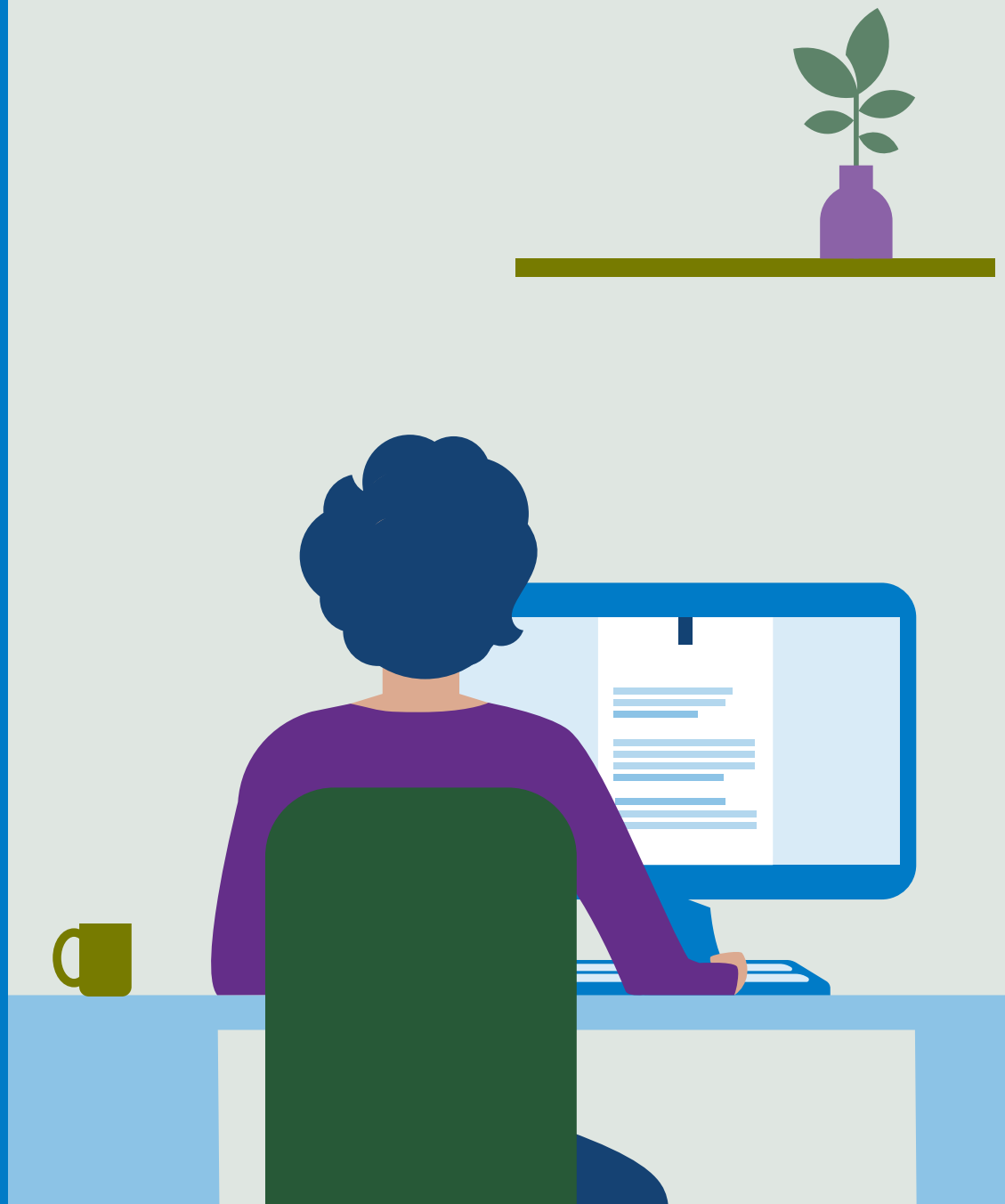
Central Authority for Scientific Procedures on Animals

Generating, breeding, genotyping, monitoring and maintaining genetically altered animals

Guideline issued by the Central Authority for Scientific Procedures on Animals



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Introduction

This guideline is a revised version of the Central Authority for Scientific Procedures on Animals (CCD) guideline dated 7 October 2016 that entered into force on 1 January 2023 (text amended April 2024). The revision implements the requirements contained in Implementing Decision 2020/569/EU (which replaced 2012/707/EU) and takes into account the recommendations contained in the EU consensus document 'Framework for the genetically altered animals under Directive 2010/63/EU on the protection of animals used for scientific purposes' issued by the European Commission in 2021. The consensus document is intended to aid interpretation of the European Directive.

