OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Draft Updated Subchronic Inhalation Toxicity: 90-Day Study

(Tracked changes mode text is new or revised text since the previous version)

SUMMARY

1. This revised Test Guideline 413 (TG 413) is designed to fully characterize test chemical toxicity by the inhalation route for a subchronic duration (90 days), and to provide robust data for quantitative inhalation risk assessments. The primary impetus for revising this test guideline was to accommodate the testing of nanomaterials. The actual design of a main study depends on the physical form of the test chemical (gas, vapour, or aerosol) and whether a range-finding study reveals that the test chemical is likely to be biopersistent in the lung and/or cause long-term lung effects. A main study is typically performed in the more sensitive sex (usually males), but both sexes may be used if justified. Groups of rodents are exposed 6 hours per day during a 90 day (13 week) period to the test chemical at three or more concentration levels, and to filtered air (negative control) and/or the vehicle (vehicle control). Animals are generally exposed 5 days per week but exposure for 7 days per week is also allowed. The main study is typically performed in the more sensitive sex, as determined from the range-finding study. Range-finding and main studies may also include one or more post-exposure periods. The duration of the post-exposure period(s) and the nature and timing of sampling intervals will be determined by the study director based upon the results of a range-finding study. Bronchoalveolar lavage is always measured when testing gases, vapours, and aerosols. The main study should also include lung burden measurements when a range-finding study demonstrates that a test chemical is likely to be retained in the lung. This guideline also allows the study director the flexibility to include satellite groups and interim sacrifices, as well as optional health assessments such as chemical toxicokinetics, and/or systemic toxicity evaluations such as immune, hepatic, neurologic and/or cardiovascular effects evaluations to better characterize the overall toxicity of a test chemical. Further considerations and guidance on including additional observations and measurements can be found in a revised edition of Guidance Document 39 on Inhalation Toxicity Testing (1).

INTRODUCTION

2. OECD Guidelines are periodically reviewed in the light of scientific progress in evaluating toxicological responses, animal welfare considerations, and changing regulatory needs. The original subchronic inhalation Test Guideline 413 (TG 413) was adopted in 1981 (1). It was revised in 2009 to reflect the state of the science and to meet current and future regulatory needs. The primary impetus for this latest revision was to accommodate the testing of nanomaterials. It is noted that nanoparticles and fine particles coexist as a continuum and that engineered nanoparticles can commonly consist of aggregated / agglomerated structures rather than as isolated nanoparticles. The most notable features of this latest version are as follows:

- This revision requires evaluations of bronchoalveolar lavage (BAL) when testing gases, vapours, and aerosols. Measurements of lung burden are also required when a range-finding study demonstrates that a test chemical is likely to be retained in the lung. BAL and lung burden measurements occur during one or more post-exposure intervals. The duration of the post-exposure period(s) and the timing of the post-exposure intervals are determined based upon results from the range-finding study.

- There is an emphasis on using comprehensive range-finding studies to provide the information necessary to design a robust main study and post-exposure period(s). When determined by the study director, the range-finding study may include evaluations of BAL, lung burden, pulmonary function, body temperature, and gender sensitivity.

- Whereas the 2009 version of TG 413 always tested in both sexes, this revision allows for testing in only the more sensitive sex in the interest of minimizing animal usage. Both sexes may be tested if justified.

The 2009 version of TG 413 required a mass media aerodynamic diameter (MMAD) of 1-3 μm with a geometric standard deviation (σg) of 1.5 to 3.0. Not only did this criteria did not allow for the testing of particles in the nanometer range, but it allowed for the testing of particles too large to reach the lungs of rodents. When particles cannot reach the alveoli, the toxicity of a test chemical can be understated. These deficiencies have been addressed by the new recommended particle size criteria of an MMAD of <2 μm with a σg of <3.

3. Subchronic inhalation toxicity studies are primarily used to derive regulatory concentrations for assessing worker risk in occupation settings. They are also used to assess human residential, transportation, and environmental risk. This guideline enables the characterization of adverse effects following repeated daily inhalation exposure to a test chemical for 90 days (approximately 10% of the lifespan of a rat). The data derived from subchronic inhalation toxicity studies can be used for quantitative risk assessments and for the selection of concentrations for chronic studies. Definitions of technical terms used in the context of this Test Guideline can be found in GD 39 (1).

INITIAL CONSIDERATIONS

4. All available information on the test chemical should be considered by the testing laboratory prior to conducting the main study in order to enhance the quality of the study, minimize animal usage, and avoid the need to repeat the study. Information that will assist in the selection of appropriate test concentrations might include the identity, chemical structure, and physico-chemical properties of the test chemical, results of any *in vitro* or *in vivo* toxicity tests, anticipated use(s) and potential for human exposure, available (Q)SAR data and toxicological data on structurally related substances, and data derived from other repeated exposure studies. When testing an aerosol, it is important to understand particle solubility and kinetics before performing a main study. If systemic toxicities (e.g., immunotoxicity, neurotoxicity, hepatotoxicity, cardiovascular are expected or are observed in the course of the study, the study director may choose to include appropriate evaluations such as a functional observational battery (FOB), measurement of motor activity, or other relevant toxicological assessments. Although the timing of exposures relative to specific examinations may be critical, the performance of these additional activities should not interfere with the basic study design.

5. If the test chemical is a nanomaterial, the specific physicochemical and morphological/dimensional characterizations are to be determined to the extent possible, along with a characterisation of solubility. Prior to conducting these characterizations, the study director should consult the Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials (3) and the Guidance Document on Sample Preparation and Dosimetry (4). In addition, each type of nanomaterial may require particular testing such as a determination of the deposition fraction in the respiratory tract (refer to paragraph 27 for additional guidance on estimating the thermodynamic diameter), solubility in biological fluids or environments, post-exposure observation for lung and pleural retention/accumulation, translocation, carry-over effects, etc.

6. Due to their physical and chemical properties, poorly soluble nanomaterial particles may exhibit 'biopersistence,' a property defined by the ability of particles to persist and/or accumulate in biological systems because of their inability to dissolve in biological fluids (e.g., fluids present in gastrointestinal tissues, lymphatics, and lung) and/or biological environments such as intracellular organelles (e.g., endosomes, phagolysosomes) and/or evade or inhibit biological clearance pathways (5) (6). Due to the potential for inhaled nanomaterials to exhibit biopersistence, they may accumulate in the lung as well as distribute broadly within the organism, and may thus require the need to measure lung burden and assess systemic toxicological effects (e.g., cardiovascular, hepatic, neurotoxicity, and immunotoxicity). This may be especially important for long-term studies where low levels of poorly soluble nanomaterials may undergo extrapulmonary translocation over a prolonged period of time. Robust lung burden information is critically needed for quantitative risk assessment determination to eliminate the potential for false negatives and to ensure pulmonary responses are due to either the specific properties of the test particles or to a non-specific response caused by an overload of low toxicity particles. Finally, information on nanomaterial lung deposition (lung burden), clearance pathways, and their fate can aid in the use of read-across between different nanomaterials as well as provide accurate information for developing nanomaterial toxicokinetic models associated with inhalation exposure to these novel chemicals. Further guidance is provided in GD 39 (1).

