

# Alternatives to Animal Experimentation: Exploring the Approval Process of Nanomaterial Safety Assessments

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## Abstract

Due to economic, practical, and ethical reasons, many researchers are promoting more widespread adoption of alternative testing methods to replace existing animal tests. The most effective way to increase the use of these alternatives is to gain regulatory approval and instatement as guidelines through the Organisation for Economic Co-operation and Development (OECD). However, determining the exact approval process has been a major obstacle for many. Through information gathered from research and interviews, the process has been broken down into three main phases: development, validation, and regulatory acceptance. This report aims to provide a simplified guide for the approval process of alternative methods with a focus on nanomaterial assessments.

## Authorship

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Ms. Bas served as the overall coordinator and primary source of contact for interviewees, the Adolphe Merkle Institute, and advisors. She was the notetaker for interviews and meetings with advisors, wrote meeting agendas, contributed significantly to the background of this report, and provided extensive editing. She kept the group on track, took care of unexpected disruptions during the interview process, scheduled an additional key interview, and offered productive content and modifications in all presentations and both reports.

### ***Nicole Burns***

Ms. Burns focused on filling in any necessary roles throughout the project. She was the main writer on the interview sections as well as the alternatives background section. She provided many edits on both grammar and content throughout the report. She also ensured that the citations were properly done and learned the APA formatting needed. As necessary, she aided in interview questions and helped to fill in gaps throughout the reports.

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Mr. Gulotta played a key role in developing the content of the project. Along with James, he took a leading position in conducting interviews to gather information about the topic at hand. He maintained a primary position in writing for both reports by writing up a significant portion of the knowledge gained, including all sections on nanomaterials. He assisted in creating and formatting some of the various diagrams and flowcharts, worked on editing and revising drafts, and played a primary position in coordinating the development of the final presentation slides.

### ***James Junker***

Mr. Junker had many responsibilities for the course of this project. As the only member with background in the subject of alternative testing methods (biomedical engineering) and nanomaterials (mechanical engineering), his technical skills were needed to guide research and the formulation of results. Along with Andrew, he took a main role in conducting the interviews – gathering and analyzing the necessary data to achieve the project goal. He had an important capacity in coordinating the content throughout the reports and developed the majority the diagrams. Furthermore, he played a primary role in the writing, editing, and organization of the journal article, this final IQP report and the final presentations.

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## Executive Summary

For hundreds of years, scientists have used animals to test the safety of chemicals, pharmaceuticals, and cosmetics prior to their approval and distribution. Although animals are commonly used, they are not the perfect test subjects, as they are expensive to maintain and replace (since generally only one test can be performed per animal), their reactions to chemicals or drugs being tested are not always the same as humans', and many people do not approve of testing on animals. As such, alternatives to animal experimentation are being developed and approved as standards to reduce, replace, and refine the use of animals during a product's testing phase.

The best way to maximize the population of users of an alternative test is to gain approval and standardization. After going through the initial research and development stages, a test can move forward through the standardization process by being validated through third party testing and becoming approved by the Organisation for Economic Co-operation and Development (OECD). Numerous levels of testing and refinement, testing across multiple laboratories, and extensive data review can take up to 10-15 years. However, this lengthy process can be condensed with the proper preparatory steps.

### *Methodology*

Three main objectives were set for this project:

1. To create a concise overview of animal experimentation and current alternatives with a focus on nanomaterials,
2. To evaluate the approval process for new alternative testing techniques and determine what challenges researchers and regulators have faced in getting these alternatives approved,
3. To prepare a report detailing the process for alternative method approval, while taking into account the complications that nanomaterials introduce. It will be submitted to the scientific journal ALTEX for possible publication.

The main motivation behind this report was to simplify and concisely state the process to follow when gaining approval of an alternative method as an OECD Test Guideline. This guide will be useful for many, as there are 36 member states within the OECD. This means that the



report, while focusing on Switzerland, will also have an impact across several countries to help test developers in the standardization of new alternative methods.

Information for this project was primarily gathered in an independent research phase and an interview phase. Following this, the information was analyzed and the final reports were developed. Before arriving in Switzerland, initial research was conducted; it focused on gathering information about the sponsor, the history of animal testing, current alternatives to animal experimentation, incentives behind the development of alternative tests, and an explanation of nanomaterials and the need to assess their safety levels. Four interviews were conducted in Switzerland to obtain information from all perspectives in the test approval process. During this phase, both test developers and regulators were asked questions suited to their individual positions. These interviews were conducted in a discussion-like manner, and the information provided explained the difficulty behind getting tests approved and standardized, an estimate of the timeline, and, from the regulators, how to actually get these tests to become an OECD Test Guideline (the highest form of approval).

The purview of this project concerned the regulatory process. The main source of information was the aforementioned interviewees. This report was developed as a tool to bridge the gap between test developers and regulators by simplifying the approval process for alternative methods.

## Findings

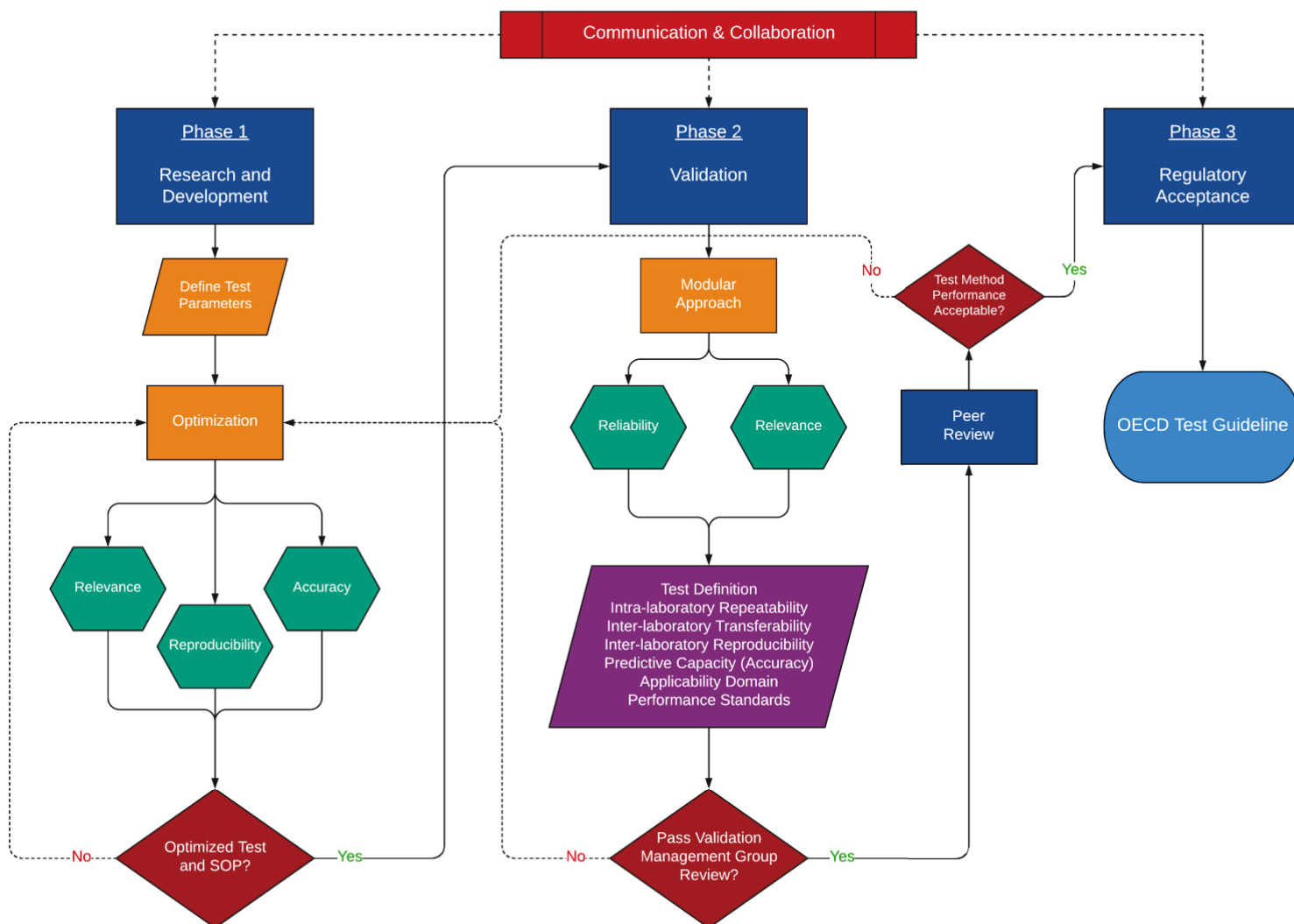


Figure ES 1: Overview of Test Approval and Standardization Process

The combination of research and interviews allowed the development of a clear outline of the steps for approval. These steps are detailed in Figure ES 1. In short, the process is as follows:

1. Determine the need for the alternative test.
2. Develop a test method that responds to this need.
3. Optimize all aspects and variables of the test.
4. Internally verify that the method is robust, reproducible, and transferable.
5. Send the test to third-party validation laboratories for formal validation study.
6. Test enters peer review process.

7. If approved, the test may be accepted by individual regulatory bodies and used by laboratories. At this point, it can be submitted for OECD review.
8. If approved, the test becomes an OECD Test Guideline. All 36 member states must follow this standard.

It was also found that one of the issues hindering the adoption of alternative methods in certain fields was the lack of biological complexity in current offerings. Animals are useful for clinical testing since they are representative of interactions between systems rather than only within one system (for example, testing a skin alternative by itself in a petri dish will not show effects on the endocrine system). It is difficult for a single alternative test to replicate these interactions. Due to these many factors that must be accounted for when replicating the human body, alternative methods are typically used in parallel rather than just one at a time.

## ***Recommendations***

1. During design, test developers should make the alternative method
  - a. Relevant: how well does it fulfill its intended purpose? Are there other applications for the test?
  - b. Accurate: to what degree does the test predict the intended outcome?
  - c. Reproducible: can the original lab and other labs replicate endpoints with precision?
2. Researchers can use resources listed in this report to better communicate throughout the process. The National Coordinator of the OECD Test Guidelines Program can help bridge the communication gap. Communication earlier in the process will allow the NC to determine the user need and help coordinate with all involved parties.
3. Due to the nature and complexity of alternative methods, especially relating to nanomaterials, the current case-by-case methodology of regulation is not yet replaceable. Thus, close collaboration between researchers and regulators will grow in importance as topics become increasingly complex.
4. When presenting to regulators, test developers should simplify their explanations of relevant material and remember that regulators may not have the same level of experience in the subject. The best way to present a new alternative method is to lay out a specific endpoint and express exactly what the method seeks to test.

## ***Conclusion***

This project has the potential for significant social impact. The main reason for constructing the report was to educate test developers on how to standardize alternatives to animal experimentation. At the moment, it is difficult to find a simple guide to this process. Dr. Chantra Eskes, Dr. Lothar Aicher, and Dr. Barbara Rothen expressed their appreciation for the report and excitement for the ideas introduced. They feel that this project will have a tangible impact on their work. As the field of alternative methods continues to grow, the content of this report should help to ensure that standards progress alongside new testing methods.

# Chapter 1: Introduction

## 1.1 Context and Motivation Behind this Project

When it comes to testing the safety of products and materials without putting humans at risk, animal testing has traditionally been the default method. However, due to ethical and practical concerns, many institutions are seeking alternative methods to replace animals. The Adolphe Merkle Institute (AMI) is one of these institutions. One aspect of their work focuses on the development of hazard assessments for nanomaterials that do not involve animals (more information on AMI can be found in Appendix C). This will provide labs with cheaper, more ethical methods to test the safety of nanomaterials. This project focuses on outlining the approval process for passing these new test methods as Organisation of Economic Co-operation and Development (OECD) Test Guidelines. This is a broad topic and a fairly daunting task without first understanding all of the different aspects of the question being addressed: What is needed for alternative testing techniques to be approved and standardized in Switzerland? To begin, it is pertinent to understand the focus of this question: alternatives to animal experimentation. However, before that question can even begin to be answered, one must first understand animal testing. Animal testing has been the standard for many years and is not easy to replace. Included is a brief history of animal experimentation, why change was and still is necessary, as well as an overview of what is currently being done to reduce and improve animal testing. With this background knowledge, it is much easier to understand the development of the alternative testing technologies and their comparison to their predecessors in terms of practicality, ethics, and economic value.

Of course, this is only the beginning. Work is being done at AMI to develop alternative models for nanomaterial hazard assessment. Nanomaterials pose additional challenges that make the regulatory process more difficult than for tests involving other substances. Therefore, it is only with a solid understanding of nanomaterials, their health risks, and the importance of hazard assessments that one may begin to venture into the complicated world of regulating these alternatives. Gaining an understanding of the different aspects of test regulations and the different organizations that play a role in creating these guidelines is vital to answering the project question and achieving the overall goal. After assessing all aspects of the process, recommendations can be

made to improve it for all parties involved and assist in the continued advancement of alternative testing methods.

## Chapter 2: Goals

### 2.1 Research Questions and Goals

This project has one overarching goal: to create a step-by-step guide for the approval of alternative methods while providing recommendations to improve the process. However, this is a very broad and deep topic with multiple facets that need to be considered. The overall project goal has been broken up into three smaller objectives, as seen in Table 1. Furthermore, questions to focus and guide inquiry into these objectives on a more specific basis have been associated with the three objectives. Together, these questions, objectives, and overall goal outline what this project will aim to address.

*Table 1: Project Goals and Questions*

<u>Overall Project Goal</u>	<u>Overall Research Question</u>
The goal of this project is to evaluate the current regulatory process for alternative testing methods in Switzerland and provide a guide for the approval of such methods with a focus on nanomaterial testing.	What is needed for alternative testing techniques, such as <i>in vitro</i> testing, to be approved in Switzerland?
<u>Project Objectives</u>	<u>Subsidiary Research Questions</u>
(1) Review of animal experimentation and current alternatives surrounding nanomaterials.	<ol style="list-style-type: none"> <li>1. What are the current alternatives?</li> <li>2. What are the pros and cons of animal testing and its alternatives?</li> <li>3. How do the alternatives which test nanomaterial safety compare to current animal testing techniques ethically and practically?</li> </ol>

<p>(2) Evaluate the approval process for new alternative testing techniques and determine what challenges researchers and regulators have faced in getting these alternatives approved.</p>	<ol style="list-style-type: none"> <li>1. Which alternative methods are already approved, which aren't, and why?</li> <li>2. What are the steps to getting a test approved?</li> <li>3. What obstacles have researchers faced in getting alternatives approved?</li> <li>4. Who is in charge of getting these alternatives passed?</li> </ol>
<p>(3) To pursue publication of a report (in the scientific journal ALTEX) detailing the process for alternative method approval, while taking into account the complications that nanomaterials introduce.</p>	<ol style="list-style-type: none"> <li>1. Why is it important to get these alternatives passed? Who does it affect?</li> <li>2. What is most relevant to the sponsor's work?</li> <li>3. What important changes need to be made to improve the approval process for alternatives?</li> </ol>

The main deliverable for this project was a guide for AMI detailing the approval process for alternative testing methods. It includes a set of recommendations on how to improve and simplify the regulatory process and expedite the approval of testing methods. The guide will be submitted for potential publication in the ALTEX journal on alternatives to animal experimentation and can be found in Appendix D.



## Chapter 3: Background and Literature Review

### 3.1 Animal Experimentation and Welfare

The focus of this project was to evaluate the approval process of new alternatives to animal testing with specific focus on nanomaterial tests. However, in order to understand the alternatives and why they are being developed, it is important to first review animal testing. This section will address:

- The broad history of animal experimentation
- The pros and cons and ethical and practical issues with using animal experimentation
- The current outlook that Switzerland and the world have on animal experimentation and the 3Rs movement

Understanding of the above permits greater insight as to the possible advantages of new alternative methods and thus is a vital portion of the Background and Literature Review section.

#### 3.1.1 History of Animal Experimentation

As early as Aristotle, humans have been testing on animals for various reasons, including medical knowledge, experimentation, and safety within various industries such as pharmaceuticals, cosmetics, and chemicals. Towards the beginning of these experiments, scientists and philosophers conducted studies in the fields of “anatomy, physiology, pathology, and pharmacology” (Hajar, 2011, p.1). In the 12th Century, a physician named Ibn Zuhr proposed (and carried out) methods to test surgical procedures on animals prior to using them on humans. During the 19th Century, ‘father of physiology’ Claude Bernard tested on animals, stating that “experiments on animals are entirely conclusive for the toxicology and hygiene of man. The effects of these substances are the same on man as on animals, save for differences in degree” (Hajar, 2011, p.1); he is credited with making animal experimentation a standard part of carrying out the scientific method. Historically, it is evident then that the importance of animal testing has been recognized.

Despite this, there have been multiple cases of lethal disaster in the past due to skipping the animal testing phase of certain pharmaceutical drugs. For example, in the late 1950s to early

1960s, the drug thalidomide remained untested until pregnant women began taking it to relieve morning sickness. As a result, “more than 10,000 children in 46 countries were born with malformations or missing limbs” (Hajar, 2011, p.1). Since then, legislation has since been put into place requiring a certain form of safety guarantee, usually in the form of animal testing, before allowing products to be released to the public.

Starting in the early 2000s, data has been collected by the German Federal Ministry of Food and Agriculture (BMEL) to track changes within the animal experimentation field, such as increased usage of vertebrates. Between 2003 and 2012, the number of non-human vertebrates being tested on in European Union countries went up by 39% (3,080,727 animals). From 2013 to 2014, this number decreased by 6.7%, but was still higher than in 2003. In 2014, out of 2,000,000 vertebrates involved in testing, 800,000 were killed (40%); 73% of these vertebrates were mice (which are not genetically close to humans but are still frequently used in testing despite evidence that there are drastic differences in reactions between mice and humans). Overall, it is clear the monumental role that animal testing has in the regulation of so many products and their safety.

One of the main major regulations in Germany specifically enacted for animal testing was enacted in 1991, which banned the use of great apes. There is a great range of animals which are used, from invertebrates and worms, to vertebrates such as apes. Invertebrates are useful since their structure and DNA are simple, while the vertebrates have great similarity of structure to humans (thus more useful in drawing results of how substances might affect humans). The vertebrate with the greatest genetic similarity to humans would be individuals from the ape lineage. However, there are many ethical concerns that are brought up by testing on such close relatives of humans, which is one of the main reasonings for banning their use in testing.

Recently, there has been a lot support for not just restricting animal experimentation but rather replacing it; further details are included in later sections.

### 3.1.2 Is There a Need for Change?

It is common knowledge that overall technology, and particularly medical technology, has improved drastically over the past century. Given time and innovation, humans have developed vaccines and safer medical equipment, refined more efficient surgical procedures, lowered death rates during operations, etc. These developments in technology have extended to developing organ tissue from various sources (such as WPI developing heart tissue from spinach in 2017) and skin

grafting. With all of the funding available and the increasing public demand, there is both financial motivation (stemming from the principles of supply and demand, not having to maintain animal welfare, and having to replace animals from experiment to experiment) and technological motivation (investing now will lead to more developed innovation later) to pursue finding alternatives to animal experimentation. Additionally, there are practical and ethical incentives to move forward with alternatives.

As stated previously, animal testing has been used for many years. However, recent studies comparing results of animal testing to use of alternatives have shown that genetically different animals do not always show realistically comparable results to humans. For example, cancer in mice has actually been cured, but these same tests done on humans proved a 95% failure rate; additionally, a series of 80 HIV vaccines had a 100% success rate among chimpanzees (non-human primates that, genetically, closely resemble humans), but, when test on humans in 200 cases, there was a 100% failure rate (Cimons, 1998). From this data, it can be concluded that the practicality of testing on animals is minimal, since the results often don't align with human reactions. Furthermore, the significant public ethical concerns over testing on animals in recent years has caused companies which test on animals to lose revenue from customers to companies which do not test on animals.

