NeoGeneStar
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NeoGeneStar™ Circulating DNA Purification Kit

For concentration and purification of circulating cell-free DNA for variable sample volumes

(Additional information and protocol suggestions for cell free DNA purification is available on NeoGeneStar website: www.NeoGeneStar.com)

Liquid Lysis Reagents with Protease

For Research Use Only.

Not for human or animal therapeutic or diagnostic use.

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Introduction

Circulating cell-free DNA fragments, usually shorter than 1000 bp, such as tumor-specific extracellular DNA fragments in the blood or fetal nucleic acids in maternal blood can be isolated from serum or plasma.[1, 4] Analysis of circulating cell-free DNA fragments can be useful in characterizing certain cancers[3] and disease states[5] as well as in fetal genetic analysis[1, 2, 4] The concentrations of circulating cell-free DNA in biological fluids such as plasma, serum, or urine can vary considerably (~50pg/ml to >100ng/ml) and depend on the individual, disease state, therapeutic regimen, and period of gestation.[5]For example, the concentrations of circulating cell-free DNA fetal DNA was shown to be 3.4% (range 0.39%-11.9%) and 6.2% (range 2.33%-11.4%) of the total plasma DNA in early and late pregnancy, respectively.[6]

The NeoGeneStar™ Circulating DNA Kits come with proprietary NeoGeneStar™ superparamagnetic beads that have been especially designed for isolation of circulating cell-free DNA in plasma, serum or urine. Cutting edge nanotechnology fabrication methods are utilized in the GMP manufacturing facility. The NeoGeneStar™ Circulating DNA Kits enable starting sample volumes from 0.2ml to 22ml and elution volumes of 50µl; which enables concentration and enrichment of circulating cell-free DNA that is usually present at low concentrations during the early stages of pregnancy, cancer or disease state.

Samples can be either freshly collected or frozen, though multiple freeze/thaw cycles should be avoided due to reduced yield and quality of the purified circulating cell-free DNA. The purified circulating cell-free DNA is then ready for use in subsequent analysis such as PCR, qPCR or Next Generation Sequencing (NGS) or long term frozen storage.

Principle and Procedures

1) Sample is fresh or proteolytically digested if frozen or using Streck BCT. (Recommend NeoGeneStar Pretreatment kit or NeoGeneStar cfDNA-STABIL kit)
2) Superparamagnetic particles bind nucleic acids in the presence of lyse/bind buffer.
3) Particles are captured, washed with wash buffer and 75% ethanol, and air dried.
4) Cell Free DNA is eluted.

This procedure is available for manual or automated processing and yields circulating cell-free DNA in about 45 minutes.

Binding Characteristics and Sample Volumes

The superparamagnetic particles bind DNA molecules from ~30 bases to > 10,000 bases.
The liquid lysis formulation NeoGeneStar™ circulating cell-free DNA Kit has been optimized for variable sample volumes.

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Kit Contents

LYS Buffer[1]	200 ml
Carrier RNA	0.25 ml
NeoGeneStar Protease	2.5 ml
NGS Beads™	1.0 ml
Wash Buffer[2]	100 ml
Elution Buffer	5 ml

[1]LYSBuffer contains chaotropic salts, which are irritants. Please wear gloves and handle with appropriate laboratory safety measures.
[2]Absolute ethanol must be added prior to use for Wash Buffer.

Storage and Stability

Store NeoGeneStar™ Circulating DNA Kit in the dark at room temperature (15-25°C) until use. Once opened, NGS Beads™ should be stored at 2-8°C for up to one year after delivery. RNA carrier should be stored at -20°C freezer upon deliver. **The kit's other components should not be frozen.**

Note Regarding Lysis Buffer and Wash Buffer

Wash Buffer must be diluted with an equal amount of absolute ethanol and stored at room temperature prior to use. In the event that the Lysis Buffer or Wash Buffer is chilled, a precipitate may form; it would then be necessary to warm the Lysis Buffer or Wash Buffer to fully dissolve the components prior to use.

Wash Buffer can be prepared in advance and stored at room temperature.

Materials Needed That Are Not Supplied:

- Low DNA binding microcentrifuge tubes
- Magnetic separation devices (for 15ml and microcentrifuge tubes)
- Absolute ethanol and isopropanol
- NeoGeneStar Pretreatment Kit.
- Tube shaker / vortexer for 15ml and microcentrifuge tubes.

