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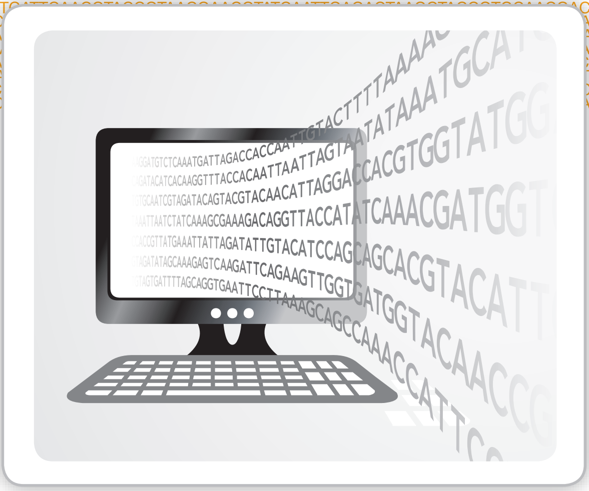
FastTrack Phasing Analysis Services Guide



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Revision History

Document	Date	Description of Change
Document # 15047619 v01	September 2015	Corrected Phased VCF, Unphased VCF, and Phasing Summary Report file names in the Analysis Deliverables chapter.
Part # 15047619 Rev. B	June 2015	Revised documentation to reflect changes in version 1.1.0 of the Illumina FastTrack Phasing Analysis Service pipeline.
Part # 15047619 Rev. A	November 2013	Initial Release.

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Chapter 1

Getting Started

Phasing Analysis Service

The Phasing Analysis Service leverages TruSeq® Synthetic Long-Read technology to complement whole-genome sequencing. Phased sequencing captures the unique content of homologous chromosomes, including mutations that can differ across chromosome copies. Phase information distinguishes between variants originating from separate chromosomes, enabling the generation of haplotype fragments for allele-specific analysis.

The FastTrack Phasing Informatics Pipeline uses algorithms designed to provide whole-genome phasing information. TruSeq Synthetic Long-Read uses the variants discovered from a Whole-Genome Sequencing (WGS) run and the base calls from a TruSeq Synthetic Long-Read Library Prep as inputs. The FastTrack Phasing Informatics Pipeline then reports haplotype blocks across the genome and phasing confidence scores in a phased VCF file.

The purpose of this document is to help you understand the Phasing Analysis Service data package you receive from Illumina. This document provides an overview of the FastTrack Phasing Informatics Pipeline 1.1.0. deliverables and the Prism v2.2 phasing algorithm.

Data Delivery

Illumina FastTrack Services currently provides data delivery through the following choices.

Illumina Hard Drive Data Delivery

Illumina FTS ships data on 1 or more hard drives. The hard drives are formatted with the NTFS file system and can optionally be encrypted.

The data on the hard drive are organized in a folder structure with 1 top-level folder per sample or analysis.

Illumina Cloud Data Delivery

Illumina FTS uploads data to a cloud container. Illumina currently supports uploads to the Amazon S3 service. Upload data are organized per upload batch by date with an Illumina_FTS prefix. For example, a sample in a batch uploaded on February 1, 2014 would be found in the container with the prefix Illumina_FTS/20140201/SAMPLE_BARCODE. Contact your FastTrack Services project manager to enable cloud delivery.

Chapter 2

Analysis Deliverables

Overview

This section details the files and folder structure for the Phasing Analysis Service results. The files and folders are named based on the unique sample identifiers. Usually, these unique identifiers are the barcodes associated with the samples in the lab, but can be a known sample ID for reference samples.

Result Folder Structure

Under each phasing sample folder, you can find the following file structure that contains analysis results.

