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DIRECTIVE 2010/63/EU ON PROTECTION OF ANIMALS USED FOR SCIENTIFIC PURPOSES

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GENETICALLY ALTERED ANIMALS

Environment

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Framework for the Genetically Altered Animals under Directive 2010/63/EU on the protection of animals used for scientific purposes

National Competent Authorities for the implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes

A working document on Genetically Altered Animals to fulfil the requirements under the Directive

- ***Replacing consensus document of 22-23 March 2012 -***

Brussels, 25-26 November 2021

In 2011, the Commission established two Expert Working Groups (EWG) 1) to develop common format for statistical reporting and 2) for the assessment of severity of procedures, to facilitate the implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes.

As a part of the results of this work, a guidance document on Genetically Altered Animals (GAA) was endorsed by the National Contact Points of the Member States for the implementation of Directive 2010/63/EU at their meeting of 22-23 March 2012, followed by the endorsement of the GA Welfare Assessment scheme (incorporated in the Annex) at their meeting of 11-12 July 2012. A corrigendum to the Annex was endorsed on 23 January 2013.

However, with the rapid technological development over the last decade, and apparent difficulties in achieving uniform understanding on when and what authorisation was required, and on how to report animals used to create and maintain GA lines, the European Commission hosted a meeting of an additional EWG on the creation, breeding and maintenance of GAA in Brussels on 27-28 June 2018. The meeting was followed by the establishment of several subgroups to develop Welfare Assessment frameworks for the most commonly used genetically altered species, and another to identify elements of information that should travel with GAA when sent between establishments or to places outside EU to ensure appropriate husbandry and care practices are in place to assist in optimal application of reduction and refinement practices.

All Members States and main stakeholder organisations were invited to nominate experts to provide input and participate in the discussions. This document has been developed through the work of all the above mentioned EWGs, discussions with the Member States as well as legal input from the Commission. It was endorsed by the National Competent Authorities for the implementation of Directive 2010/63/EU at their meeting of 25-26 November 2021.

Disclaimer:

The following is intended as guidance to assist the Member States and others affected by Directive 2010/63/EU on the protection of animals used for scientific purposes (as amended by Regulation (EU) 2019/1010 of the European Parliament and of the Council) to arrive at a common understanding of the provisions contained in the Directive and to facilitate its implementation. All comments should be considered within the context of this Directive 2010/63/EU and the Commission Implementing Decision 2020/569/EU. The content of the document does not impose additional obligations beyond those laid out in the Directive.

Only the Court of Justice of the European Union is entitled to interpret EU law with legally binding authority.

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Introduction

In 2017, Genetically Altered Animals (GAA) made up almost one third (2,59 million) of all the animals used in scientific research and testing in the EU[1]. Mice and zebra fish were the most commonly reported GAA species, although significant numbers of Xenopus, rabbits and rats were also reported, as well as smaller numbers of other species such as guinea pigs, dogs, pigs, sheep, domestic fowl and other fish species. 64 % zebra fish used in research and testing were genetically altered, and 38% of mice.

Article 1 in conjunction with Article 3 and 17 of Directive 2010/63/EU on the protection of animals used for scientific purposes, hereafter "the Directive", consider the creation and maintenance of a genetically altered animal as a scientific "procedure", if the birth or hatching may cause the animal pain, suffering, distress or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice. Consequently, in addition to animals used directly in research and testing, animals that are needed for the creation of new genetically altered (GA) lines and those used for maintaining (breeding) existing GA lines with a harmful phenotype are also both within the definition of a procedure. In 2017, in addition GAA used in research and testing, the creation and maintenance of GAA amounted to almost 1,3 million animals[2]. Furthermore, as a result of GAA creation and breeding programmes to ensure sufficient availability of the required GA lines, an additional 6.1 million animals were reported as killed without being used in a procedure[3].

It is therefore important that attention is given to the techniques of production and maintenance of GAA, and to the specific characteristics conveyed as a result of genetic alteration in order to apply the Three Rs principles in the creation, breeding, use and care practices of these animals. In addition, to facilitate uniform understanding of the Directive requirements, further guidance was considered necessary on the respective administrative procedures and reporting obligations arising from the Directive and the related Commission Implementing Decision 2020/569/EU.

Part 1 of this guidance document sets out the legal framework and formal obligations under the Directive and provides information on the key elements to be covered and detailed for the purposes of project application and evaluation to help facilitate compliance.

Part 2 considers the application of the Three Rs within activities and procedures related to the GA line creation and maintenance and which should be carefully considered by breeders, users, project evaluators and inspectors, offering some general principles to be followed.

Parts 3 and 4 of this guidance document cover the Welfare Assessment scheme for GA lines necessary to facilitate the identification of the effects of the genetic alteration in order to:

- allow the classification of the GA line according to whether it is considered to have a harmful or non-harmful phenotype;

[1] European Commission ; *2019 Statistical report, Commission Staff Working Document, SWD(2020)10 final;* (2020).
[2] European Commission ; *Commission staff working document SWD(2017) 353 final/2* ; (2017).
[3] European Commission ; *Report on the implementation of Directive 2010/63/EU, Commission Staff Working Document, SWD(2020)15 final;* (2020)

- provide tools for the monitoring of the health and welfare of the GAA;
- facilitate appropriate care and accommodation tailored to the needs of the line;
- provide the necessary care and welfare information on the GAA when animals are to be transferred to another establishment.

A number of user templates were developed in order to provide practical and useful guidance covering the following areas:

- **Section A of the Welfare Assessment Template for all species and times points** details the relevant information such as the description and name of the line, genetic alteration, assessment details (date, assessor), and provides the final prospective severity classification assigned to the line;

- **Section B of the Welfare Assessment Template for specific species** details elements and findings that are specific to the species being assessed;

- **Section C - Transfer template for the care and husbandry requirements for GAA** draws from the findings of the Welfare Assessment providing information on welfare concerns to be aware of with the line, specific housing and care needs, and/or suggestions for Refinement strategies.

All three documents together will form the necessary information that should accompany GAA when moved within, and between establishments.

Finally, Part 5 discusses the legal reporting obligations related to GAA during creation and maintenance of lines and offers further guidance to ensure compliance with both annual statistical reporting and five-year implementation reporting requirements.

Part 1: Administrative procedures involving genetically altered animals

1. Background

Since 2011, a number of Expert Working Groups have been convened to address how GAA should be considered within the context of the Directive. Outcomes from these EWGs have resulted in the endorsement of a Working document on Genetically altered animals[4], and the adoption of Commission Implementing Decision 2012/707/EU detailing inter alia the handling of GAA within statistical reporting.

On the basis of the text of the Directive, the approach taken was to separate established harmful GA lines from those considered non-harmful. This was motivated mainly by the removal of a need for a specific project authorisation and the related administrative burden for non-harmful lines for which there was no likelihood of a risk of pain, suffering, distress or lasting harm as stated in Article 3(1) definition for "a procedure". However, in this context it is important to note that irrespective of whether an activity requires an explicit *project* authorisation, all animals bred for scientific use are under the scope of the Directive and subsequently can only be bred by authorised establishments that comply with the requirements of the legislation.

In practice, the separation of harmful from non-harmful lines has caused difficulties both for the authorities and operators, and has resulted in inconsistencies of practices on whether a line is considered harmful or not, the criteria used for the decision making and the subsequent reporting by the Member States. Furthermore, the differences of approaches to project authorisations for the creation of new GA lines (varying from a single line project authorisation to projects covering multiple GA lines) have prevented the development of a level playing field for the operators, one of the key objectives of the Directive. Such issues were highlighted both in the Directive Review Report published in 2017[5] and the first EU report on the Implementation of the Directive, published in 2020[3]. Member State national contact points considered that further clarity and guidance would be beneficial. Additional clarity and precision were incorporated in the Commission Implementing Decision 2020/569/EU, replacing Decision 2012/707/EU.

This first part of the GAA guidance will look into the main principles and key elements related to the application, evaluation and authorisation of projects dealing with GAA creation and maintenance.

2. Legal framework

Directive 2010/63/EU on the protection of animals used for scientific purposes covers within its scope the creation, maintenance and use of genetically altered animals in the Union. Commission Implementing Decision 2020/569/EU provides further instructions on practical implementation.

[4] National Competent Authorities for the implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes ; *Working document on genetically altered animals* ; (2013).
[5] European Commission ; *Report in accordance with Article 58 of Directive 2010/63/EU on the protection of animals used for scientific purposes* ; (2017).

As described above, all animals used for scientific purposes, whether genetically altered (harmful or non-harmful) or conventional / wild-type are covered by the Directive. Breeders of animals are required to be authorised, and operate in compliance with the Directive. The oversight is exercised by authorities who are required to carry out regular inspections of animal breeders, suppliers and users. Activities falling under the definition of "a procedure" can only be undertaken within the context of an authorised project. Project authorisation can only be granted on the basis of a favourable project evaluation, performed by a competent authority. The project evaluation needs to ensure that the principle of the Three Rs is being complied with and that the authority is satisfied that the harms to the animals are justified by the expected benefits, taking into account ethical considerations.

To determine what activities fall within the scope of a procedure and consequently require a project authorisation, Article 3(1) of the Directive includes within the definition of "a procedure" any course of action intended, or liable, to **result in the birth or hatching of an animal or the creation and maintenance of a genetically modified animal line which may cause the animal a level of pain, suffering, distress or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice**.

As stated in Commission Implementing Decision 2020/569/EU, for the purposes of the Directive, "genetically altered animals (GAA)" include genetically modified (transgenic, knock-out and other forms of genetic alteration) and naturally occurring or induced mutant animals as per the definition in Article 3(1).

Furthermore, Article 3(1) provides the minimum threshold of pain, suffering, distress and lasting harm beyond which point the activity is considered "a procedure" and requires to be authorised within a framework of a project (Article 12(2)).

Article 17 states that "a procedure" shall be deemed to end when no further observations are to be made for that procedure or, **as regards new genetically modified animal lines, when the progeny are no longer observed or expected to experience pain, suffering, distress or lasting harm** equivalent to, or higher than, that caused by the introduction of a needle.

Commission Implementing Decision 2020/569/EU further describes that a new line is considered to be "established" once the transmission of the genetic alteration is stable (a minimum of two generations) and a Welfare Assessment is completed. Scientific input is required on what likely effects of the genetic change will cause, and when such changes are likely to manifest. Information/evidence obtained during a Welfare Assessment and other scientific input will determine whether the line will be classified as harmful/non-harmful when bred and maintained as an established line. In the context of maintenance of established lines, Article 1(2) states *inter alia* **that the elimination of pain, suffering, distress or lasting harm by the successful use** of anaesthesia, analgesia or **other methods shall not exclude** the use of an animal in procedures from the scope of this Directive. **The breeding of GA lines which retain a risk of the development of a harmful phenotype** (e.g., risk of infection due to compromised immune system) **regardless of the applied refinement** (barrier/biosecure conditions), **requires project authorisation** in line with Article 1(2), as the application of

refinement **does not entirely eliminate the risk,** but only reduces the risk in that context (the positive interventions required to reduce the risk).

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.

In rare cases, lifetime studies may determine that there are no harms nor reduction in lifespan in these age-onset disorders. If this is demonstrated, then the line could be reclassified as non-harmful, and no project authorisation would be required from that point in time.

Finally, it is important to recall the definition of a project as provided in Article 3(2) stating that "a project" means **a programme of work having a defined scientific objective** and involving one or more procedures (see Appendix II for examples).

3. Activities falling under the definition of a procedure and requiring a project authorisation

In line with the above Directive provisions, the **creation** of a new GA line is in principle considered a procedure as the consequences of creation of the new line cannot always be determined fully in advance.

An exception is when crossing/backcrossing two lines of non-harmful phenotype and where 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. This decision should be recorded clearly at the establishment where the animals are bred. When offspring are produced, it needs to be confirmed that the line does not show any harmful phenotype using a Welfare Assessment as described in section 3. All these animals remain under the protection and control of the establishment as animals bred for scientific use.

The **maintenance** of an established GA line is considered a procedure when the line carries a harmful phenotype.

In addition, during the creation and maintenance of GA lines a number of particular activities are carried out such as superovulation, vasectomy, embryo transfer, and tissue sampling for the purposes of genotyping. Most of these fall within the definition of "a procedure" as defined under Article 3(1) of the Directive.

For tissue sampling, the least invasive method should be used that provides an adequate DNA sample in terms of quality and quantity to perform a robust genotyping procedure. Whenever possible, this method should, at the same time, provide highly reliable identification/marking. When excess tissue is used from an identification/marking method this is not considered a procedure (Article 1.5 (e)).

The below table provides an overview of the most common activities and when these are required to be covered by a project authorisation.

Activity	Comment	Project authorisation required	No project authorisation required
Creation of a new GA line	Genetic manipulation of gametes or embryos	The creation of new GA line requires a project authorisation.	
	Crossing of existing lines	The creation of a new GA line by *crossing of different lines to create a new genetically altered line where* ***the phenotype of the new line cannot be determined prospectively as non-harmful*** **requires a project authorisation** as stated in Commission Implementing Decision 2020/569/EU (Annex III, Part B; Section B – Data Input Categories - point 8) for the statistical reporting obligations on the use of animals.	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. **In these cases, the competent authority has to consider the principles of decision making at the establishment and be satisfied that processes are in place so that if these predictions turn out to be incorrect then project authorisation can be quickly secured.**
	Mutagenesis	Chemical exposure or irradiation is used to induce random mutation in germ cells which in many cases are harmful to offspring. Exposure of parent and offspring requires project authorisation.	
	Spontaneous harmful mutant	Mutations arise spontaneously in all breeding and in some cases lead to harmful traits which are of scientific interest. When such animals are maintained, bred, supplied and / or used for a scientific purpose then project authorisation is required.	Harmful mutations arising in single animals / litters but which are killed immediately and the harmful trait is identified and parents not bred from again. Some inbred “wild-type” lines show occasional harmful traits, such as hydrocephalus in B6 mice. These lines

			are not maintained to investigate hydrocephalus. All animals identified with such a trait will be killed immediately. Efforts will be made to reduce the incidence of the trait by selective breeding.
Maintenance of an existing GA line	**Phenotype of the line**	Breeding of an existing harmful phenotype line. Lines which have a Welfare Assessment (as described in Part 3) which demonstrate that the line has a risk for a harmful phenotype above the minimum threshold of pain, suffering, distress or lasting harm during the lifetime of the animal. Breeding of harmful lines by crossing het x het or het x wild-type to reduce / eliminate the risk for expressing a harmful phenotype still require authorisation.	Breeding of an existing non-harmful phenotype line. Lines which have a Welfare Assessment (as described in Part 3) which demonstrate that no harms above the minimum threshold of pain suffering, distress or lasting harm are likely to occur during the lifetime of the animal e.g., some green fluorescent protein (GFP) lines. Should animals from a non-harmful GA line (i.e. not bred under a project authorisation) experience adverse effects, the Welfare Assessment should be reviewed, updated, and the line re-classified from non-harmful to harmful. Authorisation to maintain the line and to breed further animals should be sought immediately from the competent authority.
	Immuno-compromised lines	**Immuno-compromised** lines are particularly sensitive to infection as a consequence of the gene alteration and need to be kept in special housing arrangements such as a specific bio-secure environment to protect them, and can also need additional care beyond that required for conventional animals to maintain their health and well-being. Such lines are defined as being of harmful phenotype requiring project authorisation; Commission Implementing Decision 2020/569/EU,	

		Annex III, Part B, Section A General Provisions point 11. 7.	
	Age-onset lines with a harmful phenotype	Harmful phenotypes include **age-onset GA lines**, Commission Implementing Decision 2020/569/EU states in Annex III, Part B, point 11. 7 that *"… Such animals include, amongst others, those that require… additional care beyond that required for conventional animals to maintain their health and well-being."*	
	Cre/Lox lines	Breeding **of crossed Cre/Lox lines** to express harmful phenotype requires project authorisation.	Breeding of uncrossed Cre or Lox lines where no harmful phenotype is displayed does not require project authorisation.
	Induced or suppressed lines	Inducing agent has been administered to "activate" a harmful phenotype.	Lines in which the genetic modification of the phenotype is only active when treatment with inducing agents (e.g., tamoxifen, tetracycline etc.): these are considered not to have a harmful phenotype until the time of induction and are not subject to authorisation before induction.
		Lines in which a genetically based phenotype is suppressed by treatment with supressing agents (such as tetracycline) whilst they demonstrate no harmful phenotype, because a specific action needs to be taken to keep the line non-harmful therefore these require project authorisation. Commission Implementing Decision 2020/569/EU states in Annex III, Part B, Section A, point 11. 7 that *"…*	

		Such animals include, amongst others, those that require… additional care beyond that required for conventional animals to maintain their health and well-being."	
	Reporter lines		The presence of reporter genes in the genome and molecules arising from these genes do not result in a harmful phenotype per se. Therefore, breeding of lines into which only reporter genes were introduced is not subject to authorisation.
Genetic characterisation	Invasive tissue sampling	Tissue sampling by ear clipping when not carried out for the purposes of identification/marking e.g., where alternative identification method such as microchipping are used.	Surplus tissue from the identification/marking of an animal (e.g., ear marking **but excluding** tail tipping or fin clipping).
		Tissue sampling by tail tipping, or fin clipping (not methods suitable for identification/marking of individuals).	
		Tissue sampling by phalanx / toe clipping when not used as identification/marking.	Surplus tissue from the identification/marking by removal of a single distal phalanx where it is still considered the most refined method to identify individual animals such as in neonatal rodents.
		Blood sample (not method suitable for identification/marking of individuals).	
			Tissue obtained by invasive method but only after death is confirmed (post mortem).

	Non-invasive		Below minimum threshold of pain, suffering, distress or lasting harm (as defined in Article 3(1)) methods such as use of faeces, hair sampling.
			Observational methods e.g., coat colour, UV-fluorescent light.
Vasectomy		Surgical procedure required in males to allow them to be used to produce pseudopregnancy. Only the surgical procedure requires authorisation (subsequent natural mating is not a procedure, see below).	
Superovulation		Injections required for scientific purpose so above the minimum threshold of pain, suffering, distress or lasting harm, and therefore are procedures.	
Embryo transfer		Requires surgical implantation or requires insertion of tube through cervix (non-surgical embryo transfer) which is above the minimum threshold of pain, suffering, distress or lasting harm, and therefore are procedures.	
Natural mating		Natural mating where **either parent** carries a harmful phenotype. Where the cross will generate a harmful phenotype e.g., Cre/Lox crosses requires project authorisation for the birth or hatching of offspring (Art 3(1)).	Crossing/backcrossing two lines of non-harmful phenotype and where it can be reasonably expected that the new line will not result in a harmful phenotype, the requirement for a project authorisation will not apply. Post recovery use of vasectomised animals to mate to produce pseudopregnancy is not a procedure.

Rederivation		When 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 is not compromised.	When 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).
Cryopreservation		Techniques using live animals required for cryopreservation for scientific purpose when carried out for the preservation of a line.	When carried out for the preservation of a line by using frozen sperm from killed animals.

When considering individual procedures, the determination of whether project authorisation is required is derived from the objective of the activity i.e. whether it is carried out for scientific purposes, or for the welfare of that animal/or its colony. Some further clarification is provided below using two examples:

Rederivation

Microbes can be pathogenic or commensal. Many rederivations are required to get rid of commensals which may affect science. When the rederivation is not for the welfare benefit of the animal(/s) and is being done to create or retain animals / colonies of suitable quality and consistency for good science, then this is being done for scientific purpose and rederivation must comply with the Directive requirements including project authorisation, training of persons involved, etc. However, should the designated veterinarian determine that it is in the welfare interests of the animal or the colony to rederive, to eliminate pathogens from the colony then this would not be covered by the definition of "a procedure" and no project authorisation would be required under the Directive. This would be performed under the relevant veterinary legislation within the Member State. Decision making, numbers etc used should be reported in veterinary health records (E&T Framework, module 24 24.15), and procedures performed by veterinarians or persons they can legally delegate to (if relevant locally). This provides some flexibility but the decision must be properly documented and defensible under the respective legislation.

