CHAPTER 7

The In Vitro *Chromosome Aberration Test*

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Chapter Outline

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7.1 Introduction

The chromosome aberration test involves treatment of mammalian cells in culture with the test substance in the absence and in the presence of an exogenous metabolic system (S9 mix). Double-stranded DNA damage can be induced directly or, in the case of most genotoxins, indirectly as a result of errors in replication or repair of DNA lesions, leading to double-strand breaks (DSB), which are the major cause of structural chromosome aberrations [1]. Many aberrations are lethal or lost during subsequent cell divisions, so they are best observed at the first metaphase after induction. Various cultured cell lines (including CHL and CHO) can be used to test for chemical induction of DNA damage and are suitable for regulatory

testing. However, cell lines are genetically unstable and tend to lose and gain chromosomes or parts of chromosomes spontaneously, so they show a higher and more variable spontaneous rate of chromosome aberrations compared with primary diploid cell cultures such as mitogen-stimulated lymphocytes. Therefore, some laboratories prefer to use primary lymphocytes for routine testing, although these have their own practical drawbacks in terms of ease of culturing, assessment of toxicity, and slide reading (Chinese hamster cell lines have fewer and larger chromosomes than human lymphocytes). This chapter focuses on the description of the two most widely used systems: those using Chinese hamster fibroblast cell lines and those using human peripheral blood lymphocyte (HPBL) cultures, in which lymphocytes are stimulated into division using phytohemagglutinin (PHA). For the lymphocyte culture system, many laboratories use a density gradient technique to separate white blood cells from erythrocytes prior to culture; however, we describe the whole-blood technique here because it is simpler and provides a high yield of mitotic cells.

In all systems, cells are arrested at metaphase (when chromosome morphology is clearest) using colchicine or colcemid, swollen in hypotonic solution, and then fixed before being dropped onto slides, air-dried, and then stained. In this way the chromosomes of metaphase cells are well-spread in a single plane and show clear morphology. Chromosome breakage is evident in the form of various structural aberrations that are scored using high-resolution light microscopy. Increases in the proportion of aberrant metaphase cells (i.e., those showing at least one chromosome aberration) as a result of treatment are indicative of genotoxicity. In routine experiments, cells are treated with the test agent using short (3−6 h) and long exposure times (equal to 1.5 cell-cycle times). Because cell lines and lymphocytes have limited ability to metabolize xenobiotics, cultures are treated in the absence and presence of an exogenous metabolic activation system, usually S9 mix, which consists of a chemically induced rat liver S9 fraction with appropriate cofactors. Treatment in the presence of S9 is performed using only the short exposure period because S9 is somewhat toxic and rapidly loses metabolic activity after addition to the test system.

The main endpoints scored in the test are gross (i.e., observable with standard staining methods) structural aberrations. Polyploidy and any evidence of other forms of aneuploidy are recorded as incidental observations, but increases in these are not necessarily considered indicative of genotoxicity. More subtle chromosomal aberrations including translocations between chromosomes or rearrangements within a chromosome can also occur as a result of treatment with genotoxic agents, but these generally need special staining methods and/or more lengthy analysis to be recognized and therefore are not scored routinely.

7.2 History

Structural and numerical aberrations are associated with adverse health effects, including congenital abnormalities and neoplasia, and approximately 50% of human miscarriages

show chromosomal abnormalities [2]. It has been known for more than a century that chromosome aberrations can be used as a marker of exposure for both radiation and genotoxic chemicals. Early cytogenetic studies studied the effect of radiation in various meiotic and somatic tissues with limited success because of the poor quality of the preparations. Progress in the field included examination of giant salivary gland chromosomes in the F1 generation of *Drosophila* as described by the Nobel prize winner Hermann Muller in the 1920s [3]. Later, aberrations were noted at anaphase in plant root tip squash preparations soon after exposure [4], as described in reviews of the theory and history of the test by Kirkland [5] and Natarajan [2].

In the 1950s and 1960s, various technical improvements made examination of chemically induced aberrations in mammalian cells feasible. In particular, use of colchicine to accumulate cells in metaphase, hypotonic treatment to spread chromosomes, and fixation of cells in suspension were described by Ford and Hamerton in 1956 [6], while a modified method involving air-drying of fixed preparations further enhanced preservation of chromosome morphology [7]. Plant lectins including PHA were found to stimulate lymphocyte division in cultures, allowing chromosome analysis of a large number of diploid cells from human donors after simple and minor invasive sampling of peripheral blood [8]; a combination of these techniques was used to demonstrate the clastogenic (chromosome-breaking) effect of the cross-linking agent mitomycin C (MMC) in dividing lymphocytes [9,10]. Sources of exogenous metabolic activation were introduced in later studies using CHO cells to evaluate indirect acting carcinogens [11,12]. These improved methodologies enable the assay to be used in various screening studies on chemical mutagens or carcinogens in the environment, as well as food additives [11,13–18]. In approximately 1990, many laboratories, particularly in Japan, moved from using CHO to CHL cells because the latter seemed to show increased sensitivity [19] and the background rate of aberrations in some CHO cell lines was unstable. Both CHO and CHL are similar transformed Chinese hamster fibroblast cell lines, so it seems that differences in sensitivity were probably due to procedural differences; however, some CHO sublines were found to be unstable in terms of rates of aberration [5,20,21]. These points emphasize the importance of obtaining cell lines from a reliable laboratory and then ensuring they are pure and appropriately characterized before using them for testing.

More recently, many works have been published proposing standard protocols for the test [22–25], including the original (1983) OECD guidelines for the test. However, discussions continued concerning the appropriate cell type, harvest times, limits of toxicity, and indirect effects resulting from departures from physiological conditions in terms of pH and osmolality [18,26a,b,–29]. In 1994, an International Workshop on Genotoxicity Testing working group discussed various previously unresolved or controversial aspects of the study design/evaluation aspects of the then current OECD test guidelines, including metabolic activation, exposure concentrations, number of replicates, treatment and harvest times, analysis/evaluation of results,

and repeat testing [30], and reached a consensus on most of these items. Important points raised by the group included the poor reproducibility of toxicity between testing occasions and the need to delay the main sampling until approximately 1.5 cell cycles after exposure because of the cytostatic (cycle delaying) effects of clastogens at higher dose levels. In the 2000s, there was a growing concern over the lack of specificity of the mammalian cell tests in general following a series of publications that addressed the assay's performance in terms of discriminating rodent carcinogens and noncarcinogens and apparent oversensitivity. In particular, oversensitivity could be explained by much higher and more toxic concentrations being used in the *in vitro* systems than could be realistically achieved in the *in vivo* situation [31–33], which was a key point in the discussion of how to determine the relevance of *in vitro* findings to human health [34]. In an attempt to reduce these "misleading" positive results, and to allow conditions that would still maintain the element of hazard identification, new upper limits have recently been set by ICH and OECD in the two documents most often cited in justification of chromosome aberration test studies designed for regulatory submission [35,36]. At the same time, the parallel OECD test guidelines for the *in vitro* micronucleus test were similarly updated [37].

7.3 Fundamentals

Treatment of cells with DNA-damaging agents (*in vitro* or *in vivo*) can result in unrepairable lesions in both strands of DNA. This leads to chromosome breakage that can be seen on microscopic examination of metaphase preparations (Figure 7.1).

In the *in vitro* test, cultures of established mammalian cell lines or primary cultures of human or rodent cells are grown before addition of the test substance to three sets of cultures. An exogenous metabolic activation system (S9) is added to one set of cultures at the same time as the test substance. One set of cultures without S9 and one with S9 are washed free of the test substance 3–6 h later. All cultures are then incubated for a total period equivalent to 1.5 cell cycles after initiation of treatment. The third set of cultures is treated in the absence of S9 only for a continuous period equivalent to 1.5 cell cycles. About two hours prior to harvesting, all three sets of cultures are treated with an agent that arrests cells in the metaphase stage of cell division (e.g., colcemid or colchicine) when the structure of the chromosomes is most clear. The most commonly used primary cells are human peripheral blood lymphocytes (HPBL), which, like other lymphocytes, must be exposed to a plant lectin/mitogen and cultured for approximately 48 h before they are actively dividing and most sensitive to DNA-damaging agents.

The cells are then harvested, separated by centrifugation, and resuspended in hypotonic potassium chloride solution. This causes the cells to swell and enhances eventual separation of the chromosomes to facilitate analysis. The cells are fixed and washed in a mixture of methanol and acetic acid and then dropped onto glass microscope slides. Slides are stained (typically with Giemsa), mounted with coverslips, and examined by light microscopy.

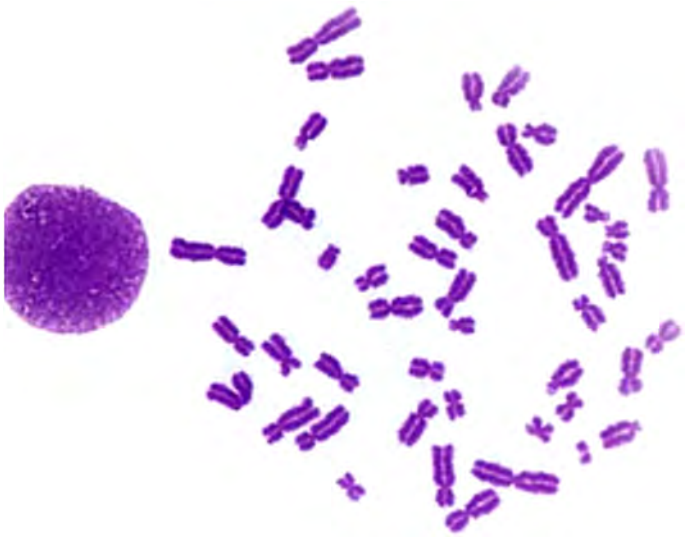


Figure 7.1
Human lymphocyte in metaphase showing a chromosome break with fragment.

The various types of structural chromosome aberration observed are tabulated; however, these all result from chromosome breakage. The main parameter used to assess genotoxicity is the percentage of cells showing structural aberrations. Apparent gaps in chromatid or chromosome structure are also observed occasionally; these are recorded but are not included in assessment of genotoxicity because they do not necessarily involve chromosome breakage.

High-quality metaphase preparations, a trained and experienced observer who is familiar with the karyotype, together with appropriate acceptance, and evaluation criteria are essential for producing reliable results [38].

A test substance formulation that causes a substantial increase in the proportion of metaphases showing chromosome aberrations is regarded as clastogenic and therefore genotoxic.

7.4 Equipment

The following is a list of specialized equipment expected to be found in a laboratory performing tests on a routine basis:

1. Autoclave
2. Binocular light microscope with high-quality, flat-field achromatic optics and parfocal objectives: medium-power plan objective (16× or similar) and high-power oil-immersion

(100×). The oil immersion lens should be plan apochromat (Planapo), although plan fluorite may be preferred if the microscope will also be used for fluorescence work. Investment in a high-quality microscope is critical to facilitate accurate assessment of chromosome damage and minimize operator fatigue. Most good modern microscopes use infinity-corrected extra-low dispersion glass lenses and Köhler illumination to optimize image quality. If you have a limited budget, then consider buying a second-hand microscope, because a good microscope will last a lifetime if properly cared for; in this case, ensure the lenses are not damaged (scratched or with deterioration of the cement between components of compound lenses). Carl Zeiss Axio Scope, Leica DM, Nikon Labophot II, and Olympus BX all have good reputations and are, arguably, the only makes worth considering

3. Vibration-proof bench for the microscope
4. Biological containment cabinet class II (externally vented)
5. Büchner funnel and Büchner vacuum flask with adapter ring (with clamp stand) connected to an aspirator or vacuum pump via vacuum tubing for vacuum filtration of diluted Giemsa stain
6. Cell maintenance log[1]
7. Centrifuge—bench-top, low-speed swing-out with adapters for 15 mL tubes. The speeds of centrifugation indicated in this chapter are for guidance and may need adjusting depending on the particular centrifuge rotor.
8. CO_2 incubator with gas supply
9. Electronic cell counter,[1] such as a Coulter counter; other electronic cell counting systems may also be suitable and much less expensive, such as Millipore Scepter™ 2.0
10. Fume hood/cupboard
11. General laboratory equipment: balances, glassware refrigerator, purified water, timer, pipette aids, and others
12. Hemocytometer with coverslips
13. Inverted microscope[1]
14. Liquid nitrogen cell storage system[1]
15. Micropipettes (adjustable, repeating, and positive displacement types; range 2−1000 μL)
16. Microscope slides with a frosted end. Plain slides can be used if an automated slide labeling/etching system is available
17. Pump for aspirations, with receiving flask/bottle
18. Racks for culture tubes
19. Slide storage system for archiving slides—cardboard systems are most economical and least liable to damage the slides

[1] Mainly for use with cell lines.

20. Stainless steel slide racks and staining dishes—cleaned by washing in 50% acetic acid and then dried before each use
21. Two-channel bench-top tally counter, such as Denominator-type or electronic
22. Vortex mixer
23. Waterbath

7.5 Consumables and Reagents

Consumables typically used in a testing laboratory (sterile where appropriate) include:

1. Blood collection tubes with sodium heparin[2]
2. Centrifuge tubes, 15 and 50 mL polypropylene with caps
3. Colchicine or colcemid
4. Coverslips, 22 × 50 mm
5. Culture medium. RPMI 1640 is used for lymphocytes and is available commercially in various forms: powder, 10× and 1× liquid, and as an autoclavable solution. The 1× liquid can usually be used as is, but other forms will need supplementing with sodium bicarbonate and/or glutamate if these are not already present. Some forms of RPMI (Dutch modification) include HEPES as a buffering agent, but this tends to be inhibitory and is best avoided. (Ham's) F-12 medium is used for CHO cells
6. Culture vessels: 25 and 75 cm^2 vented flasks (used for adhesive cell lines)[3]; vials, tubes, or plates as appropriate, such as 4 mL clear, glass, flat-bottom, screw cap vials or flat-sided culture tubes (Nunc or equivalent) for 1 and 5 mL blood cultures, respectively[2]
7. Dulbecco's phosphate-buffered saline (DPBS) without calcium or magnesium[3]
8. Fetal calf serum (FCS)/fetal bovine serum (FBS)
9. Filter papers, Whatman No. 1 to fit Büchner funnel (or cotton wool)
10. Filters units, 0.2 or 0.22 μm Luer-Lok syringe-fitting for aqueous solutions and solvents, such as Millex®
11. Fix—3 volumes methanol:1 volume glacial acetic (ethanoic) acid prepared just before each use
12. Gentamicin, aqueous 10 mg/mL (commercially available)
13. Hypotonic—0.075 M potassium chloride
14. Immersion oil for microscopy
15. Isoton II diluent for cell counting with the Coulter counter[3]
16. Labels, self-adhesive printed with code number—code numbers can be generated in Excel using the random function and then printed onto sheets of adhesive labels with

[2] HPBL.
[3] For attached cell lines including CHO.

an appropriate number of replicate labels per culture. The slide code should be printed and saved electronically to decode results later.
17. Medical wipes, such as Kimwipes
18. Microcentrifuge tubes
19. Micropipette tips, sterile
20. Pasteur pipettes—the polypropylene ones are most convenient
21. Phosphate buffer, 0.2 M, pH 7.4
22. Pipettes, sterile plastic disposable
23. Positive control agents
24. Purified water
25. Results sheets/forms
26. S9 fraction and cofactors
27. Slide mountant (Cytoseal, DPX, Permount, or similar permanent nonaqueous type)
28. Slide trays, cardboard
29. Solvents including appropriate anhydrous organic solvents; DMSO in particular is hygroscopic and can develop mutagenic impurities in the presence of small amounts of water. Pure organic solvents should be maintained in an anhydrous condition by addition of a small quantity of a compatible predried molecular sieve (type 4A in the case of DMSO) and stored well-sealed over anhydrous silica gel.
30. Stain, Giemsa solution in methanol/glycerol (see recipe in the rodent micronucleus chapter). Giemsa stain Gurr solution can also be purchased from VWR and Fisher.
31. Syringes, disposable
32. Trypan blue, 0.4% solution in DPBS
33. Trypsin 0.25% in DPBS (with or without 1 mM EDTA)[3]

Gas syringes or metering equipment, 24 mL glass anaerobic culture tubes (Bellco Glass), gas-impermeable injectable butyl rubber septum, gas bags, sealable vials, and other specialist equipment or components will be needed to test gases.

