FastPCR

Molecular Biology Software

Version 5.4 for Windows

User's manual (beta version) Monday, 23 February 2009

Overview

The FastPCR software is an integrated tools environment that provides comprehensive facilities for designing any kind of PCR primers for standard, long distance, inverse, real-time PCR (LUX and self-reporting), multiplex PCR, group-specific PCR (common primers for given N target sequences), unique PCR (design of specific (unique) PCR primers for each sequence); single primer PCR (design of PCR primers from close located inverted repeat), automatically SSR loci detection and direct PCR primers design, amino acid sequence degenerate PCR and more. The software consists of a data editor; build-in commands for probe/primers design and build automation tools. The software selects best primers with the widest range of melting temperatures that allows designing qualified primers for all PCR tasks.

The "*in silico*" PCR primers or probe searching, comprehensive pairs and individual primers analysis tests are included. The "*in silico*" oligonucleotide search is helpful for discovering target binding sites with the temperature melting calculation. A long oligonucleotide can be designed for microarray analyses and dual-labelled oligonucleotides for probes and molecular beacon.

The software utilize combinations of normal and degenerated primers for all tools and for the temperature melting calculation based on the nearest neighbour thermodynamic parameters.

FastPCR has the capacity to handle long sequences and sets of nucleic acid or protein sequences and it allowed the individual task and parameters for each given sequences and joining several different tasks for single run. It also allows sequence editing and databases analysis.

Efficient and complete detection of various types of repeats developed and applied to the program with a visualisation. FastPCR is able to perform repeat search for single sequence or for comparisons of two sequences.

The program includes various bioinformatics tools for analysis of sequences with GC or AT skew, CG content and purine-pyrimidine skew, the linguistic sequence complexity; generation random DNA sequence, restriction analysis and supports the clustering of sequences and consensus sequence generation and sequences similarity and conservancy analysis.

Manual and files for installation are available over the Internet at http://www.biocenter.helsinki.fi/bi/programs/fastpcr.htm

Web tools - java applets for comprehensive on-line primer analysis and the temperature melting calculation for normal and degenerated nucleotide combinations based on the nearest neighbour thermodynamic parameters, comprehensive oligonucleotide dilution and resuspension calculator, applet for comprehensive analysis of the list of primers with prediction of the oligonucleotides properties and self and cross dimers detection, and temperature of annealing calculation and general PCR and qPCR set-up applet available here: http://primerdigital.com/Tools/webtools.html

Availability

To academic institutions FastPCR is available for a free, provided for non-commercial research and education use only (not for reproduction or distribution, cannot be used in any commercial enterprises).

The manual, licence and files for installation are available over the Internet at:

http://www.biocenter.helsinki.fi/bi/programs/fastpcr.htm

or from PrimerDigital Ltd: http://www.primerdigital.com/

A web interface (Java) and stand-alone 64-Bit software (VS.Net C#) for Linux and Mac operation systems are currently under development.

The "FastPCR" software was currently written in Microsoft Visual Studio 6.0 and compiled to an executive file that, after installation, can be used with any version of Microsoft Windows.

For Linux and Mac "Wine" as a compatibility layer for running Windows programs and it is a completely free alternative implementation of the Windows API also for use native Windows DLLs.

Although FastPCR is not officially supported on the Linux platform at this time, users report being able to run FastPCR successfully on Linux using WINE. Linux installation, it is not need to compile source - just type:

 'wine msiexec /i FastPCR.msi' and it will install without problem. Users who want to use FastPCR on the Mac platform are encouraged to use the Virtual PC Windows Emulator from Microsoft.

All Microsoft Windows, but Windows XP, Windows Server 2003, Windows Vista and 7 (32-Bit or 64-Bit) recommended
Memory: 512-2048 MB of RAM

Reference apply to all FasPCR updates, if you use FastPCR in your work please cite. The citation of FastPCR is necessary and co-authorship for minimum 10% participation in publication result:

Kalendar R. 2008. FastPCR software for PCR design and repeat search.

[www.biocenter.helsinki.fi/bi/programs/fastpcr.htm]

Reporting Problems: If you encounter problems, please visit Web site and download the latest version to see if the issue has been resolved. It would be very helpful if you could send me a feedback report with the data and conditions you were using, in order to fix the encountered bug. Dr. Ruslan Kalendar

E-mail: ruslan.kalendar*helsinki.fi

This is the latest update for all previous FastPCR: http://www.primerdigital.com/soft/newfastpcr.zip

Manually copy and overwrite old file: **fastpcr.exe** by new one in the FastPCR folder:
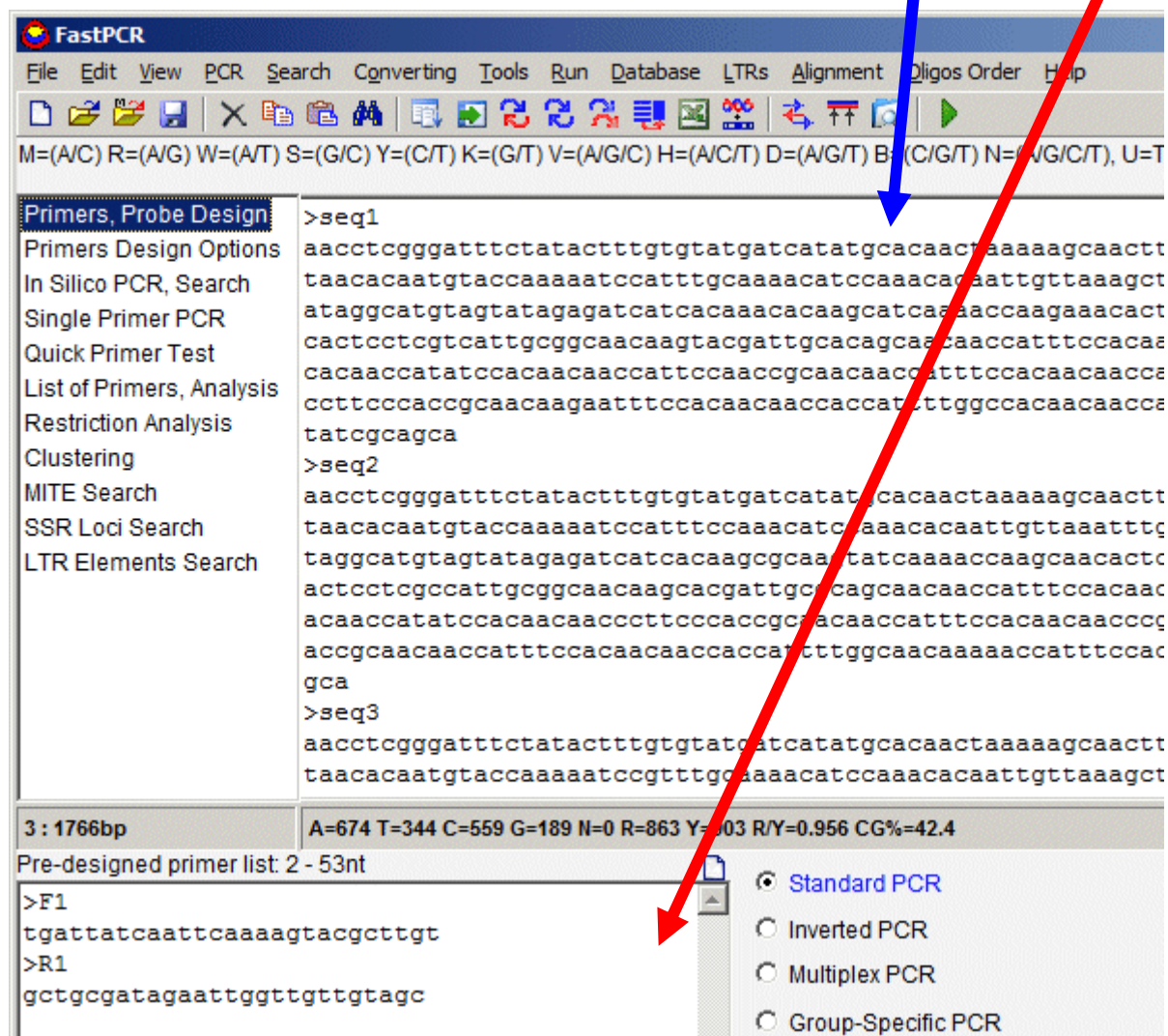
C:\Program Files\FastPCR

or

C:\Program Files (x86)\FastPCR

The program Structure

FastPCR is a software package to aid molecular biologist, students, and other in the study, manipulation and design experiment.

The logical structure of FastPCR is known below. Two text editors (boxes): **general** and **additional**:

Both editors are equally reading any sequence formats, but general editor designed for working with sequence(s) and for reading sequences from open file(s); additional editor use for controlling tools, for predesigned primer(s) or sequence(s). Import sequences from a clipboard with keyboard (Shit-Insert or Ctrl-V) and right-click mouse displays a contextual menu.

Tree View for choosing task. The result will appear in status bar and "**key F5**":

Creating an experiment

Import sequence(s), about Sequence Formats.

FastPCR normally expect to read sequence files in FASTA format. Prepare your sequence data file using a text editor (Notepad, WordPad), and save in ASCII text format (plain text) or Rich Text Format (.rtf).

You can type in FastPCR editors or import nucleotide (protein) sequence(s) from file or from the clipboard as simple text, RTF text, or FASTA format, MEGA, or alignment for BLAST, EMBL result searching, Dialign or MSF alignment, Excel sheet or Word table (two columns), the table with TAB or whitespace separators to general text editor:
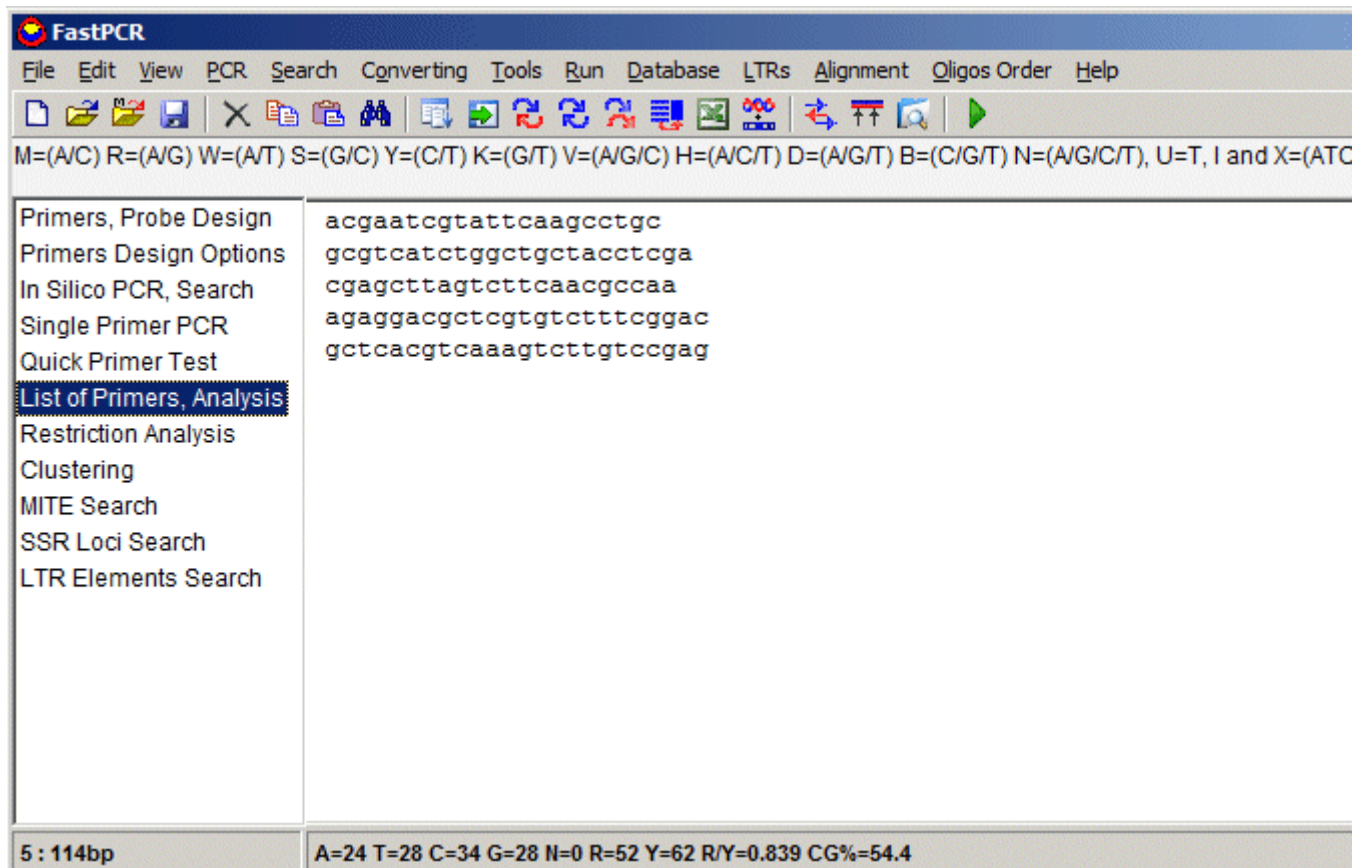
Import sequences are not different for two editors: both editors take all ways, with keyboard (Shit-Insert, Ctrl-V) and allowed work with right-click mouse displays a contextual menu.

Importing sequence(s) must be at the same format. Degenerate DNA sequences are also accepted as IUPAC code is an extended vocabulary of 16 letters which allows the description of ambiguous DNA code.

Each letter represents a combination of one or several nucleotides: M=(A/C) R=(A/G) W=(A/T) S=(G/C) Y=(C/T) K=(G/T) V=(A/G/C) H=(A/C/T) D=(A/G/T) B=(C/G/T) N=(A/G/C/T), U=T and I (Inosine).

Accepted amino acid codes: A(Ala), C(Cys), D(Asp), E(Glu), F(Phe), G(Cly), H(His), I(Ile), K(Lys), L(Leu), M(Met), N(Asn), P(Pro), Q(Gln), R(Arg), S(Ser), T(Thr), V(Val), W(Trp), Y(Tyr).

Sequence formats are simply the way in which the amino acid or DNA sequence is recorded in a computer file. Different programs expect different formats, so if you want to run a successful job, it is important to understand what the various formats look like.

When sequences are imported you may edit the sequences in general or additional editors and immediately visualize the result of editing. Press **F12** switch on-typing sequence interpretation to on or off. You can modify a nucleotide sequences by inserting, deleting and replacing sequence fragments.

Raw format (ASCII):

Like a text/plain format with any white space or digits. It read only standard [IUB/IUPAC](#) amino acid or nucleic acid codes characters and rejects anything else, low- and upper-case insensitive. Digits, spaces, TAB characters or else are removed and ignored (but Tab and space characters with combination end line character (Enter press) can be interpreted as column format). Non-alphabetic characters (for instance, punctuation characters) will made the sequence rejected as erroneous. Here are some examples of raw formatted sequence:

```
ataaattcttattttgacactcaccaaaatagtcacctggaaaacccgcttttttgtgaca
```

FASTA format description:

FASTA format (no alignment) is simple the raw sequence proceeded by definition line. The definition line begins with a ">" sign and followed immediately by a name for the sequence with using any length and amount of words.

Many sequences can be listed in the file, the format indicating a new sequence at each new '>' symbol found. It is important to press Enter at the end of each line after ">" to help FastPCR recognize the end and beginning of sequence and sequence's name. Make sure the first line starts with a ">" and has (has not) a header description. The description must be contained within one line and not run into 2 or more lines. The sequence starts directly on next line.

As for the previous raw data format, sequences must be in the standard IUB/IUPAC amino acid or nucleic acid codes, any other characters - digits, spaces, TAB characters or else are ignored, low- and upper-case insensitive:

```
>
cggccgagatcaggcgatgcatg
>
acgacgacgcagctatattacag
```

FastPCR: the alignment sequences in FASTA format will read only standard IUB/IUPAC amino acid or nucleic acid codes characters and rejects anything else, low- and upper-case insensitive. Sequence alignment (PHYLIP, NEXUS and else) is not necessary to reformat into FASTA format.

