




ARTIC primer scheme specification v3.0.0-alpha

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Abstract

Polymerase chain reaction (PCR) followed by amplicon DNA sequencing enables fast, sensitive and cost-effective molecular characterisation of target genes and genomes. PCR involves the selective amplification of a target genomic region (amplicons) using pairs of single-stranded oligonucleotide primers, that are complementary to the opposing strands flanking the target region. Multiple regions can be simultaneously amplified in a single reaction via multiplexed PCR, with multiple reactions enabling tiling amplicon sequencing (ARTIC sequencing), facilitating efficient enrichment of entire microbial genomes for whole genome sequencing. However, accurately reproducing a primer scheme and the corresponding bioinformatic analysis of amplicon sequencing data depends on knowledge of primer sequences, amplicon layout, and their coordinates with respect to a reference sequence. Analysis and reuse of amplicon sequencing data is currently hindered by the lack of a clearly defined data interchange format for primer scheme definitions, a problem highlighted by the proliferation of SARS-CoV-2 primer schemes during the COVID-19 pandemic. Here, we describe a text-based specification for describing primer sequences and locations with respect to a reference sequence. This specification formalises and expands on the existing interchange format initially used in the PrimalScheme primer design tool, and since adopted by a growing ecosystem of tooling. This specification designates the use of a primer.bed file, based on the Browser Extensible Data (BED) text format, and an accompanying reference.fasta text file for defining primer schemes, and probe-based qPCR assays. This specification is intended to facilitate the exchange of primer scheme definitions for oligonucleotide synthesis, wet-lab and bioinformatic analysis use cases.

Keywords Data standards, Primer Schemes, Amplicon Sequencing

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1. primer.bed file

A primer.bed file describes a primer scheme in machine and human-readable tabular format. Together with an accompanying reference.fasta, its purpose is to encapsulate all of the information needed to *i)* acquire the primers from suppliers or custom oligonucleotide synthesis, *ii)* combine the primers correctly to reproduce a pooled primer scheme, and *iii)* facilitate correct and reproducible bioinformatic analysis of the resulting sequencing data. It therefore incorporates both wet lab and analytical elements. This information includes primer sequences, primer pools, coordinates and orientation with respect to a reference sequence, and optionally relative primer concentrations.

1.1. Format overview

primer.bed files are tab-delimited ASCII text files. Each line can either represent a *comment line* (prefixed with “#”) or a *record line* (BedLine), representing a single unique oligonucleotide primer or probe associated with an amplicon. An amplicon comprises at least two primer record lines, each describing primers on different strands.

The format of primer.bed is based on Browser Extensible Data ([BED](#)) specification, with each oligonucleotide being treated as a genomic region, enabling compatibility with common BED file tooling.

1.2. Comment Line

Comment lines are minimally parsed, but can optionally contain scheme-level (key, value) pairs. To this end, comment lines containing a single “=” will be split, with the left and right sides representing a scheme-level key and value, respectively.

1.3. record line (BedLine) field descriptions

Column	Field name	Type	Brief description	Restrictions
1	chrom	String	Chromosome name	[A-Za-z0-9._]
2	primerStart	Integer	Primer start position (zero-based, half-open)	Positive integer (u64)
3	primerEnd	Integer	Primer end position (zero-based, half-open)	Positive integer (u64)
4	primerName	String	Primer name	[a-zA-Z0-9\._]+_([0-9]+_LEFT RIGHT PROBE)_([0-9]+)
5	pool	Integer	Primer pool	Positive integer (u64)
6	strand	String	Primer strand	[-+]
7	primerSeq	String	The nucleotide sequence in 5'→3'	ASCII non-whitespace characters
8	primerAttributes	Optional(String)	List of record-level (key, value) pairs separated by `;`. e.g. k1=v1;k2=v2	ASCII non-whitespace characters

Table 1: The column structure and description of a BedLine

1.3.1. chrom

The name of the corresponding reference sequence chromosome for the primer. This must match a valid sequence ID inside an accompanying reference sequence FASTA file, by convention named reference.fasta.

