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FY05 LDRD Final Report Molecular Radiation Biodosimetry LDRD Project Tracking Code: 04-ERD-076

I. M. Jones, M. A. Coleman, J. Lehmann, C. F. Manohar,
F. Marchetti, R. Mariella, R. Miles, D. O. Nelson, A. J.
Wyrobek

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Molecular Radiation Biodosimetry
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Irene M. Jones, Principal Investigator

I.M. Jones, M.A. Coleman, J. Lehmann, C.F. Manohar, F. Marchetti, R.
Mariella, R. Miles, D.O. Nelson, A.J. Wyrobek

Introduction

In the event of a nuclear or radiological accident or terrorist event, it is important to identify individuals that can benefit from prompt medical care and to reassure those that do not need it. Achieving these goals will maximize the ability to manage the medical consequences of radiation exposure that unfold over a period of hours, days, weeks, years, depending on dose. Medical interventions that reduce near term morbidity and mortality from high but non-lethal exposures require advanced medical support and must be focused on those in need as soon as possible.

There are two traditional approaches to radiation dosimetry, physical and biological. Each as currently practiced has strengths and limitations. Physical dosimetry for radiation exposure is routine for selected sites and for individual nuclear workers in certain industries, medical centers and research institutions. No monitoring of individuals in the general population is currently performed. When physical dosimetry is available at the time of an accident/event or soon thereafter, it can provide valuable information in support of accident/event triage. Lack of data for most individuals is a major limitation, as differences in exposure can be significant due to shielding, atmospherics, etc. A smaller issue in terms of number of people affected is that the same dose may have more or less biological effect on subsets of the population. Biological dosimetry is the estimation of exposure based on physiological or cellular alterations induced in an individual by radiation. The best established and precise biodosimetric methods are measurement of the decline of blood cells over time and measurement of the frequency of chromosome aberrations. In accidents or events affecting small numbers of people, it is practical to allocate the resources and time (days of clinical follow-up or specialists' laboratory time) to conduct these studies. However, if large numbers of people have been exposed, or fear they may have been, these methods are not suitable. The best current option for triage radiation biodosimetry is self-report of time to onset of emesis after the event, a biomarker that is subject to many false positives.

The premise of this project is that greatly improved radiation dosimetry can be achieved by research and development directed toward detection of molecular changes induced by radiation in cells or other biological materials. Basic research on the responses of cells to radiation at the molecular level, particularly of message RNA and proteins, has identified biomolecules whose levels increase (or decrease) as part of cellular responses to radiation. Concerted efforts to identify markers useful for triage and clinical applications

have not been reported as yet. Such studies would scan responses over a broad range of doses, below, at and above the threshold of clinical significance in the first weeks after exposure, and would collect global proteome and/or transcriptome information on all tissue samples accessible to either first responders or clinicians. For triage, the goal is to identify those needing medical treatment. Treatment will be guided by refined dosimetry. Achieving this goal entails determining whether radiation exposure was below or above the threshold of concern, using one sample collected within days of an event, with simple devices that first responders either use or distribute for self-testing. For the clinic, better resolution of dose and tissue damage is needed to determine the nature and time sensitivity of therapy, but multiple sampling times may be acceptable and clinical staff and equipment can be utilized.

Two complementary areas of research and development are needed once candidate biomarkers are identified, validation of the biomarker responses and validation of devices/instrumentation for detection of responses. Validation of biomarkers per se is confirmation that the dose, time, and tissue specific responses meet the reporting requirements in a high proportion of the population, and that variation among non-exposed people due to age, life-style factors, common medical conditions, variables that are not radiation related, do not lead to unacceptable frequencies of false negatives or false positives. Validation of detection requires testing of devices/instruments for accuracy and reproducibility of results with the intended reagents, sampling protocols, and users. Different technologies, each with intrinsic virtues and liabilities, will be appropriate for RNA and protein biomarkers. Fortunately, device and instrumentation development for other clinical applications is a major industry. Hence the major challenges for radiation biodosimetry are identification of potential radiation exposure biomarkers and development of model systems that enable validation of responses of biomarkers and detection systems.

Objectives

The goals of this project were to determine the current status and near term ability of RNA and protein biodosimeters to substantially improve radiation dosimetry. These goals were pursued under three major objectives:

(1) Identify and begin validation of candidate RNA and protein radiation biodosimeters. This entailed a number of activities:

Identify and prioritize potential biomarkers through literature surveys and analyses of pre-existing data.

Develop and utilize *ex vivo* model systems. That is, expose cells obtained from healthy people to radiation under controlled, laboratory conditions. In addition, assess the impact of cell proliferation on biodosimeter performance in a normal human fibroblast cell line that can be cultured to be either non-dividing or dividing, to help relate the results of studies in the proliferating lymphoblastoid

cell model system to the nonproliferating cells in blood, saliva or buccal samples obtained from people.

Evaluate a limited set of methods for identifying and monitoring protein biosimeters.

Begin validation of candidates in each system.

(2) Develop an *in vivo* radiation model for further validation of biosimeters. More specifically:

Obtain cells from radiation oncology patients before and after exposure to therapeutic radiation treatment of head and neck cancer.

Perform detailed analysis of individual radiation treatment plans to obtain estimates of the radiation doses received and perform physical dosimetry to refine dose estimates, for each site sampled.

Begin validation of leading RNA candidates.

(3) Design and build a prototype for collecting saliva and buccal cells for triage biosimetry.

Results

(1) Identification and initial validation of candidate radiation biomarkers

Overall Approach.

The goals were to utilize knowledge from the published literature and existing unpublished data sets to identify biomolecules that had potential to serve as indicators of acute radiation exposure in people, and to initiate validation of potential biosimeters in model systems with increasing fidelity to population dosimetry. The studies focused on two types of biomolecules, gene transcripts (RNA) and proteins (and protein modifications). It was judged that first generation biosimeters could be identified without extensive new research and that existing technologies could be adapted for proof-of-principle assays of RNA and protein molecules in clinics and the field, respectively.

