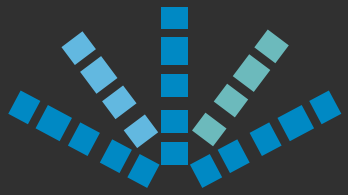
Estudio de proteínas de fusión por citometria de flujo



EuroFlow

Centro de Investigación del Cáncer, Universidad y Hospital Universitario de Salamanca, Salamanca

XVIII Congreso de la Sociedad Chilena de Hematología, La Serena, 4 - 6 de octubre de 2012

Diagnostics in hematological malignancies

1. Making the diagnosis
   Normal ↔ reactive/regenerating ↔ malignant

   Annually > 300,000 new patients with a hematological malignancy in developed countries

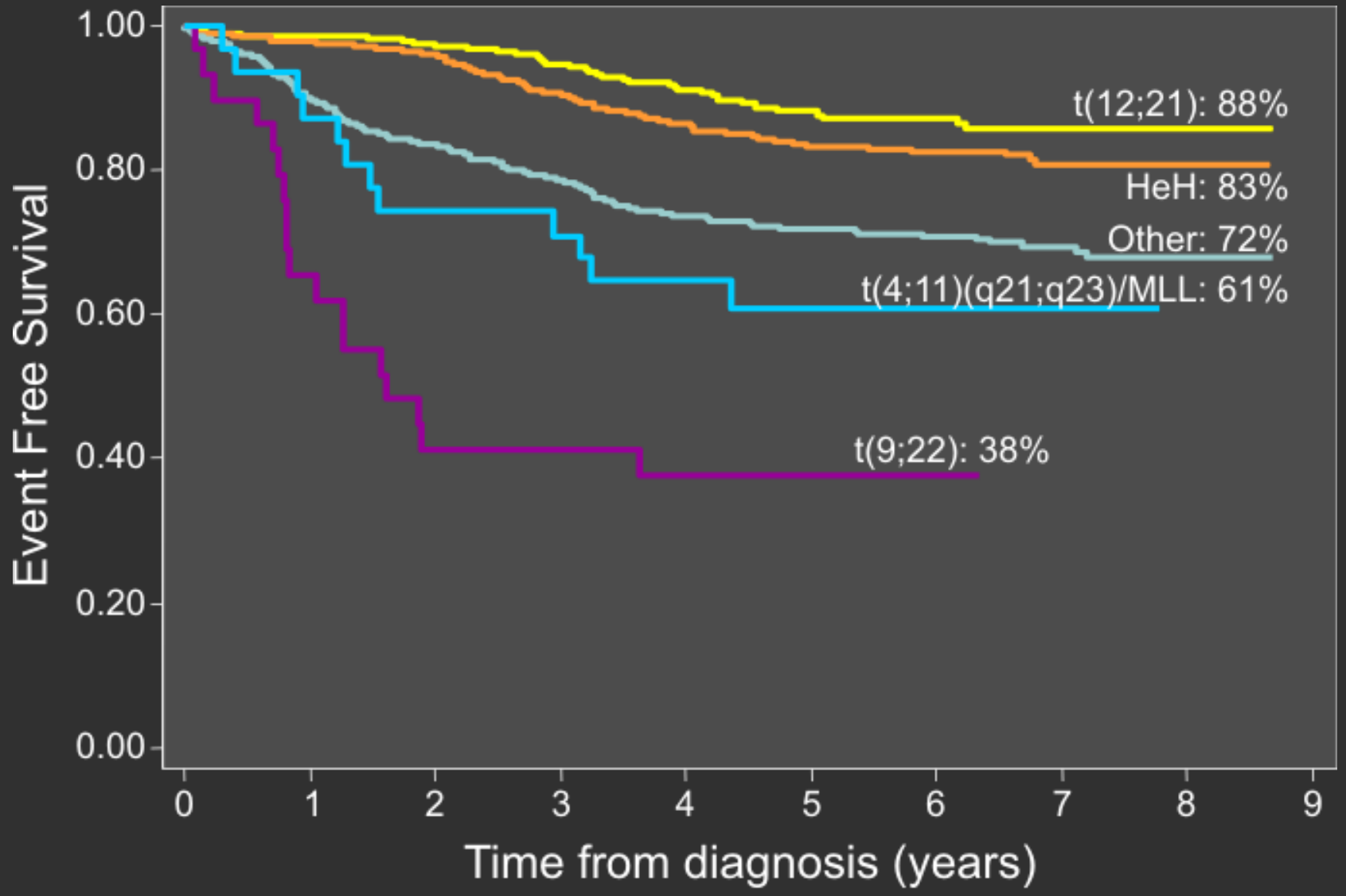
2. Classification of hematopoietic malignancies
   - relation with prognosis
   - relevance of risk-group definition in treatment protocols