1.3.2. primerStart

The start position of the primer on the chrom using BED-like zero-based, half-open coordinates.

1.3.3. primerEnd

The non-inclusive end position of the primer on the chrom using BED-like zero-based, half-open coordinates. Must be greater than `primerStart`.

1.3.4. primerName

The name of the primer in the form “{prefix}_{ampliconNumber}_{class}_{primerNumber}”.

- `prefix`: Must match regex `[a-zA-Z0-9\ -]`. See best practices
- `ampliconNumber`: The number of the amplicon for its relevant `chrom`. Must be a positive integer incrementing from 1.
- `primerClass`: The class of the primer. Must be either LEFT, RIGHT or PROBE.
- `primerNumber`: The number of the primer. Must be a positive integer incrementing from 1.

1.3.5. pool

The PCR pool the primer belongs to. Must be a positive integer incrementing from 1¹.

1.3.6. strand

The strand of the primer must be either “+” or “-”. It must correspond to the `primerClass` component of the `primerName`. LEFT and RIGHT `primerClass` must be “+” and “-” respectively, while PROBE can be either.

1.3.7. primerSeq

The sequence of the primer in the 5’ to 3’ direction. Unrestricted to contain any non-whitespace ASCII character².

1.3.8. primerAttributes

An *optional* list of (key, value) pairs used to denote additional arbitrary primer attributes, in the form of “pw=1.0;ps=10.0”. This is intentionally flexible to allow the storage of additional information. In a primer.bed file this can be represented as either an empty 8th column or only 7 columns.

1.3.8.1. Reserved keys

- `pw`: `primerWeight`. The concentration of individual primers can be altered to balance amplicon performance. Primer concentration in the PCR should be scaled by `primerWeight * [typical PCR conc]`. This is restricted to positive floating point numbers (`f64 > 0`).

1.4. Examples

1.4.1. Simple example

A seven column primer.bed file, with no `primerAttributes` or comment lines.

```

MN908947.3 100 131 example_1_LEFT_1 1 + CTCTGTAGATCTGTTCTCTAAACGAACCTT
MN908947.3 419 447 example_1_RIGHT_1 1 - AAAACGCCTTTTCAACTTCTACTAAGC
MN908947.3 344 366 example_2_LEFT_1 2 + TCGTACGTGGCTTTGGAGACTC
MN908947.3 707 732 example_2_RIGHT_1 2 - TCTTCATAAGGATCAGTGCCAAGCT

```

¹“Existing schemes/literature use refer to ‘pool 1 and pool 2’. Therefore, 1-based indexing is expected”

²“This is intentionally unrestricted (rather than IUPAC-only) to allow Primer Modification. Such as /56-FAM/{primerSeq} to represent 5’ 6-FAM fluorescent dye labelled probe”

1.4.2. Complex example

An eight column `primer.bed` file. With `primerAttributes` defined, and comment lines providing a `chrom` alias and explaining the `gc` `primerAttributes`.

```
# example scheme
# gc=fraction gc
# MN908947.3=sars-cov-2
MN908947.3 100 131 example_1_LEFT_1 1 + CTCTGTAGATCTGTTCTCTAAACGAACTTT pw=1.4;gc=0.35
MN908947.3 419 447 example_1_RIGHT_1 1 - AAAACGCCTTTTCAACTTCTACTAAGC pw=1.4;gc=0.36
MN908947.3 344 366 example_2_LEFT_1 2 + TCGTACGTGGCTTTGGAGACTC pw=1;gc=0.55
MN908947.3 707 732 example_2_RIGHT_1 2 - TCTTCATAAGGATCAGTGCCAAGCT pw=1;gc=0.44
```

