Designation: E 1398 – 91 (Reapproved 2003)

Standard Practice for *In Vivo* Rat Hepatocyte DNA Repair Assay[1]

This standard is issued under the fixed designation E 1398; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This practice covers a typical procedure and guidelines for conducting the rat *in vivo* hepatocyte DNA repair assay. The procedures presented here are based on similar protocols that have been shown to be reliable (**1, 2, 3, 4, 5**).[2]

1.2 Mention of trade names or commercial products are meant only as examples and not as endorsements. Other suppliers or manufacturers of equivalent products are acceptable.

1.3 *This standard does not purport to address all of the safety concerns associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Significance and Use

2.1 Measurement of chemically induced DNA repair is a means of assessing the ability of a chemical to reach and alter the DNA. DNA repair is an enzymatic process that involves recognition and excision of DNA-chemical adducts, followed by DNA strand polymerization and ligation to restore the original primary structure of the DNA (**6**). This process can be quantitated by measuring the amount of labeled thymidine incorporated into the nuclear DNA of cells that are not in S-phase and is often called unscheduled DNA synthesis (UDS) (**7**). Numerous assays have been developed for the measurement of chemically induced DNA repair in various cell lines and primary cell cultures from both rodent and human origin (**4**). The primary culture rat hepatocyte DNA repair assay has proven to be particularly valuable in assessing the genotoxic activity of chemicals (**8**). Genotoxic activity often results from metabolites of a chemical. The *in vitro* rat hepatocyte assay provides a system in which a metabolically competent cell is also the target cell. Most other *in vitro* short-term tests for genotoxicity employ a rat liver homogenate (S-9) for metabolic activation, which differs markedly in many important ways from the patterns of activation and detoxification that actually occur in hepatocytes. An extensive literature is available on the use of *in vitro* DNA repair assays (**9-19**).

2.2 A further advance was the development of an *in vivo* rat hepatocyte DNA repair assay in which the test chemical is administered to the animal and the resulting DNA repair is assessed in hepatocytes isolated from the treated animal (**20**). Numerous systems now exist to measure chemically induced DNA repair in specific tissues in the whole animal (**4**). The average of *in vivo* assays is that they reflect the complex patterns of uptake, distribution, metabolism, detoxification, and excretion that occur in the whole animal. Further, factors such as chronic exposure, sex differences, and different routes of exposure can be studied with these systems. This is illustrated by the potent hepatocarcinogen 2,6-dinitrotoluene (DNT). Metabolic activation of 2,6-DNT involves uptake, metabolism by the liver, excretion into the bile, reduction of the nitro group by gut flora, readsorption, and further metabolism by the liver once again to finally produce the ultimate genotoxicant (**21**). Thus, 2,6-DNT is negative in the *in vitro* hepatocyte DNA repair assay (**22**) but is a very potent inducer of DNA repair in the *in vivo* DNA repair assay (**23, 24**). A problem with tissue-specific assays is that they may fail to detect activity of compounds that produce tumors in other target tissues. For example, no activity is seen in the *in vivo* DNA repair assay with the potent mutagen benzo(a)pyrene (BP), probably because limited tissue distribution and greater detoxification in the liver yields too few DNA adducts to produce a measurable response (**3**). In contrast, BP is readily detected in the less tissue-specific *in vitro* hepatocyte DNA repair assay (**11**). An extensive literature exists on the use of the *in vivo* hepatocyte DNA repair assay (**1-3, 5, 9, 25-33**).

3. Procedure

3.1 *Treatment*:

3.1.1 All personnel must be knowledgeable in the procedures for safe handling and proper disposal of carcinogens, potential carcinogens, and radiochemicals. Disposable gloves and lab coats must be worn.

3.1.2 Any strain or sex of rat may be used. The largest database is for male Fischer-344 rats. Young adult animals are preferred. It is possible that factors such as sex, age, and strain of the rat could affect the outcome of the DNA repair

[1] This practice is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.16 on Biocompatibility Test Methods.

Current edition approved Sept. 10, 2003. Published September 2003. Originally published in 1991. Last previous edition published in 1998 as E 1398 – 91 (1998).

[2] The boldface numbers in parentheses refer to the list of references found at the end of this practice.

Copyright © ASTM International, 100 Barr Harbor Drive, PO Box C700, West Conshohocken, PA 19428-2959, United States.

experiments. Therefore, for any one series of experiments (including controls) these variables should be kept constant.

3.1.3 Administration is usually by gavage with chemicals dissolved or suspended in an appropriate vehicle such as water or corn oil, depending on solubility. An advantage of the assay is that various routes of administration may be chosen. Thus, chemicals may also be administered by intraperitoneal injection or inhalation or in the diet. For gavage administration, 0.2 to 1.0 mL of test chemical solution is administered per 100 g body weight. Controls receive the appropriate vehicle solution. Stock corn oil should be replaced with fresh monthly.

3.1.4 For DNA repair studies, animals may be taken off feed for a few hours prior to sacrifice to make the process of perfusion a little easier with less food in the stomach. The period without food should never exceed 12 h because of the possibility of altered metabolism or uptake. Water should be continuously available.

3.1.5 Dose selection will depend on the characteristics of each chemical and the purpose of the experiment. If one is investigating whether a chemical can produce a genotoxic effect in the animal, even at massive doses and by routes of administration that may overwhelm natural defense mechanisms, then high doses (such as the LD50, or higher) that do not kill the animal before the 16-h sacrifice point may be employed. Even in such a case, doses above 1000-mg/kg body weight are not recommended. In some instances hepatotoxicity at high doses may result in inhibition of cell attachment or DNA repair. More commonly, the purpose of employing the whole animal is to evaluate the genotoxic effects of realistic exposures and routes of administration in the target tissue. In this case, doses above 500 mg/kg and the intraperitoneal route of administration are not recommended. The usual range of doses is from 10 to 500 mg compound per kilogram body weight. Target doses with a new compound are usually the LD50 and 0.2 × the LD50, with 500 mg/kg as an upper limit. The potent mutagen dimethylnitrosamine (DMN) (often used as a positive control) can be detected with doses as low as 1 mg/kg. Normally, an initial range finding experiment is conducted using single animals to cover a range of times and doses. If a positive response is seen, additional experiments are conducted to establish the dose-response relationship. If no response is seen, the highest dose(s) is repeated. The final report should contain results from at least three animals per datapoint.

