Protocol guide v1.1

Cell3™ Xtract

Cell Free DNA Extraction from Plasma and Other Biological Specimens

Cell3™ Xtract kit – 16 sample kit (PRE_EXT_C3X_16)
Cell3™ Xtract kit – 48 sample kit (PRE_EXT_C3X_48)

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Updates from Version 1.5

1. Additional advise provided for dealing with complex plasma samples that do not clear the purification column after centrifugation.

Intended use

This protocol is intended to provide guidance using the Cell3™ Xtract 16 (Catalogue No. PRE_EXT_C3X_16) and 48 (PRE_EXT_C3X_48) kits. Using the Cell3™ Xtract technology high quality Cell Free DNA can be extracted from plasma and serum samples and is suitable for downstream applications such as qPCR and Next Generation Sequencing.

Key features

- Fast and simple protocol enables a 90 minute processing time with 45 minutes hands-on time, to extract cfDNA from a 1-10 ml volume. Requires centrifugation steps only. No specialist equipment such as magnets or vacuum manifolds are required.
- Flexible input volume of 1-10 ml enables increased cfDNA recovery and use of the entire sample volume.
- Kit content allows for up to 16 or 48 reactions when extracting ≤4 ml of plasma per sample. Processing of >4 ml of plasma per sample will result in a lower amount of reactions available (i.e. 10 or 32 reactions when processing 6 ml of plasma per sample; 8 or 24 reactions for 8 ml of plasma; 6 or 19 reactions for 10 ml of plasma).
- Cell3™ Xtract technology enables an elution volume of as low as 35 µl, which helps avoid the need for DNA concentration steps and assists with low input or sensitive applications such as quantitative/real-time digital PCR and Next Generation Sequencing.
- Extract cfDNA from plasma, cerebrospinal fluid (CSF), saliva, serum and amniotic fluid.
- Does not require carrier RNA, allowing for accurate and reliable quantitation of extracted cfDNA for downstream applications.

Kit contents

Cell3™ Xtract kit - 16 sample kit (catalogue no. PRE_EXT_C3X_16)

Reagent	Quantity	Storage Conditions
Proteinase K	1 x 125 mg	-20°C (after reconstitution)
Proteinase K Resuspension Buffer	1 x 7 ml	Room Temperature
5X Digestion Buffer	1 x 16 ml	Room Temperature
DNA Binding Buffer	1 x 173 ml	Room Temperature
DNA Equilibration Buffer	1 x 6.4 ml	Room Temperature
Wash Buffer	1 x 6 ml	Room Temperature
Elution Buffer	1 x 2 ml	Room Temperature
Spin columns with attached Reservoir	1 bag x 16	Room Temperature

Cell3™ Xtract kit - 48 sample kit (catalogue no. PRE_EXT_C3X_48)

Reagent	Quantity	Storage Conditions
Proteinase K	3 x 125 mg	-20°C (after reconstitution)
Proteinase K Resuspension Buffer	1 x 21 ml	Room Temperature
5X Digestion Buffer	1 x 48 ml	Room Temperature
DNA Binding Buffer	3 x 173 ml	Room Temperature
DNA Equilibration Buffer	1 x 19.2 ml	Room Temperature
Wash Buffer	2 x 9 ml	Room Temperature
Elution Buffer	1 x 6 ml	Room Temperature
Spin columns with attached Reservoir	3 bags x 16	Room Temperature

Required equipment

Water bath or heat block (55°C)

Microcentrifuge (capable of accommodating 1.5-2 ml tubes)

Swinging bucket centrifuge (capable of accommodating 15-50 ml tubes)

Storage and handling

Cell3™ Xtract kit is stable at Room Temperature; proteinase K should be stored at -20°C after reconstitution. Eluted DNA should be stored at ≤ -20°C.

Laboratory supplied consumables

15-50 ml Conical Tubes

1.5-2 ml PCR Clean Tubes

(Optional) Screw caps for spin columns: these are not required if centrifugation of spin columns in collection tubes is performed within a biosafety cabinet

Plasma isolation

Please note blood samples received in EDTA tubes (purple lid) should be stored at +4°C and processed within 24 hours of blood draw. Samples received in blood cell stabilising tubes such as the Cell3 Preserver tubes, Streck tubes or equivalent should be stored at room temperature and processed within 7 days of blood draw. The sample should not be processed if received in any other tube or if clotted.

All work should be carried out in a Class II biosafety cabinet using standard aseptic techniques to maintain sample sterility.

1. Centrifuge the blood sample at 2,000 g for 10 minutes using a swinging bucket rotor.
2. Collect the separated plasma using a 1,000 µl pipette ensuring that the buffy coat remains undisturbed.
3. Aliquot into 1.5 – 2 ml sterile 'PCR Clean' microcentrifuge tubes.
4. Centrifuge at maximum speed (minimum 10,000 g) for 10 minutes in a microcentrifuge.

