



## AlloSeq HCT

### Instructions for Use

IFU092

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# 1. Introduction

This Instructions for Use (IFU) describes the steps required to prepare a library for sequencing using the AlloSeq HCT kit in order to generate data for analysis using the AlloSeq HCT software. The software and reagent kit are part of the AlloSeq HCT product.

## 1.1. Limitations

AlloSeq HCT product is used to calculate the percent DNA (% DNA) for up to three distinct genomes present in post hematopoietic cell transplantation (HCT) samples for research use only applications. Since the test evaluates genetic differences between the recipient and donor(s), it is not possible to perform the test for an HCT transplant recipient that is a monozygotic twin to the donor.

DO NOT USE AlloSeq HCT in transplant recipients who:

- have had HCT procedure(s) which involve 3 or more genetically distinct donors
- have received a transplant from a monozygotic (identical) twin
- have had a blood transfusion that contains white blood cells within the past 30 days (washed or leukocyte-depleted RBCs are acceptable)

## 1.2. Definitions

This section provides definitions of acronyms and concepts that are useful to understand the instructions to prepare an AlloSeq HCT sequencing library.

Acronym/term	Definition
Post-transplant sample	Refers to the DNA extracted from a biological sample from blood or bone marrow from an individual after having undergone HCT for genetic chimerism analyses. Since the tissue source will present cells from the donor(s) and recipient, the DNA extracted will contain a mixture of distinct human genomes that can be evaluated with AlloSeq HCT.
Genetic contributors	Each distinct genome present in a post-transplant sample.
Locus	Specific location in a genome.
Amplicon	The PCR product from the AlloSeq HCT assay representing a genomic region containing a bi-allelic SNP of the total 202 SNPs.
Genotype	Refers to the genetic information collected for DNA samples using AlloSeq HCT for the 202 SNPs targeted by the assay.
Fastq files	Data generated by the Illumina MiSeq instrument and used in data analysis for % DNA calculation. Each sample is associated with a pair of fastq files (R1 and R2, forward and reverse reads respectively) containing the sequenced reads from a biological sample prepared using AlloSeq HCT assay.
Sample sheet	File with extension .csv that collects information needed for sequencing on the MiSeq and data analysis on AlloSeq HCT.
% DNA	The calculated percentage of the DNA fraction for each genetic contributor present in a post-transplant sample.
Data analysis	Processing of fastq files into % DNA estimates and calculation of quality and performance metrics.
Single donor	A post-transplant sample expected to contain two distinct genomes present because it is derived from an HCT recipient with a total of a single hematopoietic cell donor.
Two donor	A post-transplant sample expected to contain three distinct genomes present because it is derived from an HCT recipient with a total of two hematopoietic cell donors.

### 1.3. Overview of AlloSeq HCT

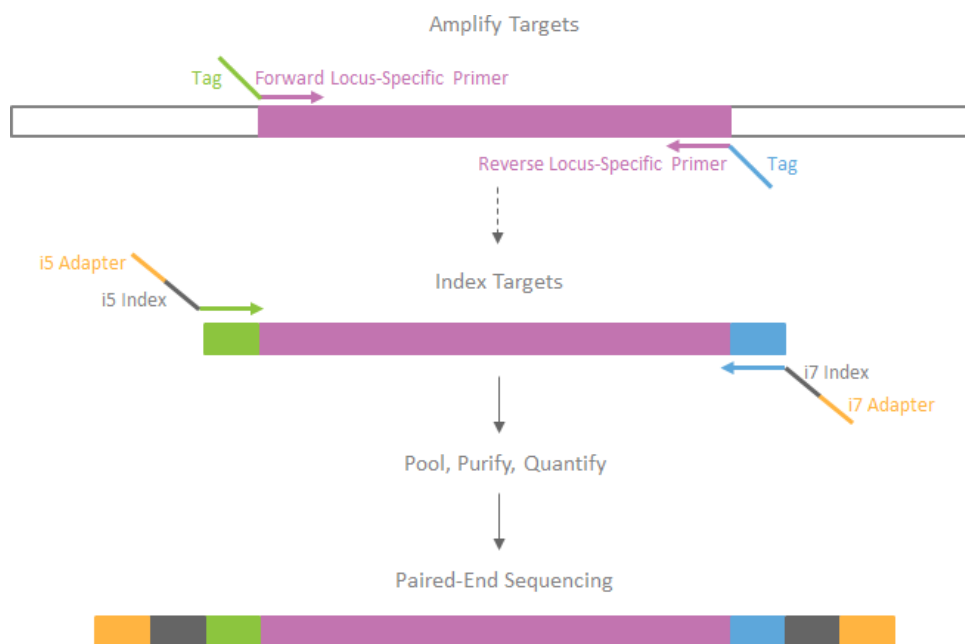
The CareDx AlloSeq HCT reagent kit and the CareDx AlloSeq HCT software (collectively referred to as CareDx AlloSeq HCT) form a system that enables relative quantification of genetic chimerism in a DNA sample derived from a hematopoietic stem cell transplant (HCT) recipient for research use only applications. Donor-derived hematopoietic stem cells have a distinct genome when compared to the recipient's hematopoietic stem cells. After HCT, the transplanted cells reconstruct the recipient's bone marrow and blood system; however, donor and recipient's cells can be detected at different levels after HCT generating genetic chimerism in the recipient.

