# AlloSeq HCT Software

## Instructions for Use

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# 1. Overview

This Instructions for Use (IFU) describes the functionality and steps required to perform data analysis using the AlloSeq HCT software. The AlloSeq HCT assay kit and the AlloSeq HCT software are collectively referred to as AlloSeq HCT.

There is a separate 'Installation Guide' document (IFU106) for the AlloSeq product family, which should be read before this IFU.

## 1.1 System and Software Overview

AlloSeq HCT is a multiplexed, one-step PCR-based solution for the preparation of sequencing libraries from DNA extracted from biological samples of hematopoietic stem cell transplant recipients and donors. The sequencing data generated (in FASTQ files) is used to calculate the % DNA from the recipient and donor(s) present in the biological sample to determine the level of genetic chimerism for the post-transplant recipient at the time the sample was collected. More information on AlloSeq HCT assay can be found in the AlloSeq HCT Assay IFU.

The 202 target Single Nucleotide Polymorphisms (SNPs) are:

(1) distributed across the 22 pairs of autosomal chromosomes in the human genome,
(2) biallelic, with an allele frequency between 0.4 and 0.6 across all populations,
(3) located in genomic regions that can be sequenced with high confidence, and
(4) not in linkage disequilibrium or associated with diseases.

Sequence data is analyzed using AlloSeq HCT software which outputs the percentage of DNA (% DNA) for up to three distinct genomes (genetic contributors) detected in post-transplant samples.

The calculation of % DNA from recipient and donor(s) present in post-transplant samples is achieved by determining the fraction of different nucleotides sequenced at each SNP location evaluated. Recipient and donor(s) pre-transplant genotypes obtained using AlloSeq HCT are required to calculate the % DNA obtained from each genetic contributor present in the post-transplant sample. Overlapping reads are merged, aligned, and counted. Results are compared to genotypes of recipient and donor(s) (informative SNPs/loci). It is recommended that genotype from all genetic contributors (recipient and donor(s)) are input into the analysis of post-transplant samples, however samples can still be analyzed with a single genotype missing (either one donor or recipient).

Once the data is analyzed by the software, the results for all samples are displayed in a user-interface window as well as output to a PDF or Excel-compatible file. The results include % DNA fractions calculated for each post-transplant sample, as well as quality control metrics that are used by the software to pass or fail a sample.

## 1.2 Intended Use

The intended use of the AlloSeq HCT products (assay kit and software) manufactured by CareDx Pty Ltd is to assist the user in monitoring engraftment following hematopoietic stem cell transplant (HSCT).

The AlloSeq HCT kit is intended to be used to measure the % donor(s) and % recipient DNA present in a single sample in allogeneic HSCT recipients.

The AlloSeq HCT software is intended to be used with data generated using the AlloSeq HCT kit.

The software is for appropriately trained, healthcare professional use only, in appropriately accredited laboratories. The AlloSeq HCT product is not used for the diagnosis of disease nor is it to be used as the sole basis for clinical decisions.

## 1.3 Limitations

**Genetic identity between donor(s) and recipients**

AlloSeq HCT is unable to perform surveillance for identical twins.

**Unknown genotypes**

- Unrelated Donor(s): -
    - It is possible to perform surveillance where the pre-transplant genotype for the recipient or one of the donor(s) is unknown. It is not possible to perform surveillance if there is more than one unknown genotype. Some of the QC Metrics require all genotypes to be present and are disabled if one of the genotypes is unknown.
    - In case of an unknown genotype, a disease relapse or loss of heterozygosity can impact results
- Related Donor(s) - all pre-transplant genotyping is required for surveillance testing.

Known anomalies are recorded in *TEC935_AlloSeq HCT Software - Known Anomalies.*

# 2. Computing Requirements and Compatibility

Refer to IFU106 AlloSeq Software Installation Guide for more details.

## 2.1 Compatible Data Files

AlloSeq HCT software is compatible with the zipped FASTQ file format (*.fastq.gz). The Illumina sequencing system generates these file formats. For more information about the FASTQ file format, for example, see the MiSeq Reporter Generate FASTQ Workflow Reference Guide (document # 15042322).

