NOTE: This document includes blood collection standards that have been researched and published as well as standards that have gained acceptance through empirical use across multiple members of the IQ 3Rs leadership group (LG) and partner CROs.

**Goal:** The goal of this project was to develop and implement harmonized guidelines for the humane collection of blood from laboratory animals in biomedical research.

**Anticipated Benefit:** Distribution and implementation of shared guidelines should 1) reduce experimental variables between studies that are/may be conducted, and in so doing, enhance the consistency and quality of the resulting experimental data, 2) enhance animal welfare and foster the 3Rs and 3) enhance biopharma and CRÓ collaborations/partnerships in designing and executing animal studies efficiently and in the spirit of employing best current practices.

**Introduction:** The harmonized blood collection guidelines provided here represent the consolidation and harmonization of institutional guidelines provided by 24 companies that are members of the IQ 3Rs LG or their CRO partners.

The guidelines provided here are, in general, appropriate for healthy, young adult animals. Compromised health/study related stress can exacerbate blood loss issues and inhibit recovery, and must carefully be taken into consideration.

The primary goals are to 1) minimize and/or alleviate pain and distress, and therefore maximize the welfare of the animal subjects and 2) provide consistent, reproducible, high quality data from animal experiments that meet scientific objectives. In the context of striving for these goals, there are many details associated with different kinds of experimental protocols in multiple species and types of institutions that must be evaluated to determine the maximum volume and frequency of blood collection that can be permitted without negatively impacting the health and welfare of the animals and/or the scientific integrity of the resulting experimental data.

The authors of this guideline hope that this document will be of value as a reference for institutions to develop and/or refine internal guidelines and will facilitate inter-institutional harmonization of blood collection guidelines.

It is the Institutional Animal Care and Use Committee (IACUC) or equivalent ethical review committee that typically assumes responsibility for the development and implementation of such guidelines to govern research conducted at the institution with the species of animals employed in that research.

**Animal Welfare Oversight:** Most guidelines clearly state that if there is a proposal by a research investigator, study director or study sponsor to exceed the recommended limit guidelines or minimum recovery periods that approval must be obtained in advance by the IACUC or equivalent. Oversight committees may also periodically monitor the effectiveness of procedures to ensure animal welfare associated with blood collection procedures (Post-approval Monitoring). Additional skill development may be required as determined by post-approval monitoring outcomes.

**Role of the Attending Veterinarian and other Veterinary Specialists:** The Attending Veterinarian or local equivalent should be consulted to provide professional advice during protocol design, execution and data interpretation. The professional guidance of a veterinarian, especially one with clinical pathology expertise, should also be sought to address any clinical or hematologic abnormalities identified in animals that may be related to blood collection procedures.
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General Principles: The acceptable quantity and frequency of blood sampling (time to recovery) from laboratory animals in biomedical research is primarily dependent on the total circulating blood volume of the animal, the red blood cell (RBC) turnover rate and the general health of the animal. Guidelines for safe blood withdrawal from laboratory animals take into account that each species has a different blood volume per unit body weight (Appendix 1). Guidelines for safe blood withdrawal also consider that after collection of maximal blood volumes from animals, the blood volume can typically be restored within 24 hours but that it takes 14-21 days for blood cell renewal/replacement from the bone marrow.

Blood loss: During blood volume calculations, it is also important to consider “accidental” blood loss that may result from obtaining a larger than intended sample volume, blood loss outside of a designated collection vial or syringe, blood loss through aspiration from an indwelling cannula that is not returned to the animal, or blood loss during attempts to achieve hemostasis (e.g. when animals do not have normal hemostasis or the sample is collected from an artery).

Restraint: Animals must always be adequately trained, restrained, tranquilized, sedated or anesthetized during blood collection procedures to minimize risk of injury to both the animal and the operator and to minimize distress and/or pain to the animal. The method of restraint selected (physical, chemical or both) must be appropriate to the species, experimental requirements, availability of appropriate restraint systems/equipment and the skill of the operators. Animals should be acclimated to restraint devices whenever feasible. Distress associated with restraint procedures and physiologic effects of chemical agents can alter blood cell counts and/or measurements of serum or plasma analytes. These potential experimental variables must be considered at the time of experimental design.

