applied biosystems

MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit

USER GUIDE

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ThermoFisher SCIENTIFIC

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Revision	Date	Description
B.0	20 February 2018	Correction to value in the Prepare Slurry table.
A.0	12 October 2017	New document instructions for MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit

Contents

Product information

Product description

The Applied Biosystems™ MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit is designed for isolation of circulating nucleic acid from cell-free human plasma samples. The kit uses Dynabeads™ MyOne™ SILANE technology and extraction chemistry, ensuring reproducible recovery of high-quality cell-free nucleic acid (cfNA), including cell-free DNA, cell-free RNA, and cell-free miRNA that is appropriate for a broad range of applications, including NGS sequencing, genotyping, qPCR, and dPCR.

This guide describes isolation of cfNA from 2- or 4-mL plasma samples. Three optimized methods are included:

- KingFisher™ Flex Magnetic Particle Processor 96DW
- KingFisher™ Duo Prime Magnetic Particle Processor
- Manual sample processing; 6 individual plasma samples can be processed in about 2 hours.

For sample sizes, see Appendix B, "Alternate methods for 1-, 3-, 5-, and 6-mL sample volume" for other plasma input protocols.

The MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit is optimized for samples that are collected in K_2EDTA and Streck Cell-Free DNA Blood Collection Tubes (BCT).

Contents and storage

Reagents that are provided in the kit are sufficient for 50 reactions from 2 mL of plasma or 25 reactions from 4 mL of plasma.

Table 1 MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit (Cat. No. A36716)

Component	Volume	Storage
MagMAX™ Cell-Free Total Nucleic Acid Lysis/Binding Solution	175 mL	15°C to 25°C
MagMAX™ Cell-Free Total Nucleic Acid Wash Solution Concentrate	66 mL	
MagMAX™ Cell-Free Total Nucleic Acid Elution Solution	21 mL	
MagMAX™ Cell-Free Total Nucleic Acid Magnetic Beads	3.5 mL	2–8°C
MagMAX™ Cell-Free Total Nucleic Acid Proteinase K (20 mg/mL)	1.5 mL	–20°C

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Table 2 Materials required for cfNA isolation (all methods)

Item	Source
Equipment	
Lab-Line Orbital shaker or similar capable of achieving 1000 rpm	MLS
Fisher Scientific™ Incubating Mini-Shaker with 50-mL conical holder	02-217-753
Adjustable micropipettors	MLS
Multi-channel micropipettors	MLS
Materials	
50-mL conical tubes	4306311
Non-stick, RNase-free, 1.5-mL microfuge tubes	4473979
Reagent reservoirs	MLS
MicroAmp Clear Adhesive Film	4306311
Reagents	
Ethanol, 100% (molecular biology grade)	MLS
Isopropanol, 100% (molecular biology grade)	MLS
Nuclease-free Water	AM9932

Table 3 Additional materials required for automated cfNA isolation

Item	Source
Instrument	
Magnetic particle processor, one of the following:	
KingFisher™ Duo Prime Magnetic Particle Processor with 6- and 12-well magnetic heads	5400110
KingFisher™ Flex Magnetic Particle Processor 96DW with 96 and 24 deep-well heads	5400630 and 5400640
Plates and combs:	
KingFisher™ deep-well 96-well plate	95040450
KingFisher™ Flex 24 deep-well plate	95040470 or 95040480

Item	Source
For Flex only: KingFisher™ Flex Magnetic Particle Processor 96 KF microplate (200 µL) KingFisher™ 96 tip comb for deep-well magnets KingFisher™ Flex 24 deep-well tip comb and plate	97002540 97002534 97002610
For Duo only: KingFisher™ Duo Prime Magnetic Particle Processor 6-tip comb, for use with KingFisher™ Flex 24 deep-well plate KingFisher™ Duo Prime Magnetic Particle Processor 12-well tip comb, for use with Microtiter 96 deep-well Plate	97003510 97003500

Table 4 Additional materials required for manual cfNA isolation

Item	Source
Equipment	
mySpin™ 6 Mini Centrifuge	MLS
Vortexer (with and without 1.5-mL tube adaptors)	MLS
DynaMag™–2 Magnet	12321D
DynaMag™–50 Magnet	12302D

Methods

Procedural guidelines

- Perform all steps at room temperature (20–25°C) unless otherwise noted.
- When mixing samples by pipetting up and down, avoid creating bubbles.
- When performing manual isolations, use 1.5- or 2.0-mL microfuge tubes to allow for vigorous mixing.
- Precipitates can occur if Lysis/Binding Solution and Wash Solution Concentrate are stored when room temperature is too cold. If there are precipitates in these solutions, warm them at 37°C for 1 hour and gently mix to dissolve precipitates. Avoid creating bubbles.
- Vortex the MagMAX™ Cell-Free Total Nucleic Acid Magnetic Beads to resuspend them before use.
- Pipet beads slowly and ensure that they remain in homogeneous suspension while pipetting. Revortexing may be required and low retention pipette tips should be used.
- Use a P100 pipette or larger when handling beads.
- The protocol is compatible with both K_2-EDTA and Streck cfDNA BCTs. K_2-EDTA tubes are the recommended tube for collection of whole blood. Centrifuge the blood to cell-free plasma as soon as possible for best results. If cell-free plasma is frozen, avoid multiple freeze-thaw cycles. Thaw plasma gently and minimize time plasma is held on ice to protect the nucleic acids from degradation.

Before use of the kit

Before first use of the kit

- Prepare fresh 80% Ethanol using Nuclease-Free Water.
- Prepare Wash Solution 1: Add 34 mL 100% isopropanol to the 66 mL of MagMAX™ Total Nucleic Acid Wash Solution Concentrate.

  After preparing the entire volume, store tightly capped at room temperature.

Before each use of kit

- Bring the MagMAX™ Cell-Free Total Nucleic Acid Magnetic Beads to room temperature and **vortex beads thoroughly**.
- Set shaking incubator to 65°C for Proteinase K digest.

Prepare the Binding and Rebinding Slurries *(automated protocols only)*

Preparing the Binding and Rebinding Slurries ensures effective bead mixing on the Kingfisher automated platforms and maximizes cfNA yield. Individual addition of the Binding and Rebinding Slurries components to the Kingfisher plates is not recommended as it decreases cfNA yield. Always prepare fresh and discard any unused portion.

- Prepare **Binding Slurry** and **Rebinding Slurry** according to the following tables.

Table 5 Binding Slurry

Binding Slurry Component	Volume of plasma per sample [1]	
	2 mL	4 mL
MagMAX™ Cell-Free Total Nucleic Acid Lysis/Binding Solution	1.5 mL	3 mL
MagMAX™ Cell-Free Total Nucleic Acid Magnetic Beads	60 µL	120 µL
Total volume	**1.56 mL**	**3.12 mL**

[1] Prepare sufficient Binding Slurry for all samples plus 10% overage.

Table 6 Rebinding Slurry

Rebinding Slurry Component	Volume per sample	Volume for 10 samples[1]
MagMAX™ Cell-Free Total Nucleic Acid Lysis/Binding Solution	440 µL	5.28 mL
MagMAX™ Cell-Free Total Nucleic Acid Magnetic Beads	10 µL	120 µL
Total volume	**450 µL**	**5.40 mL**

[1] Prepare sufficient Binding Slurry for all samples plus 20% overage.

Note: The volume of Rebinding Slurry is equivalent for all starting plasma volumes and should not be scaled.

IMPORTANT! Vortex the tube containing the Slurries until all beads are evenly distributed.

Extract cfNA using KingFisher™ Flex

Prepare cell-free plasma

1. Centrifuge the blood sample at 2000 × *g* for 10 minutes at 4°C.
2. Transfer the plasma to a new centrifuge tube, taking care not to disturb the buffy coat layer.

3. Centrifuge the plasma sample at 16,000 × *g* for 10 minutes at 4°C.

   Alternatively, the plasma sample can be centrifuged at 6000 × *g* for 30 minutes at 4°C.

