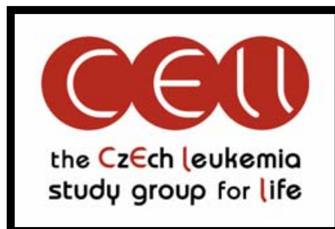
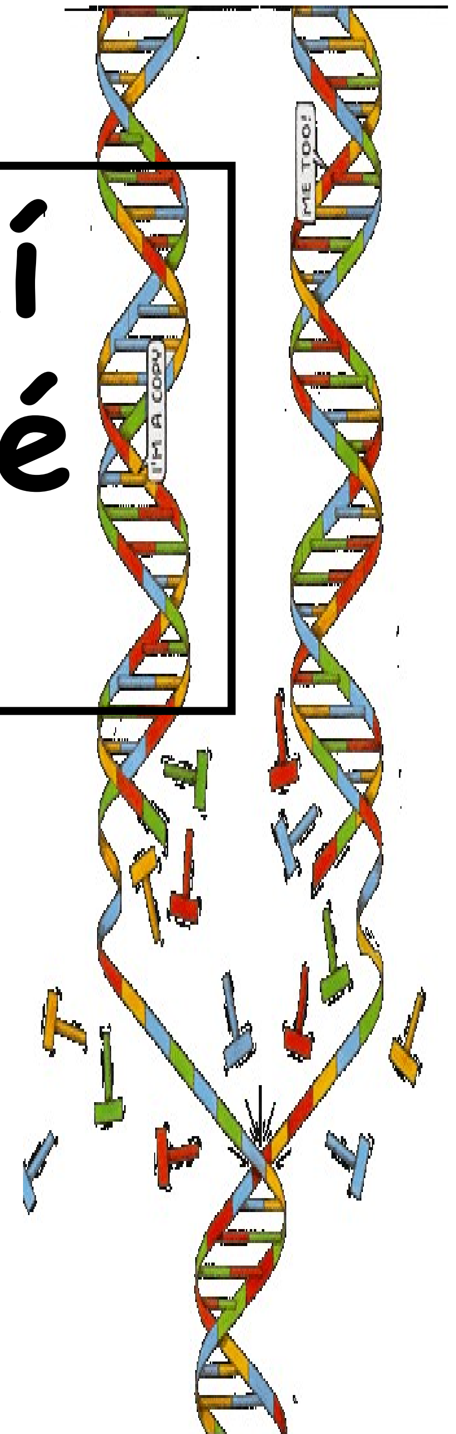
Možnosti molekulární diagnostiky v klinické praxi

Centrum molekulární biologie a genové terapie
Interní hematoonkologická klinika
Fakultní nemocnice Brno a MU Brno

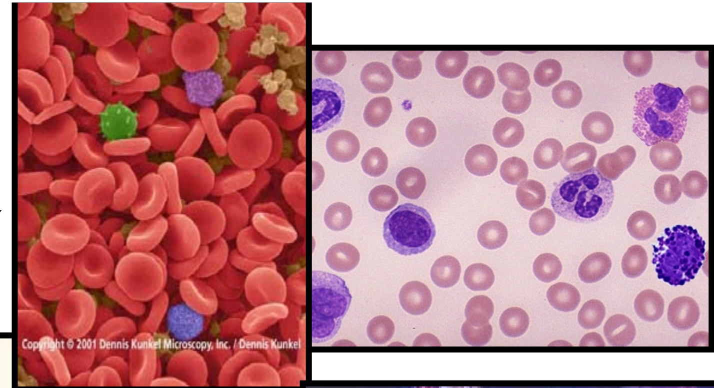




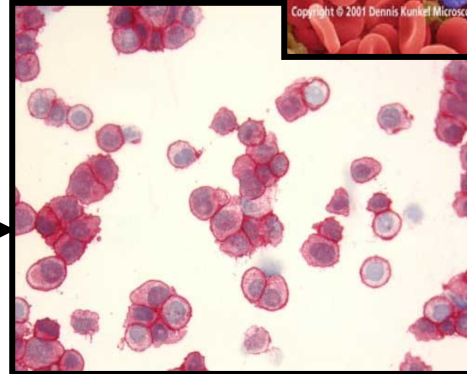
Klinická praxe

Vyšetřovací metody

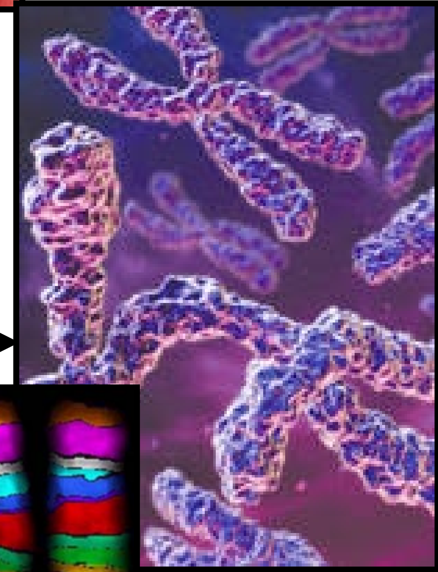
morfologické



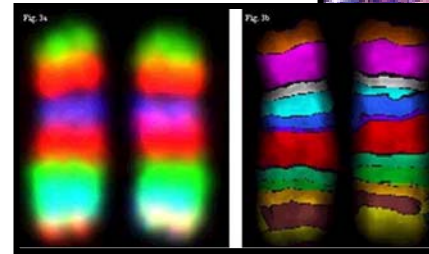
imunologické



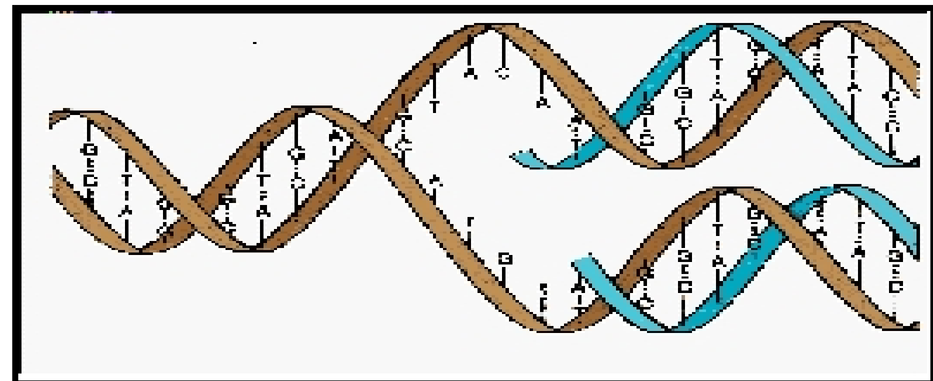
cytogenetické



molekulárně cytogenetické



molekulárně genetické



Molekulární diagnostika

- Vrozené genetické choroby - DNA změny vrozené (monogenní onemocnění, chromozomální poruchy, multifaktoriální poruchy)
- Přímá-nepřímá diagnostika
- Onkologické choroby - DNA změny získané
- Diagnostika patogenů - DNA cizorodá

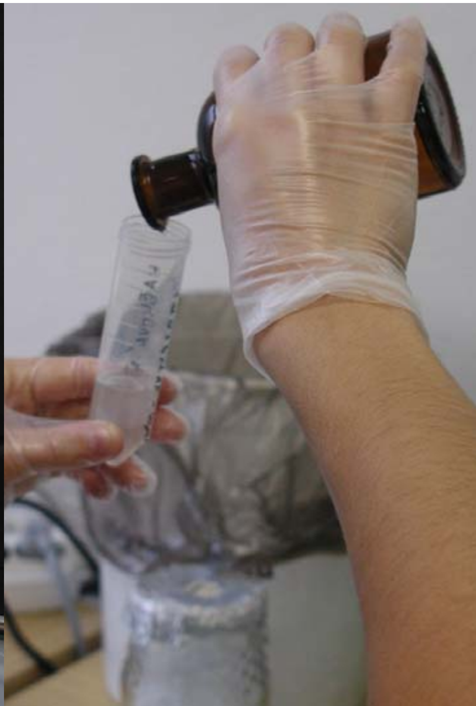
Molekulární diagnostika

- Detekce přenašečů patologické alely
- Prenatální diagnostika

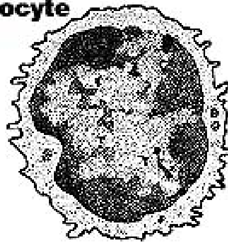
- Včasné stanovení diagnózy
- Diferenciální diagnostika
- Minimální zbytkové onemocnění

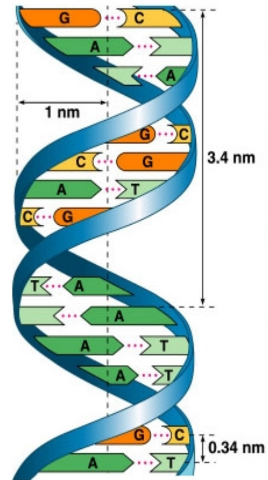
Molekulární diagnostika

- Specificita a senzitivita
- Rychlost výsledku a možnost rychlé terapeutické intervence

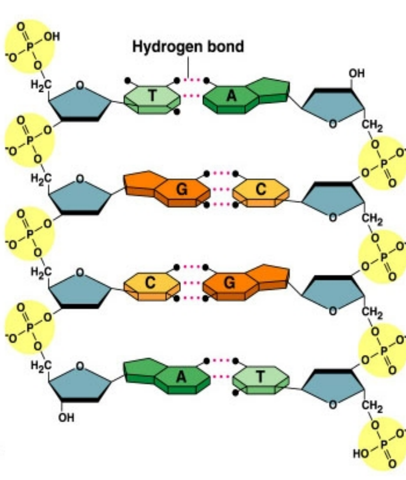


Lymphocyte

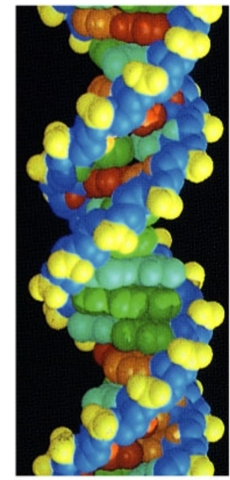




(a) Key features of DNA structure



(b) Partial chemical structure



(c) Space-filling model

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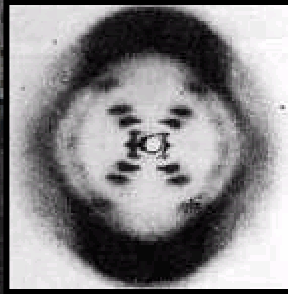
Nobel Prize in physiology/medicine in 1962

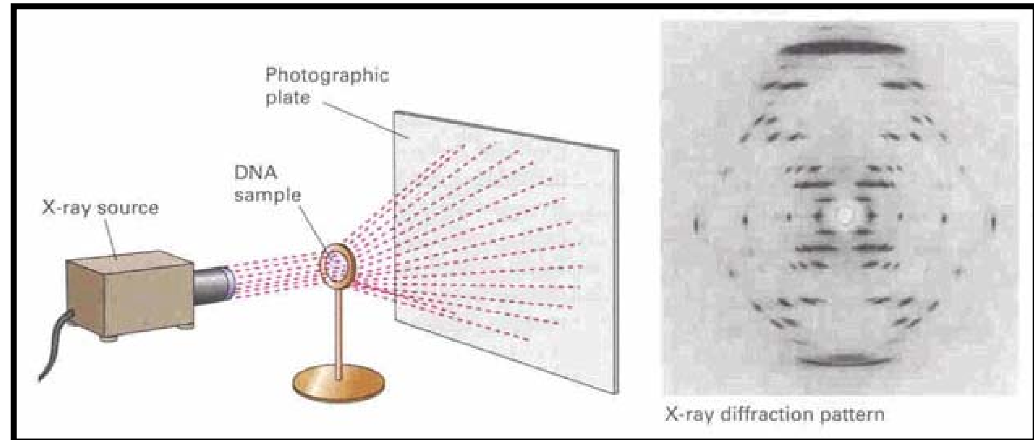
1 ctcgaggggc ctagacattg ccctccagag agagcaccca acaccctcca ggcttgaccg
61 gccagggtgt ccccttccta ccttggagag agcagcccca
121 tggga ctg cctccctcca
181 tggga cga caacactggc aaactcctac tcatccacga
241 aggcc cca gcctggcagt c
301 tagtg tggg ggtgtctctg a
361 aagcc ctcc ccccggccct g
421 tcctac tcc ctccctacac ac
481 catgtc gag cactgtggcg t
541 gtcctg aaa gggcggaagg
601 ggtg ggcag gagagccaa cg
661 gcccc ccagg gtgggacata gc
721 acagg catg gtctcatcct cc
1501 gtctggggac aggggtgtgg ggacaggggt g
1681 aggggtcccg gggacagggg tgtggggaca
1741 gggtctgagg acaggggtgt gggcacaggg
1801 gtcctgggga caggggtctg gggac
1861 tctcctggtc taatgtggaa agtggc

James Dewey Watson

Francis Harry Compton Crick

Maurice Hugh Frederick Wilkins





Rosalind Elsie Franklin (1920-1958)

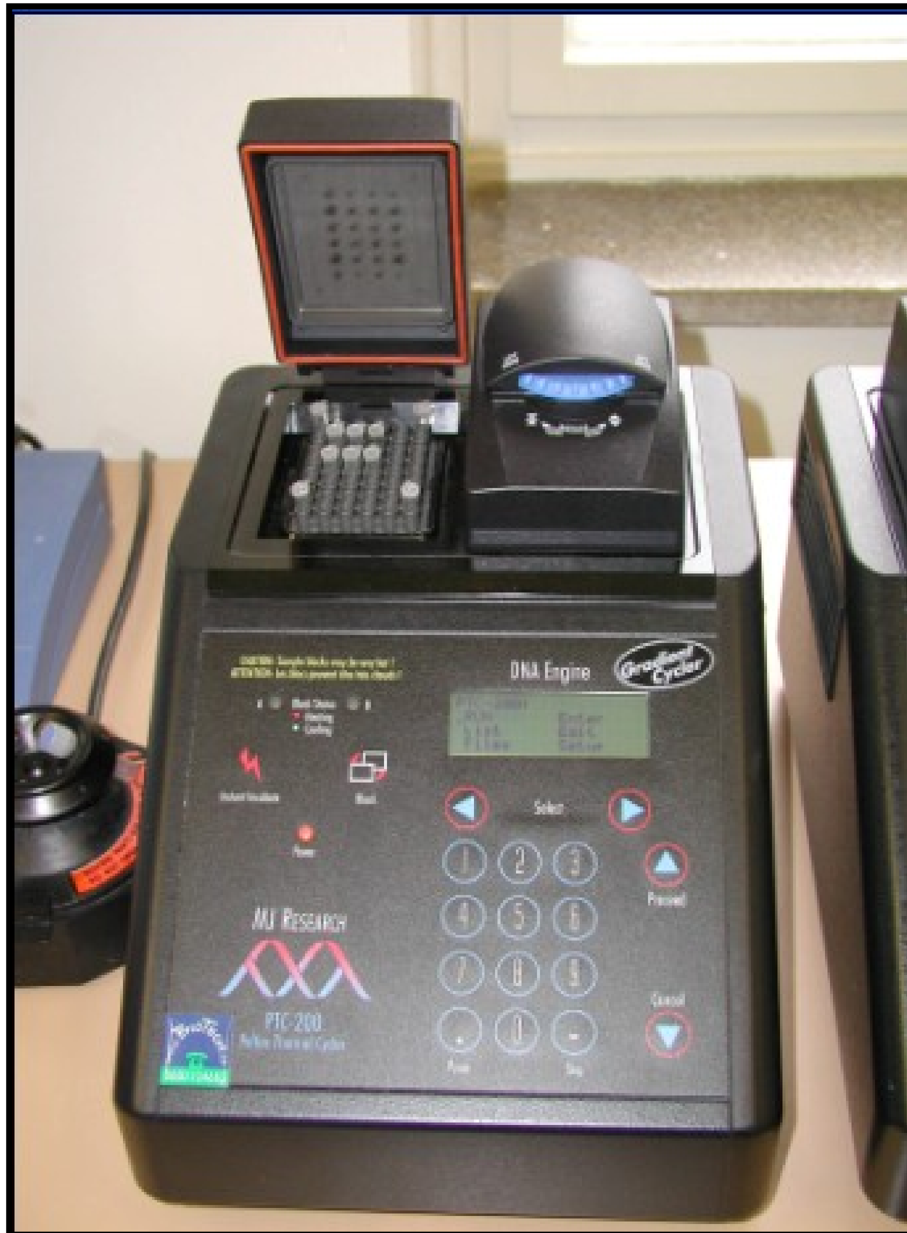
Who (really) discovered the structure of DNA?

In April of 1953, James Watson, Francis Crick and Maurice Wilkins identified the substance of life - the structure of DNA. They later shared a Nobel Prize. Their discovery depended heavily on the work of a woman, chemist **Rosalind Franklin**, whose research was used **without her knowledge or permission**. Watson's memoir of the discovery dismisses Franklin as frumpy, hostile and unimaginative. A later work by a friend casts Franklin as a feminist icon, cheated of recognition.
It was Franklin's photograph of the DNA molecule that sparked a scientific revolution. Wilkins showed Watson the photo, **Watson said, *"My jaw fell open and my pulse began to race „*** *The* photo showed, for the first time, the essential structure of DNA - the double-helix shape, which also indicated its method of replication.

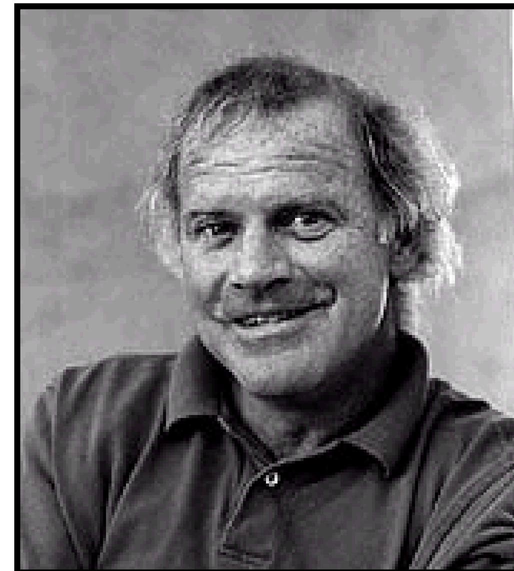
Metody molekulární diagnostiky

1. PCR
2. PCR
3. PCR

The inventor of the polymerase chain reaction while at Chiron Corporation in the late 1980s, Mullis was *R&D Magazine's 1991 Scientist of the Year* and was honored by the Nobel Committee two years later. PCR revolutionized biotechnology by allowing a fragment of genetic code to be identified and reproduced indefinitely. Applications include tests for genetic diseases and mutations, forensic research, and identification and diagnosis of viral infection. PCR helped create the new research field of molecular archaeology by allowing the study of the genetic code of extinct species. New uses for the technique are being developed every day.



PTC200 (MJR,USA,cca 250000 Kč)

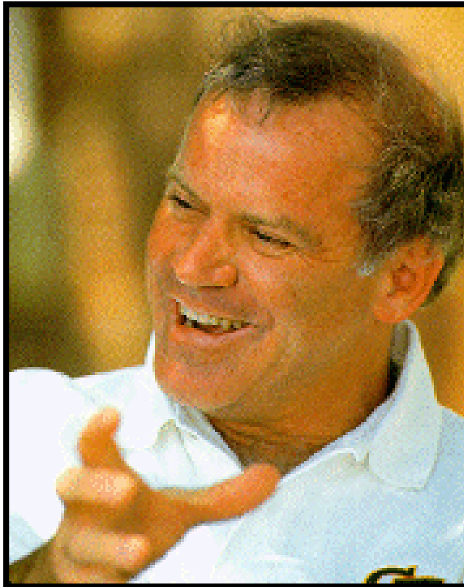


1983 K. Mullis

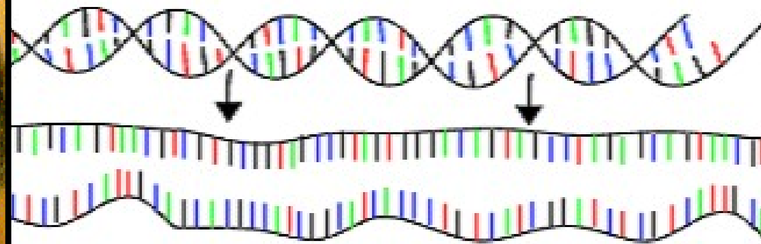
1985 publikace

1993 Nobelova cena za chemii

PCR : Polymerase Chain Reaction

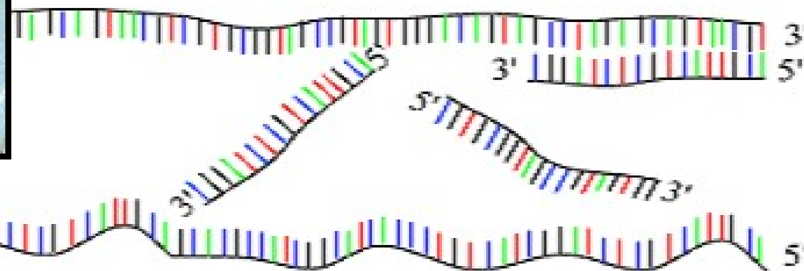


30 - 40 cycles of 3 steps :



Step 1 : denaturation

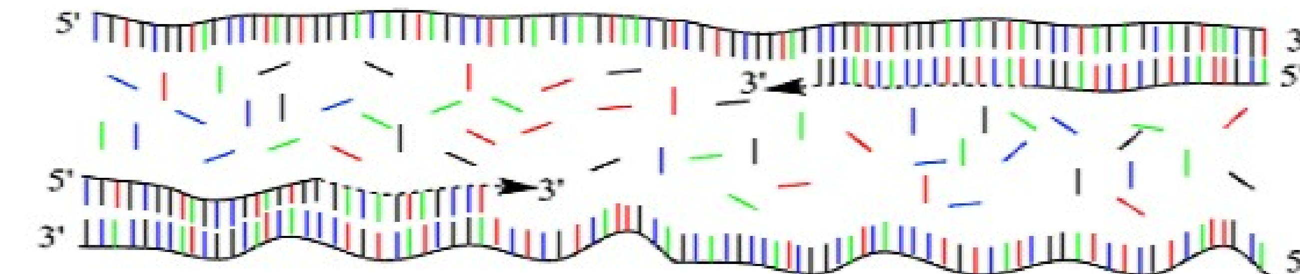
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!



