Takara Bio USA

Cogent™ NGS Analysis Pipeline User Manual

(073025)

Takara Bio USA, Inc.
2560 Orchard Parkway, San Jose, CA 95131, USA
U.S. Technical Support: technical_support@takarabio.com

United States/Canada	Asia Pacific	Europe	Japan
800.662.2566	+1.650.919.7300	+33.(0)1.3904.6880	+81.(0)77.565.6999

Table of Contents

Table of Figures

Table of Tables

I. Introduction

Cogent NGS Analysis Pipeline (CogentAP) is a bioinformatics software for analyzing RNA-seq and DNA-seq stored in FASTQ files generated from libraries prepared using select Takara Bio next-generation sequencing (NGS) reagent kits. The output from CogentAP can then be imported into Cogent NGS Discovery Software (CogentDS) for additional processing and visualizing the data.

We recommend new users to read through this document prior to starting. There is also a quick start guide available to download, which is a streamlined reference document for installation and usage of the software.

The program takes software-modified input files from sequencers and outputs the following:

- an HTML report, with results typical to single-cell analysis,
- an R data object (rds file) which can be used as input for CogentDS,
- an analysis stats (.csv) file which reports alignments stats for RNA-Seq or QC report (.html) which reports alignment stats for DNA-Seq, and
- processed data files, such as gene counts and transcript counts matrices, which can be used for further analysis (only applicable for kits other than the Shasta™ Total RNA-Seq Kit [see Table 1] and for analyses with ≤5,000 barcodes).

CogentAP uses Nextflow as the pipeline manager and can be run on a Linux server using a command-line interface.

A. What's New

Unless otherwise noted, the current version of CogentAP software contains all features included in previous versions.

- **Cogent NGS Analysis Pipeline v3.2**
  - New DNA-seq postprocess analysis option for SNV calling (Section V.C.2)
  - Support for analysis of Shasta Total RNA-Seq kit data using a single pair of FASTQ files and skipping fastq demultiplexing per barcode (Section V.B.1.c)
  - Option to generate gene body coverage plots for Shasta Total RNA-Seq analysis, human and mouse genomes only (Section V.B.1.c)
  - Support for optional depletion of Globin and RN7SL2 during RNA-Seq analysis, human genome only (Section V.B.1.e)
  - Automatic validation of input sample sheet/well-list
  - Updated stats files for UMI-based kits

NOTE: Release notes for prior versions can be found on the Cogent NGS Analysis Pipeline product page.

B. Supported NGS Products

Table 1 lists the Takara Bio products for which sequencing results can be processed by CogentAP. For processing sequencing results from Takara Bio immune profiling kits, refer to the Cogent NGS Immune Profiler.

Table 1. Applications and kits compatible with Cogent NGS Analysis Pipeline v3.2.

System	Experiment type	Kit or application
Shasta Single Cell System	Single-cell full gene-body total RNA-seq analysis	Shasta Total RNA-Seq Kit - 2 Chip
	Single-cell whole-genome amplification	Shasta Whole-Genome Amplification Kit - 2 Chip
ICELL8® cx Single-Cell System	Single-cell full gene-body total RNA-seq analysis	Shasta Total RNA-Seq Kit - 2 Chip
	Single-cell whole-genome amplification	Shasta Whole-Genome Amplification Kit - 2 Chip
	Single-cell full gene-body transcriptome analysis	SMART-Seq® Pro Application Kit - 2 Chip
Plate-based	Single-cell full gene-body transcriptome analysis (with UMIs)	SMART-Seq mRNA LP (with UMIs)
	Single-cell full gene-body transcriptome analysis (no UMIs)	SMART-Seq mRNA LP SMART-Seq mRNA SMART-Seq mRNA Single Cell LP SMART-Seq mRNA Single Cell SMART-Seq mRNA HT LP SMART-Seq mRNA HT SMART-Seq v4 PLUS Kit* SMART-Seq v4 Ultra® Low Input RNA Kit* SMART-Seq Single Cell PLUS Kit* SMART-Seq Single Cell Kit* SMART-Seq HT PLUS Kit* SMART-Seq HT*
	Strand-specific total RNA-seq for mammalian samples (with UMIs)	SMART-Seq Total RNA Pico Input with UMIs (ZapR® Mammalian) SMARTer® Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian*
	Strand-specific total RNA-seq for mammalian samples (no UMIs)	SMART-Seq Total RNA Pico Input (ZapR Mammalian) SMART-Seq Total RNA Single Cell (ZapR Mammalian) SMART-Seq Stranded Kit* SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian*
	Single-cell genome and transcriptome analysis (no UMIs)	Embgenix™ GT-omics Kit

*Product will be phased out soon. Please refer to the product page (via the hyperlink, if available) or contact your local sales representative for more information.

II. Before You Begin

A. Supported Operating Systems

CogentAP is designed to be installed on a server running Linux. The following versions of Linux have been tested and are supported for use with the software:

- CentOS 8 or higher
- RedHat 8 or higher
- Ubuntu 18.04 or higher

B. Hardware Requirements

For analyzing the output of Illumina® NextSeq® High-Output sequencing, the following server requirements (or better) are recommended:

- CPU: 24 cores
- RAM: 64 GB
- Free hard drive space:
    - For all kits apart from the Shasta Total RNA-Seq Kit - 2 Chip and Shasta Whole-Genome Amplification Kit - 2 Chip: at least 1 TB
    - For Shasta Total RNA-Seq Kit - 2 Chip and Shasta Whole-Genome Amplification Kit - 2 Chip: at least 8 times the size of the input FASTQ files (for analyses with default parameters), and 10 times the size of the input FASTQ files (for analyses that will include optional ribodepletion, immune profiling, and fusion analyses)

Testing was also done on MiniSeq™, MiSeq®, HiSeq®, and NovaSeq™ datasets.

- MiniSeq or MiSeq—less computational power may be needed than the specifications described for NextSeq output
- HiSeq or NovaSeq—requires more computational power than described for NextSeq output

Precise hardware requirements were not determined for output from these datasets. Support for performance issues of the servers in conjunction with these dataset types may be limited.

C. User Account Requirements

The account used to install CogentAP needs to have read/write (R/W) permissions for the following folders:

- Where CogentAP will be located,
- Where CogentAP will be run, and
- Where the analyses' output will be saved.

Once installed, other accounts can be used to run CogentAP, but these accounts need to have R/W permissions for the latter two folders listed above. If the software does not detect proper R/W permissions on these folders, it will terminate analysis, and an error message will describe the reason for termination.

D. Additional Hardware and Software Dependencies and Recommendations

- **Bash UNIX shell**
- **Internet connectivity on the server**

The installation process requires internet connectivity, as it sources scripts from GitHub, Bioconda, and CRAN, and downloads genome information from an Amazon S3 bucket. Please ensure that internet connectivity is available on the UNIX server while installing.

- **Conda**

  CogentAP leverages the open-source package manager Conda for installation of CogentAP and its dependencies. Any tools and applications required by CogentAP are installed through Conda inside a local environment explicitly created for CogentAP. Conda installation instructions can be found at https://conda-forge.org/download/.

  If Conda is already installed on your server, it is highly recommended to remove the existing installation and install a new version. Instructions for removing an existing Conda installation can be found at https://github.com/conda-forge/miniforge?tab=readme-ov-file#uninstallation and https://docs.anaconda.com/anaconda/uninstall/.

- **bcl2fastq/BCL Convert**

  CogentAP takes raw FASTQ files as input. Sequencer output FASTQ files can be converted to raw FASTQ files using bcl2fastq or bclconvert software from Illumina.

  The bcl2fastq software can be downloaded and installed from https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html. The bclconvert software can be downloaded and installed from https://support.illumina.com/sequencing/sequencing_software/bcl-convert/downloads.html.

- **Keyboard, monitor, and mouse directly into the server, or a remote access program**

  CogentAP must be run on the Linux server in which it is installed. If users do not have direct console access, a remote access program that enables a Virtual Network Computing (VNC) connection is required through a program such as RealVNC (realvnc.com), TightVNC (tightvnc.com), TigerVNC (tigervnc.org), or similar. Alternatively, users can connect to the Linux server using SSH. In order to keep an SSH-based analysis running when the connection is lost, users can use a screen or tmux session.

  For more information on VNC, along with other VNC clients that can be used, please see the Wikipedia entry at https://en.wikipedia.org/wiki/Virtual_Network_Computing.

E. Required Input Files

1. General Required Files

RNA-seq and DNA-seq primary analysis require the following input files.

- Paired-end read FASTQ files (converted to raw-fastq files using bcl2fastq or BCL Convert)

  NOTE: Single-end FASTQ files cannot be processed using CogentAP.

  NOTE: For experiments on Shasta, ICELL8 cx, or ICELL8 systems, generate one pair of Undetermined fastq.gz files from the sequencing run as the input, and CogentAP will use Undetermined fastq.gz files with a Well-list file for demultiplexing.

- Sample description file, which can be any one of the following:
  - For experiment results from the Shasta, ICELL8 cx, or ICELL8 systems:

- Well-list file—a text file output by the CellSelect® Software that contains well-level sample information. For more information, see Section V.A.6 of the Shasta CellSelect Software User Manual. For data obtained with the Shasta Total RNA-Seq kit, the well list comes included with the pipeline; see Section V.B.1.b for details.

- For plate-based experiment results:
  - Well-list-like format file—a text file that contains sample information, including columns "Barcode" and "Sample". Each column name is case-sensitive. The "Barcode" column contains i7 and i5 indexes concatenated with a plus-sign ("+") (e.g., TAGCGAGT+CCGTTGCG), as in the example below.

```
Sample,Barcode
GM12877,ATGTAAGT+CATAGAGT
GM12877,GCACGGAC+TGCGAGAC
GM12877,GGTACCTT+GACGTCTT
GM05067,AACGTTCC+AGTACTCC
GM06067,GCAGAATT+TGGCCGGT
GM05067,ATGAGGCC+CAATTAAC
GM08331,AGCCTCAT+TCTCTACT
GM08331,GATTCTGC+CTCTCGTC
GM08331,TCGTAGTG+CCAAGTCT
Etc…
```

    For more information about the contents of a well-list-like file, please refer to Shasta Single Cell System User Manual, Appendix C, Section A ("Wells Data Table")

  - An Illumina sample sheet—a file format used by Illumina for storing biological sample information and metadata associated with a given experiment.

    The input sample sheet/well-list file is validated by CogentAP for the following conditions:

    – The barcodes are in the proper format (except Illumina sample sheets that have separate columns for index1 and index2)

    – The sample column contains only alphanumeric characters, underscores, and hyphens. No other special characters or spaces are allowed.

    – Presence of a "Barcode" column in the well list

    – Presence of a "Sample" column in the well list

    – i7+i5 index combinations are unique

    – i7 index sequences are of a uniform length

    – i5 index sequences are of a uniform length

    If the sample sheet/well-list file does not pass validation, analysis will stop and let the user know via a message written to the console why the validation failed.

NOTE: Sample names are used by CogentAP for statistical analysis, such as clustering, and handled as a group name in the analysis. Illumina sample sheets, natively, require unique sample names for each row in the file, meaning clustering cannot be performed and may cause error messages.

If using an Illumina sample sheet as input that includes multiple experimental instances of one sample, it is recommended that the sample names in the sheet be edited to align with the Well-list-like format usage so more accurate analysis results can be provided.

If using an Illumina sample sheet as input that includes multiple experimental instances of one sample, it is recommended that the sample names in the sheet be edited to align with the Well-list-like format usage so more accurate analysis results can be provided.

2. DNA-seq Extended Analysis–SNV Calling

SNV calling analysis (Section V.C.2) on DNA-seq data requires the following input files to be provided:

- The results folder of `cogent dna analyze` for the data of interest (Section V.C.1)
- A CSV file generated by [Download Clusters] during CNV analysis in CogentDS v2.2 on the same results. For more information on generating this file, refer to Section VIII of the Cogent NGS Discovery Software User Manual, with particular attention to Section VIII.A.6.

III. Software Overview

A. RNA-seq Analysis Workflow

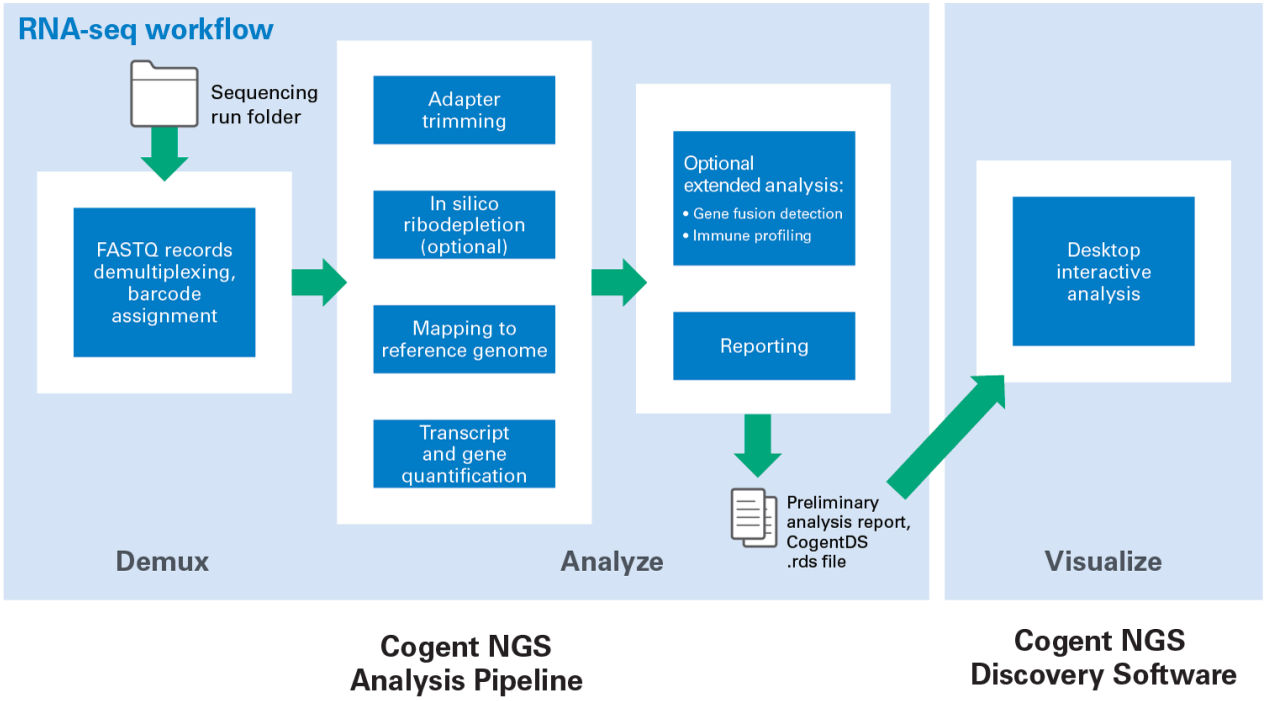


Figure 1. High-level RNA-seq analysis workflow of CogentAP and how its output can be carried over to CogentDS.

For analysis of RNA-seq data, CogentAP consists of two main parts, the demultiplexer (demuxer) and the analyzer.

- The demultiplexer extracts the barcode from the sequencing data (based on the protocol) and writes it into FASTQ files at the end of the read name. There are two options:

  - The default option splits the data up into barcode-level gzip FASTQ files, which are required for input into the analyzer.
  - The second option leaves the barcode-assigned reads in combined gzip FASTQ files. This format is incompatible with CogentAP v3.2 analysis but can be used with other third-party tools if they support such FASTQ files. For Shasta Total RNA-Seq analysis, please refer to the new analyze_direct option (Section III.B).

- The analyzer takes the data sent to it by the demultiplexer and performs the following functions:
  - Read trimming (using Cutadapt)
  - Sequencing QC metrics (optional, using FastQC)
  - Genome alignment (using the STAR aligner)
  - Ribosomal, globin, and RN7SL2 reads depletion (using SortMeRNA, optional)
  - Deduplication using unique molecular identifiers (UMIs) and unique start stop positions (USSs) (only for reagent kits that employ UMIs, using UMI-tools)
  - Gene expression and transcript expression counting (using Salmon)
  - Gene fusion detection (using STAR-Fusion, optional)
  - Immune profiling analysis (using TRUST4, optional)
  - Summarization (using custom scripts)
  - Generating an HTML report (using a build-in lite version of CogentDS)
- The optional extended analyses (gene fusion and immune profiling analysis) can be launched independently, taking input directly from the analyzer output directory.

NOTE: Not all experiment types support the optional analyses. See Section V.B.2, "Optional Extended Analysis" for more information.

B. RNA-seq Analyze Direct Workflow (For Shasta Total RNA-Seq Kit Data Only)

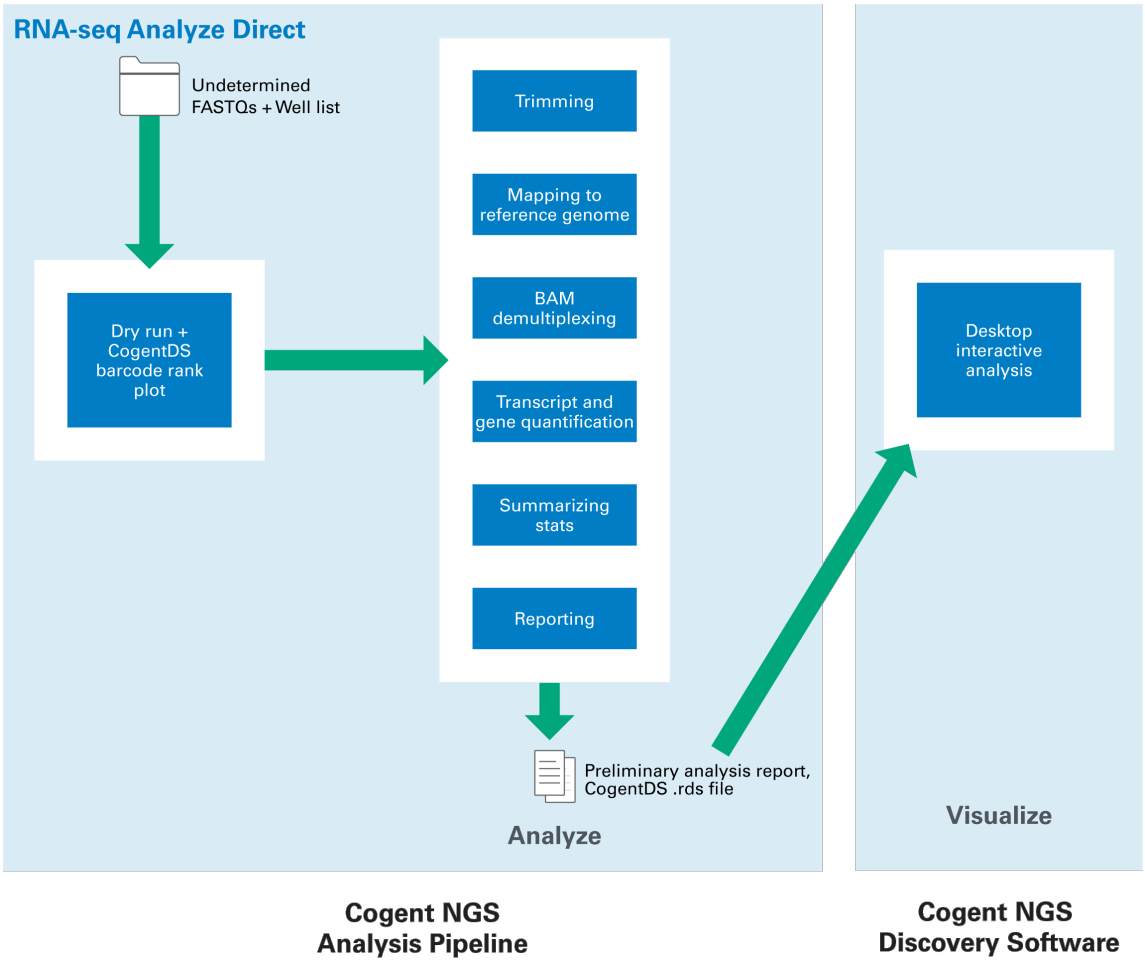


Figure 2. High-level RNA-seq Analyze Direct workflow of CogentAP and how its output can be carried over to CogentDS.

CogentAP v3.2 introduces a new workflow for analyzing data generated from the Shasta Total RNA-Seq kit that removes the need for FASTQ demultiplexing per barcode and the generation of thousands of FASTQ files during the fastq demultiplexing process. There are three main parts of the new workflow, which are summarized below.

- The demux dry run estimates the number of reads for each barcode present from the well list and outputs an estimated read counts file.
- The estimated counts file can then be analyzed in CogentDS v2.2 (scRNA application; Barcode Rank Plot mode) to generate a barcode ranks file based on algorithm defined or user-defined read threshold.
- The analyzer then uses the barcode ranks file generated by CogentDS, along with the Undetermined fastq files, to perform the following functions:
    - Read trimming (using Cutadapt)
    - Sequencing QC metrics (optional, using FastQC)
    - Genome alignment (using the STAR aligner)
    - Gene expression and transcript expression counting (using Salmon)

- Gene-body coverage analysis (optional, using RSeQC)
- Summarization of gene expression and read stats (using custom Python and R scripts)
- HTML report generation (using a build-in lite version of CogentDS)

C. DNA-seq Analysis Workflow

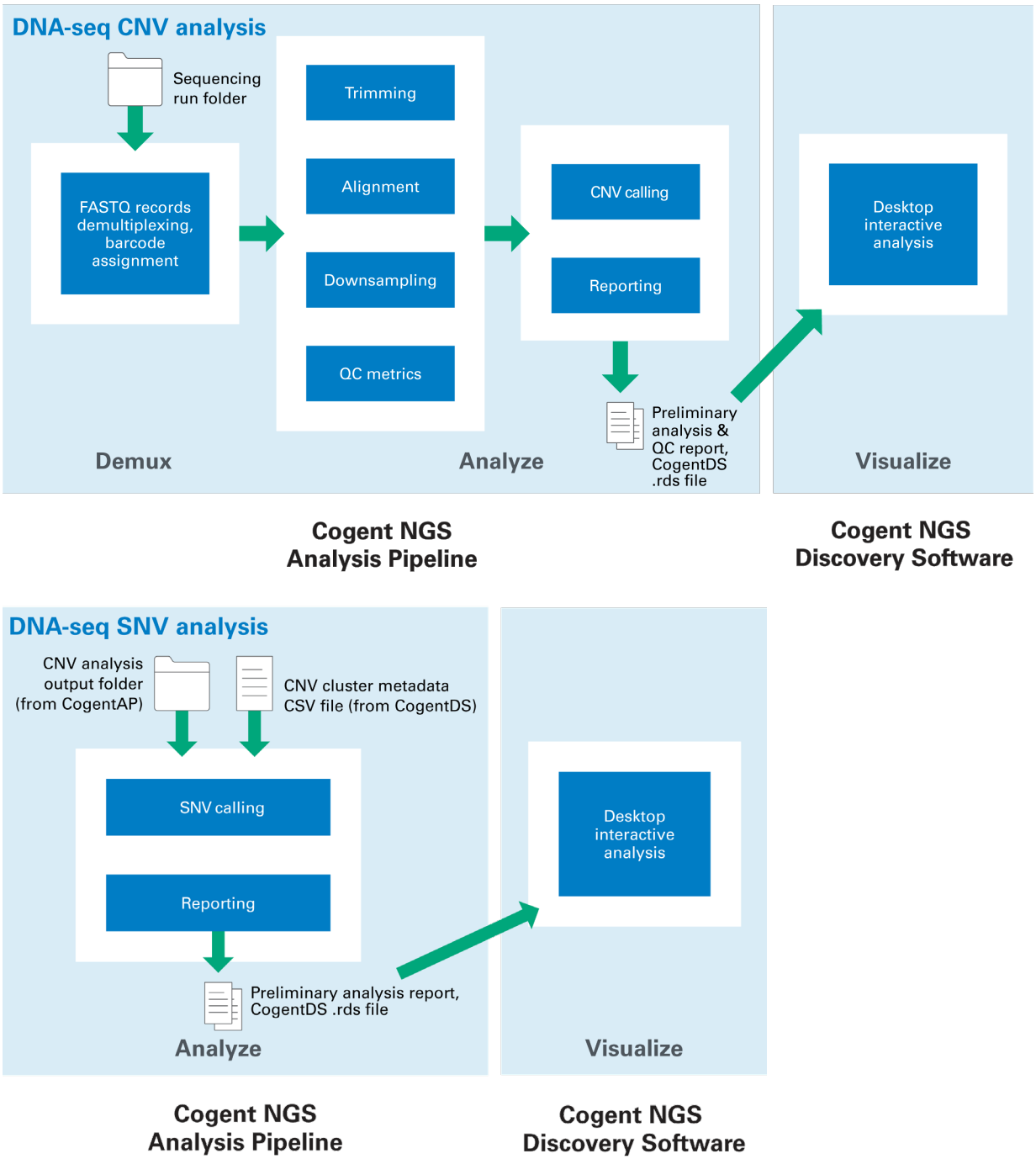


Figure 3. High-level DNA-seq analysis workflow of CogentAP and how its output can be carried over to CogentDS. CNV analysis is performed in CogentAP and CogentDS to generate required inputs for an optional SNV analysis in CogentAP.

