ForenSeq Universal Analysis Software v2.0

Reference Guide

VEROGEN PROPRIETARY
Document # VD2019002 Rev. C
February 2021

Revision History

Document #	Date	Description of Change
VD2019002 Rev. C	February 2021	Updated software descriptions to ForenSeq Universal Analysis Software v2.2, which supports the ForenSeq Kintelligence Kit with NA24385 as the positive control. • Added the ForenSeq Kintelligence assay, Verogen Kintelligence Analysis Method, and kinship reports. • Added run metrics for ForenSeq Kintelligence libraries. • Added instructions for assigning Unique Dual Index adapters, accessing the analysis folder, switching analysis methods, and printing. Updated the ForenSeq trademark to a registered trademark. Refreshed the format of the guide: • Updated the layout and formatting. • Updated fonts, table styles, and other design elements. Added guidance for failed run metrics. Added a link to the Verogen Documentation web page. Added the glossary terms clusters, intensity, intralocus balance, positions called, reference SNP ID number, short tandem repeat, typed and untyped, and Unique Dual Index. Added ambient temperature and reagent chiller temperature as potential sources of high phasing or prephasing values. Reorganized information on primary workspaces, reports, analysis methods, and reviewing sample data. Renamed the Sample Details page to Results and updated other user interface terminology as needed. Clarified that Dark Mode is the only software setting available to non-administrators. Corrected flow cell compatibility for the ForenSeq mtDNA Control Region Kit.
VD2019002 Rev. B	August 2020	Updated software descriptions for ForenSeq Universal Analysis Software v2.1, which provides the following enhancements: • Support for the ForenSeq mtDNA Whole Genome Kit • A Run, Project, and Sample History setting in System Settings • Project History and Sample History worksheets for Project and Sample Reports Updated guide format and organization, including adding an index and consolidating workspace descriptions. Revised step-by-step instructions to be more succinct and include field descriptions. Updated telephone numbers for Verogen contact information. Updated glossary term definitions and added the terms coverage plot, indel, library, mtDNA navigator, plexity, position viewer, system event, and user action. Updated figures to include call-outs highlighting user interface elements. Added information on strand bias and lost passwords. Added instructions for creating projects, assigning a sample to multiple projects, modifying user-created analysis methods, and enabling and disabling dark mode. Added another possible cause of low cluster density: belatedly combining bead-based normalized libraries and Hybridization Buffer (HT1). Noted that the analysis folder contains VCF and BAM files. Noted that the software follows SWGDAM nomenclature rules. Noted the conditions for displaying the Call or No Call filter. Clarified that each sample name must be unique in the software. Clarified that phasing and prephasing values are for Read 1 only.

Document #	Date	Description of Change
VD2019002 Rev. B	August 2020	Distributed troubleshooting information among individual sections. Moved information on analysis method settings to *Analysis Defaults for ForenSeq Universal Analysis Software v2 (Pub. No. VD20200045)*. Corrected duplicate entries of D7S820 and D16S539 in the Human Sequencing Control. Corrected the list of information included in project and sample reports. Corrected citations that appear in the appendix.
VD2019002 Rev. A	August 2019	Initial release

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Software Overview

Introduction

ForenSeq® Universal Analysis Software (UAS) v2.0 is a forensic genomics solution for run setup, sample management, data analysis, and reporting. Preinstalled on a dedicated server, the software integrates with the MiSeq FGx® Sequencing System to automatically generate FASTQ files, perform alignment, and call variants from run data.

The software analyzes two types of sequencing data for the following workflows:

- **mtDNA analysis**–For libraries prepared with the ForenSeq mtDNA Control Region Kit, the software interrogates the control region of the mitochondrial genome (mtGenome). For ForenSeq mtDNA Whole Genome libraries, the software interrogates the entire mtGenome. ForenSeq UAS v2.0 also analyzes mitochondrial DNA (mtDNA) data from libraries prepared with custom or third-party assays and sequenced on the MiSeq FGx System.
- **Kinship analysis**–For libraries prepared with the ForenSeq Kintelligence Kit, the software interrogates single nucleotide polymorphisms (SNPs) for upload to GEDmatch® PRO.

The analysis method varies for each library type and the reports are different. However, run setup is the same.

Compatibility of Run Components

Use the version of ForenSeq UAS v2.0 that is compatible with your library prep kit and a flow cell that supports the desired plexity and read coverage.

Table 1 Version compatibility

Library Prep Kit	Flow Cell[1]	Maximum Plexity[2]	Software Version
ForenSeq Kintelligence Kit	Standard	3 libraries	ForenSeq UAS v2.2, or later
ForenSeq mtDNA Whole Genome Kit	Standard	16 libraries	ForenSeq UAS v2.1, or later
ForenSeq mtDNA Control Region Kit	Micro	48 libraries	ForenSeq UAS v2.0, or later

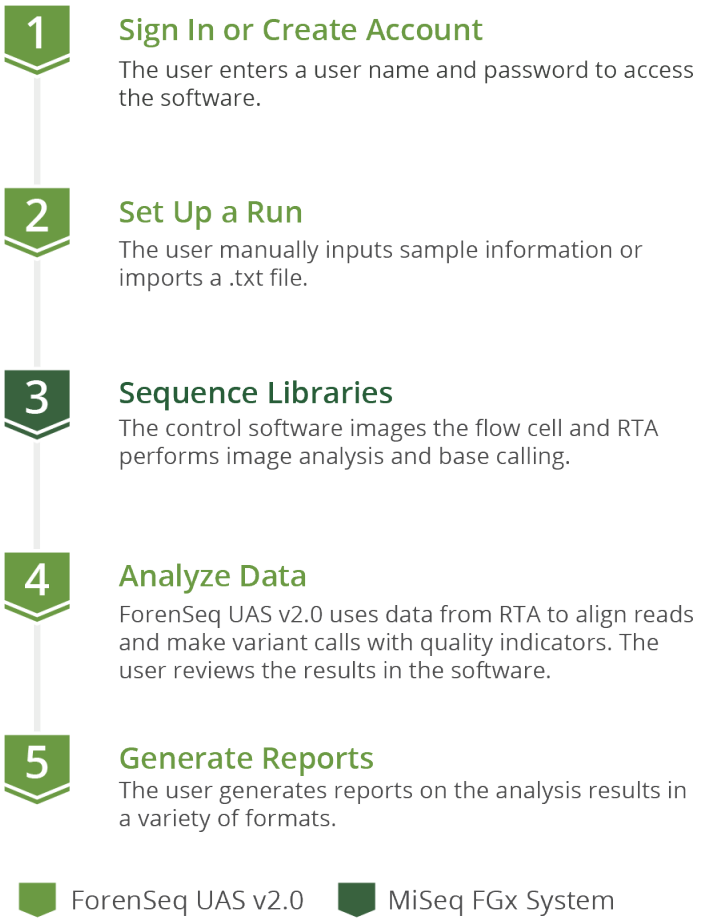
[1] The MiSeq FGx Reagent Kit provides the standard flow cell and the MiSeq FGx Reagent Micro Kit provides the micro flow cell.

[2] When using the default analysis method.

Analysis Workflow

The following diagram outlines the complete analysis workflow, which includes ForenSeq UAS v2.0 and two applications that run on the MiSeq FGx System: MiSeq FGx Control Software (MFCS) and Real-Time Analysis (RTA). For more information on these applications, see the *MiSeq FGx Sequencing System Guide (document # VD2018006)*.

Figure 1 Overview of data analysis steps



Accessing the Software

ForenSeq UAS v2.0 is a browser-based application that does not require an internet connection. Open a web browser (Chrome is recommended) on a computer with access to the same network as the MiSeq FGx System. Set screen resolution to at least 992 pixels for optimal display.

To access the ForenSeq UAS v2.0 user interface, enter the server address in the web browser address bar. If the Domain Name System (DNS) does not list the server, enter the server IP address for the network.

Create an Account

Before using ForenSeq UAS v2.0, you must create an account. The first user to create an account is automatically assigned administrator privileges. The administrator is responsible for approving new accounts. For instructions, see *Enable and Disable Accounts* on page 50.

Lost passwords are not recoverable, so creating a second administrator account is highly recommended. If the administrator password is lost and only one administrator account exists, the software must be uninstalled and reinstalled.

1. Access the network server through a web browser.
2. On the Login page, select **Register as a new user**.
3. On the Register page, complete the following fields:
   - **Email**–Enter an email address to be the user name for the account.
   - **Password**–Enter a password for the account.
   - **Confirm password**–Reenter the password for the account.
4. Select **Register** to submit your account for approval from the system administrator.

   A confirmation message appears. You can access the software after approval.

Sign In or Sign Out

1. Sign in as follows.

   a. Access the network server through a web browser.
   b. Enter your email address (user name) and password.
   c. Select **Login**.

   The software opens to the Home page.
2. When you are ready to sign out, select **Account**, and then select **Logout**.

Change a Password

1. On the navigation rail, select **Account**.
2. Select **Manage**.
3. In the Current Password field, enter your password.
4. In the New Password field, enter a new password.
5. In the Confirm Password field, repeat the new password.
6. Select **Save** to apply the new password.

Primary Workspaces

The following pages comprise the primary workspaces in ForenSeq UAS v2.0:

- **Home**–View and create runs and projects. This page functions as a system dashboard.

- **Runs**–Set up and manage runs and samples before and during a run.
- **Projects**–View and manage projects and samples after a run and generate reports.
- **Results**–Review data on a sample-by-sample basis after a run.

The software also includes printing, commenting, and other functions that supplement run setup, data analysis, and reporting. For instructions on using these supplementary features, see *Run, Project, and Sample Management* on page 40.

Home Page and Navigation

The Home page displays information and functions organized into runs and projects. Selecting a run goes to the Run Details page and selecting a project goes to Projects. Color-coded Quality Metrics icons provide an overall view of run performance. Hovering over an icon displays more detailed metrics.

The navigation rail vertically arranges icons and puts key destinations within easy reach. It appears consistently on the left side of each page so you can go to runs, projects, settings, and your account or return to the Home page.

Figure 2 Overview of the Home page

A **Navigation rail**–Icons that go to key destinations
B **Runs**–List of runs with quality metrics and options to search and create
C **Projects**–List of projects with options to search and create
D **Quality Metrics icon**–Quality metrics for a completed run
E **Scroll arrows**–Move through all runs or projects

Runs

Runs is the sequencing workspace for creating and monitoring runs and the input and organization of sample information. Selecting Runs on the navigation rail opens the Runs navigation drawer, which provides functions for creating and searching runs.

The Runs navigation drawer also lists every run in the software with the run state, number of samples in the run, flow cell type, and color-coded Quality Metrics icon. Runs are listed from newest to oldest. Use the scroll arrows to move through the complete list.

Run Details Page

Selecting a run from the Runs navigation drawer opens the Run Details page. Each Run Details page is a run-specific workspace for managing the run and samples in the run. Throughout the software, selecting a run name hyperlink goes to the Run Details page.

A table displays all samples in the run with information about each sample and options for sorting and filtering. Functions for importing, adding, searching, and removing samples appear above the table. The header section of a Run Details page displays the run name and optional description, cycles per read, and other run information. The header also includes quality metrics and sample representation and the following icons.

Table 2 Icons on a Run Details page

Icon	Name	Function
	Edit	Change the project name and add or change the optional description.
	Run Activity	See system events and user actions for the run and add comments. This icon is visible when the history setting is enabled. See *System Settings* on page 51.
	Print	Print the Run Details page.

Projects

Projects is the post-sequencing workspace for creating and managing projects, accessing analysis results, and generating reports. Selecting Projects on the navigation rail opens the Projects navigation drawer, which provides functions for creating and searching projects.

The Projects navigation drawer also lists every project in the software with the number of samples and runs in the project and the date and time of project creation. Projects are listed from newest to oldest. Use the scroll arrows to move through the complete list.

Project Side Sheet

Selecting a project from the Projects navigation drawer opens the Project side sheet and the Samples page. The Project side sheet lists all samples in the project, grouped by run. Expand the runs to see which samples are included. A green checkmark indicates an analyzed sample.

Replace the Samples page with the Reports page or PNQ page by selecting the respective icon. The Project side sheet provides the following icons for accessing these additional pages and project information.

Table 3 Icons on a Project side sheet

Icon	Name	Function
	Edit	Change the project name and add or change the optional description.
	Samples	Open the Samples page for the project.
	Reports	Open the Reports page for the project.
	Project Activity	See system events and user actions for the project and add comments. This icon is visible when the history setting is enabled. See *System Settings* on page 51.
	Print	Print the Samples page.
	PNQ	Open the PNQ page for each run in the project. Each icon letter is color-coded to provide high-level status. See *Run Metrics* on page 58.

Samples Page

The Samples page lists information for each sample in a selected project with functions for adding, reanalyzing, and searching samples. Filters narrow results by sample or assay type and sort by sample name, run name, and analysis method.

Reports Page

The Reports page lists reports in a project by creation date, starting with the most recent report. On this page, you can create reports or find and download existing reports. Sort the Date Created and File Name columns and filter the Report Type column to organize results.

PNQ Page

The PNQ page displays quality metrics, sample representation, and results for the positive and negative amplification controls. Together, these data provide an overall view of run performance. For interpretation, see *Run Metrics* on page 58.

Results Page for mtDNA

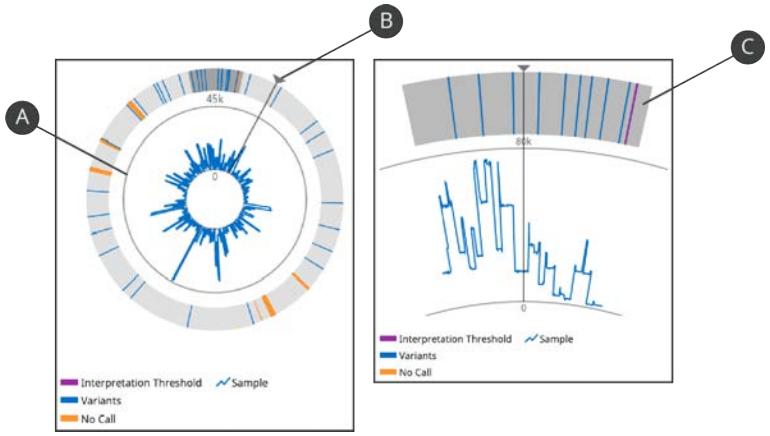
Selecting an mtDNA sample from a project opens the Results page. Each Results page is a sample-specific workspace for in-depth analysis of results. A header section displays general information about the sample and analysis with features for filtering and report generation.