Tables format description:

You can import the table from Microsoft Word or Excel sheet (or OpenOffice), or primer's list from PCR design result, or the table with TAB or whitespace separators. Software reads only first two columns with names and sequences:

1F1_234-253	agggagtagcttacctcgct	20	56.6	31.6	55	95	87
2F1_263-283	gcgaaaaccaagtgcttacct	21	55.9	29.4	47.6	95.2	101
3F1_290-313	tcctcaagcgaaaaccaatccaca	24	58.8	29.9	45.8	92.5	101
4F1_318-338	tgttcacatgtttggggacga	21	56.4	36.3	47.6	90.5	77
5F1_545-564	gcttgtaaggcaaacccaca	20	56	31	50	96	83
6F1_606-625	acgtggtactcatggtgtca	20	55.2	30	50	89	85
1R1_601-622	caccatgagtaccacgtgagtt	22	56.5	30.9	50	86.4	87
2R1_366-386	tgtgtgatcatgatgccctca	21	55.9	33.9	47.6	85.7	69
3R1_325-348	atcgacaccttcgtccccaaacat	24	60.1	30.3	50	93.3	84
4R1_262-284	gaggtaagcacttggttttcgct	23	57.6	32.1	47.8	96.5	103
5R1_234-255	caagcgaggtaagctactccct	22	58	31.9	54.5	94.5	98
1F2_32-53	cccaacggtttacctcaagggt	22	59.2	32.9	54.5	94.5	105
2F2_435-457	tcgcgaccttatgagaacgctgc	23	61.1	36.5	56.5	96.5	115
3F2_476-495	aagcagcgaccgacgaaacc	20	60.2	33.5	60	85	75
1R2_476-495	ggtttcgtcggtcgctgctt	20	60.1	36.6	60	85	75
2R2_448-470	agtcaacggcgtcgcagcgttct	23	64.7	36.6	60.9	92.2	99
3R2_160-179	ggtcgcgacatccgttccaa	20	59.7	32.6	60	95	82
4R2_33-54	tacccttgaggtaaaccgttgg	22	56.5	31.8	50	94.5	106

As for the previous raw data format, sequences must be in the standard IUB/IUPAC amino acid or nucleic acid codes, any other characters - digits, spaces or else are ignored, low- and upper-case insensitive. Tab character or spaces are used for recognition columns.

Other simple table format is with or without name for primers (probes or else); name is replaced by single space (space inside sequence not allowed) and the end of each sequence, press Enter is necessary:

```
acgaatcgtattcaagcctgc
gcgtcatctggctgctacctcga
cgagcttagtcttcaacgccaa
agaggacgctcgtgtctttcggac
gctcacgtcaaagtcttgtccgag
```

In case using sequence's name, no space inside names and sequences are allowed:

```
1 acgaatcgtattcaagcctgc
2 gcgtcatctggctgctacctcga
3 cgagcttagtcttcaacgccaa
4 agaggacgctcgtgtctttcggac
```

Software always indexing each sequence from 1 to N, therefore doesn't matter if some sequence's name are the same or absent:

```
1 acg aat cgt att caa gcc tgc
  ccg tca tct ggc tgc tac ctc ga
  cga gct agt ctt caa cgc caa
1 aga gga cgc tcg tgt ctt tcg gac
```

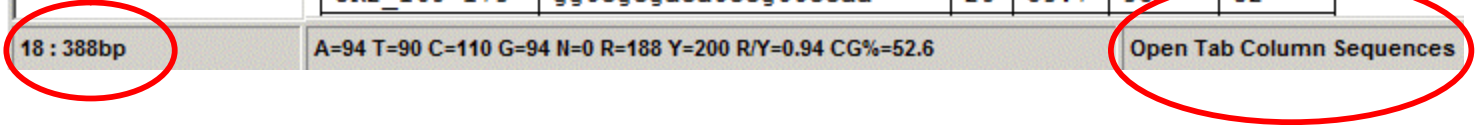To check the correct format was read, look at the information under text editor in status bar:

| 3 : 1728bp | A=472 T=485 C=420 G=351 N=0 R=823 Y=905 R/Y=0.909 CG%=44.6 | Open F/ |
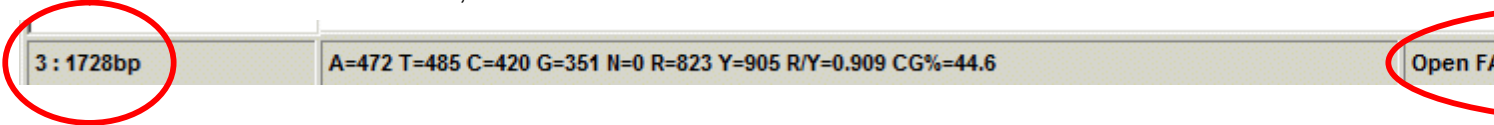
Press ![icon] ![icon] at tool bar for converting sequences to FASTA format, for checking correct format reading.

Press ![icon] at tool bar for converting sequences to IUB/IUPAC FASTA format; ![icon] at tool bar for converting sequences to original format with FASTA ">" sign.

You can import directly the result for primer's list after PCR primer design to general editor, software read only two first columns:

Primers, Probe Design	1F1_234-253	agggagtagcttacctcgct	20	56.6	95	87
Primers Design Options	2F1_263-283	gcgaaaaccaagtgcttacct	21	55.9	95.2	101
In Silico PCR, Search	3F1_290-313	tcctcaagcgaaaaccaatccaca	24	58.8	92.5	101
Single Primer PCR	4F1_318-338	tgttcacatgtttggggacga	21	56.4	90.5	77
Quick Primer Test	5F1_545-564	gcttgtaaggcaaacccaca	20	56	96	83
List of Primers, Analysis	6F1_606-625	acgtggtactcatggtgtca	20	55.2	89	85
Restriction Analysis	1R1_601-622	caccatgagtaccacgtgagtt	22	56.5	86.4	87
Clustering	2R1_366-386	tgtgtgatcatgatgccctca	21	55.9	85.7	69
MITE Search	3R1_325-348	atcgacaccttcgtccccaaacat	24	60.1	93.3	84
SSR Loci Search	4R1_262-284	gaggtaagcacttggttttcgct	23	57.6	96.5	103
LTR Elements Search	5R1_234-255	caagcgaggtaagctactccct	22	58	94.5	98
	1F2_32-53	cccaacggtttacctcaagggt	22	59.2	94.5	105
	2F2_435-457	tcgcgaccttatgagaacgctgc	23	61.1	96.5	115
	3F2_476-495	aagcagcgaccgacgaaacc	20	60.2	85	75
	1R2_476-495	ggtttcgtcggtcgctgctt	20	60.1	85	75
	2R2_448-470	agtcaacggcgtcgcagcgttct	23	64.7	92.2	99
	3R2_160-179	ggtcgcgacatccgttccaa	20	59.7	95	82

| 18 : 388bp | A=94 T=90 C=110 G=94 N=0 R=188 Y=200 R/Y=0.94 CG%=52.6 | Open Tab Column Sequences |

GCG/MSF Format:

Here, the file may begin with as many lines of comment or description as required. The comments are terminated with a line containing only two slashes.

The first mandatory line that is recognised as part of the MSF file contains the text "MSF:"; this line also includes the sequence length and type, the date and an internal check sum value. There then follows one line per sequence describing the sequence name, length, checksum and a weight value. Only one name per line is allowed; the qualifier "Name: " is followed by the sequence name. Extra characters, between the sequence names and "Len: " are acceptable if they contain no blank characters. Another blank line is added followed by a line starting with two slashes "**//**", this indicates the end of the name list. There then follows another blank line. Sequences are interleaved on separate lines with gaps represented by periods. Each sequence line starts with the sequence name which is separated from the aligned sequence residues by white space.

Here is an example of MSF file format:

```
   MSF: 865
 Name: Hordeum_vulg    Len: 725
 Name: Brachypodium    Len: 660
 Name: Phleum_prate    Len: 734
 Name: Oryza_sativa    Len: 766
 Name: Zea_mays        Len: 811

//
Hordeum_vulg      T......... .......... ....GTGATA GCCT...... .GGCTTATTA
Brachypodium      tgt....... .......... .......... .......... ..GTTTAATA
Phleum_prate      T......... .......... ....GTGATA TCCTGGCCTA GGGCTTAATA
Oryza_sativa      T......... .......... ....GTAACA TCCCGGCCTA GGGCTTAATA
Zea_mays          tgtgatatcc tggcccctgg gatgGTGATA TCCTGGCCCA AGGCTTAATA


Hordeum_vulg      GGGATGATAG ACTACTCATA TCAATAAGGa attcCTTCTT TTCCGGGAGC
Brachypodium      GGAATGATAG ACTACTTATA CCAATAAGGT GCATCTTTTT TTCCGGAAGC
Phleum_prate      GGATTGATAG GATACTCATA TCAATAAGTT GCAACTTCTT TTCCGGAAGC
Oryza_sativa      GGATTAATAG AATACTCATA TCAACAAGTT GCAACTTCTT TTCCGGAAGC
Zea_mays          GAATTAATAG AGTACTCATA CCAACAAGGT GCATCTTCTT TTTCGGAAGC
```

Sequences in MSF format are NOT analysed and all non IUB/IUPAC codes are conserved:

Press ![icon] at tool bar for converting sequences to [IUB/IUPAC](#) FASTA format, if it necessary.

Press ![icon] at tool bar for converting sequences to FASTA format in with saving "-" from alignment.

Simple Alignment format description:

The first mandatory line that is recognised as part of the Simple Alignment file contains the text "Alignment" (case not sensitive):

alignment

```
Uni_5SrRNA        1    GGGTGCGATC ATACCAGCAC TAATGCACCG GATCCCATCA GAACTCCGCA
5SrRNA_Trifo      1    AGGTGCGATC ATACCAGCAC TAATGCACCG GATCCCATCA GAACTCCGCA
5SrRNA_Ginkg      1    GGGTGCGATC ATACCAGCGT TAATGCACCG GATCCCATCA GAACTCCGCA
5SrRNA_Metas      1    GGGTGCGATC ATACCAGCGT TAGTGCACCG GATCCCATCA GAACTCCGCA
5SrRNA_Spina      1    GGGTGCGATC ATACCAGCAC TAATGCACCG GATCCCATCA GAACTCCGCA
5SrRNA_Goss2      1    GGGTGCGATC ATACCAGCAC TAATGCACCG GATCCCATCA GAACTCCACA
5SrRNA_Gossy      1    GGGTGCGATC ATACCAGCAC TAATGCACCG GATCCCATCA GAACTCCGCA
5SrRNA_Gnetu      1    GGGTGCGATA ATACCACCGC TAACGTATCG GATCCGATCA GAACTCCGTA
5SrRNA_Citru      1    GGGTGCGACC ATACCAGCAC TAATGCACCG GATCCCATCA GAACTCCGCA
5SrRNA_Lactu      1    GTGTGCGATC ATACCAGCAC TAATGCACTG AATCCCATCA GAACTCTGCA
5SRRNA_ALFAL      1    AGGTGCGATC ATACCAGCAC TAATGCACCG GATCCCATCA GAACTCCGCA


                       ********** ********** ********** ********** **********
                       ********** ********** ********** ********** **********
                       ********** ********** ********** ********** **********
                        ********* ********** ********** ********** **********
                         ******** ********** ********** ********** **********


Uni_5SrRNA       51    GTTAAGCGTG CTTGGGCGAG AGTAGTACTA GGATGGGTGA CCTCCTGGGA
5SrRNA_Trifo     51    GTTAAGCGTG CTTGGGCGAG AGTAGTACTA GGATGGGTGA CCTCCTGGGA
5SrRNA_Ginkg     51    GTTAAGCACG CTTGGGCTGG AGTAGTACTA GGATGGGTGA CCTCCTGGGA
5SrRNA_Metas     51    GTTAAGCGCG CTTGGGCCGG AGTAGTACTG GGATGGGTGA CCTCCCGGGA
5SrRNA_Spina     51    GTTAAGCGTG CTTGGGCGAG AGTAGTACTA GGATGGGTGA CCTCCTGGGA
5SrRNA_Goss2     51    GTTAAGCGTG CTTGGGCGAG AGTAGTACTA GGATGGGTGA CCTCCTGGGA
5SrRNA_Gossy     51    GTTAAGCGTG CTTGGGCGAG AGTAGTACTA GGATGGGTGA CCTCCTGGGA
5SrRNA_Gnetu     51    ATTAAGCGCG CTTGGGCTAG AGTAGTACTG GGATGGGTGA CCTCTCGGGA
5SrRNA_Citru     51    GTTAAGCGTG CTTGGGCGAG AGCAGTACTA aGATGGGTGA CCTCTTGGGA
5SrRNA_Lactu     51    GTTAAGCGTG CTTGGGCGAG AGTTGTACAA GGATGGGTGA CCCCCTGGGA
5SRRNA_ALFAL     51    GTTAAGCGTG CTTGGGCGAG AGTAGTACTA GGATGGGTGA CCTCCTGGGA
```

Sequences in Simple Alignment format are NOT analysed and all non IUB/IUPAC codes are conserved:

Press ▣ at tool bar for converting sequences to IUB/IUPAC FASTA format, if it necessary.

Press ▣ at tool bar for converting sequences to FASTA format in with saving "-" from alignment.

GenBank Format and EMBL Format:

GenBank is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences. Although there are daily exchanges of information with the EMBL Nucleotide Sequence Database, it has its own sequence format (shown below). Each GenBank entry includes a concise description of the sequence, the scientific name and taxonomy of the source organism, and a table of features identifying coding regions and other sites of biological significance (such as transcription units, sites of mutations or modifications, and repeats). Protein translations for coding regions are included in the feature table. Bibliographic references are included along with a link to the Medline unique identifier for all published sequences. Each sequence entry is composed of lines. Different types of lines, each with their own format, are used to record the various data that make up the entry.

```
ID   AF300971   standard; mRNA; PLN; 1375 BP.
XX
AC   AF300971;
XX
SV   AF300971.2
XX
DT   04-SEP-2002 (Rel. 73, Created)
DT   09-FEB-2005 (Rel. 82, Last updated, Version 3)
XX
DE   Oryza sativa DRE binding protein 2 mRNA, complete cds.
XX
KW   .
..
RL   Agricultural Science, 2-1 Ohwashi, Tsukuba, Ibaraki 305-8686, Japan
XX
CC   On Feb 7, 2005 this sequence version replaced gi:22594970.
XX
FH   Key             Location/Qualifiers
FH
FT   source          1..1375
FT                   /db_xref="taxon:4530"
FT                   /mol_type="mRNA"
FT                   /organism="Oryza sativa"
FT   CDS             334..1158
FT                   /codon_start=1
FT                   /db_xref="GOA:Q8LLU9"
FT                   /db_xref="HSSP:O80337"
FT                   /db_xref="InterPro:IPR001471"
FT                   /db_xref="UniProt/TrEMBL:Q8LLU9"
FT                   /note="drought- and salt-stress-inducible transcriptional
FT                   activator; OsDREB2"
FT                   /product="DRE binding protein 2"
FT                   /protein_id="AAN02487.2"
FT                   /translation="MERGEGRRGDCSVQVRKKRTRRKSDGPDSIAETIKWWKEQNQKLQ
FT                   EENSSRKAPAKGSKKGCMAGKGGPENSNCAYRGVRQRTWGKWVAEIREPNRGRRLWLGS
FT                   FPTALEAAHAYDEAARAMYGPTARVNFADNSTDANSGCTSAPSLMMSNGPATIPSDEKD
FT                   ELESPPFIVANGPAVLYQPDKKDVLERVVPEVQDVKTEGSNGLKRVCQERKNMEVCESE
FT                   GIVLHKEVNISYDYFNVHEVVEMIIVELSADQKTEVHEEYQEGDDGFSLFSY"
XX
SQ   Sequence 1375 BP; 374 A; 295 C; 412 G; 294 T; 0 other;
     gctggatgag ccagcagccg cccccgcccg cggttgcttc ccctccccac cacgtcaaaa        60
     cccaacccca accatgatgc tcctgcgcca ccaccaccac ccccacagcg gcgccgccac       120
     caccagcagc agctgcagcg gcggcggcgg ctgttagaga ggagggcaca caccaccacc       180
```

Sequences in GenBank format are analysed and all non IUB/IUPAC codes are removed.

MEGA format description:

http://pubmlst.org/software/analysis/start/manual/mega_format.shtml

All input data files are basic ASCII-text files, which may contain DNA sequence, protein sequence, evolutionary distance, or phylogenetic tree data. Most word processing packages (Notepad) allow you to edit and save ASCII text files. These are usually marked with a .TXT or .MEG extensions.

However, there are a number of features that are common to all MEGA data files, which are as follows.

