

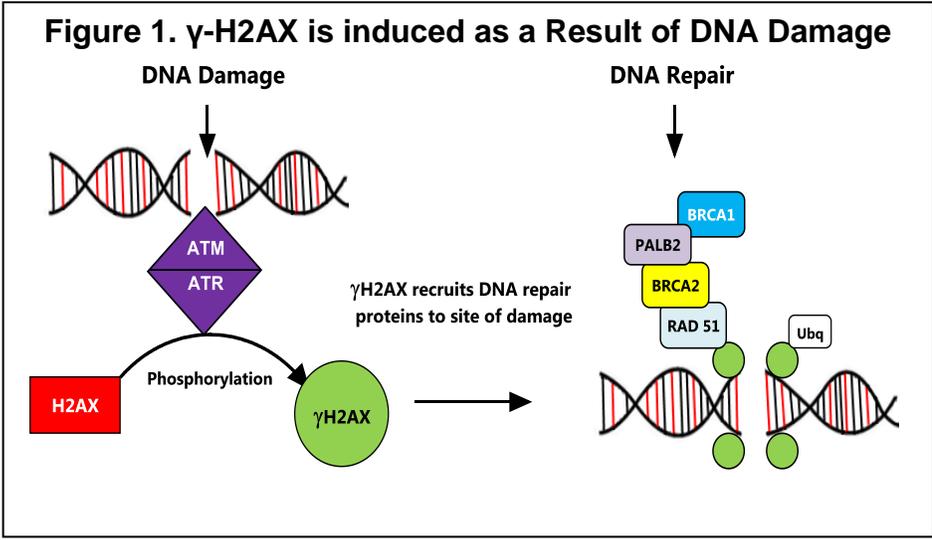
Trevigen’s γ -H2AX Pharmacodynamic Assay

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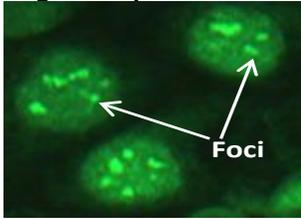
Here we describe an ELISA based Pharmacodynamic Assay (PDA) to measure the formation of γ -H2AX. Such an assay will be useful for drug screening, basic research and upcoming clinical trials providing one of many needed tools to support hypothesis-driven drug design strategies.

The development of molecularly targeted anti-cancer drugs is a result of decades of basic research [1-5] and the efforts of the human and cancer genome projects. Such therapeutics offer improved efficacy and fewer cytotoxic side effects compared to previous classes of therapies. The regulatory approval of molecularly targeted drugs such as imatinib (Gleevec), trastuzumab (Herceptin), gefitinib (Iressa), erlotinib (Tarceva), bevacizumab (Avastin), cetuximab (Erbix), sorafenib (Nexavar), and sunitinib (Sutent) has provided clinical validation for this approach. More targeted compounds such as the PARP inhibitors, based upon their synthetic lethal relationship with BRCA1/2, are currently in clinical trials demonstrating efficacy with little or no toxic side effects at therapeutic dosages [6]. The management of cancer patients is transforming from empirically based approaches to individualized treatments based on tumor specific molecular targets [4, 7, 8].

Despite new technological developments and better understanding of “cancer genes” and their associated pathways, drug discovery remains expensive, slow, and high risk [9-11]. Only 1 in 20 cancer drugs that enter clinical trials gains regulatory approval [10]. The major reasons for failure include poor therapeutic activity and unacceptable toxicity. To increase chances of success the development of the next generation of molecular cancer therapeutics and diagnostics will require the identification and validation of useful Pharmacodynamic (PD) biomarkers and assays. PD biomarkers are used to quantify the molecular and functional effects produced by a drug on its intended target which may or



