



Integrated Project

Development of a novel approach in hazard and risk assessment of reproductive toxicity by a combination and application of *in vitro*, tissue and sensor technologies

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Project summary

Validated alternative test methods are urgently required for safety toxicology of drugs, chemicals and cosmetics. While some animal tests for topical toxicity have been successfully replaced one by one by alternative methods, systemic toxicities require new test strategies in order to achieve an adequate safety level of the consumer. In the project, the European Centre for the Validation of Alternative Methods (ECVAM) takes the lead to manage the development of a conceptual framework in the area of reproductive toxicity. The involvement of all stakeholders in the Project Supervising Board including the European Consensus Platform on Alternatives (Ecopa), European regulators and Industry guarantees an efficient problem solving approach.

Reproductive toxicity offers the opportunities that: (i) to save substantial number of animals currently required in *in vivo* assays; (ii) the reproductive system can be broken down into well-defined sub-elements covering the reproductive cycle; (iii) a number of pioneering alternatives have already been developed; and (iv) the same animal experiments are carried out for drugs, chemicals and cosmetics.

The project is composed of four elements, i.e.

- (a) technological development of *in vitro*
- (b) *in silico* and sensor technologies
- (c) the strategical development of a conceptual framework
- (d) the dissemination and implementation activities

Relevance and potential impact

Reproductive toxicity refers to the adverse effects of a substance on any aspect of the reproductive cycle, including the impairment of reproductive function, the induction of adverse effects in the embryo, such as growth retardation, malformations, and death. Current regulatory data requirements that are in place to assess hazards to the reproductive system, vary considerably depending on the use category of the compound and its estimated or expected exposure. Data requirements are most stringent for pharmaceuticals, food additives and pesticides/biocides. For other chemical categories the level of data requirements increases progressively with increasing production volumes, or when other non-reproductive studies reveal indications of possible reproductive effects. Currently, a number of key phases of the reproductive cycle are covered in standardised animal tests used for regulatory purposes. These tests, however, do not provide specific information on the different aspects of the reproductive cycle. Instead, they are largely designed as apical tests where, for instance, observations of "litter size" stand for and cover: fertilisation, implantation and prenatal development.

Both Chemical Policy (REACH) and 7th amendment of the Cosmetics Directive call for the broad replacement of animal experiments on a short-term basis. Since reproductive toxicity is one of the targeted endpoints, it will be extremely important to have alternative replacement methods available within that very tight timeframe.

Concerns for the potential impact on fertility and development of so-called "endocrine disrupters" have raised a discussion on current approaches to risk assessment. A major topic was the ability of the current approaches for hazard identification to identify subtle, but nonetheless potentially severe, impairment of endocrine homeostasis in an *accurate, timely* as well as *cost-effective* way.

The overall aim of ReProTect is the integration of existing *in vitro* models and newly developed models into a test strategy that will provide detailed information on the hazard of compounds to the mammalian reproductive cycle. The test strategy will not only decrease the number of animal tests in this area of toxicology, but should also obtain more detailed information on the toxicological mechanism in different target tissues, thus providing valuable information for lead compound optimization as well as the hazard identification of substances. Furthermore, replacing a long-lasting (two generations) and costly animal experiment by *in vitro* screening methods, allows implementation at earlier stages of drug development and more efficient lead compound selection. In addition, the new approach will support the development of future testing strategies in other toxicological areas in which further organ systems and pathways are involved (e.g. carcinogenicity or chronic toxicity). In the area of basic research/drug development, single building blocks or combinations of *in vitro* tests can be individually used to answer specific questions important to the specific drug candidate.

The complementary expertise of the partners will allow the sharing of methodology developed in different centres. All partners have been working in the field of reproductive toxicology and alternatives to animal experiments and have collaborated with industrial partners. This ensures the understanding of the specific needs for industrial application of *in vitro* tests systems.