4. Transfer the supernatant to a fresh tube for Proteinase K digestion, noting the volume of plasma.

Digest with Proteinase K

1. Set up Proteinase K digestion in a 50-mL conical tube. Combine reagents in the order that is listed according to the following table.

Proteinase K Digest Component	Cell-free plasma volume	
	2 mL	4 mL
MagMAX™ Cell-Free Total Nucleic Acid Proteinase K (20 mg/mL)	30 µL	60 µL
Cell-free plasma	2 mL	4 mL
MagMAX™ Cell-Free Total Nucleic Acid Lysis/Binding Solution	1 mL	2 mL
Total volume	**3.03 mL**	**6.06 mL**

2. Incubate for 30 minutes at 65°C with shaking at 1000 rpm.

3. At the end of Proteinase K incubation, cool the sample on ice for 5 minutes, or until the mix reaches room temperature.

Set up the 24DW processing plates

During Proteinase K incubation, set up the 24DW processing plates outside the instrument according to the following table.

Note: To accommodate the large (4 mL) input volume, the digested plasma is divided evenly between matched wells on Plates 1 and 2.

Note: Ensure that the Binding Slurry is well-vortexed before adding to the plate. Pipet slowly as solution is viscous. It is not necessary to pipet sample up and down, as the instrument mixes completely. Pipetting up and down can cause loss of beads and reduce yield.

IMPORTANT! Ensure to use the volumes that are described in the following tables. Do not use the volumes that are displayed by the instrument as they are different to ensure effective mixing of viscous samples.

Table 7 Plate setup (KingFisher™ Flex Magnetic Particle Processor 24DW) for 2 mL of plasma

Plate type	Plate position	Plate ID	Reagent	Volume per well
24DW	1	Binding Plate	Digested Plasma	3 mL
			Binding Slurry	1.6 mL
	2	Wash 1 Plate	Wash Solution 1	1 mL
	3	1 mL 80% Ethanol Plate	80% Ethanol	1 mL
	4	500 µL 80% Ethanol Plate	80% Ethanol	500 µL

Plate type	Plate position	Plate ID	Reagent	Volume per well
24DW	5	Elution Plate	Elution Solution	350 µL
	6	Tip Comb	24DW Tip Comb	—

Table 8 Plate setup (KingFisher™ Flex Magnetic Particle Processor 24DW) for 4 mL of plasma

Plate type	Plate position	Plate ID	Reagent	Volume per well
24DW	1	Binding Plate 1	Digested Plasma	3 mL
			Binding Slurry	1.6 mL
	2	Binding Plate 2	Digested Plasma	3 mL
			Binding Slurry	1.6 mL
	3	Wash 1 Plate	Wash Solution 1	1 mL
	4	1 mL 80% Ethanol Plate	80% Ethanol	1 mL
	5	500 µL 80% Ethanol Plate	80% Ethanol	500 µL
	6	Elution Plate	Elution Solution	350 µL
	7	Tip Comb	24DW Tip Comb	—

Bind, wash, and elute the cfNA

1. Ensure that the instrument is set up for processing with the 24 deep-well magnetic head, then select the program on the instrument according to the following table.

Plasma input volume	Program	Run time
2 mL	**MagMAX_cfNA_Flex_2mL_v1**	26 minutes
4 mL	**MagMAX_cfNA_Flex_4mL_v1**	40 minutes

2. Press **Start** to start the program, then follow the onscreen instructions to load all plates.
3. Press **Start** to start the total nucleic acid extraction.
4. At the end of the run (the instrument beeps), take out the Elution Plate (at the loading position) and place on ice.
5. Press **Start**, then remove all remaining plates when prompted by the instrument.

Set up the 96DW processing plates

Set up the processing plates outside the instrument according to the following table.

Note: Ensure that the Rebinding Slurry is well-vortexed before adding to the plate. Pipet slowly as solution is viscous. It is not necessary to pipet sample up and down, as the instrument mixes completely. Pipetting up and down can cause loss of beads and reduce yield.

IMPORTANT! Ensure to use the volumes that are described in the following tables. Do not use the volumes that are displayed by the instrument as they are different to ensure effective mixing of viscous samples.

Plate type	Plate position	Plate ID	Reagent	Volume per well
96DW	1	Rebinding Plate	First Eluate (from 24DW Plate 6)	350 µL
			Rebind Slurry[1]	450 µL
	2	Wash 1 Plate	Wash Solution 1	1 mL
	3	500 µL 80% Ethanol Plate	80% Ethanol	500 µL
	4	100 µL 80% Ethanol Plate	80% Ethanol	100 µL
Standard KF [2]	5	Elution Plate	Elution Solution	20 µL
96DW	6	Tip Comb	96DW Tip Comb	—

[1] The volume of Rebinding Slurry is equivalent for all starting plasma volumes and should not be scaled.
[2] Kingfisher 96-well microplate.

Concentrate the cfNA

1. Ensure that the instrument is set up for processing with the 96 deep-well magnetic head, then select the program **MagMAX_cfNA_Flex_Rebind_v1** on the instrument.
2. Press **Start** to start the program, then follow the onscreen instructions to load all six plates.
3. Press **Start** to start the total nucleic acid concentration.

   Note: The **MagMAX_cfNA_Flex_Rebind_v1** program runs for 30 minutes.
4. At the end of the run (the instrument beeps), take out the Elution Plate (at the loading position) and place on ice.
5. Press **Start**, then remove all remaining plates when prompted by the instrument.

Store the purified cfNA on ice for immediate use. Alternatively, store the purified cfNA at −20°C or −80°C for long-term storage.

Extract cfNA using KingFisher™ Duo Prime

Prepare cell-free plasma

1. Centrifuge the blood sample at 2000 × *g* for 10 minutes at 4°C.
2. Transfer the plasma to a new centrifuge tube, taking care not to disturb the buffy coat layer.

3. Centrifuge the plasma sample at 16,000 × *g* for 10 minutes at 4°C.

   Alternatively, the plasma sample can be centrifuged at 6000 × *g* for 30 minutes at 4°C.

4. Transfer the supernatant to a fresh tube for Proteinase K digestion, noting the volume of plasma.

Digest with Proteinase K

1. Set up Proteinase K digestion in a 50-mL conical tube. Combine reagents in the order that is listed according to the following table.

Proteinase K Digest Component	Cell-free plasma volume	
	2 mL	**4 mL**
MagMAX™ Cell-Free Total Nucleic Acid Proteinase K (20 mg/mL)	30 µL	60 µL
Cell-free plasma	2 mL	4 mL
MagMAX™ Cell-Free Total Nucleic Acid Lysis/Binding Solution	1 mL	2 mL
Total volume	**3.03 mL**	**6.06 mL**

2. Incubate for 30 minutes at 65°C with shaking at 1000 rpm.

3. At the end of Proteinase K incubation, cool the sample on ice for 5 minutes, or until the mix reaches room temperature.

Set up the 24DW processing plates

During Proteinase K incubation, set up the 24DW processing plates outside the instrument according to the following tables.

Note: Ensure that the Binding Slurry is well-vortexed before adding to the plate. Pipet slowly as solution is viscous. It is not necessary to pipet sample up and down, as the instrument mixes completely. Pipetting up and down can cause loss of beads and reduce yield.

Note: To accommodate the large (4 mL) input volume, the digested plasma is divided evenly between matched wells of Rows A and B, on Plate 1.

IMPORTANT! Ensure to use the volumes that are described in the following tables. Do not use the volumes that are displayed by the instrument as they are different to ensure effective mixing of viscous samples.

