At the sub-cellular level cancer is certainly a genetic disorder. The molecular genetics of cancer is typically marked by mutation(s) in the oncogenes, tumour suppressor genes or micro RNA genes. These genetic changes carry potential for diagnosis of cancer by PCR. PCR can be used to diagnose the disease, predict its prognosis or monitor the disease progress and response to treatment if a cancer specific DNA marker is identified. PCR can also be used to demonstrate the association of some malignancies and viruses e.g. human papillomavirus and the cervical cancer and HTLV-1 infection and leukaemia.

**Ig gene rearrangement**

Immunoglobulin and T-cell receptor genes in the germ-line configuration are made of several individual parts called variable (V), diversity (D) and joining (J) segments. On antigenic challenge the germ line segments rearrange to form a much smaller gene. The un-rearranged Ig gene is too large for amplification by PCR. It could be amplified if the gene rearrangement brings the PCR primers for the target DNA very close to each other.

The lymphoid malignancies show clonal rearrangements of Immunoglobulin genes or T-cell receptor genes. The assembly of IgH genes from the variable (VH), diversity (DH), and joining (JH) regions creates a DNA target that is complex and unique to each patient. In the subsequent paragraphs a simple broad spectrum PCR assay is described that can pick up to 90% of the IgH gene rearrangements in patients of lymphoproliferative disorders. A forward primer complementary to the consensus JH sequence is used with a reverse primer in the VH region.

Forward primer (JH) (5pmol): 5’-AACTGCAGAGGAGACGGTGACC
Reverse primer (VH) (5pmol): 5’-CTGTCGACACGGCCGTGTATTACT

**Thermal cycling**

- Initial denaturation 94°C for 5 minutes.
- Thirty cycles each of:
  - Denaturation at 94°C for 1 minute
  - Annealing at 60°C for 1 minute
  - Extension at 72°C for 1 minute 30 seconds
- Final extension at 72°C for 3 minutes

Electrophoresis may be done on 2% agarose at 150 volts for 60 minutes or 6% mini-polyacrylamide gel at 150 volts for 30 minutes (Fig. 14.1).
Fig. 14.1. PCR for Immunoglobulin heavy chain gene rearrangement. Lane 1 shows a sharp band of clonal Ig gene rearrangement where as lane 3 shows a diffuse pattern of polyclonal gene rearrangements. Lane 2 shows a faint clonal band of minimal residual disease along with a diffuse polyclonal background in the post treatment sample of the patient at lane 1.

**Real time PCR for IgH gene rearrangement**

Forward primer (JH) (5pmol): 5′-AACGGTGCAGGGGAGGCGGTACC
Reverse primer (VH) (5pmol): 5′-CTGTCGACCCGGCCTGTATTACT
SYBR green ready reaction mix: 23μl
DNA: 2μl (~200ng)
SYBR green ready reaction mix containing dNTPs and Taq polymerase is available from many commercial sources.

**Thermal cycling**

- Initial denaturation 94°C for 5 minutes.
- Thirty cycles each of:
  - Denaturation at 94°C for 1 minute
  - Annealing at 60°C for 1 minute (read fluorescence)
  - Extension at 72°C for 1 minute 30 seconds

Melting curve analysis may be added at the end of the real time PCR protocol to differentiate between the specific and the non-specific amplification products.

**Bcl-II gene rearrangement**

Follicular lymphoma is characterized by translocation of bcl-II gene from chromosome 18 to IgH gene on chromosome 14. The fusion gene is a lymphoma specific marker that can be used in diagnosis and monitoring of disease activity.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mbr</td>
<td>5′-GAGTTGTACGTGGCCTG</td>
</tr>
<tr>
<td>mcr</td>
<td>5′-CGCTTGACTCCCTTACGTGC</td>
</tr>
<tr>
<td>s-icr</td>
<td>5′-TCGTTTCAGTAAAGTGAGTGTC</td>
</tr>
<tr>
<td>LJH</td>
<td>5′-TGAGGAGACGGTGACC</td>
</tr>
</tbody>
</table>

