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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.

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

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.

3.5.11 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] 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.