Table 9 Plate setup (KingFisher™ Duo Prime Magnetic Particle Processor 24DW) for 2 mL of plasma

Plate type	Row position	Row ID	Reagent	Volume per well
Plate 1				
24DW	A	Binding	Digested Plasma	3 mL
			Binding Slurry	1.6 mL
	B	Empty	Empty	—
	C	Wash 1	Wash Solution 1	1 mL

Plate type	Row position	Row ID	Reagent	Volume per well
24DW	D	Tip Comb	24DW 6-Tip Comb	
Plate 2				
24DW	A	1 mL 80% Ethanol	80% Ethanol	1 mL
	B	500 µL 80% Ethanol	80% Ethanol	500 µL
	C	Empty	Empty	—
	D	Elution	Elution Solution	350 µL

Table 10 Plate setup (KingFisher™ Duo Prime Magnetic Particle Processor 24DW) for 4 mL of plasma

Plate type	Row position	Row ID	Reagent	Volume per well
Plate 1				
24DW	A	Binding 1	Digested Plasma	3 mL
			Binding Slurry	1.6 mL
	B	Binding 2	Digested Plasma	3 mL
			Binding Slurry	1.6 mL
	C	Wash 1	Wash Solution 1	1 mL
	D	Tip Comb	24DW 6-Tip Comb	
Plate 2				
24DW	A	1 mL 80% Ethanol	80% Ethanol	1 mL
	B	500 µL 80% Ethanol	80% Ethanol	500 µL
	C	Empty	Empty	—
	D	Elution	Elution Solution	350 µL

Bind, wash, and elute the cfNA

1. Ensure that the instrument is set up for processing with the 6-pin magnetic head, then select the program on the instrument according to the following table.

plasma input volume	Program	Run time
2 mL	**MagMAX_cfNA_Duo_2mL_v1**	23 minutes
4 mL	**MagMAX_cfNA_Duo_4mL_v1**	40 minutes

2. Press **Start** to start the program, then follow the onscreen instructions to load Plates 1 and 2.

3. Press **Start** to start the total nucleic acid extraction.

4. At the end of the run (the instrument beeps), take out the plates, when instructed, and place on ice.

Set up the 96DW processing plate

Set up the processing plate outside the instrument according to the following table.

Note: Ensure that the Rebinding Slurry is well-vortexed before adding to the plate. Pipet slowly as solution is viscous. It is not necessary to pipet sample up and down, as the instrument mixes completely. Pipetting up and down can cause loss of beads and reduce yield.

IMPORTANT! Ensure to use the volumes that are described in the following tables. Do not use the volumes that are displayed on the instrument as they are different to ensure effective mixing of viscous samples.

Plate type	Row position	Row ID	Reagent	Volume per well
96DW	A	Rebinding	First eluate (from 24DW Plate 2, Row D)	350 µL
			Rebinding Slurry[1]	450 µL
	B	Wash 1	Wash Solution 1	1 mL
	C	500 µL 80% Ethanol	80% Ethanol	500 µL
	D	100 µL 80% Ethanol	80% Ethanol	100 µL
	E	Tip Comb	96DW 12-Tip Comb	—
	F	Empty	—	—
	G	Empty	—	—
	H	Elution	Elution Solution	20 µL

[1] The volume of Rebinding Slurry is equivalent for all starting plasma volumes and should not be scaled.

Concentrate the cfNA

1. Ensure that the instrument is set up for processing with the 12-pin magnetic head, then select the program **MagMAX_cfNA_Duo_Rebind_v1** on the instrument.
2. Press **Start** to start the program, then follow the onscreen instructions to load the plate.
3. Press **Start** to start the total nucleic acid concentration.

   Note: The **MagMAX_cfNA_Duo_Rebind_v1** runs for 30 minutes.
4. At the end of the run (the instrument beeps), take out the plate, when instructed, and place on ice.
5. Transfer the eluates into non-stick tubes on ice.

Store the purified cfNA on ice for immediate use. Alternatively, store the purified cfNA at –20°C or –80°C for long-term storage.

Extract cfNA manually

Prepare cell-free plasma

1. Centrifuge the blood sample at 2000 × *g* for 10 minutes at 4°C.
2. Transfer the plasma to a new centrifuge tube, taking care not to disturb the buffy coat layer.
3. Centrifuge the plasma sample at 16,000 × *g* for 10 minutes at 4°C.
   Alternatively, the plasma sample can be centrifuged at 6000 × *g* for 30 minutes at 4°C.
4. Transfer the supernatant to a fresh tube for Proteinase K digestion, noting the volume of plasma.

Digest with Proteinase K

1. Set up Proteinase K digestion in a 50-mL conical tube. Combine reagents in the order that is listed according to the following table.

Proteinase K Digest Component	Cell-free plasma volume	
	2 mL	4 mL
MagMAX™ Cell-Free Total Nucleic Acid Proteinase K (20 mg/mL)	30 µL	60 µL
Cell-free plasma	2 mL	4 mL
MagMAX™ Cell-Free Total Nucleic Acid Lysis/Binding Solution	1 mL	2 mL
Total volume	**3.03 mL**	**6.06 mL**

2. Incubate for 30 minutes at 65°C with shaking at 1000 rpm.
3. At the end of Proteinase K incubation, cool the sample on ice for 5 minutes, or until the mix reaches room temperature.

Bind cfNA to beads

1. Add MagMAX™ Cell-Free Total Nucleic Acid Lysis/Binding Solution to the digested plasma, according to the following table.

Starting cell-free plasma volume	Lysis/Binding Solution
2 mL	1.5 mL
4 mL	3.0 mL

2. Add well-vortexed MagMAX™ Cell-Free Total Nucleic Acid Magnetic Beads, according to the following table, then vortex briefly to mix.

Starting cell-free plasma volume	Magnetic Beads
2 mL	60 µL
4 mL	120 µL

Note: Ensure that the mixture is homogeneous and the beads are distributed evenly throughout the sample.

3. Place on a shaker for 10 minutes at 1000 rpm to mix and bind the nucleic acid to the beads.
4. Place on the DynaMag™–50 Magnet stand for 5 minutes.

IMPORTANT! Wait the entire 5 minutes before removing supernatant to ensure maximum yield.

5. Remove, then discard the supernatant without disturbing the beads.

Wash the beads

1. Resuspend the beads in 1 mL of Wash Solution 1, then mix by pipetting up and down carefully, ensuring all beads are released from the tube walls.
2. Transfer the bead slurry to a new non-stick 1.5-mL microcentrifuge tube and place on the DynaMag™–2 Magnet stand for 20 seconds. Do not discard the 50-mL conical.
3. Collect the supernatant from the bead pellet in the 1.5-mL tube and use it to rinse remaining beads from the 50-mL conical.
4. Carefully transfer the supernatant back to the 1.5-mL tube on the DynaMag™–2 Magnet stand.
5. Leave the 1.5-mL tube on the magnet stand for 2 minutes or until solution clears, then discard the supernatant, being careful not to disturb the beads.
6. Add 1 mL of 80% ethanol.
7. Vortex briefly, then centrifuge briefly.
8. Place on DynaMag™–2 Magnet stand for 2 minutes, or until solution clears.
9. Discard the supernatant.
10. Repeat steps 6–9 for a second wash with 1 mL of 80% ethanol.
11. Carefully remove, then discard the ethanol.

Elute the cfNA

1. Air-dry the beads for 5 minutes, then remove any remaining ethanol with a 10-µL pipette tip.
2. Resuspend the beads in 400 µL of MagMAX™ Cell-Free Total Nucleic Acid Elution Solution and vortex for 5 minutes at high speed.
3. Centrifuge briefly, then place the tube on the DynaMag™–2 Magnet stand to capture beads for 2 minutes.
4. Transfer the supernatant to a new 1.5-mL microcentrifuge tube.

Concentrate and wash the cfNA

1. Add 500 µL of MagMAX™ Cell-Free Total Nucleic Acid Lysis/Binding Solution.
2. Add 10 µL of well-vortexed MagMAX™ Cell-Free Total Nucleic Acid Magnetic Beads.