# 3. Experienced User Instructions

Pre-transplant samples are recommended for AlloSeq HCT, for all parties involved (Recipient and all Donors). Pre-transplant samples are required for multi-donor surveillance, for all parties involved.

1. Create Recipients, add Donors to Recipients, add Samples to Recipients and Donors.
    a. Mark pre-transplant Donor and Recipient samples as ‘genotypes.’
2. Create Batch, add Samples to Batch. ‘Generate Run Sheet’ for Batch.
3. Perform lab protocol, load sequencer.
4. When sequencing has finished, ‘Analyze Results’ for Batch, select folder with fastq files.
5. Pre-transplant samples – review Results, set ‘Approved.’
6. Post-transplant surveillance samples – review Results, set ‘Approved’ and export Results.

# 4. New User Instructions

## 4.1 Introduction

The convention in these IFUs is to capitalize ‘entities’ within the AlloSeq HCT software, such as Recipient or Sample, to draw attention to an item that is being tracked by the software.

AlloSeq HCT software allows tracking of multiple surveillance tests for the same Recipient over time. Other chimerism test results can also be entered into the software, to assist with validation.

Pre-transplant samples are recommended for AlloSeq HCT and required for multiple donor samples. AlloSeq HCT software can monitor one, two or three* Donors simultaneously, providing pre-transplant genotyping has been performed for all parties (Recipient and all Donors).

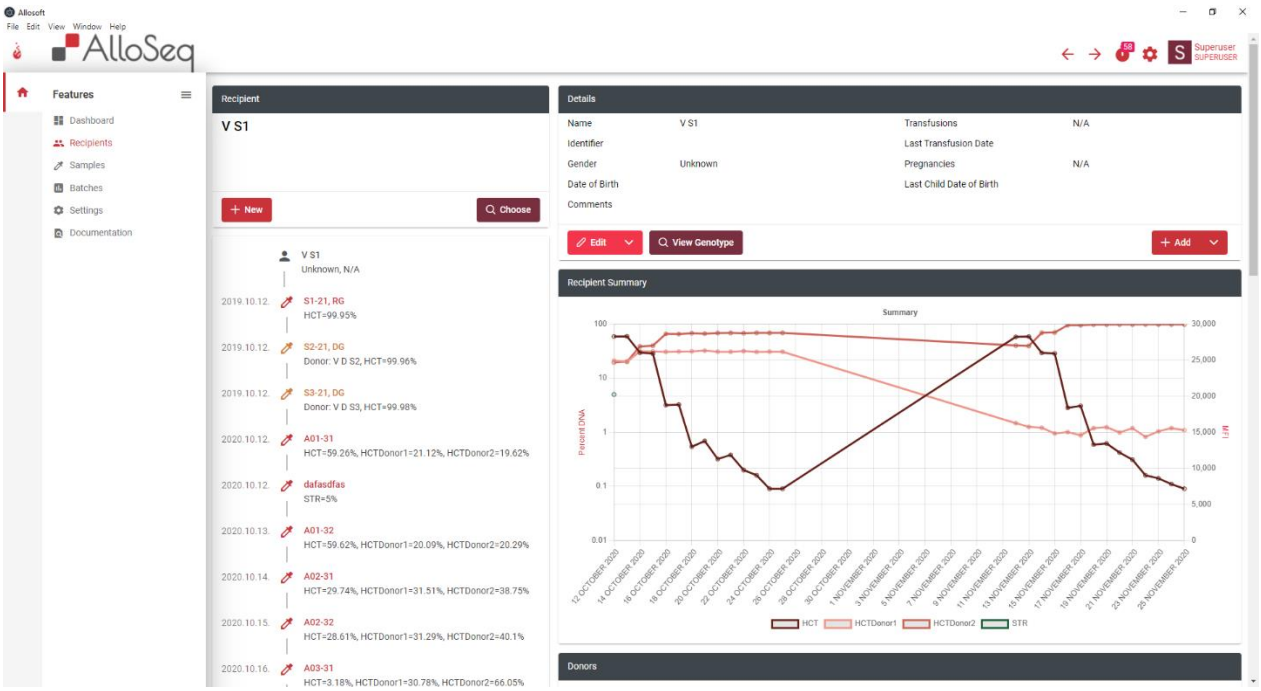
> ***Note:** There may be some rare cases where there is insufficient genetic variability between parties to track multiple related Donors.

