CHAPTER 3

Formulation of Test Articles

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3.1 Introduction

3.1.1 Safety

Genetic toxicology testing usually involves the use of compounds with unknown toxicities and highly hazardous positive control agents. It is the legal and moral responsibility of laboratory management to ensure that an appropriately equipped dedicated laboratory area is available to deal with and contain these types of materials, taking into account that they are likely to have various physical characteristics; for example, powders should not be handled in the same way as volatile liquids. In addition, appropriate PPE (clothing, masks, etc.) must be available to staff handling the materials, and procedures and training should be in place to ensure safe handling and containment. It is the responsibility of staff and management alike to ensure that the prescribed procedures are followed.

Health effects potentially resulting from inappropriate handling of chemical mutagens and cytotoxic drugs (e.g., positive controls, spindle poisons, cytochalasin) used in genetic toxicology are similar to those expected from exposure to radiation and chemotherapeutic drugs [1,2]. These may include:

1. Bone marrow disorders and blood dyscrasia
2. Fetal loss in pregnant women and malformations in the offspring
3. Loss of fertility
4. Painful gastrointestinal disorders, hair loss, nasal sores, and vomiting
5. Liver damage
6. Contact dermatitis, local toxic effects, and allergic reaction
7. Cancer initiation and promotion (one in three people develop cancer in their lifetime)

Although there may be no evidence that staff working with these chemicals have higher incidences of these conditions, minimization of exposure by ensuring appropriate control measures is essential [3]. Precautions apply especially during the formulation process because chemicals are handled in bulk and in concentrated form. In some cases, the risk of exposure can be reduced by purchasing agents in preweighed aliquots.

3.1.2 Selecting an Appropriate Formulation

Two of the first questions that need to be answered when developing a new pharmaceutical or planning a new toxicology testing program for any type of test article are:

1. What will be the route of administration?
2. What vehicles are likely to be compatible with my compound and the chosen route of administration?

Genetic toxicology studies are usually initiated at the start of the testing program and, in the case of *in vitro* studies (including the **h**uman ***E**ther-à-go-go-***R**elated **G**ene (hERG) screen for induction of cardiac arrhythmia), often involve a different vehicle. It is important that these questions are answered at the outset so that any formulation development work and associated chemical analysis to support vehicle selection covers all the studies being considered in the test program.

The vehicle and route of exposure for *in vivo* genotoxicity studies usually match the main rodent toxicology studies so that information on toxicity and, when available, information on systemic exposure can be cross-referenced. In the case of a Good Laboratory Practice (GLP) toxicology program, chemical analysis to demonstrate homogeneity, physical stability, and chemical stability, together with preliminary toxicokinetic and acute toxicity results, will be available to support vehicle, route, and dose selection for *in vivo* genotoxicity test(s).

3.2 Formulation Laboratories

Formulation laboratories should be designed to protect individual workers, prevent contamination of the environment, and, as required by GLP, the test facility as a whole.

Toxicity testing facilities are expected to have their own formulation laboratory ("pharmacy") with staff trained in preparing materials for administration to animals by common and specialized routes. However, pharmacy staff are not necessarily adept at dealing with formulations for *in vitro* studies, which usually involve small volumes and highly hazardous cytotoxic and mutagenic materials. For reasons of safety, convenience, and training, and to avoid potential contamination issues, genetic toxicology testing laboratories should have their own separate purpose-designed formulation area. In contrast, formulation (often of suspensions) for use in *in vivo* genetic toxicology work may be assigned to the main pharmacy, which will have the facilities to deal with bulk formulations and will normally be more experienced in dealing with special formulations needed for animal studies (e.g., suspensions, admixture with the diet, inhalation, and infusion). In the case of special routes of administration, the scientist should seek the advice of formulation specialists and a scientist experienced in that route to assist in dose preparation and the in-life phase of the study. Special routes will often complicate sampling and chemical analysis.

3.2.1 Designing and Equipping a Genetic Toxicology Formulation Area

The engineers involved in planning a new laboratory or refurbishing an existing area should be specialists in safe laboratory design; they and laboratory management should be aware of critical aspects of design and national requirements. These aspects, particularly with regard to handling potential mutagens, are outlined here; however, full details of laboratory design are beyond the scope of this book; readers should consult specialist reference manuals [4].

Access to the formulation area should be physically restricted to allow entry of only authorized personnel for safety reasons and to ensure the security and integrity of the test articles. Here, as in the other genetic toxicology laboratories, the flooring should be seamless, physically resilient, solvent-resistant, easy to clean, and skid-proof, and should continue for the first few inches up the wall. Poured epoxy flooring over a concrete base is probably the best option to use. The area should have an anteroom with washing facilities, where staff can change into and out of personal protective clothing when entering and leaving the area. In accord with the principles of OSHA [5], measures to ensure safety and containment should be considered in the following order:

1. Engineering controls
2. Administrative controls
3. Training and enforcement of appropriate work practices
4. Personal protective clothing and equipment

For example, protection from inhalation of chemicals primarily involves ensuring adequate air-flow, for example, powder containment and fume hoods should be used to dispense and formulate materials rather than expecting staff to formulate on the open bench while wearing uncomfortable and constrictive breathing apparatus (which might interfere with efficiency, accuracy, observation, and communication). Individual countries have their own legislation concerning transport, storage, handling, and disposal of chemicals, but useful guidance on the principles of safe handling and disposal of chemicals can be found at the following US government website: http://sis.nlm.nih.gov/enviro/labsafety.html#a1.

The air pressure in the formulation laboratory should be maintained at slightly negative pressure with respect to the anteroom using an extraction system. Fume hoods normally required a balanced air input to avoid excessive negative pressure. In general, minimum air change rates in laboratories are not subject to legal requirements, but 10 air changes per hour seems reasonable given that this figure is often used in designing animal rooms. To simplify design and maintain a standard air change rate in the laboratory, this may involve running the fume hood(s) continuously, if rates are of the constant volume type with a bypass. The extracted air should be vented on the roof with appropriate consideration of the direction of the prevailing wind and precautions should be taken to prevent backflow. The fume hood and powder containment cabinet supplier and the specialist laboratory HVAC (heating, ventilating, and air conditioning) engineer should be consulted and directly involved in the siting of equipment and design of the air-handling system. Efficient air-flow system design is extremely important because it will represent more than half the cost of the pharmacy; poor design will also result in significant additional operational costs in the form of wasted conditioned air—refer to the TSI Laboratory Design Handbook for additional information on air-flow and efficient design [6]. The incoming air should be filtered at a comfortable temperature and humidity to not interfere with the efficiency of operational staff [4,7].