   IMPORTANT! Do not use less than 10 µL of beads for the rebinding step.
3. Vortex 1.5-mL tube at high speed for 5 minutes to bind nucleic acid to beads.
4. Briefly centrifuge, then place on DynaMag™–2 Magnet stand for 5 minutes to capture the beads.
5. Discard the supernatant.
6. Add 1 mL of Wash Solution 1.
7. Vortex briefly, then centrifuge briefly.
8. Place on DynaMag™–2 Magnet stand for 2 minutes.
9. Discard the supernatant.
10. Add 1 mL of 80% ethanol.
11. Vortex briefly, then centrifuge briefly.
12. Place on DynaMag™–2 Magnet stand for 2 minutes, or until solution clears.
13. Discard the supernatant.
14. Repeat steps 10–13 for a second wash with 80% ethanol.
    Be careful to remove all the ethanol.

Elute the concentrated cfNA

1. Air-dry the beads on the DynaMag™–2 Magnet stand for 3 minutes, taking care not to over-dry the beads.
2. Tap the magnet stand gently against a flat surface to collect remaining liquid, then discard the remaining liquid.
3. Add 15 µL of MagMAX™ Cell-Free Total Nucleic Acid Elution Solution and vortex on high for 5 minutes.
4. Briefly centrifuge, then place on DynaMag™–2 Magnet stand for 2 minutes to capture the beads.
5. Transfer the eluate to a fresh 1.5-mL tube.

Store the purified cfNA on ice for immediate use. Alternatively, store the purified cfNA at −20°C or −80°C for long-term storage.

cfNA quantification

We recommend using the Agilent™ High Sensitivity DNA Kit (Cat. No. 5067-4626) to observe the cfDNA fraction of the eluate. cfDNA is fragmented dsDNA with a major peak at 170 bp.

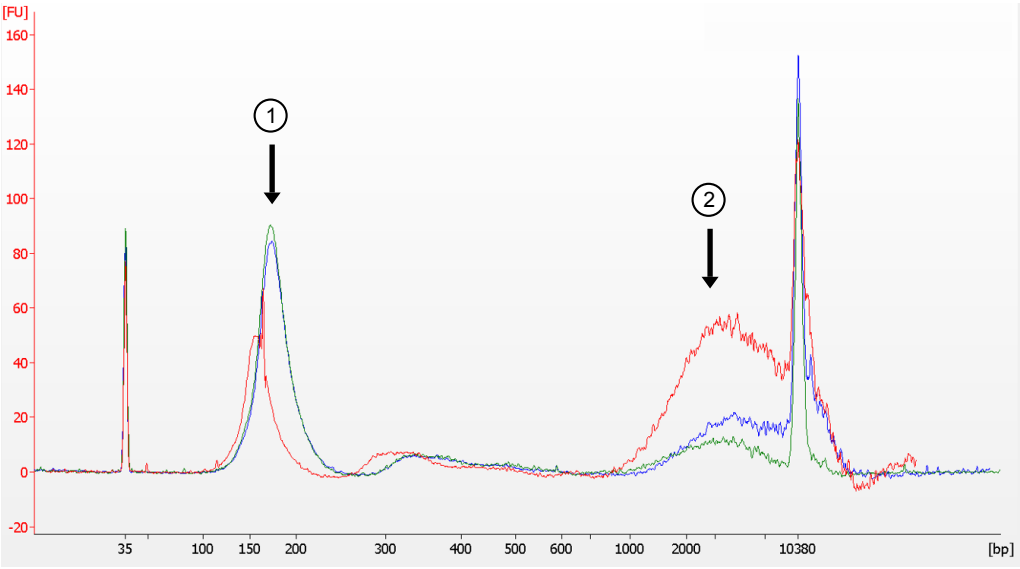

Figure 1 Matched 4 mL K_2-EDTA plasma isolations

① Cell-free DNA (100–275 bp)

② Genomic DNA

- *Blue line:* MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit Manual isolation
- *Green line:* MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit Kingfisher Flex isolation
- *Red line:* Competitor kit Manual isolation
- FU: Fluorescence units
- bp: Base pairs

We recommend using the Qubit™ dsDNA HS Assay Kit (Cat. No. Q32854) to quantify the cell-free DNA yield in the eluate. For most samples, a 2-µL input is sufficient. It is important to note that the Qubit cannot discriminate between cell-free DNA and gDNA in the sample, so running an Agilent High Sensitivity DNA analysis chip is highly recommended.

We recommend an m1 qPCR assay to observe the cfRNA in the sample. cfRNA concentrations in plasma are low and the conformation/length is not compatible with the dye in the Qubit RNA HS assay. The m1 designation indicates that the probe spans an exon-exon boundary ensuring that signal is only generated from template with correctly spliced exons. The assay will not detect signal from the cfDNA that is also present in the eluate. Perform the reverse transcription (RT) reaction according to the SuperScript™ VILO™ Master Mix (Cat. No. 11755050) protocol. The reaction volume for this step can be scaled down to 10 µL total, using 2 µL purified cfNA input. Perform the qPCR reaction according to the protocol for TaqMan® Universal Master Mix II, no UNG (Cat. No. 4440040), with sample input up to 10% of the total reaction volume. We recommend using a TaqMan assay with an appropriate amplicon length to detect cfRNA, such as Hs_99999905_m1 GAPDH (122 bases), Hs99999903_m1 ACTB 171 bases or another m1 assay target appropriate to your sample.

A Troubleshooting

Observation	Possible cause	Recommended action
Lower yield than expected	The MagMAX™ Cell-Free Total Nucleic Acid Magnetic Beads were not properly stored.	Remove the beads from the kit and store them at 2–8°C. Do not freeze the beads.
		Allow the beads to warm to room temperature before use.
	An insufficient amount of MagMAX™ Cell-Free Total Nucleic Acid Magnetic Beads were added.	Vortex the tube containing the beads thoroughly immediately before use. Ensure homogeneous distribution of beads.
		Use low-retention pipette tips for accurate pipetting of beads, buffers, and slurry mixtures.
		For automated samples: Vortex the Binding Slurry and Rebinding Slurry immediately before use. Ensure that the mix is homogeneous before adding to the sample.
		For automated samples: Add Slurry mixtures to the sample slowly, allowing the pipette tip to drain, and ejecting any remaining slurry.
	The MagMAX™ Cell-Free Total Nucleic Acid Magnetic Beads are not optimally dried.	Drying times can vary depending on the amount of beads that are used and the environment. Lower volumes of beads require less time to dry. Airflow and humidity in the immediate environment can shorten or lengthen the optimal bead drying time.
		Overdried beads stick to the wall of the plastics and can be difficult to resuspend.
		Underdried beads can carry ethanol into the eluate and negatively affect downstream applications.
	The sample contains low levels of cfDNA.	Increase the starting sample volume. See Appendix B, "Alternate methods for 1-, 3-, 5-, and 6-mL sample volume" for more information.

Observation	Possible cause	Recommended action
Lower yield than expected	*For manual samples:* Insufficient mixing of the sample with the magnetic beads during the binding step.	Vigorous mixing at the binding step is essential. After adding the Lysis/Binding Solution and the magnetic beads to the sample, shake the tube at 750–1000 rpm on the Fisher Scientific™ Incubating Mini-Shaker with 50-mL conical holder (Cat. No. 02-217-753). If this instrument is not available, the binding step can be completed on an orbital shaker capable of vigorous mixing, such as a Thermo Fisher Lab-Line Orbital Shaker. For similar benchtop mixer models with a small orbital radius, we recommend shaking at 750–1000 rpm. For shakers with a larger orbital radius, slower speeds may be sufficient as long as the beads are continuously mixed.
	Insufficient Proteinase K digestion.	Do not pre-mix the Proteinase K with the Lysis/Binding Solution before adding to the plasma sample because this can reduce Proteinase K activity and decrease cfNA yield. Add Proteinase K directly to plasma and vortex. Then add Lysis/Binding Solution, vortex, and start digest.
		Ensure that SDS has not been added to Proteinase K digest reaction as it is not necessary and reduces cfRNA yield.
	Plasma was not held on ice or was repeatedly freeze thawed.	To maximize cfRNA recovery and downstream assay functionality, thaw plasma gently and hold on ice until use. Minimize freeze thaw cycles.
	Insufficient time allowed for bead collection.	Bead loss reduces cfNA yield, allow the recommended time for bead clarification at each step to maximize yield.
Magnetic bead carryover	Loose beads present in the eluate or inadvertently transferred.	Be sure to leave the tube on the magnetic stand when removing the eluate containing the cfNA.
		If beads are carried over into the new tube, place the tube on the magnetic stand again, wait for the beads to pellet, and then transfer the sample to another tube.
		For automated samples: Ensure that Elution Solution is pipetted directly into the center of the well. If beads are carried over into the final elution, place the plate on a magnetic stand, wait for the beads to pellet, and then transfer clean eluate to a new tube or plate.
Abundance of gDNA in eluate	Hemolytic plasma, lipemic plasma, or other compromised sample types (see Figure 1).	Yields from these types of sample vary greatly from donor to donor. Process these types of sample using the automated protocol, or for the manual protocol reduce shaking at binding to 750 rpm.

