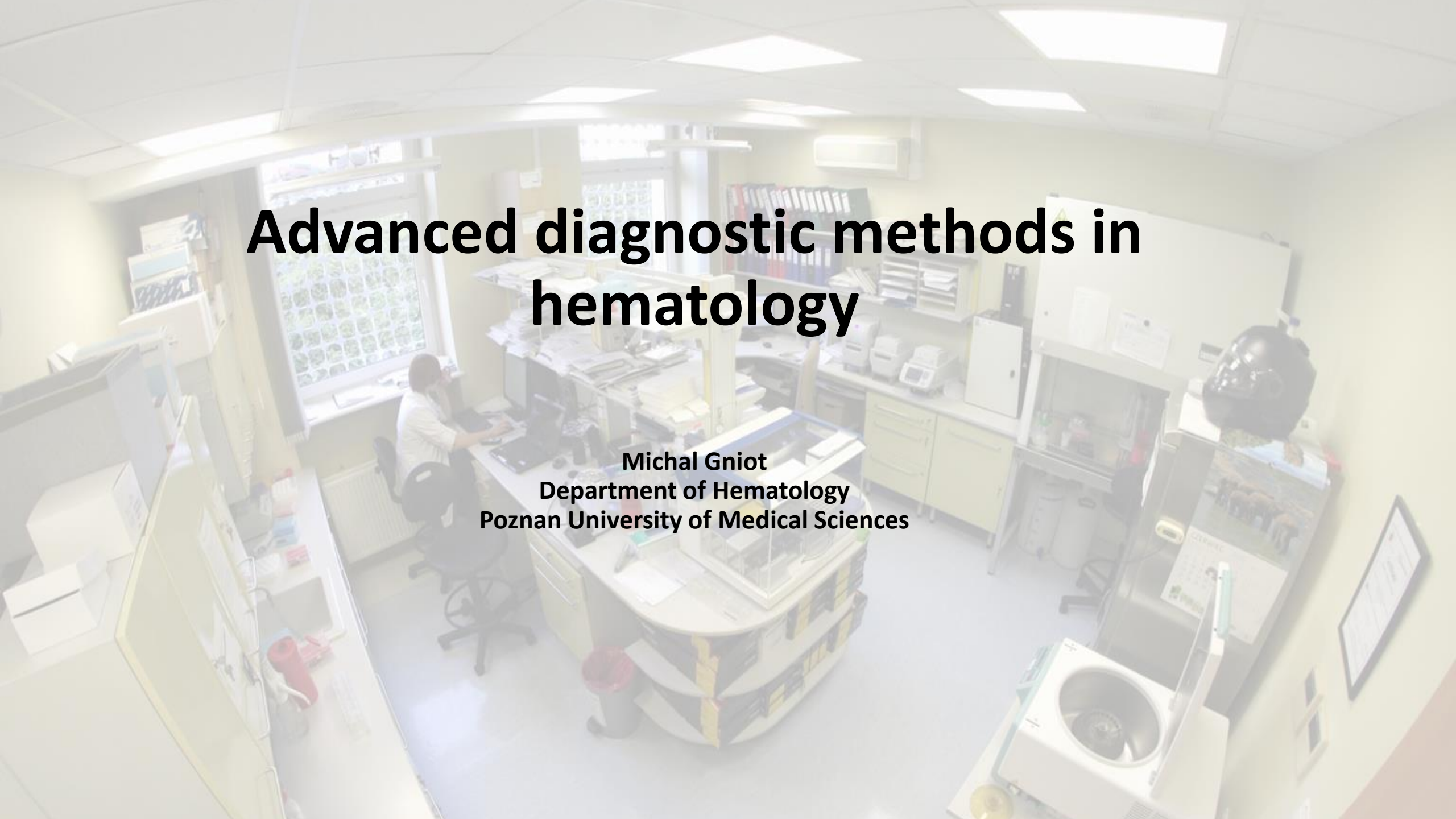


Advanced diagnostic methods in hematology

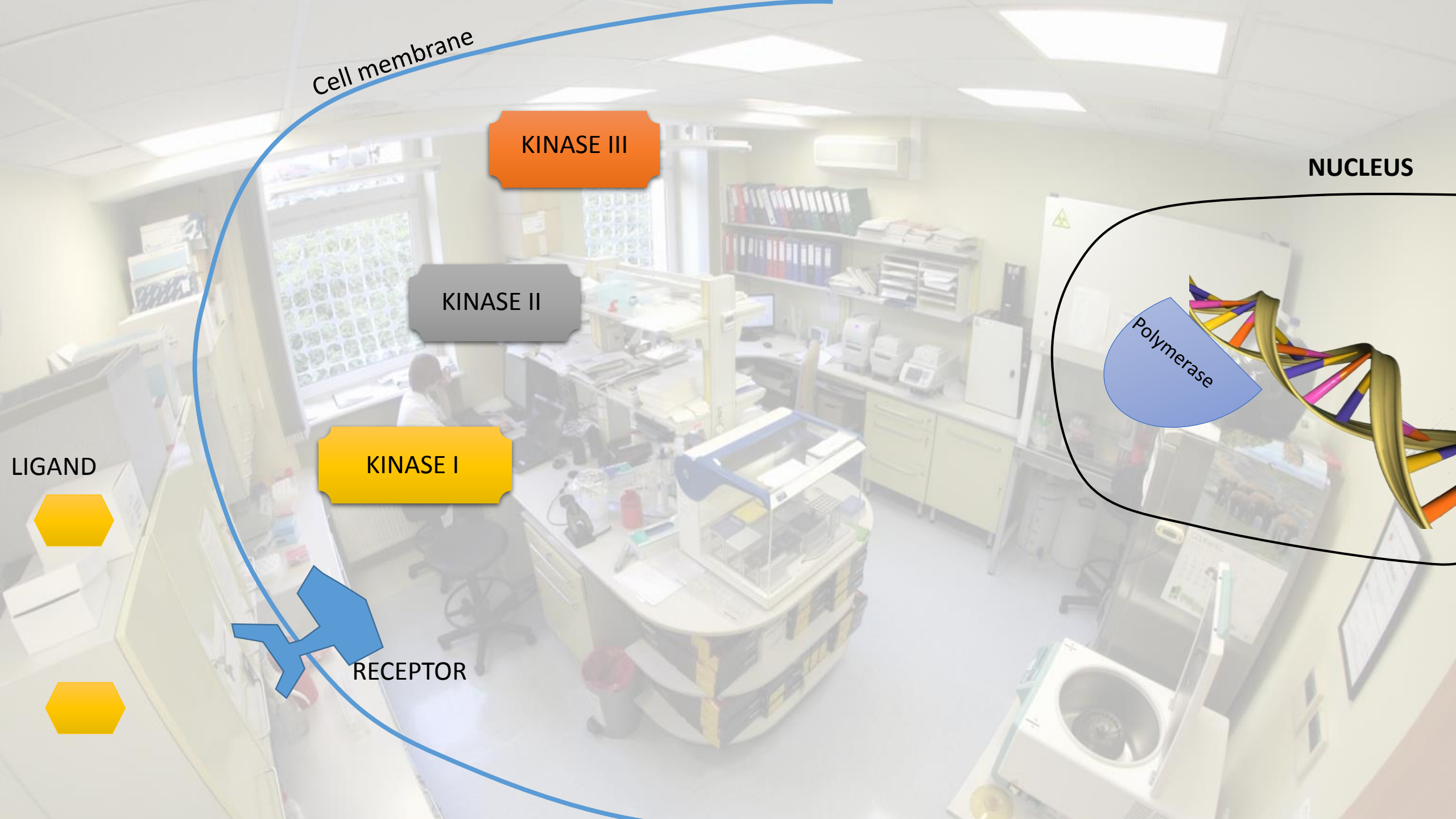
Michal Gniot
Department of Hematology
Poznan University of Medical Sciences

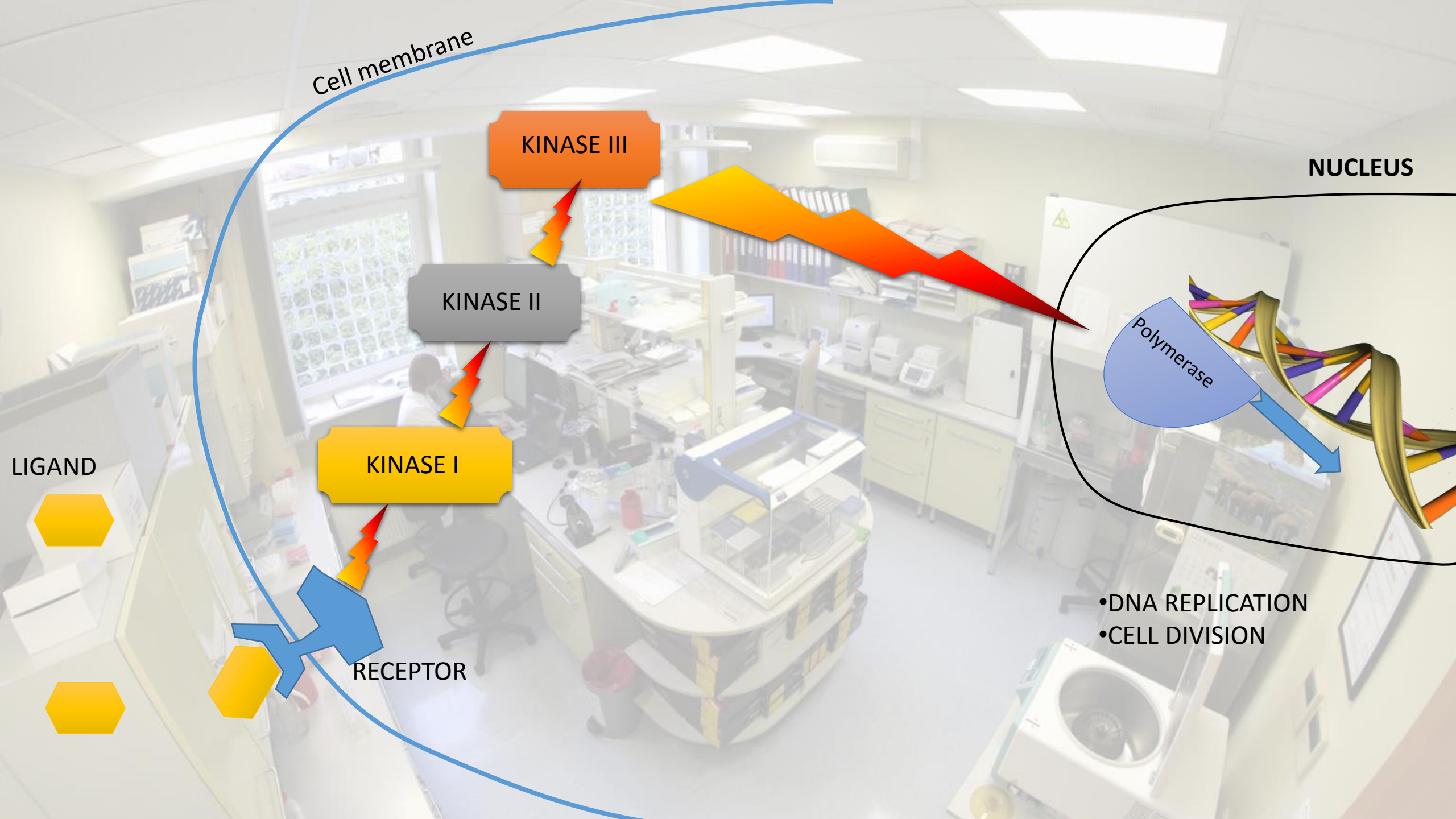
Hematological neoplasms

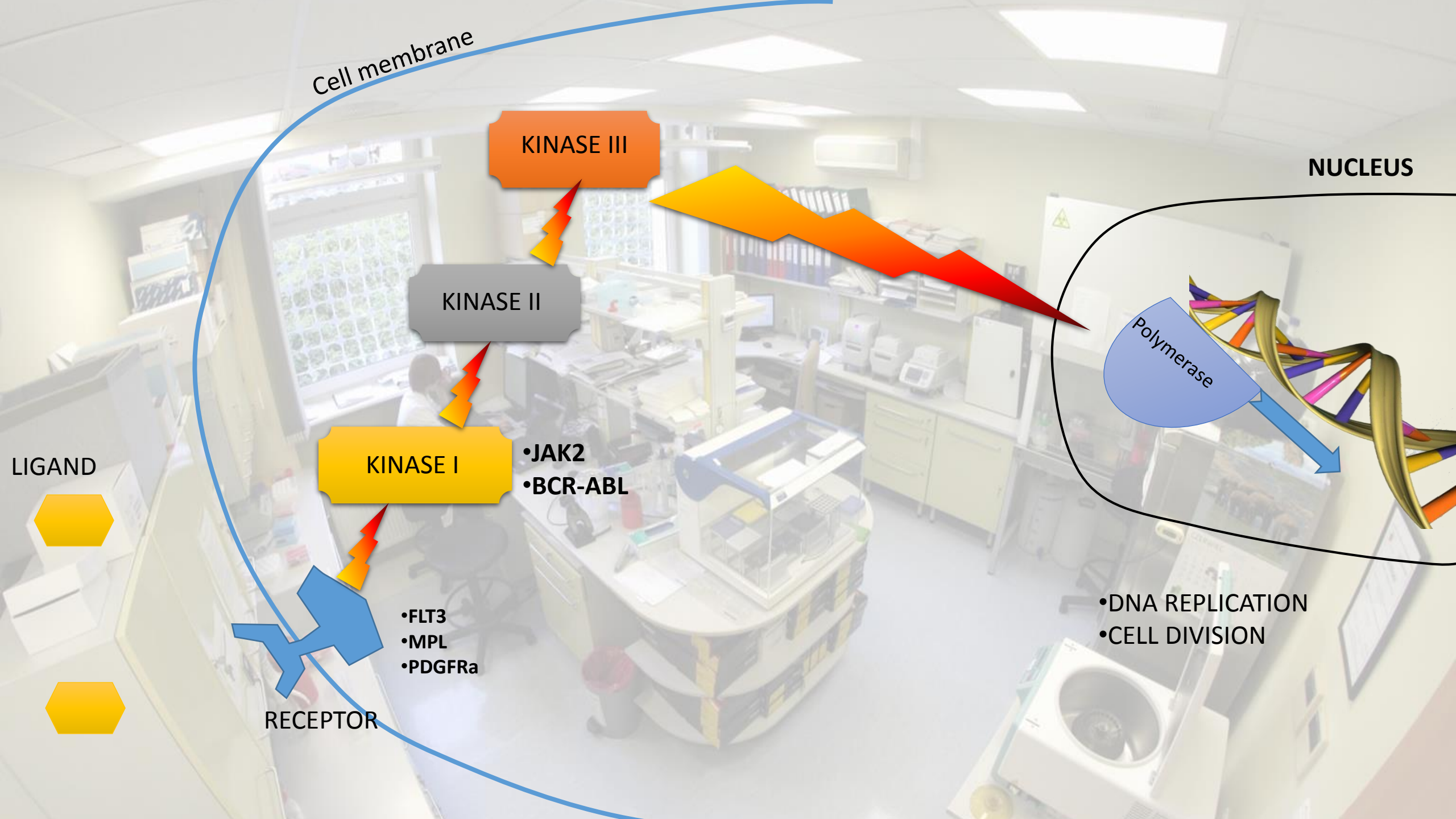
- **Clonal growth of cell population originating from myeloid or lymphoid lineage**
  - **Somatic mutation**
  - **Neoplastic cells circulating in peripheral blood**
  - **Possible abnormalities in cell maturation**
- **Examples:**
  - **Leukemias (acute and chronic, myeloid and lymphoid)**
  - **Myeloproliferative neoplasms (Polycythemia Vera, Idiopatic Myelofibrosis, Essential Thrombocythemia)**
  - **Lymphomas, myelomas**

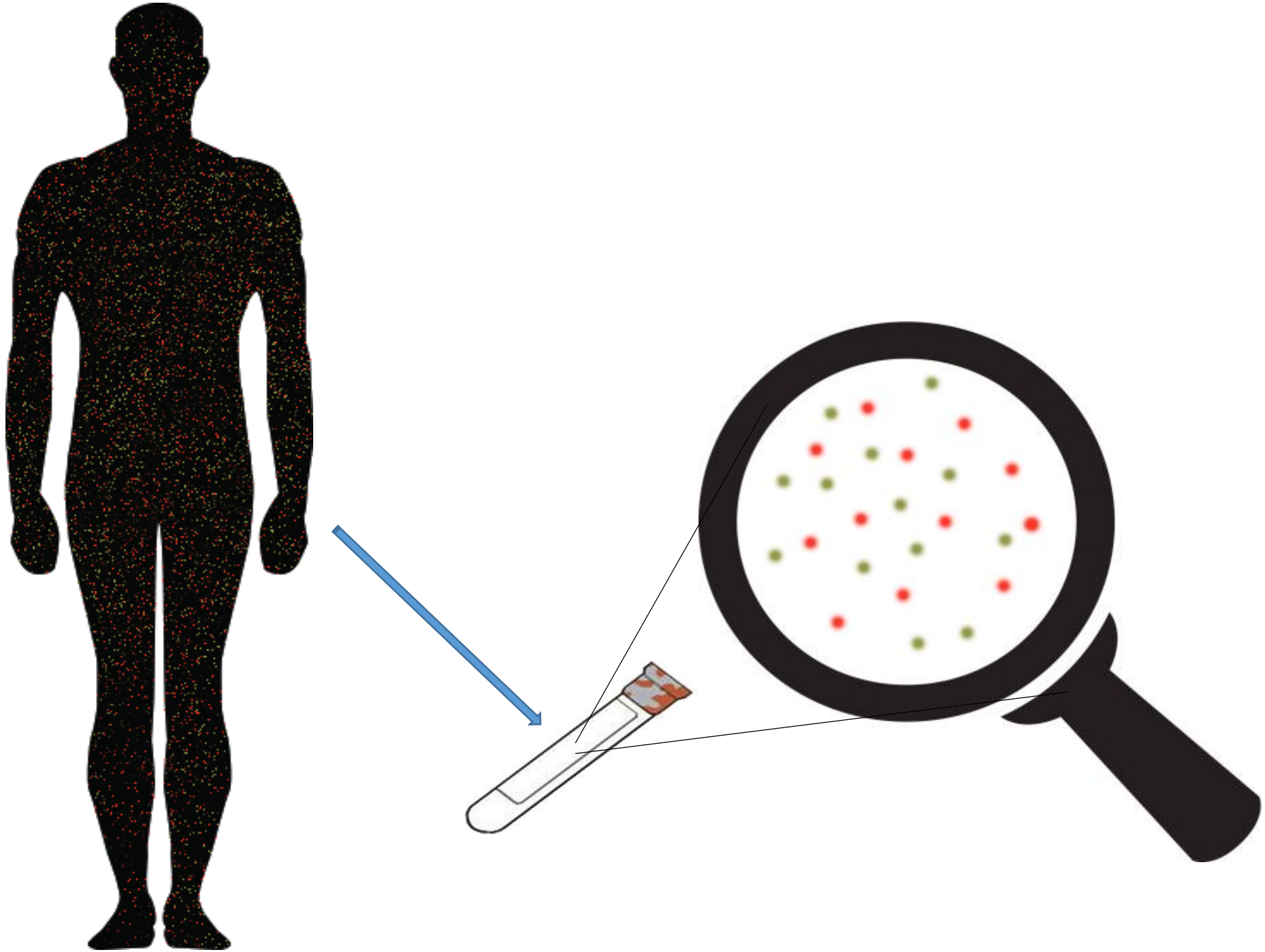
Hematological neoplasms – background

- **Somatic (accquired) mutation**
  - **Region encoding a protein which plays a role in cell signaling pathway**
  - **Change of function (usually - activation)**
  - **Point mutations, insertions-deletions, translocations**
  - **Translocations result in fusion genes**