The CCD evaluates applications for a project licence for animal procedures using various aspects as set out in the Experiments on Animals Act (Wet op de dierproeven, Wod). This guideline aims to help researchers, Animal Welfare Bodies and Animal Ethics Committees understand when a project licence is required in the Netherlands, under the Act, for activities relating to generating, breeding, monitoring and maintaining animals whose genome has been altered. The general conditions that must be met by all licence applications for these breeding projects are published in a separate Guideline: 'Defining a project'. This guideline is available for download from the CCD's website.

The guideline makes serveral references to parts of 'Framework for the genetically altered animals' that are relevant to the project licence applications. These references relate primarily to administrative procedures, including project licence requirements (Part 1), the 3Rs (Part 2) and welfare assessment schemes (Part 3).

Disclaimer: *This document may contain translations of Dutch legal texts. These are unofficial and for informational purposes only. In case of discrepancies, the original Dutch legislation prevails.*

Legal framework

The severity framework that determines whether a procedure involving a laboratory animal, for one of the purposes specified in the Animal Experiments Act, qualifies as an animal procedure is described in Article 1, paragraph 1, sub a of the Wod:

"Any use, invasive or non-invasive, of an animal for experimental or other purposes, with known or unknown outcome, or for educational purposes, which may cause the animal a level of pain, suffering, distress or lasting harm equivalent to, or greater than, that caused by the introduction of a needle in accordance with good veterinary practice. *This includes any use whose objectives or potential consequence is the birth or hatching of an animal, or* ***creating*** *and* ***maintaining*** *a genetically altered animal line*, including killing animals to use their organs, tissues or bodily fluids for a purpose identified in Article 1c;"

- In this case, '*creating*' should be interpreted as '*generating*';
- In this case, '*maintaining*' should be interpreted as '*breeding and keeping stock animals, breeding animals or laboratory animals*'.

This means that generating and breeding genetically altered animals should potentially be regarded as an animal procedure, and that the question of whether or not the activity ultimately represents such a procedure is determined by the discomfort caused.

Directive 2010/63/EU has been implemented in the Netherlands as national legislation (Experiments on Animals Act, Animal Procedure Regulation and Experiments on Animal Decree) setting out the legal framework regarding licence applications for animal procedures. This document outlines the legal framework in force in the Netherlands regarding the generation, breeding, and monitoring of genetically altered animals, as well as genotyping and the monitoring and identification of new genetic lines. An establishment licence is required in order to breed and supply laboratory animals (Experiments on Animals Act, Article 11a).

In addition to the project licence requirement, other registration requirements apply to generating, breeding and using genetically altered animals (Implementing Decision 2020/569/EU, Article 54(2)).

Definition of genetic modification (GM)/genetic alteration (GA)

Implementing Decision 2020/569/EU defines 'genetically altered animals' as follows.

a. 'genetically modified (such as transgenic, knock-out and other forms of genetic alteration) and induced mutant animals (irrespective of the type of mutation);'
b. 'animals with spontaneous deleterious mutations maintained for research for that specific genotype.'

Offspring of one or both genetic altered parent animals which do not themselves display the mutation (wild-type animals) are not considered to be 'genetically altered animals'.

Any genetic alteration involving germline transmission, including a spontaneous mutation which (whether intentional or not, and whether or not it involves a harmful phenotype) is perpetuated in a line, is by definition 'a genetic alteration'.

Licensing requirement for the generation of a newly altered genotype

This guideline observes the precautionary principle as set out in the EU consensus document 'Framework for the genetically altered animals'. As a precautionary measure, a licensing requirement is applied to the generation of a newly altered genotype, as the consequences of generating a new genetically altered genotype cannot be fully known in advance. A project licence is also required for animals which undergo an invasive procedure (such as vasectomy, superovulation, etc.) for the purpose of generating a new line. Table A on page 8 provides an overview of activities/procedures relating to generating a new altered genotype for which a project licence is and is not required. See also 'Framework for the genetically altered animals' Part 1, Section 3 "Activities falling under the definition of a procedure and requiring a project authorisation", pages 11–16 (table), and Section 4 "Flow chart for the requirements for a project authorisation for the creation and maintenance of GA lines", page 20.