The remainder of the Results page is divided into an mtDNA navigator, position viewer, and coverage plot that examine each position. Changing the position in one of these tools simultaneously updates the position in the others.

mtDNA Navigator

The mtDNA navigator displays data spanning the whole mtGenome or control region. Data include the IT, variants, calls or no calls, and coverage at various positions. The movable pointer updates the position.

Figure 3 mtDNA navigators for whole genome (left) and control region (right) results



A Maximum coverage indicator
B Movable pointer
C Control region view (filter applied)

By default, the mtDNA navigator shows the entire mtGenome. To focus on the control region only, apply the Control Region filter.

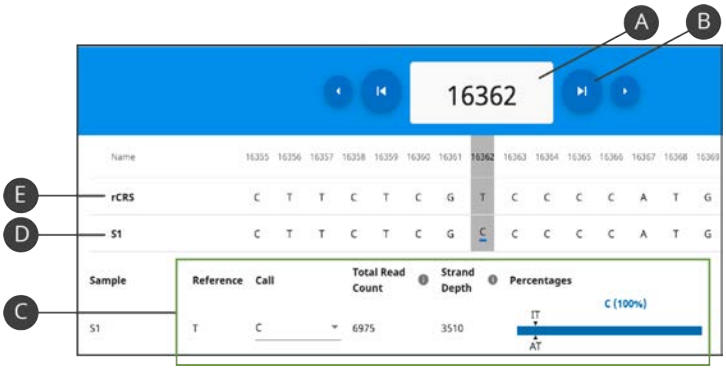
Position Viewer

The position viewer compares the sample sequence to the revised Cambridge Reference Sequence (rCRS). Color-coded dashes indicate ITs, variants, and no calls. For example, the blue dash in Figure 4 underlines the base at position 16,362 because the call (C) differs from the reference (T).

Individually select or enter each position number, or use the scroll arrows to move through pages of sequence segments and hotspots. For each selected position, the position viewer displays the following data:

- **Reference and call**–The base calls for the reference and sample.
- **Total read count**–The total number of reads from the forward and reverse strands.
- **Strand depth**–The strand (forward or reverse) read count with the majority of reads.
- **Percentages**–The percentage of each base compared to the AT and IT.

Figure 4 Position viewer set to position 16,362



A Selected position number
B Scroll arrows
C Data for the selected position
D Sample sequence
E Reference sequence

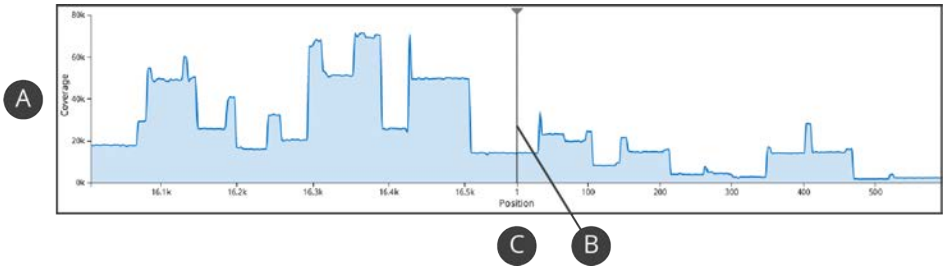
Strand Bias

If you want to evaluate strand bias, which provides an additional metric for evaluating quality, use the strand depth and total read count. The software reports strand depth as the read count from the forward or reverse strand that has the majority of reads for a base position. Subtract from the total read to determine the read count for the opposite strand.

Coverage Plot

The coverage plot displays the coverage (number of reads) for each mtDNA position. Positions are plotted along the x-axis and the y-axis shows coverage. A pointer marks the selected position. Move the pointer to shift the position.

Figure 5 Coverage at Each Position



A Coverage along the y-axis
B Movable pointer
C Positions along the x-axis

Results Page for Kinship

Selecting a kinship sample from a project opens the Results page. Each Results page is a sample-specific workspace for in-depth analysis of results. A header section displays general information about the sample and analysis and a button to create reports.

In addition to the header, the Results page for a kinship sample includes a Sample Overview section and a Sample Details section.

Sample Overview Section

The Sample Overview section displays locus call rate, total reads, gender, contributor status (XY, XX, or inconclusive), and heterozygote and intralocus balance metrics. Under these data, a table displays the percentage and total number of typed loci relative to base pair length per SNP type. The entire section is collapsible and you can edit both gender and contributor status.

Sample Details Section

The Sample Details section displays data for the SNP types listed in the following table. A card represents each locus, displaying the locus name, chromosome, allele, intensity, and intralocus balance. Each allele is color-coded: A is green, T is red, C is blue, and G is gray.

SNP Type	Description
X-SNP	Informs lineage and estimates gender in combination with Y-SNPs.
Y-SNP	Informs lineage and estimates gender in combination with X-SNPs.
Ancestry SNP (aSNP)	Estimates biogeographical ancestry.
Phenotype SNP (pSNP)	Estimates hair and eye color.
Identity SNP (iSNP)	Informs on source attribution.
Kinship SNP (kSNP)	Informs on potential biological associations (relatives).

Locus Details

Selecting a card in the Sample Details section opens locus details, which include allele intensity, thresholds, intralocus balance, and quality control (QC) indicators. Under Typed, a toggle key indicates whether the allele is typed.

- The Previous Locus and Next Locus arrows move through details for each locus.
- The Collapse icon closes locus details.

Locus Quality Indicators

Each card displays a color-coded horizontal bar: gray indicates a locus with no QC indicators and orange indicates a locus with at least one QC indicator. Any applicable indicators are displayed on the card.

Table 4 QC indicators

Symbol	Name	Description
	Allele Count	The locus has more typed alleles than expected for a single-source sample of the indicated gender.
	Unexpected Allele	The locus has an unexpected allele (non-reference or alternate) that is greater than or equal to the AT.
	Imbalanced	The locus is not homozygous, not hemizygous, and falls below the intralocus balance setting.
	Analytical Threshold	The locus has expected allele signal below the AT and no alleles greater than or equal to the IT.
	Interpretation Threshold	The locus has expected allele signal greater than or equal to the AT, but below IT.
	Not Detected	The software did not detect signal for the locus.
	User Modified	Someone edited the typed status of an allele at the locus.

Software Status

The software displays a state for runs and analyses. The state determines which modifications are permitted and are also useful for general status updates.

Run State

After a run is created and saved, the Run Details page displays the run state. The following table describes each possible state.

State	Description
Created	The run information is saved in ForenSeq UAS v2.0.
Sequencing	The run is in process on the MiSeq FGx System.
Completed	The run completed successfully.
Errored	An error prevented the run from finishing or it was manually stopped.
Paused	A user temporarily suspended the run.
Stopped	A user ended the run due to incorrect setup, poor data quality, or other issue.

Analysis State

After a sample is added to a run, the Results page displays the analysis state. The following table describes each possible state.

State	Description
Created	ForenSeq UAS v2.0 has not started analysis.
Queued	The analysis is in line to start.
Processing	Analysis or reanalysis is in progress.
Completed	Analysis has successfully completed.
Errored	Analysis has unsuccessfully completed.

Glossary

Term	Description
Amplicon	The product of PCR amplification of a targeted region of interest in a genome.
Amplicon end position	The last base position of an amplicon, including the PCR primer sequence.
Amplicon start position	The first base position of an amplicon, including the PCR primer sequence.
Analysis method	Settings and thresholds that inform the analysis of sequencing data to generate allele or variant calls and QC indicators.
Analytical threshold (AT)	The percentage defined in an analysis method that a read count must reach for the software to call a base or type an allele. Signal below the AT might be visible, but is not typed.
Assay type	The library prep kit used to process samples for sequencing and analysis.
Call	The reported base for a position (coordinate) based on the analysis method.
Cluster density	The number of clusters on a flow cell per square millimeter (K/mm²).
Clusters	A clonal group of DNA bound to the surface of a flow cell that produces one read. One template DNA strand seeds each cluster and is clonally amplified using bridge amplification until the cluster has about 1000 copies.
Clusters passing filter	The percentage of clusters that pass quality filters. For more information, see *Clusters Passing Filter* on page 61.
Control region	A subset of the human mtGenome (positions 16,024-576) containing three hypervariable regions.
Coverage	The average number of reads for a locus, allele, or variant position in a sample that align to or cover known reference bases. Sequencing coverage level (read depth) can help determine whether an allele or variant call is made with confidence.

Term	Description
Coverage plot	A graph on a Results page that displays the coverage at each position in an mtDNA sample. For details, see *Coverage Plot* on page 14.
Cycle	A chemistry step and an imaging step to call one base in a sequencing run. For cycle numbers, see *Cycles per Read* on page 63.
Deletion	A base deleted from the sample sequence that is present in the reference sequence.
Differences indicator	A software feature that highlights different allele or variants calls between two samples being compared.
Flow cell	A glass slide coated with oligos complementary to the library adapter sequences, allowing the library to adhere for a run.
Forward primer length	The number of bases in the forward PCR primer (oligonucleotide).
Forward primer start coordinate	The starting base at the 5′ end of the forward PCR primer. The base depends on the amplicon and analysis method.
Hotspot	An mtDNA position with a variant, interpretation threshold, differences, no call, call indicator, or user modification.
Human Sequencing Control (HSC)	A pre-amplified and tagged positive control added to a library pool to facilitate troubleshooting of instrument and sequencing reagent issues.
Index adapter sequence	A short stretch of oligonucleotides added to each sample during library prep for identification purposes. The software uses the oligonucleotide sequences to demultiplex pooled libraries.
Index CV	The percentage of reads assigned to each library, representing the distribution of read counts of the libraries in the run. CV is the coefficient of variation for the number of read counts across all indexes.
Indel	The insertion or deletion of bases in a genome.
Insertion	A base in the sample sequence that is not present in the reference sequence.
Intensity	The software reports signal intensity as the number of reads.
Intralocus balance	The balance of read counts between typed alleles at a heterozygous locus. The balance is measured as the intensity of the minimum intensity typed allele divided by the intensity of the maximum intensity typed allele. When intensities are identical, the intralocus balance is 100%.
Interpretation threshold (IT)	The percentage defined in an analysis method that determines the read count that must be reached for the software to call a base or type an allele. Signal below the IT but greater than or equal to the AT can be manually typed.
Library	A DNA sample that uses primers and adapters to ligate specific regions of the genome for sequencing.
Library type	The function of a library, either primer-directed sequencing or non-directed sequencing, for custom assays and analysis.

Term	Description
Minimum quality score	The Q-score reads must meet to contribute to the variant call at each DNA coordinate. This value is defined in an analysis method.
Minimum read count	The minimum read count a position must reach for a base to be called.
mtDNA navigator	A section of a Results page that displays variants and other sequencing results for an mtDNA sample. For details, see *mtDNA Navigator* on page 13.
N indicator	The quality indicator for reagent blanks and negative amplification controls.
Negative amplification control	A sample that does not contain DNA as a template for amplification.
No call	The call assigned when a position lacks read counts for a specific base.
Original call	An unmodified variant call based on the run analysis method.
P indicator	The quality indicator for positive amplification controls that use control DNA, such as HL60.
Percent A, C, G, T	The percentage of forward and reverse reads out of the total reads for the A, C, G, or T bases at the call position.
Percent del	The percentage of forward and reverse reads out of the total reads for a deletion.
Percent ref	The percentage of forward and reverse reads out of the total reads that do not support an insertion at an insertion point in mtDNA.
Percentages	A position viewer feature that shows the percentage of each nucleotide called at a position in relation to the thresholds.
Phasing	The percentage of bases in a cluster that fall behind the current cycle in Read 1. For details, see *Phasing and Prephasing* on page 62.
Plexity	The number of libraries combined in a pool for sequencing. For example, if eight libraries are combined in a pool, the plexity is eight.
Position	A base coordinate of mtDNA using the numbering convention of the rCRS, comprised of positions 1-16,569.
Position viewer	A section of a Results page that displays reference and sample sequences and data for the selected position in an mtDNA sample. For details, see *Position Viewer* on page 13.
Positions called	How many positions out of the total number of interrogated mtDNA positions, including indels, the software called a base for.
Positive amplification control	A known sequence of DNA provided in the library prep kit and prepared with forensic and reference samples to ensure that library prep and sequencing reagents are functional.
Prephasing	The percentage of bases in a cluster that jump ahead of the current cycle in Read 1. For details, see *Phasing and Prephasing* on page 62.

Term	Description
Project	A collection of analyzed results for at least one sample, and the primary workspace for viewing and modifying results and creating reports.
Revised Cambridge Reference Sequence (rCRS)	The reference genome for calling variants in the mtGenome.
Read	The sequence string of A, T, C, and G bases corresponding to an amplicon. A run can simultaneously generate millions of reads from multiple loci and multiple samples.
Reads A, C, G, T	The number of forward and reverse reads for the A, C, G, or T base at the DNA call position.
Reads del	The number of forward and reverse reads for a deletion at the call position.
Reads ref	The number of forward and reverse reads that do not support an insertion at an insertion point.
Reagent blank	An extraction control without DNA that is carried through the entire sample processing.
Read 1 (i5) primer sequence	The sequence of the PCR primer that is tagged with the Index 2 (i5) adapter sequence. Index 2 is part of the adapter sequence, as is the binding site for the Read 1 sequencing primer.
Read 2 (i7) primer sequence	The sequence of the PCR primer that is tagged with the Index 1 (i7) adapter sequence. Index 1 is part of the adapter sequence, as is the binding site for the Read 2 sequencing primer.
Reagent cartridge kit lot	The batch code to identify the batch or lot that a component of the MiSeq FGx Reagent Kit or MiSeq FGx Reagent Micro Kit was manufactured in.
Reanalysis	The process of reanalyzing a sample with another analysis method, after the original run analysis method.
Reference call	The base call of the rCRS at a given position.
Reference SNP ID number (rsID)	An identification tag the National Center for Biotechnology Information (NCBI) assigns to a group of SNPs that map to the same location.
Reverse primer length	The number of bases in the reverse PCR primer (oligonucleotide).
Reverse primer start coordinate	The starting base at the 5′ end of the reverse PCR primer. The base depends on the amplicon and analysis method.
Run	The process of clustering and sequencing libraries to generate base calls.
Run analysis method	The analysis method assigned to a sample for the initial run analysis.
Sample	The DNA input for a library prep kit, before the addition of primers and adapters.
Sample compare	A software feature that compares an mtDNA sample of interest to other mtDNA samples.
Sample of interest	A user-designated sample that is compared to other selected samples.