Site Preparation: Proper blood collection site preparation must also be considered. Collection of sterile blood samples requires aseptic collection technique, i.e. clipping or shaving of fur followed by aseptic preparation of the skin at the site prior to needle puncture of the vessel.

When short term vascular catheters or chronic, indwelling vascular cannulas and/or access ports are implanted, care must also be taken to provide asepsis at the time of implantation (e.g. clipping of fur, antiseptic cleansing of the skin and aseptic surgery) as well as during subsequent handling of the catheters/cannulas and ports to avoid implant-related infections and/or sepsis that may negatively impact animal health and welfare and/or confound interpretation of experimental data.

Protocol Preparation: Blood collection considerations to enhance animal welfare and the 3Rs and provide high quality experimental data for experimental protocols:

- Scientific objectives of the study/model and needs for blood sampling
- Analytical methods to be used that influence the blood sample volume required (NOTE: Work with bioanalytic specialists to obtain the least volume as a 3Rs refinement).
- Determine whether or not food must be withheld prior to sample collection
- Animal species, age, size, gender and general health
- Anatomic differences between species
- Presence of any condition or disease pathology in the animal such as pregnancy, concurrent menses, neonatal, advanced age, genetic mutation(s), anemia, bone marrow hypoplasia, dehydration, diabetes, inadequate nutrition, obesity, organ system pathology, concurrent microbial infection, abnormal hemostasis, effects of substances administered concurrently, or any condition that may affect cardiovascular function and/or blood cell or protein production or loss.
- If the sampling techniques or volumes require anesthesia as a terminal procedure
- Volume, sampling frequency, type of sample needed (serum, plasma, whole blood)
- Potential need to replace blood volume (whole blood, fluids, volume expanders)
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- Ability to use microsampling to limit blood collection volumes
- Blood sample quality required (sterility, tissue fluid contamination, fur contamination)
- Anesthetic, restraint procedure or blood collection method effects on blood parameter(s)
- Availability of appropriately sized needles, syringes, catheters, canulas and VAPs
- Availability of appropriate collection vials, tubes, dried blood spot cards (to include use of graduated blood collection tubes with volume markers and/or pre-measurement of the blood volume to be collected and pre-marking of blood collection tubes or use of blood spot volume/size markers for dried blood spot cards is recommended)
- Training and expertise of the staff in the preparation for, collection of, processing of and delivery of samples to the appropriate investigator or laboratory for subsequent analyses

Additional considerations for minimizing pain and/or distress and enhancing the 3Rs can be found in Appendix 2.

<table>
<thead>
<tr>
<th>Type of Samples</th>
<th>Maximum volume</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximum: Don’t exceed 18% TBV (1.3% of</td>
<td>Anecdotal: CRO partner experience</td>
</tr>
<tr>
<td></td>
<td>BW in grams)</td>
<td></td>
</tr>
<tr>
<td>Serial, Repeated Blood Samples</td>
<td>15-20% TBV</td>
<td>Adams et al., 2014, Diehl et al., 2001, Ooms et al., 2004, Raabe et al., 2011</td>
</tr>
<tr>
<td>over 24-48 hrs</td>
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</tbody>
</table>

**Recovery:** Note that we did not list recovery timeframes for acute or serial blood collection methods. Recent publications have shown data to support the hypothesis that Cynomolgus (Adams, et al., 2014), dogs (Ooms et al., 2004) and mouse (Raabe et al., 2001) erythrograms recover more rapidly after blood collection than general guidelines suggest, allowing a greater percentage of their TBV to be collected acutely and serially.

The maximum volume and frequency impacts animal health and welfare and is determined by many considerations to include species, age and general health of the animal, blood collection technique to be used and experimental goals. Since study animals may have other compromising health issues due to effects from compounds, the IACUC should only approve higher blood withdrawal volumes and frequencies with careful consideration of all of these parameters.

