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GUIDANCE DOCUMENT ON DEVELOPING AND ASSESSING ADVERSE OUTCOME PATHWAYS

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GUIDANCE DOCUMENT ON DEVELOPING AND ASSESSING ADVERSE OUTCOME PATHWAYS

Environment Directorate

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

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FOREWORD

The Adverse Outcome Pathway (AOP) methodology is an approach which provides a framework to collect, organise and evaluate relevant information on chemical, biological and toxicological effect of chemicals. This approach supports the use of a mode (and/or mechanism) of action basis for understanding adverse effects of chemicals. This guidance document intends to provide an insight into which pieces of information are necessary to identify and document an AOP and how to present them. It also provides initial assistance on how to undertake the assessment of an AOP in terms of its relevance and adequacy. A template has been included allowing authors to develop thorough AOPs and to improve consistency in AOPs developed by different stakeholders.

The document also briefly outlines the potential use for regulatory purposes of AOP. Detailed guidance on how to use AOPs for integrated testing strategies and risk assessment will be developed in the future.

This guidance document was prepared in December 2012 by the Secretariat in collaboration with the advisory group on molecular screening and toxicogenomics. Since the development of AOPs is a new activity at OECD, the guidance should be considered as a first version which will be revised as expert groups and member countries get more experience in developing and assessing AOPs. This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

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PART I

BACKGROUND

The historical paradigm for protecting humans and the environment from adverse effects of chemicals has centred primarily on whole animal toxicity testing with single chemicals of concern. However, due to the costs and time involved, it is not practical or feasible to test exhaustively all chemicals that could adversely affect humans and ecosystems. These realities have long indicated the need for scientifically sound models and tools for predicting adverse effects of chemicals based on relatively little data. However, to date, our limited knowledge about biological systems has hindered efforts to use mechanistic information as a basis for effects extrapolation. Despite this, advances in toxicogenomics, bioinformatics, systems biology and computational toxicology are to be expected; noting that the performance of such test systems (e.g. their repeatability and reproducibility) and their toxicological relevance will need to be evaluated. With these new approaches, scientists seem poised to make steps forward that may revolutionise predictive toxicology and elicit a paradigm shift in regulatory toxicity testing and risk assessment. To support this shift, the so-called Adverse Outcome Pathway (AOP) methodology is one approach toward providing a framework to collect and evaluate relevant chemical, biological and toxicological information. The purpose of providing a framework to organise information is to support the use of a mode (and/or mechanism) of action basis for understanding adverse effects. It is important to note that the AOP concept uses existing methods and links them with systems biology rather than being a completely new paradigm. Briefly, consideration of weight of evidence for AOPs builds on concepts and principles incorporated in a pre-existing evolving framework for mode of action/human relevance analysis involving large numbers of scientists internationally, including earlier work on mode of action in animals of the U.S. Environmental Protection Agency (U.S. EPA, 1999) and the World Health Organization's International Programme on Chemical Safety (IPCS) (Sonich-Mullen et al., 2001), followed by further initiatives of the International Life Sciences Institute Risk Sciences Institute (ILSI RSI) (Meek et al., 2003; Seed et al., 2005) and IPCS (Boobis et al., 2006, 2008).

The current document aims to provide the framework for consistent information gathering and organisation, including definitions for AOP-specific terminology. This document also includes a template for developing AOPs allowing individual risk assessors to develop thorough AOPs and to improve consistency in AOPs developed by different risk assessor and other stakeholders. The primary purpose of this guidance document is not to reproduce or replace the ever-expanding volume of journal articles, reports, documents, and textbooks on AOPs but to provide an introduction to the development and assessment of AOPs. In this context, an AOP is a conceptual construct that portrays existing knowledge concerning the pathway of causal linkages between a molecular initiating event and a final adverse effect at a biological level of organisation that is relevant to a regulatory decision (Ankley et al., 2010). An adverse effect occurs only when homeostasis or adaptive responses are exceeded, so that the cell or organism will not survive with impairment of function(s). In some cases, such as with mitochondrial toxicity, the adverse effect may be at a biological level of organisation that is not an apical endpoint described in a test guideline. In such cases, this describes a 'key event' that may be an important aspect of toxicity leading to many final endpoints and may not be itself a sufficient anchor in an AOP.

INTRODUCTION

Recognising the limitations of current *in vivo* testing approaches for toxicological assessment and the rapid development of new biochemical and cellular assay systems and computational predictive methods, regulators and other stakeholders have been exploring ways to integrate existing knowledge from *in vivo* tests with the results of alternative methods and other sources of information. The purpose of this integration is to identify better schemes for making regulatory decisions.

Regulatory toxicology involves many issues (e.g. hazard identification, dose response assessment, exposure assessment, risk characterisation) addressed singularly or in combination. Over the past two decades, a variety of groups have advocated systems and pathway-based approaches to define the processes by which toxicants elicit outcomes of interest in public health and environmental health. Early applications of the pathway approach were often referred to as exposure-dose-response models or biologically based dose-response models (Clewell et al., 1995; Shuey et al.,1995). In 2001, a framework for using mode-of-action (MOA) information to determine human relevance of animal data was published by the International Programme on Chemical Safety (IPCS) (Sonich-Mullin et al., 2001). The latter as adopted by OECD in OECD Environment, Health and Safety Publications. Series on Testing and Assessment No. 35 and Series on Pesticides No. 14 Guidance Notes for Analysis and Evaluation of Chronic Toxicity and Carcinogenicity Studies. Briefly, the MOA describes the key events and processes, at the different levels of biological organization starting with interaction of an agent with the cell and proceeding through functional and anatomical changes in the organism. In 2007, the United States National Academy of Science (NAS) published the *Report on Toxicity Testing in the 21st Century: A Vision and a Strategy* in which the concept of a 'toxicity pathway' was very prominent (NRC, 2007). At the centre of the vision for transforming toxicity testing described in this report is a reorientation of such testing to evaluate the responses of toxicity pathways that can be perturbed by chemical exposures in well-designed *in vitro* methods using cells, often human in origin.

Since the McKim Conferences on Predictive Toxicology in 2006, 2007, and 2008 (http://mckim.qsari.org), and in parallel to refinement of the IPCS Mode of Action framework (Boobis et al., 2006; 2008) an alternative term "Adverse Outcome Pathway" (AOP) has evolved (http://mckim.qsari.org). The text "an alternative" rather than "the alternative" is deliberately used to avoid incorrectly implying that AOP has replaced MOA. As described by Ankley and co-workers, (Ankley et al., 2010) an AOP may describe a pathway initiated via non-specific interactions (e.g. a toxicant physically residing in a bio-membrane), as well as more specific ligand-receptor interactions leading to adverse effects. Although developed for use in ecotoxicology, the AOP concept is also applicable to human health effects (Schultz, 2010). In an AOP, it is important to integrate all of the known information. The approach is based on the concept that toxicity results from the chemical first reaching and then interacting with an initial target or targets in the organism. As such, an AOP is the sequential progression of events from the molecular initiating event (MIE) to the *in vivo* outcome of interest (Fig. 1). Generally, it refers to a broader set of pathways that would: 1) proceed from the MIEs, in which a chemical interacts with a biological target (e.g. DNA binding, protein oxidation etc.), 2) continue on through a sequential series of biological activities (e.g. gene activation, or altered tissue development etc.), and 3) ultimately culminate in the final adverse effect relevance to human or ecological risk assessors (e.g. mortality, disrupted reproduction, cancer, or extinction, etc.) (OECD 2011), ENV/JM/MONO(2011)8).

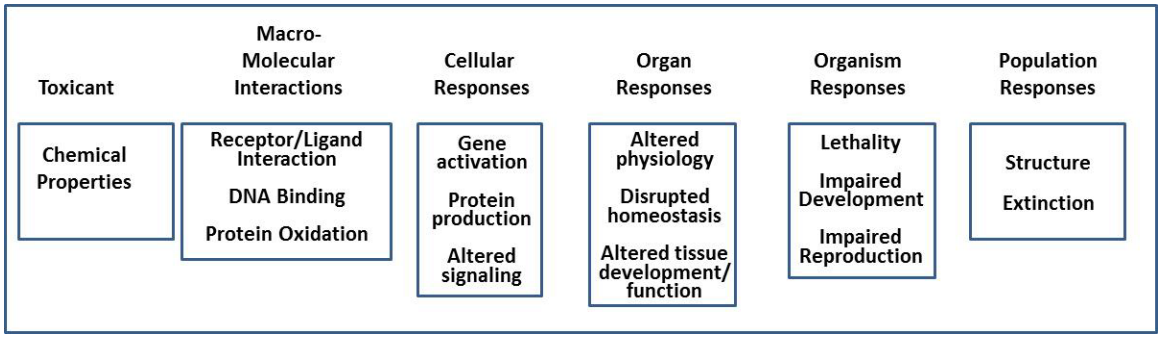

Figure1. A schematic representation of the Adverse Outcome Pathway (AOP) illustrated with reference to a number of pathways.