   → Based on differentiation characteristics and particularly on chromosome aberrations, resulting in fusion gene transcripts or aberrantly (over) expressed genes

3. Evaluation of treatment effectiveness
   Detection of minimal residual disease (MRD):

   MRD-based risk-group stratification (treatment reduction or treatment intensification)
   Annually > 400,000 follow-up samples in leukemia patients (ALL, AML, CML)

Event-free survival in childhood ALL according to chromosome aberrations



T. Szczepanski, et al. Lancet Oncology, In Press.

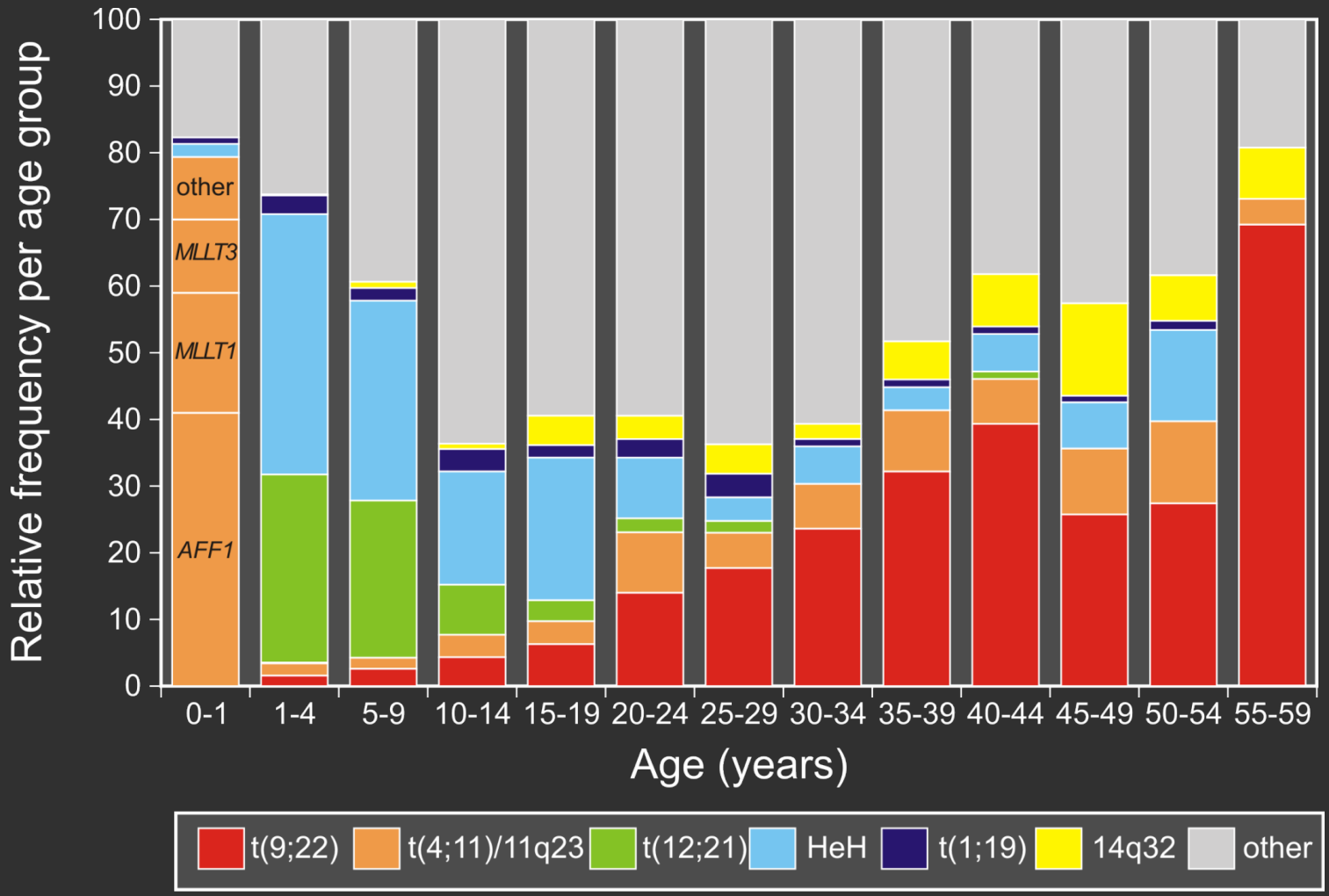
Chromosome aberrations and fusion genes in acute leukemias

