April 2019

EpiTect® Hi-C Data Analysis Portal User Guide

For use with the EpiTect Hi-C Data Analysis portal. Provides a detailed description of its HiC-Pro–based analysis pipeline, including explanations for all output files and links to relevant resources for additional information.

For Research Use Only. Not for use in diagnostic procedures.

Contents

1 Introduction

At the online GeneGlobe® Data Analysis Center of QIAGEN®, Hi-C sequencing results can be analyzed using the EpiTect Hi-C Analysis Portal. Sequencing reads are first processed through a pipeline based on the open-source HiC-Pro toolset (1) to generate a sequencing report and Hi-C contact matrices. Upon completion of the data analysis, an installation of HiGlass (2) within GeneGlobe can be used to visualize and interact with the generated contact matrices.

1.1 About this user guide

This user guide provides information about the EpiTect Hi-C Analysis Portal in the following sections:

- Introduction
- Operating Procedures
- Technical Data
- References
- Ordering Information

1.2 General information

1.2.1 Technical assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Services Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN products. If you have any questions or experience any difficulties with QIAGEN products, do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance, contact QIAGEN Technical Services via your regional technical support number, available at **www.qiagen.com/support**.

1.2.2 Policy statement

It is the policy of QIAGEN to improve products as new techniques and components become available. QIAGEN reserves the right to change specifications at any time. In an effort to produce useful and appropriate documentation, we appreciate your comments on this user guide. Please contact QIAGEN Technical Services via your regional technical support number, available at **www.qiagen.com/support**.

1.3 Intended use

The EpiTect Hi-C Data Analysis Portal is intended to be used only in combination with QIAGEN kits indicated for use with the EpiTect Hi-C Data Analysis Portal for applications described in the respective QIAGEN kit product sheets or handbooks.

The EpiTect Hi-C Data Analysis Portal is intended for research use only. Not for use in diagnostic procedures.

The EpiTect Hi-C Data Analysis Portal is intended for use by professional users trained in molecular biology techniques.

2 Operating Procedures

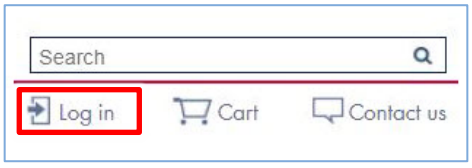
2.1 Workstation requirements

The EpiTect Hi-C Data Analysis Portal should be used with a Google Chrome® or Mozilla® Firefox® browser. The portal is not compatible with Internet Explorer®.

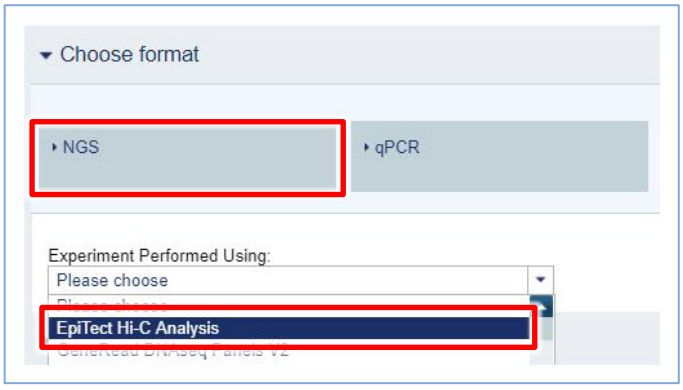
2.2 Getting started

2.2.1 Accessing the portal

1. Go to **www.qiagen.com/DataAnalysisCenter** and click **Log in**.



2. Input your QIAGEN username and password to log in. If you are a new user, select **Register now** to create an account first.
3. In "Choose format", select **NGS**. In "Experiment Performed Using", select **EpiTect Hi-C Analysis**.



You will be automatically transferred to the Hi-C Analysis portal, which has 4 tabs:

- **BaseSpace Files**: For uploading high-depth sequencing data (≥3 gigabytes per file).
- **File Upload**: For uploading low-depth sequencing data (<3 gigabytes per file) from the user's own computer.
- **File Management**: For displaying and, if necessary, deleting files uploaded to the EpiTect Hi-C Analysis Portal via the File Upload tab. **Note**: Files in the BaseSpace Files tab do not appear here.
- **Hi-C Analysis**: For analyzing data uploaded from either the BaseSpace Files or the File Upload tab.