Term	Description
Sample plate	The 96-well plate that contains libraries for sequencing.
Sample representation	The number of reads per sample for a run. These data are available after sequencing and analysis are complete.
Sample type	The function of a sample: forensic or reference sample, positive amplification control, negative amplification control, and reagent blank.
Sample well position	The column and row position in a 96-well plate containing samples or libraries. For example, position A1 indicates a well at the intersection of row A and column 1.
Short tandem repeat (STR)	Areas of the genome where short sequences of DNA (2-6 bp) are repeated, such as (GATA)n.
Single-nucleotide polymorphism (SNP)	Variation of one base pair in a DNA sequence relative to a known DNA reference sequence.
Strand depth	The read count from the forward or reverse strand that has the majority of reads for a base position.
Strand direction	The selection of a forward or reverse strand for a primer-directed library type.
System event	An action the software automatically performs and records.
Total read count	The total number of reads from the forward and reverse DNA strands for a base position.
Typed	An allele call that contributes to the locus genotype.
Unique Dual Index (UDI)	Premixed Index 1 (i7) and Index 2 (i5) adapters for dual index combinations that have no redundant sequences.
Untyped	A no-call allele that does not contribute to the locus genotype.
User action	A user interaction with the software, such as adding a sample to a run.
User-modified call	A base call that a user changed.
Variant	A base at a position in an mtDNA sequence that differs from the rCRS base and a synonym for SNP.
Whole mitochondrial genome	The entire 16,569 bp mtGenome.

Run Setup

Create a Run

1. On the Home page, select **Create Run**.
2. In the Create Run dialog box, complete the following fields:
   - **Run Name**–Enter a preferred name to identify the run.
   - **Description**–Enter a description of the run.
   - **Flow Cell Type**–Select **Standard** or **Micro**, depending on your reagent kit.
   - **Reagent Cartridge Kit Lot #**–Enter the LOT number printed on the reagent cartridge packaging.

   The user interface displays required fields with an asterisk (*).
3. Select **Save** to create the run.

   The Run Details page appears.

Edit a Run

1. On the navigation rail, select **Runs**, and then select a run.
2. Select **Edit**.
3. In the Edit Run dialog box:
   - Edit the run name.
   - Add or modify the optional run description.
   - Select a new flow cell type, **Standard** or **Micro**.
   - Add or modify the optional reagent cartridge kit lot number.

   Editing the flow cell type requires a run state of Created.
4. Select **Save**.

Add Samples to a Run

Add samples and sample information to a run using one of two methods: import a sample sheet or individually add samples. After samples are added, the Run Details page lists all samples included in the run.

All samples in a run must have the same assay type.

When adding samples to a run, you associate each sample with at least one project. If you enter the name of a new project, the software creates the project. Because a sample can be assigned to multiple projects, a run can include samples from multiple projects.

Import a Sample Sheet

1. On the navigation rail, select **Runs**, and then select a run.
2. Select **Import Samples**.
3. [Optional] Select **Sample Sheet Template** to download a .txt file that provides an example format.
4. Select **Drop Files Here**, and then navigate to the sample sheet location.
5. Select the sample sheet (.txt file), and then select **Open**.

   The sample sheet appears under the file upload area. Uploading another .txt file overrides the current file because the software accepts only one sample sheet per run.
6. Select **Next**.
7. Review the information from the uploaded sample sheet to ensure accuracy. Scroll to see all entries.
8. Select **Import** to add the samples to the run.

Assigning Unique Dual Indexes

The sample sheet includes two columns for entering index adapter names: i7Index and i5Index. When using the Unique Dual Index (UDI) adapters, enter the index name in the i7Index column only. Leave the i5Index column blank.

Assigning Projects

The InitialProjectList column of a sample sheet specifies which project a sample is assigned to. To assign multiple projects to a sample, enter each project name separated by a comma followed by a space and put quotes around the entry. For example:

```
"Project 1, Project 2, Project 3"
```

Individually Add Samples

1. On the navigation rail, select **Runs**, and then select a run.
2. Select **Add Sample**.
3. In the Add Sample dialog box, complete the following fields:

Field	Instruction
Sample Name	Enter a unique identifier for the sample. Each sample name must be unique in ForenSeq UAS v2.0, regardless of sample type.
Description	Enter a preferred description of the sample.

Field	Instruction
Sample Type	Select a sample type: • **Sample**–A forensic or reference sample • **ForenSeq Positive Amplification Control**–The control DNA (HL60 or NA24385) • **Negative Amplification Control**–The negative control (water) • **Reagent Blank**–The extraction control
Assay Type[1]	Select the library prep method: • **ForenSeq mtDNA Whole Genome**–Libraries prepared with the ForenSeq mtDNA Whole Genome Kit. • **ForenSeq mtDNA Control Region**–Libraries prepared with the ForenSeq mtDNA Control Region Kit. • **mtDNA Custom**–Libraries prepared with a custom or third-party assay. • **ForenSeq Kintelligence**–Libraries prepared with the ForenSeq Kintelligence Kit.
Index 1[2]	Select the name of an i7 index adapter.
Index 2[2]	Select the name of an i5 index adapter.
Index Combination[2]	Select the name of a premixed i7 and i5 index adapter (UDI).
Run Analysis Method	Select a method for analyzing samples in the run.
Initial Projects	Enter the name of a project to assign the sample to. To assign the sample to multiple projects, enter multiple project names.
Sample Plate	Enter a preferred name for the 96-well plate.
Sample Well Position	Enter the position of the plate well the sample occupies.

[1] Because the software allows one assay type per run, the assay type assigned to the first sample is automatically assigned to the others.

[2] Depending on assay type, the software displays either the Index 1 and Index 2 fields or the Index Combination field.

An asterisk (*) on the user interface indicates a required field.

4. Select **Save** to add the sample to the run.

Manage Samples in a Run

After adding samples to a run, you can edit sample information, change project assignments, or remove samples from the run. Use filters, sorting, and the search function to quickly find samples.

Find a Sample

1. On the navigation rail, select **Runs**, and then select a run.
2. To search for samples, enter any of the following information in the Search field:
   - Sample name
   - Description
   - Index adapter name
   - Run analysis method
3. To filter the samples, do as follows.

a. In the Sample Type or Assay Type column, select **Filter**.
b. Select at least one checkbox.
c. Reselect **Filter** to apply the selected filters.

4. To sort the samples, select a column heading.
   The Name, Description, and Index Combination columns are sortable.

Edit Sample Information

1. On the navigation rail, select **Runs**, and then select a run.
2. In the Actions column, select **Edit Sample Details**.
3. In the Edit Sample dialog box, modify the desired fields.

Field	Instruction
Sample Name	Edit the unique identifier for the sample.
Description	Enter or edit a preferred description of the sample.
Sample Type	Select a different sample type: • **Sample**–A forensic or reference sample • **ForenSeq Positive Amplification Control**–The control DNA (HL60 or NA24385) • **Negative Amplification Control**–The negative control (water) • **Reagent Blank**–The extraction control
Assay Type*	Select a different library prep method: • **ForenSeq mtDNA Whole Genome**–Libraries prepared with the ForenSeq mtDNA Whole Genome Kit. • **ForenSeq mtDNA Control Region**–Libraries prepared with the ForenSeq mtDNA Control Region Kit. • **mtDNA Custom**–Libraries prepared with a custom or third-party assay. • **ForenSeq Kintelligence**–Libraries prepared with a ForenSeq Kintelligence Kit.
Index 1*	Select the name of a different i7 index adapter.
Index 2*	Select the name of a different i5 index adapter.
Index Combination*	Select the name of a different premixed i7 and i5 index adapter.
Run Analysis Method*	Select a different run analysis method.
Initial Projects*	Enter additional projects or remove current projects. For detailed instructions, see *Modify Project Assignments*.
Sample Plate	Edit or enter a preferred name for the 96-well plate.
Sample Well Position	Edit or enter the position of the well the sample occupies.

* Editing these fields requires a run state of Created. To edit them after run completion, remove the sample from the run, make the necessary edits, and add the sample to the run again.

4. Select **Save** to apply the changes.

Modify Project Assignments

When the run state is Created, you can change which projects a sample is assigned to or assign the sample to additional projects.

1. On the navigation rail, select **Runs**, and then select a run.
2. In the Actions column, select **Edit Sample Details** for the applicable sample.
3. In the Edit Sample dialog box, edit the Initial Projects field as follows.
   - To remove a sample from a project, select **X** next to the project name.
   - To add a sample to a project, enter the project name.
   - To add a sample to multiple projects, enter multiple project names.
   - To create a project for the sample, enter a new project name.
4. Select **Save**.

Remove Samples from a Run

1. On the navigation rail, select **Runs**, and then select a run.
2. Select the checkbox of each sample you want to remove from the run.
3. Select **Remove Samples**.
4. In the Remove Samples dialog box, select **Confirm**.

Monitor a Run

During a run, monitor run status and performance from the Run Details page. See which cycle the run is on and review run quality. Green indicates passing metrics and orange indicates metrics that require further evaluation. These metrics are a subset of the run metrics used to evaluate performance after sequencing is complete.

1. On the navigation rail, select **Runs**, and then select a run.
2. Select **Quality Metrics**.
   - Review the values for cluster density and clusters passing filter.
   - Review the phasing and prephasing values for Read 1.
   - Check Read 1, Index 1, Index 2, and Read 2 to confirm that all four reads passed.
   - Check the Overall Intensity and 0 Discordant Loci icons to confirm that the HSC passed.

   For more information, see *Quality Metrics* on page 60.
3. Select **Close** to return to the run.
4. Select **Sample Representation**.
   - Compare the samples to the sample read count guideline to confirm performance.
   - Review the number of reads in parentheses next to the sample name or hover over each sample.

   For more information, see *Sample Representation* on page 64.
5. Select **Close** to return to the run.

Data Analysis

Review Run Metrics

Run metrics provide run quality, positive and negative control results, and sample representation for an overall view of run performance. Green indicates passing metrics and orange indicates metrics that require further evaluation.

For help with metrics requiring further evaluation, see *Troubleshoot a Run* on page 53. See *Run Metrics* on page 58 for detailed descriptions of each metrics.

1. On the navigation rail, select **Projects**, and then select a project.
2. Select **PNQ** for a run in the project.
3. Select **Positive Control**.
   - Review the positions called, discordance, and pass filter reads for each positive amplification control.
   - Use the scroll arrows to move through all positions in the Discordance table.
   - Select a sample name to go to the Results page.
4. Select **Negative Control**.
   - Review the number of positions called for each reagent blank and negative amplification control.
   - Select a sample name to go to the Results page.
5. Select **Quality Metrics**.
   - Review the values for cluster density and clusters passing filter.
   - Review the phasing and prephasing values for Read 1.
   - Check Read 1, Index 1, Index 2, and Read 2 to confirm that all four reads passed.
   - Check the Overall Intensity and 0 Discordant Loci icons to confirm that the HSC passed.
6. Select **Sample Representation**.
   - Compare the samples to the sample read count guideline to confirm performance.
   - Review the number of reads in parentheses next to the sample name or hover over each sample.
7. If any metrics do not pass, contact Verogen Technical Support. Collect the following items from D:\Illumina\MiSeqFGxOutput to help Verogen with troubleshooting:
   - InterOp folder
   - runInfo.xml file
   - runParameters.xml file

Review mtDNA Results

Each Results page for an mtDNA sample includes filters, fields, and buttons for a dynamic view of results for the sample. The following sections describe how to use these features to review the results. For feature descriptions, see *Results Page for mtDNA* on page 12.

Filter Results

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a sample.

   The Results page appears.
3. Select **Filter**, and then select at least one of the following checkboxes:
   - **Control Region**–Zooms in on the control region of the mtGenome.
   - **Interpretation Threshold**–Indicates positions with reads between the AT and IT.
   - **Variants**–Indicates positions with base calls that differ from the reference.
   - **Call**–For reagent blanks and negative amplification controls, indicates positions with no calls to identify areas without coverage.
   - **No call**–For samples and positive amplification controls, indicates positions with calls to identify possible contamination.

   The sample type determines whether Call or No Call filter is visible.
4. Reselect **Filter** to apply the selected filters.

   The mtDNA navigator, position viewer, and coverage plot refresh to reflect the filtered data.
5. To clear the filters:
   a. Select **Active Filter**.
   b. Deselect the checkboxes.
   c. Reselect **Active Filter**.

Set Maximum Coverage

Setting a maximum coverage filters sample data by total read count.

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a sample.
3. In the Max Coverage field, enter a read number without commas.

   The mtDNA navigator and coverage plot adjust to the specified value.

Compare Samples

You can compare and filter results among 2-9 mtDNA samples in a project. During a comparison, the mtDNA navigator and coverage plot show data for the sample of interest in gray and a selected comparison sample in blue. The position viewer displays sequences and data for the sample of interest and selected comparison sample. The name of the sample of interest appears blue.

1. Navigate to the Results page:
   a. On the navigation rail, select **Projects**.
   b. Select a project, and then select a sample to be the sample of interest.
2. On the Samples page, select a sample.
   This sample is the sample of interest.
3. On the Results page, select **Compare**.
4. In the Sample Compare dialog box, select the checkboxes of up to eight samples.
5. Select **Submit**.
   The position viewer lists the selected comparison samples. The mtDNA navigator and coverage plot refresh to show data for the sample of interest and the first selected comparison sample. A green checkmark next to a selected comparison sample indicates no differences with the sample of interest.
6. If you are comparing more than two samples, select a comparison sample in the position viewer.
   The mtDNA navigator and coverage plot refresh to compare the selected comparison sample to the sample of interest.
7. [Optional] Select **Filter**, and then select at least one of the following checkboxes:
   - **Control Region**–Zooms in on the control region of the mtGenome.
   - **Interpretation Threshold**–Indicates positions with reads between the AT and IT.
   - **Variants**–Indicates positions with base calls that differ from the reference.
   - **Call**–For reagent blanks and negative amplification controls, indicates positions with no calls to identify areas without coverage.
   - **No call**–For samples and positive amplification controls, indicates positions with calls to identify possible contamination.
   - **Differences**–Indicates call differences between the sample of interest and the selected comparison samples.

   The sample of interest determines whether the Call or No Call filter is displayed. For example, when the sample of interest is a negative amplification control or reagent blank, the software displays the Call filter. When the sample of interest is a sample or positive amplification control, the software displays the No Call filter.
8. When you are done comparing samples, select **Stop Compare**.

Edit Base Calls

1. On the navigation rail, select **Projects**.
2. Select a project, and then select an mtDNA sample.

3. In the position viewer, select a position by using the scroll arrows or entering the position.
4. Select the **Call** arrow, and then select a call. Scroll to see all available calls.

   The position viewer refreshes to reflect the modified call. An asterisk (*) marks the modified call and the original call appears below the Call list.

