

# MethSpec

## Manual

### Description

MethSpec is a simple tool that carries out evaluation of MSP primer specificity based on primer pair's sequences and parameters such as: primer concentration, ion concentration and annealing temperature. There are two alternative strategies for evaluation of MSP primers specificity used by MethSpec. The first one assumes that last nine bases at 3' end are relevant for primer specificity (9-mer approach) while the other approach uses algorithm proposed by Miura et al ( [Miura et al., 2005](#)).

The software determines SDSS (Specificity-Determining SubSequence) for forward and reverse primer and calculates stability of duplexes, expressed as dG value, that are formed with bisulfite modified methylated (target sequence), bisulfite modified unmethylated and appropriate native DNA sequence. The software carries out bisulfite modification of the input promoter sequence and localization of the primers inside it. By comparison of original input sequence and bisulfite modified one, MethSpec determines positions of modified cytosines inside primer's sequences. According to these positions program knows where mismatches are located. Alternatively, mismatch position can be specified by user. In this case position of modified cytosines should be marked with lowercase letter.

### Requirements

MethSpec is written in Java and is available as standalone and server like application. Standalone application can be installed on any operating system containing Java Virtual Machine.

### Installation

MethSpec source code and appropriate jar file are available for download at <http://www.vin.bg.ac.rs/180/tools/methspec.php> . In order to build program it is necessary to install Java Development kit (JDK). Linux users can install open JDK typing in terminal:

```
$ sudo apt-get install openjdk-7-jdk (Debian, Ubuntu)
```

```
$ su -c "yum install java-1.7.0-openjdk" (Fedora, Red Hat Enterprise Linux)
```

Windows users can download JDK using the following link: <http://www.oracle.com/technetwork/java/javase/downloads/index.html>.

If one choose to use jar file JDK installation is not necessary step.

### Usage

#### Standalone application

Methspec requires two .txt files, primers.txt and sequence.txt, to be located in the folder where executable file is. The file primer.txt contains referent primer pairs which serves to MethSpec to

calculate cut-off values. The second file is mandatory when positions of modified cytosines inside the primer sequence is not known. This file should contain promoter sequence (plain text format) where primer pair of interest is supposed to be located.

Organization of primer.txt file is depicted in Figure 1. It should be noted that user can change the file content adding new primer pairs or deleting existing ones.

| gene     | forward primer sequence     | reverse primer sequence        | Ta | b/n | m/u |
|----------|-----------------------------|--------------------------------|----|-----|-----|
| ATM MSP1 | GGAGTCGGGATtTGCgtTgtAGttAtc | ACTaAATTTCCCAaCGCAaAAaCCGCTaCG | 58 | Y   | Y   |

Figure 1. Organization of primers.txt file. Ta, annealing temperature; b/n, ability of primer pair to differentiate between bisulfite modified methylated and native sequence; m/u, ability of primer pair to differentiate between bisulfite modified methylated and appropriate unmethylated sequence. b/n and m/u columns contain description of primer pair specificity: Y- specific, N- nonspecific and – means unknown specificity. Columns are tab delimited.

The program is invoked from the command line in the following manner:

```
java MethSpecCmd <primer conc mol/l> <cation conc mol/l> <annealing T °C> <forward primer> <reverse primer>
```

or in the case of jar file usage:

```
java -jar MethSpec.jar <primer conc mol/l> <cation conc mol/l> <annealing T °C> <forward primer> <reverse primer>
```

Under default settings SDSS approach is used for primer specificity evaluation. Also, it is assumed that positions of modified cytosines is unknown to user. This means that primer sequence must contain uppercase letters only (A, C, G, T) and that sequence.txt must contain appropriate genomic sequence.