The potential impacts of the project thus are:

- A new approach for regulatory safety assessment in one of the most delicate areas, i.e. reproductive toxicity.
- The opportunity to make testing more humane and closer to the human target species, i.e. to employ human cell and tissue and substantially reduce animal use; the use of human test systems can actually increase the predictive value of test results compared to animal tests.
- Pilot the development of testing strategies for hazard assessment including the portfolio of instruments to compose, amend, and validate test strategies and test batteries.
- Develop the concepts for the validation of new technologies such as sensor technologies, QSAR and pattern-based methods such as toxicogenomics, proteomics and metabonomics.

- Develop a new type of dissemination of validated methods by e-learning and virtual training courses.
- Pilot a new type of coaching of the development of alternatives from lab bench to validation and finally regulatory consideration, by integration of all stakeholders represented in the supervising board.
- Making available a novel type of safety toxicology for drugs, chemicals and cosmetics.
- Providing reliable tests that can be used individually or combined in test strategies, which are able to answer specific questions during drug development of specific diseases related to the reproductive tissues or mechanisms.

The main innovation of the project remains the development and prevalidation of toxicological test strategies itself based on *in vitro* and *in silico* methods, and the integration into a conceptual framework.

- Conceptual framework for safety toxicology (tiered testing strategy with decision points and various branches)
- Innovative technologies to compose the test strategy (genetically engineered cells, and reporter genes) and for toxicological endpoints (proteomics, genomics, sensor technologies)
- Use of murine and human embryonic stem cells

Structure of the project

Due to the complexity of the mammalian reproductive cycle it is not possible to model the whole cycle in one *in vitro* system in order to detect chemical effects on mammalian reproduction. Therefore, the reproductive cycle has been broken down into 3 major research areas:

- Fertilization
- Implantation
- Prenatal development

Furthermore, a research area for

- Cross-cutting technologies

was formed.

The research areas consist of a different number of workpackages reflecting various aspects of reproductive toxicology. The workpackages include the development and prevalidation of *in vitro* tests that are in a various stage of test development. In some of the work packages no or only insufficiently developed *in vitro* systems are available. A workshop on the respective aspect of reproductive toxicology at the start of the workpackage, will bring together experts in these areas in order to discuss possible solutions. The development of *in vitro* models will be supported by cross-cutting technologies, which can be applied in the different research elements.

Complex tiered test strategies shall be composed and integrated in a conceptual framework for safety assessment. This entirely new approach and instrumentarium for hazard and risk assessment will have to be fully assessed as to its relevance and reliability. This conceptual framework shall be deduced and elaborated in a series of consensus workshops. In consequence, this will be utilised for other areas of systemic toxicities in the future (acute systemic, repeat-dose, chronic, carcinogenicity, toxicokinetics).

Research area 1: Fertility

Infertility is a medical problem that can be related to the man or the woman. Approximately 35 per cent of cases are due to a female problem, 35 per cent due to a male problem and in the rest of the cases the causes are unexplained. Different toxicological mechanisms involving various target organs/tissues/cells that can lead to impaired fertility have been identified. The major cellular targets covering male and female fertility have been combined in the research area “Fertility”:

- **Mature spermatozoa/Spermatogenesis (W.P. I.1.)**
Milestones: Test system for detecting germ cell mutagenicity on mature sperm cells; prevalidated test system for sperm functionality after chemical exposure.
- **Leydig cells (W.P. I.2.)**
Milestone: Genetically engineered Leydig cell line that is showing the biosynthesis of testosterone; prevalidation of model.
- **Sertoli cells (W.P. I.3.)**
Milestone: Alternative test which is detecting chemical effects on the blood testis barrier.
- **Meiotically competent oocytes (W.P. I.4.)**
Milestone: Three *in vitro* models based on bovine oocytes that can be further developed to predictive tests that can be used for analysing chemical effects on oocytes maturation, fertilisation and preimplantation of embryos.
- **Granulosa/Thecal cells (W.P. I.5.) and Folliculogenesis (W.P. I.6)**
Milestones: Two test systems that are able to detect chemical effects on the female steroidogenesis and one test system for detecting chemical effects on the folliculogenesis.