3.1.6 Thus far, no examples have been seen of a compound that produces a UDS response in female rats but not males. For those cases where a comparison has been made, males respond more strongly than females in this assay. Thus, for the purpose of routine screening only male rats need to be used.

3.1.7 Treated animals should be maintained in a ventilated area or other suitable location to prevent possible human exposure to expired chemicals. Contaminated cages, bedding, excreta, and carcasses should be disposed of safely according to standard published procedures.

3.2 *Liver Perfusion*:

3.2.1 Any proven technique which allows the successful isolation and culture of rat hepatocytes can be used. An example of one such procedure is given in 3.2.2-3.2.17.

3.2.2 DMN exhibits a maximum UDS response 1 h after treatment. However, the response remains elevated and measurable for at least 16 h after treatment. More commonly, however, chemicals (for example, 2,6-DNT and 2-acetylaminofluorene (2-AAF)) show a peak response 12 to 16 h post-treatment.The time from treatment to perfusion may be varied to obtain a time course of induced repair. The routine protocol for primary screening involves a time point between 12 and 16 h with an optional time point between 1 and 4 h.

3.2.3 Anesthetize the rat by intraperitoneal injection with a 50-mg/mL solution of sodium pentobarbital (0.2 mL per 100 g body weight) 10 min prior to the perfusion procedure. Other proven anesthetics are also acceptable. Make sure that the animal is completely anesthetized, so that it feels no pain.

3.2.4 Secure the animal with the ventral surface up on absorbent paper attached to a cork board. Fold the paper in on each edge to contain perfusate overflow. Thoroughly wet the abdomen with 70 % ethanol and wipe with gauze for cleanliness and to discourage loose fur from getting on the liver when the animal is opened.

3.2.5 Make a V-shaped incision through both skin and muscle from the center of the lower abdomen to the lateral aspects of the rib cage. Do not puncture the diaphragm or cut the liver. Fold back the skin and attached muscle over the chest to reveal the abdominal cavity.

3.2.6 Place a tube approximately 1 cm in diameter under the back to make the portal vein more accessible.

3.2.7 Move the intestines gently out to the right to reveal the portal vein. Adjust the tube under the animal so that the portal vein is horizontal.

3.2.8 Put a suture in place (but not tightened) in the center of the portal vein and another around the vena cava just above the right renal branch.

3.2.9 Perform perfusions with a peristaltic pump, the tubing of which is sterilized by circulation of 70 % ethanol followed by sterile water. Place a valve in the line so that the operator may switch from the EGTA solution to the collagenase solution without disrupting the flow. Solutions should be kept at a temperature that results in a 37°C temperature at the hepatic portal vein.

3.2.9.1 A peristaltic pump with a chargeable pump head and silicone tubing is suitable for performing the perfusion.

3.2.9.2 Begin the flow of the 37°C EGTA solution at 8 mL/min and run to waste.

3.2.10 Cannulate the portal vein with a 20 GA 1¼-in. catheter about 3 mm below the suture. Remove the inner needle and insert the plastic catheter further to about ⅓ the length of vein and tie in place by the suture. Blood should emerge from the catheter. Insert the tube with the flowing EGTA in the catheter (avoid bubbles) and tape in place.

3.2.11 Immediately cut the vena cava below the right renal branch and allow the liver to drain of blood for 1.5 min. The liver should rapidly clear of blood and turn a tan color. If all lobes do not clear uniformly, the catheter has probably been inserted too far into the portal vein.

3.2.12 Tighten the suture around the vena cava and increase the flow to 20 mL/min for 2 min. The liver should swell at this point. In some cases gentle massaging of the liver or adjusting

the orientation of the catheter may be necessary for complete clearing. At this point the vena cava above the suture may be clipped to release some of the pressure in the liver.

3.2.13 Switch the flow to the 37°C collagenase solution for 12 min. During this period, cover the liver with sterile gauze wetted with sterile saline or WEI (see Annex A1) and place a 40-W lamp 2 in. above the liver for warming. It is valuable to screen each new batch of collagenase to be ensured that it will function properly.

3.2.14 Allow the perfusate to flow onto the paper and collect by suction into a vessel connected by means of a trap to the vacuum line. The perfusate should be disposed of as hazardous waste.

3.2.15 After the perfusion is completed, remove the catheter and gauze. Remove the liver carefully by cutting away the membranes connecting it to the stomach and lower esophagus, cutting away the diaphragm, and cutting any remaining attachments to veins or tissues in the abdomen.

3.2.16 Hold the liver by the small piece of attached diaphragm and rinse with sterile saline or WEI (see Annex A1).

3.2.17 Place the liver in a sterile petri dish and take to a sterile hood to prepare the cells.

3.3 *Preparation of Hepatocyte Cultures*:

3.3.1 Place the perfused liver in a 60-mm petri dish and rinse with 37°C WEI (see Annex A1). Remove extraneous tissues (fat, muscle, and so forth).

3.3.2 Place the liver in a clean petri dish and add 30 mL of fresh collagenase solution at 37°C.

3.3.3 Carefully make several incisions in the capsule of each lobe of the liver. Large rips in the capsule lead to large unusable clumps of hepatocytes.