Cell membrane
KINASE III
KINASE II
KINASE I
LIGAND
RECEPTOR
NUCLEUS
Polymerase

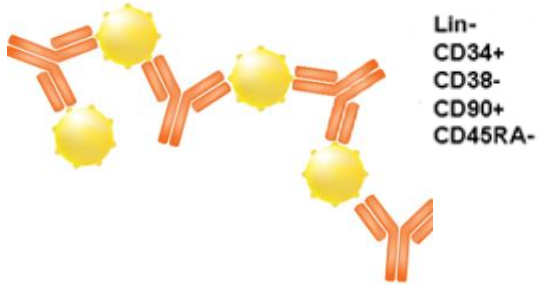

Cell membrane
KINASE III
KINASE II
KINASE I
LIGAND
RECEPTOR
NUCLEUS
Polymerase
•DNA REPLICATION
•CELL DIVISION


Cell membrane
KINASE III
KINASE II
KINASE I
•JAK2
•BCR-ABL
NUCLEUS
Polymerase
LIGAND
•FLT3
•MPL
•PDGFRa
RECEPTOR
•DNA REPLICATION
•CELL DIVISION

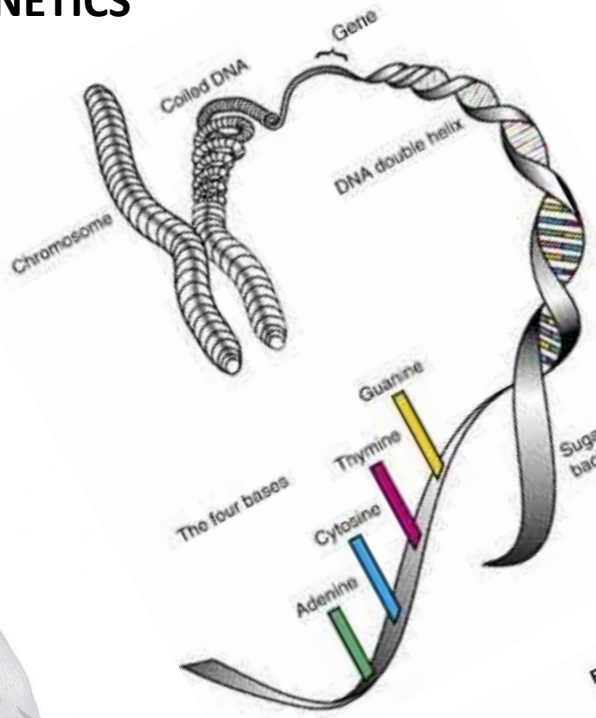


IMMUNOHISTOCHEMISRY

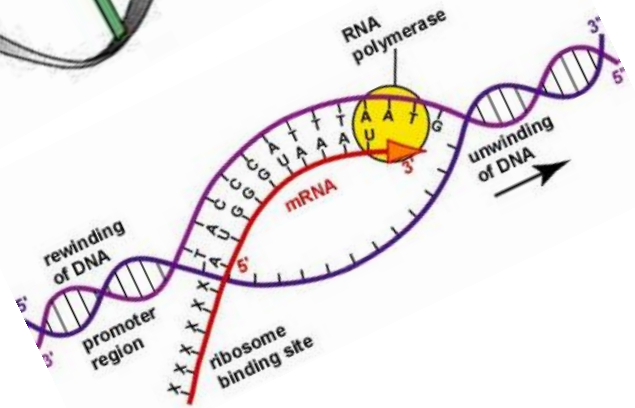
FLOW CYTOMETRY



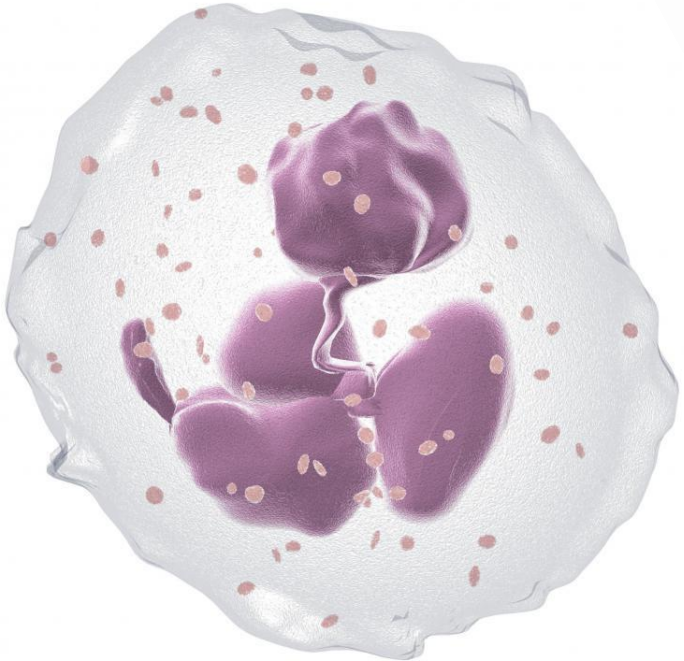
CYTOGENETICS



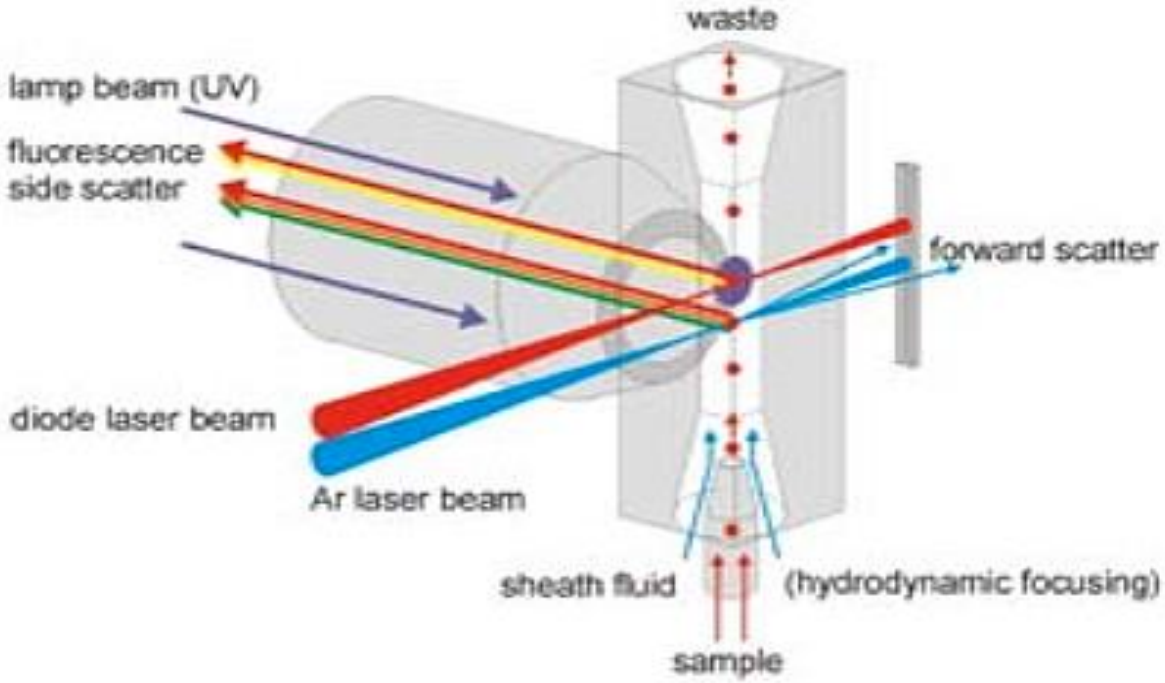
MOLECULAR BIOLOGY

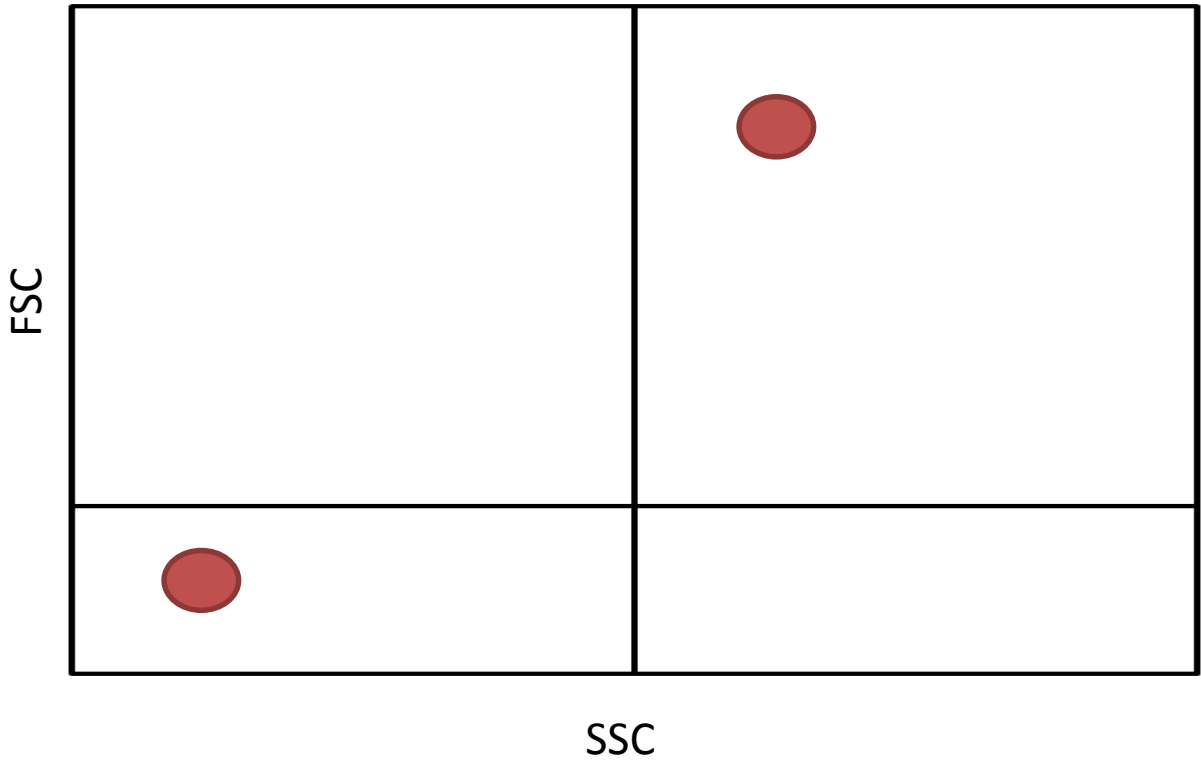


CYTOMORPHOLOGY

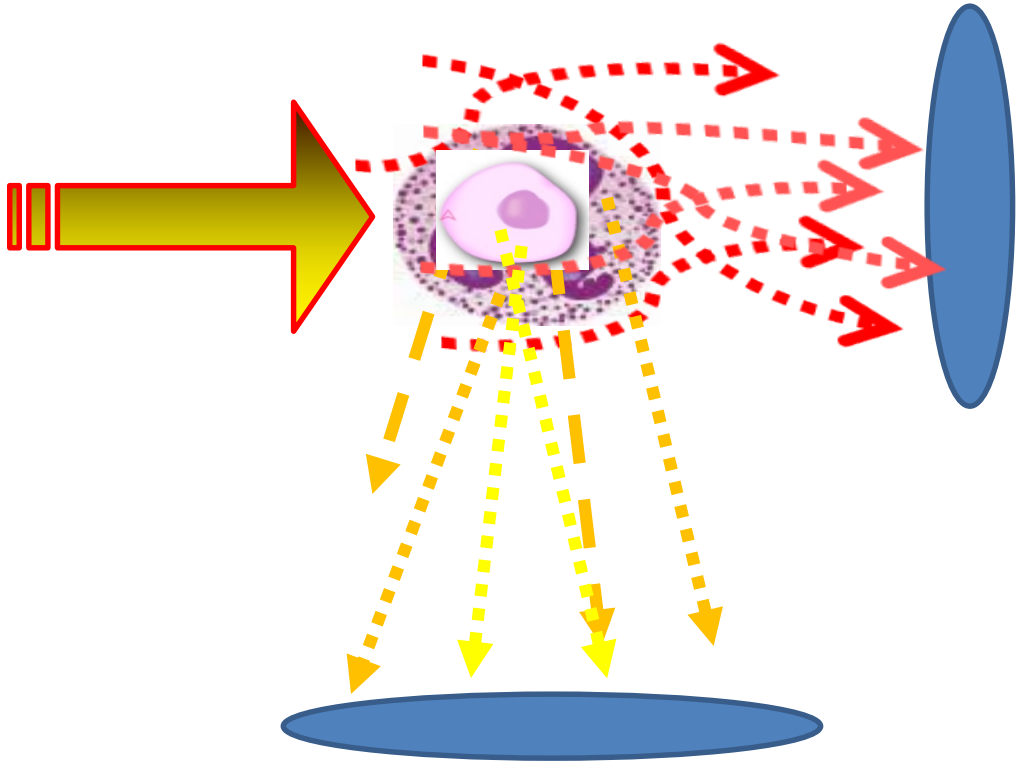


Flow cytometry





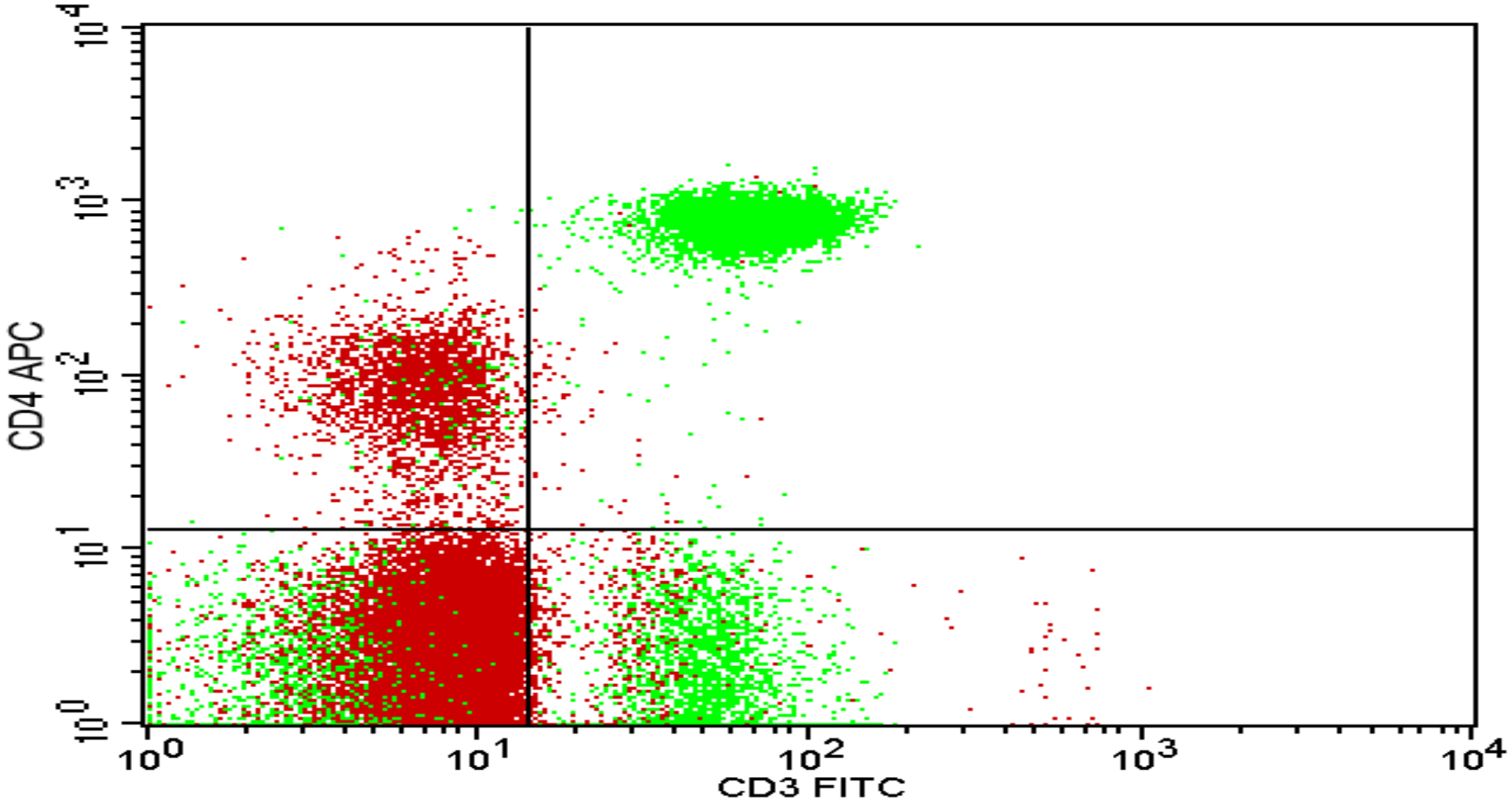
Flow cytometry



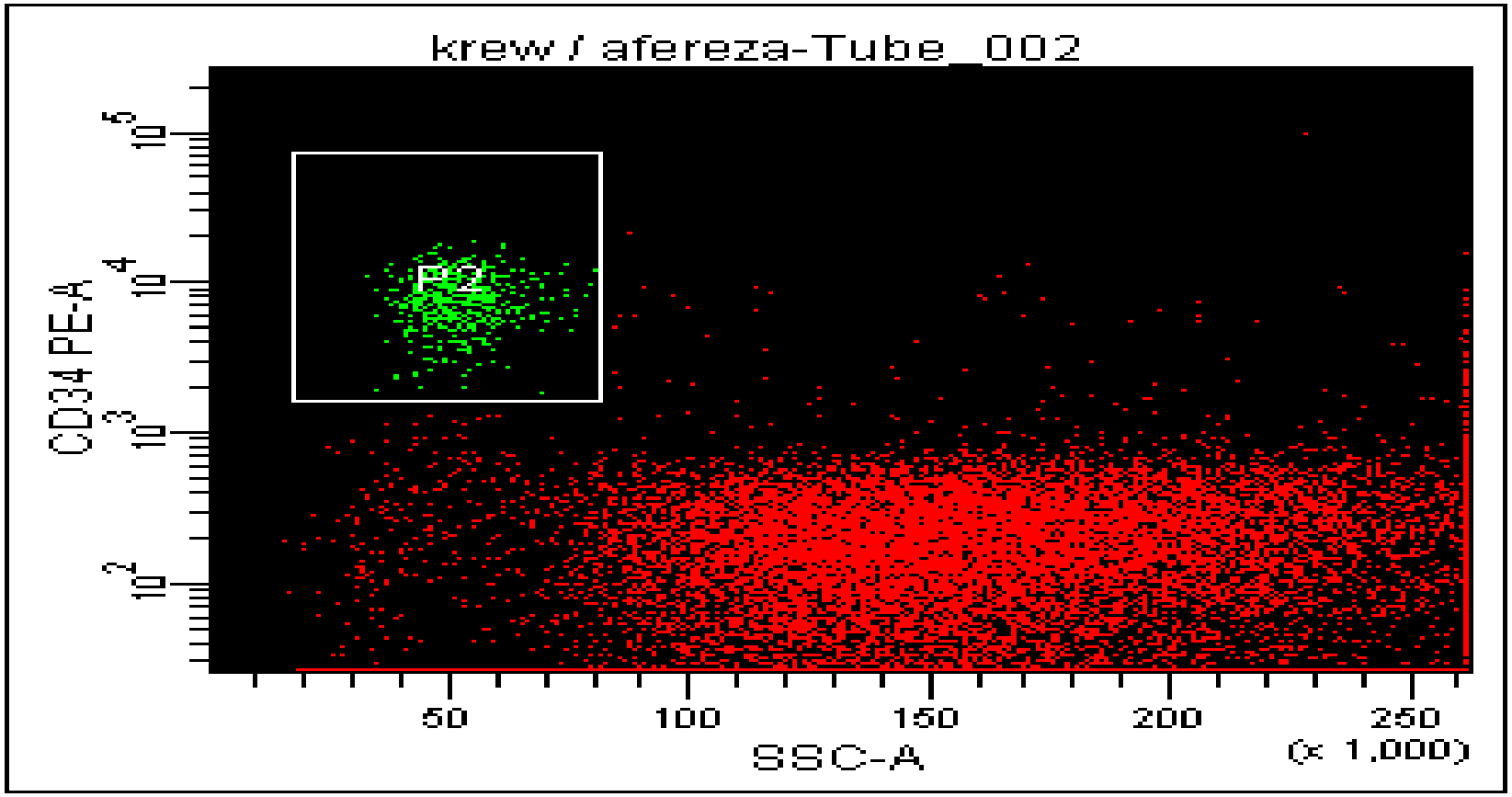
- Characterisation of hematopoietic cells in blood, marrow, CSF, exudates
- Immunophenotype describing
- Separation of cells with specific antigenity (e.g. CD34+ stem cells for transplatations)

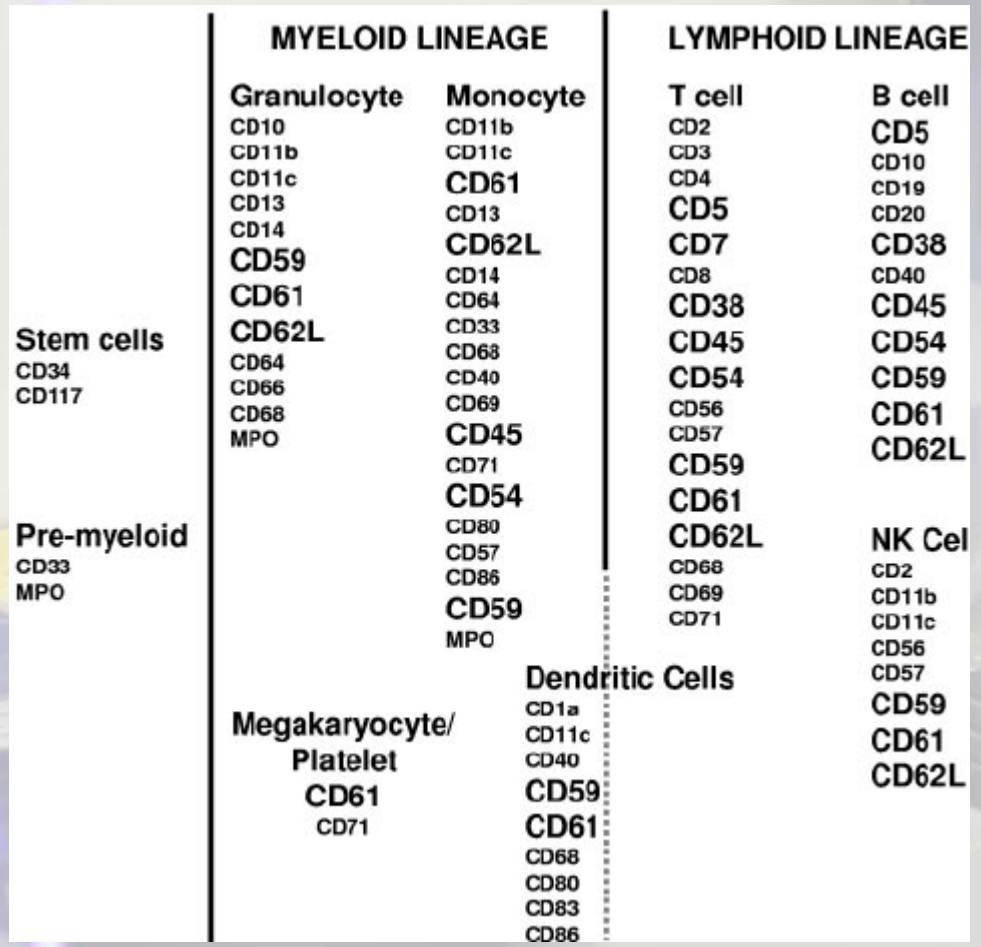
Examples:

T-helper lymphocytes (CD3+ CD4+)



CD34+ cells


krew / afereza-Tube_002
P2
CD34 PE-A
10^5
10^4