   NOTE: the second centrifugation step ensures that isolated plasma is free of cell debris, which can reduce qPCR efficiency and result in higher Ct values.
5. Taking care not to disturb the pellet retrieve the supernatant using a 1,000 µl pipette and aliquot into a fresh sterile 'PCR Clean' microcentrifuge 1.5 – 2 ml tube.
6. Plasma can be stored at -20°C for long term storage. The sample is now ready for cell free DNA extraction.

   NOTE: frozen plasma can be thawed at room temperature or in a heat block / bath at 37°C and used for cfDNA extraction.

Reagent preparation

1. Prior to use, add 6.5 ml of Proteinase K Resuspension Buffer to each Proteinase K (125 mg) tube. The final concentration of Proteinase K is ~20 mg/ml. Store at -20°C after mixing.
2. Prior to use, add 24 ml of 95-100% ethanol to the 6 ml Wash Buffer for the 16 sample kit (C3016SK); add 36 ml of 95-100% ethanol to the 9 ml Wash Buffer for the 48 sample kit (C3048LK).

Purification of cell free DNA

All procedures should be carried out in a sterile environment such as a Class II biosafety cabinet. If using saliva as input sample, see Appendix I. For sample input other than 1 ml (and not exceeding a minimum volume of 200 µl and a maximum volume of 10 ml), adjust 5X Digestion Buffer, Proteinase K and DNA Binding Buffer proportionally. Centrifuge the blood sample at 2,000 g for 10 minutes using a swinging bucket rotor.

1. Add 250 µl of 5X Digestion Buffer to every 1 ml of serum, plasma or biological fluid and mix thoroughly (see Table 1).
2. Add 100 µl of Proteinase K to every 1 ml of serum, plasma or biological fluid and mix thoroughly (see Table 1).
3. Incubate at 55°C for 30 minutes in a heat block or water bath.
4. Add two volumes of DNA Binding Buffer to the digested sample from step 3 and mix thoroughly (see Table 1).

Table 1. Summary of volumes to be used

Sample volume	1 ml	2 ml	4 ml	10 ml
5X Digestion Buffer	250 µl	500 µl	1 ml	2.5 ml
Proteinase K	100 µl	200 µl	400 µl	1 ml
Mix thoroughly and incubate at 55°C for 30 minutes				
Add DNA Binding buffer	2.7 ml	5.4 ml	10.8 ml	27 ml

5. Ensure the connection between the Reservoir and Spin Column is finger-tight and place the assembly into a 50 ml conical tube (not provided).
6. Transfer up to 10 ml of the mixture (containing DNA Binding Buffer) into the Spin Column Assembly and ensure the tube is tightly closed. Centrifuge at 1,000 g for 2 minutes. If starting from ≥ 2.5 ml of sample input, transfer the Spin Column Assembly into a fresh 50 ml tube and discard the flow-through. Repeat the procedure until the entire mixture has passed through the column.

   NOTE: occasionally, the mixture (containing DNA Binding Buffer) may not have entirely flowed through the spin column after 2 minutes of centrifugation. In this case, repeat centrifugation at 1,000 g for 2-8 minutes to ensure that all of the mixture has flowed through the spin column. If the issue persists, increase the incubation time at step 3 from 30 minutes to 60 minutes.
7. Unscrew the orange Luer Lock cap from the top of the Spin Column and discard the top Reservoir and the flow-through remaining in the 50 ml tube.

Place the Spin Column in a Spin Column Collection Tube. Add 400 µl DNA Equilibrium Buffer to the Spin Column. Centrifuge at ≥ 10,000 x g for 1 minute in a microcentrifuge. Discard the Collection Tube containing the flow-through and place the Spin Column in a fresh Collection Tube (provided).

NOTE: Centrifugation should be performed inside the biosafety cabinet. If the microcentrifuge is located outside of the biosafety cabinet, use of a screw cap (not provided) to seal the spin column is recommended.

9. Add 700 µl Wash Buffer to the Spin Column. Centrifuge at ≥ 10,000 x g for 1 minute. Discard the Collection Tube containing the flow-through and place the Spin Column in a fresh Collection Tube (provided).

10. Add 400 µl Wash Buffer to the Spin Column. Seal the Spin Column with the same Screw Cap and centrifuge at full speed for 1 minute to ensure complete removal of the wash buffer.

11. Transfer the column into a 1.5-2 ml PCR clean tube (not provided). Add ≥ 50 µl Elution Buffer directly to the column matrix. Incubate at room temperature for 3 minutes and then centrifuge at maximum speed for 1 minute.