- 📁 [SampleBarcode]/
  - 📁 Phased_results—Contains all the output files resulting from the phasing library sequencing run and analysis.
    - 🗎 [SampleName]_Phased.vcf.gz—Variant file in VCF 4.1 format with markup to indicate phased variants and haplotype blocks.
    - 🗎 [SampleName]_Unphased.vcf.gz—Variants file in VCF 4.1 format listing the variants of your sample before phasing.
    - 🗎 [SampleName]_PhasingSummaryReport.pdf—PDF report detailing sample information, sequencing metrics for phasing library, and phasing summary statistics.
    - 🗎 md5sum.txt—Checksum file to confirm file consistency.
    - 📁 [SampleName]_ShortInsertSequencing.tar.gz—Contains the original short read data for the phasing library.
  - 📁 WGS_results—Folder containing the results of the whole-genome sequencing pipeline. Refer to the folder structure described in the *Whole-Genome Sequencing Services User Guide, document # 15040892*.

Result Files

Phased VCF File

The [SampleName]_Phased.vcf.gz file contains the principal results of the FastTrack Phasing Informatics Pipeline. This file includes most of the information in the input VCF file, which is generated from the FastTrack Services Whole-Genome Sequencing Informatics Pipeline. For more information, see the *Whole-Genome Sequencing Services User Guide, document # 15040892*.

The phased VCF file differs from the input VCF in the following ways:

- Contains additional markup about the phase of variants, our confidence in the phase assignments, and on sets of phased heterozygous variants. Heterozygous variants that could not be phased are unchanged in the final VCF file.
- Does not include the variants excluded from analysis, which are based on the following criteria:
  - Indels > 50 bp in length
  - Nonpassing variants
  - Haploid
  - Nonvariant
  - Variants with nonstandard VCF formatting

The following table describes fields in the phased VCF file.

Metric	Description
Genotype (GT)	Phased variants (heterozygous and homozygous) contain a modified GT field, using a pipe symbol (|) instead of forward slash in accordance with VCF 4.1 specifications. Variants whose genotype contains a slash (/) in the phased VCF are unphased.
Emission Likelihood (EL)*	An indication of the degree of confidence that the phasing of 1 locus is correct relative to its neighbors. Although evaluation of quantitative correctness is still ongoing, the value can be interpreted as a likelihood. Values range from 0.5 (a random guess) to 1 (complete confidence).
Transition Likelihood (TL)*	The confidence in the phasing of variants between the current (heterozygous) variant locus and the previous heterozygous locus. Although evaluation of quantitative correctness is still ongoing, the value can be interpreted as a likelihood. Values range from 0.5 (random guess) to 1 (complete confidence).
Phase Set (PS)	Used in the FastTrack Phasing Informatics Pipeline to define the heterozygous variants that belong to a globally phased block set with TL > 0.95. The integer value represents the coordinate of the first locus in a given phase set. PS values are unique within a chromosome locus. Therefore, variants with the same PS value on different chromosomes are not phased relative to each other.
Local Phase Set (LPS)	Interpretation is analogous to that of PS, except that the phasing indicated by LPS annotation is based solely on the blocks determined by local phasing. In general, confidence in the relative phasing of variants that share the LPS is higher than the confidence for variants that share a PS value but not an LPS value.
Inconsistency Flag (ICF)	An indication of positions where the alleles detected in the long fragment library do not match the expected alleles from the input WGS VCF file.

*For more information, see the Phase Scoring *Overview* on page 17.

Unphased VCF File

The [SampleName]_Unphased.vcf.gz file contains a subset of variants from the input VCF that pass the filtering criteria. No additional phasing information is provided in this file. For more information, see the *Whole-Genome Sequencing Services User Guide, part # 15040892*.

Phasing Summary Report

The [SampleName]_PhasingSummaryReport.pdf file contains an overview of the metrics of the long fragments library and statistics from the phasing analysis.

Input VCF Snapshot

This section describes details related to the input whole-genome sequencing VCF file.

Metric	Description
Reference Genome	Version of human reference genome used in the analysis.
Number of Variants	Total number of indels and single nucleotide variants (SNVs) used in the phasing analysis.
Number of heterozygous variants	Total number of heterozygous indels and SNVs used in the phasing analysis.
Number of heterozygous SNPs	Total number of heterozygous SNVs used in the phasing analysis.