10^3
10^2
50
100
150
200
250
SSC-A
(× 1,000)



MARKER	DESCRIPTION
CD3	Pan T-Cell
CD4	Helper T-Cell
CD8	Cytotoxic/Suppressor T-Cell
CD19	Early B-Cell, B-Cell Specific
CD33	Early Myeloid Cells
CD34	Stem Cells, Progenitor Cells
CD38	Activated T-Cells, Early Progenitor Cells
HLA-DR	Activated T-Cell, Monocytes, B-Cell

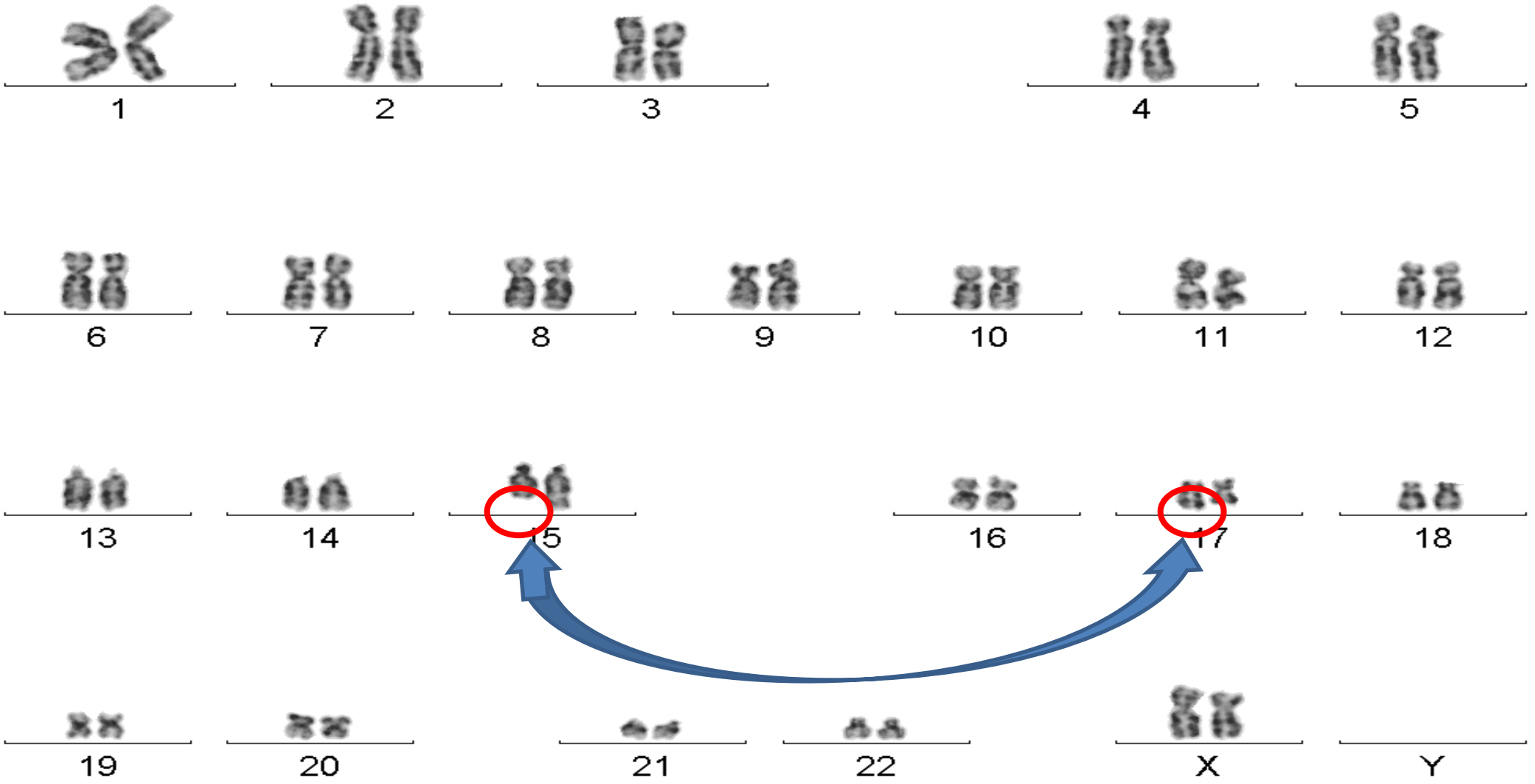
Cytogenetics

- Classical cytogenetics require culturing of bone marrow cells in order to obtain nuclei in metaphase stage.
- The chromosomes are stained using GTG (or other) banding method, photographed and analyzed in dedicated software
- Usually, 20-30 metaphases has to be assessed in comparison to normal human karyotype
- Molecular cytogenetics, FISH method (Fluorescent In-Situ Hybrydization) is a supporting method, which allows to detect specific aberrations

Aims of cytogenetic analyses

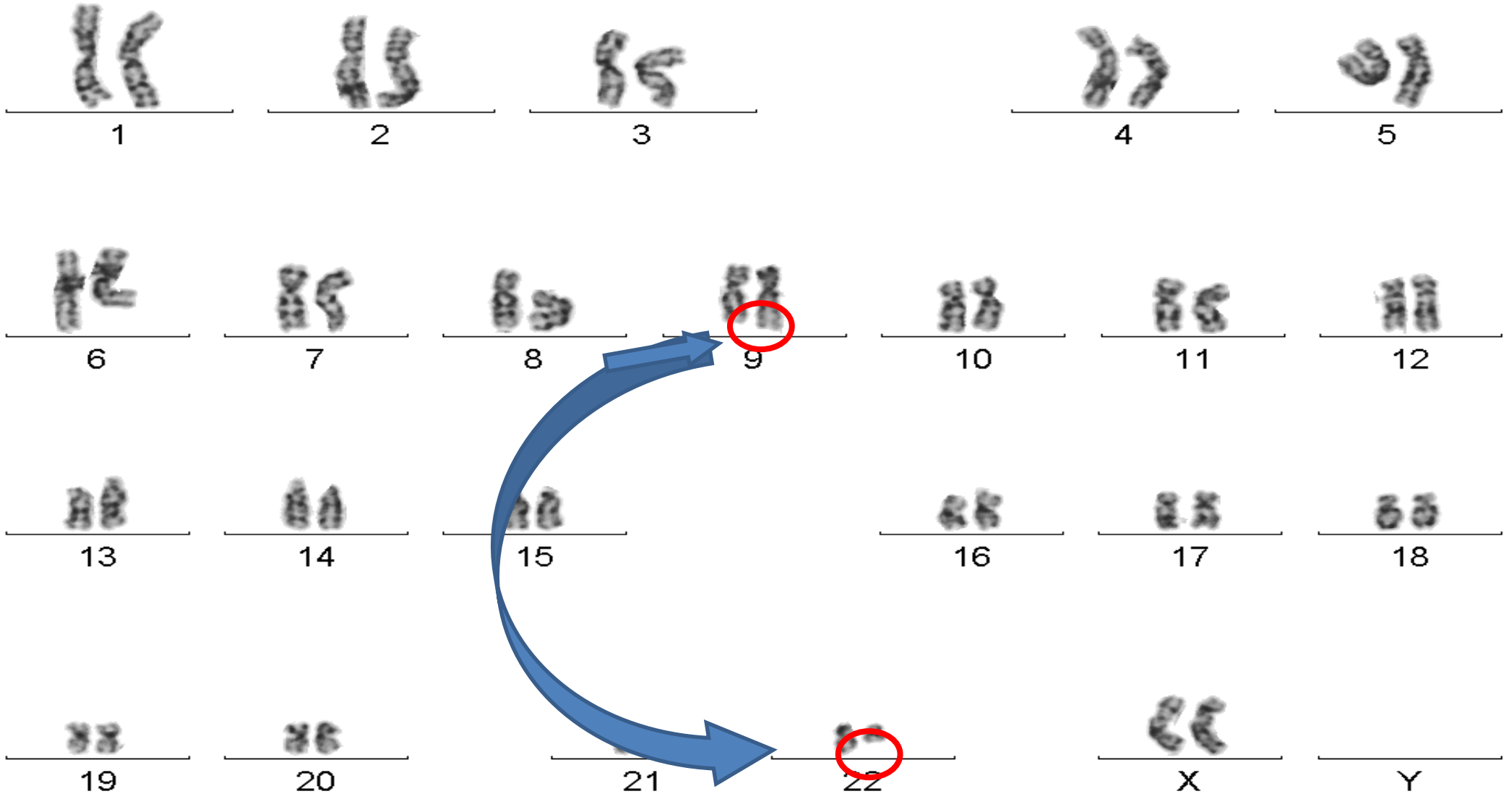
- Detection of chromosomal abnormalities characteristic to: MDS, AML, ALL, CML ...
- Observation of new, acquired abnormalities (clonal evolution)
- Prognostic factors (i.e. additional aberrations in CML)
- Monitoring of the therapy
- Post-transplantation chimerism of Y-X chromosomes