3.3.4 Gently comb out the cells constantly swirling the liver while combing. A sterile, metal, dog-grooming comb with teeth spaced from 1 to 3 mm or a hog bristle brush works well.

3.3.5 When only fibrous and connective tissue remain, remove and discard the remaining liver. Add 20 mL cold WEI (see Annex A1) and transfer the cell suspension to a sterile 50-mL centrifuge tube using a wide-bore sterile pipe. Some laboratories report successful hepatocyte preparations when 3.3.1 through 3.3.8 are conducted with media at room temperature or heated to 37°C.

3.3.6 Allow the cells to settle on ice for 5 to 10 min until a distinct interface is seen. Carefully remove and discard the supernatant by suction.

3.3.7 Bring the cells to 50 mL with cold WEI (see Annex A1). Resuspend the cells by pipeting with a wide-bore pipet. Gently pipet the suspension through a 4-ply layer of sterile gauze into a sterile 50-mL centrifuge tube.

3.3.8 Centrifuge the cells at 50 times gravity for 5 min and discard the supernatant. Gently resuspend the pellet in ice-cold WEI (see Annex A1) with a wide-bore pipet.

3.3.8.1 Some laboratories prefer to keep the cells on ice until ready for use, while others keep them at room temperature. Cells should be used as soon as possible, preferably within 1 h.

3.3.9 Determine viability and cell concentration by the method of trypan blue exclusion. The preparation should be primarily a single-cell suspension with a viability of over 60 % for control cultures. With practice and the proper technique, viabilities of about 90 % can routinely be obtained. Attachment of the cells to the substrate is an active process. Thus, if a sufficient number of cells attach to conduct the experiment, this is a further indication of the viability of the culture.

3.3.10 Place a 25-mm round plastic coverslip into each well of 6-well culture dishes. 10.5 by 22-mm plastic coverslips and 26 by 33-mm eight-chamber culture dishes can also be used. Be sure to keep the proper side up as marked on the package. Four millilitres of WEC (see Annex A1) are added to each well. Hepatocytes will not attach to glass unless the slides have been boiled. The use of collagen-coated thermanox coverslips improves cell attachment and morphology.

3.3.10.1 These procedures yield preparations consisting primarily of hepatocytes. Approximately 400 000 viable cells are seeded into each well and distributed over the coverslip by shaking or stirring gently with a plastic 1-mL pipet. Glass pipettes can scratch the coverslips.

3.3.11 Incubate the cultures for 90 to 120 min in a 37°C incubator with 5 % CO_2 and 95 % relative humidity to allow the cells to attach.

3.4 *Labeling the Cultures*:

3.4.1 After the 90-min attachment, wash cultures once with 4 mL WEI (see Annex A1) per well to remove unattached cells and debris. This is done by tilting the culture slightly, aspirating the media, and adding the fresh media at 37°C. Be careful not to direct the stream from the pipet directly onto the cells.

3.4.2 Remove the WEI (see Annex A1) and replace with 2 mL of ^{3}H-thymidine solution (100 µCi/mL). Place the cultures in the incubator for 4 h. During this period some of the DNA damage that occurred in the animal will be repaired, resulting in the incorporation of ^{3}H-thymidine.

3.4.3 Wash the cultures once with 4 mL of WEI per well, then add 3 mL of unlabeled thymidine solution (0.25 mM) to each well. Incubate cultures overnight (14 to 16 h).

3.4.4 Wash cultures twice with 4 mL WEI (see Annex A1) per well.

3.4.5 The remainder of these procedures are done with solutions at room temperature. Replace the medium with 4 mL of a 1 % sodium citrate solution and allow the cultures to stand for 10 min to swell the nuclei. The purpose for swelling the cells is that the larger nuclei are more easily scored than the unswollen nuclei where the silver grains are more bunched together. Some operators prefer to omit this step. There is no evidence that swelling the nuclei yields any significant difference in the results compared to when the nuclei are not swollen.

3.4.6 Replace the sodium citrate solution with 3 mL of a 1 to 3 ratio of acetic acid to absolute ethanol solution for 10 min to fix the cells. Repeat this step twice more for a total fixing time of at least 30 min.

3.4.7 Wash wells 2 to 6 times each with deionized distilled water.

3.4.8 Remove coverslips from the wells and place cell-side-up on the edge of the dish covers to dry in a dust-free location at room temperature.

3.4.9 When dry, mount coverslips cell-side-up on microscope slides with mounting compound. Coverslips should be

mounted about 1 cm from the unfrosted end of the slide. Give each slide a unique identifying number.

3.5 *Autoradiography*:

3.5.1 Use any proven autoradiographic technique that yields silver grains in proportion to the amount of incorporated labeled thymidine. Presented in 3.5.2-3.5.14 is a typical procedure.

3.5.2 All steps involving photographic emulsions should be done in total darkness. If absolutely necessary a safelight filter may be used sparingly.

3.5.3 Mount three of the 6 slides for each animal in plastic slide grips. Hold the other 3 slides in reserve.

3.5.4 Mount a 50-mL disposable plastic beaker with tape into a slightly larger jar full of water. Place this assembly into a 42°C water bath and allow to reach the bath temperature.

3.5.5 Kodak NTB-2 emulsion is most commonly used. The emulsion is used undiluted or can be used diluted in a 1 to 1 ratio with distilled water. If the emulsion is diluted, care should be taken to use double distilled or ultrapure water, thoroughly mix the solution, but avoid the formation of air bubbles. Undiluted emulsion saves a step and provides slightly higher grain counts. Melt emulsion in a 37°C incubator for at least 3 h. Gently pour 40 to 50 mL of the emulsion into the 50-mL disposable beaker. The unused portion can be resealed and stored under refrigeration. If one of the Ilford "K" series[3] of photographic emulsions is used, it must not be liquefied and regelled.