RNA-based biomarkers

Background The first step in gene expression is synthesis of RNA. It is established that the amount of RNA for a given gene in a cell can vary. In some cases, specific environmental conditions are known to affect the level of RNA. For example, exposure to radiation has been reported to induce increased or decreased expression of genes in a number of biological systems (Amundson et al. 2001; Snyder and Morgan, 2004). In some cases a relationship between level of RNA expression and dose of radiation has been reported, and the time dependence of the response explored (Amundson et al, 2000; Amundson and Fornace, 2001). Although a few genes have been reported to be

radiation-responsive by multiple studies employing different cell types and test parameters, the limited overlap of data sets suggested that identification of candidate RNA radiation biosimeters was not complete, and additional discovery work was justified. In addition, methods were needed for assaying RNA biosimeters in tissue samples practical for use in triage and clinics. The leading candidate samples were blood, saliva, and buccal cells (the cells lining the inside of the cheek).

Approach In this study, effort to discover new RNA biomarkers was limited to existing, unpublished data sets produced at Livermore. These data sets had been generated using RNA from human lymphoblastoid cell lines, assayed after irradiation over a range of doses and times after exposure, using Affymetrix microarrays of 22215 sequences from the human genome. It was recognized that the relevance of these cell lines to other cell types was unknown. However, the doses, times after radiation and number of independent people represented were judged to be sufficient to justify mining the data to identify new biomarkers. These candidates then would be tested, first with quantitative reverse transcript PCR (Q-RT-PCR) of RNA from the same cells, and then Q-RT-PCR using cells irradiated immediately after being obtained from healthy people to validate their utility for biosimetry. Investment in the generation of new data sets to identify additional candidate RNA biosimeters, using cells from tissue types and conditions representative of final assay deployment, was deferred until a larger program could be established.

In addition, the published literature was surveyed to identify additional leading RNA candidates. This effort enabled inclusion in the validation studies of candidates that were not discovered in the Livermore data sets. It also revealed candidates that were detected in multiple independent studies, including the unpublished Livermore studies, and hence were of high priority.

Accomplishments

Statistical analyses of Livermore microarray data

Two sets of statistical analyses were conducted to extract information about radiation biosimeters from unpublished results of microarray studies previously conducted at Livermore. The microarray data came from two separate studies, one in which responses to many different radiation doses were studied at 4 hours after exposure in 2 human lymphoblastoid cell lines (referred to here as the “dose data set”), and a second in which the response to a high and low dose were studied at multiple times after radiation in either the same two cell lines, or 6 cell lines including the other two (referred to as the “time and variation data set”).

The first statistical study performed a two-stage analysis to identify biomarkers of high dose exposure. First, gene transcripts were identified that increased or decreased at high dose exposures (200, 300 and 400cGy) either monotonically or consistently in the same direction in the dose data set relative to cells with no exposure. This stage selected 26 candidates that demonstrated a dose response in both cell lines, and 1255 that

demonstrated a consistent up or down response at all 3 doses. These candidates were confirmed in the second stage using the time and variation data set. Twenty candidates of the consolidated unique list of 1278 were found to have the predicted increase or decrease of expression 4 hours after 200 cGy in all 6 cell lines, and 10 candidates on average over the six cell lines had a statistically significant change in expression in the same direction. There were 26 nonredundant candidates (representing 23 genes) among these 30 that met one or the other criterion for differential expression at 4 hours. Analysis of the expression of these 26 candidates in the time and variation data set at 8, 24 and 48 hours after exposure to 2Gy suggested that the responses attenuated some at 24 hours and more at 48 hours. Experimental validation of these results was initiated.

The second statistical study analyzed the time and variation data set to find genes that were “high exposure biomarkers” at 4 hours or 24 hours after exposure and to determine the relationship between responses at the two times. Genes that responded to a high dose (200cGy) but not a low dose (10cGy) would provide a discrimination critical to managing medical consequences early after an exposure, where people with high dose exposure require medical evaluation right away, and others do not. Exploring the time dependence of responses would help identify the time frame in which a biomarker might be valid. Quite a few genes were initially identified as possible high exposure biomarkers in the selection analysis (145 at 4 hours and 52 at 24 hours. However, far fewer met tests for significance, 21 for 4 hours and only 2 at 24 hours. Neither of the two potential high exposure biomarkers for 24 hours after exposure was also a biomarker for exposure at 4 hours after exposure. In these analyses it was required that all 6 cell lines respond consistently, as an initial requirement for applicability to population studies. It will be important that biomarkers respond the same in almost all individuals, so that false positives and negatives are minimized. These statistical analyses were completed late in FY05, and the results have not been pursued experimentally.

The two sets of statistical analyses of microarray data provided valuable information. First, lists of candidate RNA biomarkers of high dose radiation exposure were generated that could be compared with published studies to generate more comprehensive lists of high exposure biomarkers relevant to 4 and 24 hours post exposure. These comparisons illustrated the necessity that future biodosimeter discovery studies utilize samples from all time points of interest. Second, the limitations of the microarray data sets highlighted the need for more technical replicates of each sample in microarray studies, such that technical variation could be assessed and better discrimination of biological responses achieved.

Experimental validation of candidate RNA radiation biodosimeters.

The responses of 23 of the 26 candidates identified in the first statistical analysis were verified by Q-RT-PCR (quantitative reverse transcript PCR) using RNA from lymphoblastoid cells, from one or more donors, irradiated *ex vivo* at 0, 200 and 400cGy and incubated for 24 and 48 hours post exposure.

Compilation of results of a review of the literature as well as results of the first statistical analysis led to a list of ~26 high dose RNA biomarkers, prioritized by the magnitude of

response, frequency with which the response had been detected/reported, and whether the response was evident in human cells rather than only non-human species. Several of the biomarkers identified in the Livermore lymphoblastoid microarray data sets were novel, validating the expectation that additional candidates remain to be identified.

The studies of radiation responses of a small set of RNA biosimulators in human fibroblasts revealed that the level of response after radiation tended to be higher in non-proliferating cells than proliferating cells. These results, obtained by Q-RT-PCR, were heartening as they suggested both that some biomarkers detected in the proliferating lymphoblasts would be radio-responsive in another cell type, and that the magnitude of response might be higher in the cell cycle status most common in readily sampled human tissues.