3.2.1.1 Hoods

Test article containers should be opened and contents should be dispensed only in suitable containment devices ("hoods") that are regularly monitored for containment efficiency and air-flow rate. Hoods should be solvent-resistant and easy to clean, with seamless stainless-steel work surfaces and rounded corners. As a minimum, the pharmacy should contain one 4-foot radioisotope-type fume hood. Fume hoods are used for dispensing volatile liquids and, very occasionally, gases.

Fume hoods are not suited for handling powders because the high flow, although directed away from the body of the operator, can generate an aerosol of fine particulates, leading to contamination. Most dispensing involves solids (often powders) and should be performed in a 3-foot-wide powder containment cabinet (similar to a biological containment cabinet class 2B or a laminar-flow cytotoxic drug safety cabinets) extracted via a contained high efficiency particulate arresting air (HEPA) filter to the exterior (e.g., the Xpert station supplied by Labconco). This type of hood provides a lower-speed laminar air-flow away from the operator. Any small amount of particulate generated is captured in the HEPA unit, which is changed regularly as it becomes blocked or after a specified period as per the manufacturers' instructions.

Hoods should be fitted, maintained, and cleaned in accordance with the manufacturers' instructions. They should contain only the items necessary for preparing the current formulation and no bulky items, because these would interfere with the air-flow characteristics. Balances should be situated adjacent to the hood to minimize contamination. Materials should be dispensed into suitable preweighed containers that are recapped inside the hood before being reweighed. The target weight of the dispensed material should be specified as a range so that a calculated amount of vehicle can be added to the dispensed material to achieve the required concentration rather than targeting an exact weight, which would require multiple additions and or removal of material from the weighing vial. Open containers should be held in a rack or hand-held to minimize the chance of spillage. Ancillary items needed for formulation such as pipettes can be conveniently stored adjacently on a stainless-steel cart or in racks next to the hood.

3.2.1.2 Storage equipment

Chemicals should be stored in cabinets:

1. General storage cabinet with chemicals stored in alphabetical or appropriate logical order
2. Lockable store for scheduled drugs (i.e., controlled substances such as phenobarbital)
3. Solvent cupboard
4. Refrigerator set at 4°C
5. Upright freezer set at −20°C

Individual cabinets should be extracted or placed in a glass-partitioned unit providing extraction. Positive controls and cytotoxic agents should be stored separately from test articles. Nonhazardous reagents such as buffers, glassware, and other items should be stored in normal laboratory cabinets.

When not in use, chemical containers should be protected to prevent spills or loss if dropped. Glass vials can be stored in a secondary container (e.g., see-through polycarbonate bottle containing silica gel, if appropriate) and transported in a plastic tool box between work areas.

3.2.2 Personal Protective Clothing

3.2.2.1 Standard

1. Safety glasses with protective sides or face shield
2. Nitrile, neoprene, or latex gloves should be available. The selection should be based on the main solvent being used, referring to the manufacturer's website for details of resistance. Individuals can develop skin sensitization to latex and/or the powder used by the manufacturer, so several types of glove need to be available. Gloves should be changed immediately if exposure suspected or if they become sticky or damaged.
3. Jumpsuit (polyethylene-coated paper or Tyvek® coverall/boilersuit)
4. Disposable protective sleeves, nonslip shoe covers, and head cover
5. NIOSH-approved dust mask

3.2.2.2 Capital equipment

1. Analytical balance, minimum readability 0.1 mg, capacity 300 g or more
2. Top pan balance, readability 1 mg
3. Appropriate calibrated weights to check balance calibration
4. Homogenizer: high shear with a range of probes (e.g., Silverson, Ultra-Turrax, Polytron)
5. Ultrapure water (UPW) and/or deionized water supply

Other equipment

1. pH meter and electrodes
2. Range of stainless-steel spoons, spatulas, and trullae (scoops)
3. Heated stir plates and magnetic followers ("fleas")
4. Mortar and pestle
5. Appropriate glassware including a range of graduated cylinders
6. Pipette controllers, hand-held rechargeable
7. Adjustable micropipettes (regular and positive displacement), single action, and repeater
8. Stainless-steel carts
9. A safelight for use when formulating light-sensitive materials

In addition, a range of equipment needs to be available but not necessarily present in the formulation area, including an autoclave and a laboratory glass-washer. Special formulations are beyond the scope of this chapter, but a ball mill and a set of stainless-steel sieves might be available in the main pharmacy for preparing suspensions of insoluble solids that are not received as fine powders, which is often the case with industrial compounds.

3.2.2.3 Consumables

1. A range of buffering materials, organic solvents, suspending agents, volumetric hydrochloric acid, and sodium hydroxide (stored in tightly sealed bottles to prevent loss by evaporation or reaction with carbon dioxide in the air)
2. Medical wipes and paper towel
3. Weighing paper, weigh boat
4. Aluminum foil
5. Parafilm
6. Disposable syringes and needles
7. Sterilizing filters 0.22 μm, aqueous and solvent-proof types
8. Benchkote or Bench Guard protective paper (supplied in rolls), absorbent on upper side only
9. Micropipette tips, sterile
10. Disposable sterile calibrated pipettes, 2, 5, 10, and 25 mL
11. Disposable screw-top glass vials and containers suitable for preparing and storing formulations; amber containers may be useful when using light-sensitive materials

Note that these lists are not exhaustive and other items may be required in particular cases.

3.3 Safety Data Sheets

Prior to receipt and handling of materials, the program manager in conjunction with the study director(s) is responsible for obtaining information on the test material, often from the sponsor in the case of subcontracted studies. This is used to ensure appropriate handling of the test material but can also facilitate the selection of the vehicle and dose levels. It may also help design other aspects of the study; it may even tell you whether the studies you are planning are appropriate depending on the chemical class of the material and its known or suspected biological activities. For example, volatile agents require contained exposure in the case of in vitro tests, whereas the *in vivo* micronucleus test may not be appropriate for chemicals that cause hemolysis or anemia.

Much of this information can be obtained in the form of a safety data sheet (SDS). Many countries including the United States and Europe are adopting the Globally Harmonized System of Classification and Labelling of Chemicals system developed by the

United Nations and referred to as GHS (see http://www.unece.org/trans/danger/publi/ghs/ghs_welcome_e.html). GHS has developed a standardized SDS that provides information on physical properties, chemical reactivity, and toxicity in a consistent and standardized format using the following section headings.

