PCR in Infectious Diseases

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PCR based method for detection of pathogens is very sensitive. In a clinical sample targets as small as few molecules of DNA or RNA can be detected. PCR is particularly useful in diagnosis of tuberculosis where culture takes long time or leprosy where culture may not be possible. It is also used for rapid diagnosis of life threatening common bacterial infections and for quick identification of the agents of bioterrorism like *Bacillus anthracis* and *Yersinia pestis*. Bacterial antibiotic resistance genes like MRSA (*Staph aureus*) and VRE (*enterococcus*), and MDR tuberculosis (*katG* and *rpoB* genes) can also be identified by PCR.

PCR is now the method of choice for identification of viral genomes like *hepatitis B & C*, *EBV* and *HIV* etc. An interesting application in viral diseases is in-situ PCR. The virus particles, for example *hepatitis-B* virus in the liver cells, *CMV* in lung, and *EBV* in association with lymphoma, and *HPV* in cervical cancer can be demonstrated in a smear or tissue specimen. PCR is also being used for many fungal and parasitic infections.

Points to remember in using PCR for infectious agents:

1. With the passage of time the genomes of infectious agents tend to develop subtle changes called polymorphisms. The development of DNA or RNA polymorphism may make a previously designed primer or probe ineffective. Similarly all sub-species/types of an organism or virus may not be detected by the primer or probe due to DNA polymorphism within the species. However, most infectious agents have some conserved regions of the genome. While designing a primer/probe it is important to choose only the conserved regions of the genome.
2. The presence of cross-homology (similarity) between DNA or RNA of the infectious agent and the host genome can give false positive result. More commonly cross-homology may be present between genome of the infectious agent and the organisms that may be normally present in the clinical sample. The problem of cross-homology is best addressed by BLAST searching of the primer/probe before putting them in use (Chapter 1 & 3).
3. PCR would give a positive result whether the organism in the clinical sample is dead or alive. For example a patient on anti-tuberculosis treatment may still be having dead *mycobacteria* in a clinical sample that could give a positive result on PCR.
4. PCR in itself is a very sensitive technique. But the threshold of detection of a bacterial or viral genome in a clinical sample is largely dependent on the method of extraction of DNA or RNA from the sample. The inconsistency in nucleic acid extraction from the infectious agents may be overcome by using good quality commercial kits made for this purpose.

5. Transport and storage of clinical samples for PCR is also critical. The samples with organisms/viruses where PCR would be done on DNA are fairly stable under ordinary transport and storage conditions used for culture of such agents. However, clinical samples containing RNA viruses are highly prone to degradation by RNases in the environment. Such samples must be processed as early as is possible. When delay is unavoidable the samples like serum/plasma must be separated and stored at -20^{o}C or below. Repeated freeze thawing of the samples is also damaging for the RNA.
6. If the organism in a clinical sample is low in number it may initially be grown for a short time in a culture medium before extracting its DNA.

There are numerous applications of PCR in the diagnosis of infectious disorders. But here only selected protocols involving common pathogens are described as examples.

PCR for *Mycobacterium tuberculosis*

DNA extraction

In order to overcome inconsistencies in extraction of microbial DNA it is advisable to use good quality commercial kits.

Real time PCR

A real time PCR for *Mycobacterium tuberculosis* is described. The PCR primers and the TaqMan® probe (Fig. 15.1) for the insertion sequence element IS986/IS6110 of *Mycobacterium tuberculosis* CDC1551 (GenBank accession: AE000516.2) were designed by the Primer Express® software (Applied Biosystems, USA).