may not correlate with biological and clinical effects. The effect of a drug on PD endpoints, i.e. both actions on the cognate drug target and downstream of that target in it’s immediate signaling pathway, are often a measure of a change in activity or expression of a molecular target. PD biomarkers are frequently distant to the effect of the agent, as in the case of a change in the phosphorylation of a protein by a target kinase [12]. Since PD biomarkers play important roles in deciphering disease processes and mechanisms of drug action, validated PDAs to accurately measure the effects of a drug on its target are becoming an essential component to clinical trials [13-16] and the drug development paradigm. Measuring the response of PD endpoints to therapeutic -intervention will support rational drug development approaches and improve the success of clinical trials. The commercial availability of PDAs for preclinical drug discovery will accelerate the optimization of therapeutic regimens providing insight to the process so that the correct agent and dose is chosen for clinical trial evaluations [12, 17].

Figure 2. γ -H2AX Foci

DNA Repair Pathways as Targets for Drug Development: Multiple DNA repair and DNA damage response (DDR) mechanisms detect the presence of DNA lesions and regulate cellular functions, including the cell cycle, in an effort to maintain the integrity of the genome. DNA repair pathways thereby help prevent the onset of cancer, disease and aging phenotypes [18]. It is estimated that tens of thousands DNA lesions are repaired daily by human cells [19]. If not repaired, these lesions will lead to mutations, genomic changes and may trigger apoptotic pathways. With respect to conventional cancer chemotherapies these pathways are a double-edged sword. The same pathways that maintain the stability of the genome repair the damage caused by therapeutic agents thus ameliorating the cytotoxic effects in tumor cells. Therefore the capability of the tumor cell to repair therapeutically generated DNA damage plays an important role in the effectiveness of many treatments.

It has been suggested that most tumors harbor a defect in their DNA repair capacity [20]. Knowledge of these mechanisms has enabled development of new strategies and drugs to exploit the DNA repair process for cancer treatment. This subject has recently been reviewed by [21].

Table 1 Anticancer Agents that Induce DNA Double-Strand Breaks [22]

Drugs	Mechanism of Action
Bleomycin	DSB, iron-mediated oxidative cleavage
Camptothecins Topotecan, Irinotecan indenoisoquinolines	Conversion of SSB to DSB by-TOP1 trapping of cleavage complexes and torsional forces
Doxorubicin, etoposide, mitoxantrone batracylin	DSB by trapping topoisomerase II cleavage complexes. Also indirect: ROS formation
Cisplatin, temozolomide aminoflavone trabectedin	DNA alkylation
Imatinib (gleevec)	Induction of Apoptosis via the Intracellular Pathway
5-azacytidine SAHA (vironostat)	Epigenetic modifications
PARP and DNA-PK inhibitors	interference with SSB and DSB repair
SJG-136	DNA alkylation
Tirapazamine	ROS production in hypoxic cells
TRAIL	Death receptors-mediated activation of DNA-PK and of the Extracellular Apoptosis pathway (FADD/casp8)
UCN-01 and AZD7762	Checkpoint inhibitor potentiating ionizing radiation and replication-generated DNA damage by topoisomerase inhibitors, cytidine nucleotide analogs.

Many traditional anti-cancer agents are genotoxins that directly or indirectly induce double-strand breaks in DNA. Some agents are highly reactive chemical compounds that react directly with DNA to induce damage while others may interact with DNA repair proteins or other metabolic enzymes. The accumulation of unrepaired double-strand DNA breaks in the genome is lethal to cells and is the basis for the cytotoxic effect of ionizing radiation and many cancer chemotherapeutics (Table 1). Formation of double-strand breaks leads to the activation of Ataxia Telangiectasia Mutated (ATM) protein kinase and ataxia telangiectasia-RAD3-related (ATR) kinase initiating a series of signal transduction pathways that delay the cell cycle and mobilize DNA damage response proteins including the phosphorylation of the histone H2AX at Ser 139 resulting in the formation of γ -H2AX shown in Figure 1 [23]. Accumulation of γ -H2AX at the site of damage leads to distortion of the DNA helix making it accessible to the DNA Damage Repair machinery, participates in the recruitment of DNA repair proteins [24] (Figure 1) and leads to the

