γH2AX/pH3 method for genotoxicity mode of action determination

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Section 1: Method Description

Utility of genotoxicity screening for chemical risk assessment

	Carcinogenicity assay	Genotoxicity assay
Support	Mice/Rat	Cell lines
Cost	+M€	K€
Time	2 years	2 weeks
Ethics (3Rs)		
Acceptation		↗
Predictivity		↗

Increase world needs in *in vitro* testing in compliance with new legislations (UE, USA…).

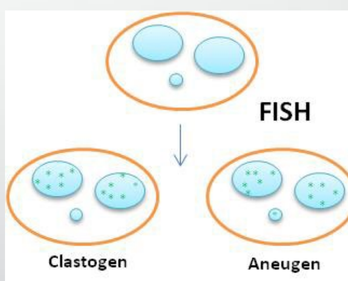
↓

Development of news genotoxicity assays.

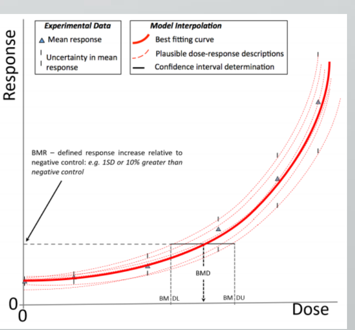
Limitations of the currently used genotoxicity assays

- **Inter and intra-specie differences** (Metabolism, DNA repair,...)

- **High-throughput** screening possibility.



- Determination of the **genotoxic mode of action**. (Aneugen/Clastogen)



- Determination of the **point of departure**.

Method introduction

- Induction of DNA damage and subsequent gene mutations is strongly correlated with **chemical carcinogenicity potential**.

- Currently **used *in vitro* battery of genotoxicity assays present a low specificity** (“false positive hit”), especially with the mammalian cell-based assay, compared with *in vivo* data.

- **Understanding the mode of genotoxic action** (aneugen or clastogen) of a genotoxic chemical is an important piece of information for chemical carcinogenesis assessment.

γH2AX/pH3 method overview

- **Histones H2AX and H3 are part of the nucleosome**, proteins directly surrounding the DNA, providing a biological context of high relevance for toxicity endpoints characterizing genotoxicity/carcinogenicity.
- The phosphorylation of H2AX histone (named γH2AX) is a very early **cellular response to DNA damage resulting from different DNA insults**. This biomarker is also a well-recognized pre-cancerous and cancerous biomarker *in vivo* and used as such in human cancer research.
- Histone H3 is phosphorylated (named pH3) during mitosis by the Aurora kinases to allow chromosome condensation and segregation and is a **biomarker of mitotic cells**.
- While an **increase in γH2AX is observed after cell treatment with clastogens, pH3 induction is observed after exposure to aneugens**, allowing an effective discrimination of clastogenic and aneugenic chemical.
- The *in vitro* γH2AX/pH3 method is based on the **quantification of these two biomarkers after cell exposure** to a tested compound. In parallel to these two biomarkers, cytotoxicity measurement permit to discriminate misleading cytotoxic chemicals. (PMID: 31289893)
- This *in vitro* method was also able to demonstrate the carcinogenic properties of radiations (ionizing and UV), bacteria producing colibactin and human virus.

Advantages of the *in vitro* γH2AX/pH3 method (1)

a) The *in vitro* γH2AX/pH3 method has been reported to be **more predictive of genotoxicity potential** (for specificity and sensitivity) than the commonly used assays (MNvit and Ames).

b) This method is the first one to permit to easily and reliably **discriminate the genotoxic mode of action** (clastogens, aneugens and misleading cytotoxic chemicals).

c) The *in vitro* γH2AX/pH3 method **can detect efficiently the different genotoxic mechanisms of action** for aneugenic and clastogenic chemicals.

d) This method is **not influenced by classical "false positive"** genotoxic chemical as apoptosis or p53 inducers (e.g. Nutlin-3).

e) The *in vitro* γH2AX/pH3 method provide **quantitative data** (potency ranking).