The first line must contain the keyword **#MEGA** to indicate that the data file is in the MEGA format:

```
#mega
!Title Adh-sequence data from 11 species;
!Format DataType=DNA indel=- CodeTable=Standard;
!Description
    Thomas, R. H., and J. A. Hunt 1993. Mol. Biol. Evol. 10(2):362-374.
    This sequence data is not neccessarily identical to
    Thomas and Hunt (1993), but contains the same species.
    Note the use of underscore ( _ ) for including a blank in
    and OTU label.
    Alignment gap symbol = '-'
    Interleaved sequences;

!Gene=Adh Property=Coding CodonStart=1;
#D._melanogaster    ATG TCG TTG ACC AAC AAG AAC GTG ATT TTC GTG GCC GGT CTG GGA
#D._pseudoobscura   ATG TCT CTC ACC AAC AAG AAC GTC GTT TTC GTG GCC GGT CTG GGC
#S._lebanonensis    ATG GAT TTG ACC AAC AAG AAC GTT ATT TTC GTT GCC GCT CTG GGC
#S._albovittata     ATG TGT ATC GCT GGC AAG AAT ATC ATC TTT GTC GCT GGT CTC GGT
#D._crassifemur     ATG TTC ATC GCT GGC AAG AAC ATC ATC TTT GTC GCT GGT CTC GGC
#D._mulleri         ATG GCC ATC GCT AAC AAG AAC ATC ATC TTC GTC GCT GGA CTC GGT
#D._affinidisjuncta ATG GTT ATC GCT AAC AGT AAC GTC ATC TTT GTC GCT GGT CTG GGT
#D._heteroneura     ATG GTT ATC GCT AAC AGT AAC ATC ATC TTT GTG GCT GGT CTG GGT
#D._mimica          ATG GTT ATC GCT AAC AGT AAC ATC ATC TTT GTG GCT GGT CTG GGT
#D._adiastola       ATG GTT ATC GCT AAC AGT AAC ATC ATC TTT GTC GCT GGT CTG GGT
#D._nigra           ATG GTT ATC GCT AAC AGT AAC ATC ATC TTT GTC GCT GGT CTG GGC


#D._melanogaster    GGC ATT GGT CTG GAC ACC AGC AAG GAG CTG CTC AAG CGC GAT CTG
#D._pseudoobscura   GGC ATT GGC CTG GAC ACC AGC CGG GAG TTG GTC AAG CGT AAT CTG
#S._lebanonensis    GGT ATT GGT CTC GAC ACG AGT CGG GAG CTC GTC AAG CGT AAT CTG
#S._albovittata     GGC ATT GGA CTG GAC ACC AAT CGC GAG ATT GTC AAG AGC GGT CCC
#D._crassifemur     GGC ATT GGA CTG GAG ACG AGT CGC GAG ATT GTC AAG AGC GGT CCC
#D._mulleri         GGC ATT GGA TTC GAC ACC AGT CGC GAG ATT GTC AAG AGC GGC CCA
#D._affinidisjuncta GGC ATT GGC CTG GAC ACC AGT CGC GAG ATT GTC AAG AGT GGC CCC
#D._heteroneura     GGC ATT GGC CTG GAC ACC AGT CGC GAG ATT GTC AAG AGC GGC CCC
#D._mimica          GGC ATT GGT CTG GAC ACC AGT CGC GAG ATT GTC AAG AGT GGC CCC
#D._adiastola       GGC ATT GGC CTG GAC ACC AGT CGC GAG ATT GTC AAG AGT GGC CCC
#D._nigra           GGC ATT GGC CTG GAC ACC AGT CGC GAG ATT GTC AAG AGT GGC CCC
```

Sequences in MEGA format are NOT analysed and all non IUB/IUPAC codes are conserved:

Press [icon] at tool bar for converting sequences to IUB/IUPAC FASTA format, if it necessary.

Press [icon] at tool bar for converting sequences to FASTA format in with saving "-" from alignment.

DIALIGN format description:

The first mandatory line that is recognised as part of the Simple Alignment file contains the text " DIALIGN" (case not sensitive):

```
Alignment (DIALIGN format):
   ==========================

Uni_5SrRNA          1    GGGTGCGATC ATACCAGCAC TAATGCACCG GATCCCATCA GAACTCCGCA
5SrRNA_Trifo        1    AGGTGCGATC ATACCAGCAC TAATGCACCG GATCCCATCA GAACTCCGCA
5SrRNA_Ginkg        1    GGGTGCGATC ATACCAGCGT TAATGCACCG GATCCCATCA GAACTCCGCA
5SrRNA_Metas        1    GGGTGCGATC ATACCAGCGT TAGTGCACCG GATCCCATCA GAACTCCGCA
5SrRNA_Spina        1    GGGTGCGATC ATACCAGCAC TAATGCACCG GATCCCATCA GAACTCCGCA
5SrRNA_Gossy        1    GGGTGCGATC ATACCAGCAC TAATGCACCG GATCCCATCA GAACTCCACA
5SrRNA_Goss2        1    GGGTGCGATC ATACCAGCAC TAATGCACCG GATCCCATCA GAACTCCGCA
5SrRNA_Gnetu        1    GGGTGCGATA ATACCACCGC TAACGTATCG GATCCGATCA GAACTCCGTA
5SrRNA_Citru        1    GGGTGCGACC ATACCAGCAC TAATGCACCG GATCCCATCA GAACTCCGCA
5SrRNA_Lactu        1    GTGTGCGATC ATACCAGCAC TAATGCACTG AATCCCATCA GAACTCTGCA
5SRRNA_ALFAL        1    AGGTGCGATC ATACCAGCAC TAATGCACCG GATCCCATCA GAACTCCGCA


                         ********** ********** ********** ********** **********
                         ********** ********** ********** ********** **********
                         ********** ********** ********** ********** **********
                          ********* ********** ********** ********** **********
                           ******** ********** ********** ********** **********
```

Sequences in DIALIGN alignment format are NOT analysed and all non IUB/IUPAC codes are conserved:

Press ![icon] at tool bar for converting sequences to IUB/IUPAC FASTA format, if it necessary.

Press ![icon] at tool bar for converting sequences to FASTA format in with saving "-" from alignment.

Blast Queue WEB alignments result format:

This format allowed reading and joining of all "**Sbjct**" sequences from BLAST result at Internet browsers. First, you need "select all" (Ctrl-A) text with graphics in the Internet browser page, then copy and paste to FastPCR. If FastPCR don't recognize directly the format of Internet browser page, you need "select all" (Ctrl-A) text with graphics in the Internet browser page, then copy and paste to Notepad and then repeat the same for this text from Notepad: select all and copy, and after this paste to FastPCR.

Press at tool bar for converting sequences to "intelligent" FASTA format in with saving "-" from alignment. Sequences in Blast Query Web format are NOT analysed and all non IUB/IUPAC codes are conserved.

Sequences in this formatted Web or text files are preceded by a line starting with a '**>**' symbol, containing the name and labels of the sequence.

```
>gi|46358504|gb|AY491681.1|  Triticum monococcum DV92 BAC 109N23, complete sequence
          Length=101101

 Score =  250 bits (126),  Expect = 2e-63
 Identities = 126/126 (100%), Gaps = 0/126 (0%)
 Strand=Plus/Plus

Query  120    TGAGGATGATGGCGACGACGATGCCGGCGACGATGAGCTTGATCTTCATGTTCTGGTACC  179
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  18496  TGAGGATGATGGCGACGACGATGCCGGCGACGATGAGCTTGATCTTCATGTTCTGGTACC  18555

Query  180    ACATCCTGCGGCGGATGCGCGTGCCCTCGGTCCTGAAATCCAAGGCGTGGTCGTGGAGCT  239
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  18556  ACATCCTGCGGCGGATGCGCGTGCCCTCGGTCCTGAAATCCAAGGCGTGGTCGTGGAGCT  18615

Query  240    GCTCGG  245
              ||||||
Sbjct  18616  GCTCGG  18621


 Score =  236 bits (119),  Expect = 2e-59
 Identities = 119/119 (100%), Gaps = 0/119 (0%)
 Strand=Plus/Plus

Query  1      TGTGGTAGATGGAGAGGACAATGACCGCGACGTTGAGCTTGACCTTCATGTTCTGGTATC  60
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  12768  TGTGGTAGATGGAGAGGACAATGACCGCGACGTTGAGCTTGACCTTCATGTTCTGGTATC  12827

Query  61     GCATCCCACAGGATCGCCCTGGGTCTTCAAATCCGAGGCATGGTCATGGAGCTGCTTGG  119
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  12828  GCATCCCACAGGATCGCCCTGGGTCTTCAAATCCGAGGCATGGTCATGGAGCTGCTTGG  12886
```
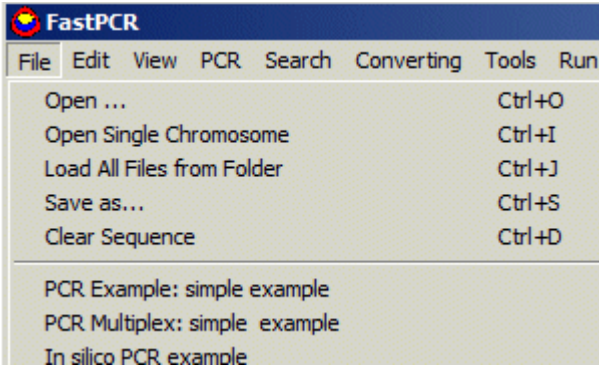
Press at tool bar for converting sequences to "not intelligent" **IUB/IUPAC** FASTA format.

Opening file or group of files

Click "File" on toolbar:

Open … (Ctrl-O) – open text or rtf file with DNA or protein sequence(s) at Fasta, GenBank, Mega, Blast etc. formats. FastPCR have general text editor similar WordPad program.

Open Single Chromosome (Ctrl-I) – is quickest way to open huge chromosome size single sequence to memory (computer memory limit, total 2Gb of RAM is necessary).

Load All Files from Folder (Ctrl-J) – this is useful way opening all text files from one folder at once. You need click on any file from folder and will see result. Especially this is reliable for read all sequences from different files. Each file will converted to FASTA format with name of the file.

FastPCR opened any text file with command line: **fastpcr.exe filename.fasta**

Software can open work with .RTF or .TXT or .FASTA (raw text) formatted files. You can associate a file type; make a file type always open by FastPCR.

View
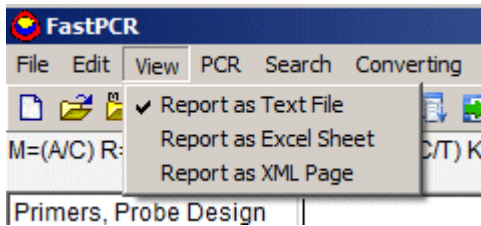
Default, all result will output to Notepad as text file (compatible with Excel), but quick way to getting output as Excel sheet or XML page is select it from View. You can select even all three way for output.

FastPCR have unknown direct limit for an amount imported sequences, but has a limit for computer memory size (2Gb) and 32-bit Windows limitation (currently, FastPCR is 32-Bit software), limits for the computer speed and a quality of the algorithm. FastPCR can work at 32-Bit (x86) and 64-Bit Windows.

Output Result

FastPCR automatically generate file's name for all new analysis at default your temporary folder:
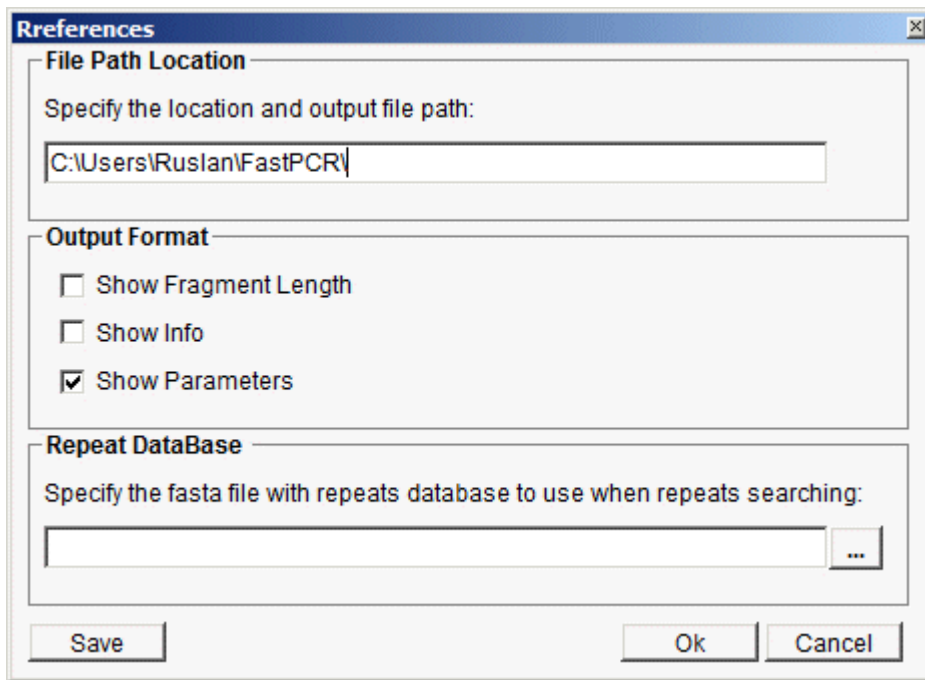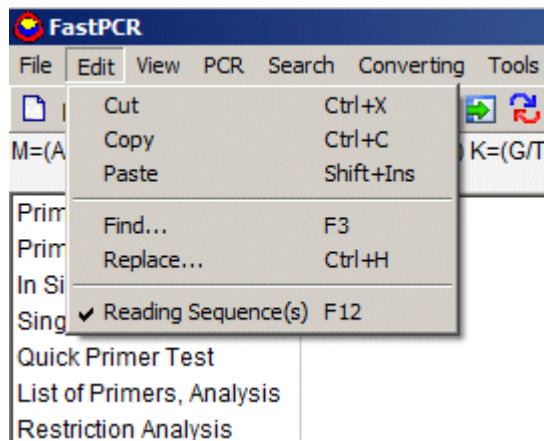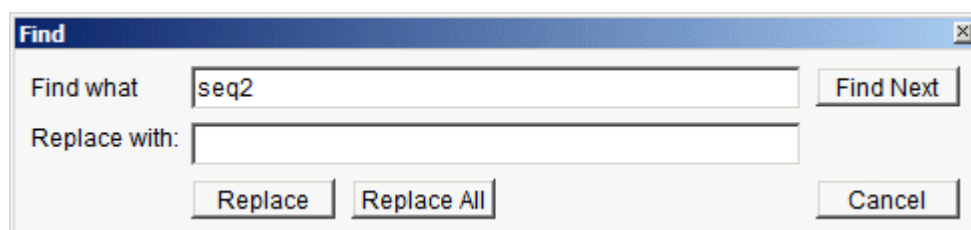

For Vista: C:\Users\xxx\FastPCR\

For XP: C:\Documents and Settings\xxx\FastPCR\;

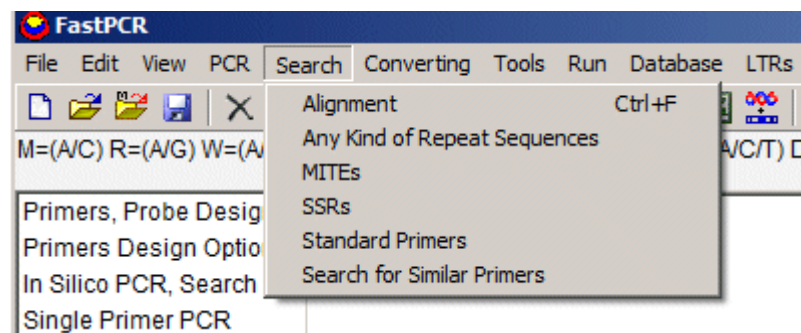You can specify any location for output file path from "Preferences".

