Precipio, Inc.

Protocol for DNA Extraction from Plasma

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Notes for this Assay

- The following protocol is presently used at Precipio, Inc. for the extraction of DNA from plasma using the QIAamp® Circulating Nucleic Acid Kit.
- For further reference, please see the manual below:

- The resulting extracted DNA can then be used in any of the Precipio ICE COLD-PCR enrichment kits.

Reagents and Equipment Required but not Supplied

1. KAPA hgDNA Quantification and QC Kit: Kapa Catalog # KK4961
2. QIAamp Circulating Nucleic Acid Kit: Qiagen Catalog # 55114
3. Pipets (adjustable)
4. Sterile pipet tips (pipet tips with aerosol barriers are recommended to help prevent cross-contamination)
5. Water bath or heating block capable of holding 50 ml centrifuge tubes at 60°C
6. Heating block or similar at 56°C (capable of holding 2 ml collection tubes)
7. Microcentrifuge for 1.7 mL tubes
8. Centrifuge, Sorvall Legend X1 or equivalent
9. Vortexer
10. 15 or 50 mL conical tubes
11. 5 mL centrifuge tubes: Eppendorf Catalog # 0030119460
12. QIAvac 24 Plus vacuum manifold: Qiagen Catalog # 19413
13. QIAvac Connecting System: Qiagen Catalog # 19419 or equivalent
14. QIAvac VacValves: Qiagen Catalog # 19408 or equivalent
15. Vacuum Pump: Qiagen Catalog # 84010 [USA and Canada], 84000 [Japan], or 84020 [rest of world] or equivalent pump capable of producing a vacuum of –800 to–900 mbar
16. Ethanol (96–100%): Sigma Aldrich Catalog # E7023
17. Isopropanol (100%): Sigma Aldrich Catalog # I9030
18. Crushed ice

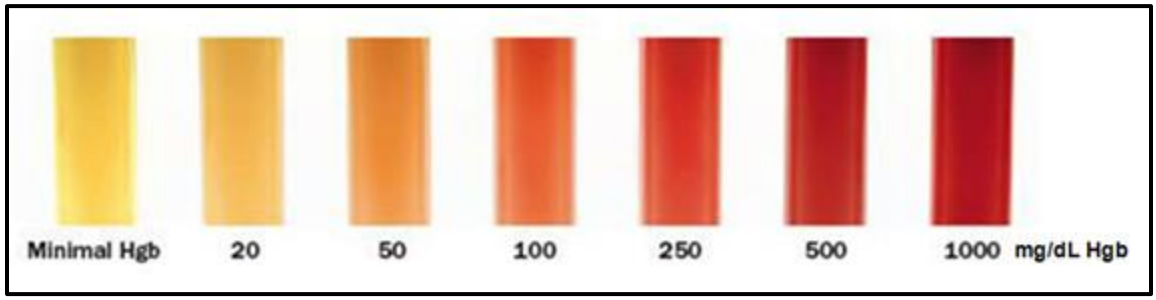
Plasma DNA Extraction Protocol

IMPORTANT! Use dedicated area/room for DNA extraction to avoid contamination from post-PCR products.

This protocol is used for purifying circulating DNA from plasma/serum using the QIAamp Circulating Nucleic Acid Kit from Qiagen and was adapted from the QIAamp Circulating Nucleic Acid Handbook.

1. Prepare buffers/reagents according to the Kit User Manual.
2. Starting From blood samples (Cell-Free DNA BCT Streck Tubes)
   a. Gently invert sample 10 times.
   b. Centrifuge samples at room temperature at 1600 x g (RCF) for 20 min.
   c. Transfer the top plasma layer carefully without disturbing the buffy coat into a clean, labeled centrifuge tube/vial.
   d. Centrifuge samples at room temperature at 16000 x g (RCF) for 10 min to remove residual cells.
   e. Determine the grade of hemolysis for the sample. If the sample has greater than 100 mg/dL Hgb, it may affect the downstream PCR amplification. See Figure 1 below for hemolysis grading.