7. Dilutions of corrosive or irritating test chemicals may be tested at concentrations that will yield the desired degree of toxicity [refer to GD 39 (1)]. When exposing animals to these materials, the targeted concentrations should be low enough to not cause marked pain and distress, yet sufficient to extend the concentration-response curve to levels that reach the regulatory and scientific objective of the test. These concentrations should be selected on a case-by-case basis, preferably based upon an adequately designed range-finding study that provides information regarding the critical endpoint, any irritation threshold, and the time of onset (see paragraphs15-17). The justification for concentration selection should be provided.

8. Animals exposed at test chemical concentrations that cause sensory irritation of the upper or lower respiratory tract may experience reflex bradypnea or a Paintal (C-fiber stimulation) reflex, respectively. Because these protective reflexes can result in marked decreases in body temperature, minute volume, and test chemical exposure, it is recommended that pulmonary function and body temperature be periodically measured when testing respiratory irritants to identify and quantify these reflexes. Further information on these reflexes can be found in GD 39 (1).

9. Moribund animals or animals obviously in pain or showing signs of severe and enduring distress should be humanely sacrificed. Moribund animals are considered in the same way as animals that die on test. Criteria for making the decision to sacrifice moribund or severely suffering animals, and guidance on the recognition of predictable or impending death, are the subject of OECD Guidance Document 19 on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints, GD 19 (7).

DESCRIPTION OF THE METHOD

Selection of Animal Species

10. Healthy young adult rodents of commonly used laboratory strains should be employed. The preferred species is the rat. Justification should be provided if other species are used.

Preparation of Animals

11. Females should be nulliparous and non-pregnant. On the day of randomization, animals should be young adults 7 to 9 weeks of age. Body weights should be within ±20% of the mean weight for each sex. The animals are randomly selected, marked for individual identification, and kept in their cages for at least 5 days prior to the start of the test to allow for acclimatization to laboratory conditions.

Animal Husbandry

12. Animals should be individually identified, preferably with subcutaneous transponders, to facilitate observations and avoid confusion. The temperature of the experimental animal maintenance room should be 22±3°C. The relative humidity should ideally be maintained in the range of 30 to 70%, though this may not be possible when using water as a vehicle. Before and after exposures, animals generally should be caged in groups by sex and concentration, but the number of animals per cage should not interfere with clear observation of each animal and should minimize losses due to cannibalism and fighting. When animals are to be exposed nose-only, it may be necessary for them to be acclimated to the restraining tubes. The restraining tubes should not impose undue physical, thermal, or immobilization stress on the animals. Restraint may affect physiological endpoints such as body temperature (hyperthermia) and/or respiratory minute volume. If generic data are available to show that no such changes occur to any appreciable extent, then pre-adaptation to the restraining tubes is not necessary. Animals exposed whole-body to an aerosol may be housed individually during exposure to prevent them from filtering the test aerosol through the fur of their cage mates. Conventional and certified laboratory diets may be used, except during exposure, accompanied with an unlimited supply of municipal drinking water. Lighting should be artificial, the sequence being 12 hours light / 12 hours dark.

Inhalation Chambers

13. Subchronic inhalation toxicity studies are always performed in dynamic chambers; the use of a static chamber is not acceptable. The nature of the test chemical and the objective of the test should be considered when selecting an inhalation chamber. The preferred mode of exposure is nose-only (which term includes head-only, nose-only, or snout-only) for studies of liquid or solid aerosols and for vapours that may condense to form aerosols. Special objectives of the study may be better achieved by using a dynamic whole-body mode of exposure, but this should be justified in the study report. To ensure atmosphere stability when using a whole-body chamber, the total volume of the test animals should not exceed 5% of the chamber volume. Principles of the nose-only and whole body exposure techniques and their particular advantages and disadvantages are addressed in GD 39 (1).

TOXICITY STUDIES

Limit Concentrations

14. The maximum concentration tested should consider: 1) the maximum attainable concentration, 2) the “worst case” human exposure level, 3) the need to maintain an adequate oxygen supply, and/or 4) animal welfare considerations. In the absence of data-based limits, the acute limits of the United Nations Globally Harmonized System (GHS) of Classification and Labelling of Chemicals may be used (i.e., 0.2 mg/L for aerosols, 1.0 mg/L for vapours, and 250 ppmV for gases). Justification should be provided if it is necessary to exceed these limits when testing gases or highly volatile test chemicals (e.g. refrigerants). The maximum concentration tested should elicit unequivocal toxicity without causing undue stress to the animals or affecting their longevity (7).

Range-Finding Study

15. The design of the main study is greatly dependent on information learned during a range-finding study. A range-finding study should always be performed unless sufficient information already exists to perform a robust main study. A range-finding study may, for example, provide information regarding analytical methods, particle sizing, systemic toxicity, kinetics, translocation of particles, discovery of toxic mechanisms, clinical pathology, histopathology, biomarkers of lung injury, gender sensitivity, BAL data, test chemical solubility in the lung, and estimations of what may be the No Observed Adverse Effects Concentration (NOAEC) and Maximum Tolerated Concentration (MTC) in a main study. The study director may use a range-finding study to identify test chemical concentrations that elicit reflex bradypnea or a Paintal (C-fiber stimulation) reflex due to respiratory tract irritation (e.g. with pulmonary function testing, body temperature, bronchoalveolar lavage, and/or histopathology of the respiratory tract), the upper concentration which is tolerated without undue stress to the animals, and the parameters that will best characterize a test chemical's toxicity. During a range-finding post-exposure period, BAL could be periodically measured when testing a gas, vapour, or aerosol (e.g., dust, mist, smoke, fume, fog, or smog). When testing an aerosol, test chemical solubility and lung burden should be periodically measured during a post-exposure period to inform the duration of the main study post-exposure period and the spacing of post-exposure sampling intervals. The range-finding study may be used to determine whether one sex is more sensitive to the test chemical. The more sensitive sex should be evaluated during the exposure and post-exposure period of the main study.

16. A range-finding study may consist of one or more test chemical concentration levels and a control group. Depending on the endpoints chosen, no more than 5 males and 5 females should be exposed at each concentration level. A range-finding study may last a minimum of 5 days and generally no more than 28 days, and may include a post-exposure period. When testing poorly soluble particles, it may be necessary for a range-finding study to be longer than 28 days to allow for a robust assessment of test chemical solubility and lung burden. The rationale for the selection of concentrations for the main study should be provided in the study report. The objective of the main study is to demonstrate a concentration-response relationship based on what is anticipated to be the most sensitive endpoint. The low concentration should ideally be a NOAEC while the high concentration should elicit unequivocal toxicity without causing undue stress to the animals or affecting their longevity (7).

