

*OECD GUIDELINES FOR THE TESTING OF CHEMICALS***90-DAY (SUBCHRONIC) INHALATION TOXICITY STUDY****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 as well as to reflect the evolving state-of-the-science for the testing of inhaled gases, vapours, and aerosols. The revision particularly addresses the actual design of the main study, its dependency on the physical form of the test chemical (gas, vapour, liquid aerosol, or solid aerosol), and whether a range-finding study or other information indicates that the test chemical deposited and retained in the lung causing adverse local or systemic effects. At a minimum, the main study comprises groups of 10 male and 10 female rodents exposed to the test chemical for 6 hours per day over a 90 day (13 week) period at three or more concentration levels, and to filtered air (negative control) or the vehicle (vehicle control). The animals are then sacrificed within 24 h after the end of the exposure period. Animals are generally exposed 5 days per week, but exposure for 7 days per week is also allowed. Bronchoalveolar lavage (BALF) is performed for all test chemicals. To achieve this, the lungs are divided: The left lung is used for histopathology, the right lung for bronchoalveolar lavage fluid (BALF) analysis. If recovery groups are planned, these should also allow for BAL analysis by lung splitting. If a test chemical is likely to be retained in the lung, the study director may consider additional post-exposure observation (PEO) periods that include lung burden measurements that can inform on lung clearance behaviour and translocation, the latter being particularly relevant in case the testing chemical is a solid nanomaterial. This test guideline also suggests additional optional investigations, such as toxicokinetics, and/or systemic toxicity evaluations (e.g., immune, hepatic, neurologic and/or cardiovascular effects evaluations) to better characterize the overall toxicity of a test chemical. Further guidance on additional observations and their analysis can be found in the revised Guidance Document 39 (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 (2). 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 is to accommodate the testing of particle aerosols including nanomaterials. It is noted that nanoparticles and fine particles coexist as a continuum and that samples of engineered nanoparticles tend to agglomerate in the test atmosphere, depending on their method of generation and their physical and chemical composition. The most notable features of this latest version are as follows:

- This revision requires specific measurements of bronchoalveolar lavage fluid (BALF) to be performed for all test chemicals, by splitting the lung for histopathology and BAL analysis. Any recovery group planned should also include BALF analysis.
 - Measurements of lung burden, which inform on pulmonary deposition and retention of particles in the lung, should be done when a range-finding study or other relevant information suggests that inhaled test particles are poorly soluble and likely to be retained in the lung. BALF analysis and lung burden measurements are performed for all test chemicals within 24 h after exposure termination and may be undertaken at one or two additional post-exposure observation (PEO) intervals. The need for additional PEOs, the duration of the post-exposure interval and the timing of the PEOs are determined by the study director based upon the purpose of the study and the results of a range-finding study and/or other relevant information (e.g., PEO-1 for determining pulmonary retention; and PEO-1, PEO-2 and PEO-3 when three lung burden measurements are needed for evaluating clearance kinetics; see Annex and GD39).
 - A range-finding study (or studies), which primarily is (are) performed to assess concentration levels for the main study should also include BALF analysis, and may also include lung burden measurements. It might also be designed to inform on gender sensitivity pulmonary function, body temperature, etc., but should be carefully planned and balanced to avoid losing robustness.
 - The 2009 version of TG 413 required particulate aerosols to have a mass median aerodynamic diameter (MMAD) of 1-3 μm with a geometric standard deviation (σ_g or GSD) of 1.5-3.0. To accommodate the testing of nano-range aerosols and to enhance deposition in the pulmonary region, a new standard should be met whenever possible: MMAD of $\leq 2 \mu\text{m}$ with a σ_g of 1-3. Justification should be provided in the study report if this standard cannot be met, including a description of efforts taken to meet it, such as milling (refer to GD 39).
3. Subchronic inhalation toxicity studies are primarily used to derive regulatory concentrations for assessing worker risk in occupational settings. They are also used to identify and assess human residential, consumer, transportation, and environmental risk. This guideline enables the characterization of adverse effects following repeated daily inhalation exposure to a test chemical for 90 days. 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 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 chemicals, and data derived from other repeated exposure studies. When testing a solid aerosol, it is useful to have information on its retention and kinetics in the lung. If systemic toxicity (e.g., immunotoxicity, neurotoxicity, hepatotoxicity, cardiovascular effects) is expected or is observed in the course of the study, the study director may choose to include appropriate evaluations of relevant toxicological endpoints. Although the timing of exposures relative to specific examinations may be critical, the performance of these additional activities should not interfere with the main study design.