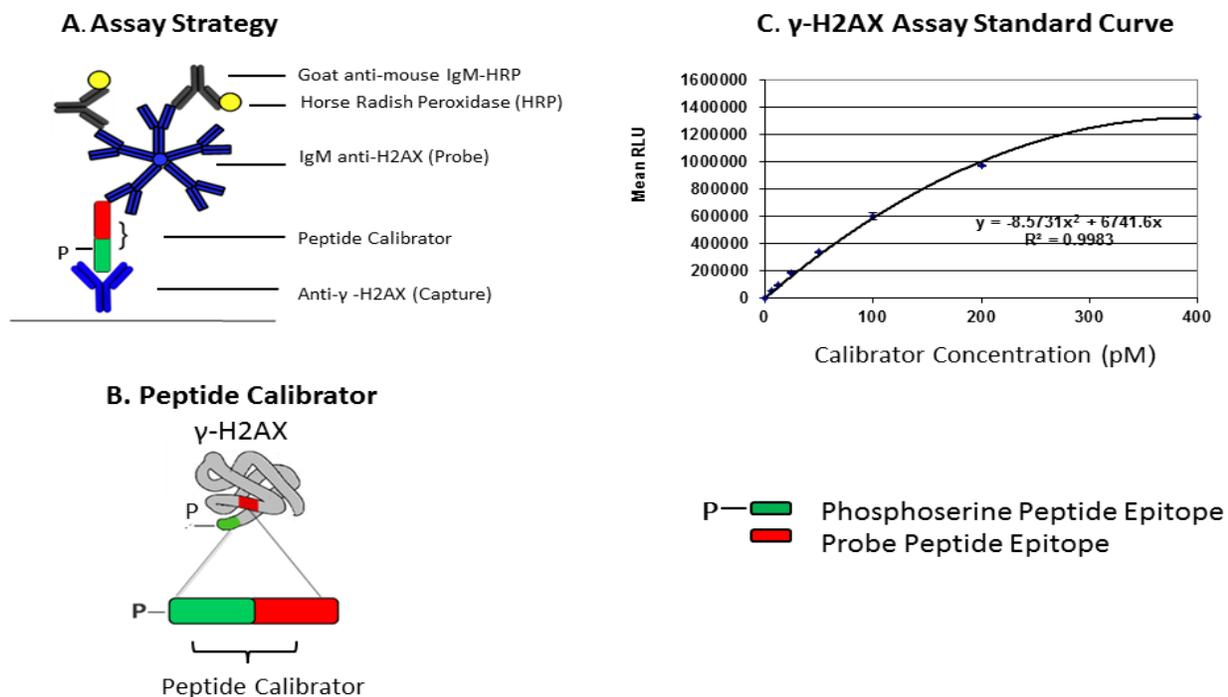
formation of immuno-recognizable foci in the nuclei (Figure 2). Clinical significance of γ -H2AX has been the subject of numerous studies [25-34] and the importance of γ -H2AX as a key pharmacodynamic end point has been discussed and demonstrated [34].

Documented variances in DNA double-strand break repair among different segments of the human population may contribute to patient specific therapeutic toxicities and therefore may warrant a more personalized approach to treatment [35]. A quantitative determination of γ -H2AX levels in response to treatment would help to establish patient specific dose regimens minimizing the toxicity [36] while maximizing the efficacy of therapy. This is especially important in monitoring radiation therapies where a significant portion of the patient's blood volume may pass through the radiation beam inducing double-strand breaks in patient's lymphocytes [37-39].

