Horizon scanning for novel and emerging *in vitro* mammalian cell mutagenicity test systems

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Abstract

The induction of gene mutation within a DNA sequence can result in an adverse impact, altering or preventing gene function. Therefore, *in vitro* evaluation of mutagenicity is an essential component of the toxicological screening process. A variety of mutagen screening tools are routinely used in genetic toxicology, which are based on selected reporter genes. These assays are however typically labour intensive and impractical for high throughput screening. Considering this, the IWGT (International Workshops on Genotoxicity Testing) sub-group on Novel & Emerging In Vitro Mammalian Cell Mutagenicity Test Systems undertook a literature search to identify new approaches for mutation detection. This review therefore focused on identifying new approaches for mutation detection that have the potential for use as a future genotoxicity screening tool. A comprehensive literature review identified genome-wide loss-of-function screening tools, next generation sequencing (NGS) mutation characterisation and fluorescence-based mutation detection methods as having significant promise as an emerging *in vitro* mammalian cell mutagenicity test system. Each of the technologies considered was assessed for its capacity to report on a wide array of heritable mutagenic changes, necessary to cover the full spectrum of genetic events imparted by substances with a broad range of modes of action. Of the technologies evaluated, NGS techniques exhibited the greatest advantages for use in a genotoxicity testing setting. However, it is important to note that the emerging techniques identified could not facilitate routine mutagenicity testing in their current format and require substantial additional optimisation and tailoring before they could be utilised as an *in vitro* mammalian cell mutagenicity test system. Additionally, new mammalian cell mutation test systems must be able to accurately and reliably detect and quantify rare events; hence any new system would require careful validation. Nevertheless, with further development emerging technologies such as NGS could become important in establishing more predictive and high-throughput regulatory hazard screening tools of the future.

Highlights

- Current mammalian *in vitro* mutagenicity assays are labour intensive and not high throughput
- Emerging techniques require substantial development and optimisation

- Next generation sequencing offers huge potential in the mutagenicity testing arena

Key words

Gene mutation, Mutation reporter screen, Next generation sequencing, Haploid cells, Trinucleotide repeat instability.

1. Introduction

The past three decades have witnessed rapid and major advances in our understanding of the mechanistic aspects of mutagenesis at the molecular, biochemical, and cellular levels. Currently, it is economically feasible to get insights into DNA sequence variations at the single base-pair level of the entire genome among various samples and cohorts. The U.S. National Cancer Institute's dictionary of cancer terms defines mutation as any change in the DNA sequence of a cell [1] . Thus, this review assumes that gene mutations are permanent alteration in the DNA sequence that may or may not have an adverse impact on the individual cell. Such alternations can vary from a single base-pair change to a large segment of the gene or multiple genes. While some DNA sequence changes result in altered gene function, we currently do not have a full understanding of the impact of all possible genomic changes.

The *in vitro* mammalian cell mutation assays currently in routine use for regulatory genetic toxicology were developed in 1970s and 80s. These screens use selectable reporter genes such that cells with certain sequence changes (mutations) can grow and form colonies in the presence of a substance that is otherwise toxic to wild-type cells. Examples of such tests include the OECD Test Guideline 476 and 490 *In Vitro* Mammalian Cell Gene Mutation Tests, based on mutation at thymidine kinase (TK) gene, hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene, and a transgene of xanthineguanine phosphoribosyl transferase (XPRT). These mammalian cell assays are labour and time intensive and not readily amenable to high throughput screening. Most importantly, these assays, by design, depend on a functional change of the reporter gene product and may not respond to all newly induced genetic alterations. For example, the X-chromosome location of the HPRT gene makes it relatively insensitive to large deletions affecting the entire chromosome, as such events tend to be lethal to the cell due to the hemizygous nature of the X-chromosome. The X chromosome location also prevents detection of mutations that occur by mitotic recombination; these events can be detected by autosomal genes such as *tk*. Thus, the HPRT, TK and XPRT mutation tests detect different spectra of genetic events. Another limiting factor of the currently used mammalian cell mutation assays is their reliance on an externally supplied metabolic activation system (such as liver homogenate preparations) to detect pro-mutagens. Furthermore, unless extensive molecular and banded karyotypic evaluation is

conducted on the mutants, the current assays provide little information on the underlying mechanism that resulted in the induction of a mutation.