5. If the test chemical is generated as an aerosol of poorly soluble material(s), dimensional, morphological and physiochemical characterisation, including the measurement of solubility, should be undertaken to the extent possible. For the characterisation of nanomaterials, 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).

6. The respirable (or alveolar) fraction of poorly soluble particles that are slowly cleared can accumulate with each consecutive exposure period. The extent of lung retention can be assessed by lung burden measurements. A portion of these particles may translocate and distribute widely within the organism. Measurements of lung-associated lymph node (LALN) burdens may indicate translocation, which may be further substantiated by target organ burden measurements. The effects of any translocation can be evaluated by considering systemic toxicological effects. Lung burden data cannot be used to exclude the relevance of toxicological findings in the experimental animal for human risk assessment. 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. When exposing animals to these test chemicals, 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 paragraphs 14-16). The justification for concentration selection should be provided.

8. Animals that are moribund 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 during a 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 (5).

DESCRIPTION OF THE METHOD

Selection of Animal Species

9. 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

10. 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 $\pm 20\%$ 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

11. 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 $22 \pm 3^\circ\text{C}$. The relative humidity should ideally be maintained in the range of 30 to 70%. 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 using nose-only exposure, the restraining tubes should not impose undue physical, thermal, or immobilization stress on the animals. Animals should be acclimated to the restraining tubes unless laboratory data demonstrate these stresses are not a concern. Animals exposed to an aerosol in a whole-body chamber 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

12. Subchronic inhalation toxicity studies are always performed in dynamic inhalation chambers. The use of a static inhalation chamber, which has no airflow, 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***

13. The maximum concentration tested should consider: 1) the maximum attainable concentration, 2) the need to maintain an adequate oxygen supply, and/or 3) animal welfare considerations. In the absence of data-based limits, the acute limits of the United Nations Globally Harmonized System of Classification and Labelling of Chemicals may be used (i.e., up to a maximum concentration of 5 mg/L for aerosols, 20 mg/L for vapours, and 20,000 ppm 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). For particles aerosol testing > 2 mg/L should only be attempted if a respirable particle size can be maintained/achieved (refer to GD 39).

Range-Finding Study

14. The design of the main study is greatly dependent on information learned during a range-finding study. A range-finding study should be performed unless sufficient information already exists to perform a robust main study. While the primary purpose of a range-finding study is to inform the selection of concentration levels for a main study, it may also provide additional information that can assure a robust main study. This is especially true when testing poorly soluble solid aerosols. A range-finding study may, for example, provide information regarding analytical methods, particle size distribution, systemic toxicity, toxicokinetics, test chemical solubility in the lung, translocation of particles, discovery of toxic mechanisms, clinical pathology (i.e., haematology/clinical chemistry), histopathology, biomarkers of lung injury, gender sensitivity, BALF data, and estimates of what may be the No Observed Adverse Effects Concentration (NOAEC), Lowest Observed Adverse Effects Concentration (LOAEC), Maximum Tolerated Concentration (MTC), and/or the benchmark concentration (BMC) in a main study. The study director should use a range-finding study to identify the upper concentration that is tolerated without undue stress to the animals, and the parameters that will best characterize a test chemical's toxicity. BAL may be performed at a range-finding study's exposure termination and periodically during a post-exposure period. When testing a solid aerosol, an assessment of the test chemical solubility and post-exposure lung burden may be performed to inform a decision on the duration of the main study post-exposure period and the spacing of post-exposure observation (PEO) time points. Also, LALN burden measurements may provide information on translocation.

15. A range-finding study may consist of one or more test chemical concentration levels and a control group. Depending on the endpoints chosen, typically no more than 5 males and 5 females should be exposed at each concentration level. A range-finding study should last a minimum of 5 days and generally no more than 28 days, and may include a post-exposure period and animal numbers should be adjusted accordingly. 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. For further guidance on range-finding, see GD 39 (1).

