

Examples on how to use the 3RsAGENT

Example 1: Generation of a new line with expected sudden cardiac death syndrome

A new line will be generated as cardiac-specific knockout of the gene of interest. The GA mouse line is expected to develop a short-term, ventricular arrhythmia followed by cardiac arrest. Information on the phenotype derives from a previous and already established line with ubiquitous knockout of the same gene. In the established line, 25% of homozygous mice died at the age of one year. The electrocardiogram of affected animals showed that the time-span between the onset of arrhythmia and the death of the animal lasts a few seconds. Mice did not show any impairment of well-being prior to the onset of arrhythmia and were found dead in cage. Animals were monitored daily and showed normal behaviour and activity levels. Histopathology did not reveal cardiac hypertrophy or dilated cardiomyopathy. A similar phenotype is expected in the new cardiac-specific knockout mouse line.

Component	Describe harm causing procedure or factor of harm	Uncertainty factor regarding harm ¹	Modulating factors of harm that influence severity ²	Severity classification ³
Genetic engineering <i>Which technique of genetic engineering is used?</i>	Use of a targeted mutation method (CRISPR/Cas9) to induce a knock-out of gene XY. Targeted mutation decreases side effects of the intended mutation.	<input type="checkbox"/> low <input checked="" type="checkbox"/> medium <input type="checkbox"/> high	Nevertheless, side effects cannot be excluded and they could influence the phenotype.	
Sterile males <i>Which method is used to produce sterile males?</i>	Males are vasectomized by surgical intervention (scrotal access, 0,5 cm cut, cauterization vas deferens) Duration 5-10 min. General isoflurane anaesthesia and systemic analgesia.	<input checked="" type="checkbox"/> low <input type="checkbox"/> medium <input type="checkbox"/> high	Refined method compared to abdominal access. Anaesthesia: Isoflurane. Analgesia: Metamizol 200 mg/kg p.o. (via drinking water 1d prior to surgery, 3d postoperative), Carprofen 5 mg/kg s.c. 1x intraoperative. Warming pad intra- and postoperative. Analgesia decreases postoperative pain and distress. Between mating cycles males are kept group-housed and can be used for multiple projects.	<input type="checkbox"/> non-harmful <input checked="" type="checkbox"/> mild <input type="checkbox"/> moderate <input type="checkbox"/> severe
Production of blastocysts <i>Which method is used to obtain blastocysts?</i>	Superovulation of 4 weeks old C57BL/6N females by injection of hormones (1x PMSG 2,5 – 5 I.U. i.p., 1 x HCG 5 – 7,5 I.U. i.p. 48 hours later). Mice are humanely killed by an overdose of anesthetics and blastocysts are harvested.	<input checked="" type="checkbox"/> low <input type="checkbox"/> medium <input type="checkbox"/> high	Prepubescent females produce more blastocysts compared to adults and less animals have to be used.	<input type="checkbox"/> non-harmful <input checked="" type="checkbox"/> mild <input type="checkbox"/> moderate <input type="checkbox"/> severe

¹ See Table 1. for the assignment of an uncertainty factor

² E.g. Refinement, for more details see 3RsAGENT: *Supplementary information and practical guidance*

³ Consider modulating factors of harm. The classification “non-harmful” may apply only for single procedures but is not applicable to the legal requirement of assigning an overall severity for the project.