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CGGAGCTGCGCGATGGCGAACTCAAGGAGCACATCAGCCGCGTCCACGCCGCCAAC
TACGGTGTTTACGGTGCCCGCAAAGTGTGGCTAACCCTGAACCGTGAGGCATCGAG
GTGGCCAGATGCACCGTCGAACGGCTGATGACCAAACTCGGCCTGTCCGGGACCAC
CCGCGGCAAAGCCCGCAGGACCACGATCGCTGATCCGGCCACAGCCCGTCCCGCCG
ATCTCGTCCAGCGCCGCTTCGGACCACCAGCACCTAACCGGCTGTGGGTAGCAGAC
```

Fig. 15.1. The primers and the TaqMan® probe for the *Mycobacterium tuberculosis* insertion sequence (IS6110): (GenBank accession: AE000516.2).

Primers:

Forward primer:	5'-GAACCGTGAGGGCATCGA
Reverse primer:	5'-ACAGGCCGAGTTTGGTCATC
TaqMan® Probe:	6 FAM 5'-CCAGATGCACCGTCGAACGGC-BHQ1
Amplified product:	64bp
Primer mix:	Make a mix of the two primers and the probe at concentration of 5pmol each (Chapter 3).

PCR protocol (per sample):

Reaction volume: 25µl

PCR mix: 20µl
Primer mix: 1µl
Taq polymerase: 0.5 units (0.1µl)
DNA: 4µl

Thermal cycling

- Initial denaturation 95°C for 10 minutes
- Forty cycles each of:
  - Denaturation at 95°C for 15 seconds
  - Annealing/extension at 60°C for 1 minute
- Take fluorescence reading at 60°C step

Endpoint PCR for *Mycobacterium tuberculosis*

The forward and the reverse primers without the probe can also be used for conventional endpoint PCR of *Mycobacterium tuberculosis*. The amplification is done as described above except that the probe is omitted from the primer mix. At the end of the PCR the amplified product may be run on 2% agarose at 100 volts for 30 minutes or 6% mini-polyacrylamide gel at 150 volts for 15 minutes (Chapter 5). Positive result is shown by 64bp amplified fragment.

PCR for *Hepatitis B virus*

DNA extraction

For consistent results the viral DNA is best extracted by silica based micro column commercial extraction kits.

Real time PCR

A TaqMan® probe based real time PCR for *HBV* targeting the conserved 5'Untranslated region of the virus (Fig. 15.2) is described.

TCAATCTTCTCGAGGACTGGGGACCCTGCACCGAACATGGAGAGCACAACATCAGG
ATTCCTAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTCTTGTTGACAAGAATCC
TCACAATACCACAGAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGA
GCACCCACGTGTCCTGGCCAAAATTCGCAGTCCCCAACCTCCAATCACTCACCAAC

Fig. 15.2. Real time PCR primers and TaqMan® probe for the conserved 5'-Untranslated region of *Hepatitis-B virus* DNA (GenBank accession: NC_003977.1).

Primers and probe:
Forward primer: 5'- GGACCCCTGCTCGTGTTACA
Reverse primer: 5'- GAGAGAAGTCCACCMCGAGTCTAGA
Probe: 6 FAM 5'- TGTTGACAARAATCCTCACCATACCRCAGA-BHQ1
(Reporter: 6 FAM, Quencher: BHQ1)

Primer mix: Make a mix of the two primers and the probe at concentration of 5pmol each (Chapter 3).

PCR protocol (per sample):

Reaction volume: 25µl
PCR mix: 20µl
Primer mix: 1µl
Taq polymerase: 0.5 units (0.1µl)
DNA: 4µl

Thermal cycling

- Initial denaturation 95°C for 10 minutes
- Forty cycles each of:
  - Denaturation at 95°C for 15 seconds
  - Annealing/extension at 60°C for 1 minute
- Take fluorescence reading at 60°C step

Endpoint PCR for *HBV*:

The forward and the reverse primers without the probe can also be used for conventional endpoint PCR of *HBV*. The amplification is done as described above except that the probe is omitted from the primer mix. At the end of the PCR the amplified product may be run on 2% agarose at 100 volts for 30 minutes or 6% mini-polyacrylamide gel at 150 volts for 15 minutes (Chapter 5). Positive result is shown by 90bp amplified fragment.

PCR for *Epstein-Barr virus (EBV)*

DNA extraction

The viral DNA is best extracted by silica based micro column commercial extraction kits.

Real time PCR

A TaqMan® probe based real time PCR for *EBV* targeting a conserved region of the virus is described (Fig. 15.3).

```
GCCCCGTCACGGTGACGTAGTCTGTCTTGAGGAGATGTAGACTTGTAGACACTGC
AAAACCTCAGGACCTACGCTGCCCTAGAGGTTTTGCTAGGGAGGAGACGTGTGTG
GCTGTAGCCACCCGTCCCGGGTACAAGTCCCGGGTGGTGAGGACGGTGTCTGTGG
TTGTCTTCCCAGACTCTGCTTTCTGCCGTCTTCGGTCAAGTACCAGCTGGTGGTC
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Fig. 15.3. DNA sequence of *Epstein-Barr virus (EBV)* genome (GenBank accession: V01555.2) showing the real time PCR primers and the TaqMan® probe.

