



# Guideline on blood collection techniques in rodents and rabbits

Swiss Animal Welfare Officer Network

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# 1 Objective of this guideline

The examination of blood parameters is essential in animal research for many different purposes. A large variety of blood collection techniques is known. However, not all of them are feasible to obtain blood samples of good quality and/or to minimize the burden to the animal caused by the collection technique. In addition, not every method is permitted in Switzerland and specification on technical details may differ between Swiss cantons.

This guideline aims to give recommendations on available state-of-the-art blood collection techniques that are in line with the Swiss regulations and that take animal welfare aspects into consideration. Since blood collection in larger species is much easier, this guideline only focuses on laboratory rodents and rabbits, which are of small size or have difficult access to vessels.

It should be kept in mind that this guideline is intended to assist in the selection of appropriate blood collection methods. However, it does not intend to highlight the overall best technique among the methods that will be described thereafter. On one hand, this is not possible because the requirements for the methods and the respective study conditions must be considered separately and different techniques must be preferred depending on the situation. On the other hand, the data available on animal welfare aspects of blood sampling in small rodents is very limited, as confirmed by a systematic review of blood sampling methods in laboratory rodents.<sup>1</sup> The authors of this systematic review also conclude that there was not enough, high-quality evidence to make any recommendations on the optimal method of blood sampling from the point of view of animal welfare and that future high-quality studies were needed.

Also this guideline describes not every technically possible technique but only focusses on the current standard methods considered to be relevant for laboratory animal research. Pictures of the techniques have not been included because pictures and videos can be found in the referenced publications.

## **2 Swiss regulations on blood collection in experimental animals**

The Swiss regulations on experimental animal use do not give specifications or rules on blood collection techniques which are legally binding. As for all other techniques, blood collection must be performed using state-of-the-art-methods with the aim to minimize the burden of the animal used for the collection. However, the Federal Food Safety and Veterinary Office has published a technical information document that aims to give guidance for the best technique choice.<sup>2</sup> However, this technical information is from 1995 and has been only partially revised in 2017. Accordingly, it does not display up-to-date techniques and still recommends methods that are no longer in use or not accepted anymore by the cantonal veterinary authorities.

### 3 General considerations on good practice of blood collection

Criteria for selection of a blood collection technique are diverse and influenced by many different factors<sup>3</sup> (and data from the literature is sometimes conflicting<sup>4-6</sup>):

**-impact on the animal:** If different techniques are available to achieve the purpose of a study, the most harmless method has to be used, to minimize the stress and damage caused to the animals. Thus, the 3R principles need to be implemented wherever it is possible. Poor bleeding technique can result in potential side effects like avoidable stress, haemorrhage, bruising, thrombosis, infection at the site of needle entry, phlebitis, scarring and nerve damage.<sup>7, 8</sup> The impact on the animal is also influenced by the volume that is taken on single occasion or with repeated bleeds.<sup>9, 10</sup> Clinical signs shown by the animals should be assessed prior to sampling.<sup>9</sup>

**-species:** the anatomy and the size of a species influences the choice of a technique. Some veins do not exist in a species (e.g. V. sublingualis in guinea pigs<sup>11, 12</sup>) or are not feasible to be used for some collection methods (e.g. tail vein bleeding in hamsters). Also, the size and constitution of the body as well as strain and sex of an animal influences the total blood volume of an animal.<sup>9, 13</sup> In addition, the body constitution may also have an influence on available blood volumes (e.g. obese mice weigh more than other mouse strains but in relation to the body weight, their total blood volume is not increased proportionally).<sup>14</sup>

**-volume:** the amount of blood needed for a certain measurement determines the selected technique<sup>9</sup>. Technical limitations of the used measurement apparatus/equipment or the number of parameters to be measured in one sample play a role here. Some blood collection techniques draw minimal volumes only, whereas others can be used if a maximal volume should be obtained from an animal. The impact on the animal is also increasing with the increasing amount of collected volume.<sup>15</sup>

**-terminal/non-terminal collection:** if a maximal volume of blood is needed from an animal and if the animal must not survive the experiment, terminal bleeding methods are an option (done under terminal anaesthesia/right after euthanasia).

**-single/repeated bleeding:** if repeated bleedings are done, the total volume of all blood collections must be considered to prevent high blood losses that an animal cannot cope with anymore (such as tissue damages through oxygen undersupply, development of anaemia or circulatory breakdown).<sup>2, 9</sup> This is also the case for single collection of large volumes. In addition, repeated bleedings also require a repetition of tissue damage to a certain extent. This burden is even more increased if general anaesthesia or strong fixation of the conscious animal is applied.