2.3 Uploading sequence files

High-depth sequencing data (≥3 gigabytes per file) must be uploaded through the BaseSpace Files tab, using Illumina® BaseSpace®.

Low-depth sequencing data may be uploaded either from Illumina BaseSpace, through the BaseSpace Files tab, or from the user's local computer, using the File Upload tab.

2.3.1 BaseSpace Files upload

1. From the EpiTect Hi-C Data Analysis Portal, select the **BaseSpace Files** tab.
2. If the User Agreement page appears, you need to accept the agreement to continue, by clicking **I Accept These Agreements** at the bottom of the page.
3. Enter login credentials for your Illumina account, and then select **Sign In**.

4. Upon successful login, you will be returned to the BaseSpace Files tab in the Hi-C Analysis portal. Select **Browse My Projects** or **Browse My Runs**, as appropriate, to continue.
5. Under the **Name** column, select the name of the project or run that you want to open.
6. Click **Grant Download Permission** to give QIAGEN limited access to your BaseSpace files.
7. Select the corresponding R1 and R2 files for each sample to be analyzed by ticking the boxes in the rightmost column.

8. Click **Select Files For Analysis**. You will automatically be transferred to the Hi-C Analysis tab. Proceed to the "Analyzing uploaded sequence files" section, page 8.

   IMPORTANT: Do not navigate away from the Hi-C Analysis tab after you have been transferred there from the BaseSpace Files tab, or the sequencing files from BaseSpace will disappear in the Hi-C Analysis tab's "Select Read Files" dropdown menu. To make the sequencing files appear again in "Select Read Files", you will need to return to the BaseSpace Files tab and initiate analysis again by clicking **Select Files For Analysis**.

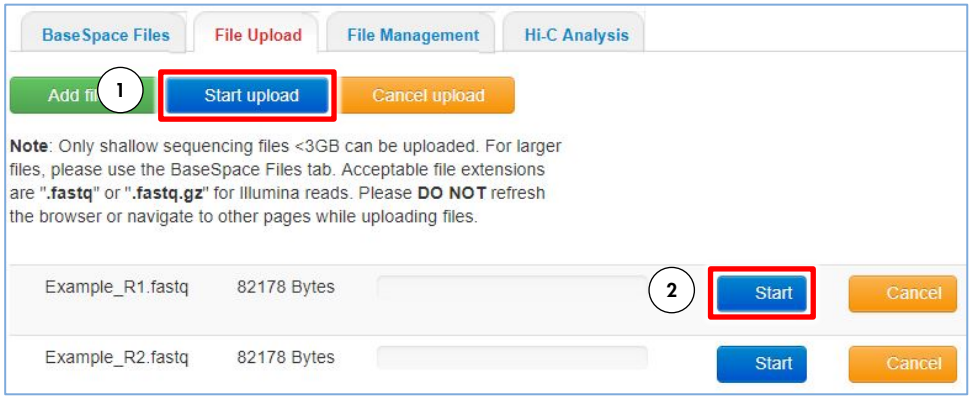
2.3.2 Local computer file upload

Note: Users may upload sequence data from their local computer only if each file is <3 gigabytes. For uploading high-depth sequencing data (≥3 gigabytes per file), Illumina BaseSpace must be used.

1. From the EpiTect Hi-C Data Analysis Portal, select the **File Upload** tab.
2. Select **Add Files** and navigate to the sequencing data files that are to be uploaded. To select multiple files, use the **Shift** or **Ctrl** key.

   Important: For each sample, 2 separate read files must be uploaded. The filename for the read file 1 must contain "_R1". The filename for the read file 2 must contain "_R2".
3. Select **Open** to link files to the EpiTect Hi-C Analysis Portal.

   Note: File upload has not begun at this point.
4. To upload all files at once, select **1: Start upload**. To upload files individually, select **2:** the blue **Start** button next to each file. Green progress bars indicate the progress of each file upload.



