

Design Primers That Yield Group-Specific Signatures

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1 Introduction

The DECIPHER function `DesignSignatures` was created to perform the most common primer design task: to efficiently amplify a shared region of DNA that will produce maximal amplicon diversity. This goal is independent of the method being used to differentiate amplicons, although some of the design details may depend on the technique being employed. `DesignSignatures` supports three common methods for distinguishing amplicons: sequencing, High Resolution Melting (HRM) analysis, and Fragment Length Polymorphism (FLP) analysis. As a case study, this tutorial focuses on designing primers to differentiate SNP alleles using HRM analysis. However, the methods described here can easily be adjusted to the other experimental techniques.

As input, we will use known sequence variants of the Isocitrate Dehydrogenase 2 (*IDH2*) gene that have been implicated in human gliomas. First, a database is constructed where the variants are identified by their unique name. Next, this database is used to design primers that will yield maximal amplicon diversity. The top scoring primers are further investigated to determine whether they can easily be distinguished experimentally. Finally, the process is repeated using a restriction enzyme to further diversify the amplicons' signatures. Note that much of the functionality described here is accessible via a web tool at <http://DECIPHER.codes>.

2 Getting Started

2.1 Startup

To get started we need to load the DECIPHER package, which automatically loads several other required packages.

```
> library(DECIPHER)
```

Help for the DesignSignatures function can be accessed through:

```
> ? DesignSignatures
```

If DECIPHER is installed on your system, the code in each example can be obtained via:

```
> browseVignettes("DECIPHER")
```

2.2 Creating a Sequence Database

We begin with a set of *IDH2* sequences in a FASTA file that is included as part of the DECIPHER package. The file contains the common IDH2 allele, and three alleles with single nucleotide polymorphisms (SNPs). Our goal is to design primers that can distinguish all four variants of the IDH2 gene. If you wish to follow along with your own FASTA file of unaligned sequences, be sure to change the path names to those on your system by replacing all of the text inside quotes labeled “<<path to ...>>” with the actual path on your system.

```
> # specify the path to your sequence file:
> fas <- "<<path to FASTA file>>"
> # OR find the example sequence file used in this tutorial:
> fas <- system.file("extdata", "IDH2.fas", package="DECIPHER")
```

Next, there are two options for importing the sequences into a database: either save a database file or maintain the database in memory. Here we will build the database in memory because it is a small set of sequences and we do not intend to use the database later:

```
> # specify a path for where to write the sequence database
> dbConn <- "<<path to write sequence database>>"
> # OR create the sequence database in memory
> dbConn <- dbConnect(SQLite(), ":memory:")
> N <- Seqs2DB(fas, "FASTA", dbConn, "")
Reading FASTA file chunk 1

4 total sequences in table Seqs.
Time difference of 0.03 secs
> N # number of sequences in the database
[1] 4
```

2.3 Defining Groups

At this point it is necessary to define groups of related sequences in the database we have just created. Ideally, groups should designate the different variants (i.e., strains or alleles) that we hope to distinguish.

In this example, we will define groups based on the allele's name, which was included in the description of each FASTA record that we imported. This is the most common case, and simply uses each sequence's name as its group name.

To assign group names directly from the sequence names (FASTA identifiers), simply run the code shown below. In more complex cases, we could use the functions `IdentifyByRank`, `FormGroups`, `IdClusters`, or `TreeLine` to define groups.

```
> # if each sequence belongs to its own group,
> # then identify the sequences with a number:
> desc <- as.character(seq_len(N)) # N is the number of sequences
> # OR get the FASTA record description:
> desc <- dbGetQuery(dbConn, "select description from Seqs")$description
> # show the unique descriptors:
> unique(desc)
[1] "IDH2" "R172K" "R172G" "R172M"
```

Now that we have unique names for our four variants, we must add them to the database as the identifier of each sequence.

```
> Add2DB(data.frame(identifier=desc, stringsAsFactors=FALSE), dbConn)
Expression:
update Seqs set identifier = :identifier where row_names = :row_names

Added to table Seqs:  "identifier".

Time difference of 0.02 secs
```