**-anaesthesia:** some blood collection techniques are only feasible in anesthetized animals (e.g. sublingual bleeding) or should be performed under anaesthesia for animal welfare reasons (e.g. blood collections in gerbils which are difficult to restrain).<sup>16</sup> As the narcosis stresses the animal (especially if repeatedly anesthetized with a narcotic gas like isoflurane<sup>17</sup>) and may influence study results through potential metabolic changes<sup>13, 18</sup>, a technique which requires anaesthesia may be excluded in some cases.

**-quality:** before blood is taken, the experimenter should define, if venous or arterial blood is needed for the measurement of parameters. As some methods (such as excision of the tail tip skin or decapitation) are mixtures of venous blood, arterial blood and tissue liquids, they may be excluded for sensible measurements.

**-microbiological quality:** if sterile samples are required, methods that allow collection under sterile conditions or prior disinfection of the skin should be used (whereas certain techniques like retrobulbar bleeding are not adequate since disinfection is not possible).

**-expertise and training:** before an unfamiliar technique is used under study conditions, the experimenter should undergo intensive training.<sup>7</sup> Training should be done by competent trainers so that the most refined and up-to-date methods are passed on. The amount of training and practice required to achieve a given level of competence in a particular technique varies from individual to individual depending on manual dexterity, prior experience, attitude, and the skills of the instructor. Retraining or additional supervision is necessary if a technique is not conducted routinely.

**-historical reference data:** several studies have shown that blood parameters change if blood is taken from another blood vessel in the same animal.<sup>19-27</sup> Therefore, new reference data is needed if an institution changes to a new blood collection technique.

**-quality management:** the number of involved experimenters also needs to be considered. All techniques can easily be performed by a single person but some techniques need close monitoring of anesthetized animals which is easier if two experimenters work together. In addition, further processing of collected sampling (e.g. centrifugation for plasma samples) or proper documentation of high-throughput collections is much easier if experimenters work in pairs. Institutional Standard Operating Procedures help to ensure that blood collections are always performed in the same way and thus, this instrument improves data quality and reproducibility.

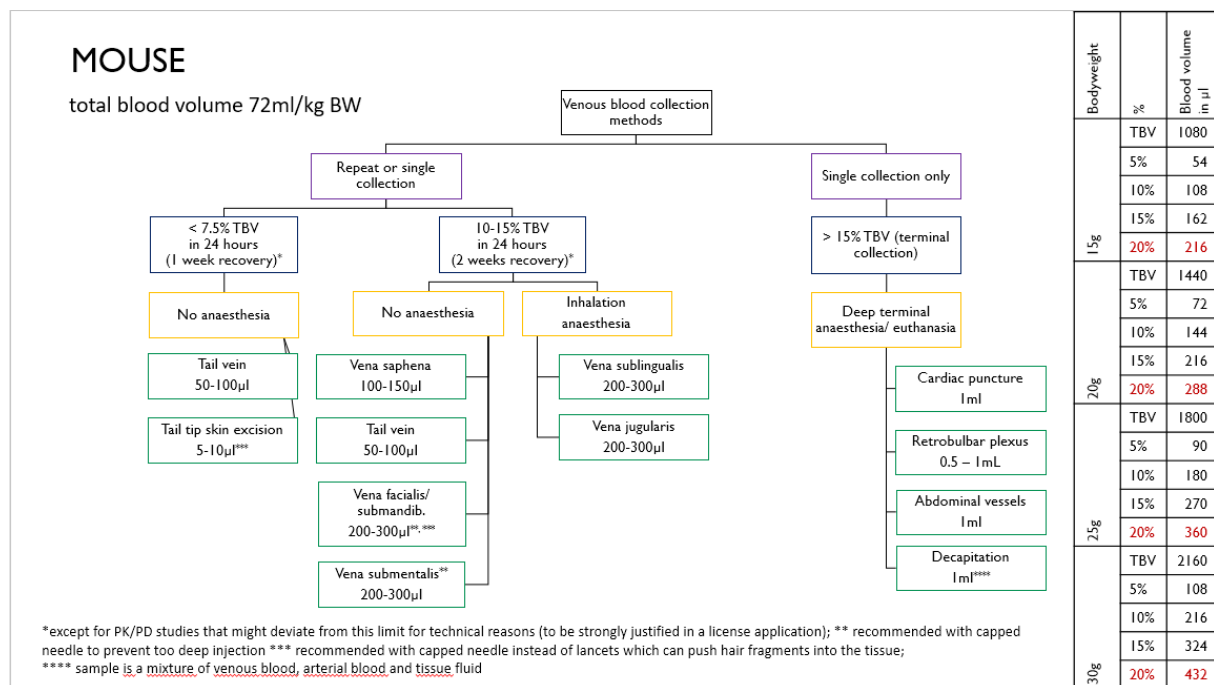
### 3.1 Recommended blood volumes to be collected