Review Kinship Results

Each Results page for a kinship sample includes filters and sortable categories to organize and locate loci for review. The following sections describe how to use these features to review results.

- For information on the Results page, see *Results Page for Kinship* on page 15.
- For information on how the software estimates gender and contributor status, see *Supporting Information* on page 73.

By default, the Results page displays results with the Typed filter and all SNP Type filters applied except X-SNP and Y-SNP. Sort 1 is set to descending Allele Count, Sort 2 is set to ascending Intralocus Balance, and Sort 3 is set to ascending Intensity.

Filter Loci

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a sample.

   The Results page appears.
3. Under Sample Details, select any of the following filters to view only the desired loci:

Filter	Effect
Chromosome	Display loci located on the selected chromosome only.
Typed	Display only loci that are typed.
Untyped	Display only loci that are untyped.
Homozygous	Display only loci that have the same allele.
Heterozygous	Display only loci that have different alleles.
QC Indicators	Displays only loci flagged with the selected QC indicators.
SNP Type	Displays only loci that belong to the selected SNP types.

4. To remove filters, select the following options:
   - **Clear Selection**–Clear all filters in a section.
   - **Default View**–Clear selected filters and restore the default filters.

Sort Loci

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a sample.

   The Results page appears.

3. Under Sample Details, select the Sort 1, Sort 2, or Sort 3 list, and then select one of the following options:

Sort Option	Effect
None	Do not apply any sorting.
Intensity	Sort loci by intensity value.
ILB	Sort loci by intralocus balance percentage.
Amplicon Size	Sort loci by amplicon size in bp.
Chromosome	Sort loci by chromosome number or letter.
SNP Type	Sort loci by SNP category.
SNP Name	Sort loci by rsID.
Allele Count	Sort loci by the number of alleles.

4. To further sort loci into ascending or descending order, select the arrow next to each sort list.
5. To clear your sorting selections and restore the defaults, select **Default View**.

Find a Locus

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a sample.
   The Results page appears.
3. In the Search field, enter the whole or partial name of the locus you want to find, and then press **Enter**.
   The Results page displays loci matching the search term.

Modify Kinship Results

Modify the results of an allele call for a kinship sample, type or untype alleles, and change the sample gender and contributor status.

A blue toggle key and green histogram bar indicate a typed allele. A gray toggle key and bar indicate an untyped allele.

Type or Untype Alleles

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a kinship sample.
3. On the Results page, under Sample Details, select a locus.
   The locus details appear.
4. Type or untype an allele using one of the following methods:
   - Under Typed, select a toggle key.
   - On the histogram, select a bar.

   The User Modified icon appears, indicating the modification. Reverting the call removes the icon.

Change the Contributor Status

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a kinship sample.
   The Results page appears.
3. In the Sample Overview section, select the Contributor Status list, and then select a status:
   - **Single-Source**–Change the sample source to one contributor.
   - **Mixture**–Change the sample is from multiple contributors.
   - **Inconclusive**–Indicate that whether the sample is from one contributor or multiple contributors is unknown.

Change the Sample Gender

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a kinship sample.
   The Results page appears
3. In the Sample Overview section, select the Gender list, and then select the applicable chromosomes:
   - **XX**–Change the sample to male.
   - **XY**–Change the sample to female.
   - **Inconclusive**–Indicate that the sample gender is undetermined.

Add Analyzed Samples to a Project

You can add analyzed samples in a project to other projects. After adding a sample to another project, sample modifications are ***not*** applied in the newly assigned project.

1. On the navigation rail, select **Projects**, and then select a project.
2. On the Samples page, select **Add Samples**.
3. In the Add Samples dialog box, in the **Search** field, enter a sample name.
4. In the search results, select the checkbox of each sample you want to add to the project.
   Each selection appears in a summary list with the corresponding analysis method.
5. To remove a sample, deselect the checkbox or select **X** in the summary.
6. Select **Save** to add the samples.

Reanalyze Samples

Reanalyze samples to apply an analysis method with user-defined parameters. The software preserves results from all analysis methods.

1. On the navigation rail, select **Projects**, and then select a project.
2. On the Samples page, select the checkbox of each sample you want to reanalyze, and then select **Reanalyze**.

3. In the Reanalyze Samples dialog box, choose one of the following options:

Reanalysis Option	Instruction
Assign the same analysis method to all samples selected for reanalysis.	Select a method from the Analysis Methods list, and then select **Apply to All**.
Assign an analysis method to each sample selected for reanalysis.	Select a method from each list in the New Analysis Method column.

   The software only lists analysis methods that are compatible with the assay used to prepare the sample.
4. Select **Next**.
5. Review the sample names, previous analysis methods, and new analysis methods.
6. To make changes, select **Previous**.
7. Select **Save** to initiate reanalysis.

   In the Projects sidebar, an icon appears next to each sample undergoing reanalysis.
8. When reanalysis is complete, select a sample to view results.

   The Results page displays results for the most recent analysis.

Switch Analysis Methods

A Results page shows results for the analysis method displayed in the Select an Analysis Method list. For reanalyzed samples, the list includes all analysis methods applied to the sample with the most recent first. Select different analysis methods to see other results.

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a sample.

   The Results page appears.
3. In the Select an Analysis Method list, select an analysis method.

   The Results page is refreshed to show results for the selected method.

Report Generation

Generating mtDNA Reports

The software compiles mtDNA results into the following reports:

- **CODIS**–Variants for the selected samples in a format compatible with CODIS.
- **EMPOP**–Specimen data, originating agency identifiers (ORIs), and other DNA profile information in a format compatible with EMPOP.
- **Project**–Results for all samples in a project compiled in one report.
- **Sample**–Results for one sample compiled in a report.

The following sections describe how to generate each type of report. For report contents, see *File Formats for mtDNA Reports* on page 67.

Create a CODIS Report

Create a CODIS report for one sample or multiple samples. Creating a report for one sample lets you choose which positions to include in the report. When creating a report for multiple samples, the software automatically includes all positions for each sample.

Create a CODIS Report for One Sample

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a sample or select **Reports**.
3. Select **Create Report**, and then select **CODIS**.
4. Edit the following CMF header fields or accept the default values.
   - **Destination Laboratory ORI**–Identifier for the agency that processes the CODIS report.
   - **Source Laboratory ORI**–Identifier for the agency that processes the specimen.
   - **Submission User ID**–Identification of the person who generates the CODIS report.
5. Select **Next**.
6. In the Specimen ID field, enter a unique identifier for the specimen or accept the default sample name.
7. In the Specimen Category list, select a preconfigured category or select **Custom** and enter the category name.
8. [Optional] Select **More Options**, and then complete the following fields:
   - **Source ID**–Specify whether the identity of the specimen contributor is known.
   - **NCIC Number**–Enter the unique number for storage in the NCIC system.
   - **ViCAP number**–Enter the unique number for storage in the ViCAP system.
   - **Specimen Comment**–Enter any additional information about the specimen.
9. Select **Next**.

10. Select the toggle key for each position you want to remove from the report.
    By default, all positions are included.
11. Select **Create** to generate the report.
12. Select **Reports** to open the Reports page.
    The Reports page lists all reports for the project with the new report on top.
13. In the Actions column, select **Download Report** to download the report as a .cmf file.

Create a CODIS Report for Multiple Samples

1. On the navigation rail, select **Projects**.
2. Select a project, and then select **Reports**.
3. Select **Create Report**, and then select **CODIS**.
4. In the Create CODIS Report dialog box, select the checkbox of each sample you want to include in the report.
5. Select **Next**.
6. Edit the following CMF header fields or accept the default values:
   - **Destination Laboratory ORI**–Identifier for the agency that processes the CODIS report.
   - **Source Laboratory ORI**–Identifier for the agency that processes the specimen.
   - **Submission User ID**–Identification of the person who generates the CODIS report.
7. Select **Next**.
8. In the Specimen ID field for each sample, enter a unique identifier for the specimen cited in the report.
9. Assign specimen categories per the following table. If you select Custom, enter a name for the custom specimen category.

Option	Instruction
Assign the same category to all samples.	In the Specimen Category list at the top of the dialog box, select a category, and then select **Apply to All Specimens**.
Assign a different category to each sample.	In the Specimen Category list for each sample, select the applicable category.

10. [Optional] Select **More Options**, and then complete the following fields:
    - **Source ID**–Specify whether the identity of the specimen contributor is known.
    - **NCIC Number**–Enter the unique number for storage in the NCIC system.
    - **ViCAP number**–Enter the unique number for storage in the ViCAP system.
    - **Specimen Comment**–Enter any additional information about the specimen.
11. Select **Create** to generate the report.
    The software returns to the Reports page, where the new report appears at the top of the list.
12. In the Actions column, select **Download Report** to download the report as a .cmf file.

Create an EMPOP Report

Create an EMPOP report for one or more samples in a project. When creating a report for one sample, you can start from the Results or Reports page.

Create an EMPOP Report for One Sample from Results

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a sample.
   The Results page appears.
3. Select **Create Report**, and then select **EMPOP** to generate the report.
4. Select **Reports** to open the Reports page.
   The Reports page lists the new report on top.
5. In the Actions column, select **Download** to download the report as a .txt file.

Create an EMPOP Report for One Sample from Reports

1. On the navigation rail, select **Projects**.
2. Select a project, and then select **Reports**.
   The Reports page appears.
3. Select **Create Report**, and then select **EMPOP**.
4. In the Create EMPOP Report dialog box, select the checkbox of the applicable sample.
5. Select **Confirm** to generate the report.
   The returns to the Reports page, where the new report appears at the top of the list.
6. In the Actions column, select **Download Report** to download the report as a .txt file.

Create an EMPOP Report for Multiple Samples

1. On the navigation rail, select **Projects**.
2. Select a project, and then select **Reports**.
   The Reports page appears.
3. Select **Create Report**, and then select **EMPOP**.
4. In the Create EMPOP Report dialog box, select the checkbox of each sample to include in the report.
5. Select **Confirm** to generate the report.
   The returns to the Reports page, where the new report appears at the top of the list.
6. In the Actions column, select **Download Report** to download the report as a .txt file.

Create a Project Report

1. On the navigation rail, select **Projects**.
2. Select a project, and then select **Reports**.

3. On the Reports page, select **Create Report**, and then select **Project**.

   If the software is analyzing samples, project report creation is disabled and a notification is displayed.
4. In the dialog box, select **Yes** to generate the report.

   The software generates the report and returns to the Reports page, where the new report appears at the top of the list. If a project includes samples with different assay types, the software generates one project report per type.
5. In the Actions column, select **Download** to download a report as an .xmlx file.

Create Sample Reports

Create one sample report at a time or simultaneously generate multiple sample reports, one for each selected sample in a project. When creating a report for one sample, you can start from the Results or Reports page.

Create One Sample Report from Results

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a sample.

   The Results page appears.
3. Select **Create Report**, and then select **Sample** to generate the report.
4. Select **Reports** to open the Reports page.

   The Reports page lists the new report on top.
5. In the Actions column, select **Download** to download the report as an .xmlx file.

Create One Sample Report from Reports

1. On the navigation rail, select **Projects**.
2. Select a project, and then select **Reports**.

   The Reports page appears.
3. Select **Create Report**, and then select **Sample**.
4. In the Create Sample Report dialog box, select the checkbox of the sample you want to create a report for. Scroll and use the arrows to see all samples.
5. Select **Confirm** to generate the report.

   The software returns to the Reports page, where the new report appears at the top of the list.
6. In the Actions column, select **Download** to download a report as an .xmlx file.

Create Multiple Sample Reports

1. On the navigation rail, select **Projects**.
2. Select a project, and then select **Reports**.

   The Reports page appears.
3. Select **Create Report**, and then select **Sample**.

4. In the Create Sample Report dialog box, select the checkbox of each sample you want to create a report for.
   - Scroll and use the arrows to move through the complete list of samples in the project.
   - Use the Delete icons under Summary to remove samples from the report.
5. Select **Confirm** to generate the reports.
   The software returns to the Reports page, where the new reports appear at the top of the list.
6. In the Actions column, select **Download** to download a report as an .xmlx file.

Generating Kinship Reports

The software compiles kinship results and other information into the following reports:

- **Phenotype and Ancestry**–Estimates of hair color, eye color, and biogeographical ancestry based on aSNP and pSNP data.
- **GEDmatch PRO**–SNP genotype calls reported on the plus strand in a format compatible with GEDmatch PRO.
- **Project**–Results for all samples in a project compiled in one report.
- **Sample**–Results for one sample compiled in a report.

The following sections describe how to generate each type of report. For report contents, see *File Formats for Kinship Reports* on page 69.

Create a Phenotype and Ancestry Report

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a sample.
   The Results page appears.
3. Select **Create Report**, and then select **Phenotype & Ancestry** to generate the report.
4. Select **Reports** to open the Reports page.
   The Reports page lists the new report on top.
5. In the Actions column, select **Download** to download the report as an .xmlx file.

Create a GEDmatch PRO Report

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a sample.
   The Results page appears.
3. Select **Create Report**, and then select **GEDmatch PRO** to generate the report.
4. Select **Reports** to open the Reports page.
   The Reports page lists the new report on top.
5. In the Actions column, select **Download** to download the report as a .txt file.

Create a Project Report

1. On the navigation rail, select **Projects**.

2. Select a project, and then select **Reports**.
3. On the Reports page, select **Create Report**, and then select **Project**.

   If the software is analyzing samples, project report creation is disabled and a notification is displayed.
4. In the dialog box, select **Yes** to generate the report.

   The software generates the report and returns to the Reports page, where the new report appears at the top of the list. If a project includes samples with different assay types, the software generates one project report per type.
5. In the Actions column, select **Download** to download a report as an .xmlx file.

Create Sample Reports

Create one sample report at a time or simultaneously generate multiple sample reports, one for each selected sample in a project.

Create One Sample Report

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a sample.

   The Results page appears.
3. Select **Create Report**, and then select **Sample** to generate the report.
4. Select **Reports** to open the Reports page.

   The Reports page lists the new report on top.
5. In the Actions column, select **Download** to download the report as an .xmlx file.

Create Multiple Sample Reports

1. On the navigation rail, select **Projects**.
2. Select a project, and then select **Reports**.

   The Reports page appears.
3. Select **Create Report**, and then select **Sample**.
4. In the Create Sample Report dialog box, select the checkbox of each sample you want to create a report for.
   - Scroll and use the arrows to move through the complete list of samples in the project.
   - Use the Delete icons under Summary to remove samples from the report.
5. Select **Confirm** to generate the reports.