The AlloSeq HCT is a targeted, next-generation sequencing (NGS) assay that utilizes differences in single nucleotide polymorphism (SNP) to measure the amount of recipient and donor-derived DNA present in a post-transplant sample. Each reagent kit contains all reagents required for the multiplex amplification of 202 loci across 24 different samples. Each amplicon covers a unique SNP, which are spread across all human autosomal chromosomes. The complete sequencer-ready amplicon is generated in a single amplification step and contains the target region, dual sample-specific indices and flow-cell adapters as shown in Figure 1. Once all sample libraries are generated and pooled, the resulting library goes through magnetic bead clean-up for removal of primers followed by quantitation. The Illumina MiSeq workflow is then followed to prepare the library for sequencing. The sequencing reaction is performed using the MiSeq v3 Reagent kit using 75 paired-end read and dual barcoding. A modified sample sheet is prepared for the sequencing reaction and data analysis of the fastq files is performed using the AlloSeq HCT Software.

The AlloSeq HCT Software has a proprietary algorithm that enables resolution of up to 3 different genomes (genetic contributors) present in a single post-transplant sample (1 recipient and up to 2 donors). Genotype information generated with AlloSeq HCT for all 3 or at least 2 of the genetic contributors present in the post-transplant sample is required and may be performed prior to or at the same time as the analysis of post-transplant samples. Based on a proprietary algorithm that uses the known population frequencies of the sequenced SNPs and expected distributions of alleles, the percentage of the different genomes present in a single sample are computed. The data analysis process is automated with less than 20 minutes hands on time. A user-friendly interface displays the % DNA fraction from each genetic contributor present in a post-transplant sample. In addition, Excel-compatible output files are generated automatically and can be integrated with LIMS systems for streamlined reporting. For more information on how to perform data analysis, refer to AlloSeq HCT Software IFU.

The AlloSeq HCT product offers the following features and technical performance:

- Fast turnaround time with:
  - 3 hours turnaround time for library preparation
  - 3 hours hands on time
  - DNA to result in up to 24 hours
- Automated data analysis software
- Compatible with Illumina MiSeq instrument and MiSeq Reagent Kit v3 (150 cycles)
- Flexible sequencing capacity with libraries containing between 8 and 48 samples
- Requires low DNA input of 10 ng and minimum concentration of 0.625 ng/μL
- Technical performance evaluated with the use of Illumina PhiX sequencing control v3 at 1%
- High sensitivity presenting lower limit of quantitation (evaluated as described in “Protocols for Determination of Limits of Detection and Limits of Quantitation”, CLSI **June 2012**) of **0.36% for post-transplant samples with two donors.**
- High precision presenting variability:
  - between experiments equal to 1.7% at 10% DNA fraction and 6.5% at 1% DNA fraction
  - within experiments equal to 1.3% at 10% DNA fraction and 4.7% at 1 % DNA fraction
- High accuracy between expected and observed DNA fraction determination with an  $R^2 > 0.99$



**FIGURE 1. SINGLE AMPLIFICATION STEP INCLUDES THE USE OF PRIMERS THAT ARE TARGET SPECIFIC AND CONTAIN SAMPLE BARCODES (I5 AND I7 INDICES) AND SEQUENCER ADAPTERS (I5 AND I7 ADAPTERS) TO GENERATE SEQUENCER-READY AMPLICONS IN ONE STEP. THE RESULTING LIBRARY IS THEN READY FOR MAGNETIC BEAD PURIFICATION AND QUANTIFICATION FOR SEQUENCING.**

## 2. AlloSeq HCT library preparation workflow

This section describes the AlloSeq HCT workflow from DNA sample to final library, including DNA sample considerations, PCR amplification, clean up and concentration determination steps.