**Real Time PCR**

**Primers:**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mbr-F</td>
<td>5′-TTAGAGAGTTGCTTTACGTGC</td>
</tr>
<tr>
<td>IgH-R</td>
<td>5′-ACTCACCTGAGGAGACGGTGAC</td>
</tr>
<tr>
<td>Mbr-Probe</td>
<td>6 FAM 5′-TTTCAACACAGACCACCCAGAGCC-TAMRA</td>
</tr>
</tbody>
</table>
Primer mix: Make the primer mix of the four primers and two probes at concentration of 5pmol each (Chapter 3).

PCR protocol:

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
- Read fluorescence at 60°C step

Bcr-abl gene rearrangements

A reciprocal translocation between the long arms of chromosomes 9 and 22 (Philadelphia chromosome) is found in >90% patients of chronic myeloid leukemia and 15–25% patients with acute lymphoblastic leukemia. This translocation transposes c-abl oncogene from chromosome 9q34 to bcr gene on chromosome 22q11. The fused bcr/abl gene provides specific marker for diagnosis and disease monitoring. The translocation can be detected by first making cDNA of the bcr-abl mRNA followed by PCR.

Common bcr-abl gene rearrangements

Over 95% of the bcr-abl transcripts in CML are between introns 13/14 on bcr gene and intron 2 on abl gene (e13a2 & e14a2). The transcripts result in protein called p210. In ALL 70% of the transcripts are e1a2 that result in protein called p190. About 25% of transcripts in ALL are e13a2 or e14a2 (Fig. 14.1). In a minority of the patients atypical transcripts may also be observed.

PCR screening strategy

Sequence of the bcr-abl gene transcripts and the PCR primers with TaqMan® probe are shown in Fig. 14.2. Three separate forward primers E-1, E-13 and E-14 are used with a common reverse primer A-2. A common bcr-abl TaqMan® probe complementary to the abl gene is used. A fragment of the abl gene just close to the breakpoint region is amplified as an internal control by a separate pair of primers.
Fig. 14.2. Homo sapiens partial mRNAs for bcr-abl e14a2 chimeric protein (GenBank: AJ131466.1).

A. Homo sapiens partial mRNA for bcr-abl e14a2 chimeric protein (GenBank: AJ131466.1).

B. Homo sapiens partial mRNA for bcr-abl e13a2 chimeric protein (GenBank: AJ131467.1).

C. Homo sapiens bcr-abl e1a2 chimeric protein mRNA, partial cds (GenBank: AF113911.1).

RNA extraction

For RNA extraction commercial TRIzol® Reagent® (Invitrogen, USA) as described in Chapter 2 gives good quality results.

Preperation of cDNA
Primer ABL-R is used for preparing the cDNA. Oligo dT primer for poly-A tail of mRNA or random hexamers may also be used for this purpose. In this experiment RNA extracted from a patient known to have bcr-abl gene rearrangement was serially diluted ranging from 100% to 50%, 25%, 12.5%, 6.25%, 3.12%, and 1.56%. A known negative RNA was also included as a negative control. cDNA was prepared from each dilution and the negative control as per the following protocol:

**Real Time PCR**

**Primers and Probes:**

**BCR-ABL**

- E-1 5'-GCAGATCTGGGCTTCCACGAT
- E-13 5'-GCATTCCGCTGACCATCAATAA
- E-14 5'-CAGCCACTGGATTTAAGCAGAGT
- A-2 (R) 5'-TCCAACGAGCGGATTCACT
- **BCR-ABL-P** 6 FAM 5'-AAAGCCCTTCAGCGGCCAGTAGCATCT-BHQ1
- **Internal control**
  - ABL-F 5'-GCTGGGTCCCAAGCAACTAC
  - ABL-R 5'-ACACAGGCCCATCGTACCA
  - **ABL-P** JOE 5'-TCACGCCAGTACAGTCTGGAGAAACA-BHQ1