Special Considerations for Toxicokinetic/Pharmacodynamic studies: In studies where blood hematology and chemistry are not critical components of the analysis, larger samples than those recommended above may be taken with IACUC or equivalent approval. *Note: More recent scientific publications have shown that up to 15% TBV can be removed in consecutive weeks in young, healthy macaques (Adams et al., 2014) and dogs (Ooms et al., 2004) and as much as 25% TBV can be collected once weekly from female C57BL/6 mice for 6 consecutive weeks and as much as 15% TBV can be collected once weekly from male C57BL/6 mice for 6 consecutive weeks without producing weight loss, behavioral changes or clinically significant anemia (Raabe et al., 2011). These data also support the notion that blood regeneration may be correlated to body mass along an allometric scale, with mice having a faster blood regeneration rate than that of larger species, including rats, dogs, and horses (Raabe et al., 2011). Additionally, volumes in excess of the recommended limits may be taken within a few hours of euthanizing rodents without adversely affecting the clinical condition of the animal or subsequent histopathology investigations when approved by the IACUC or equivalent.

3
Calculating Blood Sample Volume: Blood sample volume = body weight in g [or kg] x mean blood volume (ml/g) [or (ml/kg)] x percent of blood volume to be removed (as a decimal e.g. 10% = 0.10)

- e.g. 25 g mouse x 0.071 ml/g = 1.78 ml TBV x 0.10 = 0.18 ml
- e.g. 5.0 kg Cynomolgus monkey x 65 ml/kg = 325 ml TBV x 0.10 = 32.5 ml

<table>
<thead>
<tr>
<th>Potential Untoward Effects of Excessive Blood Loss on Animal Physiology (Single Draw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOTE: These effects will vary depending on species and overall health status</td>
</tr>
<tr>
<td>Removal of 10% of blood volume</td>
</tr>
<tr>
<td>Removal of 15-20%</td>
</tr>
<tr>
<td>Removal of 30-40%</td>
</tr>
<tr>
<td>Removal of &gt; 40%</td>
</tr>
</tbody>
</table>

Monitoring for Untoward Effects of Excessive Blood Loss on Animal Physiology: Safe blood withdrawal guidelines aim to avoid untoward effects on animal physiology. These adverse effects may be associated with collecting too large a volume of blood over a short period of time, or over long periods of time via repeated sample collections. Potential untoward effects of blood loss are listed below and can be used as warranted to monitor animals.

### Acute Blood Loss (Acute Sampling)
- Cold, pale skin and/or extremities
- Thirst, reduced urination
- Rapid, weak pulse
- Dull eyes
- Low blood pressure
- Shift of extracellular fluid to the vascular system
- Rapid, shallow breathing (tachypnea)
- Restlessness, unconsciousness
- Weakness, fatigue, collapse
- Death
- Hemorrhagic shock

### Chronic Blood Loss (Serial, Repeated Sampling)
- Anemia
- Lethargy
- Exercise intolerance (easily exhausted)
- Decreased growth rate
- Hypoproteinemia
- Extramedullary hematopoiesis
- Pale mucous membranes (tongue, gums, ear pinnae)

Monitoring for Anemia and Hypoproteinemia: By monitoring the hematocrit (HCT) and plasma total protein (TP) (+/- other parameters such as RBC count, white blood cell (WBC) count, hemoglobin, etc.), it is possible to evaluate whether an animal has sufficiently recovered from a single or multiple blood sample collections. After acute blood loss, it may take up to 24 hours for the fluid component of the blood to be replaced, and to reflect blood cell or protein loss in HCT or TP measurements.

Normal reference ranges for the measured parameters should be established for the hematology instrument used based on the species, gender, age, breed, stock, strain and vendor or source. It may, however, be necessary to obtain normal ranges from the vendor or published literature.

Maximum Frequency and Number of Sample Collections: The maximum frequency and maximum number of sample collections, per collection method and/or per animal, impacts animal welfare and is determined by many considerations to include species, age and general health of the animal, blood collection technique to be used and experimental goals. Although requested by many IQ 3Rs LG members, consensus on maximum frequency and maximum number of sample collections, per collection method and/or per animal, could not be reached.