1. *Identification*: product identifier, manufacturer or distributor name, address, and telephone number, emergency telephone number, recommended use, and restrictions on use
2. *Hazard(s) identification*: all hazards regarding the chemical and required label elements
3. *Composition/information on ingredients*: information on chemical ingredients
4. *First aid measures*: important symptoms/effects, both acute and delayed, and required treatment
5. *Fire-fighting measures*: suitable extinguishing techniques, equipment, and chemical hazards from fire
6. *Accidental release measures*: emergency procedures, protective equipment, and proper methods of containment and clean-up
7. *Handling and storage*: precautions for safe handling and storage, including incompatibilities
8. *Exposure controls/personal protection*: OSHA's Permissible Exposure Limits (PELs), Threshold Limit Values (TLVs), appropriate engineering controls, and personal protective equipment (PPE)
9. *Physical and chemical properties*: the chemical's characteristics
10. *Stability and reactivity*: chemical stability and possibility of hazardous reactions
11. *Toxicological information*: routes of exposure, related symptoms, both acute and chronic effects, and numerical measures of toxicity
12. *Ecological information*
13. *Disposal considerations*
14. *Transport information*
15. *Regulatory information*
16. *Other information*: the date of preparation or last revision

Although the SDS may imply that the material is of low toxicological concern, the information may be unreliable or, as is often the case, inadequate. Unless there is a specific hazard, it is therefore appropriate, convenient, and easier for the staff to handle these materials in the same way as they do positive controls. The scientist in charge of the study should review the SDS for content and any missing information in advance of the use of the material and advise staff of any special precautions (e.g., in the case of volatiles). This can be conveniently done using a standard section in the study protocol/plan. The scientist should also ensure the current version of the SDS is available for staff to consult; if special precautions are required, then the scientist should provide reminders prior to the study (e.g., at a prestudy meeting).

When the scientist/program manager is requesting information in the form of a SDS, the scientist/program manager should also ask for information from the sponsor/chemist regarding the compatibility and solubility of the test article with likely vehicles and relevant information from any early phase (e.g., bioavailability, *in vitro* metabolic profiling, toxicity) investigational studies. Be aware that, in the case of drugs, this information may relate to a different salt form of the test article.

3.4 Receipt of the Test Article

On receipt of the test article, the identity, batch number, expiration date, weight supplied, storage conditions, and physical appearance should be confirmed against the certificate of analysis (COA; where provided) and information given by the supplier in case of error or deterioration. The material should be stored as per the SDS and COA taking into account any additional information provided by the supplier. The receipt date of each lot of the test article should be recorded and marked on the container. These details should be presented in the protocol and in the final report. On arrival in a GLP test facility, and to continue the chain of custody history, it is a standard practice to document the receipt, appearance, location, storage conditions, and usage of test article. This test article disposition and use log should include details of the storage container and its gross weight before and after any removal of material for testing: the difference between before and after is used as a check of the weight of material in each formulation.

At the end of the test program, some of the test article may be sent for confirmation of stability under conditions of storage and use. Alternatively, stability of the test article under the stated storage conditions may already have been established or be ongoing. In any case, confirmation of stability should be stated in the final report.

3.5 Formulation Types and Planning

In GLP toxicology test programs, chemical analysis is required to demonstrate chemical stability, physical stability, and homogeneity for the planned vehicle(s) and concentration ranges to be used in the individual studies. The analytical method is usually developed and the method and formulation are validated in advance of the toxicology studies. Therefore, the nature of the formulations and concentration ranges to be covered should be determined prior to completion of this validation to avoid additional cross-validation work later.

The route of administration for standalone *in vivo* genetic toxicology studies usually matches that used for the rest of the rodent toxicology program (to facilitate dose-setting and allow use of toxicokinetic data, where available) and should be one that mimics human exposure. Oral by intragastric gavage, intravenous bolus injection or infusion,

and subcutaneous injection are common routes. Other routes or methods may be considered, such as topical (e.g., dermal, ophthalmic, inhalation, and implant) or capsule; however, if they result in low target organ exposure because of low rates of absorption or low achievable dosages, then other routes may be more appropriate. For example, when evaluating a water-soluble pharmaceutical intended for administration by inhalation, higher systemic exposure levels (in terms of concentration over time) might be achievable using subcutaneous injection or intravenous infusion. These routes may also be significantly less expensive.

For materials that are water-soluble, it usually possible to choose the same vehicle for *in vitro* as well as short-term and long-term *in vivo* studies. In this case, the vehicle will often be isotonic and buffered if given by a parenteral (nonoral) route (e.g., phosphate-buffered saline). However, (water) insoluble compounds are usually administered as suspensions for *in vivo* studies and (to enhance exposure and for practical reasons) as solutions in water-miscible organic solvents for the *in vitro* studies. In the latter case, the final maximum concentration of material in solution in the culture medium/agar is increased by the presence of the solvent ("solubilizing agent"). The disadvantage of this approach is that at least two vehicles need consideration in terms of chemical analysis and formulation validation.

Medical devices are an exception in terms of both formulation and chemical analysis. In this case readers should refer to specific guidelines and procedures published by the Food and Drug Administration (FDA) as well as the ISO 10993 series of standards. These documents undergo continual revision, so the reader should check for any updates prior to embarking on any practical work. In particular, ISO 10993-3 deals with the genotoxicity tests required and ISO 10993-12 deals with sample preparation for testing. In the case of water-insoluble medical devices, extracts are produced under exaggerated conditions using one aqueous solvent and one nonpolar solvent; common extractants are saline, culture medium without saline, and isopropyl alcohol or dimethyl sulfoxide (DMSO). Even though DMSO is polar, it is probably the most frequently used "nonpolar" solvent. Although the guidelines do not specify chemical analysis of the extracts, the manufacturer will normally be aware of the likely major components present based on in-house chemical testing of related extractable and leachable material.

3.6 *Solubility and* In Vitro *Compatibility Testing*

3.6.1 *Introduction*

The purpose of solubility testing is to determine an appropriate solvent for use in the subsequent genotoxicity assays. Subsequent compatibility testing of the solution in culture medium will establish whether the solution requires neutralization. For materials with low

aqueous solubility and toxicity, it will also establish the highest dose level to be tested in the subsequent mammalian cell test because the highest one or two dose levels used in that test should show precipitation.

In the case of the subsequent *in vitro* cytogenetic (e.g., chromosome aberration and micronucleus) tests, it is most efficient to test a wide range of dose levels up to the limit established by solubility and compatibility testing, prepare slides, perform a preliminary assessment of toxicity, and then perform detailed analysis on slides from the three or four highest dose levels not showing excessive toxicity. When the material is expected to be highly toxic or when this would involve an excessive amount of work (e.g., in the case of cell mutation assays), a preliminary toxicity test will be required prior to the main biological assay.