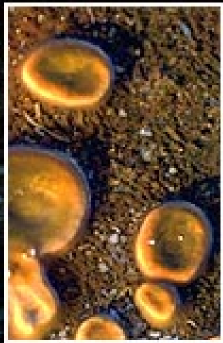
Step 3 : extension

2 minutes 72 °C

only dNTP's

(Andy Vierstraete 1999)

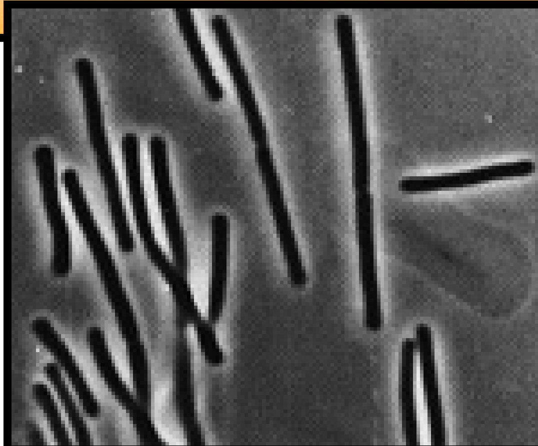
1st cycle 2^2=4 copies **35th cycle 2^{36} = 68 billion copies**

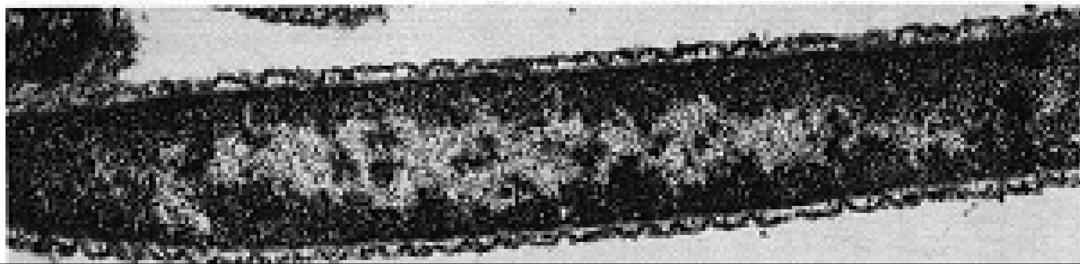


Life at High Temperatures
by Thomas D. Brock

Hot spring near Great Fountain Geyser

Yellowstone River (1964)





RESEARCH ARTICLE

Enzymatic Amplification of β-Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia

Randall K. Saiki, Stephen Scharf, Fred Faloona, Kary B. Mullis
Glenn T. Horn, Henry A. Erlich, Norman Arnheim

Abstract. *Two new methods were used to establish a rapid and highly sensitive prenatal diagnostic test for sickle cell anemia. The first involves the primer-mediated enzymatic amplification of specific β-globin target sequences in genomic DNA, resulting in the exponential increase (220,000 times) of target DNA copies. In the second technique, the presence of the β^A and β^S alleles is determined by restriction endonuclease digestion of an end-labeled oligonucleotide probe hybridized in solution to the amplified β-globin sequences. The β-globin genotype can be determined in less than 1 day on samples containing significantly less than 1 microgram of genomic DNA.*

Recent advances in recombinant DNA technology have made possible the molecular analysis and prenatal diagnosis of several human genetic diseases. Fetal DNA obtained by aminocentesis or chorionic villus sampling can be analyzed by restriction enzyme digestion, with subsequent electrophoresis, Southern transfer, and specific hybridization to cloned gene or oligonucleotide probes. With polymorphic DNA markers linked genetically to a specific disease locus, segregation analysis must be carried out with restriction fragment length polymorphisms (RFLP's) found to be informative by examining DNA from family members (*1*, *2*).

Many of the hemoglobinopathies, however, can be detected by more direct methods in which analysis of the fetus alone is sufficient for diagnosis. For example, the diagnosis of hydrops fetalis (homozygous α-thalassemia) can be made by documenting the absence of any α-globin genes by hybridization with an α-globin probe (*3–5*). Homozygosity for certain β-thalassemia alleles can be determined in Southern transfer experiments by using oligonucleotide probes that form stable duplexes with the normal β-globin gene sequence but form unstable hybrids with specific mutants (*6*, *7*).

Sickle cell anemia can also be diagnosed by direct analysis of fetal DNA. This disease results from homozygosity of the sickle-cell allele (β^S) at the β-globin gene locus. The S allele differs from the wild-type allele (β^A) by substitution of an A in the wild-type to a T at the second position of the sixth codon of the β chain gene, resulting in the replacement of a glutamic acid by a valine in the expressed protein. For the prenatal diagnosis of sickle cell anemia, DNA obtained by amniocentesis or chorionic villus sampling can be treated with a restriction endonuclease (for example, Dde I and Mst II) that recognizes a sequence altered by the β^S mutation (*8–11*). This generates β^A- and β^S-specific restriction fragments that can be resolved by Southern transfer and hybridization with a β-globin probe.

We have developed a procedure for the detection of the sickle cell mutation that is very rapid and is at least two orders of magnitude more sensitive than standard Southern blotting. There are two special features to this protocol. The first is a method for amplifying specific β-globin DNA sequences with the use of oligonucleotide primers and DNA polymerase (*12*). The second is the analysis of the β-globin genotype by solution hybridization of the amplified DNA with a specific oligonucleotide probe and subsequent digestion with a restriction endonuclease (*13*). These two techniques increase the speed and sensitivity, and lessen the complexity of prenatal diagnosis for sickle cell anemia; they may also be generally applicable to the diagnosis of other genetic diseases and in the use of DNA probes for infectious disease diagnosis.

Sequence amplification by polymerase chain reaction. We use a two-step procedure for determining the β-globin genotype of human genomic DNA samples. First, a small portion of the β-globin gene sequence spanning the polymorphic Dde I restriction site diagnostic of the β^A allele is amplified. Next, the presence or absence of the Dde I restriction site in the amplified DNA sample is determined by solution hybridization with an end-labeled complementary oligomer followed by restriction endonuclease digestion, electrophoresis, and autoradiography.

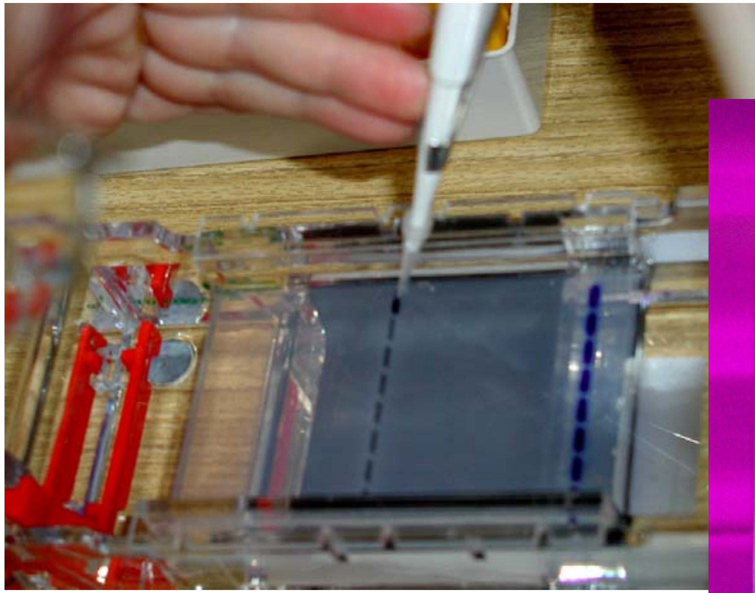
The β-globin gene segment was amplified by the polymerase chain reaction (PCR) procedure of Mullis and Faloona (*12*) in which we used two 20-base oligonucleotide primers that flank the region to be amplified. One primer, PC04, is complementary to the (+)-strand and the other, PC03, is complementary to the (−)-strand (Fig. 1). The annealing of PC04 to the (+)-strand of denatured genomic DNA followed by extension with the Klenow fragment of *Escherichia coli* DNA polymerase I and deoxynucleotide triphosphates results in the synthesis of a (−)-strand fragment containing the target sequence. At the same time, a similar reaction occurs with PC03, creating a new (+)-strand. Since these newly synthesized DNA strands are themselves template for the PCR primers, repeated cycles of denaturation, primer annealing, and extension result in the exponential accumulation of the 110–base pair region defined by the primers.

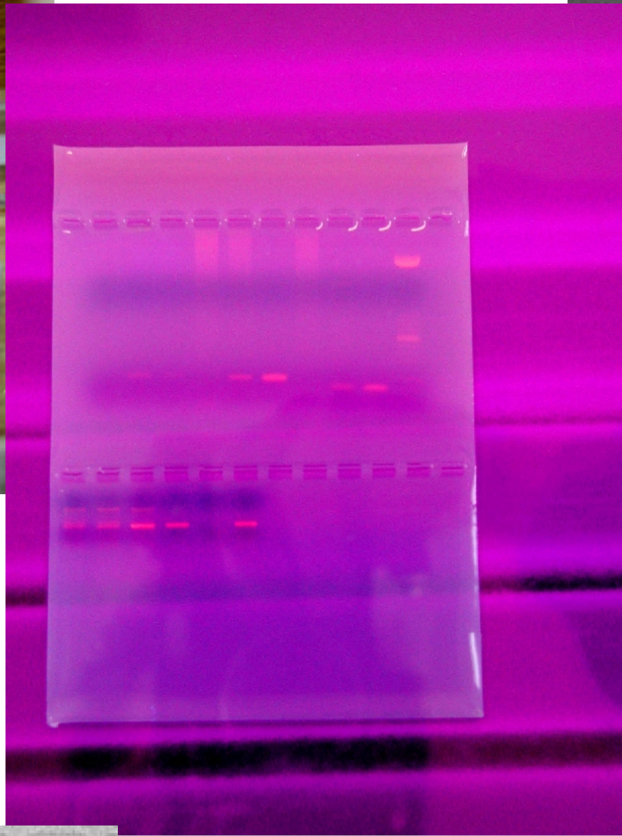
An example of the degree of specific gene amplification achieved by the PCR method is shown in Fig. 2A. Samples of DNA (1 μg) were amplified for 20 cycles and a fraction of each sample, equivalent to 36 ng of the original DNA, was subjected to alkaline gel electrophoresis and transferred to a nylon filter. The filter was then hybridized with a ^{32}P-labeled 40-base oligonucleotide probe, RS06, which is complementary to the target sequence (Fig. 1A) but not to the PCR primers. The results, after a 2-hour autoradiographic exposure, show that a fragment hybridizing with the RS06 probe

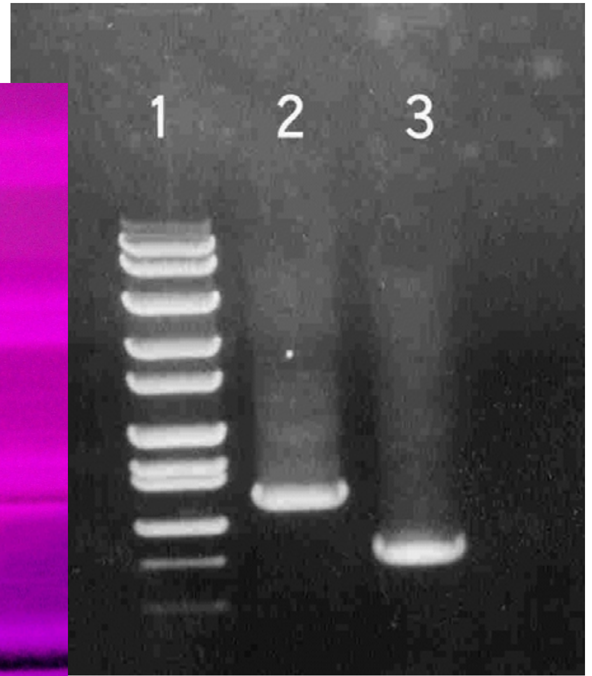
The authors are in the Department of Human Genetics, Cetus Corporation, 1400 Fifty-Third Street, Emeryville, California 94608. The present address for N.A. is Department of Biological Sciences, University of Southern California, Los Angeles 90089-0371.

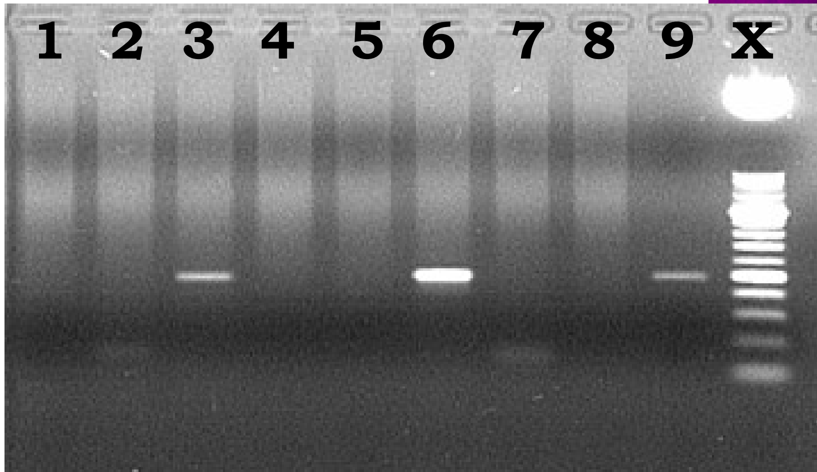
Science, 230 (4732): 1350-1354, 1985

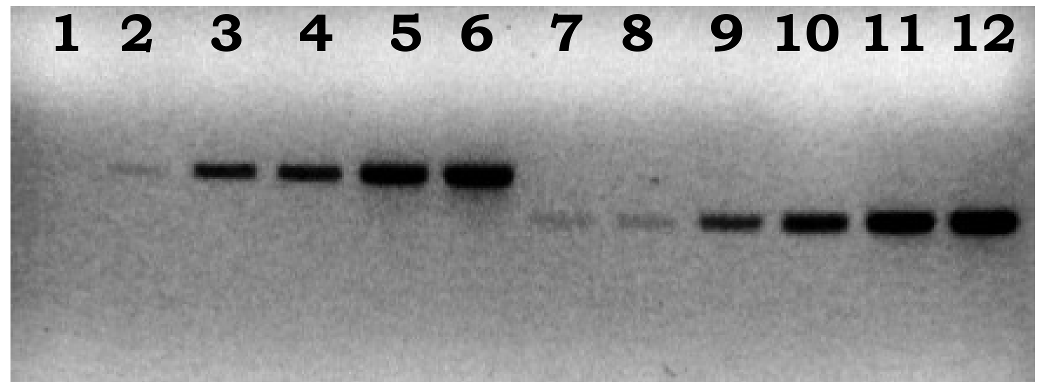
TaqDNA Polymerase, 1989






1 2 3

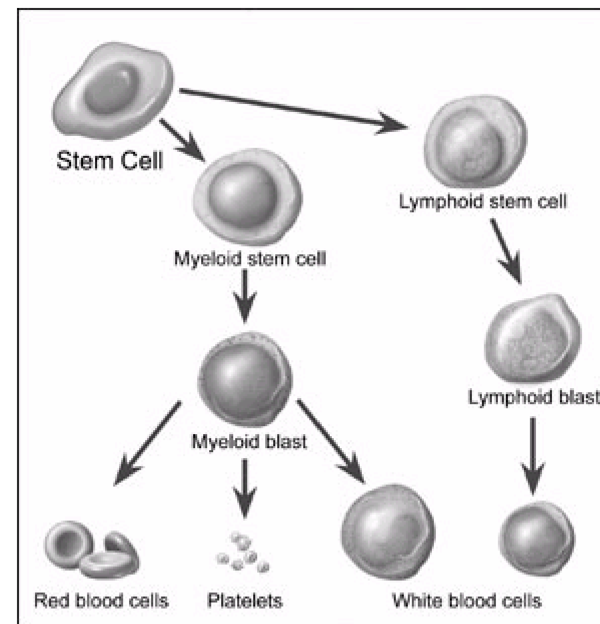

1 2 3 4 5 6 7 8 9 X


1 2 3 4 5 6 7 8 9 10 11 12

Původ hematoonkologických onemocnění

- Systémová klonální onemocnění, vznik neregulovaným dělením jediné nádorově transformované buňky
- nižší počet genetických změn nutných pro vznik, někdy stačí narušení kontroly buněčného dělení
- změna zahrnující cca 10^{6}-10^{7} bp se může projevit bez viditelné změny morfologie chromozomu

Dělení hematoonkologických onemocnění



A) Podle charakteru

- **Difúzní** (leukémie)
- **Ložisková** (lymfomy)-s nádorovými ložisky v lymfatické tkáni

B) Podle postižené krevní vývojové řady

- **myeloidní prekurzorová buňka (CML nebo AML)**
  -nádorová choroba vlastního krvetvorného systému
- **lymfoidní prekurzorová buňka (CLL nebo ALL nebo lymfomy)**
  -nádorová onemocnění imunitního systému

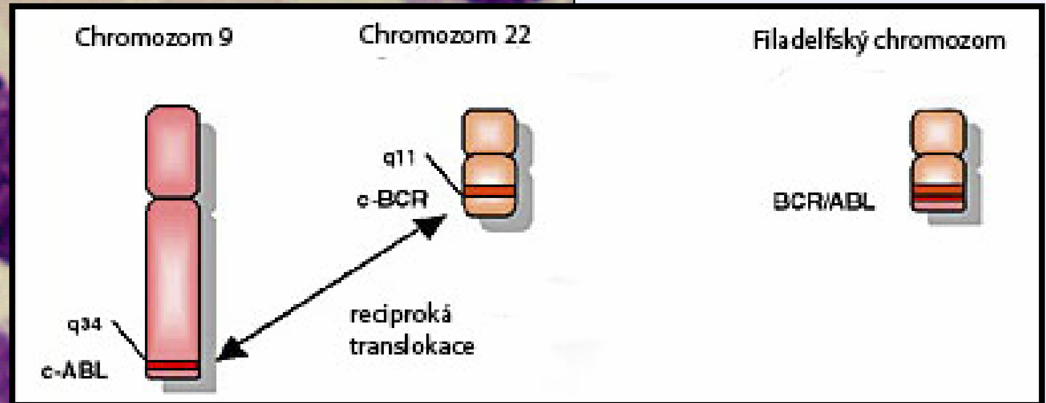
Hematoonkologická diagnóza

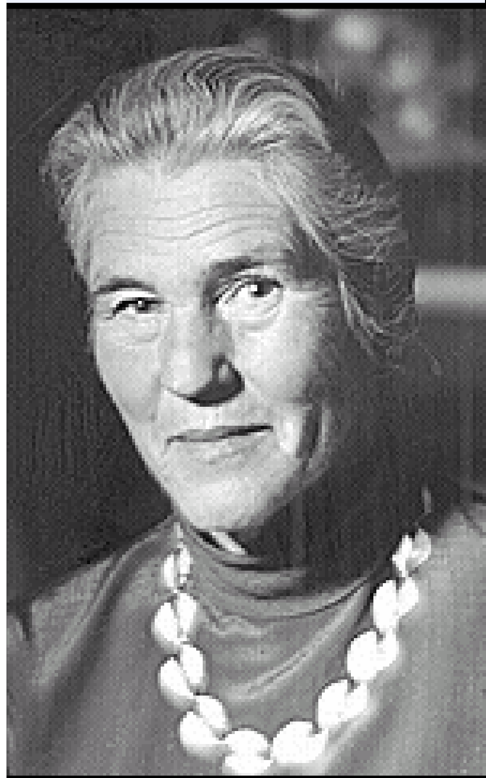
1. Specifické markery pro daný typ/subtyp
2. Nespecifické (nezávislé) markery
3. Stratifikace podle rizika

4. Detekce oportunních patogenů

Proliferace granulocytů různého stupně maturace

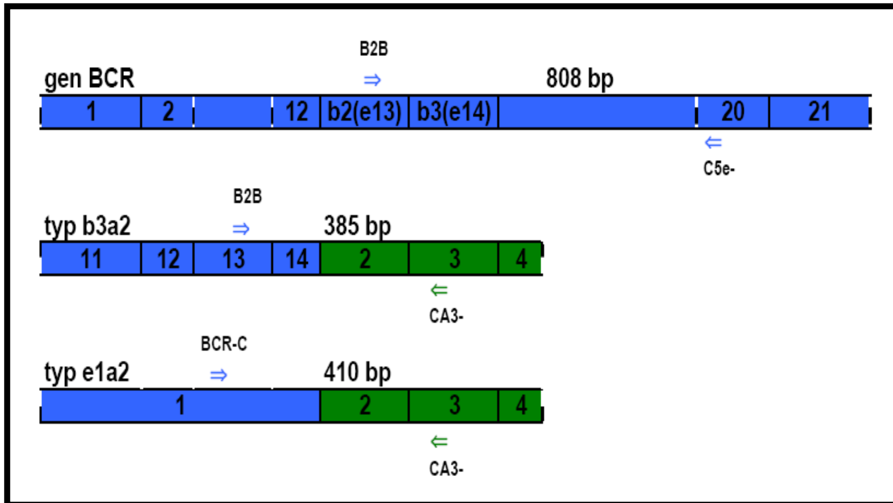
(CML)