The AOP approach, as a relatively new concept, has been broadly discussed in recent years. The Society of Environmental Toxicology and Chemistry held a Pellston Conference in 2010 which focused exclusively on AOPs. The outcome from this workshop was published as a series of six papers in Environmental Toxicology and Chemistry in 2011 (Villeneuve and Garcia-Reyero 2011; Watanabe et al.,2011; Perkins et al., 2011; Nichols et al., 2011; Celander et al., 2011; Kramer et al., 2011; ENV/JM/MONO(2012)10/PART1; ENV/JM/MONO(2012)10/PART2; Enoch and Cronin 2010; ENV/JM/MONO(2011)6; Schultz et al., 2011; Hill, 1965.; US EPA,2005; US EPA, 2011) . These papers dealt with a variety of aspects of AOPs including their derivation from existing data to techniques for reverse-engineering AOPs from genomics data. A Workshop, organised by the OECD on Using Mechanistic Information in Forming Chemical Categories was held in December 2010 in Washington DC. It resulted in a number of recommendations and conclusions for the near term (i.e. subsequent two years). These recommendations were to:

1. engage toxicologists and other scientists in discussions of AOPs in an effort to foster interactions by developing AOPs for well-established effects (e.g. skin sensitisation),
2. complete the proofs of concept that began with the December 2010 workshop by developing AOPs for the several different longer-term health and ecotoxicological endpoints,
3. develop a strategic plan for identifying, assessing and advancing AOPs and their integration into the OECD QSAR Toolbox and to include development of:

   a) an information template which can be used for developing and assessing AOPs,

   b) a set of guiding principles for assessing the completeness and acceptance of an AOP, and

   c) a format for attaining mutual acceptance of an AOP, as well as
4. harmonise the terminology associated with AOPs (OECD 2011, ENV/JM/MONO(2011)8).

In response to recommendation 2, the OECD developed an AOP for protein binding leading to skin sensitisation. Figure 2 presents the flow diagram of the pathways associated with skin sensitisation (OECD (2012), ENV/JM/MONO(2012)10/PART1; ENV/JM/MONO(2012)10/PART2)

Recently, the OECD has drawn on the experience gained in developing the AOP for skin sensitisation initiated by covalent binding to proteins (OECD 2012; ENV/JM/MONO(2012)10/PART1; ENV/JM/MONO(2012)10/PART2). Based on that experience and in an effort to address recommendations (3) and (4), the following document was developed. Since this is a new program at OECD, the guidance

given is general and many questions are likely to be raised which will only be able to be answered as more experience is gained.

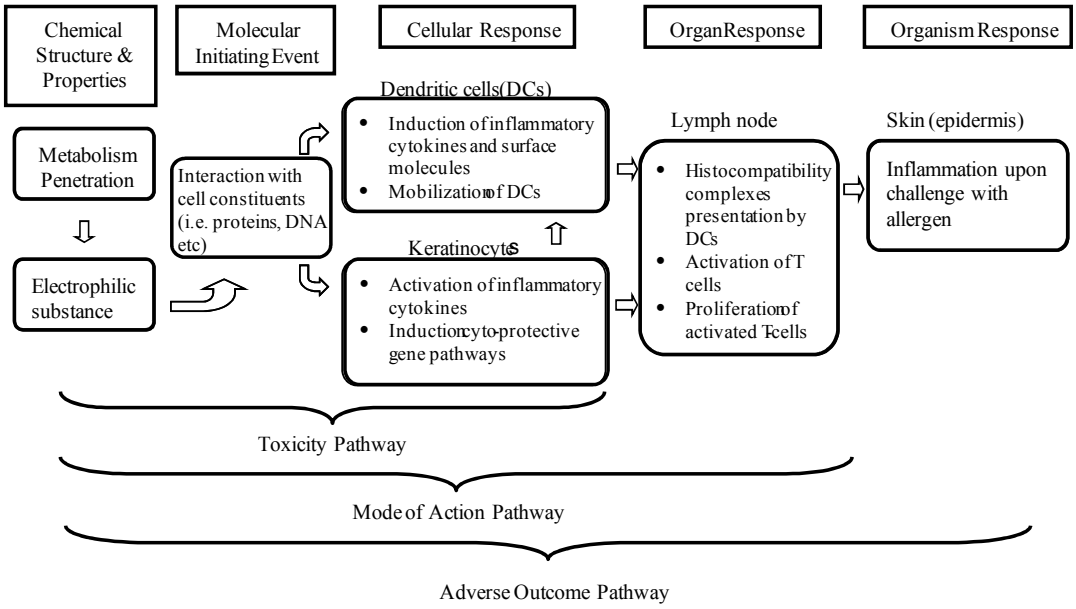

Figure 2. Flow diagram of the pathways associated with skin sensitisation (adapted from (OECD 2012, ENV/JM/MONO(2012)10/PART1).

AOPs are typically represented sequentially, moving from one key event to another, as compensatory mechanisms and feedback loops are overcome. An AOP is often applied following a so-called 'bottom-up approach', where chemistry and mechanistic information are initially used in the process of hazard identification. An AOP can also be used in a 'top-down approach', by taking the final adverse outcomes produced by well studied substances and establishing MOA, then using information to develop chemical categories such as in the International Programme on Chemical Safety (IPCS) conceptual framework for evaluating a mode of action (Sonich-Mullin et al., 2001).

Briefly, in developing an AOP, available information is collected and presented in a structured way which can aid in identifying gaps in the knowledge. Typically, this is first done at the case study level for a single chemical and then by expanding the information to a category of chemicals. Once the anchor points of the MIE and the final adverse effect are identified, the task is filling in the intermediate events in between the two anchors.

Whilst AOPs may be depicted with a single axis (e.g. level of biological organization; see Fig. 1), toxicity is multi-dimensional (e.g. gender, species), so the pathway between a MIE and the final adverse effect can vary significantly. This is especially true for more 'complex', longer-term endpoints, where effects are the result of multiple organ interactions (e.g. skin sensitisation), multiple events (e.g. repeated dose toxicity), accumulation over time (e.g. neural toxicity), or are related to a specific life stage of an organism (e.g. developmental toxicity). Nonetheless, although a number of biochemical steps are required for a toxic response to be realised, the MIEs are a prerequisite for all subsequent steps (Enoch and Cronin, 2010). With that said, it is understood that a single MIE may impact several signalling cascades and, based

on current knowledge, these signalling cascades may cause opposing events; one being adaptive and the other being maladaptive (e.g., decreased protein expression of caspace-3, with concurrent activation of caspace-6). Additionally, an AOP is based on the fact that chemical interactions are at the molecular level and not at the whole organism level. Thus, adverse effects observed *in vivo* are the result of biological cascade initiated by the chemical structure of the toxicant.

A particular MIE may lead to several final outcomes and, conversely, several MIEs may lead to the same final outcome. So neither MIEs nor final apical outcomes should be mixed together in a single AOP. Hence, where appropriate, an AOP should be designed to support an evaluation focusing on just one MIE and a single final outcome. However, it should be noted that each component of this pathway may itself be influenced by other pathways ongoing within the biological system being modelled.

The aim of this document is to provide the framework for consistent information gathering and organisation into an AOP, including a glossary of definitions for AOP-specific terminology. The document intends to provide an insight into which pieces of information are necessary to identify an AOP and how to present them. It will also provide initial assistance on how to undertake the assessment of an AOP in terms of its relevance and adequacy.

It is realised that definitions and a checklist and / or evaluation framework will need to be established to help determine sufficiency for purpose, as the level of uncertainty which can be tolerated and the level of evidence (e.g. detail, quality, and quantity of information and data) needed to be presented in the AOP depends on the targeted use of the AOP. This effort will subsequently be performed at OECD in conjunction with the Extended Advisory Group on Molecular Screening and Toxicogenomics, which has primary responsibility for approving a submitted work plan on a particular AOP, assessing the fit for purpose of the AOP and seeking member country approval.

THE DOCUMENT ALSO BRIEFLY OUTLINES THE POTENTIAL USE FOR REGULATORY PURPOSES OF AOP. DETAILED GUIDANCE ON HOW TO USE AOPS FOR INTEGRATED TESTING STRATEGIES AND RISK ASSESSMENT WILL BE DEVELOPED IN THE FUTURE.

THE USES OF AOPS

While the ultimate goal is to use AOPs in risk assessment, with the exception of a few specific cases, the level of information currently available is not sufficient to allow for risk assessment. However, a well-identified AOP, with an accurately described sequence of events through the different levels of biological organisation in organisms, provides valuable pieces of mechanistic information which can be used for many purposes (OECD 2011, ENV/JM(2011)6). By identifying and describing the key events, AOPs could inform the work of the OECD Test Guideline Programme. For example, in the Keratinosens assay (gene expression in human keratinocytes) and the h-CLAT assay (cell surface marker (CD86) expression in human monocytic cells), two methods identified in the AOP for protein binding leading to skin sensitisation have been proposed to OECD for test guidelines development. In addition, an AOP, for any given final endpoint, can be the basis for developing an integrated approach to testing and assessment (IATA) or an integrated testing strategy (ITS) for that endpoint. The application of alternative approaches, such as the read-across, where categories are first formed and data gaps filled within the category, will lead to the refinement, reduction and/or replacement of conventional *in vivo* testing.

AOPs can be inputs which address a number of decisions. While not limited to, they include: (1) priority setting for further testing, (2) hazard identification, (3) classification and labelling and, (4) risk assessment. As such, as one proceeds from (1) thru (4), the level of uncertainty which can be tolerated decreases and the level of evidence (e.g. detail, quality, and quantity of information and data) presented in supporting the AOP increases.