### 3.1.3 Switzerland's Role in Animal Rights: The 3 Rs

Currently, there are three main ethical arguments promoting continued use of animal experimentation. They focus mainly on the morality (or lack thereof) of animals. These include:

1. animals do not have a genuine moral status and are therefore not deserving of protection for their own sake,
2. all living organisms that have a similar capacity for suffering and are able to develop interests (whether humans or animals) have a comparable moral status and
3. as a "middle" position, animals have a genuine moral status, although this is subordinate to the moral status of man (Vermeulen, 2017, pg.3).

In recent years, people have become increasingly concerned regarding the cruelty with which the animals involved in these experiments are being treated. These have developed into a principle termed the "3R principle", which was the basis for the 1958 book "The Principles of Humane Experimental Technique" by William Russell and Rex Burch (Russell, 1958). The 3R

principles “[advocate] the search for the (1) *replacement* of animals with non-living models; (2) *reduction* in the use of animals; and (3) *refinement* of animal use practices” (Hajar, 2011, p. 1). These goals do not eliminate testing within industries (this would be unsafe and unrealistic); they only seek to make testing more ethical, practical, and financially appealing. These principles have been incorporated in legislation, such as in Directive 2010/63/EU, Articles 4 and 13 (Directive 2010/63/EU, 2010).

Directive 2010/63/EU was put forward on September 10th, 2010 by the European Parliament and Council to address the protection of animals being utilized in scientific testing. In reference to the 3Rs campaign, there are 3 main sections which are important. Section 27 states that, in order to reduce the number of animals used in trials for *in vitro* methods, their tissue and organs should be shared with other EU members. Section 46 states that the Community Framework Programmes for Research and Technological Development has financially invested in projects, which put the 3R principles into practice and will continue to encourage all EU members to seek alternative testing methods. Section 49 states that each project should avoid use of, specifically, non-human primates, as well as other animals, and that the aforementioned Commission will periodically check procedures to review whether the 3R principles are being utilized.

Furthermore, this particular Directive aims to “establish measures for the protection of animals used for scientific or educational purposes” (Directive 2010/63/EU, 2010). To accomplish this, the Directive addresses the following:

- (a) the replacement and reduction of the use of animals in procedures and the refinement of the breeding, accommodation, care and use of animals in procedures;
  - (b) the origin, breeding, marking, care and accommodation and killing of animals;
  - (c) the operations of breeders, suppliers and users;
  - (d) the evaluation and authorisation of projects involving the use of animals in procedures
- (Directive 2010/63/EU, 2010)

The 3R principles are directly discussed in Article 4 (Principle of Replacement, Reduction and Refinement) of this directive. Essentially, this concept aims not to eliminate animal testing in its entirety, but rather to eradicate cruelty within the industry and minimize the use of animals. The EU has chosen to abide by these guidelines, which state that those who choose to follow should

avoid and minimize the use of live animals, optimize the use and minimize the harm done to animals, and follow the guidelines of Article 13 (Directive 2010/63/EU, 2010).

In order to provide proper context for Article 4, Article 13 includes more specific details regarding the implementation of the 3Rs. Chapter 3 covers specifics of procedures; Article 13 (Choice of Methods) covers the implementation of the 3Rs guidelines in more depth, as well as clarification on addressing ethics in death (and prevention thereof) (Directive 2010/63/EU, 2010). Overall, Article 13 states that minimizing pain, long-lasting trauma, and distress are crucial for testing procedures, but that researchers should avoid compromising results from these tests. Both Articles 4 and 13 strongly emphasize the need to avoid causing unnecessary emotional and physical pain to the animals used.

In putting the 3R principles further into action, a partnership called the European Partnership to Promote Alternative Approaches to Animal Testing (EPAA) was founded in 2005. Fairly recently, the EPAA has begun to give out “3R Awards” in alternate years for significant achievements in the development of alternative experimentations. Developments such as these help lead the movement toward greater use of alternatives in the future.

## 3.2 Alternatives to Animal Experimentation

With the need for more specific and reliable testing growing, more and more organizations are developing alternatives to animal experimentation. These alternatives address ethical concerns by following the 3Rs (discussed in Section 3.1.3). Alternative tests are becoming more prevalent because of their practicality and economic efficiency. Because of this, countries around the world are trying to move their testing methods away from animals and towards their alternatives. Experimental alternatives may eventually eliminate animal testing altogether, but this field is still relatively new and there is much more work to be done before this is feasible. To evaluate what types of alternatives are currently available, this section will cover:

- Introduction to the alternatives
- Comparisons in ethics, economics, and practicality
- Current alternatives in various industries

A basic background of the current state of alternatives is also included to give a better understanding of the sponsor’s work and what they would like approved.

### 3.2.1 Introduction to the Alternative Technologies

Developing alternatives to animal testing has become an increasingly important and relevant research field in recent history. Due to increased demand for cheaper and more reliable tests, along with social groups calling for the ban of animal experimentation, institutions have been working on a variety of new alternatives. However, this brings the question: what exactly are alternatives? They can be simply defined as anything besides animal testing that produces accurate data. They range from sophisticated tests using human cells and tissues to advanced computer models. Various studies utilizing human volunteers are also considered alternatives to animal testing.

Thus far, the focus of viable research has mostly been on *in vitro* methods. This testing method utilizes human cells in an artificial environment simulating the human body. It does not require any living host. This is often achieved via cell cultures in petri dishes (displayed in Figure 1), or in advanced techniques like “organs-on-chips” that the Harvard Wyss Institute recently developed.



*Figure 1: In vitro Model of Human Lung Tissue (Jorio, 2015)*

Figure 1 shows an example of an *in vitro* lung tissue model. Micro-layers of human lung cells are placed on a permeable membrane in the petri dish to simulate pulmonary tissue of the lungs (Jorio, 2015).

These cell-based tests and models can be used to assess the safety of drugs, chemicals, cosmetics, and a variety of consumer products (PETA, n.d.). These techniques utilize human cells to simulate the environment and function of human organ tissue. By using human tissue, alternative techniques are able to produce more accurate results (compared to animal testing) by replicating the target systems to a higher genetic degree. As more and more organizations are developing alternatives, it has become evident that there are also a variety of economic benefits compared to animal testing. These aspects surrounding alternatives are discussed in the next section.

First, it is important to note that there are groups which have been tasked with developing and regulating alternatives, such as the Organisation for Economic Co-operation and Development (OECD). This particular group makes guidelines that 36 countries have chosen to follow, and are pushing to increase the availability of alternatives and decrease the need for animals (“About the OECD”, 2018). Switzerland is one of the 36 OECD members and only allows animal testing if there is no viable alternative. They are actively working to phase out animal testing as much as possible (Federal Food Safety and Veterinary Office, 2018). However, because of the fledgling state of the alternatives field, there are a lot of aspects which are, as of yet, still relatively undefined, particularly the regulatory aspect. Due to this unknown, the complex regulatory environment of alternative testing, especially relating to Switzerland, is covered in-depth in Section 3.4.3.

### 3.2.2 Comparisons in Ethics, Economics, and Practicality

There are both benefits and drawbacks to using animal testing methods versus their alternatives. One advantage of using alternatives is the lack of ethical concerns surrounding them, since using animals for testing can generally be more restrictive due to the potential misuse and endangerment of the animals. However, because alternatives are not living beings, it is much easier and more ethical to use alternatives for testing.

Another drawback of animal testing is the potential cost. This is because animals are living creatures that take up large amounts of space and require dedicated facilities and staff to properly take care of them. Over time, food for animals can be very expensive, and, in order to keep the subject in good health for testing, other items may need to be purchased, such as mouse wheels for exercise. Extra employees may need to be hired in order to properly maintain the animals because the researchers may not have time, adding to the potential cost. Additionally, animals, in general, cannot be used for multiple studies to prevent them from introducing error from outside variables,

so new animals must be purchased or raised for each new study. Due to this, alternatives may often be cheaper and easier to maintain. Alternative tests can often be created in a petri dish and require less maintenance, reducing the overall costs. Without the need to feed and take care of many animals, researchers can focus their time and money on the more important issues of their work. However, as the alternative field grows, the complexity of the technology and protocols involved will also grow. Because of this, only experts may be able to operate the various tests and techniques which would raise the amount of time and money that needs to be dedicated towards their operation and improvement. So, despite alternatives to animal testing being generally cheaper than their counterpart, they are not without their drawbacks.

Alternatives to animal testing are also much more practical than animal experimentation in many cases. There are many studies in which animals are used for testing that provide inaccurate results for humans. Because the genetic makeup of other animals can be so different from a human's, reactions toward certain chemicals or treatments can be vastly different (Hartung, 2011). In one study testing a specific antibody that seemed to have benefits for animals, for humans it resulted in "life-threatening morbidity in all six healthy volunteers" (Bracken, 2008, pg. 1), demonstrating the difficulty of using animals as subjects. These genetic differences are usually hard to predict or model, leaving humans at risk to these treatments that prove to work on other animals. A study conducted in 2004 by the Deutsches Referenzzentrum für Ethik concluded that only 70% of the dangerous effects on humans can be matched with results from animal experimentation (Vermeulen, 2017). This, among other results from studies conducted, has supported the idea that testing on animals cannot reveal every danger associated with usage of a product, due to genetic differences. Alternatives, however, use human cells and tissue to replicate the environment much more closely. This is a huge advantage for alternatives, but due to the complexity involved in growing stable organs or human systems *ex vivo* (outside the body), these alternative techniques cannot provide the same data as animal testing does. Without replicating the intricate network of interactions within living organisms, alternatives will be limited to primary stage testing and will be unable to completely replace animal testing.

For now, animals still largely have to be used in clinical trials and late stage testing. The alternatives in use thus far can model specific functions or organ membranes of the body, but they are limited in their modeling of the complex interactions between the hundreds of cell types in the human body. The alternatives tend to be more accurate for specific tissues and interactions in the



body because they specifically model human tissues, cells, and even organs, but they currently cannot go beyond that. Animals are still widely used for testing because they give the researchers an idea of how the treatment affects all of the body rather than just one section. For now, alternatives are limited to the beginning stages of trials to assess the safety of chemicals or treatments on certain types of cells while animal testing can model how the effects can interact with the rest of the body (S. Ambady, personal communication, April 4, 2018). The most reliable approach to testing currently involves using alternatives and animal testing in conjunction depending on the phase of testing.

### 3.2.3 Current Alternatives in Various Industries

There is a need for alternatives to animal testing in many different industries in order to make testing more practical and less expensive. Currently, as discussed previously, there is a push for fewer animals to be used in experimentation. However, if products or treatments are not tested on animals, there has to be some other reliable option to measure safety. According to the OECD Test Guideline 431, there is an *in vitro* method, using an organoid of reconstructed human epidermis (RHE) which can accurately test the corrosive effects a material has on human skin (OECD, 2016). This method can also be utilized to test skin irritation based on an earlier regulatory standard, OECD Test Guideline 439. Guideline 439 in particular “may be used to determine the skin irritancy of chemicals either as a stand-alone replacement test for *in vivo* skin irritation testing or as a partial replacement test within a testing strategy” (OECD, 2015, pg. 1). Thus, RHE can be used in place of animal testing and can be used to approve the use of chemicals without further testing. This alternative test can be useful to any industry or research project that may need to test the safety of a product on the skin; thus, it has far reaching implications.

In many cases, it is necessary to have accurate models that reliably simulate the inner workings and interactions of the human body, which often are more complex than a culture of a single type of tissue or cell. Reproducing the functions of specific organs is vital when conducting testing of pharmaceuticals or determining the hazard that certain particles pose. One such alternative for this, which is becoming more and more prevalent, is an organ-on-a-chip. Several institutions have been working to create chips that behave as a human organ would. These organ-on-a-chip can consist of several different cell types in order to reproduce the interactions within a human organ. This method is discussed in greater detail in section 3.3.4. Organs-on-a-chip can

potentially be used in applications such as testing the effects of a drug on the liver to make sure it isn't harmful or to test how particles inhaled would affect the lungs (Levis, 2015). This can be extremely helpful to the pharmaceutical industry to reduce cost and ensure test clinical trials are safer and more effective. "The testing strategy of the pharmaceutical industry focuses mainly on avoiding failure during clinical trials and their tremendous costs. Therefore they have a considerable interest in novel *in vitro* models able to better predict the clinical outcome" (Wick et al., 2015, p. 175). Clinical trials can be very difficult, costly, and even harmful if the drugs are not tested properly beforehand. This creates motivation to use safe and reliable alternatives such as organoids and organs-on-a-chip before moving to clinical trials. Thus, alternatives to animal testing can prove to be very useful and increase efficiency of studies if used effectively.

Another group of methods that are in use are *in silico* models. These are complex computer simulations created to model specific cell functions, iterations, or systems. An example of this is included in a study of tuberculosis: there was a model, made by the University of Surrey, which was "extremely complex, handling 848 different biochemical reactions and 726 genes" (University of Surrey, 2007, p.2). The model was created using the genome of tuberculosis bacillus, the agent which causes tuberculosis. Effects of this are generally difficult to study in lab situations because the bacilli grow very slowly, over a period of months. However, an *in silico* model of this can be 'grown' in minutes or even seconds due to the processing power of computers. A model like this can potentially be used to study how tuberculosis responds to different treatments, and can be used to create new vaccines. This alternative test method has a lot of promise, especially in the medical and pharmaceutical industries.

However, the focus of this project is the testing of nanomaterials, which overall can be tested more easily with *in vitro* methods such as organoids and cultures of human tissues than other methods (Rothen, 2017). Testing nanomaterials on specific areas to understand their effect on individual parts of the body (*in vitro*) before moving to later stage trials on animals and/or humans (*in vivo*). This allows researchers to be more prepared and to harm fewer organisms while checking for safety and hazards of materials. The intricacies and factors that are involved with nanomaterial hazard assessments will be discussed thoroughly in the next section.

### 3.3 Nanomaterials

While there are many tests involving animals for which alternatives are being developed, the main focus of the work being done by the Adolphe Merkle Institute is on alternative testing techniques for assessing the hazard of nanomaterial exposure on human tissue. This ties in with the institute's main research focus of nanomaterials and nanoscience. While this project specifically focused on the testing techniques, it is important to first understand what nanomaterials and nanoparticles are and why they might be dangerous. To cover the basics, this section will go over:

- Introduction to nanomaterials
- Defining hazard assessments in relation to nanomaterials
- The health risks of nanomaterials
- Current model systems for nanosafety assessment

The information provided in this section explains what exactly new alternative testing techniques are testing for and why they are necessary.

#### 3.3.1 Introduction to Nanomaterials

As science progresses, scientists have been able to create larger and more complex systems than ever before. The same can be said for smaller feats as well. In recent times, there has been a focus in nanoscience, specifically on nanomaterials and nanoparticles. Nanomaterials are considered to be “materials with at least one external dimension that measures 100 nanometres or less or with internal structures measuring 100 nm or less. They may be in the form of particles, tubes, rods or fibres” (Nanomaterials, 2009). Nanoparticles are generally characterized by their physical properties: size, shape, specific surface area, whether they stick to each other, smoothness and structure, as well as their chemical properties, which include molecular structure, composition, and whether it is held in a solid, liquid or gas, and its surface chemistry. The current applications of nanoscience in healthcare include targeted drug delivery, regenerative medicine, and diagnostics. However, they are also widely used in electronics, cosmetics, textiles, information technology and environmental protection (Nanomaterials, 2009).

In general, risk assessment of nanomaterials is mainly based on animal testing strategies. However, this strategy is not optimal due to the resource and time-consuming nature, leading to a

bottleneck and a backlog of materials requiring testing (Nanomaterials, 2009). Contributing to the inefficacy of animal testing is the physiological and biochemical diversity between the animal models and humans leading to poor predictive capability. Furthermore, the principle of the 3Rs (replacement, reduction and refinement), covered in earlier sections, has been rising to prominence in Switzerland and around the world, leading to significant ethical yet practical support of the replacement of animal experimentation with alternatives (Wick et al., 2015).

### 3.3.2 Nanomaterials and Hazard Assessment

There is currently a group of international academics, industrial scientists, government officials and risk assessors with the express goal of delivering “advanced and realistic tools and methods for nanomaterial safety assessment” (PATROLS, 2018). In recent years, awareness of the potential risk that exposure to engineered nanomaterials (ENM) poses to human and environmental health has greatly risen. Thus, Physiologically Anchored Tools for Realistic Nanomaterial Hazard Assessment (PATROLS), a project dedicated to standardizing nanomaterial hazard assessments, has made it their mission to provide laboratory techniques to reliably detect and assess this risk. Tools for measuring the amount of danger and being able to compare these hazards with other nanomaterials and nanoparticles will have growing importance in the coming years. There is not only pressure to develop nanomaterial safety assessments from societal and regulatory perspectives, but also from industrial points of view, mainly to avoid economic loss on investment. It is evident then that there is a tangible demand from many sectors for standardized alternative test models. Additionally, perhaps due to the variety of sources of support, these standardized test models are being developed to allow high throughput, content, and cost-effectiveness (Wick et al., 2015).

It is important to note that these hazard assessments are primary assessment tools and by no means eliminate the need for animal testing later on in a study developing nanomaterials. These alternative models are tools which may help minimize the number of animals used later on in testing by providing a better idea of where nanoparticles have dangerous effects and the degree of severity. Therefore, these alternative nanomaterial hazard assessment techniques are of great importance, but they are not yet advanced enough to allow for the termination of animal testing entirely.

When delving into the literature for this relatively new field, some oversights seem to arise. Currently, the only hazard studies for humans that have been conducted on ENMs focused more on the short-term and on high exposure, when, in actuality, exposure is often long-term and in repetitive low doses. This discrepancy poses a serious problem and will require further studies to be conducted in order to get more accurate and representative results. Further delving into human health and safety assessments, it is very common to use 2D cell monocultures to gather this data. Unfortunately, these commonly fail to capture the complex biological processes and interactions that occur within the body. The body is a complex amalgamation of an enormous variety of cell types, so creating a model using only one type will only lead to inaccurate results and models. Due to this, animal models have instead been historically relied upon to assess the hazard of nanomaterials, even with the advancement of alternative technology (PATROLS, 2018).

Similarly for ENM ecotoxicity testing, the studies have focused on short-term exposures on a small selection of organisms compared to a long-term and low-exposure approach that is closer to reality. This poses a problem because the current approaches of drug delivery, the duration of exposure, and propagation through the food chain lack environmental realism (PATROLS, 2018). Delivery and exposure duration have very direct harmful effects, but propagation through the food chain is a problem for its indirect effects. The behavior of top consumers is affected by “reducing their activity, feeding rate and changing species interactions” (PATROLS, 2018). This could very well cause secondary effects and make it impossible to contain. The potential consequences then are impossible to predict, which is unacceptable in any scientific study.

Overall, there is a clear need for testing tools to more accurately predict the adverse effects caused by long-term ENM exposure in humans and the environment (Nanomaterials, 2009). Specifically, the interests of this project and the Adolphe Merkle Institute lie in realistic and predictive 3D tissue models of the lung, gastrointestinal tract and liver for ENM safety assessment, reducing the need for animal testing. Before discussing these tests, however, it is important to take a look at what potential hazards nanomaterials and nanoparticles pose to provide better context of what the tests will be looking for.

### 3.3.3 The Health Risks of Nanomaterials

The growing ubiquity of nanomaterials and nanoparticles, while leading to many new and innovative products, has left many concerned about the very real possibility of health risks to humans. Currently, there is not much in the way of solid evidence of serious health risks. This is mostly due to a lack of accurate tests and consensus on the results (Clark, 2011). In addition to the laboratories that are conducting these tests, there are agencies such as the International Risk Management Institute and the British Health and Safety Executive which are working to gather information on the health hazards of nanomaterials and nanoparticles to protect people who may be exposed to these substances in the workplace. According to these agencies, when handled and manufactured properly, most nanomaterials and nanoparticles used in manufacturing pose no immediate known health risks (Clark, 2011). However, they still provide information as to the potential ways in which certain nanomaterials and nanoparticles may have negative effects on human health. There is undoubtedly an enormous diversity of nanomaterials across a variety of consumer, industrial, and biomedical applications. Subsequently, there are numerous methods of exposure through which nanomaterials could potentially induce harm. These include inhalation, injection, ingestion, and permeation through the skin (Wick et al., 2015).