3 Designing primers with diverse signatures

3.1 Adjusting Input Parameters

Before using our database to design primers, we must carefully consider the inputs that will be used. These inputs can have a large impact on the outcome, and therefore should be tailored to the experimental goal. Below are recommended inputs for sequencing, FLP, and HRM:

```
> # Designing primers for sequencing experiments:
> TYPE <- "sequence"
> MIN_SIZE <- 300 # base pairs
> MAX_SIZE <- 700
> RESOLUTION <- 5 # k-mer signature
> LEVELS <- 5 # max number of each k-mer

> # Designing primers for community fingerprinting (FLP):
> TYPE <- "length"
> # it is important to have a width range of lengths
> MIN_SIZE <- 200 # base pairs
> MAX_SIZE <- 1400
> # define bin boundaries for distinguishing length,
> # the values below require high-resolution, but
> # the bin boundaries can be redefined for lower
> # resolution experiments such as gel runs
> RESOLUTION <- c(seq(200, 700, 3),
                  seq(705, 1000, 5),
                  seq(1010, 1400, 10))
> LEVELS <- 2 # presence/absence of the length
```

```

> # Designing primers for high resolution melting (HRM):
> TYPE <- "melt"
> MIN_SIZE <- 55 # base pairs
> MAX_SIZE <- 400
> # the recommended values for resolution
> RESOLUTION <- seq(75, 100, 0.25) # degrees Celsius
> LEVELS <- 10

```

3.2 Select Restriction Enzymes

The following step is not applicable to sequencing experiments (i.e., the TYPE above is "sequence"), and is optional for other types of experiments. If using HRM or FLP analysis to differentiate amplicon signatures, a digestion step following PCR amplification may generate fragments with widely different signatures. This is especially useful when trying to distinguish a large number of different groups based on small variations. Potential enzymes can be selected from the built-in RESTRICTION_ENZYMES dataset, which includes all of the standard enzymes sold by New England BioLabs.

```

> ENZYMES <- NULL # required for sequencing
> # OR select restriction enzymes to consider
> data(RESTRICTION_ENZYMES) # load available enzymes
> # for this tutorial we will use the enzyme MslI
> ENZYMES <- RESTRICTION_ENZYMES["MslI"]
> ENZYMES

      MslI
"CAYNN/NNRTG"

```

3.3 Designing Primers

Now we can design primers that target all of the groups and result in maximal amplicon diversity:

```

> primers <- DesignSignatures(dbConn,
                             type=TYPE,
                             minProductSize=MIN_SIZE,
                             maxProductSize=MAX_SIZE,
                             resolution=RESOLUTION,
                             levels=LEVELS,
                             enzymes=ENZYMES)

```

Tallying 8-mers for 4 groups:

```
|=====| 100%
```

Time difference of 0.49 secs

Designing primer sequences based on the group 'IDH2':

```
|=====| 100%
```

Time difference of 123.6 secs

Selecting the most common primer sequences:

```

|=====| 100%

Time difference of 15.89 secs

Determining PCR products from each group:
|=====| 100%

Time difference of 131.86 secs

Scoring primer pair combinations:
|=====| 100%

Time difference of 1.76 secs

Choosing optimal forward and reverse pairs:
|=====| 100%

Time difference of 2.27 secs

Finding the best restriction enzyme:
|=====| 100%

Time difference of 8.69 secs

```

The output `data.frame` is sorted by the sum of "score" and "digest_score". However, we can separately look at the top scoring primers with and without using restriction enzymes.

```

> primers[which.max(primers$score),] # best primers without digestion

  forward_primer reverse_primer score coverage products
70 TGGCTGGACCAAGCCCAT GCCTTGCTACTGGTCGCCATG 0.049074....      1      4
  similar_signatures missing_signatures enzyme digest_score fragments
70                                     MslI          0          2

> primers[which.max(primers$digest_score),] # best primers with digestion

  forward_primer reverse_primer score coverage products
1 AGTCCCTGGCTGGACCAAG CTGTGGCCTTGCTACTGGTCG 0.037037....      1      4
  similar_signatures missing_signatures enzyme digest_score fragments
1                                     MslI    0.1618056          13

```

The PCR products digested with *MslI* are substantially higher scoring (digest_score) than the undigested primers (score). Note that the columns "similar_signatures" and "missing_signatures" are both empty for this primer set, indicating that all of the groups have distinct signatures after digestion with the restriction enzyme.