3.5.6 Dip a test slide. Briefly turn on the safelight and hold the slide up to it to make sure that there is enough emulsion in the cup to cover the cells and that there are no bubbles in the emulsion. Air bubbles can be removed from the surface of the emulsion by skimming the surface with a glass slide. Turn off the safelight.

3.5.7 Dip each group of slides by lowering them into the cup until they touch the bottom. Pull the slides out of the emulsion with a smooth action to a 5-s count. Touch the bottom ends of the slides to a pad of paper towels to remove the bead of emulsion on the bottom. Remember, all of these steps must take place in total darkness. Do not reuse the emulsion.

3.5.8 Hang the slide holders in a vertical position in a rack in a light-tight box for 3 to 12 h to let the emulsion dry. Pack the slides into light-tight slide boxes that contain a false bottom packed with desiccant. Seal the boxes with black electrical tape and wrap them in aluminum foil as an additional precaution against light leakage.

3.5.9 Store the sealed slides at 4°C to −20°C (−20°C is preferred) for a set amount of time. Seven to 14 days is most common; 10 days is preferred. Shorter times yield lower backgrounds; longer times produce higher counts.

3.5.10 After the exposure period, allow the slide boxes to thaw at room temperature for at least 3 h in the dark, place the slides into a rack suitable for developing and staining the slides.

[3] The sole source of supply of the apparatus known to the committee at this time is Ilford, Inc., London, England. If you are aware of alternative suppliers, please provide information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

3.5.11 Develop the slides at 15°C (56°F) for 3 min in developer. Tap the rack gently to the bottom of the developing dish several times to dislodge any air bubbles on the slides.

3.5.12 Rinse slides 30 s in 15°C water, then fix in Kodak Fixer (not Kodak Rapid-Fix, since it removes the emulsion) for 5 min with agitation every 60 s. Wash the slides in a bath with gently running water for 25 min. Exercise care to ensure that the water stream does not directly strike the slides.

3.5.13 Slides can be stained while still wet from development. Dip into methyl green Pyronin Y solution (see Annex A1) for 10 to 20 s. Follow this immediately with repeated washings in water and a final rinse in distilled water. Do not overstain the cells. Cells should have faint blue nuclei and pink cytoplasms. Overstained cells make automatic grain counting difficult. Other stains are also acceptable. Remember, the cells are still exposed at this point. Take care not to touch the slide surface.

3.5.14 Allow the slides to air-dry for at least a few hours. Mount a 25-mm square coverslip over the round coverslip using a thin layer of mounting compound. Keep the slides flat overnight to dry. They are then ready for grain counting.

3.6 *Grain Counting*:

3.6.1 Although tedious, grain counting can be done by hand. If the assay will be used routinely, an automated counting system is recommended.

3.6.2 Grain counting is best accomplished with an automated system interfaced to a microscope with high-resolution TV camera. Data can be fed directly into a computer by means of an interface. An image analyser also works well. Any other proven system that accurately counts the grains is also acceptable.

3.6.3 Normally 20 to 50 cells are counted per slide, 1 to 3 slides per animal, 3 animals per datapoint. In an initial screening experiment in which multiple doses and time points are examined, three animals per datapoint are not necessary.

3.6.4 Counting usually requires a 100× objective under oil immersion. Additional optics can be employed to further increase magnification.

3.6.5 Each slide is examined to make sure that the culture as a whole was viable. Signs of toxicity are the absence of cells or pyknotic (small, darkly stained) cells.

3.6.6 Select a patch of cells as a starting point and score cells in a regular fashion by bringing new cells into the field of view, moving only the X-axis. If the desired number of cells have not been scored before coming to the edge of the slide, move the stage 1 to 2 fields on the Y-axis and resume counting in the opposite X-direction, parallel to the first line. If upon visual scanning of the slide there appears to be any difference in response in different areas of the slide, then the counting should be done selecting patches of cells from several areas of the slide.

3.6.7 The following criteria are used to determine that a cell should not be counted:

3.6.7.1 Cells with abnormal morphology, such as those with pyknotic or lysed nuclei,

3.6.7.2 Isolated nuclei not surrounded by cytoplasm,

3.6.7.3 Cells with unusual staining artifacts or in the presence of debris, and

3.6.7.4 Cells in S-phase (will be easily recognized by the heavy labeling), and cells adjacent to S-phase cells (due to possible spillover of grains from the S-phase cell).

3.6.8 All other normal cells encountered while moving the stage must be counted without regard as to their apparent response.

3.6.9 Counts are generally made in the mode that counts the area occupied by the grains. This allows patches of grains that are touching to be counted without being mistaken by the counter as a single grain. When using the area mode, a correction factor to convert to grain counts must be used. This conversion factor must be determined for the particular counting set-up and configuration being used. To do so, count a number of areas (10 to 30 discrete grains/aperture) on the count mode and manually to determine the actual number of silver grains. Then, perform a machine area count on the same aperture area. After counting 20 to 30 areas from at least two different slides, add all the actual counts and all the area counts. The conversion factor is calculated as:

$$C = \frac{\text{actual number of grains (total)}}{\text{measured area of grains (total)}} \quad (1)$$

Thus, machine counts can be converted to actual grains by multiplying by C.

3.6.10 For each cell the following procedure in 3.6.10.1 through 3.6.10.6 is used.

3.6.10.1 Adjust the sensitivity of the machine so that only grains are being counted and so that the configuration is the same as when the conversion factor was calculated.