   The software returns to the Reports page, where the new reports appear at the top of the list.
6. In the Actions column, select **Download** to download a report as an .xmlx file.

Run, Project, and Sample Management

Create a Project

Create a project to group a selection of samples. For example: create a project of controls to compare controls from multiple projects, or group samples in a comparison to generate a project report of compared samples.

Although possible, combining mtDNA and kinship samples in a project is not recommended.

1. On the navigation rail, select **Projects**.
2. Select **Create Project**.
3. In the Create Project dialog box, complete the following fields:
   - **Project Name**–Enter a preferred name to identify the project.
   - **Description**–Optionally enter a description of the project.
4. Select **Save** to create the project.
   The new project opens to the Samples page.
5. Select **Add Samples**.
   You can add any samples in the software to the project, analyzed or not.
6. In the Add Samples dialog box, in the **Search** field, enter a sample name.
7. In the search results, select the checkbox of each sample you want to add to the project.
   Each selection appears in a summary list with the corresponding analysis method.
8. To remove a sample, deselect the checkbox or select **X** in the summary.
9. Select **Save** to add the samples.
   The Samples page lists the added samples. Runs the samples belong to appear in the Project sidebar.

Edit a Project

1. On the navigation rail, select **Projects**, and then select a project.
2. Select **Edit**.
3. In the Edit Project dialog box, do as follows.
   - Edit the project name.
   - Add or modify the optional project description.
4. Select **Save** to apply the changes.

Obtain the Analysis Folder Path

The analysis folder resides on the ForenSeq Universal Analysis Server and contains the Variant Call Format (VCF) and Binary Alignment Map (BAM) files for the analysis.

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a sample.
   The Results page appears.
3. Under Other Options, select **Analysis Folder Path** (Information icon).
   The path appears in a pop-up window.
4. When you are finished reviewing the path, select **Close**.

Printing

The Print icon converts the following pages into a printable format:

- **Run Details**–Print the header of a Run Details page, the table listing samples in the run, run quality metrics, and sample representation.
- **Samples**–Print the Samples page of a project.
- **Results**–Applicable to kinship samples only, print the header of a Results page and the Sample Overview section.

Print a Run Details Page

1. On the navigation rail, select **Runs**, and then select a run.
2. On the Run Details page, select **Print**.
   The software displays a printable view of the Run Details page.
3. Select **Print** to open the Print dialog box.
4. Choose printing options and print.
5. Select **Back to Run**.

Print a Samples Page

1. On the navigation rail, select **Projects**, and then select a project.
2. On the Project sidebar, select **Print**.
   The software displays a printable view of the Samples page.
3. Select **Print** to open the Print dialog box.
4. Choose printing options and print.
5. Select **Back to Project**.

Print a Sample Overview Section

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a kinship sample.

3. On the Results page, under Other Options, select **Print Sample Results**.
   The software displays a printable view of the Sample Overview section.
4. Select **Print** to open the Print dialog box.
5. Choose printing options and print.
6. Select **Back to Project**.

History

When the history setting is enabled, you can review activities recoded for a run, project, or sample and add comments. For example, you can comment on a sample to explain a call modification. For instructions on enabling the history setting, see *System Settings* on page 51.

The activities recorded for a run, project, or sample include system events and user actions:

- A system event is an action the software automatically performs and records.
- A user action records a user interaction with the software, such as adding a sample to a run.

Review Run Activity

1. On the navigation rail, select **Runs**.
2. Select a run, and then select **Activity** at the top of the page.
3. In the Run Activity dialog box, review system events and user actions for the run.
   The dialog box lists system events and user actions from oldest to newest.
4. Navigate using the scroll bar and arrows. Narrow results using the following fields:
   - **Search**–Search the current history.
   - **Start Date and End Date**–View the history within a specified time frame.
   - **Show System Events**–See system events and user actions for the history or see user actions only.
5. When you are finished, select **Close**.

Review Project Activity

1. On the navigation rail, select **Projects**.
2. Select a project, and then select **Project Activity** at the top of the page.
3. In the Project Activity dialog box, review system events and user actions for the project.
   The dialog box lists system events and user actions from oldest to newest.
4. Navigate using the scroll bar and arrows. Narrow results using the following fields:
   - **Search**–Search the current history.
   - **Start Date and End Date**–View the history within a specified time frame.
   - **Show System Events**–See system events and user actions for the history or see user actions only.
5. When you are finished, select **Close**.

Review Sample Activity

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a sample.
3. Select **Sample Activity**.
4. In the Sample Activity dialog box, review system events and user actions for the sample.
   The dialog box lists system events and user actions from oldest to newest.
5. Navigate using the scroll bar and arrows. Narrow results using the following fields:
   - **Search**–Search the current history.
   - **Start Date and End Date**–View the history within a specified time frame.
   - **Show System Events**–See system events and user actions for the history or see user actions only.
6. When you are finished, select **Close**.

Comment on a Run

1. On the navigation rail, select **Runs**.
2. Select a run, and then select **Activity** at the top of the page.
   The Run Activity dialog box appears.
3. To add a comment to the run history:
   a. In the Add New Comment field, type a comment.
   b. Select **Add** to save the comment.

   The comment appears as an event at the top of the history.
4. To comment on an entry in the run history:
   a. Select the system event or user action. Use the fields and scrolling to help locate the entry, if necessary.
   b. In the Edit Comment box, type a comment.
   c. Select **Save** to add the comment to the system event or user action.

   The comment appears as the last item for the entry.
5. When you are finished, select **Close**.

Comment on a Project

1. On the navigation rail, select **Projects**.
2. Select a project, and then select **Project Activity** at the top of the page.
   The Project Activity dialog box appears.
3. To add a comment to the project history:
   a. In the Add New Comment field, type a comment.
   b. Select **Add** to save the comment.

The comment appears as an event at the top of the history.

4. To comment on an entry in the project history:
   a. Select the system event or user action. Use the fields and scrolling to help locate the entry, if necessary.
   b. In the Edit Comment box, type a comment.
   c. Select **Save** to add the comment to the system event or user action.

   The comment appears as the last item for the entry.
5. When you are finished, select **Close**.

Comment on a Sample

1. On the navigation rail, select **Projects**.
2. Select a project, and then select a sample.
3. Select **Sample Activity**.
   The Sample Activity dialog box appears.
4. To add a comment to the sample history:
   a. In the Add New Comment field, type a comment.
   b. Select **Add** to save the comment.

   The comment appears as an event at the top of the history.
5. To comment on an entry in the sample history:
   a. Select the system event or user action. Use the fields and scrolling to help locate the entry, if necessary.
   b. In the Edit Comment box, type a comment.
   c. Select **Save** to add the comment to the system event or user action.

   The comment appears as the last item for the entry.
6. When you are finished, select **Close**.

Software Settings

Settings Overview

Use the following settings, which are available from the Settings icon on the navigation rail, to configure the software. All settings except Dark Mode are visible to administrators only.

- **Analysis Methods**–Create and manage analysis methods.
- **CODIS Report Defaults**–Set default values to prepopulate CODIS reports.
- **User Management**–Add users and assign permissions.
- **System Settings**–Set visibility into system events and user actions.
- **Dark Mode**–Change the color scheme of the user interface.

Analysis Methods

An analysis method is a collection of settings that inform how the software analyzes sequencing data and performs variant and allele calling. The software ships with a default Verogen analysis method for each of the following assays.

Assay Type	Default Analysis Method
ForenSeq Kintelligence	Verogen Kintelligence Analysis Method
ForenSeq mtDNA Whole Genome	Verogen mtDNA Whole Genome Analysis Method
ForenSeq mtDNA Control Region	Verogen mtDNA Control Region Analysis Method
mtDNA Custom	Verogen mtDNA Custom Analysis Method

Create an Analysis Method

The default Verogen analysis methods cannot be modified, but you can create analysis methods with the desired settings using one of two methods: copy an existing analysis method and modify the settings or start with a blank analysis method.

Performance is supported when using the default analysis methods. Perform internal validation studies as needed to develop settings for new analysis methods.

For information on how Verogen determined the default settings in the mtDNA analysis methods, see the *Analysis Settings for ForenSeq Universal Analysis Software v2.0 Technical Note (document # VD2020045)*. For the Verogen Kintelligence Analysis Method, see the *SNP Typing in Universal Analysis Software and Kinship Estimation with GEDmatch PRO Technical Note (document # VD2020058)*.

Copy an Analysis Method

1. On the navigation rail, select **Settings**.
2. Select **Analysis Methods**.
3. In the Select an Assay Type list, select an assay:
   - **ForenSeq mtDNA Whole Genome**–Create an analysis method for libraries prepared with the ForenSeq mtDNA Whole Genome Kit.
   - **ForenSeq mtDNA Control Region**–Create an analysis method for libraries prepared with the ForenSeq mtDNA Control Region Kit.
   - **mtDNA Custom**–Create an analysis method for mtDNA libraries prepared with home-brew or third-party assays.
   - **ForenSeq Kintelligence**–Create an analysis method for libraries prepared with the ForenSeq Kintelligence Kit.
4. Select **Create Analysis Method**.

   The New Analysis Method dialog box appears.
5. In the New Analysis Method Options list, select **Copy an Existing Analysis Method**.
6. In the Source Analysis Method list, select an analysis method to use as a template.

   The list displays methods that correspond to the selected assay type only.
7. In the New Analysis Method Name field, enter a unique name for the new analysis method.
8. Select **Save** to create the analysis method.

   The new method appears with the default settings from the copied method.
9. Modify the default settings as desired.

   For instructions, see *Modify Analysis Settings* on page 47.

Use a Blank Analysis Method

1. On the navigation rail, select **Settings**.
2. Select **Analysis Methods**.
3. In the Select an Assay Type list, select an assay:
   - **ForenSeq mtDNA Whole Genome**–Create an analysis method for libraries prepared with the ForenSeq mtDNA Whole Genome Kit.
   - **ForenSeq mtDNA Control Region**–Create an analysis method for libraries prepared with the ForenSeq mtDNA Control Region Kit.
   - **mtDNA Custom**–Create an analysis method for mtDNA libraries prepared with home-brew or third-party assays.
   - **ForenSeq Kintelligence**–Create an analysis method for libraries prepared with the ForenSeq Kintelligence Kit.
4. Select **Create Analysis Method**.

   The New Analysis Method dialog box appears.

5. In the New Analysis Method Options list, select **New Blank Analysis Method**.
6. In the New Analysis Method Name field, enter a unique name for the new analysis method.
7. Select **Save** to create the analysis method.
   The new method appears with blank settings.
8. Enter the desired settings for the analysis method.
   For instructions, see *Modify Analysis Settings*.

Modify Analysis Settings

You can modify the following analysis settings, depending on the analysis method:

- For a user-created mtDNA analysis method, you can edit the AT, IT, minimum Q-score, and minimum read count. If an analysis method is copied from the Verogen mtDNA Custom Analysis Method, you can also modify the library type and amplicons.
- For a user-created kinship analysis method, you can edit the AT, IT, and intralocus balance and specify which loci to include in the analysis.

Edit an mtDNA Whole Genome or Control Region Analysis Method

1. If necessary, navigate to the analysis method you want to edit:
   a. On the navigation rail, select **Settings**.
   b. Select **Analysis Methods**.
   c. In the Select an Assay Type list, select the assay that corresponds to the applicable analysis method.
   d. Select the applicable analysis method.
2. To rename the analysis method, do as follows.
   a. Select **Edit** to open the Edit Analysis Info dialog box.
   b. In the Analysis Method Name field, enter a new name.
   c. Select **Save** to apply the name change and close the dialog box.
3. Edit the following settings as desired:
   - In the Analytical Threshold and Interpretation Threshold boxes, adjust percentages.
   - In the Minimum Quality Score and Minimum Read Count boxes, adjust values.

   All percentages and values are greater than or equal to the value entered.
4. Select **Save** to apply the new settings.

Edit an mtDNA Custom Analysis Method

1. If necessary, navigate to the analysis method you want to edit:
   a. On the navigation rail, select **Settings**.
   b. Select **Analysis Methods**.
   c. In the Select an Assay Type list, select the assay that corresponds to the applicable analysis method.

d. Select the applicable analysis method.

2. To rename the analysis method, do as follows.
   a. Select **Edit** to open the Edit Analysis Info dialog box.
   b. In the Analysis Method Name field, enter a new name.
   c. Select **Save** to apply the name change and close the dialog box.
3. Edit the following settings as desired:
   - In the Analytical Threshold and Interpretation Threshold boxes, adjust percentages.
   - In the Minimum Quality Score and Minimum Read Count boxes, adjust values.

   All percentages and values are greater than or equal to the value entered.
4. In the Library Type list, select one of the following options:
   - **Non-Directed Sequencing**–Either strand can be sequenced as Read 1 or Read 2. This option is the default.
   - **Primer-Directed Sequencing**–One strand is sequenced as Read 1 and the opposite strand as Read 2.

   The software lists amplicons available for the selected library type.
5. Modify the amplicons:
   - To remove an amplicon, in the Actions column, select **Delete**.
   - To edit an amplicon, in the Actions column, select **Edit**. In the Edit Amplicon dialog box, edit the desired fields, and then select **Save**.
   - To add an amplicon, select **Add Amplicon**. In the Add Amplicon dialog box, complete the necessary fields, and then select **Confirm**.

   The user interface displays required fields with an asterisk (*).
6. Select **Save** to apply the new settings.

Edit a Kinship Analysis Method

1. If necessary, navigate to the analysis method you want to edit:
   a. On the navigation rail, select **Settings**.
   b. Select **Analysis Methods**.
   c. In the Select an Assay Type list, select the assay that corresponds to the applicable analysis method.
   d. Select the applicable analysis method.
2. To rename the analysis method, do as follows.
   a. Select **Edit** to open the Edit Analysis Info dialog box.
   b. In the Analysis Method Name field, enter a new name.
   c. Select **Save** to apply the name change and close the dialog box.
3. Select the checkbox of at least one locus to edit.
   - Enter a whole or partial locus name in the Search field to find a locus.
   - Select the checkbox of applicable SNP types to filter the list of loci.

- Select any column heading to sort the column.
- Scroll to see all loci.

The software applies any new settings to all selected loci. If you want to apply different settings to different loci, select on locus at a time.

4. Select **Edit Selected Loci**.

   The Edit Analysis Method dialog box appears.
5. To add or remove the loci from the analysis, select **Include**.

   Blue indicates that the loci are included. Gray indicates exclusion.
6. To modify the analysis thresholds, enter a value **0-100** in the applicable fields: Analytical Threshold, Interpretation Threshold, and Intralocus Balance.

   The IT value must be greater than or equal to the AT value.
7. Select **Save** to apply the new settings.