CogentAP for DNA-seq analysis also consists of two main parts, the demultiplexer and the analyzer.

- The demultiplexer extracts the barcode from the sequencing data (based on the protocol) and writes it into FASTQ files at the end of the read name. There are two options:
  - The default behavior splits the data up into barcode-level gzip FASTQ files, which are required for input into the analyzer.
  - The second option leaves the barcode-assigned reads in combined gzip FASTQ files. This format is incompatible with CogentAP v3.2 analysis but can be used with other third-party tools if they support such FASTQ files.
- The analyzer takes the data sent to it by the demultiplexer and performs the following functions:
  - Read trimming (using Trimmomatic)
  - Genome alignment (using the Bowtie2 aligner)
  - Sequencing QC metrics (using FastQC, samtools, Picard, deepTools and MultiQC)
  - Summarization (using custom scripts)
  - CNV calling and generation of CNV-related QC plots (using Ginkgo)
  - Generating QC and Analysis reports (a built-in lite version of CogentDS and custom scripts)
  - SNV calling (using Monopogen, optional)

IV. Installation & Configuration Options

Run through the steps in this section to set up the Linux server and install CogentAP.

A. Verify the Conda Installation

1. Type the following command in at the command-line prompt in any directory location on the Linux server.

   ```
   conda -V
   ```

   If Conda is successfully installed, it should return text with the version number.

   e.g.,

   ```
   conda 24.4.0
   ```

2. Check to see if the base Conda environment can be activated. Type the following command into the prompt on the server:

   ```
   conda activate
   ```

   A successful Conda install will result in a change in the prompt, as shown in Figure 4.

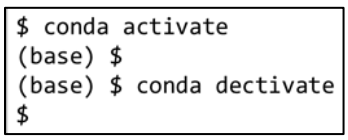


Figure 4. Screenshot of the Linux command line showing a successful check of the base Conda environment. If the Conda installation was not completed as required, both commands would return error messages.

3. If Conda is successfully installed and the prompt changed, as displayed in Figure 4, type the following command to return to the default Linux prompt:

```
conda deactivate
```

This command will take you back to the Linux prompt and out of the Conda environment.

4. Installation of miniforge3 typically adds the location of its installation to the user's system environment. This is also required for the successful installation of CogentAP.

   The following steps can be used to verify that the Conda `$PATH` is configured correctly.

   a. Open the file `.bash_profile`, which for an individual user account will be located in the home directory:

      ```
      more ~/.bash_profile
      ```

   b. Verify a line similar to the following is showing in the file:

      ```
      export PATH="/home/<USERNAME>/miniforge3/bin:$PATH"
      ```

      where `<USERNAME>` is replaced by the username of the account that installed Conda.

      e.g., username is 'myacct':

      ```
      export PATH="/home/myacct/miniforge3/bin:$PATH"
      ```

      If the line isn't displaying or the `.bash_profile` file does not exist, it will need to be manually created and populated. For more information on setting an environment variable, see a UNIX user manual or a forum post like https://stackoverflow.com/a/7502128.

B. Install Cogent NGS Analysis Pipeline v3.2

CogentAP is available for download as a compressed file from the CogentAP product page.

1. Download the installation ZIP file (`Cogent_NGS_Analysis_Pipeline_v3.2.zip`), following the directions (a) on the page seen after submitting the sign-up form on the CogentAP product page or (b) in the confirmation email sent to the email address submitted in the form.
2. Move or copy the CogentAP ZIP file onto the Linux server into the directory location where you want to install CogentAP.

   NOTE: The account logged into while doing the installation must have read/write privileges to the install directory chosen.

3. From the same directory location in Step 2, run the following two commands in the order listed:

   ```
   unzip Cogent_NGS_Analysis_Pipeline_v3.2.zip && \
   mv Cogent_NGS_Analysis_Pipeline_v3.2.zip CogentAP
   ```

   ```
   cd CogentAP
   ```

   The `CogentAP` directory contains files and directories required by the pipeline's scripts.

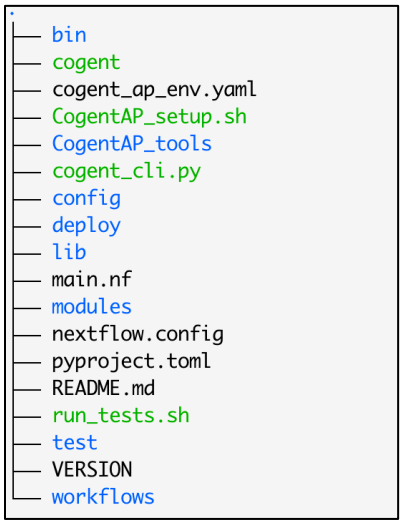


Figure 5. The sub-directory and files list of the `CogentAP` folder.

4. Run the following command to install CogentAP and its dependencies:

   ```
   bash CogentAP_setup.sh install
   ```

   Once the installation is complete, the following message will display.

   ```
   Successfully installed CogentAP pipeline and dependencies.
   Please setup genomes next. Pre-indexed genomes can be downloaded by running
   "bash CogentAP_setup.sh genome_install ${NAME}", where ${NAME} is hg38 or mm39.
   ```

Figure 6. Console message illustrating a successful CogentAP software install on the Linux server.

5. Run the following command to install the human genome build:

   ```
   bash CogentAP_setup.sh genome_install hg38
   ```

   (Optional): If you will be analyzing sequence data for *Mus musculus* (mice), run the following command to install the mouse genome build:

   ```
   bash CogentAP_setup.sh genome_install mm39
   ```

   NOTE: Each genome installation process will take approximately 1 hr to complete, depending on the computational capacity of the server and the download speed of the internet connection.

If the genome is successfully installed, a message with the text "Successfully installed genome" will be displayed. After a genome build is installed, CogentAP is ready to use.

C. (Optional) Set Up `$COGENT_AP_HOME` Environmental Variable

For ease of use, we recommend that the CogentAP install directory location be added to the `.bash_profile` as a permanent environmental variable.

Example:

If your account name is 'myacct', the absolute pathname for myacct's home directory is `/home/myacct`, and CogentAP was installed in the `~/bin` directory, edit `.bash_profile` to add the following line:

```
export COGENT_AP_HOME=/home/myacct/bin/CogentAP
```

Once added to the profile, you will either need to log out and back into the account or load the file in with the command:

```
source ~/.bash_profile
```

The phrase `$COGENT_AP_HOME` can then be used as an alias shortcut to reference `/home/myacct/bin/CogentAP`.

Example:

Running the following while logged in as 'myacct' will change directory to `~/bin/CogentAP`:

```
cd $COGENT_AP_HOME
```

NOTE: Subsequent references to `$COGENT_AP_HOME` in this document refer to the full path where the CogentAP software is installed.

D. Uninstall Previous Instances of CogentAP

NOTE: If CogentAP has never been installed on the server, skip to the next section (Section IV.E).

If an earlier version of CogentAP was installed on the server, it should be uninstalled prior to installing Cogent NGS Analysis Pipeline v3.2.

Follow the uninstall directions in the next section.

E. How to Uninstall CogentAP

CogentAP can be uninstalled by deleting the `CogentAP/` software directory from the server.

If `$COGENT_AP_HOME` has been defined in `.bash_profile`, edit the file to remove the reference to `$COGENT_AP_HOME` as well.

NOTE:

- If you've stored output files from previous analysis runs in the `CogentAP/` directory that you would like to save, make sure to move them out of the directory prior to deleting it.
- If you used an older version of this software, called mappa™ Analysis Pipeline, delete the entire `mappa/` directory to uninstall it.

V. Running the Pipeline

Before running an analysis, raw-fastq files need to be generated from the sequencer-output FASTQ files. Once the raw-fastq files are created, CogentAP can be run using the appropriate experiment type.

Tables 2 and 3 map the experiment type and corresponding kits (from Table 1) to the protocol option names/abbreviations used to select them; Table 2 is for Shasta and ICELL8 cx applications, while Table 3 lists plate-based applications.

Table 2. Shasta and ICELL8 cx experiment type options for the validated Takara Bio reagent kits.

Experiment type and supported kits	Experiment Type
Shasta, ICELL8 cx, and ICELL8 single-cell full gene-body transcriptome analysis • SMART-Seq Pro Application Kit - 2 Chip	`icell8_fla`

Shasta and ICELL8 cx single-cell full gene-body total RNA-seq analysis • Shasta Total RNA-Seq Kit - 2 Chip	`shasta_total_rna`
Shasta and ICELL8 cx single-cell whole-genome amplification analysis • Shasta Whole-Genome Amplification Kit – 2 Chip	`shasta_wga`

Table 3. Plate-based experiment type options for the validated Takara Bio reagent kits.

Experiment type and supported kits	Experiment Type
Plate-based full gene-body transcriptome analysis with UMIs • SMART-Seq mRNA LP (with UMIs)	`smartseq_fla_umi`
Plate-based full gene-body transcriptome analysis • SMART-Seq mRNA LP • SMART-Seq mRNA • SMART-Seq mRNA Single Cell LP • SMART-Seq mRNA Single Cell • SMART-Seq mRNA HT LP • SMART-Seq mRNA HT • SMART-Seq v4 PLUS Kit • SMART-Seq v4 Ultra Low Input RNA Kit • SMART-Seq Single Cell PLUS Kit • SMART-Seq Single Cell Kit • SMART-Seq HT PLUS Kit • SMART-Seq HT	`smartseq_fla`
Plate-based strand-specific total RNA-seq for mammalian samples (with UMIs) • SMART-Seq Total RNA Pico Input with UMIs (ZapR Mammalian) • SMARTer Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian	`stranded_umi`
Plate-based strand-specific RNA-seq (no UMIs) • SMART-Seq Total RNA Pico Input (ZapR Mammalian) • SMART-Seq Total RNA Single Cell (ZapR Mammalian) • SMART-Seq Stranded Kit • SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian	`stranded`
Plate-based single-cell genome and transcriptome analysis (no UMIs) • Embgenix GT-omics Kit	`shasta_wga` (genome) `smartseq_fla` (transcriptome)

Optional extended RNA-seq analysis (Section V.B.2)

Gene fusion detection and immune profiling analysis are explicitly included to leverage our full gene-body chemistry advantages over the other 3′ chemistries; refer to Section V.B.2 for how to run these extended analyses. While we provide these analyses for our other chemistries, we do not recommend it for them.

For an overview of analysis types available in CogentAP for the Takara Bio chemistries supported by the software, refer to the table on the bioinformatics portal on our website.

A. Generation of raw-fastq Files

The CogentAP demultiplexer takes one pair of raw-fastq files as input (i.e., not split by barcode). The following procedure converts the sequencer FASTQ output files into the format expected by CogentAP using bcl2fastq or BCL Convert.

1. Log in to the server that stores the sequencing run output folder. This server will typically have bcl2fastq or bclconvert installed (see Section II.D for more information about bcl2fastq and bclconvert).
2. Change to the directory where you want the raw-fastq files to be created.
3. From the server where CogentAP is installed, copy the `SampleSheet_dummy_bcl2fastq.csv` file, located in the `$COGENT_AP_HOME/config` folder, into the directory selected in Step 2.
4. Run bcl2fastq or BCL Convert:

   If running bcl2fastq, use the following syntax:

   ```
   bcl2fastq -R <RUN_FOLDER> \
    -o <RUN_ID> \
    --no-lane-splitting \
    --sample-sheet SampleSheet_dummy_bcl2fastq.csv <RUN_ID>.stdout \
    2 > <RUN_ID>.stderr
   ```

   where:

   a. `<RUN_FOLDER>` is the path to the sequencing run folder and
   b. `<RUN_ID>` is the ID automatically generated by Illumina sequencer

   NOTE: Some versions of bcl2fastq have a bug where the indexes required for demultiplexing will not be inserted into the raw-fastq if a sample sheet file is not specified in the command syntax. To prevent encountering the issue, we recommend always using the `SampleSheet_dummy_bcl2fastq.csv` option when generating the raw-fastq files from the bcl2fastq command.

   If running BCL Convert, use the following syntax template:

   ```
   bcl-convert --bcl-input-directory <RUN_FOLDER> \
    --output-directory <RUN_ID> --no-lane-splitting \
    --sample-sheet=DummySampleSheet \
    > <RUN_ID>.stdout 2 > <RUN_ID>.stderr
   ```

   where:

   c. `<RUN_FOLDER>` is the path to the sequencing run folder and
   d. `<RUN_ID>` is the ID automatically generated by Illumina sequencer

   NOTE: Templates for the DummySampleSheet for BCL Convert can be found in `$COGENT_AP_HOME/config`. Modify the read and index lengths as necessary based on the documentation for BCL Convert from Illumina's website.

   NOTE: NextSeq 1000/2000 and NovaSeq X/X Plus data are not supported by bcl2fastq and require BCL Convert.

5. Retrieve the raw-fastq files from the `<RUN_ID>` folder located in the working directory from Step 2. These are typically named in the syntax `Undetermined_*.fastq.gz`.

NOTE: To reduce downstream processing time, we recommend that the raw-fastq files are moved to a directory on the server where CogentAP is installed.

B. RNA-Seq Analysis

NOTE: See Section VI for an example of the full syntax for the command-line scripts.

For RNA-seq analysis, CogentAP starts from the main script, `cogent`, and has defined subcommands, listed below.

Demux and analyze

- `rna demux`
- `rna analyze`

Analyze (without demux, Shasta Total RNA-Seq kit data only)

- `rna analyze_direct`

Optional extended analysis

- `rna postprocess immune`
- `rna postprocess fusion`

Additional commands

- `rna add_genome`

These scripts can be launched from any location (working directory) on the Linux server where the CogentAP software is installed. The full list of arguments can be accessed using the syntax:

```
$COGENT_AP_HOME/cogent <COMMAND> -h
```

The `rna demux` and `rna analyze` commands are the core functionality of the RNA-seq analysis and are described below. Section V.B.2 covers the extended analysis options, while Section V.B.3 describes the additional available commands.

NOTE: If analyzing data from the Shasta Total RNA-Seq kit, read counts for all the barcodes in the experiment must be estimated before running downstream analysis. This process is outlined in Section V.B.1.b), "RNA Dry Run (For Shasta Total RNA-Seq Kit Data)", below.

1. Primary Analysis Commands

a) RNA Demux (For Analysis of Data from All Kits Except the Shasta Total RNA-Seq Kit)

In general, the demuxer (`cogent rna demux`) is run first to generate demultiplexed FASTQ files.

To demultiplex (demux) RNA-seq data:

```
$COGENT_AP_HOME/cogent rna demux \
  -f <FASTQ_R1> \
  -p <FASTQ_R2> \
  -b <WELL-LIST> \
```

```
  -t <EXP_TYPE> \
  -o <DEMUX_OUTPUT_DIR>
```

where:

- <FASTQ_R1> and <FASTQ_R2> are the full paths to the FASTQ files generated by an Illumina sequencing platform.
- <WELL-LIST> is the full path to the Shasta or ICELL8 system well list, Illumina's sample sheet, or TSV/CSV format file
- <EXP_TYPE> is the experiment type used (e.g., icell8_fla; refer to Tables 2 and 3 for options)
- <DEMUX_OUTPUT_DIR> is the full path of the demultiplex results directory

The full list of `rna demux` arguments are listed in Table 4 and a screenshot of the output `$COGENT_AP_HOME/cogent rna demux -h` is shown in Figure 7.

Table 4. Full list of options under `cogent rna demux -h`.

Option	Description	Default
`-h, --help`	(Optional) Produces a help message.	N/A
`-f, --fastq1`	(Required) Specifies the input Read1 (R1) FASTQ file.	N/A
`-p, --fastq2`	(Required) Specifies the input Read2 (R2) FASTQ file.	N/A
`-t, --type_of_experiment`	(Required) Specifies the experimental protocol (See Tables 2 and 3 for experimental protocols based on reagent kit).	N/A
`-o, --output_dir`	(Required) Indicates the path to the application output.	N/A
`-b, --barcodes-file`	(Required) Specifies path to the well-list file from CellSelect software or another custom file containing only barcodes that were selected for sequencing.	N/A
`--fastqc`	(Optional) Runs FASTQC to create quality reports for FASTQ files.	disabled
`-m, --mismatch`	(Optional) Specifies the number of allowed mismatched bases per barcode.	1
`-u, --umi_length`	(Optional) Overwrites the UMI length associated with the experimental type.	Calculated based on experiment type
`-n, --n_processes`	(Optional) Specifies the number of demultiplexing processes to spawn during execution. The maximum value (*N*) should not	15 (Fixed at 3 when `--no_split_fastqs` is used)

Option	Description	Default
	exceed the number of CPUs on the server.	
`--n_writers`	(Optional) Specifies the number of demultiplexing writing processes to spawn during execution. The maximum value should be less than or equal to N-2.	8 (Fixed at 1 when `--no_split_fastqs` is used)
`--no_gz`	(Optional) Specifies not to compress (gzip) output FASTQ files.	FASTQ files are compressed
`--i7_rc`	(Optional) Reverse-complement I7 Index. Default of "auto" detects and auto-corrects the reverse complement of I7 indices by certain Illumina sequencers. Otherwise, manually override with "true" or "false"*.	auto
`--i5_rc`	(Optional) Reverse-complement I5 Index. Enter "auto" to detect and auto-correct the reverse complement of I5 indices by certain Illumina sequencers. Otherwise, manually override with "true" or "false"*.	auto
`--read_buffer`	(Optional) Specifies buffer size of the data sent to each demultiplexing process in GB.	0.1
`--prog`	(Optional) Specifies number of reads to process before updating in the log file.	10,000,000
`--no_split_fastqs`	(Optional) Output merged FASTQ file(s). Barcodes are written into read names and merged into a single pair of large FASTQ files.	disabled
`--check_reads`	(Optional) Uses a specified number of reads to estimate read counts during barcode selection.	200,000,000
`--random_pick`	(Optional) Picks random reads during barcode selection.	disabled
`--seqtk_2pass`	(Optional) Uses two-pass mode to reduce memory usage during random picking but increases compute time. This can be selected if running the Cogent process on a Linux machine with memory constraints.	disabled
`--preview`	(Optional) Prints the Nextflow command without executing it to	N/A

Option	Description	Default
	verify parameters before starting the pipeline.	

*Manually specify "true" or "false" when you definitively know the orientation of I7 or I5 indexes in the well-list file. "True" automatically treats indexes as reverse-complement and corrects all indexes in the well-list file.

```
usage: cogent rna demux [-h] -f FASTQ1 -p FASTQ2 -t
                        {stranded,stranded_umi,smartseq_fla,smartseq_fla_umi,icell8_fla,smartseq_pro,shasta_total_rna} -o
                        OUTPUT_DIR -b BARCODES_FILE [--fastqc] [-m {0,1}] [-u UMI_LENGTH] [-n N_PROCESSES]
                        [--n_writers N_WRITERS] [--no_gz] [--i7_rc {auto,true,false}] [--i5_rc {auto,true,false}]
                        [--read_buffer READ_BUFFER] [--prog PROG] [--no_split_fastqs] [--use_barcodes USE_BARCODES]
                        [--check_reads CHECK_READS] [--min_reads MIN_READS] [--random_pick] [--seqtk_2pass] [--dry_run]
                        [--preview]

            Script to de-multiplex barcoded reads from sequence data stored in
            FASTQ files. User options are designed to simplify de-multiplexing
            for experiments derived from Takara Bio protocols. Barcode (and optionally
            UMI) sequences are extracted and stored in the read name. Users may
            specify whether the resulting de-multiplexed data are merged, or split
            into individual barcode-level files.

options:
  -h, --help            show this help message and exit
  -f FASTQ1, --fastq1 FASTQ1
                        Input Read1 (R1) FASTQ file.
  -p FASTQ2, --fastq2 FASTQ2
                        Input Read2 (R2) FASTQ file.
  -t {stranded,stranded_umi,smartseq_fla,smartseq_fla_umi,icell8_fla,smartseq_pro,shasta_total_rna}, --type_of_experiment {strande
d,stranded_umi,smartseq_fla,smartseq_fla_umi,icell8_fla,smartseq_pro,shasta_total_rna}
                        Experimental protocol used.
  -o OUTPUT_DIR, --output_dir OUTPUT_DIR
                        Name of output directory to store results. The directory must have write permissions, and its parent
                        directory must be accessible.
  -b BARCODES_FILE, --barcodes_file BARCODES_FILE
                        Well List file from Takara Bio's CellSelect Software (Recommended), or another custom file containing
                        only barcodes that were selected for sequencing.
  --fastqc              Run FASTQC to create quality reports for FASTQ files.
  -m {0,1}, --mismatch {0,1}
                        Number of allowed mismatched bases per barcode.
  -u UMI_LENGTH, --umi_length UMI_LENGTH
                        Overwrite UMI length. By default, length is automatically determined by experiment type.
  -n N_PROCESSES, --n_processes N_PROCESSES
                        Number of demultiplexing processes to spawn during execution.
  --n_writers N_WRITERS
                        Number of demultiplexing writing processes to spawn during execution.
  --no_gz               Do not compress (gzip) output FASTQ files.
  --i7_rc {auto,true,false}
                        Reverse-complement I7 Index (Full Length protocol only). Enter "auto" to detect and auto-correct the
                        reverse complementation of I5/I7 indices by certain Illumina sequencers. Otherwise manually override
                        with "True" or "False").
  --i5_rc {auto,true,false}
                        See help section for "--i7_rc".
  --read_buffer READ_BUFFER
                        Buffer size of data sent to each demultiplexing (worker) process in GB.
  --prog PROG           Number of reads to process before updating status in log file.
  --no_split_fastqs     Output merged FASTQ file(s). Barcodes are written into read names and merged into large FASTQ file. By
                        default output into barcode-level FASTQ files.
  --use_barcodes USE_BARCODES
                        Limit number of barcodes to this value.
  --check_reads CHECK_READS
                        Use this number of reads to estimate read counts during barcode selection.
  --min_reads MIN_READS
                        Discard barcodes with estimated read count lower than this number.
  --random_pick         Pick random reads during barcode selection, rather than analyzing the first N read pairs.
  --seqtk_2pass         Use 2-pass mode to reduce the memory when random picking.
  --dry_run             Output estimated counts of all barcodes, but do not write demultiplexed FASTQs.
  --preview             Print nextflow command without executing it.
```

Figure 7. The output of `cogent rna demux -h` at the command line.

b) RNA Demux Dry Run (For Shasta Total RNA-Seq Kit Data)

For data derived from Shasta Total RNA-Seq kit, read counts for all the barcodes in the experiment must be estimated before running the demux process. To perform the estimation, demultiplex using the `--dry_run` mode.

```
$COGENT_AP_HOME/cogent rna demux \
  --dry_run \
  -f <FASTQ_R1> \
  -p <FASTQ_R2> \
  -b <WELLLIST> \
  -t shasta_total_rna \
  -o <OUTPUT>
```

where:

- **<FASTQ_R1>** and **<FASTQ_R2>** are the full paths to the FASTQ files generated by an Illumina sequencing platform.
- **<WELLLIST>** is the full path to the Shasta Total RNA well list, located at `$COGENT_AP_HOME/config/well_list_shasta_total_rna.csv`.
- **<OUTPUT>** is a string; it will be the name of the output folder created by the analysis.