3.6.2 Choice of Solvent

The formulation needs to take into account OECD and, in the case of pharmaceuticals, ICH [8] requirements in terms of maximum dose levels; see http://www.oecd.org/env/ehs/testing/section4healtheffects.htm and http://www.ich.org/products/guidelines/safety/article/safety-guidelines.html for the latest approved and draft guidelines. If the test article is known or found to be water-soluble at 100 mM, 20 mg/mL, or 20 μL/mL (10 mM or 5 mg/mL in the case of pharmaceuticals), whichever is the lowest, then it should be dissolved in the vehicle selected for the rodent toxicology program. If not soluble at these levels, then it may be necessary to use an organic solvent. Alternatively, it may be possible to dissolve the material directly in the culture medium used for the mammalian cell test; if serum-free medium is used, then it may be possible to use the same formulation for the bacterial mutation test. Note that higher concentrations above the guideline limits may be justifiable when testing a complex mixture or qualifying a pharmaceutical containing a potentially genotoxic impurity. In addition, a correction may be needed for salt form, water, or purity. Organic solvents, where used, should generally be anhydrous to minimize potential development of solvent or test article degradation products.

Generally, aqueous suspending agents such as 1% methyl cellulose are of low toxicity and are compatible with culture medium and S9, so they can be used at the same levels as aqueous solvents. Dilute acids and dilute alkalis can also be used provided that the final formulation does not cause a substantial shift in the pH of the culture medium, because excessive departures from normal physiological conditions can cause irrelevant toxic and genotoxic effects, particularly in cultured mammalian cells. Water-miscible organic solvents are generally less compatible than aqueous vehicles (i.e., they are toxic and inhibit S9 activity), so their dose volume is normally restricted to 100 μL per plate and 1% v/v in bacterial and mammalian cell systems, respectively. Very occasionally, a nonmiscible

solvent may be used before further dilution (e.g., solubilization in toluene followed by dilution in DMSO or aqueous surfactant solution)—these solvents are generally more toxic and the final maximum level in the test system should be adjusted accordingly. Because they are not miscible and may degrade plastic culture vessels, nonmiscible solvents should not be used without dilution in a miscible solvent. Similarly, dichloromethane can be used to extract material, but the extract should not be used without evaporation because dichloromethane itself is mutagenic.

An important step in establishment of a new assay involves assessment of maximum compatible dose volumes of commonly used solvents. Otherwise, the compatibility of novel solvents will need to be evaluated in advance of their use. Note that primary cells and the preincubation version of the bacterial mutation test may be less tolerant of solvent levels. Additional solvents that have been evaluated in the bacterial mutation test are listed by Maron and associates [9].

3.6.3 Solubility Testing

Evaluation or confirmation of solubility should be performed in advance of genotoxicity testing based on information supplied by your chemist or, in the case of CROs, your sponsor—do not expect that information to be reliable because it may, for example, be based on a different salt form of a drug undergoing development. Solubility and compatibility testing are not necessarily study-specific and do not follow a formal protocol, so it may be convenient to perform them under an internal non-GLP study number. Copies of the testing procedure and results can be retained with the subsequent genetic toxicology studies, if considered appropriate.

The compatibility of solubilized material should be evaluated with the culture medium to be used in the mammalian cell test as described in the next section. In this way, a maximum practical dose can be established based on solubility and dose volume constraints. Solubility in bacterial mutation test plates does not need to be evaluated unless your facility's standard practice is to only use one nonprecipitating concentration.

When the test shows low solubility in aqueous media, the solubility in nonaqueous solvents compatible with the test system should be assessed. The preferred nonaqueous solvent is usually DMSO, but other water-miscible solvents, including methanol, ethanol, dimethyl formamide, acetone, and acetonitrile, should be tried if the material does not show adequate solubility in DMSO. For volatile solvents, including acetone or viscous solvents like polyethylene glycol, positive displacement pipettes should be used to ensure accuracy when dispensing or measuring. When the test compound exhibits inadequate solubility in aqueous or suitable nonaqueous solvents, then aqueous suspending agents (e.g., 1% methyl cellulose) may be used.

Table 3.1: A selection of vehicles that have been used in regulatory studies at Charles River Laboratories

	Bacterial	Mammalian Cell Assays
Vehicle	**μL/Plate**	**μL/mL**
10% CD		200
22% HPCD	200	200
Acetone	100	10
Acetone:THF (3:1)	75	
Acetonitrile	50	10
DMF	100	10
DMSO	100	30
Ethanol	50	20
Ethyl acetate		5
Methanol	50	30
NMP	100	
Toluene	50	
vitE/PG	200	

Notes: [a] vitE/PG 25% vitamin E TPGS/75% propylene glycol.
[b] 22% HPCD aqueous 22% w/v ß -hydroxypropylcyclodextrin in purified water.
[c] 10% CD aqueous 10% w/v sulfobutyl ether ß-cyclodextrin.
[d] NMP N-methyl-2-pyrrolidinone.

For *in vitro* studies, the test article is usually dispensed directly into a preweighed container and then a calculated volume of solvent is added to achieve the target concentration. Glass vials with a screw-top are generally the most practical because they avoid problems with static (which otherwise might causes inaccuracies in weighing), they can be resealed in the hood prior to reweighing, and they are compatible with all solvents. The solvent is added in measured and recorded increments until the test article is dissolved.

This method of formulation avoids wasting material because only the minimum amount used for dosing is needed and the material is formulated directly in the storage container. However, because the formulation is not brought to volume (e.g., in a volumetric flask or measuring cylinder), it introduces a small error because the final volume of the solution will be slightly more than the volume of solvent added. With concentrations more than 60 mg/mL (as is usually the case for mammalian cell tests using nonaqueous solvents), this error can be substantial; slightly less volume of solvent should be added to dissolve the material, and then the final volume should be measured using a variable micropipette before the solution is brought to volume. The theoretical density ("SG") of the dissolved material (typically approximately 3000 mg/mL) can then be calculated to facilitate future formulation.

If the test article is stored at low temperature, then it should be allowed to come to room temperature before dispensing to avoid condensation. Dissolution of the test article can be assisted by warming the mixture with the solvent in a water bath, vortex-mixing, or placing in a sonic bath, provided that this does not result in degradation.

The appearance of the formulation and the method of preparation should be recorded for future reference. To confirm that the material is truly in the solution, the mixture can be transferred to a flat-bottom multiwell plate and examined under an inverted phase-contrast microscope for the presence of insoluble material or centrifuged in a conical tube before examination for sedimentation.

