Guidance on the Biocidal Products Regulation

Volume III: Human health
Part A: Information requirements
Version 2, March 2022

Disclaimer

This document aims to assist users in complying with their obligations under the Biocides Regulation. However, users are reminded that the text of the Biocides Regulation is the only authentic legal reference and that the information in this document does not constitute legal advice. Usage of the information remains under the sole responsibility of the user. The European Chemicals Agency does not accept any liability with regard to the use that may be made of the information contained in this document.

Version	Changes	Date
Version 1.0	First edition	June 2013
Version 1.1	Corrigendum: - Division of the guidance in the 4 volumes of the new BPR Guidance structure Minor editorial changes	November 2014
Version 1.2	Corrigendum: - In Preface: To update the text to reflect the changes to the structure of the BPR guidance and to align the text with that in the current published Parts B+C for Volumes II, III and IV; - In Preface: to add text and links on "Applicability of Guidance"; - To amend the numbering of all sections to follow a normal sequential numbering format for the sections and to add in the heading for each section the relevant BPR Annex reference for clarification; - To relocate the "Finder" tables to follow the Table of Contents in "Notes for the Reader"; - To correct the explanation of the abbreviation ISO in the List of Abbreviations; - To delete references to the CLP transition arrangements and dates which no longer apply; To correct Figure 4 to add missing text from bottom left box.	May 2018
Version 2.0	Full revision The guidance was fully reviewed to ensure it is up to date. Major changes in the guidance were made due to the revision of information requirements by Regulation (EU) 2021/525, amending Annexes II and III to Regulation (EU) No 528/2012. The largest changes were made to the following paragraphs: - 1.1 *Skin corrosion or irritation* - 1.2 *Serious eye damage or eye irritation* - 1.3 *Skin sensitisation* - 1.6 *In vivo genotoxicity study (ADS)* – the requirement on UDS has been removed. - 1.10 *Reproductive toxicity* - 1.10.1 *Prenatal development toxicity study* - 1.10.2 *Extended one-generation reproductive toxicity study* - this new information requirement replaced the requirement of a two-generation study. - 1.10.3 *Developmental neurotoxicity* – this information requirement was changed from ADS to CDS - 1.12 *Relevant health data, observations and treatments* – this chapter was restructured and it now contains three subpoints instead of the earlier eight points. - 1.13.1 *Phototoxicity* – information is included on further test guidelines. - 1.13.3 *Endocrine disruption* and 1.13.3.1. *Specific additional studies to investigate potential endocrine disrupting properties (ADS)* – these chapters are new, reflecting the information in the Regulation (EU) 2017/2100 setting out the criteria for ED properties, in the ECHA/EFSA guidance on assessing ED properties, and the revision of the information requirements in BPR Annex II.	March 2022

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PREFACE

The Guidance on the Biocidal Products Regulation – Part A (information requirements) describes how to fulfil the information requirements set by the Biocidal Products Regulation. For an overview of all the guidance for biocides, please see the ECHA Biocides Guidance website[1].

Guidance on the applicability of new guidance and guidance related documents **for active substance approval** is provided in the document "Applicability time of new guidance and guidance-related documents in active substance approval" available on the BPC Webpage[2].

Guidance on the applicability of new guidance and guidance related documents **for product authorisation** is provided in the CA-document CA-july2012-doc6.2d (final)[3] available on the ECHA Biocides Guidance website[1].

[1] https://echa.europa.eu/guidance-documents/guidance-on-biocides-legislation
[2] Link available under Working Procedures at https://echa.europa.eu/about-us/who-we-are/biocidal-products-committee
[3] Direct link to the document: https://echa.europa.eu/documents/10162/23036409/ca-july12-doc_6_2d_final_en.pdf

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List of abbreviations

Standard term / Abbreviation	Explanation
°C	Degree(s) Celsius (centigrade)
ADME	Absorption, distribution, metabolism and excretion
ADI	Acceptable daily intake
ADS	Additional data set
AEL	Acceptable exposure level, overall systemic limit value for the human population
AOP	Adverse outcome pathways
ARfD	Acute Reference Dose
AUC	Area under the curve
BCF	Bioconcentration factor
BPD	Biocidal Products Directive. Directive 98/8/EC of the European Parliament and of the Council on the placing on the market of biocidal products
BPR	Biocidal Products Regulation. Regulation (EU) No 528/2012 of the European Parliament and of the Council concerning the making available on the market and use of biocidal products
Cat	Category
CDS	Core data set
CLH	Harmonised classification and labelling
CLP (Regulation)	Classification, Labelling and Packaging Regulation. Regulation (EC) No 1272/2008 of the European Parliament and of the Council on Classification, Labelling and Packaging of substances and mixtures
CTL	Cytotoxic T-lymphocyte
CWM	Cincinnati water maze
DG	European Commission Directorate General
DG SANTE	European Commission Directorate-General for Health and Food Safety
DIT	Developmental immunotoxicity
DNA	Deoxyribonucleic acid
DNT	Developmental neurotoxicity
DTH	Delayed type hypersensitivity
EATS	Oestrogen, androgen, thyroid, steroidogenesis (ED modalities)
EC	European Communities or European Commission
EC method	Test Method as listed in the Test Methods Regulation
ECHA	European Chemicals Agency
ED	Endocrine disruption; endocrine disruptor
EFSA	European Food Safety Agency

EU	European Union
FISH	Fluorescence in-situ hybridisation
FOB	Functional observation battery
g	Gram(s)
GC	Gas chromatography
GIVIMP	Good *in vitro* Method Practices
GLP	Good laboratory practice
h	Hour(s)
HPLC	High performance (or pressure) liquid chromatography
IATA	Integrated Approach on Testing and Assessment
IPCS	The WHO International Programme on Chemical Safety
ISBN	International standard book number
ITS	Integrated testing strategy
IUCLID	International Uniform Chemical Information Database
kg	Kilogram(s)
LD50	Lethal dose for 50% of the group of tested animals
LLNA	Murine local lymph node assay
mg	Milligram(s)
MMAD	Mass median aerodynamic diameter
mol	Mole(s)
MWM	Morris water maze
MRL	Maximum residue limit
MS	Mass spectrometry
MSCA	Member State competent authority
NAFTA	North American Free Trade Agreement
nm	Nanometre(s)
NMR	Nuclear magnetic resonance
NOAEL	No observed adverse effect level
OECD	Organisation for Economic Cooperation and Development
OPPTS	Office of Prevention, Pesticides, and Toxic Substances (U.S.-EPA)
Pa	Pascal(s)
PBK	Physiologically based kinetics
PBPK	Physiologically based pharmaco(toxico)-kinetics
pH	pH-value, negative decadic logarithm of the hydrogen ion concentration
PND	Postnatal day

PPI	Pre-pulse inhibition
PPPR	Plant Protection Products Regulation. Regulation (EC) No 1107/2009 of the European Parliament and of the Council of concerning the placing of plant protection products on the market
PT	Product-type
(Q)SAR	(Quantitative) structure activity relationship
RAAF	Read-Across Assessment Framework
RAM	Radial arm maze
REACH	Regulation (EC) No 1907/2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals
s	Second(s)
TG	Test guideline
TDAR	T-cell dependent antibody response
Test Methods Regulation	Regulation (EC) No 440/2008 laying down test methods pursuant to the REACH Regulation
UV	Ultraviolet
WHO	World Health Organisation

1. Dossier Requirements for Active Substances

Toxicological profile for human and animal including metabolism

Considerations before initiating testing

Before testing is initiated, the applicant should scrutinise all available information including open literature[4] for evidence that may indicate severe effects, serious specific system or target organ toxicity (e.g. neurotoxicity or immunotoxicity), delayed effects or cumulative toxicity. Consideration should also be given to tests already performed/submitted for the purpose of other regulatory programmes. All available information on toxicity should be taken into account when choosing the dose range for a new study.

If there is concern that an effect is not adequately covered by existing OECD Test Guidelines (TG), specialised study protocols may be used. Whenever deviating from OECD TGs, a scientific justification is required. Specialised study protocols should be designed on a case-by-case basis in order to enable an adequate characterisation of the hazards, including the dose-response, threshold for the toxic effect and an understanding of the nature of the toxic effects. Specialised study protocols may also be used to further assess already identified hazards, including investigation of the mode of action or human relevance. Where a need is identified for a modification in the study protocol to cover specific needs, this will be done in consultation with the evaluating Member State.

Both the applicants and the evaluating Member States must follow the principles of 3Rs, in line with Directive 2010/63/EU. Prior to initiating testing, applicants must consider read-across and grouping approaches (Read-Across Assessment Framework, RAAF[5]), as well as Integrated Approaches to Testing and Assessment (IATA) (OECD, 2020). In addition, existing information must be assessed even when not performed fully according to the information requirements to consider if it may be used as elements in a weight of evidence (WoE) adaptation according to BPR Annex IV. For guidance on WoE, please see the template, background document and examples published at the ECHA website: https://echa.europa.eu/support/guidance-on-reach-and-clp-implementation/formats, as well as *Guidance on the use of the weight of evidence approach in scientific assessments* (EFSA, 2017). The applicant is encouraged to follow and consider the latest developments regarding non-animal approaches and frameworks for preliminary prediction of developmental neurotoxicity. Such methods and approaches include but are not limited to the Adverse Outcome Pathways (AOP), read-across, *in silico*, *in chemico*, and *in vitro* methodologies, omics and other systems biology based approaches, and combinations of these.

The endpoints that need to be addressed for the purpose of the BPR are interlinked and in certain cases sequential testing strategy is needed to decide which tests need to be performed and in which order. This is due to the impact that the results from one study can have on the classification and labelling and the risk management measures, which can make the requirement for testing of other endpoints redundant. As an example, a carcinogenicity study would normally not need to be conducted if the substance is classified as Mutagen Category 1b.

To reduce animal use in testing, due consideration of the testing protocol is necessary while noting that the relevant study guidelines need to be followed. It is possible for two or more endpoints to be combined into a single *in vivo* study, thereby saving resources and numbers of animals used. For example, in line with the 3Rs principles, the combination of *in vivo* genotoxicity studies or integration of *in vivo* genotoxicity studies into repeated dose toxicity studies,

[4] Recommended guidance for open literature review: EFSA (2010) *Application of systematic review methodology to food and feed safety assessments to support decision making.*
[5] https://echa.europa.eu/documents/10162/614e5d61-891d-4154-8a47-87efebd1851a

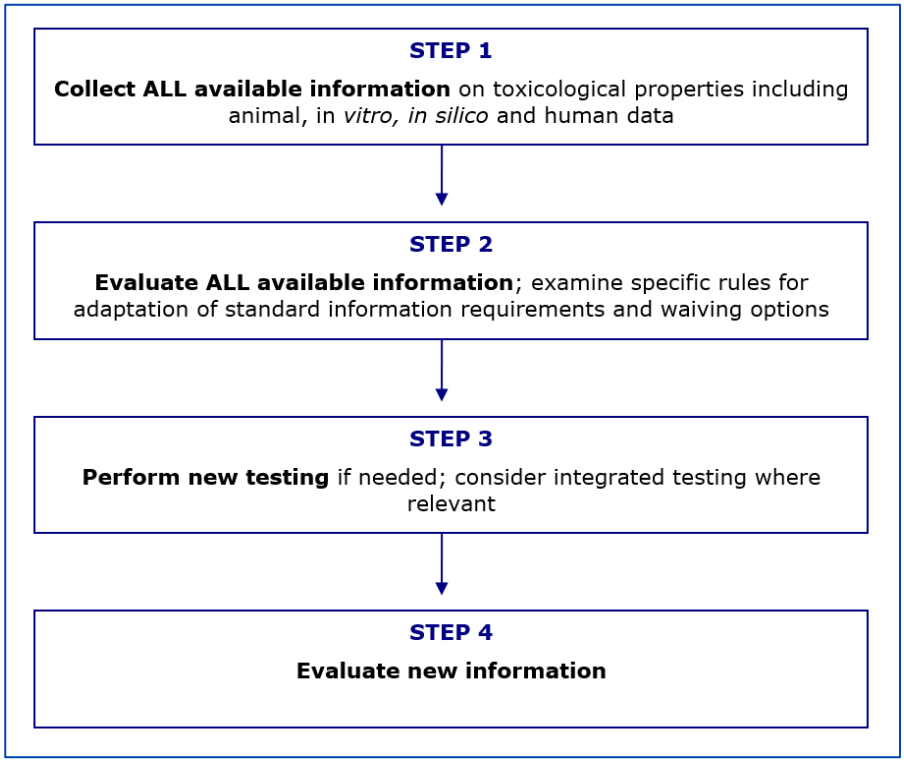
whenever possible and when scientifically justified, is strongly encouraged, as far as fulfilling the information requirements is not compromised. All experiments using vertebrate animals shall ensure that any possible pain, suffering, distress or lasting harm to the animals is eliminated or reduced to the minimum, in accordance with of Directive 2010/63/EU, noting also that stress and pain may directly or indirectly affect the study results.

The doses tested should be selected on the basis of the results of short-term testing and, where available at the time of planning the studies, on the basis of metabolism and toxicokinetic data. Dose selection should consider toxicokinetic data such as saturation of absorption measured by systemic availability of active substance and/or metabolites, as well as all other available information. *In vivo* range finding studies should be performed only if there are no suitable data available. The dose selection should ensure that the data generated are adequate for hazard identification, classification and labelling and risk assessment. Doses causing excessive toxicity should not be considered relevant to evaluations. Determination of blood concentration of the active substance (for example around Tmax) should be considered in long-term repeated dose toxicity studies. For each toxicological endpoint and the respective information requirements, all available information has to be collected and evaluated before concluding on the need to conduct further testing using integrated testing strategies (ITS) where relevant.

The Test Methods Regulation is regularly updated to follow the approval of new OECD Test Guidelines. In accordance with Point 5 of BPR Annex II, the latest version of an adopted test guideline should always be used when generating new data, independently from whether it is published by the EU or OECD. In addition to the test methods mentioned for each data requirement, new OECD validated tests should be taken into account once available in deciding the test strategy. Special attention should be given to new OECD validated methods that enable replacing or reducing the use of animals.

Figure 1 shows a schematic representation of the approach to fulfil the information requirements. For each information requirement, steps 1 and 2 need to be considered first to conclude on the need to conduct further testing using integrated testing strategies (ITS) where relevant.

Figure 1. Schematic representation of the stepwise approach to fulfil the BPR information requirements



General considerations for animal data reporting

Where submitted, historical control data should be from the same species and strain, maintained under similar conditions in the same laboratory and should be from contemporaneous studies (within a period of five years, centred as closely as possible on the date of the study). Additional historical control data not fulfilling these conditions, or from other laboratories may be reported separately as supplementary information.

The information on historical control data provided should include[6]:

(a) identification of species and strain, name of the supplier, and specific identification if the supplier has more than one geographical location;

(b) name of the laboratory and the dates when the study was performed;

(c) description of the general conditions under which animals were maintained, including the type or brand of diet and, where possible, the amount consumed;

(d) approximate age, in days, and weight of the control animals at the beginning of the study and at the time of sacrifice or death;

(e) description of the control group mortality pattern observed during or at the end of the study, and other pertinent observations (such as diseases, infections);

(f) name of the laboratory and the examining scientists responsible for gathering and interpreting the pathological data from the study;

(g) for carcinogenicity studies: a statement of the nature of the tumours that may have been combined to produce any of the incidence data.

The historical control data should be presented on a study-by-study basis giving absolute values plus percentage and relative or transformed values where these are helpful in the evaluation. If combined or summary data are submitted, these should contain information on the number of studies included and whether the current study is included, the range of values, the mean, median and, if applicable, standard deviation.

If the appropriateness of the control group of the study is in question, please refer to the considerations in OECD GD 116 (section 4.22) on the relevant details in analysing the historical control data.

Exposure assessment

Information must be provided to enable estimating the levels of exposure for users of the biocidal product and others who may be exposed following its use, including articles treated with the biocidal product where relevant. The applicant must include such information in the dossier.

The guidance on preparing the exposure assessment is provided in ECHA Guidance Vol III Parts B+C, and detailed methodological guidance is in the Biocides Human Health Exposure Methodology available at the ECHA HEAdhoc webpage[7].

Please refer also to information requirement 7.6 in BPR Annex II and information requirements

[6] This information will enable the assessment of the relevance of the historical data and the effects observed in the study provided. If some of the elements listed above are missing, this must be considered in assessing the relevance of the historical control data.
[7] https://echa.europa.eu/about-us/who-we-are/biocidal-products-committee/working-groups/human-exposure

7.10.1 and 7.10.3 in BPR Annex III, as well as sections 2.2.6 and 3.2.10 in ECHA Guidance Vol II Part A.

1.1. Skin corrosion or irritation

Table 1. Information requirement 8.1 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.1 Skin corrosion or irritation The assessment shall comprise the following tiers: (a) assessment of the available human, animal and non-animal data; (b) skin corrosion, *in vitro* testing; (c) skin irritation, *in vitro* testing; (d) skin corrosion or irritation, *in vivo* testing	The study/ies in column 1 do(es) not need to be conducted if: - the available information indicates that the substance meets the criteria for classification for skin corrosion or irritation, - the substance is a strong acid (pH≤ 2,0) or base (pH≥ 11,5), - the substance is spontaneously flammable in air or in contact with water or moisture at room temperature, - the substance meets the classification criteria for acute toxicity (Category 1) by the dermal route, or - an acute toxicity study by the dermal route provides conclusive evidence on skin corrosion or irritation adequate for classification. If results from one of the two studies listed in point (b) or point (c) in column 1 of this row already allow conclusive decision on the classification of a substance or on the absence of skin irritation potential, the second study does not need to be conducted An *in vivo* study for skin corrosion or irritation shall be considered only if the *in vitro* studies listed in points (b) and (c) in column 1 of this row are not applicable, or the results of these studies are not adequate for classification and risk assessment *In vivo* studies for skin corrosion or irritation that were carried out or initiated before 15 April 2022 shall be considered appropriate to address this information requirement

For skin corrosion/irritation, the information must be sufficient to conclude on the classification of the substance, i.e. that the criteria are met for classifying as skin corrosion (Cat. 1 of CLP) or as skin irritation (Cat. 2 of CLP), or that no classification is warranted.

The information below provides brief guidance for the assessment of skin corrosion or irritation. To support this, please refer to chapter R.7.2.6 of *REACH Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7a* where detailed information is given on the different steps/tiers, as well as on the OECD Guidance Document No. 203 on an Integrated Approach on Testing and Assessment (IATA) for skin corrosion/irritation (2017).

The testing and assessment strategy aims at identifying skin corrosion/irritation by using all the information available. A basic principle of the strategy is that the results of one study or information source are evaluated before another study is initiated. The strategy seeks to ensure that the data requirements are met in the most efficient and humane manner so that animal usage and costs are minimised.

Tier a) assessment of the available human, animal and non-animal data

In this Tier, all available information (including physico-chemical properties) should be evaluated before undertaking any new testing and to avoid, as far as possible, *in vivo* testing of corrosive

and severely irritating substances. In case new testing is needed, *in vitro* tests must be performed first, and it should be assessed whether *in vivo* testing can be completely avoided.

Further guidance regarding the assessment of existing information (physicochemical properties, grouping, (Q)SARs and expert systems, *in vitro* data, human data and animal data) is available within the *Guidance on the Application of the CLP Criteria*, ECHA Guidance Vol III Parts B+C and *REACH Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7a*.

Column 2 in Table 1 informs when studies for skin corrosion or irritation do not need to be conducted. In addition, if a good quality *in vivo* skin irritation study is already available i.e. study was carried out or initiated before 15 April 2022, it can be used to fulfil the standard information requirement.

For existing animal data, the use of methods other than those that are specified in the Annex to the EU Test Methods Regulation or the corresponding OECD methods may be accepted on a case-by-case basis. If the test was performed in other species than the rabbit, evaluation must be made with caution. Such information may be available e.g. from dermal toxicity studies in the rat or sensitisation studies in guinea pigs. One must note that the skin of the rat is less sensitive compared to rabbit skin, and the guinea pig skin is even less sensitive. Much lower exposures are employed in dermal toxicity testing and, in general, the scoring of dermal effects is performed less accurately. The results of dermal toxicity testing in rats or skin sensitisation tests in guinea pigs will not be adequate for classification for skin irritation/corrosion, unless the results indicate skin corrosivity that warrants classification as Skin Corrosive Category 1. In any other case, such information must be used in a Weight of Evidence assessment.

Existing human data include historical data that should be taken into account when evaluating intrinsic hazards of substances. New testing in humans for hazard identification purposes is not acceptable for ethical reasons. Existing data can be obtained from case reports, poison information centres, medical clinics, occupational experience, epidemiological studies and volunteer studies. Their quality and relevance for hazard assessment should be critically reviewed. However, in general, human data can be used to determine a corrosive or irritating potential of a substance. Good quality and relevant human data have precedence over other data. However, absence of incidence in humans does not necessarily overrule positive, good quality *in vitro* data or existing animal data.

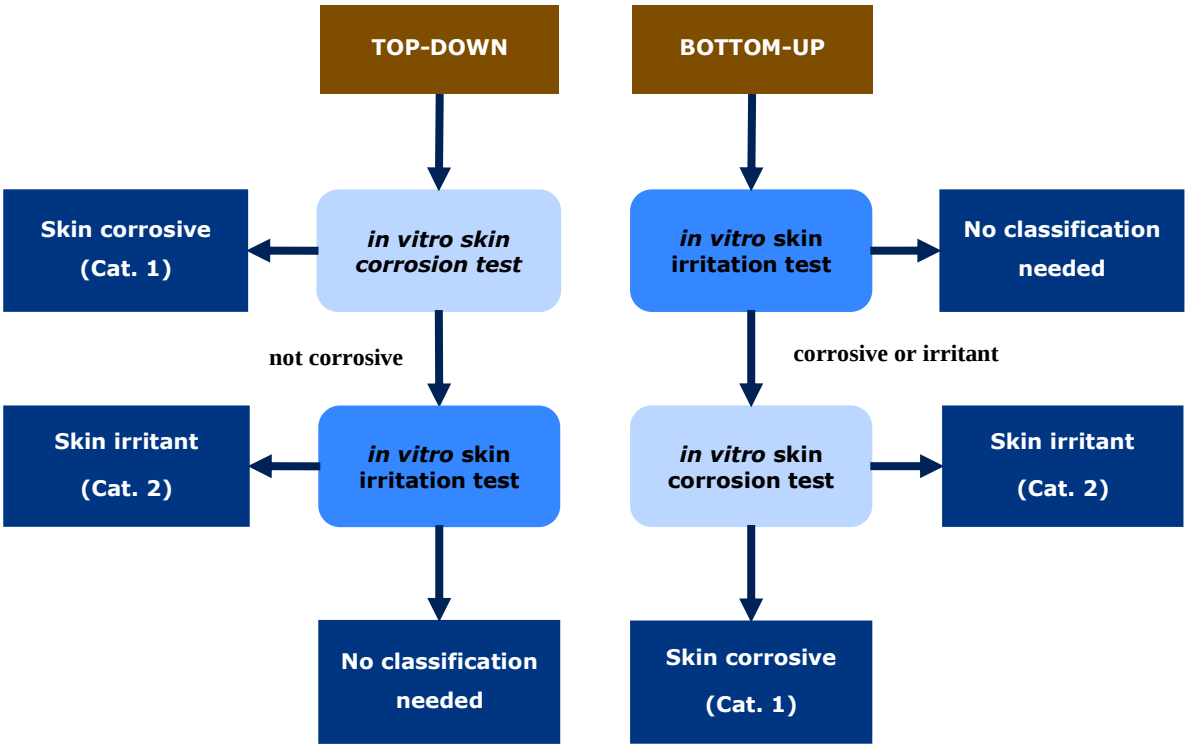
Considerations before performing further testing

If after the analysis in Tier a) further testing is needed to assess the potential for skin irritation or skin corrosion, the test methods in Tables 2, 3 and 4 should be used. Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

The tests will provide information on the degree and nature of the effects on skin especially with regard to the reversibility of responses.

New *in vitro* testing should be performed following a top-down or bottom-up approach, based on presumed properties (Figure 2). The top-down approach should be used when the available information suggests that the substance may be irritant or corrosive to the skin. The bottom-up approach should be followed when all available information suggests that the substance may not be irritant to the skin.

Figure 2. Schematic presentation of top-down and bottom-up approaches for skin corrosion/irritation.



After following this scheme, no new *in vivo* testing is normally necessary unless:

a) the available *in vitro* methods cannot be used due to substance specific limitations, or

b) the results of the *in vitro* test(s) performed do not enable a clear conclusion on classification and/or are insufficient for appropriate risk assessment.

Before performing any *in vivo* studies, it is necessary to identify any skin corrosion/irritation studies that may already be available, even if not fully equivalent to an OECD TG or an EU test method. If there are several studies and the results from such studies are consistent, they may together provide sufficient information on the skin corrosion/irritation potential of the substance.

Tier b) skin corrosion, *in vitro* testing

If after the analysis in Tier a) above, further testing is needed to assess the potential for skin corrosion, one of the test methods listed in Table 2 should be used. Before testing, consider whether corrosion or irritation would not be expected, in which case the bottom-up approach could be considered instead (see Figure 2).

Table 2. *In vitro* test methods for skin corrosion:

TEST METHOD	EU TEST METHOD / OECD TEST GUIDELINE	CLASSIFICATION ACCORDING TO CLP REGULATION
Transcutaneous electrical resistance tests	B.40 / TG 430	Cat. 1 or non-corrosive
Human skin model test(s)*	B.40bis / TG 431	Cat. 1, 1A, 1B/1C or non-corrosive
Membrane barrier test	B.65 / TG 435	Cat. 1, 1A, 1B and 1C or non-corrosive

* The test guideline contains multiple methods/protocols using reconstructed human epidermis.

The limitations and the scope of a given test method within a test guideline should be taken into account when selecting the most appropriate *in vitro* method for a particular substance and when interpreting the test results. Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

Tier c) skin irritation, *in vitro* testing

To examine the potential for skin irritation, the method(s) listed in the Table 3 below should be used.

Table 3. *In vitro* test methods for skin irritation

TEST METHOD	EU TEST METHOD / OECD TEST GUIDELINE	CLASSIFICATION ACCORDING TO CLP REGULATION
Reconstructed human epidermis test(s)*	B.46 / TG 439	Cat. 1/Cat. 2 or not classified

* The test guideline contains multiple methods/protocols using reconstructed human epidermis.

The limitations and the scope of a given test method within a test guideline should be taken into account when selecting the most appropriate *in vitro* method for a particular substance and when interpreting the test results. Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

Tier d) skin corrosion or irritation, *in vivo* testing

In vivo testing in Tier d) is required only as a last resort if the information assessed in the Tiers (a-c) above are not sufficient for concluding on the classification and/or for performing a risk assessment. In such a case, an *in vivo* skin irritation study should be performed using the test method listed in Table 4.

Table 4. *in vivo* test methods for skin corrosion/irritation

TEST METHOD	EU TEST METHOD / OECD TEST GUIDELINE	CLASSIFICATION ACCORDING TO CLP REGULATION
Acute Dermal Irritation/Corrosion test (*in vivo*)	B.4 / OECD TG 404	Cat. 1, Cat. 2 or not classified

In reporting *in vivo* information, particular attention should be given to the persistence of

irritation effects, even those which do not lead to classification. Effects such as erythema, oedema, fissuring, scaling, desquamation, hyperplasia and opacity, which do not reverse within the test period may indicate that a substance will cause persistent damage to the human skin.

1.2. Serious eye damage or eye irritation

Table 5. Information requirement 8.2 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.2 Serious eye damage or eye irritation The assessment shall comprise the following tiers: (a) assessment of the available human, animal and non-animal data; (b) serious eye damage or eye irritation, *in vitro* testing; (c) serious eye damage or eye irritation, *in vivo* testing	The study/ies in column 1 do(es) not need to be conducted if: —the available information indicates that the substance meets the criteria for classification for eye irritation or causing serious damage to eyes, — the substance is a strong acid (pH≤ 2,0) or base (pH≥ 11,5), — the substance is spontaneously flammable in air or in contact with water or moisture at room temperature, or —the substance meets the classification criteria for skin corrosion leading to classification of the substance as "serious eye damage" (category 1). If results from a first *in vitro* study do not allow a conclusive decision on the classification of the substance or on the absence of eye irritation potential (an)other(s) *in vitro* study(ies) for this endpoint shall be considered. An *in vivo* study for serious eye damage or eye irritation shall be considered only if the *in vitro* study(ies) listed in point (b) in column 1 of this row are not applicable, or the results obtained from these studies are not adequate for classification and risk assessment *In vivo* studies for serious eye damage or eye irritation that were carried out or initiated before 15 April 2022 shall be considered appropriate to address this information requirement

For serious eye damage or eye irritation, the information must be sufficient to conclude on the classification of the substance, i.e. that the criteria are met for classifying as serious eye damage (Cat 1 of CLP) or as eye irritation (Cat 2 of CLP), or that no classification is warranted.

The information below provides brief guidance for the assessment of serious eye damage or eye irritation. To support this, please refer to chapter R.7.2.11 of REACH Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7a where detailed information is given on the different steps/tiers, as well as in the OECD 2019 *Guidance Document No. 263 on Integrated Approaches to Testing and Assessment (IATA) for Serious Eye Damage and Eye Irritation*, Second Edition.

The testing and assessment strategy is aimed at the identification of serious eye damage/eye irritation by using different elements where appropriate, depending on the information available. A basic principle of the strategy is that the results of one study or from an information source are evaluated before another study is initiated. The strategy seeks to ensure that the data requirements are met in the most efficient and humane manner so that animal usage and costs are minimised.

Tier a) Assessment of the available human, animal and non-animal data

In this Tier, all available information (including physico-chemical properties) must be evaluated before undertaking any new testing and to avoid, as far as possible, *in vivo* testing of corrosive

and severely irritating substances. In case new testing is needed, *in vitro* tests must be performed first, and it should be assessed whether *in vivo* testing can be completely avoided.

Further guidance regarding the assessment of existing information (physicochemical properties, grouping, (Q)SARs and expert systems, *in vitro* data; human data and animal data) is available within the *Guidance on the Application of the CLP Criteria, ECHA Guidance Vol III Parts B+C* and *REACH Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7a*.

Column 2 in Table 5 informs when studies for serious eye damage or eye irritation do not need to be conducted. In addition, if a good quality *in vivo* eye irritation study is already available i.e. study was carried out or initiated before 15 April 2022, it can be used to fulfil the standard information requirement.

For existing animal data, the use of methods other than those specified in the Annex to the EU Test Methods Regulation, or corresponding OECD methods may be accepted on a case-by-case basis. To support this, please refer to the ECHA Guidance Vol III Parts B+C, and section 1.5.5.1.2 "*Testing data for irritation/corrosion (skin and eye)*" of REACH Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7a.

Existing human data include historical data that should be taken into account when evaluating intrinsic hazards of substances. New testing in humans for hazard identification purposes is not acceptable for ethical reasons. Existing data can be obtained from case reports, poison information centres, medical clinics, occupational experience, epidemiological studies and volunteer studies. Their quality and relevance for hazard assessment should be critically reviewed. However, in general, human data can be used to determine a corrosive or irritating potential of a substance. Good quality and relevant human data have precedence over other data. However, absence of incidence in humans does not necessarily overrule positive, good quality *in vitro* data or existing animal data.

Considerations before further testing

If after the analysis in Tier a) further testing is needed to assess the potential for serious eye damage or eye irritation, the test methods listed in Tables 6 and 7 should be used. Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

New *in vitro* testing should be performed following a top-down or bottom-up approach, based on presumed properties. The top-down approach starts with an *in vitro* test able to identify substances causing serious eye damage (Cat 1 of CLP). This approach should be used when all available information and the Weight-of-Evidence assessment indicate a high *a-priori* probability of the substance being seriously damaging to the eye. The bottom-up approach starts with an *in vitro* test able to identify substances not requiring classification for serious eye damage/eye irritation. This approach should be followed when all available information and the Weight-of-Evidence assessment indicate a high *a-priori* probability of the substance being non-irritant to the eyes.

After following this scheme, no new *in vivo* testing is normally necessary unless:

a) the available *in vitro* methods cannot be used due to substance specific limitations, or

b) the results of the *in vitro* test(s) performed do not enable a clear conclusion on classification and/or are insufficient for appropriate risk assessment.

Before performing any *in vivo* studies, it is necessary to identify any serious eye damage/eye irritation studies that may already be available, even if not fully equivalent to an OECD TG or an EU test method. If there are several studies and the results from such studies are consistent, they may together provide sufficient information on the serious eye damage/eye irritation

potential of the substance.