3.6.10.2 Place the aperture directly over the nucleus and adjust to the same size as the nucleus.

3.6.10.3 Press the count button to record the nuclear counts.

3.6.10.4 Keeping the aperture the same size, count at least one area over the cytoplasm that is adjacent to the nucleus. Press the count button to record the cytoplasmic counts. There are several methods for scoring the cytoplasmic background. The first is to always move the aperture to the right for the cytoplasmic count. If there is a problem such as no cytoplasm, then move progressively clockwise until a cytoplasmic area adjacent to the nucleus can be recorded. This method has the advantage of giving random counts. The second method is to always choose the area that appears to have the highest grain counts. This method is conservative, so that spurious positive responses due to uneven cytoplasmic counts are seldom seen. The third method is to use the highest or the average of three independent cytoplasmic counts as the cytoplasmic background. In general, cytoplasmic counts are relatively uniform throughout the cytoplasm. Accordingly, experience shows that the procedure of taking a single cytoplasmic count saves time, is consistent, and yields comparable results to that which would be obtained using three counts. The conservative approach of using the highest cytoplasmic background is the one most often used in the published literature. While each method will yield a slightly different background value for the controls, they are all acceptable. However, the same method must be used consistently throughout any one experiment.

3.6.10.5 Subtract the cytoplasmic count from the nuclear count to give the net grains/nucleus (NG) for that cell. In the case of control cells, there will usually be more grains per unit area in the cytoplasm than in the nucleus so that the NG will be a negative number. This must be reported as such. Continue counting the desired number of cells in the same manner.

3.6.10.6 At all times remember that actual grains over the nucleus are being determined. The scorer must always be aware of what is going on and must never blindly trust whatever numbers come from the machine. If a spurious count is observed, either score the cell by eye or count another cell so that the incorrect value may be corrected before the data are calculated. If good laboratory practice is being followed, any change in the dataset should be noted for the record.

4. Report

4.1 Alterations in the following parameters will affect the NG observed:

4.1.1 The concentration and specific activity of ^{3}H-thymidine used,

4.1.2 The length of cell labeling,

4.1.3 The type and extent of dilution of the emulsion, and

4.1.4 The autoradiographic exposure time.

4.2 In data analysis, one must either use a published procedure where the expected values for negative and positive responses are known, or these values must be determined for the particular conditions being used.

4.3 For the conditions described here, $\geq$5 NG has been chosen as a conservative estimate as to whether any particular cell is responding or is in repair based on historical data. In choosing an NG cutoff for designating an individual cell in repair for other conditions, one should select a value where not more than 2 % of the cells in historical control cultures would be classified as responding.

4.4 The following should be calculated for each slide:

4.4.1 The population average NG $\pm$ SD (cell to cell),

4.4.2 The percent of cells responding or in repair, and

4.4.3 The population average NG $\pm$ SD (cell to cell) for the subpopulation of cells that are in repair (optional).

4.5 The following should be calculated for each animal:

4.5.1 The population average NG $\pm$ SD or SE (slide to slide),

4.5.2 The percent of cells responding or in repair $\pm$ SD (slide to slide), and

4.5.3 The population average NG $\pm$ SD or SE (slide to slide) for the subpopulation of cells that are in repair (optional).

4.6 The following should be calculated for each data point and are the numbers presented in reports and publications:

4.6.1 The population average NG $\pm$ SD or SE (animal to animal),

4.6.2 The percent of cells responding or in repair $\pm$ SD or SE (animal to animal),

4.6.3 The population average NG $\pm$ SD or SE (animal to animal) for the subpopulation of cells that are in repair (optional), and

4.6.4 The number (n) of animals per datapoint should be reported.

4.7 A laboratory must be able to demonstrate that it can consistently obtain the expected results in hepatocytes from animals treated with positive and negative control agents to create a historical control baseline for those conditions used in

that laboratory. Having done so, it is not always necessary to run a positive control animal every time a group of animals are treated and the cells prepared. Rather, one may treat some of the cultures from the negative control animal with a positive control agent *in vitro* such as 0.01 and 0.001 mM 2-AAF or 10 and 1 mM DMN. Observation of a positive response in these cultures will assure that culture procedures, reagents, media, incubations, radiolabeled materials, photographic emulsion, and development and staining procedures were all functioning or performed properly for that experiment. However, a complete experiment in which a compound has been thoroughly evaluated should contain results from at least one *in vivo* positive control animal.

4.8 Using conditions similar to those described here with a cutoff of 5 NG for a cell in repair, historical control data from 143 rats (untreated, corn oil, water, or saline; by gavage or intraperitoneal; 2 to 48 h post-treatment) from the laboratories of B. Butterworth, D. Kornbrust, and J. Ashby yielded −3.5 ± 1.7 NG with 2.6 ± 2.6 % of cells responding. No mean value greater than zero NG was observed. No value greater than 14 % was observed for the percent of cells responding. Similarly, historical data from 68 control rats (corn oil, water, or saline; by gavage or intraperitoneal; 0.5 to 48 h post-treatment) from the laboratory of J. Mirsalis yielded −5.4 ± 2.2 NG with 2.1 ± 3.0 % of cells responding. No further precision data are presented as part of this practice. Each laboratory running this procedure should determine the reproducibility of the assay in their hands.

4.9 For the positive controls 10 mg/kg DMN (administered in water by gavage 2 h before sacrifice), or 50 mg/kg 2-AAF (administered in corn oil by gavage 12 h before sacrifice), one might expect values of 30 to 60 NG with 80 to 100 % of the cells with greater than or equal to 5 NG. No further precision data are presented as part of this practice. Each laboratory running this procedure should determine the reproducibility of the assay in their hands. For those conditions described here, if a chemical yields greater than or equal to 5 NG (population average) and greater than or equal to 20 % of cells responding, the response is considered positive. A population average between 0 NG and 5 NG would be considered a marginal response. A dose-related increase in both NG and the percentage of cells in repair are required additional information to confirm a positive response for counts less than 5 NG.