Primers:

Forward primer: 5'-AAACCTCAGGACCTACGCTGC
Reverse primer: 5'-ACAGACACCGTCCTCACCAC
Probe: FAM 5'-TAGAGGTTTTGCTAGGGAGGAGACGTGTG-BHQ1
(Reporter: 6 FAM, Quencher: BHQ1)

Primer mix: Make a mix of the two primers and the probe at concentration of 5pmol each (Chapter 3).

PCR protocol (per sample):

Reaction volume: 25µl
PCR mix: 20µl
Primer mix: 1µl
Taq polymerase: 0.5 units (0.1µl)
DNA: 4µl

Thermal cycling

- Initial denaturation 95°C for 10 minutes
- Forty cycles each of:
  - Denaturation at 95°C for 15 seconds
  - Annealing/extension at 60°C for 1 minute
- Take fluorescence reading at 60°C step

Endpoint PCR

The forward and the reverse primers without the probe can also be used for conventional endpoint PCR of EBV. The amplification is done as described above except that the probe is omitted from the primer mix. At the end of the PCR the amplified product may be run on 2% agarose at 100 volts for 30 minutes or 6% mini-polyacrylamide gel at 150 volts for 15 minutes (Chapter 5). Positive result is shown by 107bp amplified fragment.

PCR for *Cytomegalo Virus (CMV)*

DNA Extraction

DNA extraction

For consistent results the viral DNA is best extracted by silica based micro column commercial extraction kits.

Real time PCR

A TaqMan® probe based real time PCR for *CMV* targeting the human cytomegalovirus glycoprotein B gene of the virus is described (Fig. 15.4).

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CTTCTTCCCAAACGGTCAGCCATGGTGTTAACGAGACCATCTACAACACTACCCT
CAAGTACGGAGATGTGGTGGGGGTCAATACCACCAAGTACCCCTATCGCGTGTGT
TCTATGGCCCAGGGTACGGATCTTATTCGCTTTGAACGTAATATCGTCTGCACCT
CGATGAAGCCCATCAATGAAGACCTGGACGAGGGCATCATGGTGGTCTACAAACG
CAACATCGTCGCGCACACCTTTAAGGTACGAGTCTACCAGAAGGTTTTGACGTTT
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Fig. 15.4. DNA sequence of *Cytomegalovirus* glycoprotein B gene (GenBank accession: M60929.1) showing the real time PCR primers and the TaqMan® probe.

Primers:

Forward primer: 5'-AAGTACCCCTATCGCGTGTG

Reverse primer: 5'-ATGATGCCCTCGTCCAGGTC

Probe: 6 FAM 5'-TGGCCCAGGGTACGGATCTTATTCG-BHQ1
(Reporter: 6 FAM, Quencher: BHQ1)

Primer mix: Make a mix of the two primers and the probe at concentration of 5pmol each (Chapter 3).

PCR protocol (per sample):

Reaction volume: 25µl

PCR mix: 20µl

Primer mix: 1µl

Taq polymerase: 0.5 units (0.1µl)

DNA: 4µl

Thermal cycling

- Initial denaturation 95°C for 10 minutes
- Forty cycles each of:
  - Denaturation at 95°C for 15 seconds
  - Annealing/extension at 60°C for 1 minute
- Take fluorescence reading at 60°C step

Endpoint PCR

The forward and the reverse primers without the probe can also be used for conventional endpoint PCR of *CMV*. The amplification is done as described above except that the probe is omitted from the primer mix. At the end of the PCR the amplified product may be run on 2% agarose at 100 volts for 30 minutes or 6% mini-polyacrylamide gel at 150 volts for 15 minutes (Chapter 5). Positive result is shown by 116bp amplified fragment.

PCR for *HCV*

PCR of *HCV*, being RNA, is done in two steps. In the first stem *HCV* RNA is converted to cDNA and in the second step cDNA is amplified by PCR. A method targeting the highly conserved 5'-untranslated region of the virus is described. Fig. 15.5 shows a set of primers and TaqMan® probes for the 5' untranslated region of HCV genome.

CACTCCACCATGAATCACTCCCCTGTGAGGAACTACTGTCTTCACGCAGAA**AGCGT**
CTAGCCATGGCGTTAGTATGAGTGTCGTGCAGCCTCCAGGAC**CCCCCCTCCCGGGA**
GAGCCATAGTGGTCTGCGGAACCGGT**GAGTACACCGGAATTGCCAGGA**CGACCGGG
TCCTTTCTTGGATCAACCCGCTCAATGCCTGGAGATTTGGGCGTGCCCCCGCAAGA

Fig. 15.5. Sequence of 5' untranslated region of *HCV* genome (GenBank: EU678744.1) showing a set of primers and TaqMan® pobe for real time PCR.

