AlloSeq cfDNA

Instructions for Use

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Overview

1.1 Principle

The CareDx AlloSeq cfDNA kit and the CareDx AlloSeq cfDNA software (collectively referred to as CareDx AlloSeq cfDNA Assay) form a system that enables relative quantification of the donor-derived cell-free DNA (cfDNA) in a cfDNA sample derived from a transplant recipient for research applications. Cell-free DNA is fragmented DNA in the bloodstream that originates from cells undergoing injury and death.

Following cfDNA extraction from plasma, the cfDNA is amplified using multiplex PCR that includes PCR primers for 202 single nucleotide polymorphisms. The resulting PCR products are sequenced on an Illumina, Inc. (Illumina) MiSeq or MiniSeq sequencing instrument (or alternative validated Illumina sequencer), and the sequence data is analyzed using the CareDx AlloSeq cfDNA software. In the absence of donor or recipient genotype information (see below), cfDNA present as the minor cfDNA contributor in heart, kidney and lung transplant settings is assigned as 'donor-derived' (DeVlaminck et al., 2014; Khush et al., 2019; DeVlaminck et al., 2015; Grskovic et al., 2016; Bloom et al., 2017; Bromberg et al., 2017).

The CareDx AlloSeq cfDNA kit also enables the amplification and sequencing of genomic DNA to identify the genotypes of either, or both, the donor and recipient for research purposes. This step may be performed prior to or at the same time as the cfDNA test, and is required for quantification of donor-derived cfDNA in liver transplant recipients, where levels of donor-derived cfDNA, in contrast to kidney, heart or lung transplants, may represent the major fraction of the total cfDNA in the recipient (Schütz et al., 2017).

Elevated levels of donor-derived cfDNA have been shown to be associated with transplanted organ injury and rejection (Grskovic et al. 2016, Bloom et al. 2017, Bromberg et al. 2017; Khush et al., 2019; DeVlaminck et al., 2015).

This *Instructions for Use* document describes the procedure for the use of the CareDx AlloSeq cfDNA kit. All due care and attention should be exercised in the handling of the products. We recommend all users read the entire document prior to starting the procedure. Procedures for use of the CareDx AlloSeq cfDNA software can be found in the CareDx AlloSeq cfDNA Software Instructions for Use.

The AlloSeq cfDNA Assay offers the following features:

- Fast and easy sample preparation.
  Prepare up to 24 libraries in approximately 2.5 hours, with only 1.5 hours of hands-on time.

- Low DNA input.
  High data quality and robust performance with a recommended input of 10ng for both genomic DNA and cfDNA, requiring low sample concentration of 0.625 ng/µl.

- Flexible kit, sequencer, and capacity selection
  Supported formats: MiSeq Standard V3 flow cell, MiniSeq Mid Output Kit (300 cycles), and MiniSeq High Output Reagent Kit (150-cycles)
  Flexible sequencing capacity with libraries containing between 6 and 24 samples

- DNA sample to software-generated report in less than 24 hours.

1.2 AlloSeq cfDNA Kit Description

The CareDx AlloSeq cfDNA kit is intended to be used together with the CareDx AlloSeq cfDNA software to measure the relative amount of donor-derived cfDNA in solid organ transplant recipients for research use. This product is intended for use with Illumina MiSeq, MiniSeq or any other validated Illumina sequencers.

1.3 Limitations and contraindications

Limitations

- Patients who received transfusions of whole blood or other blood transfusions that contain white blood cell components within one month prior to the AlloSeq cfDNA test may have an inaccurate result. Transfusions of washed red blood cells or leukocyte-depleted, packed red blood cell transfusions do not impact the result.
- There are some indications that damage to the graft caused by invasive procedures such as biopsy may cause a short-term elevation of donor-derived cfDNA. Until definitive studies are completed, AlloSeq cfDNA should not be used on patients within 24h following a biopsy.
- In cases where a patient receives a repeat or secondary kidney with the original transplanted kidney(s) still in place, the contribution of cfDNA from the prior transplanted organ(s) to recipient's plasma is currently unknown. Data from retransplant patients in whom the prior kidney(s) remain *in situ* suggests that AlloSeq cfDNA can be used in retransplant patients in a manner similar to use in the single-kidney transplant population (Mehta, *et al.* 2019).