The IWGT sub-group on Novel & Emerging In Vitro Mammalian Cell Mutagenicity Test Systems examined the literature to identify new approaches for mutation detection, which show promise for their application in hazard identification. The focus of this sub-group was assays that were not covered by the other three sub-groups (e.g., *in vitro* Pig-a, transgenic cell lines, and improving existing assays using TK6 cells). Only assays that anchored the measured endpoint to DNA sequence changes were considered in this review; those indicator assays that only measured DNA damage signalling were excluded from consideration as they do not measure *bona fide* mutational events.

2. Materials and methods

The literature search undertaken for this review utilised the PubMed (www.ncbi.nlm.nih.gov/pubmed) and Web of Science databases (http://www.webofknowledge.com/). Initially the following key words were used as search terms:

- Trinucleotide repeat instability AND mutation
- Haploid cell line mutation
- Next generation sequencing mutation screen*
- Mutation detection AND flow cytometry
- Reporter mutation detection OR screen*
- Haploid transposon (HTP) screening
- Mutation detection
- Intragenic mutations
- Transgenic reporter genes AND mutation
- Transgenic shuttle vectors AND mutation
- PiggyBac Transposon AND mutation
- transposon mutant libraries
- Single-molecule PCR analysis (SM-PCR) AND mutation

- Expanded simple tandem repeats (ESTRs) AND mutation
- Trinucleotide microsatellites AND mutation
- Trinucleotide repeat mutagenesis
- Microsatellite mutation
- Tandem-repeat mutations
- Transgenic rodent mutation reporter (TGR)
- Characterization of mutation rate
- Haploid gene-trap mutants
- Human isogenic cell line
- Chemically induced microsatellite mutations
- Mutation reporter screen
- Sensitive small pool PCR (SP-PCR) AND mutation
- GFP Reporter AND mutation

*- Truncation search term.

Using these search terms 96,968 papers were identified in the databases, the vast majority of them considered irrelevant as they were unrelated to the subject matter of the literature search. Focus was placed on identifying articles that demonstrated novel techniques or potentially novel techniques for undertaking mammalian cell mutagenicity testing *in vitro*. Moreover, articles that focused on techniques under investigation by the other IWGT sub-groups were disregarded to avoid duplication. To narrow the focus to more relevant publications, a second search was conducted using a more restrictive set of terms. These terms were selected with the aim of narrowing the search focus to novel *in vitro* mammalian cell mutagenicity assays, whilst avoiding inclusion of articles using already established test systems and / or tests that were unrelated to mutation screening. The refined set of search terms used included the following:

- Haploid cells AND Mutation
- Mutation reporter screen

- Next generation sequencing reporter screen
- Trinucleotide repeat instability AND mutation

3. Results and discussion

The final, refined search using the four terms defined in the Methods section resulted in the identification of 3707 papers of which a total of 3684 were discarded. Papers were discarded if they were irrelevant (i.e. not applicable for application as an *in vitro* mammalian cell mutagenicity assay),were not based in mammalian systems (e.g. instead the publications focused on other organism such as plants, aquatic organisms, or microorganisms) or if they involved the currently used gene mutation assays (i.e. established mutation reporter assays that are already applied in the genotoxicity testing field). Additionally, it is important to note that an opportunity to utilise reporter screens that signify DNA damage has arisen following exposure to an exogenous agent. For example, the 'Anthem's Genotoxicity Screen' was developed to highlight the presence of increased DNA damage through the evaluation of p21, GADD153 and p53 reporter gene activity in response to genotoxic agents [2]. Although these test systems detect DNA damage signals, this does not directly mean that a mutagenic event has arisen. DNA damage signals are often induced in extensively damaged cells that may undergo either DNA repair or apoptosis. Thus, such report assays are not specifically mutation detection systems. Consequently, they were not considered further in this review as they are not capable of evaluating the induction of mutagenic events.