5. Following successful upload, files can be analyzed. To do so, proceed to the "Analyzing uploaded sequence files" section below.

2.4 Analyzing uploaded sequence files

1. In the Hi-C Analysis tab, under “Species”, select the reference genome to which the sequencing reads should be mapped.
2. In “Select Read Files”, select the sequence files you wish to analyze.

   Important: For each sample, the corresponding R1 and R2 read files must be selected.
3. Select **Create Job** to start the Hi-C analysis. Initially, the job status will appear as “queued”. Once analysis is complete, the job status will change to “done successfully” and 2 links – **Download Report** and **HiGlass Analysis** – will appear.
4. Select **Download Report** to save into your local drive a ZIP file containing the EpiTect Hi-C Analysis report.
5. Select **HiGlass Analysis** to be transferred to an installation of HiGlass where you can visualize the contact matrices generated from your Hi-C sequencing data.
6. For further details about the analysis and contents of the EpiTect Hi-C Analysis report, refer to the “Technical Data” section (page 9).

3 Technical Data

This section contains information on the EpiTect Hi-C Analysis pipeline.

3.1 Code

The code for running several steps in the Hi-C Analysis portal and for aggregating the results is publicly available in the repository qiaseq-HiC on GitHub®.

3.2 HiC-Pro

The core of the read-analysis for Hi-C reads is the published (1) pipeline named HiC-Pro. Source-code can be found on GitHub. The HiC-Pro workflow can be divided into steps presented below.

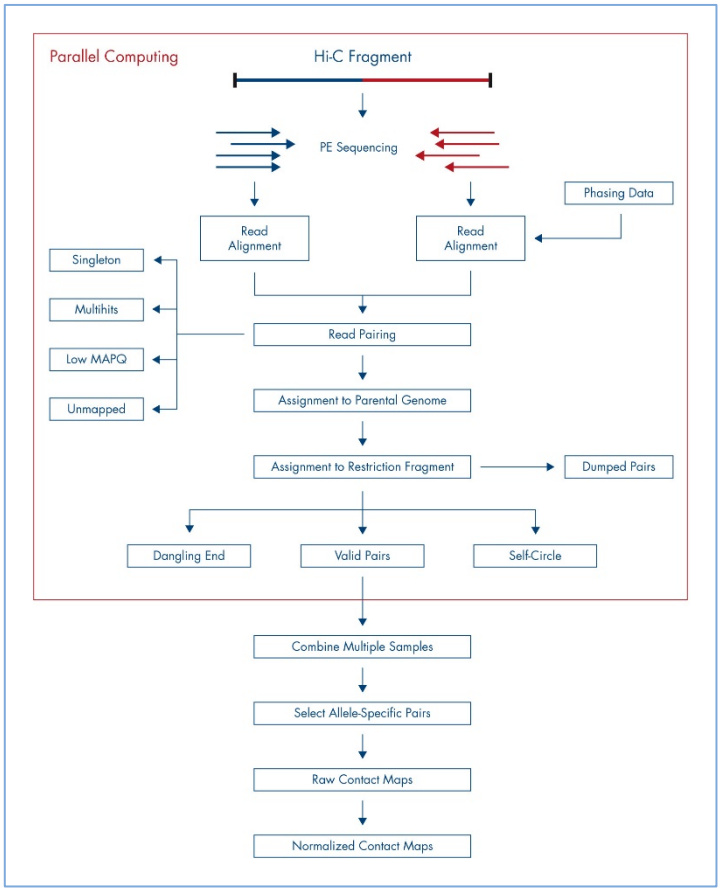


Figure 1. The HiC-Pro workflow.

3.2.1 Reads mapping

Each mate is independently aligned on the reference genome. The mapping is performed in 2 steps. First, the reads are aligned using an end-to-end aligner. Second, reads spanning the ligation-junction are trimmed from their 3' end and aligned back on the genome. Aligned reads for both fragment mates are then paired in a single paired-end BAM file. Singletons and multiple hits can be discarded according to the configuration parameters.