Tier b) Serious eye damage or eye irritation, *in vitro* testing

If after the analysis in Tier a) above further testing is needed to assess the potential for serious eye damage or eye irritation, the test methods in Table 6 below should be used. Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

Table 6: *In vitro* test methods for serious eye damage/eye irritation

TEST METHOD	EU TEST METHOD / OECD TEST GUIDELINE	CLASSIFICATION ACCORDING TO CLP REGULATION
BCOP	B.47 / OECD TG 437	Cat. 1 or not classified
ICE	B.48 / OECD TG 438	Cat. 1 or not classified
STE	B.68 / OECD TG 491	Cat. 1 or not classified
Macromolecular	N.A. / OECD TG 496	Cat. 1 or not classified
FL	B.61 / OECD TG 460	Cat. 1
RhCE	B. 69 / OECD TG 492	Not classified
Vitrigel	N.A. / OECD TG 494	Not classified

Abbreviations: BCOP = Bovine Corneal Opacity and Permeability; FL = Fluorescein Leakage; ICE = Isolated Chicken Eye; N.A. = not available; RhCE = Reconstructed human Cornea-like Epithelium Test Method; STE = Short-Time Exposure.

The limitations and the scope of a given test method within a test guideline should be taken into account when selecting the most appropriate method for a particular substance and when interpreting the test results.

The test methods mentioned above are suitable either for the direct identification of effects leading to serious eye damage (Cat. 1 of CLP) or substances not requiring classification under CLP. Currently there are no internationally adopted methods available for the direct identification of effects leading to eye irritation (Cat. 2 of CLP)[8].

If the results of one *in vitro* assay do not allow concluding on the classification of the substance or on the absence of eye irritation potential, additional *in vitro* studies may need to be performed.

Tier c) Serious eye damage or eye irritation, *in vivo* testing

In vivo testing is required only as a last resort if the information assessed in the Tiers a) and b) above are not sufficient for concluding on the classification and/or for performing a risk assessment. In such a case, an *in vivo* eye irritation study should be performed using the test method in Table 7.

[8] Currently under OECD umbrella work is ongoing on some approaches for the identification of Cat 2 eye irritants. Once formally adopted, those methods/approaches should be considered as well.

Table 7. *in vivo* test methods for serious eye damage/eye irritation

TEST METHOD	EU TEST METHOD / OECD TEST GUIDELINE	CLASSIFICATION ACCORDING TO CLP REGULATION
Acute Eye Irritation/Corrosion test (*in vivo*)	B.5 / OECD TG 405	Cat. 1, Cat. 2 or not classified

1.3. Skin sensitisation

Table 8. Information requirement 8.3 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.3 Skin sensitisation The information shall allow to conclude whether the substance is a skin sensitiser and whether it can be presumed to have the potential to produce significant sensitisation in humans (Category 1A). The information should be sufficient to perform a risk assessment where required The assessment shall comprise the following tiers: (a) assessment of the available human, animal and non-animal data; (b) skin sensitisation, *in vitro* testing. Information from *in vitro* or *in chemico* test method(s) referred to in point 5 of the introductory part of this Annex and addressing each of the following key events of skin sensitisation: (i) molecular interaction with skin proteins; (ii) inflammatory response in keratinocytes; (iii) activation of dendritic cells; (c) skin sensitisation *in vivo* testing. The Murine Local Lymph Node Assay (LLNA) is the first-choice method for *in vivo* testing. Another skin sensitisation test may only be used in exceptional cases. If another skin sensitisation test is used, justification shall be provided	The study/ies in column 1 do(es) not need to be conducted if: – the available information indicates that the substance meets the criteria for classification for skin sensitisation or skin corrosion – the substance is a strong acid (pH≤ 2,0) or base (pH≥ 11,5), or – the substance is spontaneously flammable in air or in contact with water or moisture at room temperature. *In vitro* tests do not need to be conducted if: – an *in vivo* study referred to in point (c) of column 1 of this row is available, or – the available *in vitro* or *in chemico* test methods are not applicable for the substance or the results obtained from those studies are not adequate for classification and risk assessment. If information from test method(s) addressing one or two of the key events described under point (b) in column 1 of this row allows for classification of the substance and risk assessment, studies addressing the other key event(s) do not need to be conducted An *in vivo* study for skin sensitisation shall be conducted only if *in vitro* or *in chemico* test methods described under point (b) in column 1 of this row are not applicable, or the results obtained from those studies are not adequate for classification and risk assessment *In vivo* skin sensitisation studies that were carried out or initiated before 15 April 2022 shall be considered appropriate to address this information requirement

If the substance is a skin sensitiser based on *in vitro/in chemico* testing and the results of *in vitro/in chemico* testing allow a sufficiently reliable conclusion that the substance has the potential to produce significant sensitisation in humans (Cat. 1A), no further testing is required.

If the substance is a skin sensitiser based on *in vitro/in chemico* testing, but the results of *in vitro/in chemico* testing allow a sufficiently reliable conclusion that the substance does not have the potential to produce significant sensitisation in humans, the substance can be presumed to

be a moderate skin sensitiser (Cat. 1B). In this case, no further testing is needed. However, if significant sensitisation (Cat. 1A) cannot be excluded with sufficient confidence based on *in vitro/in chemico* testing, additional information (*in silico/in vitro/in chemico*) would need to be generated to strengthen the weight of evidence. If still no reliable conclusion can be reached, as a last resort *in vivo* testing (LLNA) would need to be performed (Tier c).

According to data requirements, it is necessary to conclude whether the substance *can be presumed to have the potential to produce significant sensitisation in humans (Category 1A)*. However, in case there is already existing *in vivo* information (study initiated before 15 April 2022) that does not allow assessing the skin sensitisation potency, this information can still be used to fulfil the information requirement and no additional testing is required. In such cases, any information on skin sensitisation potency coming from such studies should be used together with existing information from other sources or with additional non-animal test data to refine the classification and risk assessment.

Tier a) Assessment of the available human, animal and non-animal data

In this Tier, all available information (including physico chemical properties) should be evaluated before undertaking any new testing. In case new testing is needed, *in vitro* tests must be performed first, and it should be assessed whether *in vivo* testing can be completely avoided.

Further guidance regarding the assessment of existing information (physicochemical properties, grouping, (Q)SARs and expert systems, *in vitro* data, human data and animal data) is available within the *Guidance on the Application of the CLP Criteria*, *ECHA Guidance Vol III Parts B+C* and *REACH Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7a*.

Column 2 in Table 8 informs when studies for skin sensitisation do not need to be conducted.

The decision on the need to test a substance for skin sensitisation when it fulfils one or more of these conditions requires expert judgment. This is because the information on skin sensitisation from the active substance will be used for the assessment of this property for products containing the substance, and it needs to be taken into account e.g. whether sub-corrosive concentrations of a substance may still have sensitising properties. For a substance that is corrosive, strong acid or strong base, the decision-making process on testing needs to take into account all the available information as specified in this tier.

If a good quality *in vivo* skin sensitisation study is already available, i.e. study was carried out or initiated before 15 April 2022, it can be used to fulfil the information requirement even if no conclusion on the skin sensitisation potency (Cat 1A or 1B of CLP) can be made.

For existing animal data, the use of methods other than those that are specified in the Annex to the EU Test Methods Regulation or the corresponding OECD methods may be accepted on a case-by-case basis, considering the reliability of the information and the relevance for classification and labelling.

When reliable and relevant human data are available, they can be useful for hazard identification and are even preferable over animal data. However, absence of incidence in humans does not necessarily overrule positive, good quality *in vitro* data or existing animal data. When human studies have been performed for safety assessment, the aim is to ensure that a specific concentration does not induce skin sensitisation, however those studies do not determine whether a substance has an intrinsic property to cause skin sensitisation. The situation is similar when diagnostic tests are carried out to see if an individual is sensitised to a specific agent, and not to determine whether the agent can cause sensitisation. The Guidance on the Application of the CLP Criteria provides extensive guidance on how to perform potency assessment based on

human data[9]. It is worth noting that sub-categorisation based on human data alone may not always be possible. Thus, all available data should be used in a weight-of-evidence approach to reach a conclusion and to avoid further testing.

Considerations before performing further testing

If after the analysis in Tier a) further testing is needed to assess the potential for skin sensitisation, the test methods mentioned in Tables 9, 10 and 11 below should be used. Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

The tests can provide information on i) whether the substance is a skin sensitiser or not, and/or ii) how potent sensitiser the substance is.

Tier b) Generation of new *in chemico/in vitro* test data

If after the analysis in Tier a) above further testing is needed to assess the potential for skin sensitisation, the test methods listed in Table 9 should be used. The limitations and the scope of a given test method within a test guideline should be taken into account when selecting the most appropriate *in vitro* method for a particular substance and when interpreting the test results. Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

As specified in the data requirement, all three key events need to be addressed. In case the *in chemico/in vitro* methods for one or more of the skin sensitisation key event(s) are not suitable for the substance, a scientific justification of that needs to be provided.

Currently there are no internationally approved stand-alone *in chemico/in vitro* methods for skin sensitisation. Therefore, information from multiple methods is always needed, either using a Defined Approach for skin sensitisation or by using a Weight of Evidence approach.

Table 9. *In chemico/in vitro* test methods for skin sensitisation

AOP KEY EVENT	TEST METHOD	EU TEST METHODS / OECD TEST GUIDELINE	OUTCOME ACCORDING TO THE TEST METHOD/GUIDELINE
Key Event 1 Peptide/protein binding	DPRA	B.59/TG 442C	Skin sensitiser (Cat 1) or non-sensitiser with complementary information
	ADRA	N.A/TG 442C	Skin sensitiser (Cat 1) or non-sensitiser with complementary information
	kDPRA*	N.A/ TG 442C	Skin sensitiser (Cat 1A) or non-category 1A (cannot differentiate between Cat 1B and non-sensitiser)
Key Event 2 Keratinocyte response	KeratinoSens™	B.60/TG 442D	Skin sensitiser (Cat 1) or non-sensitiser with complementary information
	LuSens	N.A/N.A	Skin sensitiser (Cat 1) or non-sensitiser with complementary information

[9] Please refer to Guidance on the Application of the CLP criteria, chapter 3.4.2.2 in Version 5.0 of July 2017.

Key Event 3 Monocytic /Dendritic cell response	h-CLAT	B.71/TG 442E	Skin sensitiser (Cat 1) or non-sensitiser with complementary information
	U-SENS™	B.71/TG 442E	Skin sensitiser (Cat 1) or non-sensitiser with complementary information
	IL-8 Luc Assay	B.71/TG 442E	Skin sensitiser (Cat 1) or non-sensitiser with complementary information
Defined approaches	2 out of 3	N.A/TG 497	Skin sensitiser (Cat 1) or non-sensitiser
	ITS v1 and v2	N.A/TG 497	Skin sensitiser (Cat 1, 1A and 1B) and non-sensitiser

Abbreviations: DPRA: Direct Peptide Reactivity Assay, ADRA: Amino acid Derivative Reactivity Assay, kDPRA: kinetic DPRA, h-CLAT: Human Cell Line Activation test, U-SENS™: U937 cell line activation Test, IL8-Luc assay: Interleukin-8 Reporter Gene Assay, ITS: Integrated testing strategy

Tier c) Generation of new *in vivo* test data

If after the analysis in Tiers a) and b) above further testing is needed to assess the potential for skin sensitisation, the test methods listed in Table 10 should be used. Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

Table 10. *In vivo Murine Local Lymph Node assay (LLNA)* test methods for skin sensitisation

TEST METHOD	EU TEST METHOD / OECD TEST GUIDELINE	CLASSIFICATION ACCORDING TO CLP REGULATION
Local Lymph Node Assay (LLNA)	B.46 / TG 429	Skin sensitiser (Cat. 1, 1A and 1B) or non-sensitiser
LLNA: DA.	B.50 / TG 442A	Skin sensitiser (Cat. 1) or non-sensitiser
LLNA: BrdU-ELISA or -FCM[10]	B.51 / TG 442B	Skin sensitiser (Cat. 1) or non-sensitiser

The EU method B.46/OECD TG 429 is recommended because information provided by the LLNA assay according to this method should be adequate for the assessment of the skin sensitisation potency. For the two LLNA variants there are no CLP criteria available to predict the skin sensitisation potency (Cat 1A or 1B). In case LLNA variants (EU B.50/OECD TG 442A or EU B.41/OECD TG 442B) are used to generate new information and a positive result is obtained, additional information needs to be generated to verify whether the substance warrants Cat 1A classification as specified in the legal text.

Specific limitations that may be described within the Test Guideline protocol should be taken into account before performing a test and when interpreting the test results.

[10] The LLNA assay FCM is described only in the OECD TG 442B.

If the LLNA assay is not considered suitable due to the properties of the substance to be tested, other OECD Test Guideline protocols can be used for the assessment of skin sensitisation, such as the methods in Table 11. If another *in vivo* method than LLNA is used, a scientific justification shall be provided.

Table 11. Other *in vivo* test methods for skin sensitisation

TEST METHOD	EU TEST METHOD / OECD TEST GUIDELINE	CLASSIFICATION ACCORDING TO CLP REGULATION
Guinea Pig Maximization test	B.6 / TG 406	Skin sensitiser (Cat. 1, 1A and 1B) or non-sensitiser*
Buehler Assay	B.6 / TG 406	Skin sensitiser (Cat. 1, 1A and 1B) or non-sensitiser*

* Due to the study design, potency estimation for skin sensitising substances (Cat 1A or 1B according to CLP) based on Guinea Pig Maximization study or Buehler study is rarely possible.

1.4. Respiratory sensitisation and irritation

1.4.1. Respiratory sensitisation (ADS)

Table 12. Information requirement 8.4 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.4 Respiratory sensitisation	

There are currently no standard tests and no OECD test guidelines available for respiratory sensitisation. Since an active substance identified as a skin sensitizer can potentially induce a hypersensitivity reaction, potential respiratory sensitisation and respiratory elicitation after dermal sensitisation should be taken into account when appropriate tests are available or when there are indications of respiratory sensitisation effects.

The assessment of the potential of a substance to induce respiratory sensitisation should include the assessment of the available existing information including physico-chemical properties, grouping, (Q)SARs and expert systems, *in vitro* data, human and animal data, and the outcome of immunotoxicity assessment (see section 1.13.4 of this guidance). The assessment should also consider *Guidance on the Application of the CLP Criteria* and *ECHA Guidance Vol III Parts B+C*.

The following information should be provided where available, including any details necessary for the evaluation of the information (please see also *ECHA Guidance Vol III Parts B+C*):

- Information on respiratory sensitisation or any incidences of respiratory hypersensitivity of workers or others exposed.
- Evidence that the substance can induce specific respiratory hypersensitivity will usually be based on human experience data. The clinical history data including both medical and occupational history, and reports from appropriate lung function tests related to exposure to the substance should be submitted, if available.
- Reports of other existing supportive evidence, such as:
    - Information of a chemical structure within the active substance that is related to substances known to cause respiratory hypersensitivity;

- *In vivo* immunological tests;
- *In vitro* immunological tests;
- Studies indicating other specific but non-immunological mechanisms of action; and
- Data from a positive bronchial challenge test.

1.4.2. Respiratory irritation (not in BPR Annex II)

There is no testing requirement for respiratory irritation under the BPR, and there are currently no standard tests or OECD TGs for respiratory irritation. Consequently, respiratory irritation is not included in the testing strategies suggested in this Guidance. Nevertheless, account should be taken of any existing and available data that provide evidence of the respiratory corrosion/irritation potential of a substance. One should consider if the data on dermal or ocular corrosion/irritation might contain information that is relevant for respiratory effects. Information from cases where symptoms have been associated with occupational exposures can be used on a case-by-case basis to characterise the respiratory irritation potency of a substance. Information from acute and repeated dose inhalation toxicity studies may also be considered sufficient to show that the substance causes respiratory irritation at a specific concentration level or range. The data need to be carefully evaluated with regard to the exposure conditions and sufficient documentation is required. Any confounding factors should be taken into account.

The exposure of atopic patients with bronchial asthma to some biocidal gases can result in so-called acute, unspecific hyperreactivity, an exacerbation or airway hyperresponsiveness (AHR). AHR is accompanied by adverse effects on human health and can constitute a serious health impairment especially in infants. Experimental animal testing systems for AHR are not a data requirement under BPR nor a part of an existing OECD TG, but any information on AHR should be considered for the active substance if it has the irritation potency and exposure can take place to the gas form.

Additional considerations for the evaluation of all available data with regard to respiratory irritation are provided in ECHA Guidance Vol III Parts B+C, REACH Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7a and Appendix to REACH Guidance Chapter R.8: Guidance for preparing a scientific report for health-based exposure limits at the workplace (chapter A.8-17.2.2.2.1).

1.5. Mutagenicity

Table 13. Information requirement 8.5 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.5 Mutagenicity The assessment of this endpoint shall comprise the following consecutive steps: — an assessment of the available *in vivo* genotoxicity data — an *in vitro* test for gene mutations in bacteria, an *in vitro* cytogenicity test in mammalian cells and an *in vitro* gene mutation test in mammalian cells are required — appropriate *in vivo* genotoxicity studies shall be considered in case of a positive result in any of the *in vitro* genotoxicity studies	

The testing of genotoxicity is intended to identify substances that might cause permanent transmissible changes in the amount or structure of a single gene or gene segments, a block of genes or chromosomes.

The aim of genotoxicity testing is to:

- predict genotoxic potential;
- identify genotoxic carcinogens at an early stage;
- elucidate the mechanism of action of active substances inducing germ-line mutations, which may lead to inherited disorders.

Appropriate dose levels, depending on the test requirements, should be used in either *in vitro* or *in vivo* assays. A tiered approach should be adopted, with selection of higher tier tests being dependent upon interpretation of results at each stage.

At least one *in vitro* test for gene mutations in bacteria, one test for cytogenicity in mammalian cells and one test for gene mutation in mammalian cells are required.

For substances that are short-lived reactive *in vitro* mutagens, or for which no indications of systemic availability have been presented, local genotoxicity needs to be considered. See section "Specific considerations for *in vivo* genotoxicity testing in Chapter 1.6".

Collection and evaluation of available information

For the assessment of existing information (physicochemical properties, grouping, [Q]SARs and expert systems, *in vitro* data, human data and animal data) further guidance is available within the *Guidance on the Application of the CLP Criteria* and *ECHA Guidance Vol III Parts B+C*. For further information, the following documents can be considered:

- Overview on Genetic Toxicology TGs (OECD 2017). OECD Series on Testing and Assessment, No. 238, OECD Publishing, Paris, https://doi.org/10.1787/9789264274761-en
- Clarification of some aspects related to genotoxicity assessment (EFSA 2017) https://doi.org/10.2903/j.efsa.2017.5113

Generation of new test data

If after the analysis above further testing is needed to assess the potential for genotoxicity *in vitro*, the test methods in chapters 1.5.1, 1.5.2 and 1.5.3 below should be used. Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

Testing for genotoxicity (*in vitro* assays)

The test guideline protocols to follow for the investigation of *in vitro* genotoxicity are listed below (section 1.5.1 to 1.5.3 of this guidance). These should be used taking into account some considerations described here but also taking into account the existing information for this endpoint and its assessment.

If there are indications of micronucleus formation in an *in vitro* micronucleus assay, further testing with appropriate centromere labelling should be conducted to clarify if there is an aneugenic or clastogenic response. Further investigation of the aneugenic response may be considered to determine whether there is sufficient evidence for a threshold mechanism and

threshold concentration for the aneugenic response. Please see also Sections 4 and 5 of EFSA guidance on aneugenicity assessment (EFSA, 2021).

Active substances which display highly bacteriostatic properties as demonstrated in a range finding test do not need an Ames test. Such substances should be tested in at least one *in vitro* mammalian cell test for gene mutation, i.e. in either an *In Vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene (OECD 490) or an *In Vitro* Mammalian Cell Gene Mutation Tests using the Hprt and xprt genes assay (OECD 476). If the Ames test is not performed, this should be justified.

For active substances bearing structural alerts for which the standard tests have not been optimised, additional testing may be required if the substance has given negative results in the standard test battery. The choice of an additional study or study plan modifications depends on the chemical nature, the known reactivity and the metabolism data on the structurally alerting active substance.

1.5.1. *In vitro* gene mutation study in bacteria

Table 14. Information requirement 8.5.1 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.5.1 *In vitro* gene mutation study in bacteria	

The test methods for *in vitro* gene mutation in bacteria are given in Table 15 below.

Table 15. *In vitro* test methods for gene mutation in bacteria:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Mutagenicity - reverse mutation test using bacteria*	B.13/14	TG 471

* Title of the OECD test guideline: Bacterial Reverse Mutation Test

1.5.2. *In vitro* cytogenicity study in mammalian cells

Table 16. Information requirement 8.5.2 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.5.2 *In vitro* cytogenicity study in mammalian cells	

The test methods for *in vitro* cytogenicity in mammalian cells are given in Table 17 below.

Table 17. *In vitro* test methods for cytogenicity in mammalian cells:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Mutagenicity - *In vitro* mammalian chromosome aberration test	B.10	TG 473
In vitro Mammalian Cell Micronucleus Test		TG 487

The *in vitro* cell micronucleus test is considered as the preferred method for examining *in vitro* cytogenicity in mammalian cells due to its higher sensitivity and ability to identify also the effect of aneugens provided that appropriate centromere labelling is performed in case of positive results.

1.5.3. *in vitro* gene mutation study in mammalian cells

Table 18. Information requirement 8.5.3 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.5.3 *In vitro* gene mutation study in mammalian cells	

The test methods for *in vitro* gene mutation in mammalian cells are given in Table 19 below.

Table 19. *In vitro* test methods for cytogenicity in mammalian cells:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
In Vitro Mammalian Cell Gene Mutation Tests using the Hprt and xprt genes		TG 476
In Vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene		TG 490

1.6. *In vivo* genotoxicity study (ADS)

Table 20. Information requirement 8.6 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.6 *In vivo* genotoxicity study The assessment shall comprise the following tiers: (a) If there is a positive result in any of the *in vitro* genotoxicity studies as listed in 8.5 and there are no reliable results available from an appropriate *in vivo* somatic cell genotoxicity study, an appropriate *in vivo* somatic cell genotoxicity study shall be conducted; (b) A second *in vivo* somatic cell genotoxicity study may be necessary depending on the *in vitro* and *in vivo* results, type of effects, quality and relevance of all available data; (c) If there is a positive result from an *in vivo* somatic cell genotoxicity study available, the potential for germ cell mutagenicity should be considered based on all available data, including toxicokinetic evidence to demonstrate whether the substance has the capacity to reach germ cells. If no clear conclusions about germ cell mutagenicity can be made, additional investigations shall be considered	The study/ies in column 1 do(es) not need to be conducted if: — the results are negative for the three *in vitro* tests listed in 8.5 and no other concern has been identified (e.g. metabolites of concern formed in mammals), or — the substance meets the criteria to be classified as a germ cell mutagen category 1A or 1B. The germ cell genotoxicity test does not need to be conducted if the substance meets the criteria to be classified as a carcinogen, category 1A or 1B and a germ cell mutagen category 2

Collection and evaluation of available information

For the assessment of existing information (physicochemical properties, grouping, (Q)SARs and expert systems, *in vitro* data, human data and animal data), further guidance is available within the *Guidance on the Application of the CLP Criteria* and *ECHA Guidance Vol III Parts B+C*.

Generation of new test data

If after the analysis above further testing is needed to assess the potential for genotoxicity *in vivo*, the test methods below should be used. Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

Testing for genotoxicity: *In vivo* studies in somatic cells (Tiers a-b)

Before any decisions are made on the need for *in vivo* testing, a review of the *in vitro* test results and all available information on the toxicokinetic and toxicodynamic profile of the test substance is needed. A particular *in vivo* test should be conducted only when it can be reasonably expected from all the properties of the test substance and the proposed test protocol that the specific target tissue will be adequately exposed to the test substance and/or its metabolites. If necessary, a targeted investigation of toxicokinetics should be conducted before progressing to *in vivo* testing (e.g. a preliminary toxicity test to confirm that absorption occurs and that an appropriate dose route is used).

In case *in vivo* testing with the comet assay and the micronucleus test is required, the two tests should be combined into a single acute study with appropriate modification of treatment and sampling times. The combination should not impair the validity of and the results from each individual study. Careful consideration should be given to the dosing, and tissue sampling for comet analysis alongside the requirements of tissue sampling for the mammalian erythrocyte micronucleus test. The following assays can be integrated into repeated dose toxicity studies described under section 1.9 of this guidance:

- The comet assay,
- *in vivo* micronucleus test,
- Transgenic Rodent Somatic and Germ Cell Gene Mutation Assay

In the interest of ensuring that the number of animals used in genotoxicity tests is kept to a minimum, using both males and females is not always necessary. In accordance with standard guidelines, testing in one sex only is possible when the substance has been investigated for general toxicity and no sex-specific differences in toxicity have been observed.

If the *in vitro* mammalian chromosome aberration test or the *in vitro* micronucleus test is positive for clastogenicity, an *in vivo* test for clastogenicity should be conducted using somatic cells such as metaphase analysis in rodent bone marrow or micronucleus test in rodents. The *in vivo* micronucleus test is the preferred test, as:

- it allows the investigation of both clastogenicity and aneugenicity.
- its endpoint is simple and easy to identify,
- detects the genetic alteration which is remaining after mitosis in "daughter cells".

In case of a positive result in the *in vivo* micronucleus assay, appropriate staining procedure such as fluorescence in-situ hybridisation (FISH) should be used to identify an aneugenic and/or clastogenic response. For this purpose, two sets of slides should be prepared before scoring, unless counting is done by flow cytometry.

If any of the *in vitro* gene mutation tests is positive, an *in vivo* test to investigate the induction of gene mutation should be conducted, such as the Transgenic Rodent Somatic and Germ Cell Gene Mutation Assay.

When conducting *in vivo* genotoxicity studies, only relevant exposure routes and methods (*such as* admixture to diet, drinking water, skin application, inhalation, gavage) should be used. There should be convincing evidence that the relevant tissue will be reached by the chosen exposure route and application method. Other exposure techniques (*such as* intraperitoneal or subcutaneous injection) that are likely to result in abnormal kinetics, distribution and metabolism should be justified.

The available test guideline protocols for assessing the *in vivo* genotoxic potential of a substance are listed below and reflect current state of knowledge. The choice of the most appropriate test to conduct should reflect the considerations described in this section and future recommendations or changes within the OECD Test Guideline programme for this endpoint.

The *in vivo* test methods for genotoxicity are given in Table 21 below.

Table 21. *In vivo* test methods for genotoxicity:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Mutagenicity - *In vivo* mammalian erythrocyte micronucleus test*	B.12	TG 474
Mutagenicity – *In vivo* mammalian bone-marrow chromosome aberration test**	B.11	TG 475
Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays		TG 488
In Vivo Mammalian Alkaline Comet Assay		TG 489

* Title of the OECD test guideline: Mammalian Erythrocyte Micronucleus Test
* Title of the OECD test guideline: Mammalian Bone Marrow Chromosome Aberration Test

Testing for genotoxicity: *In vivo* studies in germ cells (Tier c)

The potential to affect germ cells should always be considered for substances giving positive results in *in vivo* tests for genotoxic effects in somatic cells. This includes substances classified as Muta 2 for which data on germ cells is not available or conclusive, unless the Muta 2 substance also meets the criteria to be classified as a carcinogen, category 1A or 1B. The first step is to make an appraisal of all the available toxicokinetic and toxicodynamic properties of the test substance. Expert judgment is needed at this stage to consider whether there is sufficient information to conclude that the substance poses a mutagenic hazard to germ cells. If this is the case, it can be concluded that the substance may cause heritable genetic damage and no further testing is justified. Consequently, the substance is classified as a category 1B mutagen. If the appraisal of mutagenic potential in germ cells is inconclusive, additional investigation will be necessary. In the event that additional information on the toxicokinetics of the substance would resolve the problem, targeted (tailored) toxicokinetic investigation may be required to demonstrate whether the substance has the capacity to reach germ cells. In case germ cells testing is necessary, the type of mutation produced in earlier studies (gene mutations or chromosomal aberrations) should be considered when selecting the appropriate assay.

Alternatively, other methods can be used if deemed appropriate by expert judgment. These may

include the mammalian spermatogonial chromosome aberration test (OECD TG 483) or gene mutation tests with transgenic animals (OECD TG 488). The comet assay as described in the OECD TG 489 is, at present, not considered appropriate to measure DNA strand breaks in mature germ cells.

The available test guideline protocols for assessing *in vivo germ cell mutagenicity* of a substance are listed below according to the current state of knowledge. The choice of the most appropriate test to conduct should reflect the considerations described in this section and future recommendations or changes within the OECD Test Guideline programme for this endpoint.

The test methods for *in vivo* germ cell genotoxicity are given in Table 22 below.

Table 22. *In vivo* test methods for germ cell genotoxicity:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Mammalian spermatogonial chromosome aberration test	B.23	TG 483
Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays		TG 488

Specific considerations for *in vivo* genotoxicity testing

For substances that are short-lived, reactive, *in vitro* mutagens, or for which no indications of systemic availability have been presented, the analysis of tissues at initial sites of contact with the body is a crucial element of the testing strategy. Expert judgment should be used on a case-by-case basis to decide which tests are the most appropriate. The main options to investigate local genotoxicity are the *in vivo* comet assay and the gene mutation test with transgenic rodents. Both assays employ methods by which any tissue (containing nucleated cells) of an animal can in theory be examined for effects on the genetic material. This gives the possibility to examine site-of-contact tissues, i.e. epithelium of the respiratory or gastro-intestinal tract (e.g. nasal epithelium and lungs for inhalation; glandular stomach and duodenum for oral route) as target tissues of the assays. For any given substance, expert judgment, based on all the available toxicological information, will indicate which of these tests are the most appropriate. The route of exposure should be selected that best allows assessing the hazard posed to humans. For poorly soluble or insoluble substances, the possibility of release of active molecules in the gastrointestinal tract may indicate that a test involving the oral route of administration is particularly appropriate.

Special testing requirements in relation to photogenotoxicity may be indicated by the structure of a molecule for substances that absorb light within the range of natural sunlight (290-700 nm). If the molar extinction coefficient of the active substance and its major metabolites is less than 1.000 L × mol^{-1} × cm^{-1} (measured in methanol), photogenotoxicity testing is not required. Please see also the ICH Guidance S10 on Photosafety Evaluation of Pharmaceuticals[11].

[11] Available at https://www.ema.europa.eu/en/ich-s10-photosafety-evaluation-pharmaceuticals.

1.7. Acute toxicity

Table 23. Information requirement 8.7 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.7 Acute toxicity In addition to the oral route of administration (8.7.1), for substances other than gases, the information mentioned under 8.7.2 to 8.7.3 shall be provided for at least one other route of administration — The choice for the second route will depend on the nature of the substance and the likely route of human exposure — Gases and volatile liquids should be administered by the inhalation route — If the only route of exposure is the oral route, then information for only that route need be provided. If either the dermal or inhalation route is the only route of exposure to humans then an oral test may be considered. Before a new dermal acute toxicity study is carried out, an *in vitro* dermal penetration study (OECD 428) should be conducted to assess the likely magnitude and rate of dermal bioavailability — There may be exceptional circumstances where all routes of administration are deemed necessary	The study/ies do(es) not generally need to be conducted if: — the substance is classified as corrosive to the skin

Assessment of the acute toxic potential of a chemical is necessary to determine the adverse health effects that might occur following accidental or deliberate short-term exposure.

Administration via different routes makes an overall assessment of relative acute hazard in different exposure routes possible.

Collection and evaluation of available information

For the assessment of existing information (physicochemical properties, grouping and read-across, (Q)SARs and expert systems, *in vitro* data, human data and animal data), further guidance is available within the Guidance on the Application of the CLP Criteria, ECHA Guidance Vol III Parts B+C and in the REACH Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7a: Endpoint specific guidance.

1.7.1. By oral route

Table 24. Information requirement 8.7.1 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.7.1 By oral route The Acute Toxic Class Method is the preferred method for the determination of this endpoint	The study need not be conducted if: — the substance is a gas or a highly volatile substance

Generation of new test data

If after the analysis of all available information further testing is needed to assess the potential for acute toxicity by the oral route, the test methods below should be used. Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

The test methods for acute toxicity via oral route are given in Table 25 below.

Table 25. Test methods for acute toxicity via oral route:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Acute oral toxicity - Acute toxic class method	B.1 tris	TG 423
Acute oral toxicity - fixed dose procedure	B.1 bis	TG 420
Acute oral toxicity: up-and-down procedure		TG 425
Acute oral toxicity		TG 401*

* Acceptable only if performed before December 2002

According to the BPR data requirement, the acute toxic class method is the preferred study. However, taking into account animal welfare, in performing new studies the fixed dose procedure should be considered.

1.7.2. By inhalation

Table 26. Information requirement 8.7.2 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.7.2 By inhalation Testing by the inhalation route is appropriate if exposure of humans via inhalation is likely taking into account: — the vapour pressure of the substance (a volatile substance has vapour pressure > 1 × 10 –2 Pa at 20 °C) and/or — the active substance is a powder containing a significant proportion (e.g. 1 % on a weight basis) of particles with particle size MMAD < 50 micrometers or — the active substance is included in products that are powders or are applied in a manner that generates exposure to aerosols, particles or droplets of an inhalable size (MMAD < 50 micrometers) — the Acute Toxic Class Method is the preferred method for the determination of this endpoint	

Generation of new test data

If after the analysis of available information, and the considerations listed below, further testing is needed to assess the potential for acute toxicity by inhalation, the test methods below should be used. Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

If there is absence of information on particle/droplet size and where there is potential for exposure via inhalation from the use of biocidal products containing the active substance, an acute inhalation study should be performed.