See the next section (Reagents and Recipes) for additional components and reagents that may be needed.

7.6 Reagents and Recipes

The following reagents may be purchased from commercial suppliers such as Sigma-Aldrich, Moltox, or manufactured in-house, in which case we suggest that each recipe should be prepared using a form (an appendix to the standard operating procedure) so that appropriate details including supplier, batch number, and amounts of components can be maintained. It is convenient to create a template for each class of reagent (e.g., solution, medium, etc.).

In the following recipes, water refers to deionized reverse-osmosis purified water; other forms of purified water including distilled water may be used. Volumes of each component mentioned should be adjusted in proportion to the total volume of reagent required. Filter-sterilization normally involves the use of a 0.22 μm filter. Reagents should be labeled with identity, preparation date (or batch number), and expiry date.

Expiry dates are based on the date of preparation and should take into account the expiry dates of individual components. Expiry dates can be extended provided that results are available in the laboratory to prove the reagent is still fit for its purposes.

7.6.1 Colcemid 10 μg/mL in PBS

Colcemid 10 μg/mL in PBS is available commercial and is used to arrest cells in metaphase. Colchicine 12.5 μg/mL may also provide satisfactory results.

7.6.2 Fix

Add 750 mL of methanol to a measuring cylinder. Make up to 1 L with glacial acetic acid. Transfer to an appropriate container, seal, and mix by hand. Store at room temperature and use on the day of preparation.

7.6.3 F-12 Complete

This medium is used for culturing the CHO cells. Note that Eagle's minimum essential medium is also often used for CHL cells (e.g., [19]).

Completely thaw 100 mL of FCS in a 37°C waterbath and then mix by swirling or inversion. Add the serum and 5 mL gentamicin 10 mg/mL to 1 L of sterile (Ham's) F-12 medium by filter-sterilization and then mix well. The medium can be stored in the refrigerator for up to 2 months. Any medium that develops a precipitate should be discarded.

7.6.4 Freezing Medium 10% (CHO Cells)

Filter-sterilize one volume of DMSO into nine volumes of F-12 complete in a sterile container. Store at room temperature and use on the day of preparation. Note that this medium is diluted with an equal volume of cell suspension in normal growth medium, so the final concentration of DMSO is 5% v/v at freezing.

7.6.5 Hypotonic Solution (0.075 M KCl)

Prepare a 0.75 M potassium chloride (KCl; formula weight, 75) stock solution in advance by dissolving 56 mg of KCl per 1 mL of water and store in the refrigerator for up to 1 year. On the day of use, dilute one volume of the stock with nine volumes of water and mix well.

7.6.6 Heparin Sodium 1000 U/mL

Heparin is incorporated into RPMI complete as an anticoagulant and is commercially available as a sterile solution in saline.

7.6.7 G6P 1 M: Glucose-6-Phosphate

The solution is prepared by dissolving G6P at 260 mg/mL of water; if the sodium salt is used, then it should be dissolved at the rate of 282 mg/mL. Filter-sterilize and then store in a freezer; this expires after 1 year.

7.6.8 KMg

Potassium chloride (KCl; formula weight 75) 124 mg and magnesium chloride ($MgCl_2.6H_2O$; formula weight, 203) 81 mg comprise KMg.

Each 1 mL of solution contains 124 mg Potassium chloride (KCl; formula weight 75) and 81 mg Magnesium chloride hexahydrate ($MgCl_2.6H_2O$; formula weight 203). Dissolve the salts in water (80% of the final volume) and then make the volume with water. Autoclave or filter-sterilize the solution and then store it at room temperature in ambient light for up to 1 year.

7.6.9 NADP 0.1 M

Dissolve β-nicotinamide adenine dinucleotide phosphate sodium salt (formula weight 765) at 76.5 mg/mL of water. Filter-sterilize, store refrigerated in the dark, and use on the day of preparation.

7.6.10 PHA M Form (Phytohemagglutinin)

This is a crude extract in the form of an aqueous solution and is used for stimulating T lymphocytes into division. It is available commercially and is used at a final concentration of approximately 2% v/v in the culture medium (final concentration approximately 2−10 μg/mL in terms of solid PHA M).

7.6.11 Phosphate Buffer 0.2 M, pH 7.4

This solution is used to make the S9 mix. Mix the following two solutions in the proportions shown: sodium dihydrogen phosphate (NaH_2PO4) 0.2 M 146 mL and disodium hydrogen phosphate (Na_2HPO4) 0.2 M 854 mL.

Confirm that the pH is in the range of 7.3−7.5, and then autoclave or filter-sterilize it. Store at room temperature in ambient light. This expires after 1 year.

7.6.12 Positive Control Solutions

Stock solutions of MMC can be prepared in DMSO before use and then aliquoted in convenient amounts before storing in the freezer for up to 18 months. Frozen solutions should be completely thawed. Solutions of cyclophosphamide monohydrate (CP) are made on the day of use. Volatile positive controls should be avoided because of potential contamination of the incubator. On thawing any stored solution, ensure the material is completely dissolved before use.

7.6.13 RPMI Complete

This medium is used for culturing the HPBL. Completely thaw 100 mL of FCS in a 37°C waterbath and then mix by swirling or inversion. Filter-sterilize the serum, 5 mL of gentamycin 10 mg/mL and 4 mL of heparin sodium 1000 U/mL into 1 L of sterile RPMI 1640 medium (with $NaHCO_3$ and L-glutamine) and mix well. The medium can be stored in the refrigerator for up to 2 months.

7.6.14 S9 Fraction

The S9 fraction routinely used is identical to that described for the bacterial mutation test. It is prepared from the liver of rats that have been induced by intraperitoneal injection of Aroclor 1254 at 500 mg/kg bodyweight on one occasion or by multiple administrations of a mixture of phenobarbital with β-naphthoflavone either orally or by intraperitoneal injection to promote the levels of xenobiotic metabolizing enzymes [39−45]. S9 fraction is conventionally prepared by homogenization of liver in isotonic potassium chloride (0.15 M KCl) at a rate of 1 g wet tissue per 3 mL and then separated by centrifugation at 9000 g. S9 fraction may also be prepared using a lower proportion of 0.15 M KCl and then diluted to a standardized protein concentration (typically 40 mg/mL) based on biochemical estimation of protein content. Rarely, S9 preparations from other species (e.g., pooled human liver when testing compounds with known human-specific metabolism) or even other tissues may be included when appropriate and justified [46].

Most laboratories purchase precertified S9 fraction from a commercial source to avoid issues with handling animals and Aroclor (polychlorinated biphenyls are banned by some countries and some individual companies) and additional biochemical assays. Commercial S9 fraction can be obtained in frozen or lyophilized form in appropriately sized aliquots; alternatively, lyophilized preformulated S9 mix is available from Moltox. The quality control certificate supplied with commercial S9 should be retained with the raw study data. Frozen S9 should be stored below −70°C, thawed entirely immediately before use, and mixed well. Thawed S9 degrades fairly rapidly, so any excess should be discarded and not refrozen for later use.

7.6.15 S9 Mix

S9 mix may be prepared in the same way as for the bacterial mutation test, although some laboratories use different buffers/diluents. The concentration of S9 fraction in the S9 mix depends on the laboratory and test system. We suggest 15% and 10% v/v for CHO and HPBL, respectively, which both yield a final concentration of 2% v/v after dilution in culture medium. Typically, the final concentration of S9 fraction in the culture medium is 1−2%; higher concentrations may inhibit cell growth. In addition, S9 mix contains the following cofactors: 8 mM $MgCl_2$, 33 mM KCl, 100 mM sodium phosphate buffer pH 7.4, 5 mM glucose-6-phosphate, and 4 mM NADP [40]; therefore, each 1 mL of S9 mix contains:

	10%	15%
Water	0.335 mL	0.285 mL
Phosphate buffer 0.2 M pH 7.4	0.500 mL	0.500 mL
NADP 0.1 M	0.040 mL	0.040 mL
G6P	0.005 mL	0.005 mL
KMg	0.020 mL	0.020 mL
S9 fraction	0.100 mL	0.150 mL

All components should be sterile and added aseptically in the proportions and order listed here to a sterile container on ice, kept on ice or refrigerated, and used on the day of preparation.

Unused S9 mix should be discarded and not frozen for future use because it rapidly loses activity.

7.7 Phases in Development of the Test

Development may be conveniently divided into phases as described for the bacterial reverse mutation test: research, setup, internal validation, routine maintenance, and testing. Particular attention should be given to achieving a good mitotic index (MI) (in the case of

lymphocytes) and the quality of slide preparations in terms of spreading of metaphases, morphology, and staining. Colcemid or colchicine may be used to arrest cells in metaphase at concentrations of approximately 0.1 μg/mL and 0.25 μg/mL, respectively. Many laboratories may prefer colcemid for *in vitro* studies because it seems less toxic; during the setup phase, we recommend that you test a range of concentrations of each to determine the optimal concentration for producing a large number of well-spread readable metaphases. Note that optimized and standardized culture conditions will result in mitotic indices routinely in excess of 5% in the case of HPBL. Metaphases should be sufficiently spread so that the chromosomes do not overlap, but without bursting some of the cells (in that case, individual chromosomes will be released from the boundaries of the cells and will be found floating free), chromatids should be separated with a clear outline and fairly deeply stained with the centromeres being identifiable; the cytoplasm should be only vaguely evident in the background and lightly stained. Readable metaphases will show all these characteristics and have a chromosome number very close to the diploid (HPBL) or modal (cell lines). Particular attention should be given to standardizing conditions of fixation and slide "dropping" so that excellent preparations can be made regularly with little "fine tuning"; this especially applies to temperature and, most importantly, to humidity [22,41–44], which is usually not controlled and subject to high seasonal variations in the laboratory. You will find it useful to monitor and record the temperature and humidity (using a hygrometer) in the slide-making area for reference purposes.

Unlike cell lines, the mitotic activity of lymphocyte cultures depends on:

- the culture vessel size and shape of the bottom of the vessel; flat-bottomed vessels tend to give the best results
- the depth and volume of the culture medium
- the density of the cells

In particular, if the MI during exposure and cell harvesting is high, then this will be expected to maximize sensitivity to mutagens and it will greatly reduce time and effort involved in slide reading. We recommend that each laboratory experiments with different culture vessels as well as the density and culture volume to maximize the MI at harvest using the conditions described in this chapter as a starting point. Note that assessment of the MI in lymphocytes is not straightforward because, unlike cell lines in culture, a proportion of the cells are undergoing necrosis (see Assessment of Toxicity later). The flat-sided culture vessels that we describe for lymphocyte culture are convenient for examination and because cells can be processed and fixed *in situ*.

Once the test methodology is standardized, your laboratory will need to establish an adequate negative and positive control database; these will be regularly updated and summarized in future reports. The negative control database is particularly important

because vehicle control and test substance results in all future studies will be compared with it to confirm assay validity and help interpret any apparent increases in the incidence of aberrant cells. Initially, the negative control database should include an absolute minimum of 10, and preferably 20, experiments [36] (see also the General Recommendations chapter in this book). Incidences of aberrant cells in the negative/untreated control groups are normally similar between treatment regimes and should be pooled within one database unless there is any substantial difference. For cell lines in particular, special attention should be given to any apparent upward shifts in the incidences of aberrant metaphases and the database should only cover the past 2 or 3 years.

Consideration should be given to validating the *in vitro* versions of the chromosome aberration and micronucleus test in tandem because there is a very large overlap in culture and dosing requirements. This has the added benefit that the results of the two test systems can be compared directly.

7.8 Cell Characterization

Cell lines should be obtained from a reliable genetic toxicology laboratory that has maintained and characterized them appropriately as described here. Some cultivars (sublines) may be inappropriate for genetic toxicology testing because of genetic instability; the WBL clone of CHO cells has given good results in several laboratories and appears to be stable [47]. Appropriate characteristics of cells should be determined as listed by OECD TG473 before conducting GLP experiments. On arrival or after being rederived, cell lines should be assessed for stability of the modal chromosome number and the absence of mycoplasmal contamination, purified by cloning, characterized, and stored for later use. Once test methods have been optimized and standardized, the cell-cycle time of all cell types used in the test should be determined under typical negative control conditions in the laboratory; values obtained should be consistent with the published values. It is useful to establish representative karyotypes of cell lines on arrival for comparative purposes (e.g., with other laboratories and to check for potential shift over time); this can be performed using G-banding or related techniques [48]. Human lymphocytes have the same karyotype as other diploid human cells, and organized karyotypes showing grouping by size and centromere location are freely available online (e.g., http://www.web-books.com/MoBio/Free/Ch1C3.htm). A printed copy of the normally stained (unbanded) karyotype ideogram should be retained for training purposes and should be available in the slide reading area for potential reference.

Appropriate stocks of cell lines should be stored in the vapor phase of liquid nitrogen and appropriate clones should be used to generate frozen permanents, which are divided into two categories: master and test batch frozen permanents. Master frozen permanents are

frozen cell aliquots set aside for long-term storage and generation of new test batches. Test batches are used to inoculate cultures for routine experiments.

Details of cell line maintenance, characterization, storage, and passage (number and details of subculturing) should be maintained in the cell maintenance log book.

7.8.1 Modal Chromosome Number

Normal diploid human cells all contain 23 pairs of chromosomes (including XX or XY pairs) at metaphase, which show high consistency between cells. However, cell lines have a variable and less stable number of chromosomes. The distribution of the number of chromosomes is characteristic of a particular clone; however, especially in less stable cell lines and those grown under inappropriate conditions, this and the karyotype in general will change over the course of multiple subculturings. Cell lines used for cytogenetic studies should have a relatively stable chromosome complement and, to help achieve this, should be subcultured as little as possible (i.e., the passage number should be low) and never grown beyond log phase. To demonstrate stability on initial acceptance for use and over time, the chromosome number distribution and modal number of cell lines should be established in each laboratory and monitored. Although Chinese hamsters themselves have a stable karyotype with 22 chromosomes, CHO and CHL cell lines show a varied number of chromosomes around the modal number, with the modal number being the most common number of chromosomes. For CHO-K1 cells, this can be 20 (ATCC cell line grown at CRL) or 21 [26b], whereas a value of 21 has been reported for CHO-WBL cells [19,49], and a value of 25 is considered normal for CHL cells [13,12,14,19,26b].

Prior to routine use and as part of routine maintenance, cells should be cultured and metaphase preparations should be made and stained as described later. One hundred metaphases should be examined and the distribution of chromosome numbers should be plotted and recorded (e.g., using the distribution function in Excel) to establish the modal chromosome number. An appropriate form for recording the original results is shown here (Figure 7.2).

Note that all forms used to collect results should be appendices to SOPs and should include appropriate information in the footer, such as version date and complete electronic directory path.

If a departure from the established modal chromosome number or a more variable chromosome number is seen over time, then the cell line should be discarded and rederived. Importantly, the modal number is used to help define which metaphases are considered readable during routine testing, and a centromeric count can help distinguish structural aberrations from preparation artifacts.

Modal Chromosome Count Sheet

Exp. number:		Culture ID:		Microscope number:			
Cell number	Number of chromosomes	Cell number	Number of chromosomes	Cell number	Number of chromosomes	Cell number	Number of chromosomes
1		26		51		76	
2		27		52		77	
3		28		53		78	
4		29		54		79	
5		30		55		80	
6		31		56		81	
7		32		57		82	
8		33		58		83	
9		34		59		84	
10		35		60		85	
11		36		61		86	
12		37		62		87	
13		38		63		88	
14		39		64		89	
15		40		65		90	
16		41		66		91	
17		42		67		92	
18		43		68		93	
19		44		69		94	
20		45		70		95	
21		46		71		96	
22		47		72		97	
23		48		73		98	
24		49		74		99	
25		50		75		100	

Slide reader (init./date): _____

Distribution

No. of chromosomes												
No. of cells												

Modal No.:_____ Calculated by init./date:_____

Reviewed by (init./date): _____ Page 1 of 1

Figure 7.2
Modal chromosome number scoring sheet.