Main Study

16. The main study consists of at least three test chemical concentration levels and concurrent negative (air) or vehicle controls (see paragraph 30). As shown in the Annex,

this guideline differentiates two study designs depending on the nature of the test chemical. In addition to Option A, which is generally used for test chemicals (as gas, vapour, aerosol, or a mixture thereof), option B is used when testing chemicals that are likely to be retained in the lungs. While the schemes for options A and B shown in the annex demonstrate typical study designs, the actual design of a study is at the discretion of the study director. Information from a range-finding study and all other available, relevant data should be used to design the study and to select appropriate exposure levels (see paragraph 14).

17. Each group consists of a minimum of 10 male and 10 female rodents that are 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. When testing a chemical that is likely to be retained in the lungs (Option B in Annex), a satellite group of 5 males per concentration (i.e. at least 20 animals) should be concurrently exposed with the main study groups and evaluated at PEO-1 for lung burden measurements at exposure termination to provide information on the retained dose. In the event the range-finding study shows one sex to be more susceptible to a test chemical, the sexes may be exposed at different concentration levels in order to optimize the concentration-response as described in paragraph 20.

18. If rodent species other than rats are exposed nose-only, maximum exposure durations may be adjusted to minimize species-specific distress. A rationale should be provided when using 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.

19. 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 allow the identification of 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 lethality or persistent signs that might lead to lethality or prevent a meaningful evaluation of the results. When testing aerosols, the high concentration may be the maximally achievable level that can be reached while meeting the particle size distribution standard (see paragraph 39).
- 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.

Satellite Groups

22. Additional satellite groups may be added to the main study to evaluate recovery, persistence, delayed occurrence of toxicity, or lung burden for a post-treatment period of an appropriate length. Examples of main studies with **satellite groups are shown in the Annex**. It is noted that a study director may modify the design of a study based on the

physico-chemical characteristics and kinetics of a test chemical in order to achieve the most robust data. All satellite groups are exposed concurrently with the experimental animals in the main study and at the same concentration levels, and there should be concurrent air or vehicle controls as needed (see paragraph 29). The scheduling and design of satellite groups depend on whether the test chemical is a solid aerosol and is likely to result in lung retention following the Decision Flowchart for lung burden in the Annex. If the test chemical is likely to result in lung retention the main study is conducted as described in Option B; otherwise, the main study is conducted as described in Option A. Satellite groups can be included to evaluate recovery in Option A; Option B provides for satellite groups for the evaluation of recovery and/or for lung burden measurements.

23. Satellite recovery groups at PEO-2 consist of 5 males and 5 females per concentration in Option A and Option B. These recovery groups are exposed concurrently with the experimental animals in the main study and at the same concentration levels, and there should be concurrent air or vehicle controls as needed (see paragraph 29).

24. When testing poorly soluble solid aerosols that are likely to be retained in the lungs (Option B in the Annex), one or two additional satellite groups of 5 males per concentration may be added to measure lung burden at different post exposure time points. These additional lung burden measurements (i.e., PEO-2 and/or PEO-3) may be added to the design when the study director would like to understand the post exposure clearance kinetics of the test substance. Since three time points are generally required to provide information on clearance kinetics, lung burden measurements are performed within 24 hours after exposure termination (PEO-1) and at 2 additional PEOs (PEO-2 and PEO-3). However, the use of two time points may provide sufficient information under some circumstances, such as when the main objective is to identify whether or not clearance is very slow. Lung burden measurements are preferably performed in males, which have a higher minute volume than females and may thus have greater lung burdens.

25. Based upon the results of the range-finding study and other available relevant information, the study director should define the duration of the post-exposure period as well as the timing of PEO-2 for the recovery groups and, in case of Option B, of PEO-3 for the lung burden satellite groups (see the Annex). The scheduling of PEO-2 and PEO-3 in Option B will depend on the expected rate of lung clearance and the duration of the post-exposure recovery period. The following are other possible options:

- The study director may choose to schedule PEO-3 before the recovery group (PEO-2) (if included), if considered more appropriate.
- If the use of two post-exposure time points is considered sufficient, lung burden measurements may be performed at PEO-1 (main study) and at PEO-2 (recovery group) only, if timing for evaluation of recovery and lung clearance can be aligned to one another. The satellite group at PEO-3 can then be omitted from the study.
- The study director may choose to perform lung burden measurements at PEO-1 (main study) and at PEO-3 (satellite group) and to use both sexes of the recovery groups (PEO-2) for BALF analysis.

