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In silico toxicology protocols

Article

In silico toxicology protocols☆

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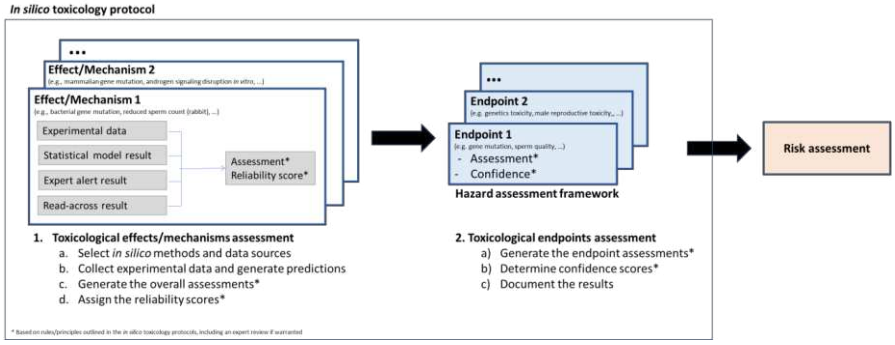
Abstract

The present publication surveys several applications of *in silico* (i.e., computational) toxicology approaches across different industries and institutions. It highlights the need to develop standardized protocols when conducting toxicity-related predictions. This contribution articulates the information needed for protocols to support *in silico* predictions for major toxicological endpoints of concern (e.g., genetic toxicity, carcinogenicity, acute toxicity, reproductive toxicity, developmental toxicity) across several industries and regulatory bodies. Such novel *in silico* toxicology (IST) protocols, when fully developed and implemented, will ensure *in silico* toxicological assessments are performed and evaluated in a consistent, reproducible, and well-documented manner across industries and regulatory bodies to support wider uptake and acceptance of the approaches. The development of IST protocols is an initiative developed through a collaboration among an international consortium to reflect the state-of-the-art in *in silico* toxicology for hazard identification and characterization. A general outline for describing the development of such protocols is included and it is based on *in silico* predictions and/or available experimental data for a defined series of relevant toxicological effects or mechanisms. The publication presents a novel approach for determining the reliability of *in silico* predictions alongside experimental data. In addition, we discuss how to determine the level of confidence in the assessment based on the relevance and reliability of the information.

152 **Graphical abstract**



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Keywords: *In silico*, *in silico* toxicology, computational toxicology, predictive toxicology, QSAR, expert alert, expert review.

Highlights

- General outline of *in silico* toxicology protocols is described
- A reliability score for predictions alongside experimental data is discussed
- A checklist for performing an expert review of the *in silico* results is outlined
- A hazard assessment framework is proposed that includes *in silico* results

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1. Introduction

In silico toxicology (IST) methods are computational approaches that analyze, simulate, visualize, or predict the toxicity of chemicals. IST encompasses all methodologies for analyzing chemical and biological properties generally based upon a chemical structure that represents either an actual or a proposed (i.e., virtual) chemical. Today, *in silico* approaches are often used in combination with other toxicity tests; however, the approaches are starting to be used to generate toxicity assessments information with less need to perform any *in vitro* or *in vivo* studies depending on the decision context. IST uses models which can be encoded within software tools to predict the potential toxicity of a chemical and in some situations to quantitatively predict the toxic dose or potency. These models are based on experimental data, structure-activity relationships, and scientific knowledge (such as structural alerts reported in the literature).

There are a number of different situations where *in silico* methods serve an important role in the hazard assessment of existing chemicals or new substances under development that would benefit from the development of *in silico* toxicology protocols. These include:

- emergency situations where rapid understanding of potential toxicological consequences from exposure is needed in the absence of existing toxicological testing data;
- cases where there is only a limited supply of a test material available;
- scenarios where there are challenges to conduct laboratory studies;
- instances where synthesis of a complex test material is not feasible; and
- situations where a less time-consuming and less expensive high-throughput approach than an experimental test is needed.

189 IST methods are one approach to generating additional information for complementing and ultimately
190 enhancing the reliability or supporting a risk assessment, including an understanding of the structural
191 and/or mechanistic basis that may contribute ideas for the rational design of new chemicals,
192 development of a testing strategy or an overall weight-of-evidence evaluation. IST inherently supports
193 the principle of the 3Rs (replacement, refinement and reduction) relating to the use of animals in
194 research (Russell and Burch, 1959; Ford 2016). Table 1 outlines fifteen specific uses of IST to illustrate
195 the diversity of applications that currently can benefit from *in silico* methods. Stanton and Kruszewski
196 (2016) recently quantified the benefits of using *in silico* and read-across methods where they
197 determined that the approach used across two voluntary high-production-volume (HPV) chemical
198 programs for 261 chemicals obviated the use of 100,000 – 150,000 test animals and saved 50,000,000
199 US$ to 70,000,000 US$.

200 The increased interest and acceptance of *in silico* methods for regulatory data submission and chemicals
201 evaluation is driving the adoption of its use for regulatory purposes. Several guidance documents have
202 been drafted to improve standardization, harmonization, and uptake of *in silico* methods by regulatory
203 authorities including the International Council for Harmonization (ICH) M7 guideline (assessment and
204 control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk)
205 (ICH M7, 2017(R1)), the European Union's Registration, Evaluation, Authorization, and restriction of
206 Chemicals (REACH) regulation (EU 2006; ECHA 2008; ECHA 2015), European Food Safety Authority
207 (EFSA) residue guidance (EFSA 2016), Canada's chemicals management plan assessments for new and
208 existing substances under the Canadian Environmental Protection Act (CEPA) (Canada 2016), and the
209 Toxic Substances Control Act (TSCA) (TSCA 2016). A number of national and international initiatives have
210 focused on developing specific documents supporting the use of *in silico* tools. The OECD has published
211 a series of (Quantitative) Structure-Activity Relationship (Q)SAR validation principles that are discussed
212 in detail in Section 2.3.2. (OECD 2004, OECD 2007) Other initiatives include the North American Free

Trade Agreement pesticides Quantitative Structure-Activity Relationship (QSAR) guidance (NAFTA 2012), considerations on the use of *in silico* approaches for assessing cosmetics ingredients (Amaral et al., 2014), European Food Safety Agency report (EFSA 2014), European Chemicals Agency REACH supporting documentation (ECHA 2008; ECHA 2016, 2017), Organization for Economic Co-operation and Development (OECD) documentation (OECD 2007; OECD 2014; OECD 2015), and the ICH M7 guideline for prediction of mutagenicity (ICH M7, 2017(R1)), along with complementary peer reviewed publications outlining the process for implementation of such computational assessments (e.g., Amberg et al., 2016; Barber et al., 2015; Powley et al., 2015; Schilter et al., 2014). Certain projects have provided substantial guidance on the documentation of the models and prediction results (JRC 2014; Patlewicz et al., 2016) as well as principles and workflows to support safety assessments (Bassan and Worth, 2008; ECHA 2015; Worth et al., 2014; Berggren et al., 2017; Amaral et al., 2017).

These prior initiatives provide a robust foundation for the current project to establish the IST protocols described here; however, several issues have hindered the general acceptance and use of *in silico* methods on a larger scale. In particular, there remains a lack of generally accepted procedures for performing *in silico* assessments for the toxicological endpoints. The lack of such procedures or protocols has led to inconsistency in the application and use of *in silico* tools across different organizations, industries, and regulatory agencies (e.g., searching databases, applying predictive models and alerts, performing an expert review/assessment, documenting and communicating the results and associated uncertainties). The use of traditional experimental evidence coupled with *in silico* information to support hazard identification and risk assessment also varies both across, and often within, organizations. Although not always, such *ad hoc* approaches may be time-consuming and the results poorly accepted. Standardization of protocols will enhance the acceptability of the methods and their results by end users. Additionally, there are misconceptions about when *in silico* predictions are appropriate to use as well as a lack of defined consensus processes for interpreting the result(s) of such

predictions (Bower et al., 2017; SCCS 2016). Some scientists view *in silico* methods as a "black box" that inhibits their ability to critically assess the predictions and their reliability. (Alves et al., 2016) Others lack expertise to interpret the results of *in silico* predictions, and some have an unrealistic expectation that an *in silico* prediction can always provide an unerring definitive assessment.

Standardization of *in silico* tool use and interpretation of results would greatly reduce the burden on both industry and regulators to provide confidence in or justification for the use of these approaches. The objective of developing IST protocols is to define *in silico* assessment principles so the results can be generated, recorded, communicated, archived and then evaluated in a uniform, consistent and reproducible manner. Incorporating these principles routinely into the use of *in silico* methods will support a more transparent analysis of the results and serves to mitigate "black box" concerns[1]. This approach is similar to guideline studies that provide a framework for the proper conduct of toxicological studies and assurance in the validity of the results (such as OECD Guidelines for the Testing of Chemicals) (OECD 2017). The development of these protocols is driven by consensus amongst leading scientists representing industry, private sector and governmental agencies. Consequently, this project provides an important step towards a quality-driven science for IST or good *in silico* practice .

