Appendix B: Recommendations for Serum and Plasma Collection, Separation and Storage

To isolate circulating, cell-free nucleic acids from whole blood samples, we recommend following these protocols, which include an initial low g-force centrifugation step to separate cells from plasma or serum followed by a high g-force centrifugation or filtration step to remove all remaining cellular debris. The latter centrifugation step significantly reduces the amount of cellular or genomic DNA and RNA in the sample. Because of the much higher abundance of RNA in cells, even small amounts of cellular debris can have a very significant effect on RNA profiling of cell-free fluids. The sooner after blood collection this removal of cellular materials is performed, the lower the risk of additional background from blood cell-derived nucleic acids released *in vitro*. Use of gel barrier tubes generally results in fewer residual cells.

The speed at which the second centrifugation step is performed will influence the recovery of different types of nucleic acid. Centrifugation at medium speed (e.g., 3000 *x g*) will effectively remove cellular material, including thrombocyte fragments and apoptotic bodies. Centrifugation at higher speed (e.g., 16,000 *x g*) may in addition remove intact chromatin from ruptured blood cells, but may also remove larger extracellular vesicles that may contain cell-free nucleic acid (especially mRNA).

Syringe filters with 0.8 µm pore size (e.g., Sartorius® Minisart® NML (cat. no. 16592) or Millipore® Millex®-AA (cat. no. SLAA033SB)) remove remaining cell fragments and debris strictly based on size, irrespective of density. These filters have dead volumes of around 100–200 µl.

Procedure: plasma separation and storage

1. Collect whole blood in BD Vacutainer® Venous Blood Collection Tubes (cat. no. 367525) containing EDTA (or any other primary blood collection tube containing EDTA as anticoagulant). Store tubes at room temperature (15–25°C) or 4°C and process within 1 hour.

   Note: Do not use heparin-containing blood collection tubes as this anticoagulant can interfere with downstream assays, such as RT-PCR.

2. Centrifuge blood samples in primary blood collection tubes for 10 min at 1900 *x g* (3000 rpm) and 4°C using a swinging bucket rotor.

3. Carefully transfer the upper (yellow) plasma phase to a new tube (with conical bottom) without disturbing the intermediate buffy coat layer (containing white blood cells and platelets). Normally up to 4–5 ml plasma can be obtained from 10 ml of whole blood.

   Note: Carryover of white blood cells and platelets from the buffy coat layer is the most likely source of cellular miRNA/RNA contamination in plasma.

   Note: Plasma can be used for cell-free nucleic acid purification at this stage. However, an additional filtration or centrifugation will remove additional cellular debris and minimize contamination of cell-free nucleic acids by gDNA and RNA derived from damaged blood cells.

4. Centrifuge plasma samples in conical tubes for 15 min at 3,000 *x g* (or 10 min at 16,000 *x g* – see above) and 4°C or pass through a 0.8 µm filter (see recommendations above).

   This will remove additional cellular nucleic acids attached to cell debris.

5. Carefully transfer cleared supernatant to a new tube without disturbing the pellet, which forms a smear along the outer side or bottom of the centrifugation tube.

6. Store at 2–8°C until further processing, if plasma will be used for nucleic acid purification on the same day. For longer storage, keep plasma frozen in aliquots at –65°C to –90°C.

7. Before using frozen plasma for nucleic acid purification, thaw at room temperature (15–25°C).

   Optional: To remove cryoprecipitates, centrifuge thawed plasma samples for 5 min at 3000 *x g* and 4°C or pass through a 0.8 µm filter. Transfer supernatant to a new tube, and begin the nucleic acid purification protocol.

Procedure: serum separation and storage

1. Collect whole blood in a primary blood collection tube with or without clot activator, but without anticoagulants such as EDTA or citrate (e.g., Sarstedt S-Monovette® Serum-Gel 9 ml tubes, cat. no. 02.1388). For complete clotting, leave tubes at room temperature (15–25°C) for 10 min to 1 h.

   Note: Tubes with clot activator can be processed after 10 min clotting time, while tubes without clot activator should be stored for at least 30 min at room temperature to allow clotting to take place.

2. Centrifuge tubes for 10 min at 1900 *x g* (3000 rpm) and 4°C using a swinging bucket rotor.

   Note: If using Sarstedt S-Monovette Serum-Gel 9ml tubes, a gel bed will form between the upper serum phase and the lower cellular phase, facilitating recovery of serum.

3. Carefully transfer the upper (yellow) serum phase to a new tube (with conical bottom) without disturbing the pellet containing cellular material. Normally up to 3–5 ml serum can be obtained from 10 ml of whole blood.

   Note: Prevent transfer of cellular material from the lower phase.

   Note: Serum can be used for cell-free nucleic acid purification at this stage. However, an additional filtration or centrifugation will remove additional cellular debris and minimize contamination acids by gDNA and RNA derived from damaged blood cells.

4. Centrifuge serum samples in conical tubes for 15 min at 3,000 *x g* (or 10 min at 16,000 *x g* – see above) and 4°C or pass through a 0.8 µm filter (see recommendations above). This will remove additional cellular nucleic acids attached to cell debris.

5. Carefully transfer cleared supernatant to a new tube without disturbing the pellet, which forms a smear along the outer side of the centrifugation tube.
6. Store at 2–8°C until further processing, if serum will be used for nucleic acid purification on the same day. For longer storage, keep serum frozen in aliquots at –65°C to –90°C.
7. Before using frozen serum for nucleic acid purification, thaw at room temperature (15 –25°C).

   Optional: To remove cryoprecipitates, centrifuge thawed serum samples for 5 min at 3000 *x g* and 4°C or pass through a 0.8 µm filter. Transfer supernatant to a new tube, and begin nucleic acid purification protocol.