Delete an Analysis Method

You can delete any user-created analysis method. However, the default Verogen analysis methods cannot be deleted.

1. On the navigation rail, select **Settings**.
2. Select **Analysis Methods**.
3. In the Select an Assay Type list, select the assay that corresponds to the analysis method you want to delete.
4. Select the applicable analysis method.
5. In the upper-right corner of the analysis method, select **More**, and then select **Delete Analysis Method**.s
6. Select **OK** to confirm the deletion.

Change the Default Analysis Method

1. On the navigation rail, select **Settings**.
2. Select **Analysis Methods**.
3. In the Select an Assay Type list, select the kit that corresponds to the analysis method you want to set as the default.
4. Select the applicable analysis method.

   A black star identifies the current default.
5. Set the selected analysis method as the default for the assay type using one of the following methods:
   - Select the empty star next to the analysis method name.
   - In the upper-right corner of the analysis method, select **More**, and then select **Set As Default Analysis Method**.

CODIS Report Defaults

To expedite report creation, define default values to appear in the CMF header of every CODIS report. You can override these values on a per-report basis.

Define CODIS Report Defaults

1. On the navigation rail, select **Settings**.
2. Select **CODIS Report Defaults**.
3. In each of the following fields, enter a default value:
   - **Destination Laboratory ORI**–Identifier for the agency that processes the CODIS report.
   - **Source Laboratory ORI**–Identifier for the agency that processes the specimen.
   - **Submission User ID**–Identification of the person who generates the CODIS report.
4. Select **Save** to apply the default values.

Set Specimen Categories

The Specimen Categories setting determines which specimen categories are available to assign to samples when creating a CODIS report. By default, all categories are available.

1. On the navigation rail, select **Settings**.
2. Select **CODIS Report Defaults**.
3. Select the Specimen Categories tab.

   This tab lists all specimen categories, divided into CODIS and Custom columns.
4. Select a specimen category to make it available or unavailable for reports.

   A blue toggle key indicates an available category.

Add Custom Specimen Categories

Add custom specimen categories to the software to supplement the predefined categories. Custom categories can be renamed, enabled or disabled, and deleted.

1. On the navigation rail, select **Settings**.
2. Select **CODIS Report Defaults**.
3. Select the **Specimen Categories** tab.
4. In the New Specimen Category Name field, enter a name of your choice.
5. Select **Add**.

   The new category appears in the Custom column and is enabled by default.

User Management

The Admin Settings page lists each account by user name. On this page, an administrator can manage permissions for each account, including granting access to new accounts and assigning access levels.

Enable and Disable Accounts

New accounts are disabled by default and must be enabled by an administrator.

1. Select **Settings**.

2. Select **User Management**.
3. Select **Enabled** to enable or disable an account.

   Blue indicates an enabled account.

Assign Access Levels

1. Select **Settings**.
2. Select **User Management**.
3. Select **Administrator** to enable or disable administrator access for an account.

   Blue identifies an administrator account.

System Settings

The System Settings page hosts the history setting. When enabled, this setting lets you view system events and user actions for runs, projects, and samples and includes these activities in project and sample reports.

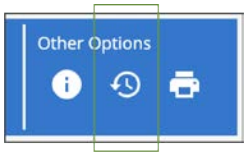
By default, the history setting is enabled. However, the software ***always records activity***, regardless of whether the setting is enabled. When enabling the setting, you can choose whether to store or delete activities that occurred since the last disabling.

Enable History

1. On the navigation rail, select **Settings**.
2. Select **System Settings**.
3. Select **Enable or disable visibility of system events and user actions recorded by the software**.
4. In the dialog box, select whether to store or delete previous system events and user actions:
   - **Permanently Delete All Previous History**–Start recording and delete all previous activity. You can access only activities recorded from this point forward.
   - **Store Previous History**–Continue recording and grant access to all activity starting from the creation of a run, project, or sample.

   An Activity icon appears on the Run Details page, Project sidebar, and Results page. Selecting the icon opens the history.

   Figure 6 Activity icon on a Results page



Disable History

1. On the navigation rail, select **Settings**.
2. Select **System Settings**.

3. Select **Enable or disable visibility of system events and user actions recorded by the software**.
   The History icon disappears from the Run Details page, Project sidebar, and Results page.

Enable or Disable Dark Mode

1. Select **Settings**.
2. Select **Dark Mode** to darken or brighten the user interface.

Troubleshooting

Troubleshoot a Run

The following sections provide recommendations for troubleshooting run problems. Most recommendations include library prep steps, which are documented in the library prep kit reference guides. Visit verogen.com/documentation to download the guide for your kit.

Low Intensity

Low intensity (number of reads) can indicate a problem with sample quantity or quality.

- Make sure that DNA concentrations are not below the targeted amount.
- Depending on the source, purify or dilute the DNA and resequence the sample.
- Resequence with fewer samples, maintaining a minimum of eight samples per run.

High Cluster Density

Review run metrics: If cluster density is high, but sample representation shows that reads are above the sample read count guideline, proceed with analysis. The following factors can result in insufficient data for analysis.

- During normalization, library from the Purified Library Plate or the Normalization Storage (LNB1) were overpipetted.
  - If the sample has a high number of alleles with quality control indicators, be sure to use correct volumes and reagents for normalization and resequence the sample.
  - If the sample volume is correct, further dilute libraries in the Pooled Normalized Libraries tube.
  - If normalization volumes or reagents were incorrect, repeat normalization and resequence.
- Too much adapter dimer in library prep.
  - If your laboratory has a Bioanalyzer or Fragment Analyzer, analyze 1-2 µl library from the Purified Library Plate to check the amount of primer dimer.
    - If the checked library has > 5% adapter dimer, repeat library purification using the 30 µl library remaining in each well of the Purified Library Plate. Adjust purification to use 30 µl Sample Purification Beads 2 (SPB2), add 32.5 µl Resuspension Buffer (RSB), and transfer 30 µl to a new 96-well PCR plate. Proceed with normalization.
    - For questions, contact Verogen Technical Support.
  - [Optional] If SPB2 is limited, purify libraries in the Purified Library Plate using a standard column cleanup method. Follow the manufacturer instructions.
- An insufficient volume of Hybridization Buffer (HT1) was added, or too much normalized library was added to HT1.
  - If the sample has a high number of alleles with quality control indicators, resequence the sample. Make sure the normalized library is sufficiently diluted.

Low Cluster Density

Review run metrics: If cluster density for the run is high, but sample representation shows that reads are above the sample read count guideline, proceed with analysis. If the sample has an insufficient number of reads due to low density, the problem might be due to the following factors.

- DNA input was too low or too degraded, or the DNA dilution was incorrect. If possible, repeat library prep with more DNA.
- A critical reagent was not added during library prep. Repeat the prep.
- LNB1 was insufficiently pipetted during normalization. Consider diluting libraries in the Pooled Normalized Libraries tube less.
- HT1 was overpipetted during library prep. Repeat sequencing using the correct volume.
- Excessive sample was lost during purification. Repeat the library prep using best practices for bead handling.
- After heating, the bead-based normalized library pool was not immediately added to HT1. If possible, repeat the prep.
- The library was not otherwise denatured properly.
  - Make sure that the final library pool is denatured with 2 N NaOH (HP3) that has a pH > 12.5.
  - Make sure that the final library pool is heat-denatured, if applicable.
- The MiSeq FGx System has a blockage.
  - Perform a maintenance wash and repeat sequencing.
  - Refill the wash tray and bottle for every wash.
  - Make all wash solutions daily.

Low Percentage of Clusters Passing Filter

If the percentage of clusters passing filter is too low, the problem might be due to the following factors.

- The cluster density is too high. See *High Cluster Density* on page 53.
- The MiSeq FGx System has a blockage.
  - Perform a maintenance wash and repeat sequencing.
  - Refill the wash tray and bottle for every wash.
  - Make all wash solutions daily.
- If the problem remains unresolved, reagents might not be performing as expected. Contact Verogen Technical Support.

High Phasing and Prephasing

The following factors can cause high phasing or prephasing.

- The cluster density is too high. See *High Cluster Density* on page 53.
- The ambient temperature is too high.
  - Verify the appropriate temperature in the *MiSeq FGx Sequencing System Site Prep Guide (document # VD2018012)*.

  - Check the MiSeq FGx System temperature logs to confirm whether the ambient temperature is too high. Contact Verogen Technical Support for help locating the logs.
- The reagent chiller temperature is too low or too high. The system displays an error.
- The MiSeq FGx System has a blockage.
  - Perform a maintenance wash and repeat sequencing.
  - Refill the wash tray and bottle for every wash.
  - Make all wash solutions daily.
- If the problem remains unresolved, reagents might not be performing as expected. Contact Verogen Technical Support.

Incomplete Run

The following factors can cause a run to stop prematurely.

- The HSC was not added during library prep. Add HSC per denature and dilute instructions and repeat the run.
- Cluster density is too high. See *High Cluster Density* on page 53.
- If an error message is displayed, contact Verogen Technical Support.

Low Quality Scores for Read 1 or Read 2

The following factors can cause low quality scores for Read 1 and Read 2.

- Phasing and prephasing are too high. See *High Phasing and Prephasing* on page 54.
- The cluster density is too high. See *High Cluster Density* on page 53.
- If the problem remains unresolved, reagents might not be performing as expected. Contact Verogen Technical Support.

Low Quality Scores for Index 1 Read or Index 2 Read

The following factors can cause low quality scores for the Index 1 Read or the Index 2 Read.

- Phasing and prephasing are too high. See *Phasing and Prephasing* on page 62.
- The cluster density is too high. See *High Cluster Density* on page 53.
- Not enough samples were sequenced, so diversity in the index reads was low.
- If the problem remains unresolved, reagents might not be performing as expected. Contact Verogen Technical Support.

Low Reads per Sample

Review sample representation: If the samples exceed the sample read count (intensity) guideline, proceed with analysis. If the samples are below the guideline, the following factors are the likely cause.

- DNA input was too low or the DNA dilution was incorrect. If possible, repeat library prep with more DNA.
- A critical reagent was not added during library prep. Repeat the prep.
- LNB1 was insufficiently pipetted during normalization.

  - If the sample has a high number of alleles with quality control indicators, resequence the sample.
  - If the sample has a low number of alleles with quality control indicators, consider diluting the libraries in the Pooled Normalized Libraries tube less and resequence the sample.
  - If the run does not pass, repeat library normalization.
- HT1 was overpipetted during library prep. Repeat library normalization.
- The libraries were not heat-denatured during library prep. Perform the heat-denature step in the Diluted Normalized Libraries tube and repeat the run.

Low Reads per Sample for the HSC

Review sample representation: If the samples exceed the sample read count guideline, proceed with analysis. If the samples are below the guideline, the following factors are the likely cause:

- HSC was not added during library prep. Add HSC per denature and dilute instructions and repeat the run.
- HT1 was overpipetted during library prep. Repeat library normalization.
- The libraries were not heat-denatured during library prep. Perform the heat-denature step and repeat the run.
- HSC was not denatured with HP3. Make sure HSC is denatured with HP3 before adding it to the library, and then repeat the run.

Troubleshoot Analysis Errors

The following sections provide recommendations for troubleshooting potential problems with the ForenSeq Analysis service or network.

Analysis Error Message

When creating an analysis results in an error message, the application might not be running or the system cannot locate the run data for analysis. Use the following steps to troubleshoot.

1. From Windows Start on the server desktop, type Services, and then select **Services app**.
2. In the Services dialog box, locate ForenSeq Analysis.
3. Check the Status column:
   - If it is blank, right-click the service and select **Start**. After the service has started, retry analysis creation.
   - If it is running, complete the remaining steps.
4. Open the log file for the analysis at the following path:
   C:\Illumina\Forenseq UAS\Analysis\logs\application.log
5. In the log file, find the message EXCEPTION OCCURRED:System.Exception.
   This message confirms that the system cannot locate the run data.
6. If the run repository is stored in a network location, make sure that the network is running.
7. Navigate to the run folder listed in the log file as RunstoragePath to make sure that the data are in the expected location.
8. Retry analysis creation.

Quality Metrics Icons Are Unavailable

When all Quality Metrics icons on the Home page are unavailable, the system might be unable to locate data or the network where data are stored is disconnected. Use the following steps to troubleshoot.

1. From Windows Start on the server desktop, type Services, and then select **Services app**.
2. In the Services dialog box, locate ForenSeq Analysis.
3. Check the Status column:
   - If it is blank, right-click the service and select **Start**.
   - If it is running, complete the remaining steps to check network connectivity.
4. Navigate to the run folder described in the configuration file.
5. Make sure that data are in the expected location.
6. Make sure that no permission changes were made to access the run folder location.

Run Metrics

Accessing Run Metrics

Available in Projects after sequencing, run metrics provide run quality, sample representation, and positive and negative control results for an overall view of run performance.

To open run metrics, select a PNQ icon on the Project side sheet. A subset of these metrics, run quality and sample representation, are also available on Run Details pages.

Positive Control

Positive control metrics provide data for each sample identified as a ForenSeq Positive Amplification Control. Every sample is analyzed and compared to this control. The software then lists each sample with values for pass filter reads and SNPs typed or positions called. Colors indicate overall success. If applicable, a table displays discordant positions or loci.

Positions Called (mtDNA)

Positions called shows the number of positions, including indels, with a base call out of the total number of positions for HL60. A short, vertical bar displays the following colors to indicate call success and concordance with HL60.

Color	Indication
	All positions have calls and are concordant.
	At least one position does not have a call or contains a discordant call.

SNPs Typed (Kinship)

SNPs typed shows the number of loci with a base call out of the total number of loci for NA24385. A short, vertical bar displays the following colors to indicate call success and concordance with NA24385.

Color	Indication
	All loci have calls and are concordant.
	At least one locus does not have a call or contains a discordant call.

Discordance Table

When a sample has calls that are discordant with the positive amplification control calls, the positive control metrics include a discordance table. For each discordant call, the table displays the position number or locus where the discordance occurred, the call observed for the sample, and the expected call.

The table displays up to 50 positions or loci. When the number of discordant positions or loci exceeds 50, use the scroll arrows to move through the table.

Pass Filter Reads

Pass filter reads displays the following colors to indicate whether the number of reads that passed filter meet the guideline of ≥ 50,000 reads for ForenSeq mtDNA libraries or 15 million reads for ForenSeq Kintelligence libraries. Reads below the guideline might still be usable. Review quality metrics and sample data to help make a determination.

Color	Indication
	The total number of pass filter reads exceeds the guideline.
	The total number of pass filter reads is below the guideline.
	The guideline for the number of reads that pass filter.