Contraindications

- Since the test evaluates genetic differences between the donor and recipient, it is not possible to perform the test for a transplant recipient that is a monozygotic twin to the donor.
- When more than two genomes are present in the recipient plasma (more than recipient + donor), contribution of cfDNA from each genome is not differentiated by the test. This includes pregnancy, due to the presence of fetal DNA in the maternal plasma and multiple-organ transplants from different donors since the grafts each introduce a unique genome (e.g. kidney/pancreas) and contribute different basal levels of cfDNA, confounding interpretation of the results.
- A recipient of multiple transplanted organs that all originated from the same donor presents a situation where elevated levels of donor-derived cfDNA could have originated from one organ or another or both. If from both, they could be contributing at different basal levels, confounding interpretation of the results. Therefore, AlloSeq cfDNA is not to be used for transplant recipients with multiple transplanted organs from the same donor.
- Recipients of allogeneic blood or bone marrow transplant who have received cells with a genome different from the recipient (e.g. non-monozygotic twin) should not receive AlloSeq cfDNA testing.

DO NOT USE AlloSeq cfDNA in transplant recipients who:

- have received a transplant from a monozygotic (identical) twin
- have had allogeneic blood or bone marrow transplant
- are pregnant
- have had multiple transplanted organs
- have had a blood transfusion that contains white blood cells within the past 30 days (washed or leukocyte-depleted RBCs are acceptable)
- have had a biopsy within past 24 hours

1.4 Sample Considerations

- For cfDNA, we recommend blood sample collection in Streck Cell-Free DNA BCT®.
- Specimens in Streck tubes that are or have been frozen or stored at or below 4°C should not be tested.

- Specimens in Streck tubes showing hemolysis above a trace/slight amount by visual inspection (approx. ≥ 50mg/dL) should not be tested.
- cfDNA extracted from serum samples is not compatible with donor-derived cfDNA testing due to the presence of genomic DNA released from recipient's cells trough clotting process.

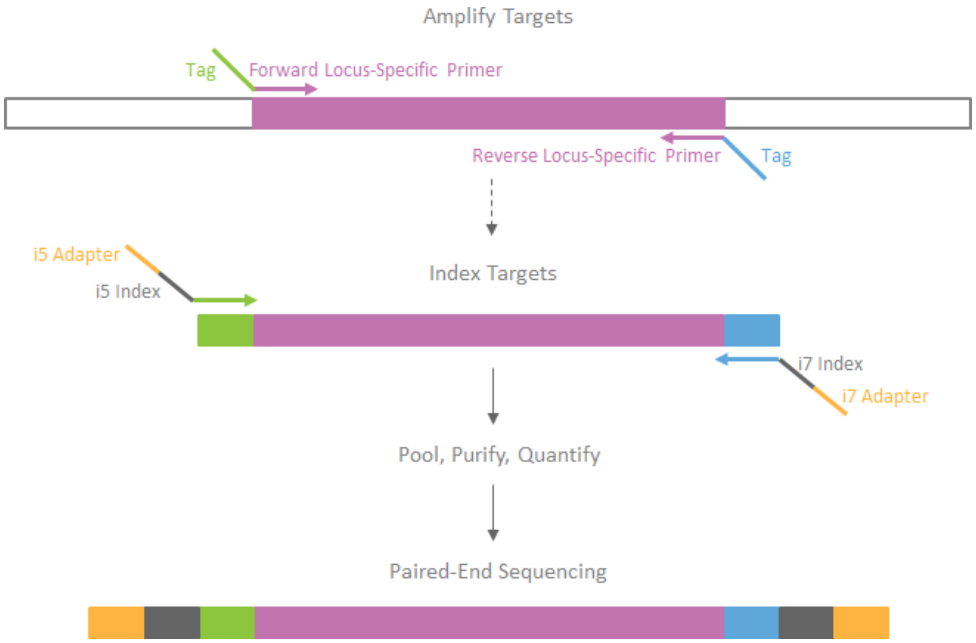
1.5 Donor-recipient Relationship

Donor-recipient relationship is used in the calculation of the AlloSeq cfDNA result. An incorrect donor relationship will affect the calculated donor-derived cfDNA result. In some cases, the difference in the dd-cfDNA calculated with a correct relationship compared to an incorrect relationship may have an impact on the interpretation of the result.