17. When selecting concentration levels for the range-finding study, all available information should be considered including structure-activity relationships and data for similar chemicals (see paragraph 4). A range-finding study should investigate likely mechanistically based endpoints, e.g. cholinesterase inhibition by organophosphates; methaemoglobin formation by erythrocytotoxic agents; thyroidal hormones (T_3, T_4) for thyrotoxicants ; or protein, LDH, or neutrophils in bronchoalveolar lavage for innocuous poorly soluble particles or pulmonary irritant aerosols. A range-finding study might also inform a decision on measurements of extra-pulmonary organ burdens.

Main Study

18. The design options for the main toxicity study are illustrated in Annex 1, including the number and sex of animals used and the parameters measured at each of the post-exposure intervals. If one sex is known to be more sensitive to a given test chemical, the more sensitive sex should be tested. If both sexes are equally sensitive, then only males should be tested (as shown in Annex 1). When a test chemical is likely to cause dissimilar responses in males and females (e.g. an estrogenic pesticide), it may be appropriate to include both sexes in the main study; in which case the sexes may be exposed

at different concentration levels in order to optimize the concentration-response as described in paragraph 16. The study director must provide justification for testing both sexes.

19. The main study generally consists of three test chemical concentration levels, and also concurrent negative (air) and/or vehicle controls (see paragraph 24). Information from a range-finding study and all other available data should be utilized in designing the study and in the selection of appropriate exposure levels (paragraphs 15-17). Each group of test animals is exposed to the test chemical for 6 hours per day on a 5 day per week basis for a period of 13 weeks (total study duration of at least 90 days). Animals may also be exposed 7 days per week. If rodent species other than rats are exposed nose-only, maximum exposure durations may be adjusted to minimise species-specific distress. A rationale should be provided when using an exposure duration less than 6 hours/day, or when it is necessary to conduct a long duration (e.g. 22 hours/day) whole-body exposure study (refer to GD 39) (1). Feed should be withheld during the exposure period unless exposure exceeds 6 hours. Water may be provided throughout a whole-body exposure.

20. The target concentrations selected should identify the target organ(s) and demonstrate a clear concentration-response:

- The high concentration level should result in a clear level of toxicity but not cause lingering signs or lethality that could prevent a meaningful evaluation. When testing aerosols, the high concentration may be the maximally achievable level that can be reached while meeting the particle size distribution criteria (see paragraphs 26 and 27).

- The intermediate concentration level(s) should be spaced to produce a gradation of toxic effects between that of the low and high concentrations.

- The low concentration level, which will ideally be a NOAEC, should produce little or no evidence of toxicity.

Interim Sacrifices

21. If interim sacrifices are planned during the main study exposure period, the number of animals at each exposure level should be increased by the number to be sacrificed before study completion. The rationale for using interim sacrifices should be provided, and statistical analyses should properly account for them.

Post-Exposure Period and Satellite Groups

22. Satellite (reversibility) animal groups may be added to the main study to observe reversibility, persistence, or delayed occurrence of toxicity during a post-exposure period, and also to measure BAL and aerosol lung burden. The study director should define the duration of the post-exposure period based on a range-finding study, but the duration should be at least 14 days. Satellite groups may be sacrificed at one or more post-exposure intervals (PEIs). Satellite groups consist of 5 animals/concentration, and they should be the same sex as in the main study (see paragraph 19). These groups are exposed contemporaneously with the experimental animals in the main study. They should be exposed at the same concentration levels as the main study groups, and there should be concurrent air and/or vehicle controls as needed (see paragraph 24).

23. When testing gases, vapours, and aerosols, satellite groups are required for BAL measurements during the post-exposure period when the range-finding study shows that the test chemical is biopersistent in the lungs and/or is likely to cause long-term lung effects (see paragraph

47). Satellite groups are also required for lung burden measurements when testing aerosols that are likely to be retained in the lungs (see paragraph 49).

Control Animals

24. Concurrent negative (air) control animals should be handled in a manner identical to the test group animals except that they are exposed to filtered air rather than test chemical. When water or another substance is used to assist in generating the test atmosphere, a vehicle control group, instead of a negative (air) control group, should be included in the study. Water should be used as the vehicle whenever possible. When water is used as the vehicle, the control animals should be exposed to air with the same relative humidity as the exposed groups. The selection of a suitable vehicle should be based on an appropriately conducted pre-study or historical data. If a vehicle's toxicity is not well known, the study director may choose to use both a negative (air) control and a vehicle control, but this is strongly discouraged. If historical data reveal that a vehicle is non-toxic, then there is no need for a negative (air) control group and only a vehicle control should be used. If a pre-study of a test chemical formulated in a vehicle reveals no toxicity, it follows that the vehicle is non-toxic at the concentration tested and this vehicle control should be used.

EXPOSURE CONDITIONS

Administration of Concentrations

25. Animals are exposed to the test chemical as a gas, vapour, or aerosol (e.g., dust, mist, smoke, fume, fog, or smog), or a mixture thereof. The physical state to be tested depends on the physico-chemical properties of the test chemical, the selected concentrations, and/or the physical form most likely present during the handling and use of the test chemical. Hygroscopic and chemically reactive test chemicals should be tested under dry air conditions. Care should be taken to avoid generating explosive concentrations. To comply with the particle size criteria described in paragraph 26, solid materials may be subjected to mechanical processes to decrease the particle size. Further guidance is provided in GD 39 (1).

Aerosol Particle-Size Distribution

26. Aerosol particle size determines the site of initial deposition in the respiratory tract and thus can significantly impact the outcome of inhalation toxicity studies. Ideally the particle size distribution of the aerosol to which animals are exposed should be similar to those present in relevant human exposure scenarios, but adjusted for rodent inhalability and respirability to allow appropriate exposure of all relevant regions of the respiratory tract. For particles primarily of concern in relation to exposure of the lower respiratory tract, the particle size distribution should meet the following recommended criteria: Mass Median Aerodynamic Diameter (MMAD) < 2 µm with a $\sigma g < 3$ (see GD 39). Although a reasonable effort should be made to meet these criteria, expert judgement should be used if they cannot be practically achieved. See also paragraph 38 which addresses particle sizing when testing vapours. Model calculations can be used to estimate how much or what fraction of an aerosol is reaching specific parts of the respiratory tract and lung.