      Figure 1. Hemolysis chart for centrifuged plasma.



   f. Determine the extraction volume to be used (1-5 mL) for the individual sample(s). Ideally, 3-4 mL is the preferred sample volume for extraction. If 3-4 mL of plasma is not available, recover as much as possible without disturbing the cell pellet
   g. Depending on the extraction volume determined, transfer 1-5 mL of plasma to a clean, labeled centrifuge tube without disturbing the cell pellet. Discard tube containing any residual cell pellets. Freeze any unused plasma at -80°C for storage, as appropriate.
3. DNA Isolation:

   IMPORTANT! Round sample volume up to the nearest milliliter to determine reagent volumes, not to exceed 5 mL.

   a. Table 1 shows the expected final lysate volume expected with the QIAgen extraction kit:

      Table 1. Final lysate volume for differing amounts of input plasma.

Volume Plasma	1 mL	2 mL	3 mL	4 mL	5 mL
Final Lysate Volume	3.7 mL	7.4 mL	11.1 mL	14.8 mL	18.5 mL

b. Add 100 µL Qiagen Proteinase K (PK) per mL of plasma extracted to each sample.
c. Prepare a master mix consisting of 795 µL Buffer ACL and 5 µL Carrier RNA (at 0.2 µg /µL) per mL of plasma extracted (1 mL = 1 reaction). When calculating for the total reactions, add one extra reaction per every 10 reactions to account for pipette loss. Invert the master mix 10 times to combine all reagents.
d. Add 800 µL of the Buffer ACL/Carrier RNA master mix per mL of plasma extracted and pulse-vortex for 30 sec.
e. Incubate at 60°C for 1 hour in a water bath.
f. During the incubation, prepare the QIAvac 24 Plus Vacuum Manifold and vacuum pump for samples.
g. Immediately following incubation, add 1.8 mL of Buffer ACB per mL of plasma extracted to the lysate and pulse-vortex for 15-30 sec.

NOTE: Add Buffer ACB within 2 min after removing samples from water bath to avoid clogging of the filter.

h. Incubate on ice for 5 min.
i. Transfer the lysate-Buffer ACB mixture into the tube extender by carefully pouring into the sterile, labeled column extender. Turn on vacuum pump and let all the lysate be drawn through the column. Close the Vac-Valves of the finished samples if the flow rates differ significantly between samples. Turn off the vacuum and release the pressure to 0 mbar. Carefully remove and discard the tube extender.

Alternatively: Transfer 700 µL lysate to the spin column and centrifuge 1 min at 6000 x g (RCF). Discard collection tube and place spin column in a clean collection tube. Repeat until all lysate has passed through the column.

j. Add 600 µL of Buffer ACW1 to QIAamp Mini Column. Leaving the column lid open, turn on the vacuum pump until all the liquid has been drawn through the column. Turn off the vacuum pump and release the pressure to 0 mbar.

Alternatively: Centrifuge 1 min at 6000 x g (RCF). Discard collection tube and place spin column in a clean collection tube.

k. Add 750 µL of Buffer ACW2 to QIAamp Mini Column. Leaving the column lid open, turn on the vacuum pump until all the liquid has been drawn through the column. Turn off the vacuum pump and release the pressure to 0 mbar.

Alternatively: Centrifuge 1 min at 6000 x g (RCF). Discard collection tube and place spin column in a clean collection tube.

l. Add 750 µL of 100% Ethanol to QIAamp Mini Column. Leaving the column lid open, turn on the vacuum pump until all the liquid has been drawn through the column. Turn off the vacuum pump and release the pressure to 0 mbar.

Alternatively: Centrifuge 3 min at 6000 x g (RCF).

m. Close the column lid and place the QIAamp Mini Column into a clean collection tube.
n. Centrifuge for 1 min at 20,000 x g (RCF). Discard collection tube and place column into a clean collection tube.
o. Open column lid and incubate at 56°C for 10 min on a heat block to dry the membrane.
p. Place the QIAamp Mini column into a clean, labeled 1.7 mL elution tube and discard the collection tube.
q. Add 30 µL of Buffer AVE to the center of the QIAamp Mini Column membrane and incubate at room temperature for 5 min.
r. Centrifuge for 1 min at 20,000 x g (RCF).

Optional: If second elution is desired, remove the spin column from the elution tube and place into another clean, labeled 1.7 mL elution tube. Repeat steps 3q – 3r. Then continue to step 3s.

s. Remove the spin column from the elution tube and discard the spin column.
t. Perform KAPA hsDNA real-time PCR or equivalent to determine the concentration of dsDNA.
u. Store extracted DNA appropriately (4°C if using immediately; -20°C if using within 1 month).