Sequencing Metrics

This section describes details related to the sequencing of reads from a TruSeq Synthetic Long-Read Library Prep.

Metric	Description
Total reads (Pass Filter)	Total number of short read pairs that pass filter.
Percent Reads Mapped	Percentage of short reads across all barcodes that align to the hg19 reference.
Cloud N50 Length	N50 value of long fragments/clouds identified across all barcodes by aligning to the hg19 reference genome. The N50 is the length for which the collection of all blocks or contigs of that length, or longer, contains half of the total bases included in blocks/contigs.

Phasing Statistics

This section describes details related to the phasing of variants.

Metric	Description
Local N50	N50 value of locally phased blocks generated without imputation from the second step of the analysis, and derived by parsing the Local Phase Set (LPS) value. The value is reported separately for haplotype blocks considering SNVs alone and both SNVs and indels.
Global N50	N50 value of globally phased blocks generated using imputation from the third step of the analysis, and derived by parsing the Phase Set (PS) value. The value is reported separately for haplotype blocks considering SNVs alone and both SNVs and indels.
Percent Phased	The overall percentage of heterozygous variants included in globally phased blocks, and derived by parsing the PS value. The value is reported separately for haplotype blocks considering SNVs alone and both SNVs and indels.

Gene Phasing Statistics

For all autosomal genes in RefSeq, we report the fraction of phased variants with EL value ≥ 0.95 that intersect the coordinates of the RefSeq genes.

Metric	Description
100% Variants phased	Number of autosomal genes on chromosomes 1–22 that have 100% of their variants with EL value ≥ 0.95 phased.
>70% Variants phased	Number of autosomal genes on chromosomes 1–22 that have at least 70% of their variants with EL value ≥ 0.95 phased.

The report file also provides 2 plots illustrating gene phasing metrics:

- **Phasing Completeness by Gene**—Provides the distribution of the fraction of variants phased per gene, over all autosomal RefSeq genes. This metric considers all variants phased across the gene, regardless of the number of haplotype blocks that span the gene.
- **End-to-End Phasing Confidence**—Provides the distribution of the minimum Transition Likelihood (TL) value within each autosomal gene in RefSeq. Phased blocks break when the TL falls below a selected accuracy threshold. Therefore, the minimum TL value per gene indicates our confidence that the gene is phased in 1 block, end-to-end, at a given accuracy threshold. Usually, most genes are phased in 1 block, indicated by the peak observed at a TL value of 1.

Short Read Output Folder

The [SampleName]_ShortInsertSequencing.tar.gz folder contains the short read output from the long fragments library sequencing run. The output files are in FASTQ format and are demultiplexed using the sample barcode, allowing a 1-base mismatch in the barcode sequence. The presence of end markers of the 5′–3′ sequence TACGCTTGCAT in short read sequences indicate one end of a long fragment. Any sequence 5′ of the end marker, or 3′ of its reverse complement, is expected to be adapter rather than sample DNA, except where the sequence TACGCTTGCAT is a native part of your sample DNA.

Data Integrity

The md5sum.txt file is provided to check the integrity of the sample files and folders. Immediately after sample quality check, the md5sums, or compact digital fingerprint, for every file in the directory tree are generated. If media failures compromise data integrity, you can use the md5sum tool to find the inconsistencies. Use the tool to compare the hash from the provided md5sum file to the hash generated from the downloaded file.

On a Unix system, you can use the following commands to perform an md5sum check, assuming the utility is installed:

```
% cd [Sample_Barcode]
% md5sum -c md5sum.txt
```

The check verifies every file in ~30–45 minutes. Any errors are listed in the output.

In Windows, there are various command line and GUI tools available to perform an md5sum check. The Cygwin tools provide a utility identical to Linux.