Standard Tools, Searching

Find Window – F3, is the quick way to find exact word in general editor.
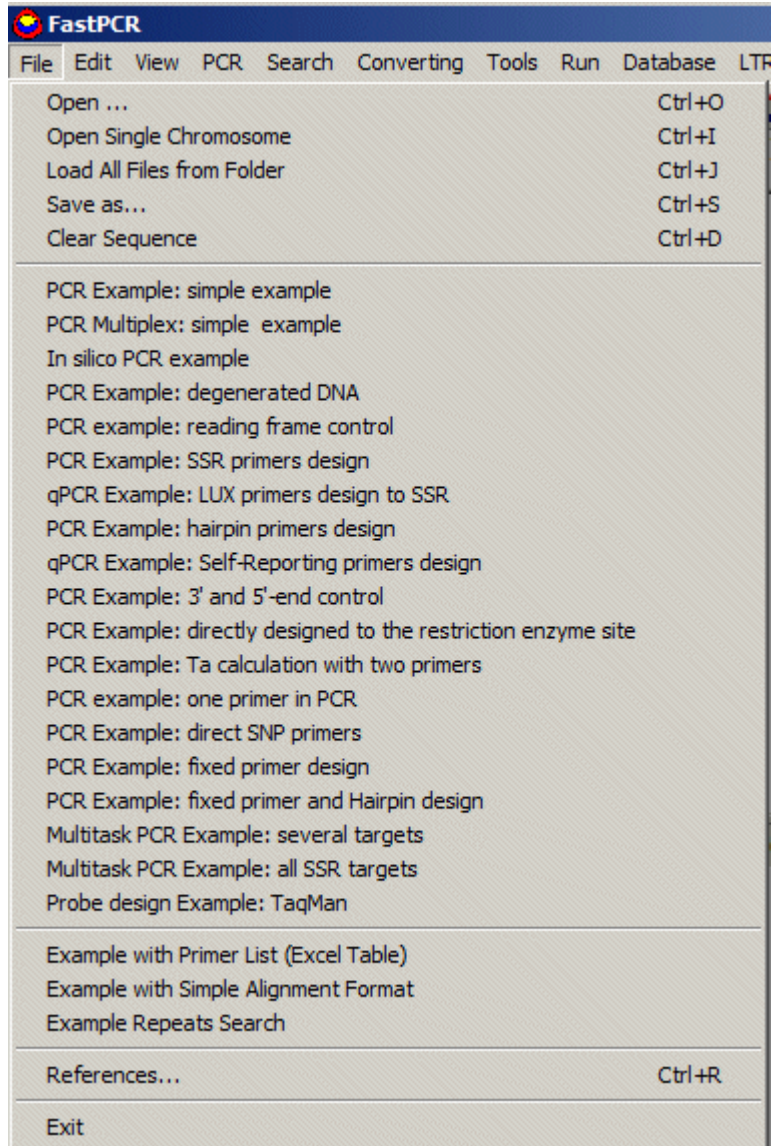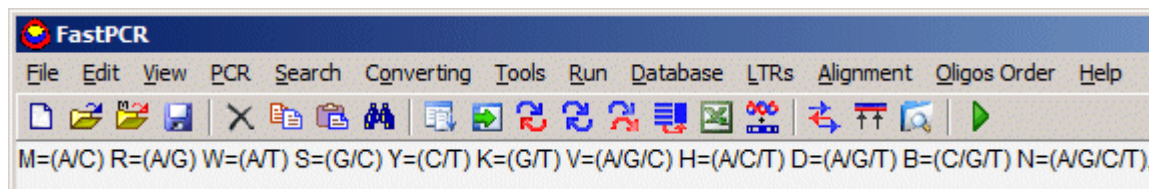

Search with alignment Ctrl-F

Examples

FastPCR have many examples, which can reach from menu bar - "File":

For starting job, need press **F5** or from tool bar press ▶

Menu toolbar:

 Clean general editor

 Open file into general editor.

 Open file into memory, sequence will not appear in general editor.

 Save text as Rich Text (.rtf)

File menu bar:

Quick sequence transformation

 Tool for converting sequences to **IUB/IUPAC** FASTA format (Ctrl-F1);

 Tool bar for converting the sequences to FASTA format with saving original sequence (alignment) (Ctrl-F2);

FastPCR program includes tools for the manipulation of DNA or protein sequences:

 Reverse (e.g. acacacc become ccacaca) (Ctrl-F3);

 Complement (e.g. acacacc become tgtgtgg) (Ctrl-F4);

 Reverse-Complement transformation (e.g. acacacc become ggtgtgt) (Ctrl-F5);

 DNA-to-protein translation in all the 6 reading frames; orientation, complementation of protein sequence; and protein-to-DNA translation (Ctrl-F6);

 Tool for joining all sequences into a single sequence (Ctrl-F7);

 Export all sequences to Excel sheet in two columns format (Ctrl-F8);

Names to Number List (Ctrl-F9) – tool for converting sequences to FASTA format with changing sequence's name to number. Default is 1, to specify initial number, type in additional editor this number;

Tab-Table Format (Ctrl-F11) – tool for converting sequences to column format with Tab separator between names;

Blank-Table Format (Ctrl-F12) – tool for converting sequences to column format with space separator between names.

Selecting and extracting fragments tool for linear sequence. Using additional editor, you can specify region(s) for extracting from sequence(s). To select fragments needs to select a beginning and an end that will be the beginning and end of the produced fragment.

Example: at additional editor type 1 100 300 550

Output: extraction, the sub-fragments between 1 and 100 and second between 300 and 550:

```
>1-100
>300-550
```

Repeats Search

Repeats searching tool can perform quick search of all kinds of repeats. This fast and effective DNA analysis algorithm was developed for the search of composite and long repeats by alignment in the following formats; direct vs. direct, direct vs. reverse, direct vs. complement, direct vs. reverse-complement. Searching in all orientations facilitates the rapid identification of unique sequence representing long terminal repeats (LTR), tandem repeats, and even new retrotransposons (such as LARD or TRIM) and transposons (such as MITEs).

kMers repeats screening – is quickest way for discovering any king of repeats, without dot plot. Similar repeats are clustered and located at the same row (Y) and coordinates (X) for nucleotide position. All different repeats are stretching on Y coordinates:

This picture is BAC clone, contains several LTR retrotransposons, first two short LTRs (TRIM element) located between 42799- and 49932+ (show with black arrow and red ovals), and then followed 3 similar long length LTRs (57065, 71331 and 85596); same solo average LTRs (99862, 128394-135527):
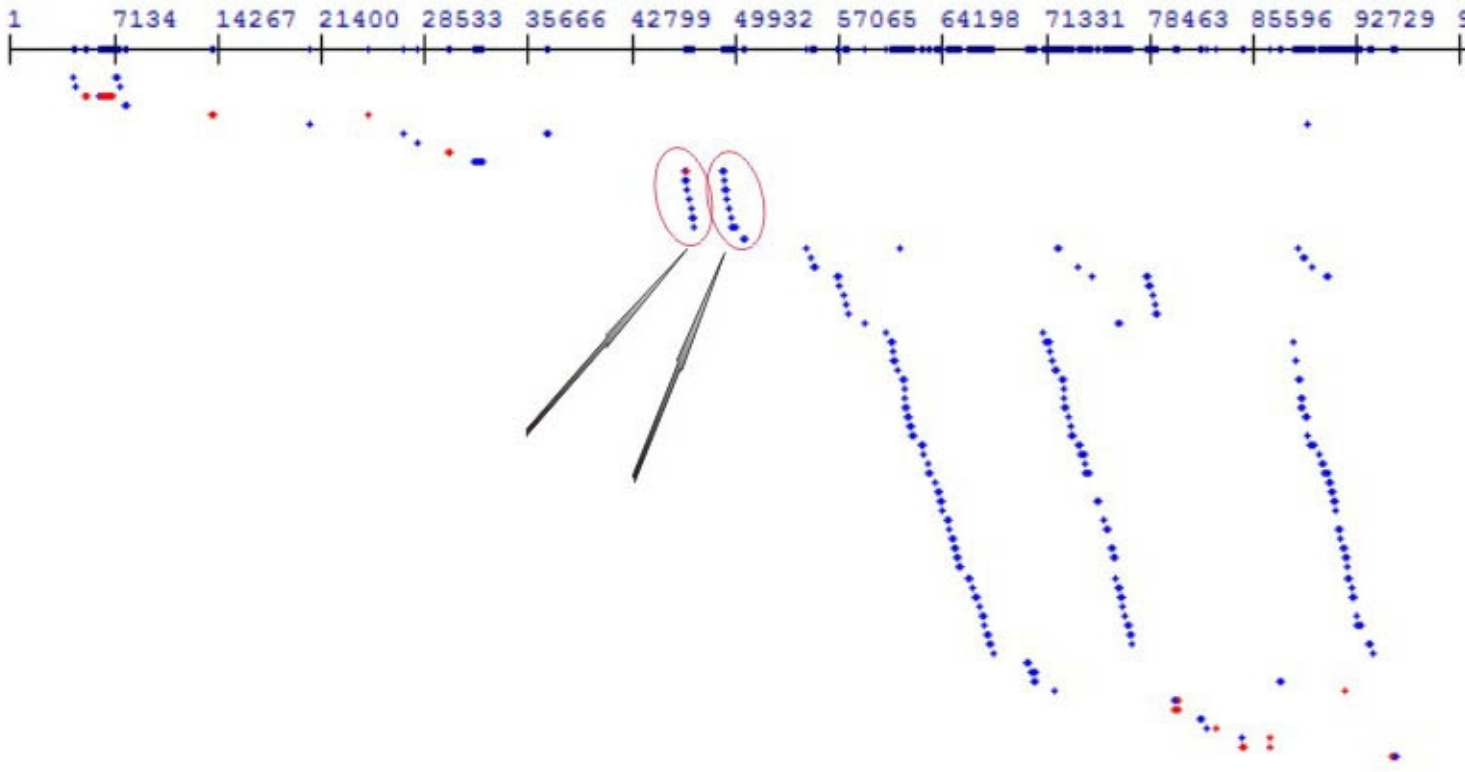
The blue dots are shown the direct orientation repeats; red dots for opposite orientation for current type of repeat.

Initial searching work size, K=7 or 10, nt – quick (or instance) method for identification repeats based on defining high-frequency k-mer (short substrings of length k) as seeds, and greedily extends each seed to a progressively longer sequence, following it clustering for consensus sequence alignment. Software is capable of detecting degenerate substrings or repeats. Value "7" is 7 bases is used, and 10 bases for value 10. Value 10 allowed much quicker analysis, but 1 Gb free RAM memory at least is necessary.

To find all degenerate repeats (or substrings) user is specified alignment parameters: initial k-mer size, minimum extended substring length with minimum similarity, maximum gap size and minimum repeat length.

Alignment Search (Ctrl-F)

This tool can help to find sequence(s) with local alignment.

FastPCR allows the searching of related DNA sequence(s) within personal databases (in a similar way as in BLAST analysis). This is also convenient for primers selection and monitoring.

Moreover, as a basis for sequence alignment, the following idea was adopted: related sequences should have sequential common homologous blocks and inversions, deletions or permutations which are looked as gaps in sequence comparisons.

Alignment algorithm started for searching of exact word (7 as default, minimum 2 base) sequence which then extended to left and to right as not complete complement segment with no gap and scoring the strings similarity. The extension is done by pairwise character comparisons. The algorithm is direct compute maximal exact segment-substrings for many candidates. Finally, segment-substrings joined to long string with gaps. For increasing detection highly degenerated substrings FastPCR allows applying the purine-pyrimidine local alignment.

In aligning the DNA sequences the program uses the similarity table for all degenerated nucleotide that increases the probability of identifying related sequences.

Primers analysis, compatibility and prediction annealing temperature

Quick Primer test:

Paste or type the primer sequence (or two primers), the program will immediately show primer characteristics:

length in bases, melting temperature, CG% content, molecular weight, the extinction coefficient (e260), nmol per 1 OD, the mass - µg per 1 OD, linguistic complexity and primer quality; minimal and maximal optimal annealing temperature for unknown PCR products. Tm calculation based on chosen the formula from "Primers Design Options".

If the primer is self-complementary, the program will show a picture of where this self-complementarity happens. All primers analysed for self-dimers formation and by your wishes primers can analysed with each other for detection cross dimers formation. A self-priming ability will also be detected and shown by the program. When the two primers are forming such a dimer, the program will also show it.

Web version for on-line primer test with Tm calculation for normal and degenerated nucleotide combinations based on the nearest neighbour thermodynamic parameters and comprehensive dilution protocol available here: http://primerdigital.com/Tools/PrimerAnalyser.html

Comprehensive analysis of the list of primers with prediction of the oligonucleotides properties, self and cross dimers detection and temperature of annealing calculation is available here: http://primerdigital.com/Tools/PrimerList.html

List of Primers

```
Primers, Probe Design      1F1_234-253  agggagtagcttacctcgct    20   56.6  31
Primers Design Options     2F1_263-283  gcgaaaaccaagtgcttacct   21   55.9  29
In Silico PCR, Search      3F1_290-313  tcctcaagcgaaaaccaatccaca     24   58
Single Primer PCR          4F1_318-338  tgttcacatgtttggggacga   21   56.4  36
Quick Primer Test          5F1_545-564  gcttgtaaggcaaacccaca    20   56    31
List of Primers, Analysis  6F1_606-625  acgtggtactcatggtgtca    20   55.2  30
Restriction Analysis       1R1_601-622  caccatgagtaccacgtgagtt  22   56.5  30
Clustering                 2R1_366-386  tgtgtgatcatgatgccctca   21   55.9  33
MITE Search                3R1_325-348  atcgacaccttcgtccccaaacat     24   60
SSR Loci Search            4R1_262-284  gaggtaagcacttggttttcgct 23   57.6  32
LTR Elements Search        5R1_234-255  caagcgaggtaagctactccct  22   58    31
                           1F2_32-53    cccaacggtttacctcaagggt  22   59.2  32
                           2F2_435-457  tcgcgaccttatgagaacgctgc 23   61.1  36
                           3F2_476-495  aagcagcgaccgacgaaacc    20   60.2  33
                           1R2_476-495  ggtttcgtcggtcgctgctt    20   60.1  36
                           2R2_448-470  agtcaacggcgtcgcagcgttct 23   64.7  36
                           3R2_160-179  ggtcgcgacatccgttccaa    20   59.7  32
                           4R2_33-54    taccttgaggtaaaccgttgg   22   56.5  31
```

```
18 : 388bp        A=94 T=90 C=110 G=94 N=0 R=188 Y=200 R/Y=0.94 CG%=52.6
```

Type of Primers Combination

⦿ All against All primers   ○ Individual primer analysis
○ Forward against Reverse (F/R) ○ All pairs against All pairs
○ All primers against Selected:

[   ]

☑ Show primers Tm for all NN thermodynamic parameters and formulas

Single or list of primers is evaluate by FastPCR for calculation Tm (default or other formula) for normal and degenerated nucleotide combinations, CG content, the extinction coefficient, nmol per OD, the mass - µg per OD, the molecular weight, linguistic complexity and primer quality. All primers analysed for self-dimers formation and by your wishes primers can analysed with each other for detection cross dimers formation.

Forward against Reverse (F/R) it is possible selectively analyse primer pair forward and reverse.

For this type of analysis, primers from the same pair must have identical names but finishing using "R or F" (in FASTA format: **>seq1R** and **>seq1F** form a pair; or EVEN simply: **>F** and **>R**). The name length and structure (including "F" and "R" inside names) are not important. Moreover, the program is not limited in one unique pair per primers: for one "forward" primer, it can be several "reverse", the same for "reverse" primer.

"**Show primers Tm for all NN thermodynamic parameters and formulas**" – allowed seeing all Tm's result for all formulas at "Primers Design Options".

PCR primers design generalities

The major aspects of primer features include unique nucleotide structure and the melting temperature. Usually PCR primers 18-35 bases in length (PCR primer or probe length can vary from 15 to 500 bases) should be designed such that they have complete sequence similarity to the desired target fragment to be amplified.

The parameters controllable by the user are primer length, melting temperature with different nearest neighbour thermodynamic parameters or simple formulas, sequence linguistic complexity and pyrimidine-purine linguistic complexity, primer CG content, CG 3'end terminal enforcement and preferable 3' or 5' ends nucleotide sequence composition in degenerated formula, polypyrimidine (T, C) or polypurine (A, G) stretches, repeats of identical bases in primers. Also other main parameters used in FastPCR for primer selection are: the general nucleotide structure of the primer such as linguistic complexity (nucleotide arrangement and composition); specificity; the melting temperature of whole primer and the melting temperature at the 3' and 5' termini; a self-complementarity test; secondary (non-specific) binding choosing primer. Software dynamically optimizing the best primer length for entered parameters.

All PCR primer (probe) design parameters are flexible and changeable according specific of analysed sequence and the task.

Primer pairs analysed for cross primer hybridization, specificity of both primers and optionally for similar melting temperature. Designing primers with balanced melting temperatures (within 1-4°C of each other) is desirable but not mandatory. The default primer design selection criteria showed at table 1.

In many cases it is necessary to use predesigned single or a list of primers (probe).

The program accept the list of predesigned oligonucleotide sequences for checking it compatibility with a newly designed primers or probes. Other case is predesigned oligonucleotides will includes as part of final PCR primer design result. The software automatically checked pimer sequences location (with local alignment) on a target sequence and add correct primers to a list of selected primers.

