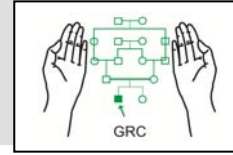


# PCR in Neoplastic Disorders

Maj Gen (R) Suhaib Ahmed, HI (M)



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.

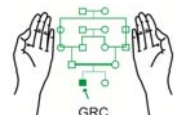
The lymphoid malignancies show clonal rearrangements of Immunoglobulin genes or T-cell receptor genes. The assembly of IgH genes from the variable (VH), diversity (DH), and joining (JH) regions creates a DNA target that is complex and unique to each patient. In the subsequent paragraphs a simple broad 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  
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.
- Thirty cycles each of:
  - i. Denaturation at 94°C for 1 minute
  - ii. Annealing at 60°C for 1 minute
  - iii. 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).



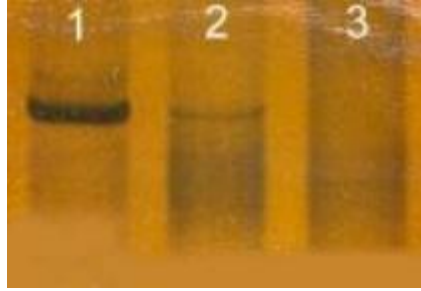


Fig. 14.1. PCR for Immunoglobulin heavy chain gene rearrangement. Lane 1 shows a sharp band of clonal Ig gene rearrangement where as lane 3 shows a diffuse pattern of polyclonal gene rearrangements. Lane 2 shows a faint clonal band of minimal residual disease along with a diffuse polyclonal background in the post treatment sample of the patient at lane 1.

### Real time PCR for IgH gene rearrangement

Forward primer (JH) (5pmol): 5'-AACTGCAGAGGAGACGGTGACC

Reverse primer (VH) (5pmol): 5'-CTGTCGACACGGCCGTGTATTACT

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:
  - 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

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.

mbr 5'-GAGTTGTACGTGGCCTG

mcr 5'-CGCTTGACTCCTTTACGTGC

s-icr 5'-TCGTTCTCAGTAAGTGAGAGTGC

LJH 5'-TGAGGAGACGGTGACC

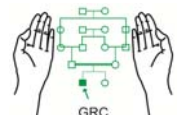
### Real Time PCR

Primers:

Mbr-F 5'-TTAGAGAGTTGCTTTACGTGGCC

IgH-R 5'-ACTCACCTGAGGAGACGGTGAC

Mbr-Probe 6 FAM 5'-TTTCAACACAGACCCACCCAGAGCC-TAMRA



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**

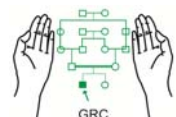
A reciprocal translocation between the long arms of chromosomes 9 and 22 (Philadelphia chromosome) is found in >90% patients of chronic myeloid leukemia and 15–25% patients with acute lymphoblastic leukemia. This translocation transposes c-abl oncogene from chromosome 9q34 to bcr gene on chromosome 22q11. 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.