Whenever blood is drawn from an animal, the species and size of the animal must be taken into account when calculating the planned withdrawal volume. In the case of repeated collections, the individual sample quantities must be added together. Only if the animal can compensate for the sampling without pathophysiological reactions, blood sampling is not considered to result into significant constraint to the animal.<sup>2</sup> These include, for example, circulatory breakdown resulting from a blood pressure drop caused by a single large amount of collected blood or the development of chronic anaemia with frequently repeated small amount withdrawals.<sup>15, 18</sup> These limits are quickly reached, especially in small laboratory rodents. According to the technical information of the Swiss Federal Food Safety and Veterinary Office, no more than 20% of the total blood volume should be withdrawn within 2 weeks.<sup>2</sup> If this limit is reached, the animal must be given an equally long recovery period. Other recommendations set the upper limit even lower as early mechanisms of compensation are already seen with a blood loss of 10% of the total blood volume.<sup>7, 9, 28, 29</sup> The calculation of the collectable volume is also complicated by the fact that the exact total blood volume of an animal is often not known. It is therefore also possible to follow a simple rule of thumb to calculate the maximum collectable amount permitted within two weeks. According to this rule of thumb 1% of the total body weight in millilitres can be collected within two weeks. Depending on the animal species, this corresponds to approximately 15-16% of the estimated total blood volume.<sup>30</sup>

## 4 How to choose the method/decision trees

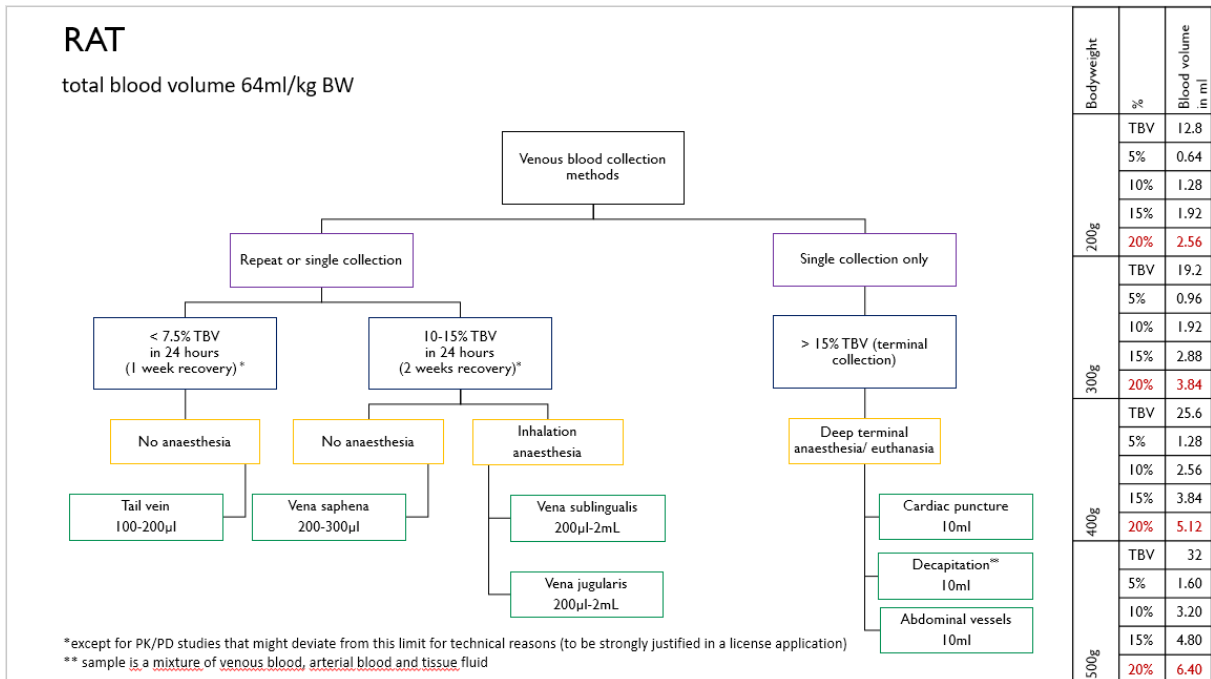
General considerations from chapter 3 are put into the following decision trees for mice and rats (Tables 1 and 2). The techniques shown are sorted according to the amount of blood to be collected with or without anaesthesia and the frequency of collection under terminal or non-terminal conditions. In addition, the right margin lists estimated values of the sampling quantity (in percent and in millilitres) in relation to the total body weight of the animal. Decision trees are only shown for mice and rats as most commonly used experimental species (recommendation for recovery period are based on published guideline of Diehl et.al.<sup>9</sup> The detailed technical description of the methods, their advantages and disadvantages as well as applicability in species are described in following chapters.