The test methods for Acute toxicity via inhalation route are given in Table 27 below.

Table 27. Test methods for acute toxicity via inhalation route:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Acute Inhalation Toxicity – Acute Toxic Class Method		TG 436
Acute Inhalation Toxicity: Fixed Concentration Procedure		TG 433
Acute toxicity (inhalation)*	B.2	TG 403

* Title of the OECD test guideline: Acute Inhalation Toxicity

When selecting an acute inhalation study, preference should be given to OECD TG 436 (according to BPR Annex II requirements) and secondarily to OECD TG 433, as these methods have been designed to use less animals than EU B.2/OECD TG 403. However, in some circumstances, e.g. if a dose-response curve is needed for risk assessment purposes, testing according to EU B.2 / OECD TG 403 may be considered appropriate (see also the OECD Guidance Document 39).

The full study using three dose levels may not be necessary if a substance at an exposure concentration equal to the limit concentrations of the test guideline (limit test) or at the maximum attainable concentration produces no compound-related mortalities.

The head/nose only exposure should be used, unless whole body exposure can be justified.

1.7.3. By dermal route

Table 28. Information requirement 8.7.3 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.7.3 By dermal route Testing by the dermal route is necessary only if: — inhalation of the substance is unlikely, or — skin contact in production and/or use is likely, and either — the physicochemical and toxicological properties suggest potential for a significant rate of absorption through the skin, or — the results of an *in vitro* dermal penetration study (OECD 428) demonstrate high dermal absorption and bioavailability	

Generation of new test data

Dermal toxicity should normally be reported for an active substance except for gases.

If after the analysis of all available information further testing is needed to assess the potential for acute toxicity by the dermal route, the following test methods should be used. Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

The test methods for acute toxicity via dermal route are given in Table 29 below.

Table 29. Test methods for acute toxicity via dermal route:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Acute toxicity (dermal)*	B.3	TG 402

* Title of the OECD test guideline: Acute Dermal Toxicity

For substances with low acute dermal toxicity, a limit test with 2000 mg/kg body weight may be sufficient.

1.8. Toxicokinetics and metabolism studies in mammals

Table 30. Information requirement 8.8 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.8 Toxicokinetics and metabolism studies in mammals The toxicokinetics and metabolism studies should provide basic data about the rate and extent of absorption, the tissue distribution and the relevant metabolic pathway including the degree of metabolism, the routes and rate of excretion and the relevant metabolites	

The generation of toxicokinetic data should be considered in light of the generation of other toxicity data (e.g. on repeated dose toxicity, mutagenicity, reproductive toxicity) to assist in the estimation of systemic exposure to the active substance and/or its metabolites and the correlation of the effects observed with internal dose estimates. This is important in establishing the mode of action of the active substance and whether administered doses cause non-linear dose response due to saturation kinetics. Such information is valuable in the derivation of assessment factors, route-to-route extrapolation and hazard characterisation, as well as in considering the validity of read-across and grouping approaches.

Collection and evaluation of available information

For the assessment of existing information (physicochemical properties, grouping, (Q)SARs and expert systems, *in vitro* data, human and animal data) further guidance is available within *ECHA Guidance Vol III Parts B+C* and the REACH Guidance on Toxicokinetics within the REACH CSA&IR, Chapter R.7c: Endpoint specific guidance.

Generation of new test data

Following the evaluation of all available data, a decision should be made on which type of kinetic

data and which test design is the most appropriate. In order to reduce the use of animals in testing, it is preferred to generate kinetic data within the toxicity studies such as repeated dose toxicity studies where possible. The sections below describe the issues to consider when designing new tests for toxicokinetics and the available techniques for the tests suitable for ADME (absorption, distribution, metabolism, elimination) estimation. See Figure 3 in Chapter 1.9, explaining how toxicokinetic data can be used in the design of repeated dose toxicity studies.

The OECD TG 417 provides the protocol for the conduct of toxicokinetic studies either as standalone test or in combination with repeated dose toxicity studies.

In vivo studies provide an integrated perspective on the relative importance of different processes in the intact biological system for comparison with the results of the toxicity studies. To ensure a valid set of toxicokinetic data, a toxicokinetic *in vivo* study has to consist of several experiments that include blood/plasma kinetics, mass balances and excretion experiments as well as tissue distribution experiments. Depending on the problem to be solved, selected experiments (e.g. plasma kinetics) may be sufficient to provide data for further assessments (e.g. bioavailability).

The high dose level administered in an ADME study should be linked to the dose levels that cause adverse effects in toxicity studies. Ideally there should also be a dose without toxic effect, which should be in the range of expected human exposure including consideration of limit of quantification. A comparison between toxic dose levels and those that are likely to represent human exposure values may provide valuable information for the interpretation of adverse effects and is essential for extrapolation and risk assessment.

In an *in vivo* study the systemic bioavailability is usually estimated by the comparison of either dose-corrected amounts excreted, or of dose-corrected areas under the curve (AUC) of plasma (blood, serum) kinetic profiles, after extra- and intravascular administration. The systemic bioavailability is the dose-corrected amount excreted, or AUC determined after an extravascular substance administration divided by the dose-corrected amount excreted, or AUC determined after an intravascular substance application, which corresponds by definition to a bioavailability of 100%. This is only valid if the kinetics of the compound is linear, i.e. dose-proportional, and relies upon the assumption that the clearance is constant between experiments. If the kinetics is not linear, the experimental strategy has to be revised on a case-by-case basis, depending of the type of non-linearity involved (e.g. saturable protein binding, saturable metabolism, etc).

Generally *in vitro* studies provide data on specific aspects of toxicokinetics such as metabolism. A major advantage of *in vitro* studies is that it is possible to carry out parallel tests on samples from the species used in toxicity tests and samples from humans, thus facilitating interspecies comparisons (e.g., metabolite profile, metabolic rate constants).

In recent years, methods to integrate a number of *in vitro* and *in silico* information into a prediction of ADME *in vivo* by the use of appropriate physiologically based kinetic (PBK) models have been developed. Such methods allow both the prediction of *in vivo* kinetics at early stages of development, and the progressive integration of all available data into a predictive model of ADME. The uncertainty associated with the prediction depends largely on the amount of available data. Applicants are encouraged to use and further develop PBK models that have become an important tool to facilitate the translation of doses that elicit biological responses in cellular systems to exposure levels *in vivo* (OECD 2021).

Information on the concentration of the active substance and relevant metabolites in blood and tissues, for example around the time to reach the maximum blood (serum/plasma) concentration (T_{max}) or other relevant toxicokinetic parameter, should be generated in short and long-term studies on relevant species to better use the toxicological data generated in terms of understanding the toxicity studies. If such information is not considered essential for the assessment, full justification should be provided.

The main objective of the toxicokinetic data is to describe the systemic exposure achieved in animals and its relationship to the dose levels and the time course of the toxicity studies. Other objectives are:

(a) to relate the achieved exposure in toxicity studies to toxicological findings and contribute to the assessment of the relevance of these findings to human health with a particular regard to vulnerable groups;

(b) to support the design of a toxicity study (choice of species, treatment regimen, selection of dose levels) with respect to kinetics and metabolism;

(c) to provide information which, in relation to the findings of toxicity studies, contributes to the design of supplementary toxicity studies.

Absorption, distribution, metabolism and excretion (ADME) after exposure by oral route

The use of *in silico* methods and physiologically based (pharmaco)kinetic (PBPK) modelling should be considered upfront in the assessment and before generating toxicokinetic data.

Absorption

Absorption is normally investigated by the determination of the test substance and/or its metabolites in excreta, exhaled air and carcass (i.e. radioactivity balance). The biological response between test and reference groups (e.g. oral versus i.v.) is compared and the plasma/blood level of the test substance and/or its metabolites is determined.

Distribution

For determination of the distribution of a substance in the body, two approaches are available at present for analysis of distribution patterns. Quantitative information can be obtained using whole-body autoradiographic techniques, or by sacrificing animals at different times after exposure and determination of the concentration and amount of the test substance and/or metabolites in tissues and organs (EC method B.36: Toxicokinetics, OECD TG 417: Toxicokinetics).

Accumulative potential

Information derived for the purpose of environmental risk assessment can be relevant for human health risk assessment and the potential for a substance to accumulate. The static bioconcentration factor (BCF) is the ratio of the concentration of a substance in an organism to the concentration in water once a steady state has been achieved. The resulting fish BCF is widely used as a surrogate measure for bioaccumulation potential. For further information, see the ECHA Guidance Vol IV Environment (Part A; Parts B+C).

If single dose toxicity and tissue distribution data are not adequate to determine the potential for accumulation, repeated dose administration may be needed to address the potential for accumulation and/or persistence or changes in toxicokinetics.

Accumulating substances can also be measured in milk and therefore additionally allow an estimation of transfer to the breast-fed pup.

Metabolism

In vitro tests can be performed using isolated enzymes, microsomes and microsomal fractions, immortalised cell lines, primary cells and organ slices. Most frequently these materials originate from the liver as this is the most relevant organ for metabolism, however, in some cases

preparations from other organs are used for investigation of potential organ-specific metabolic pathways. In the absence of standardised *in vitro* methods, generation of novel *in vitro* ADME data should be in accordance with the OECD guidance document on "Good *in vitro* Method Practices" (GIVIMP) (OECD, 2018).

When using metabolically incompetent cells, an exogenous metabolic activation system is usually added into the cultures. For this purpose, the post-mitochondrial 9000 g supernatant (S9 fraction) of whole liver tissue homogenate containing a high concentration of metabolising enzymes is most commonly employed – the donor species needs to be considered in the context of the study. In all cases metabolism may either be directly assessed by specific identification of the metabolites or by subtractive calculation of the amount of parent substance lost in the process.

In vivo toxicokinetic studies generally only determine the rates of total metabolic clearance (by measuring radiolabelled products in blood/plasma, bile, and excrements) rather than the contributions of individual tissues. It has to be taken into account that the total metabolic clearance is the sum of the hepatic and potential extrahepatic metabolism.

Excretion

The major routes of excretion are in the urine and/or the faeces (via bile and directly from the GI mucosa). For this purpose, urine, faeces and, in certain circumstances, bile are collected and the amount of test substance and/or metabolites in these excreta is measured and those accounting for 5% or more of the administered dose should be identified where possible (EC method B.36: Toxicokinetics, OECD TG 417: Toxicokinetics).

The excretion of chemicals (metabolites) in other biological fluids such as saliva, milk, tears, and sweat is usually negligible compared with renal or biliary excretion. However, in special cases these fluids may be important to study either for monitoring purposes, or in the case of milk allowing an assessment of the exposure of infants.

For volatile substances and metabolites, exhaled air has to be examined as it may be an important route of elimination.

Available data from human biological monitoring and biological marker measurement studies should be part of the assessment. Further guidance on the use of these methods is provided in *ECHA Guidance Vol III Parts B+C*.

Aspects to consider in the design of tests for toxicokinetic data generation

Information on one *in vivo* test species (normally rat), taking any gender differences into account, may be sufficient to cover absorption, distribution, metabolism and excretion after exposure by oral route. These data can provide information useful in the design and interpretation of subsequent toxicity tests. However, information on interspecies differences is crucial in extrapolation of animal data to humans and information on metabolism following administration via other routes may be useful in human risk assessments. To support this information, the applicant may consider submitting comparative *in vitro* metabolism data on different species including rats and humans.

It is not possible to specify detailed information requirements in all areas, since the exact requirements will depend on the results obtained for each particular test substance.

The studies should be designed on a case-by-case basis, considering generation of information about the kinetics of the active substance and its metabolites in relevant species after being exposed to the following conditions:

- a single oral dose (low and high dose levels);

- an intravenous dose (preferably), or if available, a single oral dose with assessment of biliary excretion (low dose level); and
- a repeated dose.

When intravenous dosing is not feasible, a justification should be provided.

A key parameter is systemic bioavailability (F), obtained by comparison of the area under the curve (AUC) after oral and intravenous dosing.

The information from the studies should include:

- rate and extent of oral absorption including maximal concentration in blood (C_{max}), AUC, T_{max} and other appropriate parameters, such as bioavailability;
- potential for bioaccumulation;
- clearance and half-lives (t½);
- distribution in major organs and tissues;
- information on the distribution in blood cells;
- chemical structure and quantification of metabolites in biological fluids and tissues;
- different metabolic pathways;
- route and time course of excretion of active substance and metabolites;
- information on enterohepatic circulation.

Any comparative *in vitro* metabolism studies should be performed on animal species to be used in pivotal studies and on human material, using microsomes or intact cells (when relevant from donors/animals of both sexes), in order to assess the relevance of the toxicological animal data, facilitate the interpretation of findings and to decide the testing strategy.

An explanation must be given or further tests should be carried out where a metabolite is detected *in vitro* in human material and not in the tested animal species.

Absorption, distribution, metabolism and excretion after exposure by other routes

Data on absorption, distribution, metabolism and excretion (ADME) following exposure by the dermal route should be provided where toxicity following dermal exposure is of concern compared to that following oral exposure. Before investigating ADME *in vivo* following dermal exposure, the need to conduct an *in vitro* dermal penetration study should be considered in order to assess the likely magnitude and rate of dermal bioavailability, also taking note of the possibility of using default values for estimating dermal uptake and excretion as described in *ECHA Guidance Vol III Parts B+C*.

Absorption, distribution, metabolism and excretion after exposure by the dermal route should be considered on the basis of the above information, unless the active substance causes skin irritation that would compromise the outcome of the study.

For volatile active substances (vapour pressure $>10^{-2}$ Pa at 20 °C) absorption, distribution, metabolism and excretion after exposure by inhalation may be useful in human risk assessments.

Dermal absorption

An appropriate dermal absorption assessment is needed. It is not always mandatory to submit experimental data. If such data are not available, as a first step default values can be used according to the EFSA Guidance Document on Dermal Absorption (EFSA, 2017).

The test methods available for skin absorption studies are given in Table 31 below.

Table 31. Test methods for skin absorption:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Skin Absorption: *In Vitro* Method (human tissue preferred over rat)	B.45	TG 428
Skin Absorption: *In Vivo* Method	B.44	TG 427

If testing to assess the likely magnitude and rate of dermal bioavailability is necessary, the OECD TG 428 for *in vitro* skin absorption should be considered first.

Percutaneous absorption depends on the partitioning of substances from the vehicle and solubility in the vehicle. OECD TG 427 and TG 428 recommend conducting tests using test preparations that are the same as (or a realistic surrogate to) those that humans may be exposed to.

In vitro methods are designed to measure the penetration of chemicals into the skin and their subsequent permeation through the skin into a fluid reservoir, as well as partition to the different skin layers and possible deposition therein. Provided that the excised skin sample is intact and its integrity has been proven by appropriate methods, it can reasonably be assumed that its barrier function to what is generally a diffusional process has been maintained *in vitro* (also after frozen storage [Harrision et al., 1984, Bronaugh 39 et al., 1986 and Steinling et al., 2001]).

Very lipophilic substances are difficult to examine *in vitro* because of their low solubility in most receptor fluids. By including the amount retained in the skin *in vitro*, a more acceptable estimation of skin absorption can be obtained. Water soluble substances can be tested more accurately *in vitro* because they diffuse into the receptor fluid more readily (OECD, 2004a).

At present, results from *in vitro* methods seem to adequately reflect those from *in vivo* experiments, supporting their use as a replacement test to measure percutaneous absorption (Lehman et al. 2011).

Advantages of the *in vivo* method (EC method B.44, OECD TG 427) are that it uses a physiologically and metabolically intact system and a species common to many toxicity studies, and it can be modified for use with other species. The disadvantages are the use of animals, the need for radiolabelled material to facilitate reliable results, difficulties in determining the early absorption phase and the differences in permeability of the preferred species (rat) and human skin. Animal skin is generally more permeable and therefore may overestimate human percutaneous absorption. The experimental conditions should also be taken into account in interpreting the results. For instance, dermal absorption studies in fur-bearing animals may not accurately reflect dermal absorption in humans.

When valid (guideline-compliant and GLP) *in vitro* studies on human skin, *in vitro* studies on animal skin and *in vivo* animal studies are available and conducted under the same experimental conditions, and the results meet the quality criteria, in particular with respect to variability, number of acceptable replicates and recovery, then the 'Triple Pack' approach can be used to

extrapolate the human dermal absorption values for risk assessment (OECD No. 156, draft) (see also section 2.6 of this guidance).

In silico models might also provide information on dermal absorption, but currently they have not gained regulatory acceptance. *In silico* models for prediction of dermal absorption for pesticides have been evaluated and reported (Kneuer et al. 2018). Mathematical skin permeation models are usually based on uptake from aqueous solution which may not be relevant for the exposure scenario being assessed. In addition, the use of such models for quantitative risk assessment purposes is often limited because these models have generally been validated by *in vitro* data ignoring the fate of the skin residue levels. However, in silico models and (Q)SARs may be useful as screening tools or for qualitative comparison of skin permeation potential, particularly within a group of closely related substances.

Considerations for test substances and analytical methodology for toxicokinetic studies

Toxicokinetic and metabolism studies can be carried out using non-labelled compounds, stable isotope-labelled compounds, radioactively labelled compounds or using dual (stable and radio-) labelling. The labels should be placed in metabolically stable positions, avoiding the placing of labels such as ^{14}C in positions from which they can enter the carbon pool of the test animal. If metabolic degradation of the test substance may occur, different labelling positions have to be taken into account to be able to determine all relevant degradation pathways. The radiolabelled compound must be of high radiochemical purity and of adequate specific activity to ensure sufficient sensitivity in radio-assay methods.

Separation techniques are used in metabolism studies to purify and separate several radioactive fractions in biota such as urine, plasma, bile and others. These techniques range from relatively simple approaches such as liquid-liquid extraction and column chromatography to more sophisticated techniques such as HPLC (high pressure liquid chromatography). These methods also allow the establishment of a metabolite profile. Quantitative analytical methods are required to follow concentrations of parent compound and metabolites in the body as a function of time. The most common techniques used are LC/MS (liquid chromatography/ mass spectroscopy) and high-performance LC with UV-detection, or if ^{14}C-labelled material is used, radioactivity detection HPLC. It is worth mentioning that kinetic parameters generally cannot be calculated from measurement of total radioactivity to receive an overall kinetic estimate. Nevertheless, to generate exact values one has to address parent compound and metabolites separately. An analytical step is required to define the radioactivity as chemical species. This is usually faster than cold analytical methods. Dual labelling (e.g. ^{13}C and $^{14}C/^{12}C$) is the method of choice for structural elucidation of metabolites (by MS and NMR spectroscopy). A cold analytical technique, which incorporates stable isotope labelling (for GC/MS [gas chromatography/mass spectroscopy] or LC/MS), is a useful combination. Unless this latter method has already been developed for the test compound in various matrices (urine, faeces, blood, fat, liver, kidney, etc.), the use of radiolabelled compound may be less costly than other methods.

In any toxicokinetic study, the identity and purity of the substance used in the test must be assured. Analytical methods capable of detecting undesirable impurities will be required, as well as methods to assure that the substance of interest is of uniform potency from batch to batch. Additional methods will be required to monitor the stability and uniformity of the form in which the test substance is administered to the organisms used in the toxicokinetic studies. Finally, methods suitable to identify and quantify the test substance in toxicokinetic studies must be employed.

In the context of analytical methods, *accuracy* refers to how closely the average value reported for the assay of a sample corresponds to the actual amount of substance being assayed in the sample, whereas *precision* refers to the amount of scatter in the measured values around the average result. If the average assay result differs from the actual amount in the sample, the assay is said to be *biased*, i.e., lacks specificity; bias can also be due to low recovery.

Assay *specificity* is perhaps the most serious problem encountered. Although *blanks* provide some assurance that no instrument response will be obtained in the absence of the test chemical, a better approach is to select an instrument or bioassay that responds to some biological, chemical, or physical property of the test chemical that is not shared with many other substances.

The assay method should be usable over a sufficiently wide range of concentrations for the substance and its metabolites. The lower limit of reliability for an analytical method has been perceived in different ways; frequently, the term sensitivity has been used to indicate the ability of an analytical method to measure small amounts of a substance accurately and with requisite precision. It is unlikely that a single analytical method will be of use for all these purposes. Indeed, it is highly desirable to use more than one method. If two or more methods yield essentially the same results, confidence in each method is increased.

1.8.1. Further toxicokinetic and metabolism studies in mammals (ADS)

Table 32. Information requirement 8.8.1 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.8.1 Further toxicokinetic and metabolism studies in mammals Additional studies might be required based on the outcome of the toxicokinetic and metabolism study conducted in rat. These further studies shall be required if: — there is evidence that metabolism in the rat is not relevant for human exposure — route-to-route extrapolation from oral to dermal/inhalation exposure is not feasible Where it is considered appropriate to obtain information on dermal absorption, the assessment of this endpoint shall proceed using a tiered approach for assessment of dermal absorption	

With the core dataset, basic information about the rate and extent of absorption, the tissue distribution and the relevant metabolic pathway including the degree of metabolism, the routes and rate of excretion and the relevant metabolites should be provided by the toxicokinetic and metabolism studies (BPR Annex II Section 8.8). Additional information might be needed based on the outcome of the toxicokinetic and metabolism study conducted in rats (ADS according to Annex II Section 8.8.1) or based on the evaluation of the toxicological and physicochemical profile of the substance.

Further toxicokinetic/metabolism studies with repeated dose administration may be necessary for example when there are indications for a potential of the active substance to accumulate, to persist or to change the toxicokinetics e.g. by induction of metabolic enzymes. Section 1.8 of this guidance provides guidance on the options available for the toxicokinetics study and its integration with the repeated dose toxicity tests.

1.9. Repeated dose toxicity

Table 33. Information requirement 8.9 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.9 Repeated dose toxicity In general, only one route of administration is necessary and the oral route is the preferred route. However, in some cases it may be necessary to evaluate more than one route of exposure. For the evaluation of the safety of consumers in relation to active substances that may end up in food or feed, it is necessary to conduct toxicity studies by the oral route Testing by the dermal route shall be considered if: — skin contact in production and/or use is likely, and — inhalation of the substance is unlikely, and — one of the following conditions is met: (i) toxicity is observed in an acute dermal toxicity test at lower doses than in the oral toxicity test, or (ii) information or test data indicate dermal absorption is comparable or higher than oral absorption, or (iii) dermal toxicity is recognised for structurally related substances and for example is observed at lower doses than in the oral toxicity test or dermal absorption is comparable or higher than oral absorption Testing by the inhalation route shall be considered if: — exposure of humans via inhalation is likely taking into account the vapour pressure of the substance (volatile substances and gases have vapour pressure > 1 × 10 – 2 Pa at 20 °C), and/or — there is the possibility of exposure to aerosols, particles or droplets of an inhalable size (MMAD < 50 micrometers)	The repeated dose toxicity study (28 or 90 days) does not need to be conducted if: — a substance undergoes immediate disintegration and there are sufficient data on the cleavage products for systemic and local effects and no synergistic effects are expected, or — relevant human exposure can be excluded in accordance with Section 3 of Annex IV In order to reduce testing carried out on vertebrates and in particular the need for free-standing single-endpoint studies, the design of the repeated dose toxicity studies shall take account of the possibility to explore several endpoints within the framework of one study

Repeated dose toxicity testing provides information on adverse effects as a result of repeated or prolonged exposure. The objectives of assessing repeated dose toxicity are to evaluate:

1. adverse effects based on human or non-human studies:

   - whether exposure of humans to a substance is associated with adverse toxicological effects occurring as a result of repeated daily exposure for a part of the expected lifetime or for the major part of the lifetime; these human studies potentially may also identify populations that have higher susceptibility;

   - whether administration of a substance to experimental animals causes adverse toxicological effects as a result of repeated daily exposure for a part or a major part of the expected lifespan; effects that are predictive of possible adverse human health effects;

2. the target organs, potential cumulative effects and the reversibility of the adverse toxicological effects;

3. the dose-response relationship and threshold for any of the adverse toxicological effects observed in the repeated dose toxicity studies;

4. the basis for risk characterisation and classification and labelling (C&L) of substances for repeated dose toxicity;

5. the mode of action (MOA) and mechanism data.

Repeated dose toxicity tests may also provide information relevant for reproductive toxicity, carcinogenicity, neurotoxicity, immunotoxicity and endocrine disruption. If new studies are performed, including relevant investigations on these effects should be considered on the basis of all the information on the substance.

For the assessment of existing information (physico-chemical properties, grouping and read-across[12], [Q]SARs and expert systems, *in vitro* data, human data and animal data) further guidance is available within the *Guidance on the Application of the CLP Criteria,* the *ECHA Guidance Vol III Parts B+C* and the practical guides[13] such as "How to use and report (Q)SARs".

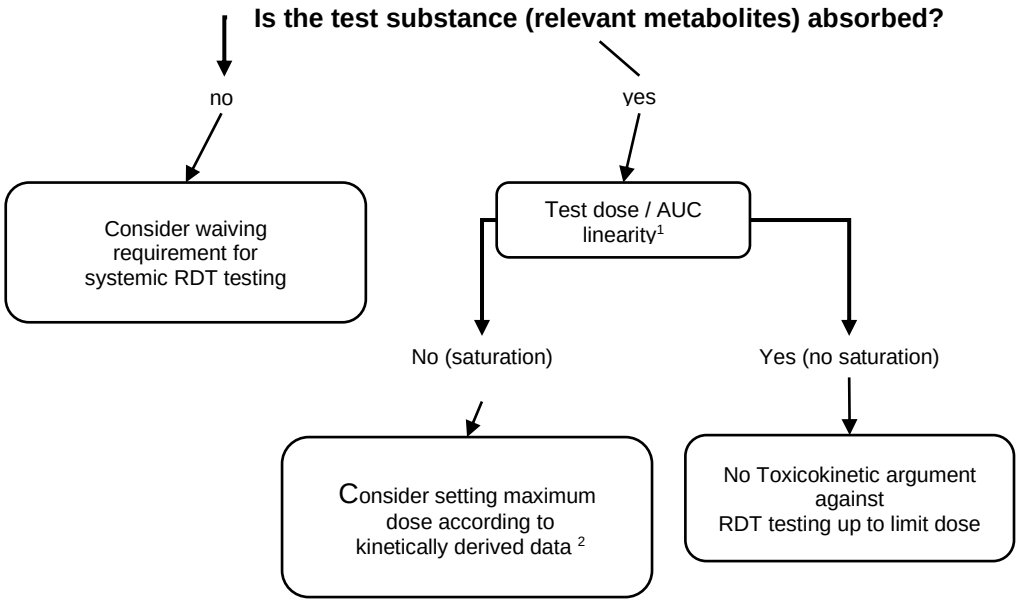
The most appropriate data on repeated dose toxicity are primarily obtained from studies in experimental animals conforming to internationally agreed test guidelines.

Where new testing is needed, please see also the general information under Considerations before initiating testing in chapter 1 and the considerations on dose range-finding studies, selection of vehicle, route of administration, and dose level selection presented in chapter 1.10.

Justification to replace the oral route by another significant route, or to require testing in addition to the oral route needs to be provided.

[12] https://echa.europa.eu/documents/10162/614e5d61-891d-4154-8a47-87efebd1851a
[13] https://echa.europa.eu/practical-guides

Figure 3. Use of toxicokinetic data in the design of repeated dose toxicity studies



[1] In the dose-range under consideration for RDT testing

[2] Meaning that the highest dose-level should not exceed the range of non-linear kinetics.

1.9.1. Short-term repeated dose toxicity study (28 days), preferred species is rat

Table 34. Information requirement 8.9.1 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.9.1 Short-term repeated dose toxicity study (28 days), preferred species is rat	The short-term toxicity study (28 days) does not need to be conducted if: (i) a reliable sub-chronic (90 day) study is available, provided that the most appropriate species, dosage, solvent and route of administration were used, (ii) the frequency and duration of human exposure indicates that a longer term study is appropriate and one of the following conditions is met: — other available data indicate that the substance may have a dangerous property that cannot be detected in a short-term toxicity study, or — appropriately designed toxicokinetic studies reveal accumulation of the substance or its metabolites in certain tissues or organs which would possibly remain undetected in a short term toxicity study but which are liable to result in adverse effects after prolonged exposure

In principle, for substances where a 90-day repeated dose toxicity study needs to be performed, an additional 28-day repeated dose toxicity study will not be required.

If a 28-day repeated dose toxicity needs to be performed, the considerations described under section 1.9.2 of this guidance regarding the generation of new test data should also be taken into account. The 28-day repeated dose toxicity study should be combined to other studies when possible to minimise animal experimentation.

Generation of new test data

If after evaluating the available information further testing is needed to assess repeated dose toxicity, the test methods below should be used.

Repeated dose toxicity (oral)

The test methods for repeated dose toxicity via oral route are given in Table 35 below.

Table 35. Test methods for repeated dose toxicity via oral route:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Repeated dose (28 days) toxicity (oral)*	B.7	TG 407

* Title of the OECD test guideline: Repeated dose 28-day oral toxicity study in rodents

Repeated dose toxicity (dermal)

If the substance is a severe irritant or corrosive, testing by the dermal route should be avoided unless it can be performed at doses that do not cause irritation or corrosion and such doses are still toxicologically relevant and the outcome can be used in risk assessment.

The test methods for repeated dose toxicity via dermal route are given in Table 36 below.

Table 36. Test methods for repeated dose toxicity via dermal route:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Repeated dose (28 days) toxicity (dermal)	B.9	TG 410

* Title of the OECD test guideline: Repeated dose dermal toxicity: 21/28-day study

Repeated dose toxicity (inhalation)

The test methods for repeated dose toxicity via inhalation route are given in Table 37 below.

Table 37. Test methods for repeated dose toxicity via inhalation route:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Repeated dose (28 days) toxicity (inhalation)	B.8	TG 412

* Title of the OECD test guideline: Subacute inhalation toxicity: 28-day study

1.9.2. Sub-chronic repeated dose toxicity study (90-day), preferred species is rat

Table 38. Information requirement 8.9.2 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.9.2 Sub-chronic repeated dose toxicity study (90 days), preferred species is rat	The sub-chronic toxicity study (90 days) does not need to be conducted if: — a reliable short-term toxicity study (28 days) is available showing severe toxicity effects according to the criteria for classifying the substance as H372 and H373 (Regulation (EC) No 1272/2008), for which the observed NOAEL-28 days, with the application of an appropriate uncertainty factor allows the extrapolation towards the NOAEL-90 days for the same route of exposure, and — a reliable chronic toxicity study is available, provided that an appropriate species and route of administration were used, or — the substance is unreactive, insoluble, not bioaccumulative and not inhalable and there is no evidence of absorption and no evidence of toxicity in a 28-day 'limit test', particularly if such a pattern is coupled with limited human exposure

Generation of new test data

If after evaluating the existing data further testing is needed to assess repeated dose toxicity, the test methods described below should be used.

Considerations for the design of the repeated dose subchronic toxicity studies

The study will be performed in a single rodent species, preferably the rat. The oral route will be used unless one of the other routes is more appropriate based on either the most relevant route of human exposure or the physico-chemical properties of the substance. The other routes should be considered especially if route-to-route extrapolation is not appropriate, and the predominant human exposure occurs via dermal and/or inhalation route. *In vivo* testing with corrosive substances at concentration levels causing corrosivity must be avoided. In the 90-day study, potential neurotoxic and immunotoxic effects (see also sections 1.13.2 and 1.13.4 of this guidance), genotoxicity by way of micronuclei formation and effects potentially related to changes in the endocrine system (see also section 1.13.3 of this guidance) must be carefully considered during the conduct of the test and reported, taking into account potential limitations when modifying test protocols in order to investigate specific effects.

Information on mode of action from structurally similar substances should also be considered in the design of repeated dose toxicity tests.

Repeated dose toxicity studies should be designed to provide information as to the amount of the active substance that can be tolerated without adverse effects under the conditions of the study and to elucidate health hazards occurring at higher dose levels. Such studies provide useful data on the risks for those handling and using biocidal products containing the active substance, among other possible exposed groups. In particular, repeated dose toxicity studies provide an essential insight into possible adverse effects of the active substance and the risks to humans as a result of repeated exposure. In addition, repeated dose toxicity studies provide information useful in the design of chronic toxicity studies.

The studies, data and information to be provided and evaluated should be sufficient to permit the identification of effects following repeated exposure to the active substance, and in particular

to further establish or indicate:

(a) the relationship between dose and observed adverse effects;

(b) toxicity of the active substance including where possible the No Observed Adverse Effect Level (NOAEL) and/or a Benchmark Dose (BMD) analysis;

(c) target organs where relevant (including immune, nervous, reproductive and endocrine systems);

(d) the time course and characteristics of adverse effects with full details of behavioural changes and possible pathological findings at post-mortem;

(e) specific adverse effects and pathological changes produced;

(f) where relevant the persistence and reversibility of certain adverse effects observed, following discontinuation of dosing;

(g) where possible, the mode of toxic action;

(h) the relative hazard associated with the different routes of exposure;

(i) relevant critical endpoints at appropriate time points for setting reference values and for assessing if criteria for classification and labelling are fulfilled, where necessary.