1.6 How Does the Assay Work?

The AlloSeq cfDNA assay is a targeted, next-generation sequencing (NGS) assay that utilizes differences in single nucleotide polymorphism (SNP) loci to measure the amount of donor-derived cfDNA relative to the total amount of cfDNA derived from a plasma sample. 202 SNP loci amplified by PCR constitute a sequencing library that is uniquely indexed and combined with other sequencing libraries (from 6 to 24 individual DNA samples) to be sequenced in a single sequencing run. Based on a proprietary algorithm that uses the known population frequencies of the sequenced SNPs and expected distributions of alleles, the percentage of the cfDNA that is derived from the transplanted organ is computed.

The assay is a single multiplex PCR in which the SNP-specific and index PCRs occur in the same reaction. The kit includes a SNP-specific oligonucleotide primer pool to amplify the targeted SNPs. In combination with index adapters, the unique cycling protocol amplifies the locus-specific regions and indexes the libraries concurrently. Indexed samples are subsequently pooled together to prepare for sequencing. After sequencing is complete, the data is analyzed using the AlloSeq cfDNA software which reports the percentage of the donor-derived cfDNA.



1.7 DNA Input Recommendations

Quantify the input DNA before beginning the protocol. We recommend using a fluorometric quantification method that uses dsDNA binding dyes, such as Qubit™ or PicoGreen®, according to the manufacturer's instructions.

It is recommended that 10ng of DNA per sample is used in the AlloSeq cfDNA kit. Dilute the DNA in RNase/DNase-free water so that the volume of DNA sample input per well is 16µl.

1.8 Cell-Free DNA Extraction

AlloSeq cfDNA measurements will be impacted by the presence of genomic DNA in the starting material. Genomic DNA is released when nucleated blood cells lyse and is difficult to remove during extraction. A cfDNA sample contaminated with genomic DNA will suppress the percent of donor-derived cfDNA measurements. To minimize contaminating cellular DNA, use the following methods to extract cell-free DNA from blood samples:

- Draw blood into two (2) Streck Cell-Free DNA BCT® per patient. At a minimum one full (≥8mL blood) Streck tube is required. These blood collection tubes are designed to stabilize nucleated blood cells to allow for high quality cfDNA extractions.
- Isolate plasma according to manufacturer's instructions (Double spin protocol 2 is recommended).
- Use QiAamp Circulating Nucleic Acid Kit (Qiagen) to extract cfDNA from plasma.

2. PCR Protocol

2.1 Protocol Information

- Follow the AlloSeq cfDNA protocol in the order shown using the specified parameters.
- Before proceeding, confirm kit contents and make sure that you have the required consumables and equipment. See Kit Contents and Consumables and Equipment in Chapter 3.

2.2 Assumptions

- Instruments are properly calibrated, maintained and under a maintenance plan as needed.
- Standard Operating Procedures (SOPs) are in place and controlled.
- Users are familiar with Qiagen's QiAamp Circulating Nucleic Acid Kit procedure to extract cfDNA (Qiagen cat no 55114), the use of the DYNAMAG-2 stand, magnetic beads-based DNA purification protocol, Qubit™ quantification (or equivalent), thermocycler program setup and Illumina sequencing reaction, including the use of PhiX reference controls. Unless specified in this IFU, refer to internal SOPs and/or Vendor's user manual for instructions.

2.3 PCR Setup

Before starting, ensure the thermal cycler is programmed to minimize plate exposure to room temperature after plate setup.

The PCR program should be set up for a reaction volume of 40 µl and requires specific ramp rate settings for the 8 first annealing (57°C) and elongation (72°C) steps. The ramp rate equivalents for different thermal cyclers and PCR cycling protocol are shown in Table 1 and Table 2. The following reagents are needed for PCR Setup:

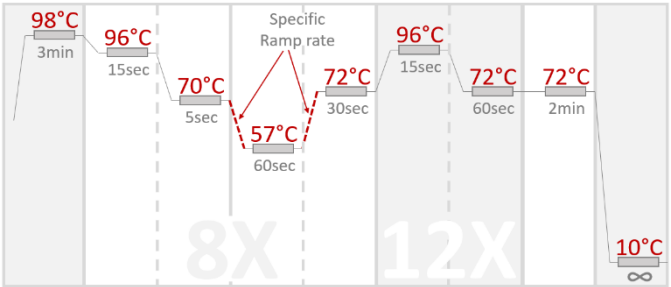
- AlloSeq cfDNA PCR Mix
- AlloSeq cfDNA PCR Enzyme
- AlloSeq cfDNA SNP Primer Pool
- AlloSeq cfDNA Index Primers (Forward, i5 and Reverse, i7)

Thaw reagents at room temperature, then place on ice. Keep reagents on ice during setup.