An essential next step was development of methods for validation of the candidate RNA biosimulators in key cell types. For peripheral blood, the *ex vivo* radiation model developed started with irradiation of whole blood and continued with isolation of white blood cells and culture at 37°C in medium with serum but no mitogens. Absence of mitogens was considered most faithful to the condition of cells in people, where few white cells are dividing. With peripheral blood, the limits of sample size were not pushed; quality and quantity of RNA was consistently better than for buccal cells due to the higher proportion of viable cells. Buccal cells were the second system developed. This choice was dictated by the plan to assay cells from radiotherapy patients, to obtain *in vivo* validation of results. The experimental work evaluated methods based on collection of sufficient material for analysis of cells from a localized position in the cheek, as necessary to link cells with site-specific dosimetry in the *in vivo* radiation model (as discussed below), and also preservation and shipping of the sample from the clinic in Sacramento to Livermore. After extensive investigation, a modified cytobrush, standard time and placement for the brushing routine, transfer of cells to RNeasyTM, followed by storage at -80°C and shipping on dry ice were adopted based on quantity and quality of RNA obtained. A method for collecting saliva was also developed. As a result of these methodological studies, buccal cell and saliva samples were collected from healthy subjects that could be used for testing detection methods.

Although no *ex vivo* irradiation studies were conducted with buccal cells due to lack of established culture protocols, Q-RT-PCR with isolated buccal cell RNA using primers for control genes was part of the quality assurance plan. It was estimated that most buccal cell samples collected with the final protocol would support Q-RT-PCR analysis in triplicate of 10 or more biomarkers assuming high enough copy number and radiation response.

Validation of RNA biosimulators in the human peripheral blood *ex vivo* model system was initiated. The goal of these studies was high quality, quantitative analysis of the radiation response of candidate RNA biosimulators in blood samples, to determine if candidates identified solely through analysis of microarray data in lymphoblastoid cell lines would demonstrate useful responses, and to extend knowledge about the applicability of published candidates. For these studies 21 genes were selected as high

priority, potential high dose biodosimeters based on published literature and analysis of unpublished Livermore data sets (discussed above) and commercial availability of primers for Q-RT-PCR. Peripheral blood samples were obtained from 16 subjects, with repeat samples obtained on different days for a subset. Cells that were exposed to 0, 200 or 400cGy were cultured for either 24 hours or 48 hours, harvested, washed and then cryopreserved at -80°C after flash freezing in liquid nitrogen. After experiments achieved good control of technical reproducibility of data in the Q-RT-PCR, high quality gene expression data for samples from 2 people for 11 genes on the candidate list were obtained and analyzed relative to a control gene. In the statistical analyses conducted, all responses were based on comparison of the relative level of the candidate in unirradiated and irradiated cells with levels of glyceraldehyde phosphate dehydrogenase (GAPDH), a gene known not to be radiation responsive, and present in high enough copy number to assure valid comparisons. Internal controls such as this one are expected to be part of most established biodosimetry assays. In addition to the responses discussed below, it was determined that in conducting Q-RT-PCR-based studies of large numbers of genes to evaluate the consistency of response among individual people, statistical randomization of samples assayed as well as multiple technical replicates in the experiments would be necessary to ensure reliable results.

The statistical analyses of the *ex vivo* blood cell model Q-RT-PCR results provided many valuable insights on the challenges ahead in identifying RNA biomarkers as robust radiation biodosimeters. Also, although the data set obtained was small and limited to peripheral blood cells, it provided preliminary validation of some RNA biomarkers. The features tested were evidence of radiation response at each dose and each time point, presence of response at both time points for each dose, and consistency of expression level between the two subjects in the absence of radiation exposure (baseline). Three of the 11 candidates tested did not demonstrate radiation responses with promising dose or time dependence. Two other candidates had responses to both 200 and 400cGy at 48 hours, but not 24 hours; the responses at 48 hours were less dramatic than for the remaining 6 candidates, and baseline gene expression differed in the two subjects for one of these candidates. The failure of these 5 candidates to meet initial needs reflects a combination of non-transferability of results from one cell type to another, variation among people, and the more stringent requirements for radiation responsiveness placed on the more quantitative PCR assays. The remaining 6 candidates demonstrated strong increases in RNA expression at both doses for both times. For 4 of these, differences were detected between responses at 24 and 48 hours after exposure to 200cGy, but were small relative to the responses to both doses and probably would neither affect general utility of the biomarker nor support determination of the time an exposure occurred. Baseline expression of 2 of the 6 candidates differed among the two subjects tested. This was of concern because an overlap of the baseline expression in some individuals with the radiation responses of other individuals would disqualify a candidate for most population applications where no sample from prior to radiation exposure (baseline) will be available for reference. One of the candidates (GADD45A) appeared to pass all tests enabled by the study design, being a consistent reporter of high dose exposure (200cGy and 400cGy) at both 24 and 48 hours, in both people tested. The radiation responsiveness

of GADD45A has been widely studied (Amundson et al., 1999a,b; Jen and Chung 2000; Tusher et al., 2001; Grace et al., 2002).

Overall, the RNA biosimulator studies of blood cells confirmed the expectation that validation of biomarkers for radiation dosimetry of populations will take a lot of effort. More extensive analysis of responses to varied doses is needed, to assess the threshold as well as maximum dose at which a response is seen. Given variation in the dose, time and person-specific gene expression responses, it is evident that panels of biomarkers will be needed to meet the basic goal of radiation dosimetry, to confidently distinguish between people who have received an exposure requiring medical follow-up from others who have received no exposure or lower doses. Different panels will be needed for each tissue, and the preferences of assays utilized in the field and clinic are likely to differ. Ultimately, thorough testing of candidate panels will be needed to understand the impact of many possible confounders such as age, gender, smoking status, disease and medication status on baseline levels of biosimulators and radiation responses.

Protein and protein modification radiation biosimulators

Background Responses to radiation exposure manifested at the protein level are of two types, changes in the amount of protein present and modifications of preexisting proteins, such as phosphorylation. Both can lead to changes in the functional capacity of the protein in a cell, and may in turn affect other proteins. A virtue of using proteins as biomarkers is that detection by relatively simple antibody-based assays (immunoassays) is possible. Technology development for immunodetection is an active field, driven by clinical needs for quick, cheap, specific tests. A liability of immunoassays is that obtaining an antibody that binds specifically and efficiently to the molecule of interest in unfractionated blood, saliva or urine, is difficult, and different antibodies may be required depending on assay format or sample type. Identification of radiation-responsive proteins has to date largely been one protein-at-a-time, driven from genetically-based differences in specific model systems. Antibodies available for proteins that have been the subject of sustained research do not necessarily meet the demands of new biosimilarity applications. Methodologies for scanning whole proteomes for radiation responses such as mass spectrometry and high resolution electrophoresis have great promise but have had limited application as yet.