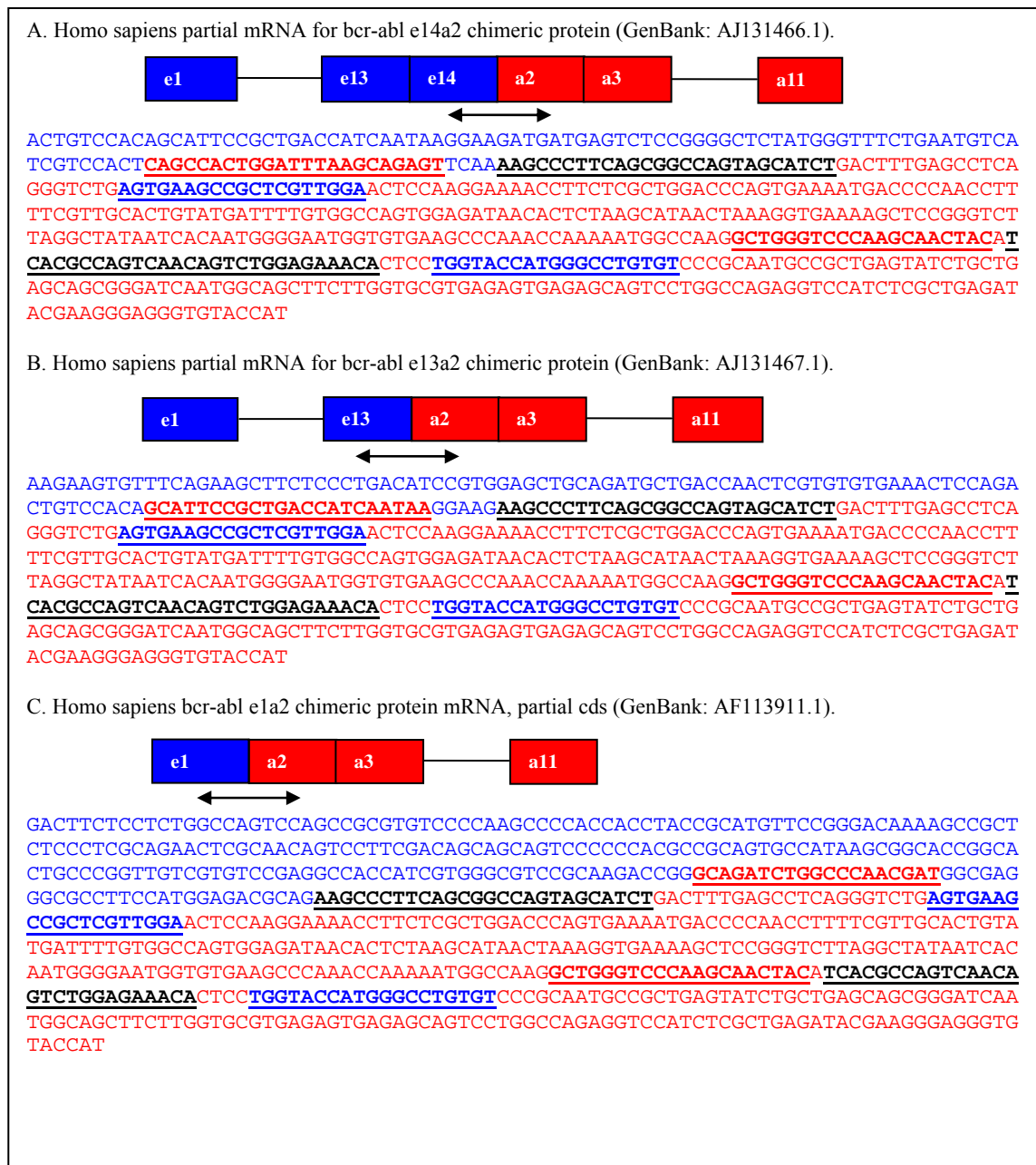
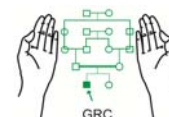


Fig. 14.2. Homo sapiens partial mRNAs for bcr-abl chimeric proteins (A-C). The PCR primers and the TaqMan probes for the bcr-abl gene rearrangement and a control fragment of the abl gene are shown. The bcr gene sequences are shown in blue whereas the abl gene sequences are shown in red.

#### RNA extraction

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

#### Preparation 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:

Primer (ABL-R) 5'-GGCCACAAAATCATACAGTGCA

Reactions conditions (per reaction):

- 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°C for 60 minutes
- RT inactivation: 70°C for 5 minutes

#### Real Time PCR

Primers and Probes:

BCR-ABL

E-1 5'-GCAGATCTGGCCCAACGAT

E-13 5'-GCATTCCGCTGACCATCAATAA

E-14 5'-CAGCCACTGGATTTAAGCAGAGT

A-2 (R) 5'-TCCAACGAGCGGATTCCT

BCR-ABL-P 6 FAM 5'-AAGCCCTTCAGCGGCCAGTAGCATCT-BHQ1

Internal control

ABL-F 5'-GCTGGGTCCCAAGCAACTAC

ABL-R 5'-ACACAGGCCCATGGTACCA

ABL-P JOE 5'-TCACGCCAGTCAACAGTCTGGAGAAACA-BHQ1

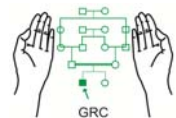
Primer mix: Make the primer mix of the six primers and the two probes at concentration of 5pmol each (Chapter 3).

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:
  - i. Denaturation at 95°C for 15 seconds



- 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.