For an example, we want to examine specificity of primer pair designed to amplify methylated allele of BRCA1 gene:

```
java MethSpecCmd 0.0000002 0.6 60 TCGTGGTAACGGAAAAGCGC AAATCTCAACGAACTCACGCCG
```

If the positions of modified cytosines is known then these positions should be marked with lowercase letters (t or a). Importantly, in this scenario MethSpec is invoked by using -s flag:

```
java MethSpecCmd 0.0000002 0.6 60 tCGTGGtAACGGAAAAGCGC AaaTCTCAaCGAaCTCACGCCG -s
```

The output is same in both cases:

Forward Primer:

Tm[primer] = 71.32 C  
Tmg[primer] = 68.81 C  
Tmu[primer] = 32.00 C  
SDS = CGGAAAAGCGC  
f = 0.046918  
Tm = 49.37 C  
Tmg = 49.37 C  
Tmu = 10.13 C

Reverse Primer:

Tm[primer] = 71.32 C  
Tmg[primer] = 52.36 C  
Tmu[primer] = 59.56 C  
SDS = aCTCACGCCG  
f = 0.034625  
Tm = 47.17 C  
Tmg = 45.91 C  
Tmu = 26.60 C

SpG = -16.84 cal/(mol\*BP)

SpU = -890.63 cal/(mol\*BP)

Reference Values for:

SpG:

Specific Primers [-1329.48, -299.85]

Nonspecific Primers [-561.68, -98.29]

Cutoffs: -561.68 and -299.85 cal/(mol\*bp)

SpU:

Specific Primers [-1717.79, -480.31]

Nonspecific Primers [-441.31, -135.01]

Cutoff = -460.81 cal/(mol\*bp)

Modified/Native is NONSPECIFIC

Methylated/Unmethylated is SPECIFIC

Where is:

T<sub>m</sub>, melting temperature of perfectly paired duplexes; T<sub>mg</sub>, melting temperature of formed duplex between MSP primer and appropriate native sequence; T<sub>mu</sub>- melting temperature of formed duplex between MSP primer and appropriate unmethylated sequence; SpG specificity factor that describes ability of primer pair to differentiate between bisulfite modified methylated and native sequence; SpU, specificity factor that describes ability of primer pair to differentiate between bisulfite modified methylated and appropriate unmethylated sequence.

In order to make MethSpec to use 9-mer approach flag -n should be used. It is important to note that the results obtained by SDSS and 9-mer approach may differ.

It is important to note that MethSpec is able to calculate annealing temperature (T<sub>a</sub>). In order to use this option MethSpec is invoked with T<sub>a</sub> value set to 0:

```
java MethSpecCmd 0.0000002 0.6 0 GCGGGGAGGACGACGAGGGC GCATCCAATATCACGCGATCTCCG
```

Option -log can be used to obtain more detailed report that include: calculated dG values for primers 3' end, total number of CpGs in the primer sequences, positions of the forward and reverse primer inside input sequence (only in the case when -s flag is not used). This report can be saved as a text file. For example, we want to save report about BRCA1 primer pair in text file brca1.txt:

```
java MethSpec 0.0000002 0.6 60 TCGTGGTAACGGAAAAGCGC AAATCTCAACGAACTCACGCCG -log>brca1.txt
```

## Server application

Use of server application is simple and intuitive. User should enter or copy/paste values and primer sequences in the appropriate fields and then click Send. The program output is identical to the output given by standalone application. However, server application has an extra feature to display results in the graphic form (Figure 2.).