A partial AOP (i.e. one where not all key events are known), such as may come from a scoping exercise, may be useful in priority setting for further testing and development. Similarly, partial AOPs may be used in hazard identification, as is currently performed with the OECD QSAR Toolbox.. At this time, physiologically-based pharmacokinetic (PBPK) modelling and toxicokinetics information on absorption, distribution, metabolism, and excretion (ADME) are out of the context of the AOP but will have to be addressed to develop a quantitative AOP required for a complete risk assessment.

A qualitative AOP is one where the key events have been identified but methods for assessing these events have not been identified and/or assessed in sufficient detail to allow for identification of the applicability domains, threshold values and/or the response relationships to other key events. In contrast, a quantitative AOP is one where the methods for assessing the key events have been identified and sufficient data generated to identify the applicability domain, threshold values and/or the response relationships with other key events. Potential uses for AOP within OECD are described in Sections 3.1 through 3.3.

Developing Chemical Categories and Further Development of the OECD QSAR Toolbox

As demonstrated, for protein binding leading to skin sensitization in Version 3.0 of the OECD QSAR Toolbox, AOPs can be used to develop and refine chemical categories. In this example, three sets of information are collated and integrated: (1) a library of *in vivo* effects typically used in assessments (e.g. EC3 values in the local lymph node assay), (2) a library of MIEs (e.g. protein binding reaction, and (3) a library of intermediate events, typically data generated using *in vitro* methods (e.g. dendritic cell surface biomarkers) (OECD 2012, ENV/JM/MONO(2012)10/PART1; ENV/JM/MONO(2012)10/PART2.). Each endpoint can, in theory, be associated with a single or multiple chemical domain(s). With regard to chemical categories, the chemical structural space covered, or applicability domain, is reliant to the chemicals assessed for the MIEs and the key events within the AOP. The addition of such relevant *in vivo* assays and data is an important part of the overall weight-of-evidence supporting the prediction.

The Test Guideline Programme

By identifying and describing the key events, the AOPs could inform the work of the Test Guideline Programme. Indeed, when the key events are identified, one could propose the development of *in vitro* and *ex vivo* assays that detect direct chemical effects or responses at the cellular or higher levels of biological organisation, as well as screening assays for targets related to the molecular initiating events identified (OECD 2011, ENV/JM/MONO(2011)8). Conversely, by linking proposals for the development of *in vitro* test methods to key events in an AOP, the relationship to hazard endpoints relevant for regulatory purposes can be established.

Development of Integrated Approaches

An AOP, for any given hazard endpoint, can be the basis for developing an integrated approach to testing and assessment (IATA) or an integrated testing strategy (ITS) for that endpoint. An AOP could assist in determining what further information (and therefore, which test, if any), would increase the certainty of linking the initiating event and adverse effect(s). Moreover, a well established AOP can be used for species-to-species comparisons. The application of IATA and ITS may also lead to the refinement, reduction and/or replacement of conventional *in vivo* testing.

DEVELOPMENT OF AN ADVERSE OUTCOME PATHWAY (AOP)

Identification of the Main Blocks of Information of an AOP

To identify the information associated with an AOP, the concept of a "template" is presented here. This template guides the acquisition of knowledge necessary to inform and evaluate an AOP. The AOP template consists of three main information blocks: the MIE (molecular initiating event), intermediate events and the final adverse effect (Fig. 2). For any AOP, each of the three main information blocks should be clearly identified.

While the development of the AOP can be started from any of these blocks, depending on what knowledge is available at the beginning of the exercise, typical AOP development begins with either the MIE or the final adverse effect. The latter reflects the fact that an AOP is anchored at its two ends by the chemical/biological interaction (i.e. MIE) and final adverse effect that is of regulatory interest (e.g. repeated dose liver fibrosis).

The MIE should explain how the chemical being assessed interacts with biological (macro) molecules. This information allows for an initial description of the molecular structure limitations for chemical category members acting in a similar manner.

The identification of the final adverse effect relevant to the assessment is another crucial aspect in the development of the AOP. It is essential to define this final adverse effect clearly, as it determines the most relevant mechanistic information and, thereby, intermediate effects related to this endpoint. Usually, the final adverse effect is associated with an *in vivo* OECD Test Guideline. A given final adverse effect will be associated with a finite set of possible MIEs. Similarly, a given MIE will be associated with a finite set of possible final adverse effects. However, each AOP will have only one MIE and one final adverse effect (i.e. the two anchors of the AOP; Fig.2).

The third block of information is the intermediate effects. From the intermediate effects, the key events in the AOP are identified. By using methods for assessing the key events (in vitro methods, test methods), scientific evidence is gathered to support or refute the AOP and add weight-of-evidence to the assessment.

To develop the AOP, different types of data can be utilised. These include: Structural alerts that reflect the types of chemicals that can initiate a pathway, *in chemico* methods that measure the relative reactivity or chemical-biological interactions, *in vitro* assays that confirm the subsequent cellular responses (e.g. gene expression), *ex vivo* and *in vivo* mechanistic tests and, ultimately, *in vivo* tests that measure the endpoint(s) that are directly relevant to the adverse effect that drives regulatory decision making (OECD 2011, ENV/JM(2011)6). This information can be used to identify key events in the AOP and provide scientific evidence supporting the AOP. Thus, the AOP provides the scientific basis for linking the effects in different dimensions (e.g. at different levels of biological organisation) to the final endpoint of the AOP.

Figure 2. A Schematic Diagram for the development of an AOP.

The method to record an AOP is yet to be fully agreed upon. It is likely that an AOP could be developed through a wiki-based tool, such as Effectopedia, which is a graphical pathway tool to record information relating to an AOP (see definition in Annex 1).

Identification of the Adverse Effects

Adverse effects can be defined based on a variety of dimensions (e.g. duration of exposure, gender, specie). While the final adverse outcome is notably an anchor of a particular AOP at the individual or population level, there is the potential for AOPs where the phenotypic expressions at higher levels of organization (e.g. organs and above) are so varied as to be of limited value in defining the AOP. For example, for AOPs used for basic cellular process, such as cell proliferation and differentiation or cellular energetics, the adverse effect may be best evaluated at the cell or tissue level. In any case, it is essential to clearly and precisely define the final adverse effect relevant to the assessment, as it is one of the anchors of the AOP. This helps to define the mechanistic sequence of events leading to this outcome. The adverse effects can also be divided into: (1) long term health endpoints, where effects are the results of multiple events (e.g. repeated dose toxicity), accumulation over time (e.g. neural toxicity) or are related specifically to a particular life stage of the organism (e.g. developmental toxicity), (2) local effects, where MIEs are likely to be closely aligned with the *in vivo* outcome (e.g. skin sensitisation, skin and eye irritation). It is essential to clearly and precisely define the final adverse effect as one of the anchors of the AOP. This helps to define the mechanistic sequence of events leading to this outcome.

Definition of the Molecular Initiating Event (at the Site of Action)

Chemical-induced perturbations of biological systems are at the molecular level. Most chemicals can interact with more than one molecular target. The molecular initiating event represents a primary anchor or "the foundation" of the AOP, therefore, it is very important to identify clearly the beginning of the cascade leading to the specified final adverse effect relevant to the assessment. Many MIEs are defined in the form of covalent binding to proteins and/or DNA. These types of MIEs are based on the principles of organic chemistry (i.e. electrophile-nucleophile reactivity). In contrast, 'receptor binding' or binding to enzymes are often based on non-covalent interaction, which are more selective in nature. Chemicals have different affinities for different targets. If internal exposure is sufficient to saturate a binding site on a receptor or enzyme, then the potency of activation or inhibition of an activity is what might drive toxicity. The understanding of the MIE allows for the definition of the properties of chemicals inducing the perturbation, such as bioavailability, structural requirements (especially for receptor binding) and metabolic transformation. The understanding of the chemistry of potential inducers helps to define the molecular structure limitations for chemical category members acting in a similar manner.

In the ideal scenario, when the MIE is well-defined, not only should the potential of a chemical to elicit that event be recognised but also the likely site of action should be noted. For example, metabolic transformation of a substance to an electrophilic species may be the same for skin sensitisation and liver fibrosis but the site of action will be different (keratinocytes versus hepatocytes). For some final endpoints, especially based on receptor binding mechanisms, the identification of the site of action is very important, as the 'conformation' and other properties of the receptor define structurally the type of molecules which can bind to it. However, there are a number of final adverse endpoints for which the identification of the site(s) of action of the molecular initiating event may be quite difficult (e.g. repeated dose) or have not been defined precisely (e.g. simple narcosis in fish). However, that does not mean the AOP, with ill-defined site(s) of action, is not useful.

Recognition of Key Events Leading to the Adverse Effect

The response matrix includes the collection of intermediate events which lie between the final adverse effect relevant to the assessment and the MIE. This matrix can be quite large but experience has shown that the relationship between adjacent events often can be identified. In an ideal scenario, the response matrix should include a relatively small, or minimal, number of key intermediate events required to establish the causal linkage/connection between the MIE (anchor 1) and the final adverse effect (anchor 2). The intermediate events of an AOP are necessarily *in vivo,* as they are leading to adverse effects in whole organisms. However, a range of *in vivo*, *in vitro* information, as well as information from high-throughput screening (HTS) assays, endpoints from high-content screening (HCS) 'omics approaches and even '*in silico*' methods, may be used to provide support and data to evaluate an AOP. As the response matrix expands, the toxicological complexity becomes apparent.