One of the most common ways in which nanoparticles can be introduced to the body is through inhalation. Due to their small size, inhaled nanoparticles can work their way deep into the lungs and potentially pose a high health risk. Evidence suggests that inhaled nanomaterials and nanoparticles have the potential to initiate inflammatory responses in the lungs, which can lead to diseases such as lung cancer. The nanomaterials that pose the greatest threat in this particular area are those classified as fibers or high aspect ratio nanomaterials (HARN). The World Health Organization defines a respirable fiber as “an object with length greater than 5 $\mu$ m, a width less than 3 $\mu$ m, and a length to width ratio (aspect ratio) greater than 3:1” (Understanding the Hazards of Nanomaterials, n.d., p.16). Additionally, any nanomaterial with at least one dimension in the nanoscale (between 1 and 100 nm) and an aspect ratio greater than 3:1 is considered a HARN. One example of such materials is carbon nanotubes, which are often used to enhance the rigidity and durability of materials. Evidence suggests that these nano-fibers and other HARNs can become trapped in the pleural cavity of the lungs for long periods of time and lead to the aforementioned lung diseases (Understanding the Hazards of Nanomaterials, n.d.). Additionally, their size allows some nanomaterials to cross cell membranes and permeate throughout the body, potentially

causing systemic health effects. This has led many to believe that nanoparticles could become the “asbestos” of the 21st century (Gwinn, 2006).

Currently, there is not much information as to the hazards posed by nanomaterials absorbed by the skin or introduced to the gastrointestinal tract. The current consensus is that, if any nanomaterials are able to permeate through the skin, the level of absorption will be low and likely not dangerous. However, more research and testing need to be done in both of these areas before a proper evaluation can be made (Understanding the Hazards of Nanomaterials, n.d.).

In addition to their size and shape, other properties of nanomaterials that may cause them to be hazardous include surface area, surface charge, chemical composition, and solubility. Surface area is a particularly important differentiator between nanoscale particles and non-nanoscale particles. For a given mass, nanoparticles have a drastically higher surface area than non-nanoscale particles of the same chemical composition. A higher surface area can cause any reaction a substance may have with another to become much more potent. Because of this, a safe mass of a certain substance may not be a safe amount for the same substance in the form of nanoparticles (Understanding the Hazards of Nanomaterials, n.d.).

Due to the small scale and differing properties of nanomaterials and nanoparticles in relation to larger scale particles of the same chemical composition, it has traditionally been difficult for scientists to determine what exact dangers they pose to human health. Recently, there has been progress made on new models and tests that are designed to better analyze toxicological nature of nanoparticles in relation to the human body (Maynard, 2011). These new tests are the main focus of the Adolphe Merkle Institute’s work.

### 3.3.4 Current Model Systems for Safety Assessment

There are multiple ways in which nanomaterials can be introduced to the body. This requires there to be just as many testing methods to determine the health risk that these nanomaterials pose. Further complicating this issue are the over “200 different cell types with distinct levels of differentiation, embedded in soft extracellular matrices, organized in different tissues and organs, regulated by complex networks and cross-talk” (Wick et al., 2015, p. 2). There are simply too many different kinds of cells in intricate environments to create comprehensive working *in vitro* models. Furthermore, they are entwined in and communicate through elaborate signaling networks. In order to try and mimic this complexity, the current cell culture models are:

1. Replacement of malignant or cancer-derived cell lines by primary or well-characterized human cell lines
2. Movement from single cell type to multi-cellular cultures
3. Movement from monolayer to organoid-like 3D models
4. Tissue preparation from explants

However, these methods fail to replicate the complex environment of the human body and the interactions within (Wick et al., 2015). The alternative models excel in creating singular aspects or functions of organs, yet fail to capture the organs in their entirety. Often this is enough, but with increasing depth of clinical studies, researchers cannot replicate the accuracy of data that animal testing provides with alternative models alone.

In recent years there have been strides towards an *in vitro* triple-cell co-culture model of the human airway wall with epithelial cells, human blood monocyte-derived macrophages, and dendritic cells. In other words, cellular models of the airway using more than just one cell type are being grown outside any living animal. These models with the multiple types of cells showed cell-cell interactions and communication strikingly similar to that of *in vivo* (living organism), systems. Recently, systems with four cell types have been developed, with epithelial and endothelial cells, macrophages, and mast cells to study the impact of both engineered and environmental particles (Wick et al., 2015). This approach shows promise in emulating the complex interactions within the human body, but there is still room for research and improvement.

Despite the recent advancements in multi-cell type culture models, the technology is not currently able to reproduce certain cellular interactions and organ systems. The liver is a vital organ in homeostasis and the filtering of foreign substances (including nanomaterials) from reaching the bloodstream. However, “although promising 3D systems have been reported, the majority of the hazard assessments of nanomaterials to date have still involved hepatocyte monocultures” (Wick et al., 2015, p. 4). In other words, the liver is still too complicated to accurately model in multi-cell type cultures. The kidneys are also central to metabolism and blood filtration, and are thus exposed to adverse metabolites, drugs, or nanoparticles. Recently, there was a study composed of a 3D organoid kidney epithelial cell system that involved “a set of toxicity indicators which accurately reflected the damage observed *in vivo*” (Wick et al., 2015, p. 4). Researchers were able to emulate a certain system of the kidney, which then produced an accurate representation of the damage seen within testing animals. This is the first confirmation that it is actually possible to



create a cell culture that produces the same results as animal testing. With a little more research, this a very promising approach.

However, despite all the success, these advanced *in vitro* systems have become increasingly time-intensive and expensive, requiring well-trained experts to take care of them. Because of this, there has been interest in the next approach of *ex vivo* tissue preparation in pursuit of predictable model systems.

Precision-cut tissue slices (PCTS) is a method which represents an *ex vivo* model of the organ by maintaining the original cells types of the tissue in their natural conformation. Basically, this is a way to look at cross-sections of tissue. Slices from different species can be prepared and compared, a useful tool in analysis of a variety of issues. However, this technique could accidentally cut the organ into slices, meaning the cells covering the surface become damaged, potentially inducing an inflammatory reaction (Wick et al., 2015). This creates problems in data retrieval and analysis. Despite this, this system has great potential and will undoubtedly be of great use upon further development.

Currently, there are emerging *in vitro* platforms based on microfluidic technologies called “organs-on-chip”, specialized towards assessing nanomaterial safety and efficacy. They are 3D organoids made of human cells which are designed to mimic the environment, structure, and key functions of human organ tissues. This unprecedented approach has the potential to improve *in vitro* model accuracy and experimental efficacy (Wick et al., 2015). Although most of the focus in this area is on more predictive preclinical alternative models, their prospective value for toxicological evaluation of chemicals, or, more specifically, nanomaterials, is undeniable.

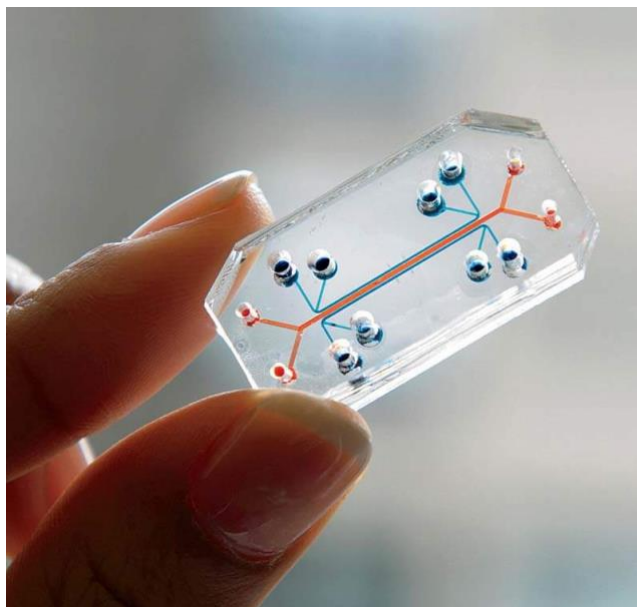


Figure 2: Example of Organ on a Chip: Lung model (Wyss Institute)

These “organs-on-a-chip” are extremely valuable due to their versatility and ability to reproduce specific aspects of the cellular microenvironment of different tissues. They are capable of reproducing microstructures with similar dimensions to those of mammalian cells and provide control of the microenvironment space and time. This allows accurate simulation of “the continuous transport of nutrients and oxygen, the dilution of the secreted cytokines and chemokines, in addition to the cellular waste products” (Wick et al., 2015, p. 5). Furthermore, the cell culture surface stiffness and the extracellular matrix can be modified. This is important because insoluble signals present in living organisms can finally be emulated with accuracy. These mechanical stimuli are present in processes such as the cyclic strain of respiratory movements or the varying pressures of arterial avenues outside of the heart.

Microfluidic technologies also provide further options in the creation of bioartificial barriers. This means researchers can now simulate *in vivo* barriers such as the liver sinusoidal barrier, the blood-brain barrier, and the gut. In place of the electrons which flow through silicon, the contents of the translucent chips push small quantities of nanoparticles past cells from lungs, intestines, livers, kidneys, or hearts. Changes in this tissue can then be observed under a microscope (Levis, 2015). These networks of tiny tubes within the chips, which let the chips mimic the structure and critical functions of organs, are called microfluidics. This can be seen in Figure 3.

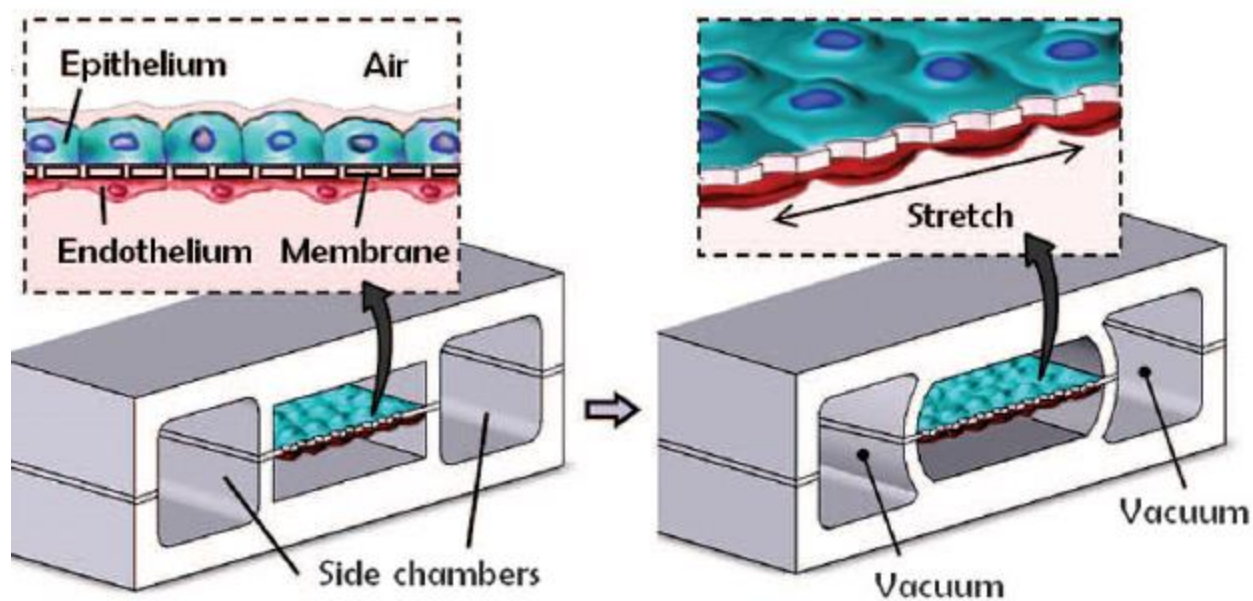


Figure 3: Microfluidic Technology Diagram (Huh, 2010)

There is a lot of evidence of the applicability of microfluidic air-liquid interface technology mimicking the alveolar barrier via the porous membranes. There have been reports of a successful attempt at realistically mimicking subchronic inhalation of multiwalled carbon nanotubes (CNTs) *in vitro*, using the air-liquid interface cell exposure (ALICE) system. This particular study was for aerosol exposures on reconstituted human bronchial tissue from healthy and asthmatic donors (Chortarea et al., 2017). This is convincing evidence of the vast and varied potential that this technology has. There have been reports on the mechanical processes of breathing and the shear stress induced by the blood stream beyond just a porous membrane. Douville et al. (2011) reported a microfluidic alveolar model that allowed for the recreation of the fluid and solid mechanical stresses taking place in the alveoli during mechanical ventilation. Alternatively, “Huh et al. reported on an innovative ‘breathing’ lung-on-a-chip device in which the air-liquid interface was reproduced, with a 10  $\mu\text{m}$ -thin, porous PDMS membrane on which epithelial and endothelial cells were cultured” (Wick et al., 2015 p. 5). The versatility of these chips in the amount of applications it has means it has great potential for further development in the future. It is by no means an exaggeration when people say that “these chips have potential to accelerate drug discovery, decrease drug-development costs, and create a future of personalized medicine to treat a wide range of diseases including cancer, liver failure, pulmonary thrombosis, and asthma” (Levis, 2015).

## 3.4 The Regulation of Test Methods

When new discoveries are made and new testing methods are developed, there are a couple of ways in which they can be assessed and introduced into widespread use. The first and fastest method is basic peer review. For methods that do not require official approval, this is the quickest and easiest way to have work reviewed and implemented by other researchers. However, for research that may pose health risks or ethical concerns, approval by an official government agency or international organization is often required. This is often a slower process than basic peer review; however, the end result is usually safer and more regulated. The main downside of official approval is the time it can take for a new method to become approved and put into use. On the other hand, official approval usually leads to more widespread use and can set the standard that all institutions will use. When it comes to alternatives to animal testing, both peer review and official approval are in use, depending on the type of test and whether the test is intended to be shared and used as a new standard. To gain a better understanding of the current processes, this section will cover:

- Current regulatory bodies and systems around the world
- The current regulations of animal testing and alternatives
- The approval process in Switzerland

These will serve as an introduction to the regulatory systems currently in use and how they apply to animal experimentation and its alternatives, as well as provide a look at an approved alternative.

### 3.4.1 Regulatory Bodies and Systems around the World

There are many areas of research and testing that require official approval, often times by a federal organization. One of the most recognizable is the FDA or Food and Drug Administration in the United States. As the name suggests, the FDA is responsible for regulating the safety of food products and pharmaceuticals, as well as other things that may pose potential health risks such as electromagnetic radiation emitting devices and veterinary products. Other countries have similar agencies such as the Medicine and Healthcare Products Regulatory Agency in the UK and Health Canada in Canada. In the United States, along with the FDA, there is also the National Institutes of Health (NIH), which is responsible for biomedical and public health research. Depending on the research, the results may need to be evaluated or approved by both the FDA and the NIH.

When it comes to animal testing, agencies including the FDA, USDA (United States Department of Agriculture), and NIH require that every institution that uses animals for federally funded research must have an Institutional Animal Care and Use Committee (IACUC). The IACUC must follow guidelines set by the Office of Laboratory Animal Welfare under NIH. This includes reviewing all laboratory work involving animals, conducting investigations into animal misuse, and submitting yearly reports to NIH (Institutional Animal Care and Use Committee Guidebook, 2002). The purpose of having each institution appoint an IACUC is to ensure the appropriate treatment of laboratory animals without the NIH needing to directly oversee each lab. Whenever a new animal experiment is proposed, it must first be reviewed and approved by the IACUC. If the committee determines that the experiment is not appropriate or that the same data can be obtained using alternative methods, it may decline the request (Ambady, personal communication, April 4, 2018).

The regulatory system for animal experimentation in Switzerland is similar to that in the United States; however, since Switzerland is much smaller, it can manage without an individual committee at each institution. Instead, the Swiss appoint cantonal committees on animal experimentation. When an application for an animal experiment is submitted, the committee reviews it and issues a rejection, conditional acceptance, or acceptance. If accepted, the cantonal veterinary office then issues a license. Ultimately, all parties report to the Federal Food Safety and Veterinary Office (Application and Authorisation, 2016). The Swiss FSVO also has a policy that animal experiments may only be performed if no alternatives are available. Additionally, “Researchers must demonstrate that the benefits to society are greater than the distress suffered by the animals involved” (Animal Experimentation, 2018, p.1).

One shared body between the United States and Switzerland is the OECD, or Organisation for Economic Co-operation and Development. The OECD is an organization with 36 member countries that sets guidelines to help share practices and standards between members to lead to collective advancement. In its own words, “The OECD provides a forum in which governments can work together to share experiences and seek solutions to common problems.” (“About the OECD”, n.d., p.2). When it comes to animal experimentation and alternatives, the OECD follows the popular 3R principles of replacement, reduction, and refinement. The OECD has released guidelines for *in vivo* methods that are specifically designed to reduce the number of animals used. Additionally, the OECD has validated and published guidelines for *in vitro* tests to replace animals

when conducting tests for genotoxicity, skin corrosion, phototoxicity, and others (Animal Welfare, 2009). Because of these guidelines and the number of countries that follow them, the OECD has become the main organization for validating alternative methods (Kandarova, 2011).

### 3.4.2 Current Regulations of Animal Testing and Alternatives

Switzerland is one of only a few countries in the world to dedicate a portion of their constitution to protecting the dignity of animals and plants, which was added in 1992. Article 80, Section 20 states that “Confederation shall legislate on the use of reproductive and genetic material from animals, plants and other organisms. In doing so, it shall take account of the dignity of living beings as well as the safety of human beings, animals and the environment, and shall protect the genetic diversity of animal and plant species” (Vermeulen, 2017). In 2003, Switzerland also questioned the ethics of genetically engineering animals in the Swiss Act on Non-Human Gene Technology. The Swiss government first utilized the term “dignity of living beings” in December 2005 (Swiss Animal Welfare Act) and again in April 2008 (Swiss Animal Welfare Ordinance) (Vermeulen, 2017). The Animal Welfare Act defines dignity as:

Inherent worth of the animal that has to [be] respected when dealing with it. If any strain imposed on the animal cannot be justified by overriding interests, this constitutes a disregard for the animal’s dignity. Strain is deemed to be present in particular if pain, suffering or harm is inflicted on the animal, if it is exposed to anxiety or humiliation, if there is major interference with its appearance or its abilities or if it is excessively instrumentalized (Vermeulen, 2017).

Article 17 states that “Animal experiments which inflict pain, suffering or harm on the animal, induce anxiety in the animal, substantially impair its general well-being or that may disregard its dignity in any other way must be limited to the indispensable minimum” (Vermeulen, 2017).

The Animal Welfare Ordinance provides very specific regulations for animal treatment. Animals are initially divided into two categories: domestic and wild. In Article 2, domestic animals are defined as:

Domesticated animals of the equine, bovine, porcine, ovine, and caprine species, excluding exotic species; domesticated yaks and water buffalo; llamas [sic] and alpacas; domestic rabbits, dogs and cats; domestic pigeons and domestic poultry,

such as domestic hens, turkeys, guinea fowl, geese and ducks (Swiss Federal Council, 2011, p. 1).

Wild animals are defined as “vertebrates, except domestic animals, and also cephalopods and decapods” (Swiss Federal Council, 2011, p. 1). These categorizations are then broken down further based upon how they are used. Farm animals are defined as “animals of species that are kept directly or indirectly for the production of food or for certain other benefit or are intended for such use” (Swiss Federal Council, 2011, p. 1), pets are defined as “animals that are kept out of interest in the animal or as a companion in the household or are intended for such use” (Swiss Federal Council, 2011, p. 1), and laboratory animals are defined as “animals that are used in animal experiments or are intended for use in animal experiments” (Swiss Federal Council, 2011, p. 1).

While there exists a lot of regulation and specified procedures for dealing with animals, when it comes to alternative testing methods, there is not as much regulation or standardization. In countries like the United States, there is no official approval process that a lab must go through in order to use a test method that does not involve animals. Without animals, there are no ethical concerns and the laboratory is free to conduct its research without being impeded by any review by a committee (Ambady, 2018).