**Table 1: Overview on acceptable blood collection in mice**



Based on course materials provided by ETH-LTK-cooperation for education and training in laboratory animal science.<sup>30</sup>

**Table 2: Overview on acceptable blood collection in rats<sup>30</sup>**



Based on course materials provided by ETH-LTK-cooperation for education and training in laboratory animal science.

Decision trees were not created for the other animal species except the mouse and rat since the number of available techniques for these species is considerably smaller. However, information is always given in the descriptions of the techniques if these techniques are also applicable in other rodents and rabbits.



## **5 Accepted blood collection methods/method description**

### **5.1 Blood collection techniques in conscious animals**

#### **5.1.1 Tail vein puncture**

The tail needs to be warmed in a water bath for a few minutes for vasodilatation (temperature-controlled with thermometer, do not exceed 42°C as tail haemorrhage or necrosis would be the consequence). Warming with red light (up to 37°C for 5 to 8 minutes)<sup>9</sup> or the use of heating pads<sup>24, 31</sup> is also possible but both methods are not ideal as the full body is heated up and the animal cannot escape the unpleasant high-temperature area.<sup>32</sup> Also overheating is possible. Prior the puncture, the tail should be swabbed with alcohol for disinfection.<sup>13</sup> Puncture of the lateral tail vein requires fixation of the animal (with a restrainer or manually if two persons work together).<sup>33</sup> For mice, a 24 to 25-gauge hypodermic needle is used<sup>32</sup>, for rats a 20 to 22-gauge hypodermic or butterfly needle is needed<sup>34, 35</sup>. Some experimenters also use a hematocrit tube to collect the blood directly from the hub of the hypodermic needle in order to get the sample faster.<sup>15, 36</sup>

It is not recommended to apply slight compression and stroking from base to tip (also called “milking”) to dilate the vessels, because this results in leukocytosis of the sample.<sup>13</sup>

After removal of the needle from the tail vein, gentle pressure with a gauze sponge should be applied on the bleeding site to ensure hemostasis.<sup>15, 36</sup>

Restrainers should be cleaned before they are used for the next animals (the smell of the previous animal could stress the animal that is thereafter fixed in the restrainer).

Although allowed by some cantons, incision of the lateral tail vein<sup>32, 37</sup> is not recommended as this technique is done with a blade and destroys the vein and the surrounding tissue is cut. Consequently, the tail can be damaged more than necessary for a successful puncture.

For multiple blood samples, the first sample should always be taken from the distal end of the tail in order to prevent perivascular clots and inflammation at the proximal end of the tail that significantly reduces blood flow to the distal portion of the vessel.<sup>35</sup>

Tail vein puncture is possible in mice, rats and gerbils. This technique is not suitable for guinea pigs and hamsters because they have no real tails.

#### **5.1.2 Tail tip skin excision**

Blood sampling from the tail tip enables the collection of small amounts of mixed blood from veins and arteries as well as some lymph.<sup>15</sup> The skin at the tip of the tail is cut with a scalpel blade.<sup>38, 39</sup> It is important to cut the skin only - not larger parts as described in some publications<sup>34, 40</sup>. Cutting more than 1mm (as described in the Swiss regulatory guideline<sup>2</sup>) or even cutting the bone or other structures of the tail is a tail tip amputation that should be avoided for animal-welfare reasons. However, this term is used in publications, but differentiation to tail tip skin excision is required. For repetition of the blood sampling, the crust can be removed. The use of a restrainer is not required in mice if animals are well handled and thus not stressed by the experimenter. For larger rats, a towel can be used to hold the animals loosely.<sup>39</sup> During the sampling the animal can be placed on the cage grid and simply held by the tail.

Tail tip skin excision is possible in mice, rats and gerbils but only superficial layers of the epidermis should be cut to avoid contraction of the skin during wound healing resulting into skin retraction and necrosis of the tail tip.

### 5.1.3 V. saphena

For saphenous vein bleeding a 24 to 25-gauge (mice, gerbils) or 20 to 22-gauge (rats, hamsters, guinea pig) hypodermic needle is used.<sup>15, 41</sup> Some authors also recommend to use lancets.<sup>42</sup> In view of higher tissue damages, hypodermic needles should be preferred over lancets. Animals need to be fixated – either by hand fixation (if two experimenter work together) or with the use of a restrainer (if one person works alone).<sup>42</sup> If a restrainer is used, animals should be trained on it to reduce the stress impact of the technique.<sup>43</sup> Alternatively, this technique can also be done under slight anaesthesia to reduce stress and to facilitate the procedure.<sup>44</sup>

The skin should be carefully shaved. For a sterile probe and better visibility of the vein<sup>9</sup>, the skin of the puncture site can be disinfected (puncture after skin has dried). Slight careful pressure is induced on the leg above the puncture site to compress the vessel for venous congestion. The visible vein is punctured and blood drips into the microtube. Usually, the lateral vein is a bit larger and more visible than the smaller medial vein.<sup>41, 45</sup> Some technicians use bland ointment to improve the blood drop formation as blood cannot seep into the fur<sup>42</sup>; however, potential effects through contamination of the collected blood sample were not described in the literature.