Before the identification of intermediate events leading to adverse effect, an understanding of the normal physiological pathways of the AOP is essential (e.g. reproductive processes, liver functions). This will help in the recognition of complex networks of processes on the different level of biological organisation which can be disrupted. During the identification of key events, a review of the existing literature is required to find out as much information as possible about the plausible mechanism and the intermediate steps leading to the final adverse effect. This aspect is crucial for the development of the AOP. Judging the reliability and relevance of key event data may include assessing the critical parameter of the study design (e.g. exposure regime, duration of exposure, sampling time(s)) for comparison and interpretation in respect to the final adverse outcome. While automated literature mining could aid and accelerate the development of an AOP, it is not required. The important aim is that AOP development should be supported by the scientific literature and how that is accomplished should be up to those developing the AOPs. Usually, multiple intermediate events are identified. The multiplicity could be a challenge in extrapolating AOPs from one species to another. Therefore, the assembled knowledge has to be filtered and associated with a particular AOP. When a key event is present in more than a single AOP, the information can be shared between the AOPs.

Key events are steps along the pathway that represent intermediate events, typically at the different levels of biological organisation. To be a key event, the intermediate step must be able to be evaluated experimentally. That is to say, the event must be able to be used in a hypothesis which can then be tested. There are no rules as to which types of data have to or can be used to support a key event. However, such data should be reliable and relevant to the final adverse effect.

There are no specifications as to how many key events have to be defined. The number clearly depends on where in the biological organisation the final adverse effect is located (e.g. organ or population level). It is intuitive that key events at different levels of biological organisation provide a greater weight-of-evidence than multiple events at the same level of organisation. However, this may not be a case where responses transmit from one cell type to another or are potentially found in different tissues and ultimately result in an adverse effect at higher levels of organisation.

Data Summation

After the compilation of all information for the adverse outcome pathway, it is necessary to report them systematically. Part II of this document presents the template on how to report the development of the AOP.

At the outset, if possible, the collected data should be used to present the whole adverse outcome pathway step–by-step, starting from a simple characterisation of the route of exposure (e.g. aqueous, dermal, vapour and chemical properties) to the identification of the molecular initiating event and site of action. After that, the responses at the macromolecular, cellular/tissue, organ, organism, and population\ecosystem levels, if relevant, should be identified; the final stage depends on the level of biological organisation of the adverse effect. At this time, physiologically-based pharmacokinetic (PBPK) modelling and toxicokinetics information on absorption, distribution, metabolism, and excretion (ADME) are out of the context of the AOP but will have to be addressed before using AOPs in risk assessment. The initial report on the knowledge relating to the AOP is often based on one of a few well-studied model toxicants. Following this report, a concise summary of the qualitative understanding of the AOP has to be undertaken. For this purpose, the key events, documentation of the experimental support for each event together with the references, and a subjective evaluation of the weight of the scientific evidence for that event need to be listed, as summarised in Table 1.

Table 1: Summary information on the key events of the AOP.

Key Events	Experimental Support (References)	Strength of Evidence
Molecular Initiating Event		
Key Event 1		
Key Event (n-1)		
Key Event n		
Adverse Effect		

An AOP may be developed based on biological research other than the use of standardized assays. However, in any case, the reporting of the experimental support for each key event and its evaluation is very important in the AOP documentation, as it is the first step in the assessment of the current usefulness of the AOP. Therefore, there is an advantage in standardising the process of evaluating the strength or weight-of-evidence by providing some criteria. For example, weight-of-evidence should consider non-positive, as well as positive results. Generally, there is a data preference of *in vivo* over *in vitro*, as well as endpoint of interest over surrogate endpoints. In order to determine the weight-of-evidence associated with a key event, there are a number of fundamental issues which must be addressed. Broadly speaking, the evidence should be based around assays that are qualitatively or quantitatively associated with the key event. As such, the following should be considered:

1. Is the assay fit for purpose?

2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question?
3. Is the assay repeatable?
4. Is the assay reproducible?

In order to be descriptive of these issues, the following points can be considered:

1. What is the level of acceptance of the assay in the scientific and / or regulatory community?
2. What is the extent of the demonstrated causal relationship between the key event and the final adverse effect? This may, for instance, be quantified in terms of the depth and breathe of the chemicals that have been tested for both the key event and the final adverse effect.

While a final classification scheme is outside the aim of this document, Table 2 presents a proposed classification for the assessment of the weight-of-evidence associated with a particular key event, or assay.

Table 2. A proposed classification of weight-of-evidence.

Weight-of-Evidence	**Extent of Development of Assay for the Key Event / Intermediate Effect**	**Relationship Between Key Event and Apical Endpoint**
Very Strong	OECD Guideline test or an assay that has progressed through a minimum of pre-validation. A large database of results for relevant chemicals supportive of the relationship between the key event and the apical endpoint.	Clear and unequivocal relationship and mechanistic basis for it.
Strong	A well developed assay, available in a form that could allow it to be submitted for pre-validation. A database of results for relevant chemicals supportive of the relationship between the key event and the apical endpoint.	General agreement that there is a strong relationship and a mechanistic basis for it.
Moderate	A robust and reliable method published in the peer-reviewed literature. A database of results for relevant chemicals supportive of the relationship between the key event and the apical endpoint.	An understanding that there is a relationship and a probable mechanistic basis for it.
Weak	An assay is available but is in the process of development. A small number of chemicals supportive of the relationship between the key event and the apical endpoint.	An understanding that there some evidence of a relationship and a plausible mechanistic basis for it.
Very Weak	The key event is identified but no assay is available.	Hypothetical or literature based.

An additional form of data summation is the flow diagram of the intermediate events associated with the AOP (see Figure 2 as an example). This graphical version of the AOP shows visually the sequence of events at the different levels of biological organisation.

AOP Assessment

In the OECD approach to developing an AOP, it is considered critical to be able to gauge its reliability and robustness. This should be done by evaluating the experimental support of the AOP. In such an assessment, the qualitative and quantitative understanding of the AOP has to be analysed. This means that key steps should be clearly identified and the degree of scientific support described, both qualitatively and (if possible) quantitatively. For the quantitative understanding of an AOP, the threshold and scale of the causal linkage between key events in the pathway play important roles. Moreover, the assessment of the quantitative understanding of an AOP should determine the response-to-response relationships required to scale *in vitro* effects to *in vivo* outcomes. Usually, the assessment of the experimental evidence and empirical data clearly support the qualitative understanding of the AOP in the identification and characterisation of the potential inducer of the final adverse effect. However, the same assessments very often reveal hurdles in predicting the relative potency of the inducer because of the lack of necessary data. Therefore, the assessment of the quantitative understanding of an AOP is more problematic than the qualitative understanding.

The first stage of the assessment of an AOP is performed during the data summation, where every key step is documented, together with the scientific evidence and its evaluation. While the establishment of an AOP will generally be the result of experimental biological research, experimental methods to challenge and test the AOP hypothesis will be crucial to its acceptance.

An additional aspect of evaluating the AOP is the implementation of the Bradford Hill criteria (Hill, 1965); US EPA, 2005) to assess the weight-of-evidence supporting the AOP. The Bradford Hill criteria have been introduced for use within the mode of action/human relevance framework and examples have been developed for this purpose within case studies (e.g. Boobis et al., 2006; 2008; Meek et al., 2003; Seed et al., 2005). In this assessment, the author of the AOP has to make a decision with regards to the following criteria:

1. concordance of dose-response relationships;
2. temporal concordance among the key events and adverse effect;
3. strength, consistency, and specificity of association of adverse effect and initiating event;
4. biological plausibility, coherence, and consistency of the experimental evidence;
5. alternative mechanisms that logically present themselves and the extent to which they may distract from the postulated AOP. It should be noted that alternative mechanisms of action, if supported, require a separate AOP;
6. uncertainties, inconsistencies and data gaps.

Confidence in an AOP

The final step in the reporting of the AOP is a statement regarding the confidence associated with this AOP. Confidence in an AOP is increased by a more comprehensive understanding of the nature of the interaction between the chemical and the biological system, coupled with mechanistic understanding of the biological response. The confidence is ascertained by addressing the following question(s):

How well-characterised is the AOP? To include addressing:

1) How well-characterised is the MIE?

2) How well-characterised is the apical outcome?

3) How well are the initiating and other key events causally linked to the outcome?

4) What are the limitations in the evidence in support of the AOP?

5) Is the AOP specific to certain tissues, life stages / age classes?

6) How much are initiating and key events conserved across species?

In summary, an AOP should be based on a single, defined MIE and linked to a stated *in vivo* hazard or final adverse effect. During the development process of the AOP, few or more toxic pathways could be determined that can be linked to the same or different MIE(s), but in the end, a single AOP linked to the specific initiating reaction should be identified.

An AOP may be considered either plausible or probable, depending upon the extent (i.e. depth and breadth) of the available scientific evidence supporting the AOP and the extent to which the key events have been experimentally tested and found to be consistent with data for other key events. Accordingly, an AOP may be considered a dynamic entity, as it can be continually updated and refined as new information is incorporated into the general understanding of the pathway. An evaluation of the scientific evidence supporting a proposed AOP can be conducted by answering a predetermined set of questions.