Research area 2: Implantation

This research area will practically start working at month 24 after starting ReProTect. Within the first 18 month reporting period promising *in vitro* models will be selected within a symposium. In addition, a workshop will be held in order to develop a test strategy that is defining the most sensitive target tissues/organs or biological mechanisms of this research area. Further partners will be recruited after the 18 months conference. The preparation of the uterus is most important for supporting the pregnancy. The rate of embryo transport and the stage of embryonic development must be synchronized with the preparation of the endometrium to a stage of receptivity for the blastocysts.

- **Endometrium (W.P. II.1.)**
The main aim of this project is to study the endometrial angiogenesis, and to study how different agents affect endothelial cells. The second aspect of female reproductive toxicity is covered by additional research on the uterine function, which should lead to a successful implantation of the embryo.
- **Implantation (W.P. II.2.)**
In vitro systems mirroring vessel formation and/or endometrial function, or placental function, would fill an important gap of test protocols mirroring the complete reproductive cycle. An *in vitro* model using human trophoblast and endometrium has been developed in

order to study the biology of attachment and invasion of the early embryo and the influence of hormonal therapy and xenobiotics.

- **Placental toxicity (W.P. II.3.)**
Proteomics and cDNA array technologies will be utilized to screen for gene regulatory and biochemical changes in the placenta that may have functional relevance. Step 2 in this project is to evaluate placental cultures with respect to changes found *in vivo*. A taskforce on placental toxicity will bring the experts in this field together in order to set up a workpackage on placental toxicity.

Research area 3: Prenatal Development

The third major effect on the female reproductive system is altering the viability and morbidity of the offspring. The research area “*Prenatal Development*” is taking this aspect into account. Developmental toxicity - taken in its widest sense - includes any effect interfering with normal development. It encompasses effects induced or manifested prenatally as well as those manifested postnatally. This includes embryotoxic/foetotoxic effects such as reduced body weight, growth and developmental retardation, organ toxicity, death, abortion, structural defects (reproductive effects) and functional defects. However, due to the complexity of the development of the unborn child only a test strategy on embryotoxicity testing will provide information that allows a hazard identification of a embryotoxic chemical.

Milestone of the research area: development of a strategy for prenatal development.

- **Early prenatal development (W.P. III.1.)**
The consortium will focus on the further development of the murine embryonic stem cell tests (EST; already validated for detecting chemical effects during cardiac cell differentiation), in order to detect also chemical effects on neural and skeletal tissues. Related toxicological endpoints based on histochemical and molecular biological parameter (e.g. flow cytometry and/or TaqMan PCR) will be developed. In addition, another objective is the enlargement of the database for the validated endpoints in the EST. A metabolic system will be evaluated by testing selected compounds. Adaptation of the murine system to human embryonic stem cells will provide a more precisely forecast of developmental toxic effects in humans in order to avoid inter-species variations in drug testing. Ultra-sensitive, differential protein expression analysis of differentiating ES cell cultures exposed to embryotoxic drugs and other chemicals will be used to identify a set of specific protein expression pattern.
Milestones: SOPs for induction of stem cell differentiation into neural cell types and chondrocytes and culturing human embryonic stem cells; list of test chemicals with reliable *in vivo* animal and human data; start of identification of embryotoxic-signatures on protein basis.
- **Late prenatal development (W.P. III.2.)**
This work package aims at the development and evaluation of an *in vitro* metabolising system as a pre-incubation step in the existing whole embryo culture (WEC) technique.
Milestone: Protocol for WEC including biotransformation system.

Research area 4: Cross-cutting Technologies

WP 4 represents a cross-cutting research area involving i) critical biological mechanisms shared by the research areas identified by WP 1-3 such as receptor interaction or biotransformation and ii) the development and assessment for possible validation of high-tech methodologies such as toxicogenomics, sensor technologies or QSARs. These approaches should be extended with proteomics and protein arrays to relate gene expression profiles to protein markers for confirmation of elucidated mechanisms.