While there is no official approval needed to be able to use an alternative test for research, some institutions may be wary of the accuracy of new alternative tests that are created. One way to remedy this is to submit the results for peer review. This allows for other institutions to test for the accuracy of the new method. However, if a new method is looking for a more official form of review, organizations such as the OECD can offer their own review and publish the new method as a guideline that it recommends for others to follow.

### 3.4.3 Regulations in Switzerland

There are two different regulatory frameworks for hazard assessments related to nanomaterial exposure based on the intended application: industrial and medical. Understanding the differences between these two styles and how these affect the regulatory environment in Switzerland will be critical to the success of the project.

Industrial nanomaterials are regulated under the REACH and OECD guidelines. Specifically, nanomaterials fall under the guidelines regulating the toxicity testing of chemicals. Additionally, within the EU (Switzerland is not officially a member, yet follows some select EU

guidelines), there has been significant support for the movement to replace the controversial and expensive animal experimentation with *in vitro* alternatives. Despite the expected difficulty in changing this regulation and being a time-intensive process, strides have already been made with a reduction on animal testing in the EU.

However, it is a much different picture for the regulatory environment of medically related nanomaterials. In contrast, the medical regulatory environment “is highly fragmented with individual national regulations” (Wick et al., 2015, p. 7). Therefore, the regulatory approach as a whole in Switzerland is not clearly defined by one environment; rather, it pulls from many sources. It is vital, then, to make sure to follow the right guidelines of a test’s specific environment when trying to establish it as a standard.

There already exists nanosafety tests dealing with the hazards of nanomaterials relating to the irritation and corrosion of skin that became OECD Test Guidelines. They are Test No. 431 and 439.



## Chapter 4: Methodology/ Implementation

The goal of this project is to evaluate the current state of alternatives to animal testing and determine what is needed for these alternatives to be approved and put into widespread use throughout Switzerland. To accomplish this goal, the following objectives were set:

- Create a concise overview of animal experimentation and current alternatives with a focus on nanomaterials,
- Evaluate the approval process for new alternative testing techniques and determine what challenges researchers and regulators have faced in getting these alternatives approved,
- Prepare a report detailing the process for alternative method approval, while taking into account the complications that nanomaterials introduce, with the possibility of future submission to the scientific journal ALTEX.

These objectives are important to the project and its goal because they help bring about the sponsor's desired outcome. Based on conversations with the sponsor, it was determined that completing these objectives most directly correlates with the project goal.

### 4.1 Schedule

Task	Week 1					Week 2					Week 3					Week 4					Week 5					Week 6					Week 7					Week 8				
	08/20-08/24					08/27-08/31					09/03-09/07					09/10-09/14					09/17-09/21					09/24-09/28					10/01-10/05					10/08-10/12				
	M	T	W	R	F	M	T	W	R	F	M	T	W	R	F	M	T	W	R	F	M	T	W	R	F	M	T	W	R	F	M	T	W	R	F	M	T	W	R	F
Meet with AMI																																								
Interview Confirmation																																								
Conduct interviews																																								
Analyze data																																								
Follow up from analysis																																								
Develop proposal																																								
Report writing																																								
Final presentation																																								

Figure 4: Project Schedule

Figure 4 details the implementation schedule for the project. Weekly meetings with AMI were scheduled for each Monday. These included giving updates to Dr. Rothen and Dr. Drasler on the progress of the report so far, along with attending their weekly meeting to discuss new projects and work within the department. Weekly meetings occurred on Thursdays with the advisors to ensure the project was on the right track.

The interview confirmation period shown above was important because two of the scheduled interviews had to be postponed. Therefore, the interview period was pushed back from the original schedule. These interviews were conducted with people from both regulatory and research backgrounds. After interviews were completed, the data obtained was organized and analyzed for further clarity in the report. After this, follow-ups were conducted with the interviewees as needed for refining and additional details. During the follow-up phase, development and writing of the report also began. From there, details were written out and finalized in a formal manner.

A final report and presentation were prepared for the project sponsor. This was a detailed presentation of the project work that described the process of passing alternative tests. There will be a discussion afterward with AMI to ensure they are satisfied with the results. The main deliverable for AMI will be a journal article aimed at test-developers with the step-by-step methodology of passing an alternative test.

## 4.2 Interviews

The following people were interviewed to gather data:

*Table 2: Interviewees and Their Institutions*

<b>Interviewee</b>	<b>Position/Institution</b>	<b>Reasoning</b>
Dr. Samuel Constant	COO of Epithelix, a company which produces an <i>in vitro</i> lung model.	Dr. Constant was contacted because of his experience as a test developer and the potential information he might have about how his company seeks approval for its <i>in vitro</i> tests.
Drs. Markus Hofmann & Christoph Studer	Current and former National Coordinators (NC) of the OECD Test Guideline Program for the Swiss Federal Office of Public Health.	Drs. Hofmann and Studer were interviewed to gain an understanding of the role of the National Coordinator in the approval process and see if they could point out any difficulties in the process that could be improved.

Dr. Lothar Aicher	Regulatory Toxicologist at the Swiss Center for Applied Human Toxicology (SCAHT), whose mission is to “advise authorities, promote research, and provide education and training in human toxicology” (SCAHT, 2018).	Having experience in both regulations and toxicology, particularly with nanomaterials, Dr. Aicher was interviewed with the goal of gaining a better knowledge of the regulator’s point of view and what intricacies nanomaterials present when developing alternative tests.
Dr. Chantra Eskes	Formerly involved with the validation of alternative tests within the European Center for the Validation of Alternative Methods (ECVAM). She is also currently the chair of the ECVAM Scientific Advisory Committee (ESAC) and is a Nominated Expert for the OECD. She heads the 3Rs Competence Center (3RCC): a new organization which promotes the education, communication, and research of the 3Rs.	Being the director of a new center specializing in the 3Rs and having experience with validation at ECVAM, Dr. Eskes was interviewed to learn more about the validation process and how this process can be more easily presented to test developers.

To begin the interviews, permission was requested to record the conversations and interviewee’s responses in developing both reports for AMI and WPI. Once permission was given, a brief introduction to the project was stated and the interviewees were asked to elaborate on their positions and experience in their own words. There was a set of interview questions which were used as a base to help guide the interviews; these can be found in Appendices A and B. However, the interviews were paced as discussions and follow up questions played the largest role in data collection.

A note-taker made a basic transcript of the interview and audio recordings were collected. These files are the main source of data which will be used to extract information or quotes.

At the end of the interviews, interviewees were asked if there were any additional resources or contacts that they felt would be provide useful information. Two of the four originally scheduled interviewees, Dr. Hofman and Dr. Aicher, provided contact information for Dr. Eskes, the new

director of the 3Rs center in Switzerland. They hailed her as the foremost expert on alternative methods in Switzerland. Contact information for all interviewees can be found in Appendix E.

### 4.3 Analysis

After conducting interviews, the information gained was organized, interpreted, and compared. Due to the lack of rigid structure and conversational nature of the interviews, it was necessary to read over the interview notes and combine information from multiple sources to piece together a cohesive idea of the current state of alternatives and the approval process they must go through. This information gave a more complete understanding of the process, though it also introduced some previously unknown complexities that are involved with new alternatives, particularly those involving nanomaterials. Because of this, a plan was developed to create a general outline of the process for approving any alternative test and a list of recommendations to assist in the process, as well as a more focused section that encompasses tests involving nanoparticles and nanomaterials that provides information on any additions or changes to the approval process.

The main deliverable to give to AMI is a report detailing the approval process of alternative test methods. This report breaks down the process step by step and includes the list of recommendations.

## Chapter 5: Results

The work done in Switzerland, described in Chapter 4, provided crucial information needed to achieve the project goals. This information consisted of detailed descriptions of the various stages of the validation and regulatory process, as well as the organizations involved and what roles they played in the process. This section covers the results of this work and describes everything from the details of the steps of the approval process, to the particular challenges presented when assessing nanomaterials.

### 5.1 Information from Interviews

Several interviews were conducted to gather data on the state of alternatives to animal testing and on the process by which a test becomes a validated standard. Both researchers and regulators were contacted to gain an understanding of the different perspectives on the challenges faced during this process. A major issue acknowledged by all interviewees was the lack of communication. Researchers were often unsure of where to begin and who to contact, while regulators were overwhelmed with the complexity of the reports provided.

The intricacy of alternative tests, along with lack of communication between test developers and regulators, make the approval process long and difficult. To analyze this issue, researchers were asked about their specific tests and the difficulties faced in gaining approval, while regulators were asked about their roles in this process. The following describes the main takeaways from each interview:

- Dr. Samuel Constant elaborated on the lack of communication between regulators and test developers and described his company's method of reaching out to customers for support in pushing for approval of their tests.
- Dr. Christoph Studer and Dr. Markus Hofmann provided insight on the process a test must go through when seeking OECD approval and explained that the first step after developing a test is to contact the National Coordinator (NC). Dr. Hofmann's office is able to expedite tests if they meet certain criteria, such as high relevance and need for the new test, which are described later on.
- Dr. Lothar Aicher elaborated on adverse outcome pathways (AOPs) and the difficulties surrounding nanomaterials. Specifically, the regulatory process is the same as other

alternatives; however, the science behind nanomaterials adds an extra degree of complexity.

- Dr. Chantra Eskes was able to clarify the process of approving alternative tests and provided resources on the modular approach to validation. Her past work at ECVAM allowed her to provide crucial information that greatly clarified the validation process that tests must go through during the approval process.

## 5.2 Approval and Standardization Process

This section covers the approval process that alternative methods must go through when seeking standardization. Outlined here are the institutions and steps involved, as well as several breakdowns and graphics to aid in simplifying the material.

### 5.2.1 Regulatory Resources

For many researchers, the official test validation and standardization process is unclear. Fortunately, there are several resources that can aid in the process. This section will focus on resources within Switzerland specifically, although some of these resources are either international or have equivalent organizations in other countries.

National Coordinator of the OECD Test Guidelines Program: Each OECD member country has a National Coordinator (NC) who represents that country's regulatory body within the OECD. The job of the NC is to review project proposals presented for standardization and select which projects to move forward through the approval process outlined by the OECD Test Guidelines Program. He coordinates all relevant aspects of the process, including having the new method tested by third-party validators (most notably ECVAM) and advocating for the method to become a new guideline. The current National Coordinator for Switzerland is Dr. Markus Hofmann, who works within the Swiss Federal Office for Public Health. For researchers within Switzerland who would like to promote a new method to become a test guideline, it is important to get in contact with Dr. Hofmann early on, such as during the development phase. Even if a new method is not yet fully developed, the NC's office can provide important information and recommendations that can make the approval process easier down the road. While the NC is an essential resource in gaining OECD approval, getting a project accepted can be a difficult task. Every year, more projects get proposed than can be accepted. In 2014, there were only 56 projects being worked on by the NC's office in

Switzerland (Hofmann, Eskes, Aicher, 2014). The acceptance of tests is determined by key factors such as relevance, importance, and feasibility. These aspects are up to the discretion of the NC. It is also important to note that the NC does not only work with alternative methods; however, the percentage of accepted tests that involve alternative methods rose from about 13% in 2007 to about 48% in 2014 (Hofmann et al., 2014). Currently, about 90-95% of proposed projects in Switzerland involve alternative methods (Hofmann, 2018).

The Organisation for Economic Cooperation and Development (OECD): The OECD is an international organization with 36 member countries whose goal is to “promote policies that will improve the economic and social well-being of people around the world” (OECD, 2018). Policies issued by the OECD deal with a range of areas, from taxation and labor, to chemical testing and research. Most relevant to alternative methods is the OECD’s capacity to standardize testing methods as guidelines that its member countries must follow. To support this, the OECD has set a standard for Good Laboratory Practice (GLP) for researchers to follow that allows for the Mutual Acceptance of Data (MAD) between members (OECD, 2018). Additionally, for research institutions, the OECD has developed a set of guidance documents that can be found online that outline specific practices to follow when conducting certain tests.

The European Center for the Validation of Alternative Methods (ECVAM): ECVAM serves as an institution that promotes and validates alternative testing methods. Most notably, it is the main center for external validation of alternative methods used by the OECD. ECVAM specializes in the independent evaluation of the relevance and reliability of tests used for assessing medicines, vaccines, medical devices, cosmetics, and household and agricultural products. (ECVAM, 2018) For a new alternative test to make its way to OECD approval, it must first go through independent validation. This is something that researchers and companies can pursue with ECVAM directly; however, it is highly advised to make contact through the country’s NC instead, as he or she will be able to better coordinate the validation process.

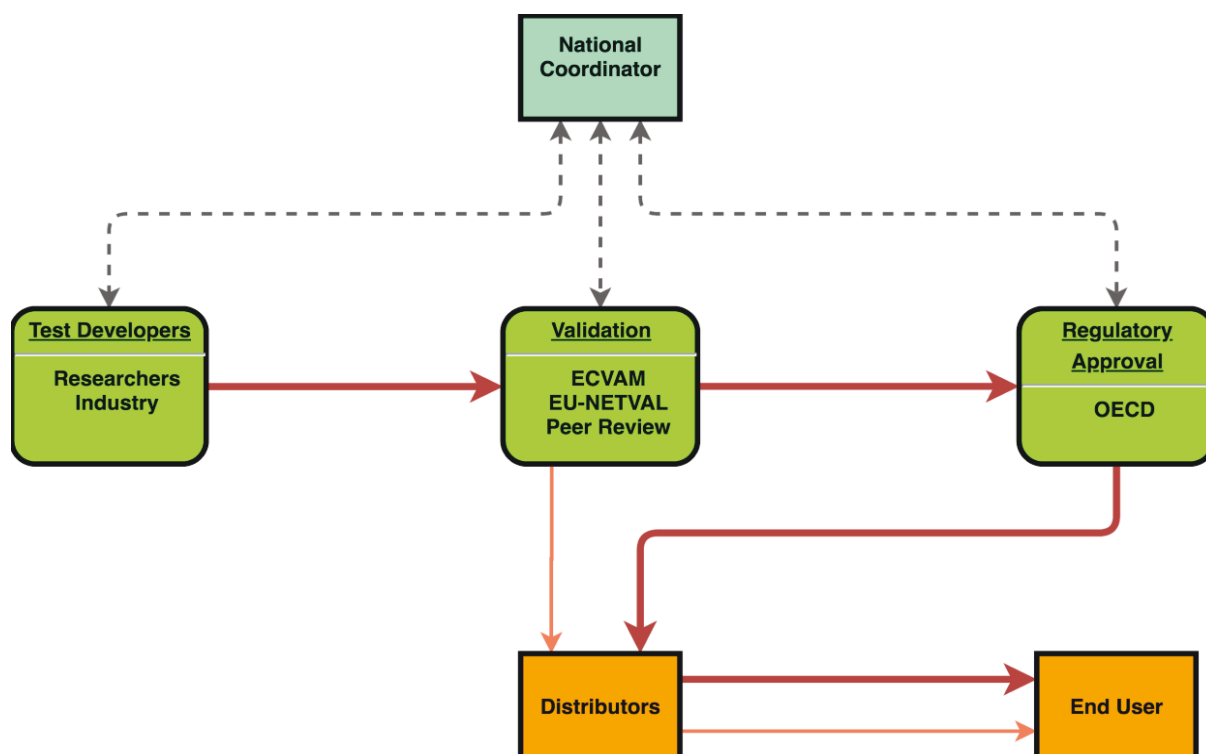
When validating a new method, it is necessary to have multiple laboratories test for transferability and variation. To accomplish this, ECVAM has organized the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL). EU-NETVAL consists of 37 laboratories that have been carefully selected and follow GLP practices. These

laboratories assist ECVAM in the assessment of new alternative testing methods (European Commission, 2017).

Swiss 3Rs Competence Center: The Swiss 3Rs Competence Center is a center which has recently opened and has a strong focus on the replacement, reduction, and refinement of animal testing. The purpose of the center is to educate on methods that involve the 3Rs, provide a place for researchers, regulators, and industry members interested in the 3Rs to network, and to subsidize projects involving the 3R practices. Even though the center is new, it is already looking to open up for a first round of project applications by the end of 2018 (3RCC, 2018). The center is also looking to establish educational programs to promote the use of 3R principles and alternative methods (Eskes, 2018). The current director of the center is Dr. Chantra Eskes. The center is poised to be a valuable resource for promoting alternative methods in Switzerland and Europe.

### 5.2.2 Regulatory Process

It is evident that the process involved is complicated so it is pertinent to first take a step back in order to visualize the different parties involved and how they interact. Figure 5 shows the big picture of the three step process to visualize which organizations are involved in each step.



*Figure 5: Flow Diagram of the Approval Process Showing the Groups Involved*



There are three main steps in the approval process: test development, validation, and regulatory approval. There are organizations involved in each of these steps. During test development, both researchers and industry developers work on creating alternative testing methods. When they feel confident in an alternative method, it moves along to external validation. At this point, organizations like ECVAM, EU-NETVAL and peer review boards become involved. They run a validation study in order to approve or reject the alternative method. It is only after the acceptance in this step that an alternative method will be elevated to regulatory acceptance where the OECD is involved. They have the role of deciding whether or not to instate the alternative method as a Test Guideline.

The following sections will go into depth on the individual steps within the regulatory process to make clear its intricacies.

### 5.2.3 Test Development and Design Control

The initial design of an alternative method is determined by the user need and several design requirements, such as versatility, reproducibility, transferability, and robustness. However, it is very difficult and highly unlikely to develop the best possible test to meet all of these requirements on initial design.

Thus, there is an optimization process involved in developing an alternative testing method. After the initial design of an alternative method, developers need to define parameters by which the alternative method is to be evaluated. These test parameters should be established using reliable past *in vivo* or *in vitro* test data to set a stable baseline. When assessing human health effects, the OECD (2005) suggests the use of human data from sources such as:

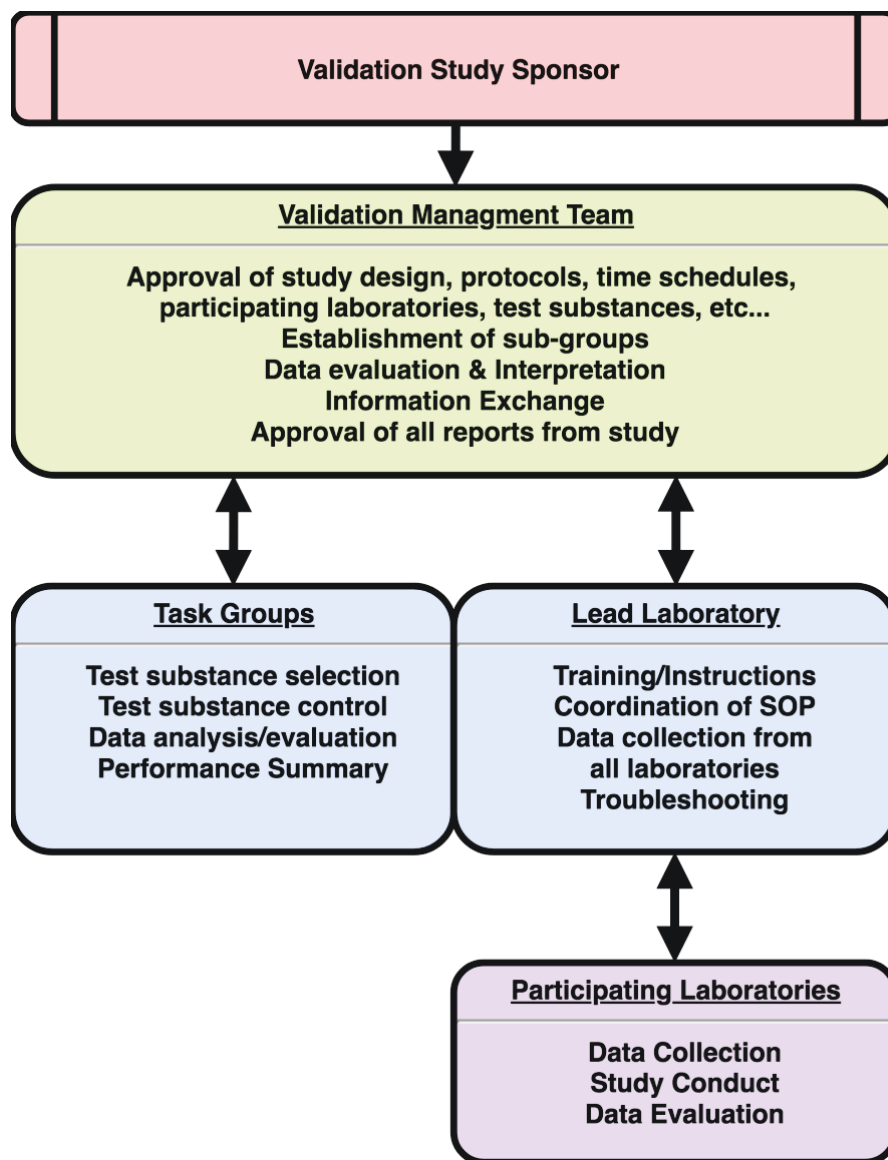
- Epidemiology
- Occupational exposure
- Accidents and cases of poisoning
- Clinical studies
- Ethically approved studies in human volunteers

It is important to continuously compare against and improve the developing alternative method using these standards. This cycle, called design control, is the optimization process that refines alternative methods and generated endpoints until they are of equal or greater significance than animal testing counterparts. This is a vital process in the development of an alternative testing method.