4.10 Within an experiment, the cytoplasmic grain count usually remains fairly constant over a wide range of test agent doses, even when the nuclear grain count increases due to DNA repair. If the chemical treatment lowers the cytoplasmic grain counts to less than half that of the control cells, this should be noted in the final report and derived NG data should be considered along with dose-response relationships for both nuclear and cytoplasmic counts in evaluating the evidence for chemically induced UDS. In this case, the results should be viewed with caution, particularly if the nuclear counts did not increase in a dose-related fashion. A small increase in NG (less than 5 NG) may have been due only to the lowered cytoplasmic background and the results are suspect. With some potent mutagens, such as DMN, the cytoplasm can be missing. However, the very large dose-related increase in the nuclear counts is clearly indicative of chemically induced DNA repair and in this specific case these cells may be scored and the result reported as such. Examination of intact cells from lower doses of chemical would confirm the positive response. This is less likely to occur in this *in vivo* DNA repair protocol compared to the *in vitro* protocol in which the cells in culture are directly treated with the test chemical.

4.11 Under some test conditions, the cytoplasmic grain counts may increase in a dose-related manner, independent of the nuclear count (which may also increase). In such situations, counting may be difficult. If chemical treatment increases the cytoplasmic count by more than double that of the control cells, this should be noted in the final report and derived NG data should be considered along with dose-response relationships for both nuclear and cytoplasmic counts in evaluating the evidence for chemically induced UDS. Increases in the cytoplasmic grain count may be due to mitochondrial DNA synthesis or repair. In the absence of a precise understanding of these effects, caution in the interpretation of such data should be exercised. This is less likely to occur in this *in vivo* DNA repair protocol compared to the *in vitro* protocol in which the cells in culture are directly treated with the test chemical.

4.12 Different situations may be encountered when conducting the *in vivo* hepatocyte DNA repair assay. There may be times when several concurrent controls will be run with the treated group. Since one laboratory can only be expected to run a modest number of animals in one day, numerous concurrent controls for every experiment are prohibitive and are not required. Nevertheless, if concurrent controls have been included, then the unpaired t-test for the equality of two means using the individual animal NG as the unit of measure is a reasonable test for statistical significance.

4.13 Generally, over the course of a few weeks, several batches of animals will be tested in order to build a database for a chemical. On each experimental day a control animal will be employed. If conditions and procedures in the laboratory remain constant, it is valid to pool those controls produced over the course of the study. The unpaired t-test or a multicomparison test such as the Dunnett's multiple range test may be used to compare the mean of the control group with the means for the treated groups for statistical significance. One may even choose to pool the entire historical control database for comparison to the treated group by the unpaired t-test. However, with a very large N, relatively small increases in the treated samples (that is, NG values less than zero), that may have no biological significance, may appear as statistically significant.

4.14 The probable reason that control NG values tend to be less than zero is that the cytoplasm (and the components therein producing the cytoplasmic background) is slightly thinner over the nucleus compared to the rest of the cell as it sits on the substrate. NG counts may vary as the result of compound-related effects on cytoplasmic grain counts. Consequently, no result may be considered positive unless the compound actually produces more grains over the nucleus than over the cytoplasm, that is, an NG value greater than zero. Knowledge of the biology of this assay dictates that in order to have any confidence in a positive DNA repair response, the

treatment must produce nuclear counts above the cytoplasmic background. Thus, for any statistical test employed, a lower limit of at least 0 NG is required for a positive response.

4.15 In the final report, the authors should clearly state their criteria and conclusions as to whether a compound was positive or negative.

ANNEX

(Mandatory Information)

A1. SOLUTIONS

A1.1 *WEI (Williams Medium E—Incomplete)*:

A1.1.1 500 mL Williams medium E; 5 mL sterile 200 mM L-glutamine; 0.5 mL of 50 mg/mL gentamycin sulfate. Some laboratories use a lower concentration of gentamicin to minimize toxicity of the antibiotic to the cells. The original pH of 7.2 of Williams medium E-based solutions should be reasonably maintained by keeping the medium sealed, under 5 % CO_2 or supplemented with HEPES buffer, or both, as indicated. Approximately 250 mL are needed per animal.

A1.2 *WEC (William Medium E—Complete)*:

A1.2.1 180 mL WEI; 20 mL heat inactivated fetal bovine serum; Heat inactivation 30 min at 56°C. Freeze aliquots. Approximately 25 mL are needed per animal.

A1.3 *0.5 mM EGTA Perfusion Solution*:

A1.3.1 100 mL Hanks balanced salt solution without Ca^{+2} or Mg^{+2}; 19 mg EGTA (ethylene glycol-bis (β-amino ethyl ether) N,N′-tetra-acetic acid). Dissolve EGTA in 0.1 mL 2 N NaOH, 0.5 mL 2 M HEPES, 0.1 mL of 50 mg/mL gentamicin sulfate. Filter sterilize the final solution. Approximately 100 mL are needed per animal.

A1.4 *Collagenase Perfusion Solution (100 Units/mL)*:

A1.4.1 500 mL WEI 2.5 mL 2 M HEPES 0.1 mL 2 N NaOH 50 000 units Type 1 collagenase. Leave in 37°C bath until dissolved (20 to 30 min). Filter sterilize. Approximately 350 mL are needed for each animal. This solution should be made up no more than 24 h prior to use.

A1.5 *^{3}H-Thymidine Solution (10 µCi/mL)*:

A1.5.1 100 mL WEI 1000 µCi (1 mL) ^{3}H-thymidine (methyl ^{3}H-thymidine, Amersham TRK.418, 40–60 Ci/mmole); 0.5 mL sterile 2 M HEPES. Make up just prior to use. ^{3}H-thymidine should be stored refrigerated and not kept or used beyond two months. Approximately 13 mL are needed per animal.

A1.6 *Unlabeled Thymidine Solution (0.25 mM)*:

A1.6.1 100 mL WEI; 6.1 mg thymidine. Filter sterilize. Approximately 20 mL are needed per animal.