For each of the four-selected search terms, the total number of hits, number of papers discarded and number of papers selected for further evaluation are highlighted in Table 1. Additionally, a contextual summary of each of the papers selected for review is provided in Table 2, coupled to a brief outline on how the techniques might be used for hazard identification and the advantages and/or disadvantages of each of the approaches.

Within Table 2 the papers selected for this review were grouped into three categories: genome-wide loss-of-function screening tools, next-generation sequencing (NGS) mutation screens and fluorescence-based mutation detection methods. Papers within the genome-wide loss of function category were based either on haploid cell or Clustered Regularly

Interspaced Short Palindromic Repeats (CRISPR) studies. The haploid studies have not directly been utilised for chemical mutagenicity screenings. However, they demonstrate the potential of using haploid cells for this purpose as they contain a single gene and thus, mutation in this reporter gene would result in measurable phenotypic change [3-8]. This would therefore offer possibilities for mutagenicity screening, including reverse mutation assays or analysis of a test compounds effect on a gene of interest. The CRISPR studies that were evaluated had not been directly applied to mutagenicity screening, but they show the potential for creating libraries of mutant cells and the isolation of specific mutants [9, 10]. The use of such a system for mutagenicity screening following exposure to an exogenous agent is questionable, as the methodology would require significant adaption for this application.

Perhaps the most promising of the three categories within Table 2 is use of NGS [11-16]. NGS technology can potentially permit high throughput genome wide mutation detection and the power to provide supporting linkage between a mutation signature with the genotoxic agents' mode of action (MoA) through identification of changes characteristic to specific MoAs. However, a significant issue with this technique at present is the high error rate associated with the sequencing procedure which would reduces the ability to detect mutations occurring at very low frequencies. NGS error rate varies, depending on the technical approach used; for example, the PCR copy consensus assay-based techniques Safe-Sequencing system (Safe-SeqS) and the Duplex sequencing method have error rates of 2.0 x 10^{-4} and 2.5 x 10^{-6} errors/base pairs (bp) respectively [11, 17, 18]. Both Safe-SeqS and duplex sequencing are capable of identifying low abundance mutations. However, they have low effective coverage because of redundant PCR amplification due to superfluous DNA replication. In comparison circle sequencing, whereby genomic DNA fragments are amplified by rolling circle amplification has an error rate of 7.6 x 10^{-6} /bp [11, 19, 20]. Circle sequencing offers the advantage that the original DNA molecule is the only template, therefore possible errors are not amplified further. Both rolling circle amplification and PCR copy consensus assays are techniques that can detect point mutations. In order to undertake analysis of genome structural variations induced by a clastogenic agent methodologies need to be used that can identify junctions of genome fragmentation [11]. Typically, this can be achieved by the breakpoint being detected in overlapping reads at that locus. This requires sequencing of multiple cells and therefore would be highly expensive. Regardless of the NGS approach

undertaken the methodology cost can be very high, making dose-response analysis expensive. Nonetheless, it is notable that the cost of NGS is continually falling and there is extensive effort currently underway to minimise the error rate. Thus with future development, NGS techniques offer significant promise as an emerging mutation detection tool that could be applied in genotoxicity assessments.

The final category was dedicated to test systems that utilised fluorescence-based mutation detection methods, typically reliant on the activation of the green fluorescent protein (GFP) gene when a mutation is induced [21-26]. This fluorescence-based technique typically detects a frame shift mutation that consequently places the GFP gene into an open reading frame, thus allowing the detection of GFP by high throughput analysis equipment such as flow cytometry. Despite the speed of analysis and the large numbers of samples that could readily be evaluated in a short space of time, there are some potentially significant disadvantages to this category of techniques. Firstly, they rely on the integration of plasmids into host cells; given that transfection efficiencies of mammalian cells vary substantially, the reproducibility of data generated in such vector-based systems may be severely compromised. Secondly, the methodology would only be able to detect mutagenic events that shifts the GFP protein into the open reading frame, thus mutagenic events that do not impact the reading frame would be undetectable.