There are three broad parameters by which an alternative method should be tested: relevance, accuracy, and reproducibility. These are key aspects of an alternative test with the goal of validation and approval as an OECD Test Guideline. However, there is no set process for this optimization phase, and it is possible to customize this process on an individual basis. It is possible, however, to check for optimization of an alternative method against the same seven modules that the validation management group will use to evaluate the method. This is often referred to as pre-validation. These modules are described in the Modular Approach section. While it is important to understand the process behind validation, it is necessary to first understand who is involved in moving the process forward.

#### 5.2.4 Validation Study Organization

A validation study begins with a sponsor, who assigns a study manager or management team to design and carry it out. This management team can then delegate various responsibilities to Task Groups. The management team also oversees the Lead Laboratory for the study, which is in charge of data collection and instructing the other participating labs on the SOP. These bodies are shown in Figure 6 and described further below.



*Figure 6: Organization of the Validation Study Tiers (OECD, 2005)*

There are several potential candidates to be a sponsor of a validation study. They include: international bodies, government entities or validation organizations for alternative methods (ECVAM, ICCVAM), national organizations, other independent organizations, or commercial sponsors. If the validation study is organized by the OECD, the sponsor may be an OECD expert group, task force, or working group/party whose members are nominated by the governments of the respective countries (OECD, 2005). These bodies oversee the validation study but play a minimal role in the day-to-day operation.

Validation study operations are overseen by the validation manager or management team. The management team must have a collective expertise on all relevant fields concerning the test

and the science behind it, along with background in management/evaluation of validation studies in order to be appointed to this position. The team has the responsibility of approving study design, protocol, test substances etc., and can delegate these decisions to specialized task groups. It also coordinates with the lead laboratory, which is in charge of training participating laboratories, coordinating the SOP, and troubleshooting any difficulties that arise during data collection. The following section details what data is being collected to test against when evaluating alternative methods.

### 5.2.5 Modular Approach

In an attempt to make the validation process more efficient, ECVAM has developed a modular approach to alternative test validation. This approach breaks down the main components of validation into seven modules. The purpose of these modules is to define what data is needed for independent validation and peer review. By having this defined list of requirements, researchers can develop tests while keeping the data needed for validation in mind, thus streamlining the process later on. Once test developers have finished development and have taken into account all of the modules, the independent validation process can begin. The seven modules are as follows:

1. Test Definition: The Test Definition states the underlying reasons for the development of the test, as well as defines any necessary information needed to properly conduct it. The specific scientific purpose of the test must be clearly stated here. Additionally, the Test Definition must include a clearly defined protocol that complies with Good Laboratory Practice and Good Cell Culture Practice. This protocol should include any necessary Standard Operating Procedures (SOPs) to allow for replication. Finally, the Test Definition must define specific endpoints and measurements, specify how to derive, express, and interpret results; as well as include a set of adequate controls (Hartung et al., 2004).
2. Intra-Laboratory Repeatability: The test must be proven to be repeatable and consistent within the same laboratory. This is usually done with different operators over time within the same laboratory setup and done at the development laboratory. Any variation in results within the same lab must be addressed (OECD, 2005).
3. Inter-Laboratory Transferability: The transferability of a test plays a crucial role in its robustness. The goal of this module is to show that the test can successfully be repeated in a laboratory other than the one that has developed the test or assisted in its optimization.

The data gathered here will provide an estimation of the training required for a naive laboratory (lab without prior experience with the specific type of test) to reproduce the test, as well as identify additional sources of intra- and inter-laboratory variation (OECD, 2005).

4. Inter-Laboratory Reproducibility: During this process, external laboratories carry out the test method and test it against a large assortment of substances. This is usually organized by ECVAM and involves three or four well-trained laboratories. The assessment of inter-laboratory variation can also be done with a more limited selection of test substances that still cover all toxic effects that the test can demonstrate. This allows the predictive capacity to be tested against a large number of substances in a single lab to save time and money (OECD, 2005).
5. Predictive Capacity: The Predictive Capacity of a test is a demonstration of how well the test can predict the reference standard and can generally be referred to as the accuracy of the test. The reference being tested against is often an existing standard such as an *in vivo* test or a human health effect. The Predictive Capacity is influenced by the quality of the standard and the range and number of substances tested. The current ECVAM process states that Predictive Capacity must be tested in at least three laboratories; however, if inter-laboratory variability has already been determined to be of acceptable levels, testing of Predictive Capacity may be done at just one laboratory (Hartung et al., 2004).
6. Applicability Domain: The Applicability Domain of a test must clearly describe the particular purposes for which the test can be applied. This includes any toxicological endpoints, chemical classes, test materials, and physicochemical properties or products that might be assessed with the test. Any changes to the Applicability Domain may require additional peer review (Hartung et al., 2004).
7. Performance Standards: At the end of the validation process, the Performance Standards of the test are evaluated. This is a compilation of essential test method components, reference substances, and accuracy and reliability values that can be used to demonstrate equivalence in performance between future tests and the current test. This can allow for faster approval of similar tests or changes to the current test in the future (Hartung et al., 2004).

### 5.2.6 Peer Review of Validation Study

Once the validation study has been completed and the Validation Management Group is satisfied that all necessary data has been collected and evaluated, a test method may move on to the independent peer review stage. This can be done in one of two ways: first, the Validation Study Sponsor may choose to organize a panel of independent peer reviewers to assess the findings of the validation study. This may be the preferred route if the test method is not being proposed as a new OECD Test Guideline. If the test method is being proposed as a new Test Guideline, the Validation Study Sponsor can still opt to organize peer review before submitting the test to the OECD, or it can submit the test without peer review and the OECD will organize the review panel. If peer review is completed prior to the test method being submitted for approval as a Test Guideline, a complete report of the independent panel must be provided, including detailed reasoning behind any conclusions and recommendations, as well as any comments made on the test method. Otherwise, the OECD will discuss and appoint a responsible party to organize any necessary peer review (OECD, 2005).

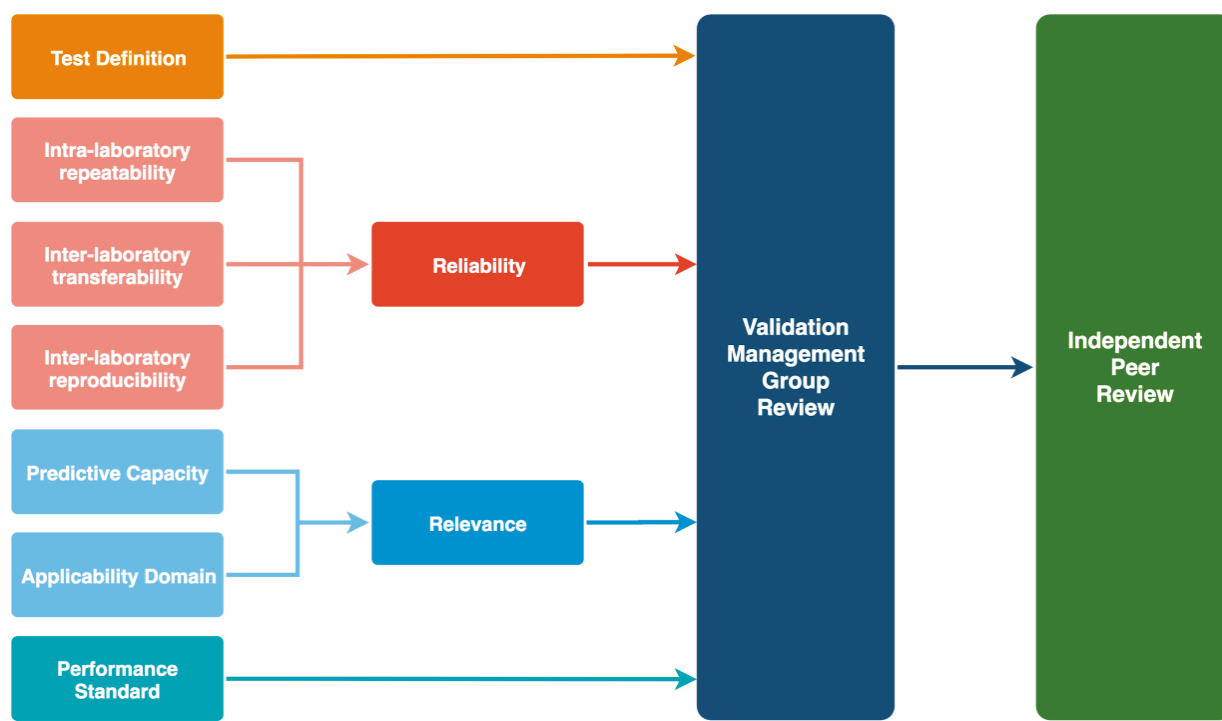
When selecting a panel of experts for peer review, there are certain criteria to follow to ensure accurate and unbiased review.

- The experts must demonstrate expertise in one or more of the scientific fields relevant to the test. The panel as a whole should contain at least one expert for each relevant field, and may contain more than one representative for any essential fields relating to the test method.
- It is important that some of the peer reviewers have experience in the development, conduct, and evaluation of validation studies for toxicology tests. When appropriate, one or more reviewers should also have an understanding of animal welfare issues and the 3R principles.
- It is crucial that all peer reviewers are independent and not subject to any conflicts of interest. This includes any previous substantial involvement in the process and any interest in the outcome of the study, such as financial gain. More minor interests may be allowed, as long as the panel as a whole is balanced and independent (OECD, 2005).

Once put together, it is up to the peer review panel to review all information from the validation process and conclude as to whether the test method has thoroughly satisfied its intended purpose and goals. The review process should be open and transparent and may allow for public comment.

At the end of review, the panel should provide a report containing an assessment of the usefulness and limitations of the test method. Questions may then be posed to the panel as the final stage of the peer review process (OECD, 2005).

After peer review has been completed, it is up to the Validation Study Sponsor to determine, based off of the panel's findings, if the test method has been validated for its intended purpose and should be recommended for regulatory acceptance. It is then up to individual regulatory bodies to determine if the validated test method is suitable for its purpose and if it may be accepted under each body's specific area of jurisdiction (Hofmann, Personal Communication, September 5, 2018).



*Figure 7: Modular Approach Validation Process*

### 5.2.7 Regulatory Acceptance

Once all seven modules have been satisfactorily completed (as judged by the Validation Management Group), the alternative testing method can enter the peer review process. This is a test conducted by an independent peer review board and is the last step in the validation of an alternative method. The board consists of qualified scientists who have not been involved in the development or validation of the test method, and who will not benefit financially or otherwise as

a result of the review (OECD, 2005). Upon completion of this step, the alternative method is officially validated and no more evaluation needs to be conducted. However, should the test fail peer review, the test would then be sent back to the development stage in an attempt to address any concerns with the test. This an unlikely event, however, as the peer review board will be evaluating on similar if not the same criteria.

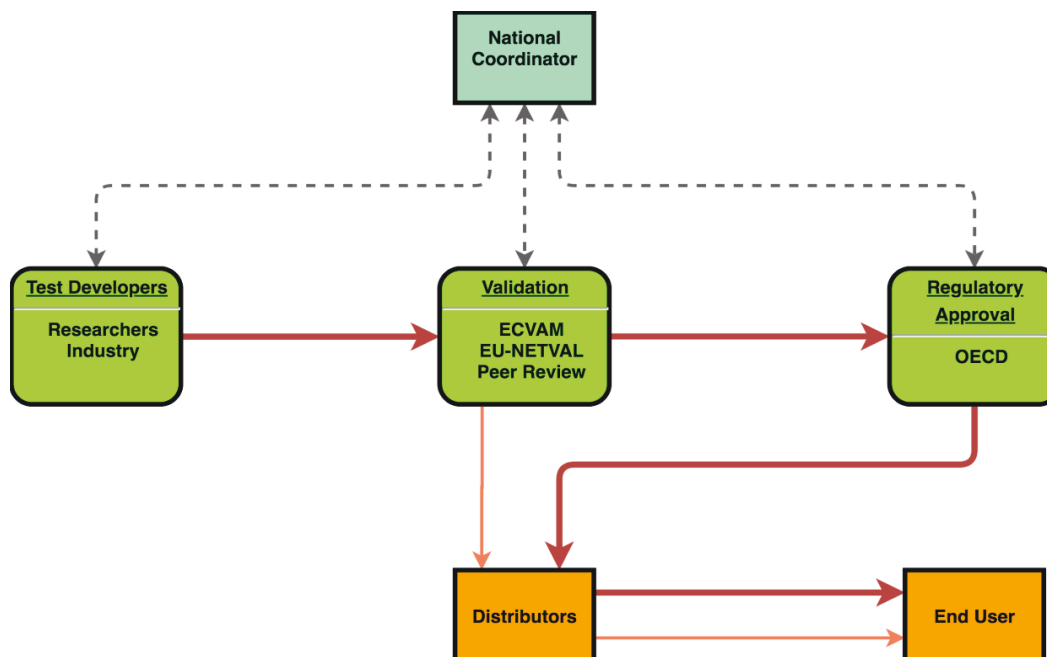
Once a test is a validated method, it can be submitted to the OECD. At this point, it is up to the regulators to review the test and data gathered during validation. In order for the alternative method to be instated as an OECD guideline, all member states must vote unanimously (C. Eskes. Personal communication, September 20, 2018). That is to say, if any singular or greater amount of country representatives do not agree on the acceptance of the alternative method, it will not become an OECD Test Guideline.

This process can be lengthy and can take up to or sometimes over a year. There are many steps for the test to go through before a definitive vote can be made. Once the decision is made, the alternative testing method will join a growing faction of internationally accepted testing methods as a standard for assessing the potential health effects of chemicals on humans and the environment.



### 5.2.8 Paths to the End User

Now that the individual steps have been coherently described, it is possible to see how they connect in the big picture.



*Figure 5: Flow Diagram of Approval Process Showing the Groups Involved*

Looking back at Figure 5, it is clear that there are two main paths to take for an alternative testing method to go from development to the end user.

The first path, and the one described in the previous sections, is the regulatory approval path through organizations such as the OECD. By going through an international organization, with many member countries, the potential population of end user increases significantly. This requires no additional effort from test developers and in countries like Switzerland, who follow the 3R Principles, alternative methods become the standard (over animal testing methods) and are put into use on a national scale.

This doesn't need to be the case, however. There is a second potential path to end users. Test developers are not obligated to submit their alternative methods to international regulatory agencies and can go directly to distributors after validation. This, however, limits the end user population, as it is up to each user's discretion to determine whether the test is acceptable and valid for their purposes or not; each country has their own agencies and practices, and it is not uncommon to see large discrepancies in their scientific regulatory policies. Thus, the end users

must decide on their own if they want to use the test without the backing of an internationally accepted organization like the OECD.

### 5.3 Challenges Surrounding Nanomaterials

Nanoscience is a relatively new field; as such, the various properties of nanoparticles are not always well documented. Due to their extremely small size and high surface-area-to-volume ratio, particles under 100 nm can have a much higher reactivity and permeate membranes that larger particles cannot. This can cause detrimental interactions that are not observed with larger particles of the same material. These differences lead to more variables that need to be tested when assessing the safety of nanoparticles. This complicates the process for developing and approving alternative tests for nanoparticle hazard assessment (L. Aicher. Personal communication, September 10, 2018).

The Swiss Center for Applied Human Toxicology (SCAHT) defines five different variables that need to be tested when assessing nanoparticles and their interactions with living tissue:

1. **Size:** on nano-scale, even small changes in size can lead to significant changes in behavior. As such, SCAHT has decided on a size variation of 10% or 10 nm for which test results are still acceptable. Any particle sizes that fall outside of the range of the tested particles must be tested separately (Aicher, 2017).
2. **Surface Chemistry:** nanoparticles may attract certain molecules or proteins that can attach to and coat the particle, creating a unique corona that may vary depending on the environment the particle is in. The coating can affect how the particle behaves: for example, by making it more stable or less likely to clump together with other particles. Any differences in surface chemistry caused by different environments must be tested to ensure proper assessment of the particle's effects (Aicher, 2017).
3. **Core Material:** the core material of a particle can affect how the corona forms and how it reacts with other chemicals. Depending on the particle, the core material can play a greater role than the surface chemistry and is an important factor to test (Aicher, 2017).
4. **Environment:** the environment that the nanoparticles interact with influences multiple factors. It affects aspects such as corona formation and whether or not the particles clump

together. As such, any environmental differences such as changes in chemistry, contents, or pH require testing (Aicher, 2017).

5. Morphology: the morphology, or shape, of the particle plays a large role in its physical interactions with living tissue. For example, differences in morphology can affect a particle's ability to permeate through membranes. Round particles can usually pass through more easily than longer, fiber-like particles. Therefore, even if the core and surface chemistry between two particles is the same, variations in shape can cause the particles to behave and interact very differently (Aicher, 2017).

When it comes to developing alternative testing methods for assessing nanomaterials, the factors listed above must be taken into account. Therefore, a test that assesses the hazard of nanoparticles on a certain organ or type of tissue must permit for all variables to be tested. These five points of variability are one of the main reasons that it is more difficult to develop alternative tests for nanomaterial assessment (Aicher, 2017). It is much easier, therefore, to develop tests for larger particles or chemicals, as small changes in these variables do not play as much of a role in determining how the substance behaves. While these variables might appear to be tedious, it is not always the case that all five need to be tested. Increases in known effects can allow variables to be omitted in testing if previously determined to be hazardous. For example, if a particular size and shape has proven to be hazardous in multiple nanoparticles, any particle being tested that has a similar shape and size can be assumed to be hazardous. Whether or not a particle is safe, however, cannot be based off of previous data and all variables must still be tested (Aicher, 2018). Some valuable resources for hazard data are verified Adverse Outcome Pathways (AOPs). AOPs are studies on how specific chemicals or materials can be hazardous through specific pathways. A database of these pathways is available online in the form of the AOP-wiki.

Since testing methods are further complicated by the properties of nanoparticles, there are additional complexities involved in the regulatory process. While an animal test can show the effects of a substance on an entire living system, alternative tests generally need to break a body system down into different sets of cell types that are tested independently. The results are then combined to determine how the substance being tested affects the system as a whole. This, along with the numerous variables presented by nanomaterials, means that multiple tests within a

comprehensive testing method must be validated to approve the overall alternative method (Aicher, 2018).

Along with the inherent complexities of nanoparticles and their testing, the largest complication in the test development and approval process at this time is a lack of knowledge about nanoparticles and their behavior. Nanoscience is still a developing field, and there is currently not a widespread understanding of the scope of the effects of nanoparticles. It is therefore difficult to get tests on these effects approved because regulators do not have an in-depth understanding of nanoscience at this time (Hofmann, 2018). Therefore, they must first gain greater comprehension of the field before approving new methods. This lack of information, along with a communication gap between researchers and regulators, is a hindrance to the spread of new alternative nanoparticle tests.