**Primer mix:** Make the primer mix of the six primers and the two probes at concentration of 5pmol each (Chapter 3).

**PCR protocol (per reaction):**

- **Reaction volume:** 25µl
- **PCR mix:** 21µl
- **Primer mix:** 1µl
- **Taq polymerase:** 0.5 units (0.1µl)
- **cDNA:** 3µl

**Thermal cycling**

- Initial denaturation 95°C for 10 minutes
- Forty cycles each of:
  - Denaturation at 95°C for 15 seconds

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PCR in Neoplastic Disorders
ii. Annealing/extension at 60°C for 1 minute
   • Take fluorescence reading at 60°C step

Result

The real time plot of the serially diluted sample and the negative control is shown in Fig. 14.3.

![Real time plot of the serially diluted RNA sample](image)

Fig. 14.3. Real time plot of the serially diluted RNA sample (100-1.56%) positive for the bcr-abl gene rearrangement and a known negative sample.

**A strategy for comprehensive molecular diagnosis of leukaemia**

Molecular diagnosis of leukaemia may be helpful in prognostic stratification and detection of minimal residual disease. The WHO classification of acute leukaemia is based on demonstration of a large number of gene rearrangements that also correspond to gross chromosomal abnormalities. A comprehensive strategy for detection of various molecular genetic abnormalities in Acute Lymphoblastic Leukaemia (ALL) and Acute Myeloid Leukaemia (AML) is described.

**RNA extraction**

Commercial TRIzol® Reagent® (Invitrogen, USA) as described in Chapter 2.

**Preparation of cDNA**

- Primer: Random hexamers at 0.2 µg/µL
- Reactions conditions (per reaction):
  - 5 X reaction buffer: 4 µl
  - 10 mmol dNTPs mix: 2 µl
  - RT Primer (10 pmol/µl): 1 µl
PCR in Neoplastic Disorders

- MMLV- Reverse Transcriptase (200 U/μl): 1 μl
- RNase inhibitor (20 U/μl): 1 μl
- Deionized water: 11 μl
- RNA: 2 μl
- Incubation: 42°C for 60 minutes
- RT inactivation: 70°C for 5 minutes

Real Time PCR
Primers and Probes

**ALL**

E2A-PBX1 t(1;19) (GenBank: M31222 & M86546)
- Forward: ENF-E2A 5'-CCAGCCTCATGCACAACCA
- Reverse: ENR-PBX1 5’-GGGCTCCTCGGATACTCAAAA
- Probe: ENP E2A FAM-5’-CCCTCCCTGACCTGTCTCGGC-BHQ1

MLL-AF4 t(4;11) (GenBank: L04284 & L13773)
- Forward1: ENF-MLL 5’-CCCAAGTATCCCTGTAAAACAAAA
- Forward2: ENF-MLL 5’-GATGGAGTCCACAGGATCAGAT
- Reverse: ENR-AF4 5’-GAAAGGAAACTTGGATGGCTCA
- Probe: ENP-AF4 FAM-5’-CATGGCCCGCCTCCTTCTGACGC-BHQ1

TEL-AML1 t(12;21) (GenBank: U11732 & D43969)
- Forward: ENF-TEL 5’-CTCTGTCTCCCCGCCTGAA
- Reverse: ENR-AML1 5’-CGGCTCGTGCCTGGCAT
- Probe: ENP-TEL FAM-5’-TCCCAATGGGCATGGCGTGC-BHQ1

m-bcr t(9;22) p190 (GenBank: X02596 & X16416)
- Forward: ENF-BCR 5’-CTGGCCCAACGATGGCGA
- Reverse: ENR-ABL 5’-CAGTCAGACCTGGGAGAT
- Probe: ENP-ABL FAM-5’-CCCTTCAGCGGCCAGTAGCATCTGA-BHQ1