Toxicokinetic data (e.g. concentration of the active substance and/or the main metabolites in blood) should be included in repeated dose toxicity studies, unless it can be justified why this is not necessary. To avoid increased animal use, the data may be derived in range finding studies where these are needed.

If nervous system, immune system, reproductive system or endocrine system are specific targets in repeated dose toxicity studies at dose levels not producing marked toxicity, supplementary studies, including functional testing, need to be considered.

Repeated dose toxicity (oral route)

The test methods for sub-chronic repeated dose toxicity via oral route are given in Table 39 below.

Table 39. Test methods for repeated dose toxicity via oral route:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Sub-chronic oral toxicity test. Repeated dose 90-day oral toxicity study in rodents.*	B.26	TG 408
Sub-chronic oral toxicity test. Repeated dose 90-day oral toxicity study in non-rodents.**	B.27	TG 409

* Title of the OECD test guideline: Repeated dose 90-day oral toxicity study in rodents
** Title of the OECD test guideline: Repeated dose 90-day oral toxicity study in non-rodents

Repeated dose toxicity (dermal route)

If the substance is a severe irritant or corrosive, testing by the dermal route should be avoided unless it can be performed at doses that do not cause irritation or corrosion and such doses are still toxicologically relevant and the outcome can be used in risk assessment.

The test methods for sub-chronic repeated dose toxicity via dermal route are given in Table 40 below.

Table 40. Test methods for repeated dose toxicity via dermal route:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Sub-chronic dermal toxicity test: 90-day repeated dermal dose study using rodent species*	B.28	TG 411

* Title of the OECD test guideline: Subchronic dermal toxicity test: 90-day study

Repeated dose toxicity (inhalation route)

The test methods for sub-chronic repeated dose toxicity via inhalation route are given in Table 41 below.

Table 41. Test methods for repeated dose toxicity via inhalation route:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Sub-chronic inhalation toxicity study: 90-day repeated inhalation dose study using rodent species*	B.297	TG 413

* Title of the OECD test guideline: Subchronic inhalation toxicity: 90-day study

1.9.3. Long-term repeated dose toxicity (≥ 12 months)

Table 42. Information requirement 8.9.3 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.9.3 Long-term repeated dose toxicity (≥ 12 months)	The long-term toxicity study (≥ 12 months) does not need to be conducted if: — Long-term exposure can be excluded and no effects have been seen at the limit dose in the 90-day study or — a combined long-term repeated dose/ carcinogenicity study (8.11.1) is undertaken

Any new long-term toxicity study and carcinogenicity study (section 1.11 of this guidance) should be combined. This section provides guidance covering both the long-term repeated dose toxicity and the carcinogenicity study. The test is required for one rodent, the rat being the preferred species. In exceptional cases and depending on the results obtained, testing in another mammalian species (rodent or non-rodent, see also section 1.9.4 of this guidance for tests in non-rodent species) may be considered.

Generation of new test data

If after the evaluation of available information further testing is needed to assess long-term repeated dose toxicity, the test methods described below should be used.

The results of the long-term studies conducted and reported, taken together with other relevant data and information on the active substance, should be sufficient to permit the identification of effects, following repeated exposure to the active substance, and in particular should be sufficient to:

- identify adverse effects resulting from long-term exposure to the active substance;
- identify target organs, where relevant;
- establish the dose-response relationship and mode of action;
- establish the NOAEL and, if necessary, other appropriate reference points.

Correspondingly, the results of the carcinogenicity studies taken together with other relevant data and information on the active substance, should be sufficient to permit the evaluation of hazards for humans to be assessed following repeated exposure to the active substance, and in particular should be sufficient:

(a) to identify carcinogenic effects resulting from long-term exposure to the active substance;

(b) to establish the species, sex, and organ specificity of any tumours induced;

(c) to establish the dose-response relationship;

(d) where possible, to identify the maximum dose eliciting no carcinogenic effect;

(e) where possible, to determine the mode of action and human relevance of any identified carcinogenic response.

If comparative metabolism data indicate that either rat or mouse is an inappropriate model for human cancer risk assessment, an alternative species should be considered.

Experimental data, including the elucidation of the possible mode of action involved and relevance to humans, should be provided where the mode of action for carcinogenicity is considered to be non-genotoxic. Suitable mode of action (MOA) studies can be considered to confirm non-relevance of the non-genotoxic MOA to humans.

Investigation of toxicokinetic parameters generated within the combined long-term toxicity study should also be considered as described also for short-term toxicity studies in section 1.9.2 of this guidance.

The test methods for long-term repeated dose toxicity are given in Table 43 below.

Table 43. Test methods for long-term repeated dose toxicity:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Chronic toxicity test*	B.30	TG 452
Combined chronic toxicity/carcinogenicity test**	B.33s	TG 453

* Title of the OECD test guideline: Chronic Toxicity Studies
** Title of the OECD test guideline: Combined Chronic Toxicity/Carcinogenicity Studies

1.9.4. Further repeated dose studies (ADS)

Table 44. Information requirement 8.9.4 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.9.4 Further repeat dose studies Further repeat dose studies including testing on a second species (nonrodent), studies of longer duration or through a different route of administration shall be undertaken in case of: — no other information on toxicity for a second non-rodent species is provided for, or — failure to identify a no observed adverse effect level (NOAEL) in the 28- or the 90-day study, unless the reason is that no effects have been observed at the limit dose, or — substances bearing positive structural alerts for effects for which the rat or mouse is an inappropriate or insensitive model, or — toxicity of particular concern (e.g. serious/severe effects), or — indications of an effect for which the available data is inadequate for toxicological and/or risk characterisation. In such cases it may also be more appropriate to perform specific toxicological studies that are designed to investigate these effects (e.g. immunotoxicity, neurotoxicity, hormonal activity), or — concern regarding local effects for which a risk characterisation cannot be performed by route-to route extrapolation, or — particular concern regarding exposure (e.g. use in biocidal products leading to exposure levels which are close to the toxicologically relevant dose levels), or — effects shown in substances with a clear relationship in molecular structure with the substance being studied were not detected in the 28- or the 90-day study, or — the route of administration used in the initial repeated dose study was inappropriate in relation to the expected route of human exposure and route-to-route extrapolation cannot be made.	

When the available data are inadequate for hazard characterisation and risk assessment, further repeated dose studies should be undertaken, including testing on a second species (non-rodent), studies of longer duration than the studies already available or through a different route of administration. However, testing should not be initiated before the evaluating competent authority has indicated that further testing is necessary. The decision on further testing should be based on expert judgement and on a case-by-case basis, ensuring that all available information is taken into account before concluding that additional testing is necessary. Where applicable, mechanistic *in vitro* studies examining the specific mechanism of action of the related substances should have preference over further animal studies.

Requiring further repeated dose toxicity studies

When all the toxicological data concern rodent species, an assessment of the data needs to be performed to understand if testing with another species is likely to provide additional information (e.g. potential of different mode of action within different species).

Further studies are not necessarily always needed when failing to identify a NOAEL. If the data are sufficient for a robust hazard assessment and for classification and labelling, the LOAEL may be used as the starting point for risk assessment.

Where the preferred animal species is an inappropriate or insensitive model, a study protocol will be identified that can be reliably performed in a more suitable animal species. It is however possible to conclude that e.g. a structural alert concerns an effect that is specific to humans and/or none of the animal models is suitable for studying this specific effect. In this case all the available information, including scientific literature and human data, will be taken into account to judge whether the risk to humans can be concluded. The human data may consist of e.g. records of worker/consumer experience, case reports, consumer tests or epidemiological studies. Whether further testing will be required will depend on a case-by-case expert judgment.

If toxicity of particular concern is already established, the substance will be classified accordingly and the appropriate risk management measures will be implemented, and therefore no further testing is required.

In some cases, data derived by protocols designed for other endpoints, as for example the OECD TG 443 (Extended One-Generation Reproductive Toxicity Study) may provide valuable information on specific effects such as immunotoxicity, neurotoxicity or endocrine disruption. Furthermore, where a need is identified for a modification in the study protocol to cover specific needs, this will be done in consultation with the evaluating competent authority. Non-standard protocols should be used only in exceptional cases, because the scientific value of such results can be questioned.

A new repeated dose toxicity study for the purpose of performing quantitative risk characterisation for local effects should not be performed by default due to the difficulty in deriving threshold levels for local effects that are also relevant for humans. The benefit from the generation of additional data for this purpose should be considered against the effectiveness of qualitative risk characterisation as another option for ensuring safe use.

Further studies might be necessary e.g. when the biocidal product is used in one or more consumer products and the (combined) exposure levels are close to toxicologically relevant dose levels where effects on humans may be expected in the relevant timeframe. Any exposure-triggered studies proposed or required should be considered on a case-by-case basis.

Effects may have been observed in substances with a clear relationship in molecular structure with the active substance, where such effects were not detected in the 28- or the 90-day study. The study protocol and the conditions in which the effects were seen in another substance will be examined in detail in order to identify the conditions in which the effect would be expected to occur for the substance to be studied. The study protocol will be selected to repeat and possibly extend the conditions where the effect has been observed.

If the route of administration in the available repeated dose study was not relevant to the expected route of human exposure, the possibility to extrapolate to the appropriate route should be considered. All available toxicokinetic information and modelling approaches should be carefully considered for this purpose.

1.10. Reproductive toxicity

Table 45. Information requirement 8.10 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.10 Reproductive toxicity For evaluation of consumer safety of active substances that may end up in food or feed, it is necessary to conduct toxicity studies by the oral route	The studies do not need to be conducted if: — the substance meets the criteria to be classified as a genotoxic carcinogen (classified both as germ cell mutagen category 2, 1A or 1B and carcinogenic category 1A or 1B), and appropriate risk management measures are implemented including measures related to reproductive toxicity, — the substance meets the criteria to be classified as a germ cell mutagen category 1A or 1B and appropriate risk management measures are implemented including measures related to reproductive toxicity, —the substance is of low toxicological activity (no evidence of toxicity seen in any of the tests available provided that the dataset is sufficiently comprehensive and informative), it can be proven from toxicokinetic data that no systemic absorption occurs via relevant routes of exposure (e.g. plasma or blood concentrations below detection limit using a sensitive method and absence of the substance and of metabolites of the substance in urine, bile or exhaled air) and the pattern of use indicates that there is no or negligible human or animal exposure, — the substance meets the criteria to be classified as reproductive toxicity category 1A or 1B: May damage fertility (H360F), and the available data are adequate to support a robust risk assessment, then no further testing for sexual function and fertility will be necessary. A full justification must be provided and documented if investigations for developmental toxicity are not conducted, or — the substance is known to cause developmental toxicity, meeting the criteria for classification as reproductive toxicity category 1A or 1B: May damage the unborn child (H360D), and the available data are adequate to support a robust risk assessment, then no further testing for developmental toxicity will be necessary. A full justification must be provided and documented if investigations for sexual function and fertility is not conducted. Notwithstanding the provisions of this column of this row, studies on reproductive toxicity may need to be conducted to obtain information on endocrine disrupting properties as laid down in 8.13.3.1.

Terminology used

The terminology explained in the Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures (CLP Regulation[14]) is used in this guidance.

For the purpose of classification and labelling, reproductive toxicity is divided into three differentiations; (i) adverse effects on sexual function and fertility), (ii) adverse effects on development of the offspring, and (iii) effects on or via lactation.

Adverse effects on sexual function and fertility include any effect of a substance that has the potential to interfere with sexual function and fertility. This includes, but is not limited to,

[14] Regulation (EC) No 1272/2008 of the European Parliament and of the Council

alterations to the female and male reproductive system, adverse effects on onset of puberty, gamete production and transport, reproductive (oestrus) cycle normality, sexual behaviour, fertility, gestation length, parturition, pregnancy outcomes, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive system.

Developmental toxicity includes, in its widest sense, any effect interfering with normal development of the organism, before or after birth and resulting from exposure of either parent prior to conception, or exposure of the developing organism during prenatal development, or postnatally to the time of sexual maturation. However, these effects can be manifested at any point in the life span of the organism.

The major manifestations of developmental toxicity include (1) death of the developing organism, (2) structural abnormality, (3) altered growth, and (4) functional deficiency.[15]

Developmental neurotoxicity (DNT) and developmental immunotoxicity (DIT) belong also under developmental toxicity.

Adverse effects on sexual function and fertility of the offspring in adulthood can be of developmental origin. Reproductive toxic effects that cannot be clearly assigned to either impairment of sexual function and fertility or to developmental toxicity shall be classified as reproductive toxicants (i.e. Repr. 1A; H360, Repr. 1B; H360 or Repr. 2; H361) without the specification (F/f and or D/d) in the hazard statement (CLP 3.7.1.1).

Effects on or via lactation are allocated to a separate single category. Please refer to Table 3.7.1(b) of Annex I of the CLP Regulation: *"substances which are absorbed by women and have been shown to interfere with lactation, or which may be present (including metabolites) in breast milk in amounts sufficient to cause concern for the health of a breastfed child, shall be classified and labelled to indicate this property hazardous to breastfed babies"*. Classification for reproductive toxicity is considered separately from effects on or via lactation.

Objectives

It is important that the hazardous properties and risks or lack of them with respect to reproduction are concluded for active substances. The information requirements have three core objectives:

- to have adequate information to conclude whether classification and labelling for adverse effects on sexual function and fertility and on development is warranted or can be with sufficient confidence excluded (e.g. by ensuring that sufficiently high dose levels have been tested);
- to have sufficient information for the purpose of risk assessment;
- to obtain information on endocrine activity/endocrine disrupting properties.

The results from reproductive toxicity studies should allow identification of specific adverse effects on reproduction for classification and labelling, identification of endocrine activity of the active substance, and derivation of points of departure for both reproductive toxicity and non-reproductive toxicity for risk assessment purposes.

In more detail, the results from required reproductive toxicity studies (and study summaries

[15] As written in 3.7.1.3 and 3.7.1.4 in Annex I to CLP (the definition for developmental toxicity is shortened here)

with numerical results) should be sufficient to:

(a) To identify and assess any specific effect on sexual function and fertility in P0 and/or P1 generations

1) for classification and labelling

2) to establish NOAELs for sexual function and fertility (P0 and P1)

(b) To identify and assess any specific effect on development (observable during pre-, peri- and postnatal periods, and including effects on developing nervous system) in F1 and/or F2 generations

1) for classification and labelling

2) to establish NOAELs for development of offspring (F1 and F2)

(c) To identify and assess any non-reproductive toxicity in parental/maternal animals;

1) To assess the potential influence of other toxicity, i.e. non-reproductive toxicity on reproductive toxicity, when reproductive toxicity co-occurs with other toxicity in order to conclude on the specificity of observed effects on reproduction;

i. Effects on reproductive toxicity (sexual function and fertility and/or development) which occur even in the presence of other toxicity are considered evidence of reproductive toxicity unless it can be unequivocally demonstrated or it is reasonable to assume that the reproductive effects are solely secondary non-specific consequences of other toxicity (CLP).

2) To identify the lowest effective dose level and the NOAEL for non-reproductive toxicity (some non-reproductive adverse effects may occur at lower doses than in other repeated dose toxicity studies with similar exposure duration); e.g. pregnant/lactating females may be more sensitive to certain effects as compared to non-pregnant animals (different or enhanced effects).

3) To assess if such effects warrant or contribute to the classification for other hazard class(es) such as STOT RE.

(d) To identify and assess effects related to endocrine activity in parental animals and offspring that can contribute to identification of endocrine disrupters.

This guidance provides advice on how the applicant can address the reproductive toxicity of the active substance and how the information requirements of BPR can be met, thereby providing data on the hazardous properties for classification purposes and for the risk assessment and endocrine activity.

Fulfilling the data requirement

Effects accentuated over generations should be reported.

Steps 1 and 2 Collection and evaluation of available information

For the assessment of existing information on the reproductive toxic properties of the substance all the relevant information should be considered together (physicochemical properties, grouping, (Q)SARs and expert systems, *in vitro* data, human data and animal data) please consult the CLP Regulation Title II. Further guidance is available within the *ECHA Guidance Vol III Parts B+C* and the *Guidance on the Application of the CLP Criteria*.

Step 3 Generation of new test data

If after the analysis in steps 1 and 2 above, further testing is needed to assess reproductive toxicity, the test methods described in chapters 1.10.1, 1.10.2 and 1.10.3 below should be used. Core information requirements include extended one-generation reproductive toxicity study (OECD TG 443) with the extension of Cohort 1B to provide mainly information on effects on sexual function and fertility, developmental toxicity observable peri- and postnatally and sometimes on effects on or via lactation. Prenatal developmental toxicity studies (OECD TG 414) in two species provide information mainly on effects interfering with normal development before birth. Furthermore, information on developmental neurotoxicity (e.g. OECD TG 426) is required. If there are specific concerns that are not addressed by the standard information requirements, additional testing might be needed to produce necessary information for hazard identification (classification and labelling) and risk management (including risk characterisation, other risk management measures), or to conclude on the ED properties (see chapter 8.13.3).

Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

Information requirements can also be fulfilled by adaptations that reduce the requirement for testing. Adaptation possibilities are specified in Column 3 of the information requirement or in BPR Annex IV.

Preliminary considerations

When planning any reproductive toxicity studies, considerations such as the properties of the test item, dose levels, vehicle, adequate study design, and animal species and strain, are needed. Some of the most relevant considerations are presented below.

(i) Dose range-finding studies

The dose range-finding studies should be reported as separate study records (in IUCLID) to provide sufficient information and justification for the doses selected for testing. The findings from a range-finding study may also support the interpretation of the results from the main study.

(ii) Selection of vehicle

Most of the test methods provide guidance on vehicle selection if that is needed. If a vehicle other than water is used, a scientific justification is needed. The vehicle should not reduce solubility, absorption, or bioavailability of the test substance. The vehicle itself should not cause any adverse effects, as that may interfere with the interpretation of the results and may invalidate the study. The vehicle must not react with the substance or interfere with toxicokinetics of the substance or affect significantly the nutritional status of the animals. The control group should receive the same vehicle and at the same dosing volume as the treated groups.

(iii) Route of administration

BPR information requirements specify that for evaluation of consumer safety of active substances that may end up in food or feed, it is necessary to conduct toxicity studies by the oral route. The selection of the route of administration focuses on identification of hazards (see the Introduction to this Guidance and REACH Guidance R7a sub-section "Selection of the appropriate route of administration for toxicity testing", under R.7.2 Human health properties or hazards) and depends on the most appropriate route for identification of the intrinsic properties of the substance.

According to the test methods for reproductive toxicity, the oral route (gavage, in diet, or in

drinking water) is the default route, except for gases. For the extended one-generation reproductive toxicity study (EU B.56, OECD TG 443) dietary administration may be an appropriate route to model human exposure. If another route of administration other than oral is used, a robust justification is required. In practice, testing via the oral route is usually performed with solids, liquids and dusts, while testing via inhalation route is usually performed with gases and liquids with very high vapour pressure. Testing via dermal route is not recommended in studying reproductive toxicity (OECD 2008b). During lactation, separating the dams from the pups for 6 hours for whole body exposure might induce additional stress on the pups that might lead to the observation of effects that are not necessarily test-item related. Deviations from the default oral route of administration must be justified, such as having information on route-specific toxicity or toxicokinetics indicating that oral administration would not be relevant for assessing the human health hazards via inhalation, which would be the main route of foreseen human exposure.

In vivo testing at concentration/dose levels causing corrosivity must be avoided. For irritating substances, the vehicle should be chosen to minimise gastrointestinal irritation. For some substances, dietary administration may allow adequate dosing without irritation compared with administration via gavage. In certain cases, irritation/corrosivity may be avoided by testing of neutral salts of alkaline or acidic substances in order to allow investigation of intrinsic properties at adequate dose levels. If immediate hydrolysis of a substance occurs, it may be possible to provide information on all the cleavage products. Such a read-across approach should be adequately justified and documented according to BPR Annex IV, 1.5 and applying the principles of Read-Across Assessment Framework, RAAF[16]. For corrosive or irritating vapours or gases for which oral testing is not possible, the highest concentration for inhalation should be chosen carefully maximising the toxicity while minimising the irritation.

Gavage dosing provides accurate information on dose levels, and the resulting toxicokinetics follow generally daily bolus dosing with high maximum concentration in blood (Cmax) and, depending on the elimination rate, daily periods with essentially no exposure are possible. Toxicity requiring high Cmax values can be observed.

Using dietary or drinking water route of administration provides less accurate information on dose levels due to loss of material due to spilling. On the other hand, the blood levels are steadier for many hours due to distribution of feed and water consumption during the day. Toxicity requiring longer effect levels per day are more easily observed. Dietary or drinking water administration is not recommended if palatability issues are seen, as reduced food or water consumption and subsequent effects may be resulting from the taste of the chemical and not its toxic properties.

Studies involving routes of administration that are not relevant exposure routes for active ingredients (e.g. intravenous or intraperitoneal injection), and resulting in unrealistically high exposure levels or eliciting local damage to the reproductive organs must be interpreted with extreme caution and on their own are not normally the basis for hazard classification or risk assessment. However, they may provide information on mechanisms/modes of action.

(iv) Selection of species

The most common species for reproductive toxicity testing is the rat. There is often good historical background information for various rat strains that may be used to support the interpretation of the results. The strain selected should have an adequate fecundity and not too high incidence of spontaneous malformations or any other specific feature that may reduce the adequacy of the strain to study reproductive toxicity of the active substance. To facilitate integrated data interpretation together with other studies, it is recommended to use the same

[16] https://echa.europa.eu/documents/10162/13628/raaf_en.pdf

(rat) strain in reproductive toxicity testing and repeated dose toxicity studies.

If there is information regarding the sensitivity of the species and strains, the most sensitive species and strain should be used, taking into account human relevance. There is no need to demonstrate the human relevance; human relevance is assumed unless demonstrated otherwise. In choosing the appropriate species and strain, consideration must be given to the suitability of the species and strain for the test protocol, and the availability of background information on the species and strain for the test protocol. The species/strain selection should be justified if the default species referred to in a test method is not used.

More information on species selection for prenatal developmental toxicity studies is given in section 1.10.1.

(v) Dose level selection

The dose level selection should ensure data generation for classification and labelling, risk assessment, and identification of endocrine disrupting properties.

The dose levels should be spaced to produce a gradation of toxic effects. If there is no evidence of toxicity at a dose of at least 1000 mg/kg bw/day in repeated dose studies, the necessary study/studies could be conducted using a control group and a single dose of at least 1000 mg/kg bw/day. However, should evidence for reproductive or developmental toxicity be found at this limit dose, further studies at lower dose levels will be required to identify a NOAEL. Expected human exposure may indicate the need to use a dose level above 1000 mg/kg bw/day[17]. The conditions for applicability of a limit test are provided in the individual test methods for reproductive toxicity. For inhalation exposure, OECD guidance document 39 may be used.

In selecting dose levels, information should be considered from existing studies, as well as from any dose range-finding studies that may need to be conducted. Toxicokinetic information may provide reasons to adjust for example the dosing route and regimen. Furthermore, toxicity and toxicokinetics in pregnant animals may differ from those in non-pregnant animals. This may cause challenges in selecting the highest dose level for the study, because the sensitivity of the animals may differ at various phases of the study.

It is important to get information about the reproductive toxicity profile of a substance including the spectrum of reproductive toxicity effects related to different dose levels as well as information to allow evaluation of the severity of reproductive toxicity of a substance.

The highest dose level should be intended to produce sufficient toxicity to provide adequate information on reproductive toxicity for the purpose of both classification and labelling (including categorisation), risk assessment and identification of endocrine activity. For classification and labelling it is important that the tested doses are sufficiently high to enable a conclusion on a

[17] CLP, Annex I, Sections 3.7.2.5.7 –3.7.2.5.9 state on the limit dose and very high dose levels the following: "There is general agreement about the concept of a limit dose, above which the production of an adverse effect is considered to be outside the criteria which lead to classification, but not regarding the inclusion within the criteria of a specific dose as a limit dose. However, some guidelines for test methods, specify a limit dose, others qualify the limit dose with a statement that higher doses may be necessary if anticipated human exposure is sufficiently high that an adequate margin of exposure is not achieved. Also due to species differences in toxicokinetics, establishing a specific limit dose may not be adequate for situations where humans are more sensitive than the animal model." Section 3.7.2.5.8: "In principle, adverse effects on reproduction seen only at very high dose levels in animal studies (for example doses that induce prostration, severe inappetence, extensive mortality) would not normally lead to classification, unless other information is available, e.g. toxicokinetics information indicating that humans may be more susceptible than animals, to suggest that classification is appropriate. Please also refer to the section on maternal toxicity (3.7.2.4) for further criteria in this area." And section 3.7.2.5.9 continues: "However, specification of an actual 'limit dose' will depend upon test method that has been employed to provide the test results, e.g. in the OECD Test Guideline for repeated dose toxicity studies by oral route, an upper dose of 1000 mg/kg has been recommended as a limit dose, unless expected human response indicates the need for a higher dose level."

lack of reproductive toxic properties warranting a classification in Repr. 1B or Repr. 2 if clear evidence warranting a category 1B on reproductive toxicity is not observed (see the CLP criteria). Therefore, the top dose selection should demonstrate an aim to induce clear evidence of reproductive toxicity (adverse effects on reproduction) without excessive toxicity and severe suffering in parental animals (e.g. prostration, severe inappetence, excessive mortality) that would compromise the interpretation of reproductive effects.

There are aspects to be considered in the dose level setting of OECD TG 414, 443 and 426. Common to all these TGs is that the lowest dose should not produce any evidence of either maternal or developmental toxicity (and allow to set the NOAEL). Dose level selection should ensure that any dose-related effect is demonstrated, also enabling the establishment of NOAELs for the most sensitive endpoint. To demonstrate dose response, the mid dose level is expected to produce observable toxic effects. However, there are some differences in the specifications for the top dose level (see below). Irrespective of the specifications in OECD TGs regarding selection of the top dose, for classification and labelling, as explained above, it is critical that the tested doses are sufficiently high to enable a conclusion on a lack of reproductive toxic properties warranting a classification in Repr. 1B or Repr. 2 if clear evidence on reproductive toxicity is not observed.

The OECD TGs 414 main specification for top dose:

- "*the highest dose should be chosen with the aim to induce some developmental and/or maternal toxicity (clinical signs or a decrease in body weight) but not death or severe suffering*"

The specifications in OECD TG 426 for top dose selection:

- "*the highest dose level should be chosen with the aim to induce some maternal toxicity (e.g., clinical signs, decreased body weight gain [not more than 10%] and/or evidence of dose-limiting toxicity in a target organ)"*

- *"the highest dose should be the maximum dose which will not induce excessive offspring toxicity, or in utero or neonatal death or malformations, sufficient to preclude a meaningful evaluation of neurotoxicity*."

For the OECD TG 443, the highest dose level should be based on toxicity (adverse effects) and selected with the aim to induce reproductive and/or other systemic toxicity, as stated in column 1 of the information requirement.

The top dose selection should not only follow the specifications in OECD TGs but also take into account the applicability for classification and labelling purposes.

There is a need to study various aspects in parents and their offspring in OECD TG 443. The study should be designed to ensure adequate assessment of the effects on sexual function and fertility, i.e. the dose levels should not be reduced in order to get a sufficient number of offspring for the assessment of developmental toxicity. Even if the amount of offspring is reduced due to effects on sexual function and fertility, any offspring available at that those level should be investigated for adverse effects on development. Also results at lower dose levels can still be used to assess if showing adverse effects on development.

It is also important that toxicity in both female and male animals is seen, to ensure that reproductive toxicity in either gender is not overlooked. If existing information, including results from a dose-range finding study, show that the sensitivity between male and female animals differs significantly, the dose setting should take these differences into account. The less sensitive sex should be tested at higher doses than the more sensitive sex.

For all of the TGs, the aim to have appropriate dose level setting has to be demonstrated.

Dose level selection must be justified and documented to allow independent evaluation of the choice made.

Considerations on mechanisms or modes of action

There is no requirement to investigate the mechanism or MoA and its relevance to humans in order to classify for reproductive toxicity. Only if it is conclusively demonstrated that the clearly identified mechanism or mode of action has no relevance for humans and other mechanisms or MoAs can be excluded, a substance that produces the adverse effects on reproductive toxicity only in experimental animals shall not be classified. Classification in category 2 may be more appropriate than category 1B when *mechanistic information* raises doubt about relevance in humans, as far as there is reassurance about the robustness and quality of the data.

Some reproductive effects may be mediated via specific maternally mediated mechanisms (e.g., reproductive effects due to chelating MoA) that may still be specific effects on reproduction and shall not be dismissed from classification for reproductive toxicity due to specific maternally mediated mechanism.

Information on mechanisms and modes of action are relevant for ED identification. Mechanistic information may also indicate a specific concern that may help identifying the most specific tests for e.g. associative learning and memory under DNT (see 1.10.3).

1.10.1. Prenatal development toxicity study (OECD TG 414) on two species

Table 46. Information requirement 8.10.1 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.10.1 Pre-natal development toxicity study (OECD TG 414) on two species, preferred first species is rabbit (non- rodent) and preferred second species is rat (rodent); oral route of administration is the preferred route	The study on the second species shall not be conducted if the study performed on the first species or other available data indicate that the substance causes developmental toxicity meeting the criteria for classification as toxic for reproduction category 1A or 1B: May damage the unborn child (H360D), and the available data are adequate to support a robust risk assessment

The prenatal developmental toxicity studies, taken together with other relevant data and information on the active substance (e.g. the developmental parameters of the EOGRTS and OECD TG 426), must be sufficient to permit the assessment of potential hazardous properties and risks on the offspring following exposure to the active substance during the development.

The prenatal developmental toxicity study (EU B.31, OECD TG 414) provides a focused evaluation of potential effects on prenatal development, although only effects that are manifested before birth can be detected. Detailed information on external, skeletal and visceral malformations and variations and other prenatal developmental effects are provided. Caesarean section allows precise evaluation of the number of foetuses affected.

Prenatal developmental toxicity should be determined in two species by the oral route. The information requirement indicates rabbit and rat as the preferred non-rodent and rodent species, respectively (also in accordance with the test method EU B.31 / OECD TG 414). Information on two species allows a comprehensive assessment of prenatal developmental toxicity. If there is information regarding the sensitivity of the species and strains, the most sensitive species and strain should be tested first, taking into account human relevance.

The prenatal developmental toxicity study in a second species can be omitted if the information already warrants classification as toxic for reproduction category 1A or 1B for development and

the available data are adequate to support a robust risk assessment.

The rabbit is the preferred species for the first prenatal developmental toxicity study. Selecting rat as the first species may be supported by arguments of being a more sensitive species than the rabbit for the specific active substance.

On the other hand, most toxicity studies are conducted in the rat, and it may therefore be considered that the first prenatal developmental toxicity study could also be conducted in this species. Findings from previous studies can be used in dose selection, or the identification of additional parameters for evaluation. In addition, the outcome of the prenatal developmental toxicity study may be helpful in the interpretation of other reproductive toxicity studies, for which the rat is generally the preferred species.

If one or both of the default species (rat and rabbit) are not suitable for prenatal developmental toxicity testing, a more suitable species considering the human relevancy should be selected for testing. An adequate justification must be provided for species other than the rat and the rabbit. The results from prenatal developmental toxicity studies are considered relevant to humans unless there is substance-specific toxicokinetic or toxicodynamic evidence showing otherwise.

Information on prenatal developmental toxicity coming from one- or multigeneration studies (such as OECD TGs 443, 416, 426, 421, 422) is not equivalent to that from the prenatal developmental toxicity study. The results from e.g. OECD TG 443 and 416 studies do not provide confidence to conclude that there is no prenatal developmental toxicity. Structural malformations and variations are not specifically investigated in one-and multigeneration studies. Therefore, information from one- or multigeneration studies do not cover the information on prenatal developmental toxicity in rodent species. However, in addition to information on prenatal developmental toxicity in two species, information on effects due to exposure during peri- and postnatal developmental periods that is obtained from one- or multigeneration studies (e.g. OECD TG 426, 443 and 426) is also relevant for developmental hazard identification and shall be assessed to conclude on classification and labelling for developmental toxicity (CLP 3.7.1.4).

The latest update of the test method for prenatal developmental toxicity in Table 47 should be used.

Table 47. Test methods for prenatal developmental toxicity:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Prenatal developmental toxicity study	B.31*	TG 414

* The EU test method is currently outdated, and the OECD TG 414 (revised in 2018) should be used for any studies to be performed.