7.8.2 Mycoplasma

All cell lines used in genotoxicity should be checked for mycoplasmal infection on arrival and regularly afterwards, for example, on generation of each new batch of frozen stock. Mycoplasma infection will result in unhealthy cells that grow more slowly than usual, but low-level infections can be difficult to detect. Cells should be checked for infection following two subculturings in antibiotic free medium. Mycoplasma can then be tested for in a number of ways, including PCR (e.g., Venor® GeM Mycoplasma Detection Kit), ELISA, immunostaining, autoradiography, or by growth in selective microbiological media, although none of these methods is infallible. The most straightforward and practical technique for most laboratories involves fluorescent staining with Hoescht, which allows direct visual identification of the DNA of mycoplasma in the cytoplasm [50,51].

7.8.3 Cell-Cycle Time

In exponentially dividing cell lines, the doubling time is a reasonable approximation of the cell-cycle time. However, lymphocytes do not greatly increase in number during culture because only a proportion of them divide, and a proportion of the dividing cells dies during the culture period. Therefore, especially in the case of lymphocytes, cell-cycle time needs to be quantified using a more appropriate technique. The most widely used method for lymphocytes (which is also appropriate for other cultured cells) involves examining the degree and pattern of quenching in cells labeled with 5-bromo-2′-deoxyuridine (BUDR) and then stained with Hoechst 33258 [52–54]. In the usual continuous labeling technique, BUDR is added to actively dividing cells (at the 48-h time point in the case of HPBL) at a final concentration of 25 μM, cells are harvested 24 h later (2 h after colcemid addition), and metaphase preparations are made in the same way as in the standard chromosome aberration test (see later). After staining with Hoescht 33258, the metaphases show distinctive patterns of fluorescence that depend on how many S-phases they have passed through in the presence of BUDR [38,53]. Note that some laboratories use Giemsa in combination with Hoescht, in which case the various metaphase populations can be distinguished by light rather than fluorescence microscopy. The proportions of first, second, and third division metaphases are determined and used to calculate the proliferative index (PI), calculated as:

$$(1 \times \mathrm{Ml} + 2 \times \mathrm{M2} + 3 \times \mathrm{M3}) \div 100\%$$

Where M1, M2, and M3 indicate the % of metaphases in the first, second, and third division stages after incorporation of BUDR in the S-phase.

The average cell-cycle time is calculated as:

$$\text{Number of hours in BUDR} \div \text{PI}$$

Using this differential staining technique, laboratories report a cell-cycle time of approximately 14.5 h for HPBL [55]. When using other cell types, it is advisable to investigate a range of concentrations of BUDR to obtain optimal staining results and minimize cytostatic effects at excessive concentrations of BUDR.

7.9 Routine Testing

7.9.1 General Considerations

Studies of nonpharmaceuticals for regulatory submission generally should follow the guidance of the latest OECD test guideline 473, whereas testing of pharmaceuticals should also take into account ICH S2 [35]; all such studies should be performed in compliance with GLP when possible. Some additional guidance on test performance is given in the FDA Redbook 2000 [56]. As in other tests, any planned deviation from these practices should be described and scientifically justified in the protocol and report; the potential impact of any unplanned deviation should be addressed in the report.

7.9.2 Dose Regimens

The test substance is usually evaluated under three different exposure conditions (regimens):

- *Short exposure 0S9.* Cultures are treated with the test substance in the absence of an exogenous metabolic activation system for 3–6 h and then washed; culture is continued for a total period of 1.5 normal cell-cycle lengths after the initiation of treatment.
- *Short exposure +S9.* Cultures are treated with the test substance in the presence of an exogenous metabolic activation system for 3–6 h and then washed; culture is continued for a total period of 1.5 normal cell-cycle lengths after the initiation of treatment.
- *Long exposure 0S9.* Cultures are treated continuously with the test substance in the absence of an exogenous metabolic activation system for 1.5 normal cell-cycle lengths after the initiation of treatment.

The three dosing regimens are illustrated graphically in the example for HPBL here. In the case of CHO cells, the initial incubation period is routinely 20 h. The total incubation period of 21 h after dosing is based on the cell-cycle time of HPBL and CHO cells in our experiments (i.e., 1.5 cell-cycle times = 21 h for untreated cultures) and may need to be adjusted depending on the cycle time determined in your laboratory under standardized conditions.

Set	Incubate	Dose	Incubate	Wash	Incubate	Colcemid	Incubate	Harvest
1	48 h	Test formulation	4 h	Wash	15 h		2 h	
2	48 h	S9 + test formulation	4 h	Wash	15 h		2 h	
3	48 h	Test formulation	Incubate 19 h without washing				2 h	

The reader should be aware that some classes of chemical, such as nitrosamines, nucleoside analogues, and other cytostatic drugs, may require inclusion of an even longer exposure time in the absence of S9 for optimal detection of clastogenic activity, in which case a fourth exposure regime may need to be included in the study. It is most efficient to test all exposure regimes in parallel rather than using sequential testing.

7.9.3 Metabolic Activation System

The metabolic activation system used for routine testing consists of S9 mix containing induced rat liver S9 fraction at 5–30% v/v (typically 10% or 15%) and is usually diluted in culture medium to a final 1% or 2% in terms of S9 fraction. The S9 mix contains NADP and G6P cofactors but does not necessarily need to include the inorganic cofactors used in for the bacterial mutation test because these are automatically incorporated in the culture medium.

Most laboratories never experience problems with S9 fraction; however, if your laboratory finds that a particular batch of S9 inhibits mitotic activity (or causes other technical problems such as particulate), you should consider prequalifying a batch of S9 fraction sufficient to cover, for example, 1 year of routine testing.

7.9.4 Test Substance Considerations

The chromosome aberration test is used to evaluate a wide range of chemicals, impurities, and biological materials. Medical devices are usually extracted and tested as per ISO standards series 10993 (in particular, Part 3 Tests for genotoxicity, carcinogenicity, and reproductive toxicity and Part 12 sample preparation and reference materials; see https://www.iso.org for current details).

It is important to gather relevant physical and chemical information regarding the nature of the test substance in advance so that appropriate methods of sample preparation and testing are used. At the same time, the chemist involved in the project may be able to give you useful information about potential solvents. Despite the efforts of ICH, OECD, ISO, and others, there are national variations and preferences in test requirements, so it is useful to consider the final use of the test substance and which regulatory bodies will be involved

when designing the study. The reader should also consult the General and Formulation chapters of this book for additional guidance.

7.9.5 Vehicle Selection and Dose Volume

The test substance will normally require dissolving, diluting, or suspending in an appropriate liquid for dosing, taking into account chemical stability and compatibility of the vehicle with the test system. The same type of formulation will normally be used for the bacterial mutation test that is often performed in parallel to the chromosome test (refer to the Formulations chapter for more details). Aqueous solvents such as water and saline are preferred vehicles and can be used at levels up to approximately 20% v/v. If solubility is slightly lower, then it may be possible to dissolve the material directly in culture medium and dose the cells (at the high dose at least) by performing a change of medium. If the test substance has low aqueous solubility (i.e., less than 5 mg/mL), then organic solvents are often used at a maximal dose of 10 μL/mL (1% final concentration in culture). Relatively nontoxic miscible organic solvents include dimethyl sulfoxide, dimethylformamide, ethanol, methanol, propanone (acetone), and acetonitrile.

Appropriate volumes of relatively nontoxic solvents are not expected to affect the background % aberrant cells substantially; nevertheless, inclusion of an untreated control group is advisable if a novel solvent is used. When working with novel solvents, it may be appropriate to perform a preliminary compatibility test using the long exposure in the absence of S9 and the short exposure with S9 ahead of study. We suggest you evaluate a range of likely solvents during the validation phase of the assay to facilitate vehicle selection later.

When, for practical reasons, the dose volume is variable and the solvent is not expected to have a significant effect on the background percentage of aberrant cells, it is justifiable to use only the maximal dose volume for the concurrent vehicle control. A different solvent and dose volume may be used for the positive control articles and will normally be standardized for that laboratory. There is no need to include a separate vehicle control for the positive control; instead, comparison of results is normally made with the (test substance) vehicle control.

7.9.6 Dose Level Selection

Prior to finalizing the protocol and performing the chromosome aberration test, solubility testing will normally be required to decide or confirm an appropriate maximum dose level for the test (see later and Formulation chapter).

Based on these guidelines, the maximum concentration of test substance assessed in the test should be the limit of toxicity, solubility, or the standard upper limit of 10 mM, 2 mg/mL,

or 2 μL/mL (or 1 mM or 0.5 mg/mL in the case of pharmaceuticals), whichever is the lowest. Doses higher than the standard upper limit may be justifiable in some cases, such as when testing mixtures or when qualifying a pharmaceutical with a suspect impurity.

ICH defines the limit of toxicity as a reduction of approximately 50% in cell growth, however, OECD is apparently more restrictive, indicating that the high dose should cause inhibition of growth by 55±5%. The measures of toxicity indicated by OECD are also appropriate for pharmaceuticals and are based on reduction in the relative rate of growth of the treated cells compared with the negative control cells, such as MI in the case of lymphocytes and relative increase in cell count (RICC) or relative population doubling (RPD) in the case of cell lines (see Assessment of Toxicity section). In practice, these measures tend to vary as a result of experimental variation, so the ±5% limits mentioned by OECD should not be too strictly applied. In particular, MI can vary quite widely between cultures. Whatever the situation, apparent effects seen only at the limit of toxicity should be interpreted with caution.

In the case of nontoxic compounds with limited solubility in culture medium, the highest concentration analyzed should produce turbidity or precipitate at the end of the treatment period. An inverted microscope should be available to facilitate observation of precipitation. It may also be useful to treat a parallel set of mock cultures without cells in those cases where precipitate might be expected to be obscured by the cells and/or the S9. In cases where precipitation is observed, the lowest concentration showing precipitate should be the highest dose selected for detailed examination of aberrations because precipitate can be carried over with the cells during washing and other procedures and cause physical or chemical toxicity or interfere with cell spreading and staining. The guidelines do not provide guidance on testing of materials that have very low aqueous solubility (e.g., many polyaromatic hydrocarbons). In these cases, every reasonable effort should be made to expose the cells to solubilized material or, in the case of mixtures or environmental samples, extracts of the material. Then, the highest dose selected for examination should not be excessively toxic or interfere with the quality of the metaphase spreads.

We suggest a standard dose interval between each concentration of approximately 2. A smaller interval may be used when the test substance is suspected of having a steep toxicity curve and is often appropriate in the case of confirmatory testing. In the following example, the test substance showed reasonable solubility in the culture medium in a preliminary assessment and therefore could be dosed at the standard limit suggested by the guidelines. Note that in the case of a test substance with a low molecular weight (MW), the dose levels should be proportionately lower, i.e., for pharmaceuticals the highest dose should be reduced to 1 mM if MW is $<$500 daltons, and for nonpharmaceuticals the highest

dose should be reduced to 10 mM if MW is <200 daltons. However, ICH also indicates "For pharmaceuticals with unusually low molecular weight (e.g., less than 200) higher test concentrations should be considered," implying that the high dose of 0.2 mg/mL would be appropriate when the MW of a drug is <200 daltons. For compounds with solubilities below these limits, the high dose should show slight precipitation in the culture medium.

Suggested default standard study design: main test

Dose Level/ Treatment	Final Concentration, μg/mL[a]		Number of Replicates		
	ICH	OECD	Short Exposure 0S9	Short Exposure + S9	Long Exposure 0S9
Vehicle	0	0	2	2	2
1/Test substance	1	4	2	2	2
2/Test substance	2	8	2	2	2
3/Test substance	4	16	2	2	2
4/Test substance	8	32	2	2	2
5/Test substance	16	64	2	2	2
6/Test substance	32	128	2	2	2
7/Test substance	64	256	2	2	2
8/Test substance	128	512	2	2	2
9/Test substance	256	1024	2	2	2
10/Test substance	500	2000	2	2	2
1/Positive control	[b]	[b]	2	2	2
2/Positive control	[b]	[b]	2	2	2
3/Positive control	[b]	[b]	2	2	2

0S9 without S9.
+ S9 with S9.
[a]Dose levels should be proportionately lower if the test substance has a low molecular weight or if more than one of the dose levels mentioned is above the limit of solubility (see previous paragraph).
[b]Standard positive control chemicals and dose levels as determined during set-up and validation work. Three dose levels of each positive control are dosed and processed to slides later for each regimen, but only a single dose showing low to slightly toxicity is subjected to detailed examination for chromosome aberrations.

Based on the aforementioned study design, you would expect the study to consist of a total of:

$$14 \text{ formulations} \times 3 \text{ regimens} \times 2 \text{ replicate cultures} = 84 \text{ cultures}.$$

In addition, a few untreated control cultures should be initiated to act as potential replacements in the event of a technical error and to check the suitability of slide-dropping conditions later. Although this can be mentioned in the protocol, no results will be reported for these cultures.

In the unlikely event that results are not available for an adequate number of dose levels due to toxicity, a supplementary test will be necessary.

In the case of extracts of medical devices where no significant amount of material is expected to be extracted from the device, some laboratories test only the extract undiluted (i.e., a single dose level); however, this does not comply with the OECD requirement to test at least three dose levels. In the case of medical devices, normally two extracts are tested, one in a polar (aqueous) solvent and one in a nonpolar organic solvent, using appropriate solvent controls for comparative purposes.

7.9.7 Positive Controls

Positive controls are used to confirm the sensitivity of the test system and the effectiveness of the S9 mix; examples given in OECD TG473 are listed here:

S9 Conditions	Chemical	CAS Number
0	Methyl methanesulfonate	66-27-3
0	Mitomycin C	50-07-7
0	4-nitroquinoline *N*-oxide	56-57-5
0	Cytosine arabinoside	147-94-4
+	Benzo[a]pyrene[a]	50-32-8
+	Cyclophosphamide[b]	50-18-0

[a]We do not recommend use of benzo[a]pyrene as a positive control for HPBL because its low solubility in culture media can result in weak clastogenic effects, lack of a clear dose-response, and carry-over with the cells after washing. The aqueous solubility of this chemical is listed as 1.6 μg/L by PubChem.
[b]Note that cyclophosphamide (MW 261) is usually supplied and used in genotoxicity tests in the monohydrate form (CAS 6055-19-2, MW 279) and should be reported as such.

To account for variability in toxicity shifts between experiments, it is recommended that three dose levels should be tested, but only one should be selected for evaluation based on observed toxicity and/or preliminary examination of the slides.

Suggested routine positive controls and guidance dose levels are listed here:

Exposure	Cell Type	Compound	Abbreviation	Concentration, μg/mL	S9
Short and long	HPBL	Mitomycin C[a]	MMC	0.05–0.20	0
Short	HPBL	Cyclophosphamide	CP	4.0–8.0	+
Short	CHO	Mitomycin C[a]	MMC	0.05	0
Short	CHO	Cyclophosphamide	CP	1.5–4.5	+
Long	CHO	Mitomycin C	MMC	0.10	0

[a]OECD TG473 and ICH indicate that the direct genotoxin (mitomycin C) may be omitted for the short treatment if the cyclophosphamide treatment and the treatment in the presence of S9 are performed at the same time as the short treatment. This may be problematic if there is a technical issue with the +S9 regime so we do not recommend it.

7.10 Standard Test Procedures

Note that procedures, media, and reagents are performed/used at room temperature unless otherwise stated. The incubator should have a stainless steel tray containing water in the

bottom to maintain high humidity (important if multiwell cultures are used). Centrifugations are performed using a bench-top centrifuge; speeds and timing should be adequate to pellet the cells but are not considered critical, and the speeds given here are for guidance and may need adjusting depending on the particular rotor. The harvest times and the long exposure time indicated in the procedures are based on the cell-cycle time estimate for CHO and HPBL cells at Charles River Laboratories, Montreal, and should be adjusted according to cell-cycle determinations in your own laboratory. However, 21 h = 1.5 cell cycles is fairly typical for these cell types (see Henderson et al. [55] for example). Longer cell-cycle times may be indicative of suboptimal growth conditions.

