Molecular Diagnosis

Nucleic acid based testing in Oncology

Objectives

- Describe uses of NAT in
  - Oncology
    - Diagnosis, Prediction, monitoring.
  - Genetics
    - Screening, presymptomatic testing, diagnostic testing, family studies.
- Identify correct test approaches
- Evaluate clinical significance of tests
- Evaluate Impact of genetic test results on individuals/families (ethical considerations)

Molecular Diagnosis

- “Nucleic Acid Testing”
  - Use of specific sequence information in nucleic acids - DNA and RNA - for clinical management

Nucleic Acid Testing - Oncology

- Gross alterations in DNA content of tumors (ploidy)
- Gain/Loss of nucleic acids
- Markers of Clonality
- Oncogene/Tumor Suppressor gene mutations
- Tumor Specific Translocations
- mRNA “molecular staging”
- Gene Expression profiling

Gain/Loss of Nucleic Acid

- **Diagnosis of specific entities:**
  - **1p/19q in oligodendroglioma; CLL, trisomy 12; 5q- refractory anemia; 3p- in renal clear cell carcinoma**
- **Prognostic/Predictive changes**
  - ***MYCN* amplification in neuroblastoma**
  - ***HER-2/neu* amplification in Breast CA**
- **Screening/monitoring for neoplasms**
  - **"Urovysion" .**

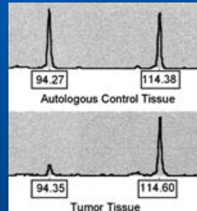
MYCN and Neuroblastoma

Feature	Type 1	Type 2	Type 3
MYCN	Normal	Normal	Amplified
DNA ploidy	Hyperdiploid or near triploid	Near diploid or near tetraploid	Near diploid or near tetraploid
1p LOH	<5%	25–50%	80–90%
14q LOH	<5%	25–50%	<5%
TRK-A exp.	High	Low	Low or absent
TRK-B exp.	Low or truncated	Low or absent	High (full length)
TRK-C exp.	High	Low or absent	Low or absent
Age	Usu. <1 year	Usu. >1 year	Usu. 1–5 years
Stage	Usu. 1, 2, 4S	Usu. 3, 4	Usu. 3, 4
3-Year survival	95%	25–50%	<5%

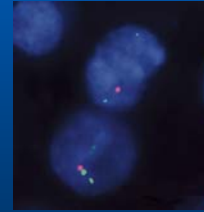
Exp., expression; Usu., usually.

Chromosome 1p, 19q and Gliomas

- 1p & 19q deletions define Oligodendroglioma.
- Detected either by: FISH or PCR –based Techniques



PCR amplification of microsatellites – normal vs tumor tissue.

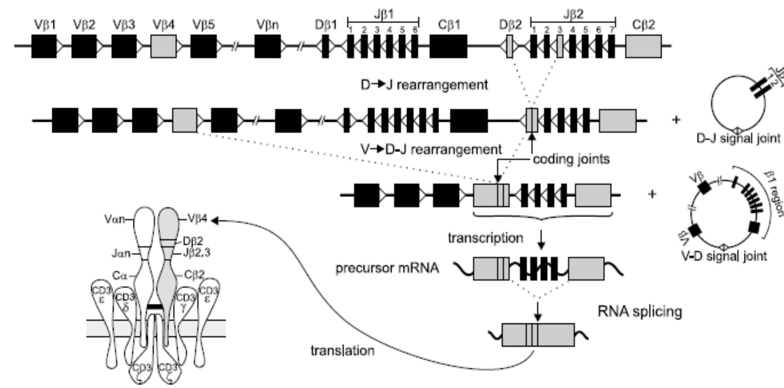


FISH: green = centromere red = 1p.