Analysis Overview

Chapter 3

Overview

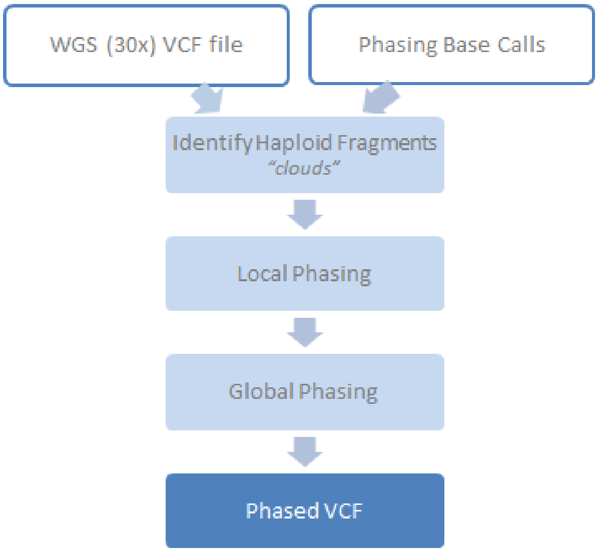
The FastTrack Phasing Informatics Pipeline works in 3 steps.

1 **Identification of Haploid Fragments**— The FastTrack Phasing Informatics Pipeline separates the sequence reads into the component 384 wells based on the barcode sequence and aligns the reads to the human reference sequence. The algorithm then detects haploid fragments of DNA, or *clouds*, in each well, and records the overlapping heterozygous variants called in the input WGS VCF file. Clouds that indicate an overlap of fragments from both maternal and paternal chromosomes are removed.

2 **Local Phasing**—The resulting fragments (up to ~10 kb long) are pooled together. Fragments that share heterozygous SNPs are chained together to reconstruct longer haplotypes. On average, these locally phased blocks are 5–10× longer than the individual clouds and provide highly accurate haplotype blocks derived entirely from the data.

3 **Global Phasing**—Using the 1000 Genomes phased reference panels for statistical imputation, the locally phased blocks are phased relative to each other to reconstruct longer haplotypes. Final block sizes are increased another 5–10× relative to locally phased blocks. Phased blocks and confidence scores are output in the phased VCF file.

Figure 1 FastTrack Phasing Informatics Algorithm Workflow



Identification of Haploid Fragments

The first stage of the phasing algorithm involves the identification of contiguous segments of haploid fragments, or clouds, in each well.

The reads from each well are aligned to the human reference genome using the BWA aligner[1] and clustered into groups separated from each other by at least 2 Kbp. Reads that cluster according to this criterion are likely to have originated from the same fragment. The heterozygous variants from the WGS VCF that intersect the long fragments are retained as input to the subsequent steps of the phasing pipeline. Fragments with the following criteria are removed from consideration:

- Insufficient sequencing read coverage
- Many variant-covering base calls with low Q-scores
- Evidence of 2 different alleles in the same fragment
- Inferred length in the top 3 percentiles

The result of this stage is a set of clouds from every well. Each cloud corresponds to an interval on the reference genome and the base calls of reads that overlap heterozygous variant calls from the input VCF. A cloud provides a tentative phasing of the set of variants that map to the original long fragment.

Reference Genome Definition

The phasing pipeline currently uses hg19 from UCSC as a reference genome (genome.ucsc.edu). The chromosome naming scheme follows the UCSC conventions of chr1-22, chrX, chrY, chrM. The pseudoautosomal region (PAR) of the Y chromosome is masked out with N's. Therefore, any mappings occurring in the PAR region map to the X chromosome. Currently, only the assembled chromosomes and mitochondria are used in the reference; none of the nonmapped contigs are included, nor are alternate haplotypes.

Table 1 hg19 PAR Regions

Name	Chr	Start	Stop
PAR #1	X	60,001	2,699,520
PAR #2	X	154,931,044	155,260,560
PAR #1	Y	10,001	2,649,520
PAR #2	Y	59,034,050	59,363,566

Local Phasing

In the next stage of the pipeline, the clouds are combined across wells. Clouds with overlapping heterozygous SNP sites are joined, resulting in blocks that are normally around 5–10× larger in size than the input clouds. Each of these blocks is analyzed to infer the phasing of the variants it covers.