3.2.2 Fragment assignment and filtering

Each aligned read can be assigned to one restriction fragment, according to the reference genome and the restriction enzyme. The next step is to separate the invalid ligation products from the valid pairs. Dangling-end and self-circles pairs are therefore excluded. Only valid pairs involving 2 different restriction fragments are used to build the contact maps. Duplicated valid pairs associated with PCR artifacts are discarded. The fragment assignment can be visualized through a BAM file of aligned pairs where each pair is flagged according to its classification.

3.2.3 Quality controls

HiC-Pro performs a couple of quality controls for most of the analysis steps. The alignment statistics are the first quality controls. Aligned reads in the first (end-to-end) step and alignment after trimming are reported. Note that in practice, we usually observe around 10–20% of trimmed reads. An abnormal level of trimmed reads can reflect a ligation issue. Once the reads are aligned on the genome, HiC-Pro checks the number of singletons, multiple hits or duplicates. The fraction of valid pairs are presented for each type of ligation product. Invalid pairs such as dangling ends or self-circles are also represented. A high level of dangling ends or an imbalance in valid pairs ligation type can be due to a ligation, fill-in or digestion issue. Finally, HiC-Pro also calculates the distribution of fragment size on a subset of valid pairs. Additional statistics report the fraction of intrachromosomal versus interchromosomal contacts, as well as the proportion of short-range (<20 kb) versus long-range (>20 kb) contacts.