3.6.4 Calculations and Checking: Small-Volume (In Vitro) Assays

The gross weight of the bulk test article should be recorded before and after removal of material for formulation. The difference in weight is expected to be very slightly more than the weight of material dispensed (e.g., there will be a slight loss on the spatula), but the apparent discrepancy should not exceed 5% of the total weight of material dispensed. The container to be used for formulation should be labeled and the gross weight recorded prior to and after addition of the test article (difference = net weight of test article) and then again after addition of the vehicle and dissolution. The net weight of the solvent divided by the specific gravity (SG; per the manufacturer's specification or the Merck index) should equal the stated volume of addition; for dilute aqueous vehicles, the SG can normally be assumed to be 1000 mg/mL. The final total volume of the formulation should be checked with a micropipette. The achieved values for these checks are expected to be within 5% of stated values under normal circumstances.

The volume of vehicle to be added to the test article is calculated as:

> Weight of test article × purity ÷ correction for content ÷ target concentration
> If there is no correction for purity or content, then those values are equal to 1 (and thus effectively ignored in the calculation). In the case when the final formulation concentration exceeds 60 mg/mL, we recommend you take into account the theoretical volume occupied by the dissolved test article so:
> volume of vehicle = weight of test article × purity ÷ correction for content ÷ target concentration − weight of test article ÷ SG
> (where SG is the notional/theoretical specific gravity of the test article determined in the solubility test, as described in the previous section.)

Examples

1. I need at least 6 mL of a 50 mg/mL stock solution of compound X in DMSO for use in a standard bacterial mutation test (top dose is 5 mg/plate using a dose volume of 100 μL/plate). If there were no correction for content or purity, then I weigh out just over 300 mg (I do not need to dispense an exact amount); in this case, I find I have added 305.0 mg of compound X, and then the required volume = 305.0 ÷ 300 = 6.1 mL DMSO. After this addition, I expect the weight of the container to increase by 6.1 × 1.100 (SG of DMSO in g/mL) = 6.71 g (±5%).

2. In the same example, the material is supplied as the hydrochloride, which has a molecular weight 1.05-times higher than the free base and contains 4% water. Because the protocol indicates that dosages should be expressed in terms of anhydrous free base, I need to prepare a stock solution at least 1.05×50 mg $\div 0.96 = 54.7$ mg/mL expressed in terms of material as supplied. So, I need to dispense at least 328 mg of material. After weighing, I find that I have added 340.1 mg of material to the formulation container, so I calculate that I need to add:

   340.1 mg $\times 0.96 \div 1.05 \div 50$ mg/mL = 6.2 mL (note that amounts are usually only expressed to an appropriate number of decimal places, dependant on the measuring device, although they do not need rounding in your calculation or spreadsheet).

   In this case, I expect the net weight of the solvent to be 6.8409 g ($\pm$ 5%).
3. I need 10.5 mL of a 200 mg/mL solution of the same hydrochloride solution for use in the mammalian cell mutation test = 10.5 mL of a 218.8 mg/mL (1.05×200 mg/mL $\div 0.96$) solution expressed in terms of material as supplied (i.e., I need at least 2297 mg of test article). Because the concentration (in terms of material as supplied) is more than 60 mg/mL, I expect the volume of the solute to make a significant contribution to the volume of the solution. I therefore need to make a correction for the notional SG of the compound, which in this example was determined to be 3.1 g/mL (3100 mg/mL) in the solubility test. In this case, I have dispensed 2330.1 mg of material so that:

   Calculated volume of DMSO = 2330.1 mg $\times 0.96 \div 1.05 \div 200$ mg/mL $-$ 2330.1 mg/mL $\div$ 3100 mg/mL = 9.9 mL.

   After adding 9.9 mL and dissolving the material, I check that the net weight of the solvent is within 5% of the target weight before measuring the total volume to confirm that it is close to 10.5 mL.

   Note that, for the purpose of calculation, you should ensure that the units (dimensions) are consistent throughout. These calculations can be quite complicated so it is best to enter the formulae into a spreadsheet that can be locked as part of an SOP for routine use. The calculations for preparation of stock solutions and dilution should be checked by a second person (ideally the scientist responsible for the study) prior to dosing.

3.6.5 Compatibility of Formulation with Culture Medium

Once an appropriate formulation has been achieved at the desired concentration in the solubility test, the formulation should be assessed for compatibility with the culture medium to be used in the mammalian cell test, if appropriate. The solubilized material is added to culture medium (complete with serum, if appropriate) in a volume calculated to reach the maximum recommended by the relevant ICH or OECD guideline(s) (e.g., 20 μL of a 200 mg/mL solution or 222 μL of a 20 mg/mL aqueous solution added to 2.0 mL medium

in the well of a 24-well plate). An inverted microscope should be used to confirm the absence of precipitate and is especially useful at low concentrations, where precipitation might otherwise not be evident. Precipitation can take many forms, including cloudiness, crystallization, and formation of a film on the surface of the medium. Note that excessive precipitation should be avoided in mammalian cell tests because precipitate is often carried over after the exposure period and can result in toxicity or interfere with the quality of slide preparations.

If a significant change in the color of culture medium occurs indicating a significant shift in pH, then the stock formulation should be neutralized by addition of an equivalent amount of alkaline or acid and the dose volume should be adjusted as necessary to avoid irrelevant toxicity that might mask genotoxicity or genotoxic effects as a result of stress [10,11]. Although older guidelines and articles refer to the potential effect of dose solutions on osmolality, osmolarity, or tonicity [12,13], the current specified limits described by ICH and OECD do not have any significant impact on the osmolality of the culture medium. Culture medium is approximately 300 mOs/kg, which is approximately equivalent to 300 mM glucose or 150 mM sodium chloride. Osmolality is proportionate to the same number of particles in solution (i.e., molecules or ions in the case of covalent or ionic compounds, respectively).

Example

The solubility of compound X was evaluated in a range of solvents; it showed low solubility in water (much less than 0.1 mg/mL), was just soluble at 100 mg/mL in DMSO, and showed lower solubility in other water-miscible solvents evaluated, including methanol, acetone, and dimethyl formamide. Therefore, DMSO was chosen as an appropriate solvent for use in the bacterial mutation test, where the standard limit dose of 5 mg/plate could be achieved using a dose volume of 100 μL of a 50 mg/mL solution.

Because Compound X was also to be tested in the *in vitro* chromosome aberration test, the compatibility of DMSO solutions of X was assessed in RPMI culture medium complete with serum (i.e., the culture medium that would be used in the test) as recorded in the table below. Culture medium was added in 2 mL aliquots to each of the 10 wells (numbered 1−10) of a 24-well microplate. A substantial amount of precipitation was seen after addition of 20 μL of the 100 mg/mL stock solution to 2.0 mL culture medium, so 10-fold and 100-fold dilutions of the stock solution were evaluated in the same way. Because precipitation was seen with the 10 mg/mL solution but not the 1 mg/mL solution, an intermediate concentration was also evaluated. The pH of wells showing any apparent

change in medium indicator color was measured. The plate was then incubated under the same conditions to be used for the subsequent chromosome aberration test to confirm that there was no change in solubility. Based on these observations, a top dose level of 2560 μg X per mL in culture medium was selected because this would show some precipitation in the culture medium (saturated solution to maximize exposure to the test article). Lower concentrations separated by an interval of two were also evaluated to ensure the dose range included at least one or two doses showing little or no toxicity.