Chromosome aberration	Fusion genes	Relative frequency per type of acute leukemia				
		Precursor-B-ALL		AML		
		children	adults	children	adults <60 y	adults >60y
t(1;19)(q23;p13)	*E2A-PBX1*	5-8%	3-4%	-	-	-
t(4;11)(q21;q23)	*MLL-AF4*	3-5%[a]	3-4%	<1%	<1%	<1%
t(9;22)(q34;q11)	*BCR-ABL* p190	3-5%	15-30%	<1%	<1%	<1%
t(9;22)(q34;q11)	*BCR-ABL* p210	1-2%	10-15%	<1%	<1%	<1%
t(12;21)(p13;q22)	*TEL-AML1*	25-30%	<2%	-	-	-
t(8;21)(q22;q22)	*AML1-ETO*	-	-	10-14%	6-8%	2-3%
t(15;17)(q22;q21)	*PML-RARA*	-	-	8-10%[b]	5-15%[b]	2-6%[b]
inv(16)(p13;q22)	*CBFB-MYH11*	-	-	5-7%	5-6%	3-4%
	TOTAL	40-45%	40-45%	25-30%	20-25%	10-12%

[a] In infant ALL, the frequency of t(4;11) can be as high as 70%.

[b] In southern European regions (ES, FR, and IT) the frequency of t(15;17) with *PML-RARA* is essentially higher than in northern European regions.

Relative frequency of well-defined genetic aberrations in ALL according to age groups



T. Szczepanski, et al. Lancet Oncology, 2010; 11:880-889

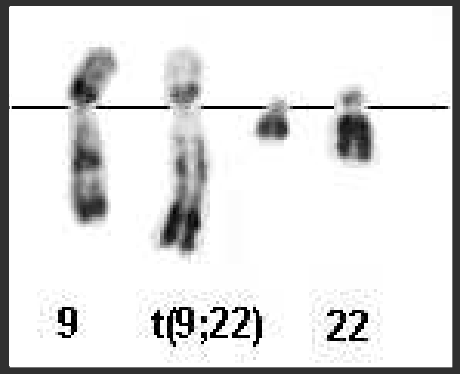
AL: GENOTYPIC-PHENOTYPIC ASSOCIATIONS

Diagnosis	Genetic lesion	Aberrant immunophenotype
BCP-ALL	t(9;22)*	$CD34^{hi}$,CD10+,$CD38^{lo}$,$CD13^{lo}$
	t(12;21)	$CD34^{het}$,CD10+,$CD20^{-}$,$CD13^{lo}$
	11q23	$CD34^{+}$,$CD10^{-}$,7.1^{+},$CD15^{+}$
AML	t(15;17)	$CD34^{-/+}$,$CD15^{-/lo}$,$CD2^{-/lo}$,$CD13^{het}$
	Inv(16)	MPO^{hi},$CD2^{-/lo}$
	t(8;21)	$CD19^{+}$,$CD56^{+}$
	11q23	$CD56^{+}$,$7.1^{-/+}$,$CD19^{-/lo}$,$CD2^{-/+}$