7.10.1 Experimental Design Spreadsheet

The experimental design spreadsheet should be generated prior to the study from a standard template file with cultures numbered sequentially to specify the treatment conditions for each culture and who did what and when. The example in Table 7.1 includes the vehicle and positive controls and all dose levels of a single substance in one test regimen (i.e., short exposure in the absence of S9). The comment column allows documentation of incidental observations such as changes in medium color and precipitation at the various phases of culture processing. The sheet also includes appropriate space to document the staff involved and dates. The design can be separated into sections:

1. The first column and second column identify the treatment regimen
2. Culture number identification is provided in the third column
3. The next columns provide information on the dose number associated with the formulated material to be dosed, as well as the amount to be administered to each culture
4. The last columns are provided for comments and staff identification for procedure accountability
5. These steps are repeated for the two remaining dose regimens (short exposure in the presence of S9 and long exposure in the absence of S9). With a design of 10 dose levels of test substance, a total of $3 \times 28 = 84$ cultures would be needed for a complete experiment.

7.10.1.1 CHO cells: routine maintenance

CHO cells are grown using standard techniques for attached cell lines, such as described by Freshney [57]. All media including DPBS and trypsin should be warmed to room temperature before use. Approximately 6 days to 2 weeks prior to the planned day of dosing, rapidly thaw a frozen vial of cells ($1.0-2.0 \times 10^6$ cells/mL/vial in complete medium with 5% DMSO) in a 37°C water bath while agitating by hand. Lay a 75 cm^2 vented flask labeled with today's date and other appropriate details (e.g., cell type, passage

Table 7.1: Experimental design spreadsheet example

Regime	Culture No.		Material	Dose No.	Dose Volume µL	Final Concentration µg/mL	Comments and Observations			
							Before Dose	After Dosing	After Exposure	At Harvest
4 h 0S9	01	02	Water	0	50	–				
Set 1	11	12	X	1	50	1				
	21	22	X	2	50	2				
Prefix*	31	32	X	3	50	4				
1	41	42	X	4	50	8				
	51	52	X	5	50	16				
	61	62	X	6	50	32				
	71	72	X	7	50	64				
	81	82	X	8	50	128				
	91	92	X	9	50	256				
	101	102	X	10	50	500				
	111	112	MMC	M1	50	0.05				
	121	122	MMC	M2	50	0.10				
	131	132	MMC	M3	50	0.20				
Performed by (initial/date)										

Explanation: Prefix prior to the culture number indicates dose regime (set number): 1 indicates 4 h without S9 (4 h 0S9); 2 indicates 4 h with S9 (4 h + S9); and 3 indicates long exposure without S9. In this case the test substance has been given the code letter X. The final concentrations shown here are typical for a relatively high-molecular-weight pharmaceutical when not limited by solubility. In similar situations, a nonpharmaceutical would be dosed at levels four-times higher. Although this study design includes duplicate vehicle control cultures, inexperienced laboratories in particular (i.e., those with a limited historical control database) should consider inclusion of an untreated pair of cultures or quadruplicate control cultures to produce more reliable results and to build their own historical control database.

number, and density or split ratio) on its side and then transfer the contents of the thawed vial before gradually adding 12 mL F-12 complete while gently agitating. Incubate the culture under standard conditions (37°C in an atmosphere containing 5% v/v CO_2). Check the condition and degree of confluence of the cells regularly and before harvesting. The cells must be subcultured before approaching confluence: if a one-tenth split is used for subculturing (i.e., one-tenth of the cells at harvest are used to inoculate another 75-cm^2 flask), then the cells will multiply by a factor of approximately 10 over the course of 3 days and therefore will need further subculturing every 3 days. To harvest the cells, remove the supernatant medium from the flask completely using a pipette, rinse the cell monolayer very gently with 10 mL DPBS (add the buffer to the side of the flask), remove the DPBS completely, and then add 5 mL trypsin 0.25% in the same way. Leave the trypsin in contact with the cells for approximately 20 seconds, stand the flask upright to drain before removing the trypsin using a pipette. Incubate the culture for approximately 10 min until the cells appear rounded when viewed under the microscope. Knock the cells into suspension by tapping the flask gently against the side of the bench and then add 12 mL

F-12 complete before dissociating the cells by repeated aspiration using a 10 mL pipette ("rough pipetting"). Transfer the cell suspension to a sterile centrifuge tube, pellet the cells by centrifugation at 500 g for 5 min, discard the supernatant, knock the cells into suspension, resuspend them in 12 mL F-12 complete, and then rough pipette to disperse any clumps. At this point the cell density can be accurately quantified, if desired, and cells can be frozen for future use, as summarized in the next paragraph. Add an appropriate volume of cells to F-12 complete to achieve the desired split (e.g., 1.2 mL cell suspension is added to 10.8 mL medium in a 75-cm^2 flask). The new culture is labeled and incubated as before. At each subculture, the passage number is increased by one and an appropriate note is made in the cell maintenance log or raw study data, as appropriate.

To perform a live ("viable") cell count (i.e., to determine cell density), dilute 100 μL of cell suspension with 100 μL trypan blue 0.4%, mix, and load a hemocytometer with the diluted suspension before counting those cells that appear bright (i.e., exclude trypan blue). Note that a normal healthy culture will contain very few dead cells. To freeze CHO cells in suspension, determine the cell density of a freshly harvested culture after dilution in complete medium and adjust the density to 2×10^6 cell/mL by dilution in F-12 complete. Dilute the suspension 1:1 with freezing medium. Dispense the cell suspensions in 1 mL aliquots into prelabeled cryovials and freeze them slowly (at 1°C/min) to avoid large ice crystal formation. This can be achieved by placing the cells in a partly insulated polystyrene foam container (e.g., manufactured in-house or Mr. Frosty™ from Thermo Scientific) before transfer to a −70°C freezer or by using a special cell-freezing insert placed inside the neck of a liquid cell storer. After overnight freezing, the ampoules should be rapidly transferred (within 2 min) to canes for storage in the gaseous phase of the liquid nitrogen cell store. Note that if ampoules are stored in the liquid nitrogen phase, shrinkage of the seal can allow liquid nitrogen to enter the vial; some makes of ampoule seem much more prone to this than others. The depth of liquid nitrogen should be monitored continually, such as by using an electronic alarm system and by performing weekly checks using a wooden 1-meter rule or similar. The insulating insert in the lid of a liquid nitrogen cell store should be replaced if it shows signs of deterioration to minimize losses of liquid nitrogen.

7.10.2 CHO Cells: Test Procedures

Refer to the experimental design to determine the number of cultures required, remembering to include some extra to act as potential replacements and for counting and slide-making checks.

Day −4 (4 days prior to dosing). Grow a sufficient number of cells in flasks as described above (see Section 7.10.1.1). These flasks would normally be inoculated 3 days prior to initiation of cultures to be used in the study.

Day −1 (1 day prior to dosing). After incubation for 3 days, once the cells have grown to approximately 50% confluence, remove the flask(s) from the incubator and record the inoculation date, passage number, percentage of confluence, and any comments appearing in the study raw data file. To harvest the cells, remove the supernatant medium from the flask completely using a pipette, rinse the cell monolayer gently with 10 mL DPBS (add the buffer to the side of the flask), remove the DPBS completely, and discard it; then, add 5 mL trypsin 0.25% in the same way. Leave the trypsin in contact with the cells for approximately 20 s by leaving the flask flat on the work surface. Remove the trypsin from the flask using a pipette and then incubate the culture for 5−10 min until the cells appear rounded when viewed under the microscope. Knock the cells into suspension by tapping the flask gently against the side of the bench and add 12 mL F-12 complete before dissociating the cells by repeated aspiration using a 10 mL pipette (rough pipetting). Transfer the cell suspension to a sterile centrifuge tube, pellet the cells by centrifugation at 500 g (1000 rpm) for 5 min, discard the supernatant, knock the cells into suspension by flicking with the fingers, resuspend them in 12 mL F-12 complete, and then rough pipette to disperse any clumps before adding enough F-12 complete to bring the suspension to the same volume as was in the original cultures (i.e., number of cultures × 12 mL). Perform a live (viable) cell count by diluting 100 μL of cell suspension with 100 μL trypan blue 0.4%, mix, and load a hemocytometer with the diluted suspension before counting those cells that appear bright (i.e., live cells exclude trypan blue).

1. *Day 1 Initiation*
   a. Prepare a sufficient volume of cell suspension by diluting an appropriate volume of the aforementioned cell suspension with F-12 complete to give a final density of 0.08×10^6 cells/mL in a sterile bottle. While continually agitating the suspension to ensure a similar number of cells are dispensed to each flask, dispense 5 mL per flask into 25-cm^2 culture flasks that have been prelabeled in accordance with the experimental design sheet. You should have three sets of culture flasks plus a few spare flasks to act as potential replacements to measure cell density at the start of treatment and to act as procedural controls during slide-making.
   b. Incubate all cultures for 20 h. Note all incubations are at 37°C in a humid atmosphere containing 5% v/v CO_2.
2. *Day 0 Dosing*
   a. Remove the flasks from the incubator approximately 20 h after initiation and, using an inverted microscope, check that the cells appear healthy and have started to grow.
   b. Harvest the cells from one of the flasks to perform a live cell as described for Day −1. In this case, however, the final cell pellet should be suspended and mixed in 500 μL of F-12 complete before diluting 100 μL with an equal volume of trypan blue 0.4% and counting. The cell count should indicate a density of approximately 0.8×10^6 cells per flask (i.e., approximately double the density at initiation).

This number is important because it represents the density of live cells at the start of treatment and will be used in the calculation of toxicity later. The cells from this culture can be discarded after the count.

c. Remove 0.650 mL of medium from the flasks for S9 treatment and then add 0.650 mL of 15% S9 mix to them immediately prior to addition of the test formulation.
d. Add vehicle and test compounds to cultures as described in the experimental design sheet. Gently swirl each flask after dosing.
e. Check medium color and precipitation in all cultures.
f. Incubate all cultures as before.

3. *Day 0 Washing: Sets 1 and 2 only*
   a. After 4 h, remove Sets 1 and 2 cultures from the incubator and make a note of color and precipitation.
   b. Completely remove the medium from each culture and add 5 mL fresh F-12 complete to each and gently agitate to resuspend any precipitate.
   c. Completely remove the medium from each culture and add 5 mL fresh F-12 complete to each.
   d. Incubate the cultures for another 15 h.
4. *Colcemid*
   a. Remove Set 3 cultures (and any remaining spare cultures) after 19 h of continuous exposure to the test formulations. Sets 1 and 2 should be removed 15 h after the start of reincubation following washing. Add 50 μL of colcemid 10 μg/mL to all cultures to achieve a final concentration of 0.1 μg/mL and mix. Slightly loosen caps (if using unvented flasks) to facilitate gas exchange and immediately return the cultures to incubator for another 2 h.
5. *Harvest*
   a. Remove the cultures from the incubator after 2 h of incubation in the presence of colcemid.
   b. Record medium color and absence or presence of precipitation and its appearance. Record the appearance of the cells as a preliminary indication of toxicity. *Note that dead cells will often slough off into the supernatant medium.*
   c. Remove the medium from the flasks.
   d. Rinse cells once with 5 mL DPBS per flask. Remove and discard the DPBS.
   e. Add 1 mL of Trypsin 0.25% to the monolayer of cells in each flask.
   f. Incubate the flasks for 5−10 min.
   g. When the cells are rounded, knock each flask to detach the cells and then stand them in an upright position to allow the cells to drain to the bottom. Add 9 mL of F-12 complete to rinse the growth surface of each flask.
   h. Transfer the suspensions to prelabeled 15 mL centrifuge tubes.
   i. Pellet cells using a centrifuge at 500 g (1000 rpm) for 5 min.

j. Discard the supernatant and then flick the cells to resuspend them in the residual medium.
k. Resuspend each culture in 7 mL F-12 complete and then mix the suspension prior to sampling as described below.
l. To determine the limit of toxicity, remove a 1.0 mL sample from each tube and place into an appropriate container; then, use this sample to perform cell counts using a Coulter counter if available. Perform a live cell count for cultures from dose levels with a count of 45−55% of the vehicle control and at least two or three lower dose levels (i.e., those cultures that might be subject to detailed examination for aberrations later). To perform a live cell count, take 100 μL of the 1.0 mL sample and dilute it with an equal volume of trypan blue 0.4%; mix and load a hemocytometer with the diluted suspension before counting those cells that appear bright (i.e., exclude trypan blue). Record the density of live and dead cells for each to calculate percentage of viability and absolute density of live cells. Perform similar live counts on the vehicle control and at least one low or slightly toxic dose level of the positive control.
m. If a Coulter counter system is not available, then live cell counts should be performed using a hemocytometer as described in the previous paragraph. In this case, the dose(s) selected for counting should be selected based on the preliminary visual assessment of toxicity performed when the cultures were removed from the incubator. Again, live/dead cell counts should be performed for all cultures for which a detailed assessment of aberrations might be performed later.
n. Cultures that show severe toxic effects (all cells dead) do not need processing beyond this point and can be discarded. Otherwise, process each tube as indicated below.
o. Pellet cells using a centrifuge at 500 g (1000 rpm) for 5 min.
p. Discard the supernatant and flick the cells to resuspend them in the residual medium.
q. Add 1 mL Hypotonic solution to each tube and mix by gentle flicking.
r. Leave the tubes at room temperature for 10 min

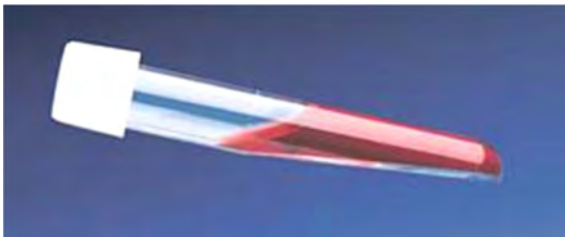
6. *Fixation*
   a. Add 1 mL Fix to the cells suspended in Hypotonic solution while gently agitating.
   b. Pellet the cells by centrifugation at 900 g (1500 rpm) for 5 min.
   c. Remove the supernatant and resuspend the cells in the residual solution by gentle flicking.
   d. Add 1 mL Fix to the suspended cells while mixing continuously.
   e. Leave tubes at room temperature for at least 30 min or overnight in the refrigerator.
   f. Pellet the cells by centrifugation at 900 g (1500 rpm) for 5 min.

g. Remove the supernatant and resuspend the cells in the residual Fix by gentle flicking.
h. Add 1 mL Fix to the suspended cells while mixing continuously.
i. Cells should be stored refrigerated at least overnight, but they may be stored for several months. If stored for an extended period, then check for evaporation and deterioration of the plastic tubes.
j. Pellet the cells by centrifugation at 900 g (1500 rpm) for 5 min.
k. Carefully remove the supernatant to avoid disturbing the pellet and resuspend the cells in the residual Fix by gentle flicking.
l. Add 1 mL Fix to each tube. At this point cells can be stored refrigerated for several months, if necessary.

7. *Slide preparation*
   a. Select appropriate cultures for slide preparation and potential detailed examination based on live cell counts. In this case, slides will need to be prepared from each of the three sets of cultures for the vehicle control, at least one nontoxic or slightly toxic dose level of the positive control and at least three dose levels of the test substance. If in doubt about the level of toxicity, it is best to prepare slides from one or two additional dose levels for potential examination. *Only those cultures that are needed for examination need to be processed to slides as detailed here.* Fixed preparations from the remaining cultures should be returned to the refrigerator in the event they are needed for examination later (e.g., to clarify borderline results).
   b. Prelabel the microscope slides with a unique identifier (e.g., experiment or study number with culture number underneath). In the case of frosted slides, this is best performed using a 2H pencil.
   c. Pellet the cells by centrifugation at 900 g (1500 rpm) for 5 min.
   d. Remove the supernatant carefully to avoid disturbing the pellet.
   e. Add at least two drops of Fix to each tube. If necessary, add additional drops of Fix to adjust the density as judged by eye; the suspension should appear slightly cloudy.
   f. Put a drop of cell suspension from a spare culture onto a clean slide using a fresh Pasteur pipette.
   g. Allow the slide(s) to air-dry and then check the degree of metaphase spreading using a microscope (phase contrast, if available). If the degree of metaphase spreading is appropriate, then continue with slide preparation until enough slides are obtained per culture to ensure an adequate number of readable metaphases (at least two slides per culture should be made). If necessary, metaphase spreading can be aided using a humid atmosphere (e.g., expose slides to vapor from a 70°C water bath during the dropping procedure).
   h. Once slide preparation is complete, add 5 mL Fix to all tubes and store refrigerated in case additional slides need to be made later (e.g., in case the required number of readable metaphases is not available from any of the original slides).