4. Recommendations and Future Outlook

Our search identified an array of technologies that are currently used to detect and analyse mutation and that are being applied in a variety of different contexts. However, none of these new approaches have been developed or are currently readily applicable for routine hazard identification. While these approaches demonstrate significant promise, particularly the NGS approaches, they require substantial development before they can be applied to evaluate the induction and characterisation of mutation for genotoxicity testing purposes. It should also be noted that none of the technologies discussed address the issue of reliance of in vitro methods on exogenous metabolic activation. It is also important to discriminate between a regulatory-driven test system versus a model for use in research mode as the requirements

can be very different when considering the demands required for routine safety assessment as opposed to hypothesis testing.

The IWGT Workgroup extensively discussed and reached consensus on the following features that would be needed for an ideal mutagenicity test system for safety assessment:

1. Novel test systems should ideally report on a wide array of heritable mutagenic changes manifested throughout the genome and cover the full spectrum of genetic events induced by substances with a broad range of modes of action.
2. Although not absolutely necessary for hazard identification as it is currently defined, it would be of substantial benefit if tests are able to detect mutations that result in disease-related phenotypes. However, assessment of overall mutational load also has value.
3. There is increasing evidence that mutations in non-coding regions can have a negative impact on cell function (e.g. transcription factor binding site disruption) and are associated with human disease and cancer. Therefore, test systems are needed that would include the identification of mutations in non-coding regions.
4. New mammalian cell mutation test systems must be able to accurately and reliably detect and quantify rare events. Accuracy and reliability would need to be carefully validated.
5. Ideally, novel test systems should be metabolically competent, thus, avoiding the necessity for exogenous metabolic activation. Nevertheless, the limitations of any *in vitro* system, relative to *in vivo* conditions, kinetics and dynamics, must be acknowledged. Novel test systems that are able to recapitulate *in vivo* complexity will likely yield improvements in predictive capacity for hazard assessment.
6. Hazard screens that have the capacity to be high throughput would be of great benefit, but this needs to be balanced against the cost effectiveness of the technology as high cost would likely retard adoption and implementation.

Despite the emergence of novel methods for identifying mutation, the new tests available are not currently in a position to move into routine safety assessment use. However, with future research to tailor the application of some of these exciting developments in the mutagenicity

testing arena, the advent of new technologies such as NGS will no doubt have an important role to play in regulatory hazard screening of the future.

5. Acknowledgements

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Table 1- Total number of papers identified, discarded and selected when chosen search terms were inputted into the PubMed and Web of Science databases

Search term	**Total number of hits**	**Number of papers discarded**	**Number of papers selected for review**
Haploid cells AND Mutation	2528	2521	7
Mutation reporter screen	502	498	4
Next generation sequencing reporter screen	17	10	7
Trinucleotide repeat instability AND mutation	660	655	5

Table 2 – Context and summary of papers identified within horizon scan for novel & emerging *in vitro* mammalian ce

Paper Reference	Context of use & cell type	Paper summary	Chemical tested (if any)	How to apply t mutagenicity t
Genome-wide loss-of-function screening tools (Haploid cells and RNA interference)				
Wutz, 2014a[3]	Introduction to what haploid cells are.	Description of the development of haploid cells and their potential use.	N/A	Almost all the p this category (h studies and CR application) we genome-wide l function screen support pheno analysis. Although the h papers do not undertake chem mutagenicity s they do demon potential of usi cell lines in par this purpose. B haploid cells, a
Wutz, 2014b [4]	Introduction to what haploid cells are (2).	Discussion on haploid development in animals, haploid mammalian embryos, mammalian haploid embryonic stem cells, developmental potential of haploid cells, haploid cells in tumours, application of haploid cells for genetic screens.	N/A	
Bürckstümmer *et al*, 2013 [5]	Creation of a library of mutant haploid cells that could be used for illustrating	The study created a library of mutant haploid cells with a gene-trap retrovirus. Using this library, a clone was identified that has a disruption to the TNFRSF1A	N/A	