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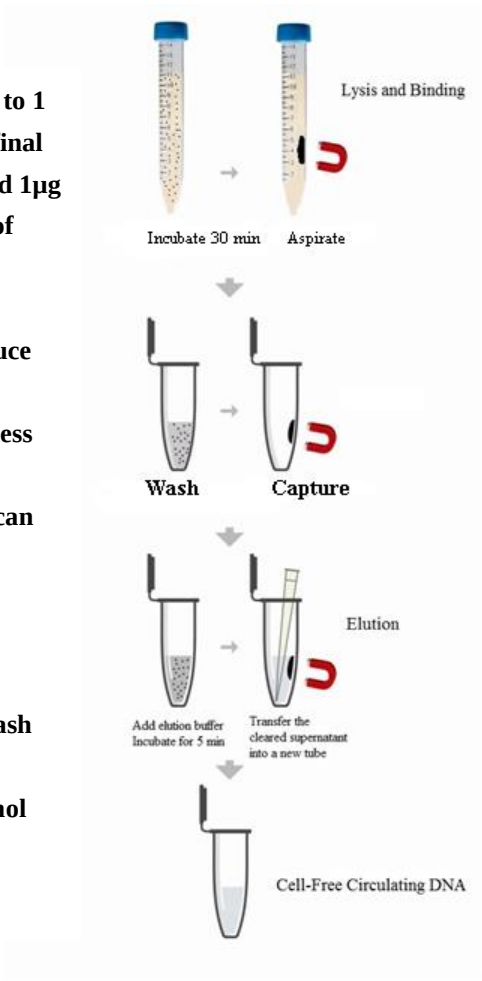
Procedure Outline

Add 3 volumes of LYS Buffer to 1 volume of sample, and 1/10th final volume of isopropanol and add 1µg carrier RNA before addition of NGS Beads™ and mix well

The isopropanol can help reduce foaming. To maximize the recovery of small fragments (less than 50 bases), the final concentration of isopropanol can be increased to 15% vol/vol.

Incubate for 30 minutes by vortexing or inverting
Wash 2 times with Diluted Wash Buffer
Wash 2 times with 80% Ethanol
Elution



Procedure of the NeoGeneStar™ Circulating DNA Kit

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Plasma Handling Guidelines:

It is recommended that K_3EDTA (lavender top) tubes be used for sample collection. Plasma preparation, centrifugation should optimally occur as soon as possible following collection and preferably within 3 hours of collection.

Samples Pretreatment Guidelines:

When Streck Cell Free DNA BCT tubes were used or the plasma was frozen, NeoGeneStar Protease digestion are recommended for optimal cfDNA purification.

Sample Volume	20x Protease Buffer	20mg/ml NeoGeneStar Protease or Proteinase K (25x)	1µg RNA carrier
200 µl	10 µl	8 µl	5µl (200ng/µl)
1.0 ml	50 µl	40 µl	5µl (200ng/µl)
2.5 ml	125 µl	100 µl	5µl (200ng/µl)
4 ml	200 µl	200 µl	5µl (200ng/µl)
8 ml	400 µl	320 µl	5µl (200ng/µl)

1) Add 20x Pretreatment buffer to the sample (as indicated in table above)
2) Add 20mg/ml NeoGeneStar Protease or Proteinase K according to the chart.
3) Add 1µg of RNA carrier per sample (5µl at 200ng/µl).
4) Mix by inversion, incubate at 55-60^{o}C for 30 minutes
5) Proceed with cfDNA purification as per original sample volume.

Note: Pretreatment can be performed for overnight incubation at 55-60^{o}C, then proceed to NGS cfDNA purification based on specified sample volume, ie. 1 ml of plasma will result in about 1.1ml after pretreatment, add 3ml of lysis Buffer to 1.1ml pretreatment sample.

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NeoGeneStar cfDNA purification Protocol:

Prepare sufficient diluted Wash Buffer and 80% Ethanol at the beginning of the procedure.

Sample Volume	LYS Buffer	Isopropanol	NGS Beads™
0.1 to 0.5ml	0.3 to 1.5ml	1/10th final volume	10 µl
0.5ml to 0.9ml	1.5ml to 2.7ml	1/10th final volume	20 µl
1ml to 5ml	3ml to 15ml	1/10th final volume	30 µl

1. For fresh or pretreated plasma, serum, CSF or urine, add 3 volumes of LYS buffer to 1 volume of sample (or entire pretreated sample) and mix thoroughly at room temperature.

2. Add 5µl of carrier RNA (200ng/µl) to the sample / lysis buffer mix. Mix thoroughly and then add 1/10th final volume of isopropanol (ie. for a 1ml sample with 3ml of lysis buffer – add 400µl isopropanol) and mix well. (carrier RNA can be added at the proteolytic digestion step.)

3. After fully resuspending NGS Beads™ by vortexing for than 1 minute, add NGS Beads™ according to table above and vortex to ensure complete mixing of the NGS Beads™ with the lysed sample. Vortex or invert for 30 minutes on a vortexer or 360° rotator. Proper shaking will result in visible foam above the liquid layer.