Advantages of the *in vitro* γH2AX/pH3 method (2)

f) This method is **faster than MNvit** test and it did not require cell cycle completion.

g) The *in vitro* γH2AX/pH3 method **can be applied to any cell type** (different species or cell lines from different organs). More than **14 different cell lines have been already tested**: TK6, HepG2, HepaRG, V79, L5178Y, CHO…

h) The use of cell lines or primary cells with different metabolism capacities enables **differentiation between directly from bio-activated genotoxins**.

i) This method can be performed with **cells cultured either in suspension, in 3D or in adherent** monolayers.

j) The use of multi well plates allows **high-throughput format**.

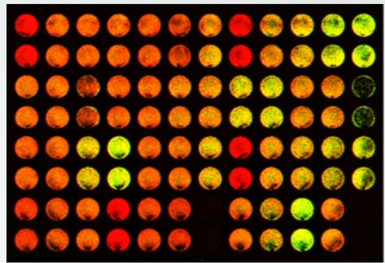
k) **Commercially available γH2AX and pH3 antibodies and kits** from different suppliers, as well as scoring services offered by CROs.

Comparison of different *in vitro* genotoxicity assays

	COMET	Micronucleus	Reporter gene assays	γH2AX/pH3
Detected DNA damage	10-100 strand break	DNA in cytoplasm	None	1-10 strand break
In vivo cancer biomarker	+/-	+/-	-	++
Cells	All	All (TK6, PBMCs)	Specific	All
High-throughput	+/-	+	++	++
Human metabolism	+	- (S9)	- (S9)	++
Genotoxicity MoA discrimination	-	-	-	++
Predictivity	-	+	+	++
Reproducibility	+/-	+	++	++
Validation	+/-	++	+	+

Quantification of γH2AX/pH3

Topic	Publications
Validation of the γH2AX assay **(Numerous model chemicals and Cell lines)**	Khoury *et al.* (2013) Env. Mol. Mut. Khoury *et al.* (2016) Mutagenesis Khoury *et al.* (2016) Arch. Tox. Khoury *et al.* (2020) Mut. Res.
Pesticides	Graillot *et al*. (2012) Env. Mol. Mut. Graillot *et al*. (2012) Mut. Res. Crepet *et al*. (2013) Toxicology
Heavy metals	Kopp *et al.* (2017) Environ Mol Mutagen.
Bisphenols	Audebert *et al*. (2011) Arch. Tox. Riu *et al*. (2011) Toxicology
Mycotoxins	Theumer et al. (2018) Toxicol lett.
Polycyclic Aromatic Hydrocarbons	Audebert *et al*. (2010) Tox. Letters Audebert *et al*. (2012) Tox. And Appl. Pharma. Liamin et al. (2017) Biochem Pharmacol. Tomasetig *et al*. (2020) Tox. Letters
Heterocyclic Aromatic Amines	Jamin *et al*. (2013) Plos One Chevereau *et al*. (2017) Arch. Tox.
Bacteria Metabolites	Martin *et al*. (2013) Plos Pathogen Andriamihaja *et al*. (2015) Free Rad. Mol Biol. Beaumont *et al*. (2016) Free Rad. Mol Biol. Beaumont *et al.* (2017) Am J Clin Nutr.
Oxidized lipids	Martin *et al*., (2013) Am. J Clin. Nut. Bastide *et al*. (2015) Cancer Research
Pyrrolizidine alkaloids	Louisse *et al.* (2019) Food Chem. Toxicol.
HepaRG cell line	Quesnots *et al.* (2016) Mutagenesis Kopp *et al.* (2020) Mut. Res.



In vitro γH2AX/pH3 method Description

- The histone H2AX and H3 phosphorylation status in the cell can be **easily quantified** with the use of **γH2AX and pH3 specific antibodies**.

- **Different quantification techniques can be used**: flow cytometry, western blot, High Content Analysis imaging, proteomic mass spectrometry.

- The γH2AX and pH3 biomarkers, and their respective antibodies used for the measurement, are **not protected by any patent or license**. Many suppliers proposed specific antibodies raised against the phosphorylated forms of histones H2AX and H3, which will serve to make the **assay readily and widely available**.