Development of γ -H2AX ELISA ASSAY: Until now induction of γ -H2AX was determined by either Western Blots or Immunofluorescence. While Western Blots provide information regarding the γ -H2AX protein they are difficult to quantitate and provide mostly qualitative data. Although image analysis software enables foci quantification, actual γ -H2AX levels cannot be determined as there is no direct relationship between foci and γ -H2AX levels after damage.

It has been shown that expression of γ -H2AX in response to therapy can be measured in PBMC [40]. Additionally, PBMCs are readily attainable from patients and their derived lysates are easily analyzed by ELISA assays which are supported by a vast commercial infrastructure and are performed by virtually all clinical and most research laboratories. However, assay development is often hampered due to the lack of independently quantifiable calibrators necessary to generate the standard curves that serve as a reference to quantify unknown levels of the PD biomarker in biological samples.

Figure 3. γ -H2AX Pharmacodynamic Assay Principle

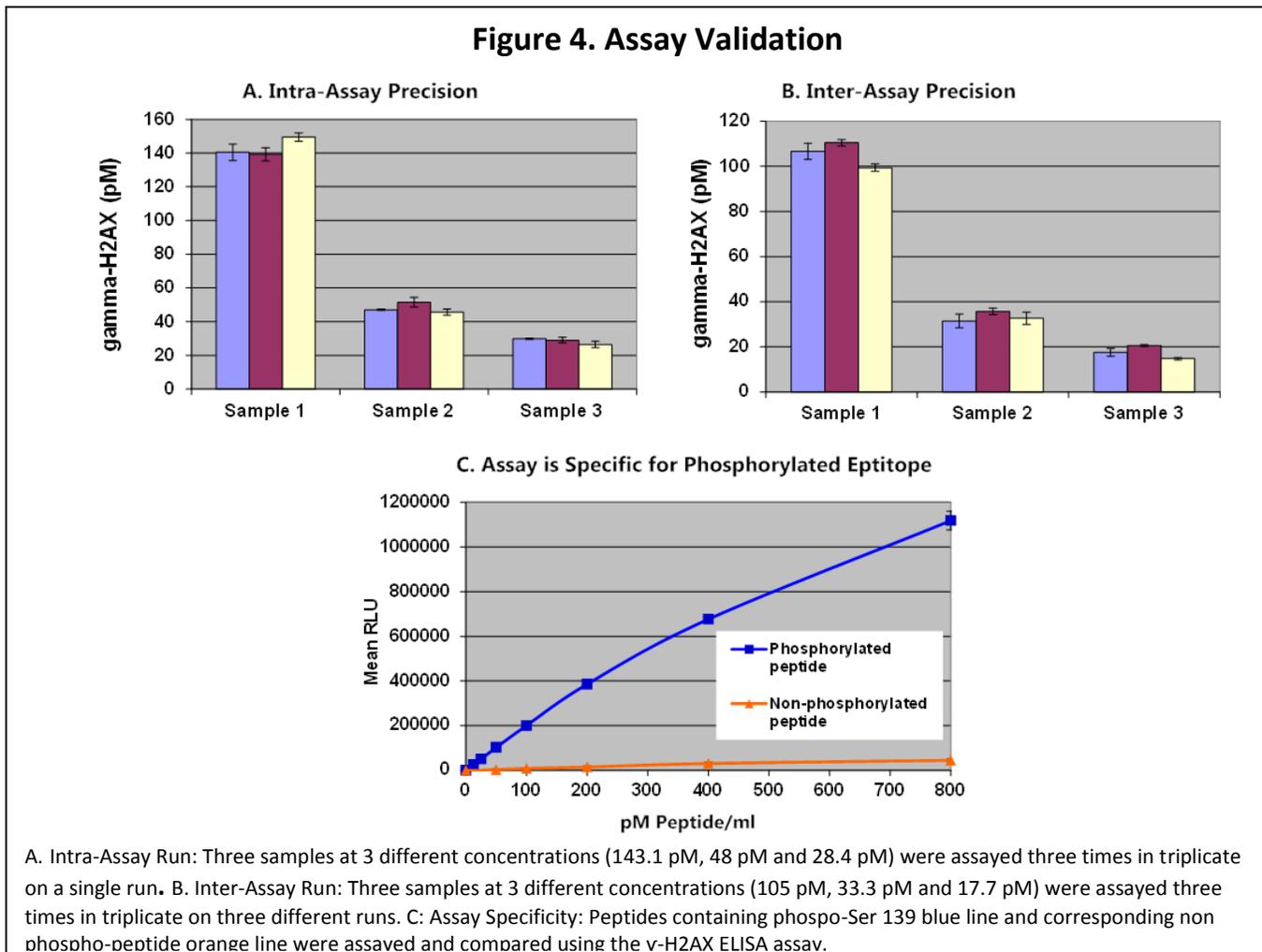


Calibrator and Antibody Development: Most laboratories have ELISA plate readers as they already perform ELISAs to detect the presence of an antibody or an antigen in test samples; however, the development of an ELISA-based PDA is often hampered due to the lack of an independently quantifiable calibrator to accurately measure the effect of a drug on a targeted PD biomarker in clinical samples. Such calibrators are required to generate standard curves for the assay

and serve as a reference to quantify the PD biomarker in biological samples. Calibration standards typically consist of known concentrations of the purified analyte. In its simplest form, a sandwich ELISA requires two antibodies (Figure 3, Panel A). One is referred to as the capture antibody, typically immobilized on a solid support. This antibody very specifically “captures” the analyte of interest permitting it to