Examples for shaking mixer:


4. Centrifuge briefly to reduce the foam, then place the 15ml tube on a magnetic stand for at least 1 minutes, the solution will clear. With the sample tube on the magnetic stand, carefully aspirate the cleared supernatant without aspirating the NGS™ Beads. A vacuum aspiration is convenient but not necessary.

5. Remove the sample tube from the magnetic stand, add 1ml of Wash Buffer (diluted 1:1 with

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absolute ethanol) into the tube and carefully rinse the NGS™ Beads and transfer to a 2.0ml low DNA binding microcentrifuge tube, add 0.4ml diluted Wash Buffer to rinse 15ml tube again and transfer to the microcentrifuge tube.

6. Place the microcentrifuge tube on a magnetic stand for at least 1 minute or until the solution clears, turn the tube over a few times while on the magnetic stand to remove the NGS™ Beads trapped on the lid. With the sample tube on the magnetic stand, carefully aspirate the cleared supernatant without aspirating the NGS™ Beads.

7. Remove the microcentrifuge tube from the magnetic stand and add 1ml of Wash Buffer (diluted 1:1 with absolute ethanol) into the tube and resuspend the NGS™ Beads by vortexing for 30 seconds.

8. Place the sample tube onto the magnetic stand for at least 1 minute or until the solution clears, turn the tube over a few times while on the magnetic stand to remove the NGS™ Beads trapped on the lid. With the sample tube on the magnetic stand, carefully aspirate the cleared supernatant without aspirating the NGS™ Beads.

9. Remove the microcentrifuge tube from the magnetic stand and add 1ml of 80% ethanol to the tube. Completely resuspend the NGS™ Beads by vortexing for 30 seconds.

10. Place the microcentrifuge tube on the magnetic stand for at least 1 minute or until the solution clears, turn the tube over a few times while on the magnetic stand to remove the NGS™ Beads trapped on the lid. With the sample tube on the magnetic stand, carefully aspirate the cleared supernatant without aspirating the NGS™ Beads.

11. Wash the NGS™ Beads with 1ml of 80% ethanol again by repeating steps 9 and 10.

12. Pulse down and aspirate again to remove as much of the liquid as possible. Keeping the tube on the magnet, air-dry the NGS™ Beads at room temperature for 10 minutes.

13. Add 30µl-100µl Elution Buffer or elution buffer of your choice and resuspend the NGS™ Beads by pipetting up and down, then incubate 10 minutes at room temperature.

14. Place sample tubes on a magnetic stand, the solution will clear in about 1 minute. Transfer the cleared supernatant into a low DNA binding microcentrifuge tube. This is the purified cfDNA. Freeze the eluted cfDNA until you are ready for your downstream analysis.

> **Note**: For certain applications, such as digital (microdroplet) PCR, it may be advantageous to briefly microcentrifuge to remove any NGS™ Beads that maybe present in the eluate.

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General Considerations/ Suggestions

Sample Collection and Plasma Preparation

Plasma should be prepared as soon as possible after collection. Generally two centrifugations are recommended. First spin 10 minutes in a clinical centrifuge at about 1,600 x g. Transfer the clear plasma to a second tube and spin at ~ 16,000 x g for 10 minutes. Carefully transfer the clear plasma to a fresh tube for immediate cfDNA purification, or stabilization for room temperature storage, or to be frozen.

Best results are obtained from fresh plasma or plasma that is pretreated and stored at room temperature until cfDNA purification. The NeoGeneStar Pretreatment / Storage Kit is recommended.

Plasma may be aliquoted, frozen and stored at -70 ^{o}C

Important: Thaw plasma at room temperature – do not warm untreated plasma to greater than 40 ^{o}C as interfering coagulation can form.
For best results, follow the proteolytic digestion in the NeoGeneStar Pretreatment kit.

K_3EDTA and K_2EDTA (lavender top) tubes

EDTA will chelate divalent ions which prevent coagulation and have the added advantage of inhibiting DNases. K_3EDTA collection tubes have liquid EDTA in solution in glass tubes. K_2EDTA collection tubes have the EDTA spray dried in plastic tubes. The K_3EDTA is preferred. The plasma should be prepared as soon as possible and preferably within 3 hour of collection. For best results, cfDNA should be purified immediately. The plasma sample can be stabilized with the NeoGeneStar pretreatment kit – cfDNA is stable for up to two weeks. Alternatively the plasma may be frozen.

Plasma Separator Tubes (PST) (light green with gel)

PST tubes contain lithium heparin and gel to prevent coagulation and to separate the plasma from the blood cells during the first clinical centrifugation. These tubes are ideal for immediate plasma preparation and stabilization. Plasma from PST tubes can be poured into NeoGeneStar pretreatment and stabilization reagents for room temperature storage or shipping. Alternatively, if minimum genomic contamination is required, the plasma from the PST tube should be subjected to a second spin and then either frozen or preferably stabilized with the NeoGeneStar Pretreatment Kit.