It is recommended that when possible, the IACUC or equivalent establish maximum frequencies for blood collection. In all cases, the maximum frequency of blood collections should be defined in the
IACUC protocol or equivalent and be approved by the IACUC or equivalent. Where applicable, blood collections should be alternated between vessels on the right and left sides to minimize repeated trauma at the same site and allow a longer period of healing for individual sites between samples. The IACUC or equivalent may also choose to establish a maximum number of unsuccessful attempts to obtain a blood sample at specific collection sites and/or per animal per collection and define associated contingencies.

**Blood Collection via Surgically Implanted Cannulas:** The use of implanted cannula(s) allows for the removal of blood samples with minimal disturbance to the animal. Blood samples may either be taken manually from the cannula or utilizing an automated collection system. If a cannula is exteriorized (e.g. animal in a jacket or harness with or without a tether), it is important to ensure that the cannula is protected from trauma that may be caused by the animal or its cage mates.

**Blood Collection via Vascular Access Port (VAP):** Consideration should be given to establishing limits on the frequency and/or total number of times a subcutaneously implanted access port can be punctured over time. Repeated puncturing of the skin over an access port may cause necrosis of the skin and subsequent exposure of the access port through the skin with associated risk of infection. It is also important to ensure aseptic preparation of the skin over the port to minimize risk of infection and ensure use of appropriate non-coring needles to maintain the integrity and useful life of the VAP.

**Exclusions:** In an effort to focus on the stated goals above, it was necessary to exclude a considerable amount of information from this guideline that remains very important to achieving the objectives stated above:

- There are animal welfare regulations, guidelines and policies that govern the use of animals in research in many countries, states, provinces, cities, etc. that must be adhered to. There was no attempt within this guideline to ensure compliance with all federal, state, provincial or local laws or guidelines since many of these differ across countries.
- Because of the huge number of well-established blood collection procedures in many different species of animals used in research, inclusion of detailed procedural descriptions within this guideline document was not possible.
- Description of the implantation and maintenance of temporary or chronic vascular catheters and access ports was not possible to include in this guideline.
- It is beyond the scope of this document to include specific recommendations for blood collection in embryonic, neonatal, pregnant or aged animals.
- These guidelines are focused on the collection of survival blood samples and does not specifically address the collection of terminal blood samples under anesthesia.
- It is also beyond the scope of this document to include detailed information about recommended procedures for sample handling, processing, storage conditions and analysis following collection of blood samples.

**Proposed Plan for Keeping the Guidelines Current:** These guidelines will be posted electronically on the IQ 3Rs LG website and be reviewed and updated by the CRO WG at least every 3-5 years. More frequent updates will be possible if new literature is published about this topic to incorporate technological, animal welfare and 3Rs-related enhancements in a timely manner.
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References:


**Appendix 1 - Estimating Total Blood Volume (TBV) by Species**

<table>
<thead>
<tr>
<th>Species</th>
<th>Range of TBV (ml/kg)</th>
<th>Recommended Average (ml/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>62-80</td>
<td>71</td>
</tr>
<tr>
<td>Rat</td>
<td>58-70</td>
<td>64</td>
</tr>
<tr>
<td>Hamster</td>
<td>64-80</td>
<td>72</td>
</tr>
<tr>
<td>Gerbil</td>
<td>59-85</td>
<td>72</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>64-90</td>
<td>77</td>
</tr>
<tr>
<td>Rabbit</td>
<td>44-70</td>
<td>56</td>
</tr>
<tr>
<td>Ferret</td>
<td>60-80</td>
<td>70</td>
</tr>
<tr>
<td>Cat</td>
<td>45-75</td>
<td>60</td>
</tr>
<tr>
<td>Dog</td>
<td>80-90</td>
<td>85</td>
</tr>
<tr>
<td>Marmoset</td>
<td>60-70</td>
<td>65</td>
</tr>
<tr>
<td>Cynomolgus</td>
<td>55-75</td>
<td>65</td>
</tr>
<tr>
<td>Rhesus</td>
<td>44-68</td>
<td>56</td>
</tr>
<tr>
<td>Swine</td>
<td>51-69</td>
<td>60</td>
</tr>
<tr>
<td>Göttingen Minipig</td>
<td>61-69</td>
<td>65</td>
</tr>
<tr>
<td>Sheep</td>
<td>60-74</td>
<td>66</td>
</tr>
<tr>
<td>Goat</td>
<td>57-89</td>
<td>70</td>
</tr>
<tr>
<td>Chicken</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>
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Adapted from Altman and Dittmer, 1974 and Joint Working Group on Refinement, 1993
Appendix 2 – Additional Considerations for Pain and/or Distress and 3Rs