27. For fine particles >1 µm, (e.g., micronized bulk material and agglomerated nanomaterials) deposition in the respiratory tract is dominated by inertial effects, including sedimentation. The relevant particle size parameter used to describe the deposition behaviour of fine particles is the aerodynamic diameter. For particles <100 nm, deposition is dominated by diffusion. The relevant particle size to describe the deposition of these nanoparticles is the thermodynamic equivalent diameter. The aerodynamic diameter is defined as the diameter of the spherical particle with a density

of 1 g/cm^3 (the density of water) that has the same settling velocity as the particle. The thermodynamic equivalent diameter of a particle is defined as the diameter of a spherical particle with the same diffusion coefficient as the particle of interest. An estimate of the thermodynamic equivalent diameter of the particles and the share of particles <100 nm, respectively, can inform the agglomeration status and efficiency of transport to the deep lung. This takes into account deagglomeration or poorly agglomerated nanomaterials (e.g., highly hydrophobic and chemically inert particles).

Test Chemical Preparation in a Vehicle

28. Ideally, the test chemical should be tested without a vehicle. If it is necessary to use a vehicle to generate an appropriate test chemical concentration and particle size, water should be used whenever possible. When a test chemical is dissolved in a vehicle, its stability should be demonstrated.

MONITORING OF EXPOSURE CONDITIONS

Chamber Airflow

29. The flow of air through the exposure chamber should be carefully controlled, continuously monitored, and recorded at least hourly during each exposure. The real-time monitoring of the test atmosphere concentration (or temporal stability) is an integral measurement of all dynamic parameters and provides an indirect means to control all relevant dynamic inhalation parameters. If the concentration is monitored real-time, the frequency of measurement of air flows may be reduced to one single measurement per exposure per day. Special consideration should be given to avoiding rebreathing in nose-only chambers. Oxygen concentration should be at least 19% and carbon dioxide concentration should not exceed 1%. If there is reason to believe that this standard cannot be met, oxygen and carbon dioxide concentrations should be measured. If measurements on the first day of exposure show that these gases are at proper levels, no further measurements should be necessary.

Chamber Temperature and Relative Humidity

30. Chamber temperature should be maintained at 22 ±3°C. Relative humidity in the animals' breathing zone, for both nose-only and whole-body exposures, should be monitored continuously and recorded hourly during each exposure where possible. The relative humidity should preferably be maintained in the range of 30 to 70%, but this may either be unattainable (e.g. when testing water based formulations) or not measurable due to test chemical interference with the test method.

Test Chemical: Nominal Concentration

31. Whenever feasible, the nominal exposure chamber concentration should be calculated and recorded. The nominal concentration is the mass of generated test chemical divided by the total volume of air passed through the inhalation chamber system. The nominal concentration is not used to characterize the animals' exposure, but a comparison of the nominal concentration and the actual concentration gives an indication of the generation efficiency of the test system, and thus may be used to discover generation problems.

Test Chemical: Actual Concentration

32. The actual concentration is the test chemical concentration as sampled at the animals' breathing zone in an inhalation chamber. Actual concentrations can be obtained either by specific

methods (e.g., direct sampling, adsorptive or chemical reactive methods, and subsequent analytical characterisation) or by non-specific methods such as gravimetric filter analysis. The use of gravimetric analysis is acceptable only for single component powder aerosols or aerosols of low volatility liquids, and should be supported by appropriate pre-study test chemical-specific characterisations. Multi-component powder aerosol concentration may also be determined by gravimetric analysis. However, this requires analytical data which demonstrate that the composition of airborne material is similar to the starting material. If this information is not available, a reanalysis of the test material (ideally in its airborne state) at regular intervals during the course of the study may be necessary. For aerosolised agents that may evaporate or sublime, it should be shown that all phases were collected by the method chosen.

33. One batch of the test chemical should be used throughout the duration of the study, if possible, and the test sample should be stored under conditions that maintain its purity, homogeneity, and stability. Prior to the start of the study, there should be a characterization of the test chemical, including its purity and, if technically feasible, the identity, and quantities of identified contaminants and impurities. This can be demonstrated by, but is not limited to, the following data: retention time and relative peak area, molecular weight from mass spectroscopy or gas chromatography analyses, or other estimates. Although the test sample's identify is not the responsibility of the test laboratory, it may be prudent for the test laboratory to confirm the sponsor's characterization at least in a limited way (e.g. colour, physical nature, etc.).

34. The exposure atmosphere should be held as constant as practicable. A real-time monitoring device, such as an aerosol photometer for aerosols or a total hydrocarbon analyser for vapours, may be used to demonstrate the stability of the exposure conditions. Actual chamber concentration should be measured at least 3 times during each exposure day for each exposure level. If not feasible due to limited air flow rates or low concentrations, one sample per exposure period is acceptable. Ideally, this sample should then be collected over the entire exposure period. Individual chamber concentration samples should deviate from the mean chamber concentration by no more than ±10% for gases and vapours, and by no more than ±20% for liquid or solid aerosols. Time to attain chamber equilibration (t_{95}) should be calculated and reported. The duration of an exposure spans the time that the test chemical is generated. This takes into account the times required to attain chamber equilibration (t_{95}) and decay. Guidance for estimating t_{95} can be found in GD 39 (1).

35. For very complex mixtures consisting of gases/vapours and aerosols (e.g. combustion atmospheres and test chemicals propelled from purpose-driven end-use products/devices), each phase may behave differently in an inhalation chamber. Therefore, at least one indicator substance (analyte), normally the principal active in the tested product formulation, of each phase (gas/vapour and aerosol) should be selected. When the test chemical is a mixture (e.g. a formulation), the analytical concentration should be reported for the total formulation, and not just for the active ingredient or the component (analyte). Additional information regarding actual concentrations can be found in GD 39 (1).

Test Chemical: Aerosol Particle Size Distribution

36. The particle size distribution of fine aerosols should be determined at least weekly for each concentration level by using a cascade impactor or an alternative instrument such as an aerodynamic particle sizer (APS). If equivalence of the results obtained by a cascade impactor and the alternative instrument can be shown, then the alternative instrument may be used throughout the study.

37. A second device, such as a gravimetric filter or an impinger/gas bubbler, should be used in parallel to the primary instrument to confirm the collection efficiency of the primary instrument. The

mass concentration obtained by particle size analysis should be within reasonable limits of the mass concentration obtained by filter analysis [see GD 39 (1)]. If equivalence can be demonstrated at all concentrations tested in the early phase of the study, then further confirmatory measurements may be omitted. For the sake of animal welfare, measures should be taken to minimize inconclusive data which may lead to a need to repeat a study.

38. Particle size measurement should be performed for vapours if there is any possibility that vapour condensation may result in the formation of an aerosol, or if particles are detected in a vapour atmosphere with potential for mixed phases.

39. When testing nanomaterials, scanning mobility particle sizers, differential mobility analyzers, or mobility particle sizers are preferred for non-fibrous and isometric nanomaterials for both aerosol exposure particle counts and size distributions. Micro-orifice uniform-deposit impactors may be used for fibrous materials to determine the exposure concentrations in terms of mass. Where technically feasible, two different methods of determining quantitative particle exposure (particle counts, size distribution, particle mass) should be used. In addition, scanning and/or transmission electron microscopy may be periodically (e.g., monthly) used for qualitative confirmation of material size and shape.