The estimated counts for each barcode can be found in the path listed below and can be imported into CogentDS to generate a knee plot for determining the optimal and/or minimum number of reads required to retain a barcode for downstream analysis:

<OUTPUT>`/demultiplexed_fastqs/demultiplexed_fastqs_counts_all.estimated.csv`

c) RNA Analyze Direct (Primary Shasta Total RNA-Seq Kit Analysis Method)

NOTE: This is the recommended method of analysis for Shasta Total RNA-Seq kit data and is not applicable to any other kit type.

FASTQ files for the Shasta Total RNA-Seq kit do not need to be demultiplexed prior to running analysis and can be analyzed using the `analyze_direct` workflow option. This workflow speeds up the analysis time and removes the need to generate separate FASTQ files for each barcode. The estimated counts file generated from the RNA Demux Dry Run (Section V.B.1.b, above) needs to be used to generate a barcode ranks output file in CogentDS (see Section VI.C, "Barcode Rank Plot" in the Cogent NGS Discovery Software User Manual).

The full list of rna analyze_direct control options are listed in Table 5 and a screenshot of the output of `$COGENT_AP_HOME/cogent rna analyze_direct -h` is shown in Figure 8.

Table 5. Full list of options under `cogent rna analyze_direct -h`.

Option	Description	Default
`-h, --help`	(Optional) Produces a help message.	N/A
`-f, --fastq1`	(Required) Specifies the input Read1 (R1) FASTQ file.	N/A

Option	Description	Default
-p, --fastq2	(Required) Specifies the input Read2 (R2) FASTQ file.	N/A
-t, --type_of_experiment	(Required) Specifies the experimental protocol (shasta_total_rna)	N/A
-o, --output_dir	(Required) Indicates the path to the application output.	N/A
-b, --barcodes-file	(Required) Specifies path to the well-list file from CellSelect software or another custom file containing only barcodes that were selected for sequencing.	N/A
-g, --genome	(Required) Allows for selection of a supported genome or custom genome that you have installed.	N/A
--cogentds_barcode_ranks	(Required) Specifies path to the barcode ranks file generated from CogentDS Barcode Rank Plot module	N/A
-G, --genome_dir	(Optional) Specifies the directory where the genome and index files are installed.	$COGENT_AP_HOME/genomes
--i7_length	(Optional) Length of the i7 index.	8
--i5_length	(Optional) Length of the i5 index.	8
--keep_intermediate	(Optional) Saves intermediate files from analysis such as BAM files.	disabled
--fastq_chunk_size	(Optional) Specifies the number of reads to keep in a FASTQ chunk for parallelization.	100000000
--bam_chunk_size	(Optional) Specifies the number of reads to keep in memory before writing to disk during bam demultiplexing.	25000000
--sample_bam_size	(Optional) Number of reads to keep per sample bam for gene-body coverage plots.	2000000
--sample_layout	(Required, defaults to a dummy layout file) Path to 96-well layout file for barcode-sample mapping.	$COGENT_AP_HOME/config/barcode_maps/dummy_sample_layout.csv
--gene_body_coverage	(Optional) Perform gene-body coverage analysis per sample	false
--resume	(Optional) Resumes a previous pipeline run with the same inputs.	N/A
--preview	(Optional) Prints the Nextflow command without executing it to verify parameters before starting the pipeline.	N/A

```
usage: cogent rna analyze_direct [-h] -g {hg38,mm39} [-G GENOME_DIR] -o OUTPUT_DIR -t {shasta_total_rna} -b BARCODES_FILE -f
                                 FASTQ1 -p FASTQ2 [--cogentds_barcode_ranks COGENTDS_BARCODE_RANKS] [--keep_intermediate]
                                 [--fastq_chunk_size FASTQ_CHUNK_SIZE] [--bam_chunk_size BAM_CHUNK_SIZE]
                                 [--sample_bam_size SAMPLE_BAM_SIZE] [--i7_length I7_LENGTH] [--i5_length I5_LENGTH]
                                 [--sample_layout SAMPLE_LAYOUT] [--gene_body_coverage {true,false}] [--preview] [--resume]

            Script to perform analysis of Shasta Total RNA-Seq data.
            This workflow skips demultiplexing of fastqs. Only data from
            Shasta Total RNA-Seq experiments are supported.
            The modules currently included are:
                - Trimming (cutadapt)
                - Alignment (STAR)
                - Counting (Salmon)
                - Summarization (TBUSA)
                - Reporting (TBUSA, CogentDS)

options:
  -h, --help            show this help message and exit
  -g {hg38,mm39}, --genome {hg38,mm39}
                        Select a supported genome or provide the name of a custom genome that you installed.
  -G GENOME_DIR, --genome_dir GENOME_DIR
                        Directory where genome and index files were installed by add_genome. [Default: $COGENT_ROOT/genomes]
  -o OUTPUT_DIR, --output_dir OUTPUT_DIR
                        Name of output directory to store results. The directory must have write permissions, and its parent
                        directory must be accessible.
  -t {shasta_total_rna}, --type_of_experiment {shasta_total_rna}
                        Experimental protocol used.
  -b BARCODES_FILE, --barcodes_file BARCODES_FILE
                        Well List file from Takara Bio's CellSelect Software (Recommended), or another custom file containing
                        only barcodes that were selected for sequencing.
  -f FASTQ1, --fastq1 FASTQ1
                        Input Read1 (R1) FASTQ file.
  -p FASTQ2, --fastq2 FASTQ2
                        Input Read2 (R2) FASTQ file.
  --cogentds_barcode_ranks COGENTDS_BARCODE_RANKS
                        Barcode Rank Plot output (.csv) from CogentDS Barcode Rank Plot module
  --keep_intermediate   Save genome and transcriptome BAM files from STAR and other intermediate files from the analysis
  --fastq_chunk_size FASTQ_CHUNK_SIZE
                        Number of reads to keep in a fastq chunk for parallelization [Default: 100000000]
  --bam_chunk_size BAM_CHUNK_SIZE
                        Number of reads to keep in memory before writing to disk during bam demultiplexing [Default: 25000000]
  --sample_bam_size SAMPLE_BAM_SIZE
                        Number of reads to keep per sample bam for gene body coverage plots [Default: 2000000]
  --i7_length I7_LENGTH
                        i7 index length [Default: 8]
  --i5_length I5_LENGTH
                        i5 index length [Default: 8]
  --sample_layout SAMPLE_LAYOUT
                        96 well layout file for barcode - sample mapping. Example sample sheet is located in
                        config/barcode_maps/dummy_sample_layout.csv.If no sample layout is provided, the dummy sample layout
                        will be used.
  --gene_body_coverage {true,false}
                        Perform gene body coverage analysis per sample [Default: false]
  --preview             Print nextflow command without executing it.
  --resume              Resume a previous pipeline run with the same inputs
```

Figure 8. The output of `cogent rna analyze_direct -h` at the command line.

NOTE: The `analyze_direct` workflow option is not compatible with fusion or immune postprocess analyses. If fusion or immune analysis is needed for Shasta Total RNA-Seq kit data, please use the conventional FASTQ demultiplexing approach listed below in Section V.B.1.d) and Section V.B.1.e).

(Optional) Gene-body coverage analysis

To perform an optional gene-body coverage analysis, the flag `--gene_body_coverage` can be set to `true` while running the `analyze_direct` workflow. Currently, gene-body coverage analysis is supported for Shasta Total RNA-Seq sequencing data with the `analyze_direct` option. Two gene-body coverage plots are reported:

- where coverage is normalized with respect to maximum value
- where coverage is normalized with respect to mean value. (see example in Figure 9 below)

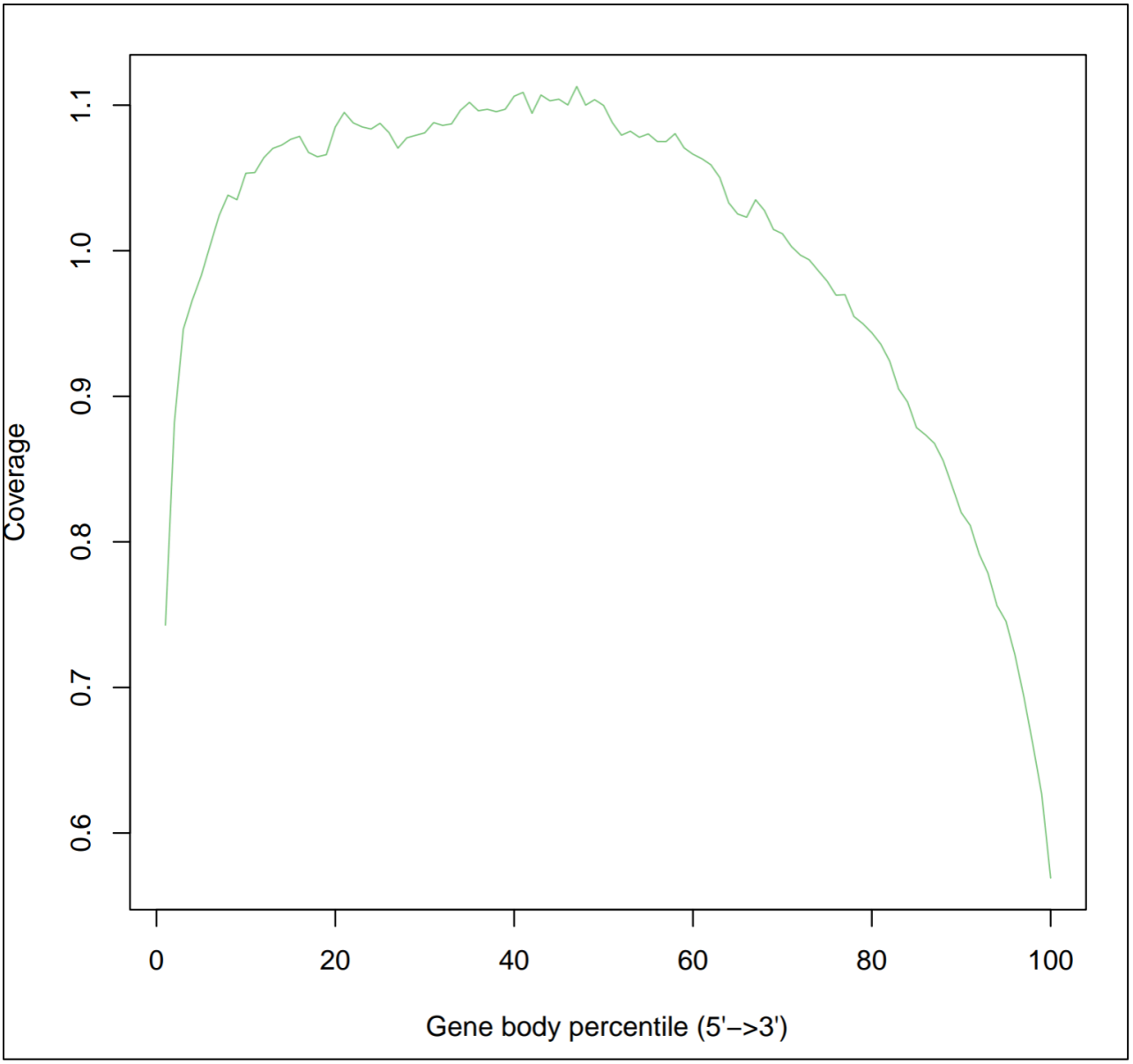


Figure 9. Gene-body coverage plot. This plot shows normalized read coverage across gene bodies (from 5′ to 3′) for each sample.

d) RNA Demux (Shasta Total RNA-Seq Kit–Second Option)

NOTE: This is the conventional analysis method for Shasta Total RNA-Seq kit data, which uses FASTQ demultiplexing. RNA Demux Dry Run (Section V.B.1.b) is required before proceeding with this method.

To demux into a pair of FASTQs per barcode, set the number of barcodes to keep (`--use_barcodes`) and/or the minimum number of reads required (`--min_reads`) to keep a barcode, based on the estimated counts of all barcodes from the demux dry run generated in Section V.B.1.b). The more stringent of these two parameters will be applied as the filter to select the barcodes in the demux process.

```
$COGENT_AP_HOME/cogent rna demux \
  --use_barcodes <NUMBER OF BARCODES TO KEEP> \
  --min_reads <MINIMUM NUMBER OF READS REQUIRED TO KEEP A BARCODE> \
  -f <FASTQ_R1> \
  -p <FASTQ_R2> \
  -b <WELLLIST> \
  -t shasta_total_rna \
  -o <OUTPUT>
```

e) RNA Analyze

The resulting directory of FASTQ files from `cogent rna demux` (Section V.B.1.d [for the Shasta Total RNA-Seq kit] or V.B.1.a [for other kits]) is used as input to run the analyzer (`cogent rna analyze`) to obtain the output files described in Section VII.

To analyze RNA-seq data:

```
$COGENT_AP_HOME/cogent rna analyze \
   -i <DEMUX_OUTPUT_DIR> \
   -g <GENOME> \
   -t <EXP_TYPE> \
   -o <ANALYSIS_OUTPUT_DIR>
```

where:

- `<DEMUX_OUTPUT_DIR>` is the full path of the demultiplex results directory
- `<GENOME>` is a name of genome build (e.g., `hg38`)
- `<EXP_TYPE>` is the experiment type used (e.g., `icell8_fla`; refer to Tables 2 and 3 for options)
- `<ANALYSIS_OUTPUT_DIR>` is the full path to the output directory created for the analysis results

The full list of `rna analyze` control options are listed in Table 6 and a screenshot of the output of `$COGENT_AP_HOME/cogent rna demux -h` is shown in Figure 10. For an example on how to run analysis on the test dataset, see Section VI.

Table 6. Full list of options under `cogent rna analyze -h`.

Option	Description	Default
`-h, --help`	(Optional) Produces a help message.	N/A
`-g, --genome`	(Required) Allows for selection of a supported genome or custom genome that you have installed.	N/A
`-o, --output_dir`	(Required) Specifies the output directory in which to store the results of the pipeline.	N/A
`-i, --input_dir`	(Required) Specifies the input directory that contains the results from `rna demux`.	N/A
`-G, --genome_dir`	(Optional) Specifies the directory where the genome and index files are installed.	`$COGENT_AP_HOME/genomes`
`--fastqc`	(Optional) Runs FASTQC to create quality reports for FASTQ files.	disabled
`-t, --type_of_experiment`	(Required) Specifies experimental protocol to be used.	N/A
`--immune`	(Optional) Generates immune profiling matrix.	disabled
`--fusion`	(Optional) Generates gene fusion matrix.	disabled
`--ribodeleption`	(Optional) Removes ribosomal RNA reads*. Options are: `auto`, `true`, or `false`.	auto
`--globindeleption`	(Optional) Removes globin/RN7SL2 reads. Options can be `true` or `false`. **NOTE:** Can only be enabled when `--ribodepletion` is also enabled.	false
`--keep_intermediate`	(Optional) Saves intermediate files from analysis such as BAM files.	disabled
`--resume`	(Optional) Resumes a previous pipeline run with the same inputs.	N/A
`--preview`	(Optional) Prints the Nextflow command without executing it to verify parameters before starting the pipeline.	N/A

***NOTE**: The `--ribodepletion` parameter is set to 'auto' by default. 'auto' mode enables in silico ribodepletion on all RNA kits except for the Shasta Total RNA-Seq kit, for which it is disabled by default. To enable ribodepletion during Shasta Total RNA-Seq kit analysis, specify the `--ribodepletion` parameter as 'true'. To disable ribodepletion in other kits, set `--ribodepletion` to 'false'.

```
usage: cogent rna analyze [-h] -g {hg38,mm39} [-G GENOME_DIR] -o OUTPUT_DIR -i INPUT_DIR [--fastqc]
                          -t
                          {stranded,stranded_umi,smartseq_fla,smartseq_fla_umi,icell8_fla,smartseq_pr
o,shasta_total_rna}
                          [--immune] [--fusion] [--ribodepletion {auto,true,false}]
                          [--globindepletion {true,false}] [--preview] [--resume]

            Script to perform counting analysis for exons and genes by fastq input data.
            The input to this script are files output by Cogent demux.
            The fastq files are expected to contain the barcode info in the read name.
            Optionally it can also contain UMI info following the BC.
            The modules currently included are:
                - Trimming (cutadapt)
                - Alignment (STAR)
                - Counting (featureCounts)
                - Summarization (TBUSA)
                - Reporting (TBUSA, CogentDS)

options:
  -h, --help            show this help message and exit
  -g {hg38,mm39}, --genome {hg38,mm39}
                        Select a supported genome or provide the name of a custom genome that you
                        installed.
  -G GENOME_DIR, --genome_dir GENOME_DIR
                        Directory where genome and index files were installed by add_genome.
                        [Default: $COGENT_ROOT/genomes]
  -o OUTPUT_DIR, --output_dir OUTPUT_DIR
                        Name of output directory to store results. The directory must have write
                        permissions, and its parent directory must be accessible.
  -i INPUT_DIR, --input_dir INPUT_DIR
                        Directory contains results from demux command. The directory must contain
                        FASTQ files after demultiplexing.
  --fastqc              Run FASTQC to create quality reports for FASTQ files.
  -t {stranded,stranded_umi,smartseq_fla,smartseq_fla_umi,icell8_fla,smartseq_pro,shasta_total_rna},
--type_of_experiment {stranded,stranded_umi,smartseq_fla,smartseq_fla_umi,icell8_fla,smartseq_pro,sha
sta_total_rna}
                        Experimental protocol used.
  --immune              Generate immune profiling matrix.
  --fusion              Generate gene fusion matrix.
  --ribodepletion {auto,true,false}
                        Counting and removal of ribosomal RNA. Enter "auto" for depletion based on
                        kit type.Otherwise manually override with "true" or "false".
  --globindepletion {true,false}
                        Counting and removal of globin and RN7SL2 RNA along with ribosomal. Only
                        applicable for the human genome.
  --preview             Print nextflow command without executing it.
  --resume              Resume a previous pipeline run with the same inputs
```

Figure 10. The output of `cogent rna analyze -h` at the command line.

2. Optional Extended Analysis

a) Gene Fusion Analysis

Gene fusion analysis is launched by the command

```
$COGENT_AP_HOME/cogent rna postprocess fusion
```

You can also launch this as an option while running the analyzer with the option `--fusion`

```
$COGENT_AP_HOME/cogent rna analyze --fusion
```

to launch gene fusion analysis at the same time. The options for gene fusion analysis can be viewed with the option `-h` (Figure 11).

```
$COGENT_AP_HOME/cogent rna postprocess fusion -h
```

The resulting `CogentDS_analysis.rds` file includes gene fusion detection and all other analysis done with the `rna analyze` command.

NOTE: The Rdata file from gene fusion analysis is only generated when run as an option when running the pipeline. When launched as a standalone gene fusion analysis, the resulting output files (in MTX format) cannot currently be used for downstream analysis with CogentAP or CogentDS.

```
usage: cogent rna postprocess fusion [-h] -i INPUT_DIR -o OUTPUT_DIR -g {hg38,mm39} [-G GENOME_DIR] [--resume] [--preview]

       A command to perform gene fusion detection analysis.
       The input to this command is result directory from analyze command.
       The directory is expected to contain junction information files (.Chimeric.out.junction) and stats.csv
       This analysis ignores UMI even if UMI enabled experiment type is specified.

options:
  -h, --help            show this help message and exit
  -i INPUT_DIR, --input_dir INPUT_DIR
                        Directory contains results from analyze command. The directory must contain genematrix and *_stats.csv.
  -o OUTPUT_DIR, --output_dir OUTPUT_DIR
                        Name of output directory to store results.
  -g {hg38,mm39}, --genome {hg38,mm39}
                        Select a supported genome or provide the name of a custom genome that you installed.
  -G GENOME_DIR, --genome_dir GENOME_DIR
                        Directory where genome and index files were installed by add_genome. [Default: $COGENT_ROOT/genomes]
  --resume              Resume a previous pipeline run with the same inputs
  --preview             Print nextflow command without executing it.
```

Figure 11. The output of `cogent rna postprocess fusion -h` at the command line.

b) Immune Profiling Analysis

Immune profiling analysis is launched by the command

```
$COGENT_AP_HOME/cogent rna postprocess immune
```

You can also launch this as an option while running the analyzer with the option `-- immune`

```
$COGENT_AP_HOME/cogent analyze --immune
```

to launch immune profiling analysis at the same time. The options immune profiling analysis can be viewed with the option `-h` (Figure 12).

```
$COGENT_AP_HOME/cogent rna postprocess immune -h
```

The resulting `CogentDS_analysis.rds` file includes detected clonotypes and all other analysis done with the `analyze` command.

NOTE: The Rdata file from immune profiling analysis is only generated when run as an option when running the analyzer. When launched as a standalone immune analysis, the resulting output files cannot be currently used for downstream analysis with CogentAP or CogentDS.

```
usage: cogent rna postprocess immune [-h] -i INPUT_DIR -o OUTPUT_DIR -g {hg38,mm39} [-G GENOME_DIR] [--resume] [--preview]

       A command for perfoming immune-profiling on split fastqs.
       The input to this script are files output by Cogent demux.
       The fastq files are expected to contain the barcode info in the read name.
       The modules currently included are:
           -- read assembly & clonotype identification (Trust4)

options:
  -h, --help            show this help message and exit
  -i INPUT_DIR, --input_dir INPUT_DIR
                        Directory contains results from analyze command. The directory must contain genematrix and *_stats.csv.
  -o OUTPUT_DIR, --output_dir OUTPUT_DIR
                        Name of output directory to store results.
  -g {hg38,mm39}, --genome {hg38,mm39}
                        Select a supported genome or provide the name of a custom genome that you installed.
  -G GENOME_DIR, --genome_dir GENOME_DIR
                        Directory where genome and index files were installed by add_genome. [Default: $COGENT_ROOT/genomes]
  --resume              Resume a previous pipeline run with the same inputs
  --preview             Print nextflow command without executing it.
```

Figure 12. The output of `cogent rna postprocess immune -h` at the command line.