Information on developmental toxicity observable during peri-postnatal period can be obtained from:

- Developmental neurotoxicity study (OECD TG 426; EU B.53);
- Extended one-generation reproductive toxicity study (OECD TG 443, EU B.56);
- Two-generation reproductive toxicity study (OECD TG 416; EU B.35).

Note regarding prenatal developmental toxicity studies and assessment of endocrine disruption:

The studies for prenatal developmental toxicity may need to be conducted to clarify endocrine activity of the substance. Conduct of the studies may be needed even if the classification criteria

for Repr 1B; H360D (adverse effects on development) are met.

OECD TG 414 has been updated with thyroid hormone and thyroid stimulating hormone analysis in dams (T4, T3 and TSH) and anogenital distance (by sex and related to weight) in foetuses to be measured in rats. Some findings, such as increased foetal weight or placental weight, considered together with litter size, can also be considered beyond the assessment of reproductive toxicity, e.g. in the assessment of endocrine disruption.

The OECD TG 414 may detect and provide diagnostic information on the effects of substances with EATS-related modes of action. The test is also sensitive to substances with retinoid mode of action, but not diagnostic of it.

1.10.2. Extended One-Generation Reproductive Toxicity Study

Table 48. Information requirement 8.10.2 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.10.2 Extended One-Generation Reproductive Toxicity Study (OECD TG 443), with cohorts 1A and 1B and extension of cohort 1B to include the F2 generation with the aim to produce 20 litters per dose group, F2 pups must be followed to weaning and investigated similarly as F1 pups. Rat is the preferred species and oral route of administration is the preferred route. The highest dose level should be based on toxicity and selected with the aim to induce reproductive and/or other systemic toxicity	A two-generation reproductive toxicity study conducted in accordance with OECD TG 416 (adopted 2001 or later) or equivalent information shall be considered appropriate to address this information requirement if the study is available and was initiated before 15 April 2022.

Table 49. The test method: extended one-generation reproductive toxicity study

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Extended one-generation reproductive toxicity study	B.56	TG 443

The extended one-generation reproductive toxicity study (EOGRTS), taken together with other relevant data and information on the active substance, must be sufficient to permit the assessment of potential hazardous properties and risks on sexual function and fertility, and development, following repeated exposure to the active substance. The study also includes certain parameters for endocrine disrupting modes of action.

Information on blood concentration of the active substance in parents and foetus/offspring may be included and reported to enhance interpretation of the results. Furthermore, the concentrations of active substance and its relevant metabolites should be measured in milk, although not required in the OECD test guideline, where adverse effects are observed in the offspring or are expected due to effects on or via lactation (for example from a range-finding study).

OECD TG 443 is a modular study design with various investigational options. For BPR, OECD TG 443 with extension of Cohort 1B is the information requirement. The extension of Cohort 1B to mate the Cohort 1B animals and produce the F2 generation is also recommended in OECD GD 150 for the identification of endocrine disruptors. This extension provides information on sexual function and fertility of the offspring of the P0 parental animals and developmental toxicity of

the second filial generation and is important for the identification of endocrine activity.

Developmental neurotoxicity is a separate information requirement (section 1.10.3) and can be fulfilled with an OECD TG 443 with Cohorts 2A and 2B and with additional investigation of cognitive functions, as specified by the minimum requirements for developmental neurotoxicity under section 1.10.3.

Information on developmental immunotoxicity belongs to additional data set, and in section 1.13.4, a common recommendation for a test battery is described which should be used to address a concern for developmental immunotoxicity. OECD TG 443 with Cohort 3 can be considered as a screening level information on developmental immunotoxicity which may need to be followed with confirmative investigations (see further details in section 1.13.4).

Important considerations regarding the study conduct are explained below. These are not clearly expressed in OECD TG 443 or OECD GD 151 and/or need to be specified to ensure data applicable to hazard classification, risk assessment and identification of endocrine activity.

Premating exposure duration

To ensure that sexual function and fertility are adequately studied, a ten-week premating exposure duration is required in P0 animals. The sexual function and fertility part of the reproductive toxicity study should be capable of providing information that is adequate for both risk assessment and classification and labelling, including categorisation. For the comprehensive assessment of effects and for the classification and labelling purpose, it is important to produce and evaluate the full spectrum of effects on sexual function and fertility. The premating exposure period must be sufficiently long to be able to provide full information on magnitudes, incidences, severities and types of all effects (MIST information) to be assessed together, not only aiming to detect the most sensitive adverse effects The most conclusive outcome can be obtained when mating is allowed after an exposure covering one full spermatogenic cycle (including sperm maturation) and folliculogenesis, and an analysis of sperm parameters, organ weights and histopathology of gonads and accessory sex organs are conducted around the same time after the same exposure history. The full spermatogenesis, without sperm maturation, takes 48-53 days in rats, (e.g. Kerr *et al*., 2006). After spermatogenesis, sperm maturation in rats takes around two weeks in epididymides. A ten-week premating exposure duration covers the full spermatogenesis and maturation meaning that the full cycle of development of sperm from spermatogonia into mature sperm is exposed. Thus, a ten-week premating exposure duration allows an assessment of the adverse effects on male sexual function and fertility by combining the information from all possible parameters in males evaluated at the same time.

Regarding females, fixed number of primordial follicles are endowed during early life and growth of these dormant follicles is initiated before and throughout reproductive life. Duration of follicle development from initial recruitment of a primordial follicle until cyclic recruitment into preovulatory follicles takes 61 days in rats (e.g., McGee and Hsueh, 2000). This follicle development is fully covered only after a sufficiently long exposure period, such as ten weeks. Therefore, for both the P0 males and females, a ten-week premating exposure duration is required before mating.

The data on F1 generation provides the most conclusive information for sexual function and fertility because the primordial germ cells develop, migrate and proliferate during embryonic development and effects to these events can be investigated only when the animals are exposed already *in utero*. Furthermore, the exposure period in F1 generation also covers the postnatal period before sexual maturation. Therefore, information also on potential effects by exposure during the developmental period on sexual function and fertility is obtained from F1 animals. This full evaluation is possible as the mating and littering of the Cohort 1B animals in an extended one-generation reproductive toxicity study (EU B.56, OECD TG 443) is specified in the data requirement 8.10.2.

It is important to expose all the developmental stages of the sperm and follicles before the mating in order to be able to detect any potential adverse effect on sexual function and fertility. Furthermore, a 10-week premating exposure duration supports interpretation of results when effects in P0/F1 generations are compared to those of P1/F2.

To allow the ten-week premating period, the exposure can be started when the animals are around 5 weeks old and mate them around 15 weeks of age.

Number of litters produced

The number of males and females mated should aim to produce 20 litters for both generations. Typically, 24 or 25 males and females are used to aim at producing 20 litters.

Investigating F1 and F2

The F2 pups must be followed to weaning and investigated similarly as F1 pups. Termination should take place at weaning (around post-natal day 20 or 21). By comparing effects and effect levels between F1 and F2, it can be deduced if developmental effects are observed at lower doses (indicating a higher sensitivity) in F2 compared to F1. Effects that are observed in filial generations only and/or there is an increase in sensitivity in filial generation(s)is a strong indication that the effects are developmental (see also CLP 3.7.1.4; developmental effects can be manifested at any point in the life span of the organism).

All investigations required for F1 pups should be also performed for F2 pups until weaning. These include:

- general observations (all signs of toxicity, morbidity, mortality),
- body weight,
- clinical observations (changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions, abnormalities of genital organs e.g. hypospadias or cleft penis),
- clinical examination of the neonates, e.g. qualitative assessment of body temperature,
- state of activity and reaction to handling,
- litter examination/parameters including number and sex of pups, stillbirths and live births,
- litter examination/parameters including presence of gross anomalies (externally visible abnormalities, including cleft palate; subcutaneous haemorrhages; abnormal skin colour or texture; presence of umbilical cord; lack of milk in stomach; presence of dried secretions),
- anogenital distance in pups (preferred: relative to square root of body weight),
- presence and number of nipples/areolae in male pups (see OECD GD 151, Section 3),
- Macroscopic examination of all organs for abnormalities,
- Retention for possible histopathology: mammary tissue and other organs as appropriate.

Furthermore, from surplus F1 pups at weaning and from F2 pups, body weight is recorded and macroscopic abnormalities investigated from all organs. The following organs are weighed: brain, spleen, thymus and other organs as appropriate and these and mammary tissues are kept for

possible histopathology.

(Developmental) neurotoxicity

Required minimum investigations on developmental neurotoxicity are specified in section 1.10.3. OECD TG 443 with Cohorts 2A and 2B and with additional investigation of cognitive functions can fulfil these minimum requirements. However, even without the specific cohorts for developmental neurotoxicity (Cohorts 2A and 2B), some parameters of (developmental) neurotoxicity are investigated in P0, Cohort 1A, F1 pups, P1 (extension of Cohort 1B) as well as F2 pups up to weaning and/or surplus pups. These comprise of:

- general observations on behavioural changes,
- clinical observations on autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern),
- changes in gait, posture, response to handling,
- presence of clonic or tonic movements,
- stereotypy (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. self-mutilation, walking backwards),
- clinical examination of the neonates, e.g. qualitative assessment of body temperature,
- state of activity and reaction to handling,
- brain weight and histopathology,
- histopathology of peripheral nerve, spinal cord and optic nerve,
- brain weight (F2 and surplus F1 pups)
- Thyroid hormones (T4 and TSH) (F2 and surplus F1 pups) (MoA).

Results on these parameters in the offspring should be assessed along with the information described in 1.10.3 and the information in P0 shall be considered along with all other relevant available information when considering the need for additional studies/investigations on adult neurotoxicity (section 1.13.2).

(Developmental) immunotoxicity

Information on (developmental) immunotoxicity belongs to ADS. The developmental immunotoxicity Cohort 3 in OECD TG 443 investigates primary IgM antibody response to a T cell dependent antigen (immunization with antigen is part of the test). However, even without specific cohort for developmental immunotoxicity (Cohort 3), some parameters of (developmental) immunotoxicity are investigated in P0, Cohort 1A and F1/F2 pups up to weaning and/or surplus pups. These comprise of:

- spleen weight and histopathology,
- thymus weight and histopathology,
- bone marrow histopathology,
- total and differential leukocyte count,

- splenic lymphocyte subpopulation analysis (CD4+ and CD8+ T lymphocytes, B lymphocytes and NK cells) using one half of the spleen,
- weight of lymph nodes associated with and distant from the route of exposure,
- histopathology on the collected lymph nodes and bone marrow.

Results on these parameters should be carefully evaluated to inform on possible indications or effects on (developmental) immunotoxicity. Possible concerns for (developmental) immunotoxicity may need to be followed-up e.g., in investigations in adults or in a standalone study for developmental immunotoxicity. Recommended parameters for a potential separate developmental immunotoxicity study are presented in chapter 1.13.4.

In case the developmental immunotoxicity Cohort 3 is included to OECD TG 443 as a screening investigation, it is important that this T cell dependent antibody response (TDAR) contains valid positive and negative controls with sufficient number of reacting animals.

Two-generation reproduction toxicity study

The two-generation reproductive toxicity study was a core information requirement for BPR until the amendment of BPR Annex II[18]. Although the two-generation reproductive toxicity study (OECD TG 416) lacks information on some parameters which are part of EU B.56 (OECD TG 443), it addresses the sexual function and fertility in two generations (P0 and F1). OECD TG 416 study or equivalent information is adequate instead of OECD TG 443 if the study is available and was initiated before 15 April 2022 and is conducted in accordance with the version of OECD TG 416 adopted 2001 or later.

If the study is conducted, e.g., for other regulation, and was initiated after 15 April 2022, the applicant may explore the possibilities to adapt the information requirement by substance specific justifications according to BPR Annex IV. When considering the relevance of old two(multi)-generation reproductive toxicity studies to address reproductive toxicity and ED, these studies will be assessed in line with BPR Annex IV, 1.1.2 adaptation rules for existing information. Thus, old existing non-guideline studies may fulfil the Column 1 core information requirement or may serve as elements in a weight of evidence adaptation according to BPR Annex IV, 1.2 to identify hazardous properties or support a category approach.

Where necessary for the assessment of the effects on reproduction and/or ED and as far as the available information is not yet sufficient for concluding on classification and labelling for reproductive toxicity, ED identification or NOAELs, supplementary studies/investigations may be required to provide information on the lacking parameters and the possible mechanisms. For further information, refer to the ECHA/EFSA ED guidance (2018).

Note regarding EOGRTS and assessment of endocrine disruption

The EOGRTS is a Level 5 *in vivo* assay providing more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism (see OECD Guidance Document 150). OECD GD 150 recommends OECD TG 443 with extension of Cohort 1B (to mate the Cohort 1B animals to produce F2 generation).

In particular, the EOGRTS includes investigations informing on oestrogenic, androgenic, thyroid-related, and steroidogenesis-related activities. For example, the EOGRTS investigates endocrine-sensitive parameters in parental animals and offspring, such as sexual function and fertility, weights and histopathology of reproductive organs/ tissues (e.g. male and female reproductive tissues/ organs, thyroid including thyroid hormone measurements, adrenals, pituitary),

[18] Regulation (EU) 2021/525

anogenital distance, and developmental landmarks such as sexual maturation. Sexual maturation should be investigated from 3 animals/sex/litter, from 20 liters per dose group.

1.10.3. Developmental neurotoxicity

Table 50. Information requirement 8.10.3 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.10.3 Developmental neurotoxicity Developmental Neurotoxicity Study in accordance with OECD TG 426, or any relevant study (set) providing equivalent information, or cohorts 2A and 2B of an Extended One-Generation Reproductive Toxicity study (OECD TG 443) with additional investigation for cognitive functions	The study shall not be conducted if the available data: — indicate that the substance causes developmental toxicity and meets the criteria to be classified as toxic for reproduction category 1A or 1B: May damage the unborn child (H360D), and — are adequate to support a robust risk assessment

The BPR data requirement describes three study options that can fulfil the information requirement:

1. OECD TG 426: Developmental neurotoxicity study,
2. Any relevant study (set) providing information equivalent to OECD TG 426, or
3. OECD TG 443 with Cohorts 2A and 2B and with additional investigation for cognitive functions.

Table 51. Test methods for developmental neurotoxicity:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Developmental neurotoxicity study		TG 426
Extended one-generation reproductive toxicity study, with Cohorts 2A and 2B and with additional investigation for cognitive functions		TG 443

Investigations for developmental neurotoxicity in these three study options include tests for clinical observations, motor activity, motor and sensory function and cognitive functions (including associative learning and memory) as well as neuropathological examination and brain weight. In this guidance, the tests or test types that are considered to constitute the indicative minimum requirements to fulfil the obligations to test developmental neurotoxicity (DNT) under BPR are described.

The information provided in this chapter is more detailed than elsewhere in this guidance because no ECHA guidance is currently available that could provide such information to help the applicant and the evaluating CA to select the adequate tests for developmental neurotoxicity.

The overview of the indicative minimum requirements for DNT by performing OECD TG 426 or 443 is given in Table 52 below. Fulfilling the information requirement by a study set equivalent to these indicative minimum requirements is also possible.

For fulfilling the indicative minimum information requirements identified in Table 52, the following aspects have been taken into account:

- The aim to investigate different nervous system functions in the most optimal manner possible
- The minimum information requirements should be achievable with both OECD TG 426 and OECD TG 443 with additional investigations for cognitive functions and even if Cohort 3 (DIT) is included in OECD TG 443
- Examples of possible animal assignments described in OECD TG 426
- Different types of associative learning and memory tests should be performed in adolescents and young adults (in OECD TG 426, OECD TG 443 or other study set), in different animals at these two time points.

Alternative test methods (a battery of *in vitro* DNT assays) are not described because an OECD guidance document for an integrated approach to testing and assessment (IATA) for DNT is still under development. However, DNT *in vitro* testing battery is not considered as an option to fulfil the minimum data requirements because it currently does not provide equivalent information to the required minimum requirements in *in vivo* tests. Although results from *in vitro* studies indicating DNT properties may strengthen the other available evidence on DNT, results from *in vitro* studies showing no indication on DNT hazard do not allow concluding on DNT properties due to limitations of *in vitro* studies as compared to information from *in vivo* studies. In addition, in *vitro* information alone is currently not sufficient for classification and labelling in accordance with the CLP Regulation. For further reading on DNT *in vitro* battery, see Sachana et al., 2021.

Table 52. Indicative minimum requirements[19] of investigations and test types to detect DNT in OECD TG 426 and OECD TG 443.

INVESTIGATIONS IN F1 GENERATION	OECD TG 426		OECD TG 443	
	TIME POINT AND MINIMUM NUMBER OF MALES AND FEMALES PER DOSE GROUP*	TEST METHOD/TEST TYPE	TIME POINT, COHORT AND MINIMUM NUMBER OF MALES AND FEMALES PER DOSE GROUP**	TEST METHOD/TEST TYPE
[1] Detailed clinical observations	Weekly during preweaning, at least every two weeks thereafter; (set 3: 20M+	Reporting changes e.g. in autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern	Weekly, all F1 animals	Reporting occurrence of e.g. secretions and excretions and autonomic activity (e.g. lacrimation, piloerection, pupil size, unusual respiratory pattern), changes in gait, posture,

[19] In general, a study meets the information requirements when it has been performed under GLP according to the required guideline and any specifications in the BPR. This table intends to help defining which tests should be performed to optimally cover the necessary parameters. Please see also the text below under "Learning and memory" concerning tests that are not recommended for investigating associative learning and memory.
This table specifies only the tests on DNT within the respective OECD TGs, but not other toxicological investigations to be performed and reported in the offspring and/or dams, such as body weight and sexual maturation. These investigations and their reporting should be performed in accordance with the applied OECD TG.

	20F)	and/or mouth breathing, unusual urination or defecation), body position, activity level, gait, posture, reactivity to handling, placing or other environmental stimuli, clonic or tonic movements, convulsions, tremors, stereotypies, bizarre behaviour or aggression		response to handling, presence of clonic or tonic movements, stereotypy (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. self-mutilation, walking backwards
[2] FOB	-	-	PND 63- 75 (cohort 2A)	See Appendix A in OECD TG 443
[3] Brain weight	PND 22 (subset 1a: 10M+10F unfixed, and subset 1b: 10M+10F fixed) and at termination (PND 70) (at least subset 3a: 10M + 10F, subset 4a: 10M+10F)		PND 21-22 (all surplus animals, cohort 2B, 10M+10F); PND 75-90 (cohort 2A, 10M+10F)	
[4] Neuropathology and morphometry	PND 22 (subset 1b: 10M+10F) and at termination (PND 70) (subset 3a: 10M + 10F)	Staining of slices containing slices of olfactory bulbs, cerebral cortex, hippocampus, basal ganglia, thalamus, hypothalamus, midbrain (tectum, tegmentum, and cerebral peduncles), pons, medulla oblongata, cerebellum; spinal cord and the PNS at PND 70 only	PND 21-22 (cohort 2B, 10M+10F); PND 75-90 (cohort 2A, 10M+10F)	2A: Staining of slices containing slices of olfactory bulbs, cerebral cortex, hippocampus, basal ganglia, thalamus, hypothalamus, mid-brain (tectum, tegmentum, and cerebral peduncles), brain-stem and cerebellum, the eyes (retina and optic nerve), peripheral nerve, muscle and spinal cord***
[5] Behavioural ontogeny	At least two measures of at least 2 behaviours during pre-weaning) (set 2: 20M+20F)	Open-field as one test for behavioural ontogeny highly recommended. The other behaviour for behavioural ontogeny should not develop at the same age.		
[6] Motor activity (including	1 or 3 (if tested for behavioural	Open-field test	Once on PND 63-75	Open-field test

habituation)	ontogeny) times during preweaning and Once on PND 60-70 (set 2: 20M + 20F) [in same animals at all time points]		(Cohort 2A, 10M + 10F)	
[7] Motor and sensory function	PND 25±2 (set 3: 20M+20F) and PND 60-70 (set 3: 20M+20F)	Acoustic startle test with PPI and short-term habituation [7a] (PND 25±2) **and** grip strength test [7b] **and** righting response test [7c] (PND 60-70)	PND 24-25 (Cohort 2A, 10M+10F); PND63-75, (Cohort 2A,10M+10F)	Acoustic startle test with PPI and short-term habituation [7a] (PND 24-25) **and** grip strength test [7b] **and** righting response test [7c] (part of FOB) (PND63-75)
[8] Learning and memory (L&M) (cognitive functions)	PND 25±2 (set 2: 20M+20F, same animals as in open-field test); PND 60-70 (set 4: 20M+20F); different animals at these two time points.	Explicit associative L&M (by MWM [8a] **or** RAM [8b]) at one time point **and** implicit associative L&M test at the other time point (by CWM [8c] **or** olfactory conditioning test [8d] **or** acquisition and retention of schedule-controlled behaviour test [8e])	PND 25±2 (Cohort 1A [10M+10F] or Cohort 3 animals, if DIT investigations are not conducted, 10M+10F); PND 60-70 (Cohort 1A animals, 10M+10F)	Explicit associative L&M (by MWM [8a] **or** RAM [8b]) at one time point **and** implicit associative L&M test at the other time point (by CWM [8c] **or** olfactory conditioning test [8d] **or** acquisition and retention of schedule-controlled behaviour test [8e])

* The animal allocation for OECD TG 426 follows that of example 3 of the OECD TG 426 with 4 sets (divided to subset a and b in some places) of 20 pups/sex/dose level (i.e. 1 male and 1 female per litter). Other animal allocations according to OECD TG 426 are possible.

**In OECD TG 443 adverse effects on sexual function and fertility may limit the number of offspring available for developmental investigations. However, the dosing should not be lowered in order to get a sufficient number of offspring. The priority of the OECD TG 443 test is to identify potential effects on sexual function and fertility and if this effect leads to an insufficient number of offspring, DNT should be investigated in OECD TG 426.

***Histopathology of fixed peripheral nerve, spinal cord and eye (and optic nerve) is also performed in Cohort 1A(B) offspring.

The sequence of tests should progress from the least invasive (e.g. observations in the home cage and open field) to the most invasive (e.g. handling assessments) to minimize the influence of stress on subsequent measures. Parameters that require descriptive measures should include a clear description of what constitutes "other than normal" and ranking or scales describing different severities of effects should be given. It is also recommended to include valid positive controls if not already available for the laboratory and setup to ensure that the technical personnel of the testing laboratory is able to correctly use the test procedures and animal model. Those results should verify that the laboratory can effectively demonstrate effects that are qualitatively and quantitatively consistent with those reported in other laboratories for the same agent, at similar doses, and under comparable conditions. This outcome provides added confidence that the absence of effects due to a treatment accurately reflects the situation rather than being due to inadequate implementation of a valid test method (i.e., a false negative). Positive control data also helps interpreting the results (Tyl et al., 2008).

For a robust and independent assessment of the study, full details are needed including a detailed description of the method and software settings, numerical results (magnitudes and

incidences of effects), severities and types of effects for all dose groups (including information on maternal toxicity) and concurrent controls on all tested parameters, even when concluding that there are no treatment-related effects. Positive and historical control data is needed to support the reliability and sensitivity of the test method. Please see paragraphs 77-85 of OECD TG 443 and 46-50 of OECD TG 426 about the data reporting. For more detailed guidance on reporting and appropriate statistical techniques, please refer to NAFTA Technical Working Group on Pesticides (TWG) on Developmental Neurotoxicity Study Guidance Document (2016) and Holson et al. Neurotoxicology and Teratology 30 (2008) 326–348. The minimum requirements of investigations are given in table 52.

The investigations are grouped below according to the main headings in OECD TG 426 into physical and developmental landmarks and functional behavioural endpoints.

Physical and developmental landmarks

OECD TG 426 and 443 require the testing of physical and developmental landmarks, including body weight, clinical observations, brain weight, neuropathology and sexual maturation, and these should be investigated accordingly. Additional developmental landmarks (e.g. pinna unfolding, eye opening and incisor eruption) can be optionally added as given in the OECD TG 426.

Effects on various parameters, even if not specific on the nervous system, may be relevant for the interpretation of the effects on the nervous system. These include e.g. pup body weight, morbidity, mortality, changes in skin, fur, eyes, mucous membranes, occurrence of some secretions, unusual signs of urination or defecation, and sexual maturation.

Even when not specific for (developmental) neurotoxicity, physical and developmental landmarks are relevant for the reproductive toxicity hazard assessment, both NOAEL/LOAEL determination and classification and labelling. Adverse effects on development include death of the developing organism, structural abnormalities, altered growth and functional deficiency, and adverse effects on sexual function and fertility include effects on onset of puberty (sexual maturation) among other effects (CLP 3.7.1.4 and 3.7.1.3).

Detailed clinical observations and FOB *([1] and [2] in Table 52)*

Clinical observations and functional observation battery (FOB) of the F1 generation should be investigated according to OECD TG 426 or 443, depending on the selected TG.

Clinical observations required in OECD TG 426 and 443, and FOB required in OECD TG 443 are often subjective evaluations, and therefore explicitly defined scores and criteria should be used. Measures that are ranked provide more information than binary (all-or-nothing) measures. A ranking or scale describing different levels of activity improve consistency across observers. More details are given in NAFTA guidance (2016).

Brain weight, neuropathology and morphometry *([3] and [4] in Table 52)*

Neuropathological evaluation and brain weight measurement of the offspring should be conducted according OECD TG 426 or 443, depending on the selected TG.

All neuropathologic alterations should be assigned a subjective grade indicating severity and their incidences should be reported. Cellular alterations (e.g., neuronal vacuolation, degeneration, necrosis) and tissue changes (e.g. gliosis, leukocytic infiltration, cystic formation) should be reported and assessed. Reporting should follow OECD GD 20 which requires that ambiguous terminology should be avoided and the nomenclature used for describing lesions and areas of the nervous system should follow standards and be as specific as possible. Further, the cell types involved in the lesion should be described to the degree possible, and attention should be paid to the distribution pattern of lesions, e.g. whether they are formed in bilateral and/or symmetrical pattern.

A performance impairment detected in a behavioural test may not be reflected in outcomes from brain pathology or brain morphometry, and vice versa. Behavioural effects may reflect e.g. effects on specific ion channels or neurotransmitters affecting nerve cell communication and such effects are not observed via standard histopathological staining procedures or morphometry. However, when planning the test set for developmental neurotoxicity testing, if there are already some neurohistopathological investigations (or any other information) indicating effects on certain areas of the nervous system, one should ensure that the function of these areas is specifically examined by selecting such behavioural test(s) from the possible alternative tests that target the function of these structures.

Functional/behavioural endpoints

Behavioural ontogeny *([5] in Table 52)*

Behavioural ontogeny of the F1 generation should be investigated according OECD TG 426. In OECD TG 443, testing behavioural ontogeny is not required, but it is recommended to test behavioural ontogeny as in OECD TG 426.

According to OECD TG 426, ontogeny of at least two selected behaviours should be measured in at least one pup/sex/litter during the appropriate age period (twice during pre-weaning), with the same pups being used on all test days for all behaviours assessed. OECD TG 426 gives righting reflex, negative geotaxis and motor activity as examples of behaviours for which their ontogeny could be assessed, and the TG strongly recommends the use of motor activity to assess behavioural ontogeny. If motor activity by open field is selected as one of the ontogeny behaviours, it will be investigated three times during pre-weaning (see below the requirements for motor activity). Generally, 20 males and 20 females per dose group should be selected for investigations (1 pup per sex per litter).

Motor activity (including habituation) *([6] in Table 52)*

Motor activity of the F1 generation should be investigated according OECD TG 426 or 443, depending on the selected TG and with the specifications below.

Motor activity should be monitored once (on PND 63-75 in Cohort 2A) according to OECD TG 443, and at least once during the pre-weaning and once on PND 60-70 according to OECD TG 426. If motor activity is tested for behavioural ontogeny, the test should be performed at least three times during the pre-weaning period. In normal conditions locomotor capacity starts to develop in rodents at around PND 13 and appears to be fully developed around PND 21 (NAFTA guidance, 2016).

The OECD TG 426 or 443 does not specify the type of test arena for assessing motor activity, other than that the motor activity testing must be conducted in automated test chambers. The open-field test is the most suitable and therefore the required test for motor activity; it is widely used to investigate hyper- or hypoactivity and habituation. As the open-field test may provide also information on the anxiety-like behaviour, movements in central and peripheral parts should be recorded and included in the analysis.

As an example, Qian et al. (2010) describe a methodology for measuring open field spontaneous activity. The length of the test session should allow the detection of potential effects on motor activity and on its habituation. It is necessary to determine precisely what measures are recorded and reported. Since fine motor movements (e.g. sniffing, scratching, grooming) do not provide a measure of locomotor or ambulatory activity, the activity test data should clearly distinguish various activity measures and their types. Software settings for defining the type and threshold for activity units can be critical for computing measures of ambulatory activity, and therefore clear reporting on data collection and computation is necessary (e.g. recording instrumentation, software versions and settings at each age). At least the distance traveled in the center,

periphery, and total (entire box), latency to enter the central area, as well as the number of rearing activity should be recorded. Activity measures should be described broken down by dose group, sex and 10-minute time blocks. Please see the NAFTA guidance (2016) for further methodological and reporting aspects as well as the normal developmental stages in the development of locomotor activity.

Motor and sensory function *([7] in Table 52)*

Minimum requirements for motor and sensory function of the F1 generation include testing for auditory startle response with pre-pulse inhibition (PPI) and short-term habituation at least once during adolescent period (PND 25±2 in OECD TG 426; or PND 24-25 in Cohort 2A in OECD TG 443), and grip strength and righting reflex in young adults (PND 60-70 in OECD TG 426; or as part of FOB on PND 63-70 in Cohort 2A in OECD TG 443). Any other test set providing equivalent information may also be used.

In the OECD TG 443 there is no heading for "motor and sensory function", but the tests investigating these functions include the auditory startle test and grip strength and righting reflex tests that are tested as part of the functional observational battery. The tests required in this section for motor and sensory function in OECD TG 426 are based on the requirements specified in OECD TG 443. The OECD TG 426 does not specify the required tests to ensure "adequate quantitative sampling of sensory modalities and motor functions" but rather provides a list of examples of tests (extensor thrust response, righting reflex, auditory startle habituation and evoked potentials).

Additional tests for motor and sensory functions are recommended especially if there is a specific concern for effects on some motor or sensory components that would not be adequately addressed by this minimum set of tests. For example, cerebellar dysfunction often correlates with abnormalities of gait synchronisation that can be sensitively measured by a rotating rod (Lane and Dunnet, 2011, vol II).

Below is a short overview of the required specific tests that investigate different modalities of sensory and motor functions and closely associated other key functions. The rotating rod test is also summarised as it is recommended as an additional test.

Acoustic startle test with pre-pulse inhibition (PPI) and short-term habituation ([7a] in Table 52)

Habituation (short- and/or long-term) and PPI of acoustic startle response can be measured within one protocol. As it is a valuable predictive model for cognitive impairment, it is important that PPI is included in the testing protocol for acoustic startle response in both OECD TG 426 and 443, although PPI is not specifically mentioned in OECD TG 443 as part of auditory startle test, and it is reviewed only in the reference of OECD TG 426 (Koch, 1999). The PPI is a simple addition to the acoustic startle and its short-term habituation test method. Acoustic startle and its short-term habituation is a sensory-motor test involving only a short neural pathway, whereas PPI adds a cognitive dimension to the test by predicting cognitive impairment involving a certain limbic circuitry (cortico-striato-pallido-pontine), that converges with the primary startle circuit in humans and rodents (Valsamis and Schmid, 2011).

Before measuring PPI, animals should always undergo startle habituation, so that startle attenuations due to habituation do not interfere with PPI measurements. Before running the habituation and PPI, the animal must adapt to the animal holder, startle box and background noise via an acclimation period. A protocol design and data analysis is described in detail in Valsamis and Schmid (2011).

Grip strength test ([7b] in Table 52)

The grip strength test measures the strength of limb flexor muscles in fore and hindlimbs that are innervated by peripheral motor nerves. The test is a specified requirement as part of the

FOB in OECD TG 443, and it should be measured also as part of the sensory and motor testing in OECD TG 426. The peak of the grip strength is measured by a grip strength meter for each rat during five trials, separated by approximately 1 min between each trial, and the average is used as the grip strength for each rat. Methodologies for measuring grip strength are well established and protocols can be found e.g. in Torii *et al*., 2010; Jeyasingham *et al* 2001.

Righting response test ([7c] in Table 52)

Righting response test is a postural reflex test and is required as part of the FOB in OECD TG 443 and should be measured also as part of the sensory and motor testing in OECD TG 426. Rats are momentarily held supine by the shoulders and hip-girdle on a flat surface and released. Normal animals will immediately turn over to recover their normal prone quadruped stance. The presence or absence of the reflex, time taken and direction of response are noted and reported. The test should be performed three times a day with an upper time limit of 3 min for each test. For each animal, the data for the three tests are averaged. Normal animals will turn in either direction with equal frequency, but they often turn away from the tester or a bright light source. It is therefore necessary to randomly change the orientation in which the animals are held: head to the left for one test and head to the right for another (Lane and Dunnet, 2011, vol II).