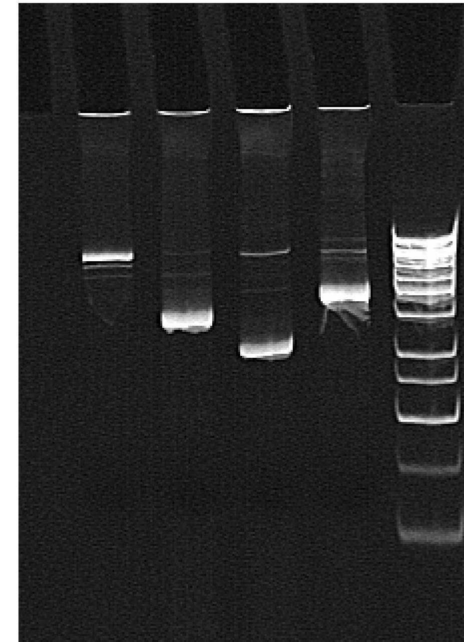




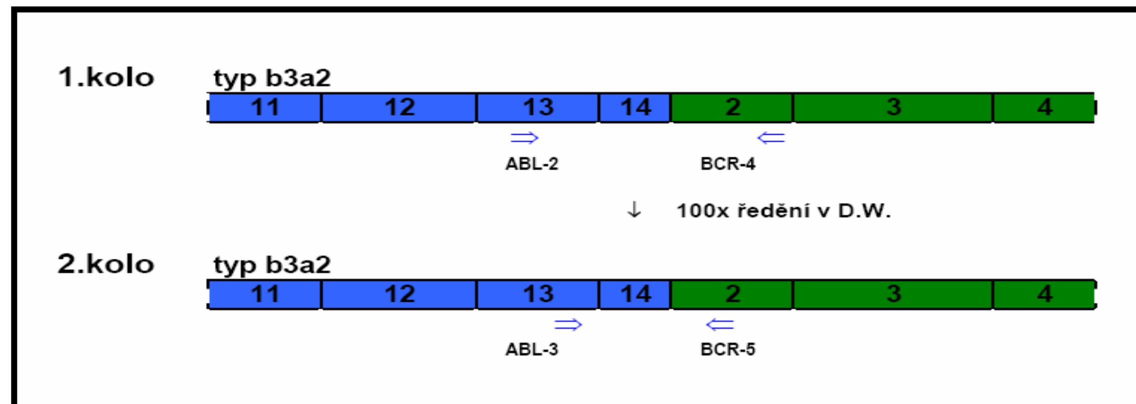
Janet D. Rowley, 1973

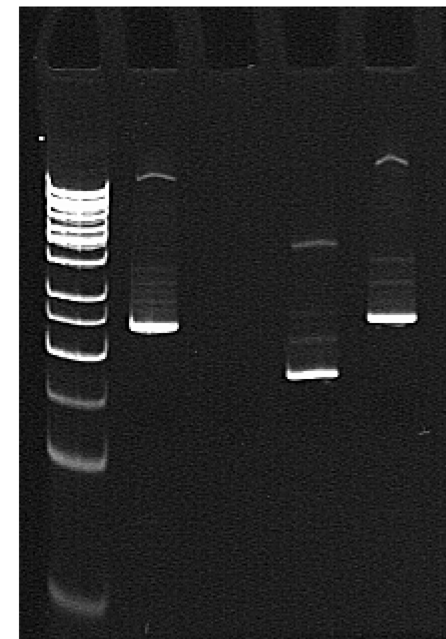
P.C. Nowell, D.A.Hungerford, 1960





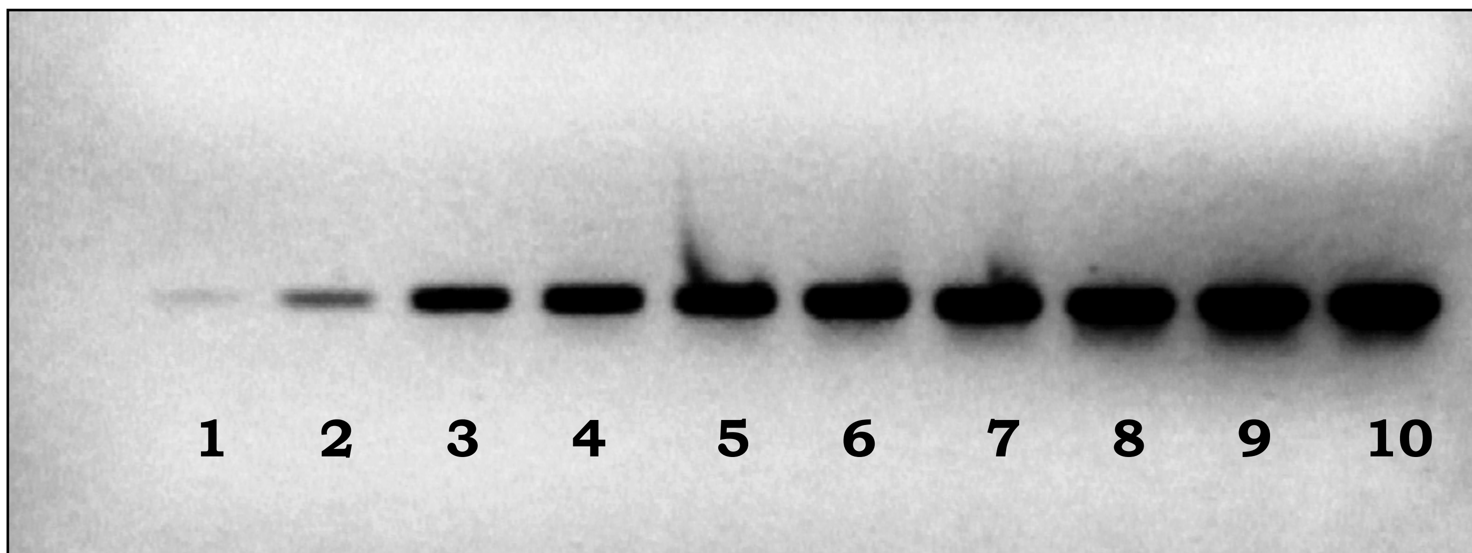
Multiplex-PCR





Dvoukolová nested-PCR

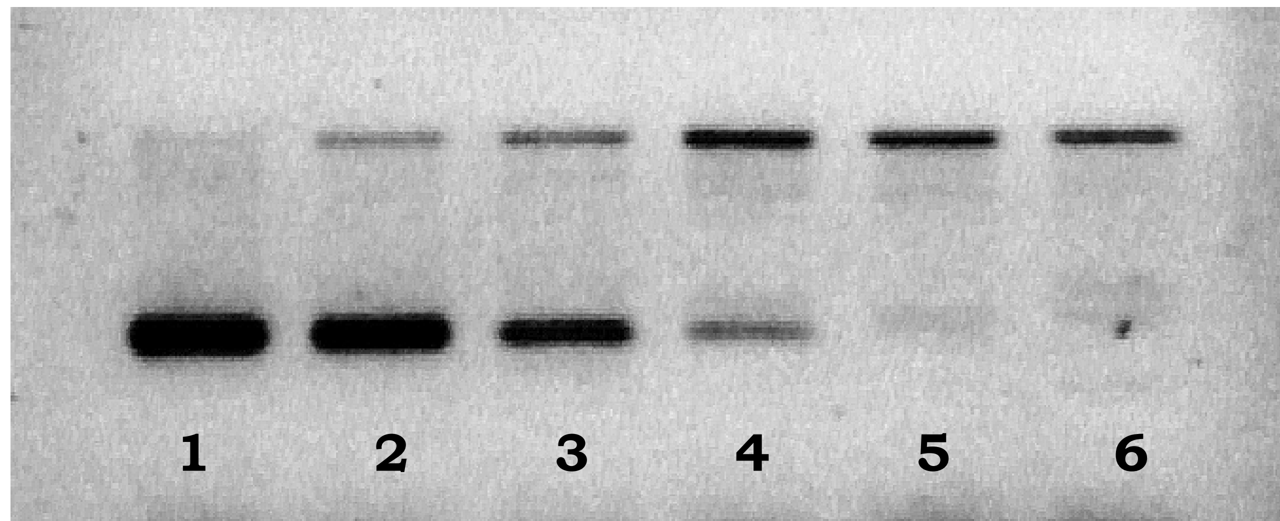
Titrační ředění templátové cDNA



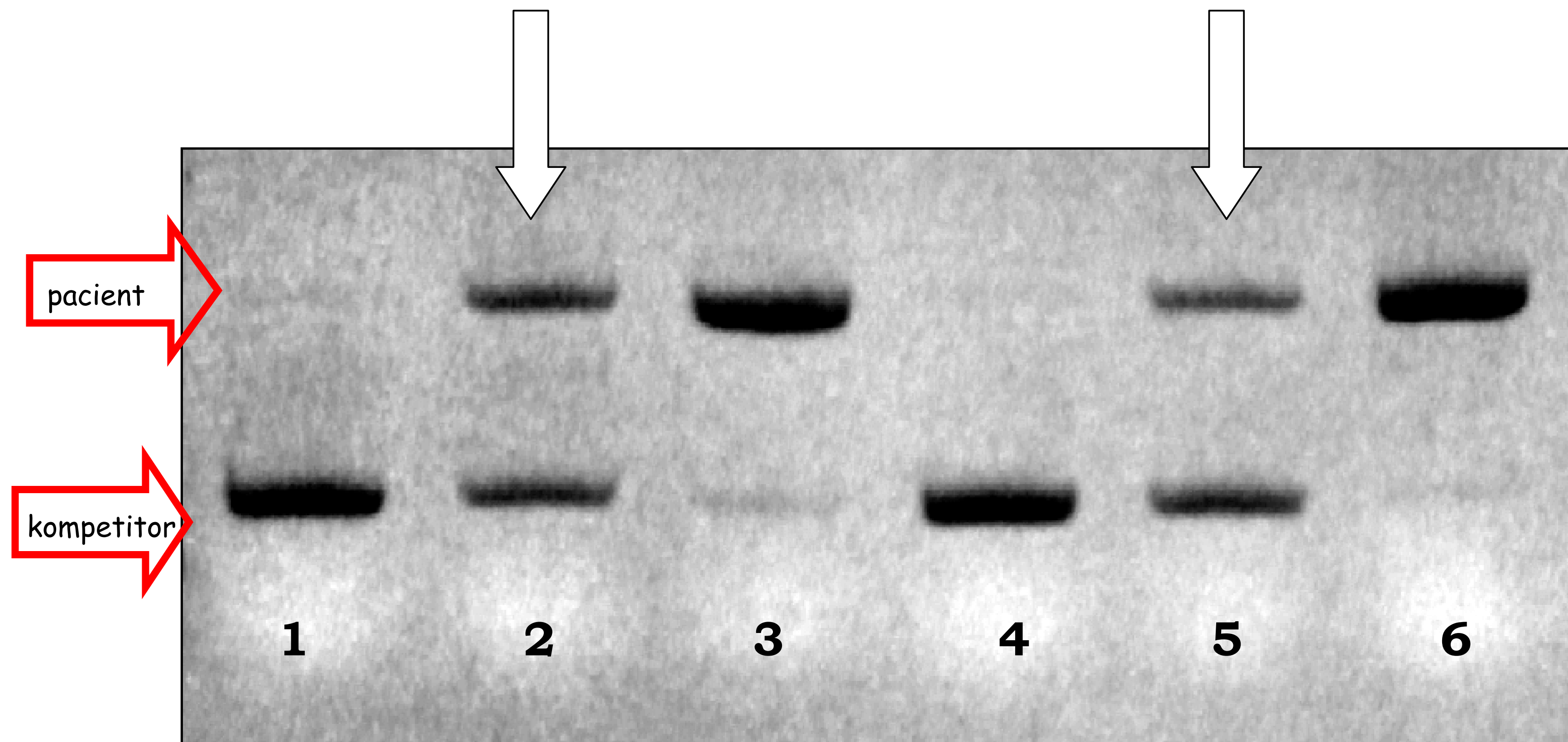
Titrační ředění kompetitorového templátu

pacient

kompetitor



Kompetitivní PCR a vyznačení bodů ekvivalence



1: Biotechnology (N Y). 1992 Apr;10(4):413-7.

Simultaneous amplification and detection of specific DNA sequences.

Higuchi R, Dollinger G, Walsh PS, Griffith R.

Roche Molecular Systems, Inc., Emeryville, CA 94608.

We have enhanced the polymerase chain reaction (PCR) such that specific DNA sequences can be detected without opening the reaction tube. This enhancement requires the addition of ethidium bromide (EtBr) to a PCR. Since the fluorescence of EtBr increases in the presence of double-stranded (ds) DNA an increase in fluorescence in such a PCR indicates a positive amplification, which can be easily monitored externally. In fact, amplification can be continuously monitored in order to follow its progress. The ability to simultaneously amplify specific DNA sequences and detect the product of the amplification both simplifies and improves PCR and may facilitate its automation and more widespread use in the clinic or in other situations requiring high sample throughput.

1: Biotechnology (N Y). 1993 Sep;11(9):1026-30. Related Articles, Links

Kinetic PCR analysis: real-time monitoring of DNA amplification reactions.

Higuchi R, Fockler C, Dollinger G, Watson R.

Roche Molecular Systems, Inc., Alameda, CA 94501.

We describe a simple, quantitative assay for any amplifiable DNA sequence that uses a video camera to monitor multiple polymerase chain reactions (PCRs) simultaneously over the course of thermocycling. The video camera detects the accumulation of double-stranded DNA (dsDNA) in each PCR using the increase in the fluorescence of ethidium bromide (EtBr) that results from its binding duplex DNA. The kinetics of fluorescence accumulation during thermocycling are directly related to the starting number of DNA copies. The fewer cycles necessary to produce a detectable fluorescence, the greater the number of target sequences. Results obtained with this approach indicate that a kinetic approach to PCR analysis can quantitate DNA sensitively, selectively and over a large dynamic range. This approach also provides a means of determining the effect of different reaction conditions on the efficacy of the amplification and so can provide insight into fundamental PCR processes.

1: Nucleic Acids Res. 1993 Aug 11;21(16):3761-6. Related Articles, Links

Allelic discrimination by nick-translation PCR with fluorogenic probes.

Lee LG, Connell CR, Bloch W.

Applied Biosystems, Division of Perkin-Elmer, Foster City, CA 94404.

Nick-translation PCR was performed with fluorogenic probes. Two probes were used: one complementary to a sequence containing the F508 codon of the normal human cystic fibrosis (CF) gene (wt DNA) and one complementary to a sequence containing the delta F508 three base pair deletion (mut DNA). Each probe contained a unique and spectrally resolvable fluorescent indicator dye at the 5' end and a common quencher dye attached to the seventh nucleotide from the 5' end. The F508/delta F508 site was located between the indicator and quencher. The probes were added at the start of a PCR containing mut DNA, wt DNA or heterozygous DNA and were degraded during thermal cycling. Although both probes were degraded, each probe generated fluorescence from its indicator dye only when the sequence between the indicator and quencher dyes was perfectly complementary to target. The identify of the target DNA could be determined from the post-PCR fluorescence emission spectrum.

ABI PRISM 7700 (Perkin Elmer/ABI) první dostupný RQ-PCR systém s laserem /1996

Light Cycler (Roche Molecular Biochemicals) extrémní rychlost, kapiláry

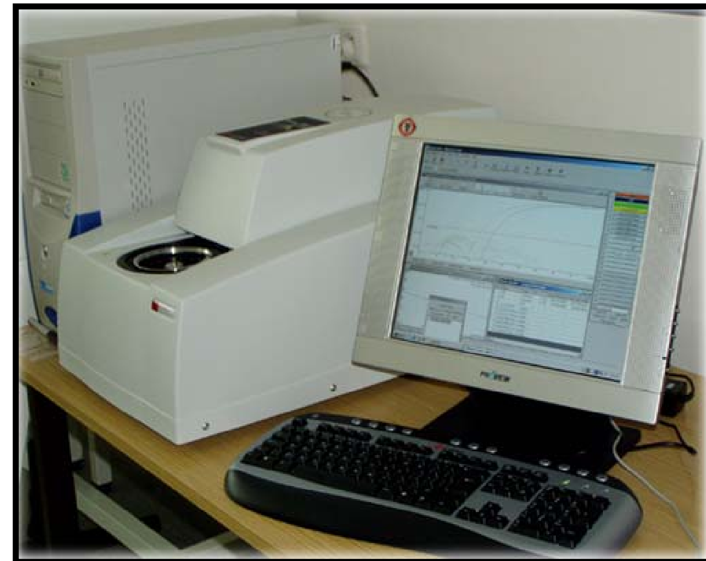
5700SDS (Perkin Elmer/ABI) halogen, CCD /1998

...

LightCycler (Roche)

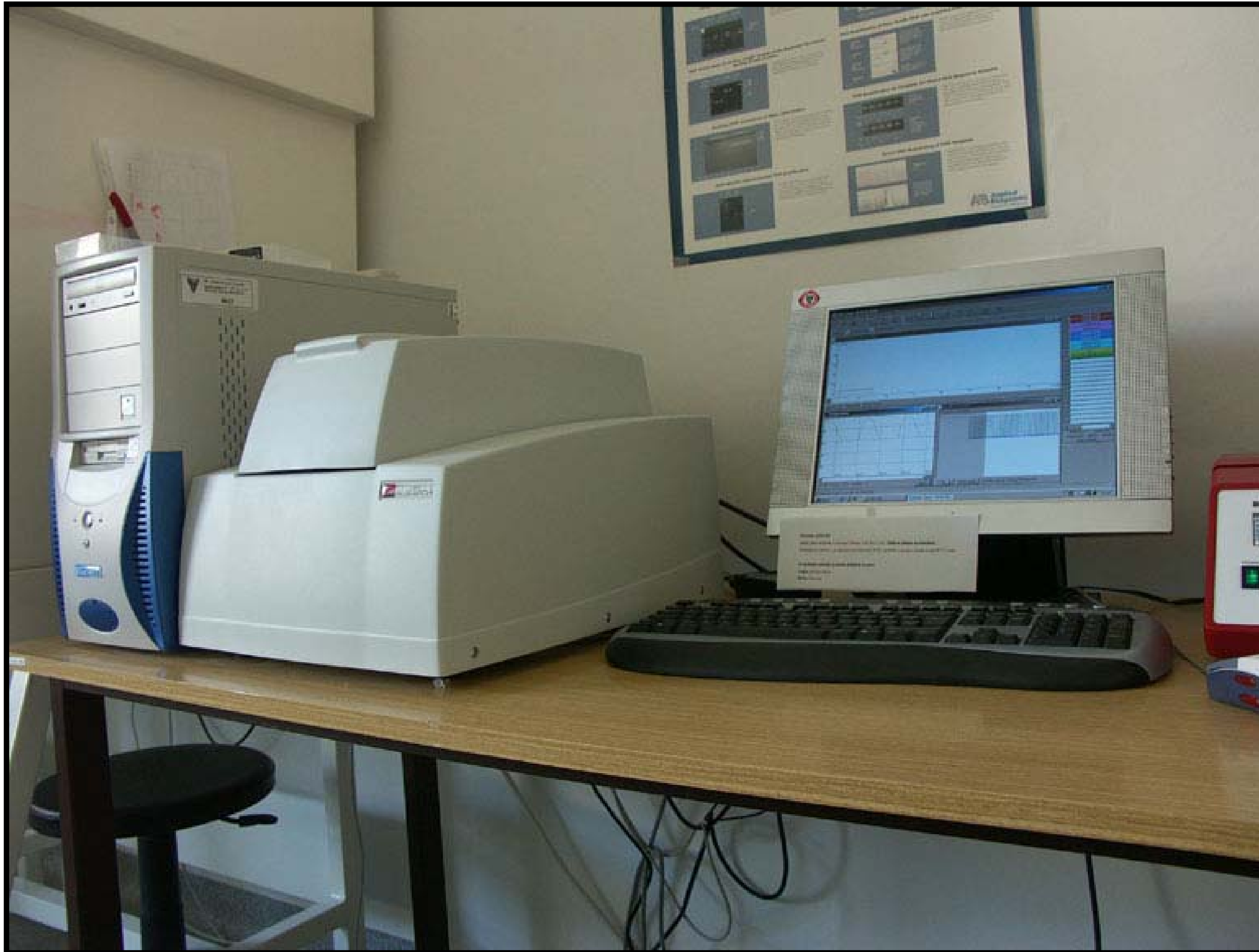
SDS 5700 (Applied Biosystems)

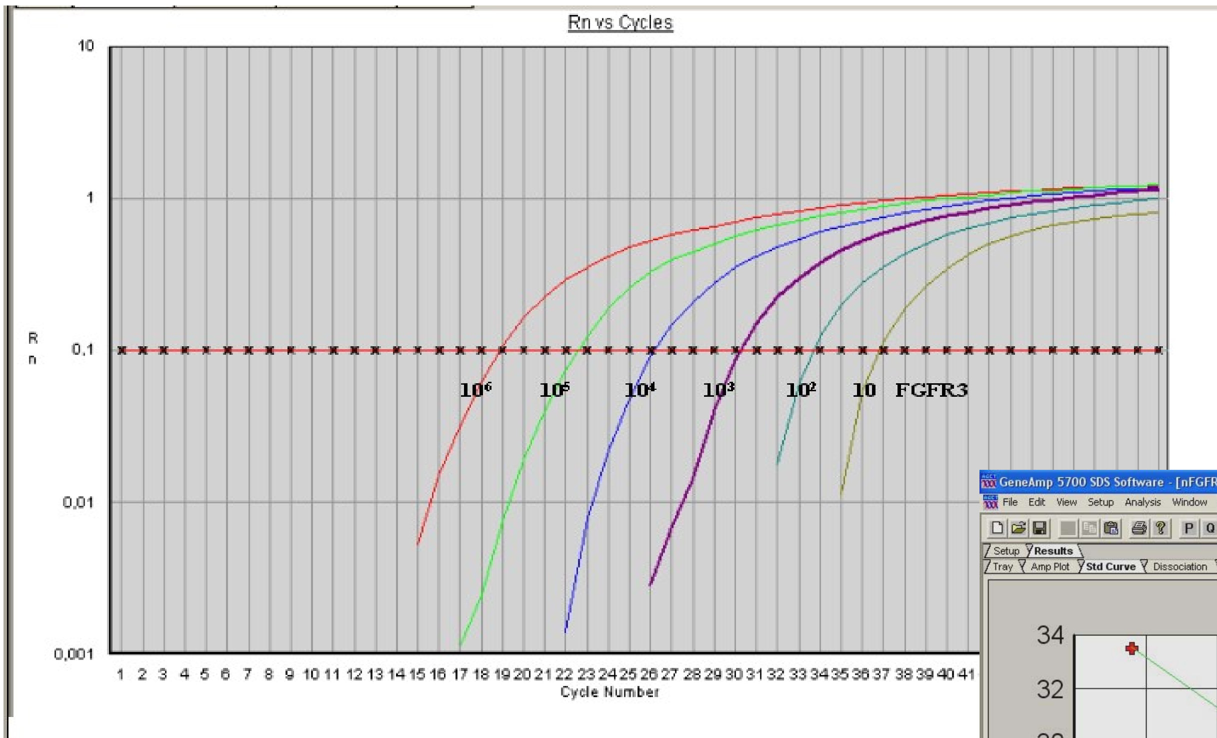
7300 System (Applied Biosystems)