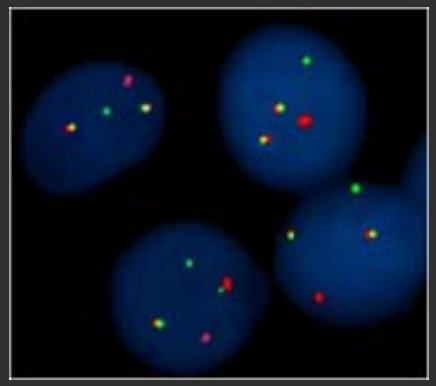
Ortuño F, Orfao A, Cytometry B, 2004

Current detection of genetic aberrancies

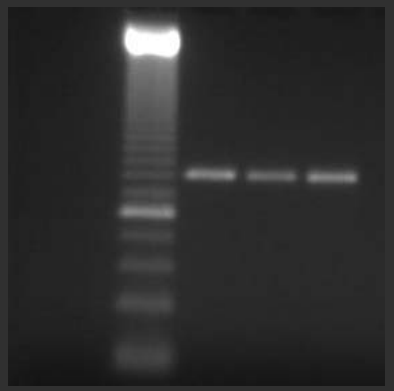
including overexpressed oncogenes and fusion genes



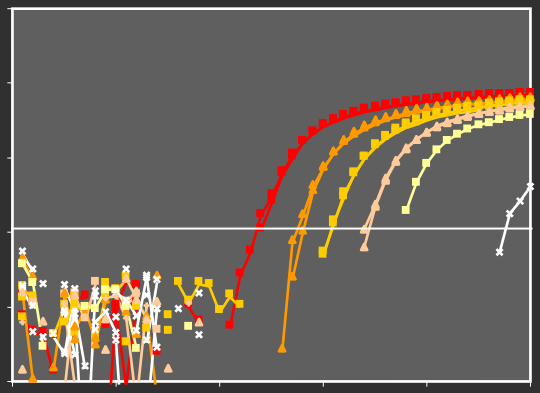
Cytogenetics



FISH



PCR



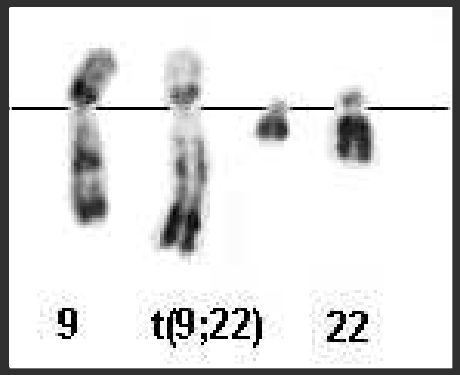
RQ-PCR

Advantages of molecular techniques:

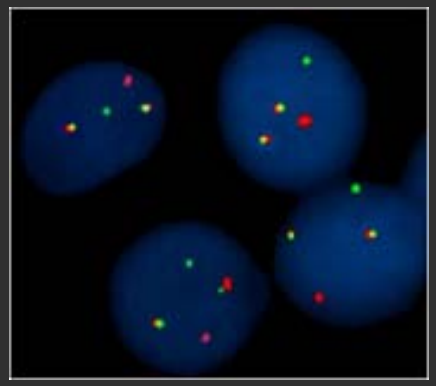
- Generally well-established;
- Cytogenetics screens total genome for visible structural aberrations;
- FISH: screening of all relevant breakpoints of targeted genes;
- PCR: most variant breakpoints are identified (size differences);
- RQ-PCR: highly sensitive and reproducible: Useful for MRD diagnostics
- Microarray, CGH, SNP: promising, but to be established !

Current detection of genetic aberrancies

including overexpressed oncogenes and fusion genes



Cytogenetics



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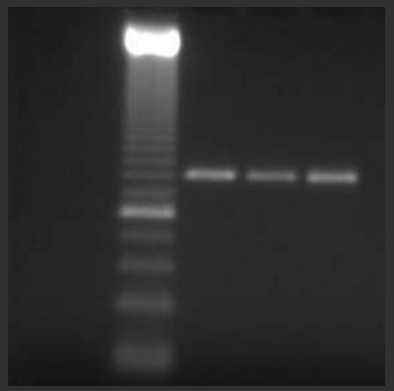
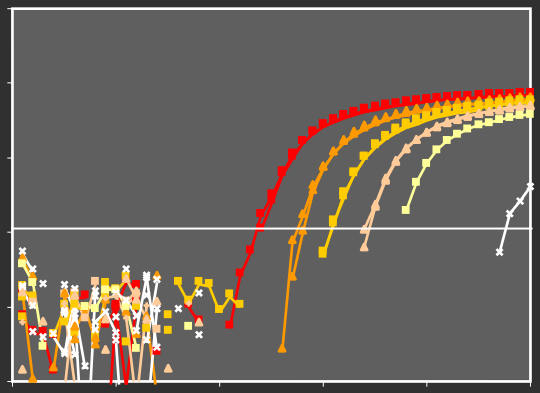