Herein, we provide a framework to develop a series of procedures for performing an *in silico* assessment to foster greater acceptance. These IST protocols are being created for a number of toxicological endpoints (e.g., genetic toxicity, carcinogenicity, acute toxicity, reproductive toxicity, developmental toxicity) as well as other related properties (e.g., biodegradation and bioaccumulation) that could impact the chemical hazard classification. Throughout this publication, these toxicological and related

[1]It should be noted that black box models may be acceptable in certain situations, such as compound filtering and virtual screening, as long as they show acceptable performance in validation studies; however, for most applications the acceptance of this class of models is low.

endpoints are referred to as “major endpoints” and the protocols are referred to as IST protocols. These protocols will support the assessment of hazards and in some cases the prediction of quantitative values, such as a No Observed Adverse Effect Levels (NOAELs); however, these protocols do not define how a risk assessment will be performed. This publication outlines the components of an IST protocol, including schematics to describe how a prediction could be performed, approaches to assess the reliability and confidence of the results, and items that may be considered as part of an expert review. This publication also outlines the process for creating the IST protocols through an international consortium comprising representatives across regulatory agencies, government research agencies, different industrial sectors, academia and other stakeholders. Specific endpoint-dependent considerations will be described in future separate publications and IST protocols (developed as a result of this process) will also be published for widespread use and for incorporation into different technology platforms.

2. *In silico* toxicology protocols

2.1 Overview

Each IST protocol describes the prediction process in a consistent, transparent, and well-documented manner. This includes recommendations on how to:

1) plan the *in silico* analyses including identifying what toxicological effects or mechanisms to predict (Section 2.2), what *in silico* methodologies to use (Section 2.3.1), and other selection criteria for the *in silico* methods (Section 2.3.2),

2) conduct the appropriate individual software predictions (Section 2.3.3) and further database searches (Section 2.5),

3) perform and document the *in silico* analysis (Sections 2.6 and 2.7) including expert review (Section 2.4), and

4) report and share the information and assessment results, including information about uncertainties (Section 2.9).

Section 2.8 provides a template for the individual IST protocols for major toxicological endpoints. IST protocols could be applicable for use with several *in silico* programs, including different *in silico* models and databases.

2.2 Toxicological effects and mechanisms

In an experimental approach, hazard is evaluated based on specific observations (toxicological effects) during toxicity studies. Often, toxicity of a chemical involves a biological event: a non-specific or specific interaction with a vital biological structure, which causes sequential perturbation of a physiological pathway at a cellular, tissue, organ and/or system level, leading to a toxicological effect observed at the organism level. Experiments evaluating the potential of a chemical to cause such a biological event (e.g., *in vitro* analysis of specific interaction with a cellular receptor or inhibition of an enzyme or non-specific cytotoxicity), may support hazard assessment and provide information about the mechanism of toxicity. Such an approach is utilized in the Adverse Outcome Pathway (AOP), where identification of a molecular initiating event supports assessment of the related adverse outcome at the organism level (Bell et al., 2016; OECD 2016a; OECD 2016b). A computational approach to hazard assessment may address the two complementary levels of hazard identification in a similar way (i.e., predicting the resulting manifestation (effect) or the molecular perturbation (mechanism) that led to the toxicological effect).

Each IST protocol defines a series of known toxicological effects and mechanisms relevant to the assessment of the major toxicological endpoint. For example, in the reproductive toxicity IST protocol,

the list of toxicological effects/mechanisms may include reduced sperm count, androgen signaling disruption *in vitro*, and so on. Within each IST protocol, these effects/mechanisms may be species and/or route of administration specific.

Figure 1 outlines a general approach to performing an *in silico* assessment. For each toxicological effect/mechanism, relevant information (as defined in the IST protocol) is collected, including any available experimental data as well as *in silico* predictions. The experimental data and/or *in silico* results are then analyzed and an overall assessment of the toxicological effect or mechanism is generated alongside a reliability score (defined in Section 2.6.2) that reflects the quality of the results. The assessment results and reliability scores for a range of relevant toxicological effects/mechanisms are then used to support a hazard assessment within the hazard assessment framework.

2.3 *In silico* predictions

2.3.1 *In silico* methodologies

Several organizations develop and make available computer software packages for predicting toxicity or physicochemical properties of query chemical(s). These systems generally contain one or more models, where each model predicts the compound's putative toxicological effect or mechanism of action. For example, a model may predict the results for bacterial gene mutation using data generated from the bacterial reverse mutation test or Ames test. These models may be revised over time as more data become available, structure-activity relationships are better characterized, and any data set used is updated. Each new or updated model is given a different version number because the results from different model versions may vary and it is important to track the source of the results. (Amberg et al., 2016)

All IST protocols will identify the toxicological effects or mechanisms to be predicted as discussed in Section 2.2. These predictions may be dichotomous (e.g., predict mutagenic or non-mutagenic compounds), quantal (e.g., Globally Harmonized System [GHS] Classification and Labeling[2] scheme) or quantitative/continuous (e.g., prediction of median toxic dose [TD_{50}] values). The specific IST protocols will detail the type of prediction(s) ideally generated.

The major *in silico* prediction methodologies include the following:

- **Statistical-based (or QSAR).** This methodology uses a mathematical model that was derived from a training set of example chemicals. The training set includes the chemicals that were found to be positive and negative in a given toxicological study (e.g., the bacterial reverse mutation assay) or to induce a continuous response (e.g., NOAEL in teratogenicity) that the model will predict. As part of the process to generate the model, physicochemical property-based descriptors (e.g., molecular weight, octanol water partition coefficient [log P]), electronic and topological descriptors (e.g., quantum mechanics calculations), or chemical structure-based descriptors (e.g., the presence or absence of different functional groups) are generated and used to describe the training set compounds. The model encodes the relationship between these descriptors and the (toxicological) response. After the model is built and validated (OECD 2007; Myatt et al., 2016), it can be used to make a prediction. The (physico)chemical descriptors incorporated into the model are then generated for the test compound and are used by the model to generate a prediction. This prediction is only accepted when the test compound is sufficiently similar to the training set compounds (i.e., it is considered within the applicability domain of the QSAR model, often considering the significance of descriptors). (Netzeva et al., 2005; Carrió et al., 2014; Patlewicz et al., 2016) This applicability domain analysis may be

[2] A chemical is assigned to a category (e.g., 1, 2, 3, 4, or 5) based on distinct ranges of quantitative values (e.g., LD_{50}). Examples of such ranges include LD_{50} <5mg/kg (i.e., category 1) or 50-300mg/kg (i.e., category 3).

344 performed automatically by some software to determine whether the training set compounds
345 share similar chemical and/or biological properties with the test chemical.
346 • **Expert rule-based (or expert/structural alerts).** This methodology uses structural rules or alerts
347 to make predictions for specific toxicological effects or mechanisms of toxicity. These rules are
348 derived from the literature or from an analysis of data sets generated by scientists. Structural
349 alerts are defined as molecular substructures that can activate the toxicological effect or
350 mechanism. The rules may also encode situations where the alert is deactivated. Expert rule-
351 based models often include a description of the toxic mechanism and examples from the
352 literature or other reference sources to justify the structural alert. A positive prediction is
353 generally made when a structural alert is present (without deactivating structural features or
354 properties) in the test compound. When no alerts are triggered for a test chemical, a negative
355 prediction may be generated for well investigated endpoints; however, additional analysis is
356 generally required to make this assessment as discussed further in Section 2.4.3.
357 • **Read-across:** Read-across uses data on one or more analogs (the "source") to make a prediction
358 about a query compound or compounds (the "target"). Source compounds are identified that
359 have a structurally or toxicologically meaningful relationship to the target compound, often
360 underpinned by an understanding of a plausible biological mechanism shared between the
361 source and target compounds. The toxicological experimental data from these source
362 compounds can then be used to "read-across" to the specific target compound(s). Read-across is
363 an intellectually-derived endpoint-specific method that provides justification for why a chemical
364 is similar to another chemical (with respect to chemical reactivity, toxicokinetics,
365 mechanism/mode of action, structure, physicochemical properties, and metabolic profile). (Wu
366 et al., 2010; ECETOC 2012; Patlewicz et al., 2013a; Patlewicz et al., 2013b; OECD 2014; Blackburn

and Stuard, 2014; Patlewicz (2014); Patlewicz et al., 2015; Schultz et al., 2015; Ball et al., 2016; ECHA 2017b)

- **Other approaches:** In certain cases, other *in silico* methodologies may be appropriate. Examples include the use of molecular dynamics (e.g., simulating interactions of a query chemical with a metabolic enzyme) and receptor binding as an indication of a possible Molecular Initiating Event (e.g., estrogen receptor-ligand docking).

Each IST protocol will include an assessment of key computational aspects and specific issues to consider. For example, when performing read-across, issues such as the data quality of the source compound(s), how to perform an assessment of non-reactive chemical features and selection of grouping approaches used to form categories will be discussed to ensure source compound(s) are sufficiently similar, both chemically and biologically, for the endpoint being considered.

Each methodology has its strengths and weaknesses, which often depend on the type of toxicological effect or mechanism being predicted. This will be discussed in the individual IST protocols. In addition, there may be cases of unique or novel compounds for which it is not possible to make a prediction or for which confidence in the predictions is so low as to render it meaningless or unhelpful.