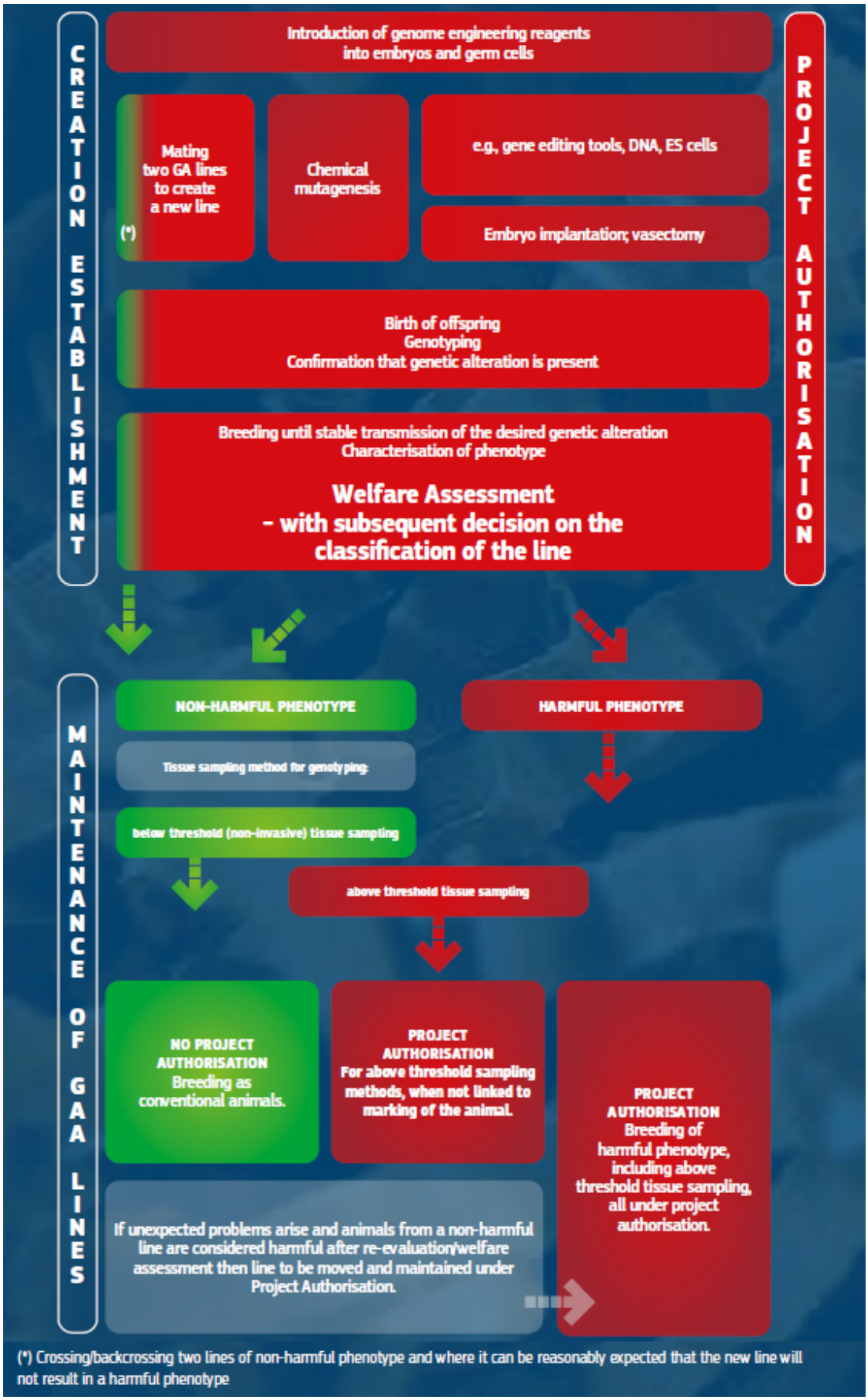
Cryopreservation

Cryopreservation (superovulation with egg / embryo retrieval after killing, or sperm harvest with freezing of gametes / embryos, reconstitution of a specific GA line) is carried out for the purpose of maintaining the scientific integrity and suitability of a GA line when e.g,. to prevent genetic drifting that has been identified in the colony. The use of live animals for cryopreservation for scientific purpose requires a project authorisation. Only when cryopreservation is done using frozen sperm from killed animals, it is outside of the definition of "a procedure".

4. Flow chart for the requirements for a project authorisation for the creation and maintenance of GA lines

The flow chart on the following page provides the key steps from the creation to establishment and maintenance of a GA line and the related requirements for a project authorisation, areas marked in red. It can be downloaded as a poster at
https://ec.europa.eu/environment/chemicals/lab_animals/pubs_posters_en.htm

Flow chart for the requirements for a project authorisation for the creation and maintenance of GA lines



5. Types of projects and authorisation processes

A uniform approach to project authorisation involving creation and maintenance of GA lines is needed in order to attain the Directive aims for a level playing field among operators across the Union. Despite efforts to facilitate uniform application of the Directive, the 2016 feedback from users for Article 58 Review of the Directive[5] suggested that significant differences were experienced in project authorisation processes with regard to GAA production varying from a requirement for a separate project proposal for each new GA line, to the greater flexibility offered by multiple generic projects where multiple lines can be created and maintained under a single project authorisation – resulting in disparities in costs of and delays in conducting research. The responses to the review also identified difficulties in moving GAA between Member States due to differing authorisation approaches and differing classifications of the lines (harmful versus non-harmful line), potentially compromising animal welfare.

Since the adoption of the Directive, gene-editing techniques have evolved greatly. One of the most significant changes, as a result of the introduction of CRISPRCas/9 techniques, concerns the accessibility of the new technologies and the rapidity of the creation process. As a result of this technology, multiple lines can be created in a fraction of the time compared to the earlier methods and allowing for selections to be made progressively as to which lines should be continued for further development. This evolution, which has occurred since the adoption of the Directive, has an even greater impact on the related administrative processes in Member States/regions where a project is authorised at the level of a single line rather than multiple lines within a given disease/research area.

In this context it is important to recall that the definition of a project is described as 'a programme of work having a defined scientific objective'. The Directive further requires that the project evaluation is carried out with the level of detail appropriate to the project whilst allowing for a realistic estimation of harms and expected benefits, and ensuring the application of the Three Rs in the project.

Article 38 calls for the necessary flexibility with regard to the degree of detail required in the project evaluation, and Article 40(4), and in some cases Article 42, provide possibilities to simplify administrative processes. The creation of new GA lines differs from maintenance of an established GAA for production purposes. However, it must be recognised that often projects contain both the creation and maintenance of GA lines.

A project application must therefore contain sufficient level of detail to allow the project evaluation to be carried out in line with the Directive obligations whilst minimising administrative burden both for the operators and authorities.

Amendments to projects on the creation and maintenance of GA lines is covered under section 7.

Activities that are required to be covered under a project authorisation include

- The creation of new GA lines (with the exception that 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).

- techniques required for creation of new lines e.g., vasectomy, superovulation;
- The maintenance of established harmful GA lines;
- Tissue sampling (irrespective of the phenotype of the line when using above threshold tissue sampling methods for the purposes of genotyping);

It is therefore important to note that even if the maintenance of a non-harmful phenotype line does not require a project authorisation, it is likely that the establishment will need to have a project authorisation for procedures such as invasive tissue sampling for the purposes of genotyping.

Recommendations

In order to harmonise and simplify administrative practises to reduce administrative burden for both the scientific community and authorities, and to ensure compliance with the Three Rs consideration should be given to

- moving from projects covering only the creation of single lines to those covering **creation of lines for a defined disease area or other focussed project theme;**
- combining all GAA related activities i.e. creation, breeding, maintenance and other GAA related procedures in a single project;
- the level of detail in the project application:
    - on one hand, to ensure that all elements that are needed to assess the compliance of the project with the Three Rs and improve animal welfare (including care and accommodation requirements) are sufficiently covered;
    - on the other, that the information requested is limited to elements relevant to the harm-benefit assessment, (including any proposed re-use);
- the use of multiple generic projects as provided in Article 40(4) of the Directive e.g., authorisations covering multiple lines required to investigate pathogenesis of motor neurone disease, where phenotypes will be similar, or can otherwise be described within an integrated programme of work.

6. Key elements in a project application for project evaluation

Project evaluation must ensure compliance with the Directive requirements including that all required elements in Directive Annex VI and on the implementation of the Three Rs in relation to these activities are included. Part 3 of this document provides a number of recommendations on ways in which Three Rs could be implemented within different activities related to the creation and maintenance of GA lines.

It is important that a project application contains sufficient level of detail to allow assessing whether such elements have been given due consideration to allow an evaluation.

Appendix II, Part A, provides examples of the type of information required for a project application to create and maintain GAA, and Appendix II, Part B, outlines issues and considerations to be given to such an application during project evaluation (PE).

7. Amendments to Project Authorisations

Article 44 of the Directive states that amendments are required only when changes may have a negative impact on animal welfare. Such changes in a project may result in increasing the numbers of animals, introduction of new species or increases to severities from those originally authorised.

In the context of projects for the creation and maintenance of GA lines, production of new lines is likely to increase the numbers required, and completion of welfare assessments may change the severities. Where new lines can be predicted at the time of application they can be included as a group e.g., lines which label neurones, or lines showing signs of muscular dystrophy. But there will be many projects which will require amendment over their lifetime, e.g., increase in demand or changes in / additional scientific direction.

Another example would be the need for a process to deal promptly with amendments where additional welfare issues beyond those initially considered have been identified. Two examples could be

1. harmful lines with higher-than-expected severity (e.g., mild prospective severity classification but found in practice that animals experience moderate severity), or
2. unexpected harmful effects in a line originally classified as non-harmful, requiring the line to be included within a project authorisation.

Structure and content of project applications and detail required for the authorisation process can have a very significant influence on the number of amendments which may be required over a five-year period.

Recommendations

- Consider the structure and key elements of the application to minimise the number of amendments whilst allowing sufficient level of detail for the project evaluation to be carried out.
- Limiting requirement for amendments only to those changes that may have a negative impact on animal welfare or where they are required to re-evaluate the harm-benefit analysis.

Part 2: Three Rs in the creation, breeding and maintenance of genetically altered animals

Application of the Three Rs within GAA creation, maintenance and breeding practices

The Three Rs (Replacement, Reduction, and Refinement) should be considered at all stages, from inception of project through creation, breeding and supply of genetically altered animal lines.

This Part 2 of the guidance highlights areas where the application of the Three Rs should be given specific consideration in this context.

1. Availability of existing lines of genetically altered animals

No line should be created if it, or an alternative which will achieve the scientific outcome is already available. However, there are a number of issues that may hinder the information of and access to already existing GA lines.

Several specialist databases of established GA lines exist. Examples of current such databases are listed in Appendix I. However, as often is the case, new databases are being continuously established whilst older ones are either being deleted or not being maintained. Furthermore, there is no consolidated platform for sharing information across different organisations or research establishments on existing lines across species and strains.

GAA models are not necessarily shared between individual research groups within establishments, and even less so externally either with research groups outside one's own facility and/or country or through open access.

There can be issues of confidentiality and/or intellectual property (IP), which delay or, in some cases, prevent access to technologies and new lines of GAA.

The health status may not be suitable. In this case, a decision should be taken as to whether it is better to rederive into animals of a suitable pathogen-free status or to recreate the line.

Recommendations:

- In order to avoid unnecessary duplication, there needs to be a systematic search through available databases on existing GA lines before a decision to generate a new one is taken. Duplication of a line should only be undertaken when there is specific scientific justification, lack of availability or a problem with access to an existing line.
- It is important to regularly review the status of databases (whether being continuously maintained) that are used for searching for existing lines, and to ensure a thorough search to identify any new databases which may have been developed meanwhile.
- A common platform for sharing information on existing lines across species and strains would be mutually beneficial to reduce the cost of duplication (time and resources) and contribute towards reduction and refinement.

- Research groups and Animal Welfare Bodies (AWB) should review internal use of GAA to ensure duplication does not occur within the establishment. Where commonly used models are held by individual groups, rationalisation to a single breeding colony allows better planning for a more efficient use of animals, reduction in surplus and greater control over issues such as genetic drift.
- Research groups should consider external supply of GAA avoiding duplication required for the breeding and management of GAA colonies which is inherent in multiple dispersed colonies. Consolidation of lines such as those carrying specific recombinases, conditional alleles or reporter genes, would improve efficiency and reduce surplus nationally and internationally.
- Efforts should be made by the user community, to improve open sharing of information on and supply of existing GA lines for mutual benefit and to update databases to which they have access.
- Standard descriptors using agreed terminology for nomenclature (http://www.informatics.jax.org/mgihome/nomen/strains.shtml) and welfare (e.g., www.mousewelfareterms.org) should be used/ for recording information on GA lines and when searching for existing GA lines.
- Repositories (databases and cryostorage facilities) should include information on phenotype, mutation design, welfare, accommodation and care needs. It would help consistency across Europe if information is included on whether the line has been classified as a harmful or non-harmful phenotype.
- The GAA user community should consider developing strategies to identify and overcome issues of confidentiality and/or IP to reduce unnecessary duplication of GA lines.
- There should be a standardised set of information, which accompanies the animals when moved to a new establishment (see Part 4 of this Guidance document).

2. Choice of methods of generating new lines of genetically altered animals

A number of methods are now available to generate new lines of GAA. The "traditional" methods of gene manipulation have been largely superseded by endonuclease mediated gene editing technologies of which CRISPR (clustered regulatory interspersed short palindromic repeat associated nucleases)/Cas has prevailed. Inevitably as genetic manipulation techniques evolve, new and more controlled methods will be employed. Mosaicism, unpredictable modifications at the target site and off-target effects remain potential problems. Specialists may be required to be brought in to overcome/minimise these problems. Random transgenesis needs good justification due to the unpredictable results of non-targeted integration into coding or regulatory genome regime. However, embryonic stem cells continue to play a role in the creation of new models, when aiming at complex genome changes, in particular homologous recombination of longer stretches of the genome.

The CRISPR/Cas9 technology has promoted and enabled the creation of new GAA in a way that makes it easier for many scientists to create a new line than by using pre-existing methodologies.

Scientific need will generally determine which method will be used. Each method offers different challenges and opportunities both for science and with regard to implementation of the Three Rs, in particular, the number of animals required. The numbers of animals are also impacted by the complexity of the desired model i.e. how many concurrent gene manipulations are needed.

Systems should be in place to validate the genes of interest and the regions of the insertion / deletion, and breeding lineages should always be traceable by clear documentation.

Genotyping assays should be specific for the genetic alteration of the strain (i.e. allele-specific) and not for a common transgene such as CRe, GFP, neo etc. as these genetic sequences are common in laboratory stocks and a generic assay will not identify when strains carrying them have been mixed up.

Recommendations:

- Consideration should be given to how the precise manipulation by the chosen method for generating new lines is optimised and unwanted molecular events can be screened and controlled for. If specialist knowledge in genetic quality control is not available locally to ensure efficient and effective production, then consideration should be given to contracting this out to others.
- There should be a careful validation that the gene of interest meets the scientific needs.
- Irrespective of the method chosen, a quality assurance component is needed to ensure the desired mutation structure is as required during creation of new lines, and that the breeding programme maintains it as expected. Quality assurance components should be considered as part of maintaining the genetic integrity of the model being produced.
- Expansion for supply of animals from lines which are not established and / or well-characterised should be avoided.
- To ensure good genetic integrity, the use of sequencing, robust genotyping protocols that can validate the lineage should be adopted.

3. Refinements in procedures involving rodents (mice and rats)

There are ample opportunities to apply Refinement during common procedures carried out for the purposes of creation and maintenance of GA lines. As the impact of these elements vary (e.g., age or line dependent), it is important that choices concerning the elements listed below are discussed and considered on a case-by-case basis.

<u>Superovulation</u>

- The background strain can impact the numbers of offspring produced and therefore impact on the number of animals used. However, the most important factor is the

scientific requirement for the final background strain on which the genetic alteration is required, in order to optimise production and avoid backcrossing;

- Oocyte and embryo production are also affected by the age and weight of females. For superovulation an immature female gives more oocytes and is preferred when generating oocytes for *in vitro* fertilisation (IVF) or embryos for cryopreservation. Therefore, young females that have not ovulated are recommended. Most units would not pair young (small) females with large potentially overvigorous males;

- For cryopreservation, the use of sperm taken from killed males should be considered before creation and freezing of embryos. It is a more refined method and results in a reduction in the number of animals required for archiving;

- Appropriate and most refined hormone stimulation regime should be ensured, including optimal timing / interval, age and weight of females considering both the scientific and animal welfare needs.

Vasectomy

- Considerations for the use of sterile males:
    - overall mating performance of genetically sterile (potentially reduced) versus vasectomised animals and
    - the impact on numbers if having to maintain a breeding colony to supply the genetically sterile males (potentially resulting in an increased number of surplus animals, unless used for other purposes);
- If vasectomised males need to be prepared, the latest scientific evidence should be consulted to consider whether the scrotal incision should be preferred over the abdominal approach;
- For efficient mating and production of pseudo pregnant females, performance of males should be monitored and replaced as required to ensure vigour and effectiveness.

Embryo transfer

- Choice of background strain impacts on mothering/rearing ability;
- Age and weight of males used to induce pseudopregnancy should be selected to avoid any negative welfare impacts on the females;
- Use of surgical versus non-surgical embryo transfer (NSET): whilst it may appear that NSET is the most refined method it is not currently suitable for very early-stage embryos (0.5 days post fertilisation) where success rates are poor. However, it should be considered for later pre-implantation embryonic stages;
- Surgical approach: it is possible to implant embryos via a single or bilateral incision. Possibilities should be considered with advice from the Designated Veterinarian, taking account of the expected successes of each approach and the differential welfare impacts.

Induction and suppression of gene activity

- The fact, that the phenotype in inducible mutants can be activated just before the intended use and is not present during the whole life of the animal, can contribute to refinement by reducing the period in which animals experience pain, suffering, distress or lasting harm. However, it must be considered that substances administered for the

suppression and induction of gene expression (e.g., tamoxifen) may themselves cause unwanted side effects, such as weight loss;
- Where an animal displays a harmful phenotype, the onset of any deleterious trait should be managed with a well-defined care package and / or strictly applied humane endpoints.

4. Genetic characterisation

Genetic characterisation is required to confirm the desired genotype of the animal. It is essential that characterisation occurs not only at creation, but also to preserve the required genotype with breeding and maintenance. Care must be taken to prevent accidental cross breeding, in particular in open cage systems. There is the potential for inadvertent contamination. Good training of investigators including good handling practices and use of secure cages, accurate animal selection and recording, and implementation of a robust breeding management information system should minimise "accidental" breeding and emphasise the importance of genetic quality/drift.

Genetic drift will occur with time. Regular refreshing either to a genetically controlled background or from cryopreserved stocks is the best practice in managing all GA lines. When generating a line in house or with a vendor, care should be taken to work with a defined background. When receiving animals or working with legacy lines, these should be thoroughly analysed before being used to generate scientific data.

The use of genetic integrity panels to assess the integrity of the background strain is essential from a scientific data perspective to avoid any confounding factors. Such care over the genetic quality should be reported and published, where appropriate, and included in transfer documentation to assure receiving institutions or organisations of the quality control applied to any given GAA model.

Genetic characterisation can be carried out in a number of different ways varying from non-invasive methods (e.g., observation) to highly invasive methods using tissue sampling (e.g., tail or phalanx (toe) clipping). The most refined methodology should be used consistent with an accurate scientific outcome.

Samples for verification of genotype may be taken from animals which die or are killed within the colony, for example surplus animals.

In some cases, even if invasive, the tissue may be obtained as a by-product from the marking of an animal e.g., ear clipping. Under the Directive, marking of the animal for identification does not fall within the definition of a procedure and, therefore, does not require to be carried out under a project authorisation.

