2.4. Blood collection

(Vincent Herbreteau, Yannick Chaval and Serge Morand)

Collecting blood is probably the most delicate task but fundamental for the serological study of rodent-borne diseases. Blood collection requires training and practice: when manipulating animals alive to limit their stress, and generally to get a maximum of blood since this operation can be easily missed.

Different techniques can be used depending on the status of the animal, the amount of blood needed, the purpose of the study and the necessity of dissection for further sampling. These techniques can be separated into three groups (Hoff, 2000):

- Blood collection not requiring anesthesia: saphenous vein (on the caudal surface of the thigh), dorsal pedal vein (on the top of the hind foot);
- Blood collection with anesthesia recommended to minimize pain and stress: tail vein, orbital sinus (or retro-orbital), jugular vein (near the throat or neck);
- Terminal procedures: cardiac puncture, posterior vena cava, axillary vessels, orbital sinus.

The different techniques on living animals can be repeated frequently, except from the orbital sinus. Anesthesia aims at reducing pain but should also be carefully managed: a low dose of isoflurane can be used until the animal is lying on its side and breathes slowly.

We only develop below the techniques that we have adopted in the field and found suitable for the study of blood parasites or the identification of antibodies against pathogens.

Recommendations:

- ***Only trained people should collect blood****, to avoid accident when manipulating syringes, to minimize stress to living animals and to obtain a maximum volume of blood.*
- ***Used needles should be placed in a sharps bin*** *(needle containers are made of puncture-resistant and leakproof plastic, and can be incinerated or autoclaved).*
- *As soon as used,* ***replace the plastic cap*** *and then remove the needle from the syringe, to avoid any injuries. Under no circumstances should needles be left on the table without their plastic cap.*
- *Used syringes without needles can be disposed of in a different container and burnt everyday.*

2.4.1. Blood collection from the base of the tail

Blood can be collected from the base of the tail on living animals when small amounts are needed.

Protocol:

1- Warm the animal and the base of the tail to increase flood flow.

2- Carefully and gently grasp the animal (see chapter 2.2), eventually place the animal in a restraining tube covering the head and front legs.

3- Disinfect the base of the tail with ethanol. Puncture the vein with a 23-25 gauge needle or use collection tubes with capillary action to collect blood.

4- Afterwards, apply pressure or use a cauterizing agent (such as silver nitrate) until bleeding stops.

2.4.2. Blood collection from the orbital sinus (retro-orbital)

Retro-orbital blood collection is also possible for small amounts of blood. This technique requires practice.

Protocol (Hoff, 2000):

1- The animal should be anesthetized first.

2- Pull the skin away from the eyeball, making the eyeball protruding out of the socket as much as possible.

3- Insert the tip of a glass capillary tube or Pasteur pipette into the corner of the eye socket underneath the eyeball, with a 45-degree angle. Apply gentle downward pressure and then release until the vein is broken and blood is visualized entering the pipette.

4- Slightly withdraw the pipette and allow the pipette to fill.

5- Before removing the pipette, cover the open end with your finger to prevent blood from spilling out of the tube. Bleeding usually stops immediately and completely when the pipette is removed.

2.4.3. Blood collection by cardiac puncture

When the dissection of the animal is required by the research protocol, cardiac puncture appears **the best method to rapidly obtain large amount of blood in aseptic conditions**. It requires practice to maximize the volume of blood collected but has proved to be very effective.

Equipment:

A suitable needle and syringe should be chosen according to the animal size (Table 1):

Table 1: Recommended choice of syringe and needles for cardiac puncture according to rodent size

	Syringe	*Needle gauge (length)*
Large-size rat	*5 cc*	*18 – 21 (1 – 1.5 inch)*
Medium-size rat	*3 cc – 5cc*	*22 – 23 (1 inch)*
Small-size rat	*1 cc (or insulin syringe)*	*24 (1 inch)*

Protocol:

We recommend a direct cardiac puncture without opening the thorax for a quick and safe process. Nevertheless, it requires practice and some prefer directly taking the blood from the visible heart, which particularly suits for smaller species.

1- Blood should be taken as soon as possible after the death of the animal as blood can coagulate rapidly.
2- Fix the needle to the syringe and carefully loosen the needle cover. Expel the air from the syringe. Eventually coat the syringe with heparin by aspirating and expelling heparin, to prevent coagulation.
3- Place the animal on its back, in a horizontal position. Clean the thoracic fur with ethanol.
4- Locate the thoracic cage and xiphoid process (the lower part of the sternum) with one hand, making sure that the animal is well positioned (i.e. the xiphoid process is well centered).
5- With the other hand, insert the needle under the xiphoid process at about 30 degrees to the left (Fig. 17). While inserting the needle, gently pull the plunger to aspirate until the heart is perforated and blood begins to flow. As soon as blood is flowing, continue pulling the plunger without moving the needle to avoid any more perforations in the heart, which will stop the flow.