2.3.2 *In silico* methods selection criteria

In silico methods selection may include the following five considerations:

1. **Relevant toxicological effects or mechanisms**. As discussed in Section 2.2, each IST protocol will define a series of toxicological effects or mechanisms relevant to a specific endpoint and appropriate *in silico* models need to be selected that predict these specific effects or mechanisms.

2. **Model validity**. Best practices for validation of (Q)SAR *in silico* models have been documented in a number of publications (Cherkasov et al.; 2014, Raies and Bajic, 2016; Myatt et al., 2016), and models built using these best practices may be preferred. The OECD has published a series of validation principles for *in silico* models (OECD 2004; OECD 2007) and valid statistical-based or expert rule-based *in silico* methods. Such (Q)SAR methods have: 1) a defined endpoint; 2) an unambiguous algorithm; 3) a defined domain of applicability; 4) appropriate measures of goodness-of-fit, robustness and predictivity; and 5) a mechanistic interpretation, if possible. Any *in silico* model must include documentation that supports an assessment of the model's scientific validity, including the toxicological effect or mechanism being predicted, version number, type of methodology, training set size and content, as well as any predictive performance information. Validation performance is documented in report formats such as the QSAR Model Reporting Format (QMRF) (JRC 2014). The level of adherence to the OECD principles and the performance statistics need to be appropriate for the purpose of the assessment.
3. **Chemical space**. Often, *in silico* models will only make predictions for specific classes of chemicals, the so called "applicability domain". The chosen *in silico* model(s) may report the applicability domain assessment to demonstrate its proficiency for this class of compounds. Vice versa, only models are ideally chosen where the query compound is in the applicability domain. (Netzeva et a l., 2005; Carrió et al., 2014; Patlewicz et al., 2016)
4. **Model combinations**. Complementary or independent *in silico* models may be selected, as concurring results increase the reliability of the prediction (as discussed in Section 2.6.2).
5. **Supporting an expert review**. For QSAR models, tools to help the expert review (see Section 2.4) include the ability to allow examination of the descriptors and weightings used in the model, underlying training set data, and how the applicability domain assessment was defined. For

expert rule-based systems, this could include how the alert was defined (including any factors that activate or deactivate the alert), any mechanistic understanding associated with the alert, citations, and any relevant known examples of alerting chemicals.

Read across may be used when there are experimental data from high quality databases for one or more substances which are similar enough to the target chemical of interest. The Read-Across Assessment Framework (RAAF), or similar published and established frameworks, may be used to document the read-across assessment and to support its scientific plausibility (ECHA 2017b; Patlewicz et al., 2013b; Blackburn & Stuard 2014; Schultz et al., 2015; Patlewicz et al., 2015). The OECD has also produced guidance on the process of grouping chemicals and other considerations as part of a read-across assessment (OECD 2014), and ECHA has generated guidelines on the process of performing a valid read-across assessment (ECHA 2008).

2.3.3 Running the *in silico* models

All *in silico* systems require an electronic representation of the chemical structure and any errors in this representation will result in invalid predictions. Therefore, it is important to ensure that the chemical structure is properly curated and entered following conventions set out by the model's developer, including appropriate representations for tautomers, aromaticity, salt forms, stereochemistry, charges, and specific functional groups (e.g., nitro or carboxylic acid groups). It is possible that different formats (i.e., SMILES *vs*. MOL files) may be processed differently. It is also important to verify that the software correctly interprets the structural representation during processing, particularly for complex molecules. For some types of chemicals, *in silico* models may not be applicable due to the structural representation or the unsuitability of the experiment assay for the specific chemical class. Examples include non-discrete chemical substances, UVCBs (unknown/variable composition, complex reaction products and

biologicals), metals, inorganics, polymers, mixtures, organometallics and nano-materials. (Mansouri et al., 2016)

Some models, such as statistical-based models, allow for prediction settings to be adjusted or turned off (e.g., they report "positive" when a value is greater than a predetermined threshold). The settings are ideally selected in a way that does not compromise the model's validity (such as changing the validation statistics of the model) and appropriately reported.

A thorough documentation of all selected models and computer software packages including, version numbers, and any parameters set, is needed as part of the materials and methods in sufficient detail to assess and potentially repeat the analysis (discussed in Section 2.9). In addition, the results need to be presented in enough detail to fully understand how they were generated and to critically assess the findings.

2.4 *In silico* expert review

2.4.1 Overview

As with *in vitro* or *in vivo* study data, *in silico* predictions may be critically assessed and an expert review of the output is often prudent (Dobo et al., 2012; Sutter et al., 2013). Frameworks for conducting an expert review ensure that it is performed in a consistent and transparent manner. Examples of such a review framework include the Office of Health Assessment and Translation (OHAT) systematic review and evidence integration (Rooney et al., 2014), weight-of-evidence assessments (ECHA 2017a), and Integrated Approaches to Testing and Assessment (IATA) (OECD 2016a; OECD 2016b).

The purpose of an *in silico* expert review is to evaluate the reliability of the prediction. The outcome of the review provides information to include in the assessment of the toxicological effect or mechanism. As part of this review, the expert might agree with, or refute, individual *in silico* predictions. In addition,

these reviews might support cases when a chemical is out of the applicability domain of the model, support the use of an equivocal prediction (i.e., there is evidence both for and against the supposition), or support cases where multiple predictions do not agree. A checklist of items to consider and report will help to ensure such reviews are performed in a consistent manner (as illustrated in Tables 2 and 3). This review may include knowledge from proprietary information available within an organization from the testing of related chemicals.

When an expert review assesses multiple predictions from different *in silico* systems, it is important to justify how they complement each other with regard to the training set (i.e., the use of relevant guideline studies plus relevant chemical classes), methodology (e.g., expert rule-based vs. statistical-based vs. read-across), or QSAR descriptor sets.

It is essential to document the reasoning and decisions of the expert review steps so they can be retraced at any time, including the information used as the basis for the review.

2.4.2 Expert review of statistical models

An expert review of a statistical-based model involves a critical assessment of how the model generated the prediction. This includes examining the weightings of the model descriptors (e.g., structural features or physicochemical properties related to toxicity), underlying data, chemical space of the training set of the model, and the experimental results for analog compounds and model performance for these analogs (e.g., nearest-neighbor list of compounds) (Amberg et al., 2016). This may also incorporate an understanding of the mechanism of toxicity or knowledge of factors that activate or deactivate the toxicity. The items described in Table 2 provide a checklist of elements to consider as part of any QSAR expert review to ensure such a review is as objective as possible, transparent and based on a consistent set of considerations. An expert review may increase the reliability of statistical model results based on one or more elements defined in Table 2.

Individual IST protocols will outline specific points to consider when performing an expert review, such as how the similarity of analogs could be assessed.

2.4.3 Expert review of expert rule-based (structural) alert systems

An expert review of the results from an expert rule-based alert system may involve inspection of the underlying information as well as external knowledge. Special emphasis needs to be placed on the assessment of chemicals where no alerts are identified in the expert alert system. When no alert is fired (i.e., it is not predicted active), it is often not reported if the prediction is negative, equivocal, or out of the applicability domain of the model and often no prediction is generated. An expert review may increase the reliability of the results based on one or more elements defined in Table 3.

2.4.4 Read-across expert review

Read-across contains an expert assessment by its nature: it requires expert judgment of the analogs, their data and extrapolation to the query chemical. For example, read-across assessments performed and documented according to the RAAF (i.e., following the detailed RAAF Assessment Elements), or similar frameworks, as discussed earlier, incorporate an expert review as part of the assessment. This type of assessment includes a strong justification for biological plausibility of any analogs selected (including an assessment of the structural differences and similarities to the target structure, and an analysis of potential metabolism). It also includes an expert assessment when a read-across prediction concludes there is an absence of effects. In addition, an assessment of supporting evidence (including the reliability of the source data), any weight-of-evidence considerations, and an assessment of any possible bias in the selection of source chemicals is required.

2.5 Assessment of available experimental data

Experimental data may have been previously generated and reported for a chemical being assessed, for example, in the literature or through a public or proprietary database. To support the identification of

experimental data, each IST protocol will identify a series of relevant study types and specific result(s) corresponding to the identified toxicological effects or mechanisms, as discussed in Section 2.2. To illustrate, in the assessment of the toxicological effect/mechanism bacterial gene mutation (part of the genetic toxicity IST protocol), the overall mutagenic or non-mutagenic results from a bacterial reverse mutation assay may be used. A more complex example is in the assessment of the toxicological effect/mechanism of sperm morphology (part of the reproductive IST protocol). Here, specific results from potentially different study types, such as one- or two- generation reproductive studies, repeated dose toxicity studies or segment I (fertility) studies, and possibly also from different species (rat, mouse, rabbit) will be applicable.