- TRAINING: Always ensure that phlebotomists are satisfactorily trained and experienced to competently conduct the blood collection procedures in the select species, to include periodic refresher training and/or re-assessment of competency, especially for procedures that are employed less commonly.
- FOOD WITHDRAWAL: Minimize the period of food withdrawal prior to sample collection, if warranted.
- BODY WEIGHTS: Obtain body weight measurements at appropriate intervals to ensure accurate calculation of total blood volume.
- HEALTH OF ANIMALS: Address needs for reduction in sample collection volume and/or frequency in animals with conditions or disease pathology as identified above (see “General Principles”).
  - Avoid collecting samples from sites showing significant signs of inflammation and/or bruising from previous sampling.
- PHYSICAL RESTRAINT: Consistently select the least distressful restraint technique.
  - Acclimate animals to the restraint technique prior to study start, if applicable.
- CHEMICAL RESTRAINT: Use tranquilizers, sedatives or anesthetic agents as warranted.
  - Use topical anesthetics at the site of vessel puncture when warranted and allow adequate contact time for effect before obtaining the blood sample.
- ASEPSIS: Ensure blood collection sites are appropriately prepared for antisepsis.
- NEEDLE/CONTAINER SIZE: Consistently choose the smallest needle size that can reasonably be used for the species and type of sample being collected.
  - If vacutainers are used for sample collection, ensure that the size of the vacutainer is appropriate for the sample volume and size of the animal.
- VASODILATION: If supplemental heat is required to facilitate vasodilation (e.g. when collecting samples from peripheral vessels in small animals), choose the least distressful method and monitor the animal continuously to avoid overheating and/or thermal injury to tissues. Ensure that the temperature is not greater than 40°C (104°F) by measuring the temperature of the warm water bath with an alarming thermometer. The use of an alarming thermometer is recommended.
  - Avoid the use of tissue irritants to facilitate vasodilation unless there is scientific justification and approval by the IACUC or equivalent.
  - Judiciously use pharmacologic agents to facilitate vasodilation.
- METHOD: Consistently select the least distressful/painful blood collection method and site.
  - Consider the use of temporary or chronic, surgically implanted vascular access cannulas/ports when frequent and/or chronic blood sample collection is necessary and offsets the distress/pain of surgical implantation.
  - When catheters or cannulas are used, ensure that they are appropriately protected with bandages, tethers, jackets, other to avoid self-trauma and/or dislodgement by the animal.
  - Use automated blood sampling systems, when appropriate.
- VOLUME: Always collect the minimum amount of blood needed to perform the subsequent assay to include the use of microsampling whenever possible (Chapman et. al., 2014).
  - Minimize the frequency and duration of collections.
- FLUID REPLACEMENT: When vascular catheter(s) are in place for blood withdrawal, consider administering an equal volume of isotonic fluid (e.g. 0.9% NaCl) IV following withdrawal of each blood sample to replace fluid loss (while taking into consideration potential dilution of circulating blood that already has proteins removed, i.e. impacting oncotic blood pressure).
  - Consult with a veterinarian to determine the appropriateness or necessity for providing warm (37°C), isotonic replacement fluids, whole blood or volume expanders before, during or after blood collections.
  - Remember that fluid volume (not cells, clotting factors, protein, etc.) can be replaced IV, SC or IP.
- HEMOSTASIS: Always provide prompt hemostasis following blood collection to minimize further blood loss and/or bruising or hematoma formation at the site.