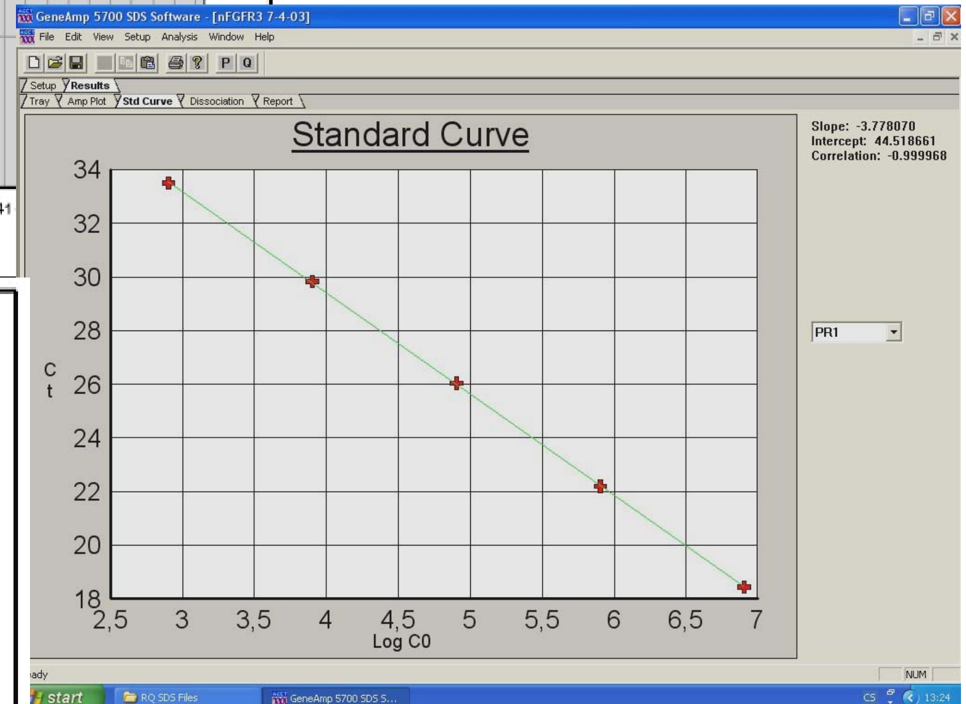
RotorGene 3000 (CorbettResearch)

RotorGene RG 3000 (CorbettResearch, Australia, cca 1 mil Kč)

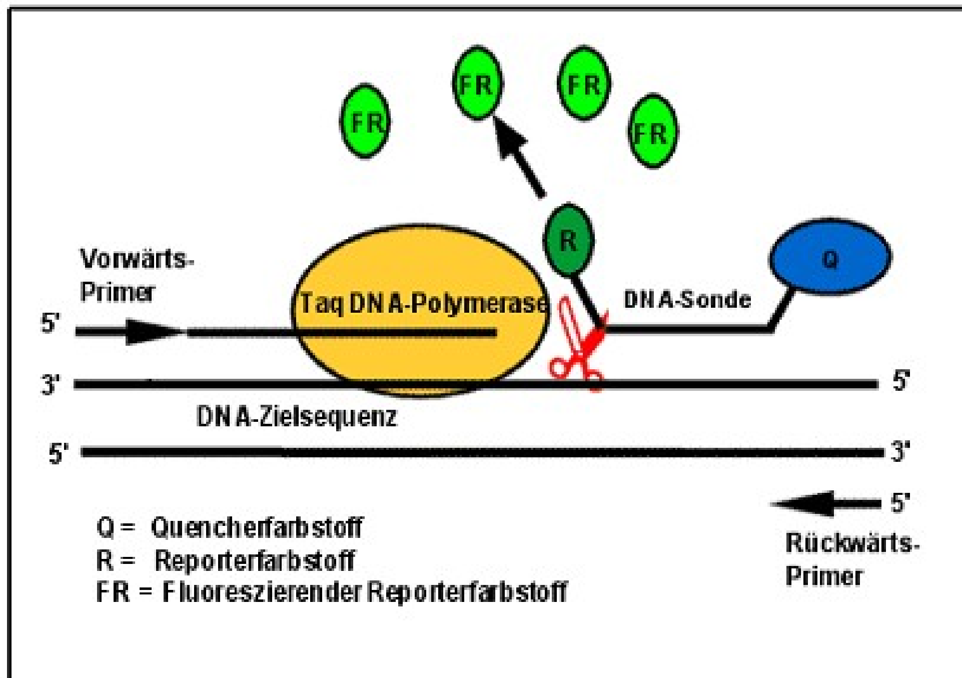




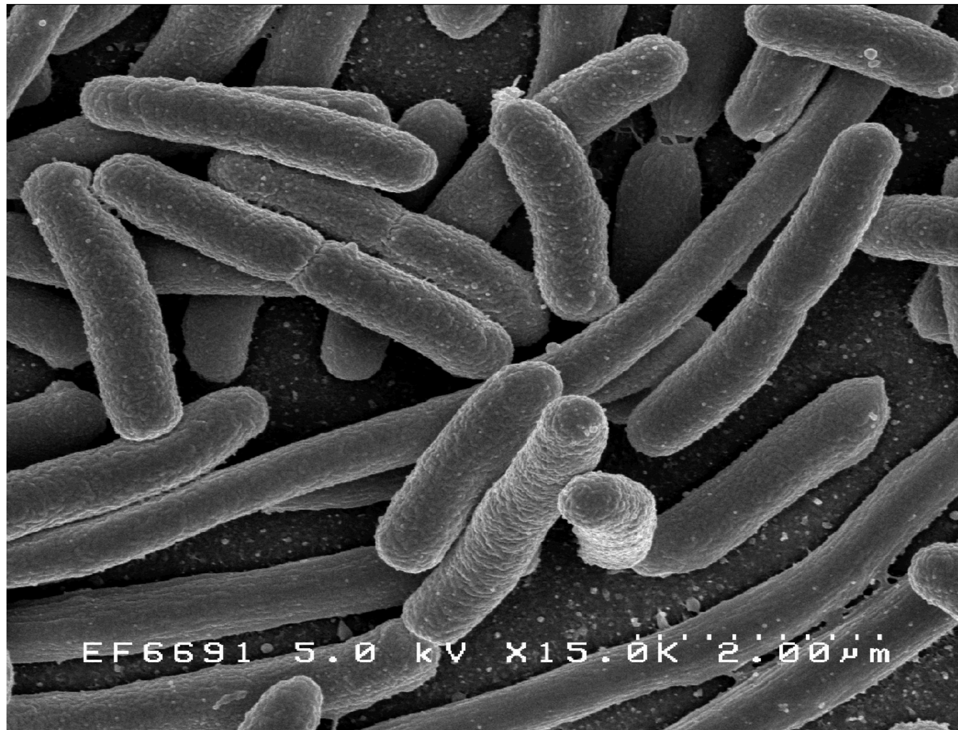
Rn vs Cycles
10
1
0,1
0,01
0,001
Rn
10^6 10^5 10^4 10^3 10^2 10 FGFR3
Cycle Number

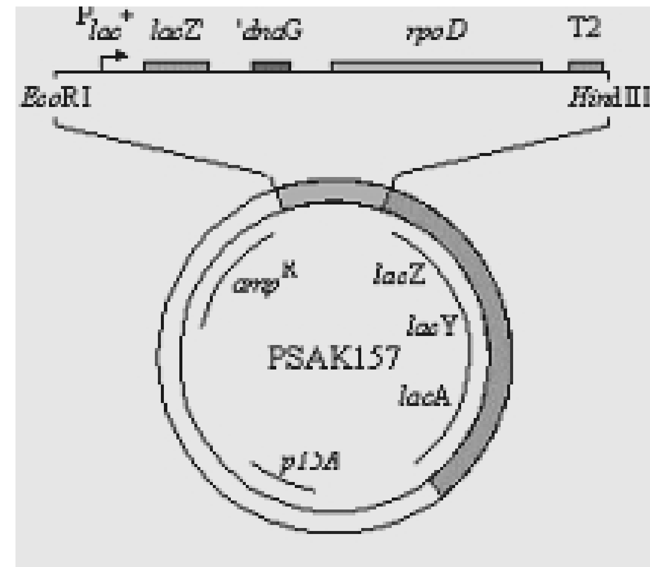


GeneAmp 5700 SDS Software - [nFGFR3 7-4-03]
File Edit View Setup Analysis Window Help
Setup Results
Tray Amp Plot Std Curve Dissociation Report
Standard Curve
Slope: -3.778070
Intercept: 44.518661
Correlation: -0.999968
PR1
Ct
Log C0



FR
R
Q
Vorwärts-
Primer
Taq DNA-Polymerase
DNA-Sonde
5'
3'
DNA-Zielsequenz
Q = Quencherfarbstoff
R = Reporterfarbstoff
FR = Fluoreszierender Reporterfarbstoff
Rückwärts-
Primer





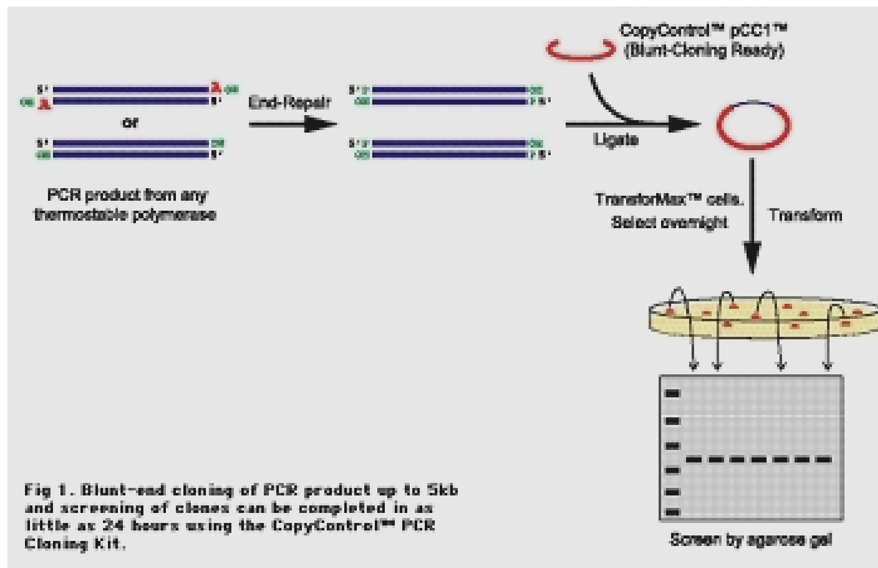
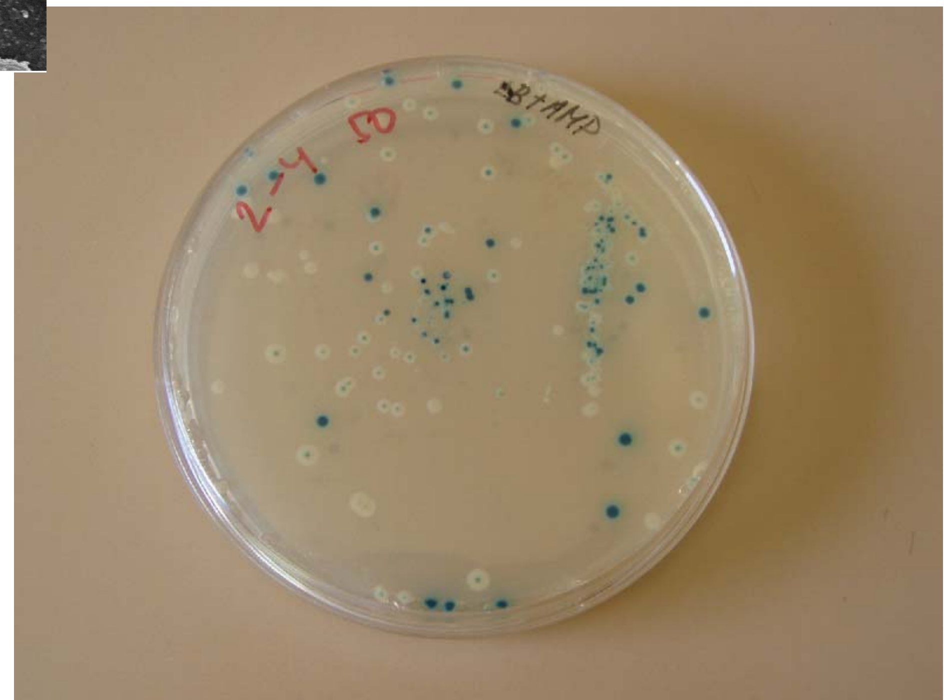
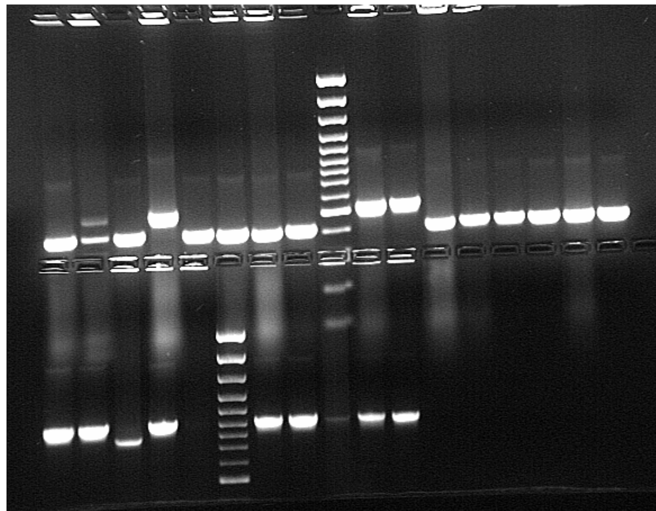


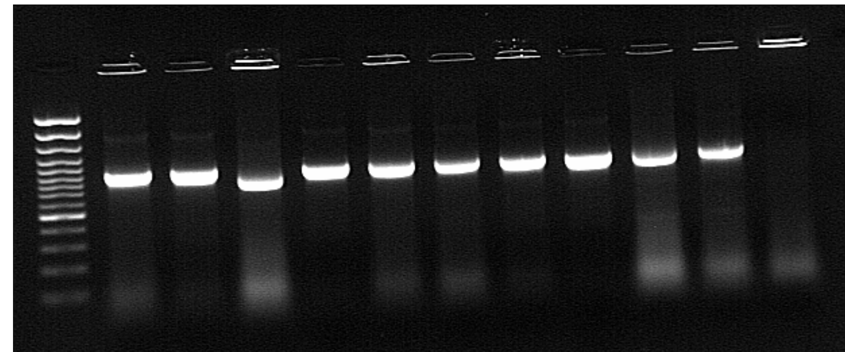
Fig 1. Blunt-end cloning of PCR product up to 5kb and screening of clones can be completed in as little as 24 hours using the CopyControl™ PCR Cloning Kit.



Příprava standardní DNA

- klonování PCR produktu do plazmidového vektoru
- transfekce plazmidu do E.coli
- izolace namnožené plazmidové DNA
-vytvoření ředící řady o známém počtu kopií sledovaného genu





Gelová elektroforéza specifického PCR produktu po rozklonování a vložení do E.coli

Control Gene Standards:

Product	Reference Name
CGRS-01	ABL
CGRS-02	B2M
CGRS-03	GUS
CGRS-04	TBP

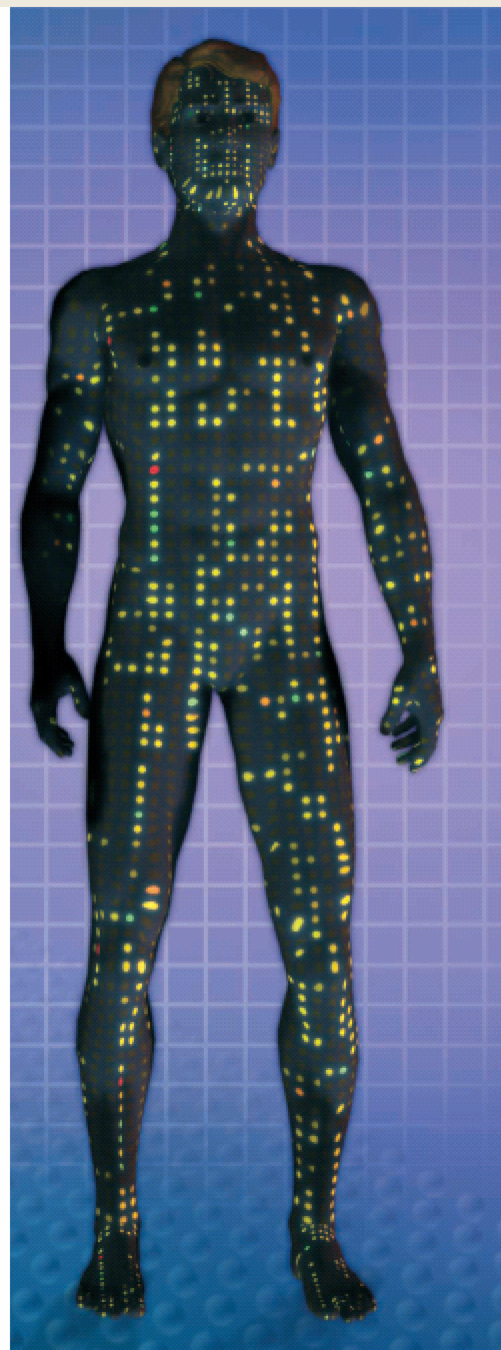
Fusion Gene Standards:

Product	Reference Name
FGRS-01	AML1–ETO
FGRS-02	CBFB-MYH11 A
FGRS-03	CBFB-MYH11 D
FGRS-04	CBFB-MYH11 E
FGRS-05	PML-RARA bcr1
FGRS-06	PML-RARA bcr2
FGRS-07	PML-RARA bcr3
FGRS-08	E2A-PBX1
FGRS-09	BCR-ABL e1a2 m-bcr
FGRS-10	BCR-ABL b3a2 M-bcr
FGRS-11	TEL-AML 1 e4e11
FGRS-12	SIL-TAL
FGRS-13	MLL-AF4 e10e4 = RS411 type
FGRS-14	MLL-AF4 e9e5 = MV411 type
FGRS-15	MLL-AF4 e11e5 = ALL-PO type
FGRS-16	MLL-AF9 type A
FGRS-17	MLL-AF9 type B
FGRS-18	MLL-AF6
FGRS-19	MLL-DUP
FGRS-20 *New*	MLL-ENL ex9
FGRS-21 *New*	MLL-ENL ex10
FGRS-22 *New*	MLL-ENL ex11
FGRS-23 *New*	MLL-AF9 ex9
FGRS-24 *New*	MLL-ELL ex9
FGRS-25 *New*	MLL-ELL ex10
FGRS-26 *New*	MLL-AF1p ex11

Bibliography

1. J. Gabert et al. Standardization and quality control studies of "real-time" quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) of fusion gene transcripts for minimal residual disease detection in leukemia - A Europe Against Cancer Program. In Press, Leukemia.

2. E. Beillard et al. Evaluation of candidate control genes for diagnosis



AML M2 s AML1/ETO (53 let)

I.

RI

%EXPRESE

1.K

2.K

3.K

2.RI

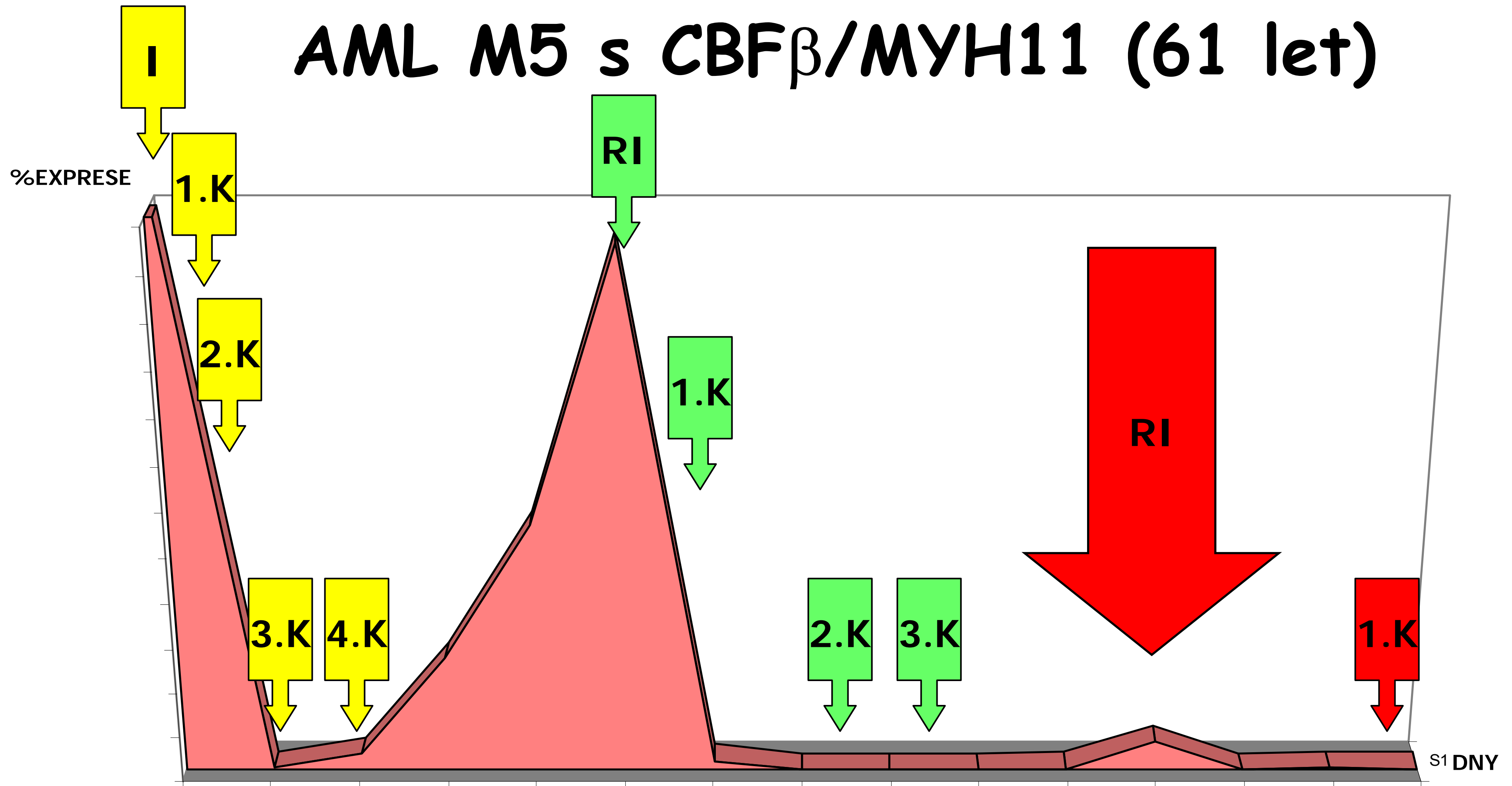
DNY

CYTOGENETIKA

MYELOGRAM

FLOWCYTOMET.