The most commonly used methods for generating a line with a new altered genotype can be classified into 4 groups[1]:

- Genetic manipulation of gametes or embryos (transgenic, knock-out, knock-in);
- Crossing of existing lines
- Mutagenesis
- Spontaneous harmful mutant

In specific cases, deviations from the precautionary principle may be allowed. The EU consensus document 'Framework for the genetically altered animals' lists the following exceptions to the licence requirements.

1. When creating a new GA line by crossing/backcrossing two lines of non-harmful phenotype and it can be reasonably expected that the new line will not result in a harmful phenotype, the requirement for a project authorisation may not apply.
2. Harmful mutations arising in single animals / litters but which are killed immediately and the harmful trait is identified and parents not bred from again.

[1] Where procedures requiring a licence are carried out on founders, the founders of a new line must be both included in a licence and registered in the annual statistical reporting. See 'Framework for the genetically altered animals' Part 3.1. "Annual statistical reporting" (page 60).

Genotyping

A project licence is required for genotyping genetically altered lines by analysing tissue obtained using an invasive method (=a method which is *above the minimum threshold of pain, suffering, distress or lasting harm during the lifetime of the animal*). Genotyping using surplus tissue obtained by identifying/marking animals as part of general activity does not require a project licence. As tail tipping and blood samples are generally unsuitable as methods of identification, these procedures almost always require a project licence. Fin clipping of zebra fish is not a suitable method of identification, as the fins regenerate quickly. Where fin clipping is used for genotyping purposes in zebra fish (starting at the independently feeding larval form), this must be included in a project licence. Table A on page 8 provides an overview of activities/procedures relating to genotyping a new altered genotype for which a project licence is and is not required. See also 'Framework for the genetically altered animals' under "Genetic characterisation" on pages 16–17.

Monitoring and 'establishing' a new generated genotype

For each new genotype, the welfare of at least the first 2 genetically stable generations must be monitored. The Welfare Assessment should be performed on a scientifically and statistically justified number of animals, but it should not be less than 14 animals, and from >1 litter / clutch. Sex differences in the phenotype should be considered for the sample size calculation if they cannot be excluded on a scientific basis. If no gender dependency of the genotype is known, assess 7 females and 7 males. Animals of representative age groups and relevant genotypes (heterozygous and homozygous) should be included in the Welfare Assessment.

The animals involved in this monitoring must be included in a project licence. The EU consensus document 'Framework for the genetically altered animals' describes the implementation criteria for welfare monitoring and the associated required clinical parameters (see 'Framework for the genetically altered animals' Part 3: Welfare assessment schemes for the most common genetically altered species, pages 33–56).

Animals of the corresponding genetic backgrounds (e.g., wild-type) or of a defined reference line should serve as controls. Each phenotypic abnormality determined in the GA line should be compared to the occurrence in the defined reference line. No project licence is required for these genetically unaltered reference animals, unless they are subjected to procedures that result in discomfort above the minimum threshold of pain.

When crossing/backcrossing two lines of a non-harmful phenotype, and where it can be reasonably expected that the new line will not result in a harmful phenotype, a project licence is not required. However, the welfare of these lines must be monitored, as later confirmation is required that there are indeed no harmful phenotypes. The welfare monitoring of these lines does not require a project licence. Neither is a licence required for lines which risk discomfort only when crossed with a different line (e.g. Cre, Lox, Crispr, Flp), as these lines only produce a functional mutation in their offspring when combined.

The monitoring period must be formally brought to an end. A line including genetically altered animals is only considered to be 'established' once transmission of the genetic alteration is stable (i.e. after 2 generations) and once a welfare evaluation has been completed. The conclusion as to whether or not a line has a harmful phenotype must be made by the establishment licence holder, following consultation with the Animal Welfare Body.

See also Table A on page 8 and 'Framework for the genetically altered animals' "Creation of a new GA line" on pages 11–12.

Licensing requirement relating to maintaining lines

When the new genetically altered line is 'established', a harmful phenotype can be considered to have been introduced if the animals' genetic predisposition inherently causes them a level of pain, suffering or distress equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice. This is equivalent to the threshold value for a procedure that requires a licence, as described in Article 1(1a) of the Experiments on Animals Act and Article 3(1) of the European Directive. In such cases, a project licence is required for the maintenance of the line.