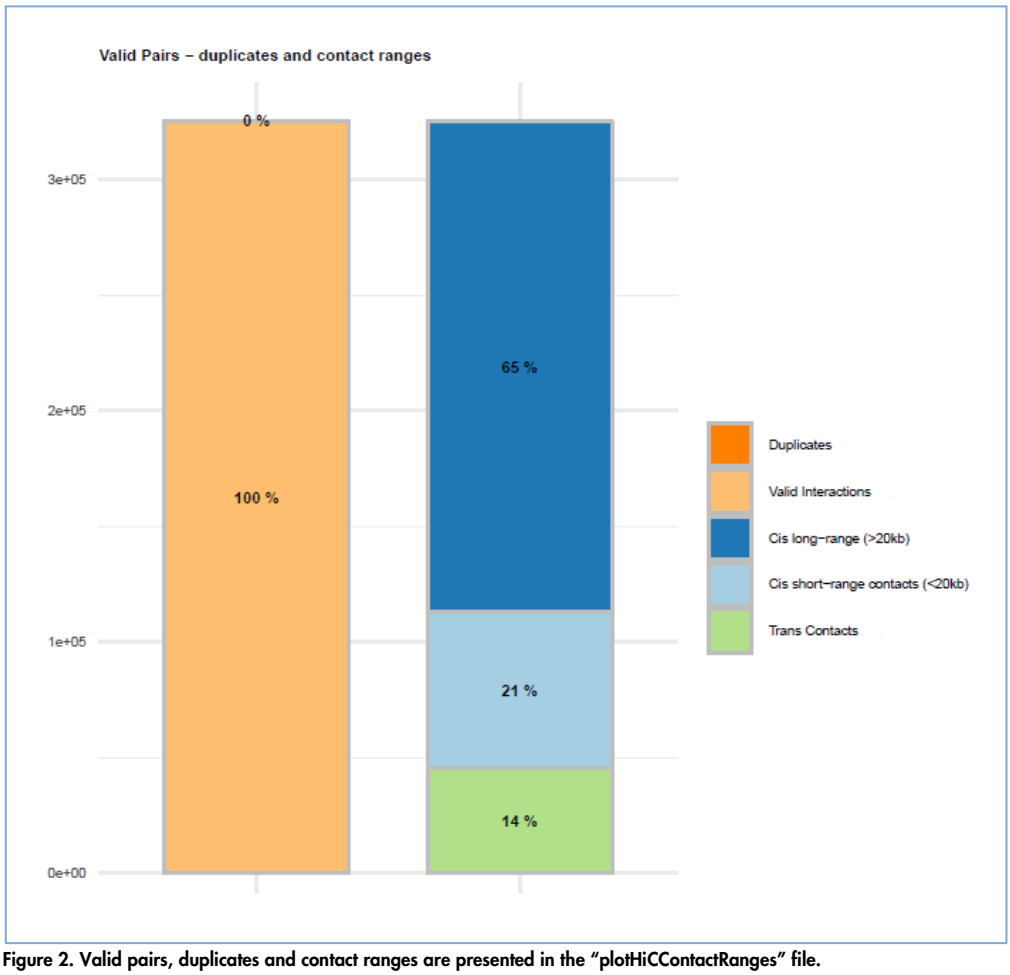


Figure 2. Valid pairs, duplicates and contact ranges are presented in the "plotHiCContactRanges" file.

All quality control plots of the abovementioned measures can be found in the subfolder named "/pic". An example of the "plotHiCContactRanges" file is presented in Figure 2.

3.2.4 Map builder

Intrachromosomal and interchromosomal contact maps are built for a resolution of 2500 bases. The genome is split into bins of equal size. Each valid interaction is associated with the genomic bins to generate the raw map.

3.3 Post processing

A few additional tools are run to convert the output files from HiC-Pro into formats that are commonly used and compatible with a range of downstream analysis tools and visualization software.

3.3.1 Pairs

A custom script is used to convert the "allValidPairs" file into the PAIRS format according to specifications defined by the 4D Nucleome Network (www.4dnucleome.org). This file includes the 2 optional columns named "frag1" and "frag2", which contain the integer index of the restriction fragment in the genome.

This file is the most comprehensive result because it contains all individual contacts at single-base resolution (no binning), including the readID of the library fragment from the input read data. The uncompressed plain-text file looks like the example below:

```
## pairs format v1.0
#sorted: chr1-chr2-pos1-pos2
#shape: upper triangle
#genome_assembly: hg38
#columns: readID chr1 pos1 chr2 pos2 strand1 strand2 frag1 frag2
BJFY6:1:1112:5915:11069        chr1    629270  chr1       81381488      -     +
                               1195    216143
BJFY6:1:2101:14333:2314        chr1    629484  chr1      242769744      -     -
                               1195    568557
BJFY6:1:2117:22179:2271        chr1    631129  chr1         634141      +     -
                               1195      1202
BJFY6:1:2116:17587:24798       chr1    631237  chr1      200809963      +     +
                               1195    461302
BJFY6:1:1110:8536:16896        chr1    631251  chr1         633698      +     +
                               1195      1199
```

The tool pairix was used to create the index ".pairs.gz.px2". The index is required before running pairix queries. Using the pairs file is a solid choice for any downstream analyses other than visualization (for that, see below).

Output files are stored in a folder named "/pairs" and the data are compressed (".pairs.gz").

3.3.2 Juicer tools (.hic)

The "pre" command of Juicer Tools (github.com/aidenlab/juicer) is run to create HIC files (located in the "/hic" subfolder) based on the PAIRS file. The HIC file is a highly compressed binary file that stores contact matrices from multiple resolutions in a clever way, allowing random access. The format is described extensively by Durand et al. in *Juicer provides a one-click system for analyzing loop-resolution Hi-C experiments* (3).

The visualization tool Juicebox (www.aidenlab.org/juicebox) uses the fast querying capabilities of HIC files to make it possible to zoom in and out of many different resolutions quickly. Juicebox is available for Windows®, macOS® and Linux, and there is even an online version.

3.3.3 Cooler (.mcool)

Cooler is a support library for a sparse, compressed binary persistent storage format called COOL, used to store genomic interaction data, such as Hi-C contact matrices. The COOL file format is a reference implementation of a genomic matrix data model using HDF5 as the container format.

The "cooler" tool (github.com/mirnylab/cooler) is run twice: First, a 2500 bp binned contact matrix generated by HiC-Pro is converted into the COOL file format running cooler load. Then, the result is enriched by "cooler zoomify" to generate a multi-resolution contact-matrix file MCOOL, stored in the subfolder named "/mcool". These files can be visualized by HiGlass (higlass.io), a feature-rich and very responsive browser-based software package (4).