DNY	0	49	88	126	152	172	180	214	250	272	285	348	388
%EXP.- BM	149	11,74	21,77	0,00003	0,03	0,04	0,02	0,09	12,68	74	204	127	133
%EXP.- PB												42	

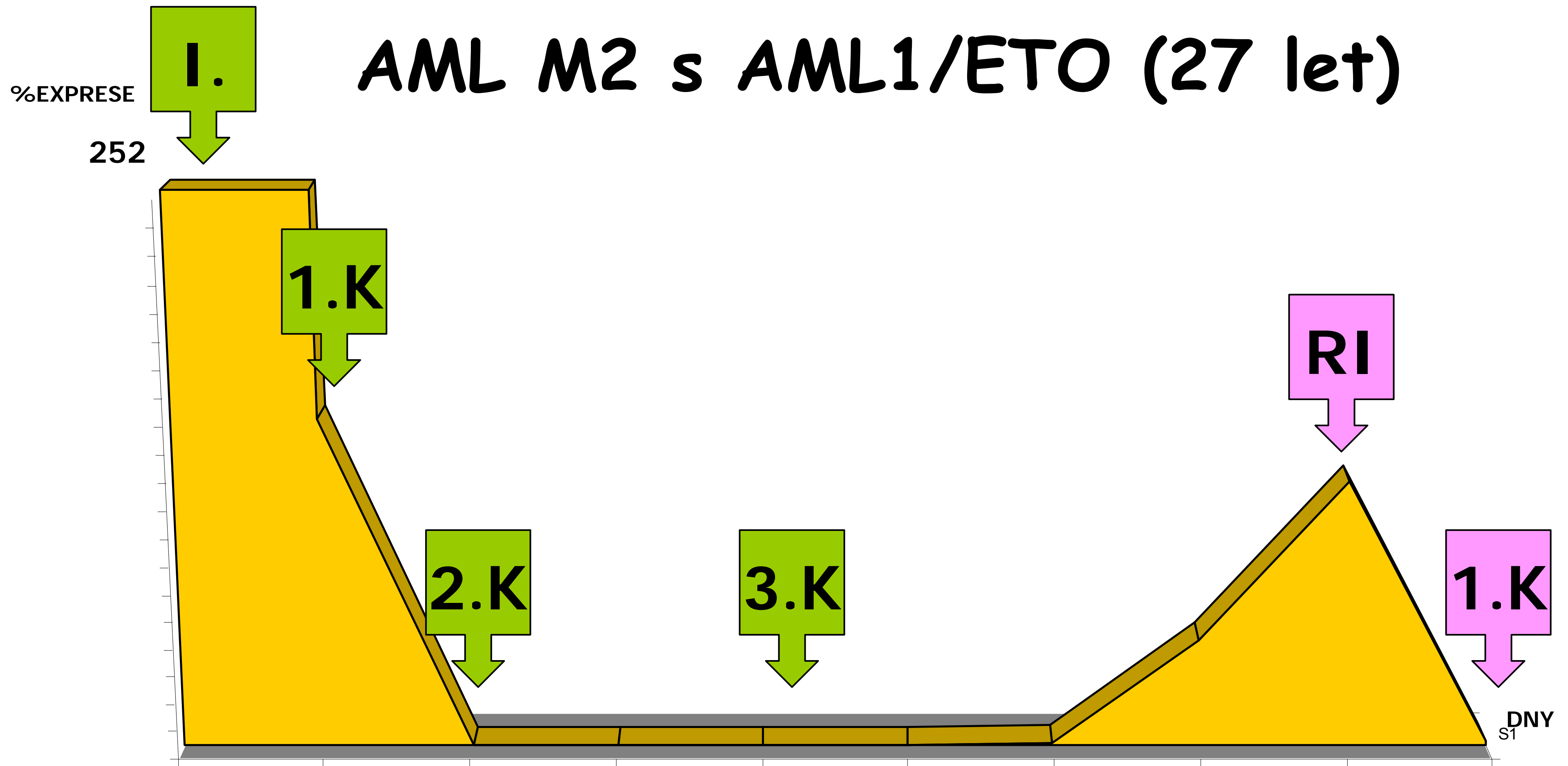


CYTOGENETIKA

MYELOGRAM

FLOWCYTOMET.

DEN	0	112	268	280	290	322	360	402	448	483	511	541	560	597	645
%EXP.- BM	130	0,18	3,54	25,41	54,92	115	1,97	0	0,025	0,007	0,15	6,3		0,38	0,16
%EXP.- PB										0,05	0,27	6,07	35,96		

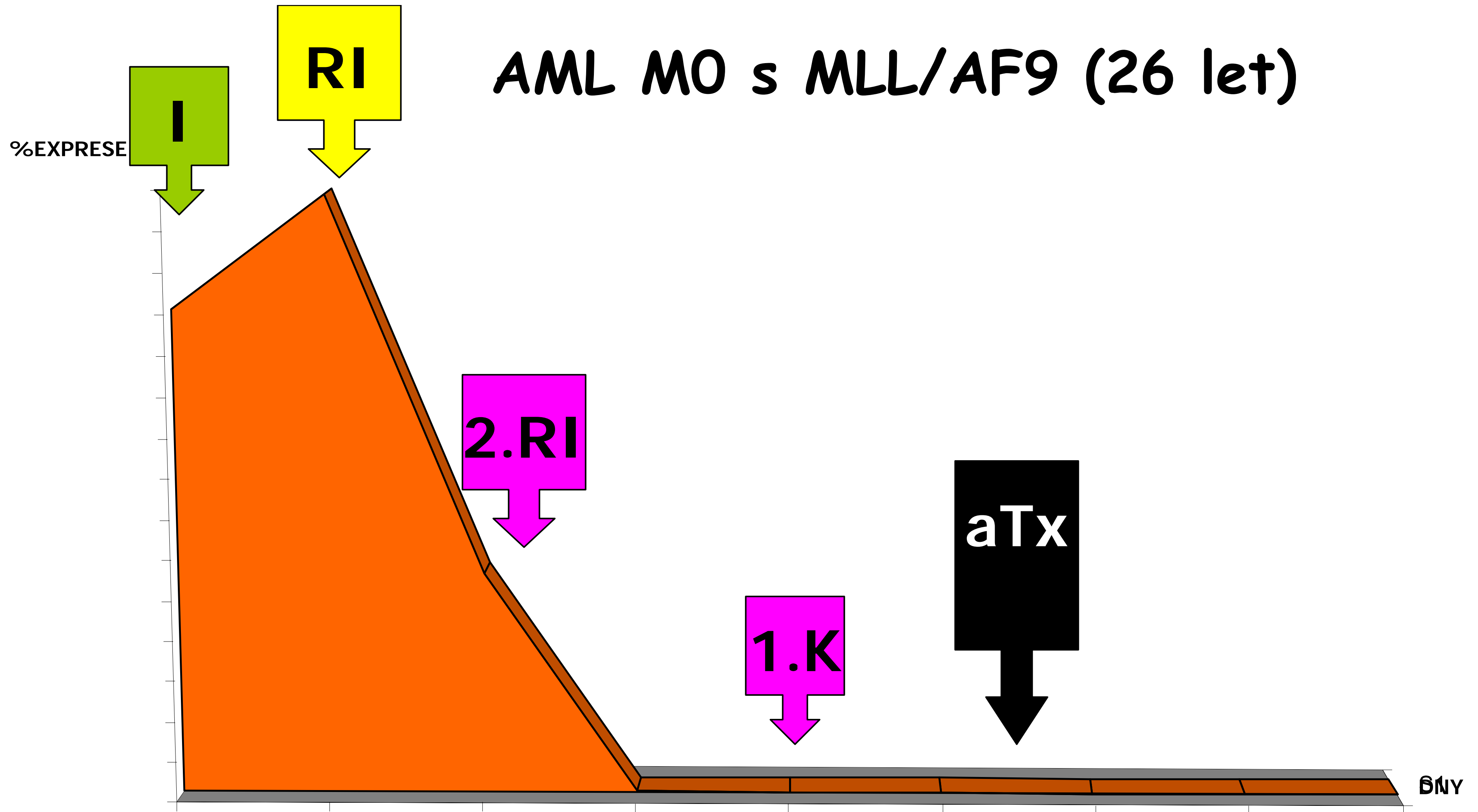


CYTOGENETIKA

MYELOGRAM

FLOWCYTOMET.

DNY	0	23	62	99	118	160	198	232	241	289
%EXP.- BM	252	1,19	0,0013	0,001	0	0	0,008	0,39	0,97	0,015
%EXP.- PB		0,74			0	0				0,01



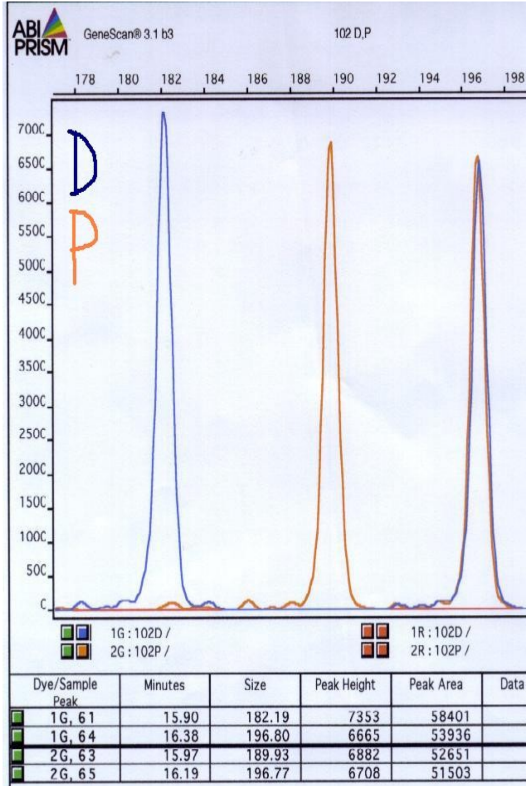
CYTOGENETIKA

MYELOGRAM

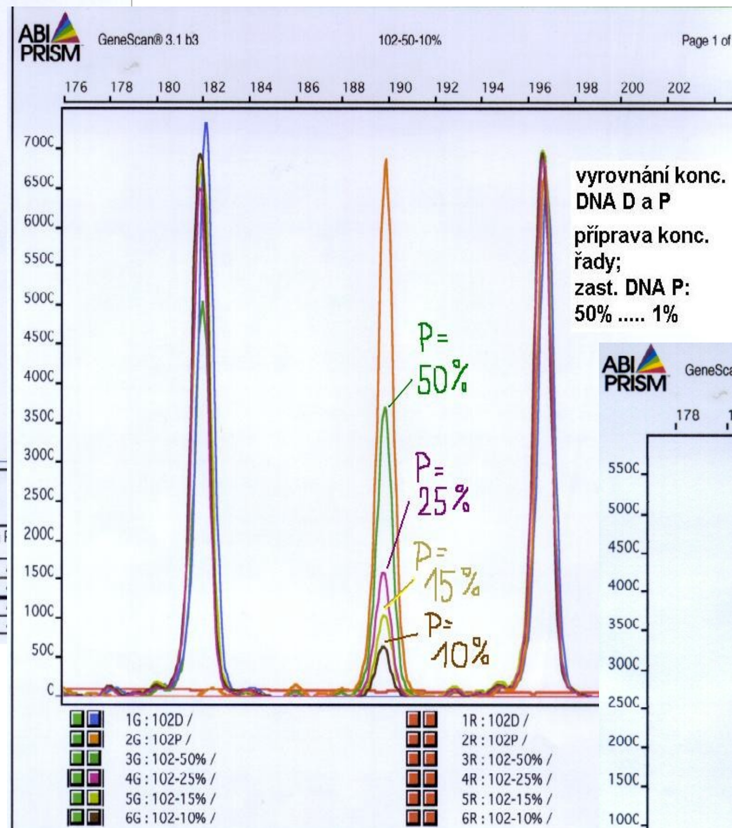
FLOWCYTOMET.

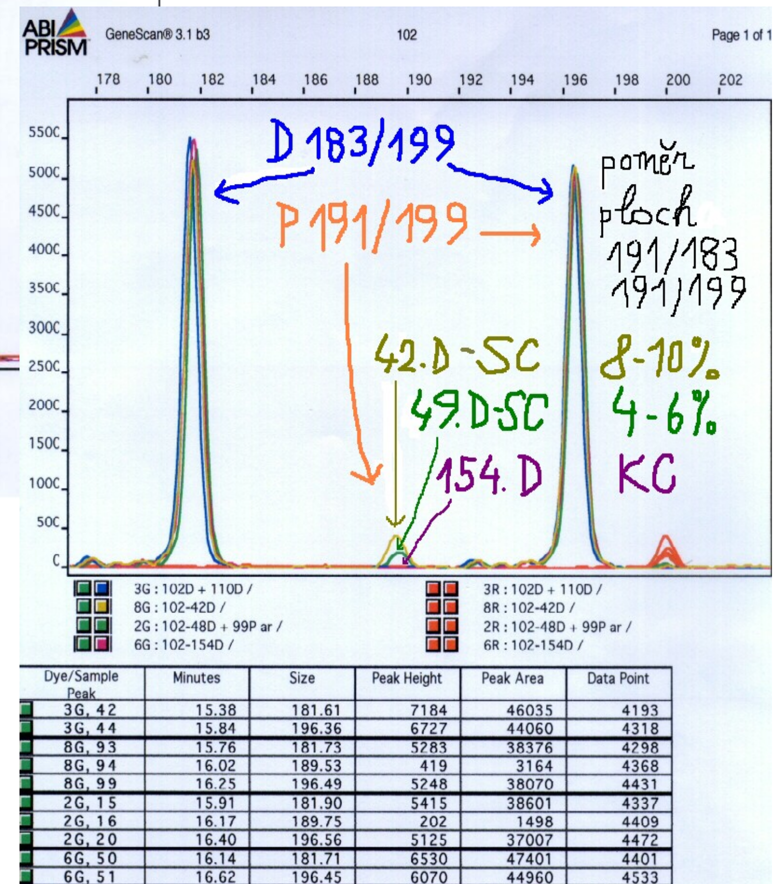
DNY	0	33	62	96	105	138	175	182	201
%EXP.- BM	60	73,98	27,49	0,02	0,07	0,07		0	
%EXP.- PB			18,69	0		0,02	0	0	0

Detekce buněčného chimérismu po HSCT

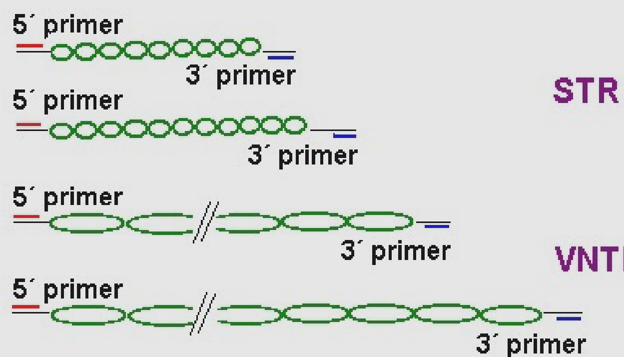


Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data P
1G, 61	15.90	182.19	7353	58401	
1G, 64	16.38	196.80	6665	53936	
2G, 63	15.97	189.93	6882	52651	
2G, 65	16.19	196.77	6708	51503	

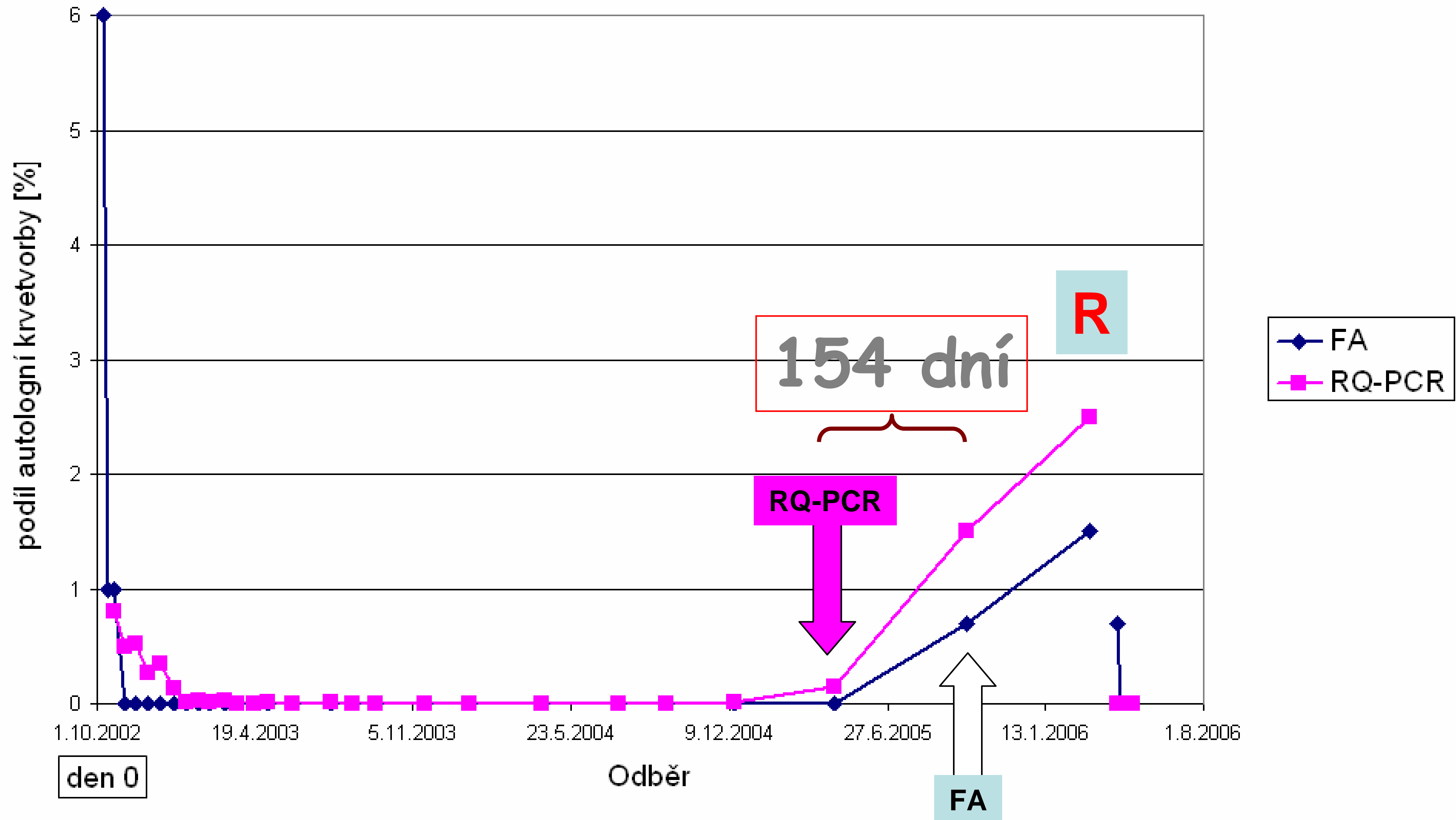




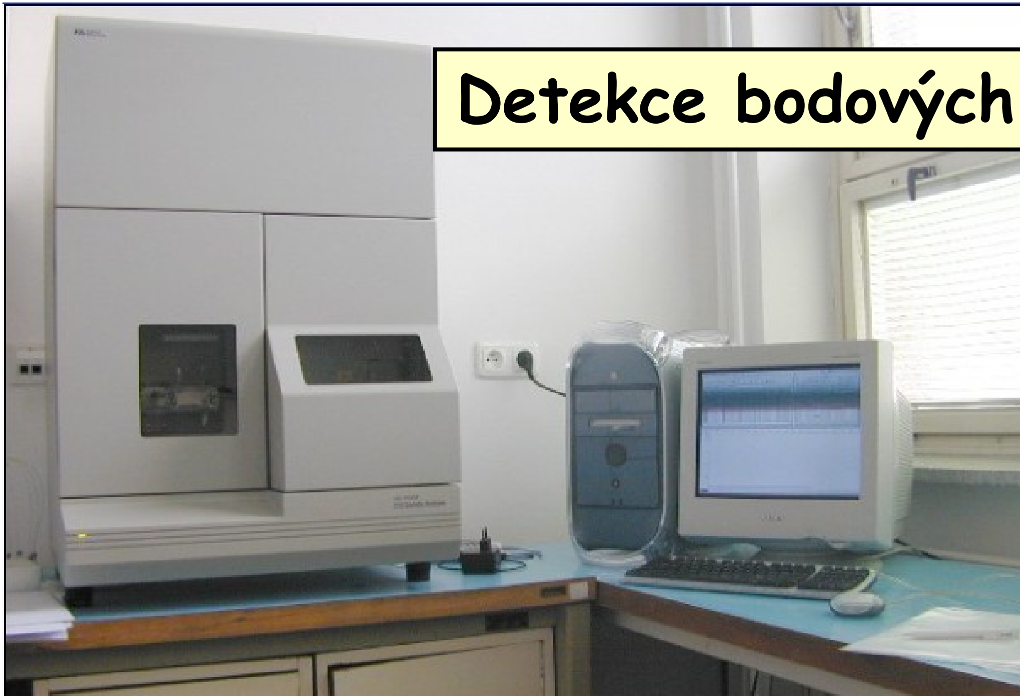
Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
3G, 42	15.38	181.61	7184	46035	4193
3G, 44	15.84	196.36	6727	44060	4318
8G, 93	15.76	181.73	5283	38376	4298
8G, 94	16.02	189.53	419	3164	4368
8G, 99	16.25	196.49	5248	38070	4431
2G, 15	15.91	181.90	5415	38601	4337
2G, 16	16.17	189.75	202	1498	4409
2G, 20	16.40	196.56	5125	37007	4472
6G, 50	16.14	181.71	6530	47401	4401
6G, 51	16.62	196.45	6070	44960	4533