### 2.1. Sample considerations

It is recommended to use high quality DNA samples in the library preparation procedure. DNA quality in this context is defined by absorbance reading ratios obtained with a low volume spectrophotometer such as NanoDrop. Below are the reference values for high quality and pure DNA at the following ratios:

1. Abs at 260/230 nm: 2-2.2
2. Abs at 260/280 nm: 1.8

While the assay is tolerant to lower DNA quality, sequencing metrics, particularly mean coverage, will be affected if a given sample in a library is of lower quality compared to the rest.

It is critical to quantify the DNA concentration of the samples before beginning the protocol. We recommend using Qubit™ as a fluorometric quantification method or equivalent methods that use dsDNA binding dyes, according to the manufacturer's instructions. The DNA sample set should have concentration normalized to 0.625 ng/μL using PCR-grade water to fulfill the 10 ng input requirement in 16 μL volume.

### 2.2. One-step multiplex PCR amplification

This section describes the steps to be followed to set up the PCR for amplification of 202 target regions per sample. The final product is a set of 202 amplicons that contain the target region, sample-specific index sequences and flow-cell adapter sequences per sample.

## Before Starting

1. Confirm kit contents, appropriate storage temperature and ensure that you have the required consumables and equipment.
2. Make sure to read and follow the manufacturer's instructions manual for the thermal cycling instruments supported for this workflow below: GeneAmp PCR System 9700 (Thermo Fisher), Veriti Thermal Cycler (Thermo Fisher), SimpliAmp Thermal Cycler (Thermo Fisher), Mastercycler® Nexus gradient (Eppendorf)
3. Follow the AlloSeq HCT protocol in the order shown using the specified parameters.
4. Prepare sample sheet for the sequencing (consult the AlloSeq HCT Software IFU for instructions).
5. Program the thermal cycler by creating a protocol with the specific parameters:
  - a) Reaction volume: 40  $\mu$ L
  - b) Pre-heat lid option to 100°C
  - c) PCR cycling protocol listed in Table 1
    - Specific ramp rate settings are required for the steps with an asterisk in Table 1. The ramp rate equivalents for the two supported thermal cycler instruments are listed in Table 2.
    - The final PCR cycling protocol is illustrated in Figure 2.
6. Reagents required for PCR setup:
  - a) AlloSeq HCT PCR Mix, Box 1 stored at -15 to -25°C
  - b) AlloSeq HCT PCR Enzyme, Box 1 stored at -15 to -25°C
  - c) AlloSeq HCT SNP Primer Pool, Box 1 stored at -15 to -25°C
  - d) AlloSeq HCT Index Plate, Box 2 stored at -15 to -25°C

NOTE: Thaw frozen 96 indices plate, perform brief vortex to mix then quick spin down the index mixtures before pipetting for PCR reaction

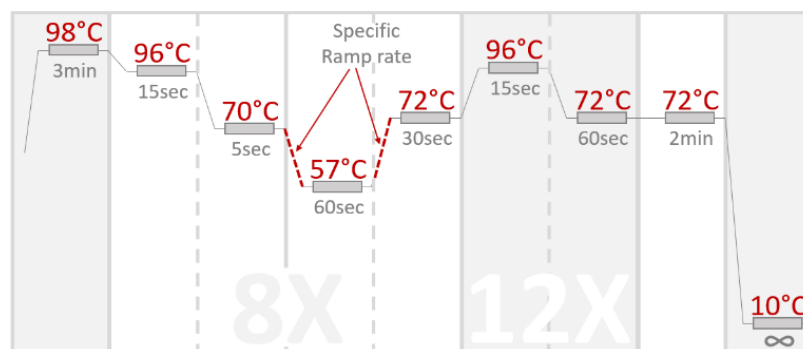
NOTE: Use alternative 48 indexes between MiSeq runs
7. Ensure DNA samples to be used in the library preparation are normalized to a final concentration of 0.625 ng/ $\mu$ L and at a minimum volume of 18  $\mu$ L to allow pipetting of 16  $\mu$ L into the final PCR plate.