   If no blood appears in the needle, the action should be repeated. If the flow stops rapidly, the heart may have been perforated on the back: slowly withdraw and adjust the needle to restore the flow.

Figure 17: Cardiac puncture (Photo: Herbreteau P.)

   As soon as enough blood is collected, release pressure on the plunger and withdraw the needle.
6- To avoid damaging blood cells, we recommend disposing of the needle before expelling blood. Slowly expel the blood from the syringe onto a filter paper or into the appropriate tube.

2.4.4. Blood collection from a dead collected rodent

It is still possible to collect blood samples from rodents, that were collected dead (e.g. from snap trappings). Under these circumstances, cardiac puncture cannot be done and the body should be opened.

Protocol:

1- Dissect the heart and cut it partly using sterile scissors.
2- Press the heart against a filter paper. Immediately hang filter paper to let it dry and avoid any contact.
3- Or, aspirate blood from the thoracic cavity using a narrow syringe or a heparin-coated Pasteur pipette. If not enough blood is available, fold a filter paper and introduce it inside the cavity, in order that it can absorb some blood.
4- Or, insert the whole heart in a vial of suitable size and add 2-3 volume of phosphate buffered saline (PBS), and freeze. The extract can be used for seroscreening.

2.4.5. Serum separation and blood storage

We recommend to prepare serum the same day as the blood is collected using a microcentrifuge (Fig. 18), and to freeze separately serum and blood clot in liquid nitrogen. This can be easily done in the field and it ensures a better preparation of blood samples.

Serum separation:

1- Place the tube containing whole blood in a micro-centrifuge and spin at 4,000 rpm for 5 minutes.

2- Eventually remove any coagulated blood using a toothpick and place back in the micro-centrifuge for a further 5 minutes at 4,000 rpm.

3- Using a 200 µL pipette and sterile tips, remove the transparent serum and place in a new labeled cryovial. Also place the remaining blood clot in another labeled cryovial.

4- Place both cryovials in liquid nitrogen (Fig. 19).

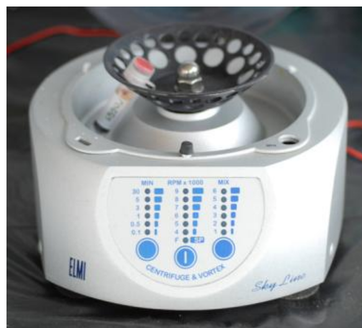


Figure 18: Example of microcentrifuge suitable to realize serum in a field laboratory (Photo: Herbreteau V.)

Figure 19: Liquid nitrogen tank suitable for field use (Photo: Herbreteau V.)

If liquid nitrogen is not available, blood can be collected on different supports, such as test tubes (that help conditioning blood) or blotting papers (that have proven easy blood storage when equipment is limited).

- **Test tubes** (Fig. 20):
- **Coagulation tubes**: filled with sodium citrate (3.8%) and used for coagulation assays. A certain quantity of blood is required to ensure a correct dilution.
- **Serum separation tubes**: used for serology. They are coated with micronised silica particles, which activate clotting. Serum can be directly aspirated from the tube.
- **EDTA** (K2 or K3) **tubes**: used for hematology examination and complete blood count (CBC).
- **Heparin tubes**: used in blood collection and anticoagulation for clinical biochemical examinations.

Figure 20: Tubes used for blood collection (EDTA, serum and coagulation tubes) (Photo: Herbreteau V.)

After collecting blood in tubes, gently invert to 180 degrees several times to react the additive with the blood. Note that tube caps are coded: red for serum tubes, light blue for coagulation tubes, purple for EDTA tubes.

- **Filter or blotting papers:**

Filter papers can be used for DNA extraction by PCR. These present several advantages:

- They are **cost-effective**,
- They **can be stored easily** in zipper bags with desiccant (suitable for studies in remote areas),
- They **require a limited amount of blood**: suitable for the smallest mammals from which only a few drops of blood can be obtained, as well as freshly-dead animals (when puncture cannot be performed).

Recommendations:

- *Use small circular or rectangular blotting papers. One cm^2 of impregnated blood is enough.*
- *Carefully expel a few drops from the syringe (after disposing of the needle). Make sure that the blood is not coagulated; otherwise it may spurt if there is too much pressure. Try to work so that the syringe is not pointed at any of your co-workers.*
- *Hang blotting papers in an open-air space for drying (Fig. 21). Use a hairdryer for drying quickly but not heat the samples.*
- *Cut away any unnecessary paper.*
- *Pack into small individual zipper storage bags. Add some desiccant or preserve in a fridge.*

Figure 21 : Blotting papers drying (Photo: Herbreteau P.)