A harmful phenotype is also considered to have been introduced if genetically altered animals, as compared to conventional/wild-type animals of the same species, must be housed or cared for in a special way in order to limit or prevent discomfort that results from genetic predisposition and exceeds the upper threshold. A project licence is also required for these lines — for example, when breeding genetically altered animals that are more sensitive to infection due to a compromised immune system. In such cases, specific barrier or biosecure conditions (which are not required for conventional animals) must be used to safeguard their health and/or welfare. Annex III, Part B, item 11.7 of Implementing Decision 2020/569/EU contains the following text: "[…] Such animals include, amongst others, those that require a specific bio-secure environment (for example, special housing arrangements to protect animals that are particularly sensitive to infection as a consequence of the gene alteration) or additional care beyond that required for conventional animals to maintain their health and well-being."

The following situations require a project licence to maintain breeding of the lines for which the phenotype has been 'established'.

- A harmful phenotype is present in offspring from the moment of birth (or during the final trimester of pregnancy);
- The (homozygous) offspring with a harmful phenotype die during or immediately after birth;
- Crossed lines that only risk introducing a harmful phenotype when crossed with another line (e.g. Cre/Lox);
- Breeding of harmful lines by crossing het x het or het x wild-type to reduce / eliminate the risk for expressing a harmful phenotype;
- Breeding in which a harmful phenotype is activated, while the animal is being bred, by administering an inducing agent;
- Breeding in which measures are taken in addition to regular housing and care to maintain the health and welfare of the genetically altered animals, where such measures would not be necessary for conventional animals. For example:
  - Breeding immunodeficient mice in a special unit;
  - Breeding animals with an 'age-onset' harmful phenotype. According to the EU consensus document, "[…] Similarly, for expected age-onset disorders, it can be predicted that adverse effects will occur later in life, as animals age e.g., hypertension. If animals are killed at times to prevent the onset of harmful effects, this does not remove the risk to the line, only to the individual animal which has been killed. Therefore, these lines must be classified as harmful, and require project authorisation to be kept.";
  - Breeding in which a harmful phenotype is suppressed, while the animal is being bred, by administering a supressing agent.

The following situations *do not* require a project licence for continued breeding, provided that no activities leading to above-threshold discomfort are carried out.

- Existing genetically altered lines with a non-harmful phenotype;
- Lines which only introduce reporter genes into the genome;
- Breeding of uncrossed Cre or Lox lines where no harmful phenotype is displayed;
- Existing lines in which the genetic modification of the phenotype is only active when treatment with inducing agents is applied (not during breeding).

See also Table A and 'Framework for the genetically altered animals' "Maintenance of an existing GA line" on pages 12–14.

Licensing requirement for the acquisition, cryopreservation, and rederivation of genetically altered lines

For these activities, the establishment must hold an establishment licence to breed/supply laboratory animals (Experiments on Animals Act, Article 11a), which implies that the facilities, staff and other elements must meet the requirements set out in the Experiments on Animals Act. The determination as to whether a project licence is required for cryopreservation and rederivation depends on the intended purpose. A project licence is required if the procedures carried out on the animals are conducted for a scientific purpose. Procedures carried out on animals for the purpose of animal health and/or welfare do not require a project licence. This concerns rederivation where pathogens are present that could make the animals ill. For cryopreservation, a project licence is required if live animals are used or if animals are killed to isolate tissues/sperm. In the case of the acquisition (potentially involving the import if animals) of a genetically altered line that has not yet been 'established', or if the necessary information is not supplied, the rules for generating a new genetic line apply.

See also Table A and 'Framework for the genetically altered animals' "rederivation" and "cryopreservation" on pages 16 and 17.

Unforeseen circumstances

Unforeseen situations may arise in which the generation of a new genetically altered line or the maintenance of an existing genetically altered line unexpectedly results in a harmful phenotype.

These situations may include the following.

- A new genotype is generated by crossing two lines, neither of which exhibit an adverse phenotype that could reasonably be expected to lead to harm (project licence not required). However, when breeding to maintain this new genotype, a harmful phenotype is found (project licence required). Where the establishment licence holder holds a generic licence to generate and breed with lines with a harmful phenotype, this line could be added to the existing licence. If this is not possible, either an amendment to the project licence or an entirely new project licence application must be submitted. Breeding must be suspended (animals are no longer paired for breeding) until the CCD grants permission. This means the number of active breeding pairs should be minimized to a 'maintenance' amount.
- A new genotype is generated that involves greater discomfort than was initially anticipated (for example, only mild discomfort was anticipated, but in practice animals experience moderate discomfort). If the level of discomfort exceeds the level for which the project licence was issued, an amendment to the project licence must be submitted. Breeding must be suspended (animals are no longer paired for breeding) until the amendment is approved or the new project licence is issued.
- A generic licence has been issued to generate and breed with lines, but the number of lines with a harmful phenotype is later shown to be greater than that stated in the project licence. If the project licence no longer covers the actual number of affected animals, an amendment to the project licence can be submitted. Breeding must be suspended (animals are no longer paired for breeding) until the amendment is approved or the new project licence is issued.