AlloSeq HCT software also allows for a combination of Sample Source and Subpopulation, so cell-separated subpopulations can be monitored separately over time if required.

## 4.2 Recipients and Donors

The starting point for AlloSeq HCT software is to create a Recipient and add Donor(s) to the Recipient. It is also possible to add to the Recipient the Transplant time points (either received or required/planned) and Treatments (known interventions that might be useful to help interpret the test results).

The Recipient screen can be reached from the main menu to the left (clicking on the house and choosing 'Recipients') or via the 'fast start' round button on the main Dashboard (the starting screen seen after logging in).




Once on the Recipient screen, you can add a new Recipient by clicking on the 'New' button. You can also search for and choose an existing Recipient. The software does not perform any duplicate checking for Recipients, so it's safer to check first to make sure the Recipient you wish to add has not already been added to the software.

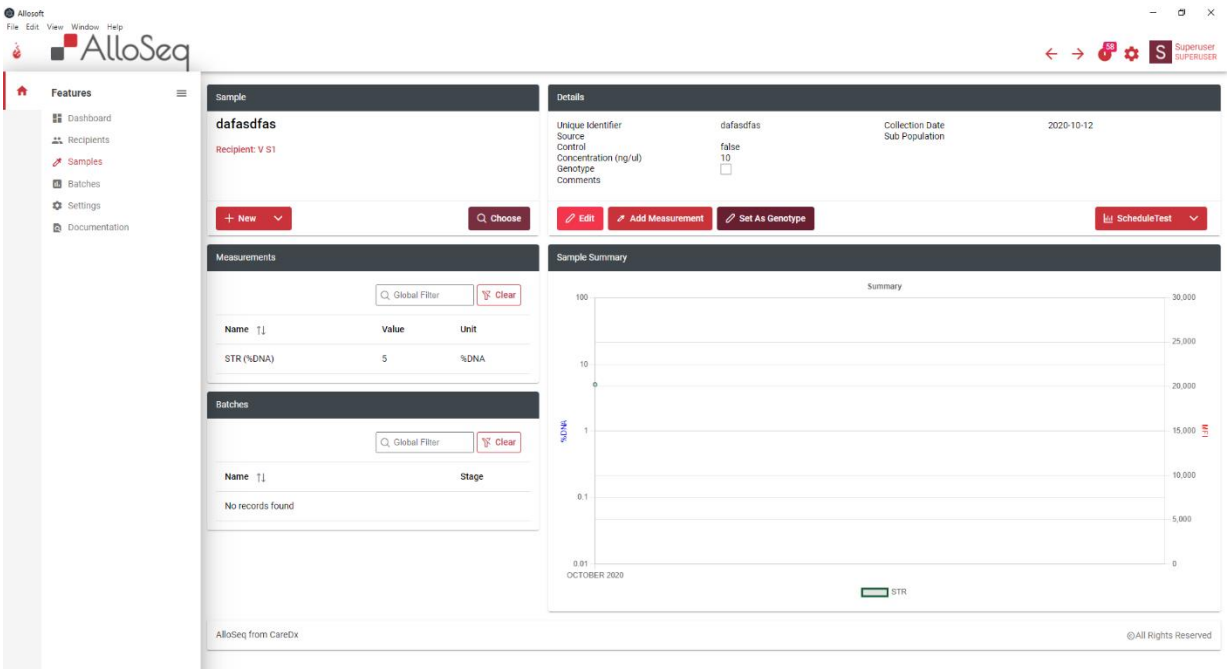
The Recipient screen contains a timeline of all the events that have been recorded for the Recipient, and a graph which summarizes all the AlloSeq HCT (and other chimerism test) measurements that have been performed. Any results marked as 'genotypes' (i.e., pre-transplant results for Recipient and Donor(s)) will not be displayed in the graph. You can add 'comments' to each Recipient, which can be useful for summarizing the current clinical state.