Approach The protein biosimilarity effort had two major elements. The first was to assess current knowledge of protein responses to radiation exposure. This effort would not only determine whether there were radiation responsive proteins that were sufficiently well characterized to merit early evaluation as radiation biosimulators but also assess gaps in knowledge and technical approaches that would have to be addressed in future efforts to advance radiation biosimilarity. The second element was to evaluate the ability of available reagents for the most promising candidates and detection technologies to support initial validation of candidate protein biosimulators and development of first generation devices for radiation triage biosimilarity.

Accomplishments

Review of current knowledge of protein responses to radiation exposure

To identify potential biomarkers for assessing individual radiation biodosimetry we conducted a review of the literature to identify mammalian proteins that have been found to respond to ionizing radiation. The literature search was confined to studies published from 1973 through 2005 that used mammalian systems, after either *in vivo* or *in vitro* radiation exposure. Review of over 300 papers yielded information on 201 genes for which proteins showed changes in the level of expression or posttranslational modifications (e.g., phosphorylation) after ionizing radiation exposure. The available research had many shortcomings for evaluation of radiation biodosimeters for population studies. For example, the majority of findings were obtained after high doses of ionizing radiation (> 4 Gy) and within 24 hr after exposure, not addressing medically important lower doses and later times. Most of the proteins had no information on the shape of the dose- or time-responses, and few proteins had data from more than one species. Further, the majority of results were conducted using cells in culture (*in vitro*), often employing tumor cell lines rather than normal cells. On the positive side, the majority of the proteins showed increased protein amounts or changes in phosphorylation status after ionizing radiation exposure (range: 1.5 – 10 fold), a pattern easier to study than a decrease. To prioritize proteins for human biodosimetry applications we considered: the number of species studied; use of normal tissues or cell lines; persistence of the response after ionizing radiation exposure, and known involvement in DNA repair, and specifically, the repair of double strand breaks, the most toxic outcome of radiation exposure. Using this approach, we developed 8 ranking groups (**Table 1**, next page). The four proteins in Priority group 1, ATM, H2AX, CDKN1A, and TP53, were identified as the top candidate ionizing radiation protein biomarkers based on their consistent response across species and their role in ionizing radiation damage response pathways. Details of this study are provided in UCRL-JRNL-218093 (publication list).

The results of this survey show that, although ultimately protein-based radiation biodosimetry should be feasible, the identification of a protein biomarker, or a panel of protein biomarkers, that provides information on whether an exposure has occurred, the dose that was received, at key times after the exposure, is in an early phase of research. There are major gaps in our knowledge about dose- and time-responses, tissue differences, inter-individual variations, and other biological factors that may affect the expression of these proteins that need to be addressed. We lack a global view of the cellular responses to ionizing radiation at the protein level comparable to that being developed for RNA molecules using RNA microarrays. The majority of the reviewed studies used traditional single gene/protein approaches, which are not sufficient to elucidate the regulation and relationships among the many cellular pathways associated with the ionizing radiation response. The rapid development of new proteomic approaches such as 2D-gel/MALDI (Zhang et al. 2003; Bo et al. 2005; Chen et al. 2005a; Chen et al. 2005b; Szkanderova et al. 2005; Tapio et al. 2005) and protein arrays (Sreekumar et al. 2001) are beginning to generate new mechanistic insight into the complexity of the cellular responses to ionizing radiation and are identifying novel biomarkers of tissue-specific damage.

Table 1. Summary of the results of prioritizing proteins as potential radiation dosimeters based on published research.

Priority group	Human	Animals	<i>In vivo</i>	<i>In vitro</i>	Normal cells	Tumor cells	Persistence	mRNA response reported same study	DNA damage related	Double strand break repair	Number
1	X	X	X	X	X	X	X	X	X	X	4
2	X	X	X	X	X	X		X	X		7
3	X		X		X		X				13
4	X		X	X	X						20
5		X	X		X		X				59
6	X			X	X						31
7	X			X		X	X				15
8	X			X		X					52
										Total	201

Experimental valuation of protein biodosimeters with available reagents and selected detection technologies

Approach These studies focused on evaluating the potential for immunoassays of protein biodosimeters with currently available reagents and existing/emerging technologies. Two technologies were evaluated, glass slide-based microarrays of antibodies for simultaneous comparison of large numbers of candidates and rapid chromatography-based lateral flow methods for a limited number of candidates. A collection of commercially available antibodies was established for ~80 top candidates identified from the review of the published literature (UCRL-JRNL-218093). Antibodies for a few high priority candidates were evaluated critically for specificity, sensitivity and applicability to assays of selected tissues by lateral flow methods. In addition, the complete collection was utilized to assess the issues affecting utilization of protein array-based assays of minimally processed samples to screen for and/or monitor biodosimeters.

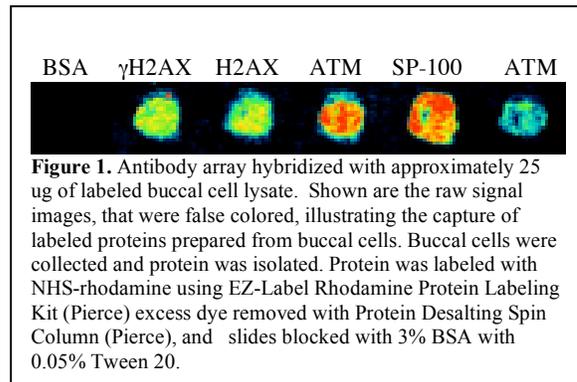
Accomplishments

Sample processing

Modifications of sample processing were identified for stabilization and detection of protein biodosimeters. For most applications, initial processing in MPER™ was satisfactory. This variable must be tested for each isolation/detection protocol.

Antibody microarrays

Using purified antigens, antibody arrays demonstrated sensitivity to the ng level for two of the top candidates, γ H2AX (the phosphorylated form of H2AX) and ATM-phos-ser1981 (**Figure 1**). This sensitivity is high, comparable to that we obtained with Biocore, a label free based-method and Luminex, a bead-based flow system using the same reagents. Specificity to pure test proteins also was high; antibodies showed little cross reactivity with the non-phosphorylated molecules.



Several aspects of performance of arrays of ~80 candidate biosimeters were tested. Samples included complex protein mixtures purified from whole cell lysates for buccal, white blood, lymphoblastoid and fibroblast cells. We also looked at crude protein preparations from saliva. The focus of these studies was evaluation of the impact of isolation and sample lysis protocols on results, including detection of known controls, ability to discriminate between normal and radiation responsive forms of lead candidates (H2AX and γ H2AX; ATM and ATM-phos-ser-1981) in samples of unirradiated cells, and assessment of background levels of other candidate biosimeters. Key findings were that human fibroblasts demonstrated the induction and time response the phosphorylated form of γ H2AX after 100cGy exposures. Relevant amounts of ATM and γ H2AX could be measured under ideal conditions in test experiments.

Evaluation of antibodies to top candidates

Western blot analyses performed with antibodies to the top candidates revealed some of the antibodies' strengths and limitations. First, results for γ H2AX. As expected based on published studies, this phosphorylated histone is radiation responsive in *ex vivo* irradiated blood cells and fibroblasts; comparisons were made 24 and 48 hours after exposure of cells that received either 0, 2, 4 Gy, or 0, 1 and 10 Gy, blood and fibroblasts, respectively. In addition, a number of important issues were identified. Apparent lack of specificity of antibody was evident in analyses against total cell protein; there were multiple bands in Western blots, and not all radiation responsive. This result indicated that either the antibody would have to be purified for the γ H2AX epitope, or an antibody with higher specificity obtained/created for studies in whole cell lysates, the expected sample for triage devices. With respect to sample choice, presence of apoptotic cells in unirradiated buccal cells from saliva led to such high levels of background that detection of a radiation response would be highly compromised. In contrast, unirradiated blood cells had low backgrounds, indicating that blood samples or other tissues with low numbers of apoptotic cells are essential for this biosimeter. Second, ATM-phos-ser 1981 was evaluated in samples from the same radiation exposure conditions as γ H2AX. ATM antibodies failed to detect antigen of the correct size in the samples used. Cross reactivity with a smaller molecular weight protein was seen (<13 kDa). It is plausible that this was

a degradation product ATM. It is known that ATM is one of the first proteins to signal radiation damage and would be an ideal biomarker when we identify the best antibodies for detection. Other antibodies tested included BAX, which is involved in apoptosis. The BAX protein was elevated in ionizing radiation exposed samples 2 hours post-exposure, but gave variable results. Two out three *ex vivo* irradiated samples showed similar results for BAX ionizing radiation-induced signal. This may be a good multiplex marker because of the induction, but more work is needed to understand the biological variation.

Production and testing of lateral flow strip units

We contracted the production of our first lateral flow units. We provided antibodies to a small set of biodosimeters, along with purified test reagents to a contractor. The contractor characterized and processed the antibodies, including concentration, tests of sensitivity, labeling of some of isolates with immuno-gold, and printing of test strips and assembled lateral flow units.

Due to failure of some of the antibodies to perform as needed, the goal for the lateral flow component was limited by the small number of units produced, all based on the antibody for γ H2AX. We verified that lateral flow could be used to detect purified γ H2AX in a sample of the volume delivered by the prototype device produced for collection of buccal cells and saliva (see below). In addition, very preliminary data showed potential for such lateral flow units, by detecting early radiation responses of γ H2AX using samples prepared from *ex vivo* ionizing radiation exposed blood cells.

This experience illustrated that performance of antibodies is highly application specific. Iterative preparation and testing of antibodies will be needed in the development of each detection method, and method specific expertise is critical.

(2) Development of a model for *in vivo* radiation in humans

Background By definition, the final validation of radiation biodosimeters, deployment for population monitoring after an accident or terrorist event, will be unscheduled. Effort to develop assays and continuously increase confidence in those available for deployment is necessary. Given that misleading biodosimetry could be worse than none, testing in a succession of models is needed. Models will be needed that provide increasingly complete evaluation of the responses of different tissues in people, or close surrogates such as non-human primates, over a wide range of exposure and sampling conditions and population characteristics.

Approach The goal was an *in vivo* model that assays cells obtained directly from ionizing radiation exposed humans, to provide initial validation of lab-based studies. Among the essential features of a model are that the cells are practical for biodosimetry such that extrapolation from one tissue to another is not required, that the exposure history of the cells is well defined, that samples can be collected from each subject both before and after exposure so that each individual is his/her own control, and that the radiation exposures cover the range of doses associated with medical consequences. The cells sampled should be healthy.

Radiotherapy patients were the obvious population to study. Many treatment plans are designed to deliver 200cGy to the diseased tissue on each day of treatment. Inevitably adjacent normal tissues receive a range of doses, varying with proximity to the tumor target. Head and neck cancer patients were selected as the population for this project. Although the exposure is only to a limited region of the body, healthy buccal cells could be sampled and reliably related to treatment plans in the dose range of interest. In addition, real-time physical dosimetry at collection sites was possible. Sample collection could be non-invasive and well tolerated on two time points, before the first therapy session (unirradiated control) and before the second therapy session, which is about 24h after the first session (irradiated sample). This feature was felt to be essential for clinician and patient acceptance necessary for success in recruiting consenting patients. Buccal cells have been considered among the lead candidates for triage biodosimetry due to expected acceptance of sampling by the general population. The goal was to assemble the experts, approvals and methods required, and conduct a pilot study of the responses of leading RNA biodosimeters in 12 patients.

Accomplishments

Initial characterization of the patient population and establishment of critical collaborations

It was determined that the number of head and neck cancer patients treated at the UC Davis Medical Center was adequate. The rate of completion of a pilot would depend both on the number of eligible patients (preferably those with no recent or concurrent chemotherapy) and the proportion of these patients consenting to participate in the study. Accrual of 12 subjects was expected to take 6 to 12 months once IRB approval was obtained.

The suitability of this patient population was determined by detailed analysis of radiation treatment plans of previously treated patients. Doses to right and left cheek buccal cells calculated for 4 patients ranged from 30 to 190cGy. To maximize the biodosimetric information from each subject in our pilot study, four sites in each individual would be sampled both before and 24 hours after treatment, sites adjacent to the upper and lower molars of both left and right cheeks. Sampling at the same sites the day before treatment would enable evaluation of radiation-independent variation in each individual.

A strong collaboration between radiation oncologists and scientists at UC Davis Medical Center and lab scientists was developed. Both groups were keenly interested in assessing individual patient responses to radiation. The commitment of clinicians was high, as

evident by their taking full responsibility for recruiting subjects, a time intensive process, and for performing the buccal cell sampling according to a set protocol. The clinicians also were essential participants in the design of the study and gaining approval from the Institutional Review Boards (IRB) for human subjects at both UC Davis Medical Center and LLNL. IRB approvals were obtained on 5/3/05 and 6/6/05, for UC Davis Medical Center and LLNL, respectively.

Recruitment, sample collection and dosimetry

A sample collection procedure for gathering cells in a 1cm² region was developed. Based on iteratively testing in healthy subjects, a site-specific sampling with a folded cytobrush and set rotation protocol was established that provided sufficient cells for analysis. Collected cells were dispersed from the brush in RNALater™. Cryopreservation was used to stabilize samples both before and during shipment (-80°C and dry ice, respectively) and archiving pending future molecular analyses (-80°C). Time did not permit tests of long-term stability at -80°C prior to recruitment of subjects, and have not been performed.

Recruitment of patient volunteers and archiving of samples began in July 2005. Accrual of patients in FY05 was limited to 4 patients, primarily due to the strong preference for patients not receiving chemotherapy. Although recruitment of patients and archival of samples from the patients is being completed by our clinical collaborators at UC Davis, biodosimeter analysis is pending future funding.

Dosimetry analyses confirmed that the clinical model of *in vivo* radiation exposure was providing samples with the desired range of exposures. Two types of dosimetric studies were performed. The first was estimation prior to treatment of doses expected to the buccal regions to be sampled based on the radiotherapy treatment plan of each patient. The second was the real time (*in vivo*) measurement of doses using MOSFET dosimeters attached to the mouthpieces worn by the patient during treatment or placed by the radiation oncologist for patients without teeth and mouthpiece. **Figure 2** (next page) illustrates the dose distribution for one patient in a treatment plan view. The target area for the treatment is on the patient's right side. Therefore the sample taken adjacent to the upper right molar received a radiation dose close to the treatment dose while the samples taken at the other three locations received less dose, depending on the distance from the target. **Figure 3** (next page) presents the dose calculations from the treatment plan and MOSFET measurements performed for two patients. The agreement of dose calculations and measurements is excellent at many points. However, for some points there are substantial deviations. They can be explained by the high dose gradient in that area, which is intentional to focus most radiation to the tumor while sparing the normal tissue but provides challenges for the *in vivo* dosimetry. Especially important for the planned evaluation of radiation biodosimeters, the doses received at the 4 sampling sites in some patients were similar, and others quite divergent, and the doses (from ~5cGy to ~170cGy) covered the range in which discrimination is sought.

Figure 2. Radiation dosimetry of sites sampled in patients included estimates from treatment plans (left) and real time physical dosimetry (right two figures).

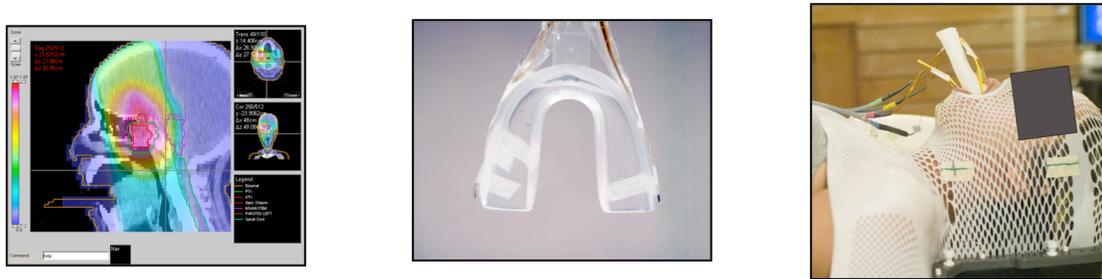
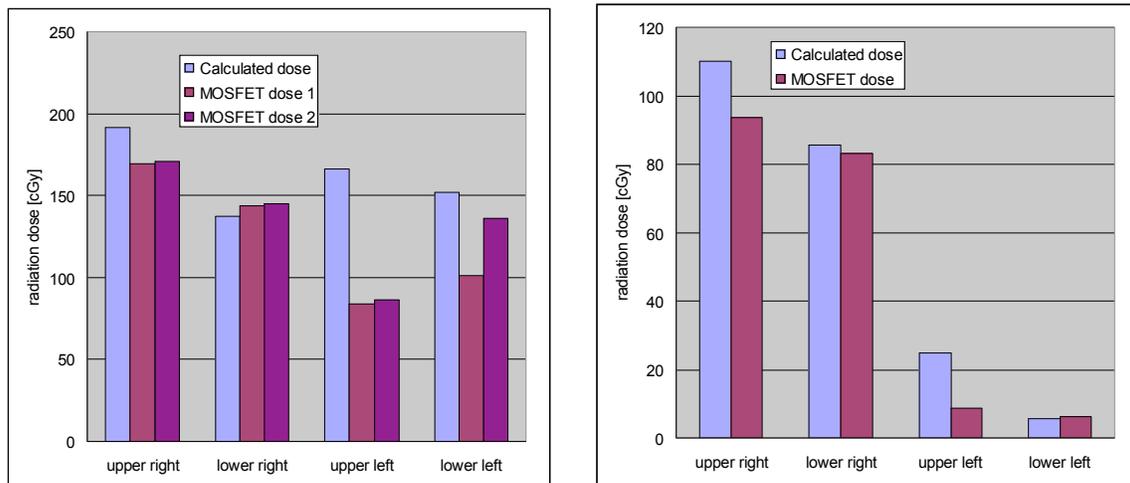


Figure 3. The dose estimates and measurements on two patients illustrate the range of doses achieved in this clinical, *in vivo* model.



(3) Development of a prototype device for collecting saliva and buccal cells for triage biodosimetry assays

Background In an instance of potential exposure of a large population to ionizing radiation, effective triage requires the capacity to efficiently prioritize people for medical follow-up based on received dose. Ideally, dose estimates for each individual would be obtained with a single measurement by first responders or trained volunteers in the community, using equipment or devices that are a standard part of community disaster response supplies. These needs translate to requirements of ease of use in the field, speed of data acquisition, clarity of results, low unit costs, and good shelf-life. Currently the only biologically-based radiation dosimetry options are evaluation of gross physiological symptoms such as severity and time of initiation of vomiting, or blood cell counts. Both

have serious shortcomings, and only the gross physiological symptoms can be utilized at this time in the field.

Approach Development of a device for triage radiation biodosimetry was envisioned as a team effort of engineers and biologists, to achieve a match between engineering and biological realities. It was agreed that immuno-assay based detection of protein biodosimeters by lateral flow would be the central principle for the detection part of the prototype device. Lateral flow devices have been commercially successful for other monitoring applications such as drug testing. Given that the long term goal was a device usable by a member of the public with limited instruction, it also was agreed that assays of saliva and buccal cell samples should be the first priority. Alternative methods for collecting and processing sample would be tested, then a first generation device would be engineered that provided for sample collection, processing and biodosimeter detection.

The goal of this effort was construction of a device that provided proof-of-principle in ability to meet sample collection, processing and detection requirements for a triage application. It was recognized that development of a device ready for deployment for radiation exposure was beyond the scope of this project. Extensive research and validation of candidate radiation responsive molecules and of antibodies and detection schemes with the specificity, sensitivity and multiplicity required for specific detection applications will be needed. Engineering capabilities are not expected to be the rate-limiting step in reaching a fieldable unit. Production of continuously improved devices for deployment will entail ongoing coupling of engineering of devices as the targets, tissues, reagents and detection methodologies evolve.

Accomplishments A variety of buccal cell and saliva sample collection methods were tested, and methods were selected for two applications, the triage device and the clinical *in vivo* model (discussed above). Methods tested included spatulas, brushes and medical sponges to enrich for cells, and collection of saliva with flow stimulated by chewing on a wad of parafilm. Samples were analyzed for RNA and protein content. Enrichment for viable buccal cells, to obtain more and higher molecular weight RNA, was best achieved with a cytobrush rotated in a limited position, as opposed to general swabbing of the mouth which tended to recover a higher proportion of the nonviable superficial cells. Repeated sampling in the same position did not irritate the tissue. It was determined that 1 ml of saliva provided enough protein from fluid and suspended cells to satisfy expected protein biodosimeter detection needs assuming low background and strong radiation responses. Rubbing of the buccal area with small piece of medical foam capable of retaining 1 ml liquid recovered sufficient cells and saliva fluid to meet initial design objectives for samples containing both saliva and buccal cells. It was concluded that the preferred collection tool would depend on whether the biodosimeters of interest were present in viable buccal cells or saliva (fluid, or fluid and cells).

To provide maximum initial flexibility, the lateral flow device package developed consisted of two parts (Disclosure of invention IL-11574). The first part is a rod-like structure which is comprised of a sample collection component on one end and a holder for a lateral flow strip on the other as shown in **Figure 4** (page 20). The second part is a

reservoir of reagent fluid also shown in Figure 4. The device works by first collecting saliva and/or buccal cells from an individual using the sample collection component then immersing the sample collection component into the reagent reservoir, then extracting the sample collection component from the reservoir and turning the rod-platform around (180 degrees) so that the tip of the lateral flow strip is immersed into the reservoir and the reagent/sample mixture wicks up into the strip to perform the assay as shown in Figure 4. The tip of the lateral flow strip that is housed in the rod-like structure protrudes slightly from the housing. This tip is immersed in the sample-reagent reservoir following introduction of the sample into the reservoir as shown in Figure 4. The liquid is wicked up the lateral flow strip containing detection reagents due to capillary forces. Flanges in the housing prevent the tip from being immersed too far into the reservoir as shown in Figure 4. A viewing window in the housing permits easy reading of the lateral flow assay results.

Flexibility of application is achieved in several ways. The sample collection component can consist of a swab, cytobrush or spatula for collecting buccal cells or a sponge (as pictured in Figure 4 for collecting both buccal cells and saliva). The reagent reservoir in which the sample collection component is immersed contains a reagent mixture that is used to wash the material from the sample collection component. The reagent mixture can contain lysing solution to help extract proteins from the buccal cells. The solution can also be used to reduce the viscosity of the saliva sample to make the lateral flow assay proceed more rapidly. As designed, the entrance to the reagent reservoir is tapered into an hour-glass shape as shown in **Figure 5** (page 20) such that a sponge collector can be squeezed while retracting the sponge from the reservoir to increase the volume of sample introduced into the reservoir. The sponge can be squeezed in the process of insertion into the reservoir to aid in mixing the sample. Sufficient height above the restriction prevents overflow of the sample. The reservoir can be sealed prior to use using a cap, a sealed lid or a septum.

The individual components of the device satisfied tests for performance. The sponge size was adjusted to obtain sufficient sample of buccal cells and saliva. The sample reservoir as described above with 1 ml of MPER™ was found to be adequate to totally lyse a sample for antigen detection. Finally, immersion of the tip of the lateral flow unit in the sample reservoir led to flow of sample through the pad, dissolving and transporting the reagent to test for sample flow, such that a strong signal developed within 5 minutes where the sample flow control antibody was printed on the strip (**Figure 6**, page 21). These protocols have been optimized for use in future sample testing with the complete lateral flow device.

Figure 4. Picture of the lateral flow housing and reagent reservoir. Left the separate pieces. Right with the lateral flow strip inserted into the reagent reservoir.

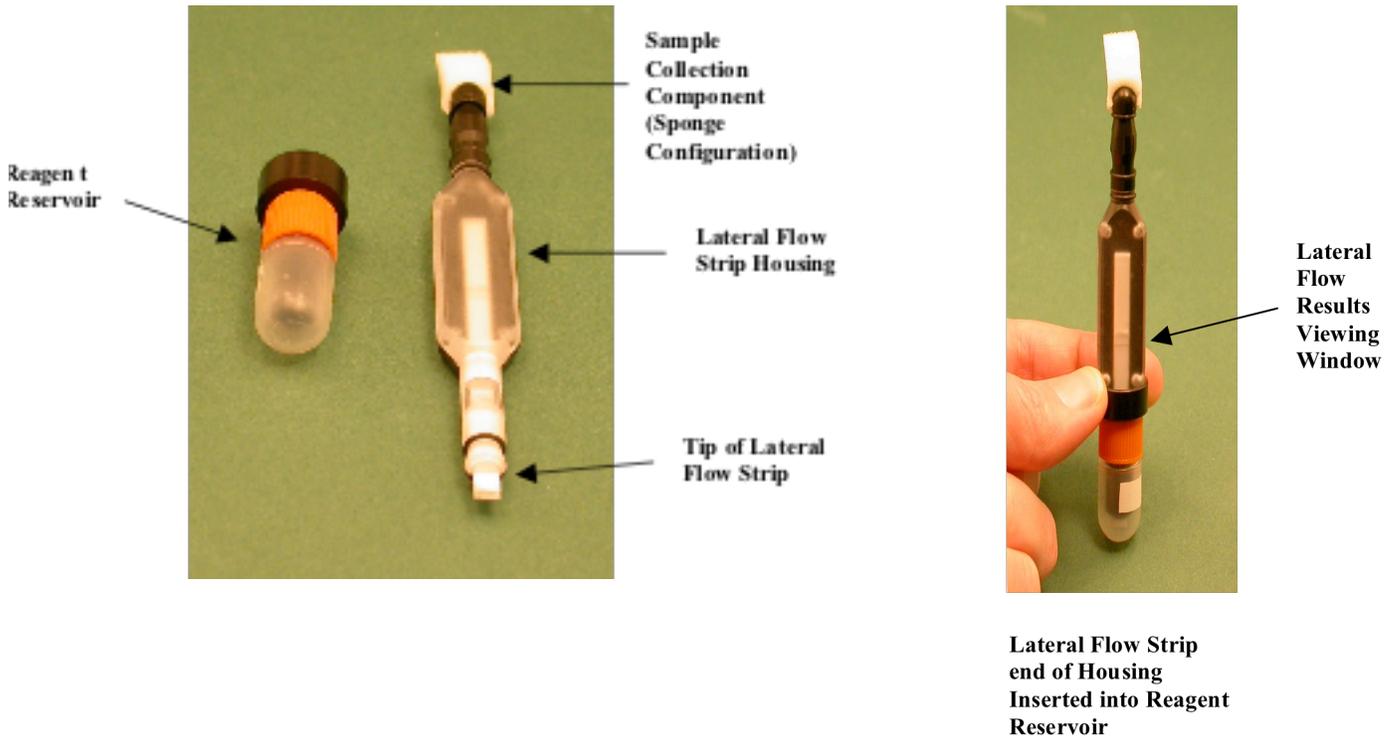


Figure 5. Detailed drawing of the reagent reservoir.

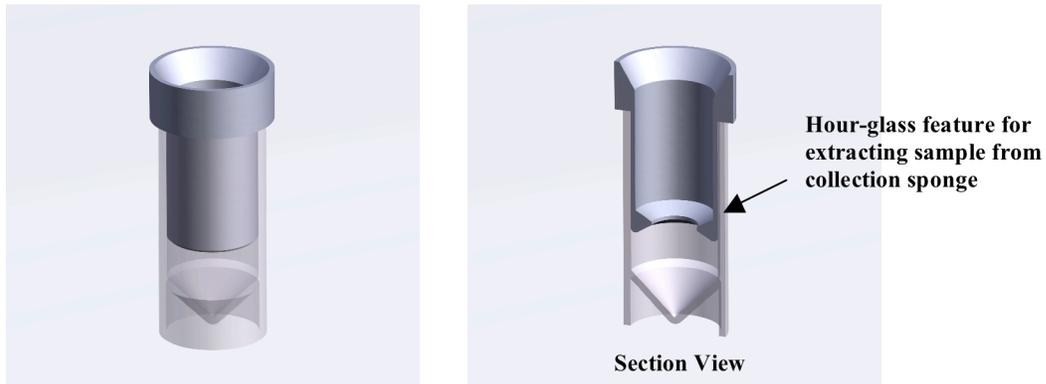
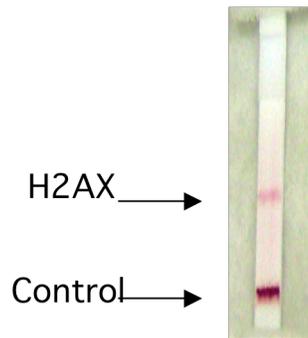


Figure 6. Demonstration of lateral flow test strip. Strip with single signal for sample flow of 10ng/ml of γ H2AX (H2AX in figure) in 1xPBS.



Future development of the device will be driven by results of testing of specific biodosimeters and detection antibodies and sample types, that may lead to adjustments of any or all of the components.

Summary

The goals of this project were to determine the current status and near term ability of biodosimeters to improve radiation dosimetry and our nation's ability to manage the consequences of accidental or terrorist radiological and nuclear events. The results obtained provide substantial encouragement that biodosimetry can make valuable contributions. There are indeed changes in cellular protein and RNA molecules that, to the extent tested, can report radiation exposure in the relevant dose and time range, and sample processing and detection technologies to measure these that can be adapted for clinical and triage applications. However, the results also demonstrated that individual biodosimeters have distinctive dose and time dependence, and that the responses of one cell type are not predictive for other tissues. As a result, it is likely that panels of radiation responsive molecules, selected for each tissue tested, will be needed to provide dosimetry over the dose range and times of interest. A further complication is that baseline values of RNA responsive molecules were found to vary among individuals. Given that pre-exposure samples will not be available, full validation of biodosimeters must ensure that variation in response to other factors which leads to this baseline variation does not lead to unacceptable false positives or negatives.

As expected at the outset, our studies also revealed that a major research and development program is needed to enhance national radiation biodosimetry capabilities. Such a program would utilize an expanded suite of discovery technologies, types of biomolecules, cell types and radiation exposure scenarios, with the goal of identifying and validating biomarkers that satisfy increasingly stringent dosimetry criteria. In addition, it might pursue development of biomarker-specific detection reagents, methods and devices. Such a program will be necessary to assure availability of well validated,

commercially produced biodosimetry assays for managing the medical consequences of a suspected or known radiological/nuclear event.

Note

Many details of results have been omitted due to the expectation that in the future resources will be identified that enable completion of data sets, validation of results, and publication in the peer reviewed literature.

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Publications

UCRL-JRNL-218093 Candidate protein biodosimeters of ionizing radiation. F. Marchetti, M. A. Coleman, I. M. Jones and A. J. Wyrobek

UCRL-TR-218890 Molecular Radiation Biodosimetry, I.M. Jones, M.A. Coleman, J. Lehmann, C.F. Manohar, F. Marchetti, R. Mariella, R. Miles, D.O. Nelson, A.J. Wyrobek (this report)

Records of invention

IL-11574, “Packaging for lateral flow strip assays for use with saliva and buccal cell samples”. R. Miles, W. Benett, F. Pearson, M. Coleman, S. Nasarbadi

IL-11383, “Ionizing radiation transcript panel for biodosimetry”, A.J. Wyrobek, M.A. Coleman, D.O. Nelson and J.D. Tucker.

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