RNA Extraction

For best and consistent results RNA extraction by commercial silica based extraction columns is recommended.

Preperation of cDNA:

Reverse transcription (RT):

Primer (HCV-R) 5'-TCCTCGCAATTCCGGTGTACTC

Reactions conditions:

- 5 X reaction buffer: 4 µl
- 10 mmol dNTPs mix: 2 µl
- RT Primer (10 pmol/µl): 1 µl
- MMLV- Reverse Transcriptase (200 U/µl): 1 µl
- RNase inhibitor (20 U/µl): 1 µl
- Deionized water: 11 µl
- RNA: 2 µl
- Incubation: 42^{o}C for 60 minutes
- RT inactivation: 70^{o}C for 5 minutes

Real Time PCR

PCR Primers:

HCV-F 5'-AGCGTCTAGCCATGGCGTTAGTAT

HCV-R 5'- TCCTCGCAATTCCGGTGTACTC

HCV Probe 6 FAM-CCCCCCTCCCGGGAGAGCCATAGT-BHQ1

Amplified Product: 109bp

Reaction volume: 25µl

PCR mix: 21µl

Primer mix: 1µl (containing each primer at 5pmol/µl concentration)

Taq polymerase: 0.5 units (0.1µl)

cDNA: 3µl

Thermal cycling

- Initial denaturation 95^{o}C for 10 minutes
- Forty cycles each of:
  - Denaturation at 95^{o}C for 15 seconds
  - Annealing/extension at 60^{o}C for 1 minute
- Take fluorescence reading at 60^{o}C step

Endpoint PCR for *HCV*

The forward and the reverse primers without the probe can also be used for conventional endpoint PCR of *HCV*. cDNA is prepared as described above using the *HCV*-R primer. The amplification is also done as described above except that the probe is omitted from the primer mix. At the end of the PCR the amplified product may be run on 2% agarose

at 100 volts for 30 minutes or 6% mini-polyacrylamide gel at 150 volts for 15 minutes (Chapter 5). Positive result is shown by a 109bp amplified fragment.

PCR for *Dengue virus*:

A pan dengue PCR is described that can identify all four genotypes of the virus. PCR of *Dengue virus*, being RNA, is done in two steps. In the first stem viral RNA is converted to cDNA and in the second step cDNA is amplified by PCR. A method using the primers and TaqMan® MGB probe (Fig. 15.6) for the highly conserved 3' untranslated region shared by the four genotypes of the *Dengue virus* genome is described. The TaqMan® MGB probe is conjugated with a minor groove binder (MGB) at the 3' end that allows higher probe Tm with a relatively short length of the probe.

DENV-1 (GenBank NC_001477.1)
AGACACAACGCAGCAGCGGGGCCCAACACCAGGGGAAGCTGTACCCTGGTGGTAAG
GACTAGAGGTTAGAGGAGACCCCCCGCACAACAACAAACAGCATATTGACGCTGGG
AGAGACCAGAGATCCTGCTGTCTCTACAGCATCATTCCAGGCACAGAACGCCAAAA
AATGGAATGGTGCTGTTGAATCAACAGGTTCT

DENV-2 (GenBank *AF038403.1*)
CAAATCGCAGCAACAATGGGGGCCCAAGGTGAGATGAAGCTGTAGTCTCACTGGAA
GGACTAGAGGTTAGAGGAGACCCCCCCAAAACAAAAAACAGCATATTGACGCTGGG
AAAGACCAGAGATCCTGCTGTCTCCTCAGCATCATTCCAGGCACAGAACGCCAGAA
AATGGAATGGTGCTGTTGAATCAACAGGTTCT

DENV-3 (GenBank *NC_001475.2*)
ATGACACAACGCAGCAGCGGGGCCCGAGCACTGAGGGAAGCTGTACCTCCTTGCAA
AGGACTAGAGGTTAGAGGAGACCCCCCGCAAATAAAAACAGCATATTGACGCTGGG
AGAGACCAGAGATCCTGCTGTCTCCTCAGCATCATTCCAGGCACAGAACGCCAGAA
AATGGAATGGTGCTGTTGAATCAACAGGTTCT