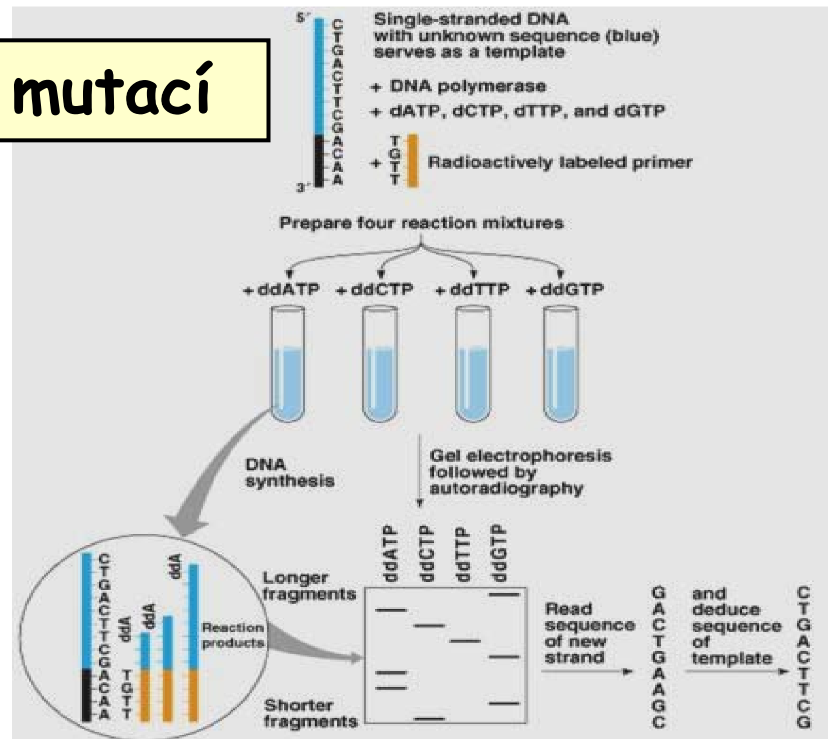


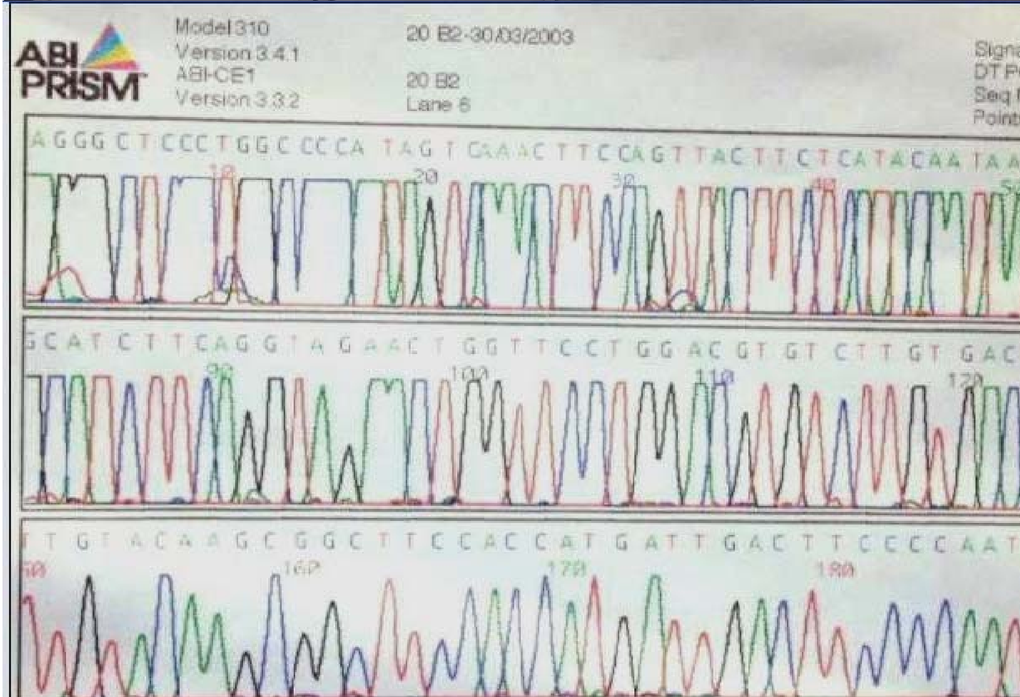
Hematologický relaps-příklad monitorování pacientů po HSCT

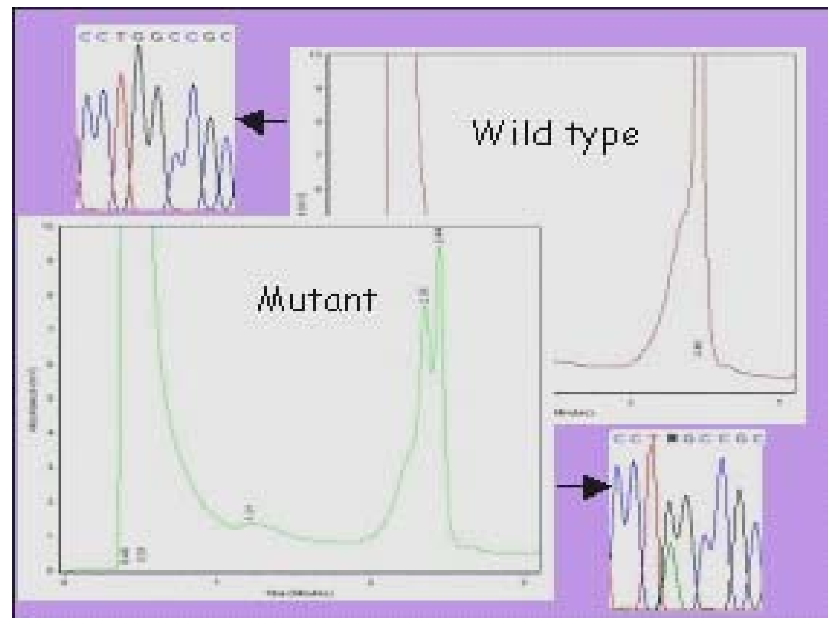


Detekce bodových mutací

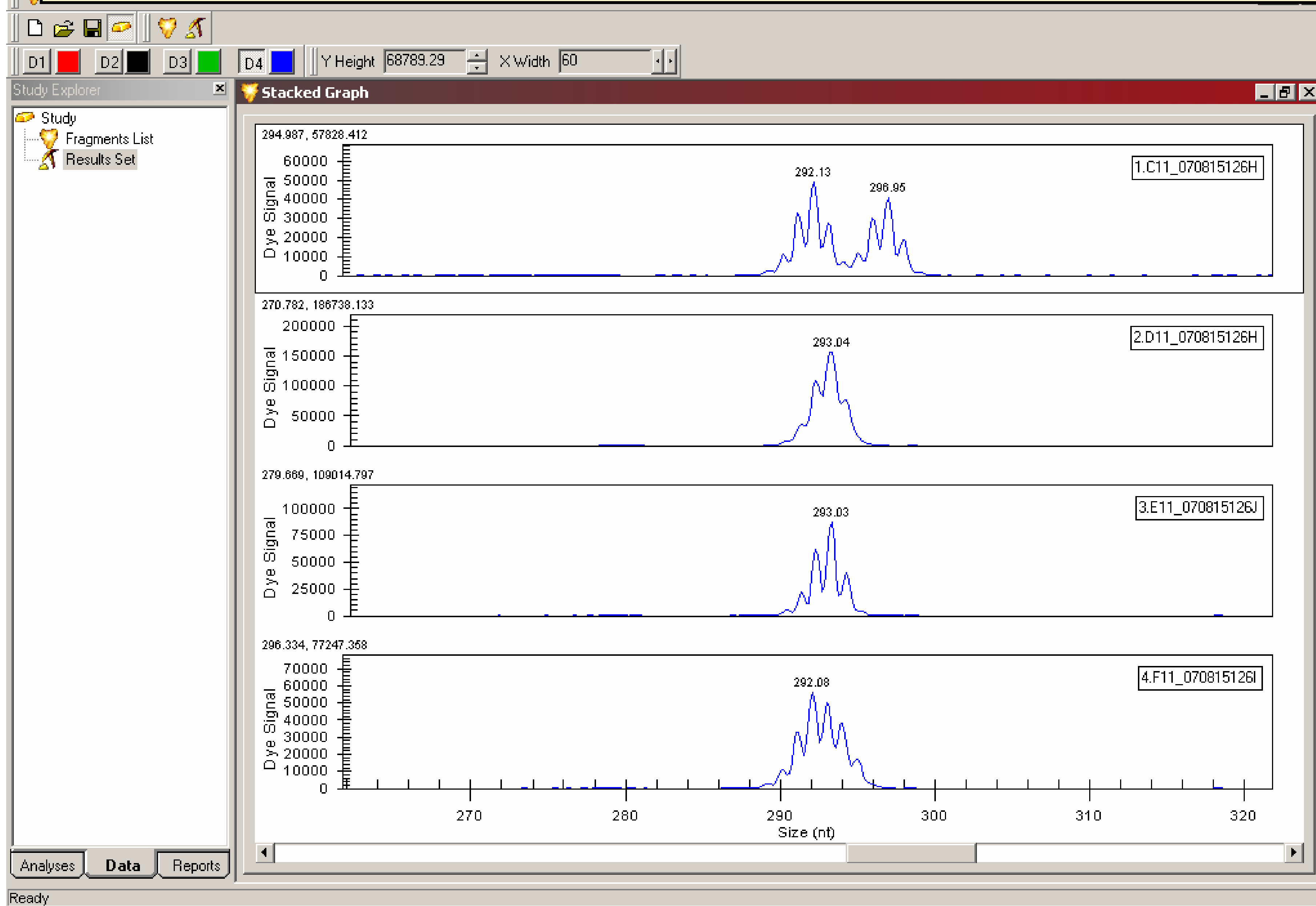








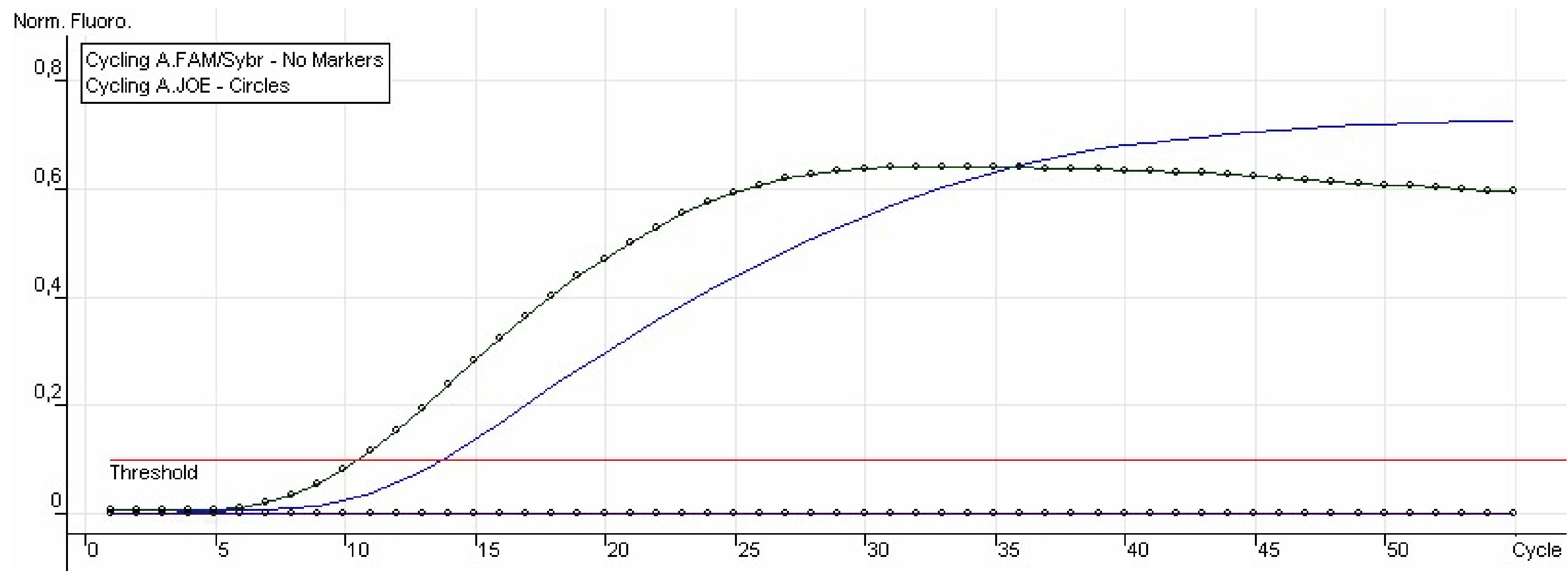
Detekce mutací metodou fragmentační analýza CEQ 8000 Genetic Analysis System (Beckman Coulter,CA)



Detekce mutace V617F JAK-2 metodou alelické diskriminace

3) **RQ – PCR**
- izolace DNA
- RQ – PCR s využitím fluorescenčně značených LNA modifikovaných hybridizačních sond (Locked Nucleic Acids)
- vyznačují se 100% alelickou diskriminací obou genotypů
- citlivost detekující 10% příměs granulocytů nesoucích mutantní alelu na pozadí zdravé populace
- fluorescenční značení FAM pro sondu s WT sekvencí a JOE pro sondu s MUT sekvencí

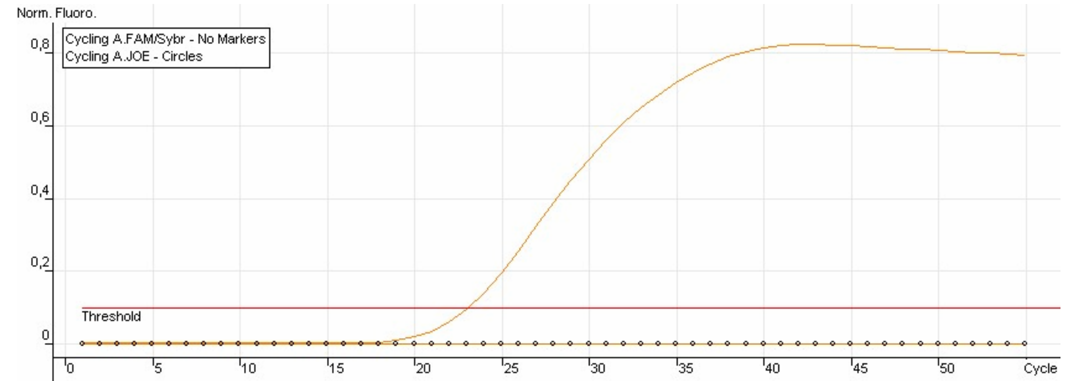
RQ – PCR analýza



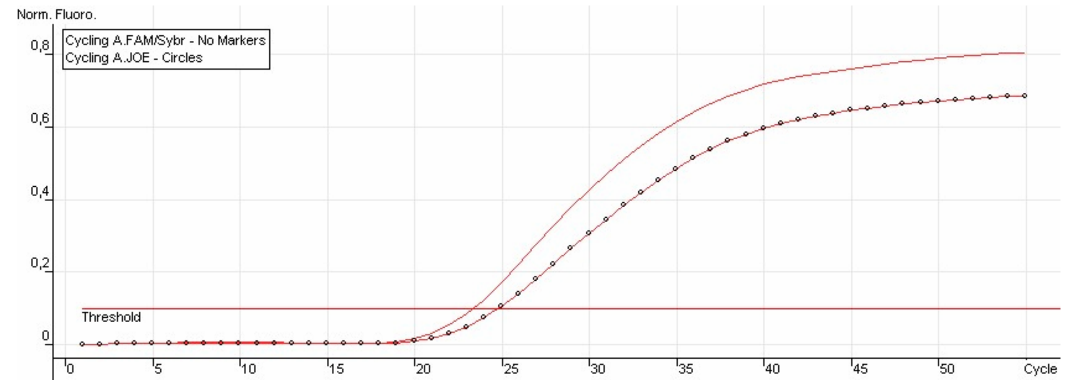
Amplifikační křivky plasmidu wt a mutant V617F

RQ – PCR analýza

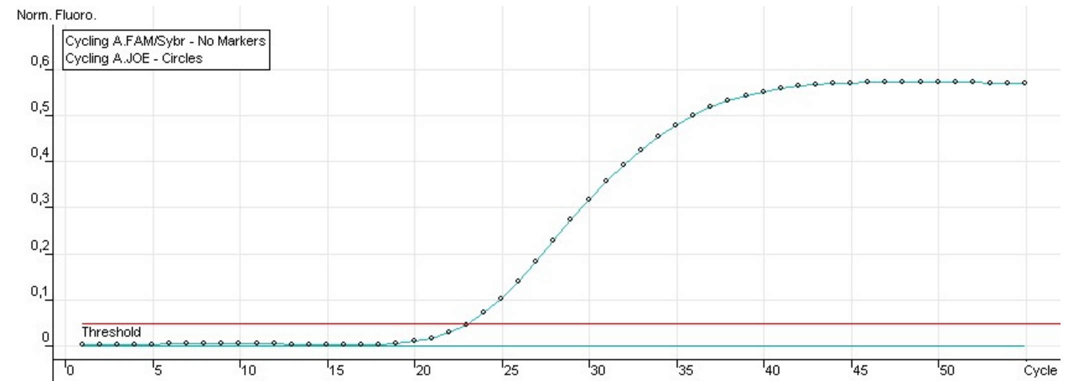
Pacient - wt



Pacient – heterozygot

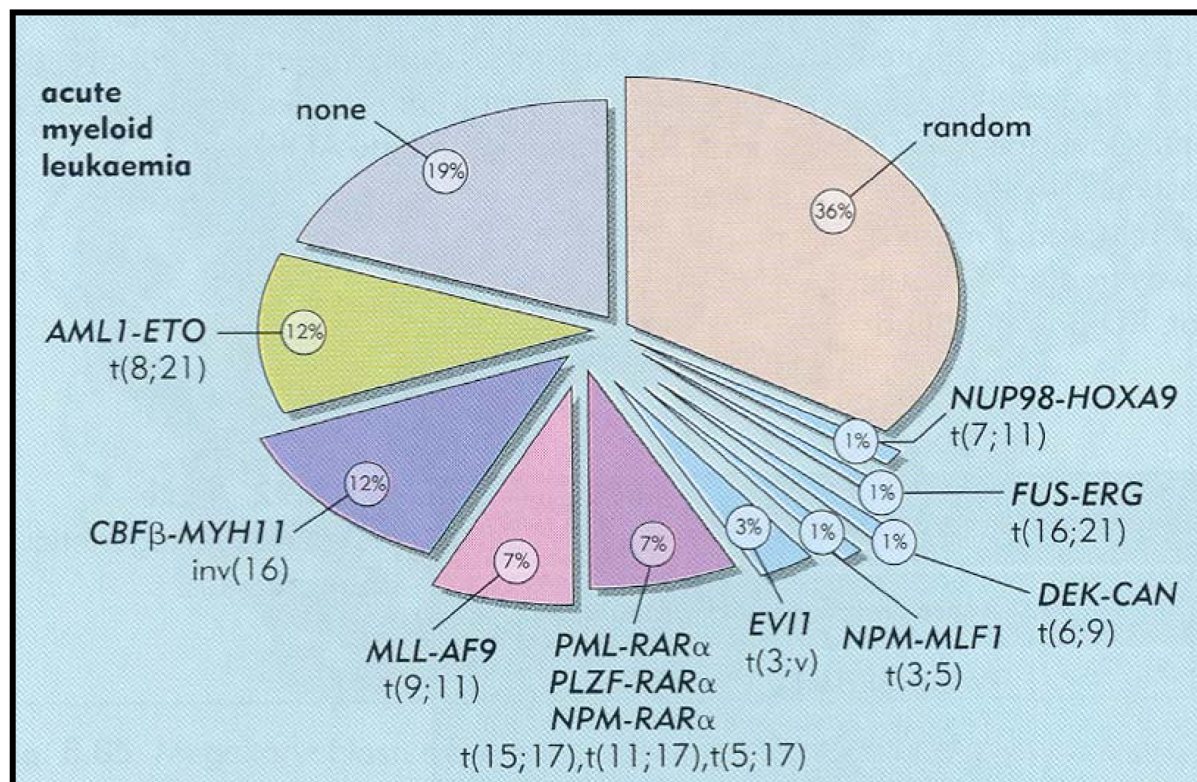


Pacient – mut. homozygot



Akutní myeloidní leukémie

- heterogenní skupina onemocnění
- různé chromozomální aberace
- 40-50% případů AML s molekulárním markerem



