



AlloSeq cfDNA Software

Instructions for Use

IFU085

Document Version Number: 7.0

Software Version: 2.2.0

Issue Date: October 2023



ASCFS1.0



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1. Overview

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

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

1.1. System and Software Overview

The AlloSeq cfDNA assay and the AlloSeq cfDNA software (collectively referred to as AlloSeq cfDNA) form a system that enables relative quantification of the donor-derived cell-free DNA (cfDNA) in a cfDNA sample derived from a solid organ transplant recipient. 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(s) and the sequence data is analyzed using the AlloSeq cfDNA software. In the absence of donor or recipient genotype information, 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).

AlloSeq cfDNA 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). 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.

1.2. Intended use

The AlloSeq cfDNA Software is intended to be used together with the AlloSeq cfDNA assay to calculate the relative amount of donor-derived cell-free DNA (%dd-cfDNA) in solid organ transplant recipients.

The product is intended for use in appropriately regulated laboratories.

The software is for appropriately trained, healthcare professional use only, in accredited laboratories and must not be used as the sole basis for clinical decisions. The AlloSeq cfDNA kits and Software are not used for the diagnosis of disease.

1.3. Compatible Data Files

AlloSeq cfDNA 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 or MiniSeq Reporter Generate FASTQ Workflow Reference Guide (document # 15042322).

1.4. Limitations

The software has not been evaluated using fastq files generated by:

- A Sequencer other than a MiSeq or MiniSeq
- A MiSeq or MiniSeq using non-default Runtime default settings
- Genetic identity between donor and recipient AlloSeq cfDNA is unable to perform surveillance for identical twins.

When no recipient or donor genotyping are provided, the software reports the minor cell free DNA fraction as the donor fraction by default. If the donor has the potential to be the major fraction (>50%), a recipient-only or donor-only pre-transplant genotype sample must be used to correctly assign the fraction to the donor. The donor relationship to the recipient must be provided: parent, child, grandparent, grandchild, sibling, half-sibling, aunt, uncle, niece, nephew, cousin, unrelated, other (specify) must be selected “Relatedness” under Donor Details. For liver transplant recipients, it’s required to have a single genotype for analysis. It’s highly recommended to have both donor and recipient genotypes, when possible, for liver recipients.

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

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

2. Experienced User Instructions

1. Create Recipients, add Donors to Recipients, add Samples to Recipients.
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. Post-transplant surveillance samples - review Results, set ‘Approved’ and export Results.

3. New User Instructions

3.1. Introduction

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

AlloSeq cfDNA software allows tracking of multiple surveillance tests for the same Recipient over time. Other test results can also be entered into the software such as donor-specific antibody (DSA) results or kidney creatinine results, to assist with visualization of the latest tests performed for the Recipient.

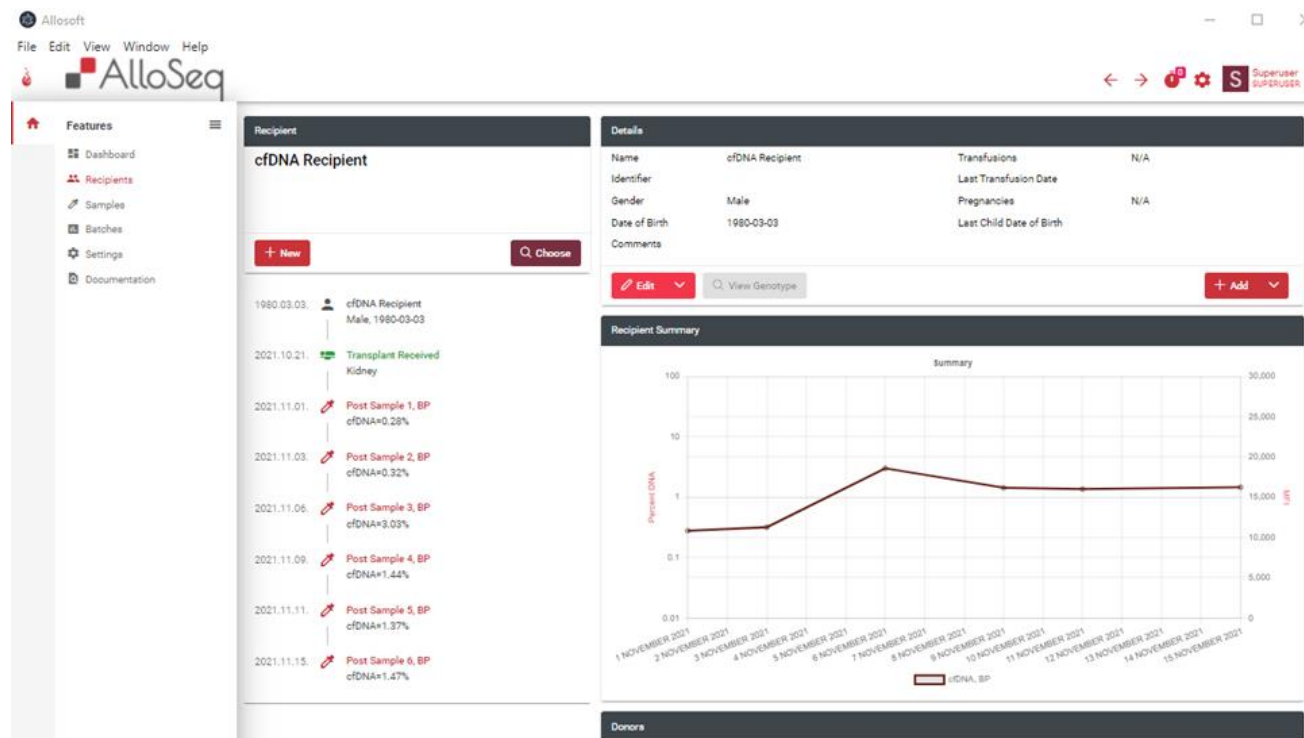
AlloSeq cfDNA has only been validated with cfDNA extracted from blood plasma samples.