7.10.3 HPBL Test Procedures

HPBL are normally grown as 5 mL cultures in prelabeled Nunc polystyrene flat-sided culture tubes incubated with the flat side down and placed in a rack. Refer to the experimental design to determine the number of cultures required, remembering to include some extra to act as potential replacements and for slide-making checks. You will need at least 0.5 mL blood per culture plus a small amount to allow for waste. All incubations described here are at 37°C in a humid atmosphere containing 5% CO_2.



HPBL 5 mL culture. *Photograph courtesy of Fisher Scientific.*

HPBL can also be successfully grown in 1 mL cultures, but this may limit the number of metaphases available for analysis later; therefore, this method is more suited to screening studies when the test substance is in limited supply. In this case, dispense 1.0 mL aliquots of the blood-medium-PHA mixture described below into clear, flat-bottom, 4 mL glass vials with screw caps for treatment in the absence of S9 and dispense 0.8 mL aliquots into vials for treatment in the presence of S9.

Day −2 (2 days prior to dosing). Initiate cultures 2 days prior to dosing using sodium-heparinized fresh blood samples obtained by venipuncture (usually from the median cubital vein on the inside of the elbow) from healthy (normally male) human volunteers; donors should be nonsmoking individuals of approximately 18−35 years of age with no known illness, recent viral infection, or recent exposure to drugs or radiation that might increase the background incidence of chromosomal aberrations. OECD TG473 indicates that there is a slight trend for increased background rates of aberration with age, which is more marked in females than males. The blood can be taken from a single donor or pooled (after dilution in culture medium) from more than one donor if a larger volume is needed. The number of donors used should be indicated in the eventual study report. Some individual donors seem to provide blood that results in higher MI cultures than others; subsequently, these donors are generally preferred.

Dilute the whole blood with RPMI complete (1 volume of blood per 9 mL medium) in a sufficiently large sterile bottle and add 1 mL PHA M solution per 50 mL of diluted blood. For reference purposes, white cell counts can be made at this point or later during culture

process by dilution of 100 μL of suspension with an equal volume of white cell counting fluid (WCF; consists of 1 mg crystal violet per 1 mL of aqueous 1.5% v/v acetic acid), which fixes and stains white cells while lysing red cells. White cells can then be counted with the aid of a hemocytometer.

Note the instructions that follow apply to 5 mL cultures; volumes of additions described here should be adjusted proportionately for 1 mL cultures (i.e., by a factor of one-fifth). We routinely include a 24-well plate containing 1 mL RPMI complete per well that is dosed and incubated in parallel with the other cultures to facilitate checks for precipitation at the start and end of each treatment period because it can be very difficult to see this in the presence of cells in the standard culture tubes.

1. *Day −2 Initiation.* Prelabel the culture tubes as per the experimental design. Dispense aliquots of the blood-medium-PHA mixture into each flat-sided culture tube while stirring the mixture regularly. Divide cultures into three sets (one for each treatment regimen) plus a 24-well plate as follows:
   a. Set 1: 5 mL/tube
   b. Set 2: 4 mL/tube
   c. Set 3: 5 mL/tube
   d. 24-well plate: 1 mL medium (without cells) per well
   e. Spare cultures: 5 mL/tube, not dosed with formulation (unless needed as replacements) but dosed with colcemid and harvested alongside Set 3 to act as a procedural control at the slide preparation stage.

   Incubate all cultures with the flat side down in racks for 48 h.
2. *Day 0 Dosing.* Remove the cultures from the incubator without disturbing them 48 ± 0.5 h after initiation.
   a. Add 1 mL of S9 mix to Set 2 cultures.
   b. Add vehicle and test compounds to cultures in accordance with the experimental design sheet. Mix the cultures well after treatment.
   c. Check medium color and precipitation in all cultures. Check the 24-well plate under a microscope for precipitate.
   d. Incubate all cultures as before.
3. *Washing.* After 4 h, remove the 24-well plate and Sets 1 and 2 from the incubator and make a note of color and precipitation.
   a. Return the 24-well plate to the incubator.
   b. Pellet the cells in culture Sets 1 and 2 by centrifugation at 500 g (1000 rpm) for 5 min.
   c. Discard the supernatant and flick the tube to resuspend the cells in the residual medium.
   d. Resuspend each culture in 5 mL fresh RPMI complete (without PHA) and mix. Slightly loosen tube caps to allow gas exchange.
   e. Incubate the cultures for another 15 h (±30 min).

4. *Day 1. Colcemid.* Remove Set 3 cultures after 19 h of continuous exposure to the test formulations. Sets 1 and 2 should be removed 15 h after the start of reincubation after washing. Add 50 μL colcemid 10 μg/mL to each culture to achieve a final concentration of 0.1 μg/mL and mix. Slightly loosen caps to allow gas exchange and immediately return the cultures to the incubator for another 2 h.
5. *Day 1 Harvest.* After 2 h of incubation in the presence of colcemid:
   a. Remove the cultures from the incubator.
   b. Check medium color in Sets 1, 2, and 3, and check precipitation in the 24-well plate using an inverted microscope. Discard the multiwell plate after recording observations.
   c. Pellet the cells by centrifugation at 500 g (1000 rpm) for 5 min.
   d. Remove the supernatant and resuspend the cells in residual supernatant by flicking (striking the side of the tubes with the fingers).
   e. Add 1 mL Hypotonic solution to each tube and mix by gentle flicking; then, add an additional 4 mL Hypotonic solution and mix gently.
   f. Put the tubes in a 37°C water bath for 10 min
6. *Fixation.* Add 1 mL Fix to the cells suspended in Hypotonic solution and mix.
   a. Pellet the cells by centrifugation at 900 g (1500 rpm) for 5 min. Note that after fixation, the cell pellet will be very small because any remaining erythrocytes will have lysed (the supernatant will have a brown coloration).
   b. Remove the supernatant and resuspend the cells in the residual solution by gentle flicking.
   c. Add 5 mL Fix to the suspended cells while mixing continuously.
   d. Leave tubes at room temperature for at least 30 min or overnight in the refrigerator.
   e. Pellet the cells by centrifugation at 900 g (1500 rpm) for 5 min.
   f. Remove the supernatant and resuspend the cells in the residual Fix by gentle flicking.
   g. Add 5 mL Fix to the suspended cells while mixing continuously.
   h. Cells should be stored refrigerated at least overnight, but they may be stored for several months. If stored for an extended period, then check for evaporation and deterioration of the plastic tubes.
   i. Pellet the cells by centrifugation at 900 g (1500 rpm) for 5 min.
   j. Carefully remove the supernatant to avoid disturbing the pellet and resuspend the cells in the residual Fix by gentle flicking.
   k. Add 5 mL Fix to each tube. Cells can be stored refrigerated for several months, if necessary.
7. *Slide preparation.* Prelabel the microscope slides with a unique identifier (e.g., experiment or study number with culture number underneath). In the case of frosted slides, this is best done using a 2H pencil.

a. Pellet the cells by centrifugation at 900 g (1500 rpm) for 5 min.
b. Remove the supernatant carefully to avoid disturbing the pellet and place the tubes upside down in a rack for at least 2 min.
c. Add at least two drops of Fix to each tube. If necessary, add additional drops of Fix to adjust the density as judged by eye; the suspension should appear slightly cloudy.
d. Put a drop of cell suspension from a spare culture onto a clean slide using a clean Pasteur pipette.
e. Allow the slide(s) to dry and then check the degree of metaphase spreading using a microscope (phase contrast, if available). If the degree of metaphase spreading is appropriate, then continue with slide-making until enough slides are obtained per culture to ensure an adequate number of readable metaphases (at least two slides per culture should be made). If necessary, metaphase spreading can be aided using a humid atmosphere (e.g., slide exposure to hot water vapors from a 70°C water bath).
f. Once slide preparation is complete, add 5 mL Fix to all tubes and store refrigerated in case additional slides need to be made later, for example, in case the required number of readable metaphases is not available from any of the original slides.

7.10.4 Slide Staining: All Cell Types

1. Place all the slides in clean stainless steel slide racks.
2. Within 1−2 h of staining, dilute neat Giemsa (Gurr's Improved R66) with 9 parts purified water and filter it through cotton wool (absorbent cotton), or, when using a Büchner filtration system, through Whatman no. 1 filter paper.
3. Pour the filtered dilute stain into a staining trough and, immediately prior to use, remove any oxide film from the surface using paper tissue.
4. Rinse the slides in three changes of purified water for at least 1 min/change.
5. Stain all the slides for 15 min in Giemsa 10% staining solution. Remove the film at the surface of the stain before removing slides.
6. Rinse the slides in one change of purified water (in-house) for at least 1 min.
7. Wash the slides in running tap water for 2 min.
8. Rinse the slides in one change of purified water (in-house) for at least 1 min.
9. Drain the slides and allow them to air-dry.
10. Working in a fume hood, mount all the stained slides permanently with 50 mm coverslips using nonaqueous mountant (e.g., Cytoseal, DPX, Permount). There is no need to clear the slides in xylene prior to mounting.
11. Allow the mountant to harden at least overnight. The slides can be transferred to a warm cabinet at approximately 50°C at this point to accelerate further hardening of the mountant.
12. Slides should be subjected to a preliminary microscopic examination to determine which dose levels from each regimen will be examined for MI.

7.10.5 Selection of Slides for Detailed Examination

To ensure that appropriate cultures are chosen for detailed examination and results are produced for vehicle and positive controls, and that at least three dose levels of the test substance are examined for each dose regimen without having to examine an unnecessary number of slides, we recommend you use the following procedures.

7.10.5.1 Cell lines

Estimates of toxicity are based primarily on reductions in the expected increase in live cell numbers compared to the concurrent vehicle control over the period between dosing (start) and cell harvest (final). In accordance with OECD guidelines for the chromosome aberration test [36] and the closely related *in vitro* micronucleus test [37], either of two formulae can be used to calculate relevant values that are termed RICCs and RPD. These are calculated as:

$$RICC\ (\%) = [(\text{Increase in number of cells in treated cultures (final} - \text{starting)}) \div (\text{Increase in number of cells in control cultures (final} - \text{starting)})] \times 100\%$$

$$RPD\ (\%) = (\text{No. of Population doublings in treated cultures}) \div (\text{No. of Population doublings in control cultures}) \times 100$$

where:

$$\text{Population Doubling} = [\log(\text{Post} - \text{treatment cell number} \div \text{Initial cell number})] \div \log 2$$

Although OECD is rather strict in its definition that, in the case of toxicity, the highest dose examined should cause a $55 \pm 5\%$ reduction in one of these, the two calculations will yield somewhat different results, so you may find it convenient to report both. Note that OECD indicates that RPD may not be appropriate for prolonged exposures (greater than the standard 1.5 cell cycles).

For each dosing regimen, the highest test substance dose chosen for detailed examination should show moderate toxicity (i.e., approximately 55 [OECD G473] or 50% [ICH S2] reduction in RICC or RPD). In that case, the two adjacent lower doses should also be selected for analysis unless the lowest dose also shows toxicity, in which case the lowest dose examined should be the highest dose not showing obvious toxic effects.

In the absence of toxicity (or any severe reduction in the density of mitotic figures), the highest dose chosen for each treatment regimen should be the lowest precipitating concentration or, in the absence of precipitate, the highest dose level tested. The adjacent two lower dose levels should also be selected for detailed examination.

Slides from all vehicle control slides should be selected for potential analysis. One dose level of the positive control from each regimen should also be selected for examination that shows no or a slight reduction in RICC or RPD. This dose should cause a moderate, but not immediately obvious, increase in the incidence of aberrant cells.

7.10.5.2 HPBL selection of slides for provisional detailed examination

For HPBL, although the MI is later determined upon slide reading, slides for encoding and examination must be chosen prior to coding by preliminary screening under the microscope. When toxicity is seen, the highest test substance dose chosen for detailed examination should show approximately 50% reduction in the MI when compared to the vehicle. In that case, one dose higher and two doses lower that that target dose should be selected to ensure the appropriate range of toxicity is covered and should include one nontoxic dose. One dose level of the positive control should also be selected for examination that does not cause a substantial reduction in the density of mitotic cells. Slides from all vehicle control slides should be selected for potential analysis.

In the absence of any substantial reduction in the density of mitotic figures, the highest dose chosen for each treatment regimen should be the lowest precipitating concentration or, in the absence of precipitate, the highest dose level tested. The adjacent two lower dose levels should also be selected for detailed examination.

7.10.6 Slide Coding

Slides are randomized and encoded by someone other than the slide reader to minimize potential operator bias. Slide code numbers can be generated using the random function in Excel and then printed onto adhesive labels. The labels should obscure the culture number but not the experiment/study number. Each slide from the same culture should be given the same code number. As the slides are read, the reader should use consecutive letters to indicate on the label and in the study records which results were obtained from which replicate slides to facilitate review. Although a label could also be placed or wrapped around the back of the slide, we do not recommend that because it makes focusing more laborious.

7.10.7 Preliminary Slide Reading

In the case of HPBL, the MI is determined by examination of at least 500 cells per culture. In this case, the following totals are scored using bench-top tally counters:

- number of nuclei (including mitoses)
- number of apparently diploid metaphases (mitoses)
- number of endoreduplicated metaphases
- number of polyploid metaphases

- number or comment on the proportion of metaphases that might not be readable due to apparent test substance effects (e.g., centromeric disruption)
- comments on slide quality that might prohibit reading, such as if the total number of readable metaphases on the slide appears low (likely test substance effect) or if there is a large number of free chromosomes (preparation artifact)

The MI is calculated as the total number of metaphases÷number of nuclei (including mitotic nuclei).

Note that lymphocyte cultures contain a proportion of dead and degrading cells. To help standardize assessment of MI between different slide readers, small pyknotic (condensed and structureless) or faintly stained nuclei should not be included in the count of normal nuclei.

The responsible scientist should review these results before deciding which slides need to be subjected to detailed examination for chromosome aberration. At this point, it may be appropriate to remove or replace some dose levels in each treatment regimen. Occasionally, the test substance will affect the quality of the metaphase spreads or reduce the absolute number of readable metaphases without causing a substantial reduction in MI. In this case, the highest dose level subjected for detailed analysis may need to be lowered accordingly; detailed and appropriate justification for this course of action should be noted in the raw data by the study director and presented in the report, with representative photographic evidence if possible.

7.10.8 Slide Scoring

7.10.8.1 Basics

To produce reliable results, the slide reader should understand the basics of chromosome morphology and how aberrations are formed. Structural aberrations fall into two main categories:

- breaks (including deletions) result from double-strand breaks (DSB) in the DNA that are not repaired
- exchanges result from two or more DSBs with inappropriate rejoining/repair of the "sticky ends" within a single chromosome ("intrachanges") or between chromosomes ("interchanges")

The majority of clastogenic chemicals cause single-strand lesions that, during the course of DNA repair, can be converted to a DSB. If the DSB persist through the S phase, then it will be replicated and becomes evident as a chromatid break at metaphase [1]. In turn, if the chromatid break is not repaired, then it can be replicated, resulting in formation of a chromosome break. However, chromatid lesions are the major type of chemically induced damage seen using the standard sampling time of 1.5 cell cycles because they become

evident at the first metaphase following induction [58]. Radiation and a few radiation-like chemicals can cause double-strand breakage directly; if this occurs after the S phase, then it will lead to chromatid lesions (breaks and exchanges); however, if it occurs prior to the S phase and is not repaired, then it will lead to chromosomal lesions involving both sister chromatids. Certain types of radiation-induced chromosome exchanges (notably dicentrics and centric rings) are accompanied by a paired fragment; in the case of chemicals, these derived aberrations are usually seen at the second metaphase after induction of damage and are not usually accompanied by fragments because acentric fragments tend to be lost during the first anaphase.

Gaps are achromatic lesions smaller than the width of one chromatid with minimum misalignment of the chromatid(s). Gaps are recorded but not scored as structural aberrations because they do not necessarily involve chromosome breakage. Note that identification criteria for gaps can vary between laboratories and countries. In addition, the width of the chromatids will depend on the degree of condensation, whereas not all chromatids have an equally clear outline. So, in a few cases, lesions classified as gaps may, in fact, be true breaks or even intrachanges.

Numerical aberrations (endoreduplication and polyploidy), if observed, are recorded and reported but are not necessarily indicative of genotoxicity. Apparent losses (in particular) or gains of one or two chromosomes in most metaphase figures are usually an artifact of slide preparation and therefore are not usually recorded or reported (i.e., the standard chromosome aberration test is not generally considered appropriate for detection of aneugenic events other than polyploidy).