Rotating rod (test recommended but not listed in table 52)

Rotating rod test is not specifically required to fulfil the minimum requirements, but due to its potential to detect e.g. basal ganglia and cerebellum dysfunctions, it is highly recommended to be performed as part of the OECD TG 426 or OECD TG 443 (or any other study set providing equivalent information). The rotarod is a horizontal cylinder that rotates about its long axis at either constant or accelerating speeds. The animal is placed on the rotating cylinder perpendicular to the direction of rotation facing away from the tester by allowing the animal to walk off the open palm onto the rotating rod. In order to maintain position on top of the rod and not fall off, it has to walk forwards synchronising stepping frequency and stride length to the speed of rotation. For each trial, the parameters recorded are total time on the rod, time walking, time spent in error (clinging or walking backwards) and time to first error (fall or cling). The trial ends either when the animal falls or 180 s is reached (Lane and Dunnet, 2011, vol II).

Automatic time-to-fall is a useful measure for general motor ability, but it is not a sensitive indicator of cerebellar function, which requires that the gait is synchronised to the speed of rotation. Rodents undertake all possible alternative strategies they can to avoid falling, such as clinging to the rod and being passively rotated or turning around, lying with their abdomen in contact with the rod and shuffling backwards. All these alternative strategies may indicate incorrect cerebellar function but will not be detected as "error" by automatic time-to-fall devices. Therefore, it is important that the experimenter records also the additional parameters specified above. An accelerating rotarod is quick and simple, but it is a less sensitive assessor of cerebellar dysfunction than is the constant speed protocol (Lane and Dunnet, 2011, vol II).

Learning and memory (cognitive functions) *([8] in Table 52)*

Learning and memory of the F1 generation should be investigated with the specifications below.

The minimum information requirements for learning and memory, a component of cognitive functions, include two different tests for associative learning and memory at two different time points. Different test types of associative learning and memory should be performed at adolescence (PND 25±2 days) and young adulthood (PND 60 and older). Different set of animals is recommended to be used. In OECD TG 443, Cohort 1A animals can be allocated to two sets of animals, 10 males and 10 females in both; the first set of animals to be tested at adolescence and the other set of animals at young adulthood. If necessary, animals from other Cohorts (such as Cohort 3 if not included to investigate developmental immunotoxicity) may be used also, taking into account the integrity of the study. For OECD TG 426, the examples of the alternative animal allocations can be followed. It is recommended to use more than 10 animals per sex if

possible, and e.g. example 3 of the OECD TG 426 may be used as the basis for animal allocation.

Two criteria for associative learning and memory tests are presented in paragraph 37 of OECD TG 426 and these should be fulfilled also if the DNT is tested as part of OECD TG 443 or by other means:

1) Learning should be assessed either as a change across several repeated learning trials or sessions, or, in tests involving a single trial, with reference to a condition that controls for non-associative effects of the training experience; and

2) The test(s) should include some measure of memory (short-term or long-term) in addition to original learning (acquisition), in the presence of a measure of acquisition obtained from the same test.

Different test types of associative learning and memory engage different brain regions, combinations of regions and neural pathways. Different tests can have also different sensitivities for observing effects on learning and memory. One of the required tests should investigate explicit associative learning and memory and the other test should investigate implicit associative learning and memory. Explicit memory (or declarative memory) is recalled consciously whereas implicit memory (or nondeclarative memory) is recalled unconsciously (Kandel, 2000).

Two examples of explicit associative learning and memory tests are the Morris water maze (MWM) test and Radial arm maze (RAM) test, both investigating allocentric spatial learning and memory. An example of one type of implicit associative learning and memory test is Cincinnati water maze (CWM) which investigates egocentric navigational learning and memory. Allocentric learning and memory in rodents is homologous to the same brain networks that in people mediate memory for people, places, facts, and events. Egocentric navigation in rodents is homologous to path finding and procedural learning and memory including skilled behaviours such as driving a car and other highly trained behaviours that become semiautomatic in people. However, the neural networks mediating egocentric and spatial navigation overlap despite partial dissociations of the two systems. (Vorhees and Williams, 2015 and 2016).

Examples of other types of implicit associative learning and memory tests are classical and operant/instrumental conditioning tests such as olfactory conditioning test, and acquisition and retention of schedule-controlled behaviour. If there is any prior information indicating a need for a specific test subtype, this should be used to select the most appropriate test. Although in OECD TG 426 also the T-maze, Biel water maze and passive avoidance test are given as examples of possible tests, these should not be selected because based on practical experience in regulatory use, they have been suspected to be insensitive for detecting developmental neurotoxicants (Levin, 2014; Vorhees and Williams, 2014; Vorhees and Makris, 2015). Below is an overview of associative learning and memory tests that may be selected to fulfil the minimum information requirements.

Morris water maze (MWM) *([8a] in Table 52)*

The MWM test studies allocentric spatial learning and memory that is a type of explicit learning and memory. MWM test involves hippocampus, entorhinal cortex and surrounding structures. The most basic MWM procedure tests allocentric learning and reference memory, but by an appropriate modification of the basic protocol it is possible to study allocentric learning and memory in more depth or with higher sensitivity or assess also other forms of learning and memory. These variants of protocols are presented, and the basic protocol is described (with troubleshooting) in detail in Vorhees and Williams (2006).

The concept behind the basic MWM is that the animal must learn to use distal cues, such as landmarks, to navigate a direct path to the hidden platform when started from different, random locations around the perimeter of the tank. MWM is an open circular pool that is filled

approximately half-way with water. The interior is as featureless as possible, and the maze is divided into four equal quadrants, and a relatively small hidden platform is positioned in the middle of one of the quadrants below the water surface in a fixed location. The animal must search in order to locate the hidden platform. The pool must be professionally constructed for MWM to ensure that there are no proximal cues undermining the goal of the test. The correct size of the tank is also one critical factor for obtaining valid spatial learning curve. Spatial learning (spatial acquisition) is assessed across repeated trials (normally 4 trials per day, inter-trial interval 15 s, repeated for 5-6 days) and reference memory (memory/probe trial) is determined by the preference for the platform area when the platform is absent (animal placed in a novel starting position to ensure that its spatial preference is a reflection of the memory of the goal location rather than for a specific swim path, tested at least 24 h after the last learning trial, trial length of 30 s recommended). Escape from water is relatively immune from motor activity (e.g. on open field) or body mass differences, making it ideal for many experimental models. In addition, the MWM has proven to be a robust and reliable test (Vorhees and Williams, 2006 and 2015).

Radial arm maze (RAM) ([8b] in Table 52)

Similar to the MWM, the RAM test studies allocentric spatial learning and memory that is a type of explicit learning and memory. However, the RAM test involves brain areas partly different from the MWM (hippocampus, frontal cortex, mediodorsal thalamic nucleus, septum, amygdala and mammillary bodies). RAM can be used with a variety of different procedures (reviewed e.g. in Levin, 2014 and Vorhees and Williams, 2016). Typically, the RAM is used as an appetitive test. In an eight arm RAM eight equally spaced arms extend from a central circular platform and four of the eight arms are baited with a food reward. Over a course of successive daily test trials, the rat is expected to learn which arms are baited (or never baited) and will efficiently retrieve the food rewards at the ends of four baited arms by using visuospatial cues in the room. The performance of the rat is measured by the time and distance to complete each trial, and by the number the animal goes down a never baited arm between trials (reference memory error) or re-entries into an arm it already visited within that trial (working memory error). RAM can be also run with aversive (water escape) motivators. (Levin, 2014; Vorhees and Williams, 2016).

Cincinnati water maze (CWM) ([8c] in Table 52)

The CWM test investigates egocentric navigational learning and memory that is a type of implicit learning and memory. Dorsal striatum is considered as the key component in mediating egocentric navigation. The CWM is an asymmetric 9-unit multiple-T labyrinthine maze that can be used to test either egocentric (body-centered) navigation if tested under infrared lighting, or combined allocentric and egocentric navigation if tested under standard light. In egocentric navigation the animal uses internal and/or near (proximal) cues. Internal cues include proprioceptive feedback from limb/joint receptors and stretch receptors in muscles and tendons that provide a sense of speed of motion that, when combined with heading or directional information and signposts about which way to turn, produce a pathway or route to and from different locations. Signs or signposts are different from landmarks; a signpost is close whereas a landmark is farther away from the organism. Although the CWM test run under the infrared light provides the most stringent test of egocentric learning and memory and is more sensitive than CWM test performed under standard light, the dark variant is more challenging, and it takes rats many trials over multiple days with multiple trial failures before learning the CWM to a proficient level of performance. The extended length of the test, when used under infrared light, may limit its applicability in a regulatory study (Vorhees and Williams, 2015 and 2016).

A day before the actual CWM test, whether using the standard light or infrared light procedure, rats must be given acclimation trials consisting of a separate straight water channel under standard light with a submerged platform at one end located in a different room than the maze. If these acclimation trials are not given, rats will find the task too difficult, give up, and never find the escape. The detailed test protocol is given e.g. in Vorhees and Williams (2016).

Olfactory conditioning test ([8d] in Table 52)

Olfactory fear conditioning test involves amygdala, the key structure for initiating and controlling fear reactions, but also playing a role in coding for the biological significance, intensity, or salience of sensory stimuli (Buettner [ed], 2017). In humans, dysregulation of function of amygdala is associated with abnormally heightened fear such as in anxiety disorders (Hakim et al., 2019; Buettner [ed], 2017). Examples of methods for olfactory fear conditioning are given in Kucharski and Spear (1984) and Crofton *et al*. (1993).

Aversive olfactory conditioning is a specific form of classical conditioning, also known as Pavlovian learning, that is a fundamental form of learning and expressed between and within species. The principle of Pavlovian fear learning is that an unpleasant unconditioned stimulus (US), such as foot shock, that produces a strong negative response, irrespective of training, gets associated with a neutral cue, odor in olfactory conditioning, that acts as a conditioned stimulus (CS). Before this association the CS is a stimulus that at first induces only a minor orienting response, but following contingent associations with the US (such that the CS predicts the occurrence of the US), the CS acquires aversive properties itself and evokes an aversive conditioned response (CR). Thereby after a certain number of pairings between the odor and foot shocks, the sole presentation of the odor will trigger a freezing reaction in the rat (Buettner [ed], 2017).

Acquisition and retention of schedule-controlled behaviour test ([8e] in Table 52)

Acquisition and retention of schedule-controlled behaviour involves dopaminergic projections to nucleus accumbens, amygdala and prefrontal cortex. Examples of protocols for fixed interval (FI) schedule of reinforcement can be found in Campbell and Haroutunian (1981) and Cory-Slechta *et al*. (1983).

Schedule-controlled behaviour is an example of operant conditioning test. Ratio schedules of reinforcement specify the number of responses that the animal must perform in order to obtain a reinforcer. In fixed ratio schedules, this number is an unchanging feature of the schedule, whereas in variable ratio schedules, it changes unpredictably from one reinforcer to the next. In progressive ratio schedules, the required number of responses is systematically increased, from one reinforcer to the next, between sessions or otherwise. Responding on progressive ratio schedules is normally well maintained under lower ratios, but the rate of responding declines with progressive increases in the ratio requirement. The ratio at which the subject stops responding is known as the breaking point (Bradshaw and Killeen, 2012).

Dopamine is considered necessary for e.g. positive reinforcement and expression of learned appetitive behaviours (reviewed for example in Fields et al., 2007), and reduced reward learning might contribute e.g. to the onset and maintenance of major depressive disorder in humans (Vrieze et al., 2014). For example, systemically administered dopamine antagonists have been shown to reduce previously learned responses in simple operant tasks such as fixed ratio 1 for food reward.

Note regarding developmental neurotoxicity studies and assessment of endocrine disruption

In OECD TG 426, ED related investigations include parameters such as open field activity, spatial learning and memory, AGD, sex distribution and results in tests with expected gender-dependent reactions that may indicate and support endocrine activity of an active ingredient together with other data. OECD TG 426 is a Level 4 study in the OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals (according to OECD GD 150), and it provides data on adverse effects on endocrine-relevant endpoints. OECD TG 426 may produce responses to EATS-modalities (oestrogen/androgen/thyroid/steroidogenesis), and non-diagnostic responses to R (retinoid-related) modalities.

1.10.4. Further studies

Table 53. Information requirement 8.10.4 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.10.4 Further studies A decision on the need to perform additional studies including those informing on the mechanisms should be based on the outcomes of the studies listed in 8.10.1, 8.10.2 and 8.10.3 and all other relevant available data	

A decision on the need to perform additional studies on additional species or strain or mechanistic studies should be based on the outcome of the studies already conducted and all other relevant information. If there is a specific concern that is not sufficiently addressed by the minimum study requirements and there is a concern that the risks associated with such hazards would not be sufficiently managed, a need for additional studies expected to provide answers to the identified concerns may be decided. The decision on additional species/strain to be tested primarily depends on consideration of all available information including the type of substance to be tested (see above in preliminary considerations of 1.10). Mechanistic studies may strengthen the WoE for reproductive toxicity when the *in vivo* evidence alone is not e.g. sufficiently convincing.

1.11. Carcinogenicity

Table 54. Information requirement 8.11 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.11 Carcinogenicity See 8.11.1 for new study requirements	A carcinogenicity study does not need to be conducted if: — the substance is classified as mutagen category 1A or 1B. The default presumption would be that a genotoxic mechanism for carcinogenicity is likely. In these cases, a carcinogenicity test will normally not be required

Carcinogenicity means the induction of cancer or an increase in the incidence of cancer occurring after exposure to a substance or mixture. Substances and mixtures which have induced benign and malignant tumours in well performed experimental studies on animals are considered as known or presumed (Category 1) or suspected (Category 2) human carcinogens, unless there is strong evidence that the mechanism of tumour formation is not relevant for humans.

Carcinogenicity testing under BPR is intended to provide information for classification and labelling[20] and for risk assessment. To conclude on appropriate classification and labelling, the available data should be considered using the criteria and guidance associated with the CLP regulation. For an appropriate risk assessment, the information on dose response has to be sufficient and should allow concluding on the existence of a threshold (see *ECHA Guidance Vol III Parts B+C).*

[20] Note that as indicated in Table 54, a carcinogenicity study does not need to be conducted if the substance is classified as mutagen category 1A or 1B. Therefore, in such cases information that would be sufficient for classification and labelling cannot be required for carcinogenicity.

Collection and evaluation of available information

For the assessment of existing information (physicochemical properties, grouping and read-across, (Q)SARs and expert systems, *in vitro* data, human data and animal data) further guidance is available within the ECHA Guidance Vol III Parts B+C, Guidance on the Application of the CLP Criteria and the practical guides[21] such as "How to use and report (Q)SARs". For guidance on WoE, please see the template, background document and examples published at the ECHA website: https://echa.europa.eu/support/guidance-on-reach-and-clp-implementation/formats, as well as *Guidance on the use of the weight of evidence approach in scientific assessments* (EFSA, 2017).

In addition to the waiving conditions indicated in the data requirement and in BPR Annex IV, the study does not need to be conducted if:

- No genotoxic potential for humans is identified in genotoxicity tests, and
- Possible mechanisms of toxicological effects observed in subchronic toxicity studies are without any indications of non-genotoxic carcinogenicity and there are no structural alerts for carcinogenicity, and
- The subchronic studies in rodents and/or non-rodents are without indication of substance related adverse effects at the limit dose level.

Generation of new test data

If further testing is needed to assess carcinogenicity, the test methods below should be used.

Table 55. Test methods for carcinogenicity:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Combined chronic toxicity/carcinogenicity study	B.33	TG 453
Carcinogenicity test*	B.32	TG 451

* Title of the OECD test guideline: Carcinogenicity study

Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

Other tests may contribute to a weight of evidence evaluation, e.g. by providing supporting information or mechanistic data.

For guidance on reporting historical control data see Section 1.

Mode of action (MoA) and human relevance

When carcinogenicity is observed, it may be necessary to further investigate the MoA and the relevance of the effect for humans. All available data, including read-across from structurally similar substances, must be carefully considered to assess if it can be concluded that the tumours

[21] https://echa.europa.eu/practical-guides

are induced by a specific mechanism.

For the purpose of elucidating a non-genotoxic mode of action and human relevance, the need for further investigations should be considered on a case-by-case basis, focusing first on mechanistic studies (see also 1.13.5). The IPCS Framework for Analyzing the Relevance of a Cancer Mode of Action for Humans (2007)[22] may be useful in considering the testing/assessment strategy.

1.11.1. Combined carcinogenicity study and long-term repeated dose toxicity

Table 56. Information requirement 8.11.1 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.11.1 Combined carcinogenicity study and long-term repeated dose toxicity Rat, oral route of administration is the preferred route. If an alternative route is proposed a justification must be provided. For evaluation of consumer safety of active substances that may end up in food or feed, it is necessary to conduct toxicity studies by the oral route	

See also section 1.9.3 of this guidance.

1.11.2. Carcinogenicity testing in a second species

Table 57. Information requirement 8.11.2 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.11.2 Carcinogenicity testing in a second species (a) A second carcinogenicity study should be conducted using the mouse as test species; (b) For evaluation of consumer safety of active substances that may end up in food or feed, it is necessary to conduct toxicity studies by the oral route	The second carcinogenicity study does not need to be conducted if the applicant can justify on the basis of scientific grounds that it is not necessary

If comparative metabolism data or any other reliable information indicate that mouse is an inappropriate model for human cancer risk assessment, an alternative species shall be considered.

[22] IPCS (2007) Boobis A.R., Cohen S.M., Dellarco V., McGregor D., Meek M.E., Vickers C., Willcocks D., Farland W.: IPCS Framework for Analyzing the Relevance of a Cancer Mode of Action for Humans in IPCS Harmonization Project Document No. 4, Part 1, IPCS framework for analysing the relevance of a cancer mode of action for humans and case-studies. http://www.who.int/ipcs/methods/harmonization/areas/cancer_mode.pdf

1.12. Relevant health data, observations and treatments

Table 58. Information requirement 8.12 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.12 Relevant health data, observations and treatments Justification should be provided if data is not available	

When no human studies/data are available, new studies on human volunteers should not be conducted.

Data and information on any effects observed in humans may provide valuable information on the validity of extrapolations from animal data to expected effects in humans, and to identify any unexpected adverse effect that could be specific to humans.

All available data and information of adequate quality following accidental or occupational exposure have to be submitted.

1.12.1. Information on signs of poisoning, clinical tests, first aid measures, antidotes, medical treatment and prognosis following poisoning

Table 59. Information requirement 8.12.1 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.12.1 Information on signs of poisoning, clinical tests, first aid measures, antidotes, medical treatment and prognosis following poisoning	

Observations and information relevant to the recognition of the symptoms of poisoning, as well as on the effectiveness of first aid and therapeutic measures must be included.

The reports should include a complete description of the exposure situations, clinical symptoms observed, therapeutic measures and clinical follow-up.

A detailed description of clinical signs and details of clinical tests (such as biomonitoring and patch tests) useful for diagnostic purposes must be included.

Symptoms of poisoning must be described, including full details of the time courses involved for all exposure routes.

First aid measures in the event of poisoning and eye contamination must be provided.

Therapeutic regimes and the use of antidotes must be described. Information based on practical experience must be provided where available, and otherwise, information must be provided based on theoretical grounds as to the effectiveness of any treatment regimes. Contraindications associated with particular regimes, particularly those relating to 'general medical problems' and conditions, must be described. The expected effects and the duration of these effects following poisoning must be described.

1.12.2. Epidemiological studies

Table 60. Information requirement 8.12.2 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.12.2 Epidemiological studies	

Four major types of epidemiological studies may be submitted: (1) analytical epidemiology studies on exposed populations, (2) descriptive or correlation epidemiology studies, (3) case reports and (4) in very rare, justified cases, controlled studies in human volunteers.

Analytical epidemiology studies are useful for identifying a relationship between human exposure and effects such as biological effect markers, early signs of chronic effects, disease occurrence, or mortality. Such studies may provide the best data for risk assessment.

Descriptive epidemiology studies examine differences in disease rates among human populations in relation to e.g. age and gender, and differences in temporal or environmental conditions. Typically, these studies can only identify patterns or trends in disease occurrence over time or in different geographical locations but cannot ascertain the causal agent or degree of human exposure.

Case reports describe a particular health condition in an individual or a group of individuals who were exposed to a substance. They may be particularly relevant when demonstrating effects that cannot be observed in experimental animal studies. In many such studies, information is lacking on critical aspects such as substance identity and purity, exposure, health status of the persons exposed and even the symptoms reported; thorough assessment of the reliability and relevance of case reports is therefore necessary.

For further information, please refer to REACH Guidance on information requirements and chemical safety assessment, Chapter R.4: Evaluation of available information.

1.12.3. Medical surveillance data, health records and case reports

Table 61. Information requirement 8.12.3 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.12.3 Medical surveillance data, health records and case reports	

The reports should include detailed information on the design of the occupational surveillance programme and exposure to the active substance and to other chemicals. Data relevant to the mechanism of the action of substance should also be included where feasible. The data may consist of published articles or unpublished medical surveys.

The following information on sensitisation should be provided where available, including any details necessary for the evaluation of the information (please see also ECHA Guidance Vol III, Parts B+C):

- Information on (respiratory) sensitisation or any incidences of (respiratory) hypersensitivity of workers or others exposed.
- Evidence that the substance can induce specific respiratory hypersensitivity will usually be based on human experience data. The clinical history data including both medical and

occupational history, and reports from appropriate lung function tests, bronchial challenge tests and allergy testing related to exposure to the substance should be submitted, if available.

- Reports of other existing supportive evidence, such as:
    - Information of a chemical structure within the active substance that is related to substances known to cause respiratory hypersensitivity;
    - *In vivo* immunological tests;
    - *In vitro* immunological tests;
    - Studies indicating other specific but non-immunological mechanisms of action.
    -

1.13. Additional studies (ADS)

Table 62. Information requirement 8.13 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.13 Additional studies Additional data which may be required depending on the characteristics and intended use of the active substance Other available data: Available data from emerging methods and models, including toxicity pathway-based risk assessment, *in vitro* and 'omic' (genomic, proteomic, metabolomic, etc.) studies, systems biology, computational toxicology, bioinformatics, and high-throughput screening shall be submitted in parallel	

Supplementary studies, where they relate to substances other than the active substance, are not a routine requirement. Decisions as to the need for supplementary studies should be made on a case-by-case basis.

Toxicity studies of metabolites

Whereas a result of metabolism or other processes, metabolites from plants or in animal products, soil, groundwater or open air differ from those in animals used for the toxicology studies or are detected in low proportions in animals, further testing should be carried out on a case-by-case basis, taking into account the amount of metabolite and the chemical structure of the metabolite compared to the parent.

Supplementary studies on the active substance

Supplementary studies should be carried out where they are necessary to further clarify the observed effects, taking into account the results of the available toxicological and metabolism studies and the most important exposure routes. Such studies may include:

(a) studies on absorption, distribution, excretion and metabolism, in a second species;

(b) studies on the immunotoxicological potential;

(c) a targeted single dose study to derive appropriate acute reference values (ARfD, AEL);

(d) studies on other routes of administration;

(e) studies on the carcinogenic potential;

(f) studies on mixture effects.

The studies required should be designed on an individual basis, in the light of the particular parameters to be investigated and the objectives to be achieved.

1.13.1. Phototoxicity (ADS)

Table 63. Information requirement 8.13.1 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.13.1 Phototoxicity	

There is possible concern of phototoxicity if the active substance:

- Absorbs light within the range of natural sunlight (290-700 nm); and
- Is liable to reach the eyes or light-exposed areas of skin, either by direct contact or through systemic distribution.

No testing is required if the molar extinction coefficient (MEC)[23] of the active substance is less than 1000 L x mol^{-1} x cm^{-1} (measured in methanol), as the active substance is not considered to be photoreactive enough to result in phototoxicity. MEC is also called molar absorptivity and it reflects the efficiency with which a molecule can absorb a photon at a particular wavelength (typically expressed as L mol^{-1} cm^{-1}) and is influenced by several factors, such as solvent. Detailed guidance on the setting and use of the coefficient and the assessment of phototoxicity is provided in the ICH Guidance S10 on Photosafety Evaluation of Pharmaceuticals[24].

The test methods in Table 64 should be used[25].

Table 64. Test methods for phototoxicity:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
In vitro 3T3 NRU phototoxicity test	B.41	TG 432
In vitro Phototoxicity - Reconstructed Human Epidermis Phototoxicity test method		TG 498
Ros (Reactive Oxygen Species) Assay for Photoreactivity		TG 495

[23] This property is assessed under point 3.6 of Annex II of BPR.
[24] Available at https://www.ema.europa.eu/en/ich-s10-photosafety-evaluation-pharmaceuticals.
[25] Work is taking place under OECD on Integrated Approaches to Testing and Assessment (IATA) on Photosafety. Once available, this IATA should be considered as well.

Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

The study should provide information on the potential of certain active substances to induce cytotoxicity in combination with light.

Examples of phototoxic active substances:

- active substances that are phototoxic *in vivo* after systemic exposure and distribution to skin;
- active substances that act as photoirritants/photosensitisers after dermal application to skin.

A positive result should be taken into account when considering potential human exposure. For photogenotoxicity see section 1.6 of this guidance.

1.13.2. Neurotoxicity (ADS)

Table 65. Information requirement 8.13.2 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.13.2 Neurotoxicity If the active substance is an organophosphorus compound or if there is an indication, knowledge of the mechanism of action or knowledge from acute or repeated dose studies that the active substance may have neurotoxic properties, additional information or specific studies (such as OECD TG 424 or OECD TG 418 or 419 or equivalent) will be required If anticholinesterase activity is detected a test for response to reactivating agents should be considered For evaluation of consumer safety of active substances that may end up in food or feed, it is necessary to conduct toxicity studies by the oral route	

Specific studies or additional specific investigations on neurotoxicity should be performed for active substances that:

- are organophosphorus compounds;
- have structural or other similarity to substance(s) capable of inducing neurotoxicity (e.g. carbamate compounds);
- induce specific indications of potential neurotoxicity such as clinical signs or effects in functional tests indicating neurotoxicity or neuropathological lesions in toxicity studies;
- have a neurotoxic mode of action unless the MoA has been demonstrated to be irrelevant to humans and other MoAs can be excluded,

and these indications of neurotoxicity are not as such sufficient for classification and labelling for neurotoxicity and/or risk management.

Indications or evidence of neurotoxicity can be acquired from the standard systemic toxicity studies, but only when neurotoxicity is so pronounced that it is visible as clinical signs (e.g. sedation, coma, convulsions) or by histopathological investigations. Lack of such effects does not indicate lack of neurotoxicity as standard repeated dose studies do not include specific and sensitive tests for neurotoxicity. Thereby also the potential effects seen in these standard systemic toxicity studies normally represent high-dose effects and when testing further by sensitive and specific methods it may be possible to detect also more subtle effects at lower doses.

If additional information or specific studies are warranted for neurotoxicity, they should provide adequate data to sufficiently investigate the neurotoxic potential of the active substance after single and repeated exposure. The data should also be useful for classification and labelling in accordance with CLP; therefore, please consult the CLP criteria for STOT SE (CLP 3.8) and STOT RE (CLP 3.9) under which neurotoxicity is assessed (note that developmental neurotoxicity is part of reproductive [developmental] toxicity and discussed in chapter 1.10.3).

Specific neurotoxicity studies often investigate the function of different components of the nervous system by specific and sensitive neurobehavioural tests and the histopathological effects in the central and peripheral nervous systems. Other possible investigations may comprise of neurophysiological (e.g. electroencephalography, electrophysiology) or biochemical studies (investigating e.g. neurotransmitter levels, receptor expression and binding).

Collection and evaluation of available information

For the assessment of existing information (physicochemical properties, grouping, (Q)SARs and expert systems, *in vitro* data, human data and animal data) further guidance is available within the *Guidance on the Application of the CLP Criteria* and *ECHA Guidance Vol III Parts B+C*.

Generation of new test data

When it is considered necessary to conduct a neurotoxicity study, it is important that the study design is discussed by the contractor/laboratory and the assessor before initiating the study, paying particular attention to the specificity and sensitivity of the protocol to be used.

If further standard 28- or 90-day studies are to be conducted, additional neurotoxicity parameters could be added if expected to be able to provide the missing information.

Neurotoxicity testing to conclude on classification and labelling and to establish a NOAEL for neurotoxicity, is required when data from standard toxicity studies or any other available information are indicative but not conclusive for neurotoxicity.

The test method for neurotoxicity study in rodents is given in Table 66 below.

Table 66. Test methods for neurotoxicity:

TEST METHOD	EU TEST METHOD	OECD TEST GUIDELINE
Neurotoxicity study in rodents	B.43	TG 424

The OECD TG 424 is intended for confirmation or further characterisation of potential neurotoxicity identified in previous studies or by other available information. It allows a flexible approach where comprehensive investigations of specific neurotoxicity endpoints by sensitive tests can be included. The dose levels should be adjusted to avoid confounding effects by general toxicity, but they should be sufficiently high to allow to conclude on potential absence of effects on the tested parameters. The procedures set out by OECD TG 424 can be used to investigate

both repeated dose and acute neurotoxicity. For STOT SE and STOT RE both reversible and irreversible effects are relevant.

The timing of the peak effect caused by the substance needs to be considered for the timing of testing different neurotoxicity parameters. The duration of exposure and time after administration needed to induce specific neurotoxic effects will depend on toxicokinetics of the substance and the underlying mechanism(s) of action.

Testing during short-term peak exposures is important for revealing acute neurotoxic effects that are often transient and to which tolerance may develop after repeated exposure. When the test compound is administered as a bolus via the intravenous, subcutaneous or oral route and causes acute neurotoxicity, it is essential to determine the time-effect course of the acute effect, and to perform measurements of acute neurotoxicity parameters at the time of the peak effect.

Where cumulative toxicity or repeated-dose effects are the primary focus, testing should precede the daily dose to rule out acute (less than 24 hour) effects. For delayed neurotoxicity a sufficiently long period between the last dose and neurotoxicity testing is required.

For example, the acute and chronic neurotoxicity associated with exposure to specific volatile organic solvents has been well identified based on human experience. The acute neurotoxic effects are investigated with acute inhalation studies designed to detect findings such as transient narcotic effects. However, long-term exposure to acute neurotoxicants may cause additional neurotoxic effects of different nature and at lower doses than the acute neurotoxic effects. To reveal these effects, repeated dose neurotoxicity studies should be performed by using sensitive and specific tests. For some neurotoxic substances only a long exposure period will elicit neurotoxic effects.

The most appropriate methods for further investigation of neurotoxicity should be determined on a case-by-case basis, guided by the effects seen in the standard systemic toxicity tests, any other available data. Methods which may be used are given in Table 67 below.

Table 67. Methods for investigation of neurotoxicity

EFFECT	METHODS
Morphological changes	Neuropathology Gross anatomical techniques Immunocytochemistry Special stains
Physiological changes	Electrophysiology Electroencephalogram (EEG) Evoked potentials
Behavioural changes	Functional observations Sensory function tests Motor function tests Cognitive function tests
Biochemical changes	Neurotransmitter analyses Enzyme/protein activity Measures of cell integrity

Several MoAs, such as acetylcholine esterase (AChE) inhibition, have been associated with neurotoxic effects. AChE may be inhibited to varying extents depending on animal or cell model,

dose, duration of exposure, and specific compound (Voorhees et al., 2016). Organophosphorus compounds and carbamates are examples of compounds that can inhibit acetylcholinesterase, but they have also other targets causing neurotoxicity (Voorhees et al., 2016; Lotti and Moretto, 2006). Exposure to high levels of organophosphorus compounds may cause cholinergic crisis in humans and animals characterised by via overstimulation of the nervous system leading to respiratory failure, flaccid paralysis, decreased blood pressure, parasympathetic discharge, and even death. Lower (repeated dose) exposure levels have been associated with neurodegenerative disease, psychiatric illness, and sensorimotor deficits in humans whereas in rodent models deficits in learning and memory, attention and impulsive behaviour and some other cognitive functions have been reported after repeated exposure to certain organophosphorous compounds (see also delayed neuropathy below) (Voorhees et al., 2016).

There are many other neurotoxic MoAs as well. Based on the MoA, it needs to be carefully considered which neurotoxicity test(s) is (are) most appropriate (specific and sensitive) to investigate the adverse effects caused by the identified MoA. For example, in rats pyrethroids may produce marked behavioural arousal, aggressive sparring, increased startle response, and fine body tremor progressing to whole-body tremor, and prostration (T syndrome) and/or profuse salivation, coarse tremor progressing to choreoatetosis, and clonic seizure (CS syndrome) by affecting the function of sodium channels, $GABA_A$ receptors and voltage-dependent chloride channels. Degeneration of dopaminergic neurons in the substantia nigra may result in Parkinson's disease-like symptoms manifested in rodents as e.g. impairments in movement initiation, weight shifting, and in postural stability, whereas a substance targeting hippocampal, amygdala and pyriform cortex neurons may cause cognitive impairment (Costa et al., 2008). When having any mechanistic information on the active substance, please consider the available information on Adverse Outcome Pathways[26] (AOPs) and investigate whether additional specific tests on neurotoxicity are warranted.