3. Adding a Genome Build

The human and mouse genome builds available from our server (Section IV.B, "Install Cogent NGS Analysis Pipeline v3.2") are recommended for use in the pipeline, but genomes of other species can be added into the software post-install.

NOTE: Extended analysis for gene fusion detection or immune profiling is not supported for custom genome builds added through this process.

To add custom genome data to CogentAP:

1. Create a copy of the file

   `$COGENT_AP_HOME/config/genome_sources/sample.config`

   and rename it

   `$COGENT_AP_HOME/config/genome_sources/<common_species_name>.config`

   where `<common_species_name>` is the name of the genome being added (e.g., `dm6`)

2. Update the following fields using a text editor:

a. Replace 'GENOME' with the value of `<common_species_name>` from Step 1 (e.g., `dm6`).

b. Replace 'ENSEMBL_GENOME_FASTA_URL' with the public URL of the FASTA file containing all the sequences (chromosomes and contigs) from Ensembl.

   Using the fruit fly genome from Ensembl.org as an example, you would replace 'ENSEMBL_GENOME_FASTA_URL' with the following URL:

   ```
   https://ftp.ensembl.org/pub/release-113/fasta/drosophila_melanogaster/dna/Drosophila_melanogaster.BDGP6.46.dna_sm.toplevel.fa.gz
   ```

c. Replace 'ENSEMBL_GTF_URL' with the public URL of the GTF file containing the annotation and, importantly, the gene information for analysis from Ensembl.

   Using the fruit fly genome from Ensembl.org as an example, you would replace 'ENSEMBL_GTF_URL' with the following URL:

   ```
   https://ftp.ensembl.org/pub/release-113/gtf/drosophila_melanogaster/Drosophila_melanogaster.BDGP6.46.113.gtf.gz
   ```

d. Replace 'PATH_TO_SORTMERNA_FASTAS' with the location of the file (path) of the FASTA files that contain ribosomal sequences to be used for ribodepletion.

e. Replace 'PATH_TO_MITO_GENES' with the location of the file (path) containing a list of mitochondrial genes in ENSEMBL format, one gene listed per line.

NOTE: As FASTA and GTF files are a standard file format, files from any source should work. However, the pipeline has only been tested on genomes downloaded from Ensembl. If a problem is encountered using files from another source, it is recommended to try importing a genome using files from Ensembl.org. For RNA-seq analysis, the line 'ginkgo_ref' in the config file can be safely removed.

3. Run the script:

```
$COGENT_AP_HOME/cogent rna add_genome \
  -g <common_species_name> \
  -G <genome_dir>
```

where `<common_species_name>` is the name of the genome being added, and `<genome_dir>` is the directory where the new genome will be stored. If `-G` is not used, the genome is stored by default in `$COGENT_AP_HOME/genomes`.

For additional help with this script, type:

```
$COGENT_AP_HOME/cogent rna add_genome -h
```

CogentAP should now be able to analyze data with the genome option `-g` set to `<common_species_name>` during `cogent rna analyze`.

4. Processing Time

The time taken by the pipeline will vary based on the hardware specifications of the server on which it is run, the size of the raw-fastq input files, and where the files are stored.

During testing, a combined demultiplexing and analysis run for data generated by MiSeq (~25M read pairs) against raw-fastq files stored locally (on the same server CogentAP was installed) typically took about 1–1.5 hr to process. A NextSeq High Output run (~400M read pairs) from local raw-fastq files typically took ~10–12 hr to complete. Input taken from a NovaSeq run (~2G read pairs, or more) will take even longer. Data generated with the Shasta Total RNA-Seq kit could take 48–60 hr to complete.

If the raw-fastq files are instead stored on a network drive, these baselines might be exceeded.

C. DNA-seq Analysis

For DNA-seq or Shasta WGA analysis, CogentAP starts from the main script, `cogent`, and has defined subcommands, listed below.

DNA demux and analyze (CNV Calling)

- `dna demux`
- `dna analyze`

Optional extended analysis

- `dna postprocess snv_calling`

Additional commands

- `dna add_genome`

These scripts can be launched from any location (working directory) on the Linux server where the CogentAP software is installed. The full list of options can be accessed using the syntax:

```
$COGENT_AP_HOME/cogent <COMMAND> -h
```

1. CNV Calling Commands

a) DNA Demux

For DNA-seq or Shasta WGA analysis, the demux process (`cogent dna demux`) is run first to generate demultiplexed FASTQ files.

Example demux command:

```
$COGENT_AP_HOME/cogent dna demux \
  -f <FASTQ_R1> \
  -p <FASTQ_R2> \
  -b <WELL-LIST> \
  -t <EXP_TYPE> \
  -o <DEMUX_OUTPUT_DIR>
```

where:

- `$COGENT_AP_HOME` is the path to the directory where CogentAP is installed
- `<FASTQ_R1>` and `<FASTQ_R2>` are the full paths to the FASTQ files generated by an Illumina sequencing platform
- `<WELL-LIST>` is the full path to the Shasta, ICELL8 cx, or ICELL8 system well list, Illumina's sample sheet, or TSV/CSV format file
- `<EXP_TYPE>` is the experiment type used (e.g., `shasta_wga`)

- `<DEMUX_OUTPUT_DIR>` is the full path of the demultiplex results directory

The full list of `dna demux` arguments are listed in Table 7 and a screenshot of the output of `$COGENT_AP_HOME/cogent dna demux -h` is shown in Figure 13.

Table 7. Full list of options under `cogent dna demux -h`.

Option	Description	Default
`-h, --help`	(Optional) Produces a help message.	N/A
`-f, --fastq1`	(Required) Specifies the input Read1 (R1) FASTQ file.	N/A
`-p, --fastq2`	(Required) Specifies the input Read2 (R2) FASTQ file.	N/A
`-t, --type_of_experiment`	(Required) Specifies experimental protocol to be used (see Table 2 and Table 3).	N/A
`-o, --output_dir`	(Required) Indicates the path to the application output.	N/A
`-b, --barcodes-file`	(Required) Specifies path to the well-list file from CellSelect Software or another custom file containing only barcodes that were selected for sequencing.	N/A
`--fastqc`	(Optional) Runs FASTQC to create quality reports for FASTQ files.	disabled
`-m, --mismatch`	(Optional) Specifies the number of allowed mismatched bases per barcode.	1
`-n, --n_processes`	(Optional) Specifies the number of demultiplexing processes to spawn during execution. The maximum value (N) should not exceed the number of CPUs on the server.	15 (Fixed at 3 when `--no_split_fastqs` is used)
`--n_writers`	(Optional) Specifies the number of demultiplexing writing processes to spawn during execution. The maximum value should be less than or equal to N-2.	8 (Fixed at 1 when `--no_split_fastqs` is used)
`--no_gz`	(Optional) Do not compress (gzip) output FASTQ files	FASTQ files are compressed
`--i7_rc`	(Optional) Reverse-complement the i7 index. `auto` detects and corrects the reverse complement of i7 indices by certain Illumina sequencers. The value can be manually specified with `true` or `false`*.	auto

Option	Description	Default
`--i5_rc`	(Optional) Reverse-complement the i5 index. `auto` detects and autocorrects the reverse complement of i5 indices by certain Illumina sequencers. The value can be manually specified with `true` or `false`*.	auto
`--read_buffer`	(Optional) Specifies buffer size of the data sent to each demultiplexing process in GB.	0.1
`--prog`	(Optional) Specifies the number of reads to process before updating in the log file.	10,000,000
`--no_split_fastqs`	(Optional) Output merged FASTQ file(s). Barcodes are written into read names and merged into a single pair of large FASTQ files.	disabled
`--use_barcodes`	(Optional) Limits the number of barcodes to the specified number.	10,000
`--check_reads`	(Optional) Use this number of reads to estimate read counts during barcode selection.	200,000,000
`--min_reads`	(Optional) Discards barcodes with estimated read count lower than this number.	1
`--random_pick`	(Optional) Picks random reads during barcode selection rather than analyzing the first N read pairs.	disabled
`--preview`	(Optional) Prints the Nextflow command without executing it to verify parameters before starting the pipeline.	N/A

*Manually specify "true" or "false" when you definitively know the orientation of i7 or i5 indexes in the well-list file. Using "true" automatically treats indexes as reverse-complement and corrects all indexes in the well-list file.

```
usage: cogent dna demux [-h] -f FASTQ1 -p FASTQ2 -t {shasta_wga} -o OUTPUT_DIR -b BARCODES_FILE [--fastqc] [-m {0,1}]
                        [-n N_PROCESSES] [--n_writers N_WRITERS] [--no_gz] [--i7_rc {auto,true,false}]
                        [--i5_rc {auto,true,false}] [--read_buffer READ_BUFFER] [--prog PROG] [--no_split_fastqs]
                        [--use_barcodes USE_BARCODES] [--check_reads CHECK_READS] [--min_reads MIN_READS] [--random_pick]
                        [--preview]

            Script to de-multiplex barcoded reads from sequence data stored in
            FASTQ files. User options are designed to simplify de-multiplexing
            for experiments derived from Takara Bio protocols. Barcode (and optionally
            UMI) sequences are extracted and stored in the read name. Users may
            specify whether the resulting de-multiplexed data are merged, or split
            into individual barcode-level files.

options:
  -h, --help            show this help message and exit
  -f FASTQ1, --fastq1 FASTQ1
                        Input Read1 (R1) FASTQ file.
  -p FASTQ2, --fastq2 FASTQ2
                        Input Read2 (R2) FASTQ file.
  -t {shasta_wga}, --type_of_experiment {shasta_wga}
                        Experimental protocol used.
  -o OUTPUT_DIR, --output_dir OUTPUT_DIR
                        Name of output directory to store results. The directory must have write permissions, and its parent
                        directory must be accessible.
  -b BARCODES_FILE, --barcodes_file BARCODES_FILE
                        Well List file from Takara Bio's CellSelect Software (Recommended), or another custom file containing
                        only barcodes that were selected for sequencing.
  --fastqc              Run FASTQC to create quality reports for FASTQ files.
  -m {0,1}, --mismatch {0,1}
                        Number of allowed mismatched bases per barcode.
  -n N_PROCESSES, --n_processes N_PROCESSES
                        Number of demultiplexing processes to spawn during execution.
  --n_writers N_WRITERS
                        Number of demultiplexing writing processes to spawn during execution.
  --no_gz               Do not compress (gzip) output FASTQ files.
  --i7_rc {auto,true,false}
                        Reverse-complement I7 Index (Full Length protocol only). Enter "auto" to detect and auto-correct the
                        reverse complementation of I5/I7 indices by certain Illumina sequencers. Otherwise manually override
                        with "true" or "false").
  --i5_rc {auto,true,false}
                        See help section for "--i7_rc".
  --read_buffer READ_BUFFER
                        Buffer size of data sent to each demultiplexing (worker) process in GB.
  --prog PROG           Number of reads to process before updating status in log file.
  --no_split_fastqs     Output merged FASTQ file(s). Barcodes are written into read names and merged into large FASTQ file. By
                        default output into barcode-level FASTQ files.
  --use_barcodes USE_BARCODES
                        Limit number of barcodes to this value.
  --check_reads CHECK_READS
                        Use this number of reads to estimate read counts during barcode selection.
  --min_reads MIN_READS
                        Discard barcodes with estimated read count lower than this number.
  --random_pick         Pick random reads during barcode selection, rather than analyzing the first N read pairs.
  --preview             Print nextflow command without executing it.
```

Figure 13. The output of `cogent dna demux -h` at the command line.

b) DNA Analyze

The directory of FASTQ files resulting from the DNA demux (Section V.C.1.a) is then used as input to run the analyzer (`cogent dna analyze`) to obtain the output files described in Section VII.

```
$COGENT_AP_HOME/cogent dna analyze \
  -i <DEMUX_OUTPUT_DIR> \
  -g <GENOME> \
```

```
  -t <EXP_TYPE> \
  -B <BIN_SIZE> \
  -r <READ_LENGTH> \
  -R <READ_FILTER> \
  -b <BARCODES_FILE> \
  -o <ANALYSIS_OUTPUT_DIR>
```

where:

- <DEMUX_OUTPUT_DIR> is the full path of the demultiplex results directory
- <GENOME> is a name of genome build (e.g., hg38)
- <EXP_TYPE> is the experiment type used (e.g., shasta_wga)
- <BIN_SIZE> is the bin size used for CNV analysis using Ginkgo. Must be either 500kb or 1mb
- <READ_LENGTH> is the read length of the input data. Must be either 76bp or 151bp
- <READ_FILTER> is the minimum number of PE reads required per barcode to be kept in downstream analysis.
- <BARCODES_FILE> is the full path to the Shasta or ICELL8 system well list, Illumina's sample sheet, or TSV/CSV format file
- <ANALYSIS_OUTPUT_DIR> is the full path to the output directory created for the analysis results

The full list of dna analyze control options are listed in Table 8 and a screenshot of the output of $COGENT_AP_HOME/cogent dna analyze -h is shown in Figure 14.

Table 8. Full list of options under `cogent dna analyze -h`.

Option	Description	Default
-h, --help	(Optional) Produces a help message and exits the application.	N/A
-g, --genome	(Required) Allows for selection of a supported genome or custom genome that you have installed.	N/A
-G, --genome_dir	(Optional) Specifies the directory where the genome and index files are installed.	$COGENT_AP_HOME/genomes
-B, --bin_size	(Required) Specifies the bin size used for Ginkgo analysis. Allowed values are 500kb or 1mb.	N/A
-r, --read_length	(Required) Specifies the read length of the input data. Allowed values are 76bp or 151bp.	N/A
-R, --read_filter	(Optional) Specifies the minimum number of paired-end reads required per barcode to retain for downstream analysis.	25,000
-b, --barcodes_file	(Required) Specifies path to the well-list file from CellSelect Software or another custom file containing only barcodes that were selected for sequencing.	N/A
-o, --output_dir	(Required) Specifies the output directory in which to store the results of the pipeline.	N/A
-i, --input_dir	(Required) Specifies the input directory that contains the results from rna demux.	N/A

Option	Description	Default
`-t, --type_of_experiment`	(Required) Specifies the experimental protocol.	N/A
`--resume`	(Optional) Resumes a previous pipeline run with the same inputs.	N/A
`--preview`	(Optional) Prints the Nextflow command without executing it.	N/A

```
usage: cogent dna analyze [-h] -g {hg38,mm39} [-G GENOME_DIR] -B {500kb,1mb} -r {76bp,151bp} [-R READ_FILTER]
                          -b BARCODES_FILE -o OUTPUT_DIR -i INPUT_DIR -t {shasta_wga} [--preview] [--resume]

            Script to perform CNV analysis by fastq input data.
            The input to this script are files output by Cogent demux.
            The modules currently included are:
                - Trimming (Trimmomatic)
                - Alignment (Bowtie2)
                - Sequencing QC (Picard/Samtools)
                - Summarization (TBUSA)
                - Reporting (TBUSA, CogentDS)

options:
  -h, --help            show this help message and exit
  -g {hg38,mm39}, --genome {hg38,mm39}
                        Select a supported genome or provide the name of a custom genome that you installed.
  -G GENOME_DIR, --genome_dir GENOME_DIR
                        Directory where genome and index files were installed by add_genome. [Default:
                        $COGENT_ROOT/genomes]
  -B {500kb,1mb}, --bin_size {500kb,1mb}
                        Bin size used for Ginkgo analysis.
  -r {76bp,151bp}, --read_length {76bp,151bp}
                        Read length of input data.
  -R READ_FILTER, --read_filter READ_FILTER
                        Minimum number of PE reads required per barcode to keep in downstream analysis.
                        [Default: 25000]
  -b BARCODES_FILE, --barcodes_file BARCODES_FILE
                        Well List file from Takara Bio's CellSelect Software (Recommended), or another custom
                        file containing only barcodes that were selected for sequencing.
  -o OUTPUT_DIR, --output_dir OUTPUT_DIR
                        Name of output directory to store results. The directory must have write permissions,
                        and its parent directory must be accessible.
  -i INPUT_DIR, --input_dir INPUT_DIR
                        Directory contains results from demux command. The directory must contain FASTQ files
                        after demultiplexing.
  -t {shasta_wga}, --type_of_experiment {shasta_wga}
                        Experimental protocol used.
  --preview             Print nextflow command without executing it.
  --resume              Resume a previous pipeline run with the same inputs
```

Figure 14. The output of `cogent analyze -h` at the command line.

2. SNV Calling Analysis (Optional)

CogentAP can perform SNV calling with pseudo-bulk BAM files containing reads from the barcodes in the CNV cluster or sample. The results from `cogent dna analyze` must be processed in CogentDS v2.2 to generate a cluster mapping file. Refer to Section VIII.A.6 of the Cogent NGS Discovery Software User Manual for more information.

SNV calling can be launched by running the following command.

```
$COGENT_AP_HOME/cogent dna postprocess snv_calling
```

Example command:

```
$COGENT_AP_HOME/cogent dna postprocess snv_calling \
-i <ANALYSIS_OUTPUT_DIR> \
-g <GENOME> \
-b <BARCODES_FILE> \
--cluster_mapping_file <CLUSTER_CSV> \
-o <SNV_OUTPUT_DIR>
```

where:

- <ANALYSIS_OUTPUT_DIR> is the full path to the output directory created for the analysis results
- <GENOME> is the name of the genome build (e.g., hg38)
- <BARCODES_FILE> is the full path to the Shasta or ICELL8 system well list, Illumina's sample sheet, or TSV/CSV format file
- <CLUSTER_CSV> is the full path to the CogentDS [Download Clusters] CSV file resulting from the CNV calling results
- <SNV_OUTPUT_DIR> is the full path to the output directory created for the SNV calling results

The full list of SNV calling options are listed in Table 9 and a screenshot of the output of `$COGENT_AP_HOME/cogent dna postprocess snv_calling -h` is shown in Figure 15.

By default, only clusters with more than 100 million reads (50 million paired reads) are selected for SNV calling. This can be changed by using the optional flag `--snv_threshold` in the `snv_calling` command.

Table 9. Full list of options under `cogent dna postprocess snv_calling -h`.

Option	Description	Default
`-g` `--genome`	(Required) Allows for selection of a supported genome or custom genome that you have installed.	N/A
`-b` `--barcodes_file`	(Required) Specifies path to the well-list file from CellSelect Software or another custom file containing only barcodes that were selected for sequencing.	N/A
`-i` `--input_dir`	(Required) Specifies the input directory that contains the results from `dna analyze`.	N/A
`-o` `--output_dir`	(Required) Specifies the output directory in which to store the results of the pipeline.	N/A
`--cluster_mapping_file`	(Required) Specifies path to the cluster mapping CSV file obtained from CogentDS.	N/A
`--cluster_by`	(Optional) Specifies the column name from the cluster mapping CSV file to use for SNV clustering.	`sample`

Option	Description	Default
`--snv_threshold`	(Optional) Specifies the minimum number of reads that needs to be in the cluster for the cluster to be selected for downstream SNV analysis.	100,000,000
`--resume`	(Optional) Resumes a previous pipeline run with the same inputs.	N/A
`--preview`	(Optional) Prints the Nextflow command without executing it.	N/A
`-h` `--help`	(Optional) Print a help message to the console.	N/A

```
usage: cogent dna postprocess snv_calling [-h] -b BARCODES_FILE -i INPUT_DIR --cluster_mapping_file
                                          CLUSTER_MAPPING_FILE --cluster_by
                                          {cnv_clusters,sample,custom_cnv_clusters}
                                          [--snv_threshold SNV_THRESHOLD] -g hg38 -o OUTPUT_DIR [--preview]
                                          [--resume]

       Script to perform SNV calling analysis for DNA data.
       The input to this script are files output by cogent dna analyze.
       The modules currently included are:
           - SNV calling (Monopogen)
           - Reporting (Monopogen)

options:
  -h, --help            show this help message and exit
  -b BARCODES_FILE, --barcodes_file BARCODES_FILE
                        Well List file from Takara Bio's CellSelect Software (Recommended), or another custom
                        file containing only barcodes that were selected for sequencing.
  -i INPUT_DIR, --input_dir INPUT_DIR
                        Directory contains results from analyze command.
  --cluster_mapping_file CLUSTER_MAPPING_FILE
                        Full path to the file containing cluster mapping from CogentDS.
  --cluster_by {cnv_clusters,sample,custom_cnv_clusters}
                        Column name in the cluster mapping file to use for SNV clustering. Default: "sample"
  --snv_threshold SNV_THRESHOLD
                        Minimum number of reads required per cluster to perform SNV analysis on that cluster.
                        [Default: 100000000]
  -g hg38, --genome hg38
                        Select a supported genome.
  -o OUTPUT_DIR, --output_dir OUTPUT_DIR
                        Name of output directory to store results. The directory must have write permissions,
                        and its parent directory must be accessible.
  --preview             Print nextflow command without executing it.
  --resume              Resume a previous pipeline run with the same inputs
```

Figure 15. The output of `cogent dna postprocess snv_calling -h` at the command line.

3. Adding a Genome Build

The human and mouse genome builds available from our server (Section IV.B, "Install Cogent NGS Analysis Pipeline v3.2) are recommended for use in the pipeline, but genomes of other species can be added into the software post-install.

To add custom genome data to CogentAP:

1. Create a copy of the file under

   ```
   $COGENT_AP_HOME/config/genome_sources/sample.config
   ```

   and rename it

   ```
   $COGENT_AP_HOME/config/genome_sources/<common_species_name>.config
   ```

   where `<common_species_name>` is the name of the genome being added (e.g., `dm6`)

2. Update the following fields using a text editor:

   a. Replace 'GENOME' with the `<common_species_name>` from Step 1 (e.g., `dm6`)

   b. Replace 'ENSEMBL_GENOME_FASTA_URL' with the public URL of the FASTA file containing all the sequences (chromosomes and contigs) from Ensembl.

   Using the fruit fly genome from Ensembl.org as an example, you would replace 'ENSEMBL_GENOME_FASTA_URL' with the following URL:

   ```
   https://ftp.ensembl.org/pub/release-
   113/fasta/drosophila_melanogaster/dna/Drosophila_melanogaster.
   BDGP6.46.dna_sm.toplevel.fa.gz
   ```

   c. Replace 'PATH_TO_GINKGO_REF' with the path to the directory containing the Ginkgo reference files for the genome.

NOTES:

– Ginkgo reference files need to be generated for the newly added genome. For instructions on generating these files, please refer to https://github.com/robertaboukhalil/ginkgo/tree/master/genomes/scripts.

– For DNA-seq analysis, the lines 'annotation_gtf_url', 'sortmerna_fastas', and 'mito_reads' in the config file can be safely removed

3. Run the script:

   ```
   $COGENT_AP_HOME/cogent dna add_genome \
     -g <common_species_name> \
     -G <genome_dir>
   ```

   where `<common_species_name>` is the name of the genome being added, and `<genome_dir>` is the directory where the new genome needs to be stored in. If `-G` is not used, the genome is stored by default in `$COGENT_AP_HOME/genomes`.