OBSERVATIONS

40. The animals should be clinically observed before, during, and after each exposure period. More frequent observations may be indicated depending on the response of the animals during exposure. When animal observation is hindered by the use of animal restraint tubes, poorly lit whole body chambers, or opaque atmospheres, animals should be carefully observed after exposure. Observations before the next day's exposure can assess any reversibility or exacerbation of toxic effects. If the study protocol includes a post-exposure period, then the animals should be observed at least once daily during this period.

41. All observations are recorded with individual records being maintained for each animal. When animals are sacrificed for humane reasons or found dead, the time of death should be recorded as precisely as possible.

42. Cage-side observations should include changes in the skin and fur, eyes, and mucous membranes; changes in the respiratory and circulatory systems; changes in the nervous system; and changes in somatomotor activity and behaviour patterns. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep, and coma. The measurement of rectal temperatures may provide supportive evidence of reflex bradypnea, a Paintal (C-fiber stimulation) reflex, or hypo/hyperthermia related to treatment or confinement. Additional assessments may be included in the study protocol such as kinetics, biomonitoring, lung function, retention of poorly soluble materials that accumulate in lung tissue, and behavioural changes.

BODY WEIGHTS

43. Individual animal weights should be recorded shortly before the first exposure (day 0), twice weekly thereafter (for example, on Fridays and Mondays to demonstrate recovery over an exposure-free weekend, or at a time interval to allow assessment of systemic toxicity), and at the time of death or euthanasia. If there are no effects in the first 4 weeks, body weights may be measured weekly for the remainder of the study. When there is a post-exposure period, animals should be weighed weekly. At study termination, all animals should be weighed shortly before sacrifice to allow for an unbiased calculation of organ to body weight ratios.

FOOD AND WATER CONSUMPTION

44. Food consumption should be measured weekly. Water consumption may also be measured. These measurements should continue when the study protocol includes a post-exposure period.

CLINICAL PATHOLOGY

45. Periodic clinical pathology assessments should be made for all exposed and control animals during the exposure and post-exposure periods and when animals are sacrificed. Clinical pathology assessments are not required for satellite animals used for lung burden and lung weight measurements. The time interval between the end of exposure and blood collection should be recorded. Sampling following the end of exposure is indicated for those parameters with a short plasma half-time (e.g., COHb, CHE, and MetHb).

46. **Table 1** lists the clinical pathology parameters that are generally required for all toxicology studies. Urinalysis is not required on a routine basis, but may be performed when deemed useful based on expected or observed toxicity. The study director may choose to assess additional parameters in order to better characterize a test chemical's toxicity (e.g., cholinesterase, lipids, hormones, acid/base balance, methaemoglobin or Heinz bodies, creatine kinase, myeloid/erythroid ratio, troponins, arterial blood gases, lactate dehydrogenase, sorbital dehydrogenase, glutamate dehydrogenase, and gamma glutamyl transpeptidase).

Table 1. Standard Clinical Pathology Parameters

Haematology

Erythrocyte count	Total leukocyte count
Haematocrit	Differential leukocyte count
Haemoglobin concentration	Platelet count
Mean corpuscular haemoglobin	Clotting potential (select one):
Mean corpuscular volume	Prothrombin time
Mean corpuscular haemoglobin concentration	Clotting time
Reticulocytes	Partial thromboplastin time

Clinical Chemistry

Glucose*	Alanine aminotransferase
Total cholesterol	Aspartate aminotransferase
Triglycerides	Alkaline phosphatase
Blood urea nitrogen	Potassium
Total bilirubin	Sodium
Creatinine	Calcium
Total protein	Phosphorus
Albumin	Chloride
Globulin	

Urinalysis (optional)

Appearance (colour and turbidity)	Total protein
Volume	Glucose
Specific gravity or osmolality	Blood/blood cells
pH	

* Because a lengthy fasting period can introduce bias in glucose measurements for the treated versus control animals, the study director should determine whether it is appropriate to fast the animals. If a fasting period is used, it should be appropriate to the species used; for the rat this may be 16 h (overnight fasting). Determination of fasting glucose may be carried out after overnight fasting during the last exposure week, or after overnight fasting prior to necropsy (in the latter case together with all other clinical pathology parameters).

BRONCHOALVEOLAR LAVAGE

47. Bronchoalveolar lavage (BAL) analysis should be performed at the end of the exposure period and at the end of each interval of the post-exposure period when testing all gases, vapours, and aerosols unless it is known that the lower respiratory tract is not a test chemical target at the concentrations tested. Typically, the left lung should be lavaged at the end of each of the post-exposure intervals. The length of the post-exposure period is determined by the study director based on findings in the range-finding study. Specific guidance on how to perform BAL can be found in GD 39. The mandatory and optional BAL endpoints are as follows:

Mandatory	**Optional**
• Lactate dehydrogenase (LDH) • Total protein or albumin • Cell counts and differentials for alveolar macrophages, lymphocytes, neutrophils, and eosinophils	• Cytokines • Chemokines • Mediators

LUNG BURDEN

48. When testing aerosols, measurements of lung burden provide 1) clarity regarding the actual deposited dose, 2) information regarding the dose-response relationship, allowing NOAEC and LOAEC concentrations to be determined, 3) easier comparisons between studies, and 4) the ability to address the issue of lung overload. Lung burden data allow risk assessors to distinguish between pulmonary responses caused by the specific properties of test particles as opposed to a non-specific response due to an overload of low toxicity particles. Ambiguity on this aspect can result in repetition of the test and an avoidable waste of animals.

49. Measurements of lung burden are required when a range-finding study demonstrates that the test chemical is retained in the lung, and when there is an analytical method available. As shown in Annex 1, satellite groups of 5 animals of the more sensitive sex are used for measurements of lung weight and lung burden (see paragraphs 19, 22, and 23). Sufficient satellite animals should be used to allow for one or more sacrifices during the post-exposure period at different intervals (i.e., post-exposure intervals, or PEIs). For illustration, Annex 1 shows the use of 2 PEIs. The satellite animals sacrificed at PEI-1 should be sacrificed 1 day after the end of the exposure period to allow for the rapid clearance of deposited test chemical via mucociliary transport from the conducting airways. For each satellite animal, both of its lungs should be weighed and evaluated for lung burden. The length of the post-exposure period and the number and spacing of the PEIs are determined by the study director based on findings in the range-finding study. Further guidance on lung burden evaluation can be found in GD 39.

OPHTHALMOLOGICAL EXAMINATION

50. Using an ophthalmoscope or an equivalent device, ophthalmological examinations of the fundus, refractive media, iris, and conjunctivae maybe performed for all animals prior to the administration of the test chemical, and for all high concentration and control groups at termination of the main study. If changes in the eyes are detected, all animals in the other groups should be examined including satellite groups.