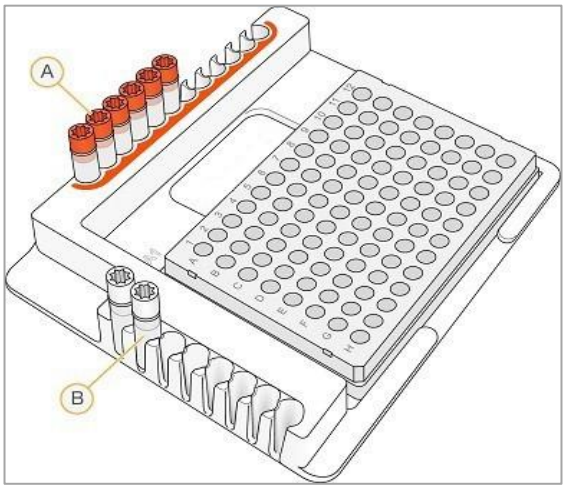
Table 1: Thermal Cycler Ramp Rate Equivalents	
ABI 9700	25%
Veriti	18%
ABI SimpliAmp	0.7°C/sec
Eppendorf Nexus	0.4°C/sec

Table 2: PCR Cycling Protocol

Choose preheat lid option to 100°C			
Temperature	Duration	Ramp Rate	Cycles
98°C	3 min	Default Ramp rate	1
96°C	15 sec	Default Ramp rate	8
70°C	5 sec		
57°C	60 sec	Use the appropriate ramp rate for your thermal cycler from Table 1 above.	
72°C	30 sec		
96°C	15 sec	Default Ramp rate	12
72°C	60 sec		
72°C	2 min	Default Ramp rate	1
10°C	∞	Default Ramp rate	1



1. Dilute sample DNA in nuclease-free water to 10ng in a final volume of 16 µl.
   Record the final DNA input amount to be used in AlloSeq cfDNA software.
2. In a PCR plate, on ice, (if using a plate holder, have the plate holder set on ice), add 4 µl of the forward index primers (i5) to the appropriate sample wells, based on the plate layout defined in the Sample Sheet.



A i7 reverse index primers
(1-6 depending on plate layout for columns 1 to 6)
B i5 forward index primers
(1-4 depending on plate layout for rows 1 to 4)

3. Add 4 µl of the reverse index primers (i7) to the appropriate sample wells.

NOTE: To reduce run-to-run sequencing contamination risks, it is recommended to rotate the i5 and i7 primers combination and well usage between runs. Performing Sequencer Maintenance Washes (as defined in the MiSeq System User Guide, Illumina part# 15027617 or in the MiniSeq System User Guide, Illumina part #1000000002695v03) on a weekly basis should also be considered to lower run-to-run sequencing contamination risks, especially when index primer rotation is not possible.

4. Add 16 µl of your prepared DNA to each sample well.
5. Prepare a Master Mix in a 1.5 ml Eppendorf tube for the appropriate number of samples needed using the following table:

Components of PCR Master-Mix (MM)		
Reagent	Volume, 1 well (µl)	Volume used to make MM for "x" number of samples, allowing for 12% recommended dead volume
AlloSeq cfDNA PCR Mix	13.0	(13.0)*x*1.12
AlloSeq cfDNA PCR Enzyme	0.8	(0.8)*x*1.12
AlloSeq cfDNA SNP Primer Pool	2.2	(2.2)*x*1.12
Total Volume	16.0	

NOTE: After addition of the enzyme, minimize exposure to room temperature as much as possible to limit unspecific amplification. For example, start thermocycler immediately after setup. Delays > 1 hr post-enzyme addition shown to decrease Q > 30.

6. Vortex Master Mix for 5 sec to mix, and centrifuge briefly.

NOTE: Aliquot Master Mix into tube strips to enable use of multichannel pipettes.

7. Add 16 µl of the above prepared Master Mix to each sample well, Apply Microseal 'B' and immediately vortex the plate for 5 sec to mix.
8. Immediately centrifuge plate at 1000 x g for 30 seconds. Check that no bubbles are present. If any, put the plate back on ice for 5 min, then repeat the centrifugation step.
9. Immediately place the plate on the thermal cycler in a post-amplification area and run the PCR cycling protocol. The PCR Cycling Protocol takes approximately 1 hour.