26. Care must be taken with the choice and timing of the PEOs to ensure that repeat studies are not required. The study director must justify the choice and timing of PEOs, clearly addressing these points.

27. BALF analysis is required at exposure termination (PEO-1) and at additional PEO's, if these are planned. If lung burden is measured (Option B), BALF analysis in the recovery group (PEO-2) will only be performed in females because males of these groups will be used for lung burden measurements (see paragraph 24).

28. Although in Option B additional groups are scheduled only for lung burden measurements (main study at PEO-1 and satellite group at PEO-2 and PEO-3), these animals may be considered for additional parameters that may aid to the understanding of the toxicokinetics and toxicity (including recovery) of the test chemical (see GD 39). These additional parameters are designated as to be determined (TBD) in the Annex. Observations made at PEO-1 may be used to choose additional parameters to be examined at PEO-3, for example, if the time duration between PEO-1 and PEO-3 allows such considerations to be made. For instance, additional histopathology may be performed in the left lung or other organs at PEO-3.

Control Animals

29. 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 a vehicle other than water is used to assist in generating the test atmosphere, a vehicle control group should be used instead of a negative (air) control group. 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. The use of both a negative and a vehicle control is discouraged unless a vehicle's toxicity is not well known. 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

30. Animals are exposed to the test chemical as a gas, vapour, or aerosol 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 standard described in paragraphs 39-42, solid materials may be subjected to mechanical processes to decrease the particle size. Further guidance on mechanical processing (milling) is provided in GD 39 (1).

Test Chemical Preparation in a Vehicle

31. Ideally, the test chemical should be tested without a vehicle, and solid aerosols should be dry-generated. 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***

32. 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

33. Chamber temperature should be maintained at $22 \pm 3^{\circ}\text{C}$. 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

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

Test Chemical: Analytical Concentration

35. The analytical concentration is the test chemical concentration as sampled at the animals' breathing zone in an inhalation chamber. Analytical 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 solid aerosols or aerosols of low volatility liquids, and should be supported by appropriate pre-study test chemical-specific characterisations. Multi-component solid aerosol concentration may also be determined by gravimetric analysis, but this requires analytical data that demonstrate the composition of airborne material is similar to the starting material. If this information is not available, a reanalysis of the test chemical (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.

36. 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 identity 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.).

37. 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. Analytical 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 $\pm 10\%$ for gases and vapours, and by no more than $\pm 20\%$ 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).

38. 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 substance 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 analytical concentrations can be found in GD 39 (1).

Test Chemical: Aerosol Particle Size Distribution

39. To ensure sufficient exposure of the lower respiratory tract, an aerosol should meet the following standard whenever possible: Mass Median Aerodynamic Diameter (MMAD) $\leq 2 \mu\text{m}$ with a geometric standard deviation (σ_g or GSD) of 1-3 for rats (see GD 39). Although a reasonable effort should be made to meet this criterion, expert judgement should be used if it cannot be practically achieved. Particle size measurement should be performed for vapours that may condense and form an aerosol. Deposition models can be used to estimate how much or what fraction of an aerosol is reaching specific parts of the respiratory tract. This can also be supported with lung burden data after a single exposure (see GD 39). When testing species other than rats, similar considerations apply regarding the requirement to use an aerosol with a particle size distribution that results in sufficient exposure of the lower respiratory tract, and the size range chosen should be justified on this basis. Justification should be provided in the study report if the MMAD standard cannot be met.

40. The particle size distribution of fine aerosols should be determined, preferably weekly, for each concentration level by using a cascade impactor or an alternative instrument such as (thermodynamic equivalent diameter or optical) particle sizers. 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.

41. Scanning mobility particle sizers, differential mobility analyzers, or aerodynamic particle sizers are preferred for non-fibrous and isometric nanomaterials both for aerosol exposure particle counts and size distributions. Micro-orifice uniform-deposit impactors may be used for all materials including fibrous materials to determine the exposure concentrations in terms of mass and size distribution. At least two different methods of determining quantitative particle exposure (i.e., particle counts, size distribution, or particle mass) should be used. Aerosols produced using nanomaterials typically consist of agglomerated particles and may thus have a particle size distribution that includes both micro and nano-sized particles. It may therefore be necessary to characterise the aerosols using a combination of the techniques indicated above. In addition, scanning and/or transmission electron microscopy should always be used periodically (e.g., monthly) for monitoring and qualitative confirmation of particle size and shape for all particulates, not just nanomaterials. Other measures such as particle surface area can be a valuable addition.