4 Assessing the Results

4.1 View the Target Sites

We can now look at the target sites of the top scoring primers and restriction enzyme.

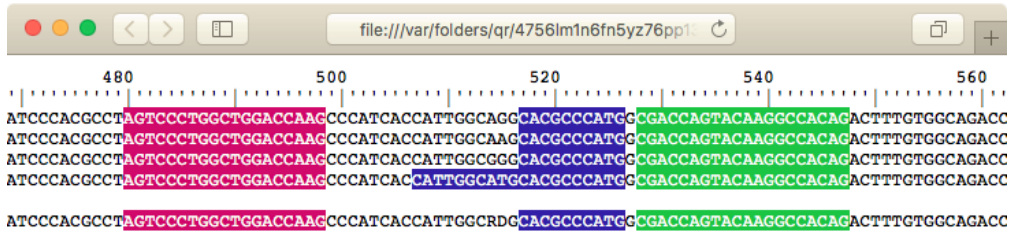


Figure 1: The forward (magenta) the reverse (green) primers situated around the SNP, with internal restriction site(s) (blue)

```
> PSET <- 1 # examine the top scoring primer set overall
> # select the first sequence from each group
> dna <- SearchDB(dbConn,
  remove="all",
  nameBy="identifier",
  clause="row_names =
    (select min(row_names) from Seqs as S
     where S.identifier = Seqs.identifier)",
  verbose=FALSE)
> f_primer <- DNASTringSet(primers$forward_primer[PSET])
> r_primer <- DNASTringSet(primers$reverse_primer[PSET])
> patterns <- c(f_primer,
  reverseComplement(r_primer),
  DNASTringSet(gsub("[^A-Z]", "", ENZYMES)))
> BrowseSeqs(dna,
  patterns=patterns)
```

We can see in Figure 1 that the forward and reverse primers target sites on both sides of the SNP, which is located approximately in the center of the amplicon. There are restriction sites next to the SNP, which will shorten the amplicons and further spread their melting curves. Furthermore, the final allele has two adjacent restriction sites, whereas the others all have one.