- **Sensor technologies (W.P. IV.1.)**

The development of a model sensor technology will focus on the detection of early developmental effects. The first phase aims to develop microelectrode arrays to monitor ion channel activity in developing embryonic stem cells. This will identify chemicals interacting with either cardiac or neural development. In the second Phase the developed arrays will be applied to embryotoxicity testing with murine embryonic stem cells, coupled with signal processing and data acquisition. Model teratogenic compounds will be used to assess the potential for application.

Milestones: Development of microelectrode arrays to monitor ion channel activity in murine embryonic stem cells.

- **Quantitative Structure/Activity Relationships (QSARs) (W.P. IV.2.)**

The development of a QSAR will focus on the *blood testis barrier*, thus complementing and supporting the building blocks Leydig cells, Sertoli cells and spermatogenesis: Data will be compiled, collated and information on receptor mediated efflux and influx across membranes will be sought. The primary sources of information will be the principal databases on toxicology and scientific literature. Data from experiments within the project will be included as well, in the initial data set and then in the data set for validation of models. In the second phase molecular descriptors for the model compounds identified, will be calculated and assessed for their variability and reproducibility. Redundancy of the parameters and methods to reduce the number of descriptors will be evaluated. Finally, models will be developed using a number of methodologies to assess their viability (multivariate analyses, neural networks, machine learning, classification systems) with an emphasis on the development of clear and transparent models suitable for regulatory purposes.

Milestone: QSAR for blood testis barrier. Further step will be the development of QSAR for placental transfer.

- **Biotransformation (W.P. IV.3.)**

The development of metabolic systems incorporated within a particular in vitro technique is a critical step for promoting the use of in vitro methods in toxicology.

In the first phase, different metabolic systems will be evaluated with the main emphasis on the human liver (cryopreserved human hepatocytes, cryopreserved precision-cut liver slices, genetically engineered cells for expression of relevant metabolic enzymes). Such systems will be optimised and compared with freshly isolated liver tissue and S9-fraction. Attention will also be given to the compatibility of the metabolic systems with the cultured tissue and cells. In the second phase, a panel of 6 compounds will be selected to evaluate the usefulness and applicability of the co-cultures. Transfer of the relevant metabolic systems to other ReProTect partners for incorporation into individual in vitro systems will be performed with the support of the technological scout. Finally, the metabolic systems will be optimised in collaboration with the partners in order to be fully compatible with the respective *in vitro* system.

Milestone: Biotransformation system that can be combined with the validated embryonic stem cell test.

- **Array technology (W.P. IV.4.)**

Genomics, proteomics and toxicogenomics will be used to complement *in vitro* assays in areas of reproductive toxicology characterized by highly complex mechanisms (late organogenesis, developmental neurotoxicity, steroid-mediated pathways). Arrays will be selected suitable for the identification and characterization of reprotoxicants, e.g. homeobox, apoptosis, folate, retinoid and steroid pathways. Basic assays on endocrine disrupters using estrogen-dependent cell lines will be complemented by gene profiling. In collaboration with WP.III.1 (early prenatal development) changes in protein expression pattern will be analysed in order to identify a toxicological signature of model teratogenic compounds. Special attention will be given to optimise gene profiling both in the presence and in the absence of metabolic activation. Array technology will be assessed as a tool to unravel mechanisms and subcellular target sites in order to predict subtle, long-lasting developmental effects (two dimensional electrophoresis gels for developmental neurotoxicity in embryonic stem cells, quality and efficiency of microarray technology in reproductive toxicology). The methodology will be prevalidated using a limited set of chemicals (maximum 6) from various chemical classes and with different embryotoxic mechanisms.

Milestones: Reports on the gene expression profile changes and arrays relevant to early prenatal, including neural, development and endocrine disruption; preliminary assessment of partnering of gene profiling with relevant toxicological endpoints; interim reports on issues relevant to quality and efficiency relevant to microarray technology in reproductive toxicology.