42. A second quantitative device, such as a gravimetric filter or an impinger/gas bubbler, should be used in parallel with 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 that may cause a study to be repeated.

OBSERVATIONS

43. The animals should be clinically observed before, during, and after each exposure period as well as during the post-exposure period(s). 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 chamber 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.

44. 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.

45. 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 evidence of hypo/hyperthermia related to test chemical treatment or confinement. Additional

assessments may be included in the study protocol such as biomonitoring (urine/faeces collection), lung function assessment, and behavioural changes.

BODY WEIGHTS

46. 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 significant body weight 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

47. 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

48. At least at the end of the administration and recovery phase 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 measurements, but if considered to be of aid in the understanding of the toxicity (including recovery) selected parameters may be assessed at PEO-2 and PEO-3 (see paragraph 29). The time interval between the end of exposure and blood collection should be recorded. Sampling following the end of exposure is indicated for parameters with a short plasma half-time (e.g., COHb, CHE, MetHb).

49. 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, such as: cholinesterase, lipids, hormones, acid/base balance, methaemoglobin or Heinz bodies, creatine kinase, bone marrow cytology, troponins, lactate 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 haematology/clinical chemistry parameters).

BRONCHOALVEOLAR LAVAGE

50. BAL should be performed at PEO-1 (within 24h at termination of exposure) and at one post-exposure interval (PEO-2) (see Annex, Option A). When testing a poorly soluble aerosol (see Option B in Annex), BAL should be performed at PEO-1 (within 24 hours of exposure termination) in both sexes and in female animals at PEO-2 if a recovery group is scheduled. The right lung is generally preferred for lavage. The duration of the post-exposure period and the timing of the PEOs are determined by the study director based on findings in the range-finding study and other available, relevant information. GD 39 provides specific guidance on optional BAL parameters and how to perform BALF (1). The mandatory BALF analysis encompasses the following parameters:

- Lactate dehydrogenase (LDH)
- Total protein or albumin
- Cell counts and differentials for alveolar macrophages, lymphocytes, neutrophils, and eosinophils

LUNG BURDEN

51. When testing poorly soluble solid aerosols, measurements of lung burden can provide clarity on the retained dose. Lung burden measurements should be done when a range-finding study or other relevant information suggests that the test chemical is, or might be, retained in the lung, and when there is an appropriate analytical method available. Males are used because they have a higher minute volume than females and may thus have greater lung burdens. As shown in the Annex (Option B), groups of 5 males per concentration are added to the main study for mandatory lung burden measurements at exposure termination (PEO-1). PEO-1 is to be scheduled approximately 24 h after termination of exposure to allow for the rapid clearance of deposited test chemical from the conducting airways via mucociliary transport. A study director has the option of measuring lung burden in satellite recovery groups at PEO-2 and PEO-3 (see paragraphs 17, 23, 24). To obtain clear information on lung clearance kinetics the same lung (the right lung is recommended) should be measured for lung burden at all post-exposure time points. The duration of the post-exposure period and the spacing of the PEOs are determined by the study director based on findings in the range-finding study and other relevant information. Further guidance on lung burden measurement and evaluation can be found in GD 39 (see also paragraph 24).

OPHTHALMOLOGICAL EXAMINATION

52. Using an ophthalmoscope or an equivalent device, ophthalmological examinations of the fundus, refractive media, iris, and conjunctivae should be 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

53. 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. Table 2 lists the organs and tissues that should be preserved in a suitable medium during gross necropsy for histopathological examination.

Table 2. Organs and Tissues Preserved During Gross Necropsy

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

* Reference to TG 407

54. The left lung should be preserved for histopathologic evaluation and the right lung should be used for BAL, though this order may be reversed. The left lung should be removed intact, weighed and instilled with a suitable fixative at a pressure of 20–30 cm of water to ensure that lung structure is maintained (6). If the study design includes the use of satellite groups for lung burden measurements, both lungs may be weighed for these animals.