Observation	Possible cause	Recommended action
Variations in cfDNA yield from donor to donor	Variation in amount of circulating cfNA. Levels of cfDNA in circulation can range from 0.5–3.5 ng/mL and cfRNA levels can range from 0.5–1.25 ng/mL of plasma in normal healthy donors.	For samples containing low levels of cfNA, increase the starting sample volume.
cfRNA not detected by Qubit	The cfRNA yield can be below the detection limit for Qubit assays.	Levels of cfRNA vary from donor to donor and can be below the limit of detection for the Qubit assay. The recommended detection method for cfRNA is qPCR. See "cfNA quantification" on page 19 for more information. The cfRNA in plasma can not have a length or structure compatible with the dyes in the Qubit kit.
cfNA yield is lower than competitor kit	Samples that are processed with competitor kits can have a higher concentration of genomic DNA contamination.	The Qubit™ dsDNA HS Assay Kit (Cat. No. Q32854) does not distinguish between cfDNA and genomic DNA, so gives higher concentration readings for samples with high genomic DNA contamination. Therefore, perform fragment analysis with the Agilent™ High Sensitivity DNA Kit (Cat. No. 5067-4626) to compare the primary cell-free peak (from 100–275 bp). Large genomic DNA fragments can be visualized >1 kb (See Figure 1 on page 19).

B Alternate methods for 1-, 3-, 5-, and 6-mL sample volume

Procedural guidelines

- Perform all steps at room temperature (20–25°C) unless otherwise noted.
- When mixing samples by pipetting up and down, avoid creating bubbles.
- When performing manual isolations, use 1.5- or 2.0-mL microfuge tubes to allow for vigorous mixing.
- Precipitates can occur if Lysis/Binding Solution and Wash Solution Concentrate are stored when room temperature is too cold. If there are precipitates in these solutions, warm them at 37°C for 1 hour and gently mix to dissolve precipitates. Avoid creating bubbles.
- Vortex the MagMAX™ Cell-Free Total Nucleic Acid Magnetic Beads to resuspend them before use.
- Pipet beads slowly and ensure that they remain in homogeneous suspension while pipetting. Revortexing may be required and low retention pipette tips should be used.
- Use a P100 pipette or larger when handling beads.
- The protocol is compatible with both K_2-EDTA and Streck cfDNA BCTs. K_2-EDTA tubes are the recommended tube for collection of whole blood. Centrifuge the blood to cell-free plasma as soon as possible for best results. If cell-free plasma is frozen, avoid multiple freeze-thaw cycles. Thaw plasma gently and minimize time plasma is held on ice to protect the nucleic acids from degradation.

Before use of the kit

Before first use of the kit

- Prepare fresh 80% Ethanol using Nuclease-Free Water.
- Prepare Wash Solution 1: Add 34 mL 100% isopropanol to the 66 mL of MagMAX™ Total Nucleic Acid Wash Solution Concentrate.

  After preparing the entire volume, store tightly capped at room temperature.

Before each use of kit

- Bring the MagMAX™ Cell-Free Total Nucleic Acid Magnetic Beads to room temperature and **vortex beads thoroughly**.
- Set shaking incubator to 65°C for Proteinase K digest.

Prepare slurries

Preparing the Binding and Rebinding Slurries ensures effective bead mixing on the Kingfisher automated platforms and maximizes cfNA yield. Individual addition of the Binding and Rebinding Slurries components to the Kingfisher plates is not recommended as it decreases cfNA yield. Always prepare fresh and discard any unused portion.

- Prepare **Binding Slurry** and **Rebinding Slurry** according to the following tables.

Table 11 Binding Slurry

Binding Slurry Component	Volume of plasma per sample [1]			
	1 mL	3 mL	5 mL	6 mL
MagMAX™ Cell-Free Total Nucleic Acid Lysis/Binding Solution	0.75 mL	2.25 mL	3.75 mL	4.5 mL
MagMAX™ Cell-Free Total Nucleic Acid Magnetic Beads	30 µL	90 µL	150 µL	180 µL
Total volume	**0.78 mL**	**2.34 mL**	**3.9 mL**	**4.68 mL**

[1] Prepare sufficient Binding Slurry for all samples plus 10% overage.

Table 12 Rebinding Slurry

Rebinding Slurry Component	Volume per sample	Volume for 10 samples[1]
MagMAX™ Cell-Free Total Nucleic Acid Lysis/Binding Solution	440 µL	5.28 mL
MagMAX™ Cell-Free Total Nucleic Acid Magnetic Beads	10 µL	120 µL
Total volume	**450 µL**	**5.40 mL**

[1] Prepare sufficient Binding Slurry for all samples plus 20% overage.

Note: The volume of Rebinding Slurry is equivalent for all starting plasma volumes and should not be scaled.

IMPORTANT! Vortex the tube containing the Slurries until all beads are evenly distributed.

Extract cfNA using KingFisher™ Flex

Prepare cell-free plasma

1. Centrifuge the blood sample at 2000 × *g* for 10 minutes at 4°C.
2. Transfer the plasma to a new centrifuge tube, taking care not to disturb the buffy coat layer.

3. Centrifuge the plasma sample at 16,000 × *g* for 10 minutes at 4°C.

   Alternatively, the plasma sample can be centrifuged at 6000 × *g* for 30 minutes at 4°C.

4. Transfer the supernatant to a fresh tube for Proteinase K digestion, noting the volume of plasma.

Digest with Proteinase K

1. Set up Proteinase K digestion in a 50-mL conical tube. Combine reagents in the order that is listed according to the following table.

Proteinase K Digest Component	Cell-free plasma volume			
	1 mL	3 mL	5 mL	6 mL
MagMAX™ Cell-Free Total Nucleic Acid Proteinase K (20 mg/mL)	15 µL	45 µL	75 µL	90 µL
Cell-free plasma	1 mL	3 mL	5 mL	6 mL
MagMAX™ Cell-Free Total Nucleic Acid Lysis/Binding Solution	0.5 mL	1.5 mL	2.5 mL	3 mL
Total volume	**1.515 mL**	**4.545mL**	**7.575 mL**	**9.09 mL**

2. Incubate for 30 minutes at 65°C with shaking at 1000 rpm.

3. At the end of Proteinase K incubation, cool the sample on ice for 5 minutes, or until the mix reaches room temperature.

Set up the 24DW processing plates

During Proteinase K incubation, set up the 24DW processing plates outside the instrument according to the following table.

Note: To accommodate the large input volume (plasma input 3, 5, and 6 mL), the digested plasma is divided evenly between matched wells on Plates 1 and 2 (for 3-mL input) or on Plates 1, 2, and 3 (for 5- and 6-mL input).

Note: Ensure that the Binding Slurry is well-vortexed before adding to the plate. Pipet slowly as solution is viscous. It is not necessary to pipet sample up and down, as the instrument mixes completely. Pipetting up and down can cause loss of beads and reduce yield.