The selection of experimental study types need focus on those that have general value based on scientific justification. This includes study types that have widespread use in risk assessments, regulatory acceptance and that follow internationally recognized test guidelines. In addition, other types of data may be considered relevant on a case-by-case basis. Numerous guidance documents discuss acceptable studies, their relevancy, and their use in hazard identification, hazard characterization and risk assessment. These include guidance documents from the ICH (ICH 2017), OECD (OECD 2017), European Food Safety Authority (EFSA) (EFSA 2017a), Scientific Committee on Consumer Safety (SCCS) (SCCS 2017), REACH /ECHA (ECHA 2008; ECHA 2015), United States Environmental Protection Agency (EPA) Office of Chemical Safety and Pollution Prevention (OCSPP 2015), and National Institute of Environmental Health Sciences (NIEHS) (NIEHS 2017) guidance documents. Such guidance documents provide a useful basis for test considerations but may not always be harmonized across legislation, industrial sector or geographical regions, as requirements may differ across guidance documents.

The IST protocols will discuss how to assess and document the experimental data and uncertainties to ensure the proper justification of the experimental results' reliability, including defining what specific

elements or fields are important to document. With older studies pre-dating existing guidelines, it will often still be possible to perform an expert review to determine the adequacy of the data, but it will be important to document specifically why the study results were considered acceptable or dismissed as unacceptable. The IST protocols will also provide recommendations on how to select a result when multiple studies (with potentially conflicting results) for the same effect or mechanism are reported.

Klimisch scores are a widely used approach adopted to support an assessment of experimental data reliability (Table 4; Klimisch et al., 1997). The Klimisch score (1 to 4) is based on factors including whether the test was compliant with the OECD principles of Good Laboratory Practices (GLP) or Good *In Vitro* Methods Practices (GIVIMP) standards (OECD 2016c), whether the data were generated using accepted test guidelines, whether the data are available for independent inspection, and the quality of the report. ECHA uses this score, for example, as part of its data submission process (ECHA 2011), and there are tools to support the assignment of Klimisch scores (ECVAM 2017; Schneider et al., 2009). Another approach to the assessment of the reliability of the experimental data is the Science in Risk Assessment and Policy (SciRAP) application, a web-based reporting and evaluation resource created to help understand how academic toxicity-related studies can be used as part of any regulatory assessment (Molander et al., 2014). An approach proposed by EFSA is a detailed analysis of different parameters of the study (e.g. statistical power; verification of measurement methods and data; control of experimental variables that could affect measurements; universality of the effects in validated test systems using relevant animal strains and appropriate routes of exposure, etc.) with detailed documentation of the process (EFSA, 2011).

2.6 Combined assessment of experimental data and *in silico* predictions

2.6.1 Toxicological effect or mechanism assessment

Reliable data, generally defined by Klimisch scores 1 or 2 reviewed by an expert (see Table 4), is ideally used for the toxicological effect or mechanism (shown in Figure 1) whenever available[3]. In the absence of adequate experimental data, results from one or more *in silico* models can be used to support assessment of the toxicological effect or mechanism. When multiple *in silico* model results, from potentially different methodologies, or QSAR models using different descriptors and/or training sets, are generated per toxicological effect or mechanism, the individual results need to be compiled to provide one overall assessment, as shown in Figure 1. This assessment may take into consideration information from any expert review of the *in silico* results, as certain results may need to be refuted. Similarly, when there are data assigned Klimisch 3 or 4 and/or there are *in silico* results, this information needs to be compiled into an overall assessment. Individual IST protocols will document such procedures.

There are multiple approaches to compile results. A cautious approach is to use the most conservative data or prediction for this assessment. For example, when predicting the results of the bacterial reverse mutation test using two models, if either model's prediction result is mutagenic then the overall assessment is mutagenic. Other options include a weight-of-evidence or consensus approach or selection of the prediction with the highest confidence (e.g., predictive probability score and relevance of analogous structures). Specific considerations per endpoint may be addressed in the individual IST protocols and may be dependent on the problem formulation.

[3] As mentioned in Section 2.5, where high quality experimental data are available (as shown in Figure 1), it may not be necessary to run *in silico* models. However, generating *in silico* predictions for chemicals with known values is sometimes performed to verify experimental results because an unexpected positive or negative experimental result in a physical assay may be explained by the presence of an active impurity or to provide additional weight-of-evidence or for other reasons.

2.6.2 Reliability scores

Reliability, in this context, is defined as the inherent quality of the experimental study (Klimisch, 1997) and/or *in silico* analysis. It is used to support any hazard assessment, in combination with other information. A reliability score (RS) is associated with the toxicological effect or mechanism assessment (as shown in Figure 1). As noted earlier, when data from the literature or other sources are considered, Klimisch scores can be used to assess the reliability of the results. However, the Klimisch framework was never intended to assess the reliability of *in silico* predictions. It is also important to note that regardless of the approach taken, reliability assessments will contain subjective decisions.

A number of general factors can affect the reliability of *in silico* results:

- **Multiple *in silico* results**: Combining results from multiple complementary or independent *in silico* tools which use different methodologies or QSAR descriptors and/or training sets, has been shown to improve overall sensitivity, but it can lower specificity by increasing false positive rates (Myatt et al., 2016). In the case of quantitative predictions, such process are overly conservative estimates. Hence, consistency across several different models can increase the reliability of the results.
- **Expert review**: A plausible and well-documented read-across (consistent with the RAAF or similar frameworks) may be acceptable as part of a REACH regulatory submission as an alternative to experimental data. A structured expert review is implicit in any read-across assessment (as discussed in Section 2.4.4). Similarly, an explicit expert review (following the elements described in Sections 2.4.2 and 2.4.3) of the *in silico* predictions can improve the reliability of the final results, especially for negative predictions. (Dobo et al., 2012)

585 To generate an overall reliability score for assessments based on experimental data and/or *in silico*
586 predictions, the Klimisch score has been adapted (as shown in Figure 2) to include an assessment of *in*
587 *silico* prediction results.

588 Experimental data assigned a Klimisch score of 1 or 2 is assigned a score of RS1 and RS2, respectively, in
589 this revised scheme. *In silico* results are not assigned a score of RS1 or RS2 since adequate experimental
590 data is preferred over *in silico* predictions. Since *in silico* results may be used directly as part of certain
591 regulatory submissions, whereas experimental data with a Klimisch score of 3 or 4 would not (or only as
592 supporting data under REACH, for example), the next two categories (RS3 and RS4) represent, in part, *in*
593 *silico* predictions. The following may be acceptable as part of a regulatory submission: (1) an adequately
594 performed read-across prediction (EU 2006), or (2) an expert review of *in silico* and/or other
595 experimental data (ICH M7, 2017(R1); EU 2006); they are assigned a reliability score of RS3. A score of
596 RS4 would be assigned when two or more predictive models are available that are complementary, with
597 concurring results (with no expert review), and no supporting literature data are available. Examples
598 include those predictive models that use either substantially different QSAR descriptors and/or QSAR
599 training sets or different *in silico* methodologies. If two or more *in silico* model results do not agree, then
600 an expert review would be required to assess the results. This review might increase the confidence in
601 the assessment, resulting in an increased reliability score of RS3. A single acceptable (as discussed in
602 Section 2.3.2) *in silico* model result, without further expert review, is afforded the same reliability score
603 of RS5 as an actual test result of lowest reliability (Klimisch 3 or 4). The *in silico* result is placed in the
604 same category as low reliability data because such models inform decisions based on a series of
605 compounds or trends However, this reliability score may be increased following expert review. This
606 reliability score closely follows the ICH M7 guideline, where submissions corresponding to reliability
607 scores RS1-RS4 would be accepted according to the guideline. In addition to this score, it may be helpful

to document any additional considerations that may be important to the overall assessment. Individual IST protocols may deviate from this scheme with appropriate justification.

2.6.3 Worked examples

Three examples from Amberg et al. (2016) illustrate how the framework described in this publication can be used for determining a toxicological effect or mechanism assessment and reliability score, based on experimental data and/or *in silico* predictions. Assessing reliability is an initial step in the overall assessment of hazard, where it will be combined with other information, including an evaluation of the relevance of the information, to support decision making.

In the example in Figure 3, no experimental data were identified. Two *in silico* models were run; the statistical-based model prediction was negative and the expert rule-based alert prediction was negative. The initial score would be RS4 based on multiple concurring prediction results; however, an expert review was performed on the results from both methodologies and the negative result was confirmed with increased reliability. The review concluded there were no potentially reactive features in the chemical. This resulted in a negative overall assessment and a reliability score of RS3 (as a result of the expert review increasing the reliability).

In the example in Figure 4, no experimental data were identified. Two *in silico* models were run; the statistical model prediction was positive and the expert alert prediction was positive. No expert review of the results was performed. The overall assessment was therefore positive and a reliability score of RS4 was assigned as a result of two concurring positive predictions using complementary *in silico* methodologies but without expert review.

In the example in Figure 5, no experimental data were identified. Two *in silico* models were run; the statistical model prediction was positive and the expert alert prediction was negative. An expert review

was performed on the results from both methodologies, refuting the statistical model's positive prediction. This review was based on an analysis of the test chemical's potential to react with DNA and the highlighted structural feature was determined to be irrelevant for the mechanism of interaction with DNA. This resulted in a negative overall assessment and a reliability score of RS3 (as a result of the expert review increasing the reliability).