55. At least 4 levels of the nasopharyngeal tissues should be examined, one of which should include the nasopharyngeal duct (7) (8) (9) (10) (11) to allow adequate examination of the squamous, transitional (non-ciliated respiratory), respiratory (ciliated respiratory) and olfactory epithelium, and the draining lymphatic tissue (NALT) (12) (13). Three levels of the larynx should be examined, and one of these levels should include the base of the epiglottis (14). 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. At least three levels of the left (or right) lung should be examined; these levels should represent different regions of the lobe. Additional guidance can be found in GD 125 (7).

56. If it is necessary to assess test chemical translocation, the particle burden of relevant organs may be determined in preserved histopathological tissue specimen. It is therefore important to consider appropriate preservation techniques to allow the application of a broad spectrum of analytical methods. Independent of preserved samples, deposition of particles in LALN should always be determined as indicator of translocation when testing poorly soluble particles.

HISTOPATHOLOGY

57. 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. The study director may choose to perform histopathological evaluations for additional concentration groups to demonstrate a clear concentration response. 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. 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. When a recovery group is used, histopathological evaluation should be performed for all tissues and organs identified as showing effects in the treated groups. Histopathology may be considered for the satellite groups used for lung burden measurements as indicated in paragraph 28. It is recommended that the left lung be used for histopathologic evaluation. An attempt should be made to correlate gross observations with microscopic findings.

PULMONARY FUNCTION

58. If the study director finds evidence of reflex bradypnea or a Paintal reflex in rodents in the range-finding study, or other information (e.g., hypothermia) indicates that these reflexes may occur, then the extent of these reflexes should be quantified in the main study by periodically measuring pulmonary function and body temperature. Further information on these reflexes and on how to perform pulmonary function studies can be found in GD 39.

DATA AND REPORTING

Data

59. Individual animal data on body weights, food consumption, clinical pathology, BALF analysis, gross pathology, organ weights, lung burden (when evaluated) and histopathology should be provided for both the range-finding and main study. 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. Any generally accepted statistical method may be used and the statistical methods should be selected during the design of the study.

Test Report

60. 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.
- Method of randomization.
- Description of any pre-test conditioning or screening 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 are relevant to particulates, in particular 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 of the inhalation chamber including its 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 analytical 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 (t95).
- 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).
- Analytical 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/m³, etc.) rather than in units of volume (ppm, ppb, etc.).
- Particle size distribution, mass median aerodynamic diameter (MMAD), and geometric standard deviation (σ_g), including their methods of calculation. Individual particle size analyses should be reported. Thermodynamic equivalent diameter (count or mass median diameter) should also be reported for aerosols test atmospheres that contain particles <100 nm.
- Analyses of the degree of NP agglomeration in the aerosol

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 for BALF analysis and lung burden measurements.

Results

- Tabulation of chamber temperature, humidity, and airflow.
- Tabulation of chamber target, analytical, and nominal concentration data.
- For aerosols atmospheres with an MMAD >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 (σ_g). 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 (σ_g). For aerosols with significant components in both the nano-size range and larger a combination of the above data will be required to fully characterise the aerosol, for such an aerosol information on the fraction of particles < 100 nm should also be provided.
- 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 fluid (BALF) data
- Tabulation of lung burden, when measured.
- Tabulation of pulmonary function data and body temperatures, when measured.
- Organ weights, gross pathology, and histopathological findings for each animal, if available.
- Report findings from optional health assessments of systemic toxicity (e.g. immunotoxicity, neurotoxicity, hepatotoxicity, cardiovascular effects).

Discussion and interpretation of results

- Particular emphasis should be given to the description of methods used to meet the criteria of this test guideline.
- The respirability of aerosol particles in light of the overall findings should be addressed, especially if the MMAD standard could not be met.
- The consistency of methods used to determine analytical and nominal concentrations, and the relation of analytical concentrations to nominal concentrations 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 (5).
- The target organ(s) should be identified.
- The BMC or NOAEC and 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.
- If applicable, detailed justification for the inclusion, design and methodology of post-observation lung burden determinations

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ANNEX TEST SCHEMES FOR TG 413¹