DENV-4 (GenBank *NC_002640.1*)
GATAAAACGCAGCAAAAGGGGGCCCGAAGCCAGGAGGAAGCTGTACTCCTGGTGGA
AGGACTAGAGGTTAGAGGAGACCCCCCCAACACAAAAACAGCATATTGACGCTGGG
AAAGACCAGAGATCCTGCTGTCTCTGCAACATCAATCCAGGCACAGAGCGCCGCAA
GATGGATTGGTGTTGTTGATCCAACAGGTTCT

Fig. 15.6. The primers and the TaqMan® MGB probe for real time PCR targeting the 3' un-translated region of four genotypes of *Dengue virus*. Minor differences in the sequence of the four dengue virus genotypes are shown as green.

RNA Extraction

For best and consistent results RNA extraction by commercial silica based extraction columns is recommended.

Preperation of cDNA:

Reverse transcription (RT):

Primer (DENV-R) 5'-ACCATTCCATTTTCTGGCGTT

Reactions conditions:

- 5 X reaction buffer: 4 µl
- 10 mmol dNTPs mix: 2 µl
- RT Primer (10 pmol/µl): 1 µl
- MMLV- Reverse Transcriptase (200 U/µl): 1 µl
- RNase inhibitor (20 U/µl): 1 µl
- Deionized water: 11 µl
- RNA: 2 µl
- Incubation: 42^{o}C for 60 minutes
- RT inactivation: 70^{o}C for 5 minutes

Real Time PCR

PCR Primers:

DENV-F 5'- GARAGACCAGAGATCCTGCTGTCT

DENV-R 5'- ACCATTCCATTTTCTGGCGTT

DENV-Pro FAM 5'-AGCATCATTCCAGGCAC-BHQ1-MGB

Amplified Product: 68bp

Reaction volume: 25µl

PCR mix: 21µl

Primer mix: 1µl (containing each primer at 5pmol/µl concentration)

Taq polymerase: 0.5 units (0.1µl)

cDNA: 3µl

Thermal cycling

- Initial denaturation 95^{o}C for 10 minutes
- Forty cycles each of:
  - Denaturation at 95^{o}C for 15 seconds
  - Annealing/extension at 60^{o}C for 1 minute
- Take fluorescence reading at 60^{o}C step

Endpoint PCR for *Dengue virus*

The forward and the reverse primers without the probe can also be used for conventional endpoint PCR of Dengue virus. cDNA is prepared as described above using the DENV-R primer. The amplification is also done as described above except that the probe is omitted from the primer mix. At the end of the PCR the amplified product may be run on 2%

agarose at 100 volts for 30 minutes or 6% mini-polyacrylamide gel at 150 volts for 15 minutes (Chapter 5). Positive result is shown by a 68bp amplified fragment.

PCR for *Malaria*

A TaqMan® probe based real time PCR for malaria targeting a conserved region of *Plasmodium falciparum, P. vivax, P. malariae*, and *P. ovale* is described (Fig. 15.7).

P. falciparum (GenBank M19172.1)
TAGTGTGTATCAATCGAGTTTCTGACCTATCAGCTTTTGATGTTAGGGTATTGGCCTAACAT
GGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAGAGGGAGCCTGAGAAATAGCTA
CCACATCTAAGGAAGGCAGCAGGCGCGTAAATTACCCAATTCTAAAGAAGAGAGGTAGTGAC
AAGAAATAACAATGCAAGGCCAATTTTTGGTTTTGTAA

P. vivax (GenBank X13926.1)
TTAGTGTGTATCAATCGAGTTCTGACCTATCAGCTTTTGATGTTAGGGTATTGGCCTAACAT
GGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAGAGGGAGCCTGAGAAATAGCTA
CCACATCTAAGGAAGGCAGCAGGCGCGTAAATTACCCAATTCTAAAGAAGAGAGGTAGTGAC
AAGAAATAACAATACAAGGCCAATCTGGCTTTGTAATT

P. malariae (GenBank M54897.1)
TAGTGTGTATCAATCGAGTTTCTGACCTATCAGCTTTTGATGTTAGGGTATTGGCCTAACAT
GGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAGAGGGAGCCTGAGAAATAGCTA
CCACATCTAAGGAAGGCAGCAGGCGCGTAAATTACCCAATTCTAAAGAAGAGAGGTAGTGAC
AAGAAATAACAATGCAAGGCCAAATTTTGGTTTTGCAA