Minimal Information Requirements for an AOP

It is important to define the minimal requirements for information associated with the AOP developed. The acceptance of the AOP requires an understanding of critical processes or key events measured along the pathway. The essential steps in establishing an AOP are the establishment of the MIE and final adverse effect, as these are the anchors of an AOP. Therefore, it is important to identify the chemical-biological interaction and the outcome elicited by this MIE. The identification and characterisation of key events depends on the level of knowledge reported about the final adverse effect. There are examples of relatively well-recognised endpoints, such as skin sensitisation, for which the AOPs are accurately developed. However, it has to be kept in mind that for many endpoints, there is a lack of relevant information allowing for the definition of the sequence of events leading to the final endpoint of interest. In this case, it is important to have a mechanistic understanding between the MIE and final adverse effect. Furthermore, it is necessary to understand the basis of normal physiology (e.g. nervous system function, reproductive processes, differentiation of tissues) of the cells, tissues, organs, etc., associated with the AOP. The AOPs identified must not contradict any steps of normal biological processes, since they need to be biologically plausible. Even if some steps are not known with certainty, the overall process must agree with what is known about the particular biology being considered (US EPA, 2011). It is important to understand the causal linkages and scaling factors between events as the pathway moves up the level of biological organisation, especially for events which affect the potency in the *in vivo* outcome (Schultz, 2010). As the process of AOP development proceeds and more are recorded, there will be a better understanding of what may, ultimately, constitute the minimum requirements of an AOP for a particular use. The absolute minimum is the MIE and the final adverse effect. However, recommendations of such minimum requirements are wanting at this time.

EXAMPLES OF THE AOP DOCUMENTATION

During the last few years, there has been a growing interest in AOPs as a transparent causal linkage between the exposure and the final adverse effect. To date, a small number of AOPs have been proposed including: Skin sensitisation initiated by covalent binding to proteins (OECD 2012, ENV/JM/MONO(2012)10/PART1; ENV/JM/MONO(2012)10/PART2); voltage gated sodium channels mediated neurotoxicity (OECD 2011, ENV/JM/MONO(2011)8); oestrogen receptor-mediated reproductive impairment (OECD 2011, ENV/JM/MONO(2011)8); acute aquatic toxicity initiated by weak acid respiratory uncoupling (OECD 2011, ENV/JM/MONO(2011)8); haemolytic anaemia induced by anilines following repeated dose exposure and nephrotoxicity induced by 4-aminophenols (OECD 2011, ENV/JM/MONO(2011)8); cardiotoxicity in fish induced by 2,3,7,8-tetrachlorodibenzeno-p-dioxin (Volz et al., 2011); limb defects induced by disruption of embryonic blood vessel development (Knudsen. and Kleinstreuer, 2011) reproductive toxicity in fish caused by activation of the estrogen receptor (Ankley et al., 2010); acute lethality in aquatic organisms associated with photoactivation of polycyclic aromatic hydrocarbons (Ankley et al., 2010); depressed egg production in fish through inhibition of vitellogenin production via multiple MIE (Ankley et al., 2010), and acute lethality of chemicals to aquatic organics via narcosis (Ankley et al., 2010). Many of the AOPs can be found in the report from a recent OECD workshop (OECD 2011, ENV/JM/MONO(2011)8). In analysing all these documents, significant differences can be identified in the documentation of the AOPs. Different levels of information are available among these reports; for some of them, no clear assessment of the AOP is made. This confirms the importance of the standardisation procedure during the development and documentation of an AOP.

The recently developed AOP for skin sensitisation initiated by covalent binding to proteins demonstrated the application of the AOP template and gave an example of the completed AOP (OECD 2012, ENV/JM/MONO(2012)10/PART1; ENV/JM/MONO(2012)10/PART2). During the development of AOPs, some problems have become apparent, such as the identification of relevant literature and the assessment of AOPs' completeness by identifying uncertainties, inconsistencies and information gaps. The primary strategy to address these problems is likely to be targeted tested.

CONCLUSION

To implement a predictive strategy for risk assessment, results from *in vitro* toxicity assays focused on cellular responses to MIEs will need to be extrapolated to effects on organisms and ultimately to populations. This can be achieved by developing the AOP which causally links an MIE with adverse effects. As they are intended to be used by the regulatory agencies, it is important to standardise the way in which AOPs will be developed and documented.

The AOP should provide a transparent, mechanistically-based framework for developing or refining chemical categories, as well as proposing and prioritising targeted *in vitro* and *in vivo* testing. By understanding the likelihood of effects at the chemical level and/or lower levels of biological organisation from structure-activity relationships (SARs) and *in chemico* and *in vitro* assays, one could efficiently determine if additional tests at higher levels of biological organisation (e.g. *in vivo* assays) are required (Meek et al., 2011). The guidance provided here, along with incorporation of evolving mode of action analysis to be presented in the Mode of Action framework updated by WHO/IPCS in 2012, will assist in advancing consideration of quantitative risk assessment in the decision making process. However, whilst the potential has been shown (e.g. the work of Meek et al., (Meek et al, 2011), the depth and breadth of

available data does not currently allow for a large proportion of decisions associated with quantitative risk assessment to be made.

As indicated by Bauch et al., not all key events in an AOP may have to be satisfied in order to make an assessment (Bauch et al., 2011). Recommendation of an AOP for a particular use will involve consideration of the information concerning the MIE, other key events, and the final endpoint, which is the basis of the assessment, as well as the weight-of-evidence for each event in the AOP. What is considered sufficient knowledge of an AOP will be use-dependent, with a greater knowledge required for applications with greater potential impact (Meek et al., 2011). For the development of an IATA or an ITS, a consistency across several levels of biological organisation, including causal linkage between the adverse effects, is likely to be required. However, for refinement of a chemical category such as is done within the OECD QSAR Toolbox, the understanding of a few or a single key event may be sufficient to group potential chemicals inducing the final adverse effect.

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PART II

THE AOP TEMPLATE

To standardise the documentation of an AOP, a scheme on how to conduct this process is proposed. The author(s) of the AOP should, if possible, fill every field in the AOP template. If the field is not pertinent to the proposed pathway, for example, the final adverse outcome is localised at the organ level, so the identification of responses on the higher level–individual or population/ecosystem is not appropriate then it should be stated as not applicable. In addition, instances where information is lacking should be stated clearly.

1. The Adverse Outcome Pathway Identifier

Name the AOP by defining a clear and concise the final adverse effect together with MIE.

2. Date of Declassification of AOP

Report the date (day/month/year) of AOP declassification.

3. Date of Updating the AOP

Indicate the date (day/month/year) of any update of the AOP. The AOP can be updated for a number of reasons, such as additions of new information and corrections of information.

4. The Introduction

Give short background on the current knowledge about the final endpoint of interest.

5. Summary of the AOP

Report briefly the knowledge about the AOP following steps:

5.1. Characterisation of the exposure

Define the route of exposure.

5.2. Characterisation of chemical properties

Identification of properties and/or processes required to initiate the MIE (bioavailability, reactivity, metabolism).

5.3. Identification of the molecular initiating event

Name and describe the MIE.

5.4. Identification of the site of action

Name the site of the chemical (re)actions which initiates the AOP.

5.5. Identification of the responses at the macromolecular level

Describe how the biochemical pathway(s) is affected by the interaction of the chemical(s) with the molecular target.

5.6. Identification of the responses on the cellular/tissue level that may be an adverse outcome or linked to the final adverse outcome

Describe the cellular/tissue outcomes, based on available information.

5.7. Identification of the responses on the organ level that may be the final adverse outcome or linked to the final adverse outcome

Describe the organ level responses, based on available information.

5.8. Identification of the responses on the organism level that may be the final adverse outcome or linked to the final adverse outcome

Describe the key organism response, based on available information.

5.9. Identification of the overall effect on the population or ecosystem that may be the final adverse outcome or linked to the final adverse outcome

Describe how the population or ecosystem is affected by the pathway.

6. Summary of the Key Events of the AOP

Summarise the qualitative understanding of the AOP by listing them in a table that summarises the key events, documentation of the experimental support for each event, and a subjective evaluation of the strength of the scientific evidence for that event (See Table 1 and Table 2).

Include also a flow diagram of the intermediate events associated with AOP (See Figure 2 as an example).

7. Scientific Evidence Underlying the AOP

Include any available information underlying the steps/key events in the AOP. This can include any type of data: in vivo, in vitro, in silico, in chemico, toxicogenomics etc. Each key event should be considered separately in a single sub-section.

8. Assessment of the AOP

8.1. Assessment of the weight-of-evidence supporting the AOP

Answer the Bradford Hill criteria:

***8.1.1.*Concordance of dose-response relationships**

Report any reference/study giving evidence of dose-response relationship.

***8.1.2.*Temporal concordance among the key events and adverse effect**

Describe the agreement between the sequences of biochemical and physiological events leading to the final adverse effect together with the evidence in the literature.

***8.1.3.*Strength, consistency, and specificity of association of final adverse outcome and MIE**

Give the scientific evidence on the causal linkage between initiating event and final adverse outcome.

8.1.4. Biological plausibility, coherence, and consistency of the experimental evidence

Explain the logic, coherence and consistency along with the experimental data supporting the AOP. Describe how the experimental evidence is logical and consistent with the mechanistic plausibility proposed by the theory explaining the initiation of the final adverse effect. If possible, describe the coherence of experimental results for multiple chemicals across different species.

8.1.5. Alternative mechanism(s) or MIE(s) that logically present themselves and the extent to which they may distract from the postulated AOP. It should be noted that alternative mechanism(s) of action, if supported, require a separate AOP.

Report other possible mechanisms that can lead to the final adverse effect and state if they can be covered by this AOP.