GROSS PATHOLOGY AND ORGAN WEIGHTS

51. All test animals, including those which die during the test or are removed from the study for humane reasons should be subjected to complete exsanguination (if feasible) and gross necropsy. The time between the end of each animal's last exposure and its sacrifice should be recorded. If a necropsy cannot be performed immediately after a dead animal is discovered, the animal should be refrigerated (not frozen) at a temperature low enough to minimize autolysis. Necropsies should be performed as soon as possible, normally within a day or two. All gross pathological changes should be recorded for each animal with particular attention to any changes in the respiratory tract.

52. Table 2 lists the organs and tissues that should be preserved in a suitable medium during gross necropsy for histopathological examination. The preservation of the **[**bracketed**]** organs and tissues and any other organs and tissues is at the discretion of the study director. The **bolded** organs should be trimmed and weighed wet as soon as possible after dissection to avoid drying. The thyroid and epididymides should only be weighed if needed because trimming artefacts may hinder histopathological evaluation. Tissues and organs should be fixed in 10% buffered formalin or another suitable fixative as soon as necropsy is performed, and no less than 24-48 hours prior to trimming depending on the fixative to be used.

Table 2. Organs and Tissues Preserved During Gross Necropsy

Adrenals
Aorta
Bone marrow (and/or fresh aspirate)
Brain (including sections of cerebrum, cerebellum, and medulla/pons)
Caecum
Colon
Duodenum
[**Epididymides**]
[Eyes (retina, optic nerve) and eyelids]
Femur and stifle joint
Gallbladder (where present)
[Harderian glands]
Heart
Ileum
Jejunum
Kidneys
[Lacrimal glands (extraorbital)]
Larynx (3 levels including the base of the epiglottis)
Liver
Lung (right lung including main bronchi and pleura)
Lymph nodes from the hilar region of the lung, especially for poorly soluble particulate test materials. For more in depth examinations and/or studies with immunological focus, additional lymph nodes may be considered, e.g. those from the mediastinal, cervical/submandibular and/or auricular regions.
Lymph nodes (distal from the portal-of-entry)
Mammary gland (female)
Muscle (thigh)
Nasopharyngeal tissues (at least 4 levels; 1 level to include the nasopharyngeal duct and the Nasal Associated Lymphoid Tissue (NALT))
Oesophagus
Olfactory bulb
Ovaries
Pancreas
Parathyroids
Peripheral nerve (sciatic or tibial, preferably close to muscle)
Pituitary
Prostate
Rectum
Salivary glands
Seminal vesicles
Skin
Spinal cord (cervical, mid-thoracic, and lumbar)
Spleen
Sternum
Stomach
Teeth
Testes
Thymus
Thyroids
[Tongue]
Trachea (at least 2 levels including 1 longitudinal section through the carina and 1 transverse section)
[Ureter]
[Urethra]
Urinary bladder
Uterus
Target organs
All gross lesions and masses

NOTE: The preservation of the [bracketed] organs and tissues and any other organs and tissues is at the discretion of the study director. The **bolded** organs should be trimmed and weighed wet as soon as possible after dissection to avoid drying.

53. All lobes of the right lung should be preserved for histopathologic evaluation and the left lung should be used for bronchoalveolar lavage (this order may be reversed if one wishes). For test chemicals that are likely to result in lung retention, entire lungs are weighed for the satellite animals used for lung burden measurement but not for the animals used for BAL and histopathology evaluation. The right lungs should be removed intact and instilled with a suitable fixative at a pressure

of 20-30 cm of water to ensure that lung structure is maintained (8). Further guidance can be found in GD 125 (8).

54. At least 4 levels of the nasopharyngeal tissues should be examined, one of which should include the nasopharyngeal duct (9) (10) (11) (12) (13) to allow adequate examination of the squamous, transitional (non-ciliated respiratory), respiratory (ciliated respiratory) and olfactory epithelium, and the draining lymphatic tissue (NALT) (14) (15). Three levels of the larynx should be examined, and one of these levels should include the base of the epiglottis (16). At least two levels of the trachea should be examined including one longitudinal section through the carina of the bifurcation of the extrapulmonary bronchi and one transverse section. Additional guidance can be found in GD 125 (9).

HISTOPATHOLOGY

55. A histopathological evaluation of all the organs and tissues listed in Table 2 should be performed for the control and high concentration groups, and for all animals which die or are sacrificed during the study. Particular attention should be paid to the respiratory tract, target organs, and gross lesions. The organs and tissues that have lesions in the high concentration group should be examined in all groups. The study director may choose to perform histopathological evaluations for additional groups to demonstrate a clear concentration response. When a satellite group is used, histopathological evaluation should be performed for all tissues and organs identified as showing effects in the treated groups. It is recommended that all lobes of the right lung be used for histopathologic evaluation. If there are excessive early deaths or other problems in the high exposure group that compromise the significance of the data, the next lower concentration should be examined histopathologically. An attempt should be made to correlate gross observations with microscopic findings.

DATA AND REPORTING

Data

56. Individual animal data on body weights, food consumption, clinical pathology, BAL, gross pathology, organ weights, lung burden (when evaluated) and histopathology should be provided for both the range-finding and main studies. Clinical observation data should be summarized in tabular form showing for each test group the number of animals used, the number of animals displaying specific signs of toxicity, the number of animals found dead during the test or killed for humane reasons, time of death of individual animals, a description and time course of toxic effects and reversibility, and necropsy findings. All results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used and the statistical methods should be selected during the design of the study.

Test Report

57. The test report should include the following information, as appropriate:

Test animals and husbandry

- Description of caging conditions, including: number (or change in number) of animals per cage, bedding material, ambient temperature and relative humidity, photoperiod, and identification of diet.

- Species/strain used and justification for using a species other than the rat. Source and historical data may be provided, if they are for animals exposed under similar exposure, housing, and fasting conditions.
- Number, age, and sex of animals. Justification should be provided if both sexes are tested in the main study.
- Method of randomization.
- Description of any pre-test conditioning including diet, quarantine, ophthalmologic examination, and treatment for disease.

Test chemical

- Physical nature, purity, and, where relevant, physico-chemical properties (including isomerization or radiolabelling). Additional characterization information that may be relevant to nanomaterials includes shape, surface area/specific surface area, surface chemistry, composition including coating and surface modifications, surface charge, particle solubility, and aggregation/agglomeration state.
- Identification data and Chemical Abstract Services (CAS) Registry Number, if known.

Vehicle

- Justification for use of vehicle and justification for choice of vehicle (if other than water).
- Historical or concurrent data demonstrating that the vehicle does not interfere with the outcome of the study.