Prognostické faktory AML

- **Fúzní geny s diagnostickým významem**
  - **PML/RARa** ® dobrá prognóza
  - **AML1/ETO** ® dobrá prognóza
  - **CBFb/MYH** ® dobrá prognóza
  - přestavby **MLL** genu ® špatná prognóza
- **změny genů s prognostickým významem**
  - interní tandemové duplikace **ITD-FLT3** genu
  - bodové mutace aktivační smyčky **FLT3** genu
  - mutace **CEBPa** genu, parciální tandemové duplikace MLL genu

Rozšiřování spektra molekulárních markerů v pozici nezávislých prognostických faktorů - snaha o **prognostickou stratifikaci** leukemických pacientů a sledování **minimální residuální choroby** i u pacientů s normálním karyotypem : (AML 40-50%!)

gen NPM-1 mutace, příznivá prognóza

gen FLT3/ITD a FLT3/D835 : negativní prognóza (AML)

gen WT-1 , tumor-supresor gen na 11p13, zvýšená exprese negativní prognóza (akutní leukémie a MDS)

mutace v genu pro myeloidní transkripční faktor **CEBPA** (CCAAT/enhancer binding protein-alfa): je zahrnut v neutrofilní diferenciaci, příznivá prognóza (myeloblastická AML, M1 a M2)

gen BAALC (brain and acute leukemia, cytoplasmic): 8q22.3, progenitorové buňky, vysoká exprese negativní prognóza (AML a CML pouze BC)

aktivující bodová mutace v genu **JAK2 V617F** (Janus kinases) nepříznivá prognóza (cMPD-PV, také AML M6 a M7)

FLT3 (FMS-like tyrosine kinase) STK1 (Stem cell kinase1) flk2 (Fetal liver kinase2)

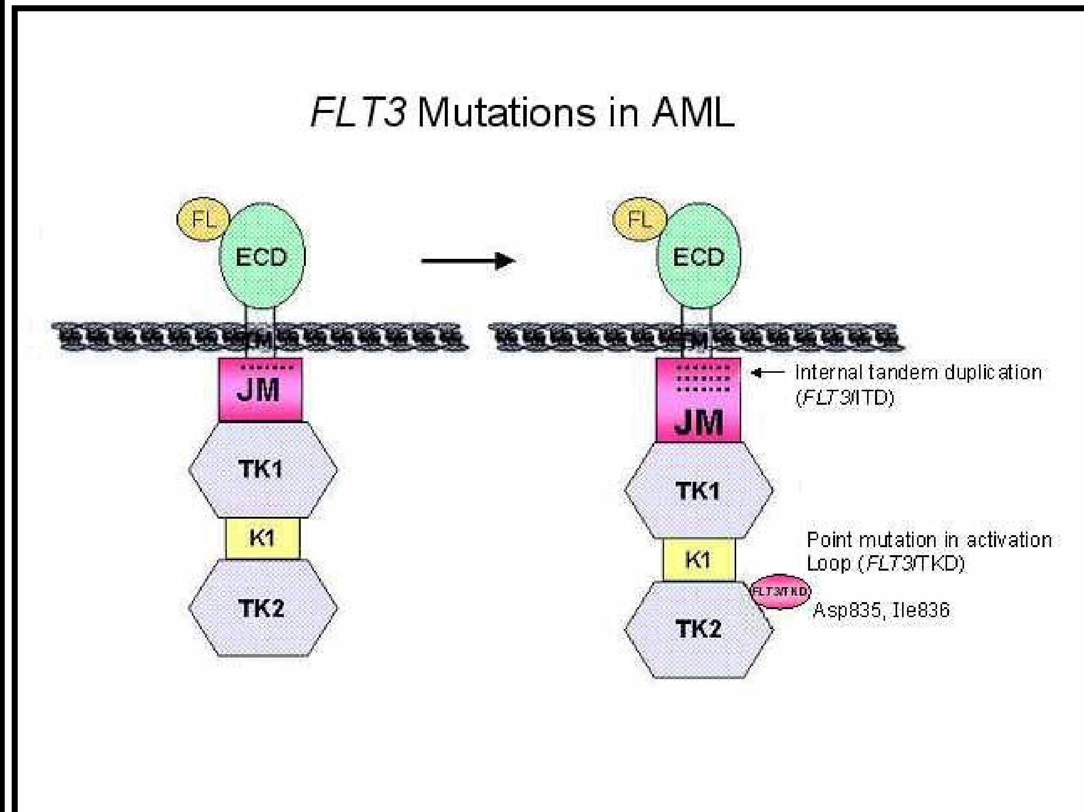
lokalizace 13q12

je členem rodiny receptorových tyrozinových kináz, exprimován na hematopoetických kmenových buňkách a také na buňkách leukemických. Mutace dvou typů popsány:

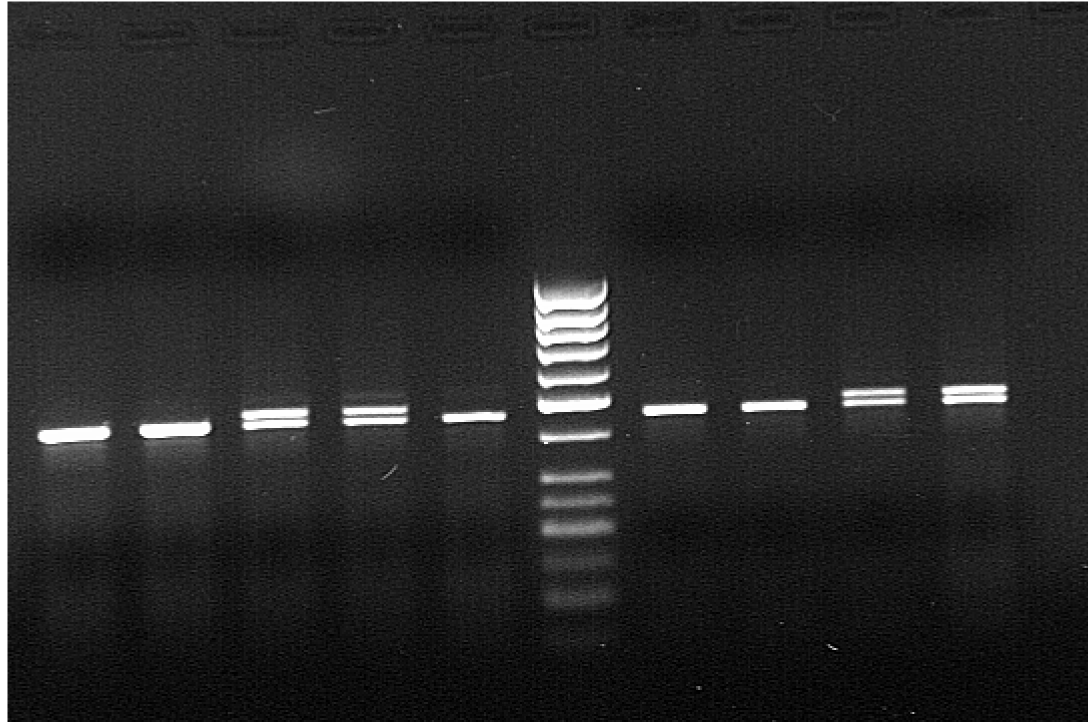
1) délkové v JM doméně (ITD, interní tandemové duplikace části genu) v exonu 14 (příp.15)

2) mutace v katalytické doméně TKD, kde kodóny D835 a I836 jsou kódovány nt GATATC, tvořící restrikční místo pro EcoRV (FLT3/D855)

Mutace jsou detekovány u ~30% AML s normálním karyotypem, představují nepříznivou prognózu často jsou považovány za sekundární aberaci a nejsou stabilní v průběhu follow-up.



RT-PCR detekce interních tandemových duplikací v exonu 12 genu FLT3



R5: 5´-TGTCGAGCAGTACTCTAAACATG-3´

12R: 5´-CTTTCAGCATTTTGACGGCAACC-3´, PCR produkt 456 bp

Kiyoi, H. et al., Leukemia, 11, 1447-1452, 1997

Restrikční analýza - Detekce mutací D835 v katalytické doméně TKD v exonu 20 (17) *genu* FLT3

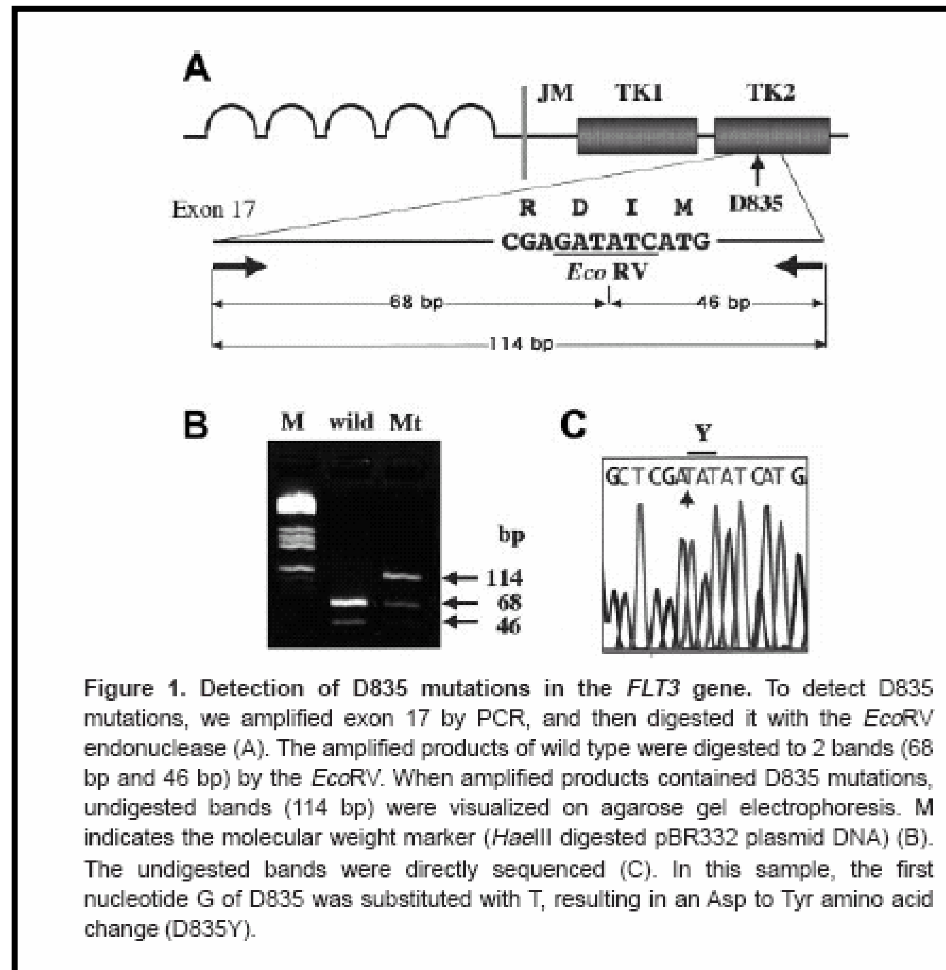
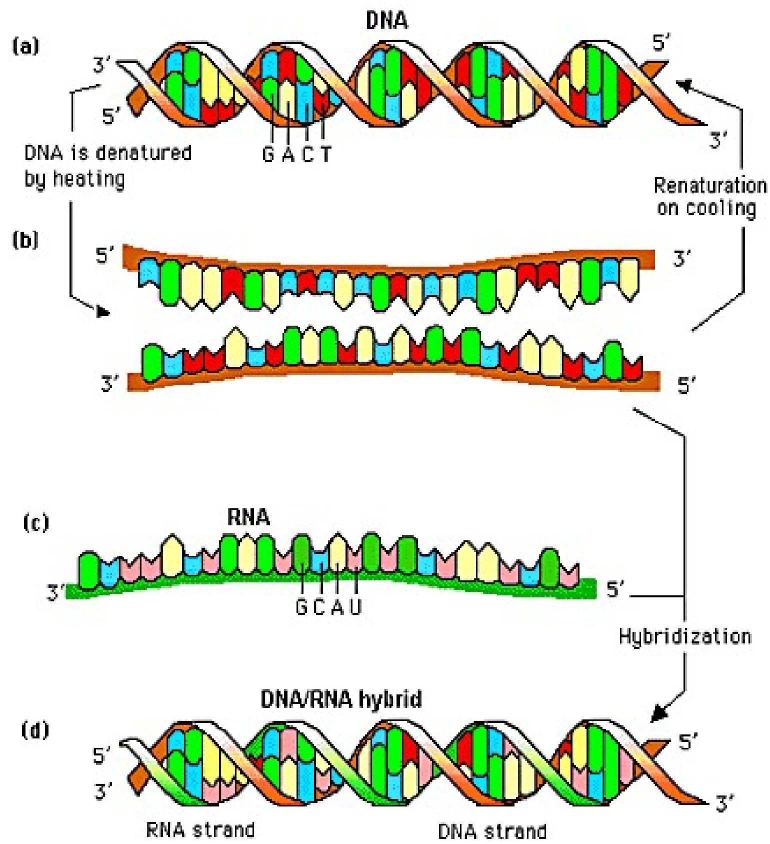
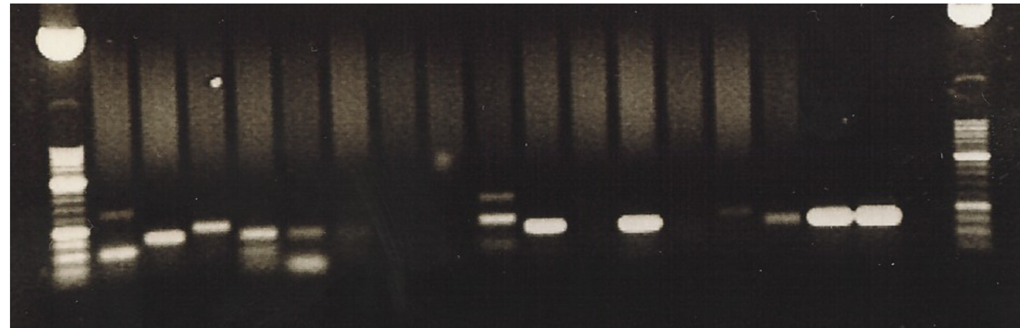


Figure 1. Detection of D835 mutations in the *FLT3* gene. To detect D835 mutations, we amplified exon 17 by PCR, and then digested it with the *Eco*RV endonuclease (A). The amplified products of wild type were digested to 2 bands (68 bp and 46 bp) by the *Eco*RV. When amplified products contained D835 mutations, undigested bands (114 bp) were visualized on agarose gel electrophoresis. M indicates the molecular weight marker (*Hae*III digested pBR332 plasmid DNA) (B). The undigested bands were directly sequenced (C). In this sample, the first nucleotide G of D835 was substituted with T, resulting in an Asp to Tyr amino acid change (D835Y).

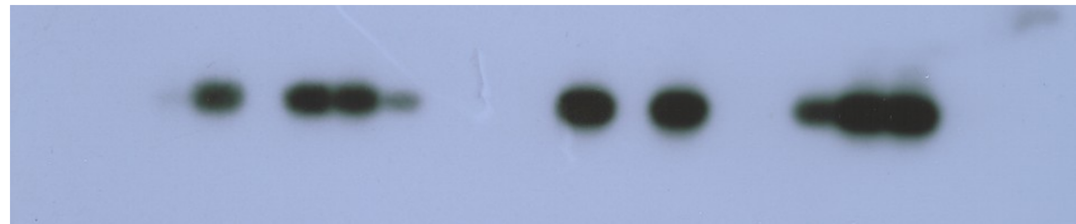
Yamamoto, Y., et al., Blood, 97, 2434-2439, 2001

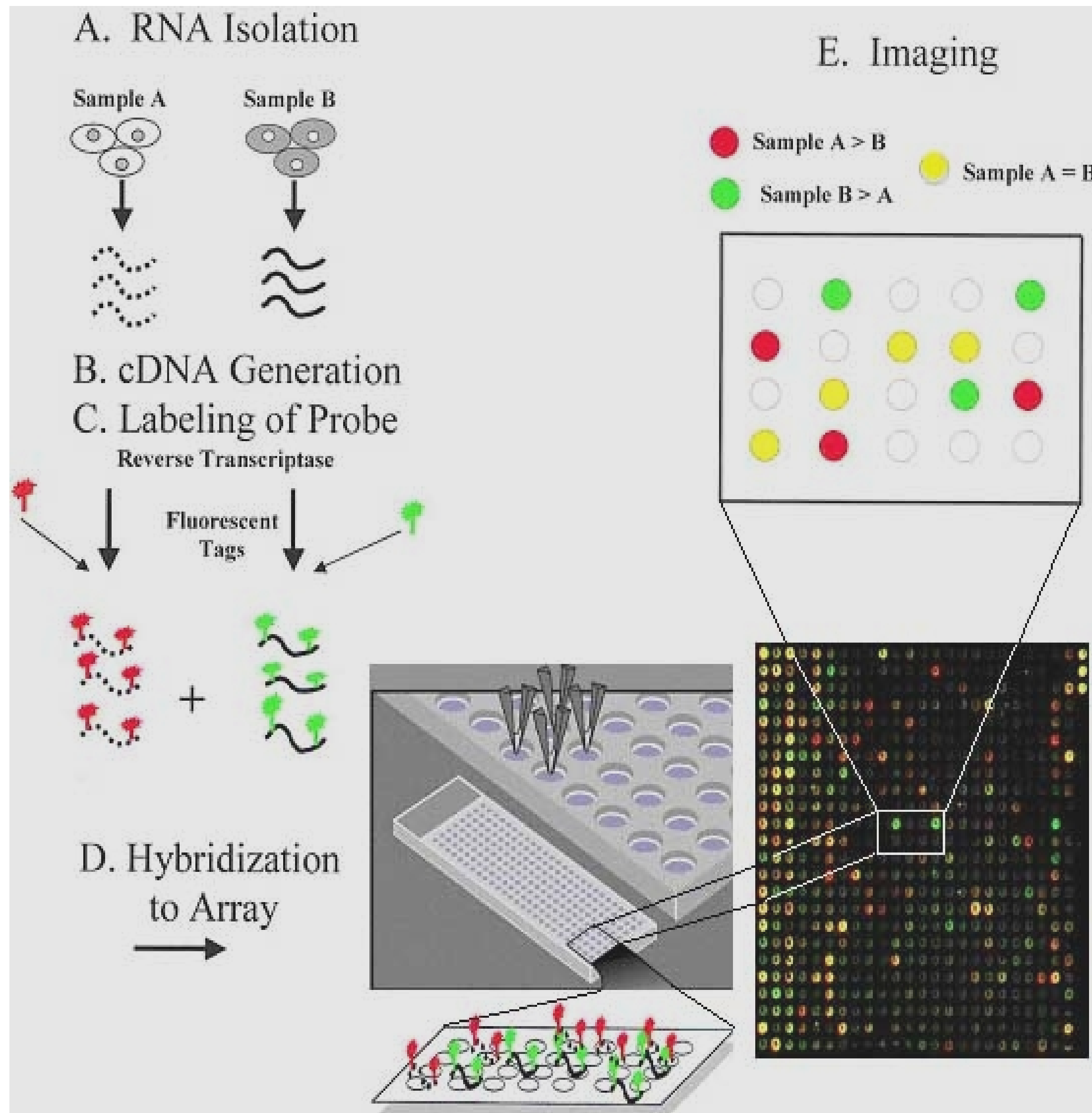


Ethidium bromide-stained agarose gel

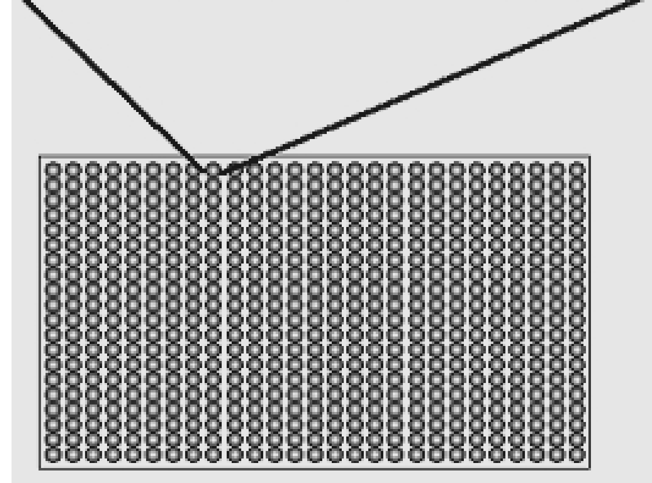


^{32}P probe





A. RNA Isolation
Sample A
Sample B
B. cDNA Generation
C. Labeling of Probe
Reverse Transcriptase
Fluorescent
Tags
+
D. Hybridization
to Array
E. Imaging
Sample A > B
Sample B > A
Sample A = B



