# FluDen Primer DB –PCR Primer Database for Influenza A and Dengue Virus

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**Abstract:** FluDen Primer Database (http://www.fludenpdb.com) has been designed and developed as a web application program to provide free access to the in-silico designed multiple potential primers for PCR detection and quantification assays for Influenza and dengue viruses. This program also permits user to submit sequence of their choice for primer design. The database contains primer records for Influenza and dengue viruses which cause infection in Humans. As of 2014 there are 142 primer sets for screening 32 genes/regions of Influenza and dengue viruses together. Application contain gene information, assay details such as oligonucleotide sequence, primer properties and reaction conditions, publication information. We have developed a resource, FluDen Primer DB which contains primer that can be used for PCR under provided amplification conditions for each primer pair. A distinguish feature of the FlueDen is the primers listed in DB are the products of PCR design application which are experimentally validated. Primers for the FluDen were designed using current genomic information available from the National Center for Biotechnology Information (NCBI).

**Keywords:** PCR, Influenza, Dengue, NetPrimer, FastPCR.

## INTRODUCTION

PCR provides alternative to culture based detection as changing approaches to clinical diagnostics [1]. A great advantage of PCR is its specificity. User can target genome with specific primers, out-performing traditional culture methods. PCR primers can be used to screen bacteria, viruses, fungi and protozoa which cause infection to humans.

Researchers every time will be designing their own primers for which already around the world thousands of primer pair exists to amplify/detect the same gene and organism, but the effort goes in vein as they are not shared to public, again for doing this every researches tend to spend valuable amount of time in understanding the bioinformatics behind designing/selecting primers to amplify/detect there gene/organism of interest. There are many parameters which needs more focus while designing their PCR experiment. a. Target gene/sequence selection – it consumes time to get aware of the information that are available in online biological database, orientation of the gene sequence, selection of desired gene sequence, format of the sequence to download, b. NCBI BLAST [2] parameters which includes selection of right database for getting the hits, type of BLAST, E-Value, bit scores c. Primer Design – selection of right online tools, bio-chemistry values which includes GC%, Tm (melting temperature), Ta (annealing temperature), primer dimer formation, cross dimer formation, runs, repeats and many more. Researches spend lot of time in understanding these concepts rather than focusing on their research work.

FluDen Primer DB online free web application was presented to public for accessing primers for detection and quantification PCR applications. FluDen database was developed to address the problem of the repeated laborious primer design and assay evaluation for quantification or detection of the same nucleic acid sequence by different individuals, which significantly prohibits standardized and uniform assays. Therefore, the database's primary objective was the deployment of web-interface to find primer information obtained from experimentally validated primer design application which can be used by all users who intend to detect or quantify Influenza A and Dengue virus.

Multiple primer design algorithms have been proposed online, but few of them have been validated experimentally. FastPCR developed by PrimerDigital Biotechnology Company [3, 4] and Netprimer, developed by PREMIER Biosoft International [5-7] are experimentally validated.

The data that are maintained in the DB include all the information required to understand the purpose of primer set and implement them in an experiment. Included information are target gene name, primer position of target gene, primer properties like Tm (melting temperature), GC%, primer length, sense and anti-sense primer sequence (in 5'-3' orientation),

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ISSN: 1927-3037/15 

amplicon size, Ta opt (Optimum annealing temperature), molecular weight, optical activity (both in nmol/A260 & µg/A260), DG or ΔG (Gibbs free energy), 3' end stability, DH or ΔH (enthalpy), DS or ΔS (entropy), and 5' end DG (Gibbs free energy), primer secondary structures including hairpins, dimers, cross dimers, palindromes, repeats and runs are for each primer sets.