The EU report on the implementation of the Directive, provided, for the first time, some information on methods used for genetic characterisation of GAA. However, due to lack of data with sufficient quality on other species, only information from mice could be analysed. The results indicated that in 2017 over half of the tissue samples were obtained as surplus material from identification/marking of the animal (89% from ear clipping and 11% from

phalanx clipping). A significant proportion of animals were subjected to invasive sampling under project authorisation, which was not surplus tissue from marking, and it appears that tail biopsy is a common method within this group. It seems likely that much of this could be replaced with a more refined method immediately.

The use of non-invasive sampling methodologies (below threshold of minimum pain, suffering, distress or lasting harm requiring project authorisation) accounted for less than 2% of all sampling, with the use of post-mortem material accounting for the majority in this category, with a few using observation, exposure to specific lighting conditions or hair sampling.

Concerning invasive methods of tissue sampling under project authorisation, tail biopsy, followed by ear biopsy were the most common (65% and 20% respectively). However, distal phalanx biopsy represented still 13% of invasive methods. It is important to note that some Member States no longer allow, or strongly discourage, the use of distal phalanx biopsy for tissue sampling. Where genotyping needs to be performed in the first week of life, distal phalanx and tail tip amputation may be the only possible methods for mice. In immature mice, pain pathways are not fully developed. Specific justification should be provided why the chosen methods are the most refined.

As with all technology, the more developed and commonplace it becomes the more efficiently it can be applied. Rapid turn round of results is essential for better colony management and planning of studies, and to ensure animals are used effectively at an optimal age.

Other issues to be considered to reduce and refine the effects on the animal include:

- use of fluorescent markers (non-invasive) to indicate when the gene is present;
- use of tissue from animals killed in the process of quarantine or rederivation
- sampling culled redundant breeders;
- tissue taken is as small as possible;
- the choice of analytical method:
    - improved accuracy allows a much-reduced sample size;
    - reliability of the method removes the need for second testing / sampling;
- saving part of tissue in the event that a resample/reanalysis is required;
- tail biopsy technique, if required, is performed before ossification and innervation is advanced (young animals);
- use of local and/or general anaesthesia and/or analgesia should be utilised as necessary to ensure the most refined methodology for each method of tissue sampling;
- for zebrafish, genotyping of larvae allows removing unsuitable surplus animals before independent feeding;
- rapid analysis which reduces the time that fish must be kept single housed to one to two days are refinements used by some.

Recommendations:

- Wherever possible, non-invasive methods of tissue sampling for genotyping should be used.

- When invasive methods are required then the most refined (least severe) should be used. Tail sampling can almost always be replaced by a more refined method, and will require very good justification to be authorised.
- The obligation to refine tissue sampling methods should be systematically addressed by persons responsible for colonies, establishments (e.g., by animal welfare bodies) and authorities tasked with project evaluation.
- When invasive methods are used for identification/marking of the animals (e.g., ear notch/punch), these should provide surplus tissue for genotyping.
- Project evaluators should ensure that adequate justification is given for the use of invasive methods which are not used for marking.
- Animal Welfare Bodies have a role to play in obtaining and sharing information on new non-invasive tissue sampling methods (e.g., non-invasive ocular (tear) sampling) and techniques to refine invasive tissue sampling methods.
- When invasive tissue sampling is used, the use of analgesia/anaesthesia should be considered (taking into consideration the potential additional harms due to the application of the anaesthetic/analgesic).
- The use of distal phalanx biopsy solely for tissue sampling should be discontinued.
- As tail biopsy, ear biopsy and removal of part or all of a digit remain the most commonly used methods in the EU, inspections should systematically address whether the most refined methods of identification/marking and tissue sampling are being used.
- Establishments should develop systems which ensure rapid return of genotype results.
- Establishments should consider whether the provision of genotyping services within their organisation or the use of external professional genotyping services are more effective and efficient.

5. Welfare assessment

A comprehensive welfare assessment will identify welfare concerns, which can be addressed through application of refinement (or reduction) strategies, including the establishment of humane endpoints.

A welfare assessment provides information on whether or not additional husbandry and/or care requirements are necessary. It also assists in the discrimination between harmful and non-harmful lines and the severity classification for harmful lines in the project authorisation.

Unexpected harmful phenotypes can be driven by the genetic alteration applied to the animal. A one-off effect / health problem needs consideration of whether or not it is related to a genetic effect as a consequence of the genetic manipulation. Further diagnostics may be warranted and the Designated Veterinarian should be involved in these discussions. However, repeated consistent signs developing in a line suggest genetic origin in many cases. Additionally, changes to the environment may also influence the nature and onset of clinical signs and application of humane endpoints.

Recommendations:

- The nature and timing of the assessments should be informed by the expected nature and time of onset of the clinical impact of the gene alteration.
- Care should be taken to monitor all aspects of the animals' wellbeing and where they impact the welfare of the animal, recorded and managed appropriately.
- The assessment should allow separation of gene effect from normal background / husbandry effects, including reproductive performance e.g., pre-weaning loss rates.
- The corresponding background strain or reference line should be used as a baseline comparison to ensure phenotypes are not confounded by background traits.
- Welfare assessments should be repeated if there is change of the environment (including a change of establishment).
- Welfare assessments should be repeated if new persistent signs are seen in the line (including a different age of the animals).

The section on Welfare Assessment Schemes (see Part 3 of this Guidance) provides more detailed information on numbers of animals and clinical parameters to be included in the welfare assessment as well as proposes standardised templates to improve consistency.

6. Breeding, care and maintenance, and managing surplus

Minimising surplus

In the EU in 2017, 12.6 million animals that were bred for scientific purposes, were killed without being used. Almost 49% of these were from either the creation of new GA lines or from the maintenance of existing GA lines.

Some animals are specifically bred for their organs and tissue. However, on the basis of information currently available from some Member States, such animals account for around 10% of all animals bred and killed without being used in procedures. At present, it is not known what proportion of animals bred for their organs/tissue are genetically altered.

Guidelines for optimal colony management (e.g., optimal number of breeding pairs/trios for maintaining GA lines, including breeding schemes, number of litters etc.) support the minimisation of animal numbers. Several organisations and Member States have published useful guidance on how to minimise surplus and improve efficiency of GAA breeding.

Self–assessment tools have been produced to review the efficiency of some aspects of GAA breeding. There are a number of common threads running through the published guidance intended to improve efficiency of production and minimise surplus.

Effective Colony management includes a number of considerations, and which should be used to benchmark good practice. These include:

- an individual identified as the primary colony manager for each colony;
- regular reviews of colony performance and management at individual colony and establishment-wide levels;

- training and support to colony managers to equip them with the skills they need, keep their skills up to date and assist them with challenging situations;
- defined strain-appropriate breeding performance indicators for each colony, and regular/continued monitoring against these;
- a methodology for assessing strain-specific tendencies, preferences and phenotypes for the planning and provision of optimum conditions for those strains;
- consideration of the environmental requirements for each strain and make strain-specific adaptations as necessary;
- consideration of the optimum strategy for maintenance of colonies, balancing genetic needs against practical constraints;
- consideration of the optimum controls for experimental crosses;
- avoid duplication of colonies by making these available across research groups;
- calculation of colony size which should be based upon the numbers required to meet scientific need and the reproductive performance of the line. Scientific need will include age, weight, sex and numbers required in a specific timeline. The reproductive performance will include consideration of mating success, litter size, mortality rates, genotype, breeding life and replacement breeder strategy.
- An assessment to determine whether surplus animals bred can be used either in other studies, or as a source for organs/tissues, in particular in relation to wild-type offspring e.g., wild-type offspring of an appropriate background may be used to create cell lines, or used for pilot studies on another project. Appropriate authorisations may need to be in place.
- To improve scientific validity, both sexes should be used where a single sex is not required for the experimental outcome. This will consequentially reduce wastage of GAA.

The use of conditional mutants (e.g., Cre x lox) and the use of inducible mutants (e.g., by tamoxifen) may contribute to refinement. The fact, that the phenotype in inducible mutants can be activated just before the intended use and is not present during the whole life of the animal can contribute to refinement by reducing the period in which animals experience pain, suffering, distress or lasting harm.

The more complex that the lines become (e.g., double or triple mutations), the lower the likely frequency of the desired genetic combination. This results in the consequential rise in animals of unsuitable genotype increasing the number of surplus animals. Although some of these other genotypes will often be used as controls for the multiple-genotype of main interest, even with optimised breeding, some surplus may be expected in these cases. Avoidance of low Mendelian ratios for animals to be studied should be built into the breeding program where possible. Consideration should be given to fixing alleles e.g., making one allele homozygous while the other remains heterozygous to bring the Mendelian ratio back from 1 in 16 to 1 in 4 study animals.

Finally, the archiving of frozen gametes and/or embryos helps reduce the number of animals bred to maintain lines that are not currently being used in procedures. It also facilitates the sharing of GA lines between researchers, providing further opportunities for reduction.

Recommendations:

- A person should be appointed within each establishment to ensure Three Rs are applied effectively within the context of GAA production and breeding.
- GAA co-ordinator should be appointed, especially within larger establishments with several independent scientific programmes and / or animal facilities, to maintain an establishment overview of the demand for and availability of GA lines.
- Colony management systems should allow, and be used to facilitate, improved matching of supply to demand.
- Monitoring and improving the efficiency of breeding and production of GA lines / strains, by development of internal benchmarks and using regular, periodic self-assessments within and between establishments, by e.g., Animal Welfare Bodies.
- Regular monitoring of genetic quality should be established for early detection of e.g., genetic drifting / accidental contamination and strategies for resolution should be in place.
- Appropriate background strains should be used in the creation of new lines to avoid, as far as possible, the need for backcrossing.
- Offspring of genetically altered parents which are genotyped as wild-type should be considered for use/re-use for other purposes e.g., supply of blood/tissues or for educational or training purposes to reduce "surplus".
- When complex models with multiple genetic alterations are required, much care and planning should be given to breeding strategies taking into consideration the mixing of different genetic backgrounds, controls and breeding numbers.
- GA lines should be archived as frozen gametes and/or embryos when not required for ongoing experiments as a routine part of establishment processes or scientific programmes that produce GAA.
- GA lines should be transferred between establishments using gametes and/or embryos instead of live animals.
- The use of commercial breeders should be considered as overall reduction may be obtained through economy of colony scale.

<u>Balancing Refinement versus Reduction</u>

Maintaining harmful homozygous lines will reduce numbers of animals required to supply demand. However, harmful lines maintained in heterozygous (het) colonies will reduce the number of animals experiencing harms, but increased numbers of animals will be required to maintain the colony. Breeding of lines by crossing het x het or het x wild-type will reduce the risk for expressing a harmful phenotype, but the numbers of animals will be increased. Such crosses still require authorisation. It is generally considered that using more animals with lower harms is more ethical.

Recommendations:

- Where welfare issues are noted only in homozygous animals, consideration should be given to the use of heterozygous animals for breeding purposes. This strategy may increase the number of surplus animals but will reduce suffering overall.
- When breeding complex GAA crosses, the method of production should be carefully planned to minimise surplus.
- Both sexes should be used to improve scientific validity and reduce wastage where a single sex is not required for the experimental outcome.

Cryopreservation

Cryopreservation has several benefits in the operation, protection and sharing of GA lines.

This should be considered whenever there is a period when animals are not required.

Cryopreservation also facilitates and promotes exchange of lines in a welfare friendly manner by transport of gametes or embryos and not live animals. It can also be used to improve health status of a colony and, during active use of the line, to improve genetic integrity. Genetic drift is stopped by cryopreservation. No animals are required for the maintenance of the line and thus cryopreservation contributes to an overall reduction of potential surplus. However, some animals will be required to restore the line.

Cryopreservation should be an integral part of a GAA breeding facility's disaster plan, e.g., if an animal facility is destroyed in a fire or there is an outbreak of a serious disease within the colony, sufficient material is available in cold storage to allow a new colony to be established.

Recommendations:

- All involved in GAA production and breeding should have access to cryopreservation services.
- Consideration should be given to the numbers and welfare costs of maintaining colonies versus cryopreservation.
- There should be regular review of breeding colonies and strategies in place for cryopreservation where strains are no longer required.
- In support of refinement and reduction, consideration should be given to the use of sperm freezing in preference to embryo freezing.
- Cryopreservation should be used to support ease of distribution and sharing of GAA models.

Part 3: Welfare Assessment schemes for the most common genetically altered species

1. Introduction

Whether a line is of a non-harmful or a harmful phenotype has direct consequences for the regulatory requirements concerning the breeding and maintenance of such a line and the subsequent reporting obligations. These are discussed in more detail in Parts 1 and 5 of this guidance.

A Welfare Assessment is required for each newly created GA line so that all necessary information for the appropriate care and accommodation for that line can be provided. Furthermore, it provides the basis for a transfer document so that all critical information is transmitted with the animals to a new establishment or new scientific group within an establishment. Finally, in combination with the predicted gene effects on the animal (for example increased susceptibility to diabetes or risk of infection), it allows for the determination of whether an established GA line can be initially categorised as having a non-harmful or harmful phenotype.

The success of a Welfare Assessment scheme depends upon the selection of indicators that

- are readily and reliably recognisable;
- are effective at providing good measures of welfare;
- are relevant to species and strain (where appropriate), stage of development, and the scientific study;
- are practical to carry out and do not overly disturb the animal and
- lend themselves to consistent measurement, interpretation and analysis.

A common approach to recording clinical observations is therefore a desirable goal as this will help in the development of consistent approaches to severity classification. This would facilitate comparisons of clinical findings between studies, and inform those involved in severity assessment and potentially, to be used to reduce severity.

2. General considerations

No additional animals should be bred for the purpose of Welfare Assessment. The assessment should be based, exclusively, on the observation of the animal, and animals should not be exposed to any interventions or other manipulations that may induce additional pain, distress, suffering, or lasting harm above the threshold of insertion of a needle.

With every newly produced genetic combination, the resulting line needs to undergo a systematic assessment. In line with the Commission Implementing Decision 2020/569/EU, the creation of a new GA line covers also the crossing of existing GA lines to create a new

genetically altered line **where the phenotype of the new line cannot be determined prospectively as non-harmful**.

Where possible, 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.

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. If the abnormality also occurs in the background strain, then this should be taken into account. Statistical tests should be used to calculate if the level of abnormality seen in the GA line is significantly higher than in the background strain (i.e. to determine if an abnormality derives from the genetic modification). In cases where a phenotype that may be due to the genetic alteration is also present in the background strain, it is likely that the number of animals assessed will have to be increased.

Data should be obtained from a minimum of two breeding cycles from the generation at which the transmission of the genetic alteration is stable.

Clinical observations cannot always reliably identify all issues of concern due to the result of genetic manipulation. However, where it is scientifically expected that the genetic alteration will impact animal welfare negatively, all such harms should be considered and included in the final determination of whether the line is harmful or not. Only where necessary and specifically justified in a project authorisation should invasive methods be used to obtain supplementary information, for example a blood sample to assess glucose levels in a putative diabetes model.

Where available, additional data from other sources should be taken into account, e.g., results from animal procedures or from publications. If additional information is available, the Welfare Assessment should be updated for the particular line.

The degree and frequency of monitoring may need to be increased from the baseline daily monitoring depending on the expected effects. The templates provided in Section A and B should be used as a basis for assessment and should be supplemented by the expected effects of the gene manipulation or if an unexpected phenotype is observed.

The results of the Welfare Assessment can only relate to the age(s) of the animals at which time the assessments are undertaken and to the specific environment in which the animals have been assessed. If parameters change, e.g., age or environment (different establishment), the Welfare Assessment should be confirmed by additional observations.

Accurate clinical and environmental records should be maintained and reviewed where deaths occur, to help prevent further mortality. Where appropriate (e.g., higher than anticipated mortality rate), post mortem examinations should be carried out to help determine the cause of death. A review of fertility can also be helpful in assessment of whether or not the genetic modification is having an effect on, for example, conception rates; dystocia; abortions; stillbirths.

The assessment of individual animals should be documented and reported with high quality systems which allow easy sharing of information with others (usually IT-supported). However, e.g., in the case of immature animals such as fish larvae, group assessment may be more appropriate. To facilitate consistent recording of assessment results, templates were developed for the most commonly used GA species (see section B).

Furthermore, it is essential that information obtained during Welfare Assessments should be summarised and reported when transferring a GA line to another research group, or to another establishment in order that Three Rs principles may be immediately applied. Further guidance is included in the GAA Transfer document, including a common template for movement within and between Member States.

The results of the Welfare Assessment should allow classification of the line either as non-harmful or harmful. In the case of a GA line being assessed as harmful, the assessment should provide the appropriate classification of severity (mild, moderate, severe), on the basis of Annex VIII of Directive 2010/63/EU. Further information can be found in the Working Document on a Severity Assessment Framework (2012).

The Welfare Assessment should be reviewed and updated as more information becomes available, with appropriate feedback to the vendor, breeder and other users, where known.

As data from more animals become available, the severity classification of the line should be reviewed in particular in lines assigned as non-harmful as increased animal numbers may highlight a biologically relevant phenotype of low penetrance that is not evident in a smaller sample size.

To ensure consistency of approach, Welfare Assessment should be undertaken by competent, experienced staff that have completed appropriate training.

3. Section A of the Welfare Assessment Template for all species and time points

Section A	Welfare Assessment Template
Name of species	
Assessed line - **Internal name**	Name, that is used within the housing facility: if applicable specify the strain number
Assessed line - **International name**	The name should be given according to international standards of nomenclature[6,7], where available.
Breeding strategy	Indicate preferred method for colony maintenance e.g., heterozygote x heterozygote; heterozygote x wild-type; homozygote x homozygote or any others forms. General assessment of reproductive performance e.g., males/females/average litter size/pre-weaning mortality/hatching success, compared with wild-type control.
Background strain	If known, the background strain should be defined, e.g., by documenting the international name.
Type of genetic alteration	Brief description of the type of the genetic alteration, the technique used and the target scheme[8]. The wild-type genetic background should be named
Information on the animals at time of assessment	Age or developmental stage of the animals, numbers (indicate if estimate e.g., larval forms), and sex distribution
Information on the housing conditions at the time of assessment	Type of housing and environment, e.g., lighting regime, temperature, humidity, environmental enrichment of cages, water characteristics for aquatic species (e.g., temperature, pH , ammonia), location in the animal unit (e.g., level of cage/tank rack) etc.
Other information relevant to assessment	Any other information which may have affected the Welfare Assessment, e.g., construction work, change in personnel, health status at time of assessment
Other relevant	Publications; links to web-sites

[6] International Committee on Standardized Genetic Nomenclature for Mice & Rat Genome and Nomenclature Committee ; *Guidelines for nomenclature of mouse and rat strains* ; (2018).
[7] Zebrafish Nomenclature Committee (ZNC) ; *ZFIN Zebrafish Nomenclature Conventions*, (2019).
[8] For the type of modification (include copy number where known and applicable), gene affected, inheritance pattern, sex-linkage etc. And/or refer/attach detailed description - ideally a relevant publication on the generation of the line (see also section "Other relevant information")

information	
Severity classification	On the basis of Annex VIII of the Directive 2010/63/EU
Source	Establishment where animals were generated, or most recently held
Assessor(/s)	
Date	

4. Section B of the Welfare Assessment Template for specific species

Genetically altered Rodent Welfare Assessment scheme

Recommendations specific to rodent Welfare Assessment:

- Soon after birth, around weaning and again following sexual maturity and older animals where later onset disease is expected.
- Animals of corresponding genetic backgrounds or a defined reference line serve as controls. During the establishment of a line, wild-type littermates, if available, are particularly suitable.
- It may be helpful to generate a growth curve for the line.

Additional considerations for neonatal animals, and newborn litters, are set out in the tables below.