Negative Control

Negative control metrics provide data for each sample identified as a negative amplification control or reagent blank. The software lists each of these samples with the number of SNPs typed or positions called and a color-coded indicator of overall success.

Positions Called (mtDNA)

Positions called shows how many positions, including indels, a base call was made for. The short vertical bar uses the following colors to indicate call success.

Color	Indication
	No positions have a base call.
	At least one position has a base call.

SNPs Typed (Kinship)

SNPs typed shows how many loci a base call was made for. The short vertical bar uses the following colors to indicate call success.

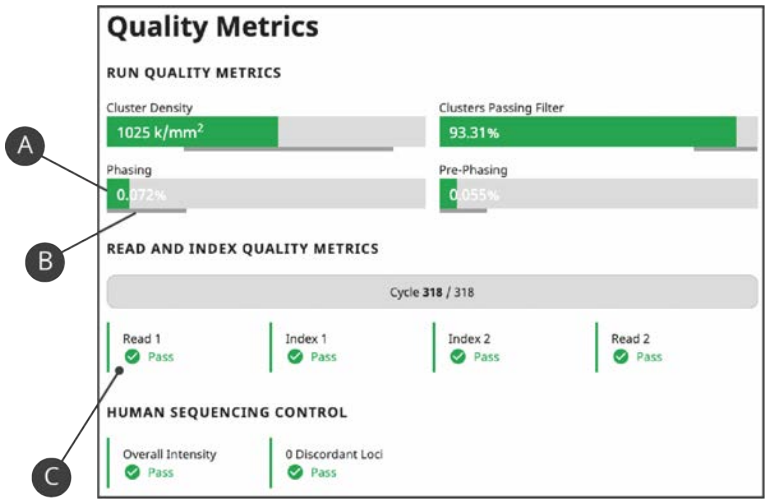
Color	Indication
	No loci have a base call.
	At least one locus has a base call.

Quality Metrics

Quality metrics monitor run quality during sequencing. After sequencing, the software preserves the quality metrics and makes them available in both Runs and Projects. In Runs, the quality metrics appear in a pop-up window. In Projects, they appear on a page.

Color-coded bars indicate results for each metric and show optimum ranges for cluster density, clusters passing filter, phasing, and prephasing. Short, vertical bars with corresponding icons indicate the quality of each read and the HSC.

Figure 7 Quality metrics overview



A Color-coded horizontal bars and values indicate run quality metrics.
B Thinner horizontal bars in dark gray indicate optimum ranges.
C Color-coded vertical bars and icons indicate the quality of each read and the HSC.

Run Quality Metrics

Run quality metrics include cluster density, clusters passing filter, phasing, and prephasing values. The following colors indicate overall quality.

Color	Indication
	Values are within the target range.
	Values require further evaluation of run quality.
	The target range for the metric.

Cluster Density

Cluster density is the number of clusters per square millimeter (K/mm²). The following table lists the target cluster density range for each assay type. Values outside these ranges can still produce results sufficient for analysis. However, substantial deviations can impact other quality metrics and decrease the amount of usable data.

Assay Type	Target Cluster Density
ForenSeq Kintelligence	700-1400 K/mm²
ForenSeq mtDNA Control Region	400-1650 K/mm²
ForenSeq mtDNA Whole Genome	400-1650 K/mm²

Clusters Passing Filter

Clusters passing filter is the percentage of clusters that passed the quality filter. This metric is based on the Illumina chastity filter, which measures quality and can detect low-quality base calls. Data appear after cycle 25.

> The chastity of a base call is the ratio of the intensity of the greatest signal divided by the sum of the two greatest signals. If multiple base calls have a chastity value < 0.6 in the first 25 cycles, reads do not pass filter.

The following table lists the target values for clusters passing filter. Values below a target can still produce results that are sufficient for analysis. However, substantial deviations from the target can impact other quality metrics and decrease the amount of data.

Assay Type	Target Value
ForenSeq Kintelligence	> 85%
ForenSeq mtDNA Control Region	≥ 80%
ForenSeq mtDNA Whole Genome	≥ 80%

Phasing and Prephasing

Phasing shows the percentage of molecules in a cluster that fall behind the current cycle in Read 1. Prephasing shows the percentage of molecules in a cluster that jump ahead of the current cycle in Read 1.

For both phasing and prephasing, lower percentages indicate higher quality run statistics. Values outside the target ranges can still produce results that are sufficient for analysis.

Assay Type	Target Phasing Value	Target Prephasing Value
ForenSeq Kintelligence	< 0.2%	< 0.15%
ForenSeq mtDNA Control Region	≤ 0.25%	≤ 0.15%
ForenSeq mtDNA Whole Genome	≤ 0.25%	≤ 0.15%

Read and Index Quality Metrics

The read and index quality metrics use the following colors and icons to indicate the status of each read and overall quality.

Table 5 Colors

Color	Indication
	The average quality of assessed reads is within the target range.
	The average quality of assessed reads is outside the target range.
	The read has not yet occurred.

Table 6 Icons

Icon	Indication
	The average quality of assessed reads is within the target range.
	The average quality of assessed reads is outside the target range.
	The read has not yet occurred.

Reads in a Run

A sequencing run completes up to four reads. Read 1 and Read 2 sequence the DNA template strands, and the Index 1 Read and Index 2 Read sequence the index adapters.

- **Read 1**–Read 1 sequencing primer is annealed to the template strand during cluster generation. RTA evaluates the first 50 cycles for quality.

- **Index 1 Read**—The Read 1 product is removed and the Index 1 sequencing primer is annealed to the same template strand as in Read 1. After index read preparation, the Index 1 Read is performed. RTA evaluates all eight cycles for quality.
- **Index 2 Read**—The Index 1 Read product is removed and the template anneals to the P5 primer grafted to the flow cell surface. The run proceeds through seven chemistry-only cycles without any imaging, followed by eight cycles of sequencing. RTA evaluates all eight cycles for quality.
- **Read 2**—The Index 2 Read product is extended to copy the original template strand. The original template strand is then removed and the Read 2 sequencing primer is annealed.

Cycles per Read

Quality metrics for each read appear after the read is complete. The number of cycles in each read depends on the assay.

Assay Type	Read	Cycles	Quality Metrics Appear
ForenSeq Kintelligence	Read 1	1-151	Cycle 152
	Index 1 Read	152-159	Cycle 160
	Index 2 Read	160-167	Cycle 168
	Read 2	168-318	After cycle 318
ForenSeq mtDNA Whole Genome	Read 1	1-201	Cycle 202
	Index 1 Read	202-209	Cycle 210
	Index 2 Read	210-217	Cycle 218
	Read 2	218-418	After cycle 418
ForenSeq mtDNA Control Region	Read 1	1-151	Cycle 152
	Index 1 Read	152-159	Cycle 160
	Index 2 Read	160-167	Cycle 168
	Read 2	168-318	After cycle 318

Human Sequencing Control

The software assesses the HSC and uses the following colors indicate the overall result.

Color	Indication
	The control meets or exceeds the criteria.
	The control does not meet at least one criterion.

When the HSC is orange, evaluate the run quality metrics to help determine whether to repeat the run. If the run quality metrics are green, indicating that values are within target ranges, you can interpret the sample with caution.

Loci

The HSC includes the following loci. The software lists any loci that do not meet intensity or genotype concordance criteria.

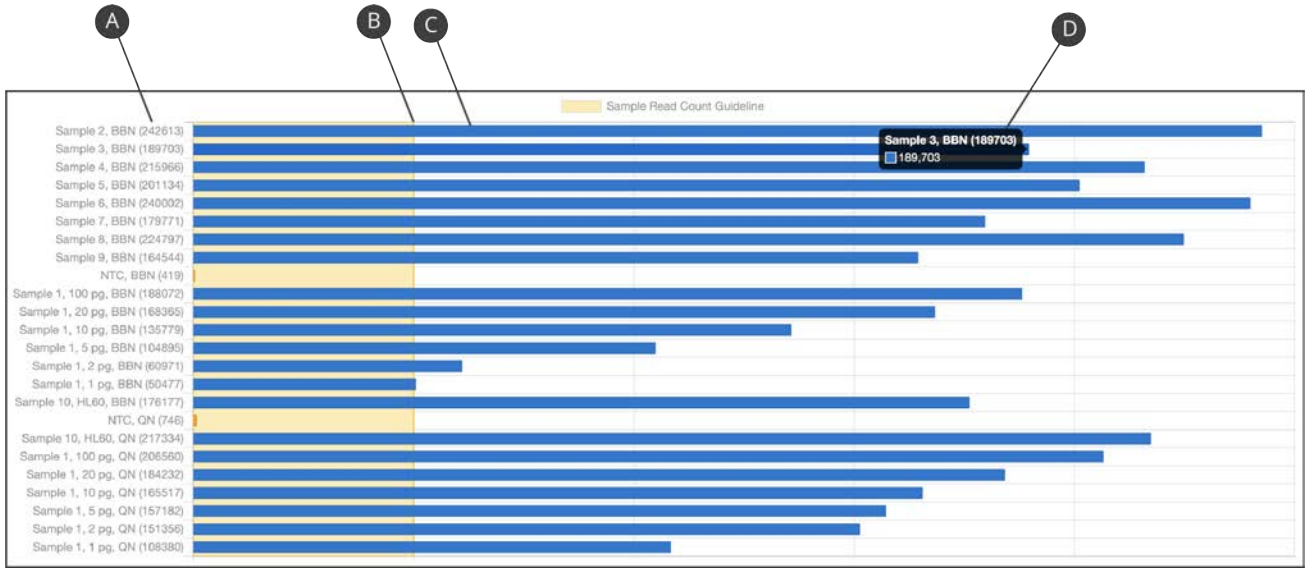
- D3S1358
- D5S818
- D7S820
- D8S1179
- D13S317
- D16S539
- D18S51
- FGA
- PentaD
- PentaE
- TPOX
- DYS391
- DYS392
- DYS460
- DXS7423
- DXS10103

Sample Representation

Sample representation is a metric that provides quantitative sample and run information. After sequencing, the software preserves sample representation data and makes it available in both Runs and Projects. In Runs, sample representation data appear in a pop-up window. In Projects, these data appear on a page.

Sample representation shows an index CV percentage for the run and the number of reads (intensity) and read distribution for each sample. These data are presented in a bar chart that also shows the sample read count guideline. Hovering over a bar displays the exact number of reads for the sample.

Figure 8 Sample Representation bar graph

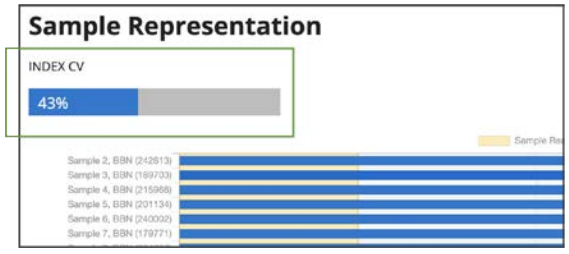


A Exact number of reads for the sample
B Threshold of the read count guideline
C Bar indicating number of reads for the sample
D Exact number of reads for the sample (hovering)

Index CV

Above the sample representation bar chart, Index CV shows a percentage that represents the distribution of read counts for all samples in the run. CV is the coefficient of variation for the number of read counts across all indexes.

Figure 9 Index CV percentage



Sample Read Count Guideline

The following table presents sample read count guidelines, which vary by assay type. As guidelines, these values are intended to help with quality reviews of the run and samples. Samples with fewer reads can still demonstrate complete coverage and calls at 100% of positions and provide enough data for interpretation.

Assay Type	Sample Read Count Guideline
ForenSeq Kintelligence	15 million reads per sample
ForenSeq mtDNA Control Region	50,000 reads per sample
ForenSeq mtDNA Whole Genome	400,000 reads per sample

The following colors indicate how sample read counts compare to the applicable sample read count guideline.

Color	Indication
	The sample read count guideline.
	The sample meets or exceeds the guideline.
	The sample does not meet the guideline and requires careful interpretation.

Report Formats

File Formats for mtDNA Reports

The following sections describe the format and contents of each type of report the software generates for mtDNA samples. For instructions on creating reports, see *Report Generation* on page 34.

CODIS Report

A CODIS report is a .cmf file that includes a CMF header, reference sequence, and specimen profile. The specimen profile provides the following information for each sample in the report:

- Specimen ID
- Specimen category
- Source ID
- Fragments

EMPOP Report

An EMPOP report is a .txt file. For each sample, the report includes a sequence string in FASTA format, variant calls in rCRS format, and names for the following items:

- Project
- Sample
- Assay
- Analysis method
- Run

Figure 10 Example EMPOP report

A Sequence string in FASTA format
B List of variant calls in rCRS format

Project Report

An mtDNA project report is an Excel workbook. The first worksheet, Variant Sample Report, provides the following project information:

- Project name and optional description
- Project creation date and time
- Run and sample counts
- Sample data:
  - Single nucleotide variant (SNV) count per sample
  - Insertion count per sample
  - Deletion count per sample
  - Call or no call count per sample
  - Manually changed calls per sample
  - Variants per sample

If applicable, the second worksheet in the project report, Project History, includes administrative details for the project and a project history log that lists time-stamped system events and user actions.

Each subsequent worksheet contains sample information (one worksheet per sample). For details, see *Sample Report*.

Sample Report

An mtDNA sample report is an Excel workbook. If applicable, the first worksheet is Sample History, which includes administrative details for the sample and a sample history log that lists time-stamped system events and user actions.

The second worksheet is named for the sample and provides the following information:

- Assay type
- Analysis method
- Run name
- Analytical and interpretation thresholds
- Minimum Q-score
- Minimum read count
- SNVs
- Insertion and deletion counts
- Call count for reagent blanks and negative controls
- No call count for samples and positive controls
- Manually changed calls
- Variant details:
  - Variant and reference calls
  - Total read count
  - Strand depth
  - Reads A, C, T, and G
  - Percent A, C, T, and G
  - Reads del
  - Percent del
  - Reads ref
  - Percent ref

File Formats for Kinship Reports

The following sections describe the format and contents of each type of report the software generates for kinship samples. In the reports, rsID numbers identify individual SNPs. For instructions on creating kinship reports, see *Generating Kinship Reports* on page 38.

Phenotype and Ancestry Report

A phenotype and ancestry report is an Excel workbook comprised of four worksheets that detail the estimates of hair color, eye color, and biogeographical ancestry: Estimation, SNP Data, SNP Balance Figure, and Settings. In the Estimation and SNP Data worksheets, a header section lists the software version, analysis method, sample gender, project creation date and time, the user who generated the report, and sample, project, and run names.