TG 413 Option A - Gases, vapours, liquid aerosols, and soluble solid aerosols

Examinations in the main study at PEO-1 and in the satellite groups at PEO-2: • Clinical observations • Body weight measurements • Food/water consumption • Clinical pathology • Gross pathology/organ weights • Lung weight-left lung • Histopathology-left lung • BALF-right lung	Exposure Groups	Main Study	Satellite Groups	Total animals
		PEO-1	PEO-2	
	0	HP (LL) + BAL (RL) 10 f/10 m	HP (LL) + BAL (RL) 5 f/5 m	
	C ₁	HP (LL) + BAL (RL) 10 f/10 m	HP (LL) + BAL (RL) 5 f/5 m	
	C ₂	HP (LL) + BAL (RL) 10 f/10 m	HP (LL) + BAL (RL) 5 f/5 m	
	C ₃	HP (LL) + BAL (RL) 10 f/10 m	HP (LL) + BAL (RL) 5 f/5 m	
		$\Sigma = 80$	$\Sigma = 40$	$\Sigma = 120$

Red = Should be done
Green = Optional

Abbreviations:

0	= control group	f	= female
C _x	= exposure concentration	m	= male
BAL	= bronchoalveolar lavage	PEO	= post-exposure observations
HP	= histopathology	PEO-1	= within one day after the last exposure day
LB	= lung burden	PEO-2	= within x weeks after the last exposure day
LL	= left lung	PEO-3	= within y weeks after the last exposure day (PEO-3 can be before or after PEO-2)
RL	= right lung	TBD	= to be determined

Notes: A successful main study depends on information obtained from a range-finding study or previous studies. All test chemicals use Option A except poorly soluble solid aerosols, which use Option B. GD 39 describes how a study director may customize these two options to optimize the hazard assessment of a test chemical.

TG 413 Option B –Poorly soluble solid aerosols

Examinations in the main study: PEO-1 (f and m): - Clinical observations - Body weight measurements - Food/water consumption - Clinical pathology - Gross pathology/organ weights - Lung weight-left lung (f and m) - Histopathology-left lung (f and m) - BALF-right lung (f and m) PEO-1 (satellite groups, m only): - Lung burden -right lung - Other parameters to be determined (TBD) by the study director	Exposure Groups	Main Study		Red = Should be done Green = Optional	Total animals		
		PEO-1					
	0	HP (LL) + BAL (RL) 10 f/10 m	LB (RL) (+ TBD) 5 m				
	C ₁	HP (LL) + BAL (RL) 10 f/10 m	LB (RL) (+ TBD) 5 m				
	C ₂	HP (LL) + BAL (RL) 10 f/10 m	LB (RL) (+ TBD) 5 m				
	C ₃	HP (LL) + BAL (RL) 10 f/10 m	LB (RL) (+ TBD) 5 m				
		$\Sigma = 80$	$\Sigma = 20$				
Examinations in the satellite groups at PEO-2 and/or PEO-3¹: PEO-2 (f and m): - Lung weight-left lung (f and m) - Histopathology-left lung (f and m) - BALF-right lung (f only) - Lung burden-right lung (m only) PEO-3 (m only): - Lung burden (right lung) - Other parameters to be determined (TBD) by the study director	Exposure Groups	Satellite Groups²		PEO-2	Satellite Groups²	Total animals	
		PEO-2					PEO-3
	0	HP (LL) + BAL (RL) 5 f	HP (LL) + LB (RL) 5 m				LB (RL) (+ TBD) 5 m
	C ₁	HP (LL) + BAL (RL) 5 f	HP (LL) + LB (RL) 5 m				LB (RL) (+ TBD) 5 m
	C ₂	HP (LL) + BAL (RL) 5 f	HP (LL) + LB (RL) 5 m				LB (RL) (+ TBD) 5 m
	C ₃	HP (LL) + BAL (RL) 5 f	HP (LL) + LB (RL) 5 m				LB (RL) (+ TBD) 5 m
		$\Sigma = 40$	$\Sigma = 20$	$\Sigma = 60$			

Red = Should be done
Green = Optional

Note: 1 The need for additional satellite groups at PEO-2 and/or PEO-3, the duration of the post-exposure interval, and the timing of the PEOs are determined by the study director based upon the purpose of the study and the results of a range-finding study and/or other relevant information. For example, PEO-1, PEO-2 and/or PEO-3 are used when multiple lung burden measurements are needed for evaluating clearance kinetics (see GD 39).