- **Receptor interaction (W.P. IV.5.)**

This workpackage will take into account the recommendations from the comprehensive report of ECVAM's sister organizations ICCVAM and NICEATM on the status of *in vitro* methods for detecting endocrine disrupting function of chemicals. Advanced test methods (PALM, MLVN, ER-CALUX, AR-CALUX) and receptor binding assays will be optimised and the prevalidation study will be initiated. Furthermore, gene expression profiles will be evaluated and the possibility of further improvements of CALUX systems through incorporation of bioconversion steps will be addressed. Preliminary development of further receptor interaction-based assays will be started, including assays for heterodimerization between ER α /ER β , ER α /AhR and ER β /AhR and assays to measure the effect of repressors and coactivators on transactivational activity of ER, AR, PR and AhR.

Milestones: Optimised tests and transferred SOPs for transcriptional assays in order to detect estrogens/antiestrogens and androgens/antiandrogens. Optimised tests and transferred SOPs for receptor binding tests. Prediction models and SOPs for PALM, MVLN, ER-CALUX, AR-CALUX and receptor binding studies.

- **GLP, GCCP and *in vitro* toxicology (W.P. IV.6.)**

The OECD Principles of GLP are general and not specific to any particular type of testing discipline. Although subject to the OECD Principles of GLP, these *in vitro* methods place special emphasis on the test system and milieu (i.e. the cells, tissues, or organs in culture), with regard to verification of identity absence of contamination or defects, replicates, performance, reproducibility over time and across different types of test substances. Furthermore, quality control of the test system is an essential element in any study conducted for regulatory purposes, to ensure that the results of the study are meaningful and comparable to data from previous studies and/or across laboratories. Procedures for formal,

systematic validation of these new alternative methods have been developed by ECVAM in Europe, and ICCVAM in the United States. In addition, OECD has published practical guidance on validation for use internationally (OECD TG No.34). Emphasis will also be given on applying Good Cell Culture Practices (GCCP), to assure high scientific quality data during all the research and development phases of ReProTect.

Management activities (W.P. V.)

By following the management structure three areas have to be managed:

- **Research and development** (prenatal development, implantation and fertility)
- **Implementation of new technology and cross cutting research activities** (sensor technologies, QSARs and array technologies)
- **Development of testing strategies for reproductive toxicology** (workshops, taskforces and consensus document)

The Supervising Board

An independent board composed of 10 members at start supervises the ReProTect project. Since the R&D character of the IP especially requires the expertise of the end-users in the respective area, experts from the regulatory community and from European industry were appointed for the board. In order to ensure the complete independence of the board, neither ECVAM as a day to day manager nor the University of Tuebingen/Germany in its role as coordinator are represented in the supervising board

The supervising board will be responsible for:

- Supporting the coordinator in fulfilling obligations towards the Commission
- Reviewing and proposing budget transfers to the coordinator
- Reviewing the progress of the project and proposing changes in work sharing, budget and participants
- Reviewing of the annual implementation plan presented by the research area leaders and the daily management group prior submission to the Commission
- Agreeing to the inclusion of additional partners
- Agreeing to the exclusion of participants
- Advising in case of problems

The Coordinator

The coordinator will be responsible for the financial coordination and will act as the intermediary to the Commission. He will submit reports to the commission. The coordinator will also be responsible for the dissemination of results to the outside world by installing and maintaining e.g. the ReProTect webpage as well as any other kind of external communication. In addition, the coordinator will be responsible for the integration of new technologies via technology scouts.

The day-to-day management

The project has the unique opportunity of the provision of the scientific/technical day-to-day management from an independent international body, i.e. ECVAM, which is part of the Commission. This body, in turn, will receive guidance from the Supervising Board of the Integrated Project. The daily management group is ensuring that there will be a continuous information flow amongst the partners including the internal dissemination of results. The management group will organize workshops, task forces and other meetings.

The research areas

Each research area is headed by a Research Area Leader who will be responsible for the development and test optimisation of the *in vitro models* in the specific research area. These tests will be used as building blocks in test strategies that are defined in workshops and taskforces. The *in vitro* models that are able to answer specific questions of the chemical effects on specific target cells/tissues or biological mechanisms will be selected only by expert judgements and after consultation with the supervising board. The participants of each research area will meet and report to the daily management team. In meetings with all research area leaders overlapping activities and the future outlines of the project will be discussed. The outcome of these meetings will be communicated to the Supervising Board by the daily management group.