Test Item: X **Phase: Compatibility with culture medium** **Vehicle:** DMSO **Reference no.:** 12345

		Init.	Date	
Medium type (complete) RPMI				**Special instructions:** None
Micropipette ID TI-234				
pH meter ID TI-456				0 Pre-incubation / Post-incubation

Well no.	Culture vol **Q** mL	Dose vol. **R mL**	Dilution factor **S = Q / R**	Volume for dilutions mL: Formulation	Volume for dilutions mL: vehicle	Formulation conc. **T** μg/mL	Final conc. **T/S** μg/mL	Pre-incubation: Medium color	Pre-incubation: pH†	Pre-incubation: observations	Post-incubation: Medium color	Post-incubation: pH†	Post-incubation: observations
1	2.0	0.020	100	-	-	100000	1000	orange	6.8	ppt	N	ND	ppt
2	2.0	0.020	100	0.100	0.900	10000	100	red/orange	6.9	ppt	N	ND	ppt
3	2.0	0.020	100	0.010	0.990	1000	10.0	N	ND	N	N	ND	-
4	2.0	0.020	100	0.025	0.975	2500	25	N	ND	sl. ppt	N	ND	sl. ppt
5													
6													
7													
8													
9													
10													
Init./Date:											Init./Date		

Key:
† measured if change in indicator color
N normal, ND not determined
ppt precipitate, sl. ppt slight precipitate

Reviewed by/date:

3.7 Formulation of Dose Solutions

Test article is usually dispensed in a powder containment cabinet (or, in the case of volatiles, in a fume hood). Clean or aseptic technique is used throughout. Formulations are prepared and diluted in the powder containment cabinet or a class 2B biological containment cabinet. The stock formulation is prepared by combining the test article with the vehicle, often at the concentration required by the high dose level (e.g., typically 50,000 μg/mL in the case of a bacterial mutation test using a dose volume of 100 μL per plate).

Suggested steps in preparation of solutions and all formulations for use in genotoxicity tests are outlined here. Although the procedural checks may seem lengthy at first glance, they minimize the chance of errors in the formulation and standardize routine calculations.

1. All formulation containers and any vials used for sampling them should be sterile (in the case of *in vitro* tests and for nonoral dosing in the case of *in vivo* studies) and uniquely identified with details of their contents (study number, date, compound name, and concentration). It is often convenient to assign code names or abbreviations to compounds and numbered dose levels (rather than absolute concentrations) for documentation and labeling—these codes should be explained in the formulation records. The stock (usually the high dose level) formulation and dilutions are usually prepared in a suitable glass bottle/vial of adequate volume to accommodate the subsequent formulation.
2. Prior to formulation, two appropriate aliquots of the vehicle should be removed. One of these is the vehicle control and should be set aside (after sampling, if required) to avoid potential contamination. The second aliquot is dispensed in appropriate volumes into the vials for subsequent preparation of lower dose levels by direct dilution from the stock test article formulation.
3. The test article container should be allowed to come to room temperature and then the gross weight should be recorded in the utilization log.
4. The gross weight of the stock formulation vial is measured and recorded.
5. A stainless-steel spatula of a suitable size is wiped with a medical wipe that has been made wet with 70% alcohol (ethanol or isopropanol, 2-propanol). The spatula is used to transfer approximately the minimum required amount of test article to the stock formulation vial. The vial is recapped and weighed to determine the net weight of test article.
6. If less than the required amount of material has been dispensed, then an additional amount is added as noted and the vial is reweighed.
7. The test article container is reweighed and the weight is recorded in the utilization log as a double-check of the amount of material dispensed. The weight of material used should be similar or slightly more than the net weight of material dispensed; any departure from this due to spillage should be recorded in the formulation record.

8. The required calculated volume of vehicle is added and the vial is reweighed to determine the net weight of vehicle added. A gravimetric check of this addition is performed using the formula:
   $(FN - N + W) + (TV \times SGV) + 1000$
   Where:
   FN = Final gross weight of formulation vial
   N + W = Weight of container + test article
   TV = Total volume of vehicle added
   SGV = Specific gravity of vehicle
9. The stock formulation should be mixed until dissolved or (in the case of suspensions) apparently homogenous. Vortex mixing, warming in a water bath, or placing in a sonic bath may be used to enhance dissolution, in which case the details should be recorded. If a stock solution is not expected to be sterile (e.g., in the case of a biological material obtained by fermentation), then it should be filtered/sterilized at this point.
10. All lower-dose formulations are prepared by direct dilution from this stock followed by mixing. When the range of concentrations is very wide, it may be appropriate to prepare the lowest dose levels by dilution from an intermediate dose level. Note that serial dilution is best avoided because this results in compound error, which is especially marked in the case of suspensions.
11. If chemical analysis of formulations is required, then any analytical and retention samples are aliquoted into labeled sterile containers at this point. Because these samples will be transferred to another laboratory or facility, labeling should be comprehensive and include (as a minimum) the study number, test material name, concentration, preparation and sampling dates, volume, site of sample (top, middle, or bottom) in the case of suspensions, and storage conditions. The samples should be accompanied by a form that is used to record the chain of custody. We recommend taking analytical samples in duplicate with back-up (retention) samples taken in triplicate, especially for suspension formulations. The analytical samples will be sent to the analytical laboratory and the back-up samples should be retained by the formulating laboratory for potential analysis (e.g., in case of loss or degradation during transfer or for confirmatory testing in the event of unexpected analytical results for the main samples).
12. The retention samples may be discarded following confirmation of acceptable results of the main samples.