   For additional help with this script, type:

   ```
   $COGENT_AP_HOME/cogent dna add_genome -h
   ```

   CogentAP should now be able to analyze data with the genome option `-g` set to `<common_species_name>` during the `cogent dna analyze` step.

4. Processing Time

The time taken by the pipeline will vary based on the hardware specifications of the server on which it is run, the size of the raw-fastq input files, and where the files are stored. Expect analysis

to take between 2–24 hr depending on the sequencing platform, the depth of the sequencing data, and the number of barcodes that are being analyzed.

If the raw-fastq files are instead stored on a network drive, these baselines might be exceeded.

D. Resuming an Analysis

CogentAP v3.2 is built on the popular Nextflow pipelining framework that allows for the option to resume a failed and/or stopped analysis. To resume an analysis that stopped in the middle of the run, the `--resume` flag can be added to the end of the same command used to run the analysis in the first place. If all the parameters are identical and the `work` directory that Nextflow created is left intact, the analysis should commence from the step where it stopped.

E. Clearing Out the Work Directory

It is generally a good idea to delete the Nextflow work directory on a regular basis. Even though CogentAP deletes all intermediate files upon successful completion of an analysis, data from older, failed, or incomplete analyses are sometimes preserved in the working directory, taking up unnecessary space. In such cases, cleaning out the work directory could help reclaim space.

VI. Test Dataset

A mini dataset file (referred to here as test dataset) is included in the CogentAP distribution package; it can be found under the main installation folder in the following sub-folder (Figure 16, below):

`$COGENT_AP_HOME/test/fixtures/experiments/ICELL8_FLA`.

This dataset can be used to test the running of the pipeline end-to-end and will provide a sample of the output files. The output (report and stats only) from the test dataset is also included in the CogentAP installation and can be found in the following folder:

`$COGENT_AP_HOME/test/fixtures/experiments/ICELL8_FLA/out_test/`.

These output files can be used to compare to the output of your test run to verify everything is working correctly.

NOTE: The test dataset should not be used for inference purposes. CogentAP output statistics and plots will only be meaningful with a real dataset.

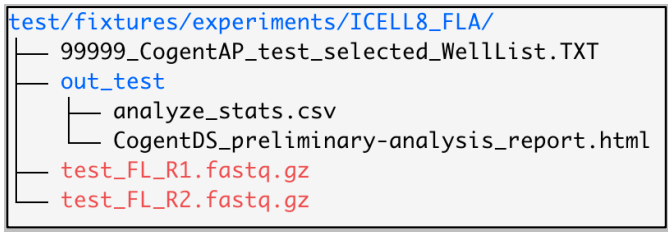


Figure 16. The `test/fixtures/experiments/ICELL8_FLA` folder under `$COGENT_AP_HOME`. The sample `*.fastq.gz` files and example output directory `out_test/` can be found there.

To start a run using test data, use the following commands to run the RNA demux and RNA analyze, respectively:

```
cd $COGENT_AP_HOME
```

```
cogent rna demux \
 -f test/fixtures/experiments/ICELL8_FLA/test_FL_R1.fastq.gz \
```

```
 -p test/fixtures/experiments/ICELL8_FLA/test_FL_R2.fastq.gz \
 -b test/fixtures/experiments/ICELL8_FLA/99999_CogentAP_test_selected_WellList.TXT \
 -t icell8_fla -o out_test
```

```
cogent rna analyze \
  -i out_test/demultiplexed_fastqs/ -t icell8_fla \
  -o out_test/analyze -g hg38
```

The test run should take ~5–10 min to complete successfully.

NOTE: If a process fails, it will automatically be retried with additional computing resources. A failure is acceptable as long as the corresponding row eventually reaches 100% completion and displays a green checkmark

VII. Output Files

The pipeline produces output files that serve two purposes:

1. Summarization of results using typical statistics and plots
2. Facilitating further analyses using our interactive R kit, Cogent NGS Discovery Software (CogentDS), or any other tertiary analysis tool

A. Output Folder Structure

The folder structure of the results folder is slightly different depending on which analysis was run and which kit was used to generate sequencing data.

1. RNA-Seq Analysis Output (For All RNA-Seq Kits Except the Shasta Total RNA-Seq Kit)

For RNA-seq analysis of sequencing data produced from all RNA-seq kits except the Shasta Total RNA-Seq kit, the contents of the demux output folder and analysis output folders will resemble Figure 17 (below).

```
├── analyze_stats.csv
├── count_matrices
│   ├── fusion_junctionCounts.csv
│   ├── fusion_spanCounts.csv
│   ├── geneCounts_exonOnly.csv
│   ├── geneCounts_exon_plus_intron.csv
│   ├── immune_clonotype_matrix.csv
│   ├── immune_metadata.csv
│   ├── immune_summary.csv
│   ├── immune_top3_clonotype_matrix.csv
│   ├── immune_top3_metadata.csv
│   ├── immune_top3_summary.csv
│   └── isoformCounts.csv
├── cutadapt_trimmed_fastqs
│   ├── GM11281_AATGGTAATAGATGAC_trimmed_R1.fastq.gz
│   ├── GM11281_AATGGTAATAGATGAC_trimmed_R2.fastq.gz
│   ├── GM11281_CCAGAGCGCGATATCC_trimmed_R1.fastq.gz
│   ├── GM11281_CCAGAGCGCGATATCC_trimmed_R2.fastq.gz
│   ├── GM11281_GCCTGAACCAATTCGG_trimmed_R1.fastq.gz
│   ├── GM11281_GCCTGAACCAATTCGG_trimmed_R2.fastq.gz
│   ├── K562_CCAATTCCCTATCGTT_trimmed_R1.fastq.gz
│   ├── K562_CCAATTCCCTATCGTT_trimmed_R2.fastq.gz
│   ├── K562_TCCAACTTCAATTCGG_trimmed_R1.fastq.gz
│   ├── K562_TCCAACTTCAATTCGG_trimmed_R2.fastq.gz
│   ├── K562_TTCTAATGCTGAGGTT_trimmed_R1.fastq.gz
│   └── K562_TTCTAATGCTGAGGTT_trimmed_R2.fastq.gz
├── fusion
│   ├── junction
│   └── span
├── gene_and_transcript_info
│   ├── gene_info.csv
│   └── transcript_info.csv
├── immune_profiling
│   ├── GM11281_AATGGTAATAGATGAC_trimmed_report.tsv
│   ├── GM11281_CCAGAGCGCGATATCC_trimmed_report.tsv
│   ├── GM11281_GCCTGAACCAATTCGG_trimmed_report.tsv
│   ├── K562_CCAATTCCCTATCGTT_trimmed_report.tsv
│   ├── K562_TCCAACTTCAATTCGG_trimmed_report.tsv
│   └── K562_TTCTAATGCTGAGGTT_trimmed_report.tsv
├── logs
│   ├── cutadapt
│   ├── salmon
│   ├── sortmerna
│   ├── star_align
│   └── star_fusion
├── report
│   ├── CogentDS_analysis.rds
│   └── CogentDS_preliminary-analysis_report.html
├── ribodepletion
│   ├── GM11281_AATGGTAATAGATGAC_trimmed_non_rRNA_R1.fastq.gz
│   ├── GM11281_AATGGTAATAGATGAC_trimmed_non_rRNA_R2.fastq.gz
│   ├── GM11281_CCAGAGCGCGATATCC_trimmed_non_rRNA_R1.fastq.gz
│   ├── GM11281_CCAGAGCGCGATATCC_trimmed_non_rRNA_R2.fastq.gz
│   ├── GM11281_GCCTGAACCAATTCGG_trimmed_non_rRNA_R1.fastq.gz
│   ├── GM11281_GCCTGAACCAATTCGG_trimmed_non_rRNA_R2.fastq.gz
│   ├── K562_CCAATTCCCTATCGTT_trimmed_non_rRNA_R1.fastq.gz
│   ├── K562_CCAATTCCCTATCGTT_trimmed_non_rRNA_R2.fastq.gz
```

Figure 17. Folders and files of the output directory for a typical RNA-seq analysis.

2. RNA-Seq Analysis Direct Output (For the Shasta Total RNA-Seq Kit)

For RNA-seq analysis of Shasta Total RNA-Seq kit data using the `analyze_direct` workflow (Section V.B.1.c), only the analyze stats file, the count matrices (if the number of barcodes in the analysis is ≤5,000), the report, gene-body coverage results (if enabled), and an RDS object created for use with CogentDS are saved in the output folder by default (Figure 18).

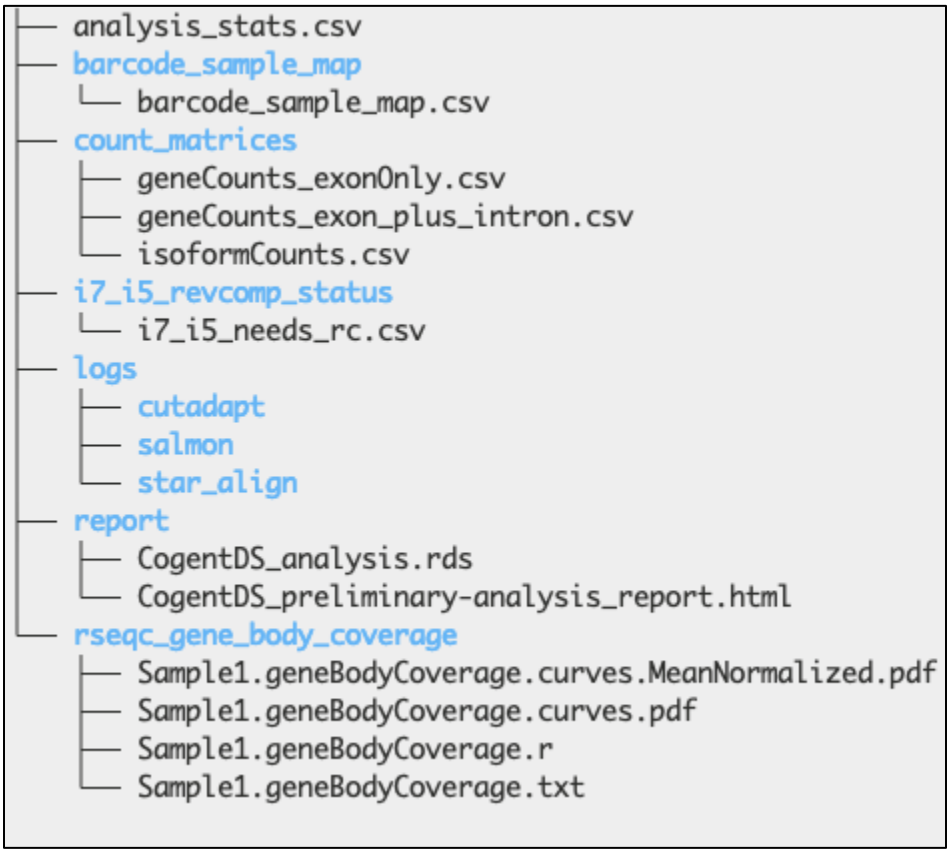


Figure 18. Folders and files of the output directory for a Shasta Total RNA-Seq kit analysis done using the `analyze_direct` workflow. This example also includes the optional gene-body coverage analysis.

If all the files from the analysis need to be saved, the `--keep_intermediate` flag can be used. This will add considerable overhead, both in analysis time and storage space utilized. Therefore, it is not recommended unless necessary.

3. RNA-Seq Analysis Output (For the Shasta Total RNA-Seq Kit, Using FASTQ-based Demultiplexing)

For RNA-seq analysis of Shasta Total RNA-Seq kit data done using the conventional FASTQ-based demultiplexing, the demux output folder remains the same, as shown in Figure 17. Due to the volume of files that get created and the amount of storage required for these files, only the analyze stats file, the count matrices, the report, and an RDS object created for use with CogentDS are saved in the output folder by default (Figure 19).

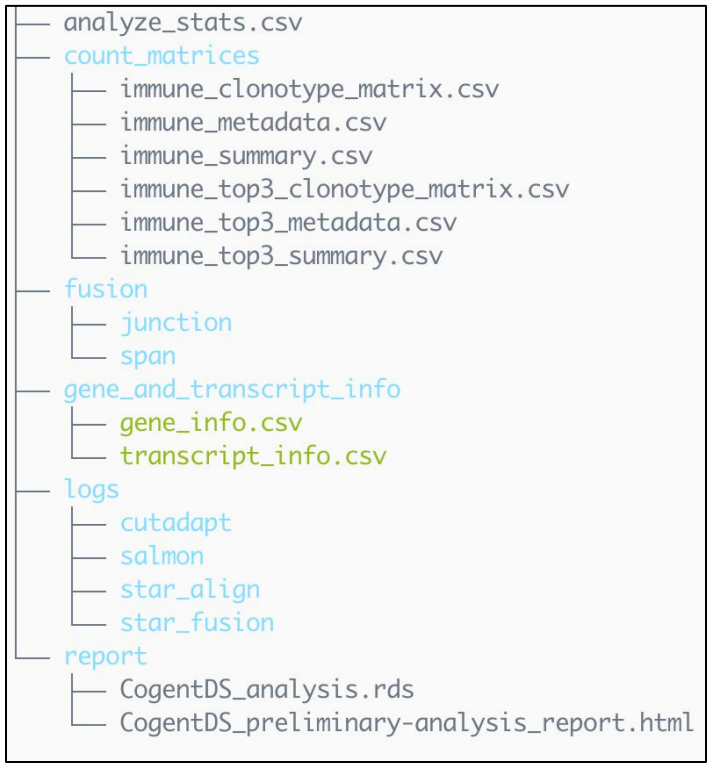


Figure 19. Folders and files of the output directory for a Shasta Total RNA-Seq kit analysis. This example also includes the optional immune and fusion analyses (using the default parameters).

If all the files from the analysis need to be saved, the `--keep_intermediate` flag can be used. This will add considerable overhead, both in analysis time, and in storage space utilized. Therefore, it is not recommended unless necessary. The directory structure from such an analysis will resemble the folder shown in the previous section in Figure 17.

4. DNA-seq CNV Analysis Output

For a typical DNA-seq analysis, the contents of the demux output folder are shown in Figure 20, which contains a demux_counts_all.csv table summarizing the number of paired-end reads for each barcode, a log file, and the pairs of fastq.gz files for each barcode.

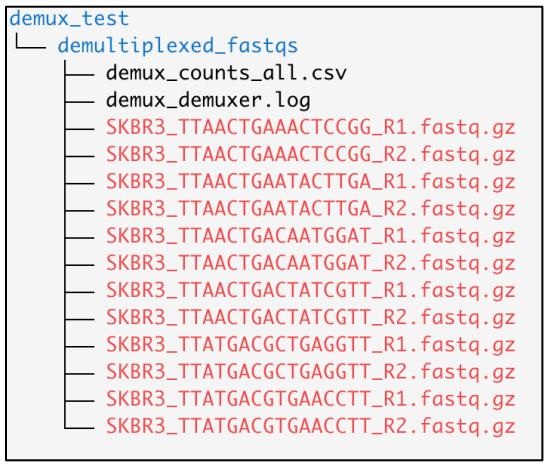


Figure 20. Folders and files of the directory for a typical Cogent DNA demux output folder.

The analysis output folders will resemble Figure 21. Specifically, the dna_report/ folder contains: the CogentDS_scDNA_analysis.rds object that can be uploaded to CogentDS for further data visualization; a dna_qc_metrics_report.html file with a summary of key QC metrics; and a CogentDS_scDNA_analysis_report.html with a summary of CNV calling related QC metrics, CNV heatmap, and CNV UMAP.

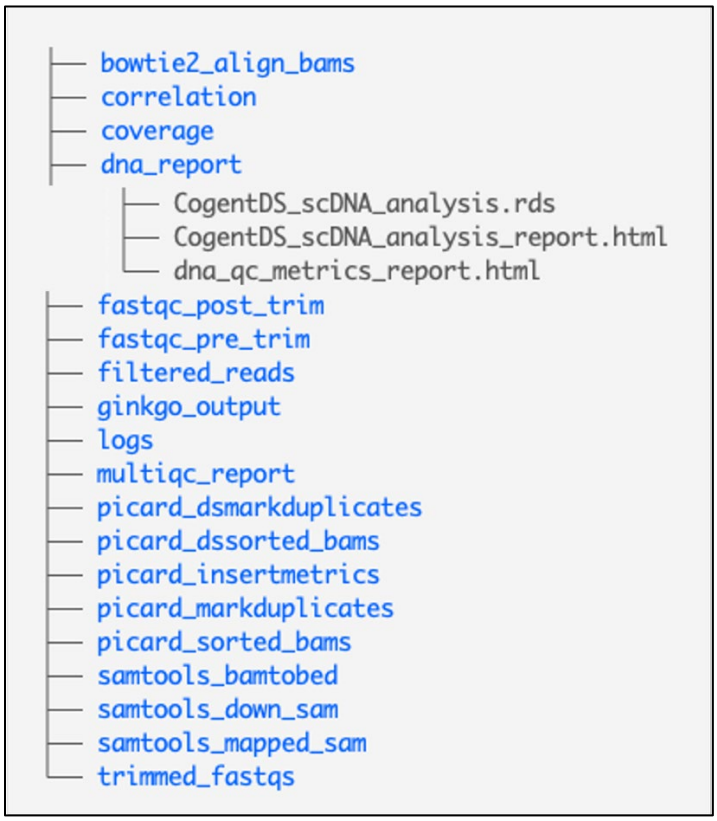


Figure 21. Folders of the directory for a typical Cogent DNA-seq analysis output folder.

5. DNA-seq SNV Calling Analysis Output

For a typical SNV calling analysis, the contents of the output folder will resemble Figure 22. The snv_report/ folder contains the CogentDS_SNV_analysis.rds object that can be opened in CogentDS for further visualization and analysis, as well as a CogentDS_SNV_preliminary-analysis_report.html file. The snv_summary folder contains the snv_summary_barplot_data.csv table which has the summary statistics of the total number of germline and somatic SNVs identified in each cluster.

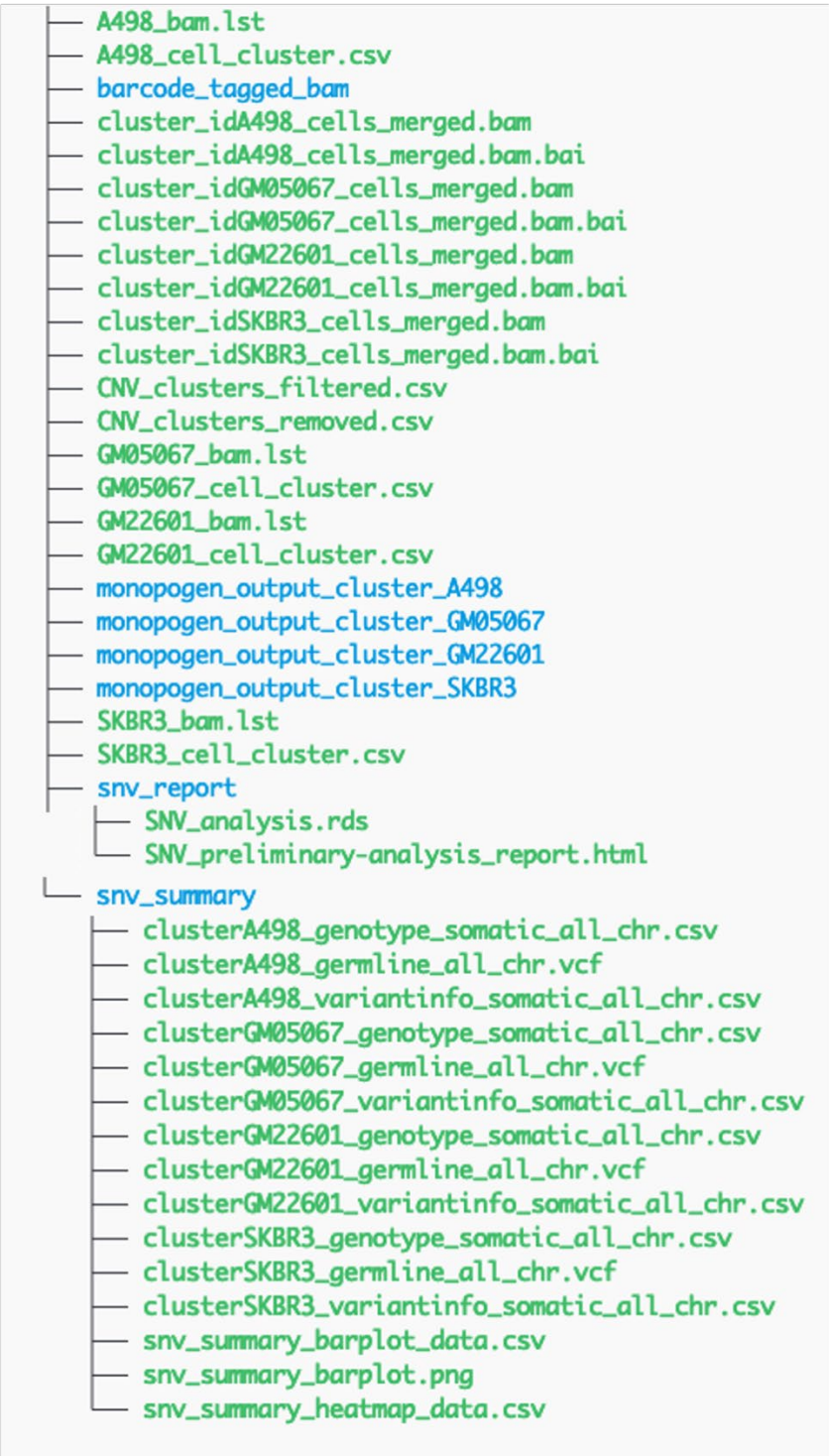


Figure 22. Folders of the directory for a typical Cogent DNA-seq SNV calling output folder.

B. HTML Report

HTML reports are generated by the same report process as CogentDS, using standard parameters, and contain the example statistics and plots listed below. For complete details, please see the Cogent NGS Discovery Software User Manual.

1. RNA-seq Analysis

NOTE: Some sections and/or plots may not be generated depending on the sample size and quality of data provided to CogentAP. Ribosomal read counts could either be present or absent in the report based on the kit used and if in silico ribodepletion was enabled during the analysis.

a) Experimental Overview and Data Statistics Plot

Experimental Overview

Experimental overview is provided to get insights into mapping stats at the experiment level. Here is how the breakdown can be evaluated. Please see: Ribosomal, Globin, and RN7SL2 reads are mapped using sortmeRNA and are removed before mapping the reads to the genome

```
##
##   ### UMI kits
##
##   Total_Mapped_Reads = Genomic_Mapped_Reads + Ribosomal_Reads + Globin_Reads + RN7SL2_Reads
##
##   Genomic_Mapped_Reads = [Genomic_Uniquely_Mapped_Reads + Genomic_Multimapped_Reads] = [Tran
scriptomic_Reads_w_UMIs + Intergenic_Reads + Discarded_Transcriptomic_Reads_STAR]
##
##   Transcriptomic_Reads_w_UMIs = Duplicated_Reads + Transcriptomic_Reads_After_Dedup
##
##   Transcriptomic_Reads_After_Dedup = Gene_Reads_After_Dedup + Discarded_Transcriptomic_Reads
_Salmon
##
##   Gene_Reads_After_Dedup = Exon_Reads_After_Dedup + Intron_Reads_After_Dedup
##
##   Undesirable = Ribosomal_Reads + Mitochondrial_Reads + Discarded_Transcriptomic_Reads_STAR
+ Discarded_Transcriptomic_Reads_Salmon + Globin_Reads + RN7SL2_Reads
##
##   Usable = Trimmed_Reads - Undesirable
##
```

Figure 23. Experimental overview of the different mapping stats that are used in the report. The stats included in the report will change based on the experiment and the options enabled during analysis.