## Chapter 6: Conclusions & Recommendations

### 6.1 Concise Guide to the Approval Process

Figure 8 shows the current step-by-step process for the approval process of alternative methods as OECD Test Guidelines.

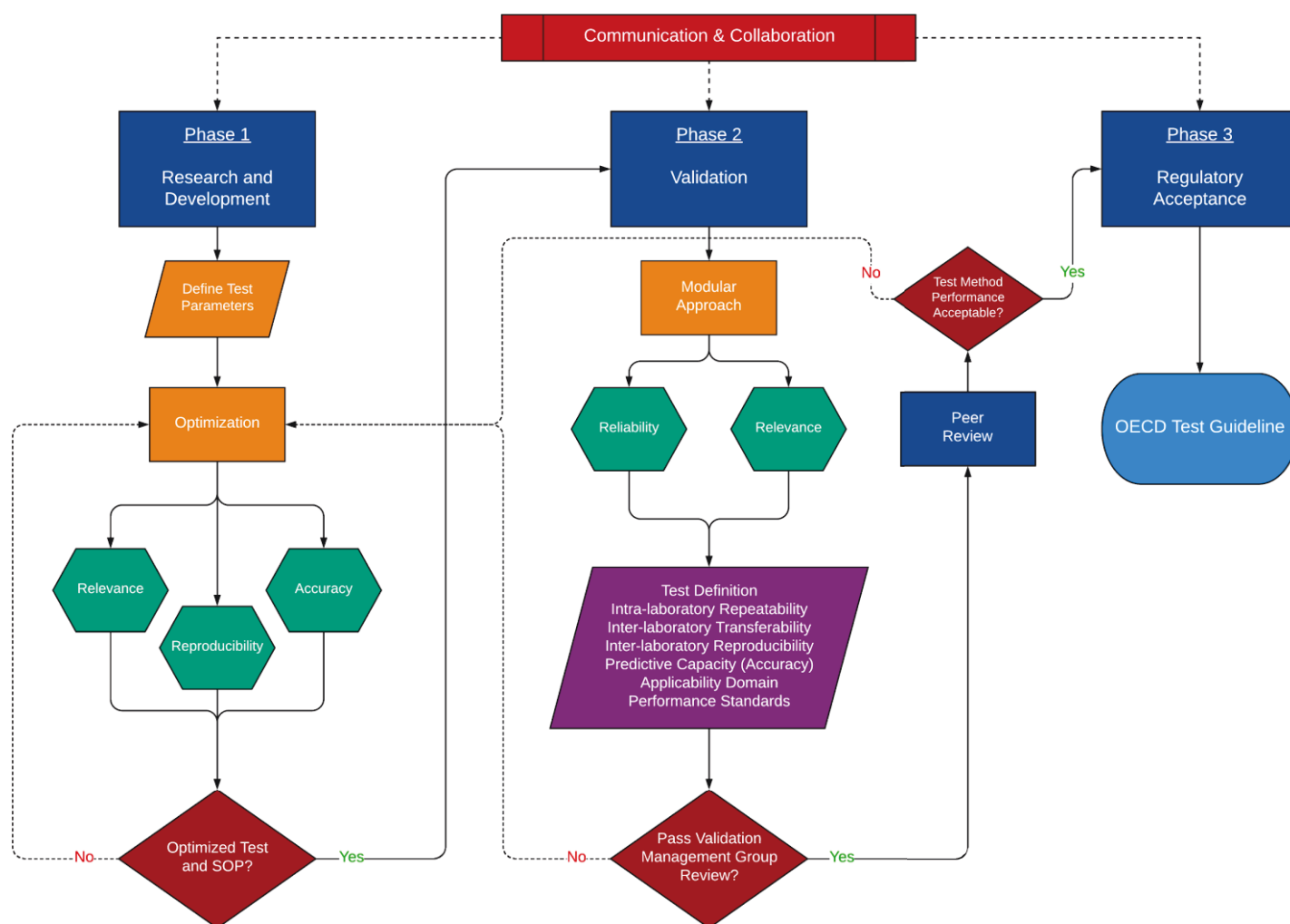


Figure 8: Pathway to OECD Acceptance

This diagram breaks up the process into three phases. The main focus of the first phase, research and development, is test development and optimization (which is cyclical in nature). By continuously testing and improving the proposed method, the alternative test and its standard operating procedure will be better prepared for the validation stage. During this phase, the design

of the test should reflect relevance, accuracy, and reproducibility. These three parameters were chosen by considering the seven test modules of the validation phase's modular approach.

This approach is used by the validation management group in phase 2 to evaluate the alternative testing method. Should the validation management group decide the test passes all seven modules, the data gathered will be sent to a board of independent peer reviewers, who make a recommendation based off of this data. This recommendation is taken into consideration in the final decision made by the validation study sponsor on whether the alternative method is validated for its intended purpose. If it is decided that the method is acceptable, it can then be sent to the OECD for potential standardization. To be instated as a Test Guideline, a method must be voted in unanimously by the OECD member countries.

## 6.2 Recommendations

Based on interviews and research conducted throughout this project, the following recommendations have been compiled:

- During design, test developers should make the alternative method
  - Relevant: how well does it fulfill its intended purpose? Are there other applications for the test?
  - Accurate: to what degree does the test predict the intended outcome?
  - Reproducible: can the original lab and other labs replicate endpoints with precision?
- Researchers should use resources listed in the regulatory resources section to facilitate communication throughout the process. The NC of the OECD Test Guidelines Program can help bridge the communication gap. Communication earlier in the process will allow the NC to determine the user need and help coordinate with all involved parties.
- Due to the nature and complexity of alternative methods, especially relating to nanomaterials, the current case-by-case methodology of regulation is not yet replaceable. Thus, close collaboration between researchers and regulators will grow in importance as topics become increasingly complex.
- When presenting to regulators, test developers should simplify their explanations of relevant material and remember that regulators may not have the same level of experience

in the subject. The best way to present a new alternative method is to lay out a specific endpoint and express exactly what the method seeks to test.

### 6.3 Further Work

One of the project sponsors expressed interest in a related field of work. While this project primarily focused on the standardization process for nanomaterial hazard assessments, a colleague of the sponsor expressed interest in *in vitro* ocular models. She gave a presentation on what she wishes to test with these models, and the scope of her proposal was deemed too big for the time remaining on this project. It is very early in its development and presents the potential for future project groups to team up with AMI again. They would coordinate with the sponsor in her work on research and development of the ocular model. This would likely take the form of an MQP that would assist in the development of a multi-cell type *in vitro* model of the ocular system. This would make an impact in the alternative testing field, as there is currently no such model available.

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# Glossary

3Rs (or 3R Principles): Replacement, Reduction, and Refinement of animal testing.

3RCC: Swiss 3Rs Competence Center

The goals of the 3RCC are to promote the usage of 3R principles, educate on the need to reduce animal testing, and support projects that implement the 3Rs.

AMI: Adolphe Merkle Institute

The Adolphe Merkle Institute is a research institute connected to the University of Fribourg that specializes in soft nanomaterials.

AOP: Adverse Outcome Pathway

A structured representation of biological events leading to an adverse effect.

CNT: Carbon nanotube

Long, thin molecules of carbon in the shape of tubes that are usually 1-3 nm in diameter and hundreds to thousands of nanometers long.

DRZE: Deutsches Referenzzentrum für Ethik (German Reference Center for Ethics in the Life Sciences)

German national center specializing in ethics in the biomedical sciences.

ECVAM: European Center for the Validation of Alternative Methods

ECVAM is a laboratory specializing in the validation and scientific promotion of alternative testing methods such as *in vitro* tests.

ENM: Engineered Nanomaterials

Nanomaterials that have been deliberately engineered and manufactured by humans to have specific properties.

EPAA: European Partnership for Alternative Approaches to Animal Testing

A European partnership whose members are committed to pooling knowledge and resources to accelerate the development, validation, and acceptance of alternative approaches to animal use in regulatory testing.

EU-NETVAL: European Union Network of Laboratories for the Validation of Alternative Methods

A network of certified laboratories organized by ECVAM to assist in the independent validation of alternative testing methods.

FOPH (BAG): Swiss Federal Office of Public Health (Bundesamt Für Gesundheit)

Swiss government agency in charge of all aspects of public health, including regulating how chemicals are tested for safety.

GLP: Good Laboratory Practice

A set of regulations defined by the OECD for non-clinical safety testing to ensure proper testing techniques and consistency between laboratories.

Guidance Documents: Documents produced by the OECD that provide specific processes for anything from the testing of particular types of chemicals, to the creation of new OECD Test Guidelines.

HARN: High Aspect Ratio Nanomaterial

A nanomaterial with a length that is significantly greater than its width, such as certain nanotubes or nanoplates.

In vitro: An alternative experimentation method involving testing in a controlled environment outside of a living organism.

In vivo: Experimentation involving a whole, living organism as opposed to a partial or dead organism.

In silico: Experimentation done via a computer simulation.

MAD: Mutual Acceptance of Data

An OECD agreement stating that any data on the testing of chemicals that has been gathered in accordance with OECD Test Guidelines and GLP shall be accepted by all member countries.

Naive Laboratory: A laboratory that has enough expertise to carry out a test undergoing validation, but does not have experience with the particular test method.

Nanomaterial: A material composed of particles in an unbound or aggregate state which, for at least 50% of the particles in the size distribution, have at least one dimension between 1 nm and 100 nm.

Nanoparticle: A particle with dimensions between 1 nm and 100 nm.

OECD: Organisation for Economic Cooperation and Development

An international regulatory agency with 36 member countries that develops regulations and guidelines with a focus on improving the economic and social well-being of people around the world.

OECD Test Guidelines: A set of standardized guidelines set by the OECD detailing methods by which to assess the safety of chemicals on human health and the environment and which are covered by the MAD agreement.

PCTS: Precision-cut tissue slices

Viable, *ex vivo* explants of tissue cut to a precise and reproducible thickness that can act as a miniature organ model for testing.

PISC: PETA International Science Consortium

PISC coordinates scientific and regulatory expertise to advance the development, use, and acceptance of *in silico* and *in vitro* testing methods.

SCAHT: Swiss Center for Applied Human Toxicology

A center that provides expert advice and services in the field of regulatory toxicology for Swiss regulatory authorities, the media, the general public, and third parties.

SOP: Standard Operating Procedure

In reference to a test method, the SOP outlines all necessary steps needed to carry out the test efficiently and accurately with detailed instructions for each process.



# Appendices

## Appendix A: Interview Questions for Research Institutions

- Have you interacted with studies involving animal experimentation or their alternatives?
  - What was your project goal and how did the animal testing or their alternatives play a role?
  - What process did you have to go through in order to get approved for the study?
  - Who were the pertinent organizations of this study?
- If you have worked with alternative testing methods, what tests/models did you use?
  - Who developed these tests/models?
  - Were these tests/models officially approved?
- Have you ever conducted hazard evaluation studies for nanoparticles or nanotoxicity?
  - Is there a standard measurement system for the severity of nanomaterial hazards?
- Have you or your institution ever worked on developing new alternative tests?
  - Have you ever sought official approval for these tests? How did/would you go about it?
  - Are there any potential issues which would make the approval process for a standard hazard assessment more difficult?
- What types of tests are better suited for your work? Please elaborate.
  - Are there certain methods that you would like to use but aren't able to?
- Is there anyone you can put us into contact with who you feel would be beneficial towards our project goal?

## Appendix B: Interview Questions for Regulatory Bodies

- Are you involved in the regulation of animal testing or their alternatives?
  - What process is used to determine these regulations?
- What general steps are involved in developing the standards that researchers are required to use involving animal testing or their alternatives?
  - Could you describe the approval process a test must go through?
  - Specifically related to nanomaterials and nanoparticles?
  - Do different tests/use cases need to be approved separately for the same model?
  - Does the approval process for hazard assessments change depending on which organ is being replicated? I.e. skin, lung, intestinal, ocular, brain tissue?
- What level of involvement does the government have in approving these standards?
- What kind of collaboration does Switzerland have with other countries or regulatory bodies in determining the standards that you hold your researchers to?
- What can researchers do to get their alternatives approved? Is there a standard process?
  - What would regulators like to see from them to expedite the process?
- Do you personally work with researchers directly to approve test methods?
  - If so, how?
- Are there any common issues when creating regulatory guidelines?
  - Does nanoscience present any unique barriers?
- Is there anyone you can put us into contact with that you feel would be beneficial towards our project goal?

## Appendix C: About the Project Sponsor

The Adolphe Merkle Institute (AMI) was established in 2007 by Dr. Adolphe Merkle, a successful local entrepreneur in Fribourg, Switzerland. He sought to strengthen the research and teaching aspects of education at the University of Fribourg by offering a 100 million Swiss Franc endowment (as a private donation) to support academic research (Adolphe Merkle Institute, n.d.).

The AMI, a branch of the University of Fribourg, is an interdisciplinary center combining soft, nano, and materials science. Their work focuses on bionanomaterials and the use of nanoparticles in biological systems. Because of this focus, the ethics behind testing various subjects can be difficult. AMI tries to limit its animal testing as much as possible and instead uses alternative methods such as *in vitro* models. Specific examples of *in vitro* models include 3D human skin and *in vitro* lung tissue models.

The institute is capable of exploring many subjects within the nanomaterials field; its employees mainly seek “Through collaborations with various industrial partners [...] to stimulate innovation, foster industrial competitiveness and more generally, improve the quality of life” (Adolphe Merkle Institute, n.d.). They engage in these issues using a team-driven approach, taking different viewpoints and strategies into account in order to produce the best research possible. The four main departments of AMI include Polymer Chemistry and Materials, BioNanomaterials, Soft Matter Physics, and Biophysics; in addition, there is a large focus on working inter-departmentally “for the successful and efficient execution of complex research projects that transcend the boundaries of traditional scientific disciplines” (“Adolphe Merkle Institute Annual Report 2014”, 2015).

The sponsors for this project are Dr. Barbara Rothen-Rutishauser, the Co-Chair of the BioNanomaterials Group, Dr. Barbara Drasler, and Dr. Roman Lehner, both Postdoctoral Researchers in the same group. The overarching goal of this BioNanomaterials group is to “[understand] and [control] all aspects from nanoparticle synthesis and ligand chemistry, colloidal behavior in complex biological fluids, and nanoparticle-cell interaction to intracellular trafficking and nanoparticle and cellular fate” (Adolphe Merkle Institute, n.d.). Advanced cell culture techniques are often used by this division along with as various types of microscopy (light, electron, atomic force) and scattering methods (light and X-ray). These practices are used “in addition to typical chemical and biological analytical methods (e.g. Calorimetry, NMR, TGA, UV-VIS, FACS, PCR etc)” (Adolphe Merkle Institute, n.d.). Dr. Rothen’s extensive knowledge and

experience in this field include conducting research and writing papers relating to the uses of Biomaterials in experimentation, both of which she has been doing since the 2000s. Similarly, Dr. Drasler has been focusing her research on promoting the use of biomaterial alternatives to animal testing. Their most recently co-authored article, *In vitro approaches to assess the hazard of nanomaterials*, was published by the scientific journal NanoImpact in 2017.

The staff of the Adolphe Merkle Institute consists of 94 full-time positions, 93 percent of which are research positions. The majority of the research staff is composed of Ph.D. students and postdoctoral researchers. As of 2016, the institute's overall expenditures were 8.8 million Swiss Francs, with 86 percent of expenditures going towards research. The institute also receives third-party funding for research projects from numerous sources, including the Swiss National Science Foundation, the European Union, the Swiss Commission for Technology and Innovation, and industrial partners. Since AMI was founded, the institute has established strategic partnerships with local businesses and organizations such as NCCR Bio-Inspired Materials, NTN Innovative Surfaces, and BioValley Alsace. Overall, AMI is a research-driven institution that has connections with other industries and businesses around them.

## Appendix D: Report for Sponsor

# Assessing the Validation and Standardization of Alternatives to Animal Experimentation

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### **Abstract**

Due to economic, practical, and scientific reasons, many researchers are promoting more widespread adoption of alternative testing methods to replace existing animal tests. These alternatives, such as *in vitro* models, are often more cost effective, can be easier to work with, and may provide more accurate results due to the use of human cells. In order for these tests to be put to use they must first be validated. Validation allows the test to be used, but does not directly lead to widespread use. The most effective way of increasing the use of these alternatives is to gain regulatory approval and instatement as guidelines through the Organisation for Economic Co-operation and Development (OECD). However, determining the exact approval process has been a major obstacle for many. Through information gathered from research and interviews, the process has been broken down into three main phases: development, validation, and regulatory acceptance. This general process applies to all alternative methods seeking approval, although the intricacies of specific fields may lead to additional work, particularly in the validation stage. This report aims to provide a simplified guide for the approval process of alternative methods with a focus on nanomaterial assessments in Switzerland.

### **Introduction**

With so many developments in the pharmaceutical, cosmetic, and chemical industries, there is a need to provide hazard assessments prior to releasing new products on the market. The default method of testing has predominantly involved animal experimentation. Over time, three main issues have been identified with the use of animals:

1. Financial strain, as animals must be provided with food, water, and shelter,

2. Ethical concerns, as the movement behind animal rights grows,
3. Incomparable genetics, as not all animal testing models have proven to be transferable to human systems.

As a response to the growing concern over these problems, several organizations have risen to create, test, and regulate alternatives to animal experimentation. A great deal of progress has been made in the conception of these alternatives; for example, *in vitro* and organ-on-a-chip methods have proven to be viable. In response to the need for regulation of these alternatives, the concept of the “3R Principles” was developed. The 3Rs involve the replacement, reduction, and refinement of animal testing. The aim of the 3R Principles is to limit animal testing while still ensuring consumer safety (3RCC, 2018).

The main regulatory institution in Europe and throughout other parts of the world is the Organisation for Economic Co-operation and Development (OECD). The OECD covers a broad range of regulations, one set of which addresses the approval and standardization of scientific testing methods as OECD Test Guidelines (OECD, 2018). It determines what criteria must be met in order for a test to be approved and will be discussed in further detail under Regulatory Resources. However, this approval process has not always been clear. One field with a significant amount of uncertainty is the development of alternative methods for testing the safety of nanomaterials. In this field there exists a significant gap in communication between test developers and regulators. To rectify this, the following information was gathered from research and interviews, then organized to provide clarity to this ongoing issue.

### **Information Sources**

Several interviews were conducted to gather data on the state of alternatives to animal testing and on the process by which a test becomes a validated standard. Both researchers and regulators were contacted to gain an understanding of the different perspectives on the challenges faced during this process. A major issue acknowledged by all interviewees was the lack of communication between test developers and regulators. Developers were often unsure of where to begin and who to contact, while regulators were overwhelmed with the complexity of the reports provided.

The intricacy of alternative tests, along with lack of communication, make the approval process long and difficult. To analyze this issue, test developers were asked about their specific

tests and the difficulties faced in gaining approval, while regulators were asked about their roles in this process.

Four sets of interviews were conducted in total, two of which were specifically within the regulatory field. The first was with two members of the Swiss Federal Office of Public health, both the former and current National Coordinator of the OECD Test Guidelines Program for Switzerland. They provided insight on the process a test must go through when seeking OECD approval and explained that the first step after developing a test is to contact the NC. He or she is able to expedite tests if they meet certain criteria, which are described later on. Next, a representative from the Swiss Center for Applied Human Toxicology elaborated on adverse outcome pathways (AOPs) and the difficulties surrounding nanomaterials. Specifically, it was stated that the regulatory process is the same as for other alternatives; however, the science behind nanomaterials adds an extra degree of complexity.

To cover the validation process, the new director of the Swiss 3R Competence Center was asked about her experience in the field and her previous work at the European Center for the Validation of Alternative Methods (ECVAM) focusing on the validation of *in vitro* methods. She was able to clarify the key steps in the approval process and provided resources on the modular approach to validation used by ECVAM.

