chemagic™ cfDNA 5k Kit

(art. No. CMG-134)

Manual Purification Protocol for 1 - 5 mL of Plasma

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Intended Use

With the **chemagic cfDNA 5k Kit,** circulating cell-free DNA (cfDNA) can be isolated from 1 - 5 mL of plasma obtained from human whole blood samples. All reagents needed for the isolation of cfDNA are included in the kit, except for nuclease-free water for dissolving Proteinase K. The kit components provide sufficient material for 40 preparations.
The kit is designed to be used manually with the **chemagic** Stand 2x12 and **chemagic** Magnetic Stand Type F. The product is intended for professional users such as technicians and physicians trained in molecular biology techniques.

Contents of the Kit

M-PVA Magnetic Beads	1 x 10 mL	**Binding Buffer 2**	2 x 150 mL
Lysis Buffer 1	1 x 15 mL	**Wash Buffer 3**	1 x 200 mL
Proteinase K (lyophilized)	5 vials	**Wash Buffer 4**	1 x 200 mL
Poly(A) RNA (lyophilized)	1 vial	**Elution Buffer 5**	1 x 10 mL
Poly(A) RNA Buffer	1 vial		

Quality Control

Each lot is tested to ensure the product meets the defined specifications according to chemagen's quality management system. Suboptimal results may be obtained if the protocol is not strictly followed.

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Functional Principle

The **chemagic cfDNA 5k Kit** is based on chemagic technology using **M-PVA Magnetic Beads** for the isolation of cfDNA. The cfDNA binds to paramagnetic beads which are magnetically separated from the sample material. During subsequent steps contaminants are removed and the purified cfDNA is transferred into an elution medium.

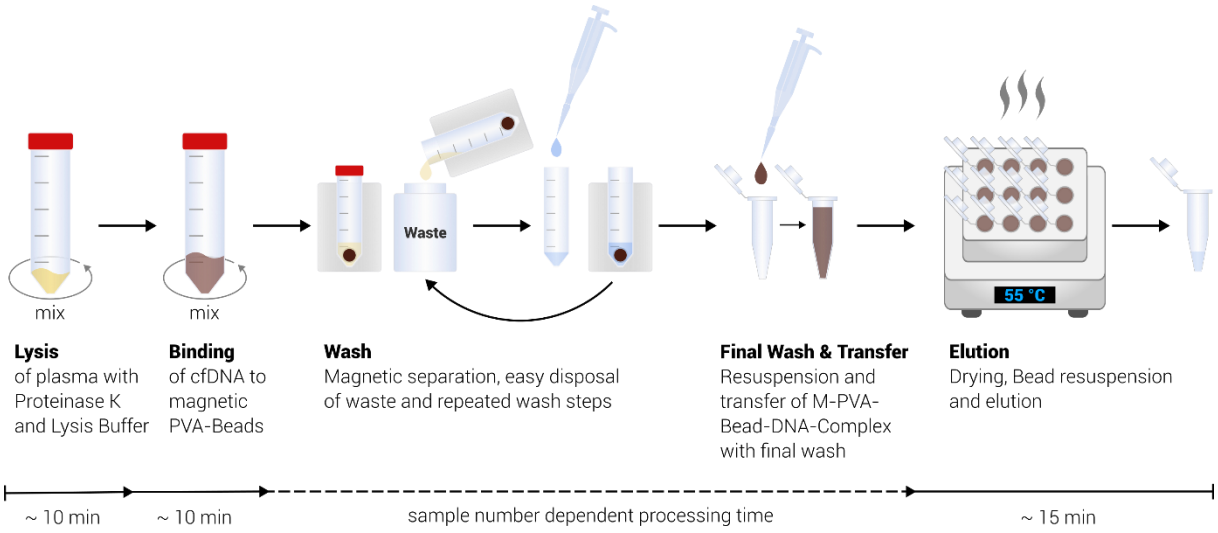

Figure 1. Functional principle of the CMG-134 cfDNA extraction kit.

Product Specifications

The kit is designed for the use with human plasma samples derived from stabilized blood collection tubes.

The kit is adapted to a manual procedure with differences regarding incubation and time efficiency compared to the **chemagic 360** platform.