In the Formulation Instruction/Record Sheet, appropriate details have been entered in advance by the responsible scientist. Additional entries made at the time of formulation are entered in the grey cells:

Example Formulation Sheet

Phase: Main Test **Study No.: ABC 123**
Special instructions: **Samples req., take vehicle samples before weighing out compound.**

Test Item: **TI X** **Vehicle:** **Dimethyl sulfoxide**
Lot: **A345** Abbreviation: DMSO
Appearance: **White powder** Supplier:
Purity/corr. factor: **1.0** Lot no.:
Theoretical SG: **Not applicable** Expiry date:
Storage:
Appearance:
Specific gravity **SGV**: **1.10**

Balance ID	Micropipette IDs

Preparation Stock 50 mg/mL DN8 Net weight vial **N mg**
Minimum weight of TI required (in mg) = **600** Weight TI **W mg**:
Vol. DMSO to add in mL = mg of TI ÷ **50.0** Vol vehicle **V mL**
Target final volume (mL): = W ÷ 50
Measured volume (mL): must be between 95% and 105% of target
Final wt of vial with TI + vehicle, **FN mg**:
Check, (**FN-N-W**) / (**TV** × **SGV**) /1000 must be between 0.95 and 1.05
Appearance of DN8

Dose No.	Formulation conc µg/mL	Vol vehicle mL	Vol DN8 mL	Total mL	*Appearance**
7	**15800**	**6.5**	**3.00**	**9.5**	
6	**5000**	**8.6**	**0.950**	**9.5**	
5	**1580**	**9.2**	**0.300**	**9.5**	
4	**500**	**9.4**	**0.095**	**9.5**	
3	**158**	**9.5**	**0.030**	**9.5**	
2	**50**	**9.5**	**0.0095**	**9.5**	
1	**15.8**	**25.0**	**0.0079**	**9.5**	

Performed by/date:

* Color and description for stock, description only for dilutions:
CL (clear liquid), SUS (suspension) or if other then describe

All formulations & vehicle prepared in glass vials & stored RT/Dark for 1 day init./date
Storage ID:
Residual formulations discarded, init./date:
Comments:

3.8 Formulation of Bulk Formulations

In the case of *in vivo* studies where formulations are usually prepared in relatively large volume, it may be easier to bring the formulation to volume in a calibrated vessel (e.g., a measuring cylinder or a calibrated beaker). However, in the case of suspensions especially, there may be some loss of material due to adherence to the measuring vessel, in which case additional precautions may be needed to ensure quantitative transfer of the test article to the final formulation container. For animal studies using suspensions, each dose level is generally prepared independently because of potential error due to adherence of the test article to formulating vessels if prepared by dilution.

If the positive control formulations for *in vivo* studies are prepared by the central pharmacy, then they should follow a protocol (SOP) appropriate for cytotoxic drugs that has been reviewed by the responsible genetic toxicology scientist for safety and accuracy reasons. Some agents (e.g., mitomycin C) are supplied in prealiquoted injectable vials that should be reconstituted by addition of an appropriate volume of solvent and do not require weighing out, thus reducing the chance of error and chemical contamination.

3.9 Formulation of Suspensions

The vehicle for formulation of emulsions (liquid in liquid preparations) may involve a combination of an aqueous suspending agent with a surfactant (emulsifying agent). Emulsions can usually be prepared and diluted, if necessary, in the same way as solutions provided that appropriate procedures are in place to ensure homogeneity (e.g., by mixing using a high-shear homogenizer at the time of preparation). Some emulsions may be unstable and separate ("crack") prior to dosing unless stirred continually. If a cracked emulsion cannot be resuspended by simple methods (e.g., multiple in version of the container) at the time of dosing, then it would normally be considered an inappropriate formulation.

In the case of solid test articles, the material must be a finely ground powder with no large particulate present. If this is not the case, then the test article will need to be ground prior to formulation. This can be done using a ball mill or a mortar and pestle.

3.9.1 Aqueous Suspending Agents

The most common suspending agents are aqueous biological polymers, including methylcellulose (MC), sodium carboxymethylcellulose (CMC), and

hydroxypropylmethylcellulose (HPMC). A range of viscosities of suspending agents is available with different molecular weights. In particular, aqueous 1% w/v MC 400 cP has many of the characteristics of an ideal vehicle:

1. Clear, colorless
2. Ideal viscosity; the most commonly used MC has a dynamic viscosity of 400 cP (centipoise) at 2% w/v in water; in comparison, water has a viscosity of approximately 1 cP at room temperature
3. Biologically inert
4. Easily sterilized
5. Not ionic
6. Can be used in combination with surfactants such as Tween 80 to improve wetting and enhance physical stability
7. Is suitable for use in long-term studies
8. Is chemically defined
9. Does not interfere with chemical analysis

Aq. 1% w/v MC can be prepared as follows:

1. Add the required amount of UPW into an appropriate glass Erlenmeyer flask with a stir bar
2. Heat the water until boiling
3. Remove from the heat and allow to cool for 5 min
4. While stirring rapidly, add the appropriate amount of methyl cellulose 400 cPs *gradually* to avoid boil out
5. Transfer the solution and stir bar to a glass bottle with a capacity of twice the volume of the MC and loosely tighten the cap
6. Autoclave the solution at 121°C, 15 psi for 20 min
7. After sterilization, remove the bottle(s) from the autoclave and stir until cold
8. Label the bottle appropriately and store refrigerated
9. Assign an expiration date of 3 months, but note that aq. MC is subject to fungal growth, so any residual amount should be discarded after use

As an alternative to these types of suspending agents, aqueous solutions of ambiphilic cyclodextrins can be used to solubilize hyrophobic compounds. These are occasionally used in toxicology studies and will probably become more widely used in the future.

3.9.2 Large Volume Suspensions

Suggested steps in formulation of large-volume suspensions of a powder in an aqueous suspending agent, as commonly needed for *in vivo* studies, are outlined here.

1. The appropriate amount of test article is dispensed onto a weighing boat (or paper) and then transferred to a mortar. Enough vehicle is added to the weighing boat to wash out any residual test article and is combined with the test article in the mortar. The test article and vehicle are mixed by grinding using a pestle. Additional vehicle is added to form a paste and grinding is continued until a smooth paste is formed. This process is referred to as "trituration" and not only mixes the materials without caking but also helps to reduce the size of aggregates and larger crystals to improve homogeneity.
2. The final required volume of the formulation (weight of test article ÷ required concentration) is calculated and then this volume of purified water is added to a clean glass beaker. The beaker is placed on a level surface and the level of the bottom of the meniscus is marked on the side of the beaker using a marker pen. The water is discarded and the beaker is dried.
3. The paste is transferred to the calibrated beaker and then additional aliquots of the vehicle are added to the mortar and pestle to wash any residual test article into the beaker.
4. Once a sufficient volume of vehicle has been added to reach the calibration mark on the side of the beaker, following gravimetric check if appropriate, the formulation is mixed thoroughly using a high-shear homogenizer (e.g., Polytron, Silverson, or Ultra-Turrax) fitted with a probe of an appropriate size.
5. The mixture is transferred to a labeled clean glass container with a sealable lid. The formulation will usually contain a large number of small bubbles that disappear over time or can be removed by placing the formulation briefly under vacuum.
6. The formulation should be checked for dosability by passing a part through the system that will be used for dosing (e.g., usually a cannula or injection needle in the case of animals studies). If the suspension is not fine enough, then it will either block the cannula or lead to formation of aggregates.
7. Typically, suspensions are stored (often refrigerated) prior to and between use, which helps maintain chemical stability and minimize any microbial growth. During this time, most suspensions will settle and solid material will aggregate ("cake") on the bottom of the storage container. Therefore, any such caked material should be thoroughly dispersed by multiple inversion and gentle shaking. Once dispersed, homogeneity should be maintained by continual shaking, multiple inversion, or stirring on a stir plate.
8. Care should be taken to avoid introduction of bubbles into the formulation just prior to use. If maintained on a stir plate, then it may still be necessary to invert the container several times prior to stirring if solid material is seen to accumulate around the lower inside edge of the formulation container.