Finally, to represent the category of test developers and industry, an executive at company known for its reliable *in vitro* lung models was asked about the challenges of developing alternative methods and how test developers go about seeking approval and standardization. He elaborated on the lack of communication between regulators and test developers and described how his company often seeks out the help of customers, such as pharmaceutical companies, for support in the approval process.

### **Regulatory Resources**

For many researchers, the official test validation and standardization process is unclear. Fortunately, there are several resources that can aid in the process. This section focuses on resources within Switzerland specifically, although some of these resources are either international or have equivalent organizations in other countries.

The Organisation for Economic Co-operation and Development (OECD): The OECD is an international organization with 36 member countries whose goal is to “promote policies that will improve the economic and social well-being of people around the world” (OECD, 2018). Policies issued by the OECD deal with everything from taxation and labor, to chemical testing and research. Most relevant to alternative methods is the OECD’s capacity to standardize testing methods as guidelines that its member countries must follow. To support this, the OECD has set a standard for Good Laboratory Practice (GLP) for researchers to follow that allows for the Mutual Acceptance of Data (MAD) between members (OECD, 2018). Additionally, for research institutions, the OECD has developed a set of guidance documents (available online) that outline specific practices to follow when conducting certain tests.

National Coordinator of the OECD Test Guidelines Program: Each OECD member country has a National Coordinator (NC) who represents that country’s regulatory body within the OECD. The job of the NC is to review project proposals presented for standardization and select which projects to push forward through the approval process outlined by the OECD Test Guidelines Program. He or she coordinates all relevant aspects of the process, including having the new method tested by third-party validators (most notably ECVAM) and advocating for the method to become a new guideline. For researchers who would like to promote a new method to become a test guideline, it is important to get in contact with the NC early on, such as during the development phase. Even if a new method is not yet fully developed, the NC’s office can provide important information and recommendations that can make the approval process easier down the road. Even with the help of the NC, who is an essential resource in gaining OECD approval, getting a project accepted can still be a difficult task. Every year, more projects are proposed than can be accepted. In 2014, there were only 56 projects being worked on by the NC’s office in Switzerland (Hofmann, Eskes, Aicher, 2014). The acceptance of tests is determined by key factors such as relevance, importance, and feasibility. These aspects are up to the discretion of the NC. It is also important to note that the NC does not only work with alternative methods; however, the percentage of accepted tests that involve alternative methods rose from about 13% in 2007 to about 48% in 2014 (Hofmann et al., 2014). Currently, about 90-95% of proposed projects in Switzerland involve alternative methods (Hofmann, 2018).



The European Center for the Validation of Alternative Methods (ECVAM): ECVAM serves as an institution that promotes and validates alternative testing methods. Most notably, it is the main center for external validation of alternative methods used by the OECD. ECVAM specializes in the independent evaluation of the relevance and reliability of tests used for assessing medicines, vaccines, medical devices, cosmetics, and household and agricultural products. (ECVAM, 2018) For a new alternative test to make its way to OECD approval, it must first go through independent validation. This is something that researchers and companies can pursue with ECVAM directly; however, it is highly advised to make contact through the country's NC instead as he or she will be able to better coordinate the validation process.

When validating a new method, it is necessary to have multiple laboratories test for transferability and variation. To accomplish this, ECVAM has organized the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL). EU-NETVAL consists of 37 laboratories that have been carefully selected and follow GLP practices. These laboratories assist ECVAM in the assessment of new alternative testing methods (European Commission, 2017).

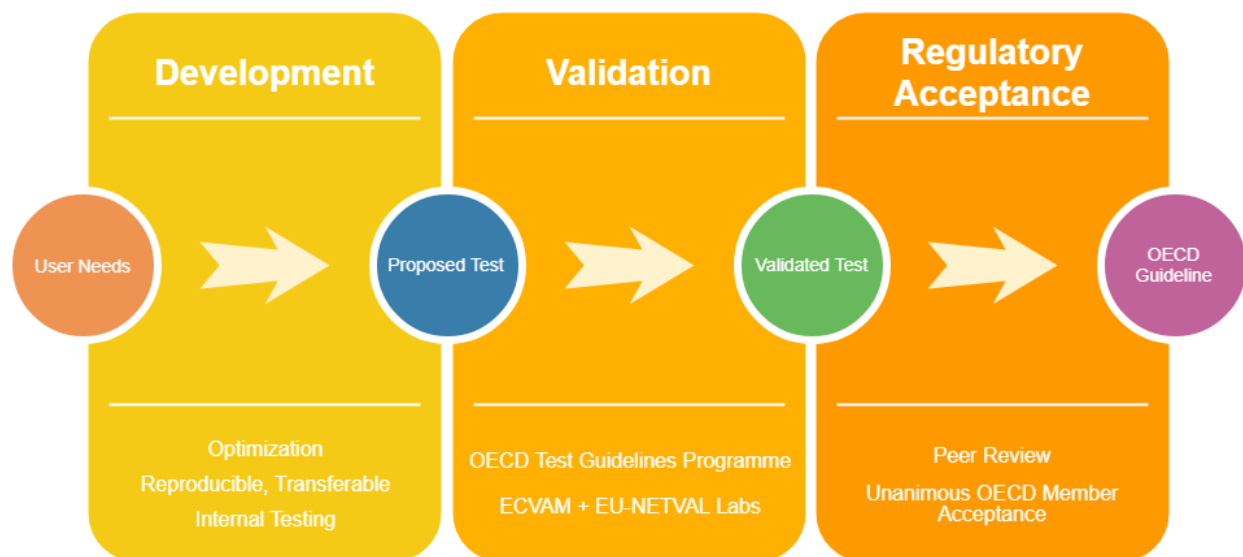
Swiss 3Rs Competence Center: The Swiss 3Rs Competence Center is a new organization with a strong focus on the replacement, reduction, and refinement of animal testing. The purpose of the center is to educate on methods that involve the 3Rs; provide a place for researchers, regulators, and industry members interested in the 3Rs to network; and to subsidize projects involving the 3R practices. While the center is very new, it is looking to open up for a first round of project applications by the end of 2018 (3RCC, 2018). The center is also looking to establish educational programs to promote the use of 3R principles and alternative methods (Eskes, 2018). The center is poised to be a valuable resource for promoting alternative methods in Switzerland and Europe.

### **The General Regulatory Process**

When considering the regulatory process surrounding alternatives to animal experimentation, it is helpful to split it up into three main steps (as seen in Figure 1):

1. Development of an alternative method
2. Validation of the method
3. Regulatory acceptance

It is necessary to follow these general steps in order to establish an alternative testing method as an OECD Guideline.



*Figure 1: Alternative Method Approval Process Diagram*

Prior to the development of the alternative test, it is important to first consider user needs. These needs originate from researchers or industry developers. A test in high demand has a greater likelihood of gaining eventual OECD approval, especially in a shorter time frame. If the test is not relevant, other tests with a more pressing need will be prioritized. With a process which can take upwards of a decade to complete, developing alternative tests with this in mind is key.

There are multiple design requirements to consider during the primary development stage of an alternative testing method. The test should be versatile and robust to be as applicable as possible while remaining reproducible. If the test cannot be used by other labs to get the same endpoint, then the alternative testing method will likely not pass external validation.

To ensure this, internal verification (or conducting a pre-validation process of the alternative method) is vital. It is imperative to make sure a given test, when distributed to other labs (around 3 or more), produces the same results. It is equally important to compare the alternative methods to endpoints generated by their animal testing counterparts, i.e. testing a particle with a known toxicity with the alternative method to verify its accuracy (Eskes, 2018). It is possible to verify the degree of accuracy and precision of an alternative test by manipulating different variables of a variety of known particles, such as concentration. Consistent and accurate

endpoints from multiple labs using well-characterized particles is a good indicator that the alternative test and standard operating procedure (SOP) are optimized.

It is only when these steps are successfully completed that an alternative test should be elevated to third-party institutions such as ECVAM and other laboratories within EU-NETVAL. These laboratories follow the procedures and testing practices set forth by the OECD to gather data to establish two aspects: the relevance and reliability of an alternative method. This data is gathered in accordance with OECD Test Guidelines using principles such as Good Laboratory Practice (GLP). This ensures that the data generated in any member country (of which there are 36) using these principles will be accepted under the Mutual Acceptance of Data (MAD) “for assessment purposes and other uses relating to the protection of human health and the environment” (OECD, 2018). This is critical for an alternative method to be considered as a validated testing method and eventually as an OECD guideline.

This data is then reviewed by a validation management group, which is in charge of accepting or rejecting the alternative method based off of seven modules or parameters of the test. Together, the modules measure the alternative method’s relevance and reliability. It is only once this is done that the alternative test can be elevated to the independent peer review process to complete validation. This group is comprised of experts in the field to ensure the results from the validation are acceptable before passing the test to regulators.

The last step in the process is regulatory acceptance. During this phase, the alternative method and data generated by third-party testing will be elevated to the OECD for review to be potentially instated as a guideline. This step is dealt with on a case-by-case basis. Thus, the potential need for an alternative test, along with its robustness and transferability, plays a role in the speed of the process. Consistent communication with the National Coordinator of the Test Guidelines Program is also important in expediting this process, as he or she works directly with the OECD and third-party institutions in determining these factors (Hofmann, 2018).

### **Organization Network**

It is evident that the process involved is complicated, so it is pertinent to first take a step back in order to visualize the different parties involved and how they interact. Figure 2 shows an overview of the three-step process to display associations among the organizations which are involved in each step.

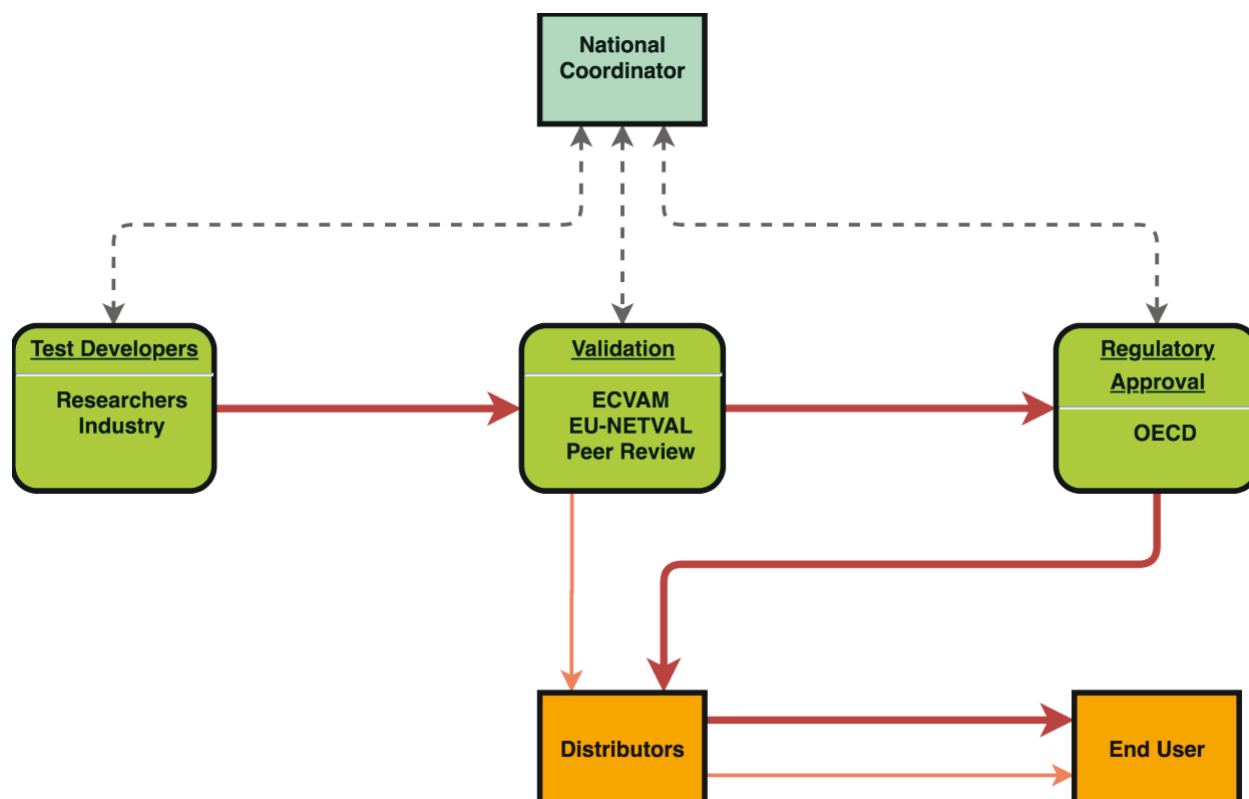


Figure 2: Overall Picture Diagram

The following sections will go in-depth on the individual steps within the regulatory process to make its intricacies clear.

### **Test Development and Design Control**

The initial design of an alternative method is determined by the user need and several design requirements, such as versatility, reproducibility, transferability, and robustness. However, it is difficult and highly unlikely to develop the best possible test on initial design.

There is an optimization process involved in developing an alternative testing method. After the initial design of an alternative method, developers need to define parameters by which the method is to be evaluated. These test parameters should be established using reliable past *in vivo* or *in vitro* test data to set a stable baseline. When assessing human health effects, the OECD (OECD 2005) suggests the use of human data from sources such as:

- Epidemiology
- Occupational exposure
- Accidents and cases of poisoning

- Clinical studies
- Ethically approved studies in human volunteers

It is important to continuously compare against standards to improve the developing alternative method. This cycle, called design control, is the optimization process that refines alternative methods and generated endpoints until they are of equal or greater significance than animal testing counterparts. This is a vital process in the development of an alternative testing method.

There are three broad parameters by which an alternative method should be tested: relevance, accuracy, and reproducibility. These are key aspects of an alternative test with the goal of validation and approval as an OECD Test Guideline. However, there is no set process for one to use during this optimization phase, and it is possible to customize this process on an individual basis. It is possible, however, to check for optimization of an alternative method against the same seven modules that the validation management group will use to evaluate the method. This is referred to as pre-validation. These modules are described in the Modular Approach section. While it is important to understand the process behind validation, it is necessary to first understand who is involved in moving the process forward.

### **Validation Study Organization**

A validation study begins with a sponsor, who assigns a study manager or management team to design and carry it out. This management team can then delegate various responsibilities to Task Groups. The management team also oversees the Lead Laboratory for the study, which is in charge of data collection and instructing the other participating labs on the SOP. These bodies are shown in Figure 3.

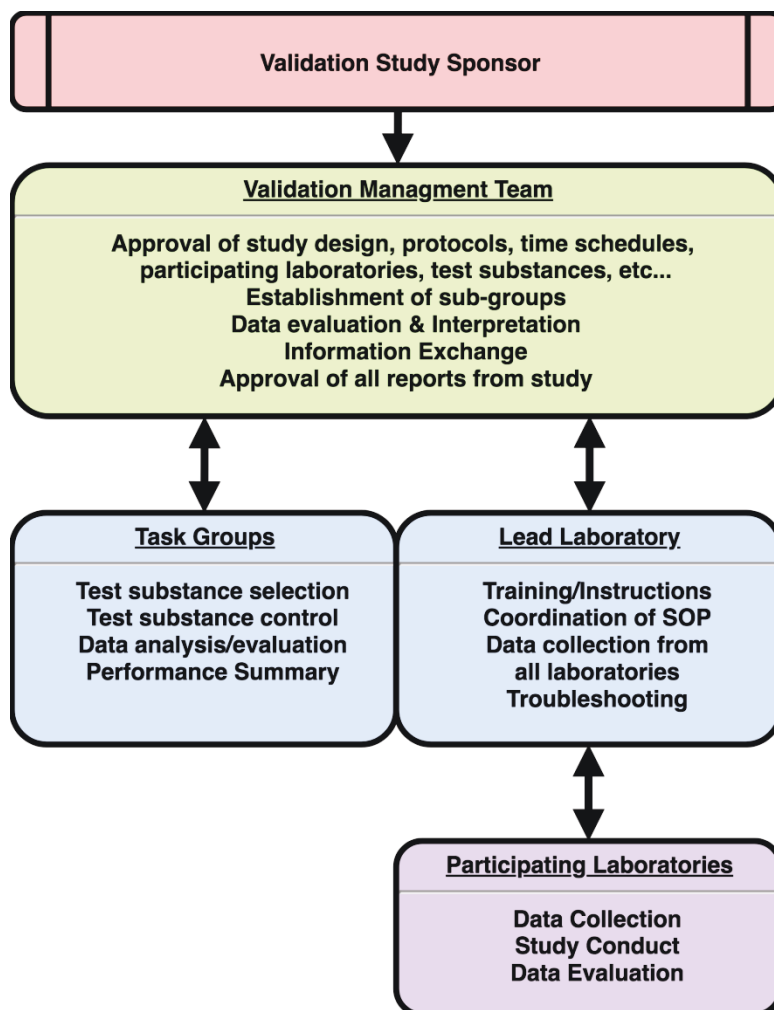


Figure 3: Validation Study Groups (OECD, 2005)

There are several potential candidates to become a sponsor of a validation study. They include: international bodies, government entities or validation organizations for alternative methods (ECVAM, ICCVAM), national organizations, other independent organizations, or commercial sponsors. If the validation study is organized by the OECD, the sponsor may be an OECD expert group, task force, working group/party whose members are nominated by the governments of the respective countries (OECD, 2005). These bodies oversee the validation study but play a minimal role in the day-to-day operation.

For this stage, the validation manager/management team takes over. They must have the collective expertise with the test, the science behind the test, and management/evaluation of validation studies in order to be appointed to this position.

## **Modular Approach**

In an attempt to make the validation process more efficient, ECVAM has developed a modular approach to alternative test validation. This approach breaks down the main components of validation into seven modules. The purpose of these modules is to define what data is needed for independent validation and peer review. By having this defined list of requirements, researchers can develop tests while keeping the data needed for validation in mind, thus streamlining the process later on. Once test developers have finished development and have taken into account all of the modules, the independent validation process can begin. The seven modules are as follows:

1. Test Definition: The Test Definition states the underlying reasons for the development of the test, as well as defines any necessary information needed to properly conduct it. The specific scientific purpose of the test must be clearly stated here. Additionally, the Test Definition must include a clearly defined protocol that complies with Good Laboratory Practice and Good Cell Culture Practice. This protocol should include any necessary SOPs to allow for replication. Finally, the Test Definition must define specific endpoints and measurements, specify how to derive, express, and interpret results; as well as include a set of adequate controls (Hartung et al., 2004).
2. Intra-Laboratory Repeatability: The test must be proven to be repeatable and consistent within the same laboratory. This is usually done with different operators over time within the same laboratory setup and done at the development laboratory. Any variation in results within the same lab must be addressed (OECD, 2005).
3. Inter-Laboratory Transferability: The transferability of a test plays a crucial role in its robustness. The goal of this module is to show that the test can successfully be repeated in a laboratory other than the one in which it was developed or which assisted in its optimization. The data gathered here will provide an estimation of the training required for a naive laboratory (lab without prior experience with the specific type of test) to reproduce the test, as well as identify additional sources of intra- and inter-laboratory variation (OECD, 2005).
4. Inter-Laboratory Reproducibility: During this process, external laboratories carry out the test method and test it against a large assortment of substances. This is usually organized by ECVAM and involves three or four well-trained laboratories. The assessment of inter-

laboratory variation can also be done with a more limited selection of test substances that still cover all toxic effects that the test can demonstrate. This allows the predictive capacity to be tested against a large number of substances in a single lab to save time and money (OECD 2005).