IMPORTANT! Ensure to use the volumes that are described in the following tables. Do not use the volumes that are displayed by the instrument as they are different to ensure effective mixing of viscous samples.

Table 13 Plate setup (KingFisher™ Flex Magnetic Particle Processor 24DW) for 1 mL of plasma

Plate type	Plate position	Plate ID	Reagent	Volume per well
24DW	1	Binding Plate	Digested Plasma	1.5 mL
			Binding Slurry	800 µL
	2	Wash 1 Plate	Wash Solution 1	1 mL
	3	1 mL 80% Ethanol Plate	80% Ethanol	1 mL

Plate type	Plate position	Plate ID	Reagent	Volume per well
24DW	4	500 µL 80% Ethanol Plate	80% Ethanol	500 µL
	5	Elution Plate	Elution Solution	350 µL
	6	Tip Comb	24DW Tip Comb	—

Table 14 Plate setup (KingFisher™ Flex Magnetic Particle Processor 24DW) for 3 mL of plasma

Plate type	Plate position	Plate ID	Reagent	Volume per well
24DW	1	Binding Plate 1	Digested Plasma	2.25 mL
			Binding Slurry	1.2 mL
	2	Binding Plate 2	Digested Plasma	2.25 mL
			Binding Slurry	1.2 mL
	3	Wash 1 Plate	Wash Solution 1	1 mL
	4	1 mL 80% Ethanol Plate	80% Ethanol	1 mL
	5	500 µL 80% Ethanol Plate	80% Ethanol	500 µL
	6	Elution Plate	Elution Solution	350 µL
	7	Tip Comb	24DW Tip Comb	—

Table 15 Plate setup (KingFisher™ Flex Magnetic Particle Processor 24DW) for 5 mL of plasma

Plate type	Plate position	Plate ID	Reagent	Volume per well
24DW	1	Binding Plate 1	Digested Plasma	2.5 mL
			Binding Slurry	1.3 mL
	2	Binding Plate 2	Digested Plasma	2.5 mL
			Binding Slurry	1.3 mL
	3	Binding Plate 3	Digested Plasma	2.5 mL
			Binding Slurry	1.3 mL
	4	Wash 1 Plate	Wash Solution 1	1 mL
	5	1 mL 80% Ethanol Plate	80% Ethanol	1 mL
	6	500 µL 80% Ethanol Plate	80% Ethanol	500 µL
	7	Elution Plate	Elution Solution	350 µL
	8	Tip Comb	24DW Tip Comb	—

Table 16 Plate setup (KingFisher™ Flex Magnetic Particle Processor 24DW) for 6 mL of plasma

Plate type	Plate position	Plate ID	Reagent	Volume per well
24DW	1	Binding Plate 1	Digested Plasma	3 mL
			Binding Slurry	1.6 mL
	2	Binding Plate 2	Digested Plasma	3 mL
			Binding Slurry	1.6 mL
	3	Binding Plate 3	Digested Plasma	3 mL
			Binding Slurry	1.6 mL
	4	Wash 1 Plate	Wash Solution 1	1 mL
	5	1 mL 80% Ethanol Plate	80% Ethanol	1 mL
	6	500 µL 80% Ethanol Plate	80% Ethanol	500 µL
	7	Elution Plate	Elution Solution	350 µL
	8	Tip Comb	24DW Tip Comb	—

Bind, wash, and elute the cfNA

1. Ensure that the instrument is set up for processing with the 24 deep-well magnetic head, then select the program on the instrument according to the following table.

Plasma input volume	Program	Run time
1 mL	**MagMAX_cfNA_Flex_2mL_v1**	26 minutes
3 mL	**MagMAX_cfNA_Flex_4mL_v1**	40 minutes
5–6 mL	**MagMAX_cfNA_Flex_6mL_v1**	53 minutes

2. Press **Start** to start the program, then follow the onscreen instructions to load all plates.
3. Press **Start** to start the total nucleic acid extraction.
4. At the end of the run (the instrument beeps), take out the Elution Plate (at the loading position) and place on ice.
5. Press **Start**, then remove all remaining plates when prompted by the instrument.

Set up the 96DW processing plates

Set up the processing plates outside the instrument according to the following table.

Note: Ensure that the Rebinding Slurry is well-vortexed before adding to the plate. Pipet slowly as solution is viscous. It is not necessary to pipet sample up and down, as the instrument mixes completely. Pipetting up and down can cause loss of beads and reduce yield.

IMPORTANT! Ensure to use the volumes that are described in the following tables. Do not use the volumes that are displayed by the instrument as they are different to ensure effective mixing of viscous samples.

Plate type	Plate position	Plate ID	Reagent	Volume per well
96DW	1	Rebinding Plate	First Eluate (from 24DW Plate 6)	350 µL
			Rebind Slurry[1]	450 µL
	2	Wash 1 Plate	Wash Solution 1	1 mL
	3	500 µL 80% Ethanol Plate	80% Ethanol	500 µL
	4	100 µL 80% Ethanol Plate	80% Ethanol	100 µL
Standard KF [2]	5	Elution Plate	Elution Solution	20 µL
96DW	6	Tip Comb	96DW Tip Comb	—

[1] The volume of Rebinding Slurry is equivalent for all starting plasma volumes and should not be scaled.
[2] Kingfisher 96-well microplate.

Concentrate the cfNA

1. Ensure that the instrument is set up for processing with the 96 deep-well magnetic head, then select the program **MagMAX_cfNA_Flex_Rebind_v1** on the instrument.
2. Press **Start** to start the program, then follow the onscreen instructions to load all six plates.
3. Press **Start** to start the total nucleic acid concentration.

   Note: The **MagMAX_cfNA_Flex_Rebind_v1** program runs for 30 minutes.
4. At the end of the run (the instrument beeps), take out the Elution Plate (at the loading position) and place on ice.
5. Press **Start**, then remove all remaining plates when prompted by the instrument.

Store the purified cfNA on ice for immediate use. Alternatively, store the purified cfNA at −20°C or −80°C for long-term storage.

Extract cfNA using KingFisher™ Duo Prime

Prepare cell-free plasma

1. Centrifuge the blood sample at 2000 × *g* for 10 minutes at 4°C.
2. Transfer the plasma to a new centrifuge tube, taking care not to disturb the buffy coat layer.

3. Centrifuge the plasma sample at 16,000 × *g* for 10 minutes at 4°C.

   Alternatively, the plasma sample can be centrifuged at 6000 × *g* for 30 minutes at 4°C.

4. Transfer the supernatant to a fresh tube for Proteinase K digestion, noting the volume of plasma.

Digest with Proteinase K

1. Set up Proteinase K digestion in a 50-mL conical tube. Combine reagents in the order that is listed according to the following table.

Proteinase K Digest Component	Cell-free plasma volume			
	1 mL	**3 mL**	**5 mL**	**6 mL**
MagMAX™ Cell-Free Total Nucleic Acid Proteinase K (20 mg/mL)	15 µL	45 µL	75 µL	90 µL
Cell-free plasma	1 mL	3 mL	5 mL	6 mL
MagMAX™ Cell-Free Total Nucleic Acid Lysis/Binding Solution	0.5 mL	1.5 mL	2.5 mL	3 mL
Total volume	**1.515 mL**	**4.545mL**	**7.575 mL**	**9.09 mL**

2. Incubate for 30 minutes at 65°C with shaking at 1000 rpm.

3. At the end of Proteinase K incubation, cool the sample on ice for 5 minutes, or until the mix reaches room temperature.

Set up the 24DW processing plates

During Proteinase K incubation, set up the 24DW processing plates outside the instrument according to the following tables.

Note: Ensure that the Binding Slurry is well-vortexed before adding to the plate. Pipet slowly as solution is viscous. It is not necessary to pipet sample up and down, as the instrument mixes completely. Pipetting up and down can cause loss of beads and reduce yield.

Note: To accommodate the large (3, 5, and 6 mL) input volume, the digested plasma is divided evenly between matched wells of Rows A and B, on Plate 1 for 3-mL input, and Rows A, B, and C, Plate 1 for 5- and 6-mL input.