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		
72°C*	30 sec	*Use the appropriate ramp rate for your thermal cycler from Table 2.	
96°C	15 sec	Default Ramp rate	12
72°C	60 sec		
72°C	2 min	Default Ramp rate	1
10°C	$\infty$	Default Ramp rate	1

**TABLE 1. PCR CYCLING PROTOCOL**

Supported thermal cyclers	Ramp Rate Equivalent
GeneAmp PCR System 9700	25%
Veriti Thermal Cycler	18%
ABI SimpliAmp	0.7°C/sec
Eppendorf Nexus (aluminum block)	0.4°C/sec

**TABLE 2. THERMAL CYCLER RAMP RATES**



**FIGURE 2. GRAPHIC VIEW OF THE PCR CYCLING PROTOCOL**

## Procedure

1. Turn on the thermal cycler instrument to ensure it is at the right temperature after the PCR plate is set up.  
NOTE: Delays of  $\geq 1$  hour in starting thermal cycling protocol of the PCR plate has shown to decrease Q30 scores.
2. Thaw the PCR Mix, SNP Primer and Index Plate at room temperature, vortex and spin down before use and keep them on ice. Confirm index plate orientation is correct with A1 well being at top left. Then pierce foil for desired index combination using a pipetting tip.
3. Note: Make sure to use a different pipet tip for each well to be pierced
4. Note: When piercing the wells, make sure not to disturb the index solution
5. Place a fresh PCR plate on ice (if using the TruSeq Index Plate Fixture, keep it on ice). Add 8  $\mu$ L of premixed i7 and i5 dual index mixtures from the 96 indices plate to the fresh PCR plate on ice using a single P10-P20 or multichannel P10-P20 pipette.  
Note: Wells are pre-filled with sufficient overage to allow blind pipetting. Make sure to touch bottom well and aspirate entire volume. Confirm with visual check.  
Note: To reduce run-to-run sequencing contamination risks, it is recommended to rotate the i5 and i7 primer combination and wells assigned between runs. Additionally, it is recommended to do a post-run sequencing wash on the MiSeq using bleach after each run (refer to manufacturer's instructions).
6. Add 16  $\mu$ L of your prepared DNA to each sample well of PCR plate.
7. Remove the PCR enzyme from  $-20^{\circ}\text{C}$ , flick it and spin it down keeping it on ice. Prepare a Master Mix in a 1.5 mL Eppendorf tube for the appropriate number of samples "N" using the following table:

Reagent	Volume, 1 well ( $\mu$ L)	Volume for "N" samples plus 12% recommended overage ( $\mu$ L)
AlloSeq HCT PCR Mix	13.0	$(13.0) \times 1.12 \times N$
AlloSeq HCT PCR Enzyme	0.8	$(0.8) \times 1.12 \times N$
AlloSeq HCT SNP Primer Pool	2.2	$(2.2) \times 1.12 \times N$
Total Volume	16.0	$(16) \times 1.12 \times N$

**TABLE 3. ONE-STEP MULTIPLEX AMPLIFICATION MASTER MIX**

8. Vortex Master Mix for 5 seconds to mix, and centrifuge briefly.
9. Add 16  $\mu$ L of the Master Mix to each sample well containing the index primers and DNA samples. Make sure to change tips after pipetting into each well.
10. Apply Microseal 'B' PCR Plate Sealing Film and immediately vortex the plate for 5 seconds to mix.
11. Immediately centrifuge plate at 1000 x g for 30 seconds.
12. 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.

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

## 2.3. Library clean up and concentration determination

### Before starting

1. Confirm kit contents, appropriate storage temperature, and ensure that the required consumables and equipment are available.
2. Make sure to read and follow the manufacturer's instructions manual for the instruments and methods recommended below in this workflow:
  - a) DynaMag-2 magnet
  - b) Qubit dsDNA HS assay kit
  - c) Qubit Fluorometer
3. Reagents required for this step:
  - a) PCR plate from the previous step
  - b) AlloSeq HCT Purification Beads, Box 3 stored at 2 to 8°C
  - c) AlloSeq HCT Resuspension Buffer, Box 3 stored at 2 to 8°C
  - d) Ethyl alcohol for molecular biology – not provided
  - e) PCR-grade water – not provided
4. Remove Purification Beads from 2–8°C storage and equilibrate to room temperature for at least 30 minutes.
5. Remove Resuspension Buffer and PCR plate from storage and keep both at room temperature. Centrifuge PCR plate at 1000 x g for 30 seconds.
6. Prepare a fresh solution of 3 mL of 80% ethyl alcohol (EtOH), which is enough to clean up a single library pool with 50% overage.

### Procedure

1. Determine the equal volume of each sample to be pooled into a single 1.5 mL Eppendorf tube to prepare a library with final volume of 120 µL. Use Table 4 as a reference.