Fresh and frozen plasma can be used. Please refer to section "**Plasma Preparation Protocol**" below.

The kit is not intended for the use with whole blood or tissue sample material. The isolation efficiency of cfDNA with other sample materials has not been investigated.

All reagents required for the cfDNA isolation are included in the kit except nuclease-free water for dissolving **Proteinase K**.

The **Elution Buffer 5** included in this kit is 10 mM Tris-HCl pH 8,0 with 0,1 mM EDTA. 10 mM Tris-HCl pH 8,0 can also be used without any protocol adjustments. Water pH 8,0 may also be used, but the yield could be slightly decreased.

Required Equipment

- CMG-134, **chemagic cfDNA 5k Kit** (40 preps)
- CMG-300, **chemagic** Stand 2x12 for 1,5 mL and 2,0 mL tubes
- CMG-302, **chemagic** Magnetic Stand Type F for 50 mL centrifuge tubes
- CMG-306, **chemagic** cfDNA stand 12 for 50 mL centrifuge tubes (optional)
  This stand allows for the simultaneous separation of 12 samples and is recommended for faster processing of multiple samples.
- (Thermo-) shaker suitable for 50 mL tubes
- Vortex mixer
- Thermoshaker for 1,5 mL tubes
- Conical 50 mL tubes, 1,5 mL tubes, 1,5 mL low binding tubes
- Centrifuge for 50 mL tubes for 50 mL centrifuge tubes (optional)
- Antifoam (Antifoam A Concentrate, Sigma, Product number A5633) (optional)
- Mini centrifuge for 1,5 mL tubes (optional)

Stability and Storage

The shelf life of the kit is 18 months. Expiry dates are noted on the kit label and on the labels of the individual kit components. All components of the unused kit can be stored at room temperature. Do not use the kit beyond the expiry date.

Once the kit has been opened, the “in use” stability of the kit is 3 months. All **Wash Buffers**, **Lysis Buffer 1**, **Binding Buffer 2, Poly(A) RNA Buffer** and the **M-PVA Magnetic Beads** of the “in use” kit can be stored at room temperature.

Binding Buffer 2, **Wash Buffer 3** and **Wash Buffer 4** contain ethanol. Longer storage of the buffers without lids should be avoided. If ethanol evaporates the optimal yield cannot be guaranteed.

Store **Poly(A) RNA Buffer, Binding Buffer 2** and **Wash Buffer 3** in the dark.

Lysis Buffer 1 may form a precipitate upon storage. If necessary, warm up to 55 °C to dissolve.

The reconstituted **Poly(A) RNA** in **Poly(A) RNA Buffer** and reconstituted **Proteinase K** are stable for 4 weeks at 4 °C.

For long-term storage we recommend storing the reconstituted **Poly(A) RNA** in **Poly(A) RNA Buffer** and **Proteinase K** in aliquots at -20 °C. Do not freeze the **Poly(A) RNA** in **Poly(A) RNA Buffer** and **Proteinase K** aliquots after thawing.

Before using equilibrate **Poly(A) RNA** and **Proteinase K** to room temperature.

The use of **Poly(A) RNA** is recommended as indicated in the detailed protocol description below. The extraction of cfDNA using this kit is possible without the use of **Poly(A) RNA.** However, the quantification of cfDNA may vary.

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Quantification Methods – Comments

In some cases, you may find traces of **M-PVA Magnetic Beads** remaining in the eluate. In such a case we recommend a short centrifugation of the samples to isolate the remnant **M-PVA Magnetic Beads** at the bottom of the vessel or perform an additional separation step using an appropriate **chemagic** magnetic stand to separate traces of particles.

During development, the performance of the automated kit was evaluated using the following quantification methods:

- qPCR on ALU115 primer set[1] on the QuantStudio® 5 Real-Time PCR System
- Qubit™ 1X dsDNA HS Assay Kit on the Qubit® 3.0 Fluorometer

cfDNA yields isolated from human plasma samples are typically in the range of 1 - 30 ng/mL of plasma and therefore critically low and may lie outside the detection parameters determined by common spectrophotometric methods.

If quantification of the extracted cfDNA is required, a PCR-based method (qPCR, ddPCR) is recommended or an electrophoretic method dedicated to cfDNA measurement, such as the LabChip® cfDNA assay where the standard used for quantitation moves close to the cfDNA peaks.