Section B Template for a Rodent Welfare Assessment

Appearance / Body Functions / Environment / Behaviours / Procedure-specific indicators / Free observations

High level categories	Areas to focus on when observing animals	Specific indicators to monitor
Appearance	**Body condition**	Weight loss/gain
		Obese
		Thin
		Body condition score, if available
	Coat and skin condition	Piloerection
		Unkempt/lack of grooming
		Greasy coat
		Hair loss
		Dehydration – skin tenting
		Skin lesions – swelling; scab; ulcer; injury/wound
		Faecal or urine staining
	Discharge	Ocular; nasal; uro-genital; porphyrin staining in some species e.g., rat
	Eyes	Sunken or 'dull', or enlarged
		Closed/semi-closed/swollen
		Damage/injury to eye (e.g., corneal ulceration)
	Mouth	Salivation
		Malocclusion/overgrown teeth
	Other	'Pain face' – e.g., semi-closed eyes and nose bulge in mice
		Abdominal constrictions
		Swollen body part, e.g., distended abdomen
Body functions	**Respiration**	Accelerated breathing (tachypnoea)
		Laboured breathing (hyperpnoea)
		Very laboured breathing (dyspnoea, gasping)

		Wheezing or other sound when breathing
	Food intake	Increased/decreased
	Water intake	Increased/decreased
	Body temperature	Increased/decreased; measured body temperature if available (contact or non-contact thermometry); colour of extremities in rodents
	Senses	Signs of impaired sight, hearing or balance
Environment	**Enclosure environment, including any litter, nesting material, enrichment items**	Presence and consistency of faeces
		Wet bedding, e.g., due to polyuria
		Presence of blood
		Whether animal is using enrichment items e.g., nesting material, chew blocks
Behaviours	**Social interaction**	Change from normal temperament - apprehensive/aggressive interactions with other animals; anxious behaviour (e.g., marked escape responses, hiding)
		Isolated or withdrawn from other animals in social group
	Undesirable behaviours	Repetitive/ stereotypic behaviour
		Barbering (rodents), trichotillomania
		Self-mutilation
		Increased aggression to humans or other animals
	Posture and mobility	Abnormal posture
		Abnormal gait; lameness; lack of movement/lethargy/reluctance to move if stimulated
		Uncoordinated movements
		Hunched abdomen; tilted head
	Other	Tremors
		Seizures/convulsions/spasms/twitches
		Vocalisation; spontaneous or invoked. *(Note - rodents, usually vocalise in the ultrasonic range, so audible vocalisations are of special concern.).*
		Mortality (or early killing due to adverse signs) before the expected lifespan or longest duration of life held

Procedure-specific indicators	These are identified on the basis of the individual project, its potential adverse effects and expected indicators of these	For example, in a Multiple sclerosis model these could include; loss of tail tone, hind limb weakness, fore limb weakness, paralysis, loss of bladder function
Free observations	A Welfare Assessment scheme should always include a facility to note any observations of unexpected negative welfare impacts.	

Additional considerations for Welfare Assessment of neonatal animals up until weaning

Criteria	**What to look for**
Clinical signs	e.g., deformities, size, skin colour, oedema growth and abnormal / delayed development (e.g., time of eye opening; growth of fur)
Behavioural signs	e.g., increased activity, aggression, excess vocalisation, lethargic/unresponsive?
Milk spot (for neonates only)	Do any pups fail to show presence of milk spot?
Maternal behaviour	Any evidence of poor mothering (e.g., cannibalism, pups scattered in cage and not retrieved, high pre-weaning losses)?
Litter	Size of litter; litter homogeneity

Genetically altered Fish Welfare Assessment scheme (bony fish, teleost fish)

Recommendations specific to fish Welfare Assessment:

The primary Welfare Assessment of genetically altered fish lines shall focus on the observation of visible alterations in the fish. If new scientific insights into the pain perception and pain-related behaviours of fish are available, they will be taken into account in any future Welfare Assessment updates.

The present guiding document applies for all teleost fish species. The name of the exact species of the assessed line should be specified. It is recommended that Welfare Assessment of teleost fish shall be conducted at least at two stages:

1. **Larval stage at the point of independent feeding**
2. **Adult, sexually mature animals**
3. **Older animals should be assessed where later onset disease is expected.**

The final assessment of genetically altered teleost fish should be based on the observations at, at least, these developmental stages.

The time point of larvae feeding independently depends on the fish species (suggested 5 days post fertilisation (dpf) for zebrafish *(Danio rerio)* (at a water temperature of 28°C) according to Commission Implementing Decision 2012/707/EU, and 12 dpf for medaka (*Oryzias latipes*) and can differ greatly depending on the breeding conditions (mostly water temperature). The principle to be followed is to use the time when the gut is open end to end *and* the fish would normally actively take food.

The time point of sexual maturity also depends on the fish species as well as the housing conditions. For zebrafish and medaka sexual maturity can be considered to be about 12-16 weeks at a water temperature of 28°C.

Occurrence of any alteration as a result of the genetic manipulation can depend on the specific housing conditions within a given facility. Thus, the housing conditions (including feeding) have to be taken into account in the Welfare Assessment, and shall be documented.

The following principles should be taken into account for the Welfare Assessment of genetically altered teleost fish:

- The assessment is exclusively based on animal observation within their housing environment (e.g., petri dish, aquarium).
- For the assessment of adult, sexually mature fish, it is not necessary to use the same individuals used for the assessment at the larval stage, since it is usually not possible to permanently mark larvae.
- All alterations shall be viewed with respect to the specific wild-type background and to the housing conditions of a given facility, which should be both documented for the final Welfare Assessment. Note: Alterations listed in the two following tables are

assumed to occur only occasionally (up to 1 %) in wild-type fish, if they are kept in a well-maintained facility in accordance with Annex III of the Directive 2010/63/EU. These figures may vary from establishment to establishment

- Appropriate wild-type mortality rates should be used to assess mortality in genetically altered fish at the population level.

Section B Template for a Fish Welfare Assessment

The observations are structured on the following six categories. Note that fish development is highly dependent on the species and housing conditions (e.g., water temperature and feeding). Observed alterations should always been viewed with respect to the specific wild-type background and the housing conditions of a given facility.

Larvae / Appearance / Body function / Behaviours / Procedure-specific indicators / Free observations

High level categories	Areas to focus on when observing animals	Specific indicators to monitor
Larvae	**Before the time of independent feeding** Indicate, if estimated values are provided	Average clutch size as number of eggs (or examples).
		Time from spawning to independent feeding (if the water temperature differs from the normal housing conditions, the exact water temperature should be recorded)
		Hatch as a percentage of all eggs of a clutch, preferably documented at 5 dpf for zebrafish.
		Survival rate of larvae at independent feeding (e.g., for zebrafish at 5 dpf, for medaka at 12 dpf) as a percentage of all hatched eggs.
		As survival of larvae stabilises at a later stage, the survival rate as a percentage of all larvae should be noted at a second time point before sexual maturation (e.g., for zebrafish at 28 dpf).
	At the time of independent feeding	Morphology; e.g., arrested / abnormal development, size, skin, fins, any form of swelling, abnormal flexion, heart oedema, unopened swim bladder
		Swimming behaviour and activity; e.g., persistent swimming at the bottom of the tank or close to the surface, position in the water
Areas to focus on when observing adult, sexually mature teleost fish		
Appearance	**Body condition**	Variability in length / overtly reduced / increased size for age (estimated)
		Emaciated
		Obese / swollen
		Altered form e.g., spinal abnormalities

		Altered or missing fins (specify which and how)
		Altered or missing gill covers (operculum)
	Scales and skin condition	Changes in scales/skin
		Reddened skin
		Lighter / darkened pigmentation
		Other changes of skin colour
		Ulcerations
		Localized swelling / tumor
Body function	**Respiration**	Increased opercular rate
		Gulping at surface
	Food intake	Altered feeding (specify)
	Other	Specify
Behaviours	**Swimming**	Increased / decreased activity (including to stimulation)
		Circling / corkscrew / spiral swimming
		Rubbing against tank side/floor, 'bumping' along the bottom of the tank
		Swimming at the bottom of the tank
		Swimming at the surface
	Social interaction	Aggression
	Other issues with respect to the population	Loss of schooling behaviour, segregation from the school
		Fertility, specified for males and females. Including the susceptibility to become "egg bound".
		Mortality (or early killing due to adverse signs) before the expected lifespan or longest duration of life held
Procedure-specific indicators	These are identified on the basis of the individual project, its potential adverse effects and expected indicators of these	
Free observations	A severity assessment scheme should always include a facility to note any observations of unexpected negative welfare impacts.	

Additional considerations for Welfare Assessment of larval forms

Assessment of larvae is carried out exclusively by observing the animal, depending on size, either by microscopy, in a petri dish or in a tank. A representative number of larvae (and clutches) shall be used for the Welfare Assessment. To complement the Welfare Assessment, specific aspects of larvae before the time of independent feeding should be taken into account.

Additional considerations for Welfare Assessment of sexually mature, adult forms

Assessment of the adult, (preferably) sexually mature fish shall be conducted by observing the animal in the tank. If possible, all observed fish should be kept within one group as separating the fish for the assessment would cause additional stress.

In general, whilst a separate assessment of the two sexes is not considered necessary, both sexes should be included in the evaluation. If there are any suggestion that observed abnormalities are sex specific, then the Welfare Assessment of adult, sexually mature fish has to be done separately for male and female animals.

Genetically altered Farm- or Mini-pig Welfare Assessment scheme

Recommendations specific to farm- or mini-pig Welfare Assessment:

The Welfare Assessment should include animals of representative age groups:

- Soon after birth, around weaning (4-5 weeks of age) and again following sexual maturity (approx. 4-6 months of age) and at additional time points as considered appropriate by a prospective review of the potential impact of the gene alteration e.g., where there is an age dependent onset of disease
- A minimum of 7 males and 7 females sampled from more than one litter (the numbers of offspring in minipig litters are small, and as the number of genetically altered breeders and number of animals are generally much lower than for other species)

Section B Template for a Farm- or Mini-pig Welfare Assessment

Appearance / Body Functions / Environment / Behaviours / Procedure-specific indicators / Free observations

High level categories	Areas to focus on when observing animals	Specific indicators to monitor
Appearance	**Body condition**	Deviations from growth curve
		Obese or larger size
		Thin or smaller size
		Deviations in body condition score
	Coat and skin condition	Deviation in skin colour
		Deviations in skin texture
		Deviations in hair quality (e.g., thick coat)
		Hair loss or alopecia
		Loose skin due to e.g., dehydration or starvation
		Skin lesions – swelling; scab; ulcer; injury/wound
		Dermatitis or eczema
	Discharge	Ocular; nasal; uro-genital or diarrhoea
	Eyes	Microphthalmia
		Swollen or closed/semi-closed
		Damage/injury to eye (e.g., corneal ulceration or sign of blindness)
	Mouth	Salivation
		Malocclusion/overgrown teeth
	Other	Malformations (e.g., skeletal deformity or abnormalities like hydrocephalus)
		Morphological, neurological or musculoskeletal abnormalities
		Swollen body part (e.g., distended abdomen) or tumours
Body functions	**Respiration**	Accelerated breathing (tachypnoea)
		Deep breathing (hyperpnoea)
		Laboured breathing (dyspnoea, gasping)
		Wheezing or other sound when breathing

	Food intake	Increased/decreased
	Water intake	Increased/decreased
	Body temperature	Increased/decreased; measured body temperature if available (e.g., contact or non-contact thermometry);
	Senses	Signs of impaired sight, hearing or balance
Environment	**Enclosure environment, including any litter, nesting material, enrichment items**	Presence and consistency of faeces
		Excessive urination
		Presence of vomit or blood
		Whether animal is using enrichment items e.g., nesting material, chew blocks
Behaviours	**Social interaction**	Does the animal exhibit the full repertoire of behaviours appropriate for the strain; including social interaction, rooting, walking, running, sleeping
		Isolated or withdrawn from other animals in social group
	Undesirable behaviours	Repetitive/stereotypic behaviour
		Prolonged inactivity
		Increased aggression to humans or other animals
	Posture and mobility	Abnormal posture (e.g., splay-legged piglets)
		Abnormal gait; lameness; lack of movement/lethargy/reluctance to move if stimulated
		Uncoordinated or impaired movements or any difficulties with orientation
		Hunched abdomen; tilted head
	Other	Rigidity or tremors
		Seizures/convulsions/spasms/twitches
		Vocalisation; spontaneous or invoked
		Mortality (or early killing due to adverse signs) before the expected lifespan or longest duration of life held
Procedure-specific indicators	These are identified on the basis of the individual project, its potential adverse effects and expected indicators of these	

Free observations	A severity assessment scheme should always include a facility to note any observations of unexpected negative welfare impacts.

Additional considerations for Welfare Assessment in neonatal animals

CRITERIA	**WHAT TO LOOK FOR**
Neonates skin colour and appearance	Do any piglets show evidence of abnormal skin colour (e.g., anaemia, poor circulation)? Do they have loose skin (indication of dehydration or starvation)? Do they have a "hairy" appearance (indication of difficulty to maintain normal body temperature)?
Activity of neonates	Any abnormal activity? Are the neonates active and moving freely? Are they breathing normally? Are the postures abnormal (e.g., splay-legged piglets)?
Neonates interaction with sow and suckling behaviour	Have the neonates received colostrum? Are they interested in and capable of suckling and appear to have normal milk consumption? Are they isolated away from the sow or the heat source? Is there fighting and aggression at the udder? Any evidence of mis-mothering?
Litter	Was the gestation at full term? Was the farrowing normal? Any abnormalities in relation to litter sizes, litter homogeneity, development and growth of piglets?

Genetically altered Chicken Welfare Assessment scheme

Recommendations specific to chicken Welfare Assessment:

It is important to include animals of representative age groups.

- Consider hatching success rates
- Soon after hatching (usually from incubator), and other appropriate times*), adult, aging or maximum age kept
- A minimum of 7 males and 7 females sampled from more than one clutch of eggs
- Comparison made wherever possible with similar non GA-animals.

*) and at additional time points as considered appropriate by a prospective review of the potential impact of the gene alteration e.g., where these is an age dependent onset of disease.

It is important to know if the bird is a layer or broiler line as this will impact upon the feeding and phenotype and may actually require environmental differences and dietary changes depending upon this.

Section B Template for a Chicken Welfare Assessment

Appearance / Body Functions / Environment / Behaviours / Procedure-specific indicators / Free observations

High level categories	Areas to focus on when observing animals	Specific indicators to monitor
Appearance	**Body condition / confirmation / growth**	Malformations, abnormal development, skeletal deformity, splayed legs
		Deviations from growth and expected size
		Body condition – layer / broiler specific
		Weight loss/gain
	Feather and skin condition	Abnormal feather development
		Poor feather condition / ruffled /dirty
		Areas of feather loss, more feather pecking than expected
		Dehydration – skin tenting
		Skin lesions – fragility / swelling; scab; ulcer; injury/wound
	Discharge	Ocular; nasal; vent (cloaca)
	Eyes	Sunken or 'dull'
		Closed/semi-closed/swollen
		Damage/injury to eye (e.g., corneal ulceration)
	Beak / digestive	Crop problems such as impacted crop
		Deformed beak, comb or wattles.
	Other	Colouration and conformation of wattle and comb – pale/red/cyanotic; firm or soft
		Swollen body part, e.g., distended abdomen
Body functions	**Respiration**	Accelerated breathing (tachypnoea)
		Deep breathing (hyperpnoea)
		Laboured breathing (dyspnoea, gasping)
		Wheezing or other sound when breathing
	Food/water intake	Increased/decreased

		Ability to find food and water at hatch as expected; needing assistance or to be kept with controls (as companions)
	Body temperature	Increased/decreased; measured body temperature if available (e.g., contact or non-contact thermometry); colour of extremities
	Senses	Signs of impaired sight, hearing or balance
	Reproduction	Abnormal rate of viable embryos on candling (lower than the expected 90%) Poor hatch rate due to inability to break the eggs open (stuck in shell) Laying performance of hen, onset of laying age, egg production rate over the laying period, egg deformities, shell consistency, spoiling of eggs.
Environment	**Enclosure environment, including any litter, nesting material, enrichment items**	Presence and consistency of faeces
		Excessive / unusual soiling of substrate
		Whether animal is using enrichment items, e.g., sand bath, perch
Behaviours	**Social interaction**	Exhibit normally the full repertoire of behaviours appropriate for the strain including preening, walking, running, scratching, dust bathing, perching, short "flight", foraging
		Unusual activity, such as hyperactivity.
		Change from normal temperament -
		Isolated or withdrawn from other birds in social group.
	Undesirable behaviours	Apprehensive/aggressive interactions with other birds; anxious behaviour (e.g., marked escape responses, hiding)
		Increased vocalisation on handling
		Repetitive/ stereotypic behaviour
		Feather pecking
		Prolonged inactivity (could indicate chronic stress or depression (anhedonia) and/or sickness/pain) particularly if linked with a hunched posture and/or ruffled or unkempt feathers.
		Increased aggression to humans or other animals
	Posture and mobility	Abnormal posture, hunched

		Difficulty with orientation
		Abnormal gait; lameness; lack of movement/lethargy/reluctance to move if stimulated
	Other	Tremors
		Rigidity
		Seizures/convulsions/spasms/twitches
		Vocalisation; spontaneous or invoked.
		Mortality (or early killing due to adverse signs) before the expected lifespan or longest duration of life held
Procedure-specific indicators	These are identified on the basis of the individual project, its potential adverse effects and expected indicators of these.	
Free observations	A severity assessment scheme should always include a facility to note any observations of unexpected negative welfare impacts.	

Part 4: Transferring welfare information on genetically altered animals

1. Key principles

When genetically altered animals are transferred within, and between, institutions it is important that specific information on their welfare needs travels with the animals. This will enable anyone caring for or using the animals to understand specific characteristics of the animals (or strains, or lines) that they are receiving along with any special requirements that they may have regarding their welfare to be able to immediately apply refinements.

The way that this information is transferred can take various formats including a paper document, an electronic file or a database. The important thing is that it provides tailored and meaningful information and is readily accessible to any person caring for a GAA at any location so that each animal (or batch of animals) receives consistent care throughout their lifetime.

What information should be transferred with the animals?

As well as all relevant data on the generation, breeding, nomenclature and genetic background **(Part A of Welfare Assessment)**, a clear description of the phenotype or any other characteristics observed during Welfare Assessment **(Part B of Welfare Assessment)** should be included in the transfer documents.

When transferring animals from non-harmful lines, it is particularly important that information on the conditions under which the original classification was made are reported, e.g., health status, environmental conditions as these changes may influence the welfare consequences of the genetic modification for the animal, resulting in reclassification from non-harmful to harmful.

To facilitate appropriate housing, care and monitoring practices, it is in addition essential that information on potential phenotype related welfare issues with the respective care and husbandry requirements are documented together with possible refinement strategies **(Part C - Care and husbandry requirements),** and provided with the animals.

The following information should be included for any genotype that might arise (i.e. heterozygotes and homozygotes) as a result of breeding from the animals being passed on to a new environment.

Relevant information should be provided on:

- **Phenotypes** – whether observed cage-side or tank-side – including the life stage / age of the animals involved;
- **Animal care –** husbandry issues which would affect the animal's health and any special animal care required (e.g., supplements, enrichment etc.);
- **Sterility** – fecundity, littering or rearing concerns;
- **Strain details** – nomenclature, genetic background, genotype information

2. Section C – Transfer template for the care and husbandry requirements for genetically altered animals

Section C - Care and husbandry requirements						
Brief explanation of the phenotype, including remedial actions and endpoints Include phenotypic abnormalities and observable traits, which may occur specifying age of occurrence (e.g., soon after birth/hatching, at weaning, independent feeding or during sexual maturation, as adult or while breeding (failure of eggs to develop, abortions, abnormal fetuses etc.)						
	Age	**What kind of signs**	**Potential welfare implications**	**Possible treatment, interventions and refinement strategies**	**Specific housing or care**	**Humane endpoint**
Clinical signs Including appearance & body functions & monitoring of environment						
Behavioural signs						

Breeding performance including viability of pups/offspring						
Premature death						

Part 5: Reporting of genetically altered animals

1. General legal framework

General reporting obligations are set out in Article 54 of the Directive. The detailed requirements are laid out in Commission Implementing Decision 2020/569/EU.