8.1.6. Uncertainties, inconsistencies and data gaps

Include any uncertainties about the experimental details, such as uncertainties regarding the differences in sensitivity of different biological targets (e.g. protein binding: cysteine versus lysine, teratogenicity: Type I pyrethroid versus Type II), the measurements of biological activity in different assays. Describe inconsistencies within the reported data, such as differences between in vivo responses for very similar chemicals, and report any data gap that causes the weakness of the AOP.

8.2. Assessment of the quantitative understanding of the AOP

Include an evaluation of the experimental data and models to quantify the molecular initiating event and other key events. If possible, describe transparent determination of thresholds and response-to-response relationship to scale in vitro and in chemico effects to in vivo outcomes.

9. Confidence in the AOP

Discuss the summary of the scientific evidence supporting the AOP by answering the following questions:

9.1. How well characterised is the AOP?

Describe how well the final adverse effect is understood qualitatively and quantitatively.

9.1.1. How well characterised is the MIE?

Describe how clearly the molecular initiating event is identified.

9.1.2. How well characterised is the AO?

Describe the relevance of the final adverse effect to the regulatory purpose.

9.1.3. How well are the initiating and other key events causally linked to the outcome?

Give short statement on the relationship between each key event and the final adverse effect.

9.1.4. What are the limitations in the evidence in support of the AOP?

Indicate any lack or disagreement in the scientific evidence supporting the AOP.

9.1.5. Is the AOP specific to certain tissues, life stages / age classes?

Indicate if there are critical life stages, where exposure must occur, to results in the final adverse effect. Or specify if there are key events along the pathway which are dependent on the life stage,

although the AOP is known to be initiated regardless of life stage. Indicate also if the AOP is associated also with age- or sex-dependence.

9.1.6. How much are initiating and key events conserved across species?

State if the key events for this AOP appear to be conserved across any group of animals (e.g. mammals) or if it appears only to be relevant for certain groups of specie.

Some of the Bradford Hill Criteria in a weight-of-evidence approach may have to be applied in a number of places including Section 6 (Table 1 and Table 2), Section 7 on scientific evidence in supporting the AOP, and also Section 9 confidence in the AOP. This will aid in revealing the weakness in the AOP and further refinements needed especially for a quantitative AOP.

10. References

List the bibliographic references to original papers, books or other documents used to support the AOP.

ANNEX I: GLOSSARY OF TERMS RELATED TO ADVERSE OUTCOME PATHWAYS

Contents

1. Introduction

Over the past half decade, a variety of approaches have been proposed to incorporate mechanistic information into toxicity predictions. These initiatives have resulted in an assortment of terms coming into common use. Moreover, the increased usage of 21st Century Toxicology, with a focus on advanced biological methods, has brought forward further terms. The resulting diverse set of terms and definitions has led to confusion among scientists and organisations. As a result, one of the conclusions and recommendations from the OECD Workshop on Using Mechanistic Information in Forming Chemical Categories (Washington DC, December 2010) was the development of a standardised set of terminology (ENV/JM/MONO(2011)8). It was recognised that such a glossary would assist in the understanding of the Adverse Outcome Pathway (AOP) concept as well as its recording, completion of the template and ultimate acceptance. Moreover, the use of a common ontology will also help to apply AOP concepts in developing QSARs and chemical categories to advance the use of predictive techniques in assessments.

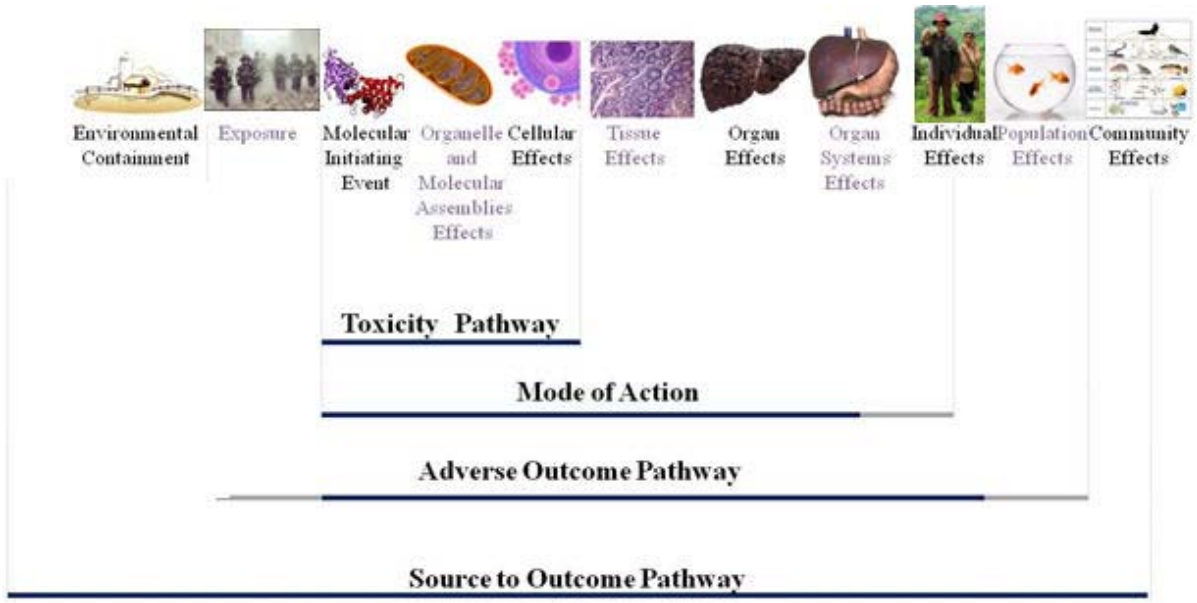

Fig. 1. Representation of the relationships between Toxicity Pathways, Mode of Action Pathways, Adverse Outcome Pathways, and Source to Outcome Pathways. The black bars represent the breadth of research common to these concepts. The gray bars represent the theoretical extent of the concepts (adapted from Croft 2010, OECD 2011 (ENV/JM/MONO(2011)8).

2. Aims

The purpose of this document is to collect existing definitions for terms relevant to the AOP concept and other pathway concepts. Whilst not inclusive, this glossary provides an illustration(s) of the various terms found to be relevant to AOP development and use during the writing of this guidance document. These terms have been collected from the literature. In many cases, there are multiple definitions of the same term, often very similar but from different sources.

Whilst the ultimate goal would be to provide a harmonised set of definitions, it is appreciated that such definitions may not be agreed upon in a formal sense in the near future. As work progresses on AOP, it will be crucial to define relevant terms precisely and clearly state the differences among them. The harmonisation of definitions is seen as a future goal.

3. Glossary

The terms are organised in alphabetic order.

Absorption (in a biological system)

Penetration of a substance into an organism and its cells by various processes, some specialised, some involving expenditure of energy (active transport), some involving a carrier system, and others involving passive movement down an (electro-)chemical gradient.

Note: In mammals, absorption is usually through the respiratory tract, gastrointestinal tract, or skin into the circulatory system and from the circulation into organs, tissues, and cells (Nordberg et al., 2004)

Adaptive Response

In the context of toxicology, the process whereby a cell or organism responds to a xenobiotic so that the cell or organism will survive in the new environment that contains the xenobiotic without impairment of function (Keller et al., 2012).

ADME

An acronym in pharmacokinetics and pharmacology for absorption, distribution, metabolism, and excretion, and describes the disposition of a pharmaceutical compound within an organism. The four processes all influence the drug levels and kinetics of drug exposure to the tissues and hence influence the performance and pharmacological/toxicological activity of the compound (Pharmacology Study Guide, 2007).

Adverse effect

A change in morphology, physiology, growth, development, reproduction, or life span of a cell or organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences (Keller et al., 2012).

Change in the morphology, physiology, growth, development, reproduction, or life span of an organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences (IPCS, 2004).

Adverse event

Occurrence that causes an adverse effect.

Note: An adverse event in clinical studies is any untoward reaction in a human subject participating in a research project; such an adverse event, which may be a psychological reaction, must be reported to an institutional review board (Duffus et al., 2007).

Adverse Outcome Pathway (AOP)

An AOP can be defined in the context of Figure 1. An AOP is a sequence of events from the exposure of an individual or population to a chemical substance through a final adverse (toxic) effect at the individual level (for human health) or population level (for ecotoxicological endpoints). The key events in an AOP should be definable and make sense from a physiological and biochemical perspective. AOPs incorporate the toxicity pathway and mode of action for an adverse effect. AOPs may be related to other mechanisms and pathways as well as to detoxification routes.

AOPs span multiple levels of biological organisation. AOPs often start out being depicted as sequential processes; however, the amount of detail and linearity characterising the pathway between a molecular initiating event and an adverse outcome within an AOP can vary substantially, both as a function of existing knowledge and assessment needs (ENV/JM/MONO(2011)8).

Representation of existing knowledge concerning the causal linkage between the molecular initiating event and an adverse outcome at the individual or population levels (Ankley et al., 2010).

Each adverse outcome pathway is a set of chemical, biochemical, cellular, physiological, behavioural, etc. responses which characterise the biological effects cascade resulting from a particular MIE. The term "adverse outcome pathway" has been selected so not to cause confusion with the term "Toxicity Pathway", which is used by the US National Research Council in its document, Toxicity Testing in the Twenty-first Century: A Vision and a Strategy, where the focus is on "omics" and high throughput *in vitro* data (Schultz, 2010).