Number of total samples in the library	Volume to pool from each sample (µL)
16	7.5
24	5
32	3.75
40	3
48	2.5

**TABLE 4. VOLUME TO BE POOLED FROM EACH SAMPLE TO PREPARE FINAL ALLOSEQ HCT LIBRARY FOR CLEAN UP.**

2. Vortex AlloSeq HCT Purification Beads until completely resuspended; visually check that there is no pellet left, and mix with pipette if needed. Briefly spin down to collect the bead suspension at the bottom of the tube. Add 100 µL of the beads to the 120 µL library tube.
3. Vortex the tube for 5 seconds on full speed and incubate for 5 minutes at room temperature.
4. Briefly spin down, then place the tube on DynaMag-2 magnet and allow beads to pellet for 5 minutes.
5. Keep the tube on the magnet and remove all supernatant with a P200 pipette. Use a P20 pipette to remove any remaining supernatant, if necessary.
6. Add 65 µL of the AlloSeq HCT Resuspension Buffer.
7. Vortex the tube for 5 seconds on full speed and incubate tube for 5 minutes at room temperature.
8. Briefly spin down, then place the tube on the magnet and allow beads to pellet for 5 minutes.



9. Keep the tube on the magnet and, without touching or disturbing the beads, transfer 60  $\mu\text{L}$  of supernatant to a new 1.5 mL Eppendorf tube.
10. Ensure AlloSeq HCT Purification Beads are thoroughly resuspended (see step 2). Add 50  $\mu\text{L}$  of the beads to the 60  $\mu\text{L}$  library supernatant.
11. Vortex the tube for 5 seconds on full speed and incubate for 5 minutes at room temperature.
12. Briefly spin down, then place the tube on the magnet and allow beads to pellet for 5 minutes.
13. Keep the tube on the magnet and remove all supernatant with a P200 pipette.
14. Keep the tube on the magnet and add 1 mL of freshly prepared 80% EtOH without disturbing the beads, then wait 30 seconds.
15. Keep the tube on the magnet and, without touching or disturbing the beads, use P1000 pipette and remove and discard the supernatant.
16. Repeat steps 14-15 for a second EtOH wash. After the second wash, use a P20 pipette to remove any remaining EtOH.
17. Keep the tube open and air dry the bead pellet for 5-10 minutes, or until no EtOH remains.
18. Add 35  $\mu\text{L}$  of the AlloSeq HCT Resuspension Buffer.
19. Vortex the tube for 5 seconds on full speed and incubate for 5 minutes at room temperature.
20. Briefly spin down, then place the tube on the magnet and allow beads to pellet for 5 minutes.
21. Keep the tube on the magnet and, without touching or disturbing the beads, transfer 32  $\mu\text{L}$  of supernatant to a new 1.5 mL Eppendorf tube.

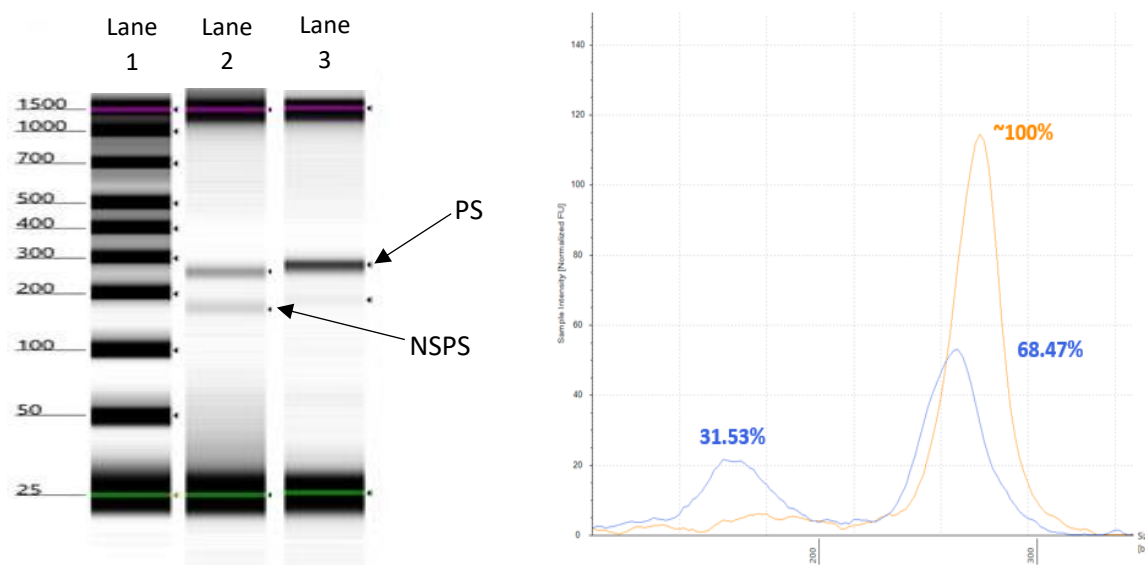
NOTE: Safe stopping point. Store cleaned library between  $-15$  and  $-25^{\circ}\text{C}$  for up to 7 days.