PCR



RQ-PCR

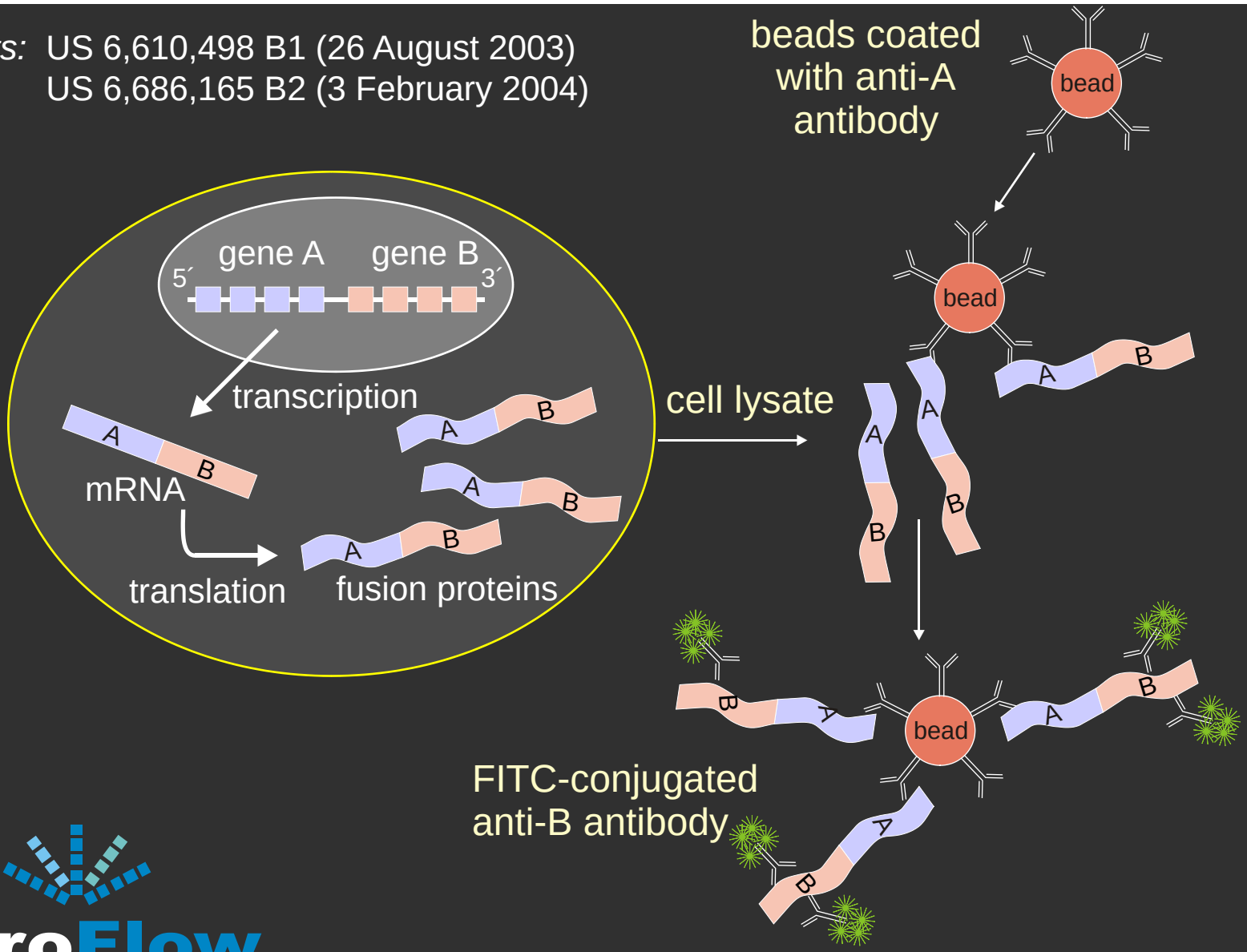
Disadvantages of molecular techniques:

- labor intensive;
- require specialized laboratories;
- time consuming (2-3 days, up to a -week)

→ Faster technique needed: at protein level?