Read Stats

	Total Counts	% (of Barcoded Reads)	% (of Trimmed Reads)
Barcoded_Reads	159,997,960	100.000	NA
Trimmed_Reads	137,441,941	85.902	100
Total_Mapped_Reads	131,201,399	82.002	95.46
Genomic_Mapped_Reads	122,877,948	76.800	89.404
Genomic_Uniquely_Mapped_Reads	117,823,361	73.641	85.726
Genomic_Multimapped_Reads	5,054,587	3.159	3.678
Transcriptomic_Reads	108,368,484	67.731	78.847
Exon_Reads	67,885,900	42.429	49.392
Intron_Reads	40,367,224	25.230	29.37
Gene_Reads	108,253,124	67.659	78.763
Intergenic_Reads	9,013,174	5.633	6.558
Discarded_Transcriptomic_Reads_STAR	5,496,290	3.435	3.999
Discarded_Transcriptomic_Reads_Salmon	115,360	0.072	0.084
Ribosomal_Reads	8,323,451	5.202	6.056
Mitochondrial_Reads	4,184,523	2.615	3.045
Usable	119,322,317	74.577	86.817
Undesirable	18,119,624	11.325	13.183

Figure 24. Overall mapping stats are reported in the read stats section of the preliminary report.

Gene Body Assignment Breakdown

	Total Counts	% (of Genomic Mapped Reads)
Genomic_Mapped_Reads	122,877,948	100.000
Transcriptomic_Reads	108,368,484	88.192
Exon_Reads	67,885,900	55.247
Intron_Reads	40,367,224	32.851
Gene_Reads	108,253,124	88.098

Undesirable Read Breakdown

	Total Counts	% (of Trimmed Reads)
Trimmed_Reads	137,441,941	100.000
Discarded_Transcriptomic_Reads_STAR	5,496,290	3.999
Discarded_Transcriptomic_Reads_Salmon	115,360	0.084
Ribosomal_Reads	8,323,451	6.056
Mitochondrial_Reads	4,184,523	3.045
Usable	119,322,317	86.817

Other Stats

	Average Stats across barcodes
No_of_Genes_Exon_plus_Intron	5,454
No_of_Genes_ExonOnly	1,920
No_of_Transcripts	2,830
Strand_Specificity	NA

Figure 25. Breakdown of read stats by gene-body assignment, undesirable reads, and other stats provided in the preliminary report.

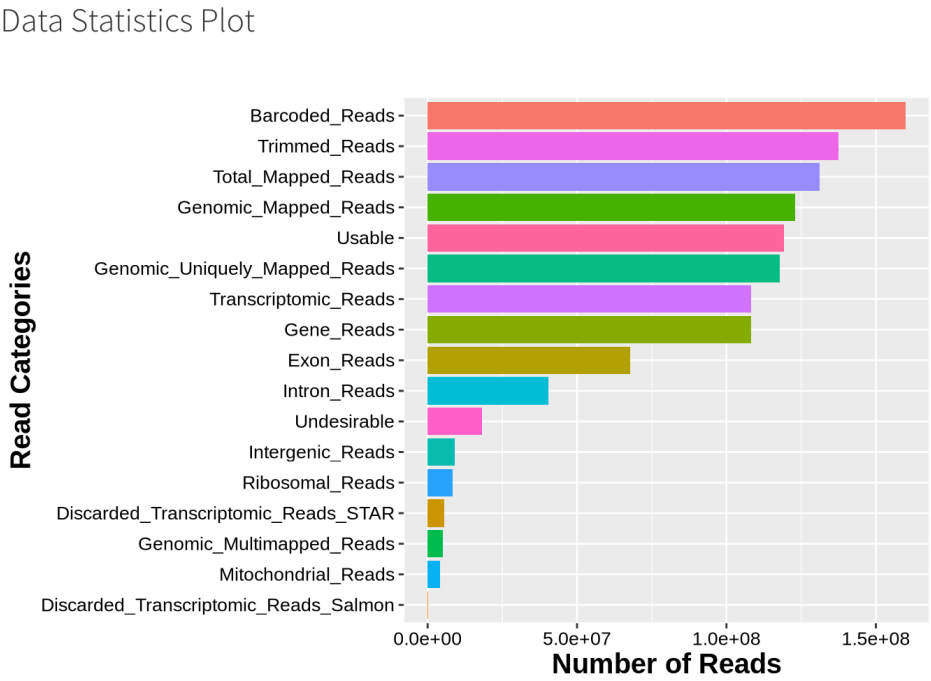


Figure 26. Example data statistics plot from the HTML report.

b) QC Analysis

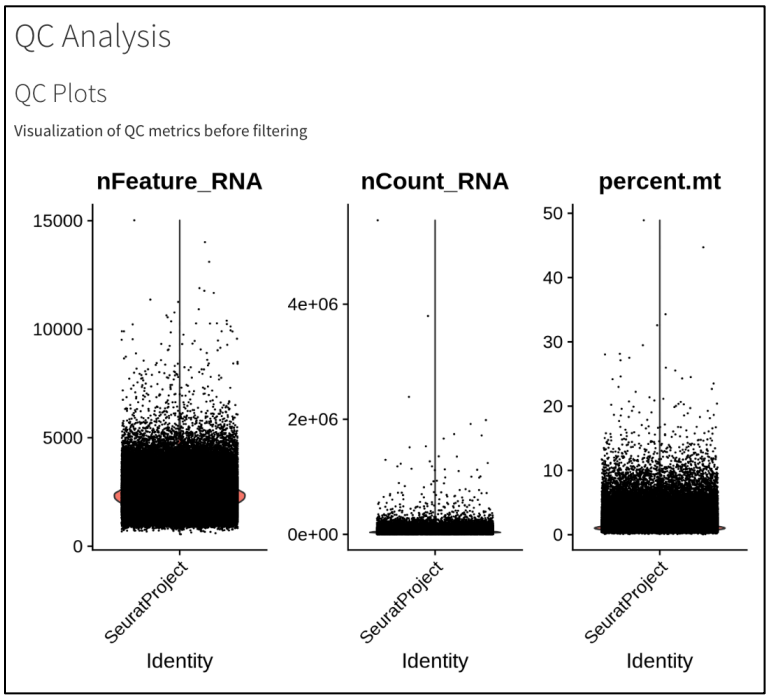


Figure 27. Example QC analysis section of the HTML report.

c) Principal Component Analysis (PCA)

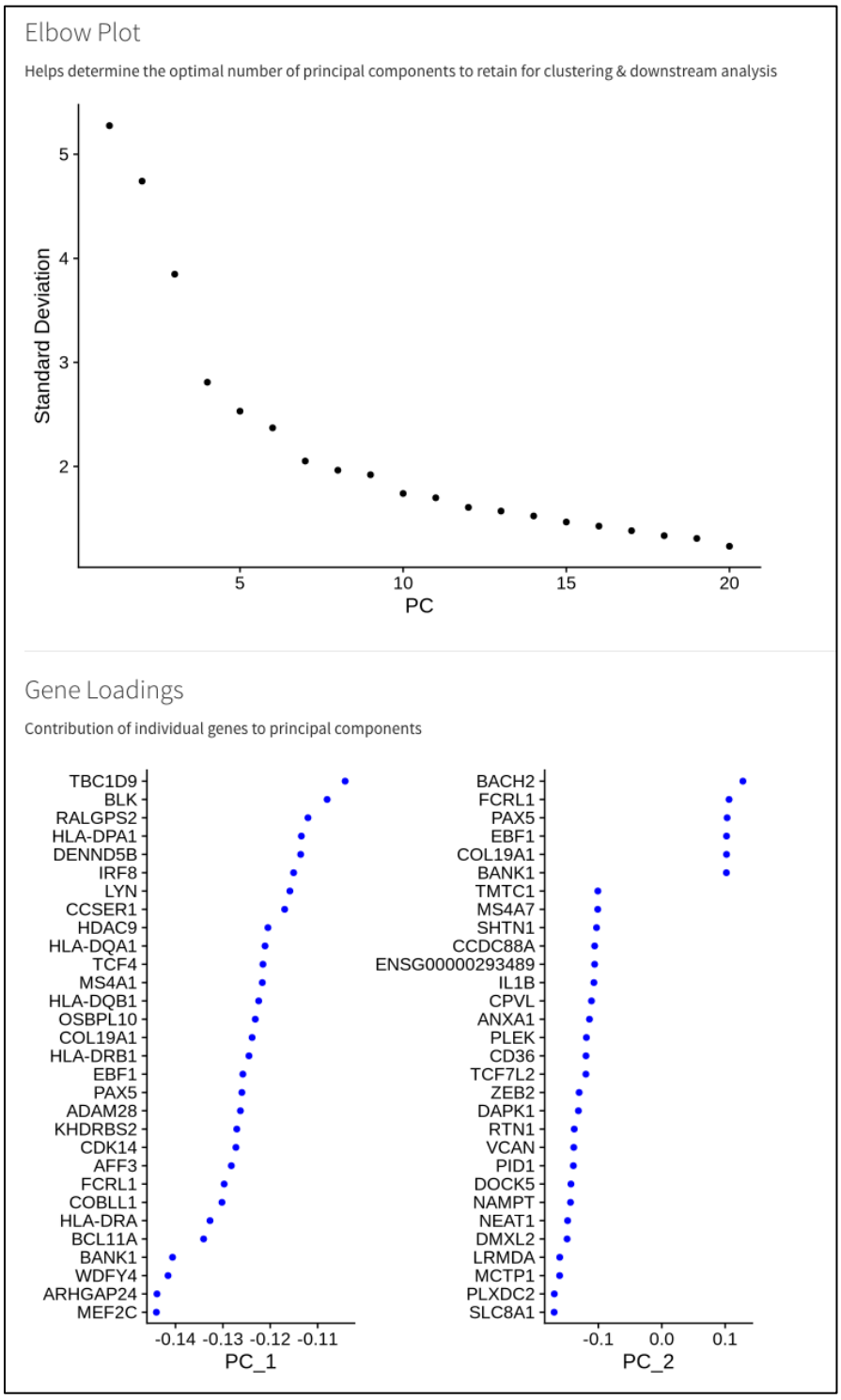


Figure 28. Example PCA analysis plots from the HTML report.

d) UMAP Plot

NOTE: UMAP plots are only generated for sequencing data from single-cell RNA-seq kits.

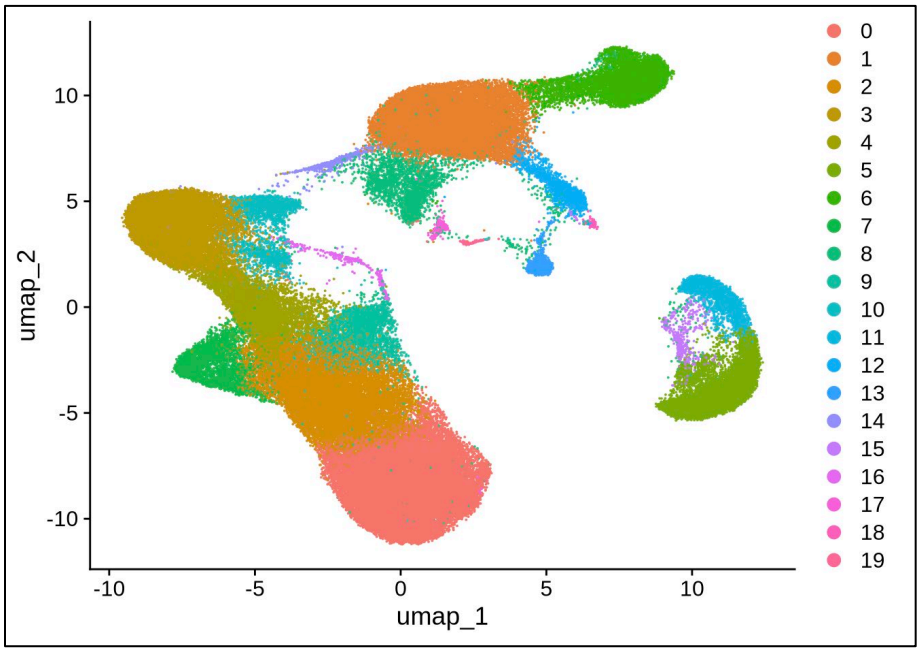


Figure 29. Example UMAP plot from the HTML report.

2. DNA-seq Analysis

NOTE: Some sections and/or plots may not be generated depending on the sample size and quality of data provided to CogentAP.

Analysis of sequencing data from DNA-seq or Shasta WGA kits results in the generation of two reports—a QC metrics report and an analysis report.

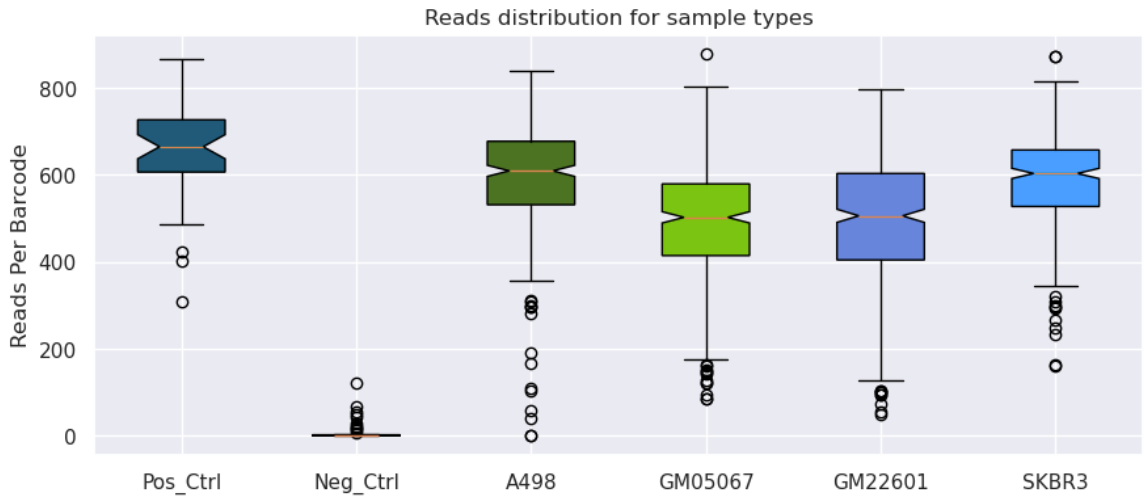
a) QC Metrics Report

Experimental Overview and Reads by Sample Type

Experimental Overview

Total Read pairs	1,000,000
Total Reads	2,000,000
Total Barcoded Reads	1,584,150
Barcoded Reads Fraction	79.21%
Total Barcodes Identified	1,527
Min Read Pairs Filter	500

Reads by Sample Type



Sample Types	Pos_Ctrl	Neg_Ctrl	A498	GM05067	GM22601	SKBR3
Barcodes Identified	46	46	321	393	424	297
Barcodes Reads	60,154	866	379,296	380,432	416,068	347,334
% of Total Barcoded Reads	3.8%	0.05%	23.94%	24.01%	26.26%	21.93%
Reads per Barcode_median	1,329	0	1,218	1,004	1,011	1,206
Reads per Barcode_mean	1,308	19	1,182	968	981	1,169
Reads per Barcode_stdDev	220	45	266	266	297	231

Figure 30. Example Experimental Overview table and Reads by Sample Type plot from the QC metrics report.

Read Statistics and Additional Metrics

Reads Statistics wells after Min Reads Filter

Sample Types	Pos_Ctrl		A498		GM05067		GM22601		SKBR3	
Passing-filter Barcodes	42		266		200		220		240	
	Reads	% Barcoded Reads	Reads	% Barcoded Reads	Reads	% Barcoded Reads	Reads	% Barcoded Reads	Reads	% Barcoded Reads
Barcoded Reads	56,920	100.00%	338,122	100.00%	234,138	100.00%	265,764	100.00%	301,340	100.00%
Trimmed Reads	56,918	100.0%	338,094	99.99%	234,116	99.99%	265,742	99.99%	301,314	99.99%
Unmapped Reads	3,186	5.6%	17,142	5.07%	12,419	5.3%	14,428	5.43%	16,178	5.37%
Mapped Reads	53,732	94.4%	320,952.0	94.92%	221,697.0	94.69%	251,314.0	94.56%	285,136.0	94.62%
Duplicate Reads	301	0.53%	1,635.0	0.48%	1,080.0	0.46%	1,189.0	0.45%	1,452.0	0.48%
Unique Reads	53,431	93.87%	319,317.0	94.44%	220,617.0	94.23%	250,125.0	94.12%	283,684.0	94.14%

Additional Metrics

Sample Types	Pos_Ctrl	A498	GM05067	GM22601	SKBR3
Insert Size (mean)	261.94	261.96	260.10	260.32	261.78
Insert Size (median)	236.50	237.00	236.00	237.00	236.00
GC Content (mean)	40.83%	40.16%	40.41%	40.44%	40.25%
GC Content (median)	41.00%	40.00%	40.00%	40.00%	40.00%
Normalized Reads per Chromosome (mean)	1.00	0.97	1.00	1.00	0.99
Normalized Reads per Chromosome (stdev)	0.10	0.27	0.13	0.12	0.43
Pearson's Correlation (mean)	0.02	0.04	0.02	0.02	0.14
Pearson's Correlation (stdev)	0.02	0.02	0.02	0.02	0.04

Figure 31. Example Read Statistics and Additional Metrics tables from the QC metrics report.

b) Analysis Report

QC Analysis Plots

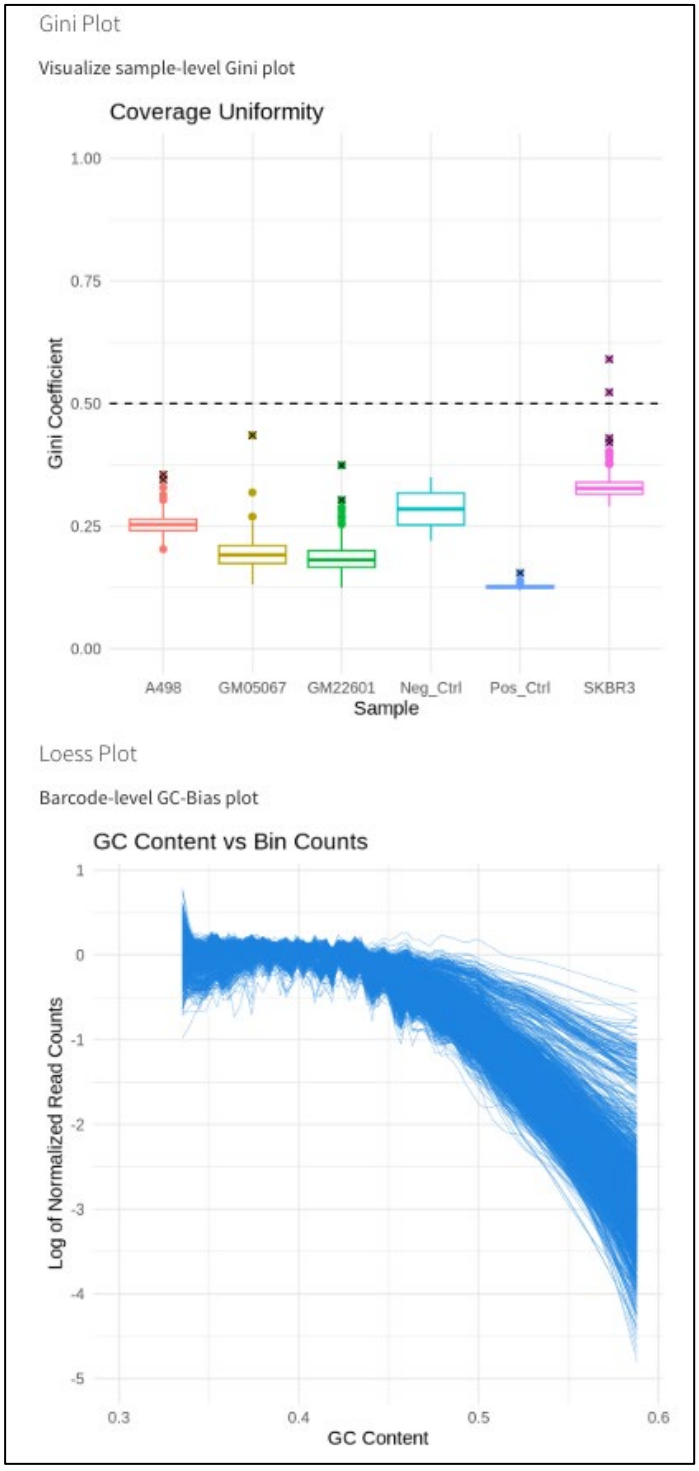


Figure 32. Example Gini Plot and Loess Plot from the DNA-seq analysis report.

CCN Heatmap

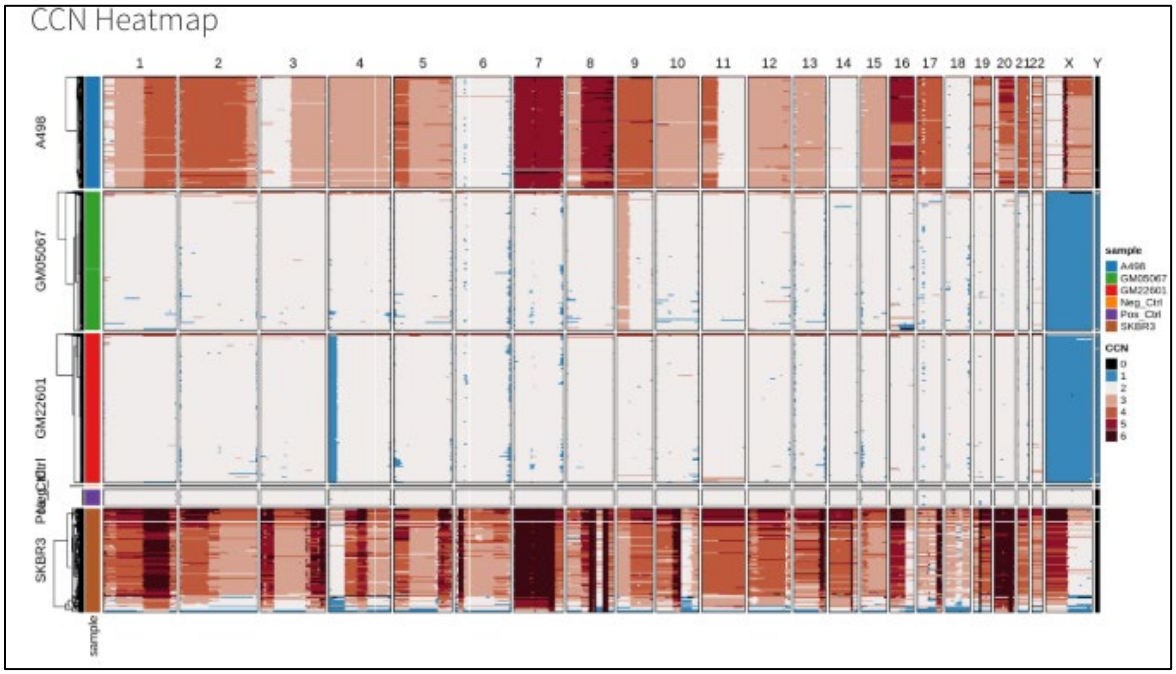


Figure 33. Example CCN Heatmap plot from the DNA-seq analysis report.