2.7 Hazard assessment framework

2.7.1 Toxicological endpoints

Figure 6 illustrates a general scheme for the prediction of a major toxicological endpoint. In this scheme, the specific toxicological effects or mechanisms are used to support the assessment of a series of toxicological endpoints. These toxicological endpoint assessments are, in turn, used in the overall assessment of the major toxicological endpoint. In Figure 6, effect/mechanism 1 is identified as being relevant to an assessment of a specific toxicological endpoint (Endpoint 1). For example, bacterial gene mutation (effect/mechanism 1) is relevant to the assessment of gene mutation (endpoint 1). Endpoint 1 is, in turn, one of the endpoints that are relevant to the major toxicological endpoint (e.g., genetic toxicity). Other identified toxicological effects or mechanisms are associated with toxicological endpoints as shown in Figure 6. For example, the mammalian gene mutation (effect/mechanism 2) is also relevant to the assessment of gene mutation*s* (endpoint 1) and clastogenicity (endpoint 2) is another endpoint to be used in the assessment of genetic toxicity (a major toxicological endpoint). Figure 6 also includes another example to illustrate how this scheme might be used to assess male reproductive toxicity.

The hazard assessment framework scheme for each IST protocol will contain different numbers of toxicological endpoints as needed to support the assessment of each major toxicological endpoint in a complete and transparent manner.

653 It is noteworthy that only the toxicological endpoints required to support a particular problem
654 formulation need to be assessed. For example, in certain applications only an assessment of gene
655 mutation may be needed (i.e., it may not be necessary to compute clastogenicity or the genetic toxicity
656 major toxicological endpoint).

657 **2.7.2 Relevance**

658 Relevance, in this context, is defined as the scientific predictivity of the each toxicological effect or
659 mechanism for the purpose of assessing a specific toxicological endpoint. As shown in Figure 6, the
660 assessment of toxicological endpoints may be based on the associated toxicological effects or
661 mechanisms. To support a transparent overall analysis, the relevance of the toxicological
662 effect/mechanism information in support of the assessment of the associated toxicological endpoint will
663 be defined in the IST protocols. This relevance will be based on the collective experience of the
664 consortium and available validation information.

665 **2.7.3 Toxicological endpoint assessment**

666 The assessment of each toxicological endpoint (as shown in Figure 6) is a function of all associated
667 toxicological effects or mechanisms and, in some cases, other toxicological endpoints. For example, in
668 Figure 6, bacterial gene mutation and mammalian gene mutation (toxicological effects or mechanisms)
669 are associated with gene mutation, whereas gene mutation and clastogenicity (both toxicological
670 endpoints) are associated with genetic toxicity. Rules or general principles for combining all associated
671 results for each endpoint will be defined in the upcoming IST protocols. For example, a rule may state
672 that if one of the associated effects/mechanisms is positive then the endpoint assessment is positive.
673 These rules or principles will take into consideration how combinations of different toxicological
674 effects/mechanisms are evaluated to generate an assessment for any toxicological endpoint which may
675 include a sequence of steps and incorporate Boolean logic.

676 **2.7.4 Toxicological endpoint confidence**

677 Confidence, in this context, is defined as a score that combines the reliability and relevance of the
678 associated toxicological effects or mechanisms. This is an additional score associated with toxicological
679 endpoints. The score may, in some cases, use other toxicological endpoint confidence scores (as shown
680 in Figure 6). This score will also take into consideration the completeness of the information available;
681 for example, the confidence score may be lowered when information on an effect or mechanism is
682 missing. It will also include complementary effects or mechanisms that need to be considered. This
683 score will be generated based on a series of general principles and/or rules defined in each IST protocol.
684 Each protocol will outline the different confidence values to generate, such as high, medium or low.

685 A confidence score is one of the most important items to generate. Different decision contexts tolerate
686 a different level of confidence in the assessment result as exemplified in the following two scenarios.

687 1) *Scenario* 1. The decision is to prioritize a large number of chemicals to screen as part of
688 product development. In this scenario, selecting a small subset of compounds using *in silico*
689 methods supports strategic resource utilization with the eventual goal of reducing overall
690 costs.
691 2) *Scenario* 2. A regulatory submission for a new cosmetic ingredient is being prepared based
692 on results from *in silico* methods.

693 Although in both scenarios, toxicological endpoint assessments generated at the highest level of
694 confidence would be preferable, Scenario 1 could still make beneficial use of lower confidence
695 predictions because the safety consequences of a false negative is lower than in Scenario 2. Therefore, a
696 risk assessment which takes into account the acceptable tolerance for a wrong prediction can be used to
697 evaluate the necessity for high confidence.

The assignment of the confidence score for each toxicological endpoint has to support the decision context(s), regulatory framework and the type of product being assessed. Minimum confidence scores for regulatory purposes may need to be set; however for other applications, the use of these scores may be based on the individual organization's risk tolerance or based on the context, a decision on the maximum permitted effort to be expended (since higher confidence score may be generated with additional resources), or an organization's internal policy for using the confidence scores for specific tasks.

2.7.5 Expert review of toxicological endpoints

In certain situations, an expert review of the toxicological endpoint assessment and/or confidence may be warranted, and specific points to consider as part of such an expert review will be detailed in the individual IST protocols. This review may take into consideration the context of the assessment, that is, the type of product being assessed and any potential regulatory framework. It may be helpful to document any additional considerations concerning the assessment and confidence to support an overall assessment.

2.8 *In silico* toxicology protocol components

Ongoing efforts are concentrated on the development of individual IST protocols for major endpoints including genetic toxicity, carcinogenicity, acute toxicity, repeated dose toxicity, reproductive toxicity, and developmental toxicity. Table 5 outlines proposed common components for these IST protocols.

2.9 Reporting formats

Standardized reporting of the results and expert review is good scientific practice and assures that when such information is communicated to regulatory authorities, it is complete, consistent and transparent;

this may avoid requests for additional information and maintain a consistent, expedient, and streamline regulatory review process. Table 6 outlines a proposed structure for the report format.

The proposed report format is more comprehensive than existing data formats by including information on overall assessment and expert reviews. For example, the "QSAR prediction reporting format" (QPRF; JRC 2014) could be used to report the individual model results (as shown in Section D of Table 6), or "QSAR model reporting format" (QMRF) can be used to report the QSAR model's details (as shown in Section H of Table 6).

The new proposed report format collects enough details on how the predictions were generated to enable another expert to repeat the process. It is also important that the reasoning and decisions of the expert review steps are transparently documented and can be retraced at any time, including the information used as their basis for conclusions.

3. Summary and outlook

IST is poised to play an increasingly significant role in the assessment of chemicals in a range of chemical exposure scenarios that have the potential to impact public health. Thus, this is an opportune time for the development of IST protocols. As expected, the quality and quantity of experimental data will vary as will the available *in silico* methods. For example, experimental data could be from a variety of sources, studies, protocols and laboratories using or not using GLP standards. Similarly, several *in silico* methods and approaches are available for assessment of toxicity. Thus, accepted selection criteria have to be defined for experimental data and *in silico* methods, for consistent and uniform use. The development of IST protocols will support the use and adoption of *in silico* methods in the same manner in which *in vitro* and *in vivo* test guidelines support the use and adoption of those assays.

Figure 7 summarizes the steps to perform an *in silico* assessment consistent with the framework defined in this publication. The key elements needed for the development of IST protocols are outlined in this publication, including: 1) how to select, assess and integrate *in silico* predictions alongside experimental data for defined toxicological effects or mechanisms, including a new methodology for establishing the reliability of this assessment, 2) a hazard assessment framework for systematic assessment of these toxicological effects or mechanisms to predict specific endpoints and assess the confidence in the results. Wherever possible, this is based on mechanistic knowledge on different biological levels of organization. (Bell et al., 2016; OECD 2016a; OECD 2016b) Overall, the IST protocols will contain information to ensure predictions are performed in a consistent, repeatable, transparent and ultimately accepted manner and will include a checklist (as defined in Section 2.4) to guide an expert review of the information. Each individual IST protocol will address how predictions will be performed in alignment with the framework discussed in this publication. These new protocols will provide specific guidance for each toxicological endpoint, including situations where no AOP or IATA is currently available. These protocols build on and fully incorporate wherever possible the considerable work previously reported, such as the OECD validation principles (see Sections 2.3.2), IATAs (see Sections 2.2), AOPs (see Sections 2.2), read-across frameworks (see Sections 2.3.2, 2.6.2), the Klimisch score (see Sections 2.5, 2.6.1, 2.6.2) and the QMRF/QPRF (see Sections 2.3.2, 2.9).

The IST protocols do not define how a risk assessment will be performed; they solely define the process which will lead to the prediction of the potential toxicity (hazard) of a chemical. Risk analysis depends on the exposure scenario, industry, regulatory framework and decision context based on the level of tolerated uncertainty and is performed in the hands of an expert.