A conceptual framework that links a molecular-level initiating event with adverse effects relevant for risk assessment (Villeneuve and Garcia-Reyero., 2011).

The sequence of events between cellular response and adverse outcome on an individual organism or population of organisms is an AOP (Watanabe et al.,2011).

Adverse response

Changes that occur that result in impairment of functional capacity, often due to an insult that exceeds the capacity of the adaptive response to permit a return to the homeostatic state. Outcomes might include changes in morphology, development, lifespan, or growth of the organism. Although harder to define at the molecular level, potentially adverse responses might include alternations in gene expression, protein synthesis, or cell regulation (Council of Canadian Academies, 2012).

Apical (final) endpoint

Traditional, directly measured whole-organism outcomes of exposure in *in vivo* tests, generally death, reproductive failure, or developmental dysfunction (Villeneuve and Garcia-Reyero ,2011). It is noted that the list noted in (Villeneuve and Garcia-Reyero, 2011) is not inclusive and other measurements (e.g. e.g. cancer or neoplasia, organ system dysfunction, immune effects can be apical endpoints.

Observable effects of exposure to a toxic chemical in a test animal. The effects reflect relatively gross changes in animals after substantial durations of exposure (North American Free Trade Agreement NAFTA, 2011).

An observable outcome in a whole organism, such as a clinical sign or pathologic state, that is indicative of a disease state that can result from exposure to a toxicant (Krewski et al., 2011).

Applicability Domain[1]

The physicochemical, structural, or biological space and information that was used to develop a (Q)SAR model, and for which that model gives predictions with a given level of reliability (Netzeva et al., 2005).

The applicability domain of a (Q)SAR model is the response and chemical structure space in which the model makes predictions with a given reliability (Netzeva et al., 2005).

The applicability domain defines the constraints of the training set compounds of a (Q)SAR model, allowing a user to choose the most suitable model, or use a given model within its own predictive capacity (Hewitt. and Ellison, 2010).

The domain of applicability of a (Q)SAR model is the chemical structure and response space in which the model makes predictions with a given reliability. It can be thought of as a theoretical region in multi-dimensional space in which the model is expected to make reliable predictions. It depends on the nature of the chemicals in the training set, and the method used to develop the model and helps the user of the model to judge whether the prediction for a new chemical is reliable or not (North American Free Trade Agreement NAFTA, 2011).

Bioavailability

Fraction of an administered dose that reaches the systemic circulation or is made available at the site of physiological activity. Usually, bioavailability of a substance refers to the parent compound, but it could refer to its metabolite. It considers only one chemical form. Please note: bioavailability and absorption are not the same. The difference between e.g. oral absorption (i.e. presence in gut wall and portal circulation) and bioavailability (i.e. presence in systemic blood and in tissues) can arise from chemical degradation due to gut wall metabolism or efflux transport back to the intestinal lumen or presystemic metabolism in the liver, among other factors (Barton et al., 2006).

Bioavailability of the toxic component (parent compound or a metabolite) is a critical parameter in human risk assessment (high-to-low dose extrapolation, route-to-route extrapolation) for derivation of an internal value from the external no observed adverse effect level. For liver effects upon oral administration, it is the oral absorption that suffices. However, for every effect other than at the portal of entry, it is the bioavailability that is in general a more reliable parameter for further use in risk assessment, not the absorption (OECD, 2010).

For the purpose of risk assessment it is essential to know the amount of chemical which is systemically available. The amount found penetrated (passing the skin barrier and absorbed by the living epidermis) has to be taken as bioavailable (Steiling et al.,2001).

Biochemical pathway

A series of reactions, typically enzyme-catalysed that are associated with a specific physiological event in a living organism (Council of Canadian Academies, 2012).

Bioinformatics

[1] All available definitions for Applicability Domain apply to QSARs; however, this term can be used for most of the current *in vitro* methods as well as for *omics*. Therefore, there is a need to develop definitions suitable for AOPs in the future.

Use of information science to integrate diverse, complex data generated by life sciences and organise it in an understandable context (Villeneuve and Garcia-Reyero, 2011).

The interpretation of complex multivariable data from High through-put screening and genomic assays in relation to target identification and effects of sustained perturbations on organs and tissues (Andersen et al.,2010).

Biomarker

A biochemical, physiological, or histological change or aberration in an organism that can be used to estimate either exposure to stressors or resultant effects (Villeneuve and Garcia-Reyero, 2011).

A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of physiological as well as pathological process or pharmacological response to a therapeutic intervention (Jain, 2010).

An indicator signalling an event or condition in a biological system or sample and giving a measure of exposure, effect, or susceptibility.

Note: Such an indicator may be a measurable chemical, biochemical, physiological, behavioural, or other alteration within an organism (Nordberg et al., 2004).

A change in a biological response (ranging from molecular through cellular and physiological responses) that can be related to exposure to, or toxic effects of, environmental chemicals (Huggett et al., 1992).

Cellular response

The binding of a chemical signals to the corresponding receptors and induces events within the cell that ultimately change its behaviour. The nature of these intracellular events differs according to the type of receptor. Also, the same chemical signal can trigger different responses in different cell types (http://global.britannica.com/EBchecked/topic/101396/cell/37445/Cellular-response).

Chemical category

A group of chemicals whose physico-chemical and human health and/or environmental toxicological properties and/or environmental fate properties are likely to be similar or follow a regular pattern as a result of structural similarity (or other similarity characteristic) (OECD, 2007).

Computational toxicology

A research area that is melding advances in molecular biology and chemistry with modelling and computational science in order to increase the predictive power of the field of toxicology (Kavlock et al., 2008).

Integration of modern computing and information technology with molecular biology to improve (United States Environmental Protection) Agency prioritisation of data requirements and risk assessment of chemicals (U.S. EPA, 2003).

A discipline at the interface of chemistry, biology, pharmacology and toxicology. It is a relatively new area of research activity that merits the attention of scientists from different fields in academia and industry. The development of accurate models for the prediction of toxic effects can only be achieved through a concerted effort involving all these disciplines (Nigsch et al.,(2009).

Distribution

Dispersal of a substance and its derivatives throughout the natural environment or throughout an organism (Nordberg et al., 2004).

Final location(s) of a substance within an organism after dispersal (Duffus et al.,2007).

Effectopedia

Effectopedia is an open knowledge aggregation and collaboration tool that provides a means of describing Adverse Outcome Pathways (AOPs) in an encyclopaedic manner. Effectopedia is designed to aid scientists with different backgrounds to work on the same AOPs describing the molecular interactions of a chemical with biological systems and the biological response models that document how molecular effects lead to adverse effects at many levels of biological organisation (Veith, personal communication).

Effectopedia is a graphical pathway tool used to aggregate knowledge that provides a means of describing adverse outcome pathways (AOPs). It is an open source, wiki-based technology that has two main interfaces – one for users and one for contributors. The user interface allows the viewing of an n-dimensional AOP in any 2D relational manner. The contributor interface supplies tools for: (1) building AOPs, (2) editing the content, (3) establishing an audit trail, (4) freezing development, and (5) participating in discussions via social networks (Schultz, personal communication).

Endpoint

The recorded observation coming from an *in chemico* method, an *in vitro* assay or an *in vivo* assay (ENV/JM/MONO(2011)8).

The measurement of a chemical or biological property. A large number of endpoints are used in regulatory assessments of chemicals. These include hydrophobicity, electrophilicity, lethality, carcinogenicity, immunological responses, organ effects, developmental and reproductive effects, etc. In QSAR analysis, it is important to develop models for individual toxicity-related endpoints (North American Free Trade Agreement NAFTA, 2011).

Excretion

Discharge or elimination of an absorbed or endogenous substance, or of a waste product, and (or) its metabolites, through some tissue of the body and its appearance in urine, faeces, or other products normally leaving the body.

Note: Excretion does not include the passing of a substance through the intestines without absorption (Nordberg et al., 2004).

Key Events

Key events are intermediate events (ones between the molecular initiating event and the apical outcome) that are toxicologically relevant to the apical outcome and experimentally quantifiable (Schultz, personal communication).

Key events are additional events further along the pathway that lead to, and are experimentally or toxicologically associated with the adverse outcome (ENV/JM/MONO(2011)8).

Key events are empirically observable precursor steps that are a necessary element of the mode of action or are a biological marker for such an element (U.S. EPA, 2005), (Boobis et al., 2008).

A key event is an empirically observable precursor step that is itself a necessary element of the mode of action or is a biologically based marker for such an element (OECD, 2008).

Levels of biological organisation

The organelle, cellular, tissue/organ and organism (and when required) population (OECD 2011, Schultz, 2010).

Atom, molecule, cell, tissue, organ, organ system, organism (individual), population, community, ecosystem, biosphere (see Figure 1) (Villeneuve and Garcia-Reyero, 2011).

Mechanism of action

Denotes the sequence of events leading from the absorption of an effective dose of a chemical to the production of a specific biological response in the target organ. Understanding a chemical's mechanism requires appreciation of the causality and temporal relationships between the steps leading to a particular toxic endpoint, as well as the steps that lead to an effective dose of the chemical at the relevant biological target(s) (Schultz, 2010).

Mechanism of action for toxicity is the detailed molecular description of key events in the induction of cancer or other health endpoints. Mechanism of action represents a more detailed understanding and description of events than is meant by mode of action (North American Free Trade Agreement NAFTA, 2011).