UMAP Plot

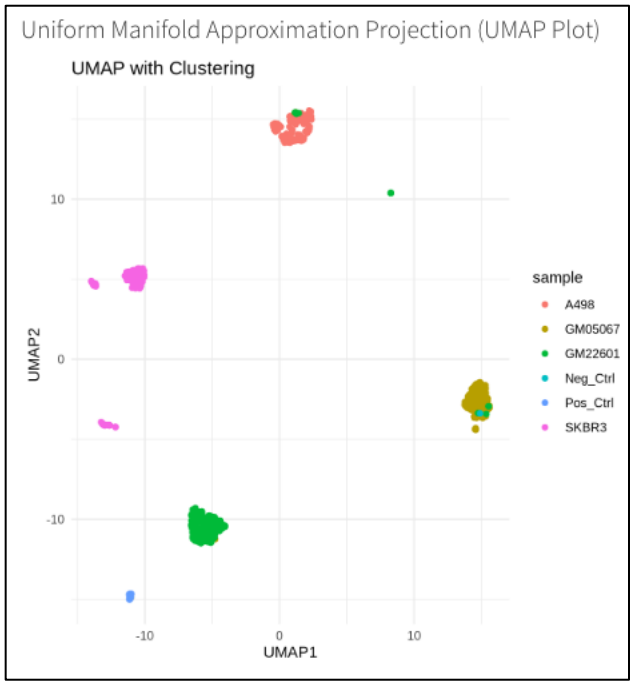


Figure 34. Example UMAP plot from the DNA-seq analysis report.

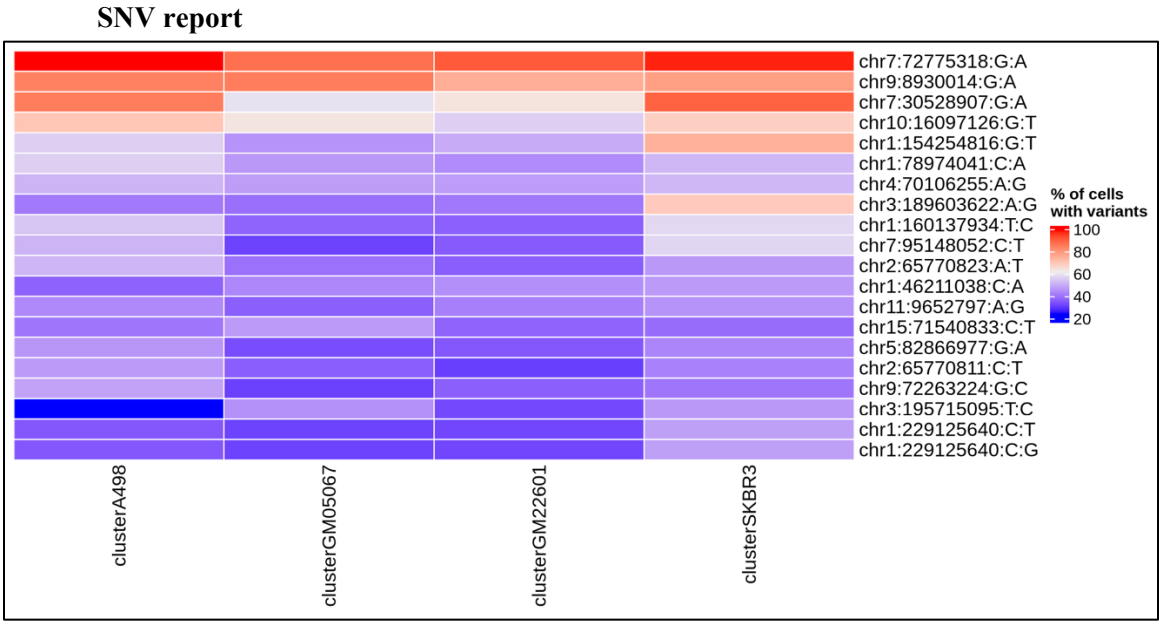


Figure 35. Example Heatmap of Variants from the SNV analysis report.

3. CogentDS Analysis Rdata Object Files

During the generation of the HTML report, an Rdata file is created with the results of the various analysis modules. This file can be used directly as input into CogentDS to perform additional analysis, saving processing time in that tool. The name of the file depends on which analysis is being run; for RNA-seq analysis, the file is called `CogentDS_analysis.rds`; for DNA-seq analysis, it is called `CogentDS_scDNA_analysis.rds`. The RNA-seq analysis rds file contains three assay objects: 'RNA' for exon and intron counts, 'Exon_RNA' for exon-only counts, and 'Transcript_RNA' for transcript counts.

C. Raw Data Files

1. RNA-Seq Analysis

CogentAP RNA-seq analysis generates several raw data files, based on the experiment type. The table below lists the possible raw output files grouped by analysis option as found within the output folder specified during the analysis run (Section VII.A). For more details about the files themselves, please refer to the Appendix.

Table 10. Raw data files generated by CogentAP RNA-seq analysis.

Analysis option	Referred to as	File name	Subfolder
(Default)	Overall Stats*	`analyze_stats.csv`†	
	Gene Info File	`gene_info.csv`	gene_and_transcript_info/
	Transcript Info File	`transcript_info.csv`	gene_and_transcript_info/

Analysis option	Referred to as	File name	Subfolder
(Default)	Gene matrix	`geneCounts_exonOnly.csv`†	count_matrices/
	Gene matrix with intron counts	`geneCounts_exon_plus_intron.csv`†	
	Transcript matrix	`isoformCounts.csv`†	
Gene fusion analysis	Junction matrix	`fusion_junctionCounts.csv`†	count_matrices/
	Spanning matrix	`fusion_spanCounts.csv`†	count_matrices/
	Junction data	`junction/barcodes.tsv.gz, junction/features.tsv.gz, junction/matrix.mtx.gz`	fusion/
	Spanning data	`span/barcodes.tsv.gz, span/features.tsv.gz, span/matrix.mtx.gz`	fusion/
Immune profiling analysis	Clonotype matrix	`immune_clonotype_matrix.csv`	count_matrices/
	Metadata	`immune_metadata.csv`	
	Summary	`immune_summary.csv`	
	Top 3 clonotype matrix	`immune_top3_clonotype_matrix.csv`	
	Top 3 metadata	`immune_top3_metadata.csv`	
	Top 3 summary	`immune_top3_summary.csv`	

*When running data from plate-based full-length transcriptome analysis with UMIs (`smartseq_fla_umi`), two stats files may be generated. Please see the Appendix, Section A for more details.

†Only generated when the number of barcodes in the analysis is ≤5,000.

2. DNA-seq Analysis

The following table lists the possible raw output files generated by CogentAP DNA-seq analysis as found within the output folder specified in the analysis run. For more information about the files themselves, please refer to the Appendix.

Table 11. Raw data files generated by CogentAP DNA-seq analysis.

Analysis option	Referred to as	File name	Subfolder
(Default)	Ginkgo Data	`data`	`ginkgo_output/`
	Ginkgo SegCopy	`SegCopy`	
	Ginkgo SegFixed	`SegFixed`	
	Ginkgo SegNorm	`SegNorm`	
	Ginkgo SegStats	`SegStats`	
(Default)	Multiqc General Stats	`multiqc_general_stats.txt`	

Analysis option	Referred to as	File name	Subfolder
	Multiqc FastQC results	multiqc_fastqc.txt	multiqc_report/multiqc_report_data/
	Multiqc Alignment Stats	multiqc_bowtie2.txt	
	Multiqc Trimming Stats	multiqc_trimmomatic.txt	
	Multiqc Picard MarkDuplicates Stats	multiqc_picard_dups.txt	
	Multiqc Picard InsertSize Stats	multiqc_picard_insertSize.txt	

D. logs Folder

The logs/ folder contains the log files generated by various tools used in the pipeline. They can be used for debugging or reference purposes.

E. BAM Files

1. RNA-Seq Analysis

The star_align_bams/ folder contains BAM files generated during the alignment step in gene and transcript expression analysis. These files are required for gene expression, transcript expression, and gene fusion analysis.

- *.Aligned.out.bam—output files from genome alignment.
- *.Aligned.toTranscriptome.out.bam—output files containing transcriptome-based alignment information that are used for gene and transcript expression analysis.
- *.Chimeric.out.junction—output files with chimeric junction information used in gene fusion analysis

2. DNA-seq Analysis

The bowtie2_align_bams/ folder contains BAM files generated during the alignment step in the WGA workflow.

Appendix A. Analysis of Raw RNA-Seq Data Files

NOTE: The information in this appendix only applies to RNA-seq analysis of sequencing data produced with RNA-seq kits, excluding the Shasta Total RNA-Seq kit, and for analyses with ≤5,000 barcodes. The raw data output files listed in this appendix are all in CSV format.

A. Default Analysis Files

Table 12. Processed data output files generated by the default CogentAP analysis command for RNA-seq analysis.

Referred to as	File name
Stats	analyze_stats.csv
Gene matrix	geneCounts_exonOnly.csv

Referred to as	File name
Gene matrix with intron counts	`geneCounts_exon_plus_intron.csv`
Gene info	`gene_info.csv`
Transcript matrix	`isoformCounts.csv`
Transcript info	`transcript_info.csv`

1. Stats File

The stats file contains barcode-level statistics across the analysis pipeline. Starting from barcoded reads, it summarizes the number of reads after each step in the pipeline: trimmed reads, genomic mapped reads, transcriptomic reads, exon/intron/intergenic reads, mitochondrial reads, ribosomal reads, etc. It also lists the number of genes detected per barcode.

Table 13 and Table 14 document all potential columns that might appear in the stats file. Not all stats files will include every column listed, as the columns shown in this file depend on the reagent kit used to generate the input data.

Table 13. Columns that will be present in the `*_stats.csv` file output by CogentAP (agnostic RNA-seq workflow). Barcoded_Reads are not output for the analyze_direct workflow.

Column name	Description
Barcode	Detected barcodes. This value will usually be the sample name from the well-list or well-list-like file.
Sample	Sample names described in the sample description file. This column is used for grouping stats/plots in CogentDS and should be filled with the type of sample it is.
Barcoded_Reads	Number of reads after demultiplexing.
Trimmed_Reads	Number of reads remaining after trimming.
Total_Mapped_Reads	Number of reads mapping to the genome and reads mapping to ribosomes, globin, and RN7SL2.
Genomic_Mapped_Reads	Number of reads mapped to the genome.
Genomic_Uniquely_Mapped_Reads	Number of reads mapped to unique genomic location.
Genomic_Multimapped_Reads	Number of reads mapped to multiple genomic locations.
Chimeric_Reads	Number of reads that have multiple subsections align to multiple distinct portions of the genome with little or no overlap.
Exon_Reads	Number of reads assigned to an exonic region.
Intron_Reads	Number of reads assigned to an intronic region.

Column name	Description
Gene_Reads	Number of reads assigned to a gene region (exon + intron).
Intergenic_Reads	Number of reads assigned to an intergenic region.
Discarded_Transcriptomic_Reads_STAR	Number of RNA reads mapped by STAR to gene locations in the genome BAM but that STAR is unable to convert to transcriptomic alignment due to incomplete agreement with transcript splice structure.
Discarded_Transcriptomic_Reads_Salmon	Number of transcriptomic reads that Salmon could not confidently assign to transcripts due to ambiguity.
No_of_Genes_Exon_plus_Intron	Number of detected genes based on reads mapped to the exon + intron region.
No_of_Genes_ExonOnly	Number of detected genes based on reads mapped to the exon region.
No_of_Transcripts	Number of detected transcripts.
Ribosomal_Reads	Number of reads assigned to a ribosomal RNA. Note: these reads are depleted before mapping to the genome.
Mitochondrial_Reads	Number of reads mapped to mitochondrial genes.
Transcriptomic_Reads	Number of reads mapped to the transcriptome.
Globin_Reads	Number of reads assigned to globin genes (optional step). Note: these reads are depleted before mapping to the genome.
RN7SL2_Reads	Number of reads assigned to RN7SL2 (optional step). Note: these reads are depleted before mapping to the genome.

Table 14 lists additional columns that will be present in the stats file when the FASTQ input files result from any kits with UMIs. Two types of output files will be generated: one using 5′ UMI-reads and one using all reads (both 5′ UMI and internal non-UMI reads, all combined for non-UMI analysis). The columns below are shown only in the stats file for the 5′ reads.

Table 14. Additional columns in the stats file protocols that utilize UMIs in the workflow.

Column name	Description
No_of_UMIs	Number of UMIs detected among the reads for the sample.
Transcriptomic_Reads_w_UMIs	Number of transcriptomic reads containing UMIs.
Transcriptomic_Reads_After_Dedup	Number of transcriptomic reads left after de-duplication (UMI+USS).

Column name	Description
Duplicated_Reads	Number of reads identified as PCR duplicates and removed during de-duplication.
Exon_Reads_After_Dedup	Number of reads assigned to an exonic region after de-duplication.
Intron_Reads_After_Dedup	Number of reads assigned to an intronic region after de-duplication.
Gene_Reads_After_Dedup	Number of reads assigned to a gene region (exon + intron) after de-duplication.
Strand_Specificity	Ratio of sequencing reads that map to the sense strand (the strand matching the annotated transcript direction) compared to the antisense strand (opposite direction).

2. Gene Matrix File

The gene matrix file (also referred to as the gene table or counts matrix) is also in CSV format and contains gene counts for each barcode, with the genes in the rows and barcodes in the columns. The file contains raw counts that can then be normalized and transformed using CogentDS. An example is shown below.

	AACCGGTTAATATTCG	AACCGGTTACTTCTAC	AACCGGTTAGAAGTAA	AACCGGTTAGCATTGA	AACCGGTTAGGCCAAG	AACCGGTTCAATGGAT
5S-rRNA	0	0	0	0	0	0
5-8S-rRNA	0	0	0	0	0	0
7SK	0	0	0	0	0	0
A1BG	0	5	0	0	0	0
A1BG-AS1	0	0	0	0	0	0
A1CF	0	0	0	0	1	0
A2M	0	0	0	5	328	3
A2M-AS1	0	0	0	0	0	0
A2ML1	0	0	0	1	0	0
A2ML1-AS1	0	0	0	0	0	0
A2ML1-AS2	0	0	0	0	0	0
A2MP1	0	0	0	0	17	0
A3GALT2	0	0	0	0	0	0
A4GALT	0	0	0	0	0	1
A4GNT	0	0	0	0	0	0
AA06	0	0	0	0	0	0
AAAS	0	1	0	0	0	0
AACS	1	0	0	0	0	0
AACSP1	0	0	0	1	0	0
AADAC	0	0	0	0	0	0
AADACL2	0	0	0	0	0	0
AADACL2-AS1	3	0	0	0	1	0
AADACL3	0	0	0	0	0	0
AADACL4	0	0	0	0	0	0
AADACP1	0	0	0	0	0	0
AADAT	0	0	0	0	0	0
AAGAB	1	0	0	0	77	10
AAK1	2	6	21	54	9	34
AAMDC	0	0	0	0	0	4
AAMP	0	32	0	0	12	15

Figure 36. Example of a gene matrix file.

3. Gene Matrix File Including Intron Counts

The gene matrix file including intron counts contains gene counts for each barcode with intron counts added to them, with the genes in the rows and barcodes in the columns. An example is shown below.

	AAGAGCGCAACTTAAC	AAGAGCGCACCGAATT	AAGAGCGCAGAAGTAA	AAGAGCGCAGACCGTT	AAGAGCGCAGATTCAT	AAGAGCGCCAATCTTG	AAGAGCGCCAATGGAT
ABCA15P	0	3	0	2	4	0	2
ABCA17P	1	10	0	0	5	1	5.001
ABCA2	0	0	0	0	0	1	0
ABCA3	3	2	1	5	2	9	1
ABCA3P1	0	0	0	0	0	0	0
ABCA4	2	9	1	8	3	1	7
ABCA5	3	17	0	9	15.53	6	16
ABCA6	15	9	2	2	13	1	3
ABCA7	14	0	0	1	0	0	0
ABCA8	1	10.504	0	1	9	4	5.025
ABCA9	2	8.187	3	2	3.145	7.638	5.769
ABCA9-AS1	0	13.309	0	0	6.855	1.362	10.207
ABCB1	25.266	31.101	0	7.194	27.25	7.663	12.259
ABCB10	1	3	13	1	3	2	3
ABCB10P1	0	0	3	0	0	0	0
ABCB10P3	0	0	0	0	0	0	0
ABCB10P4	0	0	0	0	0	0	0
ABCB11	5	12	3	7	27	7	10
ABCB4	11	8	2	4	8	6	6
ABCB5	6	23	2	20	14	5	14
ABCB6	5	0	0	0	0	0	0
ABCB7	1	6	1	0	6	4	6
ABCB8	1	0	0	0	1	0	0
ABCB9	0	3	1	0	5.04	0	3
ABCC1	17	10	5	3	6	6	16
ABCC10	2	1.012	0	0	0	0	0
ABCC11	0	5	1.014	0	1	0	3
ABCC12	0	3	0	0	2	0	3
ABCC13	6	16.001	3	9	13	8	20
ABCC2	2.002	14	2	5	5	2.004	4
ABCC3	26	4	5	2	1	0	1
ABCC4	18	43	9	8	21	17	14
ABCC5	0	13.253	0	2	5	1	2
ABCC5-AS1	1	0	0	0	0	0	0
ABCC6	0	5.27	1.229	0	1	7	0
ABCC6P1	0	2.73	3.771	1	2	0	1

Figure 37. Example of a gene matrix with intron counts file.

4. Transcript Matrix Files

The transcript matrix file contains transcript counts for each barcode, with the transcripts in the rows and barcodes in the columns. The file contains raw counts that can be normalized and transformed using CogentDS.

	AAGAGCGCAACTTAAC	AAGAGCGCACCGAATT	AAGAGCGCAGAAGTAA	AAGAGCGCAGACCGTT	AAGAGCGCAGATTCAT	AAGAGCGCCAATCTTG
CCNL2-205	17	0	0	0	0	18.327
CCNL2-217	0	0	0	0	0	0
CCNL2-213	0	0	0	0	0	0
CCNL2-212	0	0	0	0	0	0
CCNL2-203	0	0	0	0	0	1.673
CCNL2-219	0	0	0	0	0	0
CCNL2-215	0	0	0	0	0	0
CCNL2-216	0	0	0	0	0	0
CCNL2-207	0	0	0	0	0	0
CCNL2-211	0	0	0	0	0	0
CCNL2-208	0	0	0	0	0	0
CCNL2-202	0	0	0	0	0	0
CCNL2-209	0	0	0	0	0	0
CCNL2-204	0	0	0	0	0	0
CCNL2-206	0	0	0	0	0	0
CCNL2-218	0	0	0	0	0	0
MRPL20-AS1-202	0	0	0	0	0	0
MRPL20-AS1-204	0	0	0	0	0	0
MRPL20-AS1-207	0	0	0	0	0	0
MRPL20-AS1-209	0	0	0	0	0	0
MRPL20-AS1-210	0	0	0	0	0	0
MRPL20-AS1-201	0	0	0	0	0	0
MRPL20-AS1-211	0	0	0	0	0	0
MRPL20-AS1-212	0	0	0	0	0	0
MRPL20-AS1-208	0	0	0	0	0	0
MRPL20-AS1-206	0	0	0	0	1	0
MRPL20-AS1-213	0	0	0	0	0	0
MRPL20-AS1-203	0	0	0	0	0	0
MRPL20-AS1-214	0	0	0	0	0	0
MRPL20-AS1-205	27.009	0	0	0	0	0
MRPL20-201	1.991	0	0	0	0	0

Figure 38. Example of a transcript matrix file.

5. Data Output from the SMART-Seq mRNA LP (with UMIs) Kit

SMART-Seq mRNA LP (with UMIs) is a hybrid kit that generates two types of sequencing reads:

- 5′ end reads containing UMIs
- 'Internal' reads that do not contain UMIs.

CogentAP analyzes both types of reads at the same time and then generates two types of result files for gene expression analysis.

- Rdata object file, gene matrix, transcript matrix and stats files calculated using only the 5′ UMI reads, to be used for UMI-based analyses. These files include the keyword `5pUMI` in the file names.
- Rdata object file, gene matrix, transcript matrix and stats file calculated with all reads, i.e., both 5′ UMI reads and internal reads, to be used for UMI-agnostic analyses. These files include the keyword "all" in the file names.

NOTE: No result files are calculated with just internal reads.

6. Gene and Transcript Info Files

The gene info file contains the main annotations for the genes as described in the GTF file that is part of the genome build.

Table 15. Columns in the `gene_info.csv` output file.

Column name	Description
Gene_ID	Gene ID used in CogentDS, typically the Ensembl ID
Gene_Name	The gene symbol
Gene_Biotype	The gene classification
Gene_Length	The gene length, used for some normalizations.

An example file screenshot is shown below.

Gene_ID	Gene_Name	Gene_Biotype	Gene_Length
ENSG00000228037	ENSG00000228037	lncRNA	2974
ENSG00000142611	PRDM16	protein_coding	369454
ENSG00000284616	ENSG00000284616	lncRNA	5467
ENSG00000157911	PEX10	protein_coding	9834
ENSG00000260972	ENSG00000260972	lncRNA	1697
ENSG00000224340	RPL21P21	processed_pseudogene	337
ENSG00000226374	LINC01345	lncRNA	4478
ENSG00000229280	EEF1DP6	processed_pseudogene	372
ENSG00000142655	PEX14	protein_coding	158471
ENSG00000232596	LINC01646	lncRNA	22536
ENSG00000235054	LINC01777	lncRNA	12663
ENSG00000231510	LINC02782	lncRNA	4441
ENSG00000149527	PLCH2	protein_coding	79553
ENSG00000284739	ENSG00000284739	lncRNA	9345
ENSG00000171621	SPSB1	protein_coding	76639
ENSG00000272235	ENSG00000272235	lncRNA	3461
ENSG00000284694	ENSG00000284694	lncRNA	5245
ENSG00000224387	ENSG00000224387	lncRNA	959
ENSG00000142583	SLC2A5	protein_coding	53373
ENSG00000284674	LINC02781	lncRNA	8188

Figure 39. Example of a gene info file. The gene length column denotes the length of the gene from the start to the end including introns.

The transcript info file contains the main annotation for the transcripts as described in the GTF file that is part of the genome build. This file has a similar format to gene info file. In the case for transcript info file, both gene ID and transcript ID are included in the file.

Table 16. Columns in the `transcript_info.csv` output file.

Column name	Description
Transcript_ID	Transcript ID used in CogentDS, typically the Ensembl ID.
Transcript_Name	The transcript symbol.
Gene_ID	The Gene ID from which the transcript is derived.
Gene_Name	The gene symbol from which the transcript is derived.
Transcript_Biotype	The transcript classification.
Transcript_Length	The transcript length, used for some normalizations.