t(15;17) – PML-RARA in AML M3



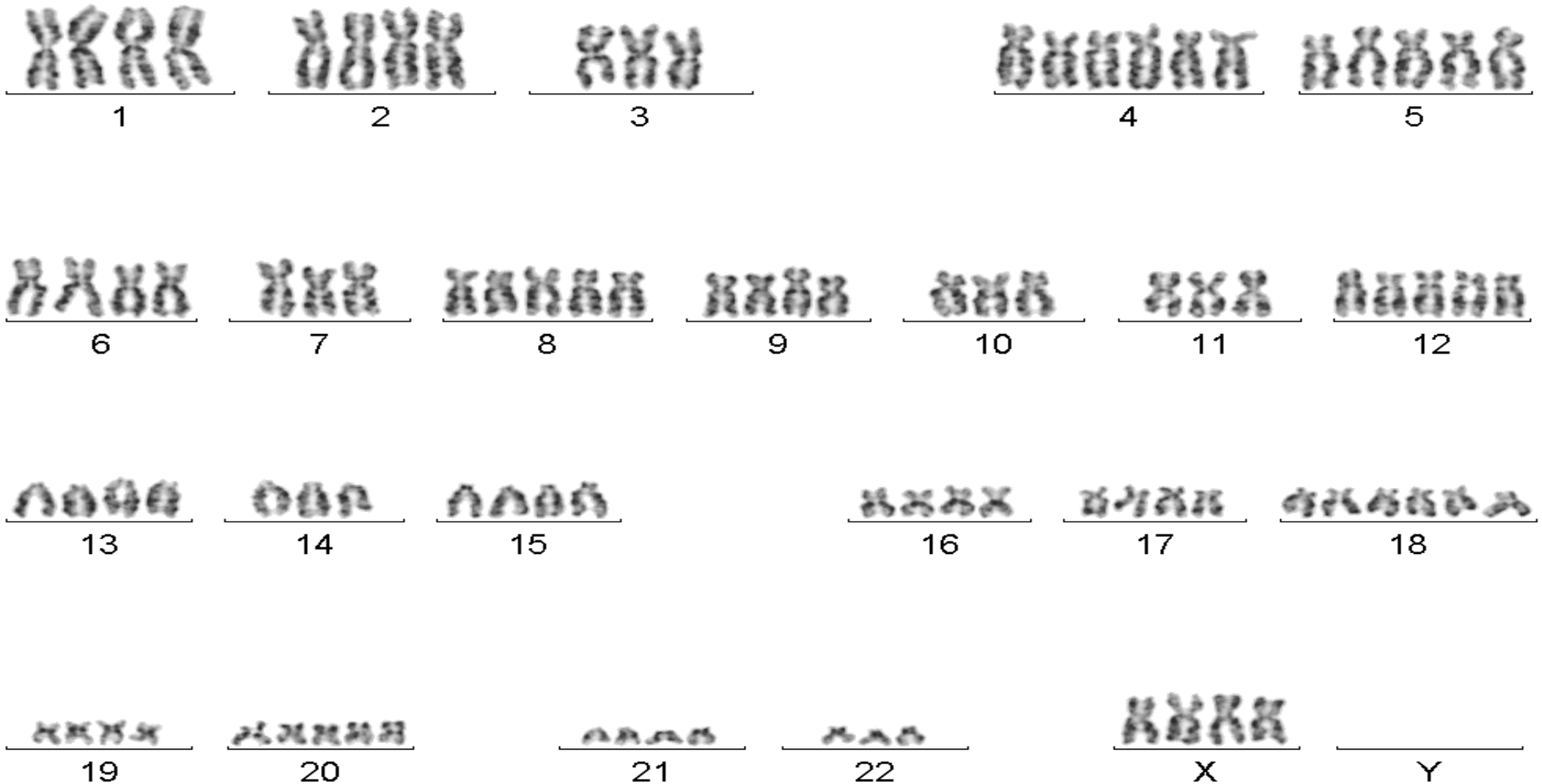
5q-

t(9;22) – Philadelphia chromosome



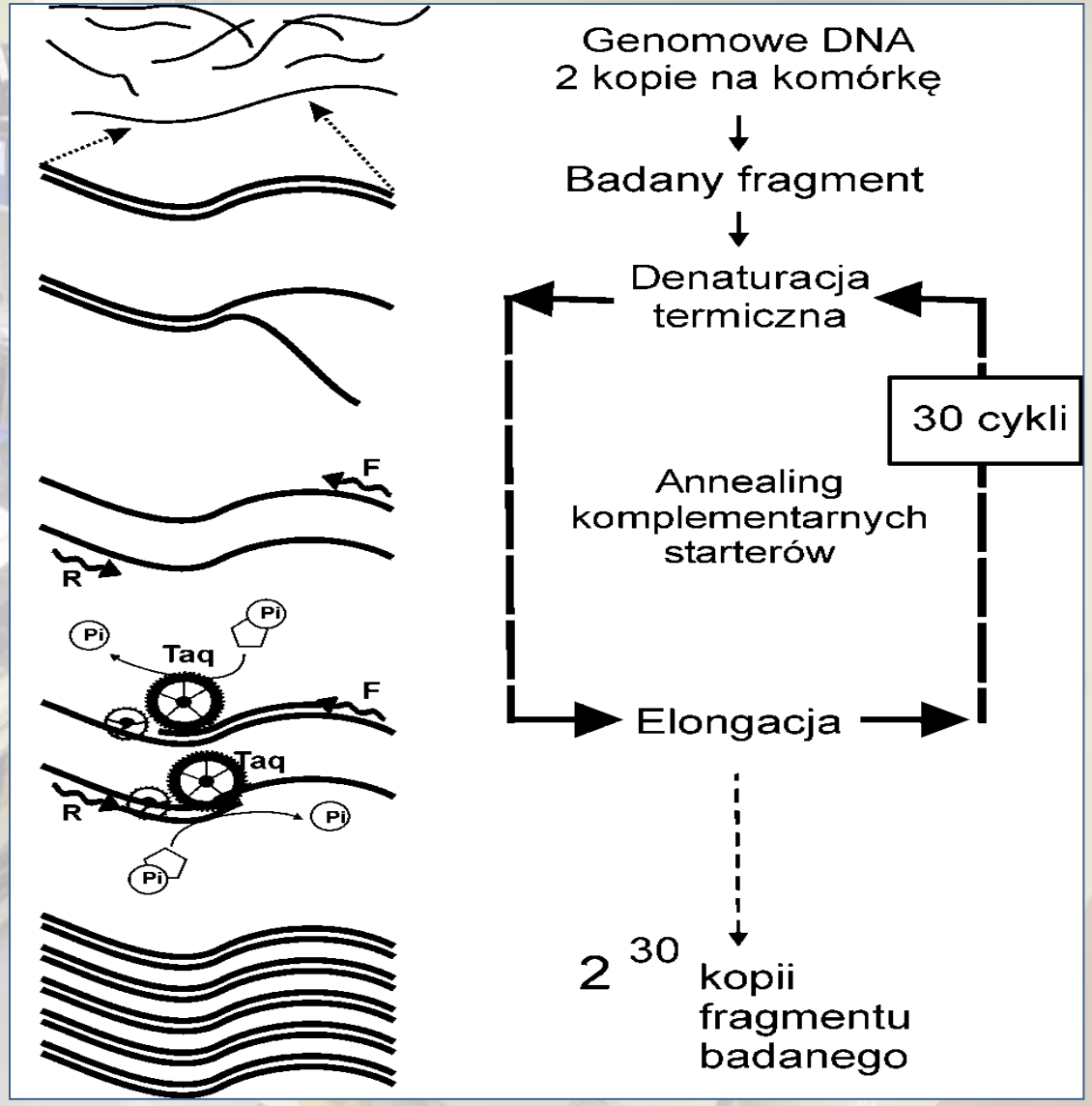
t(9;22) – FISH analysis

Polyploidy



Molecular biology

- Detection of specific molecular abnormalities
  - Fusion genes
  - Point mutations
  - In-del mutations
- Treatment monitoring
- Prognostic factors
- Most sensitive method for MRD monitoring*
- Post-transplantation chimerism



Main areas of interest

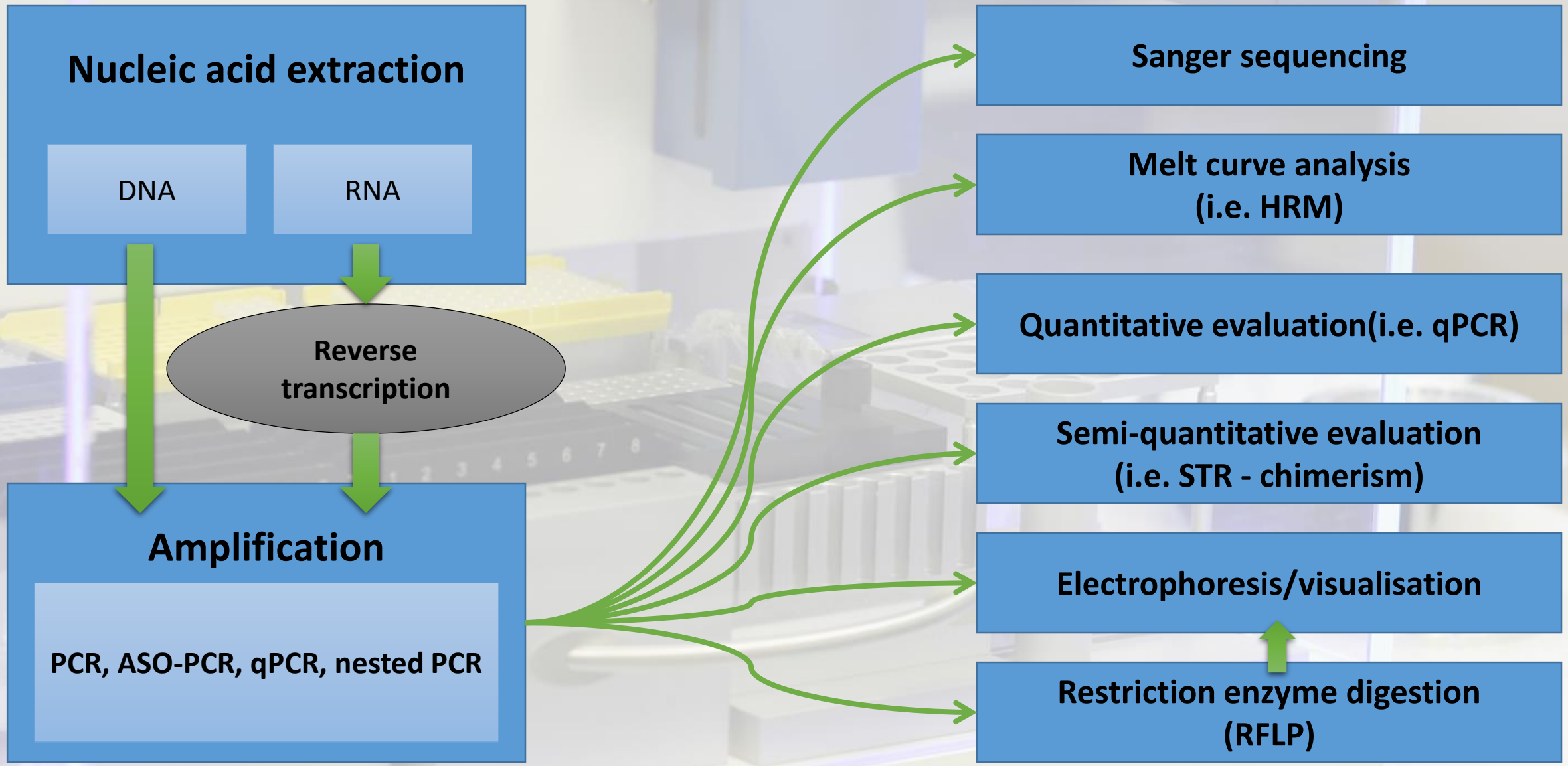
Diagnose

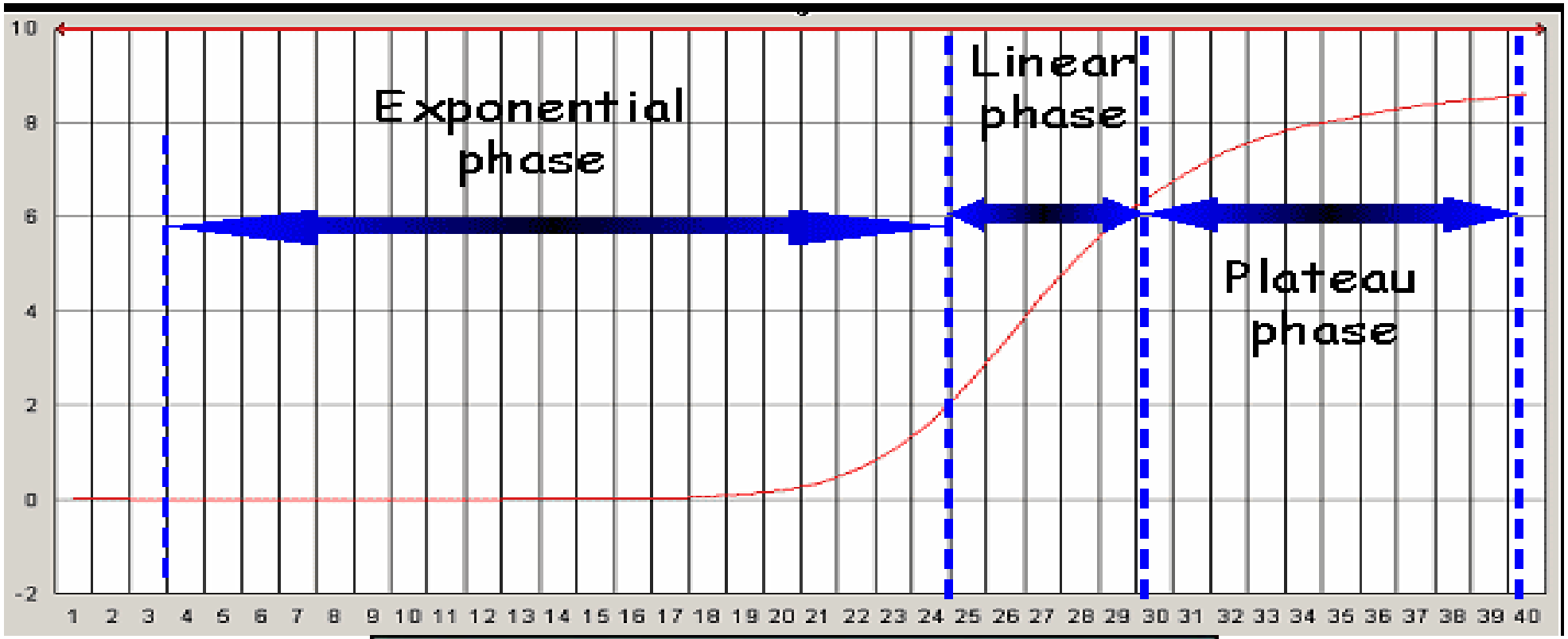
- Clasification criteria
- Prognostic factors
- Identification of molecular targets

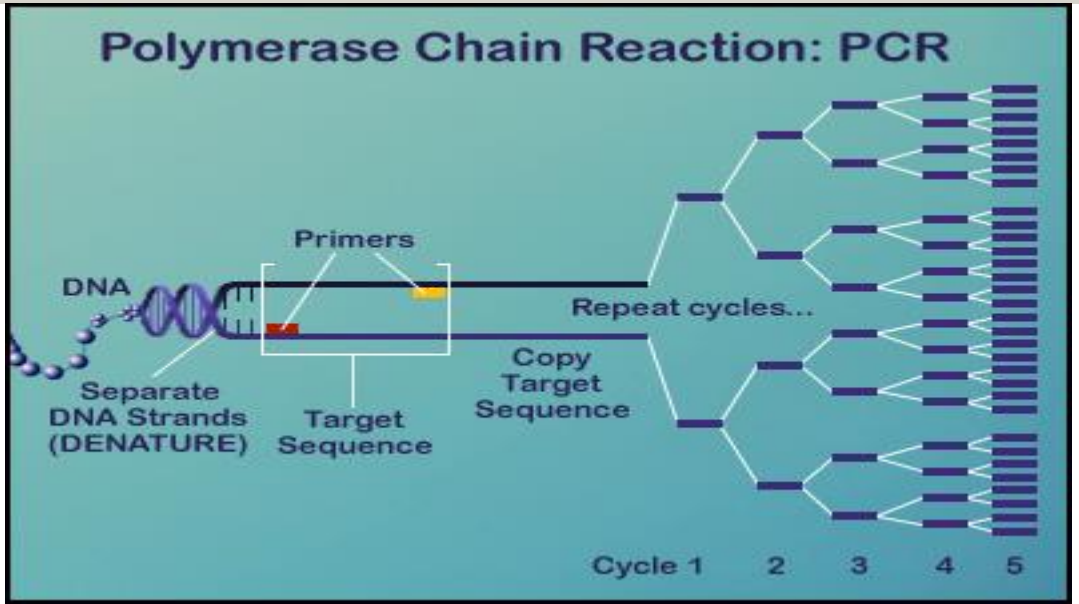
Monitoring

- Based on a specific genetic marker detected at diagnosis
- Chimerism (donor vs recipient comparison)

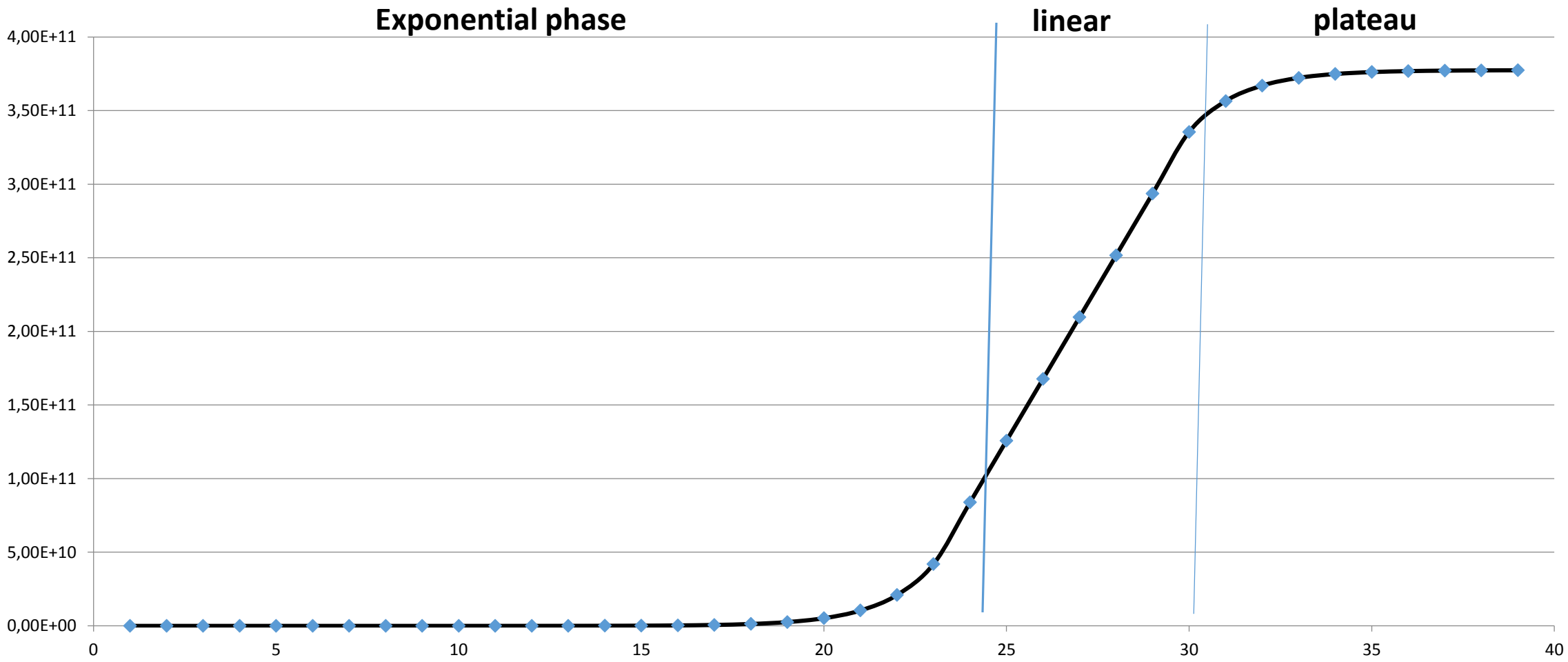
Molecular biology strategies



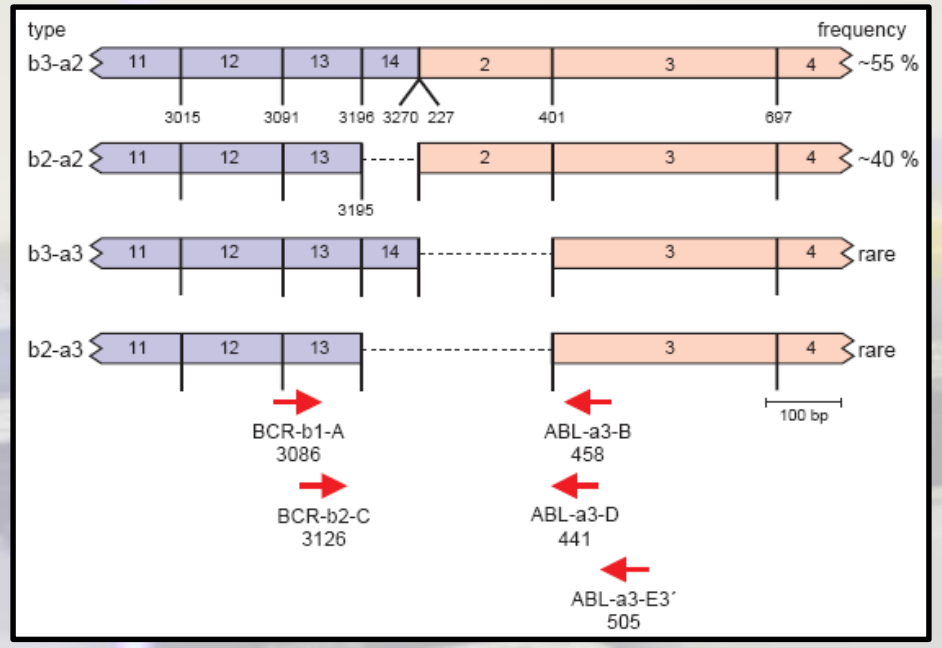

Exponential phase
Linear phase
Plateau phase
10
8
6
4
2
0
-2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40