Inhalation chamber

- Detailed description (preferably including a diagram) of the inhalation chamber including volume.
- Source and description of equipment used for the exposure of animals as well as generation of atmosphere.
- Equipment for measuring temperature, humidity, particle-size, and actual concentration.
- Source of air and system used for conditioning.
- Methods used for calibration of equipment to ensure a homogeneous test atmosphere.
- Pressure difference (positive or negative).
- Exposure ports per chamber (nose-only); location of animals in the chamber (whole-body).
- Stability of the test atmosphere.
- Location of temperature and humidity sensors and sampling of test atmosphere in the chamber.
- Treatment of air supplied/extracted.
- Air flow rates, air flow rate/exposure port (nose-only), or animal load/chamber (whole-body).
- Time to inhalation chamber equilibrium (t_{95}).
- Number of volume changes per hour.
- Metering devices (if applicable).

Exposure data

- Rationale for target concentration selection in the main study.
- Nominal concentrations (total mass of test chemical generated into the inhalation chamber divided by the volume of air passed through the chamber).
- Actual test chemical concentrations collected from the animals' breathing zone; for test mixtures that produce heterogeneous physical forms (gases, vapours, aerosols), each may be analysed separately.
- All air concentrations should be reported in units of mass (mg/L, mg/m^3, etc.) rather than in units of volume (ppm, ppb, etc.).
- Particle size distribution, mass median aerodynamic diameter (MMAD), and geometric standard deviation (σg), including their methods of calculation. Individual particle size analyses should be reported. Thermodynamic diameter should be reported for particles <100 nm.

Test conditions

- Details of test chemical preparation, including details of any procedures used to reduce the particle size of solid materials or to prepare solutions of the test chemical.
- A description (preferably including a diagram) of the equipment used to generate the test atmosphere and to expose the animals to the test atmosphere.
- Details of the equipment used to monitor chamber temperature, humidity, and chamber airflow (i.e. development of a calibration curve).
- Details of the equipment used to collect samples for determination of chamber concentration and particle size distribution.
- Details of the chemical analytical method used and method validation (including efficiency of recovery of test chemical from the sampling medium).
- Method of randomization in assigning animals to test and control groups.
- Details of food and water quality (including diet type/source, water source).
- Details of the methodology used to assess BAL and lung burden.

Results

- Tabulation of chamber temperature, humidity, and airflow.
- Tabulation of chamber nominal and actual concentration data.
- For aerosols with the majority of particles >1µm (i.e., fines), information on the aerodynamic diameter size distribution is required along with the mass median aerodynamic diameter (MMAD) and its associated geometric standard deviation (σg). For aerosols predominantly <100 nm, information on the distribution of thermodynamic equivalent diameter is required along with the count median diameter (CMD) and associated geometric standard deviation (σg).
- Tabulation of response data and concentration level for each animal (i.e., animals showing signs of toxicity including mortality, nature, severity, time of onset, and duration of effects).
- Tabulation of individual animal weights.
- Tabulation of food consumption

- Tabulation of clinical pathology data
- Tabulation of bronchoalveolar lavage (BAL) data
- Tabulation of lung burden measurements
- Tabulation of pulmonary function data and body temperatures (optional)
- Necropsy findings, organ weights, and histopathological findings for each animal, if available.

Discussion and interpretation of results

- Particular emphasis should be made to the description of methods used to meet the criteria of this test guideline, e.g., the limit concentration or the particle size.
- The respirability of particles in light of the overall findings should be addressed, especially if the particle size criteria could not be met.
- The consistency of methods used to determine nominal and actual concentrations, and the relation of actual concentration to nominal concentration should be included in the overall assessment of the study.
- The likely cause of death and predominant mode-of-action (systemic versus local) should be addressed.
- An explanation should be provided if there was a need to humanely sacrifice animals in pain or showing signs of severe and enduring distress, based on the criteria in the OECD Guidance Document on Humane Endpoints (7).
- The target organ(s) should be identified.
- The NOAEC and Lowest Observed Adverse Effects Concentration (LOAEC) should be determined.
- Descriptions of any complications that occurred in the main study that might have an impact on the results of the study.

REFERENCES

1) OECD (2009), *Guidance Document on Acute Inhalation Toxicity Testing*, Environmental Health and Safety Monograph Series on Testing and Assessment No. 39, ENV/JM/MONO(2009)28, OECD, Paris.

2) OECD (1981), *Subchronic Inhalation Toxicity Testing*, Original Test Guideline No 413, Environment Directorate, OECD, Paris.

3) OECD (2009). *Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials*. Series on the Safety of Manufactured Nanomaterials No. 15, OECD, Paris.

4) OECD (2012). *Guidance Document on Sample Preparation and Dosimetry Series on the Safety of Manufactured Nanomaterials No. 36*, OECD, Paris. OECD, Paris

5) Brown, J.S. et al. (2013). *Thoracic and Respirable Particle Definitions for Human Health Risk Assessment*. Particle and Fibre Toxicology 10:12.

6) The American Conference of Governmental Industrial Hygienists (ACGIH) (2013*). Poorly Soluble /Lung Overload*. Appendix B, pages 78 – 79. European Centre for Ecotoxicology and Toxicology of Chemicals, Technical Report No. 122, Brussels, Belgium, December 2013.

7) OECD (2000), *Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation*, Environmental Health and Safety Monograph Series on Testing and Assessment No. 19, ENV/JM/MONO(2000)7, OECD, Paris.

8) Dungworth, D.L., Tyler, W.S., and Plopper, C.E. (1985). Morphological Methods for Gross and Microscopic Pathology (Chapter 9) in *Toxicology of Inhaled Material* , Witschi, H.P. and Brain, J.D. (eds), Springer Verlag Heidelberg, pp. 229-258.

9) OECD (2010*). Guidance document on histopathology for inhalation toxicity studies supporting TG 412 (subacute inhalation toxicity: 28 day study) and TG 413 (subchronic inhalation toxicity: 90-day study).* Environmental Health and Safety Monograph Series on Testing and Assessment No. 125. ENV/JM/MONO(2010)16, OECD, Paris.

10) Young J.T. (1981) *Histopathological examination of the rat nasal cavity*. Fundam. Appl. Toxicol. 1, 309-312.

11) Harkema J.R. (1990) *Comparative pathology of the nasal mucosa in laboratory animals exposed to inhaled irritants*. Environ. Health Perspect. 85, 231-238.

12) Woutersen R.A., Garderen-Hoetmer A. van, Slootweg P.J. and Feron V.J. (1994) *Upper respiratory tract carcinogenesis in experimental animals and in humans*. In: Waalkes MP and Ward JM (eds) Carcinogenesis. Target Organ Toxicology Series, Raven Press, New York, 215-263.