22. Determine the concentration of the final library pool using a fluorometric method; Qubit™ dsDNA HS Assay Kit (Thermo Fisher PNQ32851) is recommended.

NOTE: The method recommended for quantitation has a detection range between 10  $\text{pg}/\mu\text{L}$  to 100  $\text{ng}/\mu\text{L}$ . This covers AlloSeq HCT library concentrations which can be as low as 0.1  $\text{ng}/\mu\text{L}$ . If using a different method, make sure it can provide accurate quantitation in the appropriate range.

23. **Optional**, Library Quality Control Assessment on Tape-Station:

Product Size (PS)	Non-specific product size (NSPS)
250-300 bps	<200 bps



**FIGURE 3. TAPE-STATION PROFILES. GEL: LANE 1: SIZES SCALE, LANE 2 AND BLUE CURVE: BEFORE SECOND WASH, LANE 3 AND YELLOW CURVE: AFTER SECOND WASH. THE PRESENCE OF NON-SPECIFIC PRODUCTS SHALL NOT NECESSARILY IMPACT THE SEQUENCING RUN AS LONG AS MORE PRODUCTS ARE PRESENT THAN NON-SPECIFIC PRODUCTS.**

### 3. Sequencing workflow

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

#### 3.1. 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 HCT library. Depending on the workflow of choice, follow the appropriate procedure listed below to prepare the library to be loaded into the Illumina reagent cartridge.

##### Before starting:

1. Make sure to read and follow the manufacturer's instructions manual for the Illumina MiSeq instrument.
2. Reagents required for this step:
  - a) Final sequencing library from the previous step
  - b) AlloSeq HCT 2 N NaOH, Box 1 stored at -15 to -25°C
  - c) PCR-grade water – not provided
  - d) Illumina MiSeq v3 150 cycle kit – not provided
  - e) [Optional] Illumina PhiX sequencing control v3 – not provided
3. Thaw 2 N NaOH at room temperature, then place on ice.  
NOTE: NaOH solution will readily absorb CO<sub>2</sub> from the atmosphere, altering the pH and performance of the reagent. Ensure the 2N NaOH tube is sealed when not in use.
4. Thaw and prepare Illumina sequencing reagents according to the manufacturer's specifications.
5. Generate a sample sheet using the AlloSeq HCT Software, according to the instructions in the AlloSeq HCT Software IFU.

##### Procedure (without PhiX control)

1. Prepare a dilution of the final library at 1.33 nM in AlloSeq HCT Resuspension Buffer. If necessary, use the following equation to convert the concentration from ng/μL to nM:
$$\text{Concentration (nM)} = \frac{\text{Concentration (ng/}\mu\text{L)} * 1,000,000}{120,065}$$
2. If library pool concentration is <0.16ng/ul, do not add additional resuspension buffer.
3. Prepare freshly diluted 0.2 N NaOH to use in the denaturation of the 1.33 nM library. Combine 4 μL of 2 N NaOH with 36 μL of PCR-grade water and mix thoroughly. The 0.2 N NaOH solution should be disposed of within 12 hours after preparation.
4. In a new 1.5 mL Eppendorf tube, add 9 μL of 1.33 nM library and 3 μL of 0.2 N NaOH. If library pool concentration was <0.16ng/ul, make appropriate volume adjustments to keep a 3:1 Library:NaOH ratio to ensure proper denaturation.
5. Vortex, spin down briefly, and incubate at room temperature for 5 minutes.
6. Add 588 μL of chilled HT1 to bring library concentration to 20 pM.
7. Vortex briefly and spin down. Store on ice.
8. Load 600 μL of the denatured library at 20 pM to the Illumina MiSeq sequencing cartridge previously thawed and prepared according to manufacturer's recommendations.
9. Proceed according to the Illumina MiSeq user's manual to load the flow cell, reagent cartridge, and Incorporation Buffer into the instrument to start the sequencing run. Make sure to use a sample sheet prepared using the AlloSeq HCT Software, according to the instructions in the AlloSeq HCT Software IFU.