5. Predictive Capacity: The Predictive Capacity of a test is a demonstration of how well the test can predict the reference standard and can generally be referred to as the accuracy of the test. The reference being tested against is often an existing standard such as an *in vivo* test or a human health effect. The Predictive Capacity is influenced by the quality of the standard and the range and quantity of substances tested. The current ECVAM process states that Predictive Capacity must be tested in at least three laboratories; however, if inter-laboratory variability has already been determined to be of acceptable levels, testing of Predictive Capacity may be done at just one laboratory (Hartung et al., 2004).
6. Applicability Domain: The Applicability Domain of a test must clearly describe the particular purposes for which the test can be applied. This includes any toxicological endpoints, chemical classes, test materials, and physicochemical properties or products that might be assessed with the test. Any changes to the Applicability Domain may require additional peer review (Hartung et al., 2004).
7. Performance Standards: At the end of the validation process, the Performance Standards of the test are evaluated. This is a compilation of essential test method components, reference substances, and accuracy and reliability values that can be used to demonstrate equivalence in performance between future tests and the current test. This can allow for faster approval of similar tests or changes to the current test in the future (Hartung et al., 2004).

### **Peer Review of Validation Study**

Once the validation study has been completed and the Validation Management Group is satisfied that all necessary data has been collected and evaluated, a test method may move on to the independent peer review stage. This can be done in one of two ways: first, the Validation Study Sponsor may choose to organize a panel of independent peer reviewers to assess the findings of the validation study. This may be the preferred route if the test method is not being proposed as a new OECD Test Guideline. If the test method is being proposed as a new Test Guideline, the



Validation Study Sponsor can still opt to organize peer review before submitting the test to the OECD, or it can submit the test without peer review and the OECD will organize the review panel. If peer review is completed prior to the test method being submitted for approval as a Test Guideline, a complete report of the independent panel must be provided, including detailed reasoning behind any conclusions and recommendations, as well as any comments made on the test method. Otherwise, the OECD will discuss and appoint a responsible party to organize any necessary peer review (OECD, 2005).

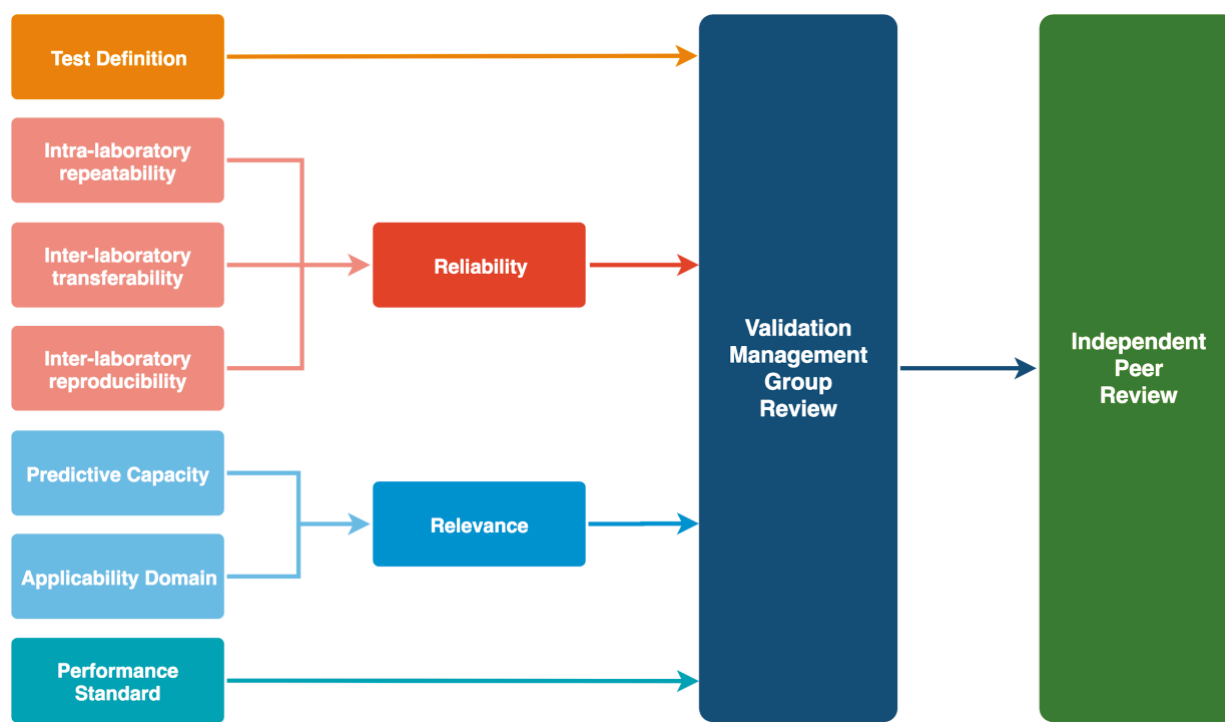
When selecting a panel of experts for peer review, there are certain criteria to follow to ensure accurate and unbiased review.

- The experts must demonstrate expertise in one or more of the scientific fields relevant to the test. The panel as a whole should contain at least one expert for each relevant field, and may contain more than one representative for any essential fields relating to the test method.
- It is important that some of the peer reviewers have experience in the development, conduct, and evaluation of validation studies for toxicology tests. When appropriate, one or more reviewers should also have an understanding of animal welfare issues and the 3R principles.
- It is crucial that all peer reviewers are independent and not subject to any conflicts of interest. This includes any previous substantial involvement in the process and any personally beneficial interest in the outcome of the study, such as financial gain. More minor interests may be allowed, as long as the panel as a whole is balanced and independent (OECD, 2005).

Once put together, it is up to the peer review panel to review all information from the validation process and come to a conclusion as to whether the test method has thoroughly satisfied its intended purpose and goals. The review process should be open and transparent and may allow for public comment. At the end of review, the panel should provide a report containing an assessment of the usefulness and limitations of the test method. Questions may then be posed to the panel during the final stage of the peer review process (OECD, 2005).

After peer review has been completed it is up to the Validation Study Sponsor to determine, based off of the panel's findings, if the test method has been validated for its intended purpose and should be recommended for regulatory acceptance. It is then up to individual regulatory bodies to

determine if the validated test method is suitable for its purpose and if it may be accepted under each body's specific area of jurisdiction (Hofmann, 2018).



*Figure 4: Modular Approach Validation Process*

### **Regulatory Acceptance**

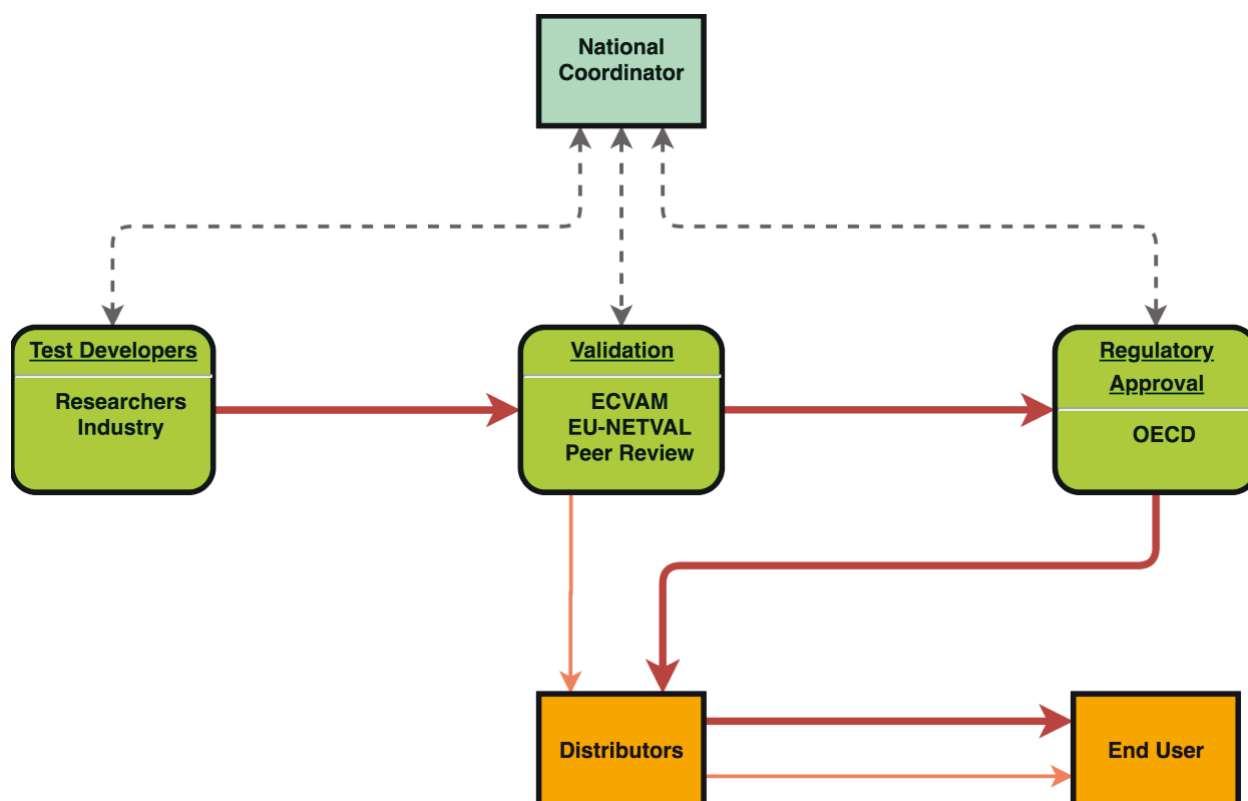
Once all seven modules have been satisfactorily completed (as judged by the Validation Management Group), and the alternative testing method has passed peer review, the method can be determined to be officially validated and no more evaluation will need to be conducted. However, should the test fail peer review, the test would then be sent back to the development stage in an attempt to address any concerns with the test. This an unlikely event, however, as the peer review board will be evaluating on similar if not the same criteria.

Once a test is a validated method, it can be submitted to the OECD. At this point, it is up to the regulators to review the test and data gathered during validation. In order for the alternative method to be instated as an OECD guideline, all member states must vote unanimously (Eskes, 2018). That is to say, if any singular or greater amount of country representatives do not agree on the acceptance of the alternative method, it will not become an OECD Test Guideline.

This process can be lengthy and can take up to or sometimes over a year. There are many steps for the test to go through before a definitive vote can be taken. Once the decision is made, the alternative testing method will join a growing faction of internationally accepted testing methods as a standard for assessing the potential health effects of chemicals on humans and the environment.

### Paths to the End User

Now that the individual steps are more understood, it is possible to see how they connect in the overall process.



*Figure 2: Overall Picture Diagram*

As seen in Figure 2, it is clear that there are two main paths to take for an alternative testing method to go from development to the end user.

The first path, and the one described in the previous sections, is the regulatory approval path through organizations such as the OECD. By going through an international organization, with many member countries, the potential population of end user increases significantly. This

requires no additional effort from test developers and in countries like Switzerland, whom follow the 3R Principles, alternative methods become the standard (over animal testing methods) and are put into use on a national scale.

This doesn't need to be the case, however. There is a second potential path to end users. Test developers do not have to submit their alternative methods to international regulatory agencies and can go straight to distributors after validation. This, however, limits the end user population, as it is at each user's discretion to determine whether the test is acceptable and valid for their purposes or not; each country has their own agencies and practices, and it is not uncommon to see large discrepancies in their scientific regulatory policies. Thus, the end users must decide on their own if they want to use the test without the backing of an internationally accepted organization like the OECD.

### **Challenges Surrounding Nanomaterials**

Nanoscience is a relatively new field; as such, the various properties of nanomaterials are not always well documented. The European Commission defines nanomaterials as “A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm” (European Commission, 2017). Due to their extremely small size and high surface-area-to-volume ratio, particles under 100 nm can have a much higher reactivity and permeate membranes that larger particles cannot. This can cause detrimental interactions that are not observed with larger particles of the same material. These differences lead to more variables that need to be tested when assessing the safety of nanomaterials. This complicates the process for developing and approving alternative tests for nanomaterial hazard assessment (Aicher, 2018).

The Swiss Center for Applied Human Toxicology (SCAHT) defines five different variables that need to be tested when assessing nanomaterials and their interactions with living tissue:

1. **Size:** on nano-scale, even small changes in size can lead to significant changes in behavior. As such, SCAHT has decided on a size variation of 10% or 10 nm for which test results are still acceptable. Any particle sizes that fall outside of the range of the tested particles must be tested separately (Aicher, 2017).

2. **Surface Chemistry:** nanomaterials may attract certain molecules or proteins that can attach to and coat the particle, creating a unique corona that may vary depending on the environment the particle is in. The coating can affect how the particle behaves, for example by making it more stable or less likely to clump together with other particles. Any differences in surface chemistry caused by different environments must be tested to ensure proper assessment of the particle's effects (Aicher, 2017).
3. **Core Material:** the core material of a particle can affect how the corona forms and how it reacts with other chemicals. Depending on the particle, the core material can play a greater role than the surface chemistry and is an important factor to test (Aicher, 2017).
4. **Environment:** the environment that the nanomaterials interact with influences multiple factors. It affects aspects such as corona formation and whether or not the particles clump together. As such, any environmental differences such as changes in chemistry, contents, or pH require testing (Aicher, 2017).
5. **Morphology:** the morphology, or shape, of the particle plays a large role in its physical interactions with living tissue. For example, differences in morphology can affect a particle's ability to permeate through membranes. Round particles can usually pass through more easily than longer, fiber-like particles. Therefore, even if the core and surface chemistry between two particles is the same, variations in shape can cause the particles to behave and interact very differently (Aicher, 2017).

When it comes to developing alternative testing methods for assessing nanomaterials, the factors listed above must be taken into account. Therefore, a test that assesses the hazard of nanomaterials on a certain organ or type of tissue must permit for all variables to be tested. These five points of variability are one of the main reasons that it is more difficult to develop alternative tests for nanomaterial assessment (Aicher, 2017). It is much easier, therefore, to develop tests for larger particles or chemicals as small changes in these variables do not play as much of a role in determining how the substance behaves. While testing these variables might appear to be tedious, it is not always the case that all five need to be analyzed. Increases in known effects can allow variables to be omitted in testing if previously determined to be hazardous. For example, if a particular size and shape has proven to be hazardous in multiple nanomaterials, any material being tested that has a similar shape and size can be assumed to be hazardous. Whether or not a material

is safe, however, cannot be based off of previous data and all variables must still be tested (Aicher, 2018). Some valuable resources for hazard data are verified Adverse Outcome Pathways (AOPs). AOPs are studies on how specific chemicals or materials can be hazardous through specific pathways. A database of these pathways is available online in the form of the AOP-wiki.

Since testing methods are further complicated by the properties of nanomaterials, there are additional complexities involved in the regulatory process. While an animal test can show the effects of a substance on an entire living system, alternative tests generally need to break a body system down into different sets of cell types that are tested independently. The results are then combined to determine how the substance being tested affects the system as a whole. This, along with the previously described variables presented by nanomaterials, means that multiple tests within a comprehensive testing method must be validated to approve the overall alternative method (Aicher, 2018).

Along with the inherent complexities of nanomaterials and their testing, the largest complication in the test development and approval process at this time is a lack of knowledge about nanomaterials and their behavior. Nanoscience is still a developing field, and there is currently not a widespread understanding of the scope of the effects of nanomaterials. It is therefore difficult to get tests on these effects approved because regulators do not have an in-depth understanding of nanoscience at this time (Hofmann, 2018). To remedy this, they must first gain greater comprehension of the field before approving new methods. This lack of information, along with a communication gap between researchers and regulators, is a hindrance to the spread of new alternative nanomaterial tests.

## **Conclusions**

It is not unusual for an alternative testing method, from conception to being instated as an OECD Test Guideline, to take upwards of a decade.. Two factors were identified as contributing to this significant time span.

1. *The complexity of the alternative field*: the scientific practices moving alternatives forward are based on recently gathered knowledge and only grow in intricacy with each discovery/invention. Thus, the risk mitigation behind these tests requires close attention to detail and thoroughness in the validation process.

2. *The communication gap between industry, researchers, and regulators*: a significant gap exists in the communication among these three parties, creating a lack of knowledge on all sides. Researchers and industry officials are unaware of the exact regulatory process for alternative methods, while regulators are overwhelmed with numerous technical reports which they have trouble understanding.

To help address these issues, a set of recommendations has been prepared.

### **Recommendations**

- During design, test developers should make the alternative method
  - Relevant
  - Accurate
  - Reproducible
- Researchers can use resources listed in the regulatory resources section to better communicate throughout the process. The NC of the OECD Test Guidelines Program can help bridge the communication gap. Communication earlier in the process will allow the NC to determine the user need and help coordinate with all involved parties.
- Figure 5 below provides an overview of the general approval process discussed earlier from the test development stage to regulatory acceptance.

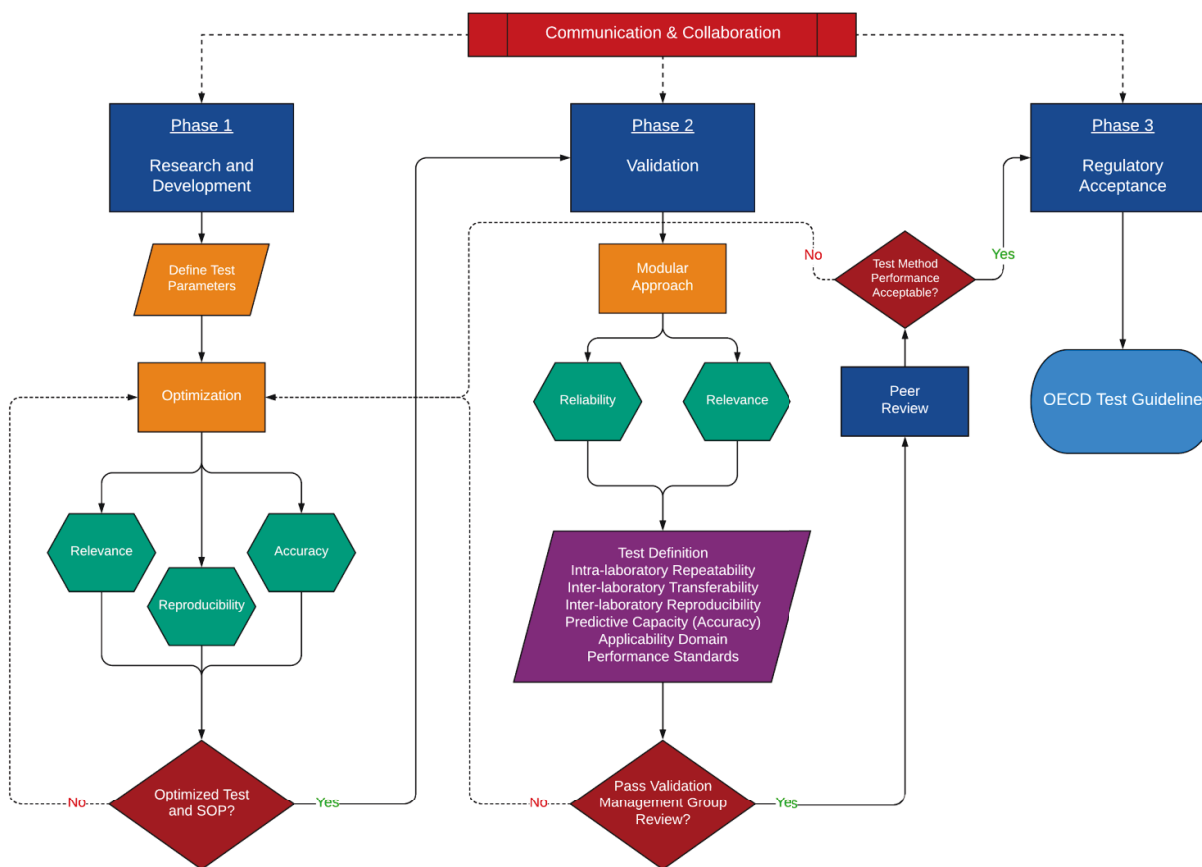


Figure 5: Pathway to OECD Acceptance

- Due to the nature and complexity of alternative methods, especially relating to nanomaterials, the current case-by-case methodology of regulation is not yet replaceable. Thus, close collaboration between researchers and regulators will grow in importance as topics become increasingly complex.
- When presenting to regulators, test developers should simplify their explanations of relevant material and remember that regulators may not have the same level of experience in the subject. The best way to present a new alternative method is to lay out a specific endpoint and express exactly what the method seeks to test.



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