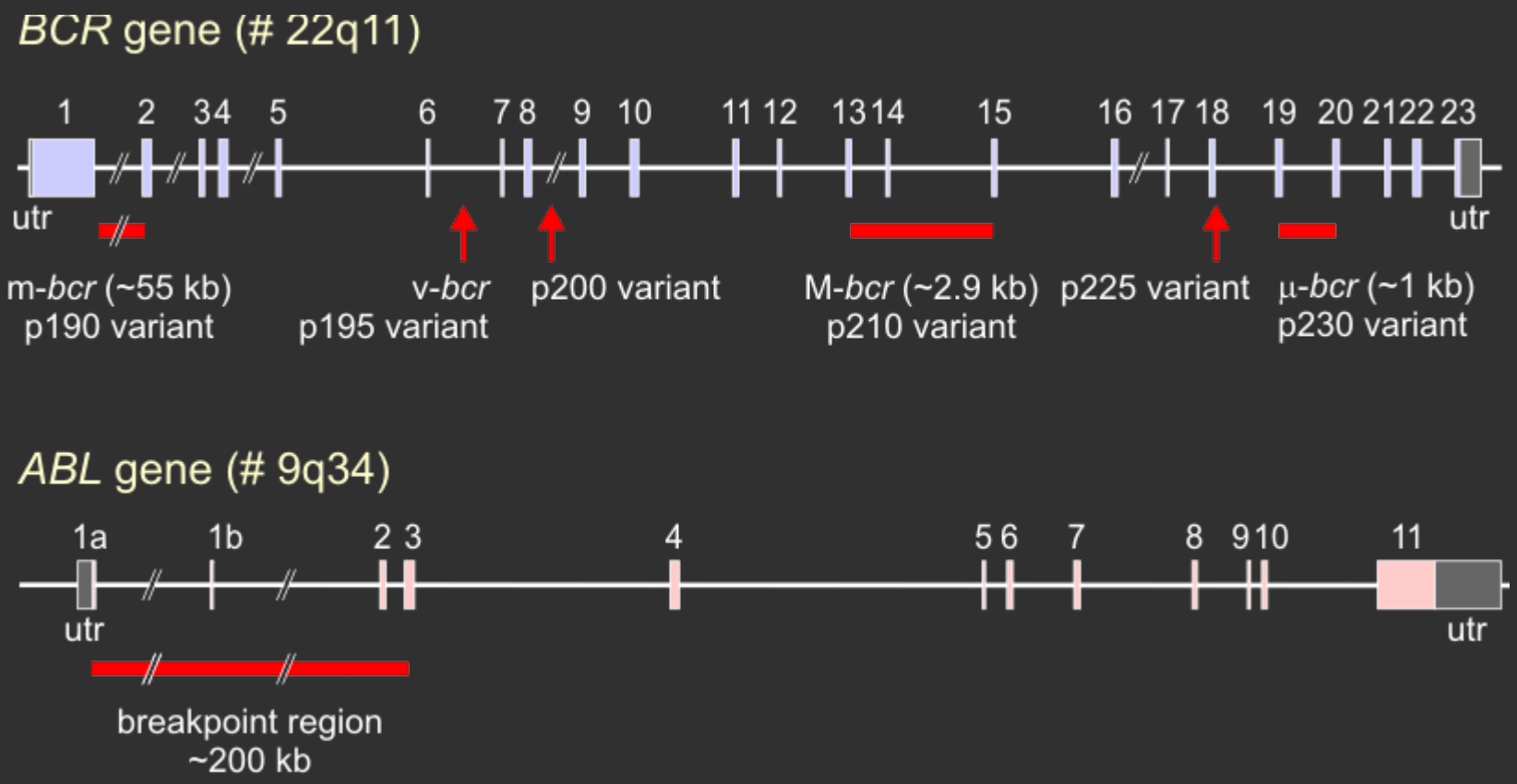
Bead-based flow cytometric assay for detection of fusion proteins