be separated from other components contained in the sample. The second antibody referred to as the probe, binds to a different epitope contained within the “captured” analyte. The probe antibody is typically coupled to a detection system that permits the accurate quantitation of the analyte bound to the capture antibody. Additionally, the assay typically requires purified analyte that is used as a calibrator to develop a standard curve for the analysis.

To use γ -H2AX protein as a calibrator would require cellular induction of γ -H2AX, immunoaffinity purification and determination of the phosphate/protein ratio. Low protein yields accompanied by unfavorable processing costs associated with such an approach does not support product commercialization. Alternatively, we synthesized a multi-epitope containing peptide possessing both capture and probe domains derived from the γ -H2AX protein sequence (Figure 3, Panel B). Using reliable computational software tools such as the immune epitope database (IEDB) [41-43] and the Epitopes Toolkit (EpiT) [44] we identified suitable epitopes for calibrator and antibody development. Selection of the appropriate epitope is complicated as many antigenic determinants may undergo post-translational modifications in response to cellular signals [45]. By applying tools such as GeneCards [46] UniProtKB /SwissProt [47] and PhosphoSitePlus [48] we identified potential modification sites within γ -H2AX.

To identify a robust probe and capture pair of antibodies we developed and auditioned both polyclonal and monoclonal antibodies to the identified epitopes. While commercially available reagents performed well in Western Blots they did not perform well in our ELISA format. Therefore, we developed our own probe and capture antibodies based on the selected epitope sequences used to develop the peptide calibrator. A typical standard curve generated using the peptide calibrator and Trevigen antibodies generated against the calibrator epitopes is shown in Figure 3, Panel C.