Restrainers should be cleaned before they are used for the next animals (the smell of the previous animal could stress the animal that is thereafter fixed in the restrainer).

### 5.1.4 Ear vein or artery

For central ear vein or ear artery blood collection in rabbits, the animals are tightly wrapped in a towel<sup>46</sup> or put in a fixation box. The ear of the animal can be warmed with the heating lamp for vasodilatation (a warming box should not be used as it has a higher risk for overheating of rabbits). In rabbits difficult to handle, EMLA cream (Lidocaine 2.5% + Prilocaine 2.5%, Streuli Pharma AG) for local anesthesia can be applied on the ear 30min before the blood collection. Before the puncture, potential rests of the cream must be removed to prevent sample contamination.<sup>47, 48</sup>

For arterial blood, a 21 to 23 gauge venous catheter or butterfly needle (depending on the size of the rabbit) is inserted into the central ear artery of rabbits (for volumes > 5ml). For volumes < 5ml, the marginal ear artery can be used alternatively.<sup>36, 49</sup>

For venous blood, the lateral (marginal) or central ear vein is punctured with 19 to 23 gauge butterfly needle, depending on the strain and size of the rabbit<sup>49</sup> after congestion of the vein. After the blood collection, constant pressure with a cotton swab should be given to the puncture site for at least two minutes.<sup>36</sup> This is even more relevant for collections from the central ear artery. Strong flat hairgrips can be put over the swab to fix the compression for several minutes, so one can proceed with the next animal while the first one is in waiting position, until the bleeding fully stops.<sup>50</sup> Incision of the ear arteria is not recommended as prolonged bleedings cannot be excluded and the artery is damaged more than necessary.

### 5.1.5 V.facialis/submandibularis

For submandibular blood collection (“cheek bleed”) anesthesia can be used as this method is considered stressful method in conscious animals.<sup>51</sup> If no other option is possible (like large volumes are required but no anesthesia is possible) it is acceptable to perform this method. In any case, 4-5mm GoldenRod Animal Lancets (Medipoint Inc, Mineola, NY, USA) should be

used instead of hypodermic needles as they prevent a puncture that goes too deep into the tissue (what leads to massive tissue damages). The size of the lancet needs to be adapted to the body size of the animal.

The animal is taken at its tail and neck and back skin for firm fixation and congestion of the head veins. With the lancet the puncture in an area approximately 3 mm caudal and 1 mm dorsal to the lateral tactile hair of the cheek is set. Dripping blood from the puncture site is collected in a microtube. If the restraint is released, bleeding stops.<sup>51, 52, 53</sup>

Note that hair fragments can be pushed into the tissue which prolongs the duration for tissue healing after this blood collection.<sup>51</sup>

It remains unclear which vein is exactly punctured by this method as several veins are located in the submandibular region of the mouse. Likely the superficial temporal and maxillary veins are targeted by this method, but potentially also the linguofacial or facial veins are hit.<sup>54</sup> However, the terminus submandibular bleeding is in use for this method.<sup>51, 54</sup> Facial<sup>40, 55, 56</sup> or cheek<sup>57</sup> bleeding are also in use as synonyms.

### 5.1.6 V.submentalis

Submental bleeding (“chin bleed”) is done in mice with the same technique as described for submandibular bleeding. Different to that, veins in in submental region are punctured with a 4-5 mm lancet.<sup>54</sup> or with a 24 to 26 gauge needle. However, different veins in this region can be punctured and it remains unclear if submental bleeding is targeting the same veins than submandibular bleeding.<sup>58</sup> Restrain the mouse with the non-dominant hand by grasping the loose skin over the shoulders and behind the ears; the skin should be tightly stretched under the chin (submental region), but not so tight as to restrict breathing.

A 4-5mm lancet or 25 to 26g needle is used to puncture over the dark area, just caudolateral to the chin on each side (memorize pictures first!), where the submental veins converge. When not readily visible, this area can be located by moving slightly rostralateral from a group of hairs (whirl) located on the midline of the throat and finding a slightly softer spot (gutter) in the tissue just medial to the facial vein. If using a needle, only the tip of the needle should enter the vessel to a shallow depth of about 3 mm. Insert and withdraw the lancet or needle in a smooth, firm motion. Blood will flow immediately. Collect sample with a capillary or a collection tube, until the target volume is reached.

Bleeding typically stops when the mouse is immediately released and the head position goes back to normal. Instant release of the animal is important to prevent unintentional oversampling of blood.