Limitations of the *in vitro* γH2AX/pH3 method

- Some test articles that cause **auto fluorescence** can interfere with the assay result with some quantification method. It is easy to recognize this with the proper controls, and it is possible to subtract out that test article-related background fluorescence.
- As with conventional *in vitro* toxicity assays, the *in vitro* γH2AX/pH3 genotoxic method does **not easily allow the evaluation of gasses**. However, some recent advances in this area will enable such investigations in near future.
- Since the γH2AX signal is expected to in part result from DNA replication or transcription blocking lesions induced by genotoxins, **cells in a proliferative state may be more appropriate** than cells in a confluence state. For pH3 signaling, cell, must be allow to reach mitosis.
- The method is **not able to detect specific aneugens with aurora kinases inhibiting activity** genotoxic MoA. However, addition of supplementary biomarkers such as polyploidy could be considered to detect them.

Section 2: Context of Use

Context of Use

A. How is your method intended to be used?

- **Screening and early selection of candidates** before entry in development by companies in-house or at service providers (e.g., for pesticides, pharmaceuticals, cosmetic ingredients). γH2AX/pH3 biomarkers quantification **can be applied as early genotoxicity screen** in parallel or as alternative to miniaturized version of the Ames test or micronucleus test in order to **predict the outcomes of the regulatory (*in vitro*) battery of genotoxicity tests.**
- **Follow-up testing and mechanistic approach** for candidates under development (e.g., for pesticides, pharmaceuticals) and marketed products (all compounds including chemicals under REACH). The *in vitro* γH2AX/pH3 method can be applied as **follow-up of the positive results in regulatory battery of *in vitro* genotoxicity assays** and to provide insight into MoA of genotoxic compounds (aneugen or clastogen).
- **Mechanistic studies and read across approaches** for retesting of marketed chemicals under REACH/EFSA and novel substances.
- **Determination of BMD and potency raking** thanks to the quantitative information by γH2AX/pH3 quantification.

Context of Use

B. What regulatory testing need does your method address?

- The *in vitro* γH2AX/pH3 genotoxicity method can contribute to a **mechanism-based, preferably animal-free, cancer assessment of chemicals**. Because of the complex and diverse mechanisms involved in carcinogenesis, it is most likely that a set of multiple *in silico* and *in vitro* tests will be required for the identification of carcinogenic propensities of a chemical. The *in vitro* γH2AX/pH3 method would be a valuable component of this set of test methods, as it has the **unique ability to reveal genotoxic modes of action** (i.e clastogenicity or aneugenicity).

- This assay can take place in a more general Integrated Approaches to Testing and Assessment (**IATA) for genotoxic carcinogens assessment**.

Context of Use

C. What regulatory space does your method address?

- The *in vitro* γH2AX/pH3 method has been used to screen genotoxicity potential of **different class of chemicals**: agrochemicals, pharmaceuticals, cosmetics, food/food additives, industrial chemicals, nanomaterials.

- The chemical space of the 800 model chemicals already tested has been analyzed and confirm a **full coverage of the chemical space**.

Context of Use

D. Has data generated by your method been used for regulatory submissions?

- The *in vitro* γH2AX/pH3 method was proposed as a complementary genotoxicity assay in a weight of evidence (WoE) approach by **WHO FAO**, european **SCCS** reglementation and **EFSA** evaluation.
- The *in vitro* γH2AX/pH3 method is currently **used by numerous pharmaceutical companies** for in-house screening and different **CRO**'s proposed this method as service. Numerous companies sell antibodies and kits to perform the method.
- Since 2022, a Detail Review Paper (DRP) and a Retrospective Performance Analysis (RPA) for the **γH2AX/pH3 method is under completion at OECD** that will contribute to a test guideline.

Section 3: Biological Relevance

Biological Relevance

A. Mechanistic understanding: How does the information provided by your method support known mechanistic knowledge of the carcinogenesis process?

- Histones H2AX and H3 are part of the nucleosome, proteins directly surrounding the DNA, providing a **biological context of high relevance for toxicity endpoints characterizing genotoxicity**.
- While an increase in γH2AX is observed after cell treatment with clastogens, pH3 induction is observed after exposure to aneugens, allowing an **effective discrimination of clastogenic and aneugenic chemical**. In parallel to these two biomarkers, cytotoxicity measurement permit **to discriminate misleading cytotoxic chemicals**.
- The γH2AX/pH3 method can be **useful in the context of an Adverse Outcome Pathway (AOP)** approach for genotoxic carcinogens and in the development of an IATA. Indeed, the **mechanistic information** that is provided by the *in vitro* γH2AX/pH3 method can be applied to **translate the molecular initiating events and cellular responses that are activated upon chemical exposure to carcinogenicity** hazards for humans.