3.2. Recipients and Donors

The starting point for AlloSeq cfDNA software is to create a Recipient and add a Donor to the Recipient. The donor “Relatedness” is required as it will have a correction factor on the result. It is also possible to add to the Recipient

the Transplant time point (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.

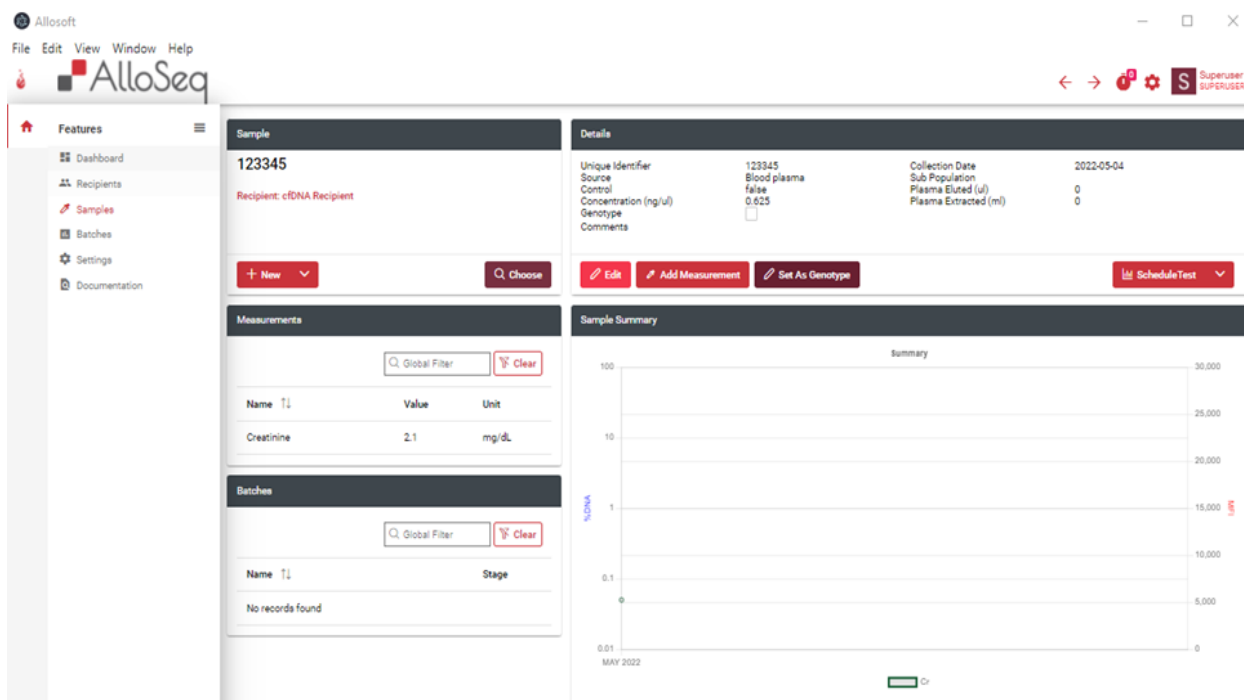
The Recipient screen contains a timeline of all the events that have been recorded for the Recipient, and a graph which summarizes all the AlloSeq cDNA (and other test) measurements that have been performed. You can add 'comments' to each Recipient, which can be useful for summarizing the current clinical state.

The Donors are listed below the graph on the Recipients screen. Here you can add new Donors and edit Donor details.

3.3. Samples

Once you have created the Recipient and Donor, 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').
- If starting on the Sample screen, you can select the 'New' button and choose to add a 'Recipient Sample', or 'Control Sample'. You will then be prompted to select a Recipient. This is the only way of adding Control Samples to the software.