No information is available on stress impact or tissue damages of this method. If lancets are used instead of needles, it cannot be excluded that hair fragments potentially could be pushed into the tissue as it is shown for submandibular bleeding.<sup>51</sup> Different to submandibular bleeding, no muscles are cut (less tissue damage) and the animal does not see the lancet as the puncture is done from ventral direction (not within the vision field of the animal).

To reduce tissue damages caused by the lancet, a stop-capped needle can be used so the needle cannot accidentally be pushed too deep (see Picture 1 below).

**Picture 1: Stop-capped needle**



Stop-capped needles can be made very easily by shortening the protective cap of the hypodermic needle with sharp scissors or a scalpel blade. The shortened cap is then placed on the hypodermic needle so that the injection depth of the needle is shortened.<sup>59</sup>

If the target volume to be taken is close to the maximum that is allowed for the body weight, and/or if the mice are very small, short-term inhalation anaesthesia of these animals prior the sampling is recommended for better control over the extracted volume and for more controlled stop of bleeding than it is possible in a conscious animal. Anaesthesia also reduces the blood flow which helps to prevent unintentional oversampling of blood.

### **5.1.1 Implantation of catheters**

Catheterization of larger vessels are useful if blood volumes are required repeatedly for a long-term period, especially if very small amounts are collected.<sup>60-62</sup> Using the catheters, stress and discomfort in the animals can be reduced as animals do not need to be restraint and punctured again. However, single housing of catheterized animals is necessary to prevent the damage of the catheters.<sup>9, 63</sup> As single housing is stressful to gregarious species, this additional constraint must be considered during the determination of the severity degree. The implantation requires a surgery under aseptic conditions and general anaesthesia.<sup>26, 57, 64, 65</sup> Alternatively, rats and mice with already implanted catheters are offered by specialized breeders. The use of such animals is recommended if institutions that have no experience in this surgery want to work with catheterized animals. On a frequent basis, catheters must be flushed with an anti-coagulant solution to prevent blockage.<sup>2</sup>

## 5.2 Blood collection techniques in anesthetized animals (non-terminal)

Techniques described in this section require anaesthesia to guarantee that the drawing is performed in a correct manner and that the stress impact on the animal is minimized.

### 5.2.1 V. sublingualis

The anesthetized animal is brought in supine position (inhalation narcosis). The tongue is pulled out of the mouth by using two fingers. The visible left or right V.sublingualis is punctured with a 24-gauge (mice, gerbil) or 22-gauge (rats, hamsters) hypodermic needle in a 30 to 45 angle.<sup>66</sup> Half of the sharp part of the needle is inserted in order to guarantee a good blood flow and to prevent unnecessary tissue damage if pushed too deep. The needle is removed then and the animal is brought in ventral position. The animal is taken with a strong neck skin fixation grip which leads to congestion of head veins. Immediately, blood drops out of the puncture site are collected into blood sampling tubes. As anesthetized animals cannot swallow, the experimenter needs to hold the animal in a strictly horizontal position while collecting blood to prevent aspiration of blood. To stop the bleeding, the animal is put on the experimental desk in ventral position and the neck skin grip is released. The animal must not be turned in supine position to prevent blood to reach the animal's trachea. The application of iron chloride on the puncture site<sup>21</sup> is neither recommended in view of the irritating effect of this substance and since it is simply not necessary as the bleeding stops after release of the neck skin grip.<sup>11, 51</sup> The mouth is cleaned from remaining blood.<sup>9, 11, 51</sup>

Sublingual bleeding can be performed with a single technician or with two technicians working in pairs. If working in pairs, the collection can be done using the same sequence as described above, except the neck skin is already grasped before puncturing the congested sublingual vein.<sup>11, 21, 51</sup> In addition, it is helpful to hold the animal's head downwards while taking it out from the anesthesia box to the desk used for puncture. In such a position, the animal's head is flushed with blood. This facilitates bleeding, especially in mice.

Anesthesia must be deep enough to allow sufficient time for the blood collection. However, too deep anesthesia leads to gasping breathing which disturbs the collection.

Although described differently in some publications<sup>67, 68</sup>, sublingual bleeding has to be performed under anesthesia for animal welfare reasons (conscious animals react with strong defence movements against the procedure). Furthermore, blood collection may fail in conscious animals as veins are not visible.<sup>67</sup>

Sublingual bleeding is not possible in guinea pigs as the vein is not apparent in this species.