Predesigned primers or probes imported in all formats accepted at generally for FastPCR from a clipboard with keyboard (Shit-Insert or Ctrl-V) and right-click mouse displays a contextual menu:
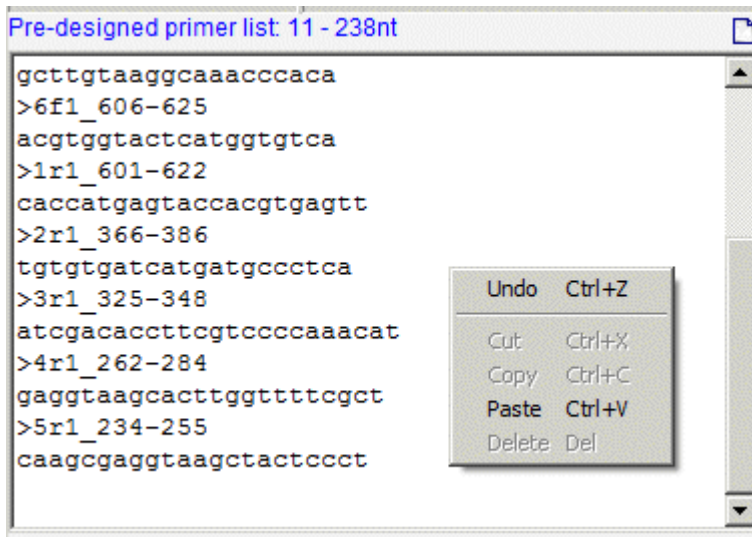
The program is able to generate either long oligomers or PCR primers for the amplification of gene-specific DNA fragments of user-defined length. Up to now, several primer/oligo design programs have been developed. All of them are specialized for either the design of PCR primers or oligomers. Our FastPCR software provides a more flexible approach of designing primers for many applications with a quality and speed. If either primers or probe have secondary binding sites that may give raise to an additional PCR product somewhere else. The evaluation of potential secondary binding sites of each primer performing with local repeats dataset sequences. The selection of the optimal target region for the design of long oligomers is performed in the same way as for PCR primers. The basic parameters in primer design are also used as

a measure of the oligomer quality; however, the thermodynamic stability of the 3' and 5' terminal bases and central part of oligomer is evaluated.

The user can vary the product size or design primer pair for whole sequence without specifying parameters or using default or pre-designed parameters.

The pre-designed parameters are specified for different situation, for example: sequence with low CG content, or long distance PCR, or degenerated sequence, or manual options.

PCR primers and probes design command lines

If necessary for an individual control for the PCR primers or probes design for each sequence individually, you can find "quick help for commands" at menu bar – Help:
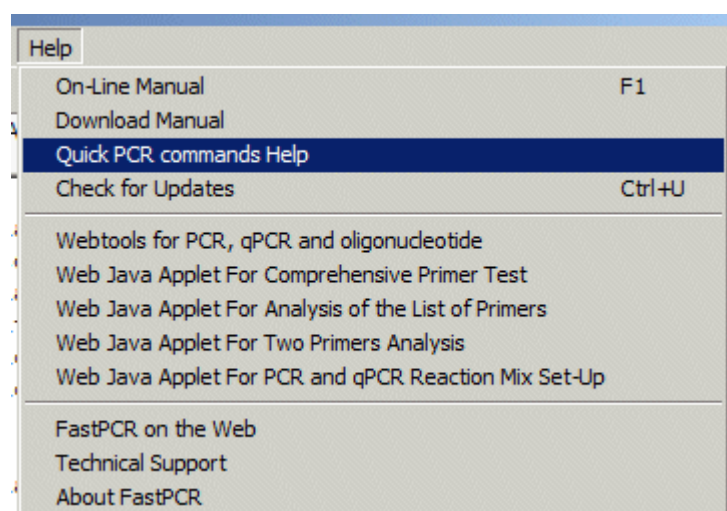
General PCR primer options you can find in menu bar – PCR or from "Tree View":
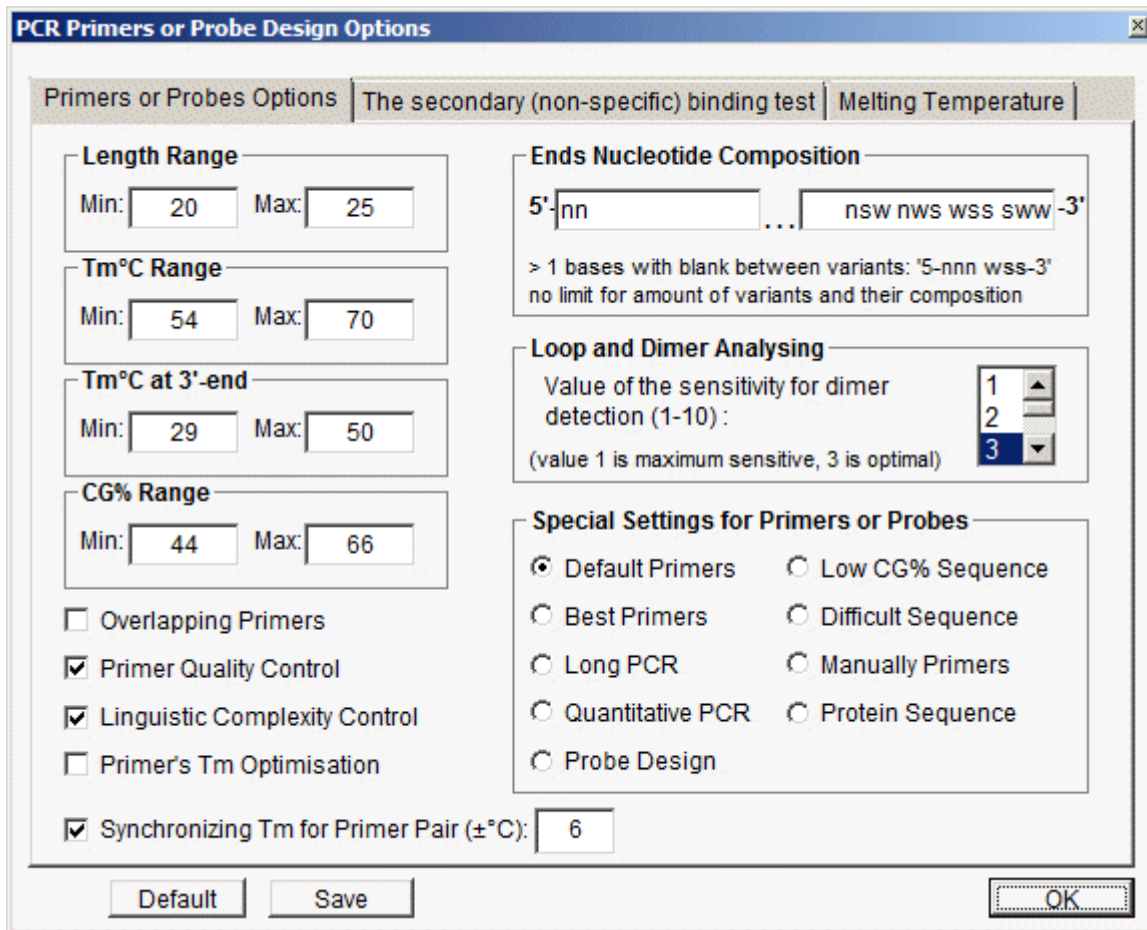
Ends Nucleotide Composition: you can chose with 5' or 3' ends is most preferable for all primers or probe. Minimal size is **2 letter (maximum – primer length)**, "5'-nn-3'" is any, "5'-sww-3'" – all variants for 5'-(C/G)(A/T)(A/T)-3'".

You can type **one or more** variants with space between them and with the same length.

Loop and Dimer Analysing: options for quick detection of the primer dimers. Value 3 is default and, probably, is optimal; value – 2 and especial 1 for very sensitive dimers detection; value more then 3 up to 10 for less sensitive dimers detection.

Primer Quality Control: help eliminate "weak" primers.

Linguistic Complexity Control: similar to "Primer Quality" quick control, analysis primer for complexity.

Primer's Tm Optimisation: allow automatically design best primer for current Tm (in practise - FastPCR select primer length from 18 to 28 bases).

Synchronizing Tm for Primer Pair: default is 4°C difference between primers in pair, but you can change from 0 (not recommend) to any (not absolute "any").

Primer quality (amplification efficiency): this abstract value of the '*primer quality*' describes thus the level of primer/PCR successfulness; this value varies from 100 for the "perfect or ideal" to 0 for the "worst" primer. A "perfect" primer has a wider range of executable temperatures. A program is select the best primer with optimal range of executable temperature, which allowed to design qualified primers (probe) for any target sequences with any CG and repeat contents.

The '*primer quality*' values of 80 and higher allow for the rapid choice of the best PCR primer pair combination. No adverse effects, due to the modification of the reaction buffer, sourced thermostable polymerases or variations in annealing temperature, have been observed on the reproducibility of PCR amplification using FastPCR-designed primers.

Save: for saving your own parameters as default after the next starting of the program. The program creates a file "**fpcr.ini**" in local user folder:

For Vista: C:\Users\xxx\AppData\Local\Temp\

For XP: C:\Documents and Settings\xxx\My Documents\;

This file can be manually edited (using a text-editor, Notepad).

Melting temperature calculation

There are several formulas for calculating melting temperatures (Tm). In all cases these calculations will give to user a good starting point for determining appropriate annealing temperatures for PCR, RT-PCR, hybridization and primer extension procedures. However, a precise optimum annealing temperature must be determined empirically.

The melting temperature (Tm) is defined as the temperature at which half of the stands are in the double-helical state and half are in the "random-coil" state. Tm for oligonucleotide for normal and degenerated (mixed or also know as wobble) nucleotide combination calculated by default following nearest neighbour thermodynamic parameters.

The melting temperature for mixed bases is calculated by averaging nearest neighbour thermodynamic parameters – enthalpy and entropy values as each mixed site and extinction coefficient is similarly predicted by averaging nearest neighbour values at mixed sites.

Recently, similar averaging Allawi's nearest neighbour thermodynamic parameters for temperature melting calculation used in new WEB tool - IDT SciTools [http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/ Integrated DNA technologies].

FastPCR allows choosing other nearest neighbour thermodynamic parameters or simple non thermodynamic Tm calculation formulas.

The melting temperature is calculated using the formula based on the nearest neighbour thermodynamic theory Tm is defined as the temperature at which half of strands are in the double-helical state and half are in the "random-coil" state.

The melting temperature is calculated using a formula based on nearest neighbour thermodynamic theory with unified dS, dH and dG parameters (Allawi and SantaLucia, 1997):

$$T_m(^{\circ}C) = \frac{dH}{dS + R\ln(c/4)} + 16.6\log_{10}[K^+] - 273.15$$

where dH is enthalpy for helix formation, dS is entropy for helix formation, R is molar gas constant (1.987cal/K mol), "c" is the nucleic acid molar concentration (determined empirically), $[K^+]$ is salt molar concentration (default value is 50 mM).

The mismatched pairs can be taken into account, the parameters being provided for DNA/DNA duplexes and the dangling ends that are unmatched terminal nucleotides being provided for DNA/DNA duplexes.
The melting temperature for primer (probe) self or cross dimer and for *in-silico* PCR experience with oligonucleotide with a mismatches to target is calculated using values of thermodynamics parameters for a nucleic acid duplex.

The non thermodynamic Tm calculation for oligonucleotides we suggest to use simple formulas, the Wallace–Ikatura rule is often used as a rule of thumb when primer T_m is to be estimated at the bench. However, the formula was originally applied to the hybridization of probes in 1 mol/L NaCl and is an estimate of the denaturation temperature (T_d):

Tm=2(A +T+U) + 4(G + C) (for short < 15 bases)
or
Tm=64.9 + 41(G + C - 16.4)/L (for longer 14 bases).

Marmur and Doty (2) originally established a formula to correlate GC content (%GC) to the T_m of long duplexes at a given ionic strength. Chester and Marshak (23) added a term to account for DNA strand length (n in base pairs) to estimate primer T_m:

$$T_m(^{\circ}C) = 69.3 + 0.41(\%GC) - \frac{650}{n}$$

The Marmur–Schildkraut–Doty equation also accounts for ionic strength with a term for the Na^+ concentration.

$$T_m(^{\circ}C) = 81.5 + 16.6\log_{10}[Na^+] + 0.41[GC\%] - \frac{b}{n}$$

Values between 500 and 750 have been used for b, a value that may increase with the ionic strength. Another modification is that of Wetmur:

$$T_m(^{\circ}C) = 81.5 + 16.6\log_{10}\left[\frac{[Na^+]}{1 + 0.7 \times [Na^+]}\right] + 0.41[GC\%] - \frac{500}{n}$$

von Ahsen et al. (2001) formula:

$$T_m(^{\circ}C) = 77.1 + 11.7\log_{10}[K^+] + 0.41[GC\%] - \frac{528}{n}$$

Two equations above assume that the stabilizing effects of cations are the same on all base pairs.
The melting temperature of the PCR product calculated using the formula:

$$T_m^{product}(^{\circ}C) = 81.5 + 16.6\log_{10}[K^+] + 0.41[GC\%] - \frac{675}{n}$$

The secondary (non-specific) binding test (alternative amplification) for primer or probe

The secondary (non-specific) binding test: the program will analyse (using quick alignment) and look for the presence of possible additional complement sites for each primer (probe), which would result in non-specific PCR product.
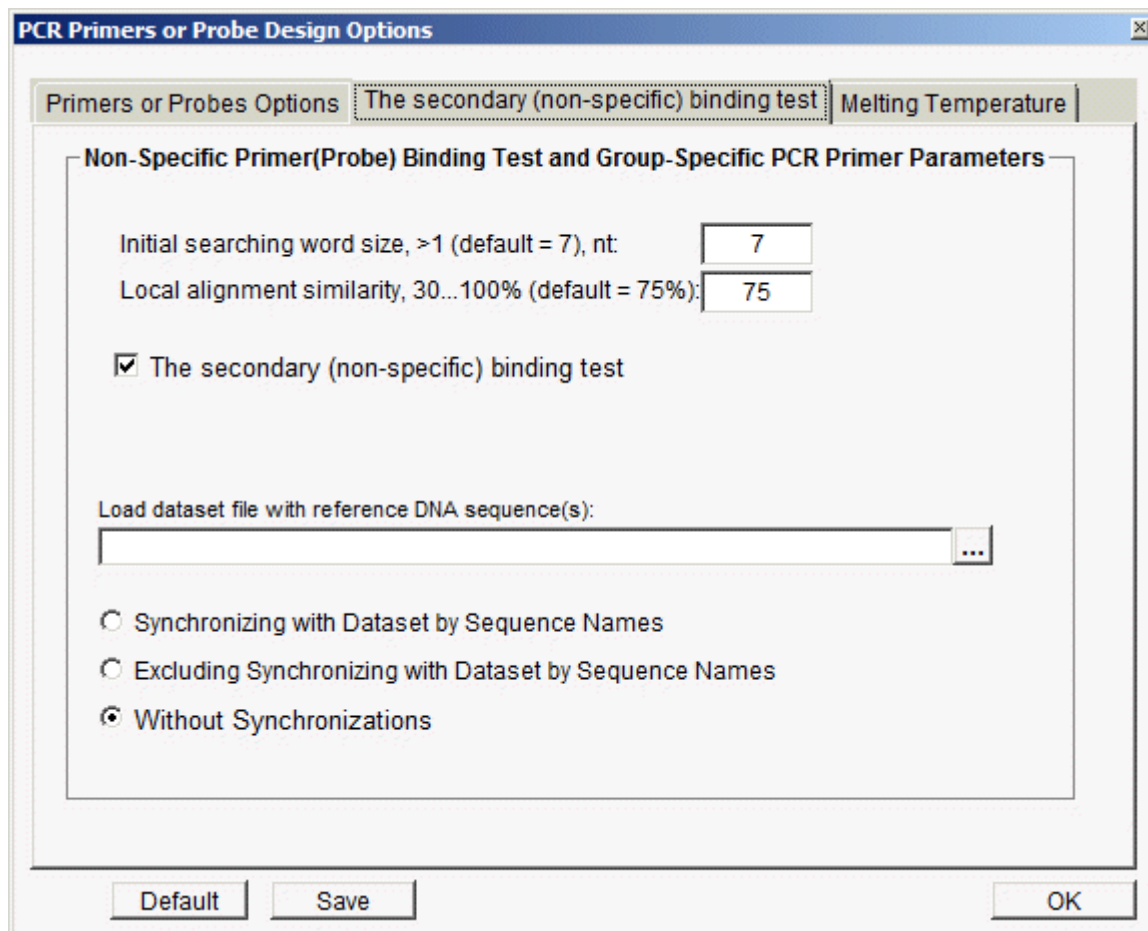
The specificity of oligonucleotides is one of most important points; the optimal primer should hybridize only with the concrete target sequence. Particularly when genomic DNA is used as the template, it is possible that primers (probe) may complement to inverted repeated regions of the genome.