The Donors are listed below the graph on the Recipients screen. Here you can add new Donors, edit Donor details, and add pre-transplant genotyping samples to the Donors (using the 'Edit' button). The Name/ID and Relatedness is required.

## 4.3 Samples

Once you have created the Recipient and Donor(s), the next task is to add Samples. This can be completed on the Samples screen (reachable via main menu and fast start button) or can also be reached from the Recipient screen.

- If starting on the Recipient screen, you can use the 'Add' button (the dropdown list contains 'Sample'), and for Donors you can click on the donor and use the 'Edit' button, which has the option to 'Add Sample'.
- If starting on the Sample screen, you can select the 'New' button and choose to add a 'Recipient Sample', 'Donor Sample' or 'Control Sample'. You will then be prompted to select a Recipient/Donor. This is the only way of adding Control Samples to the software.




When adding Samples, the only mandatory data items are the Sample identifier (this needs to be unique across all Samples), the Sample collection date and the concentration of the extracted DNA (this is currently only held for reference purposes). For HCT, you can also add a Source and Subpopulation for your sample. If you are tracking different Subpopulations from a single blood or bone marrow sample these must be entered into the software as separate Samples. Each combination of Source (including none) and Subpopulation (including none) will be tracked separately, e.g., a Sample marked as both 'Source: Whole Blood + Subpopulation: CD14', will be tracked separately to one marked only as 'Source: Whole Blood', or one marked only as 'Subpopulation: CD14'. Note that these Sample meta details can be edited at any time.

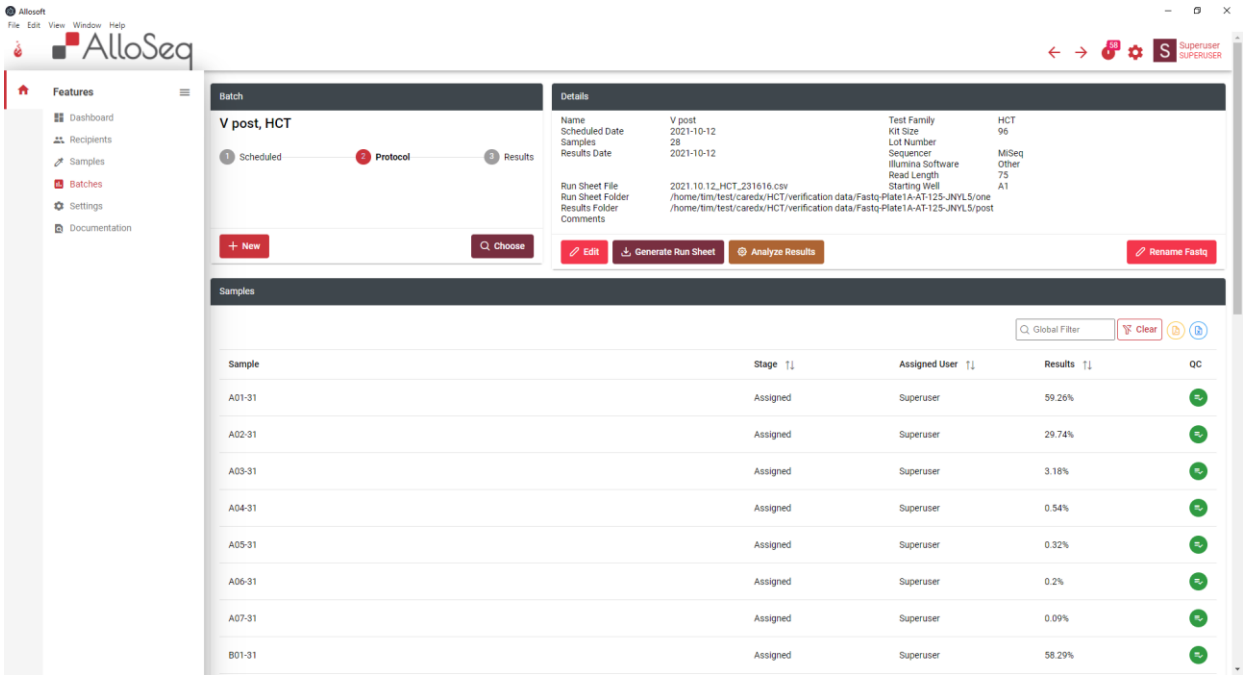
For pre-transplant Samples that will be used as the 'genotype' for the Recipient or the Donor, you can select the option to set them as the genotype while creating the sample or press the button to 'Set Genotype' once you have created the Sample. This allows the Result to be automatically assigned as a genotype once the analysis has finished.