3.9.3 Small Volume Suspensions

Very occasionally, for *in vitro* studies where a compatible solvent cannot be found, it may be necessary to formulate the material as a suspension. If the material is in the form of a homogenous fine powder with lumps or large crystals, then it can usually be formulated in a manner similar to that of small-volume solutions. To ensure homogeneity prior to use, the formulation should be thoroughly dispersed by multiple passage into a syringe fitted with an 18-G needle and then, ideally, into a syringe fitted with a 19-G or 20-G needle. If the formulation does not readily pass through the needle, then additional actions will be needed to reduce the particulate size and avoid formation of aggregates, including grinding and use of a high-shear homogenizer.

Small-volume suspensions should be thoroughly dispersed by vortex mixing and multiple inversion prior to and during sampling and/or dosing to ensure homogeneity.

3.10 Chemical Analysis and Stability

As per GLP guidelines, each test article mixed with a vehicle must be tested by appropriate validated analytical methods to determine the stability of the test article in the mixture under the conditions of storage and use in the study. This can be done either before study initiation or concomitantly. Formulations should be tested to confirm achieved concentration; additionally, suspensions should be tested for homogeneity and physical stability. Sample volumes should be representative and should not exceed those used for dosing on the study. The volumes of material involved can be substantial and must be taken into account when calculating test article and formulation requirements. If the decision is made to not include formulation analysis in the study, then this should be mentioned in the final report as a deviation to GLP. In that case, it is particularly important to describe any other factors that support achieving appropriate levels in the test system, including precipitate and signs of toxicity. A typical approach to chemical analysis is described here.

1. Development of the analytical method is necessarily non-GLP and is usually performed at the very start of the toxicology program. Ideally, the method should be specific, sensitive, robust, and reliable and should show a dose-proportionate response (linearity) over a reasonable concentration range.
2. The analytical method validation is used to confirm the suitability of the analytical method over the required concentration range for each of the formulations needed. Reliability and procedural recovery should be established at this point. The analytical method will then be used to confirm chemical stability of formulations over the period of storage and use and, in the case of suspensions, their homogeneity and physical stability. The validation should support the full range of concentrations being used in all of the studies. In the case of suspension formulations, the method will then be used

to confirm that the formulation method produces appropriately homogenous and physically stable formulations. It is therefore vital for the study scientist to communicate directly with the analytical chemist to ensure all required aspects are covered; otherwise, additional validation work (involving additional costs) will be required later. The validation should cover expected storage and use conditions (e.g., refrigerated and room temperature). The results of analysis at time zero are compared with analyses at periodic intervals after preparation (e.g., 48 h, 7, 14, and 28 days). In the case of suspensions, appropriate procedures are used to resuspend the material (e.g., multiple inversion followed by continuous stirring prior to and during sampling).

3. Duplicate samples are normally required for chemical analysis of solutions. One of these is subjected to analysis while the second acts as a backup in case of technical problems.
4. In the case of suspensions, samples are collected from the top, middle, and bottom of the formulation. Ideally, the sample size should be representative of the volume being dosed, and five samples should be collected at each level. Two of these are subjected to analysis while the remaining triplicate samples act as backup in case of problems with the initial analysis (e.g., loss or degradation of original samples, suspected technical error during original analysis, etc.).
5. The number, volume, net weight, and location of all samples should be recorded. The net weight of suspension samples should be used to confirm that the sample volume was accurate. Main samples should be sent to the analytical laboratory (which can often be at a different facility/site) with a copy of the formulation and sample details, whereas the backup samples should be retained in the formulation laboratory to minimize the potential impact of loss or deterioration of samples during transport. Again, it is important for the Study Director to communicate directly with the analytical chemist (designated as Principal Investigator in the case when the analysis is performed off-site) whose contact details should be included in the protocol. The analytical chemist should be sent copies of the protocol and any amendments.
6. If chemical analysis of formulations has demonstrated adequate stability of formulations, then *it is highly recommended* that formulations should be prepared in advance of use and chemical analysis should be used to confirm their suitability confirmed prior to dosing. This will minimize the chance of rejection of part of the study and, in the case of *in vivo* studies, waste of animals.
7. Suspension samples should be diluted in their entirety rather than subsampled to avoid procedural loss.
8. In the case that an Out of Specification (OOS) result is obtained following dose samples analysis, an investigation should be initiated and recorded. Following the completion of the investigation, the Study Director should decide and document an appropriate course of action.

Compatibility of the formulation with storage containers and administration devices (e.g., infusion lines) should be established prior to the study. For infusion studies in particular, especially at low concentrations, it may be necessary to flush the system with the formulation or increase the concentration of the formulation above nominal to ensure the final required concentration is achieved.

Example of a sampling form

Test Item:	**Phase: Main**	**Vehicle: DMSO**	**Study/Reference no.:**	**Init./date**
Mix formulations by multiple inversion then using a stir bar prior to sampling.				
Analytical samples				—
Vehicle:	2 × 1.00 mL	Remove into glass vials.		
Doses	1 to 10:	Remove 1 × 500 µL sample(s) into glass vials.		
Leave the samples alongside the formulations until the end of dosing.				
Store samples in the formulation laboratory at RT/Dark until shipment. Storage ID:_____				
Ship analysis samples at ambient/Dark to the attention of PI (see protocol).				
Retention samples				—
Vehicle:	2 × 1.00 mL	Remove into glass vials.		
Doses	1 to 10:	Remove 1 × 0.5 mL sample(s) into glass vials		
Leave the samples alongside the formulations until the end of dosing.				
Store retention samples in formulation laboratory at RT/Dark until authorization to discard is obtained from SD. Storage ID:_____				
Sample(s) IDs:_____ transferred for analysis as indicated for analysis samples. Indicate N/R if not required:				
Comments:				
			Form prepared	
			Form verified	
			Form reviewed & approved	
			SD approval to discard backup samples	
			Backup samples discarded	

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