- Estimation
  - Hair color results: brown, red, black, and blonde
  - Eye color results: intermediate, brown, blue
  - Biogeographical ancestry results (plot): the sample with distance to the nearest centroid

  - Population information for samples in the centroid: 1000 Genomes population and abbreviation, count, and data used to train models
- SNP Data
  - Allele name, whether the locus is typed, and the number of reads for each locus
  - Number of loci typed, genotype, and QC indicators for hair and eye color SNPs, common SNPs, and biogeographical ancestry SNPs

    Loci typed is the number of aSNP and pSNP loci identified out of total targeted aSNP and pSNP loci.

- SNP Balance Figure
  - Whether the allele is typed
  - Number of reads per base for each SNP with a grand total
- Settings
  - Software version
  - AT, IT, and intralocus balance for each locus

Estimating Phenotype

The software uses HIrisPlex, a multinomial logistic regression model, to determine the probabilities for hair and eye color. The software publishes these probabilities to the Estimation worksheet.[1-3]

[1] Walsh, Susan, Lakshmi Chaitanya, Lindy Clarisse, Laura Wirken, Jolanta Draus-Barini, Leda Kovatsi, Hitoshi Maeda, et al., "Developmental validation of the HIrisPlex system: DNA-based eye and hair colour prediction for forensic and anthropological usage," *Forensic Science International: Genetics* 9 (March 2014): 150-161, https://doi.org/10.1016/j.fsigen.2013.12.006.

[2] Liu, Fan, Katevan Duijn, Johannes R. Vingerling, Albert Hofman, André G. Uitterlinden, A. Cecile J.W. Janssens, and Manfred Kayser, "Eye color and the prediction of complex phenotypes from genotypes," *Current Biology* 10, no. 5 (March 2009): R192-R193, https://doi.org/10.1016/j.cub.2009.01.027.

[3] Walsh, Susan, Fan Liu, Kaye N. Ballantyne, Mannis van Oven, Oscar Lao, and Manfred Kayser, "IrisPlex: A sensitive DNA tool for accurate prediction of blue and brown eye colour in the absence of ancestry information," *Forensic Science International: Genetics* 5, no. 3 (June 2011): 170-180, https://doi.org/10.1016/j.fsigen.2010.02.004.

Estimating Biogeographical Ancestry

The software uses principal component analysis (PCA) to determine estimates of biogeographical ancestry. Data from Phase I of the 1000 Genomes Project (1000genomes.org) was used to train the model on the following super populations: European, East Asian, and African (excepting African Ancestry in Southwest USA [ASW]). For context, the unknown sample is projected with the Ad-Mixed Americans super population onto the first two pretrained components based on aSNP genotype calls.[4]

On the Estimation worksheet, the distance to the nearest centroid indicates how related a sample is to the general grouping for the centroid. For comparison, the worksheet displays the distance for 1000 Genome Project samples that contribute to the centroid. The chart on the Estimation worksheet includes one centroid for each of the major ancestries and orientational centroids at the one-quarter intervals between these groups. Centroids contextualize results and logically group populations.

[4] Abecasis, Gonçalo R., David Altshuler, Adam Auton, Lisa D. Brooks, Richard M. Durbin, Richard A. Gibbs, Matt E. Hurles, et al., "A map of human genome variation from population-scale sequencing," *Nature* 467 (October 2010): 1061-1073, https://doi.org/10.1038/nature09534.

Generating SNP Data

The SNP Data worksheet provides data for aSNPs and pSNPs. Estimating hair and eye color requires genotype results for hair color, eye color, and common SNPs. Common SNPs are SNPs that estimate hair color, eye color, and biogeographical ancestry.

If one of these SNPs is not typed, the software does not generate a result. In contrast, generating a result for biogeographical ancestry requires that only one biogeographical ancestry SNP be typed. The absence of SNPs in the biogeographical ancestry estimation diminishes the accuracy of the estimation. With each execution, the estimation model is retrained using only the aSNPs with a multilocus genotype.

GEDmatch PRO Report

A GEDmatch PRO report is a .txt file. Using human genome assembly GRCh37 (hg19) as the reference genome, the report lists each SNP locus call by rsID with the following information:

- Chromosome and position
- Allele 1 and allele 2 typed calls reported on the plus strand

Project Report

A kinship project report is an Excel workbook. A header section lists the project name, project creation date and time, and the user who generated the report. A Samples table lists each sample in the report with the following information:

- Analysis method
- Contributor status
- Gender
- Locus call rate
- Sample name and optional description
- Total sample reads

Sample Report

A kinship sample report is an Excel workbook with one worksheet per SNP type: X-, Y-, ancestry, phenotype, identity, and kinship. The workbook also includes a Settings worksheet and, if applicable, a Sample History worksheet. The Settings worksheet lists AT, IT, and intralocus balance for each locus. The Sample History worksheet includes administrative details for the sample and a sample history log listing time-stamped system events and user actions.

The header section of each SNP worksheet lists the analysis method, gender, contributor status, project creation date and time, user who generated the report, and the sample, project, and run names. Coverage information appears under the header, followed by a table listing information for each locus:

- Locus
- Genotype with allele name
- Whether the allele is typed
- Reads
- Quality control indicators

Supporting Information

Special Handling of mtDNA Data

The following sections describe how the software handles special circumstances, such as ambiguous base call codes, when analyzing sequencing data from mtDNA samples.

Consensus Calls

The software supports variant calling for multiple bases at a DNA position. When multiple bases exceed the interpretation threshold, the software reports the International Union of Pure and Applied Chemistry (IUPAC) code as the consensus for the position.

IUPAC Code	Base
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
R	A or G
Y	C or T
S	G or C
W	A or T
K	G or T
M	A or C
B	C, G, or T
D	A, G, or T
H	A, C, or T
V	A, C, or G

Ambiguous Calls

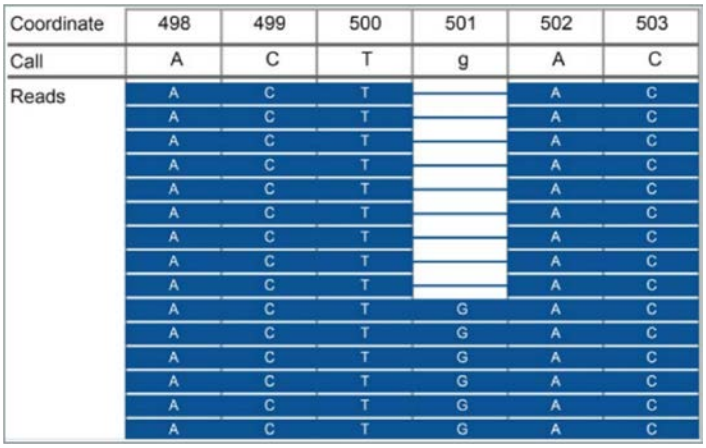
When data for the indel and a base call at a position exceed the IT, the software reports the ambiguous call code for an indel as the consensus for the position.

Base	Call
a	Adenine
c	Cytosine
g	Guanine
t	Thymine
r	a or g
y	c or t
s	g or c
w	a or t
k	g or t
m	a or c
b	c or g or t
d	a or g or t
h	a or c or t
v	a or c or g
n	Any base

Ambiguous Call Example

An example of an ambiguous call, the following figure shows an ambiguous deletion at position 501. Because an equal number of reads support a deletion call and a reference call of G, the software reports an ambiguous call of g as the consensus.

Figure 11 Ambiguous deletion at position 501



SWGDAM Nomenclature

The software follows SWGDAM nomenclature. For variant calling, differences between the reference and sample sequences are recorded as polymorphisms with the nucleotide position and DNA base difference indicated in the haplotype nomenclature (for example, 16182C).

Primer Trimming

The software trims primer sequences for ForenSeq mtDNA amplicons, which are based on a tiled primer design. This trimming allows unambiguous interrogation of mtDNA amplicon inserts without degenerate oligonucleotides. The mtDNA Variant Processor v1.0.0 application in Illumina BaseSpace Sequence Hub does not trim primers from amplicons generated from a tiled primer design.

Nuclear mtDNA Insertions

Nuclear DNA sequences homologous to mtDNA are often called nuclear mtDNA insertions (NUMTs). During library prep, these sections can be enriched alongside the respective mtDNA target. When performing alignment, ForenSeq UAS v2.0 identifies reads that map closely to sequences in the Reference Human NumtS (RHNumtS) compilation and removes these reads before further analysis.[5,6]

[5] Mishmar, Dan, Eduardo Ruiz-Pesini, Martin Brandon, and Douglas C. Wallace, "Mitochondrial DNA-like Sequences in the Nucleus (NUMTs): Insights Into Our African Origins and the Mechanism of Foreign DNA Integration," *Human Mutation* 23 no. 2 (February 2004): 125-133, https://doi.org/10.1002/humu.10304.

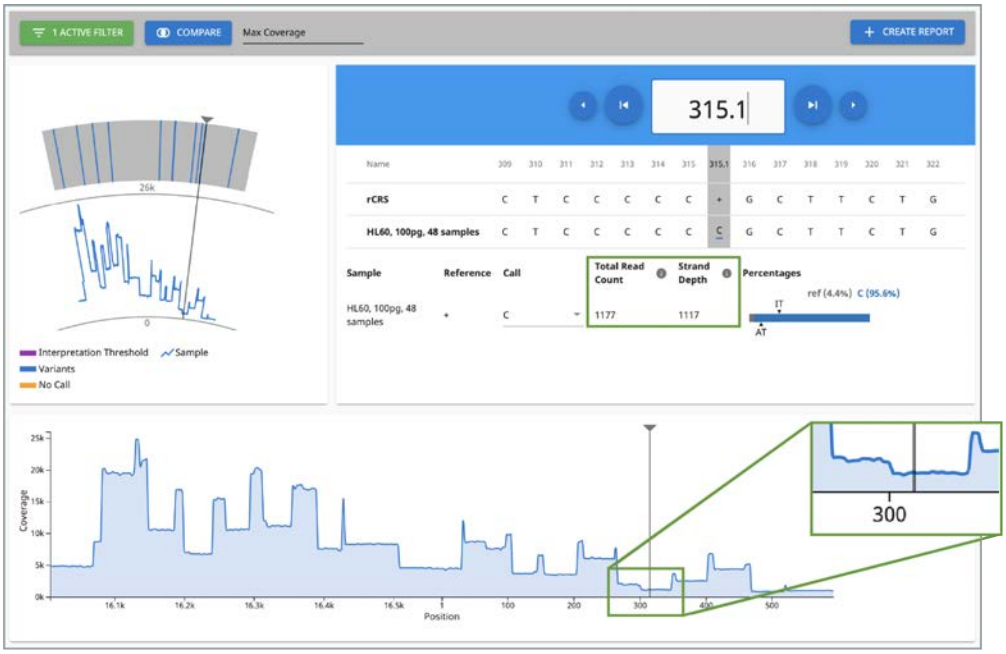
[6] Calabrese, Francesco Maria, Domenico Simone, Marcella Attimonelli "Primates and Mouse NumtS in the UCSC Genome Browser." Supplement, *BMC Bioinformatics* 13, no. S4 (March 2012): S15. https://doi.org/10.1186/1471-2105-13-S4-S15.

HVII C-Stretch Coverage

The C-stretch describes homopolymers of cytosine-stretches found in hypervariable region I (HVI) and hypervariable region II (HVII) of mtDNA. Primer pairs that amplify the region between position 262 and position 353 achieve coverage of the C-stretch (positions 303–315) in HVII. Sequences generated from the forward template strand have high accuracy and alignment. Sequences generated from the reverse template strand have high accuracy and alignment until reaching the C-stretch. Therefore, reads from the reverse strand that start sequencing at position 262 and do not meet alignment requirements are soft-clipped in ForenSeq UAS v2.0 after position 303. The soft-clipped positions are not used for base calling. As a result, approximately half the coverage (read counts) are obtained for positions 304–353 relative to positions 262–303 (on the reverse strand).[7-8]

In the following figure, a Results page shows similar total read count and strand depth at positions 262–303 and 304–353. Positions 262–303, where reads from both strands are reported, have similar coverage. Total reads for positions 304–353 are about half that of positions 262–353.

Figure 12 Results for HL60 (100 pg gDNA)



[7] Nakamura, Kensuke, Taku Oshima, Takuya Morimoto, Shun Ikeda, Hirofumi Yoshikawa, Yuh Shiwa, Shu Ishikawa, et al., "Sequence-specific error profile of Illumina sequencers," *Nucleic Acids Research* 39, no. 13 (July 2011): e90. https://doi.org/10.1093/nar/gkr344.

[8] Allhoff, Manuel, Alexander Schönhuth, Marcel Martin, Ivan G. Costa, Sven Rahmann, and Tobias Marschall. Discovering motifs that induce sequencing errors," Supplement, *BMC Bioinformatics* 14, no. S5 (April 2013): S1. https://doi.org/10.1186/1471-2105-14-S5-S1.

Contributor Status of Kinship Samples

The software applies the following logic to determine the contributor status of a kinship sample.

- Any of the following scenarios indicate a mixture:
  - The sample has ≥ 10 Y-SNPs with two alleles.
  - The sample has ≥ 10 Y-SNP loci typed and ≥ 10 X-SNPs with two alleles.
  - The sample has zero Y-SNP loci typed with ≥ 60% autosomal SNP (auSNP) heterozygosity and ≥ 50% X-SNP heterozygosity.
- If the sample has ≤ 50% auSNP heterozygosity, ≤ 40% X-SNP heterozygosity, ≥ 65% auSNP intralocus balance, and zero Y-SNPs with two alleles, the sample is single-source.
- If the sample is a negative amplification control, the call rate is < 50%, or the scenarios for mixture and single-source do not apply, the contributor status is inconclusive.

To adjust heterozygosity percentages for call rate, the observed heterozygosity is divided by the sample call rate.

Gender Estimation for Kinship Samples

The software applies the following logic to estimate the gender of a kinship sample.

- If the sample has ≥ 10 Y-SNP loci typed, the gender is male (XY).
- If the sample has zero Y-SNP loci typed and the call rate is ≥ 50%, the gender is female (XX).
- If the sample is a negative amplification control, the contributor status is mixture, or the scenarios for male and female do not apply, the gender is inconclusive.

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Technical Support

For technical assistance, contact Verogen Technical Support.

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Email	techsupport@verogen.com
Telephone	+1.833.837.6436 toll-free (North America) +1.858.285.4101 tel +44 (0) 208 054 8706 (United Kingdom)

Safety data sheets (SDS)–Available for download from verogen.com/documentation.

Product documentation–Available for download from verogen.com/documentation.

VEROGEN

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