Chemicals may have other observable effects on chromosomes (e.g., disruption of the centromere resulting in partial dissociation of the sister chromatids), which can be noted and reported as incidental observations. Although these may result in cytostatic or cytotoxic effects, they are not considered indicative of genotoxicity.

7.10.8.2 Understanding the normal karyotype

It is important to understand the structure of the individual chromosomes to facilitate identification of aberrations (e.g., chromosome breaks) and to avoid misclassification (e.g., secondary constrictions can appear similar to chromosome gaps); associations (attraction) of chromosomes via satellite bodies can give chromosome arrangements that appear similar to chromosome exchanges.

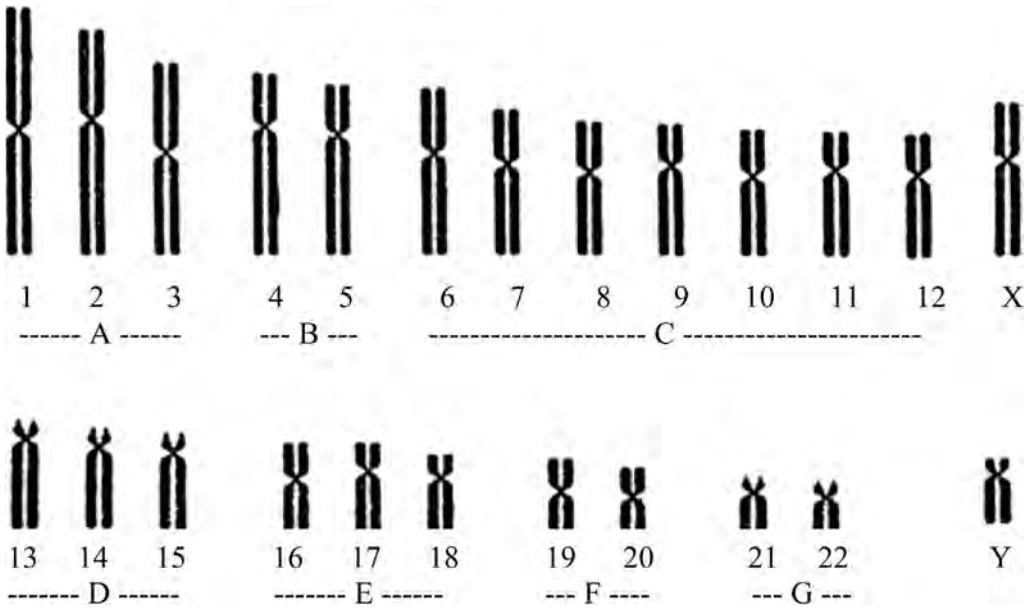
At metaphase, each pair of chromatids is joined by a constriction point called the centromere, which divides the chromosome into two arms. The short arm of the chromosome is termed the p (petite) arm and the longer arm is referred to as the q arm. The relative length of the chromosome and the ratio of the length of the p:q arms help identify the chromosome or the group of chromosomes.

- Metacentric chromosomes have the centromere near the center of the chromosome.
- Submetacentric means that the centromere is slightly off-center.
- Acrocentric means that the centromere is distant from the center.
- Telocentric means that the centromere is near one end of the chromosome.

The following descriptions refer primarily to diploid human metaphases (e.g., HPBL), but each laboratory should establish similar details (i.e., a chromosome map/karyotype) for any cell lines they use in the chromosome aberration test.

Diploid human cells contain 22 pairs of the autosomal chromosomes and a pair of sex chromosomes (XX for females and XY for males). Chromosomes are primarily identified and classified by size, with number 1 being the largest and chromosomes 22 and 21 being the smallest (they are approximately one-fourth the size of chromosome 1). The chromosomes are further classified into groups A to G:

- Group A (1−3): Large metacentrics; individually identifiable by size and p/q ratio
- Group B (4 and 5): Large submetacentrics
- Group C (6−12 and X): Medium-sized metacentrics and submetacentrics
- Group D (13−15): Medium-sized acrocentrics with satellites
- Group E (16−18): Shorter metacentric/submetacentrics
- Group F (19−20): Short metacentrics
- Group G (21, 22, and Y): Short acrocentrics with satellites on 21 and 22.



Some chromosomes often have associated morphological characteristics:

- Chromosomes 1, 9, and 16 secondary constrictions
- D and G group chromosomes satellite associations
- X and Y loss of centromeric activity
- Y chromosome is often slightly distorted

The slide reader needs to aware of these features (particularly secondary constrictions and satellite associations) to avoid misidentification of aberrations.

7.10.8.3 Routine scoring

If microscopes are equipped with a vernier scale stage, then slides should be placed on the stage in a standard orientation each time as defined in the SOP (e.g., label to the left).

The coded slides should be methodically scanned at medium power (e.g., 16× objective). Metaphase spreads that are unbroken and show good morphology are subjected to detailed analysis. Under high power (100× oil-immersion objective), the chromosomes should be well defined and should not be in an early C-anaphase state with completely separated chromatids. To avoid the analysis of cells with random chromosome loss due to preparation artifacts, only cells with the modal (cell lines) or diploid number of centromeres ± 2 in a single stage of condensation should be scored. During counting of the chromosomes, any structural aberrations or gaps should be recorded. The vernier reading of aberrant metaphases should be recorded against the aberration details. Especially during training, it can greatly facilitate review of results and correction of any misidentification if the slide reader draws a simple diagram for each recorded aberrant metaphase, indicating the approximate location within the cell of the lesion together with a stick drawing of the lesion.

Readable metaphases are identified by the following criteria:

- chromosome/centromere number within ± 2 of diploid (44−48 for HPBL) or modal number (for cell lines) in a single stage of condensation
- well-spread with minimal overlap of chromosomes and chromosome arms
- chromatids separate with centromere intact
- structure of chromosomes clear and well-defined

If, for any reason, the required total of 150 readable metaphases is not obtained, then additional slides should be prepared and examined from the reserved fixed material from that culture. Sometimes, due to technical problems with slides from a particular culture, it may be necessary to read additional metaphases from the other duplicate culture to reach the desired total of 300 readable metaphases per experimental point. In that case, this should be mentioned and justified in the report.

7.10.8.4 Classification

Metaphases are classified into the three following categories.

1. Normal metaphase (may have chromosome or chromatid gaps)
2. Those with structural aberrations
3. Numerical aberrations (polyploid, endoreduplicated, or hyperdiploid cells); these cells are not included in the readable metaphase totals

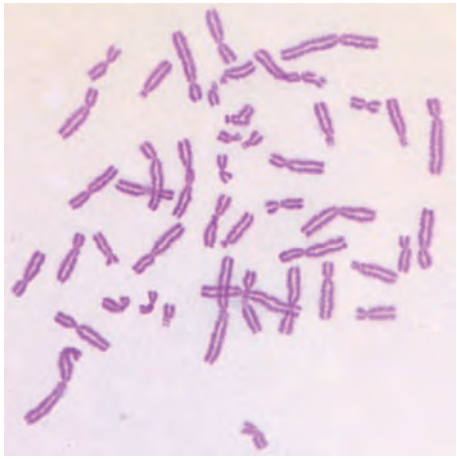
The main purpose of slide examination is to determine the proportion of metaphases with structural aberrations. A total of 300 readable metaphases (usually 150 per each of two cultures) per experimental point is examined for the presence of chromosome aberrations. Scoring less than this (e.g., when a clear positive response is evident) should be justified in the report and, ideally, the protocol.

The International System for Chromosome Aberration Nomenclature [59] is used to help classify structural aberrations into three main groups:

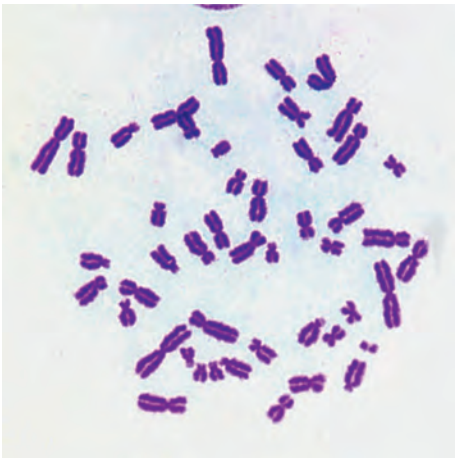
1. Chromosome
   a. breaks/deletions
   b. exchanges
2. Chromatid
   a. breaks/deletions
   b. exchanges
3. Other (complex damage)
   a. multiple (multiple aberrations in a single cell, e.g., >5)
   b. pulverized chromosome
   c. pulverized metaphase where some or all the chromosome is pulverized

The chromatid and chromosome categories are divided into two subcategories: breaks (result of a single DNA break) and exchanges (result of two or more breaks with inappropriate rejoining). These subcategories can be further subdivided, but that does not seem to provide any useful additional information in the case of routine testing. Representative examples of lesions in human lymphocytes are shown in Figure 7.3.

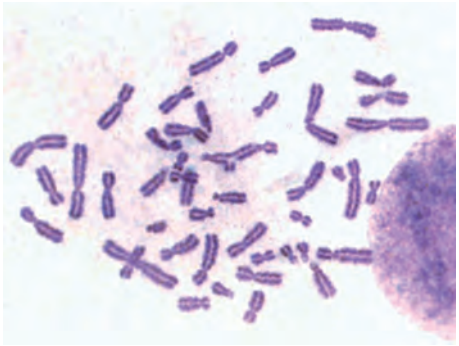
Although much of the theory of how the various aberrations are formed was developed early during the development of the test, recent technical advances including the use of specific chromosome paints have helped confirm the details [1]. An excellent review of the mode of formation of chromosome lesions, their identification, and artifacts that might be misinterpreted as aberrations was presented by Natalie Danford in 2012 [58], whose company offers training and outsourced slide reading for cytogenetic toxicology tests (see http://www.microptic.com/).



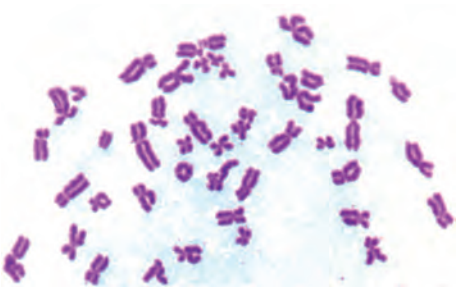
Chromatid break (and chromatid exchange)



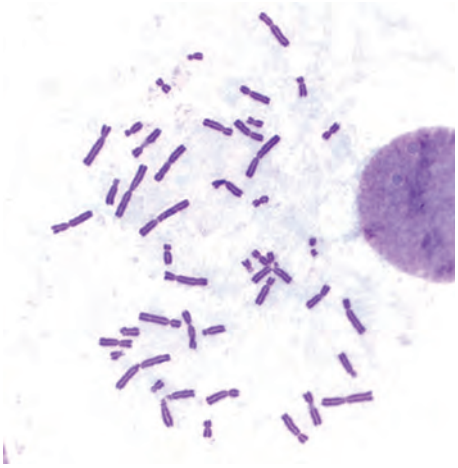
Chromosome break



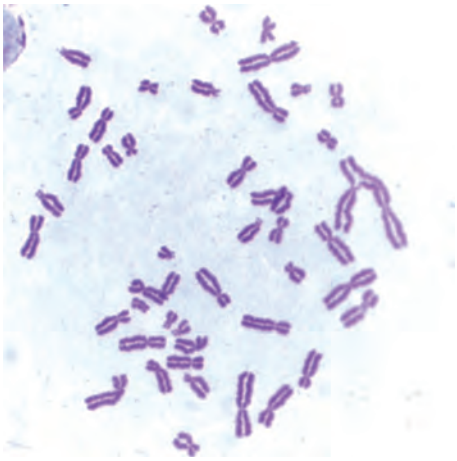
Chromatid exchange



Chromosome ring

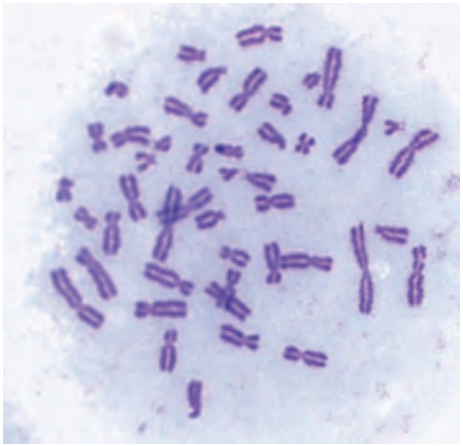


Chromatid exchange

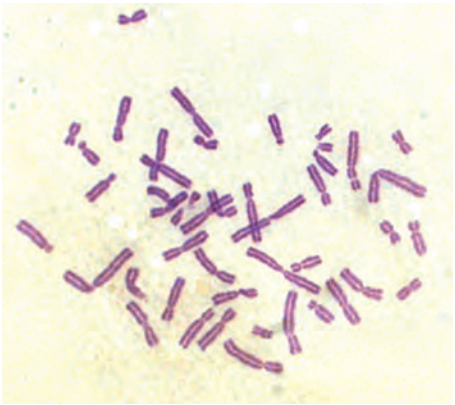


Chromatid exchange

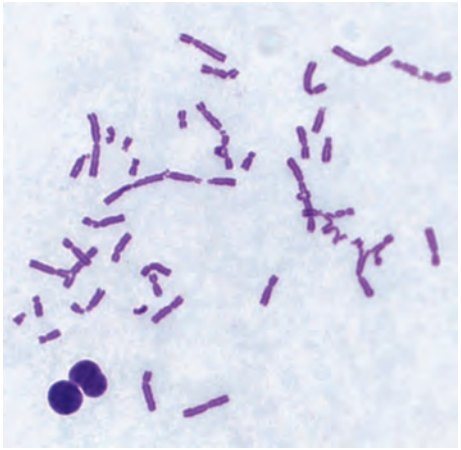
Figure 7.3
Structural aberrations and other lesions in human lymphocytes.



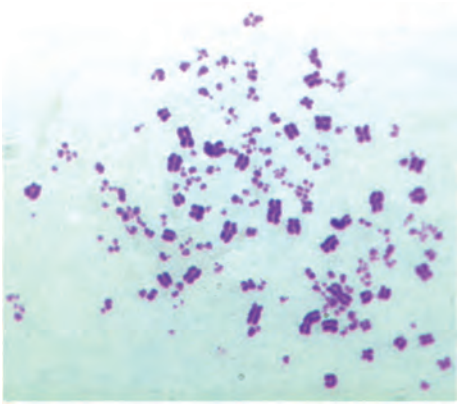
Chromatid gap



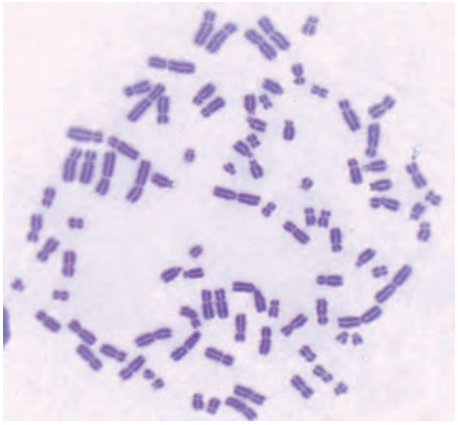
Chromosome gap



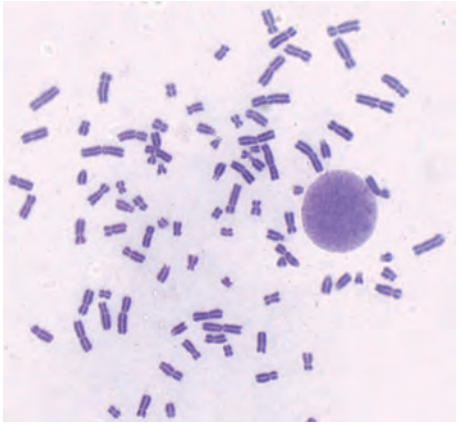
Multiple aberrations



Pulverized

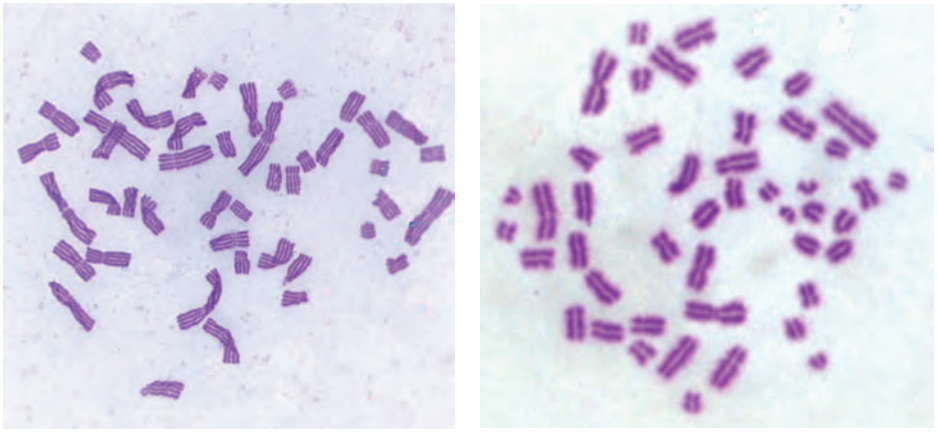


Polyploid



Polyploid

Figure 7.3
(Continued).