Molecular Markers of Clonality

- Antigen receptor gene rearrangements.
  - Southern Blotting: IgH, TCR; EBV termini.
  - PCR: Ig and TCR gene rearrangement.
- X-inactivation.
  - Human androgen receptor assay.
- Microsatellite allelotyping.
  - Altered microsatellites in lesional tissue vs germline (PCR amplification)
- Clonal Viral Integration
  - EBV termini, by Southern Blotting

Antigen Receptor Gene Rearrangement



Unique V-D-J rearrangements for each event.

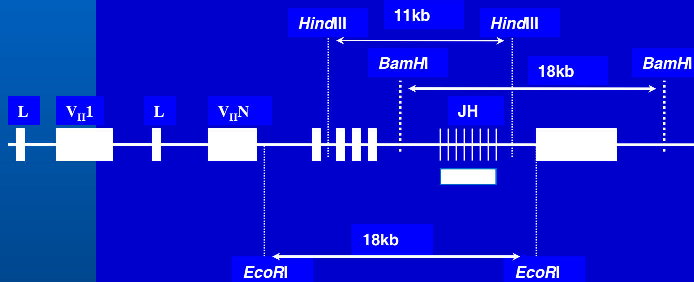
Antigen Receptor Gene Rearrangement

- **Somatic Ig & TCR gene rearrangement:**
  - **V-D-J recombination**
  - **B- & T-cell repertoire diversity**
  - **Combinatorial Diversity**
    - **Use of different V-D- and J segments**
  - **Junctional Diversity**
    - **Addition or removal of nucleotides between D-J, V-D, or V-J segments.**
      - **Greatest contribution.**

Antigen Receptor Gene Rearrangement

- Clonality Testing
  - Each lymphoma arises from a single cell w/ a unique Antigen receptor gene rearrangement.
  - Inflammatory infiltrates
    - Lymphocytes w/ different rearrangements.
  - Tests for clonality
    - Southern Blotting
    - PCR-based tests

Southern Blotting for Clonality



Immunoglobulin heavy chain locus - restriction enzyme digestion sites

Southern Blotting for Clonality

- Test based on combinatorial diversity
- Digest high DNA w/ restriction enzyme
- Separate resulting fragments by size
  - Electrophoresis (agarose gel)
- Transfer to membrane, immobilize, denature, hybridize with a probe.

PCR-based Tests for Clonality

- Primers w/c bind to relatively invariant sites flanking sites of recombination
  - No amplification w/o recombination (primer binding sites too far)
  - Polyclonal lymphoid population
    - Mixture of PCR products (combinatorial and junctional diversity)
  - Clonal lymphoid population
    - Single Predominant PCR product
    - Differentiate by
      - Plain Electrophoresis
      - Capillary Electrophoresis
      - “heteroduplex analysis”
      - Denaturation – either on gel, or w/ “real-time” PCR

a

VH DH JH

6 VH-FR1 primers

JH primer

CTGTGCAAGAGCGGGCTATGGTTCAGGGAGTTATGGCTACTACGGTATGGACGTCTGG
CTGTGCAAGAGGACGAAACAGTAACTGCCTACTACTACTACGGTATGGACGTCTGG
CTGTGCAAGAGAGATAGTATAGCAGCTCGTACAACTGGTTCGACTCCTGG
CTGTGCAAGAGATCCGGGCAGCTCGTTTTGCTTTTGATATCTGG
CTGTGCAAGAGCCTCTCTCCACTGGGATGGGGGGCTACTGG
CTGTGCAAGAGCAGCAGCTCGGCCCCCTTTGACTACTGG
CTGTGCAAGAGGACTTTGGATGCTTTTGATATCTGG
CTGTGCAAGAGGGTGGGAGCTACTAGACTACTGG
CTGTGCAAGGGTAGCTAAACCTTTGACTACTGG
CTGTGCAATATCTACTTTGACTACTGG

b

Heteroduplex analysis

monoclonal cells | monoclonal cells in polyclonal background | polyclonal cells

denaturation (94°C) / renaturation (4°C)