3.4 Output files

Every analysis job will generate 2 overview files in top-level of the results and a range of additional data files (plots and contact matrices) organized in subfolders.

3.4.1 Multi-QC HTML

MultiQC (multiqc.info) is a tool to create a single report with interactive plots for multiple bioinformatics analyses across many samples (5). The MultiQC HiC-Pro module, available at github.com/ewels/MultiQC/tree/master/multiqc/modules/hicpro, parses the results generated by HiC-Pro, and it was written by the same author as that of the HiC-Pro itself.

3.4.2 Excel® file summary

A custom script aggregates the HiC-Pro results and exports them into a Microsoft® Excel workbook. Values are presented either as an absolute number of counts or as percentages of total. The results for each Hi-C sample are organized into a single column. The Excel report is divided into several sections that represent the different processing stages of HiC-Pro.

3.4.2.1 Mapping R1/Mapping R2

Usually, a high fraction of reads is expected to be aligned on the genome (80–90%). Among them, a small percent (around 10–20%) align only after trimming. This can be the result of chimeric fragments in which reads extend over Hi-C ligation junction boundaries. An abnormal level of chimeric reads can reflect a ligation issue during the library preparation. Total_R1/R2: all R1 and R2 reads submitted to HiC-Pro.

- **Mapped_R1/R2**: all reads which aligned to the reference genome.
- **Global R1/R2**: reads which aligned to the reference genome without trimming
- **Local_R1/R2**: reads which aligned to the genome after soft trimming

3.4.2.2 Mapping: Pairs

Once R1 and R2 reads are aligned on the genome, HiC-Pro reconstructs the pairs information. The fraction of singletons or reads with multiple mapping locations depends on the complexity of the genome and the fraction of unmapped reads. The fraction of singletons is usually close to the sum of unmapped R1 and R2 reads, because it is unlikely that both mates from the same pair were unmapped.

- **Total_pairs_processed**: all read pairs analyzed by HiC-Pro
- **Unique_paired_alignments**: read pairs where each read is uniquely mapped to the reference genome
- **Multiple_pairs_alignments**: read pairs where one or both reads are mapped to multiple locations in the reference genome
- **Low_qual_pairs**: discarded read pairs where one or both reads do not pass the MIN_MAPQ threshold during mapping
- **Unmapped_pairs**: read pairs where both reads did not map to the reference genome
- **Pairs_with_singleton**: read pairs where one of the reads does not map to the reference genome
- **Unique_singleton_alignments**: read pairs where one of the reads does not map to the reference genome and one of the reads maps to reference genome once
- **Multiple_singleton_alignments**: read pairs where one of the reads does not map to the reference genome and one of the reads maps to multiple locations in the reference genome
- **Low_qual_singleton**: read pairs where one of the reads does not map to the reference genome and one of the reads does not pass the MIN_MAPQ threshold during mapping
- **Reported_pairs**: read pairs that are analyzed further to identify and characterize Hi-C interactions

3.4.2.3 Pairs: Valid Hi-C Pairs

Reported_pairs are processed further to identify valid Hi-C interaction pairs. Each aligned read can be assigned to one restriction fragment according to the reference genome and the selected restriction enzyme. Both reads are expected to map near a restriction site at a distance within the range of molecule size distribution after shearing. Fragments with a size outside the expected range can be discarded if specified but are usually the result of random breaks or star activity of the enzyme and can therefore be included in downstream analysis. Invalid ligation products, such as dangling end and self-circle ligation, are discarded. A high level of dangling-end or self-circle read pairs is associated with a low-quality experiment and reveals a problem during the digestion, fill-in or ligation steps. Only valid Hi-C interaction pairs involving 2 different restriction fragments are used to build the contact maps. Duplicated valid pairs due to PCR artifacts can also be filtered out, but it should be noted that a high level of PCR duplicates indicates poor molecular complexity and a potential PCR bias.