Endoreduplication Centromeric disruption

Figure 7.3
(Continued).

Cytogenetic analysis has a subjective component, with even highly experienced analysts differing in their interpretation of the same cell. However, there are a number of instances in which misinterpretation can occur, most commonly resulting in a normal configuration being scored as aberrant, thus indicating the importance of recognizing secondary constrictions, satellites and satellite associations, and overlapping chromatids. There are also easily missed aberrations. Analysts need to be aware of these potential situations. Unfortunately, there is no substitute for experience.

Following is a list of potential misinterpretations analysts should be aware of:

Cause	Confused With
Crossing-over of sister chromatids	Dicentric
Satellite association: two chromosomes attracted by satellite regions	Dicentric or exchange
Secondary constriction	Dicentric
Secondary constriction	Gap
Chromosomes overlapping near centromeres	Exchange (quadriradial)
Chromosome twisting and overlapping	Chromosome ring

Note that in the case of diploid cells, counting the centromeres can be a useful way of distinguishing artifacts from true lesions. Note that all these true lesions (with the exception of gaps) are relatively rare exchange events.

An example of a scoring sheet is illustrated in Figure 7.4. In this case, the standardized abbreviations for aberrations largely coincide with or are a simplification of those used by the ISCN.

Study No.(s):	**CHROMOSOME ABERRATION DATA FORM**

Slide code: Page ___ of ____ Microscope ID: Init./date:

No. of readable metaphases analysed:

Slide read	A	B	C	D	E	F	G	H
Metaphase No.	to	to	to	to	to	to	To	To

Details of aberrant metaphases *Read 200 readable metaphases per slide code unless indicated by SD.*

Metaphase No.	Vernier reading	No. of chromo-somes	Chromatid-type		Chromosome-type		Others			Gaps	
			ctb	cte	csb	cse	Mult	pvz cs	pvz cell	ctg	csg
Total, all slides this code											
Total aberrant cells all slides this code No., exclude cells with only gaps & numerical aberrations											

Ctb chromatid break cse chromosome exchange pvz cell pulverized cell

cte chromatid exchange mult >5 aberrations ctg chromatid gap

csb chromosome break pvz cs pulverised chromosome csg chromosome gap

Numerical aberrations or potential evidence of cell cycle disruption & Total number seen

	Slide replicate and Vernier reading (first 10 observed)										Total
Endoreduplication											
Polyploidy											
Centromeric disruption											
Other _____											

Comments by slide reader:

Reviewed by/date:_____

Figure 7.4
Chromosome aberration scoring sheet example.

7.11 Interpretation of Results

7.11.1 Evaluation of Toxicity

Toxicity is expressed in terms of percentage of inhibition of cell division, such as reduction of RICC or RPD in the case of cell lines and reduction in MI relative to the concurrent control for lymphocytes. Where substantial toxicity is seen (e.g., >60%) any aberration values obtained (if any) would not normally be reported or, if used to support any other findings, would be reported in parentheses.

7.11.2 Validity of the Study

For an assay to be considered valid, the vehicle/negative control incidences of metaphases showing structural chromosome aberrations should lie within or close to the historical control range, whereas the positive controls should produce a statistically significant (see next section) and substantial increase in the incidence of aberrant cells with values above the 95% tolerance limits of the laboratory historical negative/vehicle control range. The dose level reported for the positive control in each regimen should not have caused excessive toxicity (>60%).

Valid results should have been obtained for at least three dose levels of the test substance for each treatment regimen. The high dose should be justifiable in terms of the standard limit, toxicity, precipitate, or obvious effect on the quality or absolute number of metaphases, whereas the lowest dose examined should show little or no toxicity. When valid results have not been obtained for a particular regimen, it would normally be appropriate to repeat that part of the experiment; in which case, the original results do not need to be reported in detail. For repeat tests, the number of dose levels can normally be reduced based on results obtained in the initial test. It may also be appropriate to consider modifying the dose interval for a repeat test to obtain appropriate levels of toxicity.

7.11.3 Criteria for Negative/Positive/Equivocal Outcome

Although OECD TG473 mentions the use of statistical analysis for interpretation of results, it does not give any firm guidance on how those methods should be applied. Statistical analysis, when used, should only be applied to the main endpoint of the test (i.e., the proportion of metaphases showing structural aberrations). The statistical methods used assume that the experimental unit of variance is the cell, which means that, with a minimum of 300 metaphases being examined per experimental point, even quite small increases above control levels can lead to calls of statistical significance. However, in reality, small differences can result from slight variations between cultures (e.g., somewhat lower than normal incidences of aberrations being obtained for the control group in a

particular dose regimen). In addition, in a standard experiment, at least nine statistical comparisons can be made between the vehicle control and test substance groups. Therefore, applying an arbitrary critical *P*-value of 0.05 will result in at least one inappropriate call of statistical significance in approximately 50% of routine experiments as a result of chance variation. For more details, see the Statistical Analysis section of the General Recommendations chapter in this book. In summary, care should be taken to avoid inappropriate use of statistical methods and unreasonable conclusions. The interpretation criteria described here are suggested to avoid these situations.

- No statistical analysis will be performed unless the mean incidence of aberrant metaphases for any treatment with the test substance is above the expected range (i.e., beyond the upper 95% tolerance limit) for the laboratory vehicle/negative historical control database. If values are obtained beyond this limit at dose levels that are not excessively toxic, then the results from replicate cultures will be combined and compared to the results obtained for the concurrent vehicle control group from the same treatment regime using Fisher's exact test. A positive point is generally defined as a statistically significant increase ($P \leq 0.01$) in the incidence of aberrant cells for a treatment group compared with the concurrent control group, which is also above the laboratory historical negative control range (95% limits) and occurs at a dose that does not greatly exceed a 50% reduction in MI.
- The test substance is generally considered to have shown evidence of genotoxicity if there are at least two positive points. Evidence for a dose relation is also taken into account, although a dose-related response is not necessarily expected because higher doses of genotoxic agents can inhibit cell-cycle progression.
- A negative result is indicated where mean incidences of aberrant metaphase cells for the cultures treated with the test substance are within the historical control range or are not statistically significantly greater than the concurrent vehicle control.
- An equivocal response is obtained when the results do not meet the criteria specified for a positive or negative response.

Fisher's exact test can be performed in SAS or similar statistical package that has been approved by the test facility. Values should be entered in terms of absolute numbers of aberrant and nonaberrant metaphases. For example, for a control group with 3 aberrant and 297 nonaberrant metaphases and a treatment group with 13 aberrant and 287 nonaberrant metaphases, $P = 0.0098$ using a one-tailed Fisher exact test, which would be considered just significant provided the incidence for the treated group (4.3%), is above the upper tolerance limit for the laboratory historical vehicle/negative control. It may be convenient to produce a look-up table (which can be incorporated into an SOP) to avoid having to run the test on each occasion, as shown in Table 7.2 below.

Table 7.2: Fisher's exact test for 300 observations per group

Incidences in the Treated Group for 300 Observations	Incidences in the Control Group for 300 Observations															
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
	0	-	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000
	1	0.50000	0.75042	0.87563	0.93813	0.96927	0.98476	0.99246	0.99627	0.99816	0.99910	0.99956	0.99978	0.99989	0.99995	0.99997
	2	0.24958	0.50000	0.68813	0.81355	0.89180	0.93860	0.96575	0.98117	0.98977	0.99449	0.99706	0.99845	0.99918	0.99957	0.99978
	3	0.12437	0.31187	0.50000	0.65704	0.77481	0.85712	0.91180	0.94678	0.96849	0.98165	0.98946	0.99402	0.99665	0.99814	0.99897
	4	0.06187	0.18645	0.34296	0.50000	0.63764	0.74775	0.83019	0.88888	0.92902	0.95560	0.97273	0.98352	0.99019	0.99423	0.99665
	5	0.03073	0.10820	0.22519	0.36236	0.50000	0.62408	0.72749	0.80860	0.86922	0.91278	0.94306	0.96354	0.97704	0.98577	0.99130
	6	0.01524	0.06140	0.14288	0.25225	0.37592	0.50000	0.61394	0.71160	0.79081	0.85221	0.89803	0.93112	0.95436	0.97028	0.98095
	7	**0.00754**	0.03425	0.08820	0.16981	0.27251	0.38606	0.50000	0.60598	0.69871	0.77585	0.83736	0.88464	0.91986	0.94537	0.96341
	8	**0.00373**	0.01883	0.05322	0.11112	0.19140	0.28840	0.39402	0.50000	0.59953	0.68799	0.76306	0.82427	0.87247	0.90930	0.93668
	9	**0.00184**	0.01023	0.03151	0.07098	0.13078	0.20919	0.30129	0.40047	0.50000	0.59416	0.67890	0.75196	0.81263	0.86138	0.89942
	10	0.00090	**0.00551**	0.01835	0.04440	0.08722	0.14779	0.22415	0.31201	0.40584	0.50000	0.58960	0.67106	0.74221	0.80221	0.85124
	11	0.00044	**0.00294**	0.01054	0.02727	0.05694	0.10197	0.16264	0.23694	0.32110	0.41040	0.50000	0.58568	0.66422	0.73357	0.79281
	12	0.00022	**0.00155**	**0.00598**	0.01648	0.03646	0.06888	0.11536	0.17573	0.24804	0.32894	0.41432	0.50000	0.58225	0.65817	0.72585
	13	0.00011	0.00082	**0.00335**	**0.00981**	0.02296	0.04564	0.08014	0.12753	0.18737	0.25779	0.33578	0.41775	0.50000	0.57922	0.65279
	14	0.00005	0.00043	**0.00186**	**0.00577**	0.01423	0.02972	0.05463	0.09070	0.13862	0.19779	0.26643	0.34183	0.42078	0.50000	0.57653
	15	0.00003	0.00022	**0.00103**	**0.00335**	**0.00870**	0.01905	0.03659	0.06332	0.10058	0.14876	0.20719	0.27415	0.34721	0.42347	0.50000
	16	0.00001	0.00011	0.00056	**0.00193**	**0.00525**	0.01203	0.02412	0.04345	0.07167	0.10981	0.15808	0.21571	0.28112	0.35204	0.42589
	17	<.00001	0.00006	0.00030	**0.00110**	**0.00313**	**0.00750**	0.01566	0.02934	0.05021	0.07965	0.11845	0.16666	0.22349	0.28743	0.35642
	18	<.00001	0.00003	0.00016	0.00062	**0.00185**	**0.00461**	0.01003	0.01952	0.03462	0.05682	0.08726	0.12654	0.17459	0.23061	0.29318
	19	<.00001	0.00002	0.00009	0.00035	**0.00108**	**0.00281**	**0.00634**	0.01280	0.02352	0.03991	0.06325	0.09451	0.13413	0.18195	0.23717
	20	<.00001	<.00001	0.00005	0.00019	0.00062	**0.00169**	**0.00396**	**0.00829**	0.01576	0.02763	0.04516	0.06949	0.10141	0.14125	0.18879
	21	<.00001	<.00001	0.00002	0.00011	0.00036	**0.00101**	**0.00245**	**0.00530**	0.01042	0.01886	0.03178	0.05034	0.07551	0.10798	0.14796
	22	<.00001	<.00001	0.00001	0.00006	0.00020	0.00059	**0.00150**	**0.00335**	**0.00681**	0.01271	0.02206	0.03595	0.05542	0.08133	0.11423
	23	<.00001	<.00001	<.00001	0.00003	0.00011	0.00035	0.00091	**0.00210**	**0.00439**	**0.00846**	0.01511	0.02533	0.04011	0.06039	0.08693
	24	<.00001	<.00001	<.00001	0.00002	0.00006	0.00020	0.00054	**0.00130**	**0.00281**	**0.00556**	0.01023	0.01762	0.02864	0.04423	0.06524
	25	<.00001	<.00001	<.00001	<.00001	0.00004	0.00012	0.00032	0.00080	**0.00177**	**0.00362**	**0.00684**	0.01211	0.02020	0.03198	0.04831
	26	<.00001	<.00001	<.00001	<.00001	0.00002	0.00007	0.00019	0.00048	**0.00111**	**0.00233**	**0.00452**	**0.00822**	0.01407	0.02283	0.03532
	27	<.00001	<.00001	<.00001	<.00001	0.00001	0.00004	0.00011	0.00029	0.00069	**0.00148**	**0.00296**	**0.00552**	**0.00969**	0.01611	0.02551
	28	<.00001	<.00001	<.00001	<.00001	<.00001	0.00002	0.00006	0.00017	0.00042	0.00093	**0.00192**	**0.00367**	**0.00660**	0.01123	0.01821
	29	<.00001	<.00001	<.00001	<.00001	<.00001	0.00001	0.00004	0.00010	0.00026	0.00058	**0.00123**	**0.00241**	**0.00444**	**0.00775**	0.01285

(*Continued*)

Table 7.2: (Continued)

		Incidences in the Control Group for 300 Observations														
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	30	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	0.00002	0.00006	0.00015	0.00036	0.00078	**0.00157**	**0.00296**	**0.00529**	**0.00897**
	31	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	0.00001	0.00004	0.00009	0.00022	0.00049	**0.00101**	**0.00196**	**0.00357**	**0.00619**
	32	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	0.00002	0.00006	0.00014	0.00031	0.00065	**0.00128**	**0.00239**	**0.00423**
	33	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	0.00001	0.00003	0.00008	0.00019	0.00041	0.00083	**0.00158**	**0.00286**
	34	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	0.00002	0.00005	0.00012	0.00026	0.00053	**0.00104**	**0.00192**
	35	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	0.00001	0.00003	0.00007	0.00016	0.00034	0.00068	**0.00127**
	36	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	0.00002	0.00004	0.00010	0.00021	0.00044	0.00084
	37	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	0.00001	0.00003	0.00006	0.00013	0.00028	0.00055
	38	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	0.00002	0.00004	0.00008	0.00018	0.00035
	39	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	0.00002	0.00005	0.00011	0.00023
	40	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	0.00001	0.00003	0.00007	0.00014
	41	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	0.00002	0.00004	0.00009
	42	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	0.00001	0.00003	0.00006
	43	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	0.00002	0.00004
	44	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	0.00002
	45	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	0.00001
	>45	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001	<.00001

One sided *P*-values calculated using SAS versions 8.1 or 9.2. Values in bold are between 0.001 and 0.01.

7.11.4 Interpretation of Numerical Aberrations

Comments on any apparent treatment-related increases in polyploidy/endoreduplication should be made in the report. Although these may be indicative of disruption of cell-cycle progression or cell division, they are seen quite often in *in vitro* systems and are not usually indicative of genotoxic potential, so they should not be given any special weight; we suggest you include any comments about them under the title *Incidental observations* in the study report. Under some circumstances it may be appropriate to confirm the absence of related aneugenic effects *in vivo* using a rodent micronucleus test.

7.11.5 Unexpected and Borderline Results

In these cases the study director should always review the slides to check for potential artifacts and confirm the accuracy of slide reading. Borderline apparent increases in chromosome damage can occur as a result of chance variation or indirectly as a result of departures from normal physiological conditions.