The Directive contains two types of reporting obligations that involve GAA:

1) Annual statistical reporting that includes statistical data on the uses of animals including for the purposes of creation and maintenance of GAA. Activities that fall within the definition of a "procedure" require project authorisation, and animals used within projects, in general, fall under the annual statistical reporting obligations.

   The detailed requirements can be found in Annex III of the Commission Implementing Decision 2020/569/EU.

2) In addition, once every five years, Member States are required to collect information on the implementation of the Directive. This covers two specific data categories from breeders and users of GAA on the creation and maintenance of GAA covering the last year of the five-year reporting cycle:

   - all other animals that have not been accounted for within annual statistical reporting including those resulting from the creation and maintenance of GA lines (animals bred, killed and not used), and

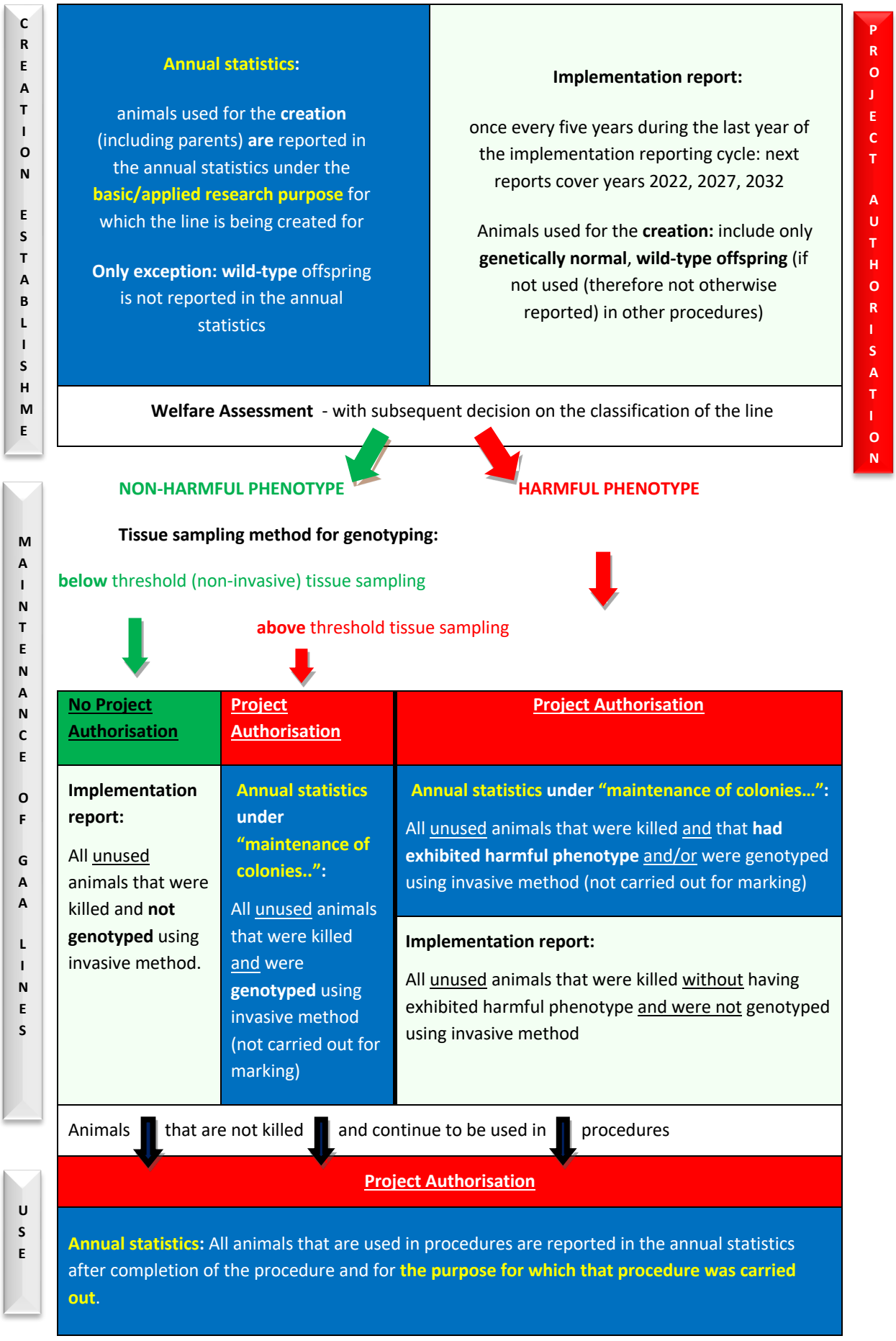
   - representative information on tissue sampling methods for the purposes of genetic characterisation (genotyping) – these animals may or may not have also been included in the annual statistical reports depending on the type of tissue sampling method used (see page 70).

The detailed requirements can be found in Annex II of the Commission Implementing Decision 2020/569/EU.

2. Flow chart for the requirements for statistical and implementation reporting for the creation, maintenance and use of GAA

The flow-chart on the following page provides a summary of the reporting requirements for both the annual statistical reporting (dark blue boxes) and five-yearly implementation reports (light green boxes).

The flow chart can be downloaded as a poster at:
https://ec.europa.eu/environment/chemicals/lab_animals/pubs_posters_en.htm


CREATION ESTABLISHME
PROJECT AUTHORISATION
Annual statistics:
animals used for the creation (including parents) are reported in the annual statistics under the basic/applied research purpose for which the line is being created for
Only exception: wild-type offspring is not reported in the annual statistics
Implementation report:
once every five years during the last year of the implementation reporting cycle: next reports cover years 2022, 2027, 2032
Animals used for the creation: include only genetically normal, wild-type offspring (if not used (therefore not otherwise reported) in other procedures)
Welfare Assessment - with subsequent decision on the classification of the line
NON-HARMFUL PHENOTYPE
HARMFUL PHENOTYPE
Tissue sampling method for genotyping:
below threshold (non-invasive) tissue sampling
above threshold tissue sampling
MAINTENANCE OF GAA LINES
No Project Authorisation
Project Authorisation
Project Authorisation
Implementation report:
All unused animals that were killed and not genotyped using invasive method.
Annual statistics under "maintenance of colonies..":
All unused animals that were killed and were genotyped using invasive method (not carried out for marking)
Annual statistics under "maintenance of colonies...":
All unused animals that were killed and that had exhibited harmful phenotype and/or were genotyped using invasive method (not carried out for marking)
Implementation report:
All unused animals that were killed without having exhibited harmful phenotype and were not genotyped using invasive method
Animals that are not killed and continue to be used in procedures
Project Authorisation
USE
Annual statistics: All animals that are used in procedures are reported in the annual statistics after completion of the procedure and for the purpose for which that procedure was carried out.

3.1. Annual statistical reporting

Legal requirements pertaining to GAA and annual statistical reporting requirements

Annex III of Commission Implementing Decision 2020/569/EU provides in

- Part A the data categories to be used;
- Part B general instructions and instructions for the detailed data categories.

For the purposes of the annual statistical reporting, animals shall only be reported **once** at the end of a procedure – generally by the project holder who completes the "use". For the purposes of statistical reporting it is important to differentiate between "use", "continued use" and "reuse" (more information in Working document on specific articles in Directive 2010/63/EU[9]).

Reporting of animals used for creating GA lines

All GA lines are commissioned for a particular scientific purpose. Procedures related to creation of new GA lines must be reported against the specific research area for which the line is being created. Therefore, discussion and documentation between those who request the line and those who create new lines must occur to ensure accuracy of reporting by the project leader.

- "Creation" is the development of a new line of GAA through deliberate/intentional gene alteration (e.g., genetic insertion/deletion/editing, chemical mutagenesis or other manipulation of a gamete or embryo, or may be by cross-breeding of two pre-existing lines)

As described in Part 1, Section 3 of this guidance, the creation of a new GA line is in principle considered a procedure. Animals used in the creation are reported in the annual statistics except, any offspring which are genotyped by non-invasive methods and which turn out to be wild-type i.e. do not carry the genetic mutation. Such wild-type animals will only be reported in the five-year Implementation Report – see page 65.

An exception is when crossing/backcrossing **two lines of non-harmful phenotype** and where 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, and subsequently, these animals are not reported in the annual statistical reports - **unless** invasive genotyping methods have been used.

When reporting animals in the annual statistical report, it is important to consider separately each of the parents and the offspring, as each may be reported differently depending on for example, the genotype and phenotype demonstrated, and fate (see table below).

Reporting of animals used for maintaining GA lines

Once the line is established, following completion of welfare assessment and categorisation of non-harmful / harmful and severity categorisation (including consideration of the heterozygotes and homozygotes), reporting should be under the category "Maintenance of

[9] https://ec.europa.eu/environment/chemicals/lab_animals/pdf/Consensus_document.pdf

colonies of established genetically altered animals, not used in other procedures", unless they are transferred for continued use in scientific procedures requiring that genotype.

Animals from a line which is categorised as **non-harmful** will be reported in the annual statistical report when invasive genotyping methods are used and the animal is killed and when kept alive and of **non-intended** genotype. This is reported as first use. Those with the **intended genotype** that continue to be used in scientific procedures requiring that genotype will be reported only at the end of the entire procedure of that continued use.

Animals from an established line which is categorised as **harmful** will be subject to reporting in the annual statistics, i.e. those animals which express harm and / or those which have been subjected to invasive genotyping methods and killed that year without subsequently being used in a scientific procedure.

Detailed information about the reporting requirements is in the table on the following page.

<u>Reporting of animals which move between procedures, projects, establishments and / or Member States</u>

Where animals move between projects, and/or move between establishments (within or between Member States), to permit accurate reporting at the end of life/procedure ("use"), information should be provided with the animals when transferred on whether the animals are

- Animals which have not undergone any procedure in the first establishment;
  - to be reported by the recipient if used in a scientific procedure, at the end of that procedure;
  - [N.B Animal not having undergone any procedure in the receiving establishment are not reported in the annual statistics, however, they need to be reported by the establishment in which they are killed if killed in the recording year for the five-year Implementation Report – see section 3.2].
- undergoing continuous use (e.g., have been genotyped using an invasive method to confirm the right genotype and being transferred to be used in a scientific procedure requiring that genotype);
  - to be reported by the recipient at the end of the procedure;
- or have completed a "use" (e.g., genotyped using an invasive method determining that the animal is not of the intended genotype) and is being transferred for re-use.
  - to be reported both by the first authorised user having genotyped the animals as its first use, and the recipient at the end of the reuse.

The following two tables list typical procedures under GAA maintenance and provide a decision schema to determine whether or not the animal should be reported in the Annual Statistics under maintenance.

1. The first table presents animals bred from an established **non-harmful phenotype** GA line.
2. The second table presents animals bred from an established **harmful phenotype** GA line

1. ANIMALS FROM THE MAINTENANCE OF AN EXISTING NON-HARMFUL PHENOTYPE LINE								
What is the genotype of the animal?	**Has the animal suffered from adverse effects due to the genotype?**	**Has the animal been genotyped using an invasive tissue sampling method not used for ID[10)]**	**Is the genotype confirmed as expected?**	**Has animal been subject to other procedures than maintenance or tissue sampling?[11)]**	**Is the animal killed during the reporting year without being used in other procedures?**	**Does the animal need to be reported in the annual statistics by the (establishment) project holder for GA line maintenance?**	**Comments**	**Reported actual severity**
Non-genetically altered	No	No	Not required	No	Yes	**NO**	Reported only once every 5 years as part of implementation report	Not required
Non-genetically altered	No	Yes	Not required	No	Yes	**YES**	Whilst information about the severity of the tissue sampling in isolation is only required in the year prior to the 5 year Implementation Report, it is recommended to note down the tissue sampling method with the related severity to facilitate five-yearly implementation report	The highest reached severity as a result of tissue sampling
Genetically altered	No	No	Irrelevant	No	Yes	**NO**	**Non-harmful GAA -** Reported only once every 5 years as part of implementation report	Not required
Genetically altered	**Unexpectedly** Yes	No	Irrelevant	No	Yes	**NO**	If adverse effects occur on several animals, Animal Welfare Assessment should be repeated and reclassification from non-harmful to harmful line should be considered, where appropriate. If reclassified to a harmful line, a project authorisation will be required for the maintenance of the line. - Reported only once every 5 years as part of implementation report	Not required

[10] This refers to all invasive tissue sampling methods where the tissue is not obtained from the marking of the animal.
[11] In the rare event where embryo transfer is required solely to remove commensal organism(s) from a breeding colony (i.e. not to improve health or the welfare of the colony but required for a scientific purpose) the procedures involved (e.g. embryo transfer, super ovulation where needed) should be included in the annual statistics with the severity recorded as the highest reached severity as a result of the said procedure.

Genetically altered	**Unexpectedly** Yes	Yes	Irrelevant	No	Yes	**YES**	If adverse effects occur on several animals, Animal Welfare Assessment should be repeated and reclassification from non-harmful to harmful line should be considered, where appropriate. If reclassified to a harmful line, a project authorisation will be required for the maintenance of the line	
Genetically altered	No	Yes	Irrelevant	No	Yes	**YES**	It is recommended to note down the tissue sampling method with the related severity to facilitate five-yearly implementation report which will be required for these animals in addition to the Annual Statistics	The highest reached severity as a result of tissue sampling
Genetically altered	No	Yes	Yes	No	No	**NO**	The invasive tissue sampling (when the **expected phenotype is confirmed** and the animal is not killed) forms the first part of **a continued use**; the end-user will record the animal in the annual statistics when the final use is completed	The severity of tissue sampling should be communicated to the end-user to be taken into account for the final reported actual severity
Genetically altered	No	Yes	Not suitable genotype	No	No	**YES**	The invasive tissue sampling (when the **expected phenotype** is **not confirmed** and the animal is not killed) is considered **the first use** of that animal; **any subsequent use is considered reuse**. It is recommended to note down the tissue sampling method with the related severity to facilitate five-yearly implementation report	The highest reached severity as a result of tissue sampling

2. ANIMALS FROM THE MAINTENANCE OF AN EXISTING HARMFUL PHENOTYPE LINE								
What is the genotype of the animal?	**Has the animal suffered from adverse effects due to the genotype?**	**Has the animal been genotyped using an invasive tissue sampling method[12)]**	**Is the genotype confirmed as expected?**	**Has animal been subject to other procedures than maintenance or tissue sampling?[13)]**	**Is the animal killed during the reporting year without being used in other procedures?**	**Does the animal need to be reported in the annual statistics by the (establishment) project holder for 'maintenance' of GAA?**	**Comments**	**Reported actual severity**
Non-genetically altered	No	No	Not Required	No	Yes	**NO**	Reported only once every 5 years as part of implementation report	N/A
Non-genetically altered	No	Yes	Not Required	No	Yes	**YES**	It is recommended to note down the tissue sampling method with the related severity to facilitate five-yearly implementation report	The highest reached severity as a result of tissue sampling
Genetically altered	No	No	Irrelevant	No	Yes	**NO**	Reported only once every 5 years as part of implementation report	N/A
Genetically altered	Yes	No	Irrelevant	No	Yes	**YES**		Highest reached severity as a result of the adverse effects due to the genotype
Genetically altered	No	Yes	Irrelevant	No	Yes	**YES**	It is recommended to note down the tissue sampling method with the related severity to facilitate five-yearly implementation report	The highest reached severity as a result of tissue sampling
Genetically altered	Yes	Yes	Irrelevant	No	Yes	**YES**	It is recommended to note down the tissue sampling method with the related severity to facilitate five-yearly implementation report	Highest reached severity taking into account both the result of the adverse effects due to the genotype and that of tissue sampling
Genetically altered	No	Yes	Yes	No	No	**NO**	**The animal is not killed, but has been subjected to** invasive tissue sampling which forms **the first part of a continued use;** the end-user will record the	Animal may/may not have experienced adverse effects as a result of the genotype. The highest severity taking into account both the effects from the genotype and tissue sampling should

[12] This refers to all invasive tissue sampling methods where the tissue is not obtained from the marking of the animal.
[13] In the rare event where embryo transfer is required solely to remove commensal organism(s) from a breeding colony (i.e. not to improve health or the welfare of the colony but required for a scientific purpose) the procedures involved (e.g. embryo transfer, super ovulation where needed) should be included in the annual statistics with the severity recorded as the highest reached severity as a result of the said procedure.

							animal in the annual statistics when use is completed.	be communicated to the end-user to be taken into account for the final reported actual severity
Genetically altered	Yes	Yes	Yes	No	No	**NO**	**The animal is not killed, but has been subjected to** invasive tissue sampling which forms **the first part of a continued use;** the end-user will record the animal in the annual statistics when use is completed.	Animal may/may not have experienced adverse effects as a result of the genotype. The highest severity taking into account both the effects from the genotype and tissue sampling should be communicated to the end-user to be taken into account for the final reported actual severity
Genetically altered	No	Yes	Not suitable genotype	No	No	**YES**	The invasive tissue sampling (when the **expected phenotype** is **not confirmed** and the animal is not killed but is kept alive for use in a different study requiring that genotype, or a study where genotype is not important) is considered **the first use** of that animal; **any subsequent use is considered reuse.** It is recommended to note down the tissue sampling method with the related severity to facilitate five-yearly implementation report	The highest reached severity as a result of tissue sampling

3.2. Implementation report every five years

Legal requirements pertaining to GAA and Member State implementation reports

Annex II of Commission Implementing Decision 2020/569/EU requires reporting every 5 years in two separate areas that involve GAA. These are

- animals that are bred, killed and not used, and which are either result of GA line creation or maintenance;
- animals that have undergone tissue sampling, irrespective of the method used for obtaining the tissue.

The detailed legal requirements are in Annex II, Part C.2 and Part D.3.1 of Commission Implementing Decision 2020/569/EU.

Animals bred, killed and not used

Once every five years, the Directive requires an exact count of all animals that are needed in support of EU research and testing. This is obtained in part through annual statistical data for that year, which includes all those animals that have been used in procedures, and, the other part to ascertain the total number, is completed once every five years, by counting all other animals bred, killed and not used in procedures. This category covers both conventional animals as well as animals from GA creation and maintenance.

In comparison with the data of 2017, the future reports require animals that were killed for obtaining organs/tissue to be identified separately.

It is important to note information provided for this data category is reported not only by **users but also by breeders of animals**.

When reporting animals from creation and breeding of GA lines for the purposes of implementation report, it is important to consider separately each of the parents and the offspring, as each may be reported differently depending on the genotype and phenotype demonstrated.

If breeding a GA line, unless it has been confirmed (e.g., by genotyping, coat colour) that it is not GA, then it should be reported as GA.

Member States use different methods for collecting these data. For the sake of clarity to the animal breeder and user community, some Member States have opted to collect these data every year.

The European Commission has created a voluntary tool to help collect these data accurately using an Excel template ("User/breeder data template for Member State Implementation Report", record type IR2). This can be used to record yearly data.

The reporting requirements are detailed in the table on page 68. However, since the reporting tools may vary from country to country, the table makes only reference to the tools provided by the Commission.

Recommendations:

- Member States should ensure that the respective competent authorities and scientific community are aware and have available the correct and up-to-date tools for the collection of data for animal bred, killed and not used in advance of the start of the 5th year of the five-year reporting cycle.

Tissue sampling

In contrast to the *exact count* of all animals bred, killed and not used, Member States are required to provide *representative* data on tissue sampling. The report on tissue sampling does not aim to identify total numbers of animals having been tissue sampled. Instead, it allows an analysis to be drawn at EU level on the type of species, the proportions of different tissue sampling methods used and their related severities in order to assess the progress in the implementation of the Three Rs for tissue sampling purposes.