For human and animals genomes, where mostly spread short interspersed repetitive element (SINE) like Alu-repeats, primer sequences complement to it will produces or ether non-specific single-primer amplified PCR products or Alu-PCR for polymorphism detection.

For plants genomes, highly copied short or long direct repeats (LTRs) can become the result on non-specific amplification or inter-repeat amplification polymorphism (IRAP, REMAP or inter MITE amplification and else).

Usually, the site-specificity of the primer can be checked by performing a sequence homology search (e.g. blastn) through all known template sequences in the public genome database such as National Center for Biotechnology Information (NCBI), but it is no necessity to scan primers though NCBI databases to prove specificity of PCR amplification (it is prejudices; any primers have many potential non-specific binding sites, but they not efficient for an exponential PCR amplification). Our experience, the almost all problems with the secondary (non-specific) primer binding can be simply solving using any the BAC sequence(s) (about 100,000 bases) from analysing genome. Other way is creation a small local specialized library of tandem and retrotransposons (especially SINE elements for animals and human genomes) repeats sequences. Repeat library created (use) from Repbase databases [http://www.girinst.org/, Jurka et al, 2005].

By default, FastPCR non-specific binding test performed inside each given sequence. Additionally software allow to do this test throw reference sequence or sequences (BAC, YAC sequences or any long sequences or own database). Primers

(probe) shown more then single location on current sequence or at least single location throw reference sequence will reject.

The non-specific primer binding test performed as default test for all primers and fully controlled by user and can be cancelled, select off: "**The secondary (non-specific) binding test**".

The secondary non-specific primer binding test based on the quick non-gaped local alignment screening primer sequence throws the reference and current sequence.

Optionally, you can synchronize the secondary non-specific primer binding test with dataset by sequences names. Program recognize that a given sequence in the screening library dataset (from loading dataset file) is the same name as the sequence for which it is designing primer and allow primers to be made even though they match that screening sequence perfectly. This would allow the same dataset to be used for both primer design and screening against without having to make a new screening database for each sequence. In other word, for a dataset that contains sequences >A, >B, >C and >D; it will use the same dataset for choosing primers and for checking primer specificity. Due to the interest in multiplex polymorphism detection based on repeated DNA, it is important to support the ability to target genomic regions rich in repetitive elements. Program does not mask repeat regions on the target and reference sequences before searching for primer (probe) binging sites. It is possible design a primer (probe) to be specific to repetitive region. Single primer amplification for primer from inverted repeats is one most efficient PCR methods detection polymorphism.

Imitation unique PCR with checked "Excluding Synchronizing with dataset by Sequence Names": load the same file in FASTA format to general editor and to "Load dataset file with reference DNA sequence(s)" and run the standard PCR.

Prepare for PCR analysis

"Primer Design Options" is general for all given sequences, as background.

For individual, selective options and task, sequences need convert to FASTA format with ">", these options have a highest priority, and they are covering the general (background) options. All these command used optionally and only for advanced tasks. In generally, you can relax and forgot about it. Press F5 show what is FastPCR found in given sequences. The result file (Excel sheet, XML page or Text) contains a list of the best primers and compatible primer combinations. It shows the type of PCR, the left and right flanking regions for primer selection, and the annealing temperature. Each primer combination includes information for primer position and length, optimal melting temperature and product length.

Any of these following commands must be written AFTER the sequence name or "> " (**these commands are not case sensitive**) and press Enter and the end of line. The commands can occupy any place in the command line.

```
/?                  load current options of PCR primers design: > /?

-npcr               standard PCR (qPCR), example:  > -nPCR

-ipcr               inverted PCR, example:  > -iPCR

-lpcr               long PCR primer design: > -lPCR

-qpcr               quantitative (real-time) PCR primer design: > -qPCR

-npd  -lnpd -rnpd   No Primer(s) Design:
                    > -npd

-pcr=no             (Primer Combinations Report) do not show in result default:
                    > -pcr=yes

(N1-N2)             specify the minimal and maximal size requested for the PCR
                    product, N1 for the shortest (minimum 30 bp), N2 for the longest
                    PCR product:
                    > (400-500)
```

```
-tagN1-N2                  to define position of the target DNA on initial N1 and final N2
                           position in DNA sequence:
                           >  -tag650-700


-pdN1-N2                   (Primer Design) design PCR primers between coordinate N1 and N2
-lpdN1-N2                  for left and right side:
-rpdN1-N2                  >  -lpd100-500 -rpd1000-1200


-tmsynchr=                 Synchronizing Tm for Primer Pair (±°C):
                           >  -tmsynchr=10


-pdN-e -lpdN-e -rpdN-e     Design PCR primers in area: from the end of sequence minus N
                           bases design left or right PCR primers from the end of sequence
                           minus N bases:
                           >  -lpd200-e
                           >  -rpd200-e


-frame/1/2/3/              Primers (probe) design fixing to the translation frames 1, 2 or 3
                           bases (according to sequence first nucleotide).
                           Left and Right Primers for frame 1:
                           >  -frame/1/

                           Left Primer only frame 1 and Right Primers frame 1 or 2:
                           >  -frame/1/12/

                           Left frame 1 or 2 and Right Primers for frame 1 or 3:
                           >  -frame/11/13/21/23/


-LUXpdN1-N2                (LUX Primer Design) design PCR LUX (self-quenched) primers for
-LUXpd                     quantitative PCR, between coordinate N1 and N2 for left or right
-lLUXpdN1-N2               sequence side. Example, design left PCR LUX primers between
-rLUXpdN1-N2               coordinate N1 and N2 (N1>N2):
-LUX =(LLUX RLUX)          >  -lluxpd100-500 -rpd1000-1200
                           >  -lluxpd -rpd1000-1200
                           >  -lluxpd -ssr/200


-mbN1-N2                   (Probe (Molecular Beacon, TaqMan, Native Hairpin and Microarray)
-TaqManN1-N2               design service) to define position of the target on initial N1
-HairpinN1-N2              and final N2 position in DNA sequence:
-MicroArrayN1-N2           >  -mb300-500
                           >  -taqman200-500


-ssr/N                     Design PCR primers to SSR loci software automatically finds
                           Simple Repeats target Sequence and will design primers: N is
                           optional value for distance before (Forward primers) and after
                           (Reverse primers) SSR loci:
                           >  -ssr/100


-loop/N                    design self-complement primers at 5'-end for qPCR (LUX primer)
-lloop/N                   optimal minimum N = 14 bases, maximal - primer size:
-rloop/N                   >  -lloop/15


-slfrep/N                  design self-reporting primers for qPCR optimal minimum N = 14
-lslfrep/N                 bases, maximal - primer size:
-rslfrep/N                 >  -lslfrep/15


-bst -lbst -rbst           design BeST PCR primers for current options design best left or
                           right PCR primers:
                           >  -bst


-any -lany -rany           design ANY PCR primers:
                           >  -any


-lowcg -llowcg -rlowcg     design PCR primers for low CG% sequence:
                           >  -lowcg
```

```
-dfs -ldfs -rdfs        design PCR primers for difficult region:
                        > -dfs


-protein
-lprotein               design PCR primers for degenerated sequences:
-rprotein               > -protein


-add5e=                 ADD additional DNA sequence to 5' or 3'end of primers (probes)
-ladd5e=                (any length).
-radd5e=                It can be useful for add selective nucleotide to 3'-end of
-add3e=                 primer, restriction site to 5'-end or else:
-ladd3e=                > -add5e=ATTCCATGGTT
-radd3e=                > -add3e=C


-mnpl=n                 (MiNimal Primer Length) for PCR primers design
-lmnpl=n                 minimal primers length for left or right PCR primers design:
-rmnpl=n                > -lmnl=20
                        > -rmnl=18


-mxpl=n                 MaXimal Primer Length) for PCR primers design maximal primers
-lmxpl=n                length for left or right PCR primers design:
-rmxpl=n                > -lmxl=30
                        > -rmxl=30


-op=yes|no              (Overlapping Primers), default (-op=yes):
-lop=yes|no             > -op=no
-rop=yes|no             > -lop=yes -rop=no


-fx3e -lfx3e -rfx3e     Design PCR primer with fixed 5' or 3' end to selected sequence:
-fx5e -lfx5e -rfx5e     > -fx3e –any
                        > -fx5e –any


-fp= -lfp= -rfp=        Pre-designed (Fixed) forward or reverse Primers, always use the
                        actual primer sequence (5'->3') without space fixed primer(s)
                        sequence, for left and right primers.
                        FastPCR will automatically detect the primer location(s) with
                        mismatches; analyzes more than one primer for both DNA chains:

                        > -fp=attccattccgcgttcga -fp=atcctacgttccgttacc

                        pre-designed forward(left)and reverse (right) PCR primer:
                        > -lfp=attccattccgcgttcga -rfp=acgttacggtatttcttgc

-snp= -lsnp= -rsnp=     PCR primers design to a certain place, it can be SNP site or
                        else:
                        > -lsnp=tggacctatY

                        > -lsnp=tggacctatcc  -rsnp=cctaattgt

-c5=nn! -c3=nn!         left and right primers (probes) 3' and 5'-ends Nucleotide
                        Composition; the program accept the universal degenerate DNA code
-lc5=nn! -lc3=nn!       minimum 2 bases (maximum is primer length) for both ends: nn -
-rc5=nn! -rc3=nn!       for any bases, example for left primers ('RRY' is equivalent to
                        aat gat agt ggt aac gac agc ggc)(accepted the sequences with
                        different lengths):

                        > -c5=tss   -c3=wss wssws sswws!

-c3enz=NameEnzyme       primers (probes) directly designed to the restriction enzyme site
-lc3enz=NameEnzyme      at 3' end. "NameEnzyme" is only one enzyme without empty sign,
-rc3enz=NameEnzyme      example (this is as the alternative command -c3=YCATG^R  =XceI):

                        > -c3enz=XceI

                        Result: 3'end of all primers contains sequences: YCATGR
```

```
-mntm=              (MiNimal Temperature of Melting) for PCR primers design
-lmntm=             minimal temperature of melting for left or right primers:
-rmntm=             > -mntm=60
                    > -lmntm=55


-mxtm=              (MaXimal Temperature of Melting) for PCR primers design
-lmxtm=             maximal temperature of melting for left or right primers:
-rmxtm=             > -lmxtm=65
                    > -rmxtm=70


-mntm10=            (MiNimal Temperature of Melting for last 10 bases) at the 3'-end
-lmntm10=           of primer minimal temperature of melting for last 10 bases at the
-rmntm10=           3'-end of primer: for left or right primers:
                    > -lmntm10=35


-mxtm10=            (MaXimal Temperature of Melting for last 10 bases) at the 3'-end
-lmxtm10=           of primer maximal temperature of melting for last 10 bases at the
-rmxtm10=           3'-end of primer, for left or right primers:
                    > -lmxtm10=50


-mncg=              (MiNimal CG%) for PCR primers design minimal CG% for left or
-lmncg=             right primers:
-rmncg=             > -mncg=50
                    > -lmncg=50


-mxcg=              (MaXimal CG%) for PCR primers design maximal CG% for left or
-lmxcg=             right primers:
-rmxcg=             > -mxcg=70
                    > -lmxcg=70


-ct=yes|no          (Optional)(Copy Test) for PCR primers repetition test repetition
-lct=yes|no         test for left or right PCR primes design:
-rct=yes|no         > -lct=yes
                    > -rct=yes


-npc=               Number of Primers Combinations show in result:
                    > -npc=10


-npr=               Number of designed Primers (maximum 100):
-lnpr=              > -npr=10
-rnpr=
```

Example 1:

Standard PCR, from 1 to 100 for left (forward) primer design, region from 500 to 600 for right (reverse) primers (PCR product between 1 to 600 bp):

```
> -lpd1-100 -rpd500-600 -npcr
```

Example 2:

Inverted PCR: from 1 to 100 for left primer design, the region to the end of sequence minus 100 bases for right primers (number-e bases, without blank).

```
> -lpd1-100 -rpd100-e -ipcr
```

Example 3:

Single side PCR primers design for left side:

```
> -rpd1000-1300
```

Example 4:

PCR primer design for the whole length (default) of a sequence for left and right PCR primer design (default analysis without command line implemented):

```
> -all
```
or
```
> -lall -rall
```
Or only raw sequence

Example 5:

PCR primer design for whole lengths of a sequence, but with a PCR product selected by size from 500 to 600 bases:

```
> (500-600)
```

Example 6:

Specific region for PCR primer design and minimal and maximal size PCR product, simultaneously:

```
> -lpd100-300 -rpd800-1000 (500-600)
```

Detailed options for PCR primer design, with a product length from 500 to 600 bp, a left primer size between 20 and 28 bp, a minimal Tm of 55°C (minimal Tm for the 10 last base of 32°C), all primers have 5'-**TSN-3'** at 5'-end and 5'-**WSS**-'3 at 3'-end; only 30 primers shown in the result file:

```
> (500-600) -mnpl=20 -mxpl=28 -mntm=55 -mntm10=32 -npr=30 -c5=tsn! -c3=wss!
```

Most of the commands are cumulative and as well as of multitasking at the same time (simultaneously design primers or probe with different parameters and for different targets within the same sequence; interaction different tasks) **Example 7**:

```
> -tag400-500/100 -tag900-1200/100 -ssr
```

PCR primers design for two targets: between 400bp and 500bp and between 900bp and 1200bp and the search for all SSR loci and design PCR primers to each of them.

Example 8:

```
> -npcr -npc=10 (400-500) -rpd100-150 -lpd450-550 -mncg=55 -q=75 -mxtm=65
```

allows the detection of primers for a normal PCR (10 combinations shown) between position 100-150 for the right on, 450-550 for the left one, each at 55% CG maximum, a maximum Tm at 65° and a threshold for quality at 75, with a final product of 400 to 500bp.

Examples for PCR primers design when either one or both primer(s) or PCR product characteristics are known:

Example 9:

For left primer already known (for normal or inverted PCR)

```
> -rpd800-1000 -lfp=tcctcaagcgaaaaccaagtgc
```

Or right primer:

```
> -lpd1-400 -rfp=ctgttgttggttacttctcggt
```

Example 10:

All possible left (forward) PCR primers with the right one already known:

```
> -lall -rfp=ctgttgttggttacttctcggt
```

Definition of the minimal and maximal size of PCR product implementation:

```
> -rfp=ctgttgttggttacttctcggt -lall (300-400)
```

```
> -rfp=ctgttgttggttacttctcggt -lpd1-400 (300-400)
```

Example 11:

Imitation the ***in silico* PCR**: both primers - forward and reverse – are fixed (getting information about **Ta** and product length) and using –**npd**:

```
> -npd -tmsynchr=10 -fp=ctgttgttggttacttctcggt -fp=attatcgcgcctattttacg
```

Examples for PCR primer design with fixed 3'-end of primer (and SNP site):

Example 12:

If it is necessary to anchor one of the primer in a specific location in the sequence (e.g. "5'-gtcaaacatgt") and that this primer 3'-end is exactly this one (i.e. "5'-gtcaaacatgt-3'"), thus it must be specified.

For left (forward) primer

```
> -lsnp=gtcaaacatgt -rall
```

For right (reverse) primer

```
> -rsnp=gtcaaacatgt -lall
```

The minimal and maximal size of the PCR product can be also implemented, as for the location of the other primer:

```
> -lsnp=gtcaaacatgt -rall (300-400)
```

```
> -lsnp=gtcaaacatgt -rpd100-300 (300-400)
```

Example 13:

If it is necessary to anchor one of the primer to a SNP-position in the sequence (e.g. "5'attccttc**Y**") and that the primer 3'-end is exactly like this (i.e. "5'-attccttc**Y**-3'"), thus it must be specified for left (forward) primer:

```
> -lsnp=attccttcY -rall
```

```
> -lsnp=Y -rall
```

or for right (reverse) primers:

```
> -rsnp=Yattccttc
```

```
> -rsnp=Yattccttc -lpd100-300
```

The minimal and maximal size of the PCR product can be also implemented:

```
> -rsnp=Rattccttc -lall (300-400)
```

```
> -rsnp=Rattccttc -lpd100-300 (300-400)
```
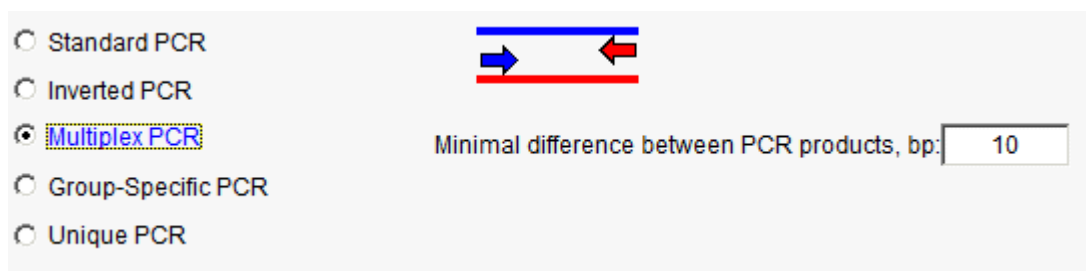
Examples for PCR primer design to SSR site:

Simple sequence repeat (SSR) loci are an important marker type for population genetic studies despite the limitation that development of novel loci requires construction and screening of genomic DNA libraries. The common practice of size fractioning genomic DNA before cloning could lead to differential representation of SSR loci within genomic libraries. In addition, linkage mapping studies have shown that small numbers of SSR markers are not randomly distributed within the genomes from which they are isolated.