MW | Monoclonal | Monoclonal | Polyclonal | H_2O

heteroduplexes

homoduplexes

c

GeneScanning

polyclonal

monoclonal

No DNA + cont Sample

Clonal

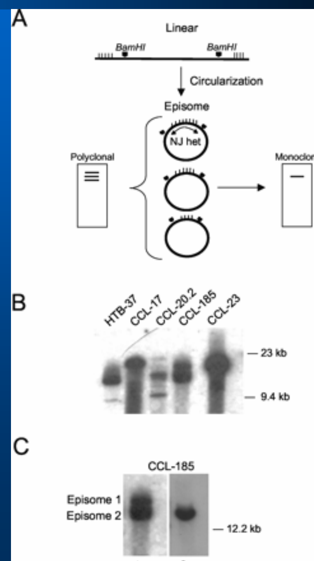
118bp

Southern Blotting vs PCR-based Tests

- Southern
  - Needs High MW DNA
  - 5-10 μg DNA
  - Labor intensive
  - Tech. demanding
  - At least 5% neoplastic cells
  - Detects most recombinations
  - Does not detect junctional diversity
- PCR
  - Can use partially degraded DNA
  - 0.1-1 μg DNA
  - Quick
  - Simple
  - May go down to 1%
  - Up to 30% of rearrangements not detected due to primer binding.
  - Junctional diversity may be used to follow clone.

Clonal EBV-Integration

- EBV-associated neoplasms:
  - Postr-transplant lymphoproliferative disorder (PTLD)
  - Nasopharyngeal CA
  - AIDS-associated “leiomyosarcoma” (pediatric)



Oncogene/Tumor suppressor mutations

- KIT/PDGFRA mutations in gastrointestinal stromal tumors
  - Target for treatment w/ Imatinib
- EGFR mutations in Lung CA
  - Adenocarcinomas in non-smokers
  - Response to treatment w/ Iressa
- PIK3CA and PTEN mutations
  - Activation of AKT pathway
  - Susceptibility to Rapamycin-type agents.

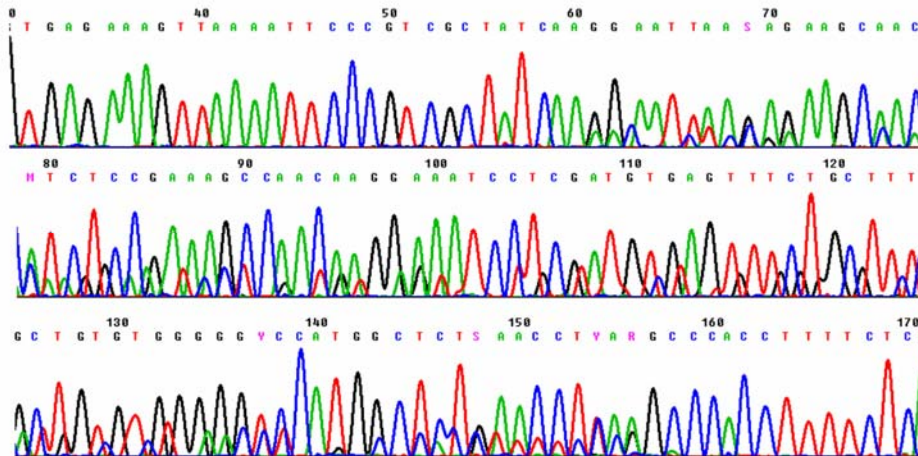
Oncogene/TSP mutations

- Oncogene mutations:
  - Activating mutations
  - Limited number of mutations/gene
  - Tests to detect specific mutations.
- Tumor Suppressor Gene mutations
  - Inactivating mutations
  - Many possible mutations
    - Occasionally hot-spots, related to gene structure and/or mutagen.

Somatic mutation tests

- Recurrent/Known mutations:
  - Tests to detect specific sequences
    - Probes, restriction enzymes, sequence-specific primers, etc.
    - Can detect mutation in minority of cells.
    - Use of sequence-specific primers can enrich for mutant sequence.
- Tests for unknown mutations
  - Mutation scanning techniques
  - Direct Sequencing.