## DENGUE VIRUS PATHOGENESIS, EPIDEMIOLOGY, MORTALITY AND DIAGNOSIS

Dengue is caused by dengue virus (DENV), a mosquito-borne flavivirus. DENV is a single stranded RNA positive-strand virus of the family Flaviviridae, genus Flavivirus. DENV causes a wide range of diseases in humans, from a self-limited Dengue Fever (DF) to a life-threatening syndrome called Dengue Hemorrhagic Fever (DHF) or Dengue Shock Syndrome (DSS). There are four antigenically different serotypes of the virus: DENV-1, DENV-2, DENV-3 and DENV-4. Here, a serotype is a group of viruses classified together based on their antigens on the surface of the virus. These four subtypes are different strains of dengue virus that have 60% - 80% homology between each other [8]. Dengue is endemic in more than 110 countries [9]. It infects 50 to 100 million people worldwide a year, leading to half a million hospitalizations [10] and approximately 12,500 - 25,000 deaths [11, 12]. Early detection is always advised for better treatment, Virus isolation, Genome detection, Antigen detection are direct and Serology igM, igG detection are indirect laboratory methods for the diagnosis of dengue infection. Specificity wise direct methods are much better than the indirect methods [13].

## INFLUENZA A VIRUS PATHOGENESIS, EPIDEMIOLOGY, MORTALITY AND DIAGNOSIS

Influenza A viruses are a major cause of morbidity and mortality worldwide and affect large segments of the population every year. The nature of their genome, formed by eight segments of single-stranded RNA. The viral genome encodes 11 proteins. On the surface of the virus particles there are two principal polypeptides, the hemagglutinin (HA) and the neuraminidase (NA), There are 16 HA and 9 NA different subtypes in the influenza A virus that circulate in humans and animals. When a virus strain with a new HA or NA subtype appears in the human population by genetic re-assortment, it usually causes a pandemic because there is no preexisting immunity against the new virus. This was the case for the three pandemics that occurred during the last century (1918, 1957, and 1968) and also for the first pandemic of the 21st century, caused by the currently circulating A (H1N1) 2009 virus, which was generated by gene re-assortment between a virus present in pigs of North America and a virus that circulates in the swine population of Euroasia [14]. The Spanish influenza pandemic of 1918-1919 caused acute illness in 25% - 30% of the world's population and resulted in the death of 40 million people [15]. The type A viruses are the most virulent human pathogens among the three influenza A, B and C types and cause the most severe disease, H1N1, which caused Spanish Flu in 1918 and Swine Flu in 2009. For both the 1918 and H5N1 viruses (which were responsible for pandemic), the hemagglutinin and the polymerase complex contribute to high virulence. Non structural proteins NS1 and PB1-F2, which block host antiviral responses, also influence pathogenesis [16].

The laboratory diagnosis of influenza and dengue virus uses a wide range of techniques including rapid immunoassays, immunofluorescence techniques, virus culture methods, and increasingly sophisticated molecular assays. Early molecular detection techniques had been labor intensive, and required separate facilities in order to prevent contamination. Those techniques have largely been supplanted by more modern methods, most notably real-time reverse transcription PCR assays, which are currently the method of choice in many laboratories for the detection and subtyping of influenza viruses [17].

## PRIMER DESIGN PROCESS

Genomic sequences were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) public repository. Nucleotide id- JN903578.1, DQ181806, AY662691.1 and JX024758.1 were the sequence used for designing primers for Dengue 1, 2, 3 and 4 strains respectively. And for Influenza A virus where HA and NA genes are specific to H1N1 subtype, GU292357, GU292363, GU292369, GU292346, GU292375, GU292381, GU292387 and GU292393 nucleotide sequence were used for designing primers for PB2, PB1, PA, HA, NP, NA, MP, NS genes respectively. Primers were covered for all entries present in the NCBI database with 100% similarity to which they were designed for.