Technology scouts shall help partnering when either a test developer group experiences the need for technological input or a technology provider offers its contribution. An additional subcontractor will be connected to the consortium in order to explore the relevance of existing sensor technologies that can be used for reproductive toxicity testing.

The strategical developments

The project will pilot the strategical discussion towards a new conceptual framework in safety toxicology. A series of workshops shall path this development; high-level experts with complementary expertise are brought together to brainstorm and define a consensus report on the status and opportunities of the respective area. The series of strategic workshops planned shall address issues linked to the challenges of setting up and later validating a whole test strategy composed of individual tests as building blocks. The workshops are organised by the management team.

Selection of new partners

New partners will be identified according to the lack of *in vitro* and *in silico* models or additional toxicological endpoints in the test strategy that are covering certain aspects of reproductive toxicology. These test systems will be identified by expert judgement and in workshops. The daily management group will propose new participants after consultation the research area leader to the supervising board. The supervising board will discuss the need and the qualification of the candidate. The inclusion needs consensus of the board members. However, if the coordinator has justified doubts about the new partner he can reject him.

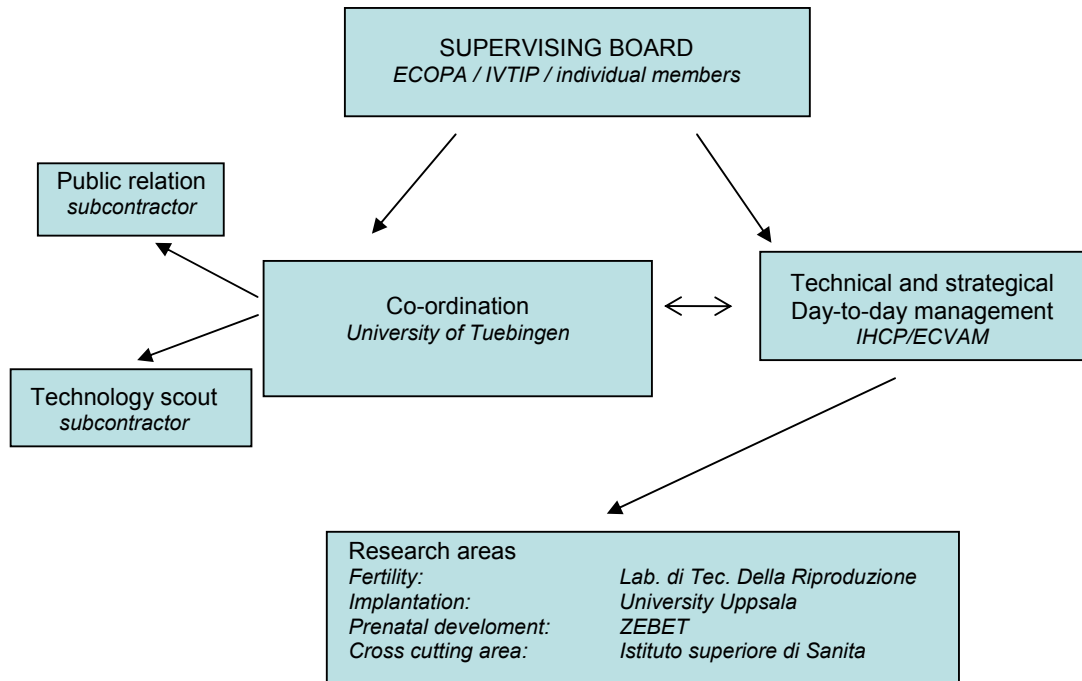


Figure 1: Organisation of the project

Webpage and training (W.P. VI.)

The organization of this work package is horizontal, integrating the scientific contributions with the purpose of sharing information inside and outside the project.

The establishment of e-learning in the ReProTect and ECVAM webpages will be used for training of potential users. The web-based training offers shall include short movies with instructions, documentations, references, frequently asked question sections, contact point references, chat forum, and questionnaires for rehearsal.