The way in which "representative data" is interpreted and collected varies greatly from one Member State to another. To reduce confusion among the user community and simplify the reporting requirements, some Member States have opted to collect all data annually and from all breeders and users. Some other Member States collected data for only the last year of the five-year reporting cycle. In some cases, data has been collected from all establishments, whilst in others, only from a representative number of establishments. In some cases, partial data (e.g., for six months during the reporting period) has been collected from all relevant establishments. The subsequent five-year implementation reports shall list the criteria used by the Member States to select and submit data to ensure that the information provided is representative. Member States should determine and inform the scientific community in good time what these criteria will be.

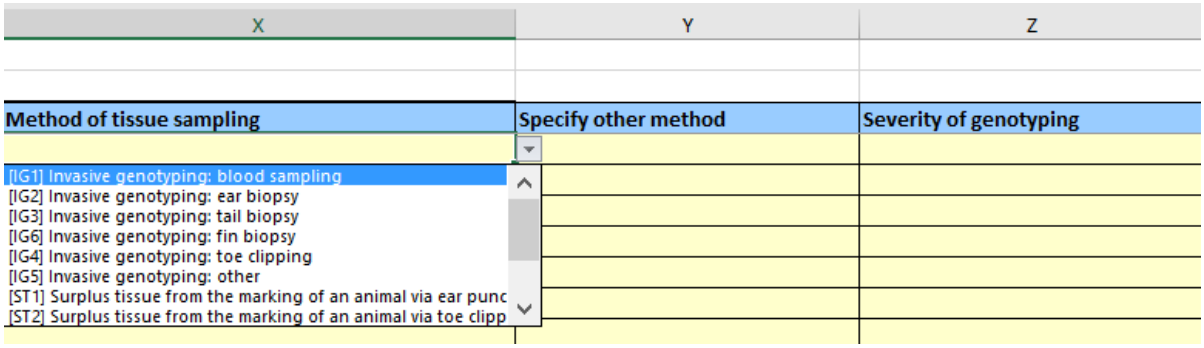
As with the data on animals bred, killed and not used, information on tissue sampling is reported by **both users and breeders of animals**.

Unlike the reporting of animals bred, killed and not used, some animals that are reported under tissue sampling may also be included in the annual statistical reporting either when an animal has undergone other scientific procedures, (even if tissue sample was taken using a non-invasive method, or surplus tissue from marking was used for genetic characterisation), or as a consequence of an invasive tissue sampling method having been used on the animal.

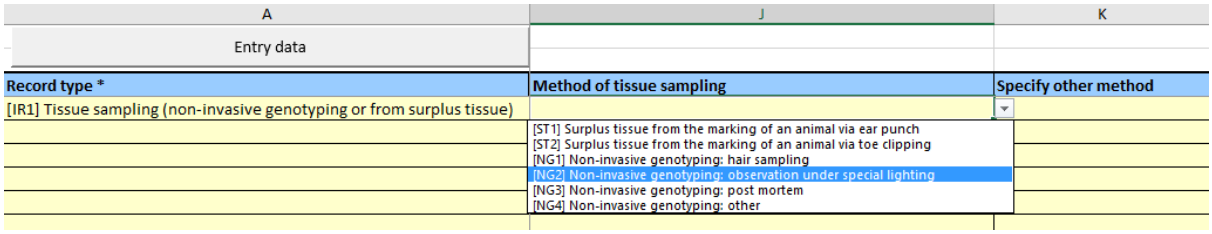
Information is required for all species that have been subject to tissue sampling. For each of the species, the numbers involved per type of method used, and, when invasive, the related distribution of severities need to be reported.

To facilitate data collection on tissue sampling methods and their related severities, the European Commission has created two voluntary, complementary tools for this purpose:

- For those **animals reported in the annual statistics**, additional voluntary fields (X-Z) in the annual statistical reporting Excel template to capture information on tissue sampling; and



- For those **animals that are <u>not</u> reported in the annual statistics**, a voluntary Excel worksheet for additional user data ("User/breeder data template for Member State Implementation Report", record type IR1) can be used to record non-invasive tissue sampling and the use of surplus material from the identification/marking of the animal.



These complementary tools, when used together, will promote accurate reporting. Should one or the other be omitted, another national tool should be made available instead to ensure all necessary information is recorded and reported for the implementation report.

Where the above tools are used in a Member State, the table below will demonstrate which of the tools should be used in which cases, how to report the actual severity related to the tissue sampling, and who should report it.

REPORTING OF TISSUE SAMPLING METHODS FOR THE PURPOSES OF FIVE-YEAR IMPLEMENTATION REPORT USING THE TOOLS PROVIDED BY THE EUROPEAN COMMISSION						
The method of genotyping	**Animal is killed after tissue sampling**	**Animal is used in a (first/continued/another)[1)] procedure after being tissue sampled**		**Reporting through voluntary Excel sheet[14]** – record type: [R1] tissue sampling	**Reporting by adding relevant information in the annual statistical report** - columns X-Z	**Comments**
		By the same establishment	**By another establishment**			
Non-invasive method or from **surplus tissue** from the marking of the animal[2)]	Yes	No	No	YES		Reported by the establishment in which animal was genotyped and killed.

[14] Voluntary Excel "User/breeder data template for Member State Implementation Report"

Non-invasive method or from **surplus tissue** from the marking of the animal[2)]	No	Yes	No		YES	Information on tissue sampling is added using columns X-Y in the annual statistical reporting, when the use of that animal in a procedure is completed
Non-invasive method or from **surplus tissue** from the marking of the animal[2)]	No	No	Yes	YES		Reporting of tissue sampling method is by the **establishment in which the animal was tissue sampled**
Invasive tissue sampling method – not from surplus tissue from the marking of the animal	Yes	No	No		YES	Tissue sampling is reported as the only use in the annual statistical reporting under maintenance or creation – the actual severity of tissue sampling will be reported in both columns "T" and "Z" [3)]
Invasive tissue sampling method – not from surplus tissue from the marking of the animal	No	Yes in a continued use requiring this genotype	No		YES	There will be 2 severities reported for this animal: the main use and the tissue sampling. The actual severity of the entire procedure (including the impact of the invasive genotyping) is reported in column "T". The severity in column "Z" should only refer to the actual severity of tissue sampling[3)]
Invasive tissue sampling method – not from surplus tissue from the marking of the animal	No	Yes in another procedure, not requiring the specific genotype	No		YES	The invasive tissue sampling is the first "use", and the subsequent use is "re-use". The first user needs to report actual severity of tissue sampling in both columns "T" and "Z". Tissue sampling is reported as the first use in the annual statistical reporting under maintenance or creation.[3)]
Invasive tissue sampling method – not from surplus tissue from the marking of the animal	No	No	Yes, in a continued use requiring this genotype		YES	Tissue sampling is reported only at the end of the entire procedure by the establishment having completed that "use". There will be 2 severities reported for this animal: the main use and the tissue sampling. The actual severity of the entire procedure (including the impact from the invasive genotyping) is reported in column "T". The severity in column "Z" should only refer to the tissue sampling[3)]. Information on the invasive tissue sampling (the method and actual severity) should have been received with the animal.
Invasive tissue sampling method – not from surplus tissue from the marking of the animal	No	No	Yes, in another procedure, **not requiring** the specific genotype		YES	Tissue sampling is reported as the "first use" by the establishment in which the animal was tissue sampled (under creation or maintenance) – the actual severity in columns "T" and "Z" therefore refers to the actual severity of tissue sampling[3)]. Information that the animal has already completed the first use should accompany animal. Reuse conditions apply.

1. First use: tissue sampling was carried out by a non-invasive method or by using surplus tissue from the marking of an animal, thus the sampling is not considered a procedure and any subsequent procedure will be considered first use;

   Continued use: animal was tissue sampled by an invasive method, the intended genotype was confirmed and the animal was used (=continued use) in a procedure that required that genotype;

   Reuse in another procedure: animal was tissue sampled by an invasive method (= first use) but subsequently used in a procedure that did not require the intended genotype.

2. Non-invasive tissue sampling/use of surplus tissue from the marking of an animal is not considered a procedure/use of animal.

3. The actual severity reported in column "Z" should only refer to the actual severity of tissue sampling in contrast to the actual severity reported in column "T" which should reflect the highest severity experience by the animal during the entire use of the animal (i.e. including the impacts from the genotype, genotyping and severity experienced during other elements of the procedure).

Recommendations:

- Member States should determine and inform the respective competent authorities and scientific community in good time what the sampling criteria will be for 'representative data' for collecting data on tissue sampling;

- Member State should ensure that the respective competent authorities and scientific community have up-to-date tools and information on requirements for the collection of data on tissue sampling.

Appendixes

Appendix I: Examples of databases of GA lines

Links controlled in March 2020.

1. Non-exhaustive list of example databases on GA lines:
   - http://www.informatics.jax.org/
   - https://www.infrafrontier.eu/
   - https://archive.har.mrc.ac.uk/index
   - https://www.mousephenotype.org/
   - http://zfin.org/
   - https://www.xenbase.org/gene/static/geneNomenclature.jsp

2. Site to screen for human genes and genetic disorders: https://omim.org

Appendix II: Project application and evaluation for the creation and maintenance of GA lines

Introduction

Articles 36-44 of Directive 2010/63/EU set out the requirements for project proposal, evaluation and authorisation.

Part A of this Appendix (based on Annex VI of the Directive) is aimed at both project applicants and evaluators. It sets out the information requirements of particular relevance to be considered in an application for the creation and/or maintenance of GA lines.

Article 37 sets out the elements required for inclusion in an application for project authorisation, namely:

- The project proposal
- A non-technical project summary, and
- Information on the elements set out in Annex VI of the Directive.

Part B of this Appendix is mainly targeted to project evaluators. It focuses on the project evaluation process highlighting key considerations and how these can be addressed during the evaluation. This part is of interest also to project applicants as it allows a better understanding of the considerations that need to be given during the evaluation process to ensure Directive obligations are met.

Further information on the requirements of the Directive can be found in the EU Guidance Document on Project Evaluation and Retrospective Assessment.

Part A: Illustrative examples of key information required in GAA project application

The Project Proposal

The proposal sets out the key scientific questions to be addressed, including the purpose of the project (as set out in Article 5). The project proposal forms the submission to the competent authority containing details of the planned work and requesting authorisation for it. Where appropriate and permissible in the Member State in question, consideration should be given to the use of multiple generic projects (https://ec.europa.eu/environment/chemicals/lab_animals/pdf/Consensus_document.pdf) and simplified administrative procedures as set out in Articles 40(4) and 42 respectively.

When breeding and maintaining only non-harmful phenotype GA lines but using invasive tissue sampling methods, a project authorisation is required. Such a project can be simple, with the main focus on the refinement and prospective severity classification of these procedures.

Projects for creation and maintenance of GAA are generally required for the purpose of basic or applied research. Such projects should set out the current state of knowledge on which this project intends to build. As appropriate, it should include goals achieved by previous projects, and which specific objectives should be achieved through this project. The scientific case should be presented concisely and supported by key references/literature review. Whilst it will not be possible to provide detailed scientific objectives where GA lines are being produced for others, the applicant must obtain information on the lines to be generated and their purpose from the users / purchasers of the animals where this is not known.

The proposal will explain why it is not possible to achieve the scientific objectives without the use of GAA, how such animals will be used, why the new GA lines are required and provide confirmation there are no other suitable lines available. A system may need to be established for service projects (see below) where information is provided by clients and reviewed.

The non-technical project summary

The template for the non-technical project summary is included as Annex I Part A of Commission Implementing Decision 2020/569/EU, and guidance on submission will be found at a later stage at https://ec.europa.eu/environment/chemicals/lab_animals/pubs_guidance_en.htm.

List of elements referred to in Article 37(1)(c) and Annex VI

The table below lists the elements from Annex VI that are required to be considered in a project application. These are then further developed below within the context of GAA to ensure that the application provides the project evaluators with sufficient information in order to be able to consider whether authorisation should be recommended.

	Annex VI element	**Numbered paragraphs where these are discussed below**
I.	Relevance and justification of the following: (a) use of animals including their origin, estimated numbers, species and life stages;	1a,1b,1c,1d,1e,2,3
	(b) procedures	1,2
II.	Application of methods to replace, reduce and refine the use of animals in procedures.	1f,1g,1h, 3
III.	The planned use of anaesthesia, analgesia and other pain relieving methods.	3c(i)
IV.	Reduction, avoidance and alleviation of any form of animal suffering, from birth to death where appropriate.	3c, 1f,1g,1h
V.	Use of humane end-points.	3c(v)

VI.	Experimental or observational strategy and statistical design to minimise animal numbers, pain, suffering, distress and environmental impact where appropriate.	3,4,5
VII.	Reuse of animals and the accumulative effect thereof on the animals.	3b(iv), 3c(vii)
VIII.	The proposed severity classification of procedures.	4
IX.	Avoidance of unjustified duplication of procedures where appropriate.	3a(i)
X.	Housing, husbandry and care conditions for the animals.	3c(iv)
XI.	Methods of killing.	3c(vi)
XII.	Competence of persons involved in the project.	6a,6b

The elements below cover both the creation and maintenance of GA lines, with the intention that they could be combined in the same project, and should be used as appropriate. The order does not follow the order of the list above but instead presents them in an order that could be followed more easily when building a project application.

Currently in Europe, the creation, breeding and use of GAA, can be described to fall within four following types of categories:

1. **Scientific project,** which includes **breeding**, **maintenance** and **use** of animals, and **may include creation of new lines** (including cross breeding of existing GA lines). All relevant information will be available to the applicant, and the application and evaluation processes should be straightforward.
2. **Project for a provision of *service type A* - Breeding and maintenance of established harmful GA lines to supply scientific research groups**, e.g., ob/ob and lepr mice. These will often be bred and maintained on a commercial basis, **often external to use establishment**. The adverse effects of the lines will be known so harms are readily considered.
3. **Project for a provision of *service type B* -** In this case, **creation, breeding and maintenance of GAA is performed as a centralised service within a research establishment.** All relevant information is readily available from the commissioning scientist, and the feedback to the service provider straightforward.
4. **Project for a provision of *service type C* -** In this case, a group with high levels of expertise is based in an establishment, which is **external to scientific user /research establishment**, and may be entirely commercial. In this case, gene constructs are sent with the request to create new animal lines, and perform all required activities to produce an established GA line, which is then usually sent to the user for breeding and maintenance. The primary function of this type of project is **the creation of new lines from gene constructs**.

This list of types of projects is not intended to be exhaustive, and other combinations of processes may be seen.

The following text addresses on one hand scientific projects and, on the other, service projects.

1. Relevance and justification of procedures

Background information is needed to understand the context of the application within the relevant scientific fields (or less commonly, the regulatory framework).

a. The overall aim of creation and/or maintenance of GA lines should be clear so that the likely achievements provide the foundation for assessment of the benefits likely to accrue from the project. Ensure the background information is specific and provides an overview of the field of use, indicating the scientific, medical, veterinary or forensic need for it. Use references (and/or regulatory guidelines) and outcomes of past work to support the main points.

For service projects, describe the service that you will be providing, and how the provision of the service will benefit the users, in terms of added advantage over doing it themselves. It must be clear which purpose(s) is/are relevant to the project, and information may need to be acquired from clients to determine purpose and evidence that there is a reasonable expectation that benefits will arise from breeding the animals.

b. **Purpose** - for projects for the creation and maintenance of GAA, procedures may only be authorised for specified purposes (Directive Article 5). From the information given in the application, it should be obvious which of these purposes apply:

(a) basic research;
(b) translational or applied research with any of the following aims:
  (i) the avoidance, prevention, diagnosis or treatment of disease, ill-health or other abnormality or their effects in human beings, animals or plants;
  (ii) the assessment, detection, regulation or modification of physiological conditions in human beings, animals or plants; or
  (iii) the welfare of animals and the improvement of the production conditions for animals reared for agricultural purposes;
(c) for any of the aims in point (b) in the development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feed-stuffs and other substances or products;
(d) protection of the natural environment in the interests of the health or welfare of human beings or animals;
(e) research aimed at preservation of the species;
(f) higher education, or training for the acquisition, maintenance or improvement of vocational skills;
(g) forensic inquiries.

Purposes (d)-(g) are very uncommonly reported relating to the use of GAA. If intending to create GAA for purposes (d)-(g), it is advisable to contact your national / regional / competent authority to determine if the purpose is assigned correctly. For projects only covering maintenance of existing lines, “maintenance” should be selected in the Non-technical project summary, and in statistical reporting. However, it is important that the

general field of use is explained in the project application background as well as in the non-technical project summary.

c. Objectives of the project

For a scientific project, the scientific objectives of the project should be clearly stated. The great majority (>95%) of new GA lines are produced for the purposes of basic research (the remainder for translational/applied research). Such lines are used to support projects authorised to investigate specific objectives in a scientific discipline. One such example could be a neurology project to investigate the genes involved in demyelination (nerve degeneration) with the intention of developing strategies for treatment of debilitating diseases such as Multiple Sclerosis. Such a project may request authorisation for the development of a number of new GA lines to investigate the pathogenesis (causes) of demyelination. Such a project could include all procedures necessary for creating, maintaining, and the subsequent scientific use of these animals, under one defined programme of work.

For a scientific project, the application would need to address issues such as:

- Why are such animals/lines needed?
- The reasons for the chosen species
- What studies will they be used for?
- What scientific outcomes / goals will be achieved by generating these animals?
- Why each of the procedures requested for creating, breeding/maintenance of these GA lines are needed.

Alternatively, for service projects, the application will need to define objectives around high quality service delivery to permit others to deliver scientific benefits.

d. Where the necessary expertise for GAA development is available, (either within the research establishment as service project type B, or in another establishment as service project type C) there may be a project application to generate new GA lines to service the needs of multiple research groups. Consideration should be given to use this expertise to provide an efficient service to meet the needs of both the internal and wider scientific community. For a service project the application should address issues such as:

- The demand for the service;
- The species which will be offered and the relevant experience with each of these;
- How the applicant will determine what the use and purpose of the animals will be (in advance of creation / maintenance);
- What advantages would this service provide to end-users;
- Why each of the procedures requested for creating, breeding/maintenance of these GA lines are needed.

e. Where appropriate and permissible in the Member State in question, GAA projects may be authorised as multiple generic projects as set out in Article 40. However, such multiple generic projects must still ensure that for each new line, the purpose of the procedures as

required by the Directive Article 5 (see 1b above) can be identified correctly within the terms of the authorisation and documented. For service projects, the internal operational process for the commissioning of new lines from the client should be described in the application, including the organisation, management and review of requests within the establishment. These processes should demonstrate sound governance and quality control of the internal decision-making process. In these cases, the internal process assures governance over the development of the new line to ensure that the local processes are sufficient to comply with the requirements of the project authorisation. Good record keeping is essential and an undertaking that the records of decision making for each line will be subsequently available and open for inspection/review by the competent authority, to ensure the continued effectiveness of the internal oversight. It is important that the total expected number of animals and the related severities are fully covered by the application and subsequent authorisation. After creation and establishment of a new line, there is often a need to maintain these established lines. Therefore, a combined creation and maintenance project should be considered, before animals are moved for continued use in a user project. Where only maintenance of established lines is required, these may be bred and maintained on a specialised breeding/maintenance project (service project type A), before being supplied to a different user as continued use under their respective authorised project. The service authorisation may be a multiple generic project, but it is improbable that the end-user project authorisation will be.

f. For scientific projects, it should be considered whether it would be more effective to have lines produced at a specialist creation site where efficiency may be greater, and therefore there would be no need to include vasectomy, superovulation or embryo recipient procedures on the scientific use project.

g. For scientific projects where lines are not created within the same research establishment, consideration should be given to transport stresses, which should be minimised where gametes/embryos cannot be imported/brought in from the other establishment. The quality of the source is expected to be high where a specialised service is being used, but the customer should apply due diligence.

h. Discussion of whether off-target effects may occur and the observational strategy to detect these should be included (Part 2 Section 2).

2. Significance and impact of potential benefits

Applications should make clear:

- **a.** What will be the benefits of the production of the GA lines?
- **b.** Who will benefit from the outcomes?
- **c.** How will they benefit or what impact will the outcomes of **this project** have?
- **d.** When (where possible) will the benefits be achieved?