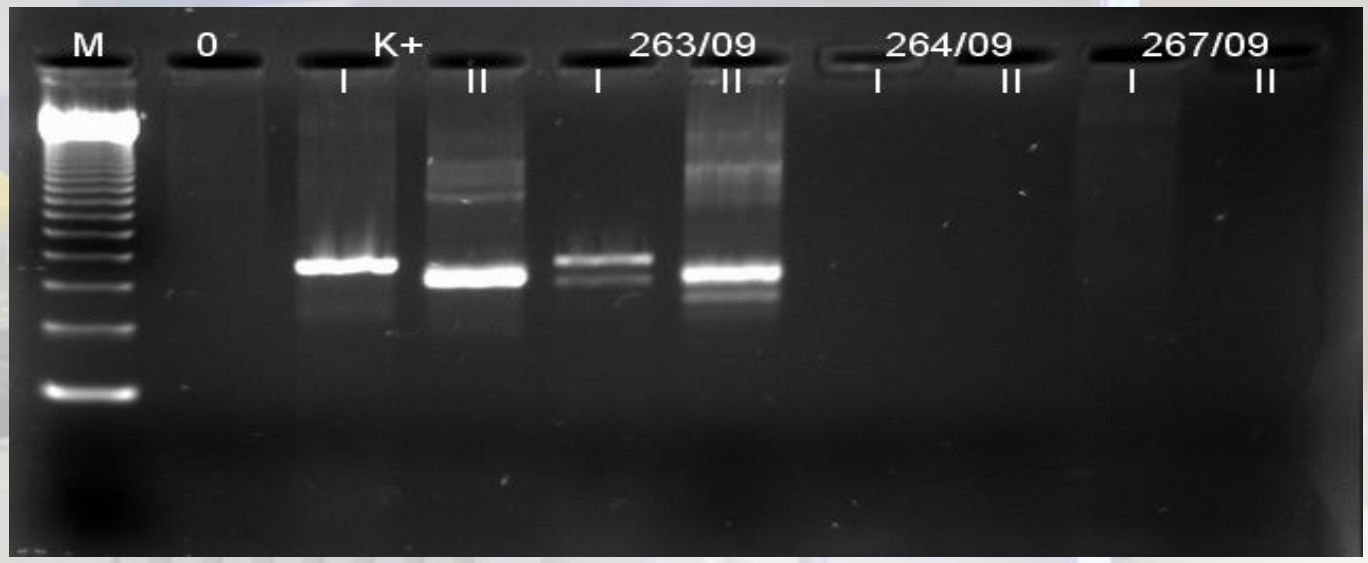

Polymerase Chain Reaction: PCR
Primers
DNA
Repeat cycles...
Copy Target Sequence
Separate DNA Strands (DENATURE)
Target Sequence
Cycle 1 2 3 4 5

Kinetics of PCR

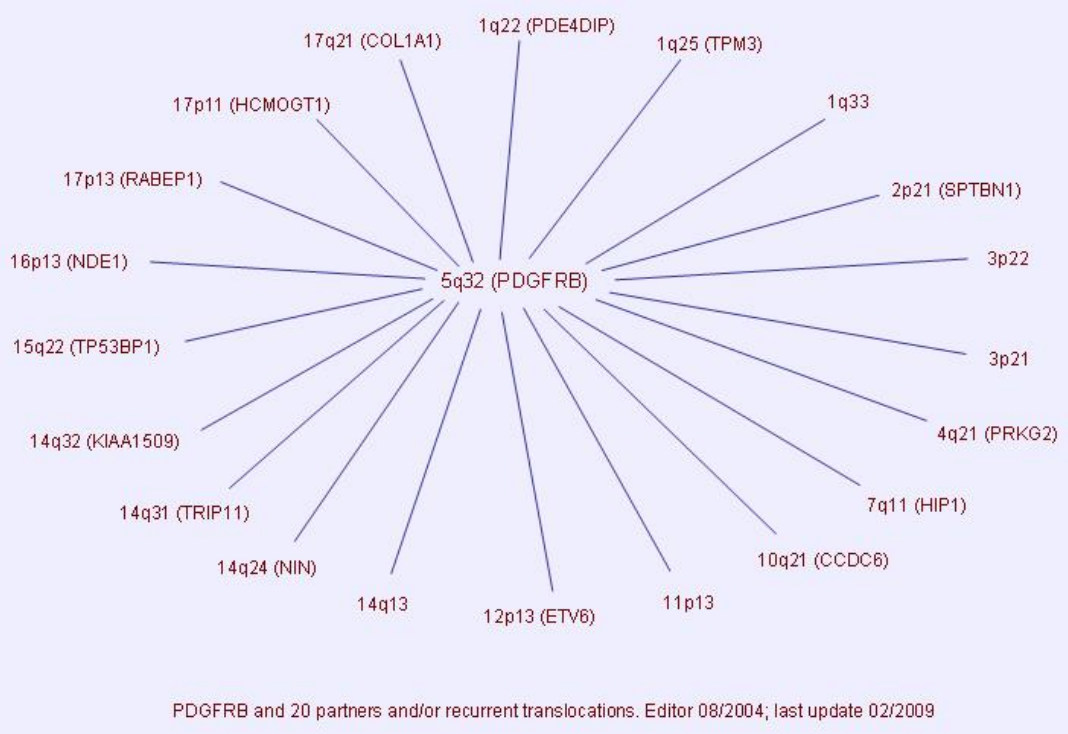


Fusion gene detection

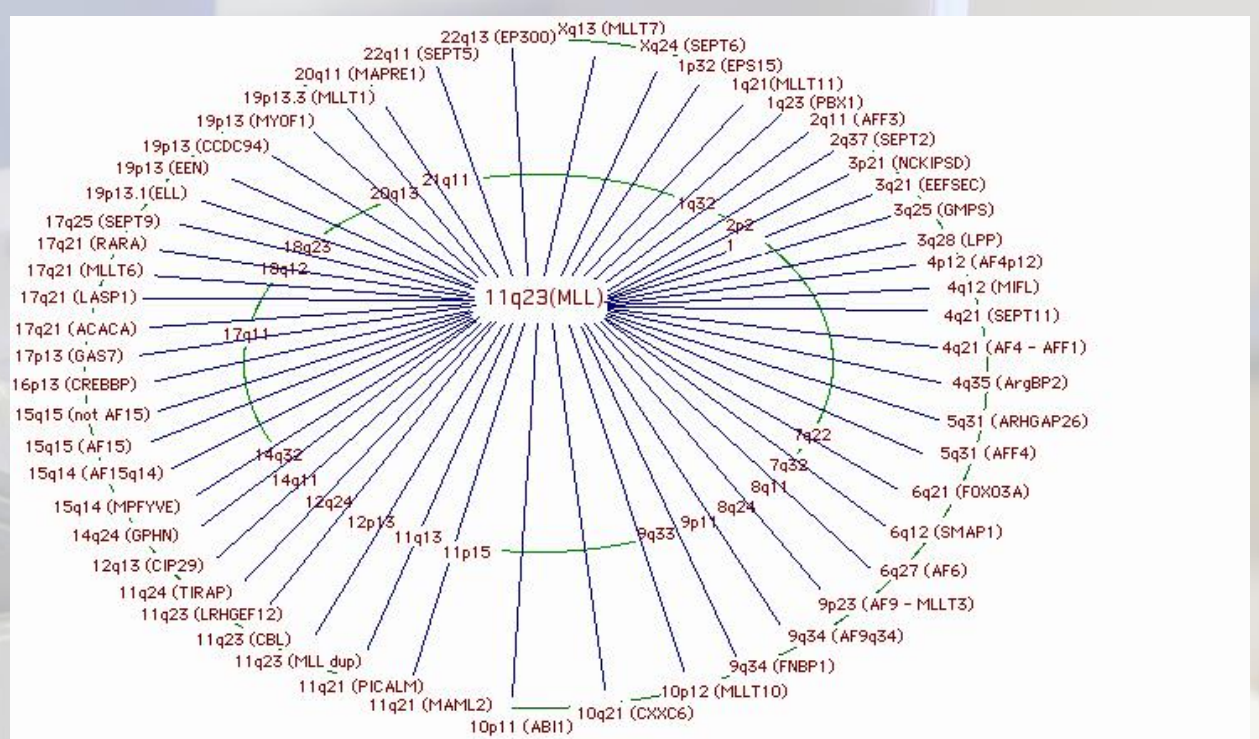




Fusion gene detection



PDGFRB and 20 partners and/or recurrent translocations. Editor 08/2004; last update 02/2009



MLL and partners - 73 recurrent translocations and 54 partner genes. Editor 06/2000; last update 10/2007

Fusion genes:

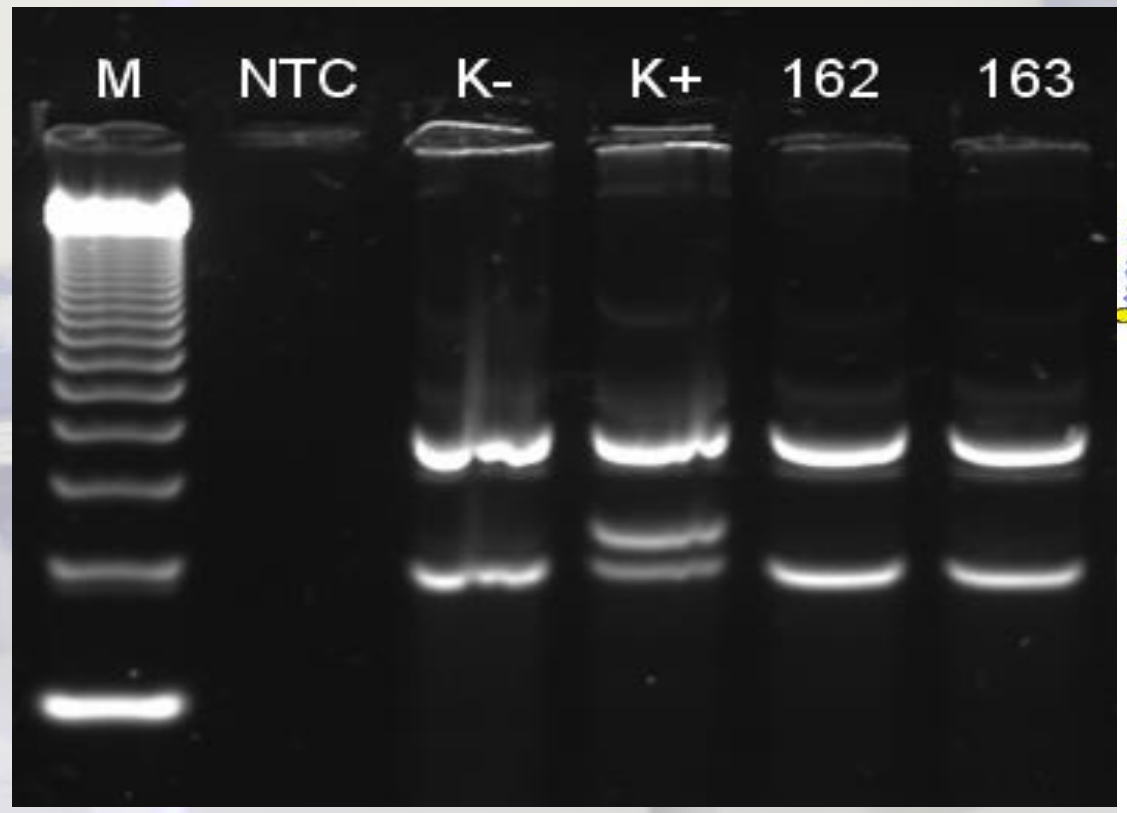
- TEL-AML1 (PCR)
- E2A-PBX (PCR)
- MLL – rearangements (multiplex PCR)
- SIL-TAL (PCR)
- BCR-ABL (multiplex PCR)
- AML1-ETO (PCR)
- PML-RARA (PCR)
- CBFB-MYH11 (PCR)
- FIP1L1-PDGFRA (PCR)

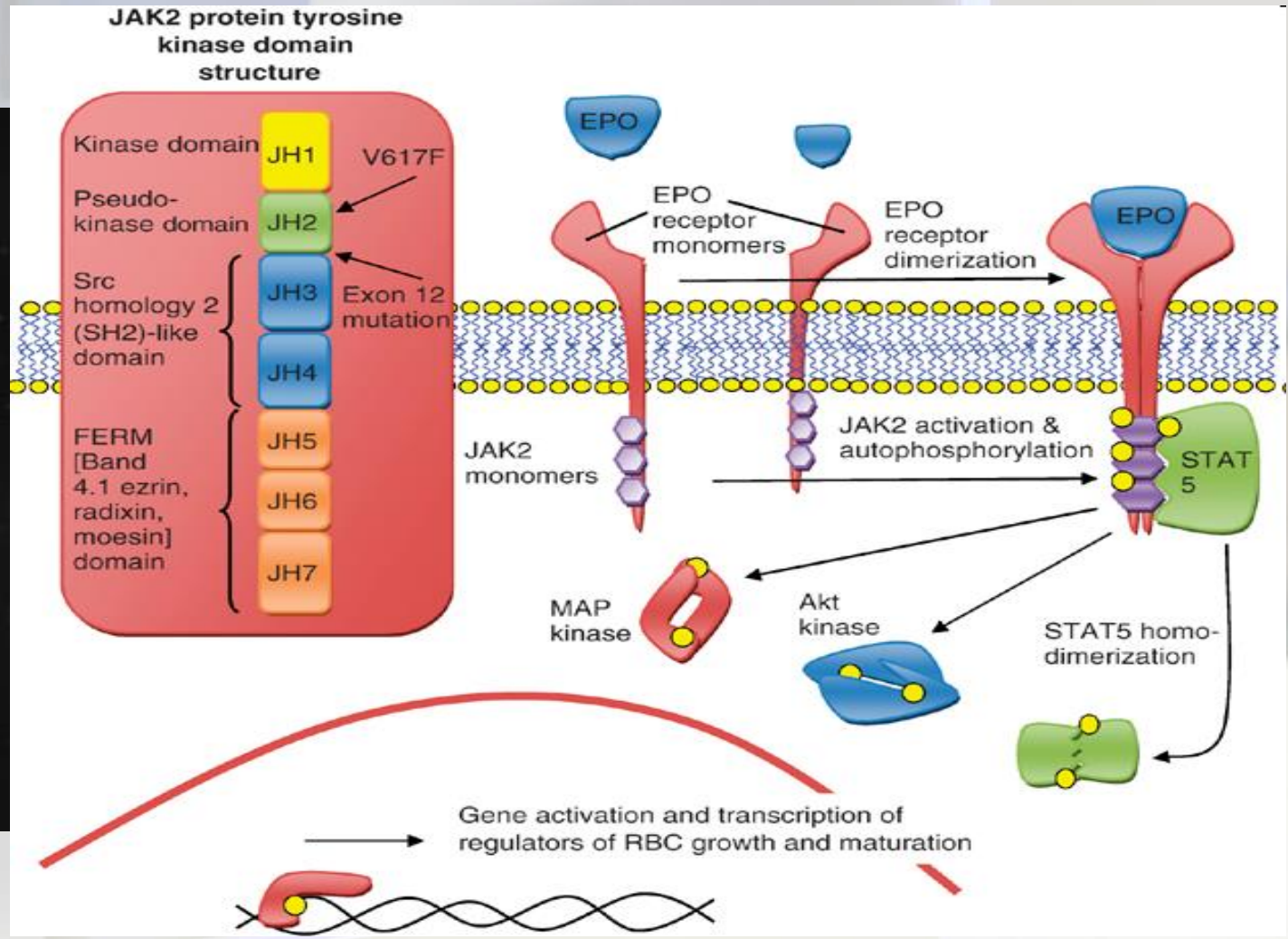
Other mutations:

- JAK2 V617F (ASO QPCR)
- JAK2 ex12 (HRM/CE/SEQ)
- MPL (HRM/SEQ)
- CALR (HRM/CE/SEQ)
- FLT3 ITD (CE)
- FLT3 TKD (RFLP)
- BRAF V600E (ARMS PCR)
- CEBPA (SEQ)
- Post transplantation chimerism (CE)

To be introduced: ASXL1, LNK, CS3FR

JAK2 V617F mutation detection





JAK2 V617F standarization

MPN & MPNr | EuroNet

Certificate of Participation

We hereby certify that:

Pracownia Biologii Molekularnej
Laboratorium Diagnostyki Hematologicznej
Poznań, Poland

Participated in the 2012 COST MPN&MPNr-EuroNet
JAK2-V617F Inter-laboratory Quality Control Study

cost

Niels Pallisgaard, Vejle, Denmark
Julia Asp, Göteborg, Sweden
Sylvie Hermouet, Nantes, France

MPN & MPNr | EuroNet

Certificate of Participation

We hereby certify that:

Laboratorium Diagnostyki Hematologicznej
Klinika Hematologii i Transplantacji Szpiku
Poznán, Polen

participated in the 2015 MPN&MPNr-EuroNet
JAK2 V617F Inter-laboratory Quality Control