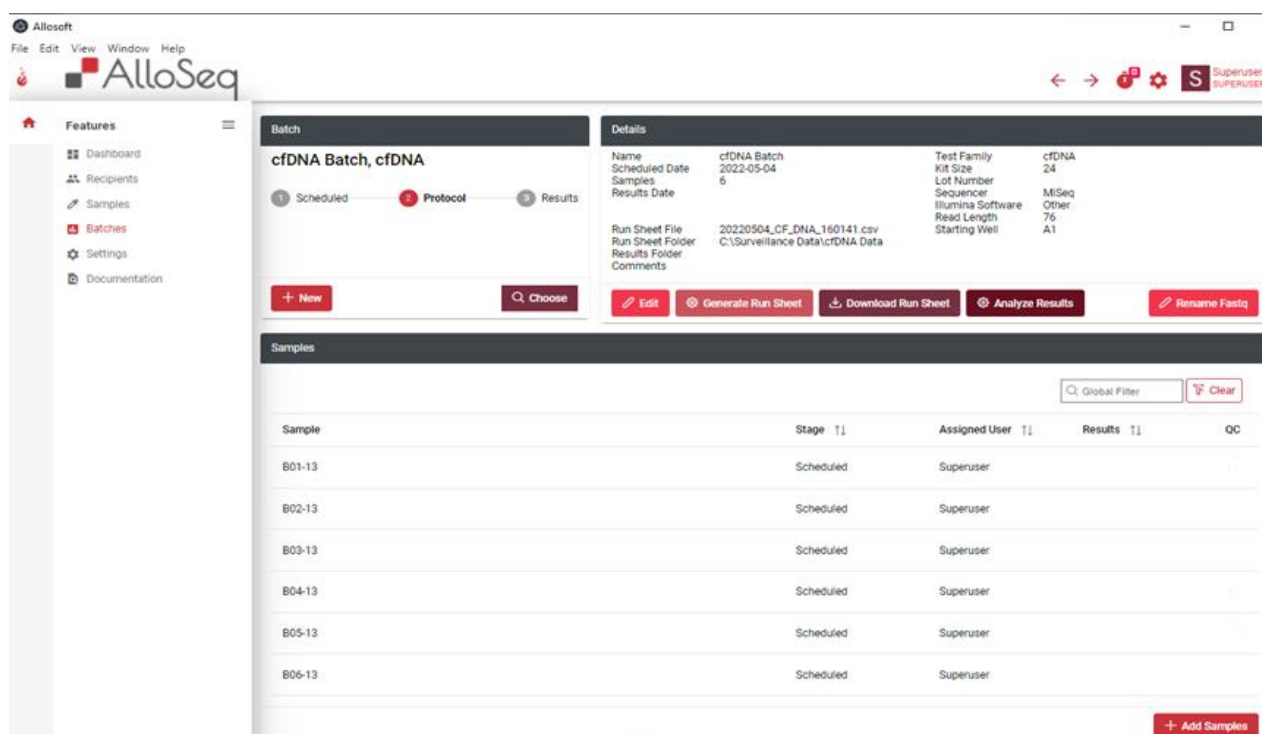
When adding Samples, the only mandatory data items are the Sample identifier (this needs to be unique across all Samples), the Sample collection date and the concentration of the extracted DNA (this is currently only held for reference purposes). Note that these Sample meta details can be edited at any time.

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 cfDNA, this is usually DSA or organ function results for the sample. You can also add 'comments' to each Sample if needed.

3.4. Batches

Batches are a group of Samples that will be prepared together using the AlloSeq cfDNA 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 'cfDNA' 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 cfDNA 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 cfDNA 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 cfDNA Lab Protocol and prepare the DNA samples for sequencing.

Platemap Feature

A platemap 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).

3.5. 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 approximately 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.

3.6. 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. For an AlloSeq cfDNA surveillance the Result will show the exact percentages detected for each party in the mix, e.g., **Recipient 99.5% (assumed), Donor 0.5% (assumed)**. The software assumes that the lower signal detected belongs to the Donor.

The AlloSeq cfDNA workflow can be summarized as:

1. 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. Only one Analysis Type is used for cfDNA:

- **Blind:** Performed automatically for all samples
 - 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 (values this high are not expected for cfDNA)

It is possible to reanalyze Results, again using the 'Analyze Results' button from the Batch screen.

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. 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	N/A	<=186	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	N/A	<151	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).

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 if is determined to be homozygous.

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.

4. 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 cfDNA 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 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. Surveillance samples with low results will likely need to be repeated.

5. References

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Interference Testing in Clinical Chemistry; Approved Guideline—Second Edition, **EP7-A2 (ISBN 1-56238-584-4)**

6. Contact Information

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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	09Jul20	Removed the Intended Use – RUO version should not have an Intended Use.
4.0	26Mar21	Updated distributor from Vienna to Stockholm in section 7.0 as per CR 2020-097
5.0	1Jun22	Release new version 2.1.1, several sections have been updated and limitations and troubleshooting sections have been added. Refer to CR 2022-028.
6.0	25Jul23	<p>Removed section 2. “Device description” as only relevant to v1 software. Updated section 1 to match the CE IFU. Added section 2 with expert user information.</p> <p>Removed section 6 “Limitations” and move to section 1.4 Limitations. Added MiniSeq as validated equipment. Added optional Platemap feature to section 3.4 “Batches” as optional feature use.</p>
7.0	18Oct23	Update section 3.2 “Recipients and Donors.” After adding Donor to Recipient, the Relatedness is required.