On the Sample screen, it is also possible to add any other Measurements you have for other test results that have been performed at the same sample/time point. For AlloSeq HCT, this is usually STR or qPCR results for the sample, which can be used to validate the AlloSeq HCT results alongside your existing technique. It is possible to add antibody testing results here. You can also add 'comments' to each Sample if needed.

## 4.4 Batches

Batches are a group of Samples that will be prepared together using the AlloSeq HCT reagents and sequenced together. You can create a Batch at any time to start planning for future weeks of testing. You can reach the Batch screen via the main menu and the fast start button. You can also add a Sample to an existing batch or create a new one from the Sample screen.

Use the ‘New’ button to create a new Batch. You will need to add a date and choose ‘HCT’ as the ‘Test Family’. The Batch name is optional but recommended to help to distinguish one Batch from another. Comments can also be added to a Batch.




Once the Batch has been created you can ‘Add Samples’ and choose which Samples from the list you wish to test in the Batch. The order of Samples in the batch will correspond to the order of the sample indexes to be used from the index plate in the AlloSeq HCT reagent kit.

> **Note:** The order of the Samples in the Batch will be the order that you add them to the Batch, the only way to change the order is to remove Samples and add them again.

Once you have added all your Samples to the Batch, you can choose the ‘Generate Run Sheet’ option, which will create a new CSV formatted run sheet file to be used on the Illumina sequencer. This can also be printed and taken into the lab to assist in matching each Sample with its index from the index plate.

> **Note:** Please be careful if opening this file in Excel – saving changes to the CSV file made in Excel may cause it to stop working for the Illumina sequencer! It’s usually safest to print this file using Excel and do any editing (normally not required) using a Text Editor such as Notepad++.

> **Note:** You can choose a ‘starting well’ for the Run Sheet – if you have chosen to break up a single AlloSeq HCT kit into multiple sequencing runs, this is how you can set a different starting well for the indexes. The indexes in the Run Sheet will be used for demultiplexing the sample data (i.e., deciding which data belongs to which sample) at the end of sequencing.

The Run Sheet can be regenerated at any time – if nothing has changed with the Batch the contents will be identical each time.

Once the Run Sheet has been generated, it’s time to proceed to the AlloSeq HCT Lab Protocol and prepare the DNA samples for sequencing.

Platemap Feature

A plate map component has been added to the software. This is an optional use for the end-user. This feature helps organise the well position on the platemap for the output file (run sheet).

## 4.5 Analysing Results

Once the sequencing has finished, there will be a folder created on the sequencer containing fastq files. There will be two fastq files per sample. We recommend copying/moving the folder with the fastq files to the server for the analysis (accessing fastq files remotely over a network can slow down the analysis).

The analysis is done by going to the Batch screen, selecting the appropriate Batch, and using the 'Analyze Results' button. This will prompt you to provide the location of the folder containing the fastq files. It will check the folder to ensure all the files for each Sample are present and then initiate an analysis. The analysis takes roughly 30 seconds per sample. Once it has finished, it will create a new 'Task' for each result – these Tasks will be assigned to the person who performed the analysis. You can see your Tasks in the main Dashboard when you log in. These Tasks can be reassigned to other users of the software if needed. These Tasks will remain open until the result has been 'Approved' by a Lab Director (or Super User).

> **Note:** Results will be displayed in the graphs on the Recipient and Sample screens regardless of approval status.