The process of developing IST protocols requires an understanding of the best practices and science across various organizations, different industries and regulatory authorities. To develop such protocols,

763 an international consortium was established comprising regulators, government agencies, industry,
764 academics, model developers, and consultants across many different sectors. This consortium initially
765 developed the overall strategy outlined in this publication. Working subgroups will develop individual
766 IST protocols for major endpoints including genetic toxicity, carcinogenicity, acute toxicity, reproductive
767 toxicity, and developmental toxicity. As each IST protocol is established, it will be reviewed internally
768 within each organization and published. This process will evolve over time, as computational technology
769 progresses, as will the assays and other information relevant to assessing these major endpoints
770 emerges. Hence, similar to other test guidelines, the IST protocols will need to be periodically reviewed
771 and updated. The implementation of IST protocols will also require user-friendly tools for performing
772 such analyses and reporting the results, education, as well as further collaboration with organizations to
773 support global adoption.

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Table Legends

Table 1: Applications of *in silico* toxicology

Table 2: Checklist of elements to consider as part of an expert review of a QSAR model result

Table 3: Checklist of elements to consider as part of an expert review of results from expert rule-based

Table 4: Summary of Klimisch scores for data reliability (adapted from Klimisch et al., 1997) (Note "restriction", as part of scores 1 and 2, indicates restricted quality)

Table 5: Common components of an IST protocol (IATA = Integrated Approaches to Testing and Assessment; AOP = Adverse Outcome Pathways)

Table 6: Elements of an *in silico* toxicology report (QMRF = QSAR Model Reporting Format)

Figure Legends

Figure 1: Overview of the IST protocol framework, showing how experimental data or *in silico* model(s) for each defined toxicological effect/mechanism are assessed and used to support a hazard assessment. (Note Effect/Mechanism N is used to illustrate that there can be any number of effects/mechanisms in each protocol)

Figure 2: Reliability of toxicity assessments based computational models and experimental data

Figure 3: Determining the bacterial gene mutation assessment and reliability score for two concurring *in silico* results with expert review

Figure 4: Determining the bacterial gene mutation assessment and reliability score for two concurring *in silico* results with no expert review

Figure 5: Determining the bacterial gene mutation assessment and reliability score where there is no experimental data available and conflicting *in silico* results

Figure 6: Hazard assessment framework

Figure 7: Summary of the IST protocol process

Tables

Table 1: Applications of *in silico* toxicology

***In silico* toxicology application**	**Discussion**
1. *Alternative to test data.*	The use of non-animal alternative methods including *in silico* approaches, may substitute for other types of tests in regulatory submissions in certain cases. Acceptable alternative methods for filling data gaps are outlined in Annex XI of the European Union's REACH regulation (EU 2006). In the United States, Frank R. Lautenberg Chemical Safety for the 21st Century Act revised the Toxic Substances Control Act (TSCA) to include predictive models and expert review as part of an overall assessment (TSCA 2016). The United States Food and Drug Administration (US FDA) Center for Devices and Radiological Health (CDRH) issued a guidance for industry and FDA staff. This guidance is on the use of International Standard ISO 10993-1 for biological evaluation of medical devices and indicates in the absence of experimentally derived carcinogenicity information, structure activity relationship modeling for these materials may be needed (CDRH 2016). The FDA draft guidance on Electronic Nicotine Delivery Devices (ENDS) also discusses the use of computational toxicology models in the absence of toxicological data for potential toxicants created by the aerosolization process (PMTA/FDA 2016). When chemicals with limited toxicity data are required to be classified and labeled for shipping or other purposes, *in silico* toxicology provides an alternative method for quickly filling the data gaps in the toxicity/safety information, such as predictions of acute toxicity to support assignment to the Globally Harmonized System of Classification and Labelling category (Freidig et al., 2007; ECHA 2015).
2. *As part of the weight-of-evidence in regulatory submissions*.	There are currently several regulatory frameworks where only specific laboratory tests for an endpoint of concern may be submitted (such as for drugs or food additives). However, in such cases, *in silico* predictions can be submitted alongside standard toxicological data to complement the assessment. This may include *in silico* assessments provided as supporting data or adjuncts to the primary *in vivo* or *in vitro* studies to give a mechanistic understanding of the observed results and/or allow a better definition of experimental needs. Additionally, *in silico* methods may be used to guide or prioritize *in vitro* testing (EU 2012). The European Union's Cosmetics Regulation (EU 2009a) prohibits the use of animal testing for products or ingredients and a complete marketing ban of such products tested as a whole or containing tested ingredients. This requires the use of alternative methods, such as IST, in the assessment of new cosmetics ingredients. In a recent memorandum, the European Commission's Scientific Committee for Consumer Safety (SCCS), which is responsible for the risk assessment of cosmetic ingredients, acknowledged the importance and limitations of *in silico* methods; the SCCS recommended that *in silico* methods be used either for internal decision making or as part of a weight-of-evidence (WOE) approach to estimate toxicity risks before embarking on any experimental testing (SCCS 2016).
3. *Mixtures assessment.*	Most exposures are not to a single chemical but rather to complex mixtures of chemicals that may be found in food, beverages, the environment, cigarette smoke, electronic nicotine delivery systems (ENDS) aerosols, botanical drugs or natural products. In certain situations, it may be possible to use *in silico* methods to assess individual components since today's *in silico* analysis can only be performed on discrete identifiable chemicals. While preliminary analytical work is required to identify all chemicals in the mixture above appropriate Analytical Evaluation Thresholds (AET) (Ball and Norwood 2012), leveraging *in silico* approaches may avoid having to synthesize or purify each of the potentially large number of mixture components to perform standard toxicological tests (Mumtaz et al., 2010). Careful consideration is required for mixtures when there are multiple chemicals for interactions, such as synergistic or additive effects that may have the same, similar or different mechanisms of action (MOA).
4. *Assessment of impurities and degradation products*.	Chemicals, such as pharmaceuticals or plant protection products, may contain low levels of impurities produced during manufacturing and degradation. Many such substances, when present at levels above accepted thresholds, need to be assessed. In most cases, mutagenicity evaluation of the impurity under question is required as a first step of the risk assessment. (Harvey et al., 2017) The ICH M7 guideline provides specific recommendations for assessing drug impurities (ICH M7, 2017(R1)), including the use of two complementary computational

	toxicology methodologies (i.e., statistical and expert based models) to predict bacterial mutagenicity.
5. *Residues of plant protection products*.	Residues of plant protection products may be evaluated as a part of residue definition for dietary risk assessment of plant protection products (EU 2009b). In this context, *in silico* methods provide a useful alternative approach. (EFSA 2016)
6. *Assessment of extractables and leachables*.	Medical devices, such as inhaled aerosols, food-contact substances, and consumer product packaging materials may pose a risk for human health due to release of potentially harmful chemicals that are used in the production of the components (Bossuyt et al., 2017). These include plasticizers, copolymers, vulcanization additives, etc. for which toxicological data is often lacking but where a risk assessment must be performed. A migration or leachables study supports the discovery, identification, and quantification of any leachables. An *in silico* toxicological assessment, in certain situations, can provide sufficient data for the risk assessment.
7. *Workers' safety and occupational health*.	Chemicals used in the manufacture of a product are assessed for mutagenicity, carcinogenicity, skin and respiratory sensitization, irritation (skin, eye and respiratory), and reproductive and developmental toxicity and possibly acute toxicity. *In silico* assessments make it possible to estimate the potential toxicity of chemicals and adopt proper engineering controls and personal protective equipment usage to protect workers who could be exposed to these substances during production, transfer, storage, and delivery processes (EU 2006). *In silico* approaches have been utilized to assess these major toxicological endpoints in the occupational safety setting. *In silico* methods to predict respiratory sensitization potential of industrial chemicals have recently been reviewed by Seed and Agius (2017).
8. *Metabolite analysis*.	Metabolites can present an increased or decreased risk of local or systemic toxicity compared with the parent chemical (Mumtaz and Durkin, 1992). While reactive or toxic metabolites may be formed by an organism, their identification, separation as well as possible synthesis for testing purposes may be challenging. *In silico* methods provide a practical alternative approach to understanding the safety profiles of this potentially large number of chemicals as well as to support the prediction of metabolites.
9. *Ecotoxicology*.	Various chemicals are discharged into the environment that may cause harm. Furthermore, the parent compounds can be transformed by hydrolysis, redox-reactions, or photolysis into numerous additional chemicals. IST methods often provide the most practical approach to assess the potential effects on the environment and wildlife species of the many chemicals that are discharged. Prediction of physicochemical parameters supports assessment of potential environment exposure to the chemical (e.g., persistence and distribution). As an example, Chen at al., 2015 describes the use of *in silico* assessment of potentially hazardous contaminants present in water.
10. *Green chemistry and safer alternatives.*	*In silico* methods can play an important role when identifying alternative chemicals that may have a safer profile than existing chemicals (Rastogi et al., 2014). This includes, for example, alternatives for use in manufacturing processes, alternative packaging/delivery materials and the use of specific additives. *In silico* methods can provide insights about structural features responsible for the toxicity of different groups of chemicals and thereby allow for the rational design of intrinsically safer chemicals.
11. *Selection of product development candidates.*	In early product discovery or development, many thousands of compounds may be evaluated. *In silico* methods may provide a helpful approach to selecting candidates, since *in silico* methods are inexpensive, rapid to perform, and high throughput. In addition, *in silico* methods can suggest which molecular substructures (toxicophores) are responsible for the predicted toxic activity, thereby supporting the optimization of future compounds (Hillisch et al., 2015; Myatt et al., 2016). Later in the product development process, a smaller number of chemicals may be selected as candidates to take forward for further development; in normal situations, preference would be given to the candidate(s) with the most advantageous safety profile(s) (Myatt et al., 2016).
12. *Emergency response situations*.	When one or more chemicals are unexpectedly released into the environment (e.g., the West Virginia chemical spill (NTP 2016)) or into a production process, it is important to quickly evaluate the potential effects on humans, wildlife, and the environment. In such emergency situations the toxicological profile of the released chemicals needs to be established as quickly as possible to support the proper emergency response and to protect emergency services staff and bystanders (Hochstein et al., 2008; Schilter, et al., 2014). In such a limited timeframe and in the