When using fluorometric quantification methods, the addition of **Poly(A) RNA** is essential for a reliable performance. Fluorometric analysis of eluates extracted without the use of **Poly(A) RNA** may lead to varying and decreased quantification data. Additionally, be careful in interpreting the results if fluorometric methods are used for quantification as not only cfDNA but the total DNA is measured.

Qualitative Methods – Comments

During development the performance of this kit was evaluated using the following qualitative method:

- DNA NGS 3K Assay with the LabChip® GX Touch/GXII Touch

To assess fragment distribution of the extracted cfDNA, fragment analysis systems may be used. A major peak at around 150 - 170 bp is expected for high quality mononucleosomal cfDNA, in some cases a smaller peak at around 300 bp representing dinucleosomal cfDNA fragments is also present.

The use of fragment analysis systems for cfDNA quantification with this assay did not lead to reproducible results as it relies on an upper marker peak for quantification. Upper marker peak areas

[1] ALU115 primer sequences obtained from: T.B. Hao, British Journal of Cancer (2014) 111, 1482–1489

(marker included in fragment analyzer kits) vary according to different extraction chemistries. Variations of upper marker peak areas within one fragment analysis run may result in miscalculations. Therefore, we recommend the use of a smaller ~50 bp marker (similar to that used in the LabChip® cfDNA assay) which migrates closer to cfDNA peaks for more accurate quantification.

Plasma Preparation Protocol

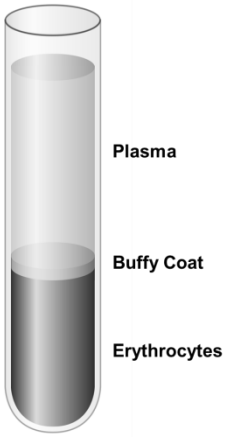
It is recommended to prepare plasma as fresh as possible (max. 5 days after blood draw). Longer storage of blood prior to plasma preparation may lead to poor separation results and higher background from genomic DNA.

A double centrifugation protocol during plasma preparation is recommended to minimize the potential for contamination of plasma with cells and genomic DNA. Transfer of any other blood components (buffy coat or red blood cells) should be avoided while separating the plasma fraction.

Please refer to the tube manufacturers specifications (max. centrifugation speed) for more information.

From 10 mL of whole blood approximately 4 - 5 mL plasma can be expected.

General Plasma Preparation Protocol



1. Centrifuge whole blood collection tube at 2.000 x g for 20 minutes.
2. Aspirate plasma carefully and at least 2 - 3 mm above the buffy coat layer, without disturbing the layer and transfer it into a new appropriate tube.
3. Centrifuge the plasma sample at 3.300 x g for 30 minutes.
4. Carefully transfer the supernatant to a fresh tube without disturbing the pellet, at least 2 - 3 mm above the pellet.
5. For direct use, storage of plasma sample at 2 - 8 °C for up to 10 hours is possible, for long-term storage -80 °C is recommended.
6. Before cfDNA extraction, equilibrate plasma to room temperature. Thaw the frozen plasma storage tubes at room temperature for approximately 30 minutes (until thawed) or using a water bath (25°C to 30°C) for 10 minutes. Ensure that the tubes are thoroughly thawed before cfDNA extraction is started. If there are precipitates in the thawed plasma, dissolve them by inverting the tubes.

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Processing Steps in Detail

Before You Start

- Dissolve lyophilized **Poly(A) RNA** by adding **440 µL of Poly(A) RNA Buffer** to the **Poly(A) RNA** tube and mix thoroughly.
- Dissolve lyophilized **Proteinase K** in 1,25 mL nuclease-free water.
- Preheat thermoshaker for 1,5 mL tubes to 55°C.

! ***See "Stability and Storage"***

- A homogenous suspension of the **M-PVA Magnetic Beads** must be ensured to guarantee the correct **M-PVA Magnetic Bead** concentration. Mix the bottle containing the **M-PVA Magnetic Beads** vigorously and check the bottom of the bottle for **M-PVA Magnetic Beads** sedimentation before dispensing. Otherwise, optimal cfDNA extraction performance cannot be ensured.