The study director may consider various courses of action:

- Accept the results and report them accordingly
- Rereading of the slides by a second slide reader
- Making and reading of additional slides to clarify borderline results
- Performing a confirmatory test using the affected dose regimen, usually over a narrower dose range and using a narrower dose interval. If the original results does not fully meet the criteria for a positive result and the confirmatory test shows no evidence of clastogenicity, then the final conclusion would be negative (i.e., that the test substance is not considered to have shown evidence of genotoxicity in the test system). The study director should also consider the possibility of an error or event (unrelated to the test substance) that might have caused an unrepeatable result. Whatever the situation, it is not advisable to conclude that a test substance is genotoxic on the basis of apparent increases in the incidence of aberrant cells at a single experimental point without supporting evidence.

Usually the study director will discuss any proposed additional work (other than preliminary or standard checks) with the study monitor before producing a protocol amendment to describe the reasons for the additional work, the work itself, and the final decision criteria.

7.11.6 *Follow-up* In Vivo *Testing*

In cases where there is evidence of clastogenicity or induction of numerical aberrations, it may be appropriate to establish the relevance of the result using a rodent micronucleus test (provided systemic exposure is expected), sometimes in combination with

examination of at least one other potential target and relevant organ (e.g., duodenum and liver using the comet assay).

7.11.7 Reporting

Readers should refer to the General Recommendations chapter of this book to establish the general layout and contents of the report. The experimental completion date is usually defined as the last day on which results were obtained directly from the test system; in the case of the chromosome aberration test, that would normally be the date of completion of slide reading. In accord with OECD TG473 and good practice, other specific details that should be mentioned include:

- methods used for assessing pH (not usually necessary unless indicator in the medium shows color change), osmolality, and precipitation following addition of test formulation to the culture medium
- any adjustment of test substance formulation to avoid pH changes
- Cells:
  - type and source of cells
  - karyotype features and suitability of the cell type used
  - modal number of chromosomes, for cell lines
  - methods of ensuring absence of mycoplasma for cell lines
  - information on cell-cycle time, doubling time, or proliferation index
  - number and sex of blood donors, age, whole blood or separated lymphocytes, mitogen used
  - number of passages, if available, for cell lines
  - methods for maintenance of cell lines
- Test conditions:
  - culture conditions, cell density at initiation, culture vessel
  - concentration of test chemical expressed as final concentration in the culture medium (e.g., μg or mg/mL or mM)
  - Rationale for selection of vehicle
  - Dose volumes
  - rationale for selection of concentrations and number of cultures, including, for example, toxicity results and solubility limitations
  - composition of media, CO_2 concentration if applicable, humidity level
  - incubation temperature
  - incubation time
  - duration of treatment
  - identity of the metaphase-arresting substance, concentration, and duration of exposure
  - harvest time after treatment

  - cell density at seeding, if appropriate
  - type and composition of metabolic activation system (source of S9, method of preparation of the S9 mix, the concentration or volume of S9 mix, and S9 in the final culture medium, quality controls of S9)
  - positive and negative control substances, final concentrations for each condition of treatment
  - methods of slide preparation and staining technique used
  - criteria for acceptability of assays
  - criteria for determining readability of metaphases
  - classification system for aberrations and gaps
  - number of metaphases analyzed
  - methods for the measurement of toxicity
  - any supplementary information relevant to cytotoxicity and method used
  - criteria for considering studies as positive, negative, or equivocal
- Results:
  - cell density at start of treatment and at harvest (cell lines)
  - cytotoxicity measurements (e.g., RPD, RICC, MI) and other observations, if any
  - results of pH or osmolality checks, if any
  - change in color of medium, signs of precipitation, and point it was observed plus similar information on pH or osmolality, if assessed
  - results of chemical analysis (if performed) together with any other evidence supporting exposure of the cells to the test substance
  - rationale for selection of dose levels for analysis
  - changes in ploidy (polyploid cells and cells with endoreduplicated chromosomes, given separately), if seen
  - number of cells scored, number of cells with chromosomal aberrations, and type of chromosomal aberrations given separately for each treated and control culture, including and excluding gaps
  - statistical analysis (indicate one-tailed) and *P*-values, if any
  - concentration-response relationship, if any
  - concurrent negative (solvent) and positive control results (concentrations and solvents)
  - historical negative (solvent) and positive control data, with ranges, means, standard deviations, and 95% control limits for the distribution, as well as the number of experimental points
- Discussion of the results
- Conclusions
- References

Note that it is important to discuss any observations that support exposure of the cells to the test substance (e.g., precipitate and toxicity), especially when no supporting formulation analysis has been presented in the report.

7.11.7.1 Results tables

Aberrations and related values (incidental observations) are reported as integer values as in Table 7.3. Typically, MI and percentage of aberrations are reported to one decimal place, whereas RMI is reported to the nearest percentage (e.g., 83%). If statistical analysis is

Table 7.3: Report appendix: tabulated results for individual cultures

Treatment	Concentration, μg/mL	MI	No. of Cells Examined	No. of Aberrant Cells	No. of Aberrations					Incidental Observations[a]				
					b	e	B	E	Other	g	G	P	N	C
4-h treatment in the absence of S9 (0S9)														
Water	–	11.2	150	1	0	0	1	0	0	0	0	0	0	0
		11.0	150	1	1	0	0	0	0	1	0	0	0	0
Test item	128	12.6	150	1	0	0	1	0	0	2	0	0	0	0
		12.0	150	2	2	0	0	0	0	1	0	0	0	0
	256	11.8	150	2	2	0	0	0	0	0	0	0	0	0
		12.0	150	0	0	0	0	0	0	0	0	0	0	0
	500	5.8	150	3	2	0	1	0	0	1	0	0	0	0
		8.2	150	2	1	0	1	0	0	0	0	0	0	0
MMC	0.1	12.2	150	15	9	2	5	0	0	7	3	0	0	0
		10.0	150	13	9	1	3	1	0	8	2	0	0	0
4-h treatment in the presence of S9 (+S9)														
Water	–	9.6	150	0	0	0	0	0	0	2	0	0	0	0
		10.0	150	1	1	0	0	0	0	2	0	0	0	0
Test item	128	10.6	150	2	1	0	1	0	0	3	0	0	0	0
		9.2	150	0	0	0	0	0	0	2	0	0	0	0
	256	11.2	150	4	3	0	1	0	0	5	0	1	0	0
		11.0	150	2	1	0	1	0	0	1	0	0	0	0
	500	12.4	150	1	0	0	1	0	0	0	0	0	0	0
		11.8	150	3	3	0	0	0	0	2	0	0	0	0
CP	6	6.6	150	31	34	4	3	0	0	16	3	0	0	0
		5.2	150	23	27	2	4	1	0	10	2	0	0	1
21-h treatment in the absence of S9 (0S9)														
Water	–	12.4	150	3	2	0	1	0	0	2	0	0	0	0
		12.0	150	4	2	0	2	0	0	2	0	0	0	0
Test item	128	8.8	150	4	3	0	1	0	0	4	1	0	0	1
		11.0	150	3	0	0	3	0	0	7	1	0	0	1
	256	10.2	150	0	0	0	0	0	0	4	0	0	0	0
		12.0	150	1	1	0	0	0	0	3	0	0	0	0
	500	6.0	150	3	2	0	1	0	0	7	0	0	0	0
		5.8	150	5	2	0	3	0	0	9	0	0	0	0
MMC	0.05	12.4	150	12	6	1	5	0	0	12	3	0	0	0
		12.0	150	10	5	1	5	0	0	9	2	0	0	0

MI, Mitotic Index; b, e, g, Chromatid break, exchange, gap; B, E, G, Chromosome break, exchange, gap; Other, includes pulverized chromosomes and cells with >5 aberrations; P, Polyploidy; E, Endoreduplication; C, Centromeric disruption.
[a]g, G, P, and C are excluded from the calculation of % aberrant cells.

Table 7.4: Example summary table: HPBL

Treatment	Concentration, μg/mL	MI	RMI %	No. of Cells Examined	Aberrant Cells		No. of Aberrations					Incidental Observations[a]				
					%	ρ	b	e	B	E	Other	g	G	P	N	C
4-h treatment in the absence of S9 (0S9)																
water	-	11.1	100	300	0.7		1	0	1	0	0	1	0	0	0	0
Test item	128	12.3	111	300	1.0		2	0	1	0	0	3	0	0	0	0
	256	11.9	107	300	0.7		2	0	0	0	0	0	0	0	0	0
	500[b]	7.0	63	300	1.7		3	0	2	0	0	1	0	0	0	0
MMC	0.1	11.1	100	300	9.3[c]	< 0.01	18	3	8	1	0	15	5	0	0	0
4-h treatment in the presence of S9 (+S9)																
water	-	9.8	100	300	0.3		1	0	0	0	0	4	0	0	0	0
Test item	128	9.9	101	300	0.7		1	0	1	0	0	5	0	0	0	0
	256	11.1	113	300	2.0		4	0	2	0	0	6	0	1	0	0
	500	12.1	123	300	1.3		3	0	1	0	0	2	0	0	0	0
CP	6.0	5.9	60	300	18.0[c]	< 0.01	61	6	7	1	0	26	5	0	0	1
21-h treatment in the absence of S9 (0S9)																
water	-	12.1	100	300	2.3		4	0	3	0	0	4	0	0	0	0
Test item	128	9.9	82	300	2.3		3	0	4	0	0	11	2	0	0	2
	256	11.1	92	300	0.3		1	0	0	0	0	7	0	0	0	0
	500[b]	5.9	49	300	2.7		4	0	4	0	0	16	0	0	0	0
MMC	0.05	12.2	101	300	7.3[c]	0.34	11	2	10	0	0	21	5	0	0	0

MI, Mitotic Index; RMI, Relative Mitotic Index (vehicle = 100%); b, e, g, Chromatid break, exchange, gap; B, E, G, Chromosome break, exchange, gap; Other, includes pulverized chromosomes and cells with >5 aberrations; P, Polyploidy; N, Endoreduplication; C, Centromeric disruption; *P*, 1-sided percentage probability using Fisher exact test where ≤1% is considered significant.

[a]g, G, P, N, and C are excluded from the calculation of % aberrant cells.

[b]Slight yellow precipitate seen at the end of the treatment period is consistent with appearance of the test substance.

[c]Substantial increase compared to concurrent vehicle control beyond 95% limits of the historical negative control range.

performed, then probability values may be reported to two significant figures; critical limits are expressed to one significant figure (e.g., $P = 0.058$ and $P < 0.01$).

It is useful to include a single table summarizing the most important results obtained in the test as well as the results of any statistical analysis, as in Table 7.4.

In this theoretical case, the test substance was a pharmaceutical; therefore, the top dose chosen for detailed analysis was the standard limit, although precipitation and toxicity were also seen at this level in the absence of S9 mix. The test substance has not shown any evidence of clastogenicity/genotoxicity, whereas the positive controls have caused substantial and highly significant increases in the incidence of metaphases with structural chromosome aberrations confirming the sensitivity of the test and the efficacy of the S9 mix.

Tables showing results in more detail are best presented as appendices, as shown in the corresponding Table 7.3.

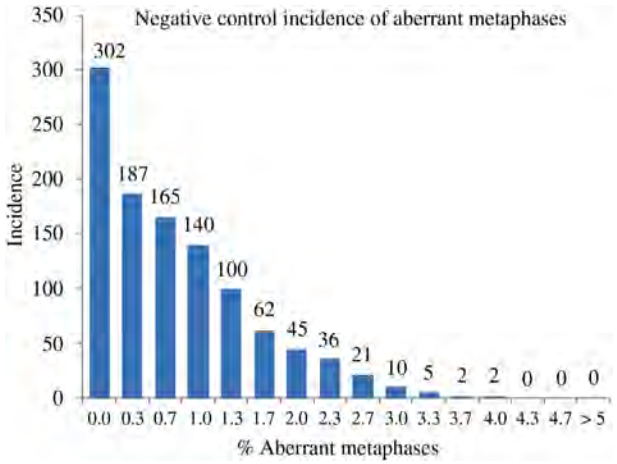


Figure 7.5

Example layout of theoretical laboratory historical control results. The laboratory historical mean incidence of aberrant metaphases for negative/vehicle control cultures is 0.83%, with a population standard deviation of 0.7; 95% of observed mean values lie within the range of 0—2.2%. These results are for 1077 groups of duplicate cultures with a total of 300 metaphases being analyzed for each group and they are taken from QA-audited experiments performed during the period from April 2014 to April 2016 and completed prior to the present study.

7.11.8 Historical Control Results

Relevant historical control results should be presented in the report to:

1. confirm the proficiency of the laboratory
2. demonstrate that negative and positive control results obtained for the present study are within normal limits and meet acceptance criteria specified in the protocol
3. help determine whether results for the test substance are within normal (tolerance) limits for unaffected cultures as defined in evaluation criteria specified in the protocol.

As indicated by OECD TG473, it is not sufficient to just list the upper and lower limits of the range of control values; the report must also show means, standard deviations, and 95% control limits for the distribution, as well as the number of experimental points (groups). It is particularly important to show the distribution for the negative/vehicle database because of evaluation criteria mentioned. The best way to summarize historical control data and their distribution is in a graphical form that can be included in an appendix to the report as per Figure 7.5.

7.11.9 Testing of Volatile and Gaseous Compounds

When testing volatile or gaseous chemicals, exposure should take place in sealed culture tubes to ensure adequate exposure. In the case of HPBL, this can be accomplished by

transferring the cultures (with S9 mix where appropriate) to gas-tight 24 mL glass anaerobic culture tubes (Bellco Glass) immediately prior to dosing. Note that positive controls do not need to be transferred unless you decide to include gaseous or volatile positive controls. Seal the tubes with gas-impermeable, butyl rubber septum (injectable) stoppers and aluminum seals; when appropriate, perform subsequent procedures in a fume hood. Volatile liquids or dilutions of them can be injected directly through the septa.

In the case of gases, an adequate volume of gas should be transferred to a gas-tight (e.g., Tedlar®) bag with an injectable septum. An appropriate volume of air is then removed from each tube using a syringe fitted with a fine needle just prior to dosing. This volume of air is calculated as 19 mL × E% ÷ (100% − E%), where E is the target concentration of gas and 19 mL is the approximate volume of air in the culture tube. A gas syringe fitted with a fine needle is used to remove the calculated required volume of gas (calculated as 19 mL × E%) from the gas bag via the injectable septum, and then this is injected directly into the culture tube. In the case of gases, we recommend a maximum dose of 50% v/v, in which case 19 mL air is removed (effectively half) and replaced by 9.5 mL gas.

In the case of volatiles and gases, culture tubes are then incubated at 37°C for the normal period and then vented in the fume hood before transferring back to standard culture tubes (i.e., after 4 h for Sets 1 and 2 and after 19 h and immediately prior to addition of colcemid in the case of Set 3). Cultures are then processed as usual.

7.12 Screening Versions of the Test

The methods described in the main body of this chapter largely relate to routine test performance using 5 mL cultures, although the HPBL test procedures section also describes the use of 1 mL cultures in 4 mL glass vials. Similarly, 1 mL cultures can be used with adherent cell lines like CHO; in this case, they can be grown in 24-well plates that make dosing and handling easier. The main advantage of these miniaturized versions is that they can meet all the requirements of the OECD test guidelines, including the specified upper concentration limit, while using one-fifth the amount of test substance and other components, including S9. The disadvantage is that the cell yield is proportionately lower, which may mean that the specified number of readable metaphases (300 per experimental point) is not always available for analysis, particularly at moderately toxic dose levels. Miniaturized versions are therefore suitable for non-GLP screening, especially when test substance supply is limited, but they are not necessarily suitable for routine regulatory testing. If you intend to set-up the HPBL in a different culture format, then you should optimize the cell density and culture volume to maximize the yield of mitotic cells.

7.13 Automation

Automatic (computerized robotic) metaphase finder systems that can capture images and stage locations of cells potentially suited for detailed analysis are available (e.g., Metafer http://www.metasystems-international.com/metafer/msearch). In this case, the slide reader reviews the images and selects those cells that appear suitable for analysis, and the system automatically moves the slide on the microscope to the appropriate location. Some systems can also automatically rank metaphases in terms of quality and can determine MI automatically. Therefore, they greatly reduce scanning and slide reading time. However, they are necessarily expensive, so they should only be purchased when throughput merits the expenditure.

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