Transcript_ID	Transcript_Name	Gene_ID	Gene_Name	Transcript_Biotype	Transcript_Length
ENST00000424215	ENST00000424215	ENSG00000228037	ENSG00000228037	lncRNA	2974
ENST00000511072	PRDM16-206	ENSG00000142611	PRDM16	protein_coding	365175
ENST00000607632	PRDM16-210	ENSG00000142611	PRDM16	retained_intron	117409
ENST00000378391	PRDM16-203	ENSG00000142611	PRDM16	protein_coding	366225
ENST00000514189	PRDM16-208	ENSG00000142611	PRDM16	protein_coding	365132
ENST00000270722	PRDM16-201	ENSG00000142611	PRDM16	protein_coding	369419
ENST00000512462	PRDM16-207	ENSG00000142611	PRDM16	protein_coding_CDS_not_defined	195995
ENST00000463591	PRDM16-204	ENSG00000142611	PRDM16	protein_coding	142787
ENST00000509860	PRDM16-205	ENSG00000142611	PRDM16	protein_coding	37803
ENST00000378389	PRDM16-202	ENSG00000142611	PRDM16	protein_coding_CDS_not_defined	9177
ENST00000606170	PRDM16-209	ENSG00000142611	PRDM16	retained_intron	857
ENST00000641871	ENST00000641871	ENSG00000284616	ENSG00000284616	lncRNA	5467
ENST00000288774	PEX10-201	ENSG00000157911	PEX10	protein_coding	8601
ENST00000447513	PEX10-202	ENSG00000157911	PEX10	protein_coding	8591
ENST00000650293	PEX10-209	ENSG00000157911	PEX10	nonsense_mediated_decay	8396
ENST00000507596	PEX10-204	ENSG00000157911	PEX10	protein_coding	7703
ENST00000510434	PEX10-206	ENSG00000157911	PEX10	nonsense_mediated_decay	6018
ENST00000508384	PEX10-205	ENSG00000157911	PEX10	protein_coding	5268
ENST00000515760	PEX10-208	ENSG00000157911	PEX10	protein_coding_CDS_not_defined	1422

Figure 40. Example of a transcript info file. The transcript length column denotes the length of the transcript from the start to the end of the genomic coordinates including introns.

B. Gene Fusion Files

NOTE: These files are only generated when the gene fusion option for RNA-seq analysis (Section V.B.2.A) is used for kits excluding the Shasta Total RNA-Seq kit, and for analysis with ≤5,000 barcodes.

Table 17. Raw data output files generated by CogentAP fusion analysis. Files can be found in the `count_matrices/` subfolder (for the csv files) and `fusion/` subfolder (for the gz files) of the output folder defined during the analysis run.

Referred to as	File name(s)
Junction matrix	`fusion_junctionCounts.csv`
Spanning matrix	`fusion_spanCounts.csv`
Junction Data	`junction/barcodes.tsv.gz`, `junction/features.tsv.gz`, `junction/matrix.mtx.gz`
Spanning Data	`span/barcodes.tsv.gz`, `span/features.tsv.gz`, `span/matrix.mtx.gz`

1. Junction Matrix File

The junction matrix file contains junction read counts for each barcode. In the table, each gene fusion is a row, with the index barcodes (i.e., a cell) in the columns. The table values represent the number of reads detected tagged with the specified barcode that contains the corresponding fusion.

GeneFusion	AACCGGTT	AACCGGTT	AACCGGTT	AACCGGTT	AACCGGTT	AACCGGTT	AACCGGTT	AAGGTCTG	AAGGTCTG	AAGGTCTG...
TPTE2P2--MALAT1	71	52	44	59	43	0	13	86	92	11
TPTE2P2--RPL37	13	9	5	9	13	0	8	5	33	4
MSH2--MRPS18A	9	0	0	0	0	0	0	0	0	0
TPTE2P2--PPIAP29	7	3	1	1	2	0	0	2	0	2
GPAT2--PFN1	9	0	0	1	1	0	0	0	0	1
WDR35--PFN1	7	0	0	0	5	0	2	1	7	2
RPS28--CHST2	5	0	0	0	0	0	0	0	0	0
TPTE2P2--B2M	7	1	10	2	13	7	4	1	13	4
CCNL1--YWHAQ	4	0	0	0	0	0	0	0	0	0
SEPTIN9--MALAT1	2	0	0	0	0	0	0	0	0	0
KNG1--RN7SL2	5	2	0	0	0	1	4	3	1	3
KNG1--AL627171.4	5	2	0	0	0	1	4	3	1	3
TPTE2P2--ACTG1	5	0	0	0	0	0	0	0	0	2
STAT1--CHSY1	5	0	0	0	0	0	0	0	0	0
PXN--ARAP2	3	0	0	0	0	0	0	0	0	0
ACTB--MYL12A	4	0	0	0	0	0	0	0	0	0
UCK2--MEAF6	3	0	0	0	0	0	0	0	0	0
AL135905.2--LMAN2	4	0	0	0	0	0	0	0	0	0
PLEK--OBSCN	6	0	0	0	0	0	0	0	0	0
TMEM59--GRK6	3	0	0	0	0	0	0	0	0	0
...										

Figure 41. Example of a junction matrix file.

2. Spanning Matrix File

The spanning matrix file contains spanning read counts for each barcode, with the gene fusion in the rows and barcodes/cells in the columns. Each value is the number of paired-end reads containing the sequences of both genes that form the corresponding fusion.

GeneFusion	AACCGGTT	AACCGGTT	AACCGGTT	AACCGGTT	AACCGGTT	AACCGGTT	AACCGGTT	AAGGTCTG	AAGGTCTG	AAGGTCTG...
TPTE2P2--MALAT1	0	0	0	0	0	0	0	0	0	0
TPTE2P2--RPL37	0	0	0	0	0	0	0	0	0	0
MSH2--MRPS18A	16	0	0	0	0	0	0	0	0	0
TPTE2P2--PPIAP29	0	0	0	0	0	0	0	0	0	0
GPAT2--PFN1	0	0	0	0	0	0	0	0	0	0
WDR35--PFN1	0	0	0	0	0	0	0	0	0	0
RPS28--CHST2	8	0	0	0	0	0	0	0	0	0
TPTE2P2--B2M	0	0	0	0	0	0	0	0	0	0
CCNL1--YWHAQ	6	0	0	0	0	0	0	0	0	0
SEPTIN9--MALAT1	14	0	0	0	0	0	0	0	0	0
KNG1--RN7SL2	0	0	0	0	0	0	0	0	0	0
KNG1--AL627171.4	0	0	0	0	0	0	0	0	0	0
TPTE2P2--ACTG1	0	0	0	0	0	0	0	0	0	0
STAT1--CHSY1	7	0	0	0	0	0	0	0	0	0
PXN--ARAP2	11	0	0	0	0	0	0	0	0	0
ACTB--MYL12A	5	0	0	0	0	0	0	0	0	0
UCK2--MEAF6	9	0	0	0	0	0	0	0	0	0
AL135905.2--LMAN2	0	0	0	0	0	0	0	0	0	0
PLEK--OBSCN	4	0	0	0	0	0	0	0	0	0
TMEM59--GRK6	4	0	0	0	0	0	0	0	0	0
...										

Figure 42. Example of a spanning matrix file.

These files are automatically used to generate fusion overlays in the final CogentDS Rdata file provided that the `--fusion` parameter was enabled during the full analysis (see Section VII.B.3). In the case of standalone fusion analysis, these files cannot be directly imported into CogentDS in the current version of the software but may be enabled in future versions.

C. Immune Profiling Files

NOTE: These files are only generated when the immune profiling option for RNA-seq analysis (Section V.B.2.b) is used.

Table 18. Raw data output files generated by CogentAP immune analysis. Files can be found in the `count_matrices/` subfolder of the output folder defined during the analysis run.

Referred to as	File name
Clonotype matrix	`immune_clonotype_matrix.csv`
Metadata	`immune_metadata.csv`
Summary	`immune_summary.csv`
Top 3 clonotype matrix	`immune_top3_clonotype_matrix.csv`
Top 3 metadata	`immune_top3_metadata.csv`
Top 3 summary	`immune_top3_summary.csv`

1. Clonotype Matrix

The clonotype matrix file contains clonotype counts for each barcode, with the clonotype by rows and barcodes (i.e., cells) in the columns.

The clonotype is defined as joining of V, D, and J genes, constant regions (C), and CDR3 amino acid (CDR3aa) sequences, connected by the dollar-sign (`$`) symbol.

```
<V gene>$<D gene>$<J gene>$<constant region><$CDR3 aa>
```

A period (`.`) is used in place of a segment that doesn't exist in the clonotype.

Examples:

```
TRBV20-1*01$TRBD2*02$TRBJZ-7*01$TRBC$CSAGSGRGGRAVEQYF

IGKV4-1*01$.$IGKJ4*01$IGKC$CQQYYSTPALTF
```

In the second clonotype, the D gene isn't present, so the period is used.

Table 19. Columns in the `*_clonotype_matrix.csv` output file.

Column name	Description
V-D-J-C-CDR3aa	The string of V, D, and J genes, constant region, and CDR3 amino acid segment details, concatenated by the $ symbol.
<barcode1> **…** **…** **…** **<barcode*N*>**	Subsequent columns correspond to the joint clonotype segments identified for the barcode listed in the column header. The cell values are a count of the clonotype reads found for the V-D-J-C-CDR3aa combination.

An example file screenshot is shown below.

V-D-J-C-CDR3aa	AATGGTAAT	CATAATGGT	CGCGGTCGT	CGCGGTCGT	TTGTAATAG	CGTAATGGT	CGAAGTCGT	CGTTGTCGT
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$CARVFDAEISTGYYLPYYFDYW	0	3	0	0	0	3	0	0
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$CARVFDDEFSTGYYLPYYFDYW	0	5	0	0	0	5	0	0
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$CARVFDDEISTGYYIPYYFDYW	0	10	0	0	0	10	0	0
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$CARVFDDEISTGYYLPYYFDYW	0	35521	0	0	0	0	0	0
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$CARVFDDENSTGYYLPYYFDYW	0	3	0	0	0	3	0	0
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$CARVFGDEISTGYYLPYYFDYW	0	5	0	0	0	5	0	0
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$CARVLDDEISTGYYLPYYFDYW	0	5	0	0	0	5	0	288
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$CGRVFDDEISTGYYLPYYFDYW	0	7	0	0	0	7	0	0
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$RARVFDDEISTGYYLPYYFDYW	0	16	0	0	0	16	0	0
IGKV1-12*01$.$IGKJ5*01$IGKC$CQQASSFPPVTF	3	0	0	0	3	0	0	0
IGKV4-1*01$.$IGKJ4*01$IGKC$CQQYYSTPALTF	0	0	0	166	0	0	0	0
IGKV4-1*01$.$IGKJ4*01$IGKC$CQ_YYSTPALTF	0	0	0	4	0	0	0	3
IGKV4-1*01$.$IGKJ4*01$IGKC$RQQYYSTPALTF	0	0	0	3	0	0	0	0
IGKV4-1*01$.$IGKJ4*01$IGKC$SQQYNSTPALTF	0	0	0	2	0	0	0	1
IGLV3-19*01$.$IGLJ2*01$IGLC$CNSRDTSDNHLVF	0	7731	0	0	0	532	0	0
TRAV8-2*01$.$TRAJ12*01$.$CVVSPMASSYKLIF	0	0	2	0	0	0	2	0
TRAV8-2*01$.$TRAJ12*01$TRAC$CVVSPMDSSYKLIF	0	0	5	0	0	0	5	0
TRBV20-1*01$TRBD2*02$TRBJ2-7*01$TRBC$CSAGSGRGGRAVEQYF	0	0	22	0	0	0	22	0

Figure 43. Example of a clonotype matrix file.

2. Top3 Clonotype Matrix

The top3 clonotype matrix file is identical in format to the clonotype matrix file, but only contains information based on the three clonotypes identified with the maximum reads in the dataset, which correspond to the top3 rows of an intermediate raw clonotype file.

3. Metadata

The metadata file contains clonotypes and their V, D, J, and C segment details, corresponding CDR3 amino acid sequences, and two columns with a boolean value 'Y' or 'N' to mark if the clonotype is a light or heavy chain. Similar to the clonotype_matrix file above, the clonotype is defined as joining of V, D, and J genes, constant region (C), and the CDR3 amino acid sequences (CDR3aa), connected by the $ symbol.

Table 20. Columns in the `*_metadata.csv` output file.

Column name	Description
V-D-J-C-CDR3aa	The string of V, D, and J genes, constant region, and CDR3 amino acid segment details, concatenated by the $ symbol.
V	V segment of the clonotype.
D	D segment of the clonotype.
J	J segment of the clonotype.
C	C segment of the clonotype.
CDR3aa	CDR3 amino acid segment of the clonotype.
Light Chain	Boolean value (Y or N). A 'Y' value designates the clonotype as a light chain.
Heavy Chain	Boolean value (Y or N). A 'Y' value designates the clonotype as a heavy chain.

An example file screenshot is shown below.

V-D-J-C-CDR3aa	V	D	J	C	CDR3aa	Light Chain	Heavy Chain
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$CARVFDAEISTGYYLPYYFDYW	IGHV4-61*01	IGHD3-9*01	IGHJ4*02	IGHG1	CARVFDAEISTGYYLPYYFDYW	N	Y
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$CARVFDDEFSTGYYLPYYFDYW	IGHV4-61*01	IGHD3-9*01	IGHJ4*02	IGHG1	CARVFDDEFSTGYYLPYYFDYW	N	Y
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$CARVFDDEISTGYYIPYYFDYW	IGHV4-61*01	IGHD3-9*01	IGHJ4*02	IGHG1	CARVFDDEISTGYYIPYYFDYW	N	Y
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$CARVFDDEISTGYYLPYYFDYW	IGHV4-61*01	IGHD3-9*01	IGHJ4*02	IGHG1	CARVFDDEISTGYYLPYYFDYW	N	Y
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$CARVFDDENSTGYYLPYYFDYW	IGHV4-61*01	IGHD3-9*01	IGHJ4*02	IGHG1	CARVFDDENSTGYYLPYYFDYW	N	Y
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$CARVFGDEISTGYYLPYYFDYW	IGHV4-61*01	IGHD3-9*01	IGHJ4*02	IGHG1	CARVFGDEISTGYYLPYYFDYW	N	Y
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$CARVLDDEISTGYYLPYYFDYW	IGHV4-61*01	IGHD3-9*01	IGHJ4*02	IGHG1	CARVLDDEISTGYYLPYYFDYW	N	Y
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$CGRVFDDEISTGYYLPYYFDYW	IGHV4-61*01	IGHD3-9*01	IGHJ4*02	IGHG1	CGRVFDDEISTGYYLPYYFDYW	N	Y
IGHV4-61*01$IGHD3-9*01$IGHJ4*02$IGHG1$RARVFDDEISTGYYLPYYFDYW	IGHV4-61*01	IGHD3-9*01	IGHJ4*02	IGHG1	RARVFDDEISTGYYLPYYFDYW	N	Y
IGKV1-12*01$.$IGKJ5*01$IGKC$CQQASSFPPVTF	IGKV1-12*01	.	IGKJ5*01	IGKC	CQQASSFPPVTF	Y	N
IGKV4-1*01$.$IGKJ4*01$IGKC$CQQYYSTPALTF	IGKV4-1*01	.	IGKJ4*01	IGKC	CQQYYSTPALTF	Y	N
IGKV4-1*01$.$IGKJ4*01$IGKC$CQ_YYSTPALTF	IGKV4-1*01	.	IGKJ4*01	IGKC	CQ_YYSTPALTF	Y	N
IGKV4-1*01$.$IGKJ4*01$IGKC$RQQYYSTPALTF	IGKV4-1*01	.	IGKJ4*01	IGKC	RQQYYSTPALTF	Y	N
IGKV4-1*01$.$IGKJ4*01$IGKC$SQQYNSTPALTF	IGKV4-1*01	.	IGKJ4*01	IGKC	SQQYNSTPALTF	Y	N
IGLV3-19*01$.$IGLJ2*01$IGLC$CNSRDTSDNHLVF	IGLV3-19*01	.	IGLJ2*01	IGLC	CNSRDTSDNHLVF	Y	N
TRAV8-2*01$.$TRAJ12*01$.$CVVSPMASSYKLIF	TRAV8-2*01	.	TRAJ12*01	.	CVVSPMASSYKLIF	Y	N
TRAV8-2*01$.$TRAJ12*01$TRAC$CVVSPMDSSYKLIF	TRAV8-2*01	.	TRAJ12*01	TRAC	CVVSPMDSSYKLIF	Y	N
TRBV20-1*01$TRBD2*02$TRBJ2-7*01$TRBC$CSAGSGRGGRAVEQYF	TRBV20-1*01	TRBD2*02	TRBJ2-7*01	TRBC	CSAGSGRGGRAVEQYF	N	Y

Figure 44. Example of a clonotype metadata file.

4. Top3 Metadata

The top3 metadata file is identical in format to the metadata file, but only contains information based on the three clonotypes identified with the maximum reads in the dataset, which correspond to the top3 lines of raw clonotype output file.

5. Summary

The summary file contains barcodes, total reads, and clonotypes identified per barcode. It has 62 columns, which include four components: barcode, total reads, clonotype category marks, and segment call details.

- The first two columns in the table are the barcode and total reads.
- Columns 3–14 are clonotype categories, which mark and summarize if a clonotype category was identified for the barcode, i.e.,
  - (For TCR): TRA, TRB, TRD, and TRG
  - (For BCR): IGG, IGD, IGA, IGM, IGE, IGH, IGK, and IGL

If a category is not identified, the cell value is left blank.

- Columns 15–62 are listed V, D, and J genes and constant region details. For example: IGHV-1, IGHD3-1, IGHJ4 and IGHG1 for the IGH category (i.e., four columns per clonotype category).

Table 21. Columns in the `*_full_summary.csv` output file.

Row #	Column name	Description
1	Barcode	The barcode sequence of a single cell.
2	Total_Reads	Number of reads with clonotype type identified for the corresponding barcode.
3–6	TRA, TRB, TRD, TRG	Categories for T-cell (TCR) chain types. The cell value will match the column header if the chain type is detected with the given barcode. If it is not detected, the cell will be blank.
7–14	IGG, IGD, IGA, IGM, IGE, IGH, IGK, IGL	Categories for B-cell (BCR) chain types. The cell value will match the column header if the chain type is detected with the given barcode. If it is not detected, the cell will be blank.

Row #	Column name	Description
15–18	TRA_V, TRA_D, TRA_J, TRA_C	(TCR) V, D, and J genes and constant region identified within the TRA chain type.
19–22	TRB_V, TRB_D, TRB_J, TRB_C	(TCR) V, D, and J genes and constant region identified within the TRB chain type.
23–26	TRD_V, TRD_D, TRD_J, TRD_C	(TCR) V, D, and J genes and constant region identified within the TRD chain type.
27–30	TRG_V, TRG_D, TRG_J, TRG_C	(TCR) V, D, and J genes and constant region identified within the TRG chain type.
31–34	IGG_V, IGG_D, IGG_J, IGG_C	(BCR) V, D, and J genes and constant region identified within the IgG chain type.
35–38	IGD_V, IGD_D, IGD_J, IGD_C	(BCR) V, D, and J genes and constant region identified within the IgD chain type.
39–42	IGA_V, IGA_D, IGA_J, IGA_C	(BCR) V, D, and J genes and constant region identified within the IgA chain type.
43–46	IGM_V, IGM_D, IGM_J, IGM_C	(BCR) V, D, and J genes and constant region identified within the IgM chain type.
47–50	IGE_V, IGE_D, IGE_J, IGE_C	(BCR) V, D, and J genes and constant region identified within the IgE chain type.
51–54	IGH_V, IGH_D, IGH_J, IGH_C	(BCR) V, D, and J genes and constant region identified within the IgH chain type.
55–58	IGK_V, IGK_D, IGK_J, IGK_C	(BCR) V, D, and J genes and constant region identified within the IgK chain type.
59–62	IGL_V, IGL_D, IGL_J, IGL_C	(BCR) V, D, and J genes and constant region identified within the IgL chain type.

6. Top3 Summary

The top3 summary file is identical in format to the summary file, but only contains information based on the three clonotypes identified with the maximum reads in the dataset, which correspond to the top3 lines of raw clonotype output file.

Appendix B. Analysis of Raw DNA-seq Data Files

A. Default Analysis Files

Table 22. Processed data output files generated by the default CogentAP analysis command for DNA-seq analysis.

Referred to as	File name
Ginkgo Data	`data`
Ginkgo SegCopy	`SegCopy`
Ginkgo SegFixed	`SegFixed`
Ginkgo SegNorm	`SegNorm`
Ginkgo SegStats	`SegStats`
Multiqc General Stats	`multiqc_general_stats.txt`
Multiqc FastQC results	`multiqc_fastqc.txt`

Referred to as	File name
Multiqc Alignment Stats	`multiqc_bowtie2.txt`
Multiqc Trimming Stats	`multiqc_trimmomatic.txt`
Multiqc Picard MarkDuplicates Stats	`multiqc_picard_dups.txt`
Multiqc Picard InsertSize Stats	`multiqc_picard_insertSize.txt`

B. Ginkgo Output Files

The output files from Ginkgo—`data`, `SegCopy`, `SegFixed`, `SegNorm` and `SegStats`—contain various metrics from the single-cell CNV analysis performed.

- Ginkgo Data—a tab-delimited file containing the raw read counts per bin per cell.
- Ginkgo SegCopy—a tab-delimited file containing final copy number estimates per bin per cell.
- Ginkgo SegFixed—a tab-delimited file containing read counts per bin per cell after segmentation, but before ploidy adjustment.
- Ginkgo SegNorm—a tab-delimited file containing read counts per bin per cell after GC-lowess normalization.
- Ginkgo SegStats—a tab-delimited file containing basic stats on read counts per bin for each cell.

C. Multiqc Output Files

The multiqc output files are the raw data files used to generate the tables and figures within the QC Metrics Report (Section VII.B.2).

- Multiqc General Stats—a tab-delimited file containing an overview of key values, taken from all the modules that were used in the analysis for each cell.
- Multiqc FastQC results—a tab-delimited file containing sequencing quality metrics for each cell.
- Multiqc Alignment Stats—a tab-delimited file containing alignment statistics derived from Bowtie2 for each cell.
- Multiqc Trimming Stats—a tab-delimited file containing trimming statistics derived from Trimmomatic for each cell.
- Multiqc Picard MarkDuplicates Stats—a tab-delimited file containing statistics derived from Picard MarkDuplicates for each cell.
- Multiqc Picard InsertSize Stats—a tab-delimited file containing statistics derived from Picard InsertSize metrics for each cell.

Contact Us	
Customer Service/Ordering	**Technical Support**
tel: 800.662.2566 (toll-free)	tel: 800.662.2566 (toll-free)
fax: 800.424.1350 (toll-free)	fax: 800.424.1350 (toll-free)
web: takarabio.com/service	web: takarabio.com/support
e-mail: ordersUS@takarabio.com	e-mail: technical_support@takarabio.com

Notice to Purchaser

Our products are to be used for **Research Use Only**. They may not be used for any other purpose, including, but not limited to, use in humans, therapeutic or diagnostic use, or commercial use of any kind. Our products may not be transferred to third parties, resold, modified for resale, or used to manufacture commercial products or to provide a service to third parties without our prior written approval.

Your use of this product is also subject to compliance with any applicable licensing requirements described on the product's web page at takarabio.com. It is your responsibility to review, understand and adhere to any restrictions imposed by such statements.

This document has been reviewed and approved by the Quality Department.