## 4.6 Reviewing Results

There is a very high-level summary of the quality of each Result visible in the Dashboard and the Batch screen – a color-coded button with green, yellow, or red color. If the Result contains a warning (yellow) or error (red) then there will be a reason for this 'Quality Control' (QC) failure displayed in the Quality Control tab in the Results screen.

Results can be viewed (and should be reviewed) individually, however, it's usually more informative to start by reviewing the Result summarized with all the other results for a Recipient in the Recipient screen. You can get to the Result screen to see the Result detail by clicking on the Sample identifier in the timeline on the Recipient screen (this will take you to the Sample screen if you do not yet have a Result for this Sample).

You can also get to the detail of each Result from the main Dashboard by selecting the item in the Task list and either 'View Results' from the 'Workflow' menu or choose 'Results' from the 'View' menu. You can also get to the Results via the Sample screen (right-click on the item in the 'Tests' list and 'View Results').

Once in the Result screen, the details of the analysis are available. The Summary Result is displayed. This will differ in its display depending on the metadata available.

- If this is a AlloSeq HCT surveillance result with known genotypes already assigned for Recipient and Donor(s) then the Result will show the exact percentages detected for each party in the mix, e.g., **Recipient 0.5%, Donor 99.5%**.
- If this is a pre-transplant Recipient genotyping Result (i.e., the Sample is set as a 'genotype') then the software will automatically assign the majority signal as Recipient.
- If it is a pre-transplant Donor genotyping Result (i.e., the Sample is as a 'genotype') the software will automatically assign the majority signal as Donor.

The software assumes that pre-transplant genotyping will have been done in advance for AlloSeq HCT Recipients and Donors. If you have a single Batch with a mixture of pre- and post-transplant Samples, the pre-transplant Samples (genotypes) will be analyzed first and then any post-transplant Samples afterwards.

The AlloSeq HCT workflow can be summarized like this:

1. Use the software to create pre-transplant genotype samples for Recipient and Donor(s), mark these samples as 'genotypes'
2. Use the assay to prepare these samples, sequence them and use the software to analyze the results
3. Use assay and software to prepare and analyze the post-transplant surveillance Samples for the Recipient

The Results summary displays which 'Analysis Type' was used for the analysis. There are two Analysis Types:

- **Blind:** Performed automatically for all samples (for AlloSeq HCT mainly used for pre-transplant genotyping)
    - Includes an 'outlier detection' mechanism, to automatically filter out any statistical outlier results

    - Limitations – cannot tell you who is who in the mix, cannot handle multiple donors and has a 'blind spot' in the 30-70% range (which is why genotyping and subsequent Targeted analysis is recommended for AlloSeq HCT)
- **Targeted:** Performed automatically if (surveillance) samples belong to Recipients with Recipient and/or Donor genotypes.
    - Requires deep sequencing data
    - It has no weak spots but does require pre-transplant genotyping to have been performed
    - Will **not** be performed for Samples/Results that are already marked as being a genotype, only the Blind analysis will be performed

It is possible to reanalyze Results, again using the 'Analyze Results' button from the Batch screen. There is an option on this screen to 'Perform Only Meta Analysis' – this means that the basic (and time-consuming) analysis of the fastq data will not be performed again, only the meta (Blind/Targeted) analysis that follows the basic analysis. This can be useful for rapidly reanalyzing surveillance samples where the genotypes were not set correctly in advance.

While reviewing results, there are few items that are particularly important:

- The main Result, with standard deviation across all informative markers
- Total Reads Processed – usually limited by the software to a maximum of 3m reads per Sample. If it's lower than 300k reads, it will trigger a quality warning (indicating potential loss of sensitivity for very low values).
- Avg Marker Coverage – the average amount of fastq reads covering each marker
- QC (Quality Control) Status – will say Passed if everything is OK
- QC Reason – will appear if QC status is 'Warning or Failed'. The reasons for a QC issue can viewed in the Quality Control tab.