	gene function by their phenotypes. KBM7 - stable near-haploid cell line subcloned from a CML patient sample containing the BCR-ABL1 gene fusion.	gene which encodes for the TNF receptor. The cells therefore would no longer respond to TNF stimulation and were unable to undergo TNF induced apoptosis.		which directly phenotypic cha be compensate second copy of (as in diploid ce offers various p for mutagenicit such as isolatio mutants for rev mutation scree direct analysis of the test com particular gene interest.
Pettitt *et al*, 2013 [6]	Creation of a large haploid library of random mutants by piggyBac transposon mutagenesis. Mouse embryonic stem cells	This study created a library of haploid mutants using piggyBac transposon mutagenesis. The library was tested by the exposure to 6-thioguanine which allowed identification of mutations in the DNA mismatch repair pathway. The study also isolated PARP1 mutants which were subsequently used to identify the role of PARP1 in olaparib toxicity.	N/A	The CRISPR pap demonstrate th to create librar mutant cells ar of specific mut this may be les detecting rand mutagenicity e required for ge screens.
Rong *et al*, 2015 [7]	Human haploid cells mutagenized by integration of gene trap vectors to	Mutagenized haploid cells were treated with a bacterial pore-forming toxin, aerolysin, which binds to GPI-anchored proteins for targeting to the cell	N/A	

	identify genes required for GPI biosynthesis. Human HAP1 cells derived from haploid KBM7 cells (CML haploid cells)	membrane. Cells that showed low surface expression of CD59, a GPI-anchored protein, were further enriched for. This screen identified 23 gene regions that when mutated are expected to decrease surface expression of GPI anchor proteins.	
Tokunga *et al*, 2014 [8]	Use of haploid mouse ESCs for identification of mutants defective in steps of the GPI-anchor biosynthetic pathway	The study mutagenized ESCs with N ENU. Then a phenotypic screen of mutants defective in different steps of the GPI-anchor biosynthetic pathway that results in an alpha-toxin resistant phenotype, was conducted. The investigation identified 115 mutant alleles that were defective in the pathway using this technique.	ENU (0.25 or 0.2 mg/ml for 2h) to induce mutation for phenotypic screening for loss-of-function mutants.
Koike-Yusa *et al*, 2014 [9]	Use of CRISPR-associated systems to introduce genome wide-targeted mutations in mouse ESCs.	This study created a large mouse genome-wide lentiviral CRISPR gRNA library to express 87897 gRNAs targeting 19150 mouse protein coding regions. This produced genome-wide	N/A

		mutant mouse ESC libraries that were used for recessive screens using alpha-toxin and 6-thioguanine, which identified 4 unknown genes involved in the resistance to these treatments.		
Wang *et al*, 2014 [10]	Loss-of-function genetic screening approach for positive & negative selection using a genome-scale lentiviral sgRNA library. KBM7 cells & HL60 cells (a pseudo-diploid human leukemic cell line).	CRISPR-Cas9 system was used to create a mutant library in two human cell lines. These were subsequently screened by exposure to 6-thioguanine to identify expected members of the DNA mismatch repair pathway.	N/A	
Next generation sequencing (NGS) mutation screens				
Maslov *et al*, 2015 [11]	Review paper discussing the application of NGS for genotoxicity testing.	Paper discusses potential NGS approaches for mutation screening, the pitfalls of the technique and what we need to overcome with further research to move	N/A	NGS based t are not wid the genetic field. This te potentially

		the use of NGS in mutagenicity testing forward.		power to er genome wic and charact mutation. A it has the po provide dat the linkage mutational profiles witl genotoxic a However, te advances th down cost a discriminati background mutations a chemically i events to m abundance mutation ar required.
Poon *et al*, 2014 [12]	Review paper that discusses mutation detection in human tumours using NGS.	Paper discusses mutation signatures in human tumours, the value of characterising those signatures by NGS and implications for surveillance and prevention of cancer. The authors state that they 'envision development of a wide-ranging compendia of mutation signatures from tumours and a concerted effort to experimentally elucidate the signatures of a large number of mutagens'.	N/A	
Wang *et al*, 2016 [13]	Development of an ultra-sensitive NGS platform called "Easy Mutation Frequency detection platform" ("EasyMF") and incorporating it with a widely used supF shuttle vector-	The paper's sequencing technique was developed to analyse low frequency mutations caused by DNA damage treatments. This was undertaken by transfecting a UV damaged plasmid into human 293T cells and allowing replication to occur. The mutations were amplified and identified by NGS. Emphasis was placed investigating mutations in the Poln and REV1 genes	UV (220 J/m^3, plasmid exposure time not given) Average mutation frequency was 1.0E-04	