	absence of previously generated data, *in silico* approaches may be a practical option for rapid hazard identification.
13. *Prioritizing testing of chemicals*.	*In silico* approaches can help prioritize *in vitro* and *in vivo* toxicology testing, based upon the chemical's exposure and prediction of toxicity; they are an important aspect of the work at several organizations such as the US EPA, National Toxicology Program, Environment and Climate Change Canada and ECHA (Schwetz 1995). *In silico* methods may be used to prioritize (based on potential toxicological liabilities) the order in which a series of toxicological studies will be performed (Myatt et al., 2016).
14. *Rationalization of in vivo or in vitro study results*.	As mentioned previously in the description of the *in silico* application titled "*As part of the weight-of-evidence in regulatory studies"*, results from quantitative structure-activity relationship (QSAR) models (toxicophore information, chemical fragments or physicochemical properties) may be used in conjunction with biological data to infer a mechanism of action (MOA), molecular initiating event (MIE), or mode of toxicity as part of an adverse outcome pathway (AOP) (Martin et al., 2015; Ellison et al., 2016). Information from *in silico* methods can also be used to tailor an *in vivo* study, e.g., by inclusion of additional endpoints. When existing experimental data on a compound are equivocal or when not all relevant safety information are available or accessible, *in silico* data may be used as additional information as part of the weight-of-evidence approach in reaching a more informed decision (Kruhlak et al., 2012).

1101 **Table 2: Checklist of elements to consider as part of an expert review of a QSAR model result**

Expert review elements	Considerations
A. Inspection of model output	• A review of the applicability domain information provided by the model's software might increase or decrease reliability in the prediction. • The results of the QSAR model might include a score (e.g., a probability of a positive outcome). The prediction reliability may be increased where a score indicating a high likelihood can be justified through an expert review of the available information.
B. Analysis of structural descriptors and corresponding training set data (see Note A)	• As part of the process of building a QSAR model, structural descriptors are selected (often automatically) when there is a statistical association to the (toxicological) data to be predicted; however, the selected descriptors might not be biologically meaningful for the predicted toxicological effect/mechanism, as discussed in Powley (2015). This assessment may be supported by inspecting the training set examples that match the descriptors wherever possible. An expert review may determine the result is incorrect if other structural moieties in the training set examples are more likely responsible for the biological activity, (i.e., the descriptors identified were coincidental and in fact irrelevant) (Amberg et al., 2016). • Another scenario is when the structural descriptors map to experimental data that is incorrect and attributable to known problems with an assay. Again, these features may be discounted if they are not relevant to the toxicological effect or mechanism and this may lead to a reversal of the overall assessment. For example, chemicals containing acid halides may give false positive results due to possible interaction with the solvent DMSO in the Ames assay (Amberg et al., 2015). • Descriptors identified as significant by the model that are also present in the query compound may be associated with a biological mechanism. An expert review may evaluate whether the mechanism is plausible for the query compound, including potential metabolism consideration. For example, does the highlighted feature represent a known reactive group or a known toxicophore? This analysis may lead to an increase in prediction reliability. • In some systems, it is possible to inspect the training set's experimental data and references for those examples that are primarily used in the prediction. An assessment of these full studies for these examples (as discussed in Section 2.5) could be used to justify an increase in the reliability of the prediction result. • The structural diversity of the underlying chemicals for each significant descriptor may be reviewed as part of an expert review. Structural features that map to a large number of structurally diverse compounds would provide additional evidence that the toxicological effects or mechanisms associated with the descriptor could be extrapolated across different chemical classes (increasing reliability in the prediction), whereas a structural feature whose underlying data constitutes a congeneric series might not, especially if the query compound is structurally distant (decreasing reliability in the prediction).
C. Analysis of physicochemical descriptors used by model (see	• Is there any supporting information from the literature or elsewhere to support any correlation between the physicochemical properties

Note B)	identified as significant by the model and the toxicological effect/mechanism? • An evaluation of the quality of the experimental data of the training set chemicals used for building of the model (e.g., if a guideline study was used to generate these data) may increase the reliability of the prediction result.
D. Assessment of other information	• An evaluation of the performance of the model for structurally similar substances with known activity (selected by the user or provided by the system) might affect the evaluation of the reliability of the prediction.

(Note A: items to consider when the QSAR model includes structure-based descriptions; Note B: items to consider when the QSAR model includes physicochemical descriptors)

1105 **Table 3: Checklist of elements to consider as part of an expert review of results from expert rule-**

1106 **based**

Expert 4review elements	Considerations
A. Alert score or qualitative output	• The results from the alert system might include information related to the likelihood of a positive outcome (e.g., precision of the alert). The reliability of the prediction may be increased when such a score can be justified through an expert review of the information provided.
B. Justification of negative prediction	• Additional considerations may be important where no alerts are identified in the test chemical. Such analysis may focus on similar analogs as well as other chemicals containing the different structural elements of the test chemical to verify there is no potential toxicity attributable to these fragments, such as additional reactive features. Such analysis may be used to evaluate the reliability of the negative prediction. • If a negative prediction has a structure of concern, a further inspection of the rules may determine why the compound was not included to elucidate the underlying cause for firing no alert. Is the prediction really negative, equivocal, or not in of the applicability domain of the model?.
C. Reliability of the mechanism of toxicity	• Although the presence of a structural alert increases the potential of the chemical to exert a toxicological effect or mechanism, this effect may depend on other features of the molecule. If a mechanism of toxicity is proposed for the structural alert, then an expert may assess the plausibility of the mechanism for the query compound. For example, the presence of other substituents in the molecule may impact the activity, potentially deactivating the alerting structure. This may include metabolism considerations.
D. Inspection of chemicals and experimental data matching the alert	• The reliability of the prediction can be assessed by the quality of the experimental data of the reference set substances used to make the prediction (e.g., if a guideline study to generate these data). • The structural diversity of the matching chemical may also be considered. For example, alerts that match diverse structures may increase the reliability over alerts where the matching chemicals are from a tight congeneric series. This is especially true when the reference set examples are structurally dissimilar from the query chemical. • Review of the scientific literature to support the alert to understand the strengths and limitations of the experimental data supporting it.

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Table 4: Summary of Klimisch scores for data reliability (adapted from Klimisch et al., 1997) (Note "restriction", as part of scores 1 and 2, implies restricted quality)

Score	Description	Summary
1	Reliable without restriction	• Well documented and accepted study or data from the literature • Performed according to valid and/or accepted test guidelines (e.g., OECD) • Preferably performed according to good laboratory practices (GLP)
2	Reliable with restriction	• Well documented and sufficient • Primarily not performed according to GLP • Partially complies with test guideline
3	Not reliable	• Inferences between the measuring system and test substance • Test system not relevant to exposure • Method not acceptable for the endpoint • Not sufficiently documented for an expert review
4	Not assignable	• Lack of experimental details • Referenced from short abstract or secondary literature

1112 **Table 5: Common components of an IST protocol (IATA = Integrated Approaches to Testing and**

1113 **Assessment; AOP = Adverse Outcome Pathways)**

Introduction	• Describe the major toxicological endpoint being assessed • Outline the general hazard assessment framework, including how a series of toxicological effects or mechanisms are related to one or more endpoints • Provide citations to any applicable AOPs or IATAs used
***In silico* methodologies and models**	• Identify toxicological effects or mechanisms that might realistically be predicted • Define what *in silico* methodologies are appropriate to use • Specify additional considerations as to what constitutes an acceptable model • Discuss issues to be considered as part of any read-across analysis
Experimental data	• Define specific study types and result(s) relevant to each toxicological effect or mechanism • Define and justify the relevance of the information to the assessment of the toxicological endpoint (defined in the hazard assessment framework) • Define specific factors to consider when assessing the results and documenting the reliability of any available data or reference specific test guideline(s) • Identify sources of data that may be considered
Toxicological effects or mechanisms assessment and reliability scores	• Describe how each toxicological effect or mechanism assessment may be generated from available experimental data and/or *in silico* prediction(s) • Define additional items to consider as part of an expert review • Discuss any endpoint specific issues to consider as part of the reliability score
Toxicological endpoint assessment and confidence	• Describe the toxicological endpoints that will be used as part of the hazard assessment framework • Describe the rules or principles for determining each endpoint assessment, based on the associated effect/mechanisms or other endpoints • Define the rules or principles for determining each toxicological endpoint confidence, based on the relevance and reliability (from associated effects/mechanisms) or confidence (from associated endpoints) • Identify points to consider as part of any expert review
Reporting	• Define a format for a report of the results, expert review and conclusions
Other considerations	• Case studies