EGFR: in-frame 15bp Exon19 deletion



Direct sequencing: mixture of tumor and normal – lower mutant peaks

Tumor-specific translocations

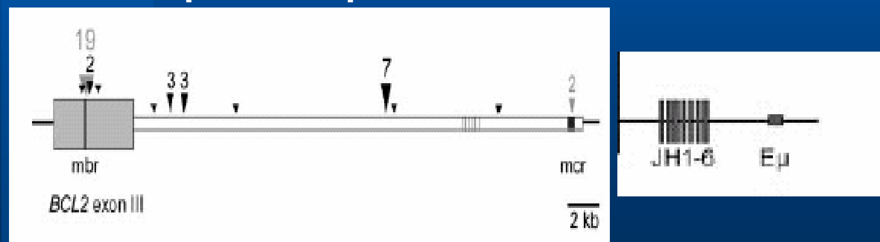
- Translocations w/o fusion gene products
  - Dysregulation of gene expression, no fusion mRNA
  - Detect translocation by:
    - Classical cytogenetics, FISH, Southern Blotting or DNA PCR.
  - DNA PCR for translocations
    - Requirement for recurrent breakpoints OR
    - Long range PCR.

Translocations w/o Fusion RNA

Tumor	Translocation	Activated Gene	Mechanism of Activation
B-All/Burkitt	t(8;14)(q24;q32)	MYC	Relocation to IgH locus
Large Cell Lymphoma	t(3;14)(q27;q32)	BCL6	Relocation to IgH locus
Mantle Cell Lymphoma	t(11;14)(q13;q32)	Cyclin D1	Relocation to IgH locus
Follicular B-cell lymphoma	t(14;18)(q32;q21)	BCL2	Relocation to IgH locus
T-cell ALL	t(8;14)(q24;q11)	MYC	Relocation to TCR α/δ locus
T-cell ALL	t(1;14)(p32;q11)	TAL1	Relocation to TCR α/δ locus

DNA PCR for translocations: BCL2

- t(14;18)(q32;q21)
  - BCL2 placed under immunoglobulin enhancer – overexpressed – loss of apoptosis – follicular lymphoma .
  - 14q32 breakpoints constant.
  - 18q21 breakpoints variable:



DNA PCR for translocations

- Variable breakpoints:
  - Not all translocations will be amplified by a limited primers sets.
    - E.g., BCL2 – 60%, BCL1 – 50%
  - Need for “long range” PCR to increase detection
    - Technically difficult.
    - Needs high quality DNA – will not work on paraffin.

Translocations w/ fusion RNA - Heme

TUMOR	Translocation	Gene fusion
Chronic myelogenous leukemia	t(9;22)	BCR-ABL(p210)

Acute promyelocytic leukemia	t(15;17);
	t(11:17)(q23;q21);
	t(5;17) (q35;q21);
	t(11;17)(q13;q21)
	der(17)
		PML-RAR
		PLZF-RAR
		NPM-RAR
		NUMA-RAR
		STAT5b-RAR

AML	t(8;21)(q22;q22)	AML1-ETO
AML and ALL (esp. infants and post-Rx)	11q23	MLL-(~40 partners)
Anaplastic large cell lymphoma (pediatric)	t(2;5)(p23;q35)	NPM-ALK
ALL	t(9;22)	BCR-ABL(p190)
MALT lymphoma	t(11;18)	API2-MLT

Translocations w/chimeric products: solid tumors

Tumor	Translocation	Product
Ewing's Sarcoma	t(11;22); t(21;22); t(7;22); t(12;22)	EWS/FLI1; EWS/ERG; EWS/ETV1; EWS/ETV4
Alveolar Rhabdomyosarc.	t(1;13); t(2;13)	PAX7/FOXOA1; PAX3/FOXOA1
Synovial sarcoma	t(X;18)	SYT/SSX1
DSRCT	t(11;22)	EWS/WT1
Myxoid/round cell liposarcoma	t(12;22)	CHOP/FUS
Clear cell sarcoma soft parts	t(12;22)	EWS.ATF-1
Extraskeletal myxoid chondrosarc	t(9;22)	EWS/TEC