10. Safe Stopping Point. You can leave the plate on the thermal cycler up to 48hrs at 10C hold step, if needed. Then proceed with clean-up procedure below.

2.4 PCR Product Clean Up

The following reagents are needed for PCR product clean up:

- AlloSeq cfDNA Purification Beads
- AlloSeq cfDNA Resuspension Buffer

Before you begin, make sure that you have done the following:

- Remove Purification Beads from 2–8°C storage and equilibrate to room temperature (at least 30 minutes). Prior to use, vortex beads until complete resuspension (visually check that no pellet is left un-resuspended) then spin down briefly to get all the beads at the bottom of the tube.
- Remove Resuspension Buffer from 2–8°C storage.
- Remove PCR plate from 2–8°C storage or thermal cycler and equilibrate to room temperature. Centrifuge at 1000 x g for 30 seconds.
- Prepare 80% ethanol, 3 ml total.

2.4.1 Mix the AlloSeq cfDNA Purification Beads and PCR Product

1. Determine the volume of each sample to pool into a final volume of 120 µl, as in the examples in the table below:

Number of samples prepped	Volume of each sample to pool (µl)
10	12
15	8
16	7.5
24	5

2. Using the volume determined above, pool the required number of samples into one 1.5 ml Eppendorf tube.
3. Add 100 µl AlloSeq cfDNA Purification Beads to the tube of pooled samples.
4. Vortex the tube for 5 seconds on full speed and incubate tube for 5 minutes at room temperature.
5. Place the tube on DYNAMAG-2 stand and allow beads to pellet for 5 minutes.
6. Keep the tube on the magnetic stand and remove all supernatant with a p200 pipette.

2.4.2 EtOH Washes

1. Add 1 ml of the freshly prepared 80% EtOH to the tube without disturbing the beads. Make sure to leave the tube on the magnetic stand.
2. Wait 30 seconds and then remove and discard the supernatant, without disturbing the beads.
3. With a p1000 pipette, remove and discard the residual supernatant.
4. Repeat steps 1-3 to complete a second 80% EtOH wash. For the final wash, use a smaller displacement pipette to remove any remaining EtOH.
5. Air dry for 5 minutes, with the caps off (tube open).

2.4.3 Resuspend and Elute

1. Remove the tube from the magnetic stand and add 35 µl of the AlloSeq cfDNA Resuspension Buffer.
2. Vortex the tube for 5 seconds on full speed and incubate tube for 5 minutes at room temperature.
3. Place the tube on DYNAMAG-2 stand and allow beads to pellet for 5 minutes.
4. Without touching or disturbing the beads, transfer 32 µl of supernatant, using a smaller displacement pipette, to a new 1.5 ml Eppendorf tube.
5. Safe stopping point. Store between -15 and -25°C for up to 7 days.
6. Determine the concentration of the pool using a fluorometric method such as PicoGreen® or QubitTM

NOTE: Pool concentration can be as low as 0.3ng/µl. Modify quantification method protocol if needed to ensure measurements are in the range of detection. Typically, using 4 µl of library pool with 196 µl of Buffer+dye solution is sufficient to be in Qubit quantification range.

2.5 Sequencing workflow

This section will describe the steps required to prepare the final library to load into the Sequencing instrument. Please refer to the Illumina Sequencer instructions manual for complete information on the workflow and operation of the instrument to start a sequencing run.

Unless specified, the following instructions are specific to the MiSeq instrument.

Additional instructions specific to the MiniSeq instrument are described in sections 2.5.2 and 2.5.3.

2.5.1 Library Denaturing and Dilution for Sequencing

In this step of the workflow the user must decide whether to include Illumina PhiX sequencing control v3 at 1% final concentration in the cfDNA library. Depending on the workflow of choice, different protocols as listed below should be followed to prepare the library to be loaded into the Illumina reagent cartridge.