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### 5.2.2 V. jugularis puncture

The anesthetized animal (inhalation narcosis) is fixed by grasping the loose skin of the back firmly with fingers. The head is elevated without spreading the submaxillary gland. Shaving around the thoracic region/ventral side of the neck of pigmented animals is recommended. The vein can be easily identified in albino animals. In pigmented animal, the slight depression at the junction of the pectoral muscles and the M. sternocephalicus is visualised. To minimize interference due to respiratory movements and for better accessibility of the puncture site, the corresponding front limb is placed between the index and the middle finger, meanwhile the thumb and the ring finger gently fix the ipsilateral ear.<sup>69</sup> A 25 to 27-gauge needle connected to a 1-mL syringe is inserted dorsoventrally towards the sternal manubrium into the jugular vein through the pectoral muscle below the sternoclavicular junction (1-3mm deep and 2-4mm

laterally to the sternoclavicular junction).<sup>15, 69</sup> In guinea pigs a 24 gauge 5/8-in needle with 1-mL syringe is used.<sup>15, 70</sup> Slow blood withdrawal avoids the collapse of the vessel.<sup>15</sup> Immediately after blood collection, gentle pressure is applied to the site to stop the bleeding. This blood collection method is performed by two technicians.<sup>71</sup>

### **5.2.3 V. facialis/submandibularis**

4-5mm GoldenRod Animal Lancets (Medipoint Inc, Mineola, NY, USA) should be used instead of hypodermic needles as they prevent a puncture that goes too deep into the tissue (what leads to massive tissue damages). The size of the lancet needs to be adapted to the body size of the animal.

The anesthetized animal is taken at its tail and neck and back skin for firm fixation and congestion of the head veins. With the lancet the puncture in an area approximately 3 mm caudal and 1 mm dorsal to the lateral tactile hair of the cheek is set. Dripping blood from the puncture site is collected in a micro-tube. If the restraint is released, bleeding stops.<sup>51, 52</sup> Slight pressure on the puncture site can be applied in order to prevent the development of hematomas.

Note that hair fragments can be pushed into the tissue which prolongs the duration for tissue healing after this blood collection.<sup>51</sup> In order to lower the stress impact on animals, anaesthesia prior submandibular blood collection can be considered.<sup>51</sup>

Special caution should be taken in animals with impaired blood coagulation. In FVIII knockout mice lacking coagulation factor VIII, excessive haemorrhages were observed after a single puncture of the submandibular vein leading to euthanasia of 60% of the mice within four hours after the blood collection.<sup>72</sup>

## **5.3 Terminal blood collection techniques in deeply anesthetized or euthanized animals**

### **5.3.1 Retrobulbar venous plexus**

Retrobulbar bleeding is carried out in the anaesthetized mouse using a 20 µL glass micro-haematocrit capillary. The capillary is inserted at a 45° angle into the medial corner of the eye. Grasping the neck skin leads to adequate congestion of the head veins. Blood drops from the capillary end into a micro collection tube. Bleeding stops if the neck skin is released and the capillary is removed. Retrobulbar bleeding is known to be responsible for massive tissue damage as diverse structures of the eye region (bulbus, muscles, optic nerve, Harderian gland, fat tissue) are directly or indirectly damaged by this method.<sup>9, 11, 23, 73-75</sup> This is also the case, if carried out by experienced technicians.<sup>8</sup> Consequently, negative effects using this blood collection method were observed in animals after recovery from anesthesia.

Therefore, this method is only recommended as terminal technique in mice and gerbils. Animals do not recover from anesthesia and as much blood as possible is taken from them (so they die during the procedure due to the breakdown of the circulatory system caused by the massive blood loss). Ensure death at the end of the blood sampling by either decapitation or induction of a bilateral pneumothorax.

For non-terminal procedures in rare exceptional cases, some labs apply a drop of 0.5% proparacaine hydrochloride ophthalmic solution 5 min prior to sampling to the anesthetized animal in order to have an analgetic effect after the animal's recovery from anesthesia.<sup>24</sup> It remains unclear how long the analgetic effect is seen in animals, to what extent this could lead to contamination of the sample or unwanted side effects in the animal (ear tear dropping).

Isoflurane anesthesia in rats and hamsters is not long-lasting enough to guarantee that the animal dies through circulatory breakdown under blood collection before it recovers from anaesthesia. Therefore, this method is not recommended in other species than mice and gerbils as less stress- and painful methods are available. However, this method is acceptable when carried out under a deep injection anesthesia in rats or hamsters and death is confirmed afterwards via e.g. exsanguination, decapitation or bilateral opening of the thorax.

