- Essential for the synthesis of glycosylphosphatidylinositol (GPI) anchors.
- GPI anchors are required for the attachment of surface proteins on hematopoietic cells (e.g. CD59).
- Absence of these surface proteins can serve as markers of *Pig-a* gene mutation.

The *Pig-a* gene mutation assay uses fluorochrome-conjugated antibodies specific to CD59 to score the frequency of mutant phenotype erythrocytes (RBC^{CD59-}) and mutant phenotype immature erythrocytes (RET^{CD59-}).

The objective of this study was to evaluate the rat *in vivo Pig-a* gene mutation assay performance through the conduct of an in-house validation study prior to subsequent use in regulatory toxicology studies.

Experimental Design

- Male Sprague-Dawley rats, approximately 7-weeks old, were treated with *N*-ethyl-*N*-nitrosourea (ENU), a genotoxic compound known to induce *Pig-a* mutation[2-3].
- Rats were treated daily, at approximately 24 hour intervals, for three consecutive days with 0, 20 or 40 mg/kg ENU via oral gavage.
- Blood samples were collected between Day 29 and Day 71 following treatment initiation.
- Samples were analyzed for *Pig-a* gene mutation using the MutaFlow Kit® , as previously described[4-5].
- Frequencies of mutant RBC and RET were calculated as previously described [4].
- Validation testing:
  - Intra-assay variability: 3 samples processed and analyzed 3 times each in a single run.
  - Inter-assay variability: the same three samples were processed and analyzed in three independent runs, on the same day.
  - Sample storage stability: 3 samples processed and analyzed freshly (Day 0) and at ~24-h intervals for up to 96 h.
  - Assay ruggedness: two analysts conducted independent runs with the same 3 samples.
  - Assay robustness: Same samples were tested under standard or slightly modified experimental conditions.
  - %CV= (standard deviation ÷ mean) × 100;%RE= ((measured value – benchmark value) ÷ benchmark value) × 100

Figure 1. Sample Processing Overview

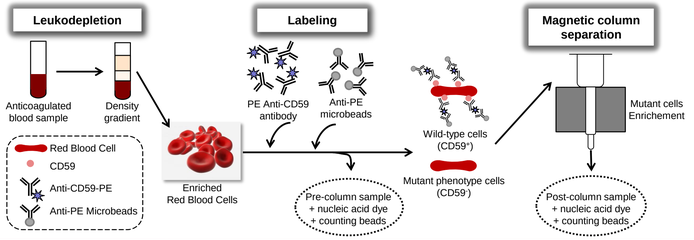
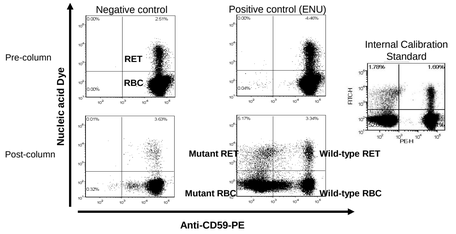


Figure 2. Flow Cytometry Data Analysis



Results

Table 1. Intra-Assay and Inter-Assay Variability

Treatment	Mean Values (SD)			%CV		
	% RET	Mutant RBC (× 10^{-6} RBC)	Mutant RET (× 10^{-6} RET)	% RET	Mutant RBC (× 10^{-6} RBC)	Mutant RET (× 10^{-6} RET)
Intra-assay						
Vehicle control*	2.6 (0.07)	0.4 (0.07)	0.2 (0.21)	2.8	n/a	n/a
ENU (20 mg/kg)	3.1 (0.06)	137.0 (3.84)	148.3 (7.03)	1.9	2.8	4.7
ENU (40 mg/kg)	2.9 (0.12)	425.8 (20.63)	330.7 (6.07)	4.0	4.8	1.8
Inter-assay						
Vehicle control	2.4 (0.18)	0.4 (0.08)	0.6 (0.83)	7.6	n/a	n/a
ENU (20 mg/kg)	2.9 (0.18)	139.6 (4.56)	153.7 (9.15)	6.2	3.3	5.9
ENU (40 mg/kg)	2.8 (0.15)	462.7 (78.35)	374.4 (77.30)	5.3	16.9	20.6

* Mean from two replicates.
n/a: not applicable due to low mutant cell frequencies in vehicle control samples.

Table 2. Blood Sample Storage Stability

Measurement	Treatment	Benchmark values	%RE			
		Day 0	24 h	48 h	72 h	96 h
%RET						
	Vehicle control	2.1	-4.8	-9.5	-9.5	-14.3
	ENU (20 mg/kg)	2.4	0.0	4.2	-4.2	0.0
	ENU (40 mg/kg)	2.5	-12.0	0.0	-12.0	-12.0
Mutant RBC (× 10^{-6} RBC)						
	Vehicle control	0.5	n/a	n/a	n/a	n/a
	ENU (20 mg/kg)	190.1	6.2	7.4	1.3	-3.3
	ENU (40 mg/kg)	373.7	-1.8	1.4	-0.8	-0.7
Mutant RET (× 10^{-6} RET)						
	Vehicle control	2.2	n/a	n/a	n/a	n/a
	ENU (20 mg/kg)	191.1	7.7	9.0	9.5	2.4
	ENU (40 mg/kg)	465.0	2.3	1.0	5.5	7.3