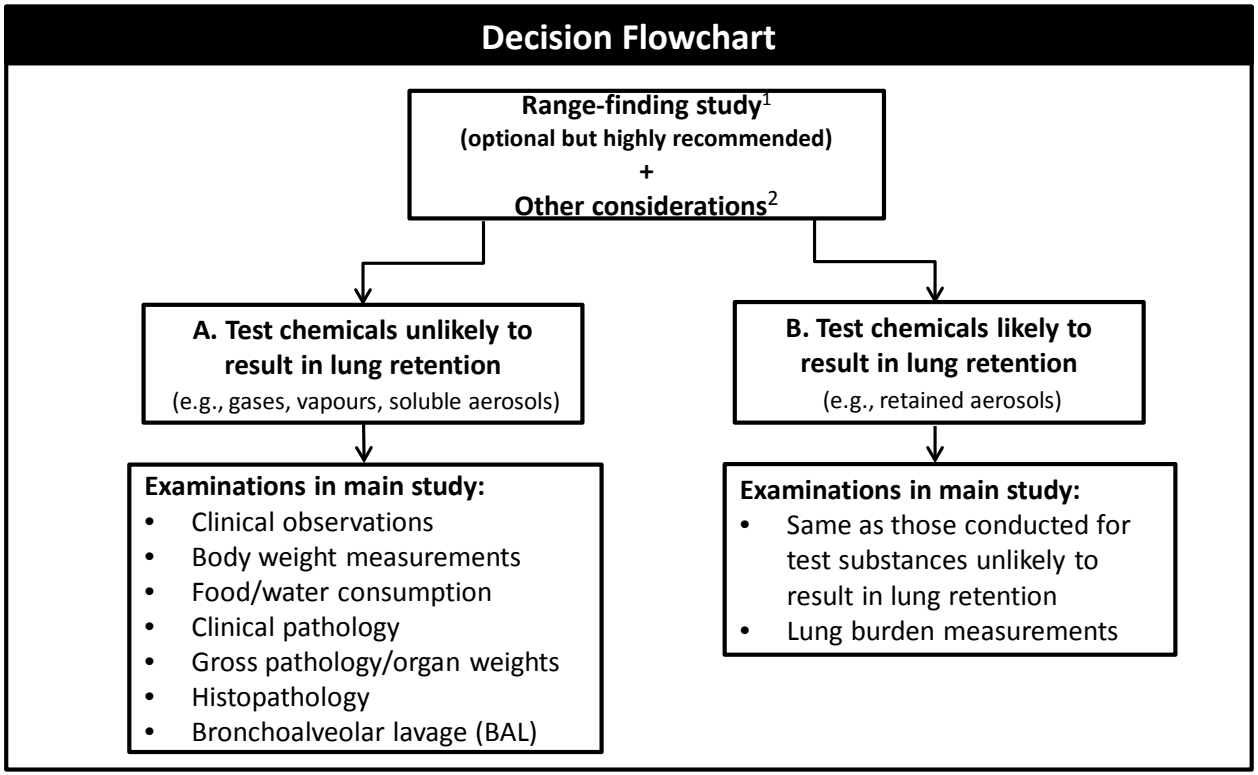
13) Mery S., Gross E.A., Joyner D.R., Godo M. and Morgan K.T. (1994) *Nasal diagrams: A tool for recording the distribution of nasal lesions in rats and mice*. Toxicol. Pathol. 22, 353-372.

14) Kuper C.F., Koornstra P.J., Hameleers D.M.H., Biewenga J., Spit B.J., Duijvestijn A.M., Breda Vriesman van P.J.C. and Sminia T. (1992) *The role of nasopharyngeal lymphoid tissue*. Immunol. Today 13, 219-224.

15) Kuper C.F., Arts J.H.E. and Feron V.J. (2003) *Toxicity to nasal-associated lymphoid tissue*. Toxicol. Lett. 140-141, 281-285.

16) Lewis D.J. (1981). *Mitotic Indices of Rat Laryngeal Epithelia*. Journal of Anatomy 132(3). 419-428.

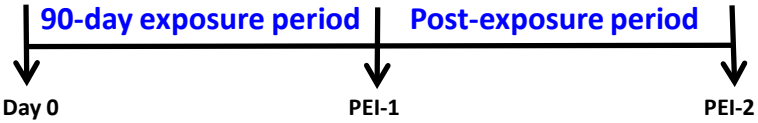
ANNEX 1



Footnotes:

1. A successful main study depends on information learned from a range-finding study including data on analytical methods applied to measurements of airborne particles (if applicable) and lung particle deposition/burden, analyses of aerodynamic particle size distribution, concentration selection, BAL, clinical pathology and histopathology, gender sensitivity, biomarkers of lung injury, assessing mechanisms of injury, and the degree of particle solubility in the lung at cumulative lung burdens observed in repeated inhalation exposure studies.
2. Other considerations may be related to the test article such as its: identify, chemical structure, purity, and key physicochemical properties (solubility, size range, agglomeration, morphology, surface area, reactivity, coatings, etc.);, results of any *in vitro* or *in vivo* toxicity tests; anticipated use(s) and potential for human exposure; available (Q)SAR data and toxicological data on structurally related substances; and data derived from acute inhalation toxicity testing.

TG 413
Main Study



A **Test chemicals unlikely to result in lung retention** (e.g., gases, vapours, soluble aerosols)	**C_0 - 10♂** **C_1 - 10♂** **C_2 - 10♂** **C_3 - 10♂** **Total=40**	Within 1 day of exposure termination, examine animals for the following: • Clinical observations • Body weight measurements • Food/water consumption • Clinical pathology • Gross pathology/organ weights • Histopathology–all lobes of the right lung • Bronchoalveolar lavage (BAL)–left lung	**C_0 - 5♂** **C_1 - 5♂** **C_2 - 5♂** **C_3 - 5♂** **Total=20**
B **Test chemicals likely to result in lung retention** (e.g., retained aerosols)	**C_0 - 10♂** **C_1 - 10♂** **C_2 - 10♂** **C_3 - 10♂** **Total=40**	Within 1 day of exposure termination, examine animals for the following: • Clinical observations • Body weight measurements • Food/water consumption • Clinical pathology • Gross pathology/organ weights • Histopathology-all lobes of the right lung • Bronchoalveolar lavage (BAL)-left lung	**C_0 - 5♂** **C_1 - 5♂** **C_2 - 5♂** **C_3 - 5♂** **Total=20**
	C_0 - 10♂ **C_1 - 10♂** **C_2 - 10♂** **C_3 - 10♂** **Total=40**	**Satellite groups:** Examine animals for the following 1 day after exposure termination: • Lung burden (both lungs) • Lung weight (both lungs)	**C_0 - 5♂** **C_1 - 5♂** **C_2 - 5♂** **C_3 - 5♂** **Total=20**

NOTES: The more sensitive sex should always be used. Use males when there is no sex difference. The main study should include at least one Post-Exposure Interval (PEI-1) but a study director may include additional PEIs as needed. The use of two PEIs is shown above. The duration of the post-exposure period and the spacing of PEIs is at the discretion of the study director. Satellite groups (in **red**) are used when a range-finding study indicates the test chemical is likely to be retained in the lung and/or cause long-term lung effects.