Example 14: SSR target find and design PCR primers for it. Software automatically finds all SSR locuses and design around them PCR primers (200 bases for left and right side from SSR loci) :

```
> -ssr/200
```

Multiplex and degenerate primer design

Multiplex PCR is an approach commonly used to amplify several DNA target regions in a single PCR reaction and it is great technology for an endless list of applications. The greatest potential for multiplex PCR is the acceleration of the number of reactions that can be carried out simultaneously. Further improvement can be achieved by selecting the optimal set of primers that maximize the range of common Tm.

FastPCR quickly calculates multiplex PCR primer pairs for given target sequences. The speed of calculation depends from amount of target sequence and amount primer pair for each of them.

Other way for design compatible multiplex PCR primer pairs is use the predesigned as references for new designed primers.

The user can also input options for the PCR product involving the minimum product size differences among the set of designed primer pairs. It also allows to set primer design conditions individually for each given sequences or using common options. The individual setting have highest priority to PCR primer or probe design than general settings. The result includes primer sequences for individual sequences, their compatible primer pairs with product size and annealing temperature and final result for compatible primer pairs for each sequence with all information includes primer pair sequences, product size and annealing temperature. It is ideally to design all primer pairs with near equal annealing temperature in single reaction. For most cases the multiplex PCR conditions are resisting to a small variation (up 6°C) of

Ta between all primer pairs and PCR products. Synchronizing Tm for primer pair user can control from "Primer Design Options" or with command: **-tmsynchr=**10.

The annealing temperature must be optimal in order to guarantee effective amplification of the targets genomic sequence while minimizing the risk of unspecific amplification. To amplify the target genomic sequence effectively, the primers "quality" and properties should be highest. PCR primer design for multiplex PCR can be performed for standard or inverted PCR pairs or both of them. A minimum of two sequences must be implemented for this analysis.

The program will find the compatible primer pairs for each sequence and will make a continuous numbering of pairs for all investigated sequences:

```
Compatible combination of pair primers for multiplex PCR:  21
1F1_234-253  2R1_366-386    153bp         56.4°C
1F2_32-53  1R2_476-495      464bp         59.5°C
1F3_7-30  1R3_479-498       492bp         59.7°C
1F1_234-253 agggagtagcttacctcgct    20    56.6°C      55.0% 87
2R1_366-386 tgtgtgatcatgatgccctca   21    55.9°C      47.6% 69
1F2_32-53   cccaacggtttacctcaagggt  22    59.2°C      54.5% 105
1R2_476-495 ggtttcgtcggtcgctgctt    20    60.1°C      60.0% 75
1F3_7-30    gtgtgggtattttcagttgccgca   24   60.1°C      50.0% 104
1R3_479-498 tccggatccaacggtcacga    20    59.9°C      60.0% 109
```

Options for dimer analysing are in "Primer Design Options" user can find in menu bar – PCR or from "Tree View". Value 3 is default and, probably, is optimal; value – 2 and especial 1 for weak and strong dimer detection; value more then 3 up to 10 for detection only strong and vary stable dimmers, but missing weak and medium dimers.

! No limits for multiplex PCR, but the recursive approach have the potential to cause a stack overflow exception.

in silico PCR or primers (probes) searching

The prediction appropriated short or long primer (probe) annealing site is only one way for PCR product prediction.

Primer can bind many predicted sequences in template, but only sequences with few mismatches (1 or 2 depends from place and nucleotide) at 3'end of primer can be used for polymerase extension. The last 10-12 bases at 3'end of primer are sensitive to initiation of polymerase extension and general primer stability on binding template site. Single mismatch at these last 10 bases at 3'end of primer depends from the position and the structure can slightly reduced the primer binding and PCR efficiency.

This software allows simultaneously testing single primer or list of the individual primer or probe with any length thorough multiplex target sequences. This test control by primer complementarity to target sequence performed with fast no gap alignment.

Probe with not complemental tail on 5'end and on 3'end to target is not problem for performing this test.

Oligonucleotides with degenerated sequence are fine for performing this test.

The probable PCR product can fond for linear and cycle molecular, for standard, inverted PCR and for multiplex PCR. This "*in silico*" tool is very attractive for quick analysing primer or probe through target sequences, for determination primer (probe) location, orientation, efficiency of binding, complementarity and **Tm calculation**.

Enter primer sequences: Paste the primer sequence(s) in the additional editor in any format acceptable for FastPCR.

Restrict analysis to F/R primer pairs: the program will recognise paired primers (Forward F Reverse R) (see below).

For this type of analysis, primers from the same pair(s) must have identical names but finishing using "R or F" (e.g. >seq1R and >seq1F form a pair; or EVEN: >F and >R). The name length and structure (including "F" and "R" inside names) are not important. Moreover, the program is not limited in one unique pair per primers: for one "forward" primer, it can be several "reverse", the same for "reverse" primer:

```
1aF    agggagtagcttacctcgct      20     56.6  31.6  55.0  95.0   87
2bF    gcgaaaaccaagtgcttacct     21     55.9  29.4  47.6  95.2   101
3cF    tcctcaagcgaaaaccaatccaca      24     58.8  29.9  45.8  92.5   101
1aR    ggtttcgtcggtcgctgctt      20     60.1  36.6  60.0  85.0   75
2bR    agtcaacggcgtcgcagcgttct 23     64.7  36.6  60.9  92.2   99
3cR    ggtcgcgacatccgttccaa      20     59.7  32.6  60.0  95.0   82
```

The Output file will be presented as follows:

In silico Primer(s) search for: >1

```
>1af
5'-agggagtagcttacctcgct

Position:  234 -> 253 20bp 100 %   Tm = 56.6°C

5-agggagtagcttacctcgct->
   ||||||||||||||||||||
cctccctcatcgaatggagcgaacag

>2bf
5'-gcgaaaaccaagtgcttacct

Position:  263 -> 283 21bp 100 %   Tm = 55.9°C

5-gcgaaaaccaagtgcttacct->
   |||||||||||||||||||||
ttcgcttttggttcacgaatggagcga

>1af
5'-agggagtagcttacctcgct
>1ar
5'-ggtttcgtcggtcgctgctt
 PCR product size: 898bp

>2bf
5'-gcgaaaaccaagtgcttacct
>2br
5'-agtcaacggcgtcgcagcgttct
 PCR product size: 844bp

>3cf
5'-tcctcaagcgaaaaccaatccaca
>3cr
5'-ggtcgcgacatccgttccaa
 PCR product size: 526bp
```

Group-specific PCR primers (family-specific primer set) and unique PCR

The group-specific amplification also call as family specific or universal amplification is most important tool for comparative studies of genes and genomes, including studies of evolution and cloning new sequences. The specific sequences that link to concrete organism can be discovered by DNA polymorphism in these conservative genome regions (genes, transposable or repeat elements). For detection DNA polymorphism in relative sequences will help with design PCR primers around this polymorphic region.

Usually, group-specific primer sets were designed by first generating a multiple alignment and then manually identifying most conservative regions for primer design. This way is slow, but easy visual and understandable. FastPCR design no degenerated PCR primers to amplify a conserved (or polymorphic) region of all interesting sequences.

The overall strategy of designing gene-specific PCR primers is divided into two phases: standard PCR design for current sequence and then test complementarity these primers in other sequences.

The test primer complementarity performed with fast no gap local alignment includes parameters for amount of mismatches at the 3'-end of primers and primers similarity to target sequence.

Users can specify the alignment parameters (the same as for *in silico* PCR) for primers searching – "initial searching word size, >3 (default = 7), nt", important length of 3'-end, 5...20, nt for testing mismatches, minimal complement primer length (>12, nt) and the local similarity (default = 80%)".

An output pages contains the group-specific PCR primers from each sequence and second page show compatible primers combination with product size and temperature annealing.

FastPCR automatically designs larger sets of universal primer pairs for all given sequences, identifies conservative regions without sequence alignment and generates suitable primers for all given sequences. All steps of algorithm are automatic and you can influence to the general options for primer design and alignment options.

FastPCR will work with any source of sequences as long as it is possible to found short consensus sequences. The quality of primer design is dependent on both on sequence relationship, phylogenetic similarity and suitability of the consensus sequence to the design of any good primers. Software is able to generate group-specific primers for each sequence independently, that suit for all sequences.

Unique PCR

The strategy for a unique PCR primer design is opposite to the group-specific PCR primers (probes) design. This case program search unique regions within a DNA sequence and automatically designing primers with minimal user intervention and maximum flexibility. Primer alignment parameters are similar as for *in silico* PCR and group-specific PCR primers.

The primers with mismatches are more efficient in PCR then the degenerated primers. This concerns the degenerated primers designed from protein sequences.

Design and properties of LUX (fluorogenic) PCR primers

LUX™ (Light Upon eXtension) Primers are an easy to use, highly sensitive, and efficient method for performing real-time quantitative PCR (qPCR) and RT-PCR (qRT-PCR). Each primer pair in the LUX™ system includes a fluorogenic primer with a fluorophore attached to its 3′ end and a corresponding unlabeled primer. The fluorogenic primer has a short sequence tail of 6–8 nucleotides on the 5′ end that is complementary to the 3′ end of the primer. The resulting hairpin secondary structure provides optimal quenching of the fluorophore (see the figure below). When the primer is incorporated into double-stranded DNA during PCR, the fluorophore is dequenched and the signal increases by up to 10-fold.

Fluorogenic primers were designed to increase their fluorescence intensity when incorporated into the double-stranded PCR product. This design is based on a previous study that demonstrated the effects of the primary and secondary structure of oligonucleotides on the emission properties of the conjugated fluorophores. Briefly, the design factors are the presence of either a C or G as the terminal 3' nucleotide of the primer, the fluorophore being attached to the second or third base (T) from the 3' end, the presence of one or more Gs within the 3 nt flanking the labeled nucleotide and, for hairpin primers, the existence of a 5' tail that is complementary to the 3' end of the primer. The 5' tail forms a blunt-end hairpin at temperatures below its melting point. The stem of the hairpin primers have a G ranging from –1.6 to –5.8 kcal/mol.

Example 15: SSR target find and design PCR LUX primers for it. Software automatically finds longest SSR loci and design around them PCR primers (200 bases for left (LUX primers) and right side from SSR loci) :

```
> -ssr/200 –lluxpd
```

Probes design

FastPCR allows the design of highly efficient probes for *TaqMan* and Molecular Beacons assays as for other short or long single oligonucleotides probes (experimentally tested).

The *TaqMan* and Molecular Beacons assays both use a reporter and a quencher dye attached to the probe. The oligonucleotide probe hybridizes to the target sequence, and the reporter and quencher are separated either by the exonuclease activity of the *Taq* polymerase (5'-to-3' polymerisation-dependent exonuclease replacement activity) for *TaqMan*, or because of conformation changes upon hybridization to the target sequence for the Molecular Beacons.

TaqMan Design

TaqMan probes provide an indirect measure of hybridization, as the signal continues to be generated once the reporter and quencher are separated by the exonuclease activity of the polymerase. *TaqMan* probes must be designed (if possible) with a **GC-content of 40-60%, a high complexity, no dimer with primers, a high Tm (60-65°C) and a probe length of 20 to 29 bp**.

Probes are covalently conjugated with a fluorescent reporter dye (generally the 6-carboxy-fluorescein [**FAM**]; excitation 494 nm, emission 518 nm) and a non fluorescent quencher dye (Black Hole Quencher [**BHQ-1**], 4'-(2-Nitro-4-toluyldiazo)-2'-methoxy-5'-methyl-azobenzene-4''-(N-ethyl)-N-ethyl-2-cyanoethyl-(N,N-diisopropyl)) at the most 5' and most 3' base, respectively.

The BHQ family of quenchers was developed to provide excellent spectral overlapping over the entire range of commonly used reporter dyes. They efficiently quench across the electromagnetic spectrum from 480 nm into the near infrared IR-making it possible to use reporter dyes that emit anywhere within this range.

Molecular Beacon Design

Molecular Beacons are oligonucleotide probes that can report the presence of specific nucleic acids in homogenous solutions. They are useful in situations where it is not either possible or desirable to isolate the probe-target hybrids from an excess of the hybridization probes, such as in real-time monitoring of polymerase chain reactions in sealed tubes, or in detection assay of RNAs within living cells. Molecular Beacons are hairpin or self-dimer shaped molecules (see below) with an internally quenched fluorophore whose fluorescence is restored when they bind to a target nucleic acid. They are designed in such a way that the loop portion of the molecule is a probe sequence complementary to a target nucleic acid molecule. The stem is formed by the annealing of complementary arm sequences on the ends of the probe sequence. A fluorescent moiety is attached to the end of one arm and a quenching moiety is attached to the end of the other arm. The stem keeps these two moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by energy transfer. Since the quencher moiety is a non-fluorescent chromophore and emits the energy that it receives from the fluorophore as heat, the probe is unable to fluoresce. When the probe encounters a target molecule, it forms a hybrid that is longer and more stable than the stem and its rigidity and length preclude the simultaneous existence of the stem hybrid. Thus, the molecular beacon undergoes a spontaneous conformational reorganization that forces the stem apart, and causes the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence.

Molecular Beacon example sequence: **6-FAM** 5'-CGCTGCtgtcgcgaccttatgagaacgcGCAGCG -3' **BHQ1**

The underlined sequence at the 5' and 3' ends identifies the arm sequences that are complementary.

The length of the probe sequence (18-30 nt) is chosen in such a way that the probe target hybrid is stable in the conditions of the assay. The stem sequence (5-8 nt) is chosen to ensure that the two arms hybridize to each other but not to the probe sequence.

PCR amplification protocol

The higher quality of primers is help to save PCR efficiency at changing PCR conditions. PCR reaction can set up in room temperature and performed without hot-start enzymes.

The range of optimal annealing temperature (Ta) was calculated Tm of primer or optionally plus 9-10°C, and in practice PCR efficiency was tested with gradient annealing temperature using MasterCycler Gradient (Eppendorf). For primer combinations with very different Tm, the optimal annealing temperature was chosen according to lowest Tm primer (primer with CG content higher then 50% is tolerant to wide annealing Ta, from 50°C up to 70°C).

PCR steps – the primers binding (usually from 55°C) and the polymerase extension (usually from 60°C to 72°C), we recommend to join into one step as 60°C. This step includes primer binding the target and polymerase extension at once; the recommended time for this step is 1 second for each 50 bases of PCR product.

The denaturation of genomic DNA is easy with short step at 95-96°C, 5-20 seconds.

The PCR was performed in a 25 µl reaction mixture containing 25-50 ng DNA, 1x PCR buffer (with 2 mM $MgCl_2$), 0.2-1 µM of primer (for primer combinations – maximum 1 µM is total concentration), 0.2 mM dNTPs, 1 U Taq DNA polymerase and optimal additional 0.04U pfu DNA Polymerase (for long products amplification).

The PCR machine was programmed for amplification short and long (1,000-6,000 bases) amplicons: 1 cycle at 95°C 3 min, 27-30 cycles for 95°C (10-15 sec), 60°C (60 sec), 68°C (10-90 sec); and final extension step 68°C 5 min.

Amplification protocol for short (100-500 bases) amplicons: 1 cycle at 95°C 3 min, 27-30 cycles for 95°C (10 sec) and 60°C (5 sec); final extension step 68°C 2 min.

Amplification was performed in PTC-100 Programmable Thermal Controller (MJ research Inc., Bio-Rad Laboratories, USA) or a Mastercycler Gradient (Eppendorf AG, Germany) in 0.2 ml tubes or 96-well plates.

General PCR and qPCR set-up we publish here: http://primerdigital.com/Tools/ReactionMixture.html

Calculation of optimal annealing temperature

Optimal annealing temperature for PCR calculated using our modified formula from Rychlik et.al.1990:

$$T_a^{Opt} = 0.7 T_m^{\mathrm{Pr}\, oduct} + 0.3 T_m^{\mathrm{Pr}\, imer} - 14$$

According to our experience with PCR, almost all high quality value primers designed by FastPCR in default conditions can be use at the annealing temperature from 60 to 68 without loosing PCR efficiency. Differences in values of melting temperature calculated using different nearest neighbour thermodynamic parameters do not play important role in calculation of the PCR optimal annealing temperature. **The "excellent" primers show stable efficient amplification in vary PCR annealing temperature.** The longer PCR product needs to amplify the higher annealing temperature use with the same primer's combination.