Several QC Metrics are measured, this will vary from one sample to the next depending on the sample type and analysis type performed. The following table summarizes these QC metrics.
**Note:** Outliers are detected if the difference between the value and the mean is greater than 5x the standard difference. Markers with less than 50 reads or with a third significant signal will fail locus-level quality checks.

| Name | Analysis | Sample Type | Pass | Warn | Fail | Description |
|---|---|---|---|---|---|---|
| Too Many Outliers | Blind | All | <2 | >=2 | >10 | Statistical outliers in the marker results are automatically detected and excluded. If there are too many outliers this may be an indication of sample contamination / mix up. |
| Markers Passing Filters | Blind | All | >=186 | <186 | <101 | The total number of markers that pass all locus-level quality control metrics. Minimum threshold is 92% (186 markers) for a sample to pass. |
| Uniformity | Blind | All | >=151 | <151 | <81 | For a sample to pass the uniformity filter ≥75% (151) of the 202 markers must have a coverage higher than 20% of the mean. |
| Average Marker Coverage | Blind | All | >=400 | <400 | <250 | The average number of reads covering each marker. Too few reads will lead to a loss of sensitivity (warning) or an unreliable result (failed). |
| Total Reads | Blind | All | >300k | <=300k | <=150k | Total number of reads processed. Too few reads will lead to a loss of sensitivity (warning) or an unreliable result (failed). |
| No Unique Markers | Targeted | 2+ Genotypes | >1 | N/A | 0 | There are no unique markers found for one or more parties, surveillance is not possible. |
| Insufficient Heterozygous Markers | Targeted | 2+ Genotypes | >5 | <=5 | N/A | Warning if there are very few uniquely heterozygous markers available. |
| Unexpected Minor Signal | Targeted | 2+ Genotypes, no missing Genotypes | 0 | <=2 | >2 | There should be no significant (>0.5%) second signal in positions that are identically homozygous for all parties. Detects contamination and sample mix ups. |
| Heterozygous Imbalance | Targeted | 2+ Genotypes, no missing Genotypes | 0 | <=2 | >2 | Identically heterozygous positions for all parties should be 50:50 in the result. High numbers of imbalanced markers (outside 70:30 range) is a possible indication of sample mix up or contamination. |
| Standard Deviation Too High | Targeted | No missing Genotypes | <=5 | >5 | >10 | A high standard deviation in the results is unexpected and may be an indication of sample mix up, contamination or missing genotype information. |

| | | | | | | |
|---|---|---|---|---|---|---|
| Noise | Meta Analysis | Genotype | <0.5% | >=0.5% | >=25% | Pre-transplant genotypes are expected to be 100% recipient or donor. Mixed samples will be unsuitable for use as a genotype. |
| Insufficient Genotypes | Targeted | 3+ Parties | <=1 | N/A | >1 | Samples with two or more donors can only be missing at most one genotype. More genotyping information is required for analysis. |

By default, only the most informative SNPs will be displayed. If you wish, you can choose other options for filtering the SNP list, including seeing all the SNP results.
A SNP will be regarded as informative in one of these two cases:

- It is heterozygous in one party, and identically homozygous in all other parties, for example AT in the recipient and AA in all donors
- It is homozygous in one party, and oppositely homozygous in all other parties, for example TT in one donor and AA in the recipient and all other donors

The 'Statistics' tab provides extra data points for those with a deeper interest in the underlying bioinformatics.

Once you are happy with the Results, you can send these for Approval to the Lab Director to 'Approve and Export' the Results. The Results can be exported in CSV, TSV or PDF formats. Multiple Results can be exported together from the main Dashboard, or from the Task List.

# 5. Troubleshooting

If all the QC Metrics are green/passed, there should be no need for any troubleshooting of results.

Yellow/warning QC Metrics are an indication that there is something in the result which requires manual inspection. The results in this case will not be affected and can be reported. Warning QC metrics can also provide background information if other QC Metrics have failed.

Red/failure QC Metrics indicate that the result is in doubt. It may in some cases still be possible to accept and report results with QC failures, see below for examples.