Where a project authorisation is requested which is designed to produce multiple, perhaps diverse, GA lines for different research purposes, (typically service projects), including multiple generic projects, the main benefits may be the provision of a high quality efficient and effective service for customers by highly experienced specialists delivering precise, high quality GAA with welfare harms and animal numbers minimised.

For applications for the creation (and maintenance) of GA lines for the purposes of basic research (science projects), there should be a commitment to dissemination of results.

For service authorisation applications, there should be a commitment to minimise surplus, and share lines where feasible.

3. Adoption / inclusion of methods to replace, reduce and refine the use of animals in procedures

Detailed information on the means of addressing the Three Rs can be found in part 2 of this guidance document. It is essential that the applicant demonstrates in the application that all relevant aspects have been considered.

Examples of key elements are discussed below.

a. Replacement.

The application should make it clear why the use of the animals is necessary, what alternatives have been considered and why they cannot be used. This section should also put the animal work in context within the overall scientific programme i.e. which alternatives are being used for aspects of the project and what contributions are these making.

For example, it may include:

i. To avoid unnecessary duplication, what searching has been/will be continued to be done to identify if the lines are/become available elsewhere.

ii. Consideration of the use of types of animals which are outside of the scope of the Directive such as *Drosophila, Caenorhabditis elegans.*

b. Reduction

This should include consideration of experimental or observational/breeding strategy to minimise animal numbers. Statistical design rarely plays a role in the **creation phase** of GAA. However, good colony management matching supply to demand is key to reduction. Examples of issues to be covered:

i. Consideration of the most appropriate gene technology to deliver scientific objective most efficiently;

ii. How monitoring numbers of animals, gametes, offspring etc. will be used to create and breed animals efficiently;

iii. Consideration of cryopreservation to reduce the requirement for maintaining so many live animals;

iv. Details of any proposed reuse of animals and the accumulative effect thereof on the animals – e.g., wild-type offspring which have been genotyped using an invasive method, which are then used for superovulation to obtain wild-type eggs for genetic manipulation;
v. Use of animals for organs/tissues;
vi. Clarification of how the specified number of animals requested has been estimated e.g., number of lines to be bred/created, numbers of surgical recipients requested etc.

c. Refinement

Generic comments that refinements will be applied will not be sufficient. Applicants should explain how animal use will be refined in the planned procedures, including the reduction, avoidance and alleviation of any form of animal suffering, from birth to death where appropriate, including:

i. the planned use of anaesthesia, analgesia and other pain-relieving methods for surgical procedures required for creation of a new GA line: e.g., vasectomy, surgical embryo transfer;
ii. Life stages to be used – including the use of very young females for breeding/egg harvest;
iii. Choice of tissue sampling methods – use of surplus tissue from marking or where invasive methods proposed, justification of why non-invasive methods are unsuitable, and what methods of local anaesthesia/analgesia will be used;
iv. Housing, husbandry and care conditions for the animals – e.g., immunocompromised animals will be kept in barrier conditions to reduce likelihood of infection, use of specific food e.g., wet mash for longer for expected small-for-age pups than normal, increased housing temperature for nudes / hairless animals;
v. Use of humane end-points – particularly important for harmful phenotypes, especially where harms have a high impact. Age-related humane endpoints may be significant – e.g., kill before 6 months of age when [phenotype] is first seen;
vi. Methods of killing – the choice of killing methods must be the most refined e.g., Annex IV method used to kill recipient mothers after weaning of litters produced by surgical implantation. In some cases, where tissues are required from GAA, a method not listed in Annex IV may need to be included in a breeding and maintenance authorisation as an exemption if this is the only "use". For methods not listed in Annex IV, specific justification should be provided e.g., the use of perfusion fixation is required to preserve microanatomy in some scientific cases;
vii. Other fates of animals - fate of the retired breeding animals, re-use of wild-type animals;

viii. Description of the processes in place to ensure uptake of emerging refinement techniques during the lifetime of the project.

4. Severity classification of procedures:

Once the procedures are finalised, along with all of the refinements which are to be applied, then it should be possible to assign prospective severities to each procedure.

For science projects, prospective severity will include consideration of the severity of the continued use as well as the impacts of the line itself. In some cases, where there is no breeding or maintenance on the authorisation, the process for obtaining severity up to the point of supply from the breeder should be discussed, as this is a consideration required for reporting actual severity at the end of the procedure.

The application needs to include information on the procedures which will be used during the creation and maintenance of GA lines, the adverse effects which may be caused, and the methods which will be used to minimise the effects on the animals.

For type A service projects, the harms of invasive genotyping which is not used for identification, and maintenance for which will be the welfare impacts of the GA lines themselves will need to be considered.

For type B and C service projects, in addition to harms as described for type A service projects for breeding and maintenance, harms from the creation procedures (including the unpredictability of harms in new GA lines) need to be considered.

Each procedure should have a severity classification proposed, which reflects the highest severity expected. Information on the target gene should allow an informed decision to be made of the likely worst-case scenario for any individual animal. Only when no informed decision can be made should the precautionary allocation of the prospective severity be to the "severe" classification, but early endpoints should be described to reduce harms to the minimum necessary.

For the procedures being applied to the animals, information is required on

- Frequency/duration of procedures;
- Likelihood of adverse effects;
- Severity level and methodology to minimise severity;
- Monitoring regime; welfare assessment protocols;
- Humane end-points and triggers for interventions.

For the routinely required procedures such as embryo transfer, superovulation, surgical implantation of embryos, and offspring produced with harmful phenotype or invasive tissue sampling, explain the potential adverse effects, the methods employed to reduce these, such as analgesia, and ensure that the most refined methods are used.

When creating new lines, consideration should be given to the potential adverse effects on the offspring. These may be anticipated using information on the genes being altered, or from

information on other lines with similar alterations. However, unexpected adverse effects may arise in some lines – where these exceed the severity predicted there may be a need to seek amendment to the project.

The severities should be assigned in line with the respective Assignment Criteria in Section II and examples in Annex VIII, and the EU severity framework guidance, which includes a GAA case example (Model 6, page 62).

In addition to the maximal severities for each procedure (prospective severity classification), it is important to demonstrate what are the realistically expected harms of the entire project taking the whole project and all of the procedures into account which will facilitate the project evaluator in performing the harm-benefit assessment. The table from the Non-technical Project Summary may provide a useful means to summarise this:

What species and numbers of animals are expected to be used? What are the expected severities and the numbers of animals in each severity category (per species)?	**Species**	**Estimated total numbers**	**Estimated numbers per severity**			
			Non-recovery	**Mild**	**Moderate**	**Severe**
	Mice	5400	0	5000	300	100
	Zebrafish	10500	0	7500	2500	500

5. Environmental impact

Reduce environmental impact where appropriate – other than not releasing GAA this is rarely, if ever, of significance for this type of project.

6. Harm-benefit analysis

Harm-benefit analysis performed by evaluators includes a determination of the likelihood of successful achievement of the proposed benefits. It needs to be demonstrated in the application that the benefits exceed the harms.

a. The field of GA in animals is rapidly evolving and requires specific knowledge and expertise by the persons involved in the project. This is in addition to general competence requirements, and provides the necessary information to enable the evaluation of the likelihood of success. This includes skills, knowledge and experience to make the project run efficiently and effectively, including the experience which will enable the choice of gene manipulation/gene-editing methods and maintaining the integrity of the lines once they have been established. The previous track record of the research group and the quality and reliability of the work that has led up to the project is relevant. Whilst prior experience is not

necessary, the likelihood of success of a new research worker is lower and this should be considered by the evaluators in the harm-benefit analysis taking into account expertise available to the project. New researchers in the field will not be prevented from acquiring authorisations, providing the likelihood of success of what they are requesting is sufficiently high. Applications for service projects, including those which are classified as multiple generic projects, must demonstrate that there are sufficient skills and experience to make decisions about whether work requested by others (for type B inside, or type C outside the establishment) can legitimately be done under the proposed project (see section 1 for examples of the types of decisions that may need to be made by the applicant).

b. Successful outcomes are also likely to be increased if the project is adequately resourced, in terms of staff, facilities and finance.

c. The demonstration that benefits will exceed harms is more straightforward for applications for specific scientific areas. It may be more difficult if the application is for a multiple generic project. In this case, the value of the service itself should be demonstrated, along with the likely benefits to science of the GA lines which will be included within the project authorisation framework.

Part B: Illustrative example of the evaluation of GAA project proposals

This is a 2-step process. When an evaluator is providing an opinion on the application, they should check that the information outlined above is included within the application. This is the verification laid out in 1. below. When all the information is included, then progression to the evaluation (in 2. below) can occur, but without sufficient information a complete evaluation cannot occur. For each relevant section, they should cross-refer to the elements above to ensure that the quality of information provided allows a decision to be made on each criterion and that it complies with the legal requirements. The considerations and outcomes below will vary for the types of authorisations as described in Part A. In some cases, questions and examples of evaluation outcomes apply to all types. Some examples are given where there are differences.

1. The project evaluation needs to **verify** that the project meets the following criteria:

	Criteria required in the project evaluation:	**Considerations on how project evaluators can comply with verification requirements**	**Examples of evaluation outcomes addressing required key elements**
(a)	the project is justified from a scientific or educational point of view or required by law;	For science projects, the project evaluator needs sufficient information to be able to decide whether the science is worth doing. **OR** For service projects, including multiple generic projects, the evaluators need sufficient information on: - how the justification for the use of animals will be determined during the life-cycle of the project on a line/group by line/group basis, and - how the scientific (or other) benefits will be established and - the benefit arising from a specialised service for others.	This project will provide animals with mutations in pathways which are known or hypothesised to be linked to the development of demyelination. The animals so produced will be used to describe further the pathways with the hope that therapeutic targets may be identified for future development. It is expected that several of these targets will be identified within the 5-year timespan of this project. These targets will be made known to other scientists and potential pharma/biotech companies by publication in scientific journals. There is a high level of scientifically evaluated funding for this work. The group has published in high quality journals to date and have shown good evidence of progress in the last 5 years, suggesting that the likelihood of achievement is high. **OR** For type A service project, the lines to be bred and maintained are all established lines related to obesity. An undertaking is given that animals will only be supplied to authorised establishments, where a project authorisation for this work is in place by supply

			of project authorisation number. This group have experience in efficient breeding processes which match supply to demand. OR For type B and C service project, the applicant is a renowned expert in the area of transgenic technology and has contributed to best practice guidelines in this field. All the procedures requested have been performed over the last 10 years and there is evidence of refinements and measures which have reduced surplus numbers. There has been much improvement activity from this group in ensuring quality of lines and genetic integrity. Good governance systems are described to ensure that each new line is not already available elsewhere and the benefits of production of each line will exceed the harms expected by the creation procedures required. Production of each line is funded by the client, who will be checked to be a legitimate scientist/user with a high expectation of quality scientific outcome. Templates of records of internal processes should generate all necessary information for decision making and it is stated that they will be available for inspection.
(b)	the purposes of the project justify the use of animals; and	The decision here involves a consideration of the expected benefits, and a determination of the potential use of alternatives.	Given that demyelination causes a range of diseases which induce disability from a young age, and often premature death, and the opportunity provided by this project to identify potential therapeutic targets to improve outcomes, the creation, breeding and maintenance, and use of genetically altered animals for this purpose is justified. The applicant has demonstrated by searches, and the evaluators agree, that there are no alternative methods which can replace the use of genetically altered animals for this project.
(c)	the project is designed so as to enable procedures to be carried out in the most	The evaluator needs to be convinced that they are not aware of any refinements which could be included (and still achieve the science/education/	Sufficient information is provided on monitoring of animals for signs relating to signs of demyelination and appropriate

	humane and environmentally sensitive manner possible.	regulatory outcomes). Environmental impacts are not likely to be relevant for this type of project.	endpoints are included for evaluation to be performed (see below).
In some cases, early versions of the project will not contain sufficient information to be able to verify these issues. In these cases, specific comments relating to the deficiencies should be returned to the applicant and they should be invited to complement/amend the application. In most cases, such comments should be combined with any additional information which is required to allow the project evaluation to be completed.			

2. The project evaluation shall consist in particular of the following:

	Elements required in the project evaluation:	**Examples on considerations on how project evaluator can comply with evaluation requirements**	**Examples of evaluation outcomes addressing required key elements**
(a)	an evaluation of the objectives of the project, the predicted scientific benefits or educational value;	The project evaluator needs to determine from the information in the application whether the science is worth doing. For example: will the creation of the GAAs described provide insight into the disease process or consequences? Is it likely that if knowledge was available, this would lead to advancement towards therapies for patients with this disease? Are the models likely to have validity? What would be lost if this work was not done? **OR** For service projects including multiple generic projects, what advantages are there from using a centralised service? Will creation and maintenance be more effective and efficient, if run in this way? Will there be welfare advantages to the animals? Is the decision making about which lines should be produced robust? Whether the justification of the use of animals will be determined on a line/group by line/group basis, and how the scientific (or other) benefit of each line will be established.	*For a science project:* This project will create lines of GA mice which will be used to investigate the pathways relating to myelin deposition, and/or related inflammatory processes. Advances in knowledge are expected which will inform potential treatments. Whilst treatment development is not expected during the course of this project, significant knowledge should be added to the literature which will enable others to focus on likely targets which may improve quality and quantity of life in these patients in the long term. *Or* *For a service project:* The skills of this team should provide an efficient and effective service of creation and maintenance of high-quality GA lines, which will provide the basis for development of science, both basic and applied to specific disease areas. It is stated that each line will only be produced after careful consideration of the specific benefits (within the context of the defined disease area) which will be likely to occur, and the purposes of production will be defined as listed within Article 5 and can subsequently be reported as detailed in Commission Implementing Decision 2020/569/EU.
(b)	an assessment of the compliance of the project with the requirement of	Each of these should be considered separately. Does the applicant reference any databases mentioned in the (*Part 2; Annex 1)* or otherwise demonstrate that they are aware of the 3Rs in this field?	

	i. replacement ii. reduction iii. refinement	i.	Have any searches been done for pre-existing lines? Is the case made that creation is necessary? Are the evaluators aware of any relevant alternative methods that are not excluded by the applicant as unsuitable? In the opinion of the evaluators, does the applicant make a valid and complete case that animals are required?	i. <u>For a science project</u>: Much work is being done by this group using cell lines and mixed cultures, but because nerve tissue is a complex interaction of cell types, even the multicellular structurally directed *in vitro* techniques cannot replicate all of the components under investigation at this time. <u>For all project types</u>: The group has referenced databases which will be searched for pre-existing lines.
		ii.	Does the applicant adequately address how they will make supply meet demand? Is there discussion of colony management strategies suggesting quality control will be robust and surplus animals will be minimised and/or re-used when appropriate? <u>For science projects</u>: Does the text provided in the application give reassurance that the fewest animals will be used to achieve robust science? OR <u>For service projects</u>, does the text provided in the application give reassurance that the fewest animals will be used to match supply with demand?	ii. <u>For all project types</u>: Strategies have been described which suggest that all appropriate measures, such as background strain and colony breeding management, will be taken to reduce numbers to a minimum required whilst ensuring genetic integrity and reduction of genetic drift in the lines produced. <u>For science project and service project types B and C</u>: Cryopreservation is discussed. It is clear from the (creation) production data that the systems are as efficient as other groups or in many cases better. There is evidence over the past 5 years of updating and improving practices to increase these efficiencies and to reduce surplus. It is expected that this will continue throughout the next 5 years.
		iii.	Considerations in 1(c) will have verified that the most humane methods will be used. This section should follow on from this and evaluators should be able to determine the impacts expected from each of the procedures included. The applicant should have explained the procedures and their	iii. <u>For a science project</u>: Where demyelinated GA lines are to be used, weakness is expected, unless controlled/prevented by the proposed disease modifying test substances. For control animals and where test product is ineffective, staging is described and stages aligned with the scientific outputs required for each of the objectives, with killing at onset of forelimb weakness as demonstrated by low grip strength, expected for 80% of the animals of these GA lines. 20% will be kept until the first definitive signs of hind limb weakness. Neither stage will have any

		adverse effects on animals, so that the overall harms caused to the animals can be determined. For all projects: the evaluator should determine whether sufficient refinements are to be applied to the breeding of animals with harmful phenotypes. The evaluator should consider the likelihood of unexpected adverse effects in new GA lines. For service projects types B & C and relevant science projects: whether sufficient refinements are to be applied to superovulation, vasectomy, transplant of embryos into recipients, the evaluator needs to understand the relevant proportions of animals which will suffer and to what degree and duration, taking account of the refinements and endpoints to be applied. Page 23 of Working Document on Project Evaluation and Retrospective Assessment may be of value. This knowledge should feed into the next section allowing determination of severity classification.	observable impact on ability to feed, drink, or groom. When hind limb weakness is present locomotion will be reduced. Monitoring 4 times a day will be in place when forelimb weakness is seen to ensure limited duration (no more than 18 hours) of hind limb weakness. Impacts of administration of test products should be mild and transient due to the administration method only, given the detailed information available on safety of these test therapeutics. AND/OR For science and service projects: The genotype of most of the breeding animals should not induce any harms providing that the biosecurity barrier described in the application remains intact, which historically it has been. Some animals would show weakness if maintained longer than 6 months, but animals for use are to be transferred to project/protocol x at 6 weeks of age and will be normal by cage-side assessment up to this time. Breeding animals from this line will be replaced at or before 6 months of age. GAA welfare reports are to be maintained for each line with a harmful phenotype assuring good monitoring and communication. For relevant science projects and for service projects B and C: Because grimace scale monitoring with appropriate analgesia as determined by the Designated Veterinarian is to be used, then good pain control can be expected for all animals and so impacts of implantation and vasectomy should be low with full recovery of normal behaviours within 24 hours. Superovulation causes momentary pain in each animal with each of the injections, but the impact should be minimal.