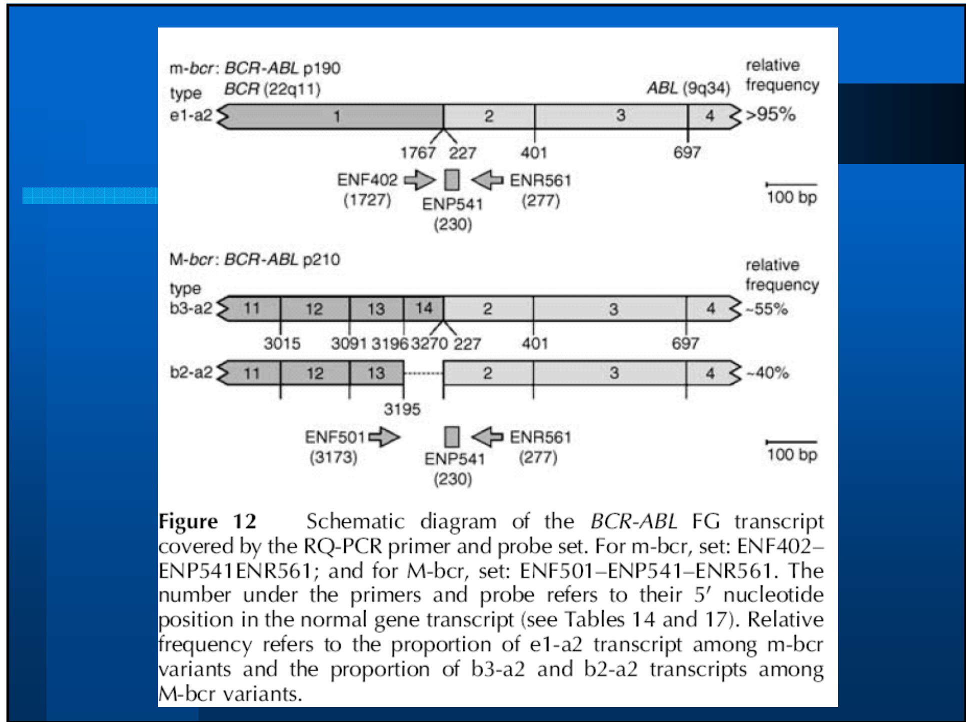
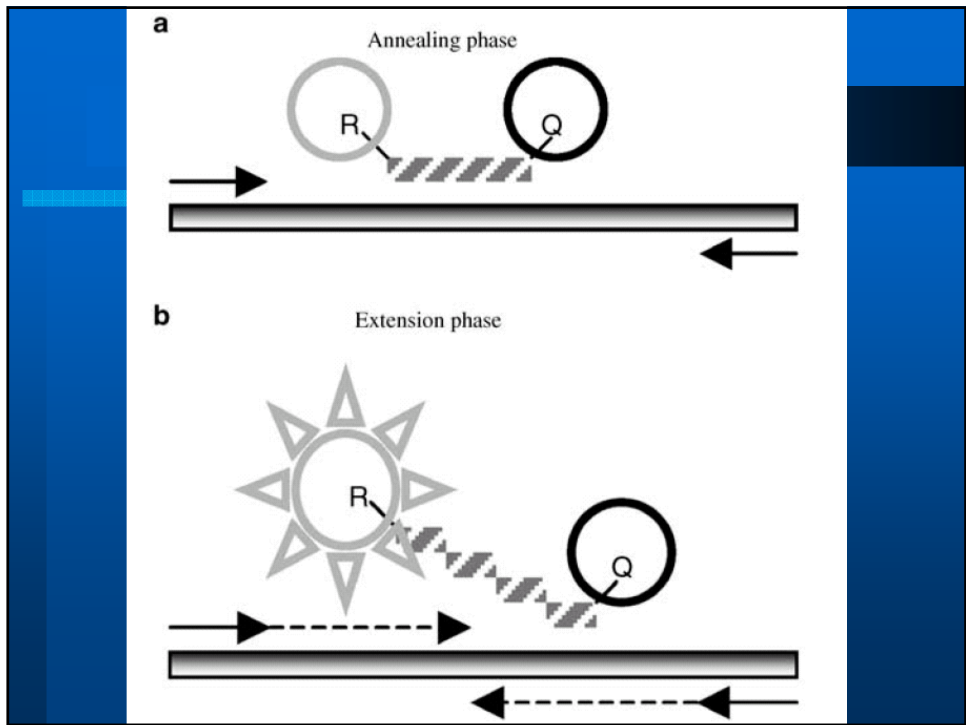