A complete and detailed understanding of each and every step in the sequence of events that leads to a toxicity outcome, underlying the MOA (ECETOC, 2007).

Metabolism

Sum total of all physical and chemical processes that take place within an organism from uptake to elimination.

In a narrower sense, the physical and chemical changes that take place in a substance within an organism, including biotransformation to metabolites (Duffus et al., 2007).

Metabolomics

The study of chemical processes involving metabolism. Metabolomics is different from transcriptomics and proteomics because it is not related to the transcription-translation paradigm.[2] It is based on the idea that the chemical composition of biological fluids reflects the health of an organism (Schultz, personal communication).

Metabolomics deals with endogenous metabolite profiles of tissues or organs derived from mass spectrometry or nuclear magnetic resonance spectrometry analyses of plasma or homogenates. Metabolic profiling can give an immediate picture of the physiological state of tissue (OECD ,2008).

Global analysis of small molecule metabolites and their relative abundance, generally through nuclear magnetic resonance and mass spectroscopy (Villeneuve and Garcia-Reyero, 2011).

[2] Another clarifying observation that could be included is the estimated numbers of transcripts (~100,000), proteins (~ 1,000,000) and endogenous metabolites (~ 2,400) that comprise the transcriptome, proteome and metabolome, respectively. The magnitude of these numbers helps illuminate the challenges in using these techniques for AOP discovery and development.

The study of the products of biological processes. Such products change in response to such things as nutrition, stress, and disease states (National Research Council US., 2007).

Evaluation of cells, tissues, or biological fluids for changes in endogenous metabolite levels that follow *exposure* to a given substance, in order to determine the metabolic processes involved, to evaluate the disruption in intermediary metabolic processes that results from exposure to that substance, or to determine the part of the genome that is responsible for the changes (Nordberg et al., 2004)

Mode of action

The definition of MOA has evolved over time with experience in its application. MOA is currently defined by WHO as "A biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data. A mode of action describes key cytological and biochemical events – that is, those that are both measurable and necessary to the observed effect – in a logical framework." World Health Organization (2009) Environmental Health Criteria 240: Principles and Methods for the Risk Assessment of Chemicals in Food. WHO, Geneva, (Definitions page A-25). http://www.who.int/entity/foodsafety/chem/principles/en/index.html

Molecular Initiating Event

The initial point of chemical-biological interaction within the organism that starts the pathway (ENV/JM/MONO(2011)8).

Direct interaction of a chemical with specific biomolecules (Villeneuve and Garcia-Reyero, 2011).

The molecular level, chemical-induced perturbation of a biological system ((Schultz, personal communication).

Chemical interaction at a molecular target leading to a particular adverse outcome (Schultz, personal communication).

The seminal interaction (e.g. DNA-binding, protein oxidation, or receptor/ligand interaction) of a chemical with a biological target (Schultz, personal communication).

Molecular screening

Molecular screening combines rapid screening methods with toxicogenomics with the objective of applying biochemical and cellular genomic methods to category analysis. The premise of molecular screening of toxicity is driven by interactions with cellular targets of one form or another so to initially assess toxicity, one must identify the proper target of concern and an appropriate assay is needed to assess the likelihood of interaction with the chemical(s) of concern (OECD, 2008).

Non-apical endpoint

Alternative, suborganism-level, *in vitro* responses, biomarkers, QSARs, genomics (Villeneuve and Garcia-Reyero, 2011).

Intermediate event or step at a level of biological organization below that of the apical endpoint (Schultz, personal communication).

Pathway perturbation

Critical alteration of a toxicity pathway by an environmental agent or its metabolites that can impair normal biological function to such an extent that an adverse health effect may occur (Krewski et al.;2011).

Pharmacological or toxicological Screening

Pharmacological or toxicological screening consists of a specified set of procedures to which a series of compounds is subjected to characterize pharmacological and toxicological properties and to establish *dose–effect* and *dose–response* relationships (Duffus et al., 2007).

Proteomics

Proteomics deals with cell and tissue-wide expression of proteins encoded by a genome. After transcriptomics, proteomics is the next step in omics studies. It is more complicated than genomics because while a particular genome is more or less constant, the proteins that are produced differs from one cell type to another and from time to time in the same cell type (OECD, 2008).

Proteomics confirms the presence and quantifies the protein. Merrick and Bruno have termed a distinct set of expressed proteins that distinguish between health, toxicity or disease as "toxicity signature" (Merrick and Bruno, 2004).

Global analysis of proteins in a sample and their relative abundance or modifications (Pharmacology Study Guide, 2007).

The study of proteomes, which are collections of proteins. Proteins carry out the functions encoded by genes (National Research Council US., 2007).

Rapid screening methods

Rapid screening methods include techniques which assess molecular properties or *in vitro* responses. They range from simple structure-activity analyses to high-throughput *in chemico* and cellular assays, to mid-level throughput *in vitro* and ex *vivo* assays (OECD, 2008).

Response matrix

The response matrix is the collection of intermediate events which lie between the adverse effect of interest and the MIE (Schultz, personal communication).

Site of action

The site of action can be the biological molecule which interacts with chemical or can refer to the more specific site on the macromolecule of interest, such as the ligand binding domain of a receptor. The site of action also can be viewed in the context of the particular cell or tissue type in which the molecular initiating event takes place (Schultz, personal communication).

Source to Outcome Pathway

The Source to Outcome Pathway can be defined in the context of Figure 1. As such it relates to the complete understanding of the effects of a chemical substance from environmental contamination through to effects at the community level. It incorporates the AOP concept and hence toxicity pathways and MoA.

The continuum or cascade of measurable events starting from release into the environment and ending at an adverse outcome (U.S. EPA, 2005).

Structural alerts

Structural alerts are atom-based fragments which, when present in a molecule, are an indication that a compound can be placed into a particular category (Schultz, 2010).

System biology

Study of the mechanisms underlying complex biological processes as integrated systems of many diverse, interacting components.

Note: It involves (1) collection of large sets of experimental data (by high-throughput technologies and/or by mining the literature of reductionist molecular biology and biochemistry), (2) proposal of mathematical models that might account for at least some significant aspects of this data set, (3) accurate computer solution of the mathematical equations to obtain numerical predictions, and (4) assessment of the quality of the model by comparing numerical simulations with the experimental data (Duffus et al.,2007).

System biology is defined as the biology of dynamic interacting networks. It requires the use of variety of analytical platforms as well as bioinformatics, data integration, and modelling (Jain, 2010).

Study of relationships and flow of biological information between elements of biological systems, with the goal of understanding and predicting emergent properties of those systems (Hood. and Perlmutter, 2004).

Toxicity Pathway

The toxicity pathway can be defined in the context of Figure 1. As such it relates to the perturbation of a normal biochemical pathway from the molecular initiating event to the cellular effect. It is at the heart of the MoA and AOP concepts, however it is not linked directed to an apical effect.

Cellular response pathways that, when sufficiently perturbed, are expected to result in adverse health effects are termed *toxicity pathways* (NRC, 2007).

After the toxic chemical reaches a target tissue, a molecular initiating event occurs that results in a cellular response, which has been called a toxicity pathway (Watanabe et al.,2011).

Toxicogenetics

Study of the influence of hereditary factors on the effects of potentially toxic substances on individual organisms (Duffus et al., 2007).

Toxicogenomics

Toxicogenomics is an integration of conventional toxicology, bioinformatics methods and genomics and is defined as the study of the response of a genome to hazardous chemicals. (OECD, 2008).

Toxicogenomics uses the three major -omics technologies: transcriptomics, proteomics and metabolomics. (U.S. EPA, 2005).

Toxicogenomics is defined as the application of genomic technologies (for example, genetics, genome sequence analysis, gene expression profiling, proteomics, metabolomics, and related approaches) to study the adverse effects of environmental and pharmaceutical chemicals on human health and the environment. Toxicogenomics combines toxicology with information-dense genomic technologies to integrate toxicant-specific alterations in gene, protein, and metabolite expression patterns with phenotypic responses of cells, tissues, and organisms. Toxicogenomics can provide insight into gene environment interactions and the response of biologic pathways and networks to perturbations. Toxicogenomics may lead to information

that is more discriminating, predictive, and sensitive than that currently used to evaluate toxic exposure or predict effects on human health (National Research Council US., 2007).

Scientific subdiscipline that combines toxicology with genomics to determine how an organism's genetic make-up influences its response to a toxic substance (Duffus et al., 2007).

Transcriptomics

Transcriptomics deals with genome wide scale mRNA expression using DNA microarray and other high through put technologies that can estimate quantity of mRNA. (U.S. EPA, 2005).

The study of transcriptomics examines the expression level of mRNAs in a given tissue, organ or other cell population, using DNA microarray and other high-throughput technologies that can estimate the quantities of mRNAs (National Research Council US, 2007).

Transcriptomics (or gene expression profiling) is the study of mRNA—the intermediary step between genes and proteins that indicates genes that are active (as opposed to dormant or silent) (National Research Council US., 2007).

Transcriptomics (also referred to as expression profiling) uses DNA microarrays (commercially available arrays or custom ones) and a DNA copy of RNA is made using reverse transcriptase. In expression profiling gene profiles are clustered into a gene expression signature. The rationale is such signatures are more sensitive and accurate methods than outcomes (e.g. histopathology) from traditional test guidelines (Merrick and Bruno, 2004).

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