P. ovale (GenBank: L48987.1)
TAGTGTGTATCAATCGAGTTTCTGACCTATCAGCTTTTGATGTTAGGGTATTGGCCTAACAT
GGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAGAGGGAGCCTGAGAAATAGCTA
CCACATCTAAGGAAGGCAGCAGGCGCGTAAATTACCCAATTCTAAAGAAGAGAGGTAGTGAC
AAGAAATAACAATACAAGGCCATTTCATGGTTTTGTAA

Fig. 15.7. Sequence and the primers for real time PCR targeting the Small Subunit (SSU) RNA gene of the four subspecies of malarial parasite.

DNA extraction:

Commercial or Chelex based method on whole blood collected in EDTA (Chapter 2).

Real Time PCR

Forward primer:	5'-ACATGGCTATGACGGGTAACG
Reverse primer:	5'-TGCCTTCCTTAGATGTGGTAGCTA
Probe:	6 FAM 5'-TCAGGCTCCCTCTCCGGAATCGA-BHQ1
	(Reporter: 6 FAM, Quencher: BHQ1)

Primer mix: Make a mix of the two primers and the probe at concentration of 5pmol each (Chapter 3).

PCR protocol (per sample):

Reaction volume: 25µl
PCR mix: 22µl
Primer mix: 1µl
Taq polymerase: 0.5 units (0.1µl)
DNA: 2µl

Thermal cycling

- Initial denaturation 95°C for 10 minutes
- Forty cycles each of:
  i. Denaturation at 95°C for 15 seconds
  ii. Annealing/extension at 60°C for 1 minute
- Take fluorescence reading at 60°C step

Endpoint PCR

The forward and the reverse primers without the probe can also be used for conventional endpoint PCR of Malaria. The amplification is done as described above except that the probe is omitted from the primer mix. At the end of the PCR the amplified product may be run on 2% agarose at 100 volts for 30 minutes or 6% mini-polyacrylamide gel at 150 volts for 15 minutes (Chapter 5). Positive result is shown by 84bp amplified fragment.

PCR for *Leishmania donovani*

A TaqMan® probe based real time PCR for *Leishmania donovani* glucosephosphate isomerase gene (Fig.15.8) is described.

GTACGGCAGCACCGCCTCTGTCTCCGCGCCGAAAAAGTTGTTGTACCAGATGCCGA
CCAAAGCCAGCATCATCGGCAGGTTCTGCTCCGTCGGTGCAGACGCAAAGTGGTTA
TCCATCACGTGCGCGCCAGTCAGGAACTCCACAAAGTTGTCGTAGCCGATCGAAAG
CATCACGGAGAGACCGATGGCGGACCACACAGAGTAGCGACCACCGACCCAGTCCC

Fig. 15.8. DNA sequence of *Leishmania donovani* isolate WR 378 glucosephosphate isomerase gene (GenBank: AY974201.1).

DNA extraction

DNA extraction by phenol chloroform or chelex method from 0.2ml bone marrow aspirate or archival bone marrow smears (Chapter 2).

Real Time PCR

Forward primer: 5'-CCAGATGCCGACCAAAGC

Reverse primer: 5'-CGCGCACGTGATGGATAAC
Probe: 6 FAM 5'-ATCGGCAGGTTCTGCTCCGTCG-BHQ1

(Reporter: 6 FAM, Quencher: BHQ1)

Primer mix: Make a mix of the two primers and the probe at concentration of 5pmol each (Chapter 3).

PCR protocol (per sample):

Reaction volume: 25µl
PCR mix: 22µl
Primer mix: 1µl
Taq polymerase: 0.5 units (0.1µl)
DNA: 2µl

Thermal cycling

- Initial denaturation 95°C for 10 minutes
- Forty cycles each of:
  - Denaturation at 95°C for 15 seconds
  - Annealing/extension at 60°C for 1 minute
- Take fluorescence reading at 60°C step

Endpoint PCR for *Leishmania donovani*

The forward and the reverse primers without the probe can also be used for conventional endpoint PCR of *Leishmania donovani*. The amplification is done as described above except that the probe is omitted from the primer mix. At the end of the PCR the amplified product may be run on 2% agarose at 100 volts for 30 minutes or 6% mini-polyacrylamide gel at 150 volts for 15 minutes (Chapter 5). Positive result is shown by a 82bp amplified fragment.

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