Bioinformatics Tools
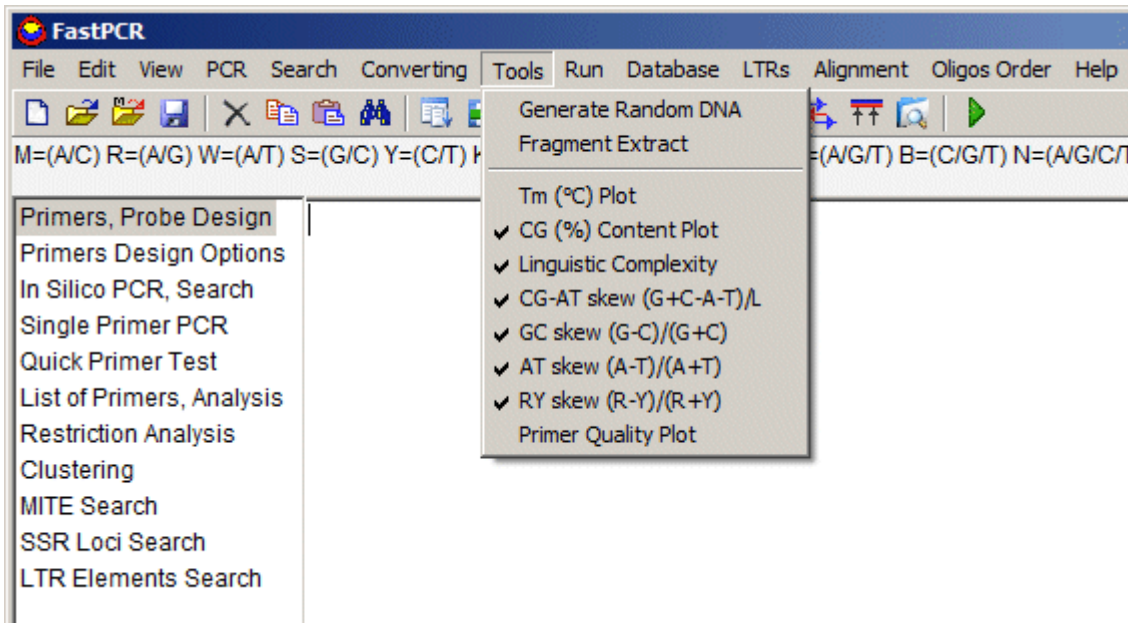
Using additional editor, you can select the length for generation random DNA sequence with "Generate Random DNA". Random DNA is useful for a design artificial primer, DNA adaptors.

Select one or more tools for simultaneous analysis: Tm, CG%, Linguistic Complexity, CG-AT skew, CG skew, AT skew, RY skew and Primer Quality; and use additional editor for selecting widow length (10 to 100,000) and press F5.

Primer Quality Plot is maximum 50 bases and 10 minimum bases widow length.

Output result as .XLS Text file, compatible for Excel or Open Office, also you can select at "View" – "report as Excel Sheet":
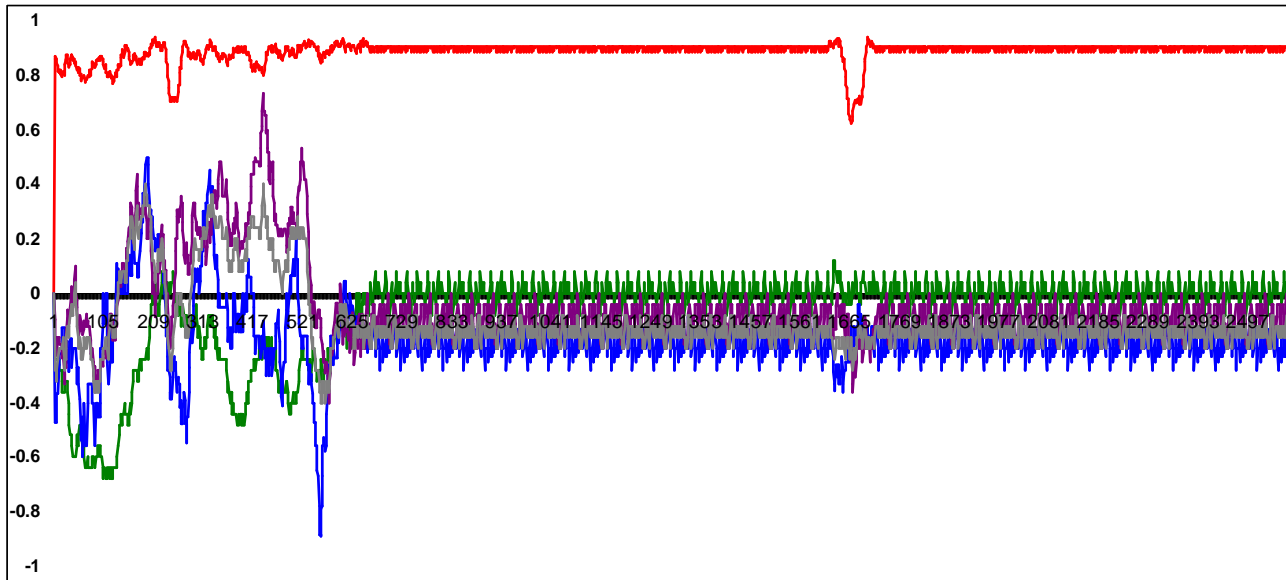
Database

FastPCR has powerful database search capabilities. To perform a databases search, press "Database" from menu bar:
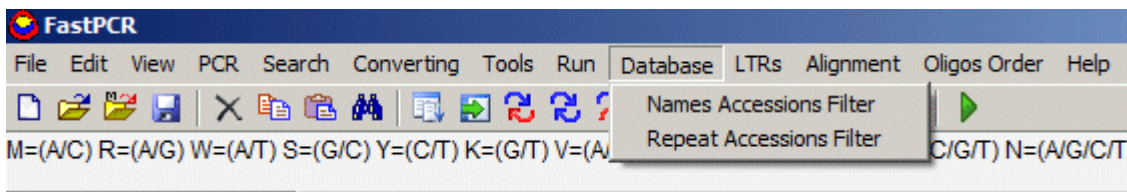
Depending on the object type the appropriate database search dialog box appears allowing you to define search conditions and perform the actual search.

Name dialog box appears: searching and getting accessions contains some words **inside accession's names**:
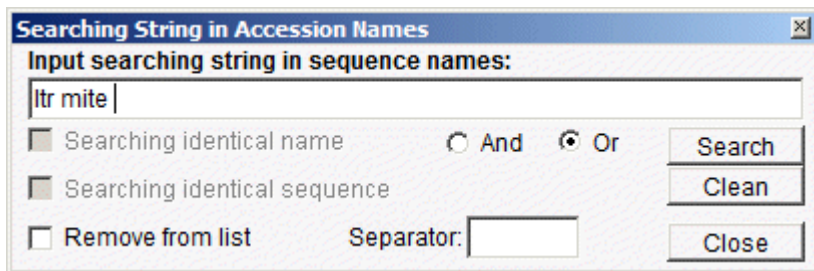
The boxes are connected with logical AND, the search will accept accessions which simultaneously satisfy the conditions. The conditions are connected with logical OR, the conditions in that box are satisfied if at least one condition is satisfied.

"Input searching string in sequence names" field is box giving key words to search for. The accessions that have any one of the key words shown in this box will be found by the search. Key words added with "separator", like space character or any else. The clear the key Words box, press the "Clean" button.

For example, accessions in FASTA format (any formats are applicable) in use:

>gnl|trep|trep1000 dna transposon, mite, stowaway, "hades_be704509-1"; complete element
tacttcctccgtcccaaaataagtatctcaactttgtactggctttagtacaaaattatactaagcttaagacacttattttgggccgga
gagagta
>gnl|trep|trep1001 dna transposon, mite, stowaway, "hades_be704981-1"; complete element
tacttcctccgttccaaaatagatgtcatctattttggnacggacggagta
>gnl|trep|trep1002 dna transposon, mite, stowaway, "hades_bf293216-1"; complete element
tactccctccgttctaaaataaatgattcaattttatactaactttagtataaagttgggtcatctattttggaacggagggagta
>gnl|trep|trep1003 dna transposon, mite, stowaway, "hades_bf588685-1"; complete element
tactccctctgttccaaaatagatgactcaactttgtactatatttgggaacagagggagta
>gnl|trep|trep1004 dna transposon, mite, stowaway, "hades_bf628687-1"; complete element
Tacttcctccgtcccaaaataagtgatttaaaaatgactcagttttgtactaactttagtacaaaattgagtcacttattttggaacaga
gggaata
…………

All accessions found will be show contains searching string: **ltr** or **mite** (if any).

Sequence database search

Searching for similarity **in sequences or identical accession names**:
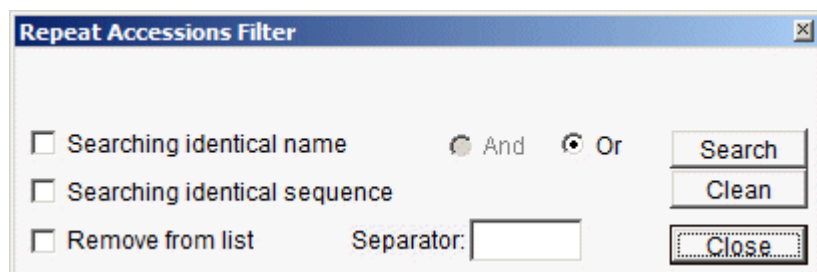
Alignment
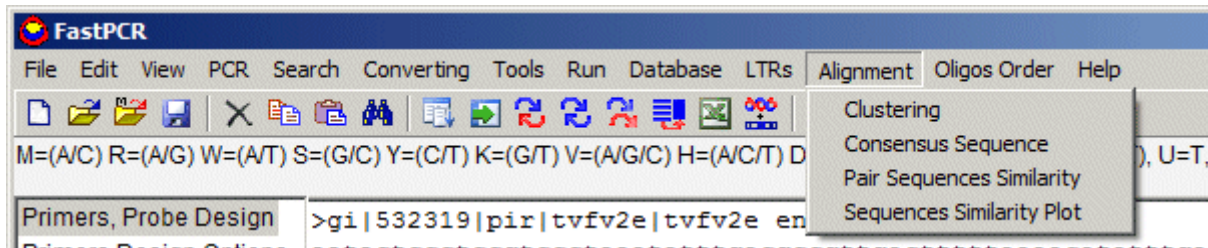
Accepted sequences in alignment format: MEGA, GCG/MSF, Simple Alignment DIALIGN formats, can use with this analysis.

Pair Sequence Similarity: comparative analysis each sequences with each other, output as the matrix:

```
d._melanogaster
d._pseudoobscura
s._lebanonensis
s._albovittata
d._crassifemur
d._mulleri
d._affinidisjuncta
d._heteroneura
d._mimica
d._adiastola
d._nigra

100.0 89.6 84.8 81.5 81.9 83.1 81.0 81.2 81.9 80.6 80.8
89.6 100.0 85.4 81.6 81.5 85.0 82.3 82.7 82.9 81.6 82.9
84.8 85.4 100.0 80.2 80.1 84.2 81.5 81.4 82.1 81.4 82.8
81.5 81.6 80.2 100.0 93.4 84.4 87.1 87.1 87.4 87.1 87.1
81.9 81.5 80.1 93.4 100.0 84.1 87.1 86.2 86.6 86.4 86.7
83.1 85.0 84.2 84.4 84.1 100.0 86.8 86.8 86.6 86.5 86.9
81.0 82.3 81.5 87.1 87.1 86.8 100.0 97.5 95.7 95.7 93.8
81.2 82.7 81.4 87.1 86.2 86.8 97.5 100.0 96.3 95.9 94.2
81.9 82.9 82.1 87.4 86.6 86.6 95.7 96.3 100.0 95.9 94.6
80.6 81.6 81.4 87.1 86.4 86.5 95.7 95.9 95.9 100.0 93.4
80.8 82.9 82.8 87.1 86.7 86.9 93.8 94.2 94.6 93.4 100.0
```

Sequence Similarity Plot: you need to select the windows length for calculation average local similarity for all given sequences. Result for Excel sheet:
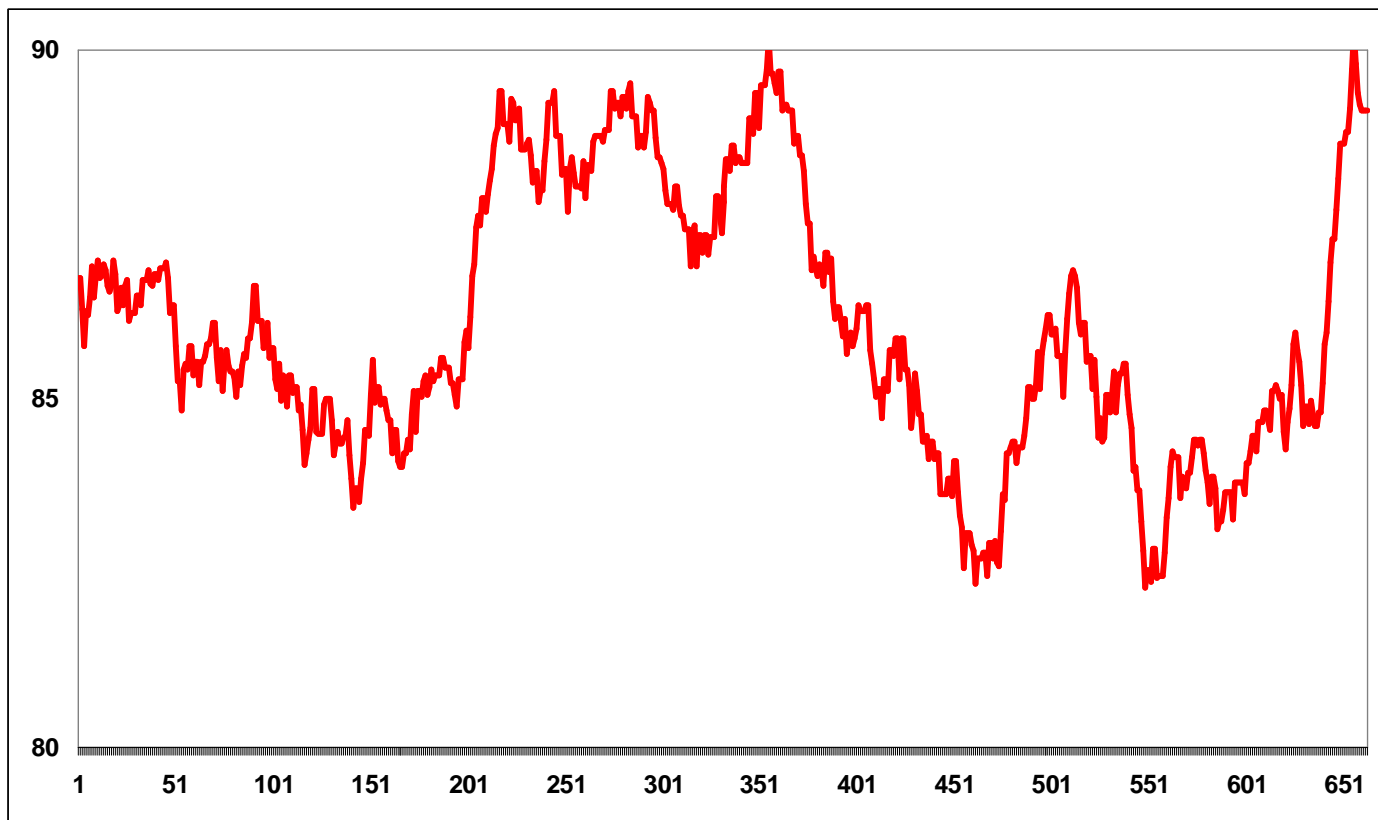
The Restriction Endonuclease Analysis

FastPCR have imported restriction endonucleases databases from several sources: New England Biolabs, Fermentas and SibEnzyme.

Each year FastPCR release contains update for new enzymes.

"**Input specified sequence**" – searching sequence must be in the standard IUB/IUPAC nucleic acid codes, any other characters - digits, spaces, TAB characters or else are ignored, low- and upper-case insensitive; palindromic or non-palindromic recognition site.

Software show restriction cleavage point on given sequence.

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