The following reagents are needed for denaturation and dilution:

a) Final sequencing library from the previous step
b) AlloSeq cfDNA 2 N NaOH, Box 1 stored at -15 to -25°C
c) PCR-grade water – not provided
d) Illumina MiSeq v3 150 cycle kit, MiniSeq Mid Output Kit (300 cycles) or MiniSeq High Output Reagent Kit (150-cycles)– not provided
e) [Optional] Illumina PhiX sequencing control v3 – not provided

Before you begin, make sure that you have done the following:

- Thaw 2 N NaOH at room temperature, then place on ice.
- Prepare freshly diluted 0.2 N NaOH to use for PhiX and sample denaturation: Mix 4 µl of 2N NaOH with 36 µl of Laboratory grade water. Use this fresh dilution within 12 hours.
- Thaw sequencing cartridge in room temperature water bath as specified in the sequencing system guide.
- Place thawed HT1 on ice to chill.
- [Optional] Denature and dilute sequencing control PhiX to 20 pM, as follows:
    - In a 1.5 ml Eppendorf, mix 2 µl 10 nM PhiX and 2 µl 0.2 N NaOH.
    - Vortex to mix, spin, and incubate at room temperature for 5 minutes.
    - Add 996 µl HT1 to dilute your denatured PhiX to a final concentration of 20 pM PhiX.
- Generate a sample sheet using the instructions in the Software IFU.

  NOTE: NaOH solution will readily absorb CO_2 from the atmosphere, altering the pH and performance of the reagent. Ensure the 2N NaOH tube is sealed when not in use.
- If sequencing on MiniSeq instrument, refer to sections 2.5.2 and 2.5.3 to specify Run settings and Samples.

Procedure (without PhiX control)

1. Dilute the final library to 2 nM in AlloSeq cfDNA Resuspension Buffer. If the final library concentration is in ng/µl, use the following equation to convert the concentration to nM:

$$Concentration\ (nM) = \frac{\text{Concentration (ng/µl)} * 1{,}000{,}000)}{120{,}065}$$

2. Prepare freshly diluted 0.2 N NaOH to use in the denaturation of the 2 nM library: Mix 4 µl of 2 N NaOH with 36 µl of PCR-grade water. The 0.2 N NaOH solution should be used within 12 hours of preparation.
3. In a 1.5 mL Eppendorf tube, add 15 µl of 2 nM library and 5 µl of 0.2 N NaOH.
4. Vortex, spin down briefly, and incubate at room temperature for 5 minutes.
5. Add 980 µl of chilled HT1 to bring library concentration to 30 pM.
6. Vortex briefly and spin down.
7. **If using a MiSeq instrument**, Load 1,000 µl of the denatured library at 30 pM to the Illumina MiSeq sequencing cartridge previously thawed and prepared according to manufacturer's recommendation.
8. **If using a MiniSeq instrument**, dilute further the denatured library at 30 pM, from step 5, down to 3pM, by adding 100 µl of the denatured library at 30pM from step 5 to 900 µl of chilled HT1. Vortex briefly and spin down. Load 1,000 µl of the diluted denatured library at 3 pM to the Illumina MiniSeq sequencing cartridge previously thawed and prepared according to manufacturer's recommendation.
9. Proceed according to the Illumina Sequencer user's manual to load the flow cell and reagent cartridge into the instrument to start the sequencing run. Make sure to use a previously prepared sample sheet according to the instructions in the AlloSeq cfDNA Software IFU.

Procedure (with PhiX control)

1. Dilute the final library to 2 nM in AlloSeq cfDNA Resuspension Buffer. If the final library concentration is in ng/µl, use the following equation to convert the concentration to nM:

$$Concentration\ (nM) = \frac{\text{Concentration (ng/µl)} * 1{,}000{,}000)}{120{,}065}$$

2. Prepare freshly diluted 0.2 N NaOH to use in the denaturation of the 2 nM library: Mix 4 µl of 2 N NaOH with 36 µl of PCR-grade water. The 0.2 N NaOH solution should be used within 12 hours of preparation.
3. In a 1.5 mL Eppendorf tube, add 15 µl of 2 nM library and 5 µl of 0.2 N NaOH.
4. In a 1.5 mL Eppendorf, mix 2 µl 10 nM PhiX and 2 µl 0.2 N NaOH.
5. Vortex both tubes, spin down briefly, and incubate at room temperature for 5 minutes.
6. Add 980 µl of chilled HT1 to the library tube to bring the concentration to 30 pM. Vortex briefly and spin down.
7. Add 996 µl of chilled HT1 to PhiX tube to bring the concentration to 20 pM. Vortex briefly and spin down.
8. Add 10 µl of the 20 pM PhiX solution to 1,000 µl of the denatured library from step 6. Vortex briefly and spin down.
   NOTE: Final PhiX concentration in the library is approximately 1%.
9. **If using a MiniSeq instrument**, dilute further the denatured library at 30 pM containing PhiX (from step 8) down to 3pM, by adding 100 µl of the denatured library at 30pM to 900 µl of chilled HT1. Vortex briefly and spin down.
10. Load 1,000 µl of the final library from step 9 to the Illumina sequencing cartridge previously thawed and prepared according to manufacturer's recommendation.