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1115 **Table 6: Elements of an *in silico* toxicology report (QMRF = QSAR Model Reporting Format)**

Section	Content
Title page	- Title (including information on the decision context) - Who generated the report and from which organization - Who performed the *in silico* analysis and/or expert review, including their organization - Date when this analysis was performed - Who the analysis was conducted for
Executive summary	- Provide a summary of the study - Describe the toxicity or properties being predicted - Include a table or summary showing the following: o The chemical(s) analyzed o Summary of *in silico* results, reviewed experimental data and overall assessment for each toxicological effect or mechanism o Summary of toxicological endpoint assessment and confidence o Summary of supporting information
Purpose	- Specification of the problem formulation
Materials and methods	- QSAR model(s), expert alerts, and other models used with version number(s) and any parameters set as part of the prediction (e.g., QMRF format) - Databases searched with version number(s) - Tools used as part of any read-across with version number(s)
Results of Analysis	- Details of the results and expert review of the *in silico* models and any experimental data, including results of the applicability domain analysis - Report of any read-across analysis, including source analogs and read-across justifications
Conclusion	- Summarize the overall analysis including experimental data, *in silico* methods and expert review - Final prediction that is based on expert judgment
References	- Complete bibliographic information or links to this information, including test guidelines referred to in the experimental data, etc.
Appendices (optional)	- Full (or summary) study reports used or links to the report, detailed (or summary) *in silico* reports, reports on the models used (e.g., QMRF reports)

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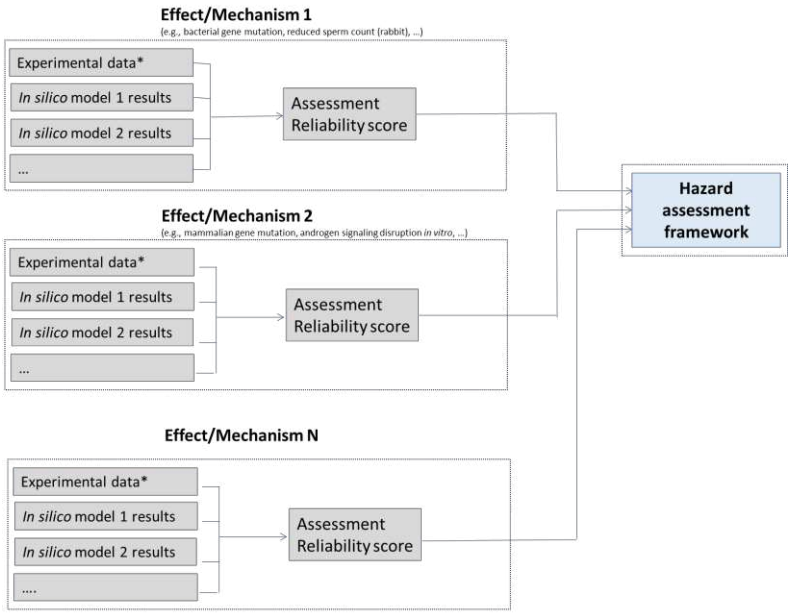
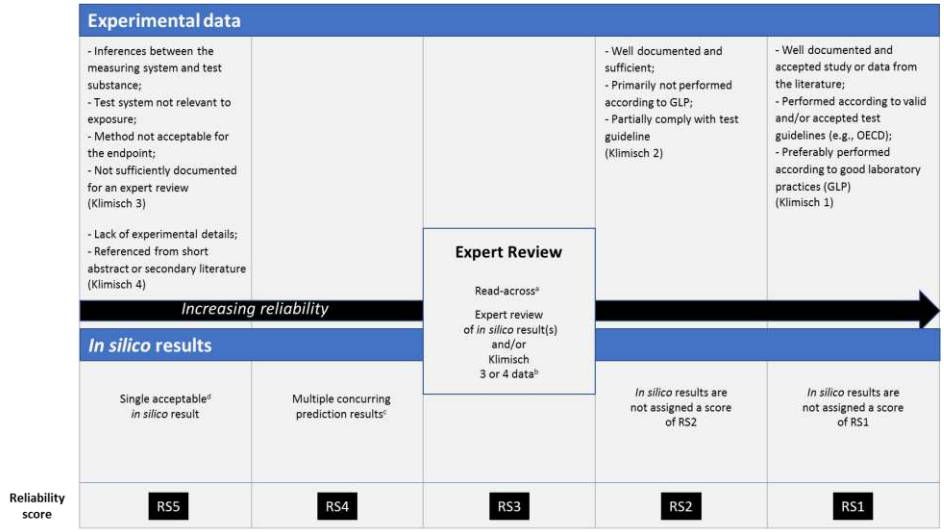
Figures

Figure 1: Overview of the IST protocol framework, showing how experimental data or *in silico* model(s) for each defined toxicological effect/mechanism are assessed and used to support a hazard assessment. (Note Effect/Mechanism N is used to illustrate that there can be any number of effects/mechanisms in each protocol)



* From the literature, database or study report

1125 **Figure 2: Reliability of toxicity assessments based on computational models and experimental data**



a. Read-across performed according to the RAAF or similar
b. Expert review resulting in increased confidence
c. 2+ concurring results from different methodologies or QSAR descriptor sets or training sets
d. Based on the selection criteria provided in Section 2.3.2

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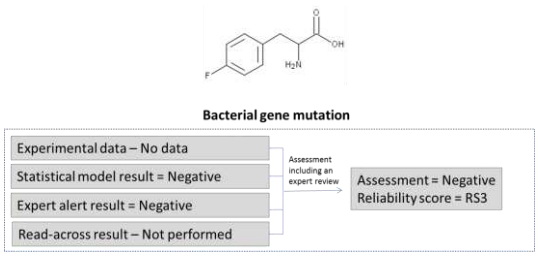
Figure 3: etermining the bacterial gene mutation assessment and reliability score for two concurring *in silico* results with expert review



1133 **Figure 4:** Determining the bacterial gene mutation assessment and reliability score for two concurring *in*

1134 *silico* results with no expert review

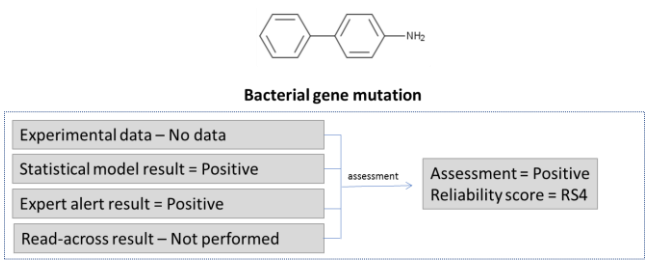


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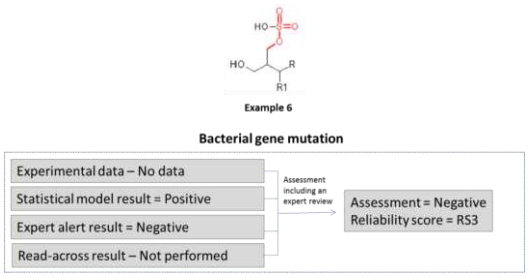
Figure 5: Determining the bacterial gene mutation assessment and reliability score where there is no experimental data available and conflicting *in silico* results



1141 **Figure 6: Hazard assessment framework**

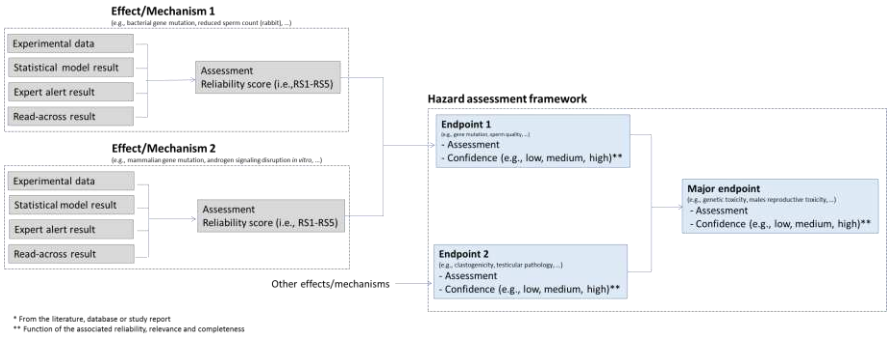


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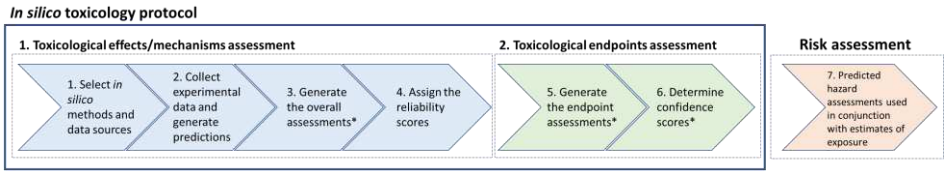
Figure 7: Summary of the IST protocol process



* Based on rules/principles outlined in the IST protocols, including an expert review if warranted