The retrobulbar venous plexus is located caudally in the bone orbita behind the eye bulbus, the eye muscles, Harderian gland and fat tissue.<sup>11, 12</sup> As it is located within the bone orbita but not behind it, the former names of this method orbital bleeding or retro-orbital bleeding are not correct although used in some publications.<sup>26, 27, 33, 36, 54, 58, 75-77</sup>

### 5.3.2 Abdominal aorta

Deeply anaesthetize the mouse and place it in dorsal recumbency, wet the abdominal area with water or alcohol. Open the abdominal cavity with surgical scissors by making a large V-cut through the skin and abdominal wall. Extend the incision line from the pubis over the sternum. Locate the widest part of the abdominal aorta by shifting the intestines to one side and the liver cranially. Alternatively, the intestines may be exteriorized using a gauze sponge. Insert a 23-26 gauge needle into the aorta. Once the needle is inserted, aspirate on the syringe plunger, ensuring that blood is drawn into the syringe. When the procedure has been completed, cut with scissors through the aorta and the diaphragm to ensure death.<sup>78</sup>

In hamsters a medial incision that opens the abdomen is recommended and a 23 gauge needle is used.<sup>36, 79</sup>

### 5.3.3 Cardiac puncture

Cardiac puncture is possible in deeply anesthetized animals<sup>9</sup> or in CO<sub>2</sub>- euthanized animals (immediately after confirmation of the death of the animal). For deep and long-lasting anaesthesia, high-dosed Pentobarbital can be used. Inhalation anaesthesia is not adequate for this method as animals recover too early. Optionally in mice and rats<sup>80</sup>, either the chest is quickly opened with a scissor and the heart directly punctured or the needle with syringe is inserted through the skin between the 5<sup>th</sup> and 6<sup>th</sup> rib in a 45° angle towards the right heart ventricle, if the animal lies on the right side.<sup>81</sup> Alternatively, the mouse is lying on its back, so that the 20 to 22 gauge needle<sup>36</sup> passes the diaphragm laterally to the xyphoid and medially at a 20–30° angle to the animal's horizontal axis to the sternum in the direction of the heart.<sup>82</sup>

While the needle is slowly advanced into the heart, very slight negative pressure on the barrel of the syringe should be applied to ensure that blood flows into the hub of the needle when the tip has entered one of the chambers of the heart. Advancing or retracting the needle tip may be necessary to obtain a maximal volume.<sup>36</sup>

The first option (euthanized animal) guarantees that the right ventricle of the heart is punctured (red dark ventricle), but takes a few seconds longer. The second option (anesthetized animal) is quicker but needs more training and contamination with tissue cells (e.g. of the lungs) is possible. Furthermore, if the second option is chosen, death needs to be confirmed in the end by either opening the chest cavity for bilateral pneumothorax or decapitation.<sup>36</sup>

In deeply anesthetized hamsters, the puncture is done from the side with the 23 gauge needle inserted in a 30° angle into the 4th to 5th intercostal junction, directly at the apex beat of the heart.<sup>36, 79</sup> This technique can also be used in deeply anesthetized guinea pigs using a 20 to 25-gauge hypodermic needle at a 30 to 45° angle towards the heart ventricle.<sup>65</sup>

If this method is done in deeply anesthetized animals, it must be guaranteed that the animal never recovers from anaesthesia during the procedure until it dies due to the massive blood loss and circulation breakdown.

Cardiac puncture is a suitable technique to obtain a single, large, good quality sample from an euthanized rabbit or a rabbit under deep terminal anesthesia if coagulation parameters, a separate arterial or venous sample or cardiac histology are not required. A sample of 60 to 200 ml of blood can be obtained depending on the size of the rabbit and whether the heart is still beating. Alternatively, in deeply anaesthetized the rabbits (Ketamine/Xylazine mix heavily overdosed) the heart is entered with a needle (14G TSK cannula), connected with a connecting tube to a 60 ml syringe, from the sternum, in a 30° angle. After blood withdrawal, the animal is injected with Pentobarbital administered intracardially, to ensure death. Videos of the procedures in several species are online available.<sup>83</sup>

### **5.3.4 Decapitation**

With this method, the animal's head is separated from the neck using a sharp instrument. The quick and effective cutting is carried out with specially developed devices (guillotines) by well-trained personnel, only.<sup>81</sup> It is particularly important to ensure that the knife cuts the animal's neck close to the head. For animal welfare reasons, mice, rats and hamsters older than 2 weeks have to be under general anaesthesia before decapitation takes place or have to be killed with another method than decapitation.<sup>84</sup>

In animals up to 2 weeks old, a short, strong scissor stroke can also be used<sup>84</sup>. Anaesthesia is not mandatory but highly recommended for animals older than 5 days (mice, rats, hamsters) based on an animal welfare point of view. In any case, it is important to clean the device thoroughly after each animal killed, so that the following animal is not stressed by traces or the smell of blood.

Decapitation enables the rapid collection of a maximum amount of blood. However, the sample is a mixture of venous, arterial and tissue fluids.<sup>81</sup>

It should be noted that decapitation can also lead to psychological stress<sup>85</sup> on the person performing it.<sup>86</sup> This is particularly the case if animals are not anaesthetised and/or have to be regularly killed using this method.



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