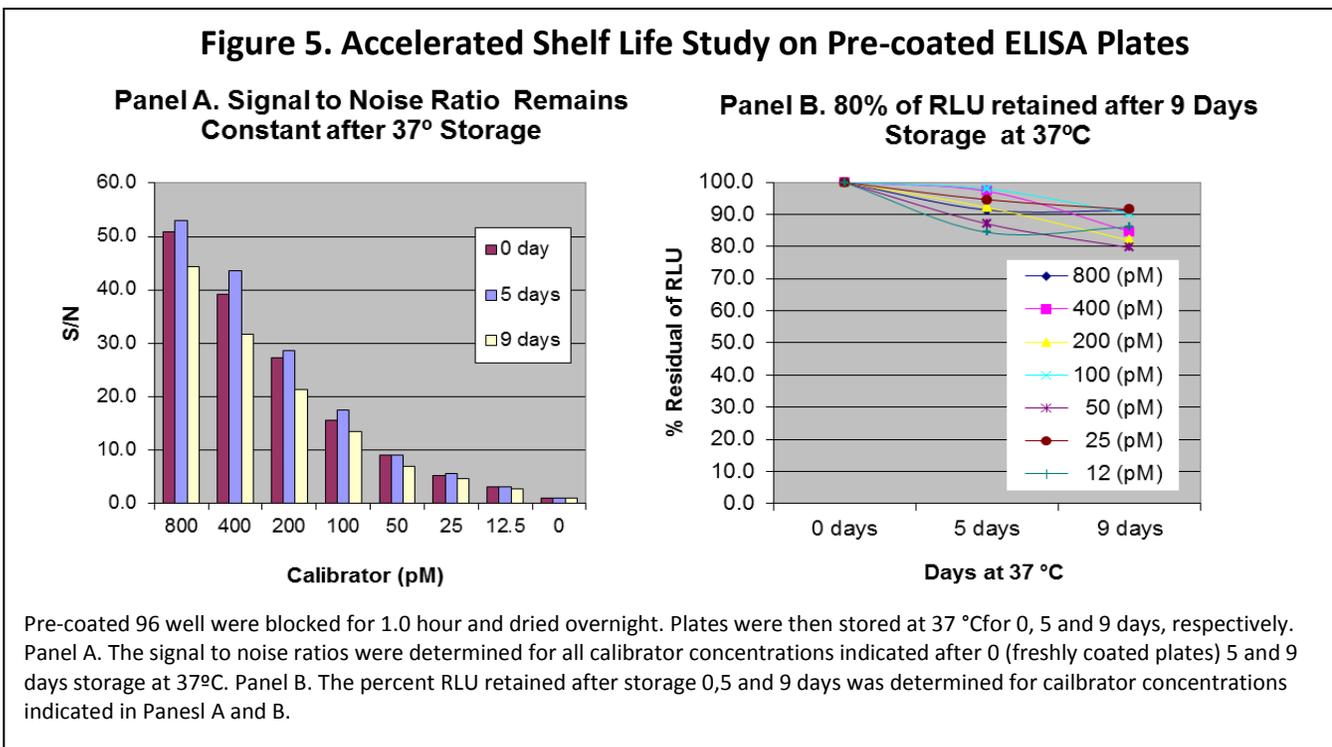


Validation of γ -H2AX Pharmacodynamic Assay: Precision of the assay was determined by standard methods. In Figure 4, the intra assay precision refers to assay performance across multiple plates within a single run while inter assay precision refers to the performance in three separate runs. Both the intra assay (Panel A) and inter assay (Panel B) coefficient of variation were calculated to be less than 10% which is within the US Pharmacopeia recommended guidelines for ELISA assays. Critical to the success of this assay is the specificity of the phospho-Ser 139 γ -H2AX antibody. Cross reactivity to H2AX would result in high backgrounds resulting in poor assay signal-to-noise ratios. Specificity (Figure 3, Panel C) was determined by testing the phosphorylated epitope and separately with the same non-phosphorylated peptide sequence. The γ -H2AX PDA kit includes a 96 well plate pre-coated with an affinity purified polyclonal antibody directed to phospho-Ser 139 γ -H2AX. Coated plates are manufactured in an environmentally controlled facility to assure lot-to-lot consistency. During the development of the kit Trevigen has developed a proprietary buffer formulation, greatly reducing background levels therefore increasing signal-to-noise ratios. Custom manufacture is available for assuring single lot availability for critical studies. In addition to assay precision we validated the assay for the following criteria:

Sensitivity: Our data indicates that the assay has dynamic range of 10 pM to 800 pM and a signal to noise ratio of 200. The lowest limit of detection (LLD) is routinely 5 pM.