Streck BCT Tubes (brown and black tiger top)

The Streck BCT is a blood collection tube with a preservative and crosslinker that stabilizes nucleated blood cells. Samples are stable for up to two weeks at room temperature. Do not freeze Streck plasma.

Important: Streck plasma should be proteolytically digested prior to cfDNA purification.

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Procedural Suggestions / Hints for successful cfDNA purification

1) Be sure that the sample is thoroughly mixed during the cfDNA capture with the magnetic particles. Vortex (about 300 to 500 rpm) and occasionally invert to ensure that the magnetic particles are well dispersed.
2) FOAMING will occur during the magnetic particle binding. It is important not to lose (or leave behind) the magnetic particles. Ideally, the 15ml centrifuge tube should be centrifuged at low speed (about ~1,000 x g) for about 5 minutes to reduce the foam and drive the magnetic particles out of the foam, then continue with the magnetic purification. Alternatively, after the 30 minutes of vortexing during the cfDNA binding step, let the tubes stand on the magnetic stand for at least 5 to 10 minutes to allow the foam and magnetic particles to settle.
3) Be careful not to lose the magnetic particles when aspirating and transferring.
4) Use Elution Buffer provided in the kit for best cfDNA recovery. Elevated elution temperature (about 60^{o}C) can improve cfDNA recovery – especially of longer molecules.
5) For some downstream applications, the final eluate should be spun to ensure that the magnetic particles have been removed and will not interfere.

cfDNA Quantitation and Qualitative Analysis

cfDNA can be quantitated and analyzed by:

Electrophoresis: BioAnalyzer, TapeStation, Fragment Analyzer (Typically there will be a peak around ~170 base pairs – example data shown on following page)

Fluorescence: Life Technologies Qubit™ dsDNA HS Assay Kit (Cat. No. Q32855), when carrier RNA is added during cfDNA purification, cfDNA concentration may not be accurate.

Quantitative PCR: Ideally, PCR Amplicons of several sizes and genomic frequency will be analyzed. Shorter amplicons (about 60 bases) will yield the highest genomic equivalents.

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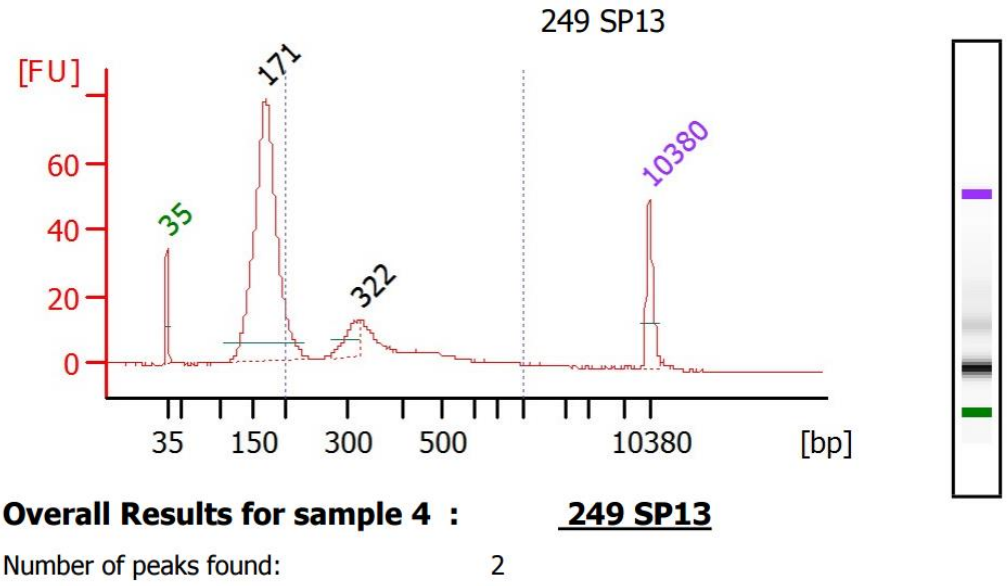
Example of Agilent™ High Sensitivity Analysis trace of cfDNA. Note the typical cfDNA pattern of nucleosomal sized fragments at ~170 base pairs and ~ 320 base pairs.



Example of Agilent™ High Sensitivity Analysis trace of spiked-in DNA Ladder. A DNA MW marker (ladder from 75bp to 700 bp) was spiked in normal plasma and recovered using the NeoGeneStar cfDNA purification kit. Recovery of spiked DNA is typically greater than 80%.