2.5.2 Specify Run Settings for a MiniSeq

1. Set the analysis configuration settings on the MiniSeq to the desired output folder (refer to the Illumina local Run Manager Software Guide).
2. Create a run on Local Run Manager using the FASTQ Generator Analysis (refer to the Illumina local Run Manager Generate FASTQ Analysis Module Workflow Guide).
    a. Select **TruSeq Amplicon** from the Library Kit drop-down list.
    b. Select **Dual-Indexed** for the number of index reads.
    c. Specify **Paired End** as the Read Type.
    d. Enter the number of cycles (eg 76 x 8 x 8 x 76).
3. Vortex both tubes, spin down briefly, and incubate at room temperature for 5 minutes.

2.5.3 Specify Samples for a MiniSeq run

Refer to the Local Run Manager Generate FASTQ Analysis Module Workflow Guide for general instructions on the two methods for entering samples (entering samples manually or importing samples).

3. Supporting Information

The protocol described in this guide assumes that you have reviewed the contents of this section, confirmed workflow contents, and obtained all required consumables and equipment.

3.1 AlloSeq cfDNA Kit Contents and Storage Requirements

Ensure that you have all the reagents identified in this section before proceeding to the library preparation procedures. All these reagents have a 1-year shelf-life from date of manufacturing.

Reagents Part 1 of 3, Store at -15 to -25°C

Quantity	Reagent	Tube size/type	Cap color
1	AlloSeq cfDNA PCR Mix	2ml	Red
1	AlloSeq cfDNA PCR Enzyme	0.5ml	Red
1	AlloSeq cfDNA SNP Primer Pool	0.5ml	Red
1	AlloSeq cfDNA 2N NaOH	0.5ml	Red

Reagents Part 2 of 3, Store at -15 to -25°C

Quantity	Reagent	Tube size/type	Cap color
1 set	AlloSeq cfDNA Index Primers	FluidX tube	White or Orange

Reagents Part 3 of 3, Store at 2-8°C

Quantity	Reagent		
1	AlloSeq cfDNA Purification Beads	2ml	clear
1	AlloSeq cfDNA Resuspension Buffer	2ml	clear

3.2 Safety

Follow general laboratory safety practices and clean room contamination prevention practices when performing this procedure. Through the CareDx Pty Ltd risk management process, all risks have been mitigated to an acceptable limit. Instructions for Use must be followed, including the workbooks provided, to prevent hazardous use scenarios. Hazardous materials are present in this kit. Please consult the Safety Data Sheets and take all required precautions in handling and disposal. Wear proper PPE at all times, including gloves, safety glasses and a lab coat.

NaOH is a corrosive substance and should be handled with care. Follow proper waste guidelines for disposal.

KIT COMPONENT	SYMBOL/PICTOGRAMS	SAFETY WARNING
2N NaOH Contains Sodium hydroxide		**Signal word** Danger **Hazard statements** H314 - Causes severe skin burns and eye damage **Precautionary Statements - EU (§28, 1272/2008)** P260 - Do not breathe dust/fume/gas/mist/vapours/spray P264 - Wash face, hands and any exposed skin thoroughly after handling P280 - Wear protective gloves/protective clothing/eye protection/face protection P301 + P330 + P331 - IF SWALLOWED: rinse mouth. Do NOT induce vomiting P303 + P361 + P353 - IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/ shower P363 - Wash contaminated clothing before reuse P310 - Immediately call a POISON CENTER or doctor P304 + P340 - IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing P305 + P351 + P338 - IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing P405 - Store locked up P501 - Dispose of contents/container in accordance with local, regional, national, and international regulations as applicable
AlloSeq cfDNA Resuspension Buffer Contains EDTA		**Signal word** Danger **Hazard statements** H319 - Causes serious eye irritation **Precautionary Statements - EU (§28, 1272/2008)** P264 – Wash skin thoroughly after handling P280 – Wear protective gloves/protective clothing/eye protection/face protection P337 + P313 – If eye irritation persists: Get medical advice/attention. P305 + P351 + P338 – IF IN EYES: Rinse cautiously with water for several minutes.