The following table lists volumes of the components to be used, depending on starting plasma sample volume.

Component	Plasma Sample	
	1 - 2 mL	3 - 5 mL
Poly(A) RNA	1,3 µL	1,3 µL
Proteinase K	100 µL	150 µL
Lysis Buffer 1	100 µL	250 µL
M-PVA Magnetic Beads	60 µL	150 µL
Binding Buffer 2	3 mL	6,25 mL
Drying Step	2 min at 55 °C	5 min at 55 °C
Elution Buffer 5	30 - 60 µL	50 - 100 µL

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Protocol Steps

To avoid foaming and facilitate easy removal of supernatant with chemagic™ Magnetic Stands, the use of antifoam **from the recommended manufacturer** is advised. Alternatively, follow recommended steps below to remove foam.

1. Prefill the 50 mL sample tube with 5 µL Antifoam A concentrate and the indicated volume of **Proteinase K, 1,3 µL dissolved Poly(A) RNA** and **Lysis Buffer 1.** Add the **plasma sample** equilibrated to room temperature. Vortex briefly for **10 seconds** and incubate the sample for **10 minutes at room temperature while mixing on a (thermo-) shaker (300 rpm)**.

   Proteinase K, Poly(A) RNA and **Lysis Buffer 1** can be premixed by transferring **Proteinase K** to an appropriate vessel first and then adding **Poly(A) RNA** followed by the **Lysis Buffer 1** (choose the appropriate volume of **Proteinase K** / **Poly(A) RNA** / **Lysis Buffer 1** to ensure you have enough for the number of isolations). The three components have to be pipetted in the given order and resuspended as soon as **Lysis Buffer 1** is added to avoid precipitation.

Do not vortex the premix as this leads to foaming. Mix by gentle pipetting.

! ***The Proteinase K activity will decrease after incubation longer than 10 minutes in Lysis Buffer 1. Ensure that all samples are mixed with Proteinase K / Poly(A) RNA / Lysis Buffer 1 within this time.***

2. Make sure that the **M-PVA Magnetic Beads** are shaken and in suspension. No bead slurry should be visible at the bottom of the bottle – shake vigorously if necessary. Add the indicated volume of **M-PVA Magnetic Beads** and **Binding Buffer 2** to each sample. Vortex briefly and incubate for **10 minutes** while mixing on a **(thermo-) shaker (300 rpm) at room temperature**.
3. Following binding incubation, place the tube on the **chemagic Magnetic Stand Type F for 5 minutes** and if necessary, until the solution clears, and **M-PVA Magnetic Beads** are completely separated. If necessary, remove any foam by pipetting (when no antifoam was used). Afterwards, discard the supernatant by pouring the tube now fixed within the **chemagic Magnetic Stand Type F (Figure 1)**.

   Alternatively: Briefly centrifuge (1 min, 4000 rpm) the lysis/binding tube to remove any foam (when no antifoam was used) and to separate the M-PVA Magnetic Beads. Keep the tube on the magnet for another minute and discard the supernatant by pouring as described previously.

Multi-sample processing with the chemagic cfDNA stand 12 (CMG-306): Following binding incubation, unscrew the lid of the 50 mL tubes and place all tubes on the **chemagic cfDNA stand 12 for 5 minutes** and if necessary, until the solution clears, and **M-PVA Magnetic Beads** are completely separated. If necessary, remove any foam by pipetting (when no antifoam was used). Afterwards, transfer the individual tubes to the **chemagic Magnetic Stand Type F**, separate for 30 seconds and discard the supernatant by pouring the tube now fixed within the **chemagic Magnetic Stand Type F.**

All following wash steps can be performed in the same manner.