- **Reported_pairs**: read pairs that are analyzed further to identify and characterize valid Hi-C interaction pairs
- **Dangling_end_pairs**: unligated fragments where both reads mapped to the same restriction fragment
- **Religation_pairs**: ligation of juxtaposed restriction fragments
- **Self-circle_pairs**: fragments ligated to themselves, where both reads mapped to the same restriction fragment in an inverted orientation
- **Single-end_pairs**: singletons that are filtered out during mapping
- **Filtered_pairs**: read pairs that are filtered during mapping
- **Dumped_pairs**: any pairs that do not match the filtering criteria on inserts size or restriction fragments size, or for which we were not able to reconstruct the ligation product
- **Valid_interaction_pairs**: all valid Hi-C interaction pairs that remain after filtering out invalid species
- **Valid_interaction_rmdup**: all valid Hi-C interaction pairs that remain after PCR duplicates are removed

3.4.2.4 Valid Hi-C Pairs: Strand Bias

Because the ligation is a random process, 25% of each valid ligation class is expected.

- **Valid_interaction_pairs**: all valid Hi-C interaction pairs that remain after invalid species have been removed
- **Valid_interaction_pairs_FF**: valid Hi-C interaction pairs in which R1 and R2 are from the same DNA strand and are oriented in the same direction (forward)
- **Valid_interaction_pairs_FR**: valid Hi-C interaction pairs in which R1 and R2 are from different DNA strands and face inward
- **Valid_interaction_pairs_RF**: valid Hi-C interaction pairs in which R1 and R2 are from different DNA strands and face outward
- **Valid_interaction_pairs_RR**: valid Hi-C interaction pairs in which R1 and R2 are from the same DNA strand and are oriented in the same direction (reverse)

3.4.2.5 Valid Hi-C Pairs: Interaction Distances

An important quality metric is the fraction of intrachromosomal and interchromosomal interactions, as well as long-range (>20 kb) versus short-range (<20 kb) intrachromosomal interactions.

- **Valid_interaction_pairs**: all valid Hi-C interaction pairs that remain after invalid species are removed
- **Trans_interaction**: all valid Hi-C interaction pairs where R1 and R2 map to different chromosomes (interchromosomal interactions)
- **Cis_interaction**: all valid Hi-C interaction pairs where R1 and R2 map to the same chromosome (i.e. *cis* or intrachromosomal interactions)
- **Cis_shortRange**: *cis* interactions where the distance between R1 and R2 is <20 kb
- **Cis_longRange**: *cis* interactions where the distance between R1 and R2 is ≥20 kb

3.4.2.6 Global Yield (Hi-C)

- **Cis_longRange (from total)**: percentage of valid long-range *cis* Hi-C interaction pairs from all read pairs
- **Cis_longRange (from reported)**: percentage of valid long-range *cis* Hi-C interaction pairs from all mapped read pairs
- **Cis_longRange (from valid)**: percentage of valid long-range *cis* Hi-C interaction pairs from all valid Hi-C interaction pairs

3.4.3 Subfolders

The additional output files are generated per sample and can be found in folders as explained above. Please refer to sections above, which explain the files and their format.

3.5 Quality control of Hi-C NGS libraries by shallow sequencing

Prior to costly deep sequencing, users are advised to sequence Hi-C NGS libraries at low depth (<1 million reads) for quality control purposes. Low-depth sequencing data can be processed at the EpiTect Hi-C analysis portal, and the generated sequencing report can be used to assess the quality of Hi-C libraries.

3.5.1 Characteristics of a high-quality Hi-C NGS library

- Greater than 80% valid Hi-C interaction pairs (after removal of PCR duplicates)

  According to guidelines by Rao et al. (6), Hi-C libraries where >20% of the paired-end reads are not valid Hi-C interactions are likely to be the result of failed restriction, fill-in, or ligation steps, and are therefore not good candidates for deeper sequencing.
- Low percentage of read pairs deriving from a single restriction fragment

  A high-quality Hi-C library for mammalian genomes typically has less than 1–4% unligated, dangling ends and less than 1–2% self-ligated circles.
- Greater than 40% long-range *cis* interactions (>20 kb)