Structure of the implementation plan

The project started with a kick-off meeting, which was organized by the daily management team in Ispra, Italy. The structure of the project and the aim were presented as well as major challenges for alternative methods such as the chemical policy were discussed. The kick-off meeting clarified what are the general requirements to the different assays and to the conceptual framework as a whole to be met in order to receive regulatory acceptance by international regulatory authorities.

Other instruments that will be implemented in order to ensure the objectives of the project are technical and strategical workshops. The main aim of the workshops is the development of test strategies in the different areas such as fertility, implantation and prenatal development. It will be discussed how the various models will fit into the test strategies and what are the important

toxicological endpoints that have to be developed. It will be discussed which kind of toxicological information can be provided by the different test systems, and how the assays can be improved. Strategic workshops will also monitor how many chemicals can be tested. A panel of test chemicals will be identified that are affecting different areas of the reproductive cycle. A technical meeting of the research areas will be held in order to review the progress of the work and to discuss the next steps.

In addition, a symposium will be held giving the partners the opportunity to present their result to experts. Furthermore, researchers are presenting *in vitro* systems or technologies that provide toxicological information on a reprotoxicant that cannot be obtained by existing systems. In particular, experts will be invited that are working on placental toxicology, implantation, uterine function and epididymal epithelium and spermatogenesis.

The first 18 months of the project will be divided in three main activities that ensures the development and prevalidation of alternative test methods:

- Further development and prevalidation of advanced tests that are acting as individual building blocks within the test strategy. After prevalidation, the tests will be handed over to ECVAM in order to be considered for formal validation.
- Further development of promising tests, which will not be able to enter in the formal validation process within the first 18 months.
- Identification of emerging tests to set up new workpackages.

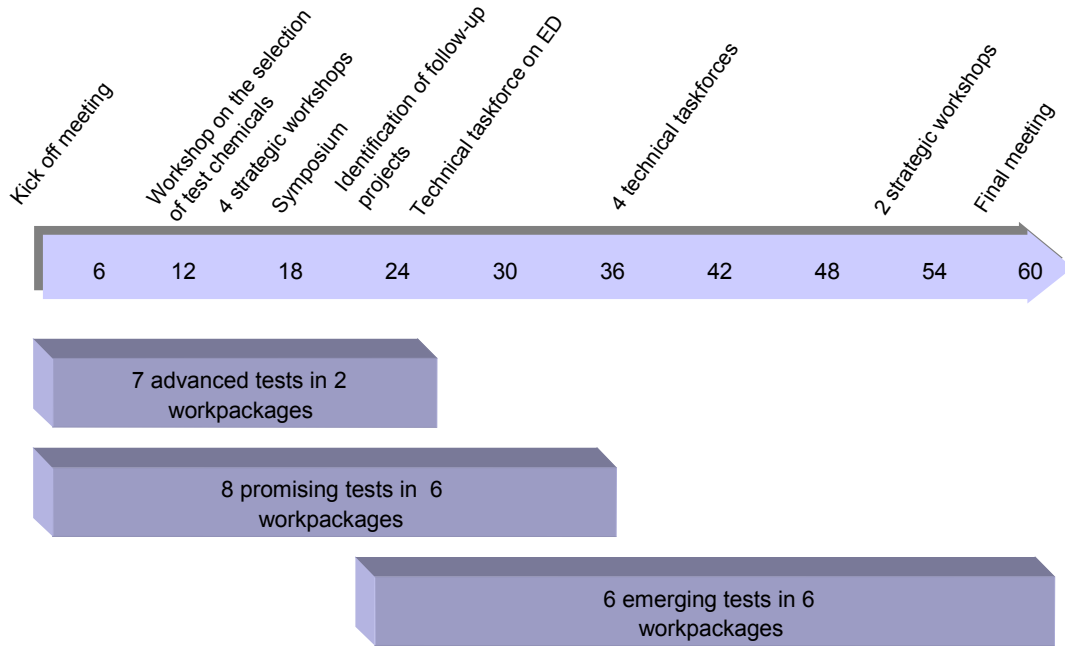


Figure 2: Timescale of the meetings, workshops and conferences.