TCCTTTCCGG AACGGTTGGC GTCTGCGCAC GGCGGTGTGG GGCATGACAT
GCCGCCCCAG GAACAACCCC GACACGGCTT TAAGCCTCTC AAATCGCTGT
AGACATCATC TTTACGTGCT TGGCTTGCCC TGCCACCATT AGGGCTGTTC
CCGCGACGAC TCGCCATTCA ACCTCAGTCC TTCGGGTTGA GCGAGTGGGT
CGCGCGCAAG GTGCGAATGG GTCGCGCGCA AAGTGTTGCG CTGGCTGTAT
TATATGCTGC CTATAGCGAG ACTAACGACC CACACTTTCA CACAAGGATT
TCCCGCTAAT GGGTACCTCG CGTCAGGACC TTGACGCAAG CGCGCCTTCG
GTTGGCCCCA AGCTTGCTAG GACTACTTAT CTTGAGCTCA TTTAACATCC
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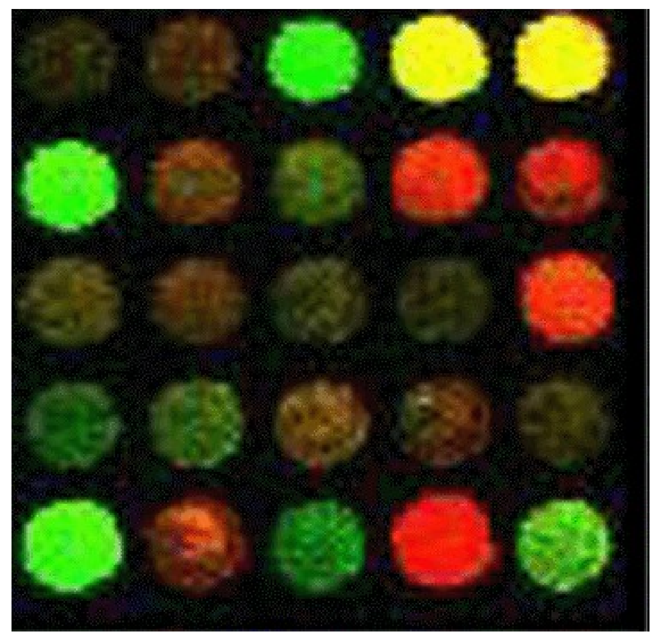
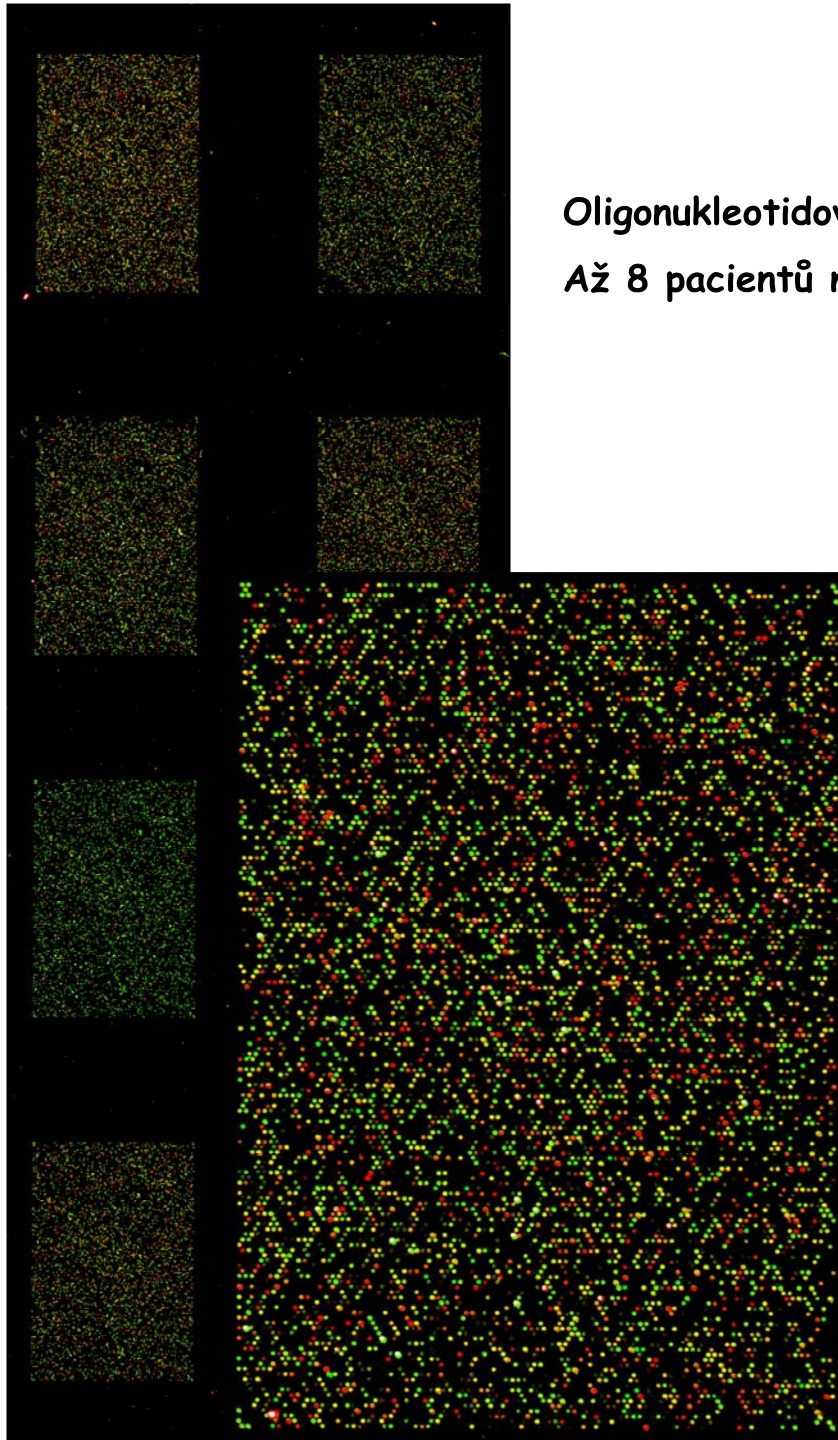
GTTGACAAAG CGTGGAGACA TCGATACCTC TGTGTCAGCG GCCACAAATC



CLL

Oligonukleotidové DNA čipy pro stanovení exprese > 19.000 genů

Geny s největšími rozdíly v expresi mezi CLL pacienty s mutovaným a nemutovaným IgVH

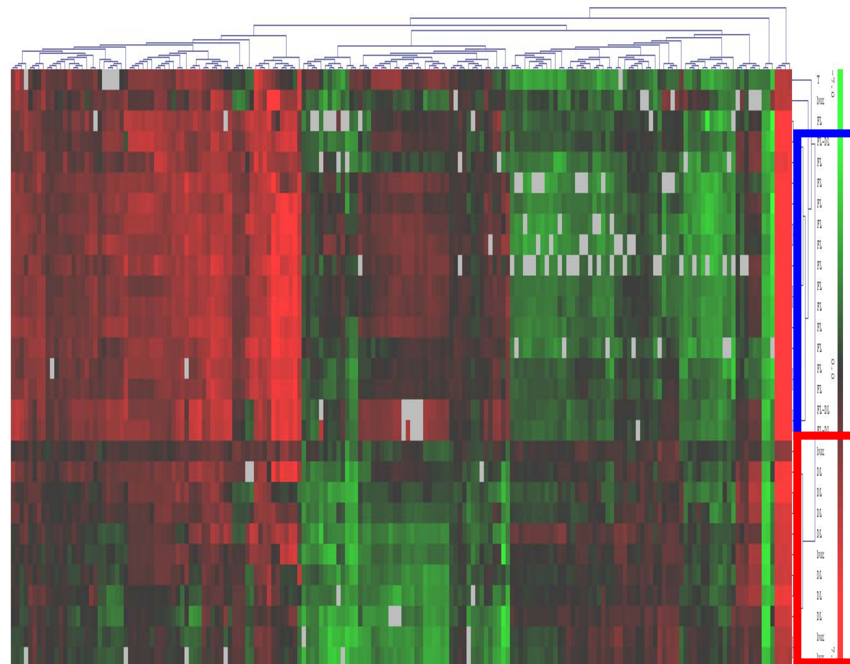


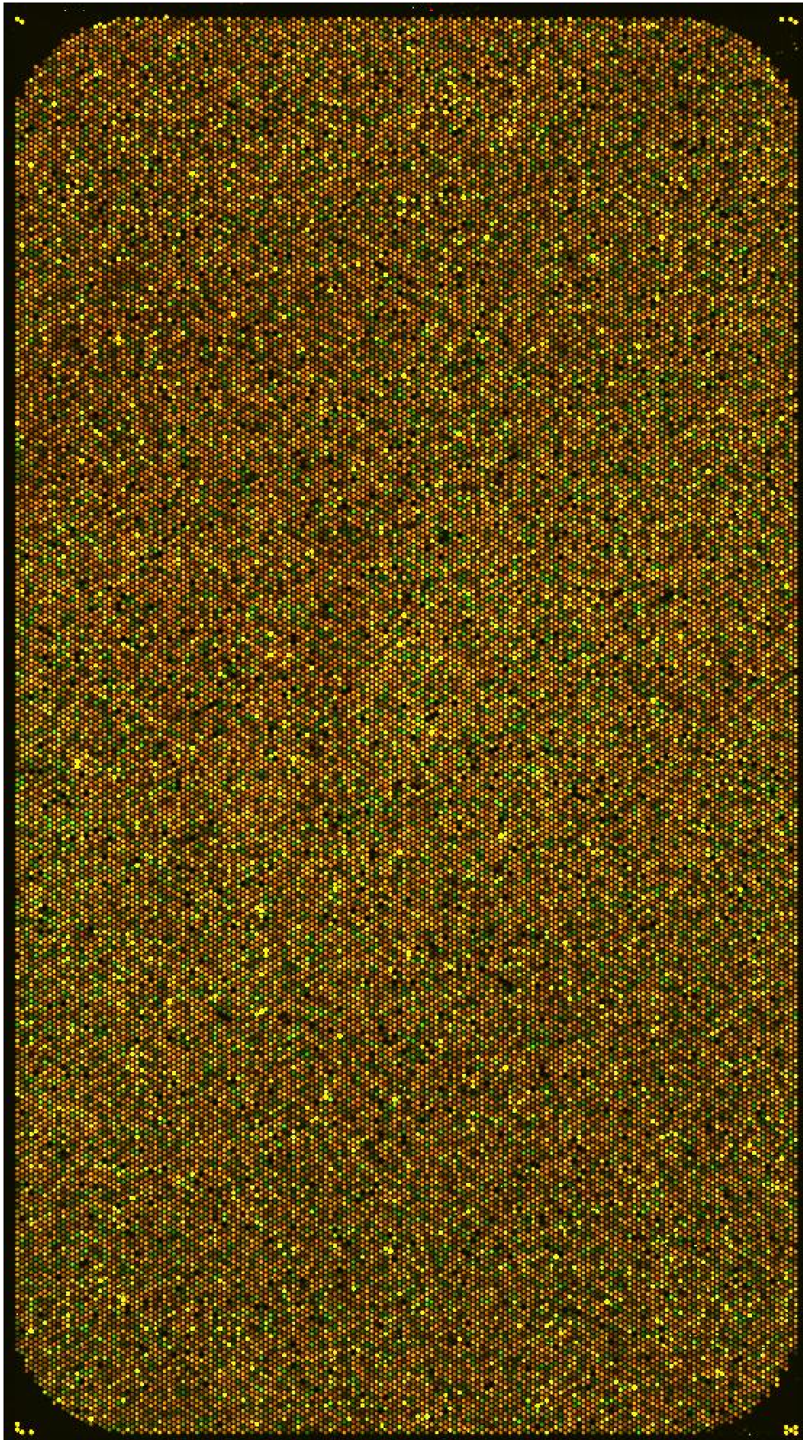
LymfoChip

Oligonukleotidové DNA čipy s 15.000 sondami pro 4.000 genů

Až 8 pacientů na jednom čipu

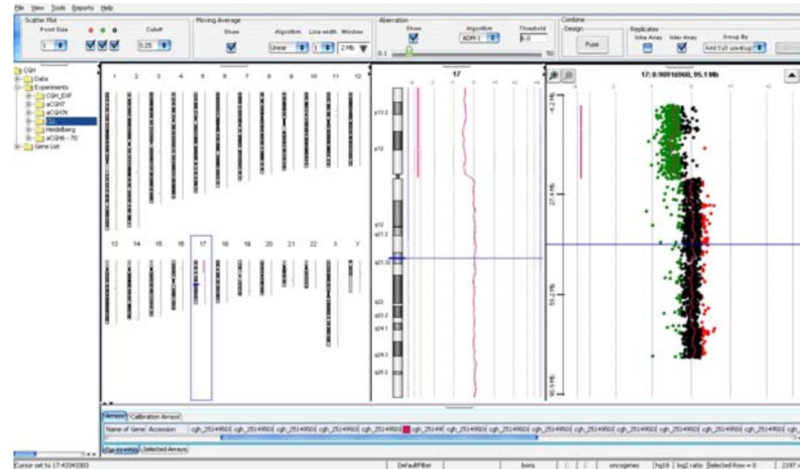
Geny s rozdílnou expresí u pacientů s Folikulárním lymfomem a DLBCL nebo Burkittovým lymfomem



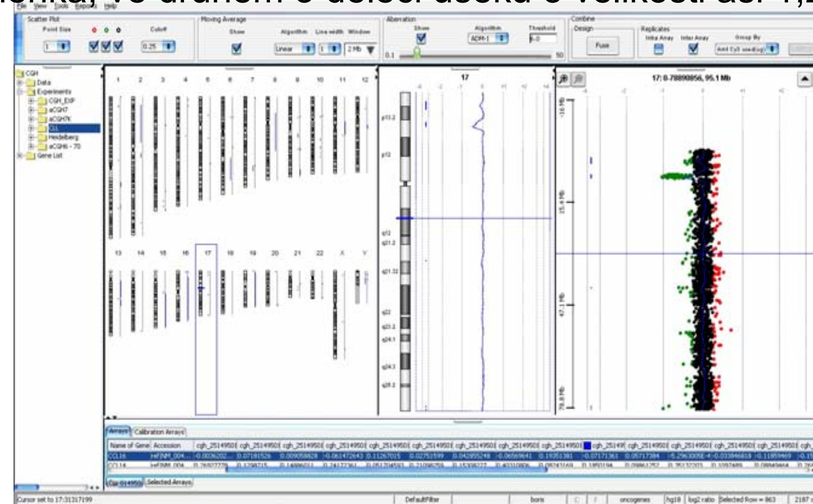


ArrayCGH

Oligonukleotidové DNA čipy >40.000 sond rovnoměrně pokrývajících všechny chromozomy



Různý rozsah delece krátkého raménka chromozomu 17 u dvou pacientů s CLL – v prvním případě jde o deleci celého raménka, ve druhém o deleci úseku o velikosti asi 1,2 Mb



CML treatment in the future

Genomic analysis

(classification and response)

Imatinib-responsive → **Imatinib**

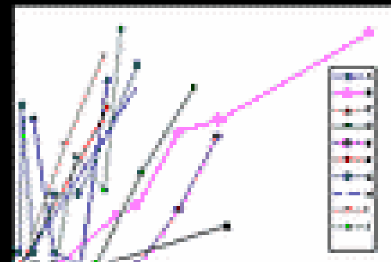
Imatinib-unresponsive → **Transplant**

Imatinib → **Transplant**

MRD monitoring

"Hi-risk" of relapse

Bcr-abl

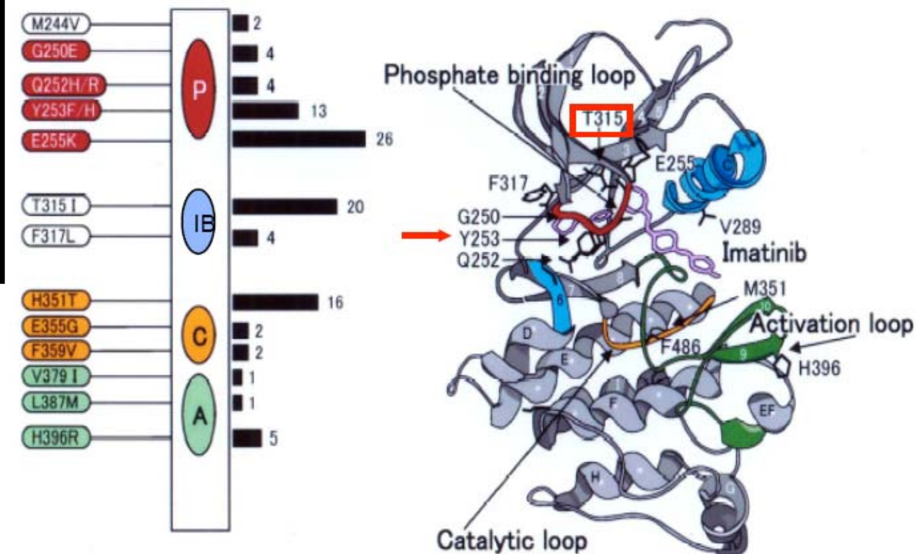


time

New inhibitory molecules

- Abl kinase inhibitors
  - AMN 107 (Novartis Pharma)
- Abl/Src inhibitors
  - Dasatinib (Bristol Myers Squibb)
  - AP23464 (Ariad Pharmaceuticals)
  - SKI-606 (Wyeth-Ayerst)
  - AZD-0530 (Astra-Zeneca)
  - Pyridopyrimidines (PD family, Pfizer)
- Abl/Lyn inhibitor - NS-187 (Nippon Shinyaku)
- Abl substrate inhibitor - ON 012380 (Onconova)

Point mutations in the Abl kinase domain



After Ohyashiki et al, 2004

Nástup nových terapeutických možností přináší obrovské úspěchy...

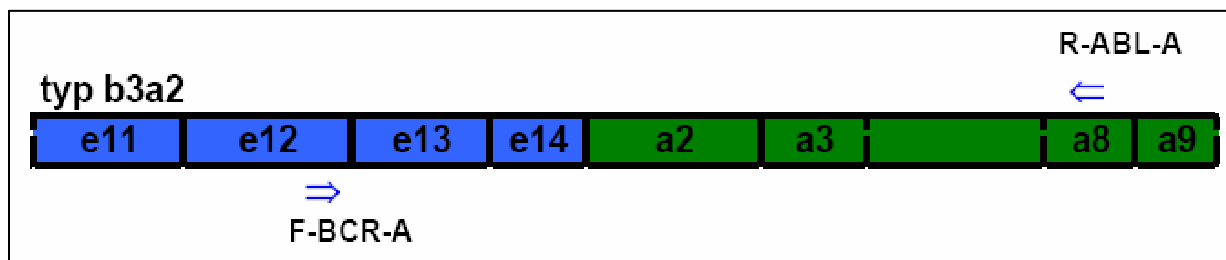
Mutace v ABL kinázové doméně

- Kinázová doména ABL: zahrnuje aminokyseliny 220-498
- **Mutace v oblasti P-loop**:
  - způsobují změnu konformace, vedou k aktivní konformaci, špatná prognoźa, G250E, Q252H, E255K
- **T315I mutace**: vodíková vazba s imatinibem, allosterické působení na vazbu léku
- **Mutace katalytické oblasti**:
  - **M351T mutace**: destabilizace autoinhibiční konformace Abl, porušením vazby s SH2, F359C
- **Mutace v oblasti A-loop**:
  - zabraňují dostat se do „vypnutého" stavu, F382L, H396R

Schéma struktury Abl kinázové domény

Detekce bodových mutací

Amplifikace BCR-ABL Expand High Fidelity Enzyme (Roche)
Primery F-BCR-A (exon e12/e13) x R-ABL-A (exon a8)



Sekvenace oblasti exonů **a4-a8** na AbiPrism 310 sekvenátoru
za použití primeru ABL-ALT a BigDye®Terminator v1.1 Seq.kit
Srovnání získaných sekvencí se standardními sekvencemi X16416

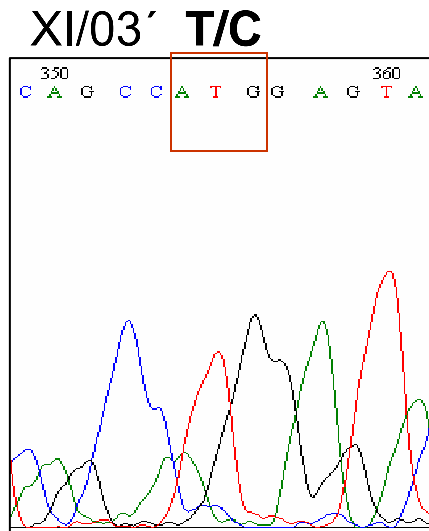
Název primeru	Sekvence 5´→ 3´
ABL-ALT*	5´-GCG CAA CAA GCC CAC TGT CTA TGG-3´
F-BCR-A*	5´-GAG CAG CAG AAG AAG TGT TTC AGA-3´
R-ABL-A	5´-CTC TAG CAG CTC ATA CAC CTG GG-3´

Gorre M.E. et al. 2001 Science*, Soverini S. et al. 2004 Clin.Chem.*

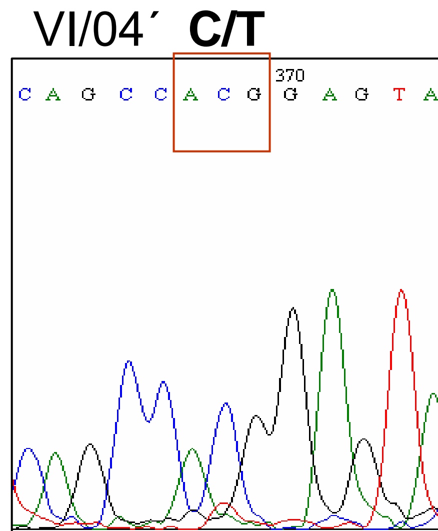
Vývoj mutace u pacienta rezistentního na imatinib s mutací Met351Thr

ATG→ACG

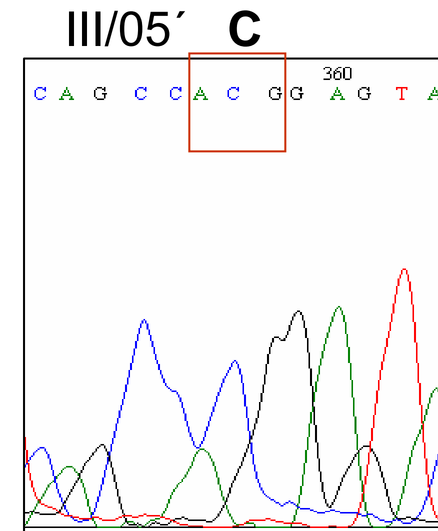
XI/03´ **T/C**



VI/04´ **C/T**



III/05´ **C**



rok léčby: pouze hematologická remise

bez cytogenetické remise

akcelerace nemoci

Granulocyty a erytroblasty (BM normal)