4.2 Plot the Signatures

Next we will compare the signatures of the top scoring primers with and without digestion. The simplest way to accomplish this is to use the **Biostrings** function `matchLRPatterns` to find the amplicons. Refer to section 2.1 above for how to easily obtain the code shown below:

```
> PSET <- which.max(primers$score) # top scoring without digestion
> f_primer <- DNASTring(primers$forward_primer[PSET])
> r_primer <- DNASTring(primers$reverse_primer[PSET])
> r_primer <- reverseComplement(r_primer)
> ids <- dbGetQuery(dbConn, "select distinct identifier from Seqs")
> ids <- ids$identifier
> if (TYPE=="sequence") {
  signatures <- matrix(0, nrow=4^RESOLUTION, ncol=length(ids))
}
```

```

} else if (TYPE=="melt") {
  signatures <- matrix(0, nrow=length(RESOLUTION), ncol=length(ids))
} else { # TYPE=="length"
  signatures <- matrix(0, nrow=length(RESOLUTION) - 1, ncol=length(ids))
}
> colnames(signatures) <- abbreviate(ids, 15)
> for (i in seq_along(ids)) {
  dna <- SearchDB(dbConn, identifier=ids[i], remove="all", verbose=FALSE)
  amplicons <- matchLRPatterns(f_primer, r_primer,
                              MAX_SIZE, unlist(dna),
                              max.Lmismatch=2, max.Rmismatch=2,
                              Lfixed="subject", Rfixed="subject")
  amplicons <- as(amplicons, "DNAStrngSet")
  if (length(amplicons)==0)
    next

  if (TYPE=="sequence") {
    signature <- oligonucleotideFrequency(amplicons, RESOLUTION)
    signatures[, i] <- colMeans(signature)
  } else if (TYPE=="melt") {
    signature <- MeltDNA(amplicons, "melt curves", RESOLUTION)
    # weight melting curves by their amlicon's width
    signature <- t(signature)*width(amplicons)
    signatures[, i] <- colSums(signature)/sum(width(amplicons))
  } else { # TYPE=="length"
    signature <- .bincode(width(amplicons), RESOLUTION)
    for (j in signature[which(!is.na(signature))])
      signatures[j, i] <- signatures[j, i] + 1/length(signature)
  }
}
> if (TYPE=="sequence") {
  d <- dist(t(signatures), "minkowski", p=1) # L1-Norm
  TreeLine(myDistMatrix=as.matrix(d), method="UPGMA", showPlot=TRUE)
  mtext(paste(RESOLUTION, "-mer Profile Distance", sep=""),
        side=2, padj=-4)
} else if (TYPE=="melt") {
  matplot(RESOLUTION, signatures, type="l",
          xlab="Temperature (degrees Celsius)", ylab="Average Helicity")
} else { # TYPE=="length"
  if (length(ids) > 20) {
    plot(NA,
         xlim=c(0.5, length(ids) + 0.5), ylim=range(RESOLUTION),
         xlab="Group Index", ylab="Amplicon Length",
         yaxs="i", xaxs="i")
    axis(1, at=1:length(ids), labels=FALSE, tck=-0.01)
  } else {
    plot(NA,
         xlim=c(0.5, length(ids) + 0.5), ylim=range(RESOLUTION),
         xlab="", ylab="Amplicon Length",
         yaxs="i", xaxs="i", xaxt="n")
    axis(1, at=1:length(ids), labels=abbreviate(ids, 7), las=2)
  }
}