    NOTE: the total yield of extracted cfDNA may be improved by eluting with Elution Buffer pre-heated to 60-70ºC. If eluting in volumes between 35 and 50 µl, it is recommended to load the eluate back to the membrane of the Spin Column, incubate for 3 minutes at room temperature and centrifuge again at maximum speed for 1 minute. Elution buffer is made up of 10 mM Tris-HCl, pH 8.5, 0.1 mM EDTA.
    If water is used to elute cfDNA, ensure that the pH is >6.0.

    Eluted DNA can be used immediately for downstream applications or stored at ≤ -20ºC.

    NOTE: as serum and plasma usually contain low quantities of DNA, the use of spectrophotometers (such as the Nanodrop) is not recommended for DNA quantification. High sensitivity techniques, such as qPCR and Qubit should be used to measure DNA quantity. Tapestation and Bioanalyzer should be used to analyse DNA fragment size and distribution.

Appendix I – cell free DNA extraction from saliva

Cellular and / or cell free DNA can be extracted from saliva using the following protocols.

A) Purification of total (ie cellular and cell free) DNA from saliva.

1. Transfer up to 1 ml of saliva into a microcentrifuge tube (if the input of saliva is ≤200 µl) or a conical tube (if the input of saliva is >200 µl).
2. Add 0.5 ml of 5x Digestion Buffer for each ml of saliva used (see Table 2).
3. Add 0.5 ml of molecular biology grade water for each ml of saliva and mix thoroughly (see Table 2).
4. Add 100 µl of Proteinase K per ml of saliva and mix thoroughly (see Table 2).
5. Incubate at 55ºC for 30 minutes.
6. Add one volume of DNA Binding Buffer to the digested sample and mix thoroughly (see Table 2).
7. Add one volume of 95-100% ethanol to the new mixture and mix thoroughly (see Table 2).

Table 2. Summary of volumes to be used

Saliva volume	200 µl	500 µl	750 µl	1 ml
5X Digestion Buffer	100 µl	250 µl	375 µl	0.5 ml
H20	100 µl	250 µl	375 µl	0.5 ml
Proteinase K	20 µl	50 µl	75 µl	100 µl
Mix thoroughly and incubate at 55ºC for 30 minutes				
Add DNA Binding buffer	420 µl	1.05 ml	1.58 ml	2.1 ml
Add Ethanol (95-100%)	840 µl	2.1 ml	3.15 ml	4.2 ml

To continue processing the lysate, proceed to step 5 of the "Purification of Cell Free DNA" paragraph.

B) Purification of cell free DNA from saliva.

1. Dilute the starting saliva sample with an equal volume of isotonic solution (e.g. 1x PBS) and centrifuge the diluted saliva sample in a microcentrifuge tube or a conical tube at 5000 g for 10 minutes to remove intact cells.
2. Without disturbing the loose cell pellet, carefully transfer the saliva supernatant to a new microcentrifuge tube or a conical tube.

   NOTE: the cellular pellet can be processed separately (see section C below) or discarded.
3. Transfer up to 5 ml of saliva supernatant into a microcentrifuge tube or a conical tube.

   NOTE: use a microcentrifuge tube if processing ≤200 µl of cell free saliva supernatant; use a conical tube if processing >200 µl of cell free saliva supernatant.
4. Add 0.5 ml of 5x Digestion Buffer for each ml of saliva supernatant used (see Table 3).
5. Add 0.5 ml of molecular biology grade water for each ml of saliva supernatant and mix thoroughly (see Table 3).
6. Add 100 µl of Proteinase K per ml of saliva supernatant and mix thoroughly (see Table 3).
7. Incubate at 55ºC for 30 minutes.
8. Add one volume of DNA Binding Buffer to the digested sample and mix thoroughly (see Table 3).
9. Add one volume of 95-100% ethanol to the new mixture and mix thoroughly (see Table 3).

Table 3. Summary of volumes to be used

Saliva volume	200 µl	1 ml	3 ml	5 ml
5X Digestion Buffer	100 µl	0.5 ml	1.5 ml	2.5 ml
H20	100 µl	0.5 ml	1.5 ml	2.5 ml
Proteinase K	20 µl	100 µl	300 µl	500 µl
Mix thoroughly and incubate at 55ºC for 30 minutes				
Add DNA Binding buffer	420 µl	2.1 ml	16.3 ml	10.5 ml
Add Ethanol (95-100%)	840 µl	4.2 ml	12.6 ml	21.0 ml

To continue processing the lysate, proceed to step 5 of the "Purification of Cell Free DNA" paragraph.

C) Purification of genomic DNA from cellular pellet recovered after centrifugation of saliva in section B, step 2.

1. Resuspend the pellet in 200 µl of isotonic solution.
2. Add 100 µl of 5x Digestion Buffer.
3. Add 100 µl of molecular biology grade water.
4. Add 20 µl of Proteinase K and mix thoroughly.
5. Proceed to step 7 of section B above.

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