Biological Relevance

B. Reference compounds: What are well-characterized and understood compounds that can be used or were used to assess the scientific validity or transferability of your method?

- **786 reference chemicals have already been tested and published:** 36 aneugens (4,6%), 411 clastogen (52,3%), 17 aneugen/clastogen (2,2%), 322 non-genotoxic (41%).
- Compounds with **different genotoxic mechanism and mode of action**:
  - **Aneugens**: kinases inhibitors, tubulin binders…
  - **Clastogens**: oxidative stress, bulky DNA adducts, topoisomerase inhibitor, inter-crosslink, dNTPs pool imbalance, nucleoside analogues, alkylation, intercalating agent, DNA repair inhibitor…
  - **"False" positives** (apoptosis or p53 inducers (e.g. Nutlin-3)).
- The *in vitro* γH2AX/pH3 method demonstrated an *in vitro* genotoxicity **predictivity of 94%** (sensitivity 98%; specificity 91%). (PMID: 31289893)

Biological Relevance

C. Comparison to existing laboratory animal methods

- Although at the moment *in vivo* genotoxicity testing is under most regulatory jurisdictions an integral part of the hazard assessment of novel chemicals and materials, reliable *in vitro* assays can **contribute to a reduction of unnecessary animal testing** following false negative or misleading positive *in vitro* genotoxicity test results.
- With an important accuracy (more than 90%), the *in vitro* γH2AX/pH3 method aim to **"replace" and "reduce" animal testing** by improving the prediction and interpretation of the *in vitro* (human cell based) genotoxicity assays, and by reducing the need for *in vivo* follow-up testing (genotoxicity and/or carcinogenicity testing).
- The mechanistic insight into the genotoxic properties of compounds (aneugen or clastogen) can **contribute to a refinement of the follow-up *in vivo*** testing strategy.
- The use of **human metabolic competent cell lines** (HepaRG, HepG2) coupled with the *in vitro* γH2AX/pH3 method permit to **avoid the use of S9 rat liver extract** as metabolizing system.
- The *in vitro* γH2AX/pH3 method is already used in **read-across studies** for chemicals, thereby reducing both *in vitro* and *in vivo* genotoxicity testing.

Section 4: Technical Characterization

Technical Characterization

A. How have the sources of variability been evaluated?

- As mentioned in points C and D, the *in vitro* γH2AX/pH3 method has been **extensively validated by intra and inter-laboratories studies** in different cell models and with different techniques of quantification of the biomarkers have been applied.

- **Different cell culture conditions** (cells in suspension or adherent), cell models (**2D or 3D**) and quantification technique have been applied to the *in vitro* γH2AX/pH3 method since the first development of the assay in 2008.

Technical Characterization

B. How has robustness been evaluated?

- As mentioned in points C and D, the *in vitro* γH2AX/pH3 method has been **extensively validated by intra and inter-laboratory studies** in different cell models with different techniques of quantification of the biomarkers.

- All these studies have demonstrated the **high robustness** of the *in vitro* γH2AX/pH3 method with **more than 90% concordance between labs**. (PMID: 31289893)

Technical Characterization

C. How has intra-laboratory reproducibility been evaluated?

- Since 2008 and the first experiment conducted at INRAE laboratory, the assay has been performed in this laboratory by more than ten different experimenters with consistently **high intra-laboratory reproducibility (superior to 95%) using positive controls as benchmark**.

Technical Characterization

D. How has transferability been evaluated?

- Transferability of the method was first assessed in collaboration with **five academic laboratories** (RIKILT, Netherlands; BPI, Greece; BfR, Germany; IPBS-CNRS, France; INSERM, France) using a **standard operating procedure (SOP)**. Testing results from the different laboratory were highly similar.

- An **extensive inter-laboratory validation** of the *in vitro* γH2AX/pH3 method was published with **seven different private companies** (Litron, Pfizer, Servier, Orion, Sanofi-Aventis, Bayer and Roche Pharma) using 84 chemicals. The validation has been performed largely according to OECD Guidance document 34. An overall **concordance between companies of 92 %** was achieved with a sensitivity of 92 % and a specificity of 96 %. (PMID: 29106658)

Closing/Contact

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