Three methods for DNA extraction from filter papers were compared by Bereczky *et al.* in 2005. They show that the sensitivity of detection was dependent on the parasite density, the type of filter paper and the DNA extraction method. For 3MM® Whatman filter paper, the sensitivity was 100% with the Tris-EDTA buffer-based extraction, and lower with the standards methods: 73% with the methanol extraction, and 93% with Chelex® extraction (Bereczky et al., 2005).

Tris-EDTA buffer-based extraction is rapid, simple and inexpensive (Bereczky et al., 2005).

Protocol (adapted from: Bereczky *et al.*, 2005):

1- Excise the area of filter paper with the blood spot using a clean scalpel on a piece of glass. Transfer the piece of filter paper to a sterile 1.5 mL Eppendorf® tube using forceps.

2- Extraction (3 methods)

- Tris-EDTA buffer-based extraction:
  a. Prepare Tris-EDTA buffer: 10 mM Tris, pH 8.0 (Tris-base plus Tris-HCl) and 0.1 mM EDTA in distilled water, at room temperature.
  b. Place the filter paper punch in an Eppendorf® tube, and soak in 65 µL of TE buffer,
  c. Incubate at 50°C for 15 minutes.
  d. Mash punches, and change pipette tip for each punch.
  e. Heat at 97°C for 15 minutes to elute the DNA.
  f. Remove the liquid condensed on the lid and the wall of the tubes by centrifugation (2−3 seconds).
  g. Keep the DNA extract at 4°C for use within a few hours or store at −20°C.

- Methanol extraction:
  a. Soak the piece of filter paper in 125 µL of methanol
  b. Incubate at room temperature for 15 minutes.
  c. Remove the methanol and dry the samples
  d. Add 65 µL of distilled water.
  e. Mash punches, and change pipette tip for each punch.
  f. Heat at 97°C for 15 minutes to elute the DNA.

- Chelex extraction:
  a. Incubate overnight at 4°C in 1 mL of 0.5% saponin in phosphate buffered saline (PBS).
  b. Wash for 30 minutes in PBS at 4°C
  c. Transfer into new tubes containing 25 µL of stock solution (20% Chelex-100 and 75 µL of distilled water) and vortex for 30 seconds.
  d. Heat at 99°C for 15 minutes to elute the DNA.
  e. Vortex, and centrifuge at 10,000 × g for 2 minutes.
  f. Transfer the supernatants (65 µL) into new tubes.

3- Store the supernatant at -20°C (short term) or at -70°C (long term).

4- Use 1-5 µL of supernatant per 20 µL PCR reaction.

- **FTA cards** (adapted from Whatman website: http://www.whatman.com/FTAElute.aspx)

FTA® Elute Cards (Fig. 22) can be used for virtually any cell type (blood, cultured cells, bacteria, plasmids, microorganisms, solid tissue, viral particles, M13 Plaques, etc.) and a wide range of applications: multiplex PCR, sequencing after PCR Amplification, whole genome amplification quantitative PCR, genotyping, genetic identification, molecular diagnostics, etc.

The FTA® Elute matrix is chemically treated to lyse cells upon contact causing the release of nucleic acids.

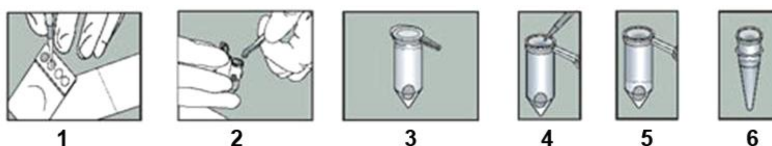
Source: www.whatman.com

Figure 22: Whatman FTA® Card

FTA® cards are expensive but present several advantages:

- Very small amount of blood can be used since the minimum volume required is 12-40 µL.
- Cards can be stored at room temperature and easily shipped.
- Security: organisms, including blood borne pathogens, are rapidly inactivated. It eliminates the risk of contamination and allows an easy transportation.
- Sample processing requires a simple hot water elution procedure to isolate DNA. Eliminates the cost of using a purification kit.

Protocol:



1- Apply sample to FTA® Elute matrix; dry thoroughly.

2- Punch a 3 mm disc and place in a microcentrifuge tube.

3- Rinse punch in 500 µL water, pulse vortex 3 times for 5 seconds.

4- Remove water and centrifuge for 5 seconds; pipette off excess water.

5- Add 30 µL sterile water, heat at 95°C for 30 min; pulse vortex 60 times; centrifuge.

6- Use 5-10 µL eluted DNA in a PCR mixture.

Herbreteau Vincent, Chaval Y., Morand S. (2011).

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In : Herbreteau Vincent (ed.), Jittapalapong S. (ed.), Rerkamnuaychoke W. (ed.), Chaval Y. (ed.), Cosson J.F. (ed.), Morand S. (ed.)

Protocols for field and laboratory rodent studies.

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