n/a: not applicable due to low vehicle control benchmark values

Table 3. Ruggedness Testing

Treatment	Mean Values		
	% RET	Mutant RBC (× 10^{-6} RBC)	Mutant RET (× 10^{-6} RET)
Analyst 1			
Vehicle control	2.6	0.4	0.2
ENU (20 mg/kg)	3.1	137.0	148.3
ENU (40 mg/kg)	2.9	425.8	330.7
Analyst 2			
Vehicle control	2.2	0.5	1.8
ENU (20 mg/kg)	2.7	144.5	164.3
ENU (40 mg/kg)	2.6	435.9	371.1
%RE			
Vehicle control	-13.7	n/a	n/a
ENU (20 mg/kg)	-12.0	5.5	10.8
ENU (40 mg/kg)	-9.3	2.4	12.2

n/a: not applicable due to low mutant cell frequencies in vehicle control samples.

Table 4. Robustness Testing – Assay Format

Treatment	Mean Values		
	% RET	Mutant RBC (× 10^{-6} RBC)	Mutant RET (× 10^{-6} RET)
Tube-based			
Vehicle control	3.6	0.2	0.3
ENU (20 mg/kg)	4.0	165.2	242.4
ENU (40 mg/kg)	4.5	311.1	499.4
Plate-based			
Vehicle control	3.6	0.2	0.7
ENU (20 mg/kg)	4.1	158.0	234.7
ENU (40 mg/kg)	4.4	327.1	524.1
%RE			
Vehicle control	0.0	n/a	n/a
ENU (20 mg/kg)	2.5	-4.4	-3.2
ENU (40 mg/kg)	-2.2	5.1	4.9

n/a: not applicable due to low mutant cell frequencies in vehicle control samples.

Table 5. Robustness Testing – Assay Conditions

Measurement	Treatment	Benchmark values	%RE			
		Standard Conditions	Modified Experimental Conditions			
			A	B	C	D
%RET						
	Vehicle control	3.2	6.2	6.2	3.1	-3.1
	ENU (20 mg/kg)	2.6	3.8	11.5	3.8	0.0
Mutant RBC (× 10^{-6} RBC)						
	Vehicle control	0.6	n/a	n/a	n/a	n/a
	ENU (20 mg/kg)	233.4	4.2	-10.3	-16.5	-2.5
Mutant RET (× 10^{-6} RET)						
	Vehicle control	0.4	n/a	n/a	n/a	n/a
	ENU (20 mg/kg)	277.1	1.5	-24.1	-21.5	-5.4

Condition A: Increased blood volume;
Condition B: Decreased antibody concentration;
Condition C: Change in incubation temperature with nucleic acid dye solution;
Condition D: Increased incubation time with antibodies and anti-PE microbeads.
n/a: not applicable due to low vehicle control benchmark values

Conclusions

- Mutant cell frequencies remained low (≤ 3.2 ×10^{-6} mutant cells) in vehicle control samples, as reported in the literature[6].
- ENU treatment induced a dose-related and persistent increase in mutant phenotype erythrocytes (both mature RBC and reticulocytes).
- Mutant cell frequencies in ENU treated animals (20 and 40 mg/kg/day) were similar to the literature[3-4].
- The %RET values obtained in vehicle controls were comparable to ENU treated animal values indicating that ENU did not cause bone marrow toxicity.
- All validation parameters tested passed:
  - ✓Intra-run variability %CV was ≤ 4.8%.
  - ✓Inter-run variability %CV was ≤ 20.6%.
  - ✓Sample stability of up to 4 days.
  - ✓Assay robustness was confirmed through small deliberate changes to assay procedure (blood volume; antibody concentration; incubation times and temperatures; assay format).
  - ✓Assay showed acceptable ruggedness.
- The method was considered suitable for use in GLP toxicology studies.

References

1. ICH Harmonised Tripartite Guideline S2 (R1), Step 4. (2011) Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use.
2. Phonethepswath, S., Franklin, D., Tourous, D.K., *et al.* 2010. *Pig-a* mutation: kinetics in rat erythrocytes following exposure to five prototypical mutagens. *Toxicol. Sci.*, 114, 59-70.
3. Dertinger, S.D., Phonethepswath, S., Weller, P. *et al.* 2011. International *Pig-a* gene mutation assay trial: evaluation of transferability across 14 laboratories. *Environ. Mol. Mutagen.*, 52, 690-698.
4. Dertinger, S.D., Phonetheswath, S., Avlasevich, S.L. . *et al.* 2012. Efficient monitoring of *in vivo Pig-a* gene mutation and chromosomal damage: Summary of 7 published studies and results from 11 new reference compounds. *Toxicol. Sci.*, 130, 328-348.
5. Dertinger, S.D., Phonethepswath, S., Avlasevich, S.L. *et al.* 2014. *Pig-a* gene mutation and micronucleated reticulocyte induction in rats exposed to tumorigenic doses of the leukemogenic agents chlorambucil, thiotepa, melphalan, and 1,3-propane sultone. *Environ. Mol. Mutagen.*, 55, 299-308.
6. Gollapudi, B. B., Lynch, A. M., Heflich, R. H. *et al.* 2014. The *in vivo Pig-a* assay: a report of the International Workshop on Genotoxicity Testing (IWGT) Workgroup. *Mutat. Res.*, in press. doi: 10.1016/j.mrgentox.2014.09.007.