IMPORTANT! Ensure to use the volumes that are described in the following tables. Do not use the volumes that are displayed by the instrument as they are different to ensure effective mixing of viscous samples.

Table 17 Plate setup (KingFisher™ Duo Prime Magnetic Particle Processor 24DW) for 1 mL of plasma

Plate type	Row position	Row ID	Reagent	Volume per well
Plate 1				
24DW	A	Binding	Digested Plasma	1.5 mL
			Binding Slurry	800 µL
	B	Empty	Empty	—

Plate type	Row position	Row ID	Reagent	Volume per well
24DW	C	Wash 1	Wash Solution 1	1 mL
	D	Tip Comb	24DW 6-Tip Comb	
Plate 2				
24DW	A	1 mL 80% Ethanol	80% Ethanol	1 mL
	B	500 µL 80% Ethanol	80% Ethanol	500 µL
	C	Empty	Empty	—
	D	Elution	Elution Solution	350 µL

Table 18 Plate setup (KingFisher™ Duo Prime Magnetic Particle Processor 24DW) for 3 mL of plasma

Plate type	Row position	Row ID	Reagent	Volume per well
Plate 1				
24DW	A	Binding 1	Digested Plasma	2.25 mL
			Binding Slurry	1.2 mL
	B	Binding 2	Digested Plasma	2.25 mL
			Binding Slurry	1.2 mL
	C	Wash 1	Wash Solution 1	1 mL
	D	Tip Comb	24DW 6-Tip Comb	
Plate 2				
24DW	A	1 mL 80% Ethanol	80% Ethanol	1 mL
	B	500 µL 80% Ethanol	80% Ethanol	500 µL
	C	Empty	Empty	—
	D	Elution	Elution Solution	350 µL

Table 19 Plate setup (KingFisher™ Duo Prime Magnetic Particle Processor 24DW) for 5 mL of plasma

Plate type	Row position	Row ID	Reagent	Volume per well
Plate 1				
24DW	A	Binding 1	Digested Plasma	2.5 mL
			Binding Slurry	1.3 mL
	B	Binding 2	Digested Plasma	2.5 mL
			Binding Slurry	1.3 mL
	C	Binding 3	Digested Plasma	2.5 mL
			Binding Slurry	1.3 mL
	D	Tip Comb	24DW 6-Tip Comb	

Plate type	Row position	Row ID	Reagent	Volume per well
Plate 2				
24DW	A	Wash 1	Wash Solution 1	1 mL
	B	1 mL 80% Ethanol	80% Ethanol	1 mL
	C	500 µL 80% Ethanol	80% Ethanol	500 µL
	D	Elution	Elution Solution	350 µL

Table 20 Plate setup (KingFisher™ Duo Prime Magnetic Particle Processor 24DW) for 6 mL of plasma

Plate type	Row position	Row ID	Reagent	Volume per well
Plate 1				
24DW	A	Binding 1	Digested Plasma	3 mL
			Binding Slurry	1.6 mL
	B	Binding 2	Digested Plasma	3 mL
			Binding Slurry	1.6 mL
	C	Binding 3	Digested Plasma	3 mL
			Binding Slurry	1.6 mL
	D	Tip Comb	24DW 6-Tip Comb	
Plate 2				
24DW	A	Wash 1	Wash Solution 1	1 mL
	B	1 mL 80% Ethanol	80% Ethanol	1 mL
	C	500 µL 80% Ethanol	80% Ethanol	500 µL
	D	Elution	Elution Solution	350 µL

Bind, wash, and elute the cfNA

1. Ensure that the instrument is set up for processing with the 6-pin magnetic head, then select the program on the instrument according to the following table.

plasma input volume	Program	Run time
1 mL	**MagMAX_cfNA_Duo_2mL_v1**	23 minutes
3 mL	**MagMAX_cfNA_Duo_4mL_v1**	40 minutes
5–6 mL	**MagMAX_cfNA_Duo_6mL_v1**	50 minutes

2. Press **Start** to start the program, then follow the onscreen instructions to load Plates 1 and 2.
3. Press **Start** to start the total nucleic acid extraction.
4. At the end of the run (the instrument beeps), take out the plates, when instructed, and place on ice.

Set up the 96DW processing plate

Set up the processing plate outside the instrument according to the following table.

Note: Ensure that the Rebinding Slurry is well-vortexed before adding to the plate. Pipet slowly as solution is viscous. It is not necessary to pipet sample up and down, as the instrument mixes completely. Pipetting up and down can cause loss of beads and reduce yield.

IMPORTANT! Ensure to use the volumes that are described in the following tables. Do not use the volumes that are displayed on the instrument as they are different to ensure effective mixing of viscous samples.

Plate type	Row position	Row ID	Reagent	Volume per well
96DW	A	Rebinding	First eluate (from 24DW Plate 2, Row D)	350 µL
			Rebinding Slurry[1]	450 µL
	B	Wash 1	Wash Solution 1	1 mL
	C	500 µL 80% Ethanol	80% Ethanol	500 µL
	D	100 µL 80% Ethanol	80% Ethanol	100 µL
	E	Tip Comb	96DW 12-Tip Comb	—
	F	Empty	—	—
	G	Empty	—	—
	H	Elution	Elution Solution	20 µL

[1] The volume of Rebinding Slurry is equivalent for all starting plasma volumes and should not be scaled.

Concentrate the cfNA

1. Ensure that the instrument is set up for processing with the 12-pin magnetic head, then select the program **MagMAX_cfNA_Duo_Rebind_v1** on the instrument.
2. Press **Start** to start the program, then follow the onscreen instructions to load the plate.
3. Press **Start** to start the total nucleic acid concentration.

   Note: The **MagMAX_cfNA_Duo_Rebind_v1** runs for 30 minutes.
4. At the end of the run (the instrument beeps), take out the plate, when instructed, and place on ice.
5. Transfer the eluates into non-stick tubes on ice.

Store the purified cfNA on ice for immediate use. Alternatively, store the purified cfNA at −20°C or −80°C for long-term storage.

Extract cfNA manually

Prepare cell-free plasma

1. Centrifuge the blood sample at 2000 × *g* for 10 minutes at 4°C.
2. Transfer the plasma to a new centrifuge tube, taking care not to disturb the buffy coat layer.
3. Centrifuge the plasma sample at 16,000 × *g* for 10 minutes at 4°C.
   Alternatively, the plasma sample can be centrifuged at 6000 × *g* for 30 minutes at 4°C.
4. Transfer the supernatant to a fresh tube for Proteinase K digestion, noting the volume of plasma.

Digest with Proteinase K

1. Set up Proteinase K digestion in a 50-mL conical tube. Combine reagents in the order that is listed according to the following table.

Proteinase K Digest Component	Cell-free plasma volume			
	1 mL	**3 mL**	**5 mL**	**6 mL**
MagMAX™ Cell-Free Total Nucleic Acid Proteinase K (20 mg/mL)	15 µL	45 µL	75 µL	90 µL
Cell-free plasma	1 mL	3 mL	5 mL	6 mL
MagMAX™ Cell-Free Total Nucleic Acid Lysis/Binding Solution	0.5 mL	1.5 mL	2.5 mL	3 mL
Total volume	**1.515 mL**	**4.545mL**	**7.575 mL**	**9.09 mL**

2. Incubate for 30 minutes at 65°C with shaking at 1000 rpm.
3. At the end of Proteinase K incubation, cool the sample on ice for 5 minutes, or until the mix reaches room temperature.

Bind cfNA to beads

1. Add MagMAX™ Cell-Free Total Nucleic Acid Lysis/Binding Solution to the digested plasma, according to the following table.

Starting cell-free plasma volume	Lysis/Binding Solution
1 mL	0.75 mL
3 mL	2.25 mL
5 mL	3.75 mL
6 mL	4.5 mL

2. Add well-vortexed MagMAX™ Cell-Free Total Nucleic Acid Magnetic Beads, according to the following table, then vortex briefly to mix.