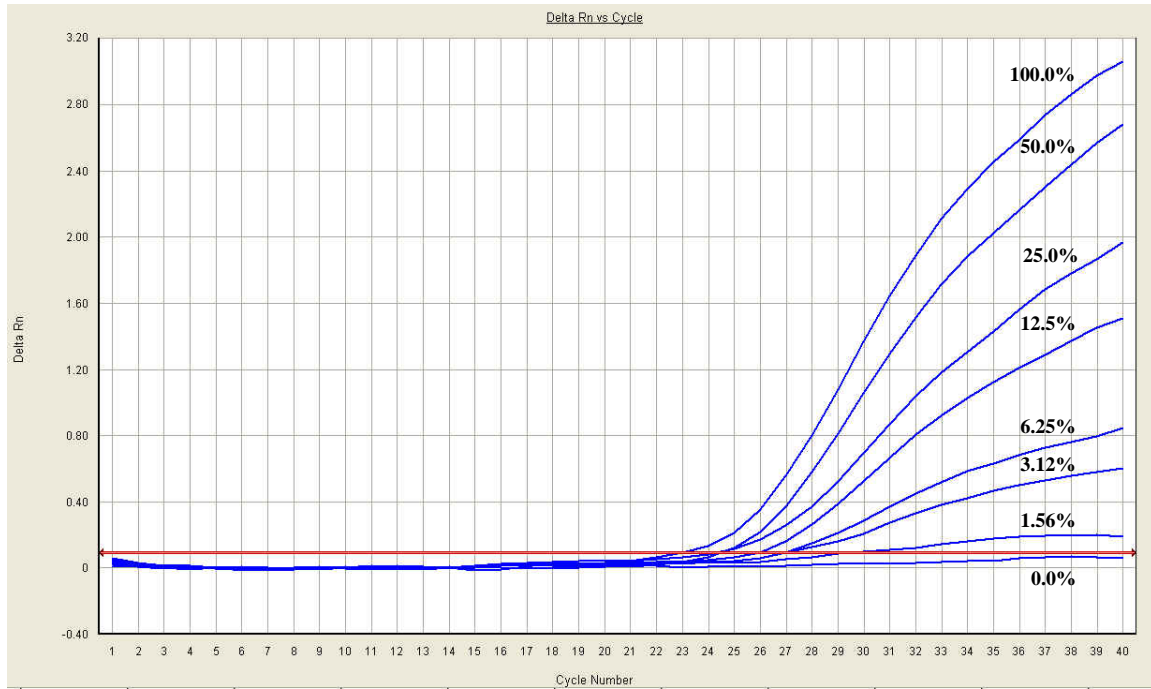


Fig. 14.3. Real time plot of the serially diluted RNA sample (100-1.56%) positive for the bcr-abl gene rearrangement and a known negative sample.

### **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

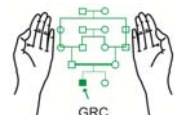
Commercial TRIzol® Reagent® (Invitrogen, USA) as described in Chapter 2.

#### 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



- 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'-CCCTCCCTGACCTGTCTCGGCC-BHQ1

##### MLL-AF4 t(4;11) (GenBank: L04284 & L13773)

Forward1: ENF-MLL 5'-CCCAAGTATCCCTGTAAAACAAAAA  
 Forward2: ENF-MLL 5'-GATGGAGTCCACAGGATCAGAGT  
 Reverse: ENR-AF4 5'-GAAAGGAAACTTGGATGGCTCA  
 Probe: ENP-AF4 FAM-5'-CATGGCCGCCTCCTTTGACAGC-BHQ1

##### TEL-AML1 t(12;21) (GenBank: U11732 & D43969)

Forward: ENF-TEL 5'-CTCTGTCTCCCCGCCTGAA  
 Reverse: ENR-AML1 5'-CGGCTCGTGCTGGCAT  
 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'-CACTCAGACCCTGAGGCTCAA  
 Probe: ENP-ABL FAM-5'-CCCTTCAGCGGCCAGTAGCATCTGA-BHQ1

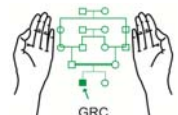
##### M-bcr t(9;22) p210 (GenBank: X02596 & X16416)

Forward: ENF-BCR 5'-TCCGCTGACCATCAAYAAGGA  
 Reverse: ENR-ABL 5'-CACTCAGACCCTGAGGCTCAA  
 Probe: ENP-ABL FAM-5'-CCCTTCAGCGGCCAGTAGCATCTGA-BHQ1

##### AML

##### SIL-TAL1 del(1) (GenBank: M74558 & S53245)

Forward: ENF-SIL 5'-CGCTCCTACCCTGCAAACA  
 Reverse: ENR-TAL 5'-CCGAGGAAGAGGATGCACA  
 Probe: ENP-SIL FAM-5'-ACCTCAGCTCCGCGGAAGTTGC-BHQ1



PML-RARA t(15;17) (GenBank: M73778 & X06538)