## Procedure (with PhiX control)

1. Prepare a dilution of the final library at 1.33 nM in AlloSeq HCT Resuspension Buffer. If necessary, use the following equation to convert the concentration from ng/μL to nM:

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

2. If library pool concentration is <0.16ng/ul, do not add additional resuspension buffer.
3. Prepare freshly diluted 0.2 N NaOH to use in the denaturation of the 1.33 nM library. Combine 4 μL of 2 N NaOH with 36 μL of PCR-grade water and mix thoroughly. The 0.2 N NaOH solution should be disposed of within 12 hours after preparation.
4. In a new 1.5 mL Eppendorf tube, add 9 μL of 1.33 nM library and 3 μL of 0.2 N NaOH. If library pool concentration was <0.16ng/ul, make appropriate volume adjustments to keep a 3:1 Library:NaOH ratio to ensure proper denaturation.
5. In another new 1.5 mL Eppendorf tube, mix 2 μL 10 nM PhiX and 2 μL 0.2 N NaOH.
6. Vortex both tubes, spin down briefly, and incubate at room temperature for 5 minutes.
7. Add 582 μL of chilled HT1 to the library tube to bring the concentration to 20 pM.
8. Add 996 μL of chilled HT1 to PhiX tube to bring the concentration to 20 pM.
9. Vortex briefly and spin down both library and PhiX tubes. Store on ice.
10. Add 6 μL of the 20 pM PhiX solution to the 20 pM library tube. Vortex briefly and spin down. Store on ice.  
NOTE: Final PhiX concentration in the library is approximately 1%.
11. Load 600 μl of the library at 20 pM to the Illumina MiSeq sequencing cartridge previously thawed and prepared according to manufacturer's recommendations.
12. Proceed according to the Illumina MiSeq user's manual to load the flow cell, reagent cartridge, and Incorporation Buffer into the instrument to start the sequencing run. Use a sample sheet prepared using the AlloSeq HCT Software, according to the instructions in the AlloSeq HCT Software IFU.

### Sequencing Metric Note: Typical values observed in post-run analysis

Instrument	Average cluster density (K/mm2)	Passing filter	Mapped Reads
MiSeq	800-1800	>85%	>75%

## 4. Additional information

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

### 4.1. AlloSeq HCT kit contents and Storage Requirements

When stored at the temperature specifications below, the kit components can be used until the expiry indicated on the outer kit containers. After use, the kits/components should be returned immediately to storage conditions.

These kits are NOT to be used beyond their expiry date.

Reagent	Quantity	Tube type
AlloSeq HCT PCR Mix	1	2 mL
AlloSeq HCT PCR Enzyme	1	0.5 mL
AlloSeq HCT SNP Primer Pool	1	0.5 mL
AlloSeq HCT 2 N NaOH	1	0.5 mL

**TABLE 5. ALLOSEQ HCT REAGENT BOX 1, STORE AT -15 TO -25°C**

Reagent	Quantity	Well Volume	Plate Seal
AlloSeq HCT index plate	1	200 µl	Pierceable foil

**TABLE 6. ALLOSEQ HCT REAGENT BOX 2, STORE AT -15 TO -25°C**

Reagent	Quantity	Tube type
AlloSeq HCT Purification Beads	2	2 mL
AlloSeq HCT Resuspension Buffer	2	2 mL


**TABLE 7. ALLOSEQ HCT REAGENT BOX 3, STORE AT 2 TO 8°C**


## 4.2. AlloSeq HCT Safety

Follow good laboratory practices (safety and contamination prevention) 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.

Please consult the Safety Data Sheet and take all required precautions in handling and disposal. For additional detail on all hazardous materials contained in the AlloSeq HCT kit, please refer to TEC513\_AlloSeq HCT Safety Data Sheet at <http://www.caredx.com>.

KIT COMPONENT	SYMBOL/PICTOGRAMS	SAFETY WARNING
<b>2N NaOH</b> Contains Sodium hydroxide		<p><b>Signal word</b> Danger</p> <p><b>Hazard statements</b>            H314 – Causes severe skin burns and eye damage            H318 – Causes serious eye damage</p> <p><b>Precautionary Statements – EU, US and AU Regulation</b>            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. Call a POISON CENTER or doctor.            P405 – Store locked up            P501 – Dispose of contents/container in accordance with local, regional, national, and international regulations as applicable</p>

KIT COMPONENT	SYMBOL/PICTOGRAMS	SAFETY WARNING
<b>AlloSeq HCT</b> <b>Resuspension</b> <b>Buffer</b> Contains EDTA		<b>Signal word</b> Danger  <b>Hazard statements</b> H319 – Causes serious eye irritation  <b>Precautionary Statements – EU, US and AU Regulation</b> 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. Remove contact lenses, if present and easy to do. Continue rinsing. Call a POISON CENTER or doctor.

### 4.3. Additional consumables and equipment required

The consumables and equipment listed below are required for the library preparation workflow but not included in the AlloSeq HCT Kit.