Accuracy: Test samples of known γ -H2AX concentration were spiked with three different levels of γ -H2AX and analyzed for recovery before and after spiking. The calculated overall mean of assay accuracy is between 100% \pm 10%.

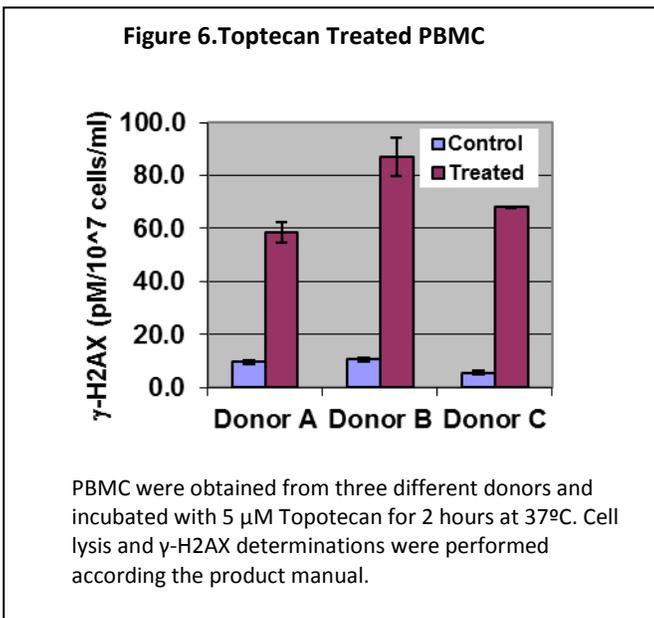
Dilution Linearity: Test samples were serially diluted in the Assay Buffer and subsequently measured by the assay. Dilution recovery is assessed by comparing observed vs. expected values based on undiluted samples. The calculated overall mean of dilution recovery is between 100% \pm 10%.



Product Stability: Accelerated shelf life studies on pre-coated assay plates were performed according to Scheel [49]. Typically, pre-coated plates were incubated at 37°C for 5 and 9 days. After incubation, assay specifications for pre-coated plates and freshly coated plates (0 days) were determined at calibrator concentrations from 0-800 pM. In Figure 5, Panel A we observed that the signal to noise ratio at all calibrator concentrations remained sufficiently high to enable reliable results over at least one year of storage at 4°C. At the same time, Figure 5, Panel B shows that over the same storage time at least 80% of the Relative Light Units (RLU) were retained at all calibrator concentrations indicating the

product can be stored for at least one year. The remaining kit components, when stored under the recommended conditions, are also stable for minimally one year. The data indicates that multiple kits can be stored during prolonged studies without compromising assay results.

As described above many therapeutic approaches rely on the accumulation of double-strand breaks in the genome of targeted cancer cells. Since it has been established that PBMC serve as a surrogate tissue for the formation of γ -H2AX in tumor cells we demonstrated that we could detect γ H2AX in Topotecan treated lymphocytes from three different donors, shown in Figure 6. At this point, it is too early to know if different responses are the result of genetic differences between individuals or assay variations. Experiments are underway to determine if genetic differences exist and if so, could they be exploited to personalize patient treatment?



From experience with our PARP PDA, we know that many customers, when performing clinical trials, wish to store samples prior to assay. Our data indicate that cellular lysates can be stored for at least six months at -70°C with no loss of signal. Additionally, we have developed methodology to freeze treated cells without altering γ -H2AX measurements when compared to their fresh counterparts.

In summary, we have presented data describing the first validated γ -H2AX pharmacodynamic assay. In addition to monitoring the effect of drugs on specific pharmacodynamic endpoints, we believe the assay will have value in establishing drug toxicity [36] as a radiation biomonitor [37-39] and a tool for epidemiological studies[50].

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