	based mutagenesis system. REV1 knocked down and Polη knocked down 293T cells (human embryonic kidney cells)		
Wright *et al*, 2016 [14]	Use of an NGS method based on amplicon sequencing for mutant analysis during cell line development. CHO cells	This study developed a method for the identification of genetic mutations in cloned CHO cells expressing a biotherapeutic protein. Total RNA was isolated from cell samples, cDNA amplified and subsequently analysed by NGS.	N/A
Zhang *et al*, 2015 [15]	In this study, the authors explored the use of RNA-sequence technology (NGS) for the characterization of	To cause an increase in cell mutation rate CHO cells were culture with a mitogenic selection reagent, methotrexate, prior to sequencing. Analysis of the heavy chain and light chain of the mAb was undertaken, coupled to use of GAPDH as the house keeping gene. When cells were	Methotrexate (0, 20 or 80 nM for 3-4-day passage period)

	the mutation rate in a stably transfected CHO cell line expressing a recombinant monoclonal antibody (mAb) under extensive *in vitro* passaging.	sequenced the study noted an elevated mutation rate with increasing passage number when 80nM methotrexate was applied.		
Getta *et al*, 2017 [16]	Study that uses NGS to detect mutations in leukaemia patient bone marrow.	The study compared a 28 panel NGS technique for mutation identification with the more commonly used multi-parameter flow cytometry technique. The paper stated that by using a 28 NGS panel their study allowed broad coverage of known mutation hotspots and a median number of 2 mutations was detected in patients at diagnosis.	N/A	
Fluorescence-based mutation detection methods				
Dobrovolsky *et al*, 2002 [21]	Development of a double transgenic CHO cell line that contains: Plasmid 1)	The double transgenic cells were treated with gamma-radiation, MNU or MMS. If a mutation occurred in the repressor gene and no functional repressor was	Gamma-radiation (400, 500, 800rad);	Fluorescence b for mutation d offer the oppo high throughpu

	coding for GFP under the control of a Tet-responsive promoter that contains repressor-binding operator sequences; and Plasmid 2) Tet-repressor.	synthesized, the cells expressed GFP. Mutation events were therefore measured by FACS. Due to the nature of the technique only large-scale mutations were detectable. Generally, the mutation frequency data were not clear cut. Issues with low / variable cloning efficiency could affect sensitivity, specificity and reproducibility.	MNU (200µM); MMS (750µM)	following treat potential muta techniques rely activation of a following a mu event such as a mutation in a t nucleotide rep shifts the gene open reading f These techniqu unable to dete mutagenic eve point mutation promise as a sc tool.
Chatterjee, *et al,* 2015 [22]	TNR mutagenesis was used to assess mutation events induced by environmental stress. GFP(CAG)89 cells derived from T-REx HEK293 cells	This study aimed to investigate if environmental stress induced mutagenesis in TNRs in human GFP(CAG)89 cells derived from T-REx HEK293 cells. These cells carry a chromosomal mini-gene with a CAG89 tract repeat in the middle of it. If a mutation of this tract repeat is induced it will cause robust expression of GFP that can be measured by FACS. During the study cells were exposed to heat, cold, hypoxia or oxidative stress and the frequency of GFP+ cells quantified by FACS.	Heat, cold, hypoxia or oxidative stress	

		The study demonstrated that environmental stress was capable of inducing mutations in CAG repeats.	
Chatterjee *et al*, 2016 [23]	This is a follow on from the previous Chatterjee paper to assess the mechanism of environmentally induced TNR mutagenesis.	The study used the same assay in [22] to investigate which DSBR pathways might play a role in environmental stress-induced TNR mutagenesis. The investigation demonstrated that a knock down of alt-NHEJ components XRCC1, LIG3, and PARP1 suppresses stress-induced TNR mutagenesis.	N/A
Healy *et al*, 2006 [24]	Flow cytometric detection of tandem repeat mutations induced by various chemical classes Embryonic murine C3h10t1/2 cells and DNA mismatch repair (MMR) proficient embryonic	To facilitate detection of genotoxicity from environmental mutagen exposure, this study generated an *in vitro* enhanced GFP reactivation assay that quickly and effectively detects frameshift mutations in tandem repeat sequences. Two cell lines (C3h10t1/2 and MC2a) were transfected with GFP reporter plasmids which contain an out of frame GFP sequence. A frameshift mutation in these sequences consequently results in GFP revertants, which can be quantified	H_2O_2 (0, 0.001, 0.01, 0.1 or 1 mM for 1h); TPA (0, 100, 325,650, or 1325 nM for 48h); BPDE (0, 0.5, 1, 2 or 3 μM for 1h);