(c)	an assessment and assignment of the classification of the severity of procedures;	The applicant is required to provide an opinion on the prospective classification of the severity of each of the procedures. The project evaluators need to check, or determine and reassign severities to each of the procedures (or series of procedures) so that it is aligned with Annex VIII, adapted to the specific case in question in line with Section II of that Annex, and any supplementary Guidance from EU and/or MS. Where this is a checking process, a confirmation should be made that the submitted assignments are correct.	We [the evaluators] concur with the view of the applicant in relation to the severities assigned to the most of the procedures as listed. However, there has been some debate as to the likely severities of some of the demyelinating lines, particularly where the alteration and interventions induces progression to the severe clinical condition. We are convinced by the arguments presented regarding monitoring, staging and application of early endpoints at stages, and having considered relevant comparison information in Annex VIII, EU guidance on severity examples and the Zintsch paper[15] we consider that procedure x should be assigned a prospective severity of moderate rather than severe as assigned by the applicant. We concur that procedures y and z should be severe. The staff (scientific and technical) have high levels of experience in these signs and detection as described is expected to be good. OR For service project type B: We concur with the view of the applicant in relation to the severities assigned to the procedures as listed. The maintained lines will not exceed the moderate severity as any expected to become severe will be transferred to the user > 6 weeks prior to the expected onset of more significant signs. Undertakings are given that welfare assessments will accompany shipments of animals, to allow appropriate reporting by the end-user. Unexpected adverse effects will result in humane killing.
(d)	a harm-benefit analysis of the project, to assess whether the harm to the animals in terms of suffering, pain	**This should be done after ALL other aspects of the evaluation have been completed, as it requires all the information to be assimilated.** Evaluators should review pages 25-27 of the Working document on Project Evaluation and Retrospective Assessment. Evaluators should ensure that benefits	For a science project: This applicant has not held an authorisation in the past, but has worked within another group experienced in this field with these species thus improving the likelihood of success. She has undergone all required training as laid out in the E&T Framework document. The team is small, but we expect that the objectives, as described, can be delivered. Pubmed

[15] Zintzsch A, Noe E, Reißmann M, Ullmann K, Krämer S, Jerchow B, Kluge R, Gösele C, Nickles H, Puppe A, Rülicke T; Guidelines on severity assessment and classification of genetically altered mouse and rat lines ; (2017).

	and distress is justified by the expected outcome taking into account ethical considerations, and may ultimately benefit human beings, animals or the environment;	relating to the field of science of the programme are realistic and specific to the described procedures on GAA creation, breeding, and use (where included). Where relevant, an evaluation should be made of the advantages of a centralised service using expertise and track record of the group, over other usually less skilled groups performing the creation, breeding and maintenance. The evaluator of the project application is required to consider the harms which may/are likely to be experienced by the animals during the course of the project. The harms discussed above need to be considered accounting for - Likelihood of adverse effects in offspring especially in creation; - Monitoring regime; welfare assessment protocols taking account of the refinements proposed; - Severity level and methodology to minimise severity; - Humane end-points and triggers for interventions; - Frequency/duration of procedures; The evaluators should also consider the total numbers of animals requested, and the numbers which are predicted to suffer at the prospective severity (maximal) and the proportions which will suffer to a lesser extent to evaluate the likely total harms from the project.	searches on the applicant reveal some publications in this field including in two very high impact journals. The described level of monitoring and use of score sheets should assure that appropriate endpoints are applied to limit harms to no more than the maximum described in this application. The applicant has undertaken to train new staff in the staging of the disease and supervise them directly until competent. It is proposed that up to 5400 mice and 10000 zebrafish will be used in this project, ~400 for surgical procedures and the remainder for breeding. Only about 20% of the breeding animals are expected to show moderate signs, and 4% may be severe, with a large proportion appearing normal to cage/tank side assessment. In our opinion, the benefits of the increase in knowledge of the effect of manipulating pathway p on the process of demyelination to scientists in this field, which will be disseminated by publications, presentations and collaborations, outweigh the predicted harms as described. For a service project, type B: This applicant has a good history of providing an effective and efficient production of lines requested by clients. Returning clients attest to this quality, particularly bearing in mind that there are cheaper options. PubMed searches on a small number of the lines created and supplied over the last 5 years show that there is high quality scientific output in prestigious journals providing benefit for the scientific community in a variety of fields. The group has been involved in training scientists in the field of genetic integrity and how to breed lines to ensure the most robust science. This is a high-quality team and their proven capability to deliver outputs which deliver GA lines efficiently and effectively to those less able to deliver such high-quality lines. The level of monitoring, training and use of score

		The likelihood of success should be evaluated, by considering the knowledge and skills available and whether the group is likely to apply the refinements and monitor appropriately. The history of publications and/or production of GAAs previously is relevant. Are there any novel developments proposed which may be less likely to succeed than established methods? In some cases, although likelihood of success is lower, the potential impact of the benefits if it does succeed may be very high – e.g., novel techniques.	sheets assure that appropriate endpoints are applied to limit harms to no more than the maximum described in this application. It is proposed that up to 5400 mice and 10000 zebrafish will be used in this project, ~400 for surgical procedures and the remainder for breeding. Only about 20% of the breeding animals are expected to show moderate signs, and 4% may be severe, with a large proportion appearing normal to cage/tank side assessment. For a service proven capability to deliver outputs which deliver GA lines efficiently and effectively to those less able to deliver such high-quality lines. The level of monitoring, training and use of score sheets assure that appropriate endpoints are applied to limit harms to no more than the maximum described in this application. It is proposed that up to 5400 mice and 10000 zebrafish will be used in this project, ~400 for surgical procedures and the remainder for breeding. Only about 20% of the breeding animals are expected to show moderate signs with a large proportion appearing normal to cage/tank side assessment. In our opinion, the benefits to science, to reduction and to animal welfare of using this high-quality team for this service to other scientists outweigh the expected harms as described.
(e)	an assessment of any justification referred to in:		
	Art 6 - Methods of killing	It is unlikely that there will be any specific justifications / exemptions required, which are different to those required in any project.	
	Art 7 Endangered species	Not likely to be relevant to GAA projects.	
	Art 8 Non-human primates	Uncommon, but if requested, a careful justification needed (see https://ec.europa.eu/info/sites/default/files/research_and_innovation/ege/ege_ethics_of_genome_editing-opinion_publication.pdf).	

	Art 9 Animals taken from the wild	Not relevant to these projects	
	Art 10 Animals listed in Annex I need to be bred for use in procedures unless justified	If requested, a careful justification needed.	
	Art 11 Stray and feral animals	Not likely to be relevant to GAA projects.	
	Art 12 Procedures should be carried out in an establishment	There is no reason for this type of project to be carried out outside an establishment. There is other legislation requiring containment of GAA.	
	Art 14 Use of anaesthesia and analgesia	These may be specifically covered in the refinement section and/or with adverse effects of protocols. Project evaluators should determine whether it is clear that anaesthesia and analgesia will be given for surgical procedures used in creation, (embryo recipients, vasectomy). Also, for genotyping unless the method is momentary and giving the analgesia/anaesthesia would be more traumatic.	Clear descriptions of the types of anaesthesia/analgesia along with monitoring regimes are described including grimace scales. Advice from the designated veterinarian is described to have been taken on a case-by-case basis. The veterinarian has shown good and up to date knowledge of modern practice in this area. This provides confidence that the animals will have optimal anaesthetic and analgesic regimes. Anaesthesia has been considered for ear marking and reported to be more traumatic than the procedure itself in this case where experienced staff perform a single ear clip by punch method, a view with which we concur.
	Art 16 Reuse	Any animals bred for use will be transferred to a use protocol (continued use and not reuse). Reuse can occur, for example animals genotyped using an invasive method but which turn out to be wild-type, but which are not required as controls. It would be reduction if these animals can be used for a different purpose. If this is requested, then it must be clearly demonstrated that the	Unusually, re-use of some wild-type animals is planned. Although genotyped by ear clipping, this group uses ID chips to facilitate reporting of on-going monitoring and therefore the method of tissue sampling for genotyping is considered a use. Those wild-type animals which have been assigned an actual severity of mild and which are to be reused will be seen by the designated veterinarian who will advise whether the animals' general state of health has been fully restored. Only those which pass these two criteria are requested to be reused for procedures of no more than

		conditions in Article 16 will be complied with (severity constraints and determination by a veterinarian).	moderate severity. This is compliant with the legal requirements and can be authorised.
	Art 33 Care and accommodation requirements need to be fulfilled	As well as compliance with Annex III of the Directive for all animals, any proposed single housing of vasectomised mice should be described and justified. It should be stated that all animals which are or may be immunocompromised will be kept in IVCs (most refined method) if this is appropriate. Any particular husbandry and/or care requirements which are pertinent to the particular GA line(s) to be authorised need to be included.	It is stated that all animals will be kept at Annex III standards with the following exceptions: single housing of mature adult vasectomised males has been justified on the grounds that separation during mating may not permit remixing of sibling groups in times when they are not in breeding pairs. We concur that this may be necessary for some individuals, and that the research group is taking what measures it can to retain social housing where possible. Single housing of some animals following study allocation is expected. Pairing with wild-type animals is being attempted within the experimental design criteria, but this has not yet been perfected and so some single housing in this context is expected to be justified at this time. It will not last longer than 3 weeks, as scientific endpoints will be achieved by then. Homozygous pups from line x are known to be particularly small and therefore weaning will not occur before day 28.
(f)	a determination as to whether and when the project should be assessed retrospectively	This will not usually be needed for this type of project. However, if a severe breeding protocol is needed then a retrospective assessment will be required.	For science project: Retrospective assessment (RA) is required because of severe procedure y. For service project: No retrospective assessment (RA) is required. [Provided there are no severe procedures foreseen, which is usually the case.]

Appendix III: Bibliography

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Appendix IV: Glossary of terms

Terms	Meaning
Age onset	Age at which signs of a disease or disorder first appear in an individual.
Allele	One of several alternative forms of a gene occupying a given locus on a chromosome.
AWB	Animal welfare body
Background strain	The strain of wild type mouse used to create the gametes/embryos for genetic modification and which is used for backcrossing.
CA	Competent Authority
Commensal	An organism which lives on or within another organism, and derives benefit thereof without injuring or benefiting the other.
Conditional line	GA line which contains mutations that can be introduced/activated in a spatially restricted manner (e.g., using CRE-LOX technology, see below).
Conventional	Context specific. In the case of animal housing this would be open topped cages with lower levels of disease hygiene control and health screening, and excluding e.g., individually ventilated cages, rederivation.
Creation of new GAA	The development of a new GA line through deliberate/intentional gene alteration (e.g., genetic insertion/deletion/editing, chemical mutagenesis or other manipulation of a gamete or embryo, or may be by crossbreeding of two pre-existing lines).
CRE-LOX	Molecular biology technique used to carry out deletions, insertions, translocations and inversions at specific sites in the genome. Allows for tissue-specific and/or time-specific editing, which CRISPR-Cas9 cannot. It requires crossing of two GA lines to activate the genetic change.
CRISPR-Cas9	Molecular biology technique allowing for site-specific genome editing in virtually any organism.
Cryopreservation	A strategy for preserving samples of animal genetic materials (usually sperm, oocytes, or embryos) at very low temperatures.
Dpf	Days post fertilization

DNA	Deoxyribonucleic acid – the substance that genes are made of
Distal phalanx biopsy	The removal of a single distal phalanx of one toe, used in limited justified circumstances to genotype and identify immature animals.
Dystocia	“Difficult or obstructed labour”: when a foetus does not exit the pelvis during birth due to physical factors, despite the uterus contracting.
Ear Biopsy	Collection of ear tissue
Ear Notch/Punch	Rodent identification/genotyping technique using a special punch either to produce a small (0.5 to 2 mm) notch near the edge of the ear or to punch a hole in the middle of the ear. The position of the mark determines the ID number with a predetermined scheme (e.g., scheme described by Hogan *et al.* in “Manipulating the Mouse Embryo - A Laboratory Manual, CSH laboratory Press 1994” allows for identification of up to 99 different animals).
ES cells	Embryonic stem cells (ES cells or ESCs) are pluripotent stem cells derived from the inner cell mass of a blastocyst, an early-stage pre-implantation embryo.
EWG	Expert Working Group
Established line	A new strain or line of genetically altered animals is considered to be "established" when transmission of the genetic alteration is stable, which will be a minimum of two generations, and an initial welfare assessment completed, along with the determination of whether this is a harmful or non-harmful line.
GAA	Genetically altered animals - include genetically modified (transgenic, knock-out and other forms of genetic alteration) and naturally occurring or induced mutant animals.
Gametes	Gametes are an organism's mature reproductive cells (sperm, ova).
Genetic Characterization	The detection and description of the presence of certain genetic traits (e.g., alleles) in an organism.
Genetic integrity	The quality attributed to an animal model population when its genetic make-up is faithful to the original line. Maintaining the genetic

	integrity of an animal research model is essential to improve reproducibility between research experiments. Integrity can be threatened due to genetic contamination, genetic drift, unintentional selection and mislabelling. Integrity can be assured through genetic monitoring (methods include biochemical markers, phenotypic analysis, and, more recently, microsatellite DNA and single nucleotide polymorphism (SNP) analysis) and proper colony management.
Genetic Integrity Panel	Set of defined and unique points throughout the genome of the required background strain to be controlled through genetic monitoring to assess genetic integrity. Bases, sequences will be evenly distributed on the autosomal chromosomes.
Genotyping	The process of determining differences in the genetic make-up (genotype) of an individual by examining the individual's DNA sequence using biological assays and comparing it to a reference sequence. Genotyping is not a method of identification.
GFP	Green fluorescent protein: frequently used as a reporter of expression (e.g., in a particular cell type as the GFP can be visualized by fluorescence microscopy).
Harmful line	Animal line with harmful phenotype.
Harmful phenotype	An animal or line which is likely to experience, as a consequence of the genetic alteration, pain, suffering, distress or lasting harm equivalent to, or higher than that caused by the introduction of a needle in accordance with good veterinary practice.
Heterozygote	An individual with different alleles at a particular locus.
Homozygote	An individual with the same allele at corresponding locus on the homologous chromosomes.
Hydrocephalus	A condition in which an accumulation of cerebrospinal fluid occurs within the brain. This typically causes increased pressure inside the skull.

Immunocompromised line	A line genetically altered to be unable to develop a normal immunological response to the presence of foreign antigens.
Inducible line	A line genetically altered to contain silent genetic information which can be induced (i.e. activated) in a temporally restricted manner by the scientist. The gene of interest is only expressed after activation by giving the animal a specific induction agent (e.g., tetracycline). Until the gene is expressed in this way no adverse effects from the gene manipulation will occur.
IP	Context specific. Intellectual property or intraperitoneal.
Isogenic	Two lines are said to be isogenic when having the exact same genetic make-up. It extends to cases with the same genetic make-up but with one gene different (the one that is studied).
IVCs	Individually ventilated cages.
IVF	*In vitro* fertilization.
Legacy Line	Older animal line which was produced in a manner that could not guarantee monoclonality.
Lifetime studies	Scientific and welfare observations performed on GA and control wild-type animals during the whole lifespan expected for the control wild-type animals.
Line	Sequence of generations of individuals that transmit and inherit a series of genetic factors that determine individual characteristics.
Lepr	Mice homozygous for the diabetes spontaneous mutation (Leprdb) manifest morbid obesity, chronic hyperglycemia, pancreatic beta cell atrophy and become hypoinsulinemic.
MS	Member State
Mendelian ratio	The ratio of occurrence of various phenotypes in any cross involving characters under the control of one genetic marker on a single locus (Mendelian character). Example: when two parents (P-generation) which differ in one genetic characteristic for which they are both homozygous (carrying the same allele on both chromosomes) are mated with each other, all offspring in the first generation (F1) are equal

	to the examined characteristic in genotype (all are heterozygous) and phenotype showing the dominant trait. When individuals of the F1-generation are crossed, the offspring in the F2-generation differ in genotype and phenotype. In a dominant-recessive inheritance an average of 25% are homozygous with the dominant trait, 50% are heterozygous showing the dominant trait in the phenotype (genetic carriers), 25% are homozygous with the recessive trait and therefore express the recessive trait in the phenotype. The genotypic ratio is 1 : 2 : 1, the phenotypic ratio is 3 : 1.
Microphthalmia	A developmental disorder in which one or both eyes are abnormally small.
Mosaicism	The presence of two or more cell lineages with different genotypes in the same individual. Mosaicism can arise in a single individual as the result of a postzygotic mutation. Alternatively, mosaicism can arise from the introduction of ES cells into a blastocyst which results in the development of one individual composed of cells from different lineages.
Multiple Generic Project	From the Working document on specific articles in Directive 2010/63/EU, article 40: “some classes of projects involve a series of standard procedures being applied for a particular purpose. These are sometimes referred to as 'multiple generic projects’. The procedures are generally well-established and the likely consequences on the animals are well-understood and can be minimised appropriately. There are unlikely to be particular novel or contentious issues raised during project evaluation. As in the case of simplified administrative procedure under Article 42, the procedures considered under multiple generic projects are required to satisfy regulatory requirements OR needed for production OR diagnostic purposes with established methods.”
Naïve animals	Animals used in an experimental set up which have been previously unused.
NCP	National contact point

NSET	Non-surgical embryo transfer. A technique involving insertion of a soft flexible tube through the cervix in mice to implant embryos (blastocysts) into the uterus.
NTS	Non-technical project summary: To ensure that the public is informed, objective information concerning projects using live animals has to be made publicly available in lay language. (Directive 2010/63/EU, Article 43)
OB/OB or Obese Mouse	A mutant mouse that eats excessively due to mutations in the gene responsible for the production of leptin and becomes profoundly obese. It is an animal model of type II diabetes.
Off-target effect, OTE	Off-target effect: nonspecific and unintended genetic modifications, including activity of the gene in a non-target tissue, or disruption of completely different gene by random insertion of genetic material into it causing its disruption.
Phenotype	The observable physical properties of an organism; these include the organism's appearance, development, and behaviour.
Phenotype Penetrance	The proportion of individuals displaying a specific trait (phenotype) associated with an allele.
Procedure	Article 3 of Directive 2010/63/EU defines *Procedure* as «*any use, invasive or non-invasive, of an animal for experimental or other scientific purposes […] which may cause the animal a level of pain, suffering, distress or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice.*». As such, the Directive considers the creation and maintenance of a genetically altered animal as a *scientific "procedure"*, if the birth or hatching may cause the animal pain, suffering, distress or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice.
Procedure severity classification	From the final report (2009) of the Expert working group on severity classification of scientific procedures performed on animals: "A severity category is to be assigned to each

	procedure. This will assist the harm-benefit analysis of the project. The severity of a procedure is determined by the degree of pain, suffering, distress or lasting harm expected to be experienced by the animal during the course of the procedure. The procedure consists of a combination of one or more technical acts carried out on an animal which may cause that animal pain, suffering, distress or lasting harm. The assignment of the severity category takes into account any intervention or manipulation of an animal within a defined procedure. The severity category shall be assigned based on the most severe effects likely to be experienced by an individual animal after applying all appropriate refinement techniques."
Rederivation	Removal of adventitious organisms, such as viruses, bacteria, and parasites from research animal lines, usually involving superovulation of infected stock and reimplantation of embryos into surrogate mothers which are of high health status.
Reporter gene	Reporter genes are genes that enable the detection or measurement of gene expression. They can be fused to regulatory sequences or genes of interest to report expression location or levels. Reporter genes include genes that code for fluorescent protein and enzymes that convert invisible substrates to luminescent or coloured products.
Reporter line	Animal line which carries a reporter gene to be able to follow gene expression *in vivo*.
Retrospective assessment	An evaluation at the end of the project by the CA of the following: (a) whether the objectives of the project were achieved; (b) the harm inflicted on animals, including the numbers and species of animals used, and the severity of the procedures; and (c) any elements that may contribute to the further implementation of the requirement of replacement, reduction and refinement. See Directive 2010/63/EU, Article 39.

Service project	Project for the provision of a service to other scientific users to create and characterise GA lines until established, and / or breed and maintain established harmful GA lines to supply scientific research groups.
Strain	Synonymous with “line”
Superovulation	The process of inducing a female to release more eggs than usual.
Suppressed line	Line in which one gene activity has been turned off (in contrast to “induced line”).
Tail biopsy / tipping	An older procedure for genotyping transgenic animals which entails cutting of the distal portion of the animal's tail.
TALENs	Transcription activator-like effector nucleases (TALEN) are restriction enzymes that can be engineered to cut specific sequences of DNA.
Three Rs / 3Rs	Replacement, Reduction, Refinement
Use, Reuse and Continued use	- The “use” of an animal within a project extends from the time the procedure (or first procedure/technique in a series) is applied to it, to the time when the observations, or the collection of data (or other products) for a particular scientific purpose (usually a single experiment or test), are completed. - “Reuse” is a term to indicate the subsequent use of an animal which has already completed a procedure (or series of procedures/techniques) for a particular scientific purpose. Article 16 on reuse defines it as a use when a different animal on which no procedure has previously been carried out could also be used. Article 16 also defines the circumstances under which an animal may be reused. - “Continued use” is a term not included in the Directive but can be used to describe the situation when the single “use” of an animal extends over more than one project or across different procedures within the same project. It is further explained in Annex III of Decision 2020/569/EU Part B.2.2.3. This arrangement can simplify project applications and avoid undue repetition. Continued use is common with GAAs from a “breeding” procedure to a “use” procedure.

Vasectomy	Surgical procedure to sterilize an animal during which the spermatic cord is transected to prevent passage of sperm and the testicles remain.
Welfare Assessment	A comprehensive welfare assessment allows the identification of welfare concerns. It will identify the need for specialised husbandry/care, and it can assist in discrimination between harmful and non-harmful lines. See more detailed definition in section 5, part 2 of this Guidance. Details on how to perform Welfare Assessment can be found in part 3 of this Guidance.
Wild-type (can be abbreviated as WT in research documents)	An animal that has had no genetic alteration made to it.
ZFNs	Zinc finger nucleases (ZFNs) are synthetic proteins used for gene targeting.

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