M-bcr t(9;22) p210 (GenBank: X02596 & X16416)
- Forward: ENF-BCR 5’-TCCGCTGACCACATCAAYAAGGA
- Reverse: ENR-ABL 5’-CAGTCAGACCTGGGAGAT
- Probe: ENP-ABL FAM-5’-CCCTTCAGCGGCCAGTAGCATCTGA-BHQ1

**AML**

SIL-TAL1 del(1) (GenBank: M74558 & S53245)
- Forward: ENF-SIL 5’-CGCTCCTACCTGCAAACA
- Reverse: ENR-TAL 5’-CGCAGGGAAGAGATGCACA
- Probe: ENP-SIL FAM-5’-ACCTCAGCTCCTCGGGAAGTTGC-BHQ1
PML-RARA  t(15;17) (GenBank: M73778 & X06538)
Forward1: ENF-PML  5’-TCTTCTTGCCCAACAGCAA
Forward2: ENF-PML  5’-ACCTGGATGGACCGCCTAG
Forward3: ENF-PML  5’-CCGATGGCTTCGACGAGTT
Reverse: ENR-RARA  5’-GCTTGTAGATGCAGGGTGAG
Probe: ENP-RARA  FAM-5’-AGTGCCCAGCCCTCCCTCGC-BHQ1

CBFB-MYH1  inv(16) (GenBank: L20298 & D10667)
Forward: ENF-CBFB  5’-CATTAGCACAACAGCCTTTGA
Reverse1: ENR-MYH11  5’-AGGGCCCAGCTTGGACTT
Reverse2: ENR-MYH11  5’-CTCTGTAAAGCACTCCCTGTGA
Reverse3: ENR-MYH11  5’-CTCCTGCTGCAGCTCTTGAT
Probe: ENP-CBFB  FAM-5’-TCTGCGTGTCTCTCCGAGCT-BHQ1

AML1-ETO  t(8;21) (GenBank: D43969 & D14289)
Forward: ENF-AML1  5’-CACCTACACACAGCCATCAAA
Reverse: ENR-ETO  5’-ATCCACAGGTAGCTGCGCATT
Probe: ENP-AML1  FAM-5’-AACCTCGAAAATCGTACTGAGAAGCACTCCA-BHQ1

Primer and Probe mixes:
Make the respective primer and probe mixes of each molecular defect in a separate tube at concentration of 5pmol each (Chapter 3).

PCR protocol (per reaction):
- Reaction volume: 25μl
- PCR mix: 21μl
- Primer mix: 1μl
- Taq polymerase: 0.5 units (0.1μl)
- cDNA: 3μl

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
  iii. Take fluorescence reading at 60°C step

P53 gene mutations:
The gene for tumor suppressor protein p53 is located on chromosome 17. It is mutated and/or deleted in >50% of human cancers. A large number of p53 gene mutations and deletions have been described in various cancers.

The mutations in p53 gene may be screened by DGGE, SSCP or melting curve analysis (Chapter 5). Final confirmation of the mutations is done by genomic sequencing (Chapter 10).
A strategy for screening of mutations in exon 5-8 of p53 gene by melting curve analysis is described:

**Primers Sequence and annealing conditions**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a</td>
<td>5’-CAACTCTGTCTCCTTCTTCTTCTTCTAC</td>
<td>5’-AGCCATGGCAGGGACGGCG</td>
<td>65–60°C touchdown 0.5°C/cycle for 10 cycles</td>
</tr>
<tr>
<td>5b</td>
<td>5’-CTCCTGCCCCGACCCGC</td>
<td>5’-CTAAGAGCAATCAGTGGAGAATCAGA</td>
<td>65–60°C touchdown 0.5°C/cycle for 10 cycles</td>
</tr>
<tr>
<td>6</td>
<td>5’-CAACCACCTTAAACCCCTCCT</td>
<td>5’-AGACGCACAGGCTGGTTCG</td>
<td>68–58°C touchdown 1.0°C/cycle for 10 cycles</td>
</tr>
<tr>
<td>7</td>
<td>5’-AGGCGCAGCTGGCCTCATC</td>
<td>5’-GAGGCCGCACTGGCCTTCATC</td>
<td>68–58°C touchdown 1.0°C/cycle for 10 cycles</td>
</tr>
<tr>
<td>8</td>
<td>5’-AACCTGAGTTTCTTCATGCTTTG</td>
<td>5’-AAATCTGAGGCATAACTGACCTT</td>
<td>63.5–58.5°C touchdown 0.5°C/cycle for 10 cycles</td>
</tr>
</tbody>
</table>