A1.7 *Methyl-Green Pyronin Y Solution*:

A1.7.1 Add 7.45 g Na_2HPO_4 to 66 mL Methanol. Add distilled water to bring volume to 263 mL. Stir until dissolved. Add 5 g citric acid to 59 mL Methanol. Add distilled water to bring volume to 240 mL. When both solutions are dissolved, mix and add 12.5 µL phenol, 125 mg resorcinol, and 5 g methyl-green pyronin Y. Allow for 2 weeks before use. Keep in a dark bottle and filter before each use. Discard after approximately 6 months or when an obvious decrease in staining intensity is observed.

REFERENCES

(**1**) Ashby, J., Lefevre, P. A., Burlinson, B., and Penman, M. G., "An Assessment of the *in vivo* Rat Hepatocyte DNA Repair Assay," *Mutation Research*, Vol 156, 1985, pp. 1–18.

(**2**) Butterworth, B. E., "Measurement of Chemically Induced DNA Repair in Hepatocytes *in vivo* and *in vitro* as an Indicator of Carcinogenic Potential," *The Isolated Hepatocyte: Use in Toxicology and Xenobiotic Biotransformations*, Rauckman, E. J., and Padilla, G. M., Eds., Academic Press, Inc., Orland, FL, 1986, pp. 241–264.

(**3**) Mirsalis, J. C., Tyson, K. C., and Butterworth, B. E., "The Detection of Genotoxic Carcinogens in the *In Vivo–In Vitro* Hepatocyte DNA Repair Assay," *Environmental Mutagenesis*, Vol 4, 1982, pp. 553–562.

(**4**) Mitchell, A. D., and Mirsalis, J. C., "Unscheduled DNA Synthesis as an Indicator of Genotoxic Exposure," *Single-Cell Mutation Monitoring Systems*, Ansari, A. A., and de Serres F. J., Eds., Plenum Publishing Corp., New York, NY, 1984, pp. 165–216.

(**5**) Hamilton, C. M., and Mirsalis, J. C., "Factors that Affect the Sensitivity of the *in vivo-in vitro* Hepatocyte DNA Repair Assay in the Male Rat," *Mutation Research*, Vol 189, 1987, pp. 341–347.

(**6**) Roberts, J. J., "The Repair of DNA Modified by Cytotoxic, Mutagenic and Carcinogenic Chemicals," *Advances in Radiation Biology*, Vol 7, 1978, pp. 211–436.

(**7**) Rasmussen, R. E., and Painter, R. B., "Radiation-Stimulated DNA Synthesis in Cultured Mammalian Cells," *Journal of Cell Biology*, Vol 9, 1966, pp. 11–19.

(**8**) Mitchell, A. D., Casciano, D. A., Meltz, M. L., Robinson, D. E., San, R. H. C., Williams, G. M., and von Halle, E. S., "Unscheduled DNA Synthesis Test: A Report of the "Gene-Tox" Program," *Mutation Research*, Vol 123, 1983, pp. 363–410.

(**9**) Butterworth, B. E., Earle, L. L., Strom, S., Jirtle, R., and Michalopoulos, G., "Induction of DNA Repair in Human and Rat Hepatocytes by 1,6-Dinitropyrene," *Mutation Research*, Vol 122, 1983, pp. 73–80.

(**10**) Casciano, D. A., and Gaylor, D. W., "Statistical Criteria for Evaluating Chemicals as Positive or Negative in the Hepatocyte/DNA Repair Assay," *Mutation Research*, Vol 122, 1983, pp. 81–86.

(**11**) Probst, G. S., McMahon, R. E., Hill, L. E., Thompson, C. Z., Epp, J. K., and Neal, S. B., "Chemically-Induced Unscheduled DNA Synthesis in Primary Rat Hepatocyte Cultures: A Comparison with

Bacterial Mutagenicity Using 218 Compounds," *Environmental Mutagenesis*, Vol 3, 1981, pp. 33–43.

(**12**) McQueen, C., and Williams, G. M., "Methods and Modifications of the Hepatocyte Primary Culture/DNA Repair Test," *Handbook of Carcinogen Testing*, Milman, H. A., and Weisburger, E. K., Eds., Noyes Publications, Park Ridge, NJ, 1985, pp. 116–129.

(**13**) Waters, R., Ashby, J., Burlinson, B., Lefevre, P., Barrett, R., and Martin, C., "Unscheduled DNA Synthesis," *UKEMS (The United Kingdom Environmental Mutagen Society), Report of the UKEMS Sub-Committee on Guidelines for Mutagenicity Testing*, UKEMS, Swansea, United Kingdom, 1984, pp. 63–87.

(**14**) Williams, G. M., "Carcinogen Induced DNA Repair in Primary Rat Liver Cell Cultures: A Possible Screen for Chemical Carcinogens," *Cancer Letters*, Vol 1, 1976, pp. 231–236.

(**15**) Williams, G. M., "Detection of Chemical Carcinogens by Unscheduled DNA Synthesis in Rat Liver Primary Cell Cultures," *Cancer Research*, Vol 37, 1977, pp. 1845–1851.

(**16**) Williams, G. M., "Further Improvements in the Hepatocyte Primary Culture DNA Repair Test for Carcinogens: Detection of Carcinogenic Biphenyl Derivatives," *Cancer Letters*, Vol 4, 1978, pp. 69–75.

(**17**) Williams, G. M., Bermudez, E., and Scaramuzzino, D., "Rat Hepatocyte Primary Cell Cultures—III, Improved Dissociation and Attachment Techniques and the Enhancement of Survival by Culture Medium," *In vitro*, Vol 13, 1977, pp. 809–817.

(**18**) Williams, G. M., and Laspia, M. F., "The Detection of Various Nitrosamines in the Hepatocyte Primary Culture/DNA Repair Test," *Cancer Letters*, Vol 66, 1979, pp. 199–206.