## PARAMETERS FOR PRIMER PAIR DESIGN

(i) Primer sequence containing bases other than "A", "T", "G" and "C" were discarded. (ii) Amplicon length depends on experimental need. For standard

PCR, amplicon size is near 500bp and for qPCR target length is closer to 100bp. (iii) Melting temperature (Tm) values provides the stability between primer-template DNA duplex. Primers with melting temperatures in the range of 52-58 $^{o}$C generally produce the best results. Primers with melting temperatures above 65$^{o}$C have a tendency for secondary annealing. The GC content of the sequence gives a fair indication of the Tm. The two primers of a primer pair should have closely matched melting temperatures for maximizing PCR product yield. The difference of 5 $^{o}$C or more can lead to no amplification. (iv) The GC content (the number of G's and C's in the primer) of primer should be 40-60%. (v) A repeat is a di-nucleotide occurring many times consecutively and should be avoided because they can misprime. A maximum number of di-nucleotide repeats acceptable are 4 di-nucleotides. (vi) Primers with long runs of a single base should generally be avoided as they can misprime. A maximum number of runs accepted is 4bp. Presence of the secondary structures produced by intermolecular or intramolecular interactions can lead to poor or no yield of the product. They adversely affect primer template annealing and thus the amplification. They greatly reduce the availability of primers to the reaction. (Vii) 3' End Stability is the maximum ΔG value of the five bases from the 3' end. An unstable 3' end (less negative ΔG) will result in less false priming. (Viii) Hairpins are formed by intramolecular interaction within the primer and should be avoided. Optimally a 3' end hairpin with a ΔG of -2 kcal/mol and an internal hairpin with a ΔG of -3 kcal/mol is tolerated generally. (ix) Self Dimer is formed by intermolecular interactions between the two (same sense) primers, where the primer is homologous to itself. Generally a large amount of primers are used in PCR compared to the amount of target gene. When primers form intermolecular dimers much more readily than hybridizing to target DNA, they reduce the product yield. Optimally a 3' end self-dimer with a ΔG of -5 kcal/mol and an internal self-dimer with a ΔG of -6 kcal/mol is tolerated generally. (x) Cross dimers are formed by intermolecular interaction between sense and antisense primers, where they are homologous. Optimally a 3' end cross dimer with a ΔG of -5 kcal/mol and an internal cross dimer with a ΔG of -6 kcal/mol is tolerated generally. (xi) Good PCR product yield with minimum false product production and primer annealing to template genomic strand depends on Optimum annealing temperature (Ta Opt). All primers falling within the range for all parameters show stable efficient amplification in vary PCR annealing temperature. The default condition scan is used at the annealing temperature from 56 to 68 without losing PCR efficiency. (xii) To improve specificity of the primers it is necessary to avoid regions of homology. Primers designed for a sequence must not amplify other genes in the mixture. Commonly, primers are designed and then BLASTed (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to test the specificity and cross-reactivity.

**Table 1: FluDen Primer DB as 124 Primer Pairs for all 14 Regions of Dengue virus Strains 1, 2, 3 and 4**

| Genomic Region\Strains | Dengue 1 | Dengue 2 | Dengue 3 | Dengue 4 |
|---|---|---|---|---|
| Flavi capsid | 2 | 2 | 2 | 1 |
| FlaviPropep | 2 | 2 | 1 | 1 |
| Flavi M | 2 | 2 | 2 | 2 |
| FlaviGlycoprot | 2 | 2 | 2 | 2 |
| Flavi E_C | 2 | 2 | 2 | 2 |
| FlaviE_stem | 2 | 2 | 2 | 2 |
| FlaviNS1 | 2 | 2 | 2 | 2 |
| FlaviNS2A | 1 | 2 | 2 | 1 |
| FlaviNS2B | 2 | 2 | 2 | 1 |
| Peptidase S7 | 2 | 2 | 2 | 2 |
| DEXDc | 2 | 2 | 2 | 2 |
| DEXDc - ATP-binding region | 2 | 2 | 2 | 2 |
| HELICc | 2 | 2 | 2 | 0 |
| Flavi NS4A | 2 | 2 | 2 | 0 |
| Flavi NS4B | 2 | 2 | 2 | 0 |
| FtsJ | 2 | 2 | 2 | 2 |
| Flavi NS5 | 2 | 2 | 2 | 2 |