Delayed polyneuropathy studies

Delayed polyneuropathy studies should provide sufficient data to evaluate if the active substance may provoke delayed polyneuropathy after acute and/or repeated exposure by inhibition of neuropathy target esterase (NTE). Organophosphate-induced delayed polyneuropathy (OPIDN) results from exposure to certain organophosphorus compounds. It is characterised by distal degeneration of some axons of both the peripheral and central nervous systems occurring 1-4 weeks after single or short-term exposures (Lotti and Moretto, 2005). The condition is characterized by motor weakness, fatigue and paralysis and sensory numbness, tingling, and pain. OPIDN has been attributable to inhibition of neuropathy target esterase (NTE), rather than AChE, as inhibition of AChE is not necessary for the development of OPIDN (Woltje, 2015). Also, some carbamates are known to inhibit neuropathy target esterase (NTE) (Lotti and Moretto, 2006). A repeated exposure study for delayed neuropathy may be waived unless there are indications that the compound accumulates and significant inhibition of NTE or clinical/histopathological signs of OPIDN occur at around the hen LD_{50} as determined in the single dose test.

Delayed neurotoxicity tests in the laying hen after acute and repeated exposure (OECD TG 418 and OECD TG 419) should be performed for active substances of similar or related structures to those capable of inducing delayed polyneuropathy such as organophosphorus compounds, unless there is already sufficient information to conclude on neurotoxicity.

Test methods for delayed neuropathy:

- OECD TG 418: Delayed Neurotoxicity of Organophosphorus Substances Following Acute Exposure. (EC method B.37 Delayed neurotoxicity of organophosphorus substances after

[26] https://aopwiki.org/

acute exposure)

- OECD TG 419: Delayed Neurotoxicity of Organophosphorus Substances: 28-day Repeated Dose Study (EC method B.38 Delayed neurotoxicity of organophosphorus substances 28-day repeated dose study)

In OECD TG 418, a single dose of the test substance is administered orally to domestic hens, NTE (and potentially AChE) activity is assayed 24 and 48 h after dosing, the animals are observed for 21 days for ataxia, paralysis and other behavioural abnormalities, and 21-days after exposure histopathological examination of selected neural tissues is performed. In OECD TG 419, the exposure period is 28 days, NTE (and potentially AChE) activity is assayed 24 and 48 h after the last dosing, the animals are observed for 14 days after the last dose and after which the histopathological examination is performed.

1.13.3. Endocrine disruption

Table 68. Information requirement 8.13.3 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.13.3 Endocrine disruption The assessment of endocrine disruption shall comprise the following tiers: (a) An assessment of the available information from the following studies and any other relevant information, including *in vitro* and *in silico* methods: (i) 8.9.1 A 28-day oral toxicity study in rodents (OECD TG 407); (ii) 8.9.2 A 90-day oral toxicity study in rodents (OECD TG 408); (iii) 8.9.4 A repeated dose oral toxicity study in non-rodents (OECD TG 409); (iv) 8.10.1 A prenatal developmental toxicity study (OECD TG 414); (v) 8.10.2 An extended one-generation reproductive toxicity study (OECD TG 443) or two-generation reproductive toxicity study (OECD TG 416); (vi) 8.10.3 A developmental neurotoxicity study (OECD TG 426); (vii) 8.11.1 A combined carcinogenicity study and long-term repeated dose toxicity study (OECD TG 451-3); (viii) A systematic review of the literature including studies on mammals and non-mammalian organisms; (b) If there is any information suggesting that the active substance may have endocrine disrupting properties, or if there is incomplete information on key parameters relevant for concluding on endocrine disruption, then additional information or specific studies shall be required to elucidate: (1) the mode or the mechanism of action; and/or	Where sufficient weight of evidence to conclude on the presence or absence of a particular endocrine disrupting mode of action is available: —further testing on vertebrate animals for that effect shall be omitted for that mode of action, —further testing not involving vertebrate animals may be omitted for that mode of action. In all cases, adequate and reliable documentation shall be provided

(2) potentially relevant adverse effects in humans or animals	
For evaluation of consumer safety of active substances that may end up in food or feed, it is necessary to consider the oral route and conduct animal studies by the oral route	

This data requirement (8.13.3 Endocrine disruption) is a core data requirement although it is placed under 8.13 Additional studies (ADS). This discrepancy is due to the change in the legislation, as Regulation (EU) 2021/525 changed this data requirement from ADS to CDS.

This guidance should be read in conjunction with OECD Guidance No. 150 (OECD 2012) and the ECHA/EFSA Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009 where the testing strategy is further elaborated.

The applicant should include a collation of the relevant information and reporting of the lines of evidence for each modality, as recommended in the ECHA/EFSA guidance.

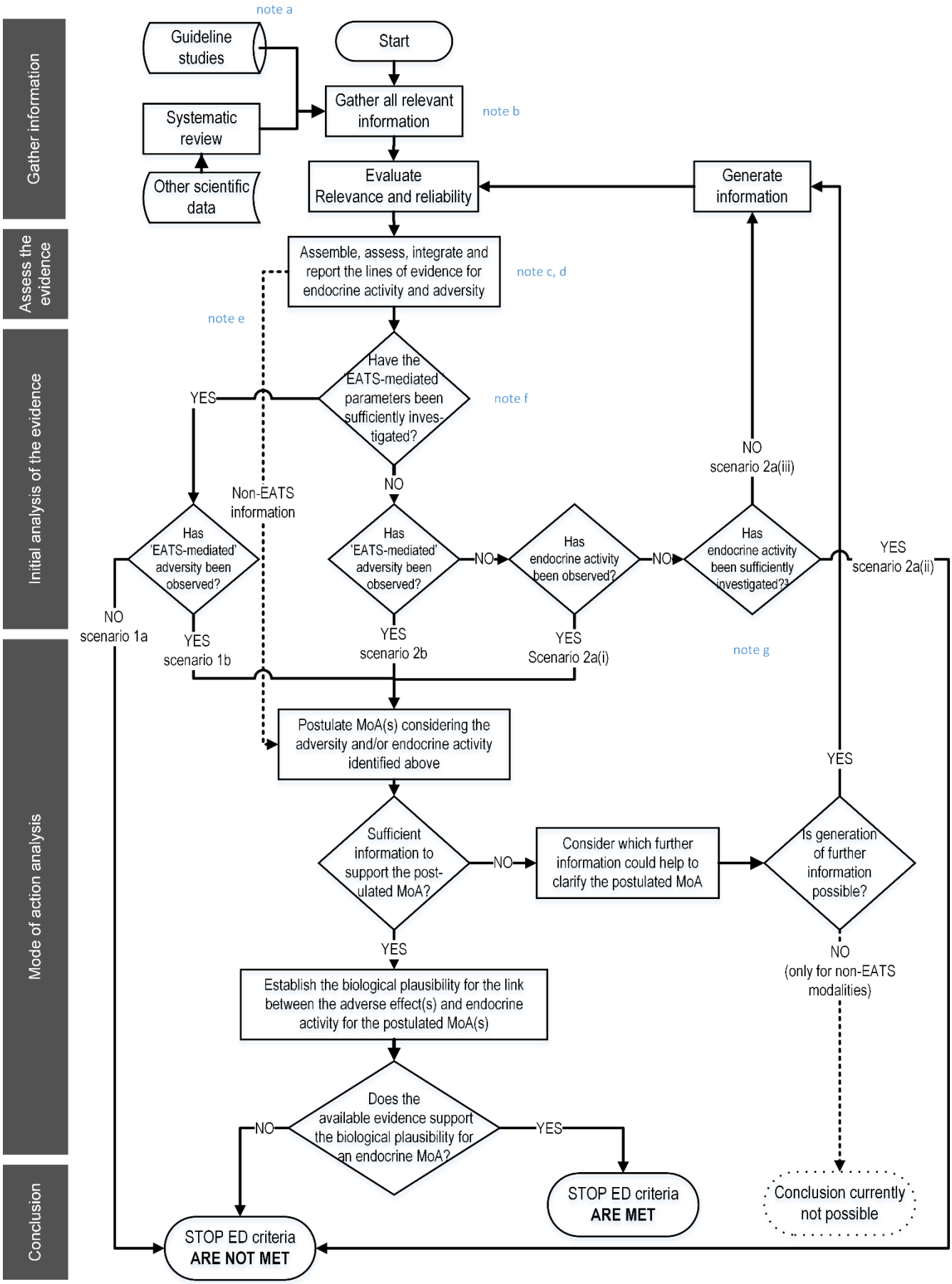
Objectives

For each biocidal active substance, a conclusion on the ED properties is required. This guidance provides advice on the tests that an applicant can or should perform to address the ED properties of the active substance and to conclude whether the ED criteria are met or not. For the ED assessment, sufficient information is needed to either conclude whether the active substance causes adverse effects and/or whether the substance has the ability to interfere with the endocrine system (i.e. show endocrine activity). Because of this, the objectives of the information requirements are:

- to have sufficient information to conclude whether adverse effects occur, and/or
- to have sufficient information to conclude whether the substance shows endocrine activity

When both adversity and activity are observed, the criteria are met if the adverse effects are a (plausible) consequence of interference with the endocrine activity. If sufficient information is available to show either lack of adversity or lack of endocrine activity, the conclusion can be that the ED criteria are not met. Similarly, when the adverse effects cannot be linked plausibly to the endocrine activity in the mode of action analysis, the substance does not meet the ED criteria. Note that while in practice the main focus will be on any of the four modalities (EATS) addressed in the ECHA/EFSA ED guidance, the ED criteria themselves are not limited to a particular modality. For indications of endocrine adversity and/or activity not covered (fully) in the current data requirements, additional data might need to be generated using dedicated testing protocols.

Figure 4: Flow chart illustrating the ED assessment strategy. The figure is from the ECHA/EFSA Guidance (2018) for the identification of endocrine disruptors – for notes and scenarios, please see this guidance. The assessment strategy illustrated in the flow chart is applicable both for humans and non-target organisms and is driven by the availability of 'EATS-mediated' parameters as these provide evidence for both endocrine activity and the resulting potentially adverse effects. However, there may be situations where the 'EATS-mediated' parameters are insufficiently investigated. In such cases, it may be possible to follow the assessment strategy using the 'sensitive to, but not diagnostic of, EATS' parameters, without the need to generate additional information on EATS-mediated parameters i.e. in case of scenarios 2a(i) or 2b. If the required data are available, it is in principle possible to establish endocrine disrupting MoA(s) on the basis of parameters indicating 'sensitive to, but not diagnostic of, EATS' potential adversity and EATS endocrine activity.


Gather information
Assess the evidence
Initial analysis of the evidence
Mode of action analysis
Conclusion
note a
Guideline studies
Systematic review
Other scientific data
Start
Gather all relevant information
note b
Evaluate Relevance and reliability
Generate information
Assemble, assess, integrate and report the lines of evidence for endocrine activity and adversity
note c, d
note e
Have the EATS-mediated parameters been sufficiently inves-tigated?
note f
YES
NO
NO scenario 2a(iii)
Non-EATS information
Has 'EATS-mediated' adversity been observed?
Has 'EATS-mediated' adversity been observed?
Has endocrine activity been observed?
Has endocrine activity been sufficiently investigated?a
YES scenario 2a(ii)
NO scenario 1a
YES scenario 1b
YES scenario 2b
YES Scenario 2a(i)
note g
Postulate MoA(s) considering the adversity and/or endocrine activity identified above
Sufficient information to support the post-ulated MoA?
NO
Consider which further information could help to clarify the postulated MoA
Is generation of further information possible?
YES
NO (only for non-EATS modalities)
YES
Establish the biological plausibility for the link between the adverse effect(s) and endocrine activity for the postulated MoA(s)
Does the available evidence support the biological plausibility for an endocrine MoA?
NO
YES
STOP ED criteria ARE MET
Conclusion currently not possible
STOP ED criteria ARE NOT MET

General overview of the assessment strategy

This section contains an overview of the assessment strategy to determine whether a substance meets the definition of an endocrine disruptor according to the ED criteria. More detailed information on the assessment strategy and relevant test methods can be found in *ECHA/EFSA Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009* and OECD Guidance No. 150 (OECD 2012).

While the ED criteria (Regulation (EU) 2017/2100) cover all endocrine-disrupting MoAs, the ECHA/EFSA Guidance focuses mainly on effects caused by the EATS (estrogenic, androgenic, thyroidal and steroidogenic) modalities. This is because these pathways are currently the best understood, i.e. with a relatively good mechanistic understanding of how substance-induced perturbations may lead to adverse effects via an endocrine-disrupting MoA. In addition, standardised test methods for *in vivo* and *in vitro* testing are currently available only for these modalities. However, there may be situations where it is possible to conclude on ED properties also for non-EATS modalities.

To facilitate the assessment, the ECHA/EFSA Guidance has grouped the parameters investigated in the standard test methods depending on the type of information they provide. The groups are:

- ***In vitro* mechanistic** – parameters measured *in vitro* that provide information on endocrine activity (e.g. by binding to and activating a receptor or interfering with hormone production).

- ***In vivo* mechanistic** – parameters measured *in vivo* that provide information on endocrine activity (e.g. changes in hormone levels or effects in a specific tissue known to be mainly under endocrine control).

- **EATS-mediated** – parameters measured *in vivo* that may contribute to the evaluation of adversity, while at the same time (due to the nature of the effect and the existing knowledge) they are also considered indicative of an EATS MoA and thus (in the absence of other explanations) also imply underlying *in vivo* mechanistic information.

- **Sensitive to, but not diagnostic of, EATS** – parameters measured *in vivo* that may contribute to the evaluation of adversity, however, due to the nature of the effect and the existing knowledge, these effects cannot be considered diagnostic on their own of any one of the EATS modalities.

Steps in the assessment strategy

The starting point for the ED assessment strategy is that the other regulatory requirements for the (active) substance are met and that the information is available. It is recognised that there may be situations where the available information does not sufficiently cover all endocrine modalities (i.e. they are 'not sufficiently investigated' according to the ED guidance) to reliably conclude on the ED properties. In such cases, additional data generation is usually required.

The assessment strategy is based on the three conditions stipulated in the ED criteria – endocrine activity, adversity and a biologically plausible link between the two – and on the grouping of the parameters as described above. The 'EATS-mediated' parameters drive the assessment strategy because, by providing evidence for both endocrine activity and the resulting potentially adverse effects, they are considered indicative of an endocrine MoA. Parameters which are considered as 'sensitive to, but not diagnostic of, EATS' and 'EATS-mediated' parameters are normally investigated in the same tests. If there is no adversity seen in the 'EATS-mediated' parameters, but adversity is observed in the same study in parameters considered 'sensitive to, but not diagnostic of, EATS', then this adversity is not likely to be caused by alterations of the EATS modalities. There may be situations where the 'EATS-mediated' parameters are not sufficiently

investigated (e.g. tests carried out according to outdated guidelines), and in such cases, any adversity observed in parameters considered 'sensitive to, but not diagnostic of, EATS', cannot be dismissed.

The assessment strategy is applicable both for humans and non-target organisms and in both cases, Figure 4 illustrates the steps of the assessment. Each of the steps outlined in the figure are described below.

Gather information. In this step, all available relevant information (including *in vitro* and *in silico* methods) is gathered, evaluated for relevance and reliability, and extracted and reported in the dossier/CAR. Relevant information is expected to be provided based on the existing data requirements (see 8.13.3 Endocrine disruption in the BPR), supplemented with the relevant studies present in the dossier. In addition to the studies in the assessment report, additional information must be identified by performing a systematic literature review. The systematic review should focus on information relevant for the ED assessment coming from *in vivo*, *in vitro* and *in silico* studies. More information is provided in Appendix F of the ECHA/EFSA ED Guidance and EFSA (2010) *Application of systematic review methodology to food and feed safety assessments to support decision making*.

Assess the evidence. The information is assembled into lines of evidence, integrating information for both adversity and endocrine activity for each of the EATS modalities. The lines of evidence are assessed and reported in the dossier/CAR. If there is indication of non-EATS-related endocrine activity and/or effects, this should be taken forward to the MoA analysis step because the questions asked in the next step are tailored to the EATS modalities.

Initial analysis of the evidence. This step includes a decision tree. The decisions are driven by the availability of 'EATS-mediated' parameters and/or evidence of endocrine activity. This first step is to assess whether the available evidence already allows concluding that a substance does not meet the ED criteria, or whether a more detailed analysis and/or additional information is needed to conclude on the ED properties.

MoA analysis. This step aims to establish if there is a biologically plausible link between observed adverse effects and endocrine activity. Different situations are outlined. Depending on the available evidence, the applicant and the assessor need to identify the information that may need to be generated to further investigate the adversity or the endocrine activity, or any potential alternative MoA(s). In this step, it should be further investigated whether it is possible to establish a plausible link between non-EATS endocrine activity and observed adversity, or whether further information could be generated to clarify whether there is a non-EATS endocrine-disrupting MoA. As recommended in the ED guidance, the applicant should utilise (published) MoAs and adverse outcome pathways in the assessment (see OECD AOP Knowledge Base[27]) whenever possible. These may be useful to structure the information and facilitate the following steps of the assessment strategy.

Conclusion on the ED criteria. In this step, the conclusion is made whether the ED criteria are met with respect to humans. The conclusion is transparently documented, including the remaining uncertainties.

If a conclusion cannot be made whether the substance meets the ED criteria, then additional information or specific studies shall be required. These are specified in the chapter below.

[27] http://aopkb.org/

1.13.3.1. Specific additional studies to investigate potential endocrine disrupting properties (ADS)

Table 69. Information requirement 8.13.3.1 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.13.3.1 Specific additional studies to investigate potential endocrine disrupting properties may include, but are not limited to the following: (a) the mammalian toxicity studies listed in 8.13.3(a); (b) the *in vitro* assays: (i) Estrogen receptor transactivation assay (OECD TG 455); (ii) Androgen receptor transactivation assay, (OECD TG 458); (iii) H295R steroidogenesis assay (OECD TG 456); (iv) the Aromatase assay (human recombinant) OPPTS 890.1200; (c) Uterotrophic bioassay in rodents (OECD TG 440) and Hershberger bioassay in rats (OECD TG 441); (d) Pubertal development and Thyroid Function in Intact Juvenile or Peripubertal Male Rats (OPPTS 890.1500). The decision to carry out studies in mammals shall be taken based on all available information, including a systematic review of the literature (including information on endocrine disrupting effects in non-target organisms) and the availability of suitable *in silico or in vitro* methods	

Point 8.13.3 of Annex II to the BPR states that *if there is any information suggesting that the active substance may have endocrine disrupting properties* (or if available information is incomplete)*, then additional information or specific studies shall be required to elucidate: (1) the mode or the mechanism of action and/or; (2) potentially relevant adverse effects in humans or animals.* Point 8.13.3.1 of Annex II to the BPR specifies which additional studies to consider.

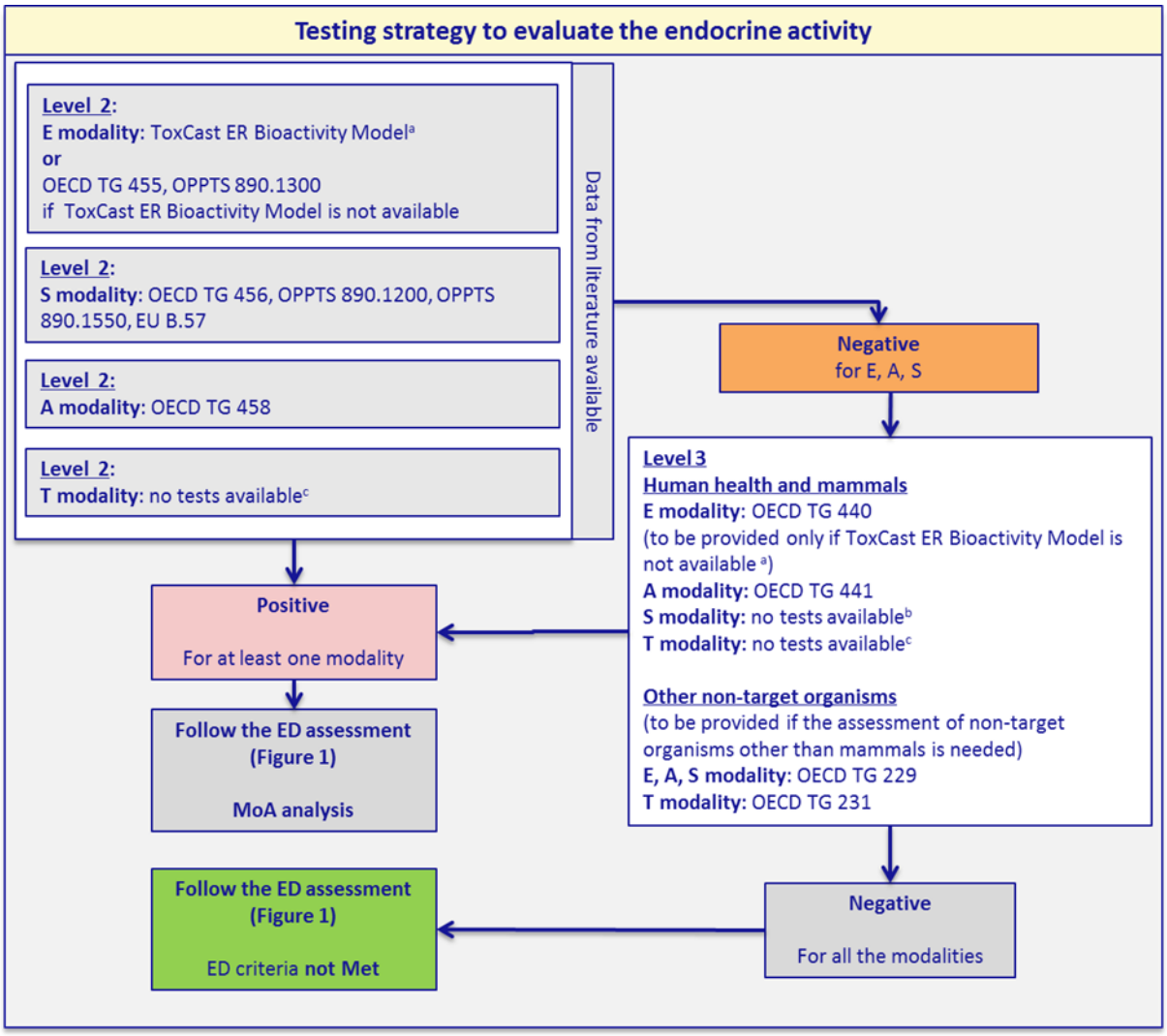
If additional data needs to be generated, there are several test methods available that investigate specific endocrine modalities and/or further investigate potentially endocrine related adverse effects. The decision on which additional studies to carry out depends on what information is missing for a robust conclusion on ED properties.

Note that the methods mentioned under 8.13.3.1 (a) generally provide information on adversity, while the systematic literature review from 8.13.3(a)(viii) can provide information on both the adversity and endocrine activity. The studies listed under 8.13.3.1(b), 8.13.3.1(c) and 8.13.3.1(d) generally provide information on endocrine activity only, though exceptions may apply.

Point 8.13.3.1 of Annex II to the BPR further specifies that, in all cases, *the decision to carry out studies in mammals shall be taken based on all available information, including a systematic review of the literature (including information on endocrine disrupting effects in non-target organisms) and the availability of suitable in silico or in vitro methods*. The ECHA/EFSA guidance recommends to first explore the modality with the strongest positive evidence. However, to exclude ED properties, all EATS modalities must be sufficiently investigated in terms of endocrine

activity or endocrine related adversity. In case generation of additional data is warranted, in line with the legal requirement and general desire to limit animal testing as much as possible, it is recommended to investigate endocrine activity first, starting with *in vitro* and *in silico* methods and approaches where applicable. A general strategy for investigating endocrine activity is given in Figure 5.

Figure 5. Strategy to investigate EATS-related endocrine activity in the context of the ED assessment. From ECHA/EFSA Guidance (2018). Note that the testing strategy also includes non-mammalian tests: since both mammalian and non-mammalian tests can inform on endocrine activity, all are included in the assessment as discussed in the ED guidance.



Point 8.13.3.1(a). The existing information might give important information on endocrine activity and/or disruption *in vivo*, based on the mammalian toxicity studies listed in 8.13.3 (a). This will most likely be based on parameters measured *in vivo* that may contribute to the evaluation of adversity, while at the same time (due to the nature of the effect and the existing knowledge) are also considered indicative of an EATS MoA. Therefore, these endpoints would imply an underlying endocrine mode of action (in the absence of other explanations). In addition, some parameters measured *in vivo* may contribute only to the evaluation of adversity, because on their own, these effects cannot be considered diagnostic of any of the EATS modalities.

Some other parameters that are measured *in vivo* are usually not considered adverse, while providing information on endocrine activity. For example, changes in hormone levels are considered indicative of perturbation of the endocrine system, while not necessarily leading to

an adverse effect.

Point 8.13.3.1(b). If further testing is needed for a robust conclusion on ED properties, the first step in the generation of new data shall be focused on investigating endocrine activity. The *in vitro* test methods in Table 70 should be used. All assays are in principle required, unless it is possible to conclude that the substance meets the ED criteria. This is because each assay investigates a different aspect of endocrine activity. Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

Table 70. In vitro assays for EATS modalities:

MODALITY ASSESSED	TEST METHOD	OECD TEST GUIDELINE
E modality	Performance-based test guideline for stably transfected transactivation *in vitro* assays to detect estrogen receptor agonists and antagonists	TG 455
A modality	Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals	TG 458
S modality	H295R steroidogenesis assay	TG 456
	The Aromatase assay (human recombinant)	OPPTS 890.1200
T modality	Currently there are no validated OECD TGs to investigate the T modality specifically. However, several assays are described in the scientific literature. In addition, repeated dose toxicity studies inform on potential interference with the T modality, i.e. thyroid hormones and HDL/LDL cholesterol levels and the weight and histopathology of the thyroid gland.	

Point 8.13.3.1(c). If the *in vitro* information is positive and sufficient to complete a MoA analysis, additional data might not be needed. However, if the available *in vitro* (and *in silico*) information is negative, the endocrine activity still needs to be further investigated using OECD CF level 3 *in vivo* assays (see Figure 5). Specifically, the assays in Table 71 should be considered.

Table 71. Level 3 *in vivo* assays:

TEST METHOD	OECD TEST GUIDELINE	REMARKS
Uterotrophic Bioassay in Rodents: A short-term screening test for oestrogenic properties	TG 440	See also OECD GD 71 for how to investigate anti-estrogenic effects
Hershberger Bioassay in Rats: A Short-term Screening Assay for (Anti)Androgenic	TG 441	See also OECD GD 115 for how to investigate anti-androgenic effects

Properties		

Before deciding on the need for *in vivo* testing, a review of the *in vitro* test results and all available information on the toxicokinetic and toxicodynamic profile of the test substance is needed.

While a conclusion on the absence of E-related endocrine activity will normally require a negative OECD TG 440 study, the ED guidance currently describes a specific case where *in vitro* information can be sufficient to waive the Uterotrophic assay. However, this is not universally accepted.

Point 8.13.3.1(d). The hazard identification of thyroid disruptors is currently hampered by a lack of internationally validated test methods to investigate substance that alter thyroid homeostasis. Nevertheless, a data package that fulfils the information requirements should in most cases be sufficient to conclude on the T mediated adversity for a biocidal active substance. This is because the data is expected to include an assessment of thyroid histopathology, which is generally considered to be among the most sensitive and reliable means to detect thyroid disruption. In 8.13.3.1 (d) *Pubertal development and Thyroid Function in Intact Juvenile or Peripubertal Male Rats (OPPTS 890.1500)* is listed to investigate T mediated effects in more detail. The male assay is designed to detect interference with both the HPG and HPT axes. As a result, it will detect substances that interfere with the androgen and thyroid pathways, as well as effects on steroidogenesis. While the male assays can also detect estrogen receptor mediated effects, its accuracy on this is currently unknown. Note that while this assay is listed in the ECHA/EFSA ED guidance, it is not included in the testing strategy for endocrine activity. For a more detailed discussion on consideration on how to assess the potential for thyroid disruption for human health, see Appendix A of the ED guidance.

1.13.4. Immunotoxicity and developmental immunotoxicity (ADS)

Table 72. Information requirement 8.13.4 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.13.4 Immunotoxicity and developmental immunotoxicity If there is any evidence from repeat dose or reproductive toxicity studies that the active substance may have immunotoxic properties, then additional information or specific studies shall be required to elucidate: (1)the mode or the mechanism of action; and/or (2) potentially relevant adverse effects in humans or animals. For evaluation of consumer safety of active substances that may end up in food or feed, it is necessary to consider the oral route and conduct animal studies by the oral route	

Immunotoxicity investigations should focus on:

- The potential to induce adverse effects involving the immune system;
    - with special attention to the adverse immunotoxic outcome among susceptible and

vulnerable groups;

- clarifying the type of the adverse immunotoxic outcomes when possible;
    - hypersensitivity, immunosuppression, autoimmunity, or unintended stimulation of immune responses;
    - impact on the developing immune system.

Collection and evaluation of available information

For the assessment of existing information (non-human data: physicochemical properties, grouping, (Q)SARs and expert systems, *in vitro* data; human data and animal data) further guidance is available within the *ECHA Guidance Vol III Parts B+C* and the *Guidance on the Application of the CLP Criteria*).

The guidance for the evaluation of all available information before conducting new tests is available in *ECHA Guidance Vol III Parts B+C* and is largely based on the WHO/IPCS Guidance on Immunotoxicity for Risk Assessment (WHO, 2012).

Current standard animal studies provide information from an unchallenged immune system, without functional tests, which can give only indications of immunotoxicity. Inclusion of functional tests is needed to adequately assess the immunotoxic potential of active ingredients (WHO/IPCS guidance for Immunotoxicty risk assessment for chemicals (WHO, 2012)).

Generation of new test data

If immunotoxicity potential is identified tests consisting of a more specific confirmatory set of studies or in-depth mechanistic studies, is carried out to confirm and further characterize the endpoint. It is worth noting that further testing to investigate immune function should be conducted only if the outcomes of such studies can be interpreted in relation to the risk assessment for the substance of interest. In addition, the need for further testing to characterise effects of concern for immunotoxicity has to be considered on a case-by-case basis.

It should be considered that the conduct of the repeated dose toxicity tests and the reproductive toxicity tests should be performed in a way that allows evaluation of immunotoxicity potential (e.g. Repeated dose toxicity according to US EPA OPPTS 870.7800 [Health Effects Test Guidelines Immunotoxicity] including parameters for immunotoxicity and OECD TG 443 - extended one generation toxicity test- may be conducted with the developmental immunotoxicity cohort). However, a separate study may be needed for confirmatory results of developmental immunotoxicity.

Whether the immunotoxic properties should be investigated in adults or in the developing organisms, or both, should be considered on a case-by-case basis taking into account the various aspects affecting the decision, for example, the target population, toxicokinetics and mode of action. Generally, a study in developing organisms is recommended as a more conservative approach.

Immunotoxicity observed in animals exposed during adulthood only may trigger the need to investigate also potential for developmental immunotoxicity unless substance specific information is provided why these effects or mode of action would not be relevant in developing organism. Findings from repeated dose toxicity studies (see Section 1.9) in adult animals may serve as triggers to investigate developmental immunotoxicity, e.g. thymus weight and histopathology, bone marrow histopathology, total and differential leukocyte count, histopathology on the collected lymph nodes. In addition, if the classification criteria for STOT are met, based on studies in adults, this is not an adaptation rule allowing the omission of investigations on developmental immunotoxicity but rather a trigger for a concern. This is due

to expected higher sensitivity of the developing organisms (see e.g. Dietert, 2014), which may lead to a lower point of departure and/or to hazard classification for development.

A classification to Repr. 1B or 2 may be necessary if the effects are considered to be of developmental origin, i.e. due to exposure during development. Sensitivity has been evaluated in animal studies for nine reviewed (immuno)toxicants and, according to the authors, the developing immune system was found to be at least as sensitive or more sensitive than the general (developmental) toxicity parameters (Hessel et al., 2015).

The test methods to be used for further immunotoxicity studies will depend also on the weight of evidence analysis. Different test methods can be employed for assessing immune suppression, immune stimulation and autoimmunity as well as developmental immunotoxicity.

Reviews of principles and methods for immunotoxicity are available from WHO/IPCS:

- WHO/IPCS Environmental Health Criteria (EHC) 180, Principles and Methods for Assessing Direct Immunotoxicity Associated with Exposure to Chemicals (WHO, 1996)
- WHO/IPCS Environmental Health Criteria (EHC) 212, Principles and Methods for Assessing Allergic Hypersensitization Associated with Exposure to Chemicals (WHO, 1999)
- WHO/IPCS Environmental Health Criteria (EHC) 236, Principles and Methods for Assessing Autoimmunity Associated with Exposure to Chemicals (WHO, 2007)
- WHO/IPCS Guidance for immunotoxicity risk assessment for chemicals, Harmonisation project document No 10 (WHO, 2012)

Below a list of methods that can be considered for further immunotoxicity testing is provided. This list is not exhaustive but provides the methodological aspects to consider on a case-by-case basis.