The local phasing algorithm finds the haplotypes and cloud-to-haplotype assignments that best fit the data (the maximum likelihood answer), using an algorithm based on Hidden Markov Models (HMM). The algorithm works iteratively, by finding and scoring the best partial solution for positions 1 through k for each possible choice of phasing and cloud-to-haplotype assignments at position k. The partial solution is based on the best partial solutions at positions 1 through k-1.

Phasing and cloud assignments at position k are scored by considering the quality scores of the alleles in the clouds at k and also requiring that cloud assignments are ‘consistent’.

For a cloud assignment to be consistent, we require that fragments that span positions k and k-1 are assigned to the same haplotype. Ignoring the cloud assignments for simplicity, we find the best possible phasing of positions 1 through k such that position k is phased 0|1 or 1|0 (chromosome A | chromosome B).

The best phasing of 1..k with phasing 0|1 at position k is obtained by comparing extensions of the following:

- The best solution for 1..k-1 with 0|1 phasing at k-1
- The best solution for 1..k-1 with 1|0 phasing at k-1

When analysis has finished with the final position N, the local phasing algorithm compares the best solution with 0|1 phasing at N against the best solution with 1|0 phasing at N. The algorithm then determines the optimal phasing. The optimal phasing of the final position also aids in determining the optimal phasing of all positions.

Global Phasing

Using a statistical algorithm, the local blocks that have been computed are phased relative to each other to form long haplotype contigs.

The global phasing algorithm is based on a hidden Markov model (HMM), which extends statistical phasing algorithms such as IMPUTE2[2] and SHAPE-IT[3]. The locally phased blocks are viewed as an *imperfect mosaic* from a reference panel of prephased haplotypes from the 1000 Genomes project[4]. The algorithm assigns the most likely phase to each locally phased block. This assignment is based on the best matches of the local blocks to the individuals in the reference panel and patterns of linkage disequilibrium inferred statistically from the panel.

In addition to finding the optimal phase, the confidence scores (Emission Likelihood (EL) and Transition Likelihood (TL)) are derived. These scores represent the likelihood of a switch or single-site error between the current SNP and the previously phased SNP. Each heterozygous variant that is given a TL score < 0.95 causes the previous phase set, if any, to end and typically starts a new phase set. An exception to this rule occurs when breaking a block at a variant with TL < 0.95 results in a singleton block (the next heterozygous locus also has TL < 0.95 or the current heterozygous locus is the last of the chromosomes). In this case, the preceding phase set is ended, but no phase set is output for the singleton variant.

The scores can also be used to break the inferred haplotypes into blocks at a given level of accuracy. For applications requiring highly accurate haplotype blocks, haplotype contigs can be broken at a higher threshold, resulting in shorter but more accurate blocks. Alternatively, a more-aggressive reconstruction can be obtained by breaking blocks at a lower threshold.

References

1 Li H. and Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 26, 589–595 (2010).

2 Howie, B.N., Donnelly, P. & Marchini, J. A Flexible and Accurate Genotype Imputation Method for the Next Generation of Genome-Wide Association Studies. PLoS Genet 5, e1000529 (2009).

3 Delaneau, O., Zagury, J. & Marchini, J. Improved whole-chromosome phasing for disease and population genetic studies. Nat Meth 10, 5–6 (2012).

4 McVean, G.A. et al. An integrated map of genetic variation from 1,092 human genomes. Nature 491, 56–65 (2012).

Phase Scoring

Chapter 4

Overview

The Emission Likelihood (EL) and Transition Likelihood (TL) values in the phased VCF file provide the degree of confidence in phasing for each phased heterozygous variant. The scores indicate 2 different modes of possible error in phasing.