Figure 12 Schematic diagram of the *BCR-ABL* FG transcript covered by the RQ-PCR primer and probe set. For m-bcr, set: ENF402–ENP541ENR561; and for M-bcr, set: ENF501–ENP541–ENR561. The number under the primers and probe refers to their 5′ nucleotide position in the normal gene transcript (see Tables 14 and 17). Relative frequency refers to the proportion of e1-a2 transcript among m-bcr variants and the proportion of b3-a2 and b2-a2 transcripts among M-bcr variants.



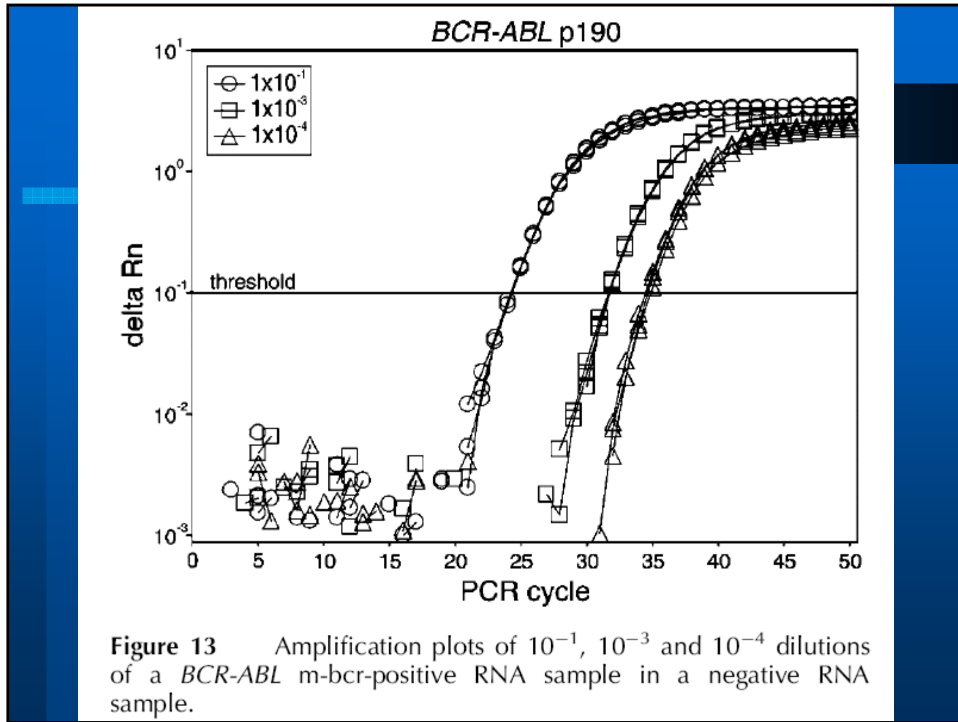


Figure 13 Amplification plots of 10^{-1}, 10^{-3} and 10^{-4} dilutions of a *BCR-ABL* m-bcr-positive RNA sample in a negative RNA sample.