For screening of mutations in each exon use the respective pair of primers in separate tubes.

For BRCA1 and BRCA2 mutations:

**BRCA1** & **BRCA2 (BReast Cancer)** tumour suppressor genes are located on chromosomes 17 and 13 respectively. A large number of mutations have been described in the two genes that are strongly associated with familial breast and ovarian cancer.

The mutations may be screened by DGGE, SSCP and melting curve analysis (Chapter 5). Final confirmation of the mutations is done by genomic sequencing (Chapter 10).

A strategy for screening of mutations in exon 2 & 20 of BRCA1 and exon 11 of BRCA2 genes by melting curve analysis is described:

**Primers**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5’-AAAAGATATAGATGTATGTTTTGCTAATGTGT</td>
<td>5’-TCCCAATTAAATACACTCTTGTGCTGA</td>
</tr>
</tbody>
</table>
PCR in Neoplastic Disorders

BRCA1-F 5'-GAGTGGTGGGGTGAGATTTTTGTC
Exon 20-R 5'-CCTGATGGGTTGTGTTTGGTTTCT
BRCA2-F 5'-CGAAAATTATGGGCAGTTGTTTACG
Exon 11-R 5'-GCTTTCCACTTTGCTGACTAAATCCA

For screening of mutations in each exon use the respective pair of primers in separate tubes.

Forward primer concentration: 5pmol
Reverse primer concentration: 5pmol
SYBR green ready reaction mix: 23μl
DNA: 2μl (~200ng)

Thermal cycling programme
- Initial denaturation 94°C for 5 minutes.
- Thirty cycles each of:
  i. Denaturation at 94°C for 1 minute
  ii. Annealing at 60°C for 1 minute (read fluorescence)
  iii. Extension at 72°C for 1 minute 30 seconds
- Select melting curve analysis form software of real time thermal cycler

JAK-2 mutation (V617F)

A simple PCR for identification of JAK-2 mutation by ARMS is described:

Primers:
JAK2-Mutant-F (5pmol) 5'-AGCATTTGGTTTTAAATTATGGAGTATATT
JAK2-Control-F (5pmol) 5'-ATCTATAGTCATGCTGAAAGTAGGAGAAAAG
JAK2-Common-R (10pmol) 5'-CTGAATAGTCCTACAGTGTTTTCAGTTTTCA

Amplified products:
- Mutation: 203bp
- Control: 364bp

Primer mix: Make the primer mix of the three primers at concentration of 5pmol each (Chapter 3).
Reaction volume: 25μl
PCR buffer: 22μl
Taq polymerase: 0.5 units (0.1μl)
Primer mix: 1μl (5pmol of each primer/μl)
DNA: 2μl (~200ng)

Thermal cycling
- Initial denaturation 94°C for 5 minutes
- Twenty five cycles of:
  i. Denaturation at 94°C for 40 seconds
  ii. Annealing at 58°C for 40 seconds
  iii. Extension at 72°C for 1 minute

Electrophoresis: Mini 6% polyacrylamide gels at 150V for 40 minutes
Staining: Silver nitrate
Result: Fig. 14.3.
Fig. 14.3. Silver stained PAGE of PCR for JAK-2 mutation. Arrow pointing at the lanes 3-6 show positive while the lanes 1, 2 & 7 show negative result.

Bibliography