Patents: US 6,610,498 B1 (26 August 2003)
US 6,686,165 B2 (3 February 2004)



EuroFlow

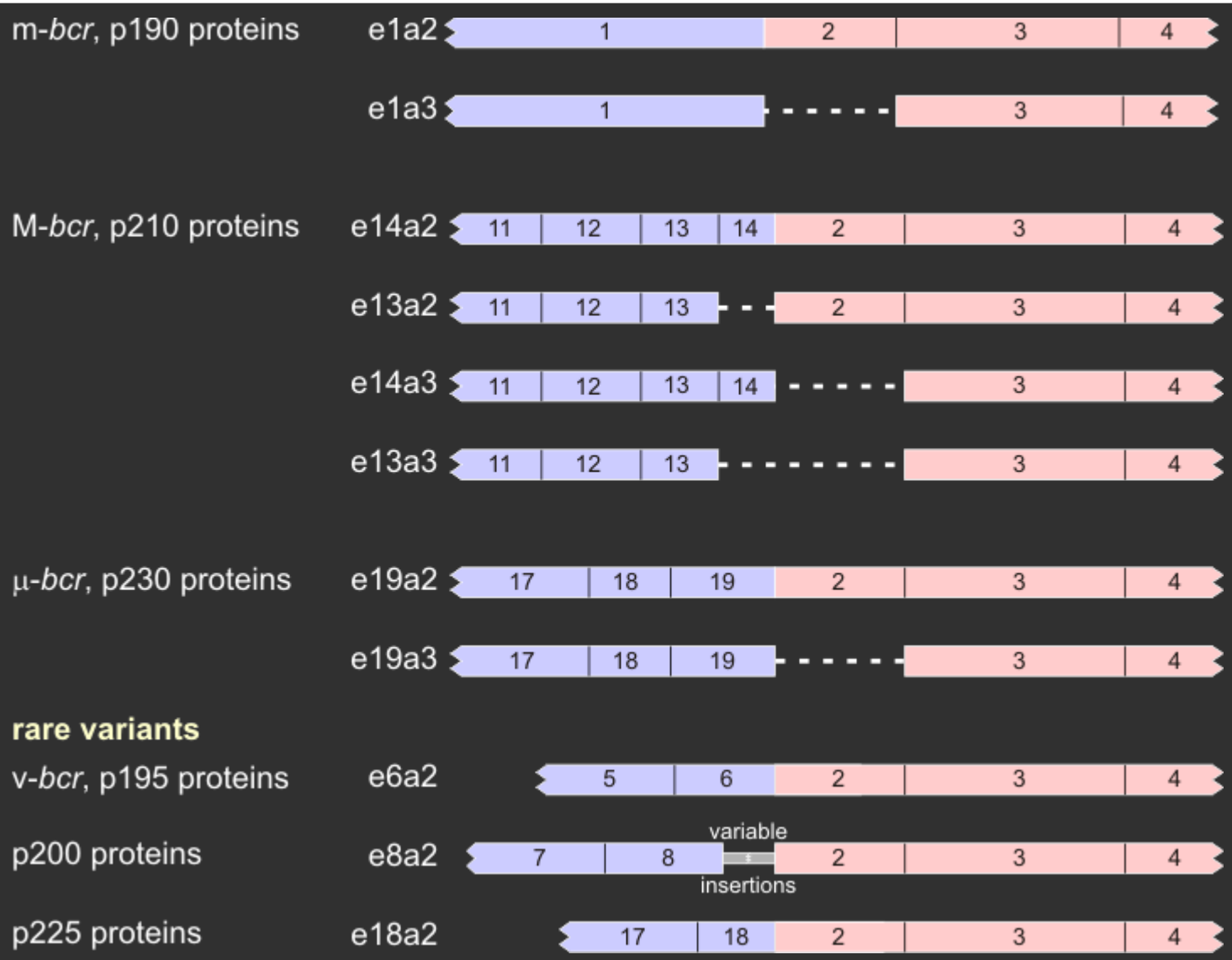
Breakpoint regions in t(9;22)(q34;q11) with *BCR* and *ABL* genes



EuroFlow

F. Weerkamp, et al. Leukemia 2009; 23: 1106-1117.