- **Single-site error**—The EL score addresses the possibility of a single-site error at a locus, which means that the inferred phasing of one locus is wrong relative to its neighbors.
- **Switch error**—The TL score addresses the possibility of a switch error, which means that the relative phase of variants before a given locus is wrong compared to the phase of variants at and after the locus.

The following examples illustrate the accurate haplotype and the 2 types of errors.

Table 2 Accurate Haploytype and Error Type Examples

Description	Example

Accurate Haplotype—Haplotypes arbitrarily labeled 0 and 1	`00000000000000000000000000 haplotype 1`
	`11111111111111111111111111 haplotype 2`

Single-site Error—Indicated by *	`00000010000000000000100000 haplotype 1`
	`11111101111111111111011111 haplotype 2`
	`      *             *`

Switch Error—Indicated by |	`00000000000000111111111111 haplotype 1`
	`11111111111111000000000000 haplotype 2`
	`              |`

Intended Use of Phase Scoring

The Emission Likelihood and Transition Likelihood scores allow the algorithm to assign scores to individual loci, rather than all pairs of loci.

For example, a given locus can be weakly phased but confidence in the phasing between variants separated by that locus can remain high. This situation can arise if a locus has low coverage and/or poor quality base calls, but the clouds spanning the variant connect other variants with better coverage. In this case, you can have a low single-site confidence (a low EL score) and a high confidence in phasing spanning these loci (a high TL score)

These scores can help answer the following questions.

Table 3 Phase Scoring Questions and Answers

Question	Answer
How confident can we be in the reported relative phasing of 2 adjacent sites? If 2 sites are on the same chromosome and there are no intervening loci for which phasing is reported, these 2 sites are 'adjacent'.	Calculate the combined probability (PQ1) of a switch error between the 2 (TL value of the second site) and a single-site error at either locus (EL values of both sites). Thus, for loci i and i+1, the relative phasing confidence can be estimated as follows: $P_{Q1} = TL_{i+1} * EL_{i} * EL_{i+1}$
How confident can we be in the reported relative phasing of 2 distal sites?	Calculate the combined probability (PQ2) of switch errors anywhere between the 2 loci (TL value at the 2 loci and between) and no single-site error at either locus (EL value at the 2 sites). Thus, for loci i and i+n, the relative phasing confidence can be estimated as follows: $P_{Q2} = TL_{i} * TL_{i+1} * TL_{i+2} * \ldots * TL_{i+n} * EL_{i} * EL_{i+n}$

Using Phase Scoring to Extract Globally Phased Blocks

In the provided phased VCF files, the default Transition Likelihood (TL) cutoff of TL ≥ 0.95 is used to determine Phase Set (PS) annotations. The PS annotations are used as a starting point to define haplotype blocks, which are all variants on a given chromosome with the same PS value.

A locus with a low EL score usually receives a PS annotation even though it is not confidently phased relative to the other members of the phase set. Before phasing 2 variants relative to each other, make sure that the 2 loci both have PS annotations with the same value and make sure that both loci have EL scores ≥ 0.95.

Some applications require greater confidence or more aggressive (less confident) phasing. To extract phased blocks using alternative probability cutoffs, you can use the TL and EL scores together to parse Globally Phased Blocks from the VCF file.

If a set of loci on the same chromosome meet the following criteria, then they are phased relative to each another.

- All SNPs have TL scores ≥ an alternative cutoff, except for the first locus in the set. This alternative cutoff is relatively high for greater confidence and relatively low for more aggressive phasing.
- For all loci in the set, EL scores are ≥ an alternative cutoff (relatively high for greater confidence and more aggressive phasing).

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 4 Illumina General Contact Information

Website	www.illumina.com
Email	techsupport@illumina.com

Table 5 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Australia	1.800.775.688	Netherlands	0800.0223859
Austria	0800.296575	New Zealand	0800.451.650
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000

Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

Product documentation—Available for download in PDF from the Illumina website. Go to support.illumina.com, select a product, then select **Documentation & Literature**.

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