**Deep Sequencing is required for maximum sensitivity**

AlloSeq HCT is unable to provide the highest levels of sensitivity if insufficient sequencing data has been created by the preparation and sequencing of a sample. The sample will be marked with a QC failure due to the QC Metrics for Average Marker Coverage and/or Total Reads in this case. However, if the result is higher than 1% then any loss of sensitivity will not have a major impact and the result can likely still be reported (unless other QC metrics have also failed). If the result is lower than 1% it is recommended to repeat the sample (repeat the preparation of the sample starting from the beginning of the assay protocol).

**Noisy Genotypes**

A pre-transplant genotype sample may fail the Noise QC Metric, indicating that the sample is not 100% recipient or donor as expected. This sample may still be usable as a genotype in this case as the software has some tolerance built-in for this. It is recommended to check the genotype using the 'View Genotype' function on the Recipient screen before using this sample as a genotype for subsequent surveillance samples.

**Noisy Batch**

If multiple genotype samples in a Batch fail with the Noise QC metric and/or multiple surveillance samples fail with the 'Unexpected Minor Signal' QC metric this is likely due to index contamination caused by using the same sample indexes as the previous sequencing run and/or not cleaning the sequencer between runs using the guidelines from the Assay IFU. If this case the genotypes will still be usable and surveillance samples with higher results (>10%) can likely still be reported, however low levels of contamination like this will requires surveillance samples with low results to be repeated.

**Sample Contamination**

The Unexpected Minor Signal QC metric can detect contamination over 0.5% in a sample. This QC Metric is only available if all pre-transplant genotypes are known. If the result is low and this QC Metric has failed the sample should be repeated.

**Sample Mix-Up**

If the Unexpected Minor Signal QC metric fails along with Heterozygous Imbalance and/or Standard Deviation too high this is likely not the correct sample for the recipient, so there has been a mix up with the samples. Attention should be paid to other samples in the same Batch as two samples could have been swapped by accident. It may be possible to resolve this mix-up without repeating the samples however we recommend contacting CareDx technical support and asking for their assistance.

Failed

| Name | Status | Algorithm | Sample Type | Value Detected | Pass Threshold | Warn Threshold | Fail Threshold | Description |
|---|---|---|---|---|---|---|---|---|
| Unexpected Minor Signal | | Targeted | 2+ Genotypes | 55 | 0 | <=2 | >2 | There should be no significant (>0.5%) second signal in positions that are identically homozygous for all parties. Detects contamination and sample mix ups. |
| Heterozygous Imbalance | | Targeted | 2+ Genotypes | 37 | 0 | <=2 | >2 | Identically heterozygous positions for all parties should be 50:50 in the result. High numbers of imbalanced markers (outside 70:30 range) is a possible indication of sample mix up or contamination. |
| Standard Deviation Too High | | Targeted | All | 30.08 | <=5 | >5 | >10 | A high standard deviation in the results is unexpected and may be an indication of sample mix up, contamination or missing genotype information. |

# 6. Contact Information

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**Technical Support and reporting serious incidents:**
Email: techsupport-labproducts@caredx.com

Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established

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

**Related Products:**
AlloSeq HCT

**Method history**

| Version | Date | Modification |
|---|---|---|
| 1.0 | 31Mar22 | First version of the CE IFU for AlloSeq HCT Software, |
| 2.0 | 02May22 | Add Importer details (symbol). Move limitations to Section 1.2. Add Section 2. Remove QC metric for insufficient homozygous markers. Add Section 5 troubleshooting. |
| 2.1 | 11May22 | Updated to correct Qarad postcode. |
| 3.0 | 30Aug22 | Release 2.1.1, removed restriction on double parent analysis, refined thresholds for 3 QC metrics<br>Section 10: Addition of vigilance reporting requirements. |
| 4.0 | 25July23 | Updated 1.2 Limitations – genotypes required for surveillance samples with related donors. Updated 4.1 Recipient & Donors – For Donor details, relatedness is required if any genotypes are missing. Added optional Platemap feature to section 4.4 “Batches” as optional feature use |
| 5.0 | 24Oct23 | Addition of CH-REP details. |
| 6.0 | 11Jan24 | Updated 1.3 Limitations - In cases of an unknown genotype, a disease relapse or loss of heterozygosity can impact results. |