Niels Pallisgaard, Roskilde, Denmark
Julia Asp, Göteborg, Sweden
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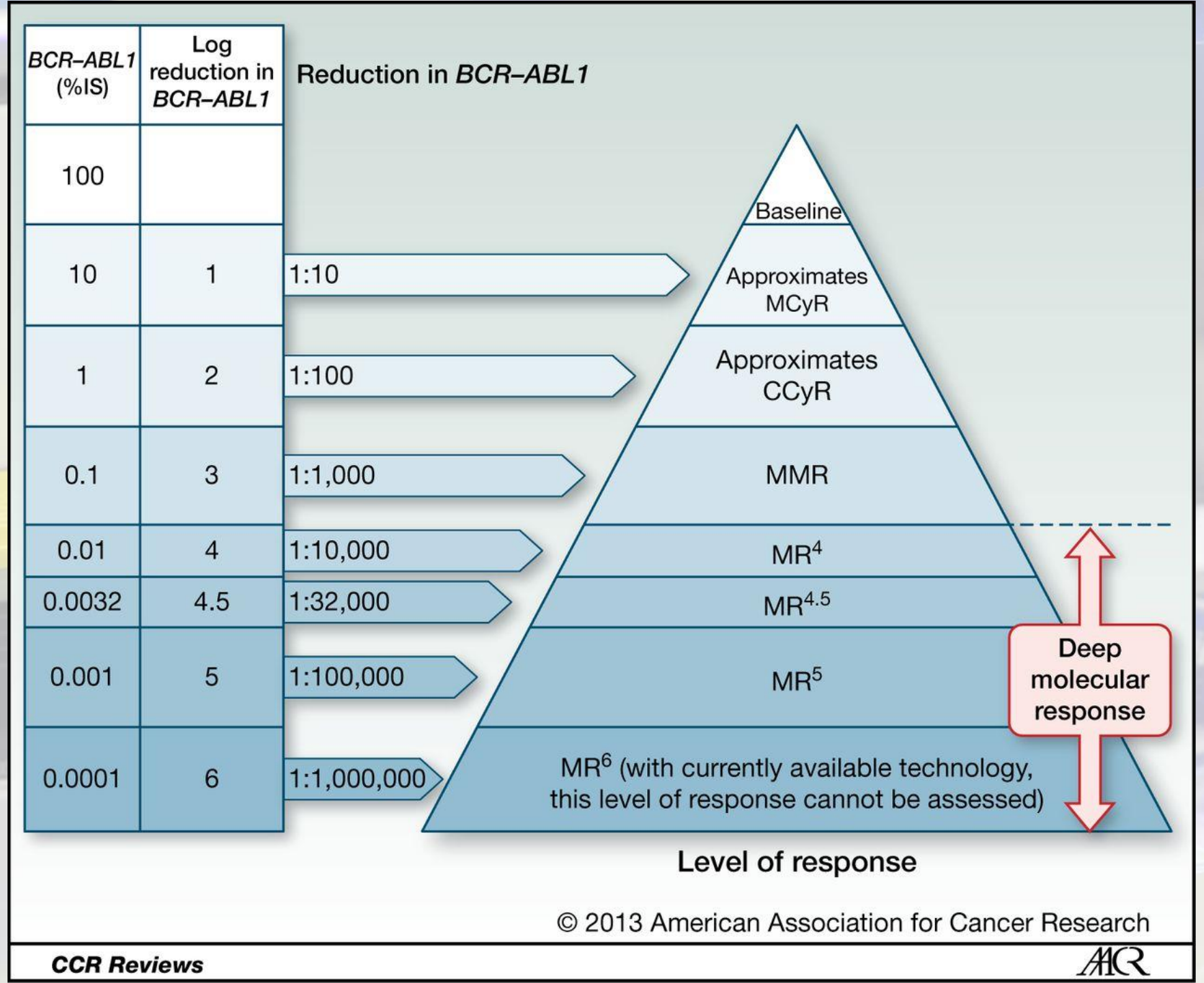
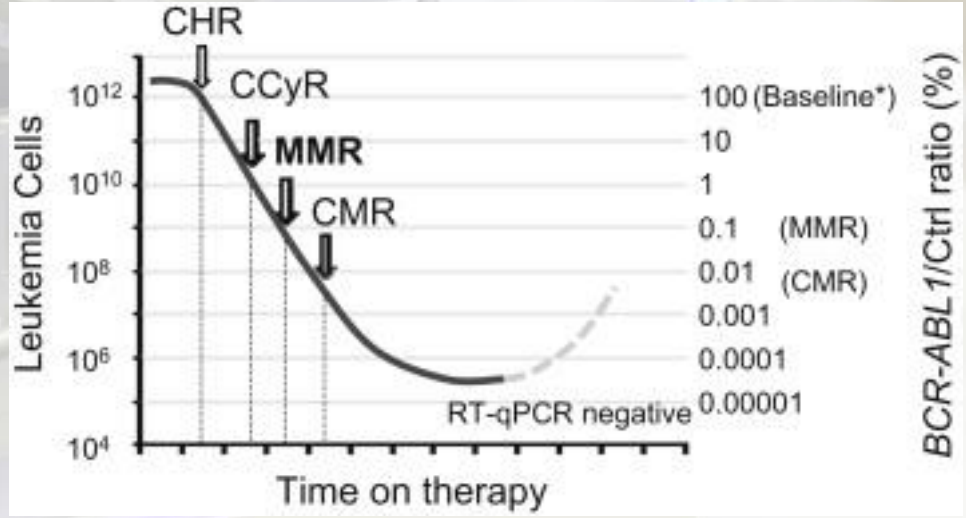


© 2013 American Association for Cancer Research

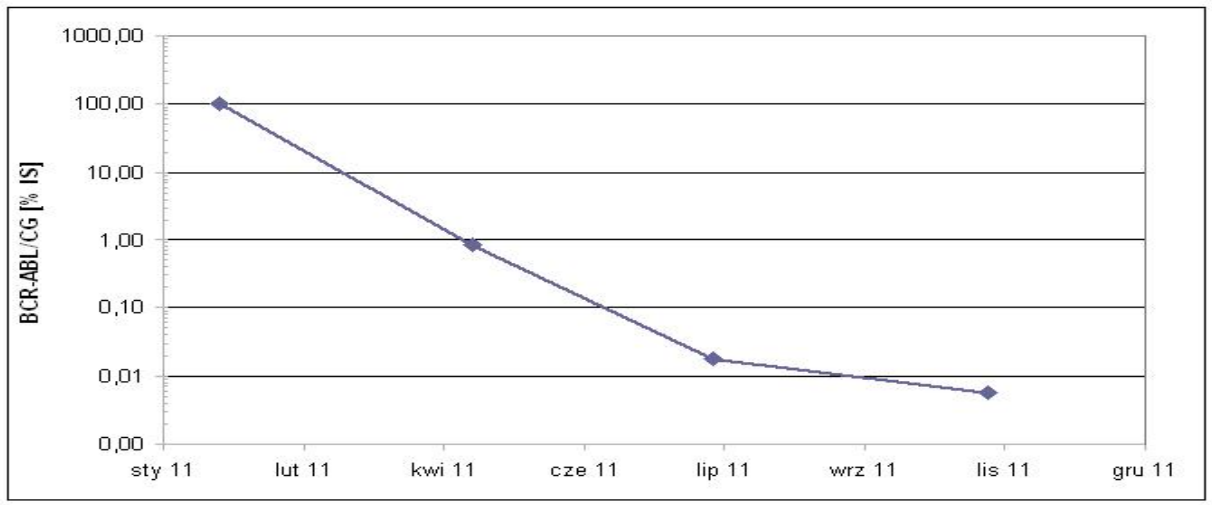
CML – recommendations based on molecular and cytogenetic analyses

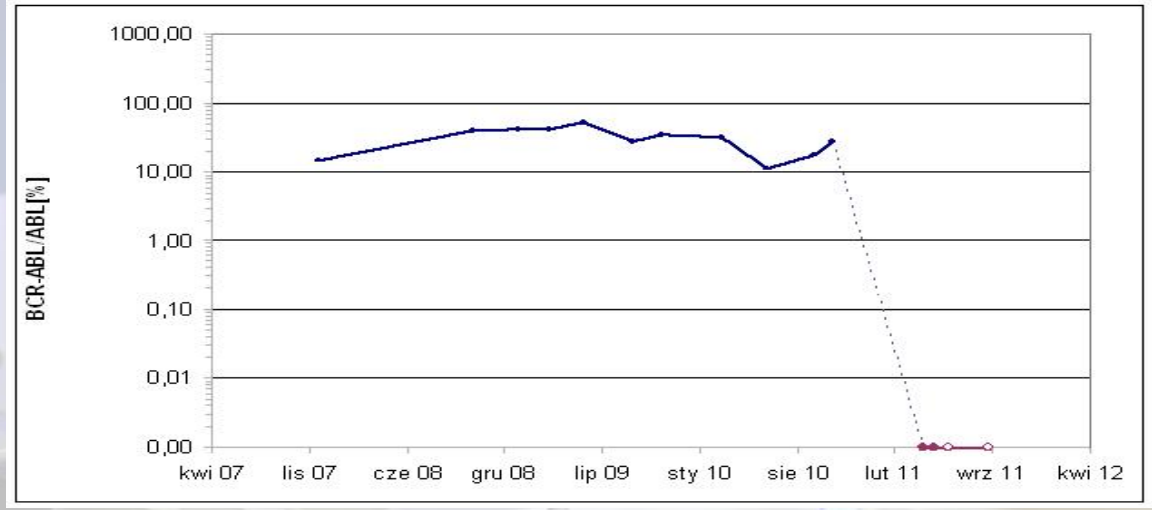
OPTIMAL RESPONSE
BCR-ABL trancript level below:

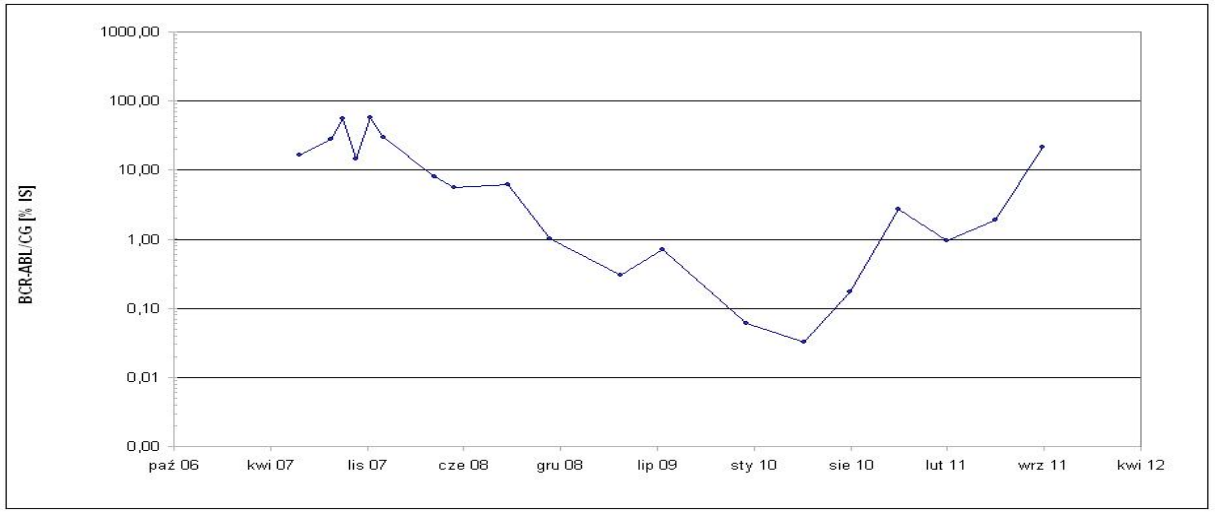
10% after 3 months
1% after 6 months
0,1% after 12 months

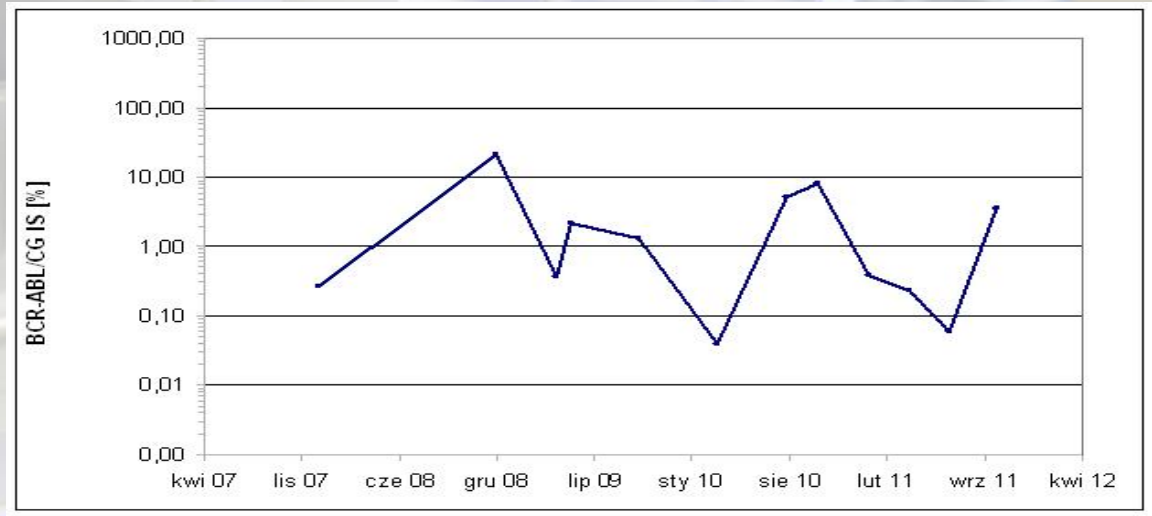


BCR-ABL monitoring in CML



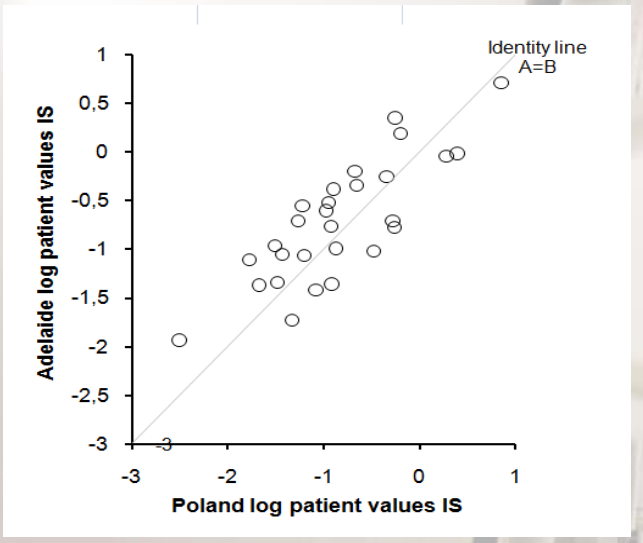


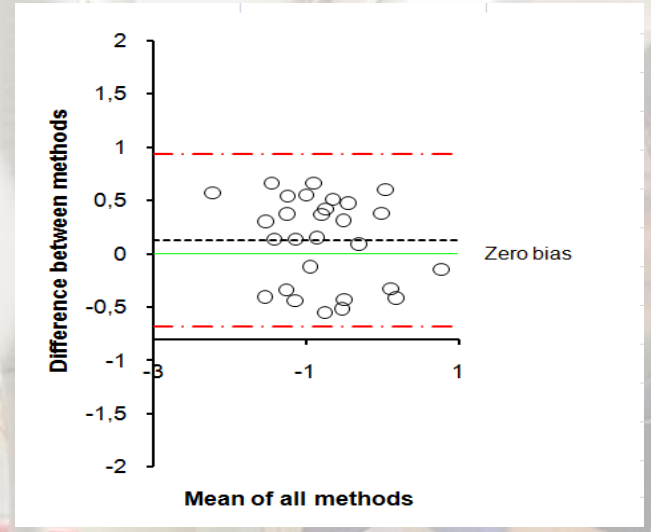




BCR-ABL standarization

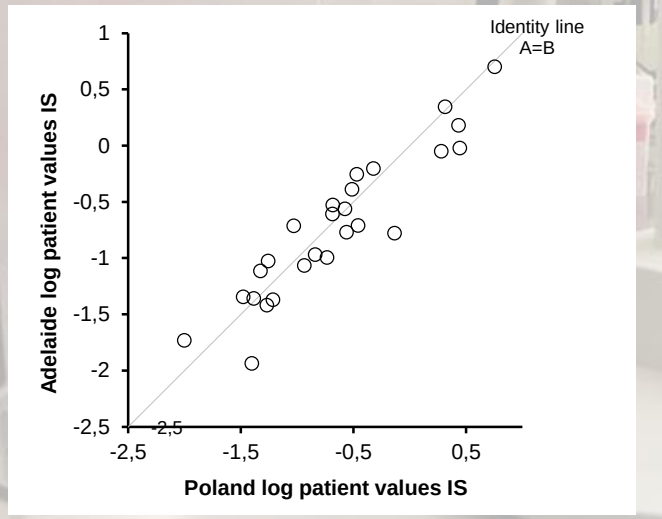
2009

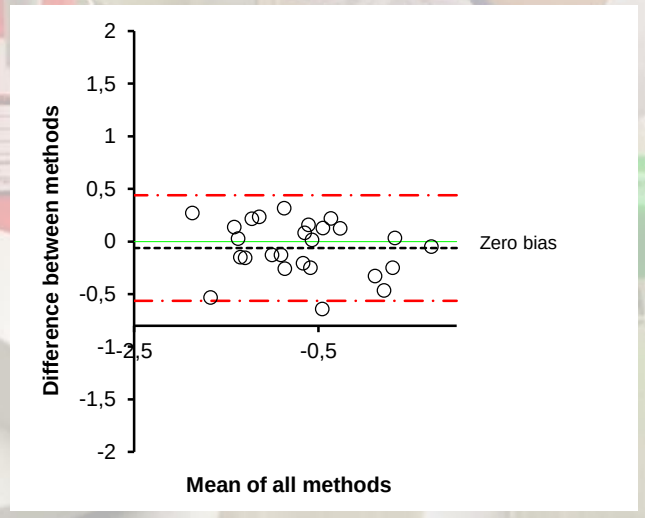




8/28 within 2-fold (29%)
19/28 within 3-fold (68%)
28/28 within 5-fold (100%)

2011

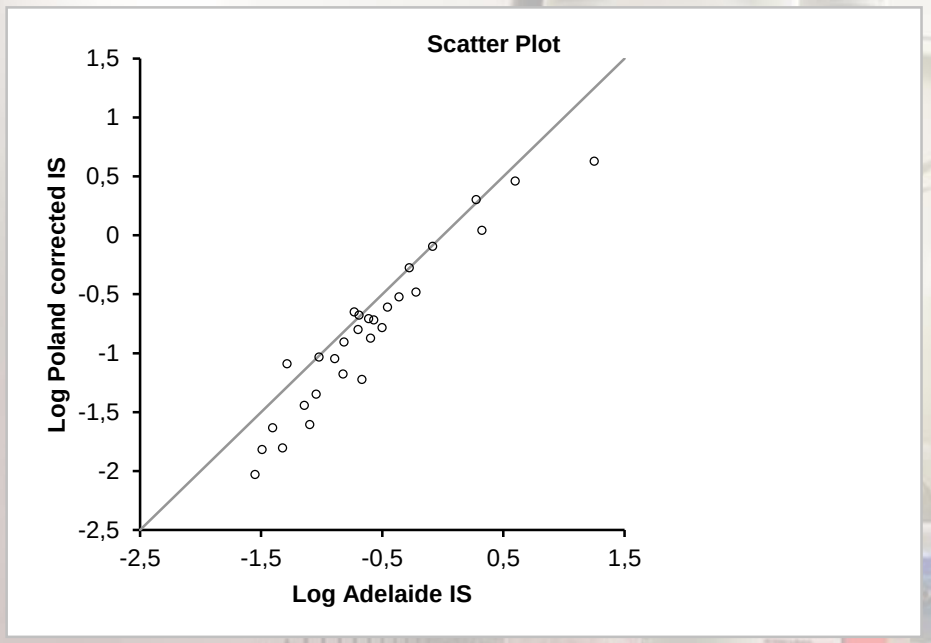




21/26 within 2-fold (81%)
24/26 within 3-fold (92%)
26/26 within 5-fold (100%)

BCR-ABL standarization

2016



This one resulted in change of CF from 1 to 1,89

EUTOS MR4.5

Data summary GUSB control gene : overall score and score per category

Lab Number	Final Score	10% cell line GUSB copy number	10% cell line BCR-ABL Detected	10% cell line %BCR-ABL IS value	CMR cell line GUSB copy number	CMR cell line BCR-ABL detected	aRNA GUSB copy number	aRNA BCR-ABL detected	cDNA GUSB copy number	cDNA BCR-ABL copy number	cDNA ratio	Audit data
40												
12												
48												
37												
2												
41												
46												
25												
29												
38												
43												
1												

Scoring criteria:

Green = able to detect MR$^{4.5}$ in a high proportion of samples

Orange = able to detect MR$^{4.5}$ in a proportion of samples

Red = unable to detect MR$^{4.5}$ in most samples

EUTOS for CML

European Treatment and Outcome Study

ELN LeukemiaNet European

Certificate

EUTOS MR$^{4.5}$ Performance Evaluation

Laboratorium Diagnostyki Hematologicznej Szamarzewskiego 82/84, Poznan, Poland

Institution

PASS: Can detect MR$^{4.5}$ in a high proportion of samples

22nd December 2014

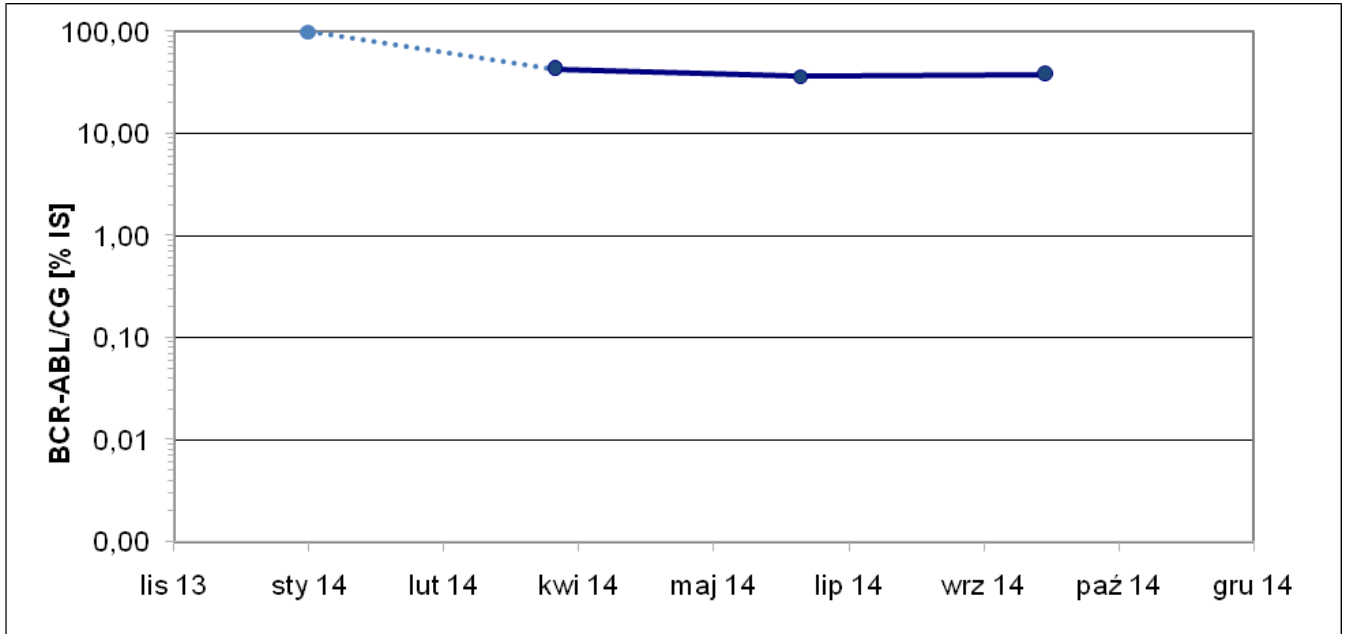
Date of Validation

Prof. Nicholas CP Cross, Salisbury, UK
Prof. Dr. Martin C. Müller, Mannheim, Germany
Prof. Fabrizio Pane, Naples, Italy
Prof. Dr. Andreas Hochhaus, Jena, Germany

Reference Laboratory:
National Genetics Reference Laboratory (Wessex), Salisbury District Hospital, Salisbury, SP2 8BJ, UK

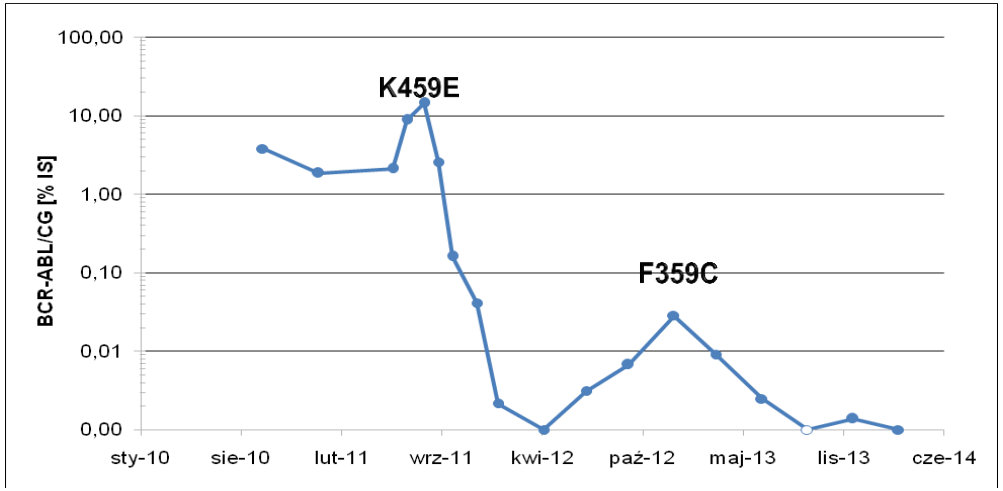
TKI resistance in CML

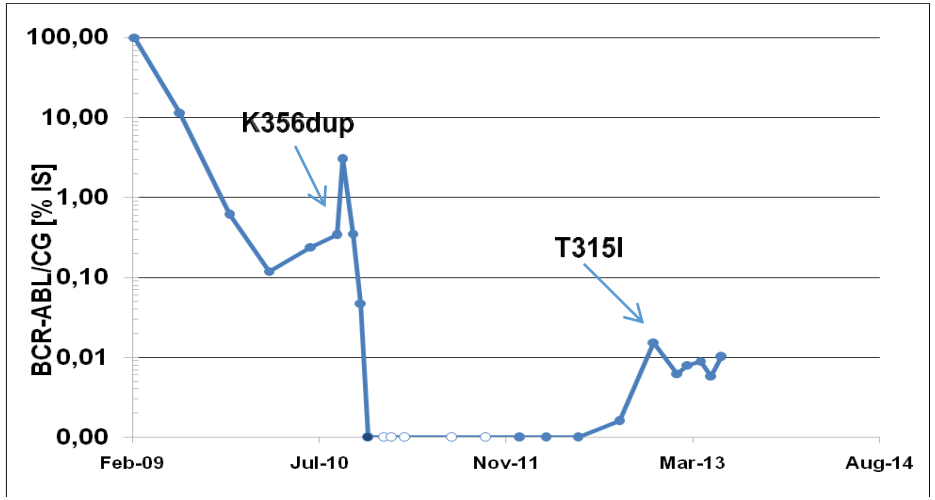
- Primary resistance – no significant improvement after introduction of TKI therapy
  - Usually not related to BCR-ABL kinase domain mutations



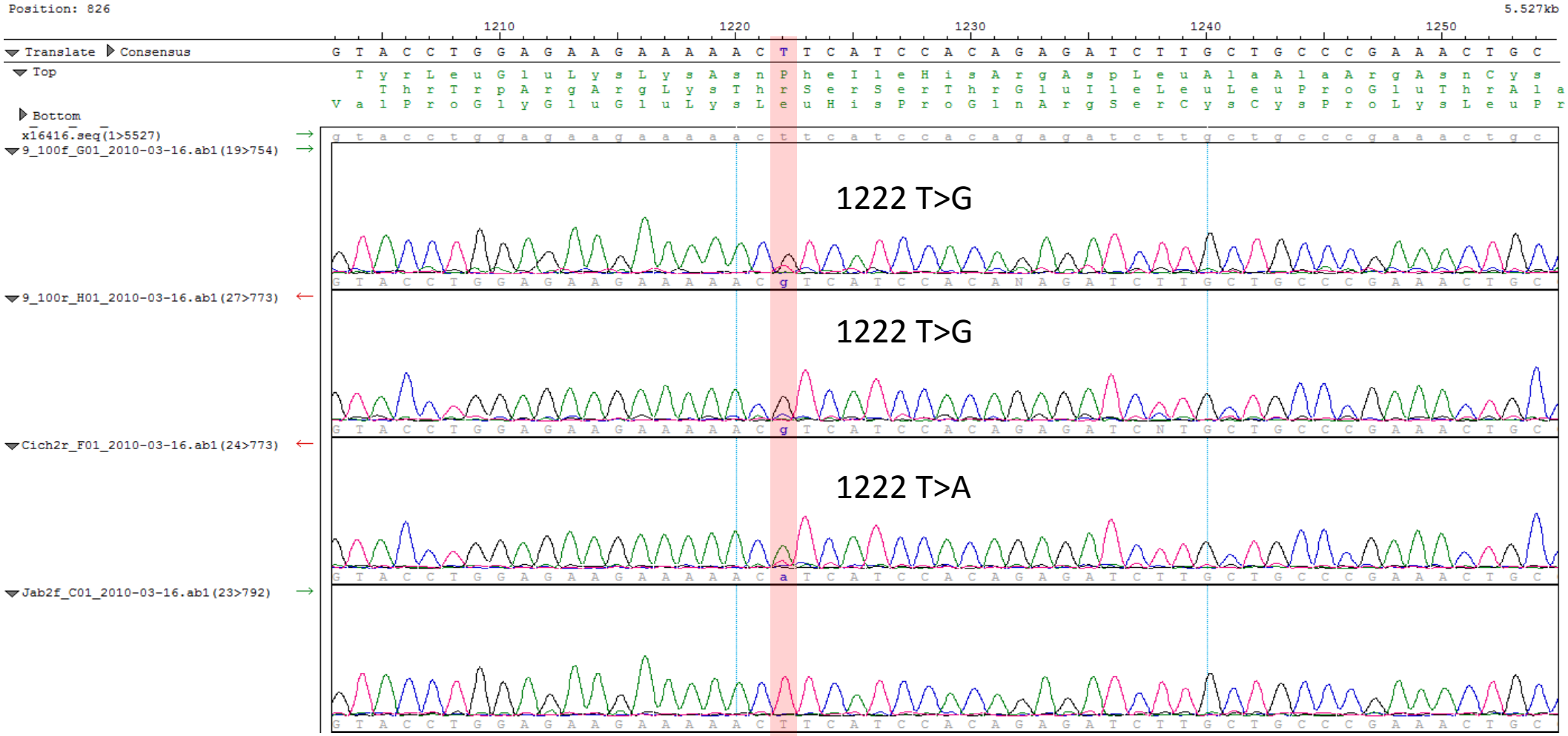
TKI resistance in CML

- Acquired resistance – an increase of BCR-ABL transcript level after an initial reduction
  - Approx 50% of cases are related to BCR-ABL KD mutations
  - Before sequencing of BCR-ABL KD we have to be sure that patient has taken a full dose of TKI!





BCR-ABL KD mutations



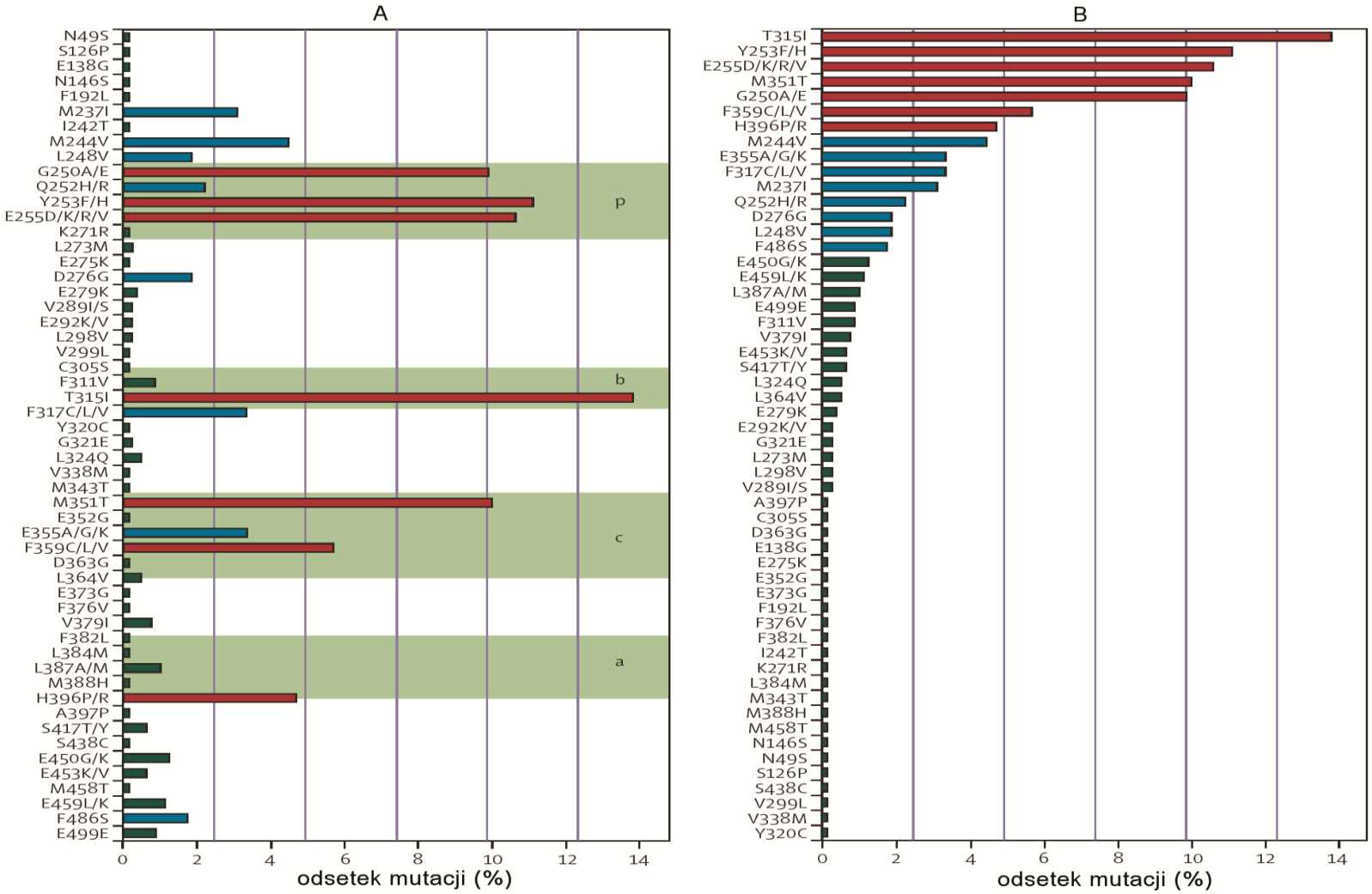
F359V

F359V

F359I

WT

BCR-ABL KD mutations



Apperley i wsp. 2007

BCR-ABL KD mutations

Wartości GI_{50} dla zmutowanych linii komórkowych Ba/F3

	imatynib (nM)	nilotynib (nM)	dazatynib (nM)
natywna forma BCR-ABL	260	13	0.8
M244V	2000	38	1.3
G250E	1350	48	1.8
Q252H	1325	70	3.4
Y253F	3475	125	1.4
Y253H	>6400	450	1.3
E255K	5200	200	5.6
E255V	>6400	430	11
V299L	540†	BD	18†
F311L	480	23	1.3
T315A	971	61	125†
T315I	>6400	>2000	>200
F317L	1050	50	7.4
F317V	350†	BD	53†
M351T	880	15	1.1
E355G	2300‡	BD	1.8§
F359V	1825	175	2.2
V379I	1630	51	0.8
L387M	1000	49	2
H396P	850	41	0.6
H396R	1750	41	1.3

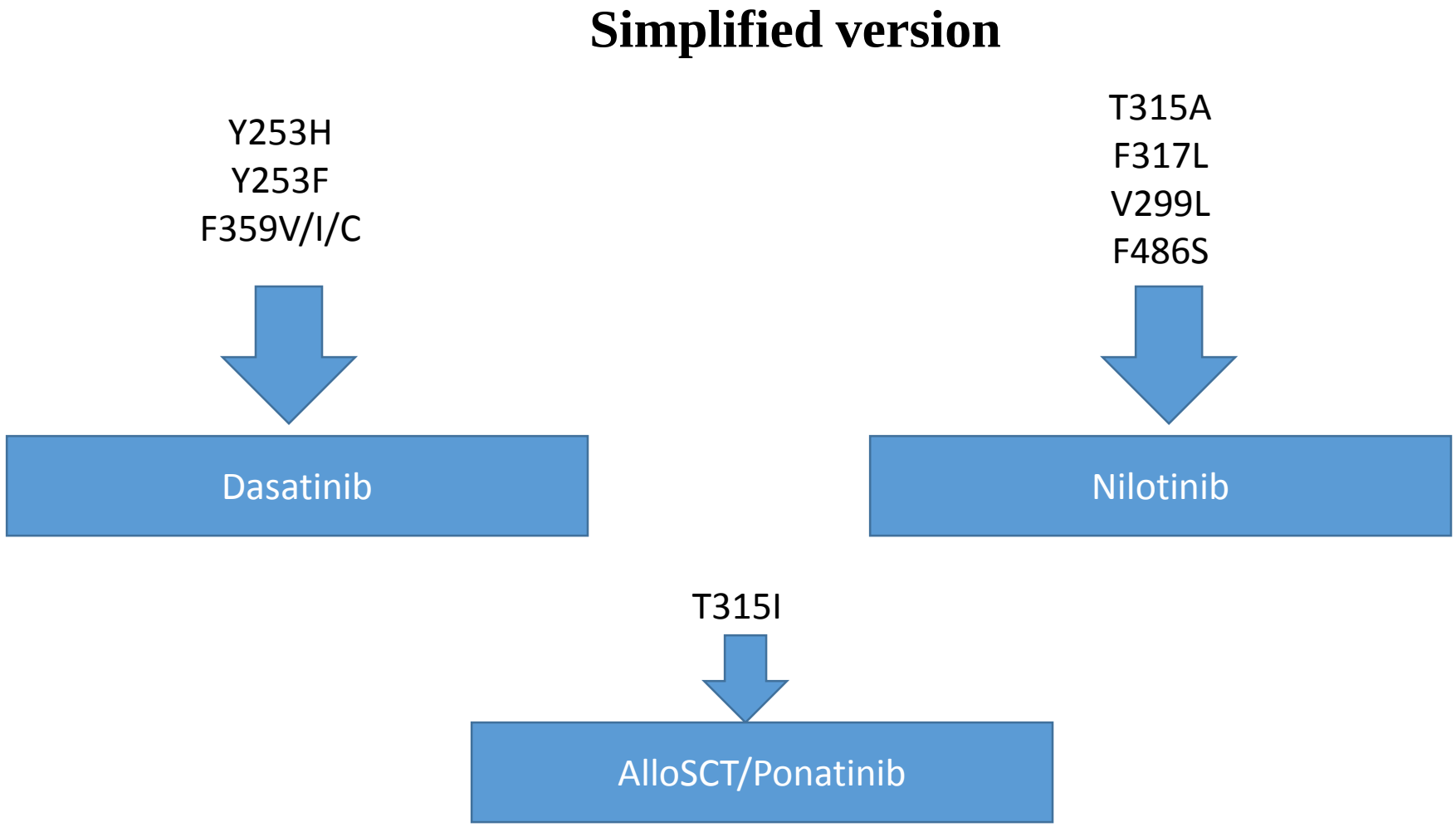
wrażliwy
średnio wrażliwy
niewrażliwy
BD brak danych

	zmiana IC_{50} (krotność, WT=1)			
	bozutynib	imatynib	dazatynib	nilotynib
WT	1	1	1	1
L248V	2.97	3.54	5.11	2.80
G250E	4.31	6.86	4.45	4.56
Q252H	0.81	1.39	3.05	2.64
Y253F	0.96	3.58	1.58	3.23
E255K	9.47	6.02	5.61	6.69
E255V	5.53	16.99	3.44	10.31
D276G	0.60	2.18	1.44	2.00
E279K	0.95	3.55	1.64	2.05
V299L	26.10	1.54	8.65	1.34
T315I	45.42	17.50	75.03	39.41
F317L	2.42	2.60	4.46	2.22
M351T	0.70	1.76	0.88	0.44
F359V	0.93	2.86	1.49	5.16
L384M	0.47	1.28	2.21	2.33
H396P	0.43	2.43	1.07	2.41
H396R	0.81	3.91	1.63	3.10
G398R	1.16	0.35	0.69	0.49
F486S	2.31	8.10	3.04	1.85