Appendix 1 Table A - Overview of Licensing Requirements for Genetically Altered Lines

License Required	License Not Required
Creation of a new genetically altered line	
• Creation of a new genetically altered line • Crossing of genetically altered lines • Biotechnological procedures (e.g., tissue sampling, superovulation, embryotransfer, vasectomy) • Maintenance of a spontaneous mutant • Chemical mutagenesis	• Crossing or backcrossing two lines with a non-harmful phenotype, where it can reasonably be expected that the resulting new line will not display a harmful phenotype • Spontaneous harmful mutations arising in single animals / litters but which are killed immediately and the harmful trait is identified and parents not bred from again.
Birth of offspring with a mutation	Birth of offspring without a mutation
Animals with a mutation kept in breeding until stable transmission of the genetic alteration	Birth of offspring from crossings/backcrossings where no welfare-impairing phenotype occurring or expected
Specify in the project application: • *Parents in case of above-threshold discomfort* • *All offspring until the line is considered 'established'*	*Note: If any above-threshold procedure is performed, a project license is required*
Genetic Characterization	
Determining the genetic alteration using tissue obtained via an invasive method (e.g., fin clipping in zebrafish)	Determining the genetic alteration using surplus tissue collected during identification/marking of the animal
Phenotype characterization using an above-threshold method (e.g., blood sampling)	Characterization/observation of phenotype using a below-threshold method (e.g., feces collection or hair sampling)
Specify in the project application: • *Parents in case of above-threshold discomfort* • *Offspring in case of above-threshold discomfort*	
Welfare Assessment	
Mandatory monitoring	Mandatory monitoring of lines resulting from crossings/backcrossings without welfare impairment where no welfare-impairing phenotype is expected
Establishing the line; determining whether a welfare-impairing phenotype is present and classification of discomfort (mild, moderate, severe)	Conventional/wild-type background animals used as reference for assessing the welfare of the new genetically altered line
Specify in the project application: • *Minimum 7 male and 7 female animals across at least 2 stable generations* • *Animals of representative ages* • *Animals with relevant genotypes* • *Above-threshold discomfort for the welfare assessment*	*Note: If any above-threshold procedure is performed, a project license is required*

License Required	License Not Required
Maintenance of an existing genetically altered line	
Genetically altered line with a welfare-impairing phenotype, including: Breeding of harmful lines by crossing het x het or het x wild-type to reduce / eliminate the risk for expressing a harmful phenotype Lines that, compared to conventional/wild-type animals, require special housing or care (e.g., immunodeficient lines in IVC cages) Breeding of animals with age-onset welfare-impairing phenotypes Crossed Cre or Lox lines that may result in welfare-impairing phenotypes after crossing Animals in breeding where the welfare-impairing phenotype is activated via an inducing agent Animals in breeding where the welfare-impairing phenotype is suppressed via a suppressing agent	Established genetically altered lines without a welfare-impairing phenotype, including: Lines with only reporter genes in the genome Non-crossed Cre or Lox lines without a welfare-impairing phenotype Lines where the phenotype is only activated by an inducing agent during the experiment (not in breeding)
Specify in the project application: *Parents in case of above-threshold discomfort* *Offspring in case of above-threshold discomfort*	*Note: If any above-threshold procedure is performed, a project license is required*
Rederivation	
Rederivation carried out solely for scientific purposes e.g., where the immune responses may be affected by the pathogen(s) present, but where the health of the animals are not compromised.	Rederivation carried out for the benefit of the health and/or welfare of the colony i.e. when it is necessary that the pathogen is removed because the animals will suffer ill health if this is not done e.g., Mouse Hepatitis Virus (MHV).
Specify in the project application: *Parents, foster, and/or donor animals*	*Note: Advice/opinion of the designated veterinarian is required*
Cryopreservation	
Techniques using live animals required for cryopreservation when carried out for the preservation of a line.	Use of frozen sperm collected from killed animals

This is a publication of the Central Authority for Scientific Procedures on Animals (CCD).

The CCD is the sole competent authority in the Netherlands authorised to grant and issue project licences for conducting animal procedures.

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