Embryo transfer <i>Which method is planned for embryo transfer?</i>	Surgical unilateral embryo transfer (0,5 cm lateral cut) in pseudopregnant nurse females. Wound closure with suture and clips. Duration 5-10 min. General isoflurane anaesthesia and systemic analgesia.	<input checked="" type="checkbox"/> low <input type="checkbox"/> medium <input type="checkbox"/> high	Anaesthesia: Isoflourane; Analgesia: Metamizol 200 mg/kg p.o. (via drinking water 1d prior to surgery), Carprofen 5 mg/kg s.c. 1x intraoperative. Warming pad intra- and postoperative. Analgesia decreases postoperative pain and distress.	<input type="checkbox"/> non-harmful <input type="checkbox"/> mild <input checked="" type="checkbox"/> moderate <input type="checkbox"/> severe
Phenotypic characteristics <i>Which phenotype is expected?</i>	Sudden cardiac death syndrome caused by short-term arrhythmia and followed by cardiac arrest.	<input type="checkbox"/> low <input checked="" type="checkbox"/> medium <input type="checkbox"/> high	Structured welfare assessment to identify animals with impairments, if possible. See detailed plan below.	<input type="checkbox"/> non-harmful <input checked="" type="checkbox"/> mild <input type="checkbox"/> moderate <input type="checkbox"/> severe
Hygienic and husbandry conditions <i>How do local hygienic and husbandry conditions influence phenotypic characteristics?</i>	Mice are kept in IVCs under SPF conditions according to FELASA (except Helicobacter spp., Pasteurella pneumotropica, Murine Norovirus) Hygienic conditions do not influence the phenotype.	<input type="checkbox"/> low <input checked="" type="checkbox"/> medium <input type="checkbox"/> high	No special requirements.	<input checked="" type="checkbox"/> non-harmful <input type="checkbox"/> mild <input type="checkbox"/> moderate <input type="checkbox"/> severe
Breeding scheme and surplus animals <i>Which breeding scheme is planned to use? How will surplus animals be handled?</i>	F0: chimeras, F1: het x het, F2 hom x hom. After establishing the line, homozygous breeding scheme, all offspring animals can be used for analysis.	<input type="checkbox"/> low <input checked="" type="checkbox"/> medium <input type="checkbox"/> high	Because of the precision of the targeted mutation method, it is not necessary in all cases to establish more than one line to identify possible side effects which appear less likely compared to the use of random integration methods. Old breeding animals will be used for educational purposes.	
Genotyping and tissue sampling <i>Which method for tissue sampling will be used?</i>	Animals are marked by ear punching and tissue is used for genotyping.	<input checked="" type="checkbox"/> low <input type="checkbox"/> medium <input type="checkbox"/> high	A combination of identification and tissue sampling method is used and, therefore, only one procedure is necessary. After establishing the line, no genotyping is necessary according to breeding scheme hom x hom	<input type="checkbox"/> non-harmful <input checked="" type="checkbox"/> mild <input type="checkbox"/> moderate <input type="checkbox"/> severe

Note: fields highlighted in grey colour are not applicable.

Prospective severity classification for the project (overall severity⁴)

mild moderate severe

With overall uncertainty of

low medium high

<p>Plan for welfare assessment (see Table 2)</p>	<p>Structured welfare assessment planned <input type="checkbox"/> yes <input type="checkbox"/> no If yes, describe your plan.</p> <p>The welfare assessment is planned according to the medium uncertainty factor of the project. The assessment will be done by a scientist of the group and animal caretakers will be informed about the expected phenotype. If animal display any clinical signs during husbandry routine, the responsible scientist has to be informed.</p> <p><i>Time-points of assessment:</i> litter of neonatal animals within the first 5 days after birth, at weaning, every 4 weeks afterwards. If unexpected clinical signs occur, the frequency will be adapted and veterinarian involved.</p> <p><i>Parameters of assessment:</i> general appearance, body weight measurement once per month, clinical signs for cardiac insufficiency, e.g. oedema, reduced general appearance, laboured breathing</p> <p><i>Endpoints:</i> If clinical signs of cardiac decompensation occur (e.g.), animals will be sacrificed and a necropsy will be made.</p> <p>The retrospective evaluation of the welfare assessment will be discussed with the veterinarian. The plan for further breeding will be communicated to the responsible animal caretaker.</p>
<p>Retrospective evaluation is planned</p>	<p><input checked="" type="checkbox"/> yes <input type="checkbox"/> no</p>

⁴ The recommended prospective severity classification assigned to procedures should be based on the highest severity anticipated for any animal on the study (see European Commission. Working Document on a Severity Assessment Framework. Available online: http://ec.europa.eu/environment/chemicals/lab_animals/pdf/Endorsed_Severity_Assessment.pdf).

Example 2: Breeding a genetically altered mouse line to study breast cancer

A transgenic mouse line has been bred for several years at a research institute and the phenotype is well known and characterized. Homozygous mice of both sexes develop autochthonous mammary gland tumors and multifocal adenocarcinomas develop over the entire mammary fat pad. Primary tumors metastasize to lymph nodes and the lung with over 80% incidence in female mice. An early onset of palpable tumors in female mice is known and occurs with a mean latency of 53 days of age. Males also develop tumors with a later age of onset. Homozygous female mice show a loss of lactation ability. Animals will be humanely killed when reaching the following endpoints: 20% body weight loss (correction with tumor weight) or Body Condition Score 2, size or location of tumors interferes with the ability to move, tumor volume more than 1500 mm³, reduced general health condition.

Animals are observed daily and a clinical examination is performed once a week. If a mammary gland tumor reaches 1000 mm³ of volume, tumors are measured daily. Moreover, special attention is paid to tumor development in organs other than the mammary gland.