Primer properties like Tm, GC%, primer length, sense and anti-sense primer sequence (in 5'-3' orientation), amplicon size, Ta opt are from FastPCR http://primerdigital.com/fastpcr.html application for each primer sets [3, 4]. Molecular weight, optical activity (both in nmol/A260 & µg/A260), DG (Gibbs free energy), 3' end stability, DH(enthalpy), DS (entropy), and 5' end DG(Gibbs free energy), Primer Secondary Structures including Hairpins, dimers, cross dimers, palindromes, repeats and runs are obtained from NetPrimer [5-7]. http://www.premierbiosoft.com/netprimer/netprlaunch/Help/xnetprlaunch.html.

## PRIMER STATISTICS

The database contains primer records for Influenza and dengue viruses which cause infection in Humans. As of 2014 from Table **1** and **2**, there are 142 primer sets for screening 32 genes/regions of Influenza and dengue viruses together.

**Table 2: FluDen Primer DB as18 Primer Pairs for all 8 Genes of Influenza A virus H1N1 Strain (Primer Sequence Used from NCBI for Influenza A/Blore/NIV236/2009(H1N1))**

| Influenza A Genes | # Primer Pair | NCBI Nucleotide Sequence ID |
|---|---|---|
| PB2 | 3 | GU292357 2275 bp cRNA |
| PB1 | 2 | GU292363 2271 bp cRNA |
| PA | 2 | GU292369 2148 bp cRNA |
| HA | 2 | GU292346 1698 bp cRNA |
| NP | 2 | GU292375 1494 bp cRNA |
| NA | 3 | GU292381 1407 bp cRNA |
| MP | 2 | GU292387 979 bp cRNA |
| NS | 2 | GU292393 835 bp cRNA |

## CONCLUSION

FluDen Primer Database is first of its kind present online for providing free primer database for PCR based Influenza and dengue virus detection and quantitation and can be accessed at www.fludenpdb.com. Primers stringently tested and selected in-silico for specificity and non cross-reactivity by BLASTing it in NCBI non-redundant database.

Algorithm developed by FastPCR-PrimerDigital Biotechnology Company for primer design been In-silico and experimentally validated [3, 4], here you will find references to articles which cite FastPCR by other articles in journals, patents and PhD thesis http://primerdigital.com/fastpcr/citations.html. Hence primers hosted in FluDenDB are reliable and requires very less PCR optimization.

Primers obtained from FastPCR are analyzed for all primer secondary structures including hairpins, self-dimers, and cross-dimers in primer pairs using NetPrimer (product of PREMIER Biosoft http://www.premierbiosoft.com/citations/netprimer.html) - a free online web-based application. This ensures the availability of the primer for the PCR reaction as well as minimizing the formation of primer dimer. The program also eases quantitation of primers by calculating primer molecular weight and optical activity [5] [6] [7].

It is tough task to get all the primer listed in FluDen DB to satisfy all the primer parameters. Values for some of the parameters go slightly out of range. Excellent primers are not the only criterion for the success of PCR. Although great care has been given to design FluDenDB primers, the success rate is not 100%. In that case, please try a different primer pair for the same gene or other primers provided for the same organism.

## SOFTWARE REQUIREMENT

Flu and Dengue DB is fully supported by all recently updated versions of internet browsers like IE, Mozilla and Google chrome on Windows. More information is provided in the Help/FAQ section of Flu and Dengue DB http://www.fludenpdb.com/help.html.

## FUTURE DIRECTIONS

Flu and Dengue Primer DB will continually be updated with many more markers for the same and also for other microorganism in the database which cause infections to humans.

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Received on 23-07-2015 Accepted on 01-10-2015 Published on 03-12-2015

DOI: http://dx.doi.org/10.6000/1927-3037.2015.04.03.2