Forward1: ENF-PML 5'-TCTTCCTGCCCAACAGCAA  
Forward2: ENF-PML 5'-ACCTGGATGGACCGCCTAG  
Forward3: ENF-PML 5'-CCGATGGCTTCGACGAGTT  
Reverse: ENR-RARA 5'-GCTTGTAGATGCGGGGTAGAG  
Probe: ENP-RARA FAM-5'-AGTGCCCAGCCCTCCCTCGC-BHQ1

CBFB-MYH1 inv(16) (GenBank: L20298 & D10667)

Forward: ENF-CBFB 5'-CATTAGCACAAACAGGCCTTTGA  
Reverse1: ENR-MYH1 5'-AGGGCCCGCTTGGACTT  
Reverse2: ENR-MYH1 5'-CCTCGTTAAGCATCCCTGTGA  
Reverse3: ENR-MYH1 5'-CTCTTTCTCCAGCGTCTGCTTAT  
Probe: ENP-CBFB FAM-5'-TCGCGTGTCTTCTCCGAGCCT-BHQ1

AML1-ETO t(8;21) (GenBank: D43969 & D14289)

Forward: ENF-AML1 5'-CACCTACCACAGAGCCATCAAA  
Reverse: ENR-ETO 5'-ATCCACAGGTGAGTCTGGCATT  
Probe: ENP-AML1  
FAM-5'-AACCTCGAAATCGTACTGAGAAGCACTCCA-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

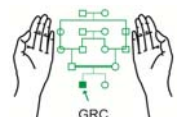
Thermal cycling

- 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

Exon5a-F	5'-CAACTCTGTCTCCTTCCTTCTTCCTAC
Exon5a-R	5'-AGCCATGGCACGGACGCG
	65–60°C touchdown 0.5°C/cycle for 10 cycles
Exon5b-F	5'-CTCCTGCCCCGGCACCCGC
Exon5b-R	5'-CTAAGAGCAATCAGTGAGGAATCAGA
	65–60°C touchdown 0.5°C/cycle for 10 cycles
Exon6-F	5'-CAACCACCCTTAACCCCTCCT
Exon6-R	5'-AGACGACAGGGCTGGTTGC
	68–58°C touchdown 1.0°C/cycle for 10 cycles
Exon7-F	5'-AGGCGCACTGGCCTCATC
Exon7-R	5'-GAGGCTGGGGCACAGCA
	68–58°C touchdown 1.0°C/cycle for 10 cycles
Exon8-F	5'-GACCTGATTCCTTACTGCCTCTTG
Exon8-R	5'-AATCTGAGGCATAACTGCACCCTT
	63.5–58.5°C touchdown 0.5°C/cycle for 10 cycles

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

### **BRCA1 and BRCA2 mutations**

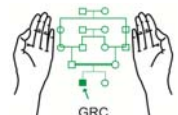
BRCA1 & BRCA2 (Breast Cancer) tumour suppressor genes are located on chromosomes 17 and 13 respectively. A large number of mutations have been described in the two genes that are strongly associated with familial breast and ovarian cancer.

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

BRCA1-F	5'-AAAAGATATAGATGTATGTTTTGCTAATGTGT
Exon 2-R	5'-TCCCAAATTAATACTCTTGTGCTGA



BRCA1-F 5'-GAGTGGTGGGGTGAGATTTTTGTC  
 Exon 20-R 5'-CCTGATGGGTTGTGTTTGGTTTCT  
 BRCA2-F 5'-CGAAAATTATGGCAGGTTGTTACG  
 Exon 11-R 5'-GCTTTCCACTTGCTGTAATAATCCA

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'-AGCATTGGTTTTAAATTATGGAGTATATT

JAK2-Control-F (5pmol) 5'-ATCTATAGTCATGCTGAAAGTAGGAGAAAAG

JAK2-Common-R (10pmol) 5'-CTGAATAGTCCTACAGTGTTCAGTTTCA

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)

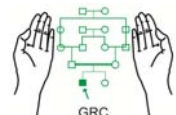
Thermal cycling

- 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.



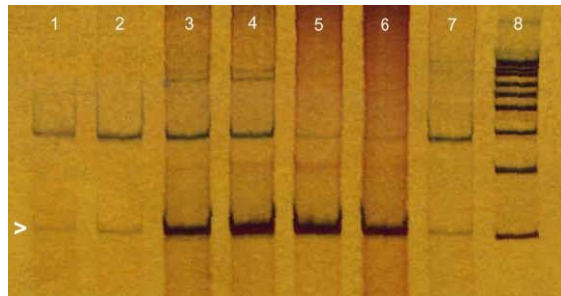


Fig. 14.3. Silver stained PAGE of PCR for JAK-2 mutation. Arrow pointing at the lanes 3-6 show positive while the lanes 1,2 & 7 show negative result.

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