4. Resuspend M-PVA Magnetic Beads in 2 mL of **Wash Buffer 3** by vortexing (10 – 30 seconds) and place the tube on the **chemagic Magnetic Stand Type F for 30 seconds** or until the solution clears and **M-PVA Magnetic Beads** are completely separated. Discard the **Wash Buffer 3** supernatant by pouring as described previously.
5. Remove the tube from the magnetic separator and repeat step 4 (vortex for 10 seconds).
6. Repeat the washing procedure (step 4) using 2 mL **Wash Buffer 4** once (vortex for 10 seconds).
7. Remove the tube from the magnet position. Resuspend the beads in **600 µL Wash Buffer 4** by pipetting, then transfer the **M-PVA Magnetic Beads/DNA Complex** to a 1,5 mL tube in **the chemagic Stand 2x12** (**non-magnet position**). Use an additional **600 µL of Wash Buffer 4** to rinse the 50 mL tube and transfer any residual beads to the corresponding 1,5 mL tube and mix gently by pipetting 10 times. Place the 1,5 mL tube into the **magnet position for at least 90 seconds**. Aspirate and discard the supernatant by pipetting (*use 200 µL pipette tip to remove last traces*).

! ***Make sure that there is no residual liquid and bead slurry left in the lid, alternatively liquid can be shortly spun down.***

8. After removing the last traces of the supernatant, dry the bead pellet for the **indicated time** (*see page 8*) **at 55 °C in a thermoshaker** (*open tube lid*).

! ***Make sure that the bead pellet is dried, extend drying time by another 1 – 2 minutes if necessary. The dried bead pellet should have a matte surface but no cracks (Figure 2).***

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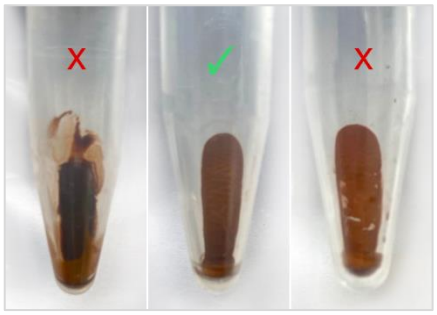

Figure 2. Bead pellet drying stages (left to right): the bead pellet is still wet and smears when the tube is twisted (x); dry bead pellet with a matte surface and slightly lighter color (✓); bead pellet with cracks should be avoided (x).

9. Add desired volume of **Elution Buffer** to the tube and thoroughly resuspend the **M-PVA Magnetic Beads/DNA Complex** by pipetting (10 - 15 times).
10. Incubate the suspension for **5 minutes at 55 °C** with gentle agitation in a thermoshaker **(500 rpm)**.
11. Following DNA elution, place the tube in a **chemagic Stand 2x12 (magnet position) for 2 minutes** or until all the **M-PVA Magnetic Beads** have separated from the eluate. Transfer the **eluate** containing the purified DNA to a clean low binding tube.

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Safety Information

To avoid injuries working with the kit components always wear safety glasses, disposable gloves and protective clothing. For detailed information please refer to the according Safety Data Sheet.

Reagent 1: **M-PVA Magnetic Beads** No hazardous substances in reportable concentrations, which must be labeled.
Reagent 2: **Lysis Buffer 1** **Substance(s):** Sodium dodecyl sulfate 20% ($CH_3(CH_2)_{11}OSO_3Na$) **CAS No.:** 151-21-3 ($CH_3(CH_2)_{11}OSO_3Na$)
Reagent 3: **Binding Buffer 2** **Substance(s):** Guanidine thiocyanate 35 - 45% ($C_2H_6N_4S$), dissolved in ethanol 20 - 40% (EtOH) **CAS No.:** 64-17-5 (EtOH), 593-84-0 ($C_2H_6N_4S$)
Reagent 4: **Wash Buffer 3** **Substance(s):** Guanidine thiocyanate 15 - 25 % ($C_2H_6N_4S$), dissolved in ethanol 30 - 50% (EtOH) **CAS No.:** 64-17-5 (EtOH), 593-84-0 ($C_2H_6N_4S$)
Reagent 5: **Wash Buffer 4** Substance(s): aqueous ethanol solution 70 - 90% (C_2H_5OH) **CAS No.:** 64-17-5 (EtOH)
Reagent 6: **Elution Buffer 5** No hazardous substances in reportable concentrations, which must be labeled.
Reagent 7: **Poly(A) RNA Buffer** **Substance(s):** Guanidine thiocyanate 30 - 40%($C_2H_6N_4S$) **CAS No.:** 593-84-0 ($C_2H_6N_4S$)
Reagent 8: **Poly(A) RNA** No hazardous substances in reportable concentrations, which must be labeled.

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