Starting cell-free plasma volume	Magnetic Beads
1 mL	30 µL
3 mL	90 µL
5 mL	150 µL
6 mL	180 µL

Note: Ensure that the mixture is homogeneous and the beads are distributed evenly throughout the sample.

3. Place on a shaker for 10 minutes at 1000 rpm to mix and bind the nucleic acid to the beads.

4. Place on the DynaMag™–50 Magnet stand for 5 minutes.

IMPORTANT! Wait the entire 5 minutes before removing supernatant to ensure maximum yield.

5. Remove, then discard the supernatant without disturbing the beads.

Wash the beads

1. Resuspend the beads in 1 mL of Wash Solution 1, then mix by pipetting up and down carefully, ensuring all beads are released from the tube walls.

2. Transfer the bead slurry to a new non-stick 1.5-mL microcentrifuge tube and place on the DynaMag™–2 Magnet stand for 20 seconds. Do not discard the 50-mL conical.

3. Collect the supernatant from the bead pellet in the 1.5-mL tube and use it to rinse remaining beads from the 50-mL conical.

4. Carefully transfer the supernatant back to the 1.5-mL tube on the DynaMag™–2 Magnet stand.

5. Leave the 1.5-mL tube on the magnet stand for 2 minutes or until solution clears, then discard the supernatant, being careful not to disturb the beads.

6. Add 1 mL of 80% ethanol.

7. Vortex briefly, then centrifuge briefly.

8. Place on DynaMag™–2 Magnet stand for 2 minutes, or until solution clears.

9. Discard the supernatant.

10. Repeat steps 6–9 for a second wash with 1 mL of 80% ethanol.

11. Carefully remove, then discard the ethanol.

Elute the cfNA

1. Air-dry the beads for 5 minutes, then remove any remaining ethanol with a 10-µL pipette tip.
2. Resuspend the beads in 400 µL of MagMAX™ Cell-Free Total Nucleic Acid Elution Solution and vortex for 5 minutes at high speed.
3. Centrifuge briefly, then place the tube on the DynaMag™–2 Magnet stand to capture beads for 2 minutes.
4. Transfer the supernatant to a new 1.5-mL microcentrifuge tube.

Concentrate and wash the cfNA

1. Add 500 µL of MagMAX™ Cell-Free Total Nucleic Acid Lysis/Binding Solution.
2. Add 10 µL of well-vortexed MagMAX™ Cell-Free Total Nucleic Acid Magnetic Beads.

   IMPORTANT! Do not use less than 10 µL of beads for the rebinding step.
3. Vortex 1.5-mL tube at high speed for 5 minutes to bind nucleic acid to beads.
4. Briefly centrifuge, then place on DynaMag™–2 Magnet stand for 5 minutes to capture the beads.
5. Discard the supernatant.
6. Add 1 mL of Wash Solution 1.
7. Vortex briefly, then centrifuge briefly.
8. Place on DynaMag™–2 Magnet stand for 2 minutes.
9. Discard the supernatant.
10. Add 1 mL of 80% ethanol.
11. Vortex briefly, then centrifuge briefly.
12. Place on DynaMag™–2 Magnet stand for 2 minutes, or until solution clears.
13. Discard the supernatant.
14. Repeat steps 10–13 for a second wash with 80% ethanol.
    Be careful to remove all the ethanol.

Elute the concentrated cfNA

1. Air-dry the beads on the DynaMag™–2 Magnet stand for 3 minutes, taking care not to over-dry the beads.
2. Tap the magnet stand gently against a flat surface to collect remaining liquid, then discard the remaining liquid.
3. Add 15 µL of MagMAX™ Cell-Free Total Nucleic Acid Elution Solution and vortex on high for 5 minutes.
4. Briefly centrifuge, then place on DynaMag™–2 Magnet stand for 2 minutes to capture the beads.
5. Transfer the eluate to a fresh 1.5-mL tube.

Store the purified cfNA on ice for immediate use. Alternatively, store the purified cfNA at –20°C or –80°C for long-term storage.

cfNA quantification

We recommend using the Agilent™ High Sensitivity DNA Kit (Cat. No. 5067-4626) to observe the cfDNA fraction of the eluate. cfDNA is fragmented dsDNA with a major peak at 170 bp.

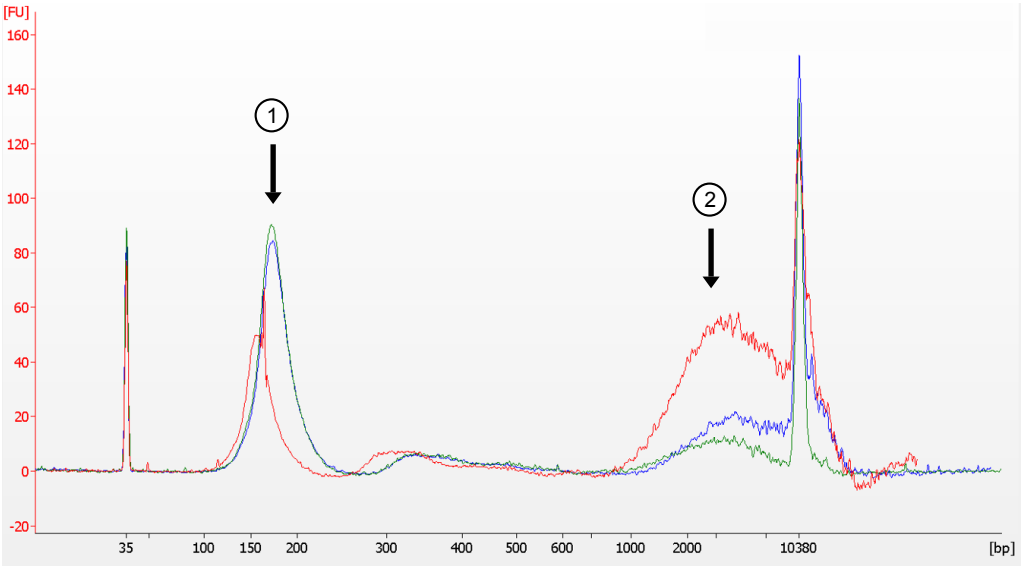

Figure 2 Matched 4 mL K_2-EDTA plasma isolations

① Cell-free DNA (100–275 bp)

② Genomic DNA

- *Blue line:* MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit Manual isolation
- *Green line:* MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit Kingfisher Flex isolation
- *Red line:* Competitor kit Manual isolation
- FU: Fluorescence units
- bp: Base pairs

We recommend using the Qubit™ dsDNA HS Assay Kit (Cat. No. Q32854) to quantify the cell-free DNA yield in the eluate. For most samples, a 2-µL input is sufficient. It is important to note that the Qubit cannot discriminate between cell-free DNA and gDNA in the sample, so running an Agilent High Sensitivity DNA analysis chip is highly recommended.

We recommend an m1 qPCR assay to observe the cfRNA in the sample. cfRNA concentrations in plasma are low and the conformation/length is not compatible with the dye in the Qubit RNA HS assay. The m1 designation indicates that the probe spans an exon-exon boundary ensuring that signal is only generated from template with correctly spliced exons. The assay will not detect signal from the cfDNA that is also present in the eluate. Perform the reverse transcription (RT) reaction according to the SuperScript™ VILO™ Master Mix (Cat. No. 11755050) protocol. The reaction volume for this step can be scaled down to 10 µL total, using 2 µL purified cfNA input. Perform the qPCR reaction according to the protocol for TaqMan® Universal Master Mix II, no UNG (Cat. No. 4440040), with sample input up to 10% of the total reaction volume. We recommend using a TaqMan assay with an appropriate amplicon length to detect cfRNA, such as Hs_99999905_m1 GAPDH (122 bases), Hs99999903_m1 ACTB 171 bases or another m1 assay target appropriate to your sample.

C

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

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  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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