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

Consumable	Manufacturer/Supplier	Catalog number	Quantity
1.5 mL microcentrifuge tubes	General lab supplier	NA	50
FluidX External Thread Screw Caps	Brooks Life Sciences	68-53100-Z6N (orange) 68-53100-Z1N (white)	960 caps ea
20 µL, 200 µL, 1000 µL barrier pipette tips	Rainin or equivalent	Multiple	2 boxes of 96
Conical centrifuge tubes (15 mL)	General lab supplier	NA	1
Ethyl alcohol, 200 proof, for molecular biology	Sigma-Aldrich	E7023-500 mL	1
96-well PCR Plates	General lab supplier	NA	1 plate
Microseal 'B' adhesive seals	Bio-Rad	MSB1001	1 pack of 96
PCR-grade water, 10 mL	General lab supplier	NA	1
Qubit™ dsDNA HS Assay Kit	Thermo Fisher	Q32851	1
MiSeq Reagent kit v3 (150-cycle)	Illumina	MS-102-3001	1
[Optional] PhiX Sequencing Control v3	Illumina	FC-110-3001	1

**TABLE 8. REQUIRED CONSUMABLES NOT SUPPLIED IN THE ALLOSEQ HCT KIT.**

Equipment/Instrument	Manufacturer
20 µL, 200 µL, 1000 µL pipettes	Rainin or equivalent
20 µL, 200 µL multichannel pipettes	Rainin or equivalent
MiSeq System	Illumina
TruSeq Index Plate Fixture Kit (2 fixtures)	Illumina, FC-130-1005
DynaMag-2 Magnet	Thermo Fisher, catalog # 12321D
Minicentrifuge	General lab supplier
Microplate centrifuge	General lab supplier
Vortexer	General lab supplier
Qubit Fluorometer	Thermo Fisher

Equipment/Instrument	Manufacturer
One of the following 96-well thermal cyclers:	
- GeneAmp PCR System 9700	Applied Biosystems (discontinued)
- Veriti Thermal Cycler	Thermo Fisher, catalog #4375786
- SimpliAmp Thermal Cycler	Thermo Fisher, catalog #A24811
- Mastercycler® Nexus gradient (aluminum block)	Eppendorf, catalog #6331000025

**TABLE 9. EQUIPMENT AND INSTRUMENTS RECOMMENDED FOR ALLOSEQ HCT SEQUENCING LIBRARY PREPARATION.**

#### 4.4. Relevant references

The list below contains instruction manuals referenced through the AlloSeq HCT library preparation workflow. Read through them carefully before starting the procedure to ensure correct use of the equipment/instrument. Make sure to check online for updated versions of the documents listed.

GeneAmp PCR System 9700, Base Module User's Manual, Thermo Fisher  
Applied Biosystems Veriti Thermal Cycler User Guide, Thermo Fisher  
Applied Biosystems SimpliAmp Thermal Cycler User Guide, Thermo Fisher  
Mastercycler Nexus Operating Manual, Eppendorf  
MiSeq System Guide, Illumina  
MiSeq System Denature and Dilute Libraries, Illumina

## 5. Contact Information

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For more information please refer to the CareDx website (<https://www.caredx.com/contact-us/>).

### Related products:

AlloSeq HCT Software

## Method history

Version	Date	Modification
1.0	21Oct21	First version of the AlloSeq HCT 96 IFU – RUO.
2.0	21Nov21	Updated procedure to incorporate index plates. Updated library pool dilution when concentration is below <0.16ng/ul and keepin library pool:denaturation to a 3:1 ratio. Section 4.1: Remove shelf life, add details of storage conditions and product not to be used after expiry. Deleted cap colour and reagent volume from kit contents. Section 4.2: link to CareDx.com, remove clean room contamination practices.
3.0	16May22	Typographical error correct for section 3.1 with phix control step 7 changed to 582ul. Remove transplant samples with single donor. Udpate copyright date. Update Section 1.3 to reflect results from CE Verification studies.
4.0	16Nov22	Clarified Eppendorf Nexus aluminum blocks (ZD-2233) A. Falvey added instruction to section 2.3 (Procedure) steps 4, 8, 12 and 20 to briefly spin down the tube content prior to placing on the magnet. This is to clarify that the operator should not spin down the tube after vortexing, which is standard procedure. It should be incubated for 5 minutes, with the spin performed immediately prior to placing on the magnet. This brings the IFU in line with IFU092-1_AlloSeq HCT Workbook – RUO
4.1	01Mar24	Correction of Unit of Measure for concentration in Section 3