1.4.3. qPCR example

An eight column `primer.bed` file. Showing a fictional qPCR assay. The specific dyes and quenchers are (optionally) included in the comment lines.

```
# example multiplexed-qPCR assay
# gc=fraction gc
# /3BHQ_1/=Black Hole Quencher 1
# /56-FAM/=FAM
# /5HEX/=HEX
target1 2010 2030 iad3_1_LEFT_1 1 + AAAGGTCAGTCAACCCGTTC pw=1
target1 2035 2060 iad3_1_PROBE_1 1 - /56-FAM/GCGTTGTTCAATTGCCTTGCTGATT/3BHQ_1/ pw=19.1
target1 2903 2923 iad3_1_RIGHT_1 1 - TCGGGCCACCGCGTATGAAG pw=1
target2 5167 5187 rfw1_1_LEFT_1 1 + TCGTAGCATGGACTCGATGA pw=1
target2 5271 5296 rfw1_1_PROBE_1 1 + /5HEX/TGATCCGCGTTTACTGTTCGACGCG/3BHQ_1/ pw=20.2
target2 5301 5321 rfw1_1_RIGHT_1 1 - GTTTACCAAGGAACCATCCA pw=1
```

1.5. primer.bed best practices

Best practices are not required by the specification; however they are strongly recommended.

1.5.1. Use dedicated tooling

While CSV parsing modules should be compatible with parsing bedfiles, they do not carry out valuation, and require additional work to parse `primerAttribute` and `primerNames`. [primalbedtools](#) is an open source Python package that carries out parsing, schema validation and conversion, and common operations on `primer.bed` files.

1.5.2. Use unique names

The `prefix` component of `primerName` should be as unique as possible (ideally a short UUID, i.e. 359ba5) and different for each `chrom` and each scheme generation run. Using `prefix` such as “scheme” or “sars-cov-2” might seem tempting, however, it will result in a freezer/LIMS full of identical `primerNames` leading to confusion and pooling mistakes. As an example a primer labelled `scheme_1_LEFT_1` could belong to any scheme.

1.5.3. The comment lines

The comment line’s key=value pattern undergoes limited validation in the specification, and therefore, tooling should implement robust error handling and should avoid using the comment line for critical metadata. A suitable use case might be to document custom `primerAttributes` or providing human-readable aliases for different `chroms`.

2. reference.fasta file

A reference.fasta file contains the DNA sequences of all the primary-reference genomes, used in primer scheme generation. Its purpose is to provide a reference genome and coordinate system for use in reference-based assembly and consensus generation.

2.1. Format overview

reference.fasta files are typical ASCII-encoded .fasta [format files](#), with text representing the nucleotide sequence of the reference. Each genome starts with a header line (starting with >) that denotes the id of the genome, followed by lines of nucleotide data.

All chrom fields of the record lines must have a corresponding ID in the reference.fasta.

2.2. Examples

2.2.1. Single fasta

```
>MN908947.3
ATTAAAGGTTTATACCTTCCA...
```

The corresponding primer.bed file contain BedLines with the chrom MN908947.3.

2.2.2. Multi fasta

```
>MN908947.3
ATTAAAGGTTTATACCTTCCA...
>NC_006432.1
CGGACACACAAAAGAAAGAAA...
```

The corresponding primer.bed file should contain BedLines with the chrom MN908947.3 and NC_006432.1.

2.3. reference.fasta best practices

Best practices are not required by the specification; however they are strongly recommended.

2.3.1. Use high-quality genomes

The genome contained in the reference.fasta file is commonly used for reference-based assembly. Therefore, using a genome with large numbers of Ns or ambiguous bases can lead to consensus sequence errors.

2.3.2. Use DNA genomes

DNA sequences are expected and should be the default. As by the nature of PCR, the amplicons and corresponding sequencing data should be DNA. However, RNA is allowed due to possible unforeseen applications.

2.3.3. Use canonical/publicly available genomes

The reference.fasta will need to be shared to reproduce the downstream analysis. Therefore, using property or restricted will inhibit sharing.

3. Further comments

3.1. Use encompassing metadata standards

This specification simply lays out the structure and formatting of the `primer.bed` and `reference.fasta` file, the minimal files used to replicate the primer pools, and the analysis used in multiplexed PCR.

For true reproducibility, each primer scheme should have an explicit name and a semantic versioning system to track changes to the scheme. Therefore, larger metadata standards are required, such as `primal-page` with [PrimalScheme Labs](#) or `primaschema` with [pha4ge primer-schemes](#).