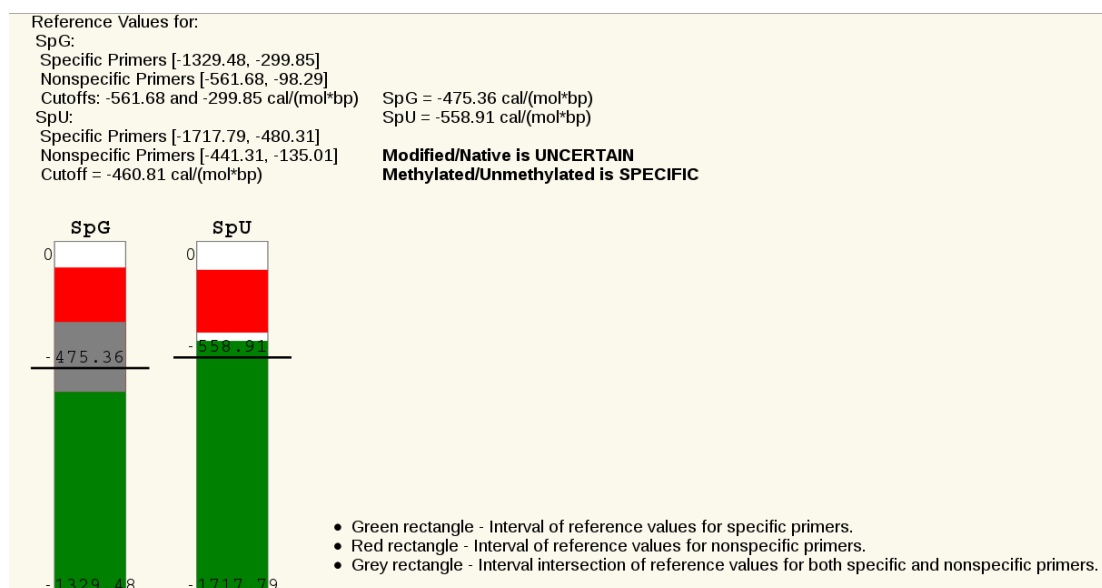


Figure 2. Graphical representation of the results.

### Additional features

MethSpec has ability to check if primer properties is in accordance with general rules for MSP primer design. This means that program will issue warning in the case that some of the rules were ignored during primer design. In the example below, program warns user that forward primer has poor ability to differentiate between bisulfite modified and native sequence:

```
Primer concentration = 0.0000002 mol/l
Cation concentration = 0.6 mol/l
Annealing temperature = 60 °C
Forward primer 5'-tAtTGC GGAGTGCGGGTC-3'
Reverse primer 5'-TCGaCGaaCTCCCGACGa-3'
9-mer approach = NO
```

#### Evaluation of primer specificity

##### Warning:

Forward primer does not contain any modified cytosine at its 3' end! This primer can not differentiate between native and modified DNA sequence!

| Forward Primer:       | Reverse Primer:       |
|-----------------------|-----------------------|
| Tm[primer] = 70.03 C  | Tm[primer] = 70.51 C  |
| Tmg[primer] = 67.17 C | Tmg[primer] = 51.38 C |
| Tmu[primer] = 42.64 C | Tmu[primer] = 39.22 C |
| SDSS = AGTGC GGTC     | SDSS = CTCCCGACGa     |
| f = 0.027074          | f = 0.014988          |
| Tm = 46.06 C          | Tm = 44.50 C          |
| Tmg = 46.06 C         | Tmg = 42.33 C         |
| Tmu = 10.02 C         | Tmu = 8.61 C          |

In order to facilitate analysis of the primer pair specificity, MethSpec provides option for downloading appropriate gene sequence from Ensembl. This option can be used if the program is invoked with -e flag:

```
java MethSpecCmd 0.0000002 0.6 0 GCGGGGAGGACGACGAGGGC GCATCCAATATCACGCGATCTCCG -e
ATM
```

or

```
java MethSpecCmd 0.0000002 0.6 60 GCGGGGAGGACGACGAGGGC GCATCCAATATCACGCGATCTCCG -e
ENSG00000149311
```

It is possible to check if primer pair has ability to amplify a fragment from appropriate pseudogene sequence. This option should be invoked with -m and -p flags. Using -m flag user defines maximal allowed number of mismatches along whole primer sequence or/and SDSS part of primer. Flag -p requires Ensembl ID of the gene, according to this information program searches for appropriate pseudogene sequence. Below is an example :

```
java MethSpecCmd 0.0000002 0.6 60 GCtCCCAGGAACCAAAAtCGC CGCTCGTaCGCGTaCTTGCC -s -p
ENSG00000182263 -m 2 1
```