```

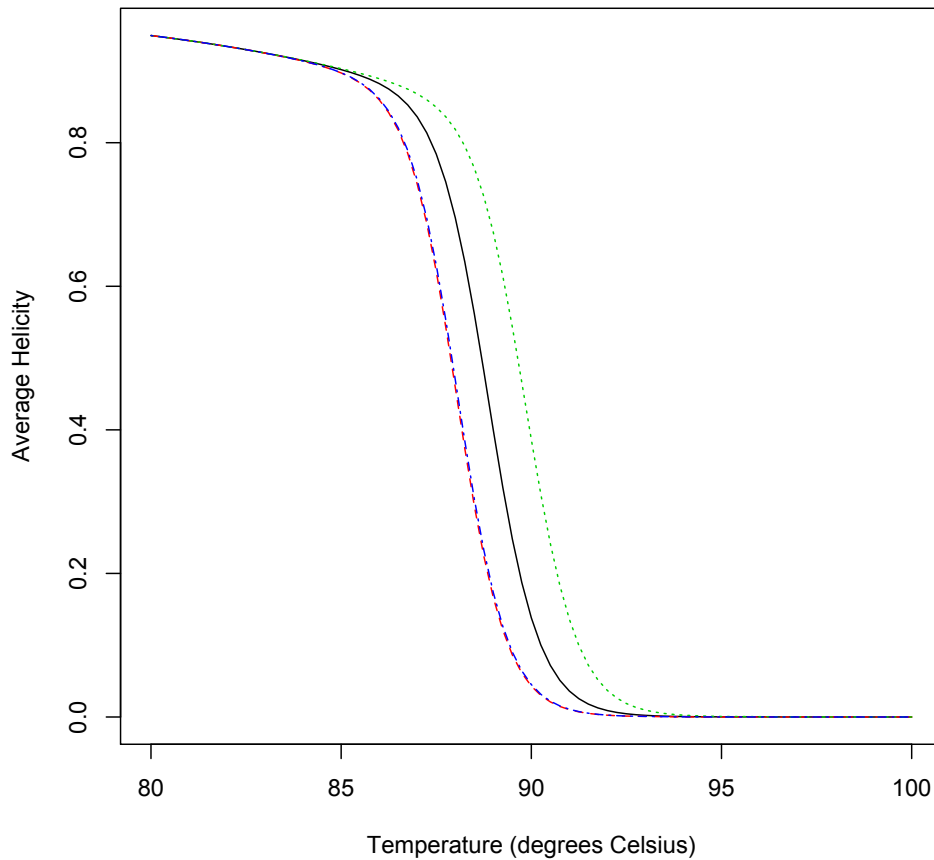


Figure 2: Melting curves for the amplicons without digestion

```

}
xaxs <- RESOLUTION[-1] - diff(RESOLUTION)/2 # average lengths
for (i in seq_along(ids)) {
  w <- which(signatures[, i] > 0)
  if (length(w) > 0)
    segments(i - 0.45, xaxs[w], i + 0.45, xaxs[w], lwd=2)
}
}

```

Figure 2 shows that two alleles will be indistinguishable when using these primers because their melting curves are nearly overlapping. We will now compare the result after using a restriction enzyme. Note that this is only possible because we previously specified a restriction enzyme in the input to `DesignSignatures`.

```

> PSET <- which.max(primers$digest_score) # top scoring with digestion
> f_primer <- DNASTring(primers$forward_primer[PSET])
> r_primer <- DNASTring(primers$reverse_primer[PSET])

```



```

> r_primer <- reverseComplement(r_primer)
> enzyme <- RESTRICTION_ENZYMES[primers$enzyme[PSET]]
> signatures[] <- 0 # initialize the results matrix used previously
> for (i in seq_along(ids)) {
  dna <- SearchDB(dbConn, identifier=ids[i], remove="all", verbose=FALSE)
  amplicons <- matchLRPatterns(f_primer, r_primer,
                              MAX_SIZE, unlist(dna),
                              max.Lmismatch=2, max.Rmismatch=2,
                              Lfixed="subject", Rfixed="subject")
  amplicons <- as(amplicons, "DNAStrngSet")
  if (length(amplicons)==0)
    next
  digested <- DigestDNA(enzyme, amplicons, strand="top")
  digested <- unlist(digested) # convert to DNAStrngSet

  if (TYPE=="melt") {
    signature <- MeltDNA(digested, "melt curves", RESOLUTION)
    # weight melting curves by their fragment's width
    signature <- t(signature)*width(digested)
    signatures[, i] <- colSums(signature)/sum(width(digested))
  } else { # TYPE=="length"
    signature <- .bincode(width(digested), RESOLUTION)
    for (j in signature[which(!is.na(signature))])
      signatures[j, i] <- signatures[j, i] + 1/length(signature)
  }
}
> if (TYPE=="melt") {
  matplot(RESOLUTION, signatures, type="l",
          xlab="Temperature (degrees Celsius)", ylab="Average Helicity")
} else { # TYPE=="length"
  if (length(ids) > 20) {
    plot(NA,
         xlim=c(0.5, length(ids) + 0.5), ylim=range(RESOLUTION),
         xlab="Group Index", ylab="Amplicon Length",
         yaxs="i", xaxs="i")
    axis(1, at=1:length(ids), labels=FALSE, tck=-0.01)
  } else {
    plot(NA,
         xlim=c(0.5, length(ids) + 0.5), ylim=range(RESOLUTION),
         xlab="", ylab="Amplicon Length",
         yaxs="i", xaxs="i", xaxt="n")
    axis(1, at=1:length(ids), labels=abbreviate(ids, 7), las=2)
  }
  xaxs <- RESOLUTION[-1] - diff(RESOLUTION)/2 # average lengths
  for (i in seq_along(ids)) {
    w <- which(signatures[, i] > 0)
    if (length(w) > 0)
      segments(i - 0.45, xaxs[w], i + 0.45, xaxs[w], lwd=2)
  }
}
}