	fibroblast cell line MC2a	by FACS. The cell lines were treated with H_2O_2, TPA, BPDE, ENU, 9AA and two controls: acetone and ethanol. All chemicals induced an increase in GFP revertants, with the assay responding to a range of classes of mutagenic and carcinogenic compounds. The responses were not linked to cytotoxicity.	ENU, 9AA (0, 2, 8 or 16 μM for 4h)
Slebos *et al*, 2002 [25]	Mutation induction in tetranucleotide repeats. Human colorectal carcinoma cancer cell line RKO	The main goals of this study were to assess the effects of DNA damage on mutation frequency in microsatellite sequences and to determine any sequence-specific responses to DNA damage that may explain sporadic microsatellite mutations observed in carcinogen- related human cancer. Using the GFP reporter assay the study demonstrated that DNA damage can differentially increase the number of these mutations, depending on the agent and on the microsatellite repeat unit. RKO cells were transfected with plasmids containing microsatellite repeat units that shift the reading frame of the GFP	Gamma irradiation (5 Gy/min for 1 min or 10 Gy/min for 1 min); MNNG (5, 25, 50 μM for 1h); t-butyl hydrogen peroxide 1, 2, 3 mM for 1h);

		downstream. This allowed for detection of microsatellite slippage mutations induced by several DNA-damaging agents via quantification of GFP revertants using FACS. RKO cells were subsequently treated with gamma irradiation, BPDE, MNNG, t-butyl hydrogen peroxide, and UV irradiation and assayed for GFP-positive cells 48 h later. All mutagenicity tests induced increased GFP positive cells.	BPDE (0.5, 1, 2 μM for 1h); UV irradiation (15, 60 and 100 J/m^3 exposure time not given)
Santillan *et al*, 2014 [26]	Use of GFP-based fluorescence assay for the assessment of CAG repeat instability. T-Rex 293 cells	The study describes a GFP-based fluorescence assay for assessment of CAG promoter repeat instability. The assay exploits an engineered intronic CAG repeat tract that interferes with expression of an inducible GFP mini-gene. GFP function was impaired by repeat expansion in a length- dependent manner. The intensity of fluorescence varies inversely with repeat length, allowing estimates of repeat tract changes in live cells. T-Rex 293 cells were transfected with the GFP-Pem plasmid with a CAG89 repeat	

tract. CAG repeat instability was subsequently induced with doxycycline allowing for assessment between repeat length and GFP fluorescence. CAG repeats were also cleaved by the addition of zinc finger nuclease. The fluorescence assay, however, offers the possibility of directly detecting CAG repeat expansions.

TNFRSF1A = tumour necrosis actor receptor superfamily member 1A, TNF = tumour necrosis factor, CML = chronic mye
cluster region Abelson proto-oncogene, PARP1 = poly adenosine diphosphate –ribose polymerase 1, GPI = glycosylp
stem cells, ENU = N-ethyl-N-nitrosourea, gRNA = guide ribonucleic acid, sgRNA = single guide ribonucleic acid, UV = u
ovary cells, mAb = monoclonal antibody, MoA = Mode of action, MNU = N-methyl nitrosourea, MMS = methyl m
activated cell sorting, TNR = trinucleotide repeats, alt-NHEJ = alt-nonhomologous end joining, DSBR = double stranded
TPA = 12-O-tetradecanoyl-phorbol-13-acetate, BPDE = benzo-a-pyrene-diol-epoxide, 9-AA = 9-aminoacridine, MNNG =
GFP = green fluorescent protein.