  Rao et al. provide guidelines to assess the quality of Hi-C libraries in their supplementary material (extended methods) accompanying their excellent publication (6):

  > *A crucial metric is the percentage of long-range intrachromosomal contacts. In successful Hi-C libraries, we found that at least 15% of unique reads were long-range intrachromosomal contacts. Lower values usually indicated that the experiment had failed. If more than 40% of unique reads are long-range intrachromosomal contacts, a library was considered a good candidate for sequencing. If the fraction was above half, a library was considered an excellent candidate for sequencing.* ***In general, this value was one of the statistics we found most important to scrutinize in performing cost-effective high-depth Hi-C.***

- Greater than 40% *cis/ trans* ratio

  In the nucleus, chromosomes are partitioned in territories where individual chromosomes are physically separated in space. For this reason DNA contacts typically occur at a higher frequency within chromosomes (*cis*) than between chromosomes (*trans*). This property of genome organization can be exploited as a useful proxy for evaluating the quality of Hi-C data. Noise from random background ligation (due to ruptured nuclei) will affect both *cis* and *trans* interactions similarly and result in a lower ratio between *cis* and *trans* interactions. *Cis/ Trans* ratios are dependent on genome size and number of chromosomes; but for human genomes, ratios of 40–60% are considered a sign of high-quality Hi-C experiments (7). *Cis/ Trans* ratio is defined as:

  [Cis_longRange / (Cis_longRange + Trans_interaction)] x 100% (see section 3.4.2.5)

- No strand-orientation bias

  Hi-C chimeras can be broken up into 4 classes distinguished by the strand orientation of read pairs: FF, FR, RF, and RR (see section 3.4.2.4). If the chimeras are a result of random proximity ligation of chromatin, then close to 25% of each class of chimera is expected.

References

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2. Kerpedjiev, P. et al. (2018) HiGlass: web-based visual exploration and analysis of genome interaction maps. Genome Biol. **19**, 125.
3. Durand, N.C. et al. (2016) Juicer provides a one-click system for analyzing loop-resolution Hi-C experiments. Cell Syst. **3**, 95–98.
4. Kerpedjiev, P. et al. (2018) HiGlass: web-based visual exploration and analysis of genome interaction maps. Genome Biol. **19**, 125.
5. Ewels, P., Magnusson, M., Lundin, S. and Käller, M. (2016) MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics **32**, 3047–3048.
6. Rao, S.S.P. et al. (2014) A three-dimensional map of the human genome at kilobase resolution reveals principles of chromatin looping. Cell **159**, 1665–1680.
7. Lajoie, B.R., Dekker, J. and Kaplan, N. (2015) The Hitchhiker's Guide to Hi-C Analysis: Practical guidelines. Methods **72**, 65–75.

Ordering Information

Product	Contents	Cat. no.
EpiTect Hi-C Kit (6)	For 6 Hi-C reactions: Buffers and reagents for cell lysis, Hi-C digestion, Hi-C end-labeling, Hi-C ligation, chromatin decross-linking and purification, purification of fragmented DNA, streptavidin pulldown of Hi-C fragments and NGS library prep (end repair, A-addition, phosphorylation, adapter ligation and library amplification); for use with Illumina instruments; includes 6 adapters with different barcodes	59971
Related Products		
For use with Illumina instruments		
QIAseq Library Quant Assay Kit	Laboratory-verified forward and reverse primers for 500 x 25 µl reactions (500 µl); DNA standard (100 µl); dilution buffer (30 ml); (1.35 ml x 5) GeneRead qPCR SYBR® Green Mastermix	333314
For assessing NGS library quality		
QIAxcel Advanced Instrument	Capillary electrophoresis device: includes computer, QIAxcel ScreenGel Software and 1-year warranty on parts and labor; fully automates sensitive, high-resolution capillary electrophoresis devices for analyzing up to 96 samples per run	9001941
QIAxcel DNA High Resolution Kit (1200)	QIAxcel DNA High Resolution Gel Cartridge, buffers, mineral oil, QX Intensity Calibration Marker, 12-tube strips	929002

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Notes

Notes

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