```

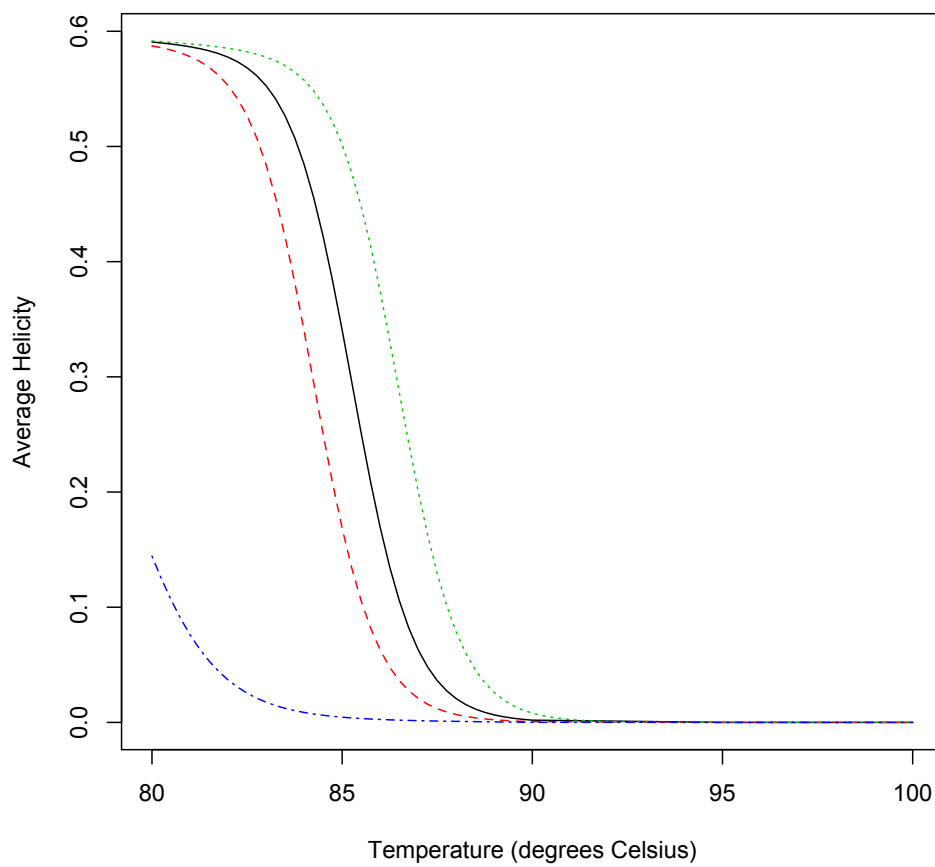


Figure 3: Melt curves for the amplicons after digestion

Figure 3 shows that the two amplicons which had nearly identical melt curves can be easily distinguished after restriction digest.

4.3 Finishing Up

Finally, we can order the top scoring forward and reverse primers for synthesis and try them out in a PCR reaction! The primers should be synthesized in the same orientation given in the output (`primers`).

5 Session Information

All of the output in this vignette was produced under the following conditions:

- R version 4.2.0 RC (2022-04-19 r82224 ucrt), x86_64-w64-mingw32
- Running under: Windows Server x64 (build 20348)
- Matrix products: default
- Base packages: base, datasets, grDevices, graphics, methods, parallel, stats, stats4, utils
- Other packages: BiocGenerics 0.42.0, Biostrings 2.64.0, DECIPHER 2.24.0, GenomeInfoDb 1.32.0, IRanges 2.30.0, RSQLite 2.2.12, S4Vectors 0.34.0, XVector 0.36.0
- Loaded via a namespace (and not attached): DBI 1.1.2, GenomeInfoDbData 1.2.8, KernSmooth 2.23-20, RCurl 1.98-1.6, Rcpp 1.0.8.3, bit 4.0.4, bit64 4.0.5, bitops 1.0-7, blob 1.2.3, cachem 1.0.6, cli 3.3.0, compiler 4.2.0, crayon 1.5.1, fastmap 1.1.0, memoise 2.0.1, pkgconfig 2.0.3, rlang 1.0.2, tools 4.2.0, vctrs 0.4.1, zlibbioc 1.42.0