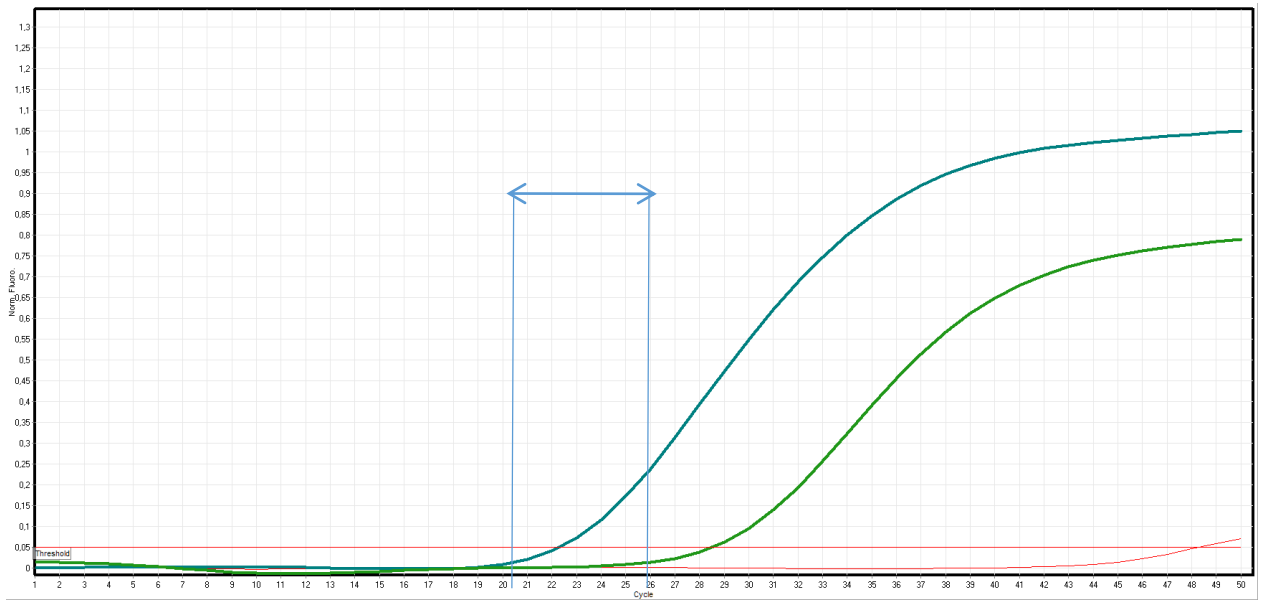
wrażliwy	≤2
umiarkowanie oporny	2.01-4
oporny	4.01 - 10
bardzo oporny	>10

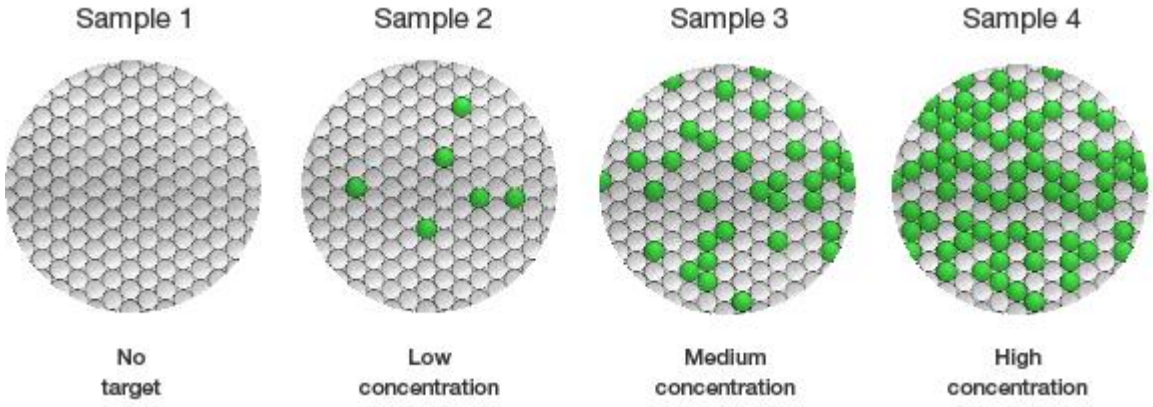
O'Hare i wsp. 2007, Redaelli i wsp. 2009

BCR-ABL KD mutations



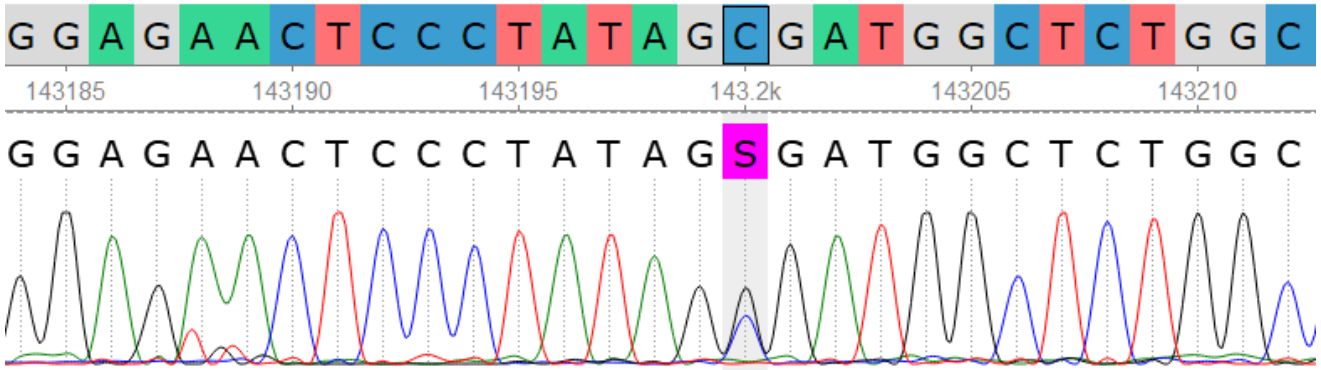
qPCR vs ddPCR

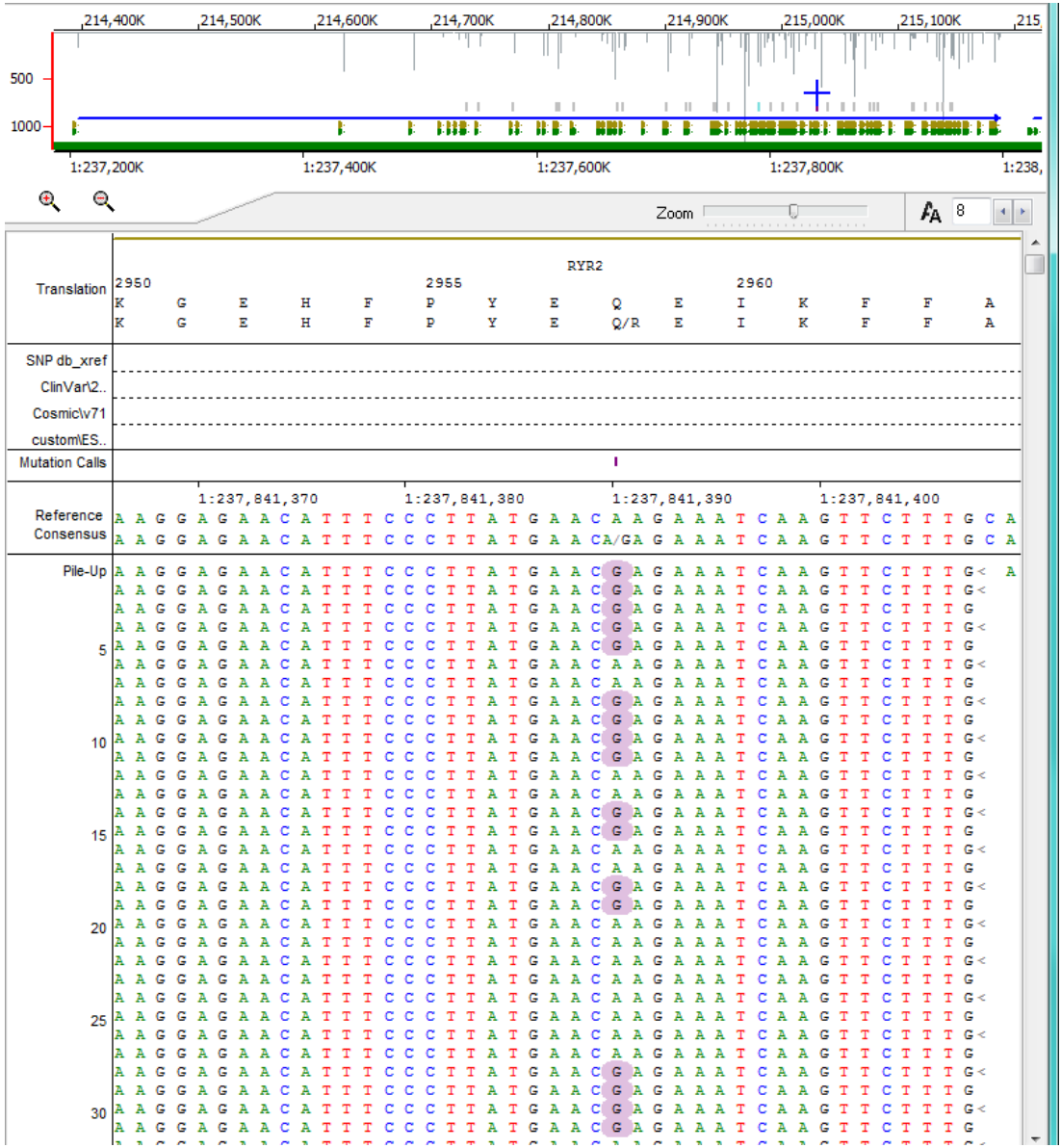




Biorad

Sanger vs NGS





NGS

Roche
454

Illumina

Oxford Nanopore

Pacific Biosciences

ThermoFisher IonTorrent

Archer DX

ALL

ABL1	ABL2	AICDA	BCL11B	BCL2	BCL6	BCR
BLNK	BRAF	CD274	CHD1	CREBBP	CRLF2	CSF1R
CTLA4	DNM2	DNTT	EBF1	EPOR	ETV6	EZH2
FBXW7	FGFR1	FLT3	HOXA10	HOXA9	IDH1	IDH2
IKZF1	IKZF2	IKZF3	IL7R	IRF4	IRF8	JAK1
JAK2	JAK3	KDM6A	KLF2	KMT2A	KRAS	LMO1
LYL1	MLLT4	MPL	MYC	NF1	NOTCH1	NRAS
NT5C2	NTRK3	NUP214	NUP98	P2RY8	PAG1	PAX5
PBX1	PDCD1	PDCD1LG2	PDGFRA	PDGFRB	PICALM	PTK2B
PTPN1	PTPN11	RAG1	RAG2	RUNX1	SEMA6A	SETD2
SH2B3	SOX11	STAT3	STAT5B	STIL	TAL1	TCF3
TLX1	TLX3	TYK2	WT1	ZCCHC7		

Legend:

- SNV/Indel
- Expression
- Fusion, splicing or exon-skipping
- Internal tandem duplication (ITD)
- CNV

Heme 2

ABL1	ABL2	ALK	BCL11B	BCL2	BCL3	BCL6
BCR	BIRC3	CBFB	CCND1	CCND2	CCND3	CD274
CDK6	CDKN2A	CEBPA	CEBPD	CEBPE	CEBPG	CHD1
CHIC2	CIITA	CREBBP	CRLF2	CSF1R	CTLA4	DEK
DUSP22	EBF1	EIF4A1	EPOR	ERG	ETV6	FGFR1
FOXP1	GLIS2	ID4	IKZF1	IKZF2	IKZF3	IRF4
IRF8	JAK2	KAT6A	KLF2	KMT2A	MALT1	MECOM
MKL1	MLF1	MLLT10	MLLT4	MUC1	MYC	MYH11
NF1	NFKB2	NOTCH1	NTRK3	NUP214	NUP98	P2RY8
PAG1	PAX5	PDCD1	PDCD1LG2	PDGFRA	PDGFRB	PICALM
PML	PRDM16	PTK2B	RARA	RBM15	ROS1	RUNX1
RUNX1T1	SEMA6A	SETD2	STIL	TAL1	TCF3	TFG
TP63	TYK2	ZCCHC7				

Legend:

- SNV/Indel
- Expression
- Fusion, splicing or exon-skipping
- Internal tandem duplication (ITD)
- CNV

Archer DX – PAN heme

ABL1	ABL2	AICDA	AKT3	ALK	ASB13	ASXL1
BATF3	BAX	BCL11B	BCL2	BCL2A1	BCL3	BCL6
BCR	BIRC3	BLNK	BMF	BMP7	BRAF	BTK
CALR	CARD11	CBFB	CBL	CCDC50	CCND1	CCND2
CCND3	CD274	CD44	CD79B	CDC25A	CDK6	CDKN2A
CDKN2B	CEBPA	CEBPD	CEBPE	CEBPG	CHD1	CHIC2
CIITA	CREB3L2	CREBBP	CRLF2	CSF1R	CSF3R	CTLA4
CYB5R2	DCK	DEK	DENND3	DLEU1	DNM2	DNMT3A
DNMT3B	DNTT	DUSP22	E2F2	EBF1	EIF4A1	ENTPD1
EPOR	ERG	ETV6	EXOC2	EZH2	FAM216A	FBXW7
FGFR1	FGFR2	FGFR3	FLT3	FOXP1	FUT8	GATA1
GATA2	GLIS2	GNAS	HOXA10	HOXA9	ID4	IDH1
IDH2	IKZF1	IKZF2	IKZF3	IL16	IL7R	IRF4
IRF8	ITPKB	JAK1	JAK2	JAK3	KAT6A	KDM6A
KIAA0101	KIT	KLF2	KMT2A	KRAS	LIMD1	LMO1
LMO2	LRMP	LYL1	LZTS1	MAL	MALT1	MAML3
MECOM	MKL1	MLF1	MLLT10	MLLT4	MME	MPL
MUC1	MYBL1	MYC	MYD88	MYH11	NEK6	NF1
NFKB1	NFKB2	NME1	NOTCH1	NOTCH2	NPM1	NRAS
NT5C2	NTRK3	NUP214	NUP98	P2RY8	PAG1	PAICS
PAX5	PBX1	PDCD1	PDCD1LG2	PDGFRA	PDGFRB	PHF6
PICALM	PIM1	PIM2	PLCG1	PLCG2	PML	PPAT
PRDM16	PRKAR2B	PTK2B	PTPN1	PTPN11	PYCR1	RAB29
RAG1	RAG2	RANBP1	RARA	RBM15	RHOA	ROS1
RUNX1	RUNX1T1	S1PR2	SEMA6A	SERPINA9	SETBP1	SETD2
SF3B1	SH2B3	SH3BP5	SLC29A1	SOX11	SRSF2	STAT3
STAT5B	STAT6	STIL	STRBP	TAL1	TCF3	TFG
TLX1	TLX3	TNFRSF13B	TNFSF4	TP63	TYK2	U2AF1
WT1	XPO1	ZCCHC7				

Legend:

- SNV/Indel
- Expression
- Fusion, splicing or exon-skipping
- Internal tandem duplication (ITD)
- CNV

199
Gene targets

1,054
# of GSP2s

>90%
Unique molecular on-target %

2.5 hours
Hands-on time

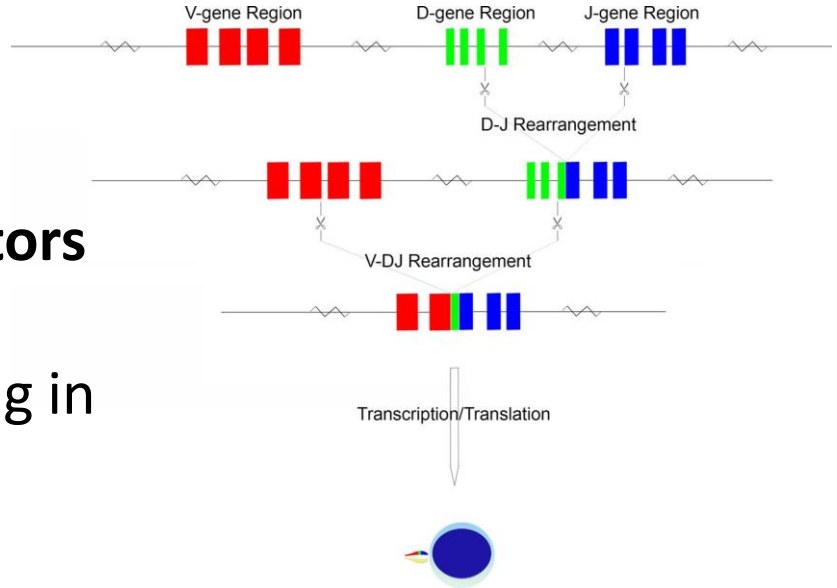
≥10ng
Input nucleic acid required*

4.5 million
Recommended # of reads

9 hours
Total time

Illumina and Ion Torrent
Platform

VDJ rearangements



- Mechanism of recombination which occurs on early stage of lymphocyte development
- Plays role in forming of **antibodies (B-cells)** and **T-cell receptors (TCR)**
- VDJ recombination – name derived from regions participating in rearrangement
    - V – variable, D – diversity, J – joining
- In lymphoid malignancies, it can be used as the clonality marker
- Clonal rearrangements can be detected with molecular techniques
- Once detected, certain rearrangement pattern **can be used for MRD monitoring with ultimate sensitivity**
- Due to the complexity of the problem, highly specialized laboratory is required for this analyses