Immune Suppression

- US EPA OPPTS 870.7800 Health Effects Test Guidelines Immunotoxicity
- Functional investigations as described under Additional Immunotoxicity Testing below

Immune stimulation including hypersensitivity (skin and respiratory sensitisation)

- *In chemico*/*in vitro* skin sensitisation assays or LLNA as a last resort (see sensitisation section)
- Functional investigations as described under Additional Immunotoxicity Testing below

Autoimmunity

- Functional investigations as described under Additional Immunotoxicity Testing below

Additional Immunotoxicity Testing (adopted from ICH S8)

- T-cell Dependent Antibody Response (TDAR)
- Immunophenotyping
- Natural Killer Cell Activity Assays
- Host Resistance Studies

- Macrophage/Neutrophil Function
- Assays to Measure Cell-Mediated Immunity

Developmental Immunotoxicity

- Protocols for independent developmental immunotoxicity studies with exposure during development and functional investigations (such as described under Additional Immunotoxicity Testing above) during development and/or adulthood
- Developmental immunotoxicity cohort in an OECD TG 443: Extended One-Generation Reproductive Toxicity Study[28]

Developmental immunotoxicity studies are designed to provide information on the potential functional and morphological hazards to the immune system arising in the offspring from exposure of the mother during pregnancy and lactation. For an independent developmental immunotoxicity study there is currently no available internationally accepted protocol, such as an OECD TG. However, protocols, considerations and recommendations for independent developmental immunotoxicity studies have been published e.g. by Ogungbesan et al., (2019), Boverhof et al., (2014), Collinge et al., (2012), WHO (2012), DeWitt et al., (2012a and 2012b), Gupta (2011, page 219-225), Dietert and DeWitt (2010), Rooney et al., (2009), De Jong and Van Loveren (2007), Dietert and Holsapple (2007), Holsapple et al., (2005). These studies investigate changes in immune response due to effects on the innate or acquired immune system. As immune response may also be affected by the function of other organs such as liver, kidneys and the endocrine system, toxic effects on these organs in offspring may also be reflected in changes in immune response. No single immune parameter is able to reflect the entire complex and intricate function of immune system and so, integration of findings of different tests is relevant to evaluate the relevance of the results on substance exposure.

The selection between the choices should be based on scientific and substance specific considerations taking into account which method adequately addresses the scientific concern with least amount of animals and investigations.

Some examples of aspects of these considerations are presented below. The nature and/or severity of the identified concern may provide guidance to select between a separate study or inclusion of parameters to other studies or a Cohort 3 in an OECD TG 443. Other aspects to consider may include statistical power and the investigations included. It should be considered whether the parameters/Cohort 3 or a separate study best address the particular concern identified. The outcome of a separate developmental immunotoxicity study may differ from that of the developmental immunotoxicity Cohort 3 in an OECD TG 443, if the exposure scenarios and set ups are different.

Important aspects to be considered for study designs are 1) sufficient statistical power, 2) separate analysis for males and females to assess potential sex differences, 3) selection of sensitive parameters, and 4) selection of representative time points for each investigation, and continuous exposure starting from implantation until investigations of immune parameters.

Although it is possible to combine the investigations for developmental immunotoxicity with reproduction toxicity studies, this approach may limit the statistical power (number of animals available; e.g. OECD TG 443) and investigations for sex differences. Furthermore, dose level setting of a study for sexual function and fertility may not be optimal for investigating developmental immunotoxicity.

[28] See also chapter 1.10.2, subheading *(Developmental) immunotoxicity*

As a common recommendation the test battery should include the following investigations:

a) Humoral immunity / antibody formation: T-cell dependent antibody response (TDAR) – PND 45 or older;

b) Cell-mediated (antigen-specific) immune responses: Delayed type hypersensitivity assay (DTH); *AND/OR* Cytotoxic T-lymphocyte (CTL) response; *AND/OR* NK cell assay;

c) Lymphoid organ weights (considered important to characterize effects; to be assessed together with a) and b));

d) Histopathology of immune organs;

e) Supporting information: haematology, cytokine production, flow cytometric immunophenotyping of lymphocyte sub-populations.

Developmental immunotoxicity investigations in an OECD TG 443 with DIT cohort (Cohort 3) investigates less parameters with limited statistical power. The parameters investigated are TDAR in Cohort 3 (10 males and 10 females), and lymphoid organ weights, histopathology, and splenic lymphocyte subpopulation analysis in Cohort 1A (CD4+ and CD8+ T lymphocytes, B lymphocytes, and natural killer cells). Cohort 3 contains 10 males and 10 females from different litters where possible per group and TDAR (IgM) is investigated at PND 56±3. For lymph node, bone marrow and splenic lymphocyte analysis the statistical power is 10 animals/sex /group in Cohort 1A, for other lymphoid organs (thymus, spleen and the adrenal glands) the statistical power is 20 animals/sex/group. Investigation from Cohort 1A are done at postnatal week 13.

Due to limited parameters and statistical power, the results from Cohort 3 in an OECD TG 443 cannot be considered as definitive but rather as screening results which may lead to confirmative investigations. Therefore, where a concern for developmental immunotoxicity is identified, it is recommended to investigate this using a testing battery described above with a sufficient statistical power such as 20 animals/sex/group (representing 20 litters). Due to lack of OECD TG for DIT, a detailed description of the test method used should be given with justifications for the selected investigations and conditions.

Effects considered as adverse will be relevant to hazard classification and the human health risk assessment, providing an N(L)OAEL, unless there is information to show that effects seen in these studies could not occur in humans. Due to a complexity of the endpoint, adversity should preferably be based on a holistic analysis of data by grouping similar parameters.

Note regarding developmental immunotoxicity and assessment of endocrine disruption

Sex differences in effects may indicate hormonal co-influence to the parameter measured.

1.13.5. Further mechanistic studies (ADS)

Table 73. Information requirement 8.13.5 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.13.5 Further mechanistic studies A decision on the need to perform additional studies should be based on all relevant data	

This data may be relevant in the weight of evidence assessment with other information to assess the toxicological properties of a substance, as it can provide information on the mode of action

(MoA) of the chemical. It can also provide information that can be used for refinement in the evaluation of mixtures.

Studies of the mechanisms of toxicity/mode of action may provide useful supplementary information when there are indications that the active substance may have effects on e.g. carcinogenicity (genotoxic and non-genotoxic MoAs are relevant for the classification of carcinogens in accordance with CLP), reproduction, neurotoxicity or immunotoxicity. Such studies may in some cases be used in concluding that the effects observed in experimental animals are not relevant to humans. For ED identification mechanistic studies may be needed (see section 1.13.3). In addition, information on the MoA/mechanisms may clarify the observed sex differences in effects (potential information on endocrine activity), differences between toxicity in different life stages (e.g., sensitivity during development or elderly animals), or an underlying cause (e.g., immunotoxicity) for other effects.

As a general principle, the effects observed in animal studies are considered relevant for humans unless there is sufficient information to prove the contrary. In order to conclude that the adverse effects are not relevant for humans, it is necessary to establish that the adverse effects are caused by a MoA that is not relevant for humans and it must be also possible to exclude other MoAs for the adverse effects seen.

1.14. Studies related to the exposure of humans to the active substance (ADS)

Table 74. Information requirement 8.14 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.14 Studies related to the exposure of humans to the active substance	

Toxicity of degradation products, by-products and reaction products related to human exposure.

Information is required on the toxic effects of substances generated from an active substance, other than mammalian metabolites, in normal use of biocidal product.

The decision as to the need for these data should be made on a case-by-case basis by expert judgment. Where human exposure is significant, toxicity testing may be needed.

These data may be relevant for many product-types for example: product-types 1 and 2 (reaction products with water when the substance is used for human hygiene purposes or reaction products with water or other materials released in water or air when the substance is used for the treatment of bathing waters), product-type 5 (substances produced in a reaction with drinking water), product-types 6, 7, 9 and 10 (residuals in treated materials), product-type 8 (irritating and sensitising effects of chemical compounds, such as metal salts, developed on the surface of the treated wood) and product-type 18 (products, which may produce harmful substances with water during gassing).

1.15. Toxic effects on livestock and pets (ADS)

Table 75. Information requirement 8.15 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.15 Toxic effects on livestock and pets	

For livestock and pets, an estimation of toxic effects and exposure via different exposure routes (e.g. inhalation, licking, skin contact and ingestion of poisoned bait) is required. In exceptional cases, toxicity testing in livestock and pets may be required. Toxic effects for livestock and pets should be estimated or studied if the substance is to be used in spaces in which animals are housed, kept or transported or exposure is possible via drinking water or feeding stuffs. Information on lethal doses for different species, symptoms of poisoning, details of the time courses in case of poisoning and antidotes should also be submitted, if available.

These data may be relevant e.g. for the following product-types:

- 3 (substances used for veterinary hygiene purposes)
- 4 (disinfection of surfaces and equipment)
- 5 (drinking water)
- 8, 10 (treated materials in areas in which animals are housed, kept or transported)
- 14, 15, 23 (ingestion of baits)
- 16, 17 (contaminated drinking water)
- 18, 19 (repellents to be used for veterinary hygiene purposes, residential indoor use).

1.16. Food and feeding stuffs studies including for food producing animals and their products (milk, eggs and honey) (ADS)

Table 76. Information requirement 8.16 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.16 Food and feeding stuffs studies including for food-producing animals and their products (milk, eggs and honey) Additional information related to the exposure of humans to the active substance contained in biocidal products	

Evaluation of residues in food and feed from biocidal uses requires information on the nature of residues as well as quantification of residues, which is covered by data requirements listed under this endpoint in Annex II of the BPR (and the endpoint 8.10 in Annex III of the BPR). Normally standard residue study with radiolabeled compounds or other study providing equivalent information, designed to reflect the realistic use conditions of the biocidal product, would be necessary to identify residue composition. Chapter 5 of the ECHA Guidance VoL III Parts B+C and Guideline on risk characterization and assessment of maximum residue limits (MRL) for biocides (EMA/CVMP/SWP/90250/2010), provides indications on the guidelines that would support the identification of the residue composition. The guidance recommends applying OECD TG 507, "Nature of the Pesticide Residues in Processed Commodities – High Temperature Hydrolysis".

Dietary Risk Assessment (DRA) follows a stepwise approach with each step leading to a more realistic estimate of residue amounts in foods. Lower-level steps generally involve calculation models populated with default values in the first tier, with the possibility of including additional data in higher tiers. With few exceptions, data from product- and use-specific residue studies with foods are only necessary if lower tiers fail to exclude a consumer risk. In addition, Maximum

Residue Limits (MRLs) must be set according to the criteria outlined in the Commission Note[29].

The basic use categories for DRA are "animal husbandry", "biocide-food contact (professional use)" and "biocide-food contact (non-professional use)". Depending on the use category, different calculation models and residue study designs apply. While some required information, e.g. metabolism in livestock and degradation during food processing is related to the active substance itself, other data are connected to the intended use of the respective biocidal product (e.g. supervised residue trials). The former can be submitted at the stage of the evaluation for active substance approval, while the latter must be generated at the product authorisation stage.

Guidance to address the non-professional uses (Chapter 5) and animal husbandry (Chapter 6) are included in the ECHA Guidance VoL III Parts B+C. Guidance on professional use is under development and will be included as an additional chapter in the same guidance. These guidance documents provide the methodology to estimate the transfer of biocidal active residues into food and indications on the studies to be performed to allow the identification of the residues and to support refinement options (for example, studies to allow the quantification of transfer factor or to estimate the rinsing efficiency). Apart from the above, there is currently no guidance for dietary risk assessment specifically for biocides. Methodologies developed by other Agencies may be used to perform dietary risk assessment. In addition, guidance documents developed by other Agencies, e.g. on metabolism in livestock and degradation during food processing, may be used to support the assessment for biocides.

1.16.1. Proposed acceptable residue levels i.e. maximum residue limits (MRL) and the justification of their acceptability (ADS)

Table 77. Information requirement 8.16.1 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.16.1 Proposed acceptable residue levels i.e. maximum residue limits (MRL) and the justification of their acceptability	

For product-type 5, any relevant legislation relating to acceptable or unacceptable residues in drinking water must be taken into consideration in the justification, including Directive 2020/2184/EC on the quality of water intended for human consumption.

For product-type 21, any directions or restrictions at the Community or national level related to residues in fish and shellfish intended to be used as food or feeding stuffs must be taken into consideration in the justification.

Please refer also to the Commission Note[24] above.

1.16.2. Behaviour of the residue of the active substance, its degradation products and, where relevant, its metabolites on the treated or contaminated food or feeding stuffs including the kinetics of disappearance (ADS)

Table 78. Information requirement 8.16.2 according to BPR Annex II:

[29] CA-March17-Doc.7.6.c-Final: *An interim approach for the establishment of maximum residue limits for residues of active substances contained in biocidal products for food and feed and specific migration limits in food contact materials*. See link under "related links" in https://echa.europa.eu/guidance-documents/guidance-on-biocides-legislation.
Direct link:
https://ec.europa.eu/health/sites/health/files/biocides/docs/2017_interimapproach_maximumresiduelimits_en.pdf.

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.16.2 Behaviour of the residue of the active substance on the treated or contaminated food or feeding stuffs including the kinetics of disappearance Residue definitions should be provided where relevant. It is also important to compare residues found in toxicity studies with residues formed in food-producing animals and their products, as well as food and feed	

Residue definitions should be provided where relevant. It is also important to compare residues found in toxicity studies with residues formed in food-producing animals, their product as well as food and feed.

Residue definition should be provided when indirect exposure via food cannot be excluded.

1.16.3. Overall material balance for the active substance (ADS)

Table 79. Information requirement 8.16.3 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.16.3 Overall material balance for the active substance Sufficient residue data from supervised trials on food-producing animals and their products, as well as food and feed, to demonstrate that residues likely to arise from the proposed use would not be of concern for human or animal health	

Point 8.16.3 of Annex II to the BPR states that *sufficient residue data from supervised trials on food producing species and their products as well as food and feed to demonstrate that residues likely to arise from the proposed use would not be of concern for human or animal health.*

1.16.4. Estimation of potential or actual exposure of the active substance to humans through diet and other means (ADS)

Table 80. Information requirement 8.16.4 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.16.4 Estimation of potential or actual exposure of humans to the active substance and residues through diet and other means	

Expected consumer exposure via diet should be studied taking into account the average consumption of different food types and drinking water.

1.16.5. If residues of the active substance remain on feeding stuffs for a significant period of time or also residues found in food of animal origin after treatment on or around food producing animals (ADS)

Table 81. Information requirement 8.16.5 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.16.5 If residues of the active substance occur in or on feeding stuffs for a significant period of time or are found in food of animal origin after treatment on or around food-producing animals (e.g. direct treatment on animals or indirect treatment of animal houses or surroundings) then feeding and metabolism studies in livestock shall be required to permit evaluation of residues in food of animal origin	

Point 8.16.5 of Annex II to the BPR states that [….] *(e.g. direct treatment on animals or indirect treatment of animal houses or surroundings) then feeding and metabolism studies in livestock shall be required to permit evaluation of residues in food of animal origin.*

1.16.6. Effects of industrial processing and/or domestic preparation on the nature and magnitude of residues of the active substance

Table 82. Information requirement 8.16.6 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.16.6 Effects of industrial processing and/or domestic preparation on the nature and magnitude of residues of the active substance	

Provide information as implied by the title.

1.16.7. Any other available information that is relevant (ADS)

Table 83. Information requirement 8.16.7 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.16.7 Any other available information that is relevant It may be appropriate to include information on migration into food, especially in the case of treatment of food contact materials	

Point 8.16.3 of Annex II to the BPR states that *it may be appropriate to include information on migration into food, especially in the case of treatment of food contact materials.*

For instance information from other chemical programmes on ADI, MRL or relevant residues.

1.16.8. Summary and evaluation of data submitted under 8.16.1. to 8.16.7. (ADS)

Table 84. Information requirement 8.16.8 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.16.8 Summary and evaluation of data submitted under 8.16.1 to 8.16.8	

It is important to establish whether the metabolites found in food (from animals or plants) are the same as those tested in toxicity studies.

Otherwise values for risk assessment (e.g., ADI) are not valid for the residues found

Please follow the guidance in section 1.16 of this guidance.

1.17. Tests to assess toxic effects of metabolites from treated plants (ADS)

Table 85. Information requirement 8.17 according to BPR Annex II:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.17 If the active substance is to be used in products for action against plants including algae then tests shall be required to assess toxic effects of metabolites from treated plants, if any, where different from those identified in animals	

The need to provide this information should be assessed on a case-by-case basis, considering all available information on plant metabolites and their toxicity. Where testing is needed, the testing strategy should be agreed between the applicant and the eCA.

2. Dossier Requirements for Biocidal Products BPR Annex III, Title 1, 8 Toxicological Profile for humans and animals

Toxicological profile for humans and animals

This section describes the information requirements for biocidal products for the assessment of the toxicological profile for humans and animals.

Where new testing is needed, please see also the general information under *Considerations before initiating testing* in chapter 1.

According to BPR Article 62(1), testing on vertebrates shall be undertaken only as a last resort, and testing on vertebrates shall not be repeated for the purposes of BPR. In considering the need to perform testing, it is necessary to first consider if sufficient and reliable information is already available on each of the components of the mixture for classification and labelling according to CLP. For this purpose, according to BPR Article 70, the Safety Data Sheets (SDS) of all components need to be provided and must comply with the requirements as laid down in the REACH Regulation. In this context, the calculation method and bridging principles need to be considered. Furthermore, the need for testing has to be considered in light of any synergistic effects expected.

2.1. Skin corrosion or irritation

Table 86. Information requirement 8.1 according to BPR Annex III:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.1 Skin corrosion or irritation The assessment shall comprise the following tiers: (a) assessment of the available human, animal and non-animal data; (b) skin corrosion, *in vitro* testing; (c) skin irritation, *in vitro* testing; (d) skin corrosion or irritation, *in vivo* testing	Testing of the product or mixture does not need to be conducted if: — there are sufficient valid data on each component of the product or mixture to allow its classification in accordance with Regulation (EC) No 1272/2008, and synergistic effects between any of the components are not expected, — the product or mixture is a strong acid (pH≤ 2,0) or base (pH≥ 11,5), —the product or mixture is spontaneously flammable in air or in contact with water or moisture at room temperature, — the product or mixture meets the classification criteria for acute toxicity category 1 by the dermal route, or —an acute toxicity study by the dermal route provides conclusive evidence on skin corrosion or irritation adequate for classification. If results from one of the two studies listed in points (b) or (c) in column 1 of this row already allow conclusive decision on the classification of product or mixture or on the absence of skin irritation potential, the second study does not need to be conducted An *in vivo* study for skin corrosion or irritation shall be considered only if the *in vitro* studies listed in points (b) and (c) in column 1 of this row are not applicable, or the results of these studies are not adequate for classification and risk assessment and the calculation method or bridging principles laid down in Regulation (EC) No 1272/2008 are not applicable *In vivo* studies for skin corrosion or irritation that were carried out or initiated before 15 April 2022 shall be considered appropriate to address this information requirement

Please follow section 1.1 of this guidance. Note also the principles outlined in the first chapter in Section 2.

2.2. Serious eye damage or eye irritation

Table 87. Information requirement 8.2 according to BPR Annex III:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.2 Serious eye damage or eye irritation The assessment shall comprise the following tiers: (a) assessment of the available human, animal and non-animal data; (b) serious eye damage or eye irritation, *in vitro* testing; (c) serious eye damage or eye irritation, *in vivo* testing	Testing on the product or mixture does not need to be conducted if: —there are sufficient valid data available on each component of the product or mixture to allow its classification in accordance with Regulation (EC) No 1272/2008, and synergistic effects between any of the components are not expected, — the product or mixture is a strong acid (pH≤ 2,0) or base (pH≥ 11,5), —the product or mixture is spontaneously flammable in air or in contact with water or moisture at room temperature, or — the product or mixture meets the classification criteria for skin corrosion leading to its classification as "serious eye damage" category 1 If results from a first *in vitro* study do not allow a conclusive decision on the classification of the product or mixture or on the absence of eye irritation potential (an)other(s) *in vitro* study(ies) for this endpoint shall be considered An *in vivo* study for serious eye damage or eye irritation shall be considered only if the *in vitro* study(ies) under point (b) in column 1 of this row are not applicable, or the results obtained from these studies are not adequate for classification and risk assessment and the calculation method or bridging principles laid down in Regulation (EC) No 1272/2008 are not applicable *In vivo* studies for serious eye damage or eye irritation that were carried out or initiated before 15 April 2022 shall be considered appropriate to address this information requirement

Please follow section 1.2 of this guidance. Note also the principles outlined in the first chapter in Section 2.

2.3. Skin sensitisation

Table 88. Information requirement 8.3 according to BPR Annex III:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.3 Skin sensitisation The information shall allow to conclude whether the substance is a skin sensitiser and whether it can be presumed to have the potential to produce significant sensitisation in humans (Category 1A). The information should be sufficient to perform a risk assessment where required The assessment shall comprise the following tiers:	Testing on the product or mixture does not need to be conducted if: —there are sufficient valid data available on each component of the product or mixture to allow its classification in accordance with Regulation (EC) No 1272/2008, and synergistic effects between any of the components are not expected, —the available information indicates that the product or mixture should be classified for skin sensitisation or skin corrosion, — the product or mixture is a strong acid (pH≤ 2,0) or base

(a) assessment of the available human, animal and non-animal data; (b)skin sensitisation, *in vitro* testing. Information from *in vitro* or *in chemico* test method(s) conducted in accordance with point 5 of the introductory part of this Annex and addressing each of the following key events of skin sensitisation: (i) molecular interaction with skin proteins; (ii) inflammatory response in keratinocytes; (iii) activation of dendritic cells. (c) skin sensitisation *in vivo* testing. The Murine Local Lymph Node Assay (LLNA) is the first-choice method for *in vivo* testing. Another skin sensitisation test may only be used in exceptional circumstances. If another skin sensitisation test is used, scientific justification shall be provided.	(pH≥ 11,5), or —the product or mixture is spontaneously flammable in air or in contact with water or moisture at room temperature. *In vitro* tests do not need to be conducted if: — an *in vivo* study referred to in point (c) in column 1 of this row is available, or — the available *in vitro or in chemico* test methods are not applicable for the product or mixture or the results obtained from these studies are not adequate for classification and risk assessment. If information from test method(s) addressing one or two of the key events described in point (b) in column 1 of this row already allows for classification of the substance and risk assessment, studies addressing the other key event(s) do not need to be conducted An *in vivo* study for skin sensitisation shall be considered only if *in vitro* or *in chemico* studies referred to in point (b) in column 1 of this row are not applicable, or the results obtained from these studies are not adequate for classification and risk assessment and the calculation method or bridging principles laid down in Regulation (EC) No 1272/2008 are not applicable *In vivo* studies for skin sensitisation that were carried out or initiated before 15 April 2022 shall be considered appropriate to address this information requirement

Please follow section 1.3 of this guidance. Note also the principles outlined in the first chapter in Section 2.

Any limitation of the additivity method specified in the Guidance on the Application of the CLP Criteria (ECHA) for sensitisation with regard to addressing sub-corrosive concentrations with sensitising potential should also be considered (see also section 1.3 of this guidance).

2.4. Respiratory sensitisation and irritation

2.4.1. Respiratory sensitisation (ADS)

Table 89. Information requirement 8.4 according to BPR Annex III:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.4 Respiratory sensitisation	Testing on the product/mixture does not need to be conducted if: — there are valid data available on each of the components in the mixture to allow classification of the mixture according to the rules laid down in Directive 1999/45/EC and Regulation (EC) No 1272/2008 (CLP), and synergistic effects between any of the components are not expected

Please follow section 1.4.1 of this guidance. Note also the principles outlined in the first chapter in Section 2.

2.4.2. Respiratory irritation (not in BPR Annex III)

Please follow section 1.4.2 of this guidance. Note also the principles outlined in the first chapter in Section 2.

2.5. Acute toxicity

Table 90. Information requirement 8.5 according to BPR Annex III:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.5 Acute toxicity	Testing on the product/mixture does not need to be conducted if:
— Classification using the tiered approach to classification of mixtures for acute toxicity in Regulation (EC) No 1272/2008 is the default approach	— there are valid data available on each of the components in the mixture to allow classification of the mixture according to the rules laid down in Directive 1999/45/EC and Regulation (EC) No 1272/2008 (CLP), and synergistic effects between any of the components are not expected

Please follow section 1.7 of this guidance. Note also the principles outlined in the first chapter in Section 2.

2.5.1. By oral route

Table 91. Information requirement 8.5.1 according to BPR Annex III:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.5.1 By oral route	

Please follow section 1.7.1 of this guidance. Note also the principles outlined in the first chapter in Section 2.

2.5.2. By inhalation

Table 92. Information requirement 8.5.2 according to BPR Annex III:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.5.2 By inhalation	

Please follow section 1.7.2 of this guidance. Note also the principles outlined in the first chapter in Section 2.

2.5.3. By dermal route

Table 93. Information requirement 8.5.3 according to BPR Annex III:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.5.3 By dermal route	

Please follow section 1.7.3 of this guidance. Note also the principles outlined in the first chapter in Section 2.

2.5.4. Biocidal products that are intended to be authorised for use with other biocidal products

Table 94. Information requirement 8.5.4 according to BPR Annex III:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.5.4 For biocidal products that are intended to be authorised for use with other biocidal products, the risks to human health, animal health and the environment arising from the use of these product combinations shall be assessed. As an alternative to acute toxicity studies, calculations can be used. In some cases, for example where there are no valid data available of the kind set out in column 3, this may require a limited number of acute toxicity studies to be carried out using combinations of the products	

Please note the principles outlined in the first chapter in Section 2.

2.6. Information on dermal absorption

Table 95. Information requirement 8.6 according to BPR Annex III:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.6 Information on dermal absorption Information on dermal absorption when exposure occurs to the biocidal product. The assessment of this endpoint shall proceed using a tiered approach	

It is not always mandatory to submit experimental data. If such data are not available, as a first step default values can be used according to the EFSA Guidance Document on Dermal Absorption (EFSA, 2017). The OECD Guidance Document on Percutaneous absorption/penetration (OECD, 2004a) and the EFSA Guidance on dermal absorption (EFSA, 2017) should be followed where applicable for the estimation of dermal absorption both for the biocidal product and the active substance (section 1.8 of this guidance). Note also the principles outlined in the first chapter in Section 2.

The following Test Guidelines are available for the conduct of skin absorption studies:

- EC method B.45 Skin Absorption: *In Vitro* Method.
- OECD TG 428: Skin Absorption: *In Vitro* Method.
- EC method B.44 Skin Absorption: *In Vivo* Method.
- OECD TG 427: Skin Absorption: *In Vivo* Method.

If testing to assess the likely magnitude and rate of dermal bioavailability is necessary, the OECD TG 428 for *in vitro* skin absorption should be considered first.

Before new studies are commenced, it should be checked whether the intended use is safe when the appropriate default value is applied. If no experimental data are available, studies with

similar formulations should be looked for. If valid studies have been performed with the same formulation for which authorisation is to be granted, these results should be used with a preference for an *in vitro* study on human skin.

Dermal absorption can be measured *in vitro* and/or *in vivo*. If valid studies with the relevant formulation are available, their results should be directly used for risk assessment. However, any deviations from OECD TG 427 and OECD TG 428 require justification, including an assessment of the impact of the deviation. Acceptable studies should be in full compliance with OECD TGs 427 (*in vivo*) or 428 (*in vitro*) or at least similar to them in all main aspects, based on expert judgement. The applicant should ensure that all relevant information is provided in the study report, e.g. regarding the use of tape stripping. It must be acknowledged that both guidelines leave a certain degree of freedom to modify the study design.

When valid (guideline-compliant and GLP) *in vitro* studies on human skin, *in vitro* studies in animals and *in vivo* animal studies are available and conducted under the same experimental conditions, and the results meet the quality criteria, in particular with respect to variability, number of acceptable replicates and recovery, then the 'Triple Pack' approach can be used to extrapolate the human dermal absorption values for risk assessment (OECD No. 156, draft). *In vitro* studies on human skin are however considered sufficiently predictive and conservative and should be normally used for the risk assessment – a complete "triple pack" including testing in living animals is not required but available triple pack data may be used to refine the assessment. *In vivo* studies on rats or *in vitro* studies on rat skin as "stand alone" information may also be used, acknowledging however that this will result in clear overestimation of dermal absorption in humans in the vast majority of cases.

Percutaneous absorption depends on the partitioning of substances from the vehicle and solubility in the vehicle. OECD TG 427 and TG 428 recommend conducting tests using test preparations that are the same as (or a realistic surrogate to) those that humans may be exposed to.

Other types of studies (e.g. in human volunteers) could be taken into consideration in exceptional cases but in general their use is not recommended.

In some cases, it may also be possible to estimate dermal absorption on the basis of existing information that comes from other sources. Mostly, this will be extrapolation of experimental data obtained with a similar formulation, but in this case strict and transparent rules should be followed as to when another formulation or product can be considered similar. Expert judgment will always be needed in these cases. A detailed justification and expert judgment is necessary if less frequently used approaches are used, such as the application of QSARs or a comparison of the results obtained in oral and dermal toxicity studies.

Dermal absorption can vary depending on the formulation, as well as due to other products that are present on the skin. This is most relevant for biocidal products that are applied on the skin. If any information of such interactions is available, this should be included in the assessment. This would normally be considered in the need of risk management measures to avoid increased systemic exposure due to other products that enhance dermal absorption.

2.7. Available toxicological data relating to non-active substances (i.e. substances of concern) and a mixture that a substance of concern is a component of

Table 96. Information requirement 8.7 according to BPR Annex III:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.7 Available toxicological data relating to: (a) non-active substance(s) (i.e. substance(s) of concern); and (b)a mixture that a substance(s) of concern is a component of Tests listed in Section 8 of the table in Title 1 of Annex II shall be carried out for the substance(s) of concern or a mixture that a substance(s) of concern is a component of if insufficient data are available and cannot be inferred through read-across, *in silico* or other accepted non-testing approaches	Testing on the product or mixture does not need to be conducted if all of the following conditions are met: — there are valid data available on each of the components in the mixture to allow classification of the mixture in accordance with the rules laid down in Regulation (EC) No 1272/2008, — a conclusion can be made whether the biocidal product can be considered as having endocrine disrupting properties, — synergistic effects between any of the components are not expected

Please note the principles outlined in the first chapter in Section 2.

2.8. Food and feedingstuffs studies (ADS)

Table 97. Information requirement 8.8 according to BPR Annex III:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.8 Food and feedingstuffs studies	

Please note the principles outlined in the first chapter in Section 2.

2.8.1. Feeding and metabolism studies in livestock (ADS)

Table 98. Information requirement 8.8.1 according to BPR Annex III:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.8.1 If residues of the biocidal product remain in or on feedingstuffs for a significant period of time, then feeding and metabolism studies in livestock shall be required to permit evaluation of residues in food of animal origin	

Please follow section 1.16 of this guidance. Note also the principles outlined in the first chapter in Section 2.

2.8.2. Residues in food (not in BPR Annex III)

If intended use of the biocidal product may lead to transfer of residues into foods, studies on the

nature of residues and studies on residue levels may be required.

Please follow section 1.16 of this guidance. Note also the principles outlined in the first chapter in Section 2.

2.9. Effects of industrial processing and/or domestic preparation on the nature and magnitude of residues of the biocidal product (ADS)

Table 99. Information requirement 8.9 according to BPR Annex III:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.9 Effects of industrial processing and/or domestic preparation on the nature and magnitude of residues of the biocidal product	

The objective of these studies is to establish whether breakdown or reaction products arise from residues in the raw products during processing which may require a separate risk assessment.

Depending on the level and chemical nature of the residue in the raw commodity, a set of representative hydrolysis situations (simulating the relevant processing operations) should be investigated, where appropriate. The effects of process other than hydrolysis may also have to be investigated, where the properties of the active substance or metabolites indicate that toxicologically significant degradation products may occur as a result of these processes. The studies are normally conducted with a radio-labelled form of the active substance.

Please follow section 1.16 of this guidance. Note also the principles outlined in the first chapter in Section 2.

2.10. Other test(s) related to the exposure to humans (ADS)

Table 100. Information requirement 8.10 according to BPR Annex III:

INFORMATION REQUIRED	SPECIFIC RULES FOR ADAPTATION FROM STANDARD INFORMATION
8.10 Other test(s) related to the exposure to humans Suitable test(s) and a reasoned case will be required for the biocidal product In addition, for certain biocides which are applied directly or around livestock (including horses) residue studies might be needed	

Please follow sections 1.14 and 1.16 of this guidance. Note also the principles outlined in the first chapter in Section 2.

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