For additional detail on all hazardous materials contained in the AlloSeq cfDNA kit, please refer to TEC510_AlloSeq cfDNA Safety Data Sheet at http://www.caredx.com.

3.3 Consumables and Equipment

The consumables and equipment listed below are required for performing the assay but are not included in the AlloSeq cfDNA Kit.

The protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Consumables Required, Not Supplied

Consumable	Supplier/Catalog #	Quantity
Qiagen QIAamp Circulating Nucleic Acid Kit	Qiagen catalog # 55114	1
1.7 ml microcentrifuge tubes	General lab supplier	50
20 µl, 200 µl, 1000 µl pipettes set	General lab supplier	1
20 µl, 200 µl multichannel pipettes set	General lab supplier	1
20 µl, 200 µl, 1000 µl barrier pipette tips	General lab supplier	2 boxes
Conical centrifuge tubes (15 ml)	General lab supplier	1
Ethanol 200 proof (absolute) for molecular biology	Sigma-Aldrich, part # E7023 or equivalent	1
96-well PCR Plates	General lab supplier	1 plate
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001 or equivalent	2
PCR-grade water	General lab supplier	10 ml
Fluorescence-based quantification reagents such as Qubit High Sensitivity or PicoGreen®	Thermo Fisher, catalog #Q32851 Thermo Fisher, catalog #P7581	One kit
Index Fixture Plate (REUSABLE)	Illumina, FC-130-1005	1
MiSeq V3 (150 cycle) sequencing reagent kit	Illumina, MS-102-3001	1
MiniSeq Mid Output Kit (300 cycles)	Illumina, FC-420-1004	1
MiniSeq High Output Reagent Kit (150-cycles)	Illumina, FC-420-1002	1

General Lab Equipment Required, Not Supplied

Equipment	Supplier/Catalog #	Quantity
Illumina MiSeq or MiniSeq	Illumina	1
DYNAMAG-2 Magnet	Thermo Fisher, catalog # 12321D	1
Microcentrifuge	General lab supplier	1
Microplate centrifuge	General lab supplier	1
Vortexer	General lab supplier	1
One of the following 96-well thermal cyclers:		1
• GeneAmp PCR System 9700	Applied Biosystems (discontinued)	
• Veriti Thermal Cycler	Thermo Fisher, catalog #4375786	
• SimpliAmp Thermal Cycler	Thermo Fisher, catalog #A24811	
• Mastercycler® Nexus gradient	Eppendorf, catalog #6331000025	
Microplate Shaker	Qinstruments, part #1808-0506	1
One of the following (for library quantification):		1
• Qubit	General lab supplier	1
• Fluorescence plate reader	Thermo Fisher, catalog #Q33216	1

4. Contact Information

Manufacturer:

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Website: http://www.caredx.com

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Repeat kidney transplant recipients with active rejection have elevated donor-derived cell-free DNA, **Mehta, *et al.* Am J Transplant. 2019 May;19(5):1597-1598**

Method history

Version	Date	Modification
1.0	24Oct19	First version of the AlloSeq cfDNA IFU issued through MasterControl as LQ-10028.
2.0	13Nov19	AlloSeq cfDNA added to SVN and Doc ID updated to IFU084. Updated safety precautions in accordance with EU standards. Other minor formatting.
3.0	20Jan20	Added resuspension buffer to warnings and added 'research applications' to section 1.1 Removed reference to PCR ProFlex
3.1	20Oct20	Added Note regarding use of NaOH.
4.0	14Jan21	Section 1: added reference to MiniSeq, added more information for flexible kit Section 1.2/2.3: added reference to MiniSeq Section 2.4.3: added step 6 Section 2.5: Updated all parts of this section Section 5.3: Added MiniSeq to consumables required table
5.0	26Mar21	Updated distributor from Vienna to Stockholm in section 4.0 as per CR 2020-097.
6.0	17Jun21	Updated section 2.2, table 2, table 9 and section 4.4 with SimpliAmp and Nexus thermocyclers. Updated Section 2.4 to remove option to wait until liquid is clear to ensure the stated time is followed.
7.0	04May22	Updated cap colour of box 1 components from clear to red. See CR 2021-083. Updated tube size for purification beads per CR 2022-010. Updated AlloSeq logo.