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

This standard is issued under the fixed designation E1397; 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 vitro* hepatocyte DNA repair assay. The procedures presented here are based on similar protocols that have been shown to be reliable (**1-6**)[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 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.4 *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 the recognition and excision of DNA-chemical adduct followed by DNA strand polymerization and ligation to restore the original primary structure of the DNA (**7**). 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) (**8**). 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 (**9**). The primary rat hepatocyte DNA repair assay developed by Williams (**10**) has proven to be particularly valuable in assessing the genotoxic activity and potential carcinogenicity of chemicals (**11**), (**12**). Genotoxic activity is often produced by reactive metabolites of a chemical. The *in vitro* rat hepatocyte assay provides a system in which a metabolically competent cell is itself the target cell for measured genotoxicity. Most other 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* hepatocyte DNA repair assays (**2**, **3**, **6**, **13-28**).

3. Procedure

3.1 *Liver Perfusion:*

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 proven technique which allows the successful isolation and culture of rat hepatocytes can be used. An example of one such procedure is given in 3.1.3-3.1.20.

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

3.1.4 Anesthetize the rat by intraperitoneal injection with a 50-g/mL solution of sodium phenabarbitol (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.

3.1.5 Wet the abdomen thoroughly with 70 % ethanol and wipe with gauze for cleanliness to discourage loose fur from getting on the liver when the animal is opened.

3.1.6 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 the skin and attached muscle back over the chest to reveal the abdominal cavity.

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

3.1.8 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.

[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 at the end of this practice.

3.1.9 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.

3.1.10 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 (see Annex A1) without disrupting the flow. Keep solutions at a temperature that results in a 37°C temperature at the hepatic portal vein.

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

3.1.12 Begin the flow of the 37°C EGTA solution (see Annex A1) at 8 mL/min, and run to waste.

3.1.13 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 the vein and tie in place by the suture. Blood should emerge from the catheter. Insert the tube with the flowing EGTA (see Annex A1) in the catheter (avoid bubbles) and tape in place.

3.1.14 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.

3.1.15 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.

3.1.16 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.

3.1.17 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.

3.1.18 After the perfusion is over, remove the catheter and gauze. Carefully remove the liver 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.

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

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

3.2 *Preparation of Hepatocyte Cultures:*

3.2.1 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).

3.2.2 Place the liver in a clean petri dish and add 30 mL of fresh collagenase solution (see Annex A1) at 37°C.

3.2.3 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.

3.2.4 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 apart, or a hog bristle brush works well.

3.2.5 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 (see Annex A1) using a wide-bore sterile pipet. Some laboratories report successful hepatocyte preparations when 3.2.1-3.2.8 are conducted with media at room temperature or heated to 37°C.

3.2.6 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.

3.2.7 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.

3.2.8 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.

3.2.9 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.

3.2.10 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, it is a further indication of the viability of the culture.

3.2.11 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. Add 4 mL of WEC (see Annex A1) 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.

3.2.12 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.

3.2.13 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.

3.3 *Labeling the Cultures:*

3.3.1 After the attachment period, wash the 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.

3.3.2 Prepare chemical solutions in ^{3}H-thymidine solution (WEI containing 10 µCi/mL ^{3}H-thymidine) (see Annex A1). Serial dilutions are generally employed. If employed, solvents for the test substance, such as dimethyl sulfoxide (DMSO) or

ethanol, should not exceed a 1 % final concentration. Most investigators try to limit the DMSO concentration to at or below 0.5 %, because of borderline toxic effects on some hepatocyte cultures at DMSO concentrations of 1 %. Both medium alone and solvent controls should be included in the experimental design. Concentrations of the test substance should be chosen that go just beyond cytotoxicity to about 1000-fold below the cytotoxic concentration. Cytotoxicity can be determined by trypan blue dye exclusion or lactic dehydrogenase (LDH) release of the cultures or by morphological examination of the fixed and stained cells at the end of the experiment. Typical concentration ranges are from 10 to 0.001 mM. Relatively insoluble substances should be tested up to their limit of solubility. Freely soluble, nontoxic chemicals should not be tested at concentrations beyond 10 mM. A dose of 10 mM dimethylnitrosamine (DMN) is required to produce a strong DNA repair response in the assay. In contrast, 1,6-dinitropyrene induces DNA repair at concentrations as low as 0.00005 mM.

3.3.3 Remove the WEI (see Annex A1) and replace with 2 mL of ^{3}H-thymidine solution (see Annex A1) containing the dissolved test chemical. Place the cultures in the incubator for 16 to 24 h. During this period the compound may be metabolized. If DNA damage occurs, it will be repaired, resulting in incorporation of the ^{3}H-thymidine (see Annex A1).

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

3.3.5 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.

3.3.6 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 twice more for a total fixing time of at least 30 min.

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

3.3.8 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.

3.3.9 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.

3.3.10 At this point, the cultures can be examined for gross cytotoxicity. If chemical treatment at the higher doses has resulted in there being no cells on the slides, they need not be subjected to autoradiography.

3.4 *Autoradiography:*

3.4.1 Any proven autoradiographic technique that yields silver grains in proportion to the amount of incorporated labeled thymidine may be used. Presented in 3.4.2-3.4.14 is a typical procedure.

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

3.4.3 Mount slides for each dose in plastic slide grips. Duplicate slides may be held in reserve.

3.4.4 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.

3.4.5 Kodak NTB-2[3] emulsion is most commonly used. The emulsion may be used undiluted or can be diluted to a 1 to 1 ratio with distilled water. If the emulsion is diluted, take care to use double distilled or ultrapure water; thoroughly mix the solution but avoid 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"[4] series of photographic emulsions is used, it must not be liquefied and regelled.

3.4.6 Dip a test slide. Briefly turn on the red 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.

3.4.7 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 that all of these steps must take place in total darkness. Do not reuse the emulsion.

3.4.8 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 disiccant. Seal the boxes with black electrical tape and wrap them in aluminum foil to ensure no light leaks.

3.4.9 Store the sealed slides 4°C to −20°C (−20° 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.

3.4.10 After the exposure, 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 Kodak NB-2 emulsion, available from International Biotech, Inc., New Haven, CT 06535. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

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

3.4.11 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.

3.4.12 Rinse slides for 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. Care should be exercised to ensure that the water stream does not directly strike the slides.

3.4.13 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 that the cells are still exposed at this point. Take care not to touch the slide surface.

3.4.14 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 now ready for grain counting.

3.5 *Grain Counting:*

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

3.5.2 Grain counting is best accomplished with an automated system interfaced to a microscope with a 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.

3.5.3 Normally 20 to 50 cells are counted per slide, 1 to 3 slides per treatment, 2 experiments per data point. In an initial screening experiment in which multiple doses are being examined, only doses up to the minimally toxic dose as judged by cell morphology need be counted and repeats only need cover the active, noncytotoxic concentrations.

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

3.5.5 Examine each slide 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. Note the highest concentration of chemical that was not toxic to the cultures as a whole.

3.5.6 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, the stage is moved 1 to 2 fields on the Y-axis and counting resumes 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.

3.5.7 The following criteria are used to determine those cells that should not be counted:

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

3.5.7.2 Isolated nuclei not surrounded by cytoplasm,

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

3.5.7.4 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).

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

3.5.8 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. Now, 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.

3.5.9 For each cell the procedure in 3.5.9.1-3.5.9.6 is used.

3.5.9.1 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.

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

3.5.9.3 Press the count button to record the nuclear counts.

3.5.9.4 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,