Component	Describe harm causing procedure or factor of harm	Uncertainty factor regarding harm ⁵	Modulating factors of harm that influence severity ⁶	Severity classification ⁷
Genetic engineering <i>Which technique of genetic engineering is used?</i>	not applicable for this specific case	<input type="checkbox"/> low <input type="checkbox"/> medium <input type="checkbox"/> high	-	
Sterile males <i>Which method is used to produce sterile males?</i>	not applicable for this specific case	<input type="checkbox"/> low <input type="checkbox"/> medium <input type="checkbox"/> high	-	<input type="checkbox"/> non-harmful <input type="checkbox"/> mild <input type="checkbox"/> moderate <input type="checkbox"/> severe
Production of blastocysts <i>Which method is used to obtain blastocysts?</i>	not applicable for this specific case	<input type="checkbox"/> low <input type="checkbox"/> medium <input type="checkbox"/> high	-	<input type="checkbox"/> non-harmful <input type="checkbox"/> mild <input type="checkbox"/> moderate <input type="checkbox"/> severe
Embryo transfer <i>Which method is planned for embryo transfer?</i>	not applicable for this specific case	<input type="checkbox"/> low <input type="checkbox"/> medium <input type="checkbox"/> high	-	<input type="checkbox"/> non-harmful <input type="checkbox"/> mild <input type="checkbox"/> moderate <input type="checkbox"/> severe
Phenotypic characteristics <i>Which phenotype is expected?</i>	Multifocal mammary gland tumors and metastasis to lung and lymph nodes in both sexes.	<input checked="" type="checkbox"/> low <input type="checkbox"/> medium <input type="checkbox"/> high	Endpoints: 20% body weight loss (correction with tumor weight) or Body Condition Score 2, size or location of tumors interferes with the ability to move,	<input type="checkbox"/> non-harmful <input type="checkbox"/> mild <input checked="" type="checkbox"/> moderate <input type="checkbox"/> severe

⁵ See Table 1. for the assignment of an uncertainty factor

⁶ E.g. Refinement, for more details see *3RsAGENT: Supplementary information and practical guidance*

⁷ Consider modulating factors of harm. The classification “non-harmful” may apply only for single procedures but is not applicable to the legal requirement of assigning an overall severity for the project.

	Reduction of lactation observed in nursing females 2 weeks postpartum.		tumor volume more than 1500 mm ³ , reduced general health condition. Supporting measure: Lactating mice are fed with high-energy nutritional supplement.	
Hygienic and husbandry conditions <i>How do local hygienic and husbandry conditions influence phenotypic characteristics?</i>	Mice are kept in IVCs under SPF conditions according to FELASA (except Helicobacter spp., Pasteurella pneumotropica, Murine Norovirus) Hygienic conditions does not influence the phenotype.	<input checked="" type="checkbox"/> low <input type="checkbox"/> medium <input type="checkbox"/> high	No special requirements.	<input checked="" type="checkbox"/> non-harmful <input type="checkbox"/> mild <input type="checkbox"/> moderate <input type="checkbox"/> severe
Breeding scheme and surplus animals <i>Which breeding scheme is planned to use? How will surplus animals be handled?</i>	Tumor-free hemizygous transgenic male x noncarrier female to ensure lactation ability. 25% hemizygous and 75% noncarrier offspring.	<input checked="" type="checkbox"/> low <input type="checkbox"/> medium <input type="checkbox"/> high	Noncarrier can be used for further breeding (females) and as control animals for experiments. Transgenic male breeders are used several times, but are humanely killed at the onset of tumor development.	
Genotyping and tissue sampling <i>Which method for tissue sampling will be used?</i>	Animals are marked by ear punching and tissue is used for genotyping.	<input checked="" type="checkbox"/> low <input type="checkbox"/> medium <input type="checkbox"/> high	A combination of identification and tissue sampling method is used and, therefore, only one procedure is necessary.	<input type="checkbox"/> non-harmful <input checked="" type="checkbox"/> mild <input type="checkbox"/> moderate <input type="checkbox"/> severe

Note: fields highlighted in grey colour are not applicable.

Prospective severity classification for the project (overall severity⁸)

mild moderate severe

With overall uncertainty of

low medium high

Plan for structured welfare assessment (see Table 2)	Structured welfare assessment planned <input type="checkbox"/> yes <input checked="" type="checkbox"/> no If yes, describe your plan
Retrospective evaluation is planned	<input checked="" type="checkbox"/> yes <input type="checkbox"/> no

⁸ The recommended prospective severity classification assigned to procedures should be based on the highest severity anticipated for any animal on the study (European Commission. Working Document on a Severity Assessment Framework. Available online: http://ec.europa.eu/environment/chemicals/lab_animals/pdf/Endorsed_Severity_Assessment.pdf).