(**19**) Williams, G. M., Laspia, M. F., and Dunkel, V. C., "Reliability of the Hepatocyte Primary Culture/DNA Repair Test in Testing of Coded Carcinogens and Noncarcinogens," *Mutation Research*, Vol 97, 1982, pp. 359–370.

(**20**) Mirsalis, J., and Butterworth, B., "Detection of Unscheduled DNA Synthesis in Hepatocytes Isolated from Rats Treated with Genotoxic Agents: An *In Vivo–In Vitro* Assay for Potential Mutagens and Carcinogens," *Carcinogenesis*, Vol 1, 1980, pp. 621–625.

(**21**) Rickert, D. E., Butterworth, B. E., and Popp, J. A., "Dinitrotoluene: Acute Toxicity, Oncogenicity, Genotoxicity, and Metabolism," *CRC Critical Reviews in Toxicology*, Vol 13, 1984, pp. 217–234.

(**22**) Bermudez, E., Butterworth, B. E., and Tillery, D., "The Effect of 2,4-Diaminotoluene and Isomers of Dinitrotoluene on Unscheduled DNA Synthesis in Primary Rat Hepatocytes," *Environmental Mutagenesis*, Vol 1, 1979, pp. 391–398.

(**23**) Mirsalis, J. C., and Butterworth, B. E., "Induction of Unscheduled DNA Synthesis in Rat Hepatocytes Following *In Vivo* Treatment with Dinitrotoluene," *Carcinogenesis*, Vol 3, 1982, pp. 241–245.

(**24**) Mirsalis, J. C., Hamm, T. E., Sherrill, J. M., and Butterworth, B. E., "Role of Gut Flora in the Genotoxicity of Dinitrotoluene," *Nature* (London), Vol 295, 1982, pp. 322–323.

(**25**) Beije, B., and Ashby, J., "Use of an *in vivo/in vitro* Rat Liver DNA Repair Assay to Predict the Relative Rodent Hepatocarcinogenic Potency of 3 New Azo Mutagens," *Carcinogenesis*, Vol 6, 1985, pp. 611–615.

(**26**) Butterworth, B. E., Bermudez, E., Smith-Oliver, T., Earle, L., Cattley, R., Martin, J., Popp, J. A., Strom, S., Jirtle, R., and Michalopoulos, G., "Lack of Genotoxic Activity of Di(2-Ethylhexyl)Phthalate (DEHP) in Rat and Human Hepatocytes," *Carcinogenesis*, Vol 5, 1984, pp. 1329–1335.

(**27**) Doolittle, D. J., Bermudez, E., Working, P. K., and Butterworth, B. E., "Measurement of Genotoxic Activity in Multiple Tissues Following Inhalation Exposure to Dimethylnitrosamine," *Mutation Research*, Vol 141, 1984, pp. 123–127.

(**28**) Doolittle, D. J., Sherrill, J. M., and Butterworth, B. E., "Influence of Intestinal Bacteria, Sex of the Animal, and Position of the Nitro Group on the Hepatic Genotoxicity of Nitrotoluene Isomers *in vivo*," *Cancer Research*, Vol 43, 1983, pp. 2836–2842.

(**29**) Hellemann, A. L., Maslansky, C. J., Bosland, M., and Williams, G. M., "Rat Liver DNA Damage by the Nonhepatocarcinogen 3,2′-Dimethyl-4-Aminobiphenyl," *Cancer Letters*, Vol 22, 1985, pp. 211–218.

(**30**) Joachim, F., and Decad, G. M., "Induction of Unscheduled DNA Synthesis in Primary Rat Hepatocytes by Benzidine-Congener-Derived Azo Dyes in the *in vitro* and *in vivo/in vitro* Assays," *Mutation Research*, Vol 136, 1984, pp. 147–152.

(**31**) Kornbrust, D. J., Barfknecht, T. R., and Ingram, P., "Effect of Di(2-Ethylhexyl)Phthalate on DNA Repair and Lipid Peroxidation in Rat Hepatocytes and on Metabolic Cooperation in Chinese Hamster V-79 Cells," *Journal of Toxicology Environmental Health*, Vol 13, 1984, pp. 99–116.

(**32**) Kornbrust, D., and Dietz, D., "Aroclor 1254 Pretreatment Effects on DNA Repair in Rat Hepatocytes Elicited by *In Vivo* or *In Vitro* Exposures to Various Chemicals," *Environmental Mutagenesis*, Vol 7, 1985, pp. 857–870.

(**33**) Steinmetz, K. L., Tyson, C. K., Meierhenry, E. F., Spalding, J. W., and Mirsalis, J. C., "Examination of Genotoxicity, Toxicity and Morphologic Alterations in Hepatocytes Following *In Vivo* or *In Vitro* Exposure to Methapyrilene," *Carcinogenesis*, Vol 9, 1988, pp. 959–963.

ASTM International takes no position respecting the validity of any patent rights asserted in connection with any item mentioned in this standard. Users of this standard are expressly advised that determination of the validity of any such patent rights, and the risk of infringement of such rights, are entirely their own responsibility.

This standard is subject to revision at any time by the responsible technical committee and must be reviewed every five years and if not revised, either reapproved or withdrawn. Your comments are invited either for revision of this standard or for additional standards and should be addressed to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee, which you may attend. If you feel that your comments have not received a fair hearing you should make your views known to the ASTM Committee on Standards, at the address shown below.

This standard is copyrighted by ASTM International, 100 Barr Harbor Drive, PO Box C700, West Conshohocken, PA 19428-2959, United States. Individual reprints (single or multiple copies) of this standard may be obtained by contacting ASTM at the above address or at 610-832-9585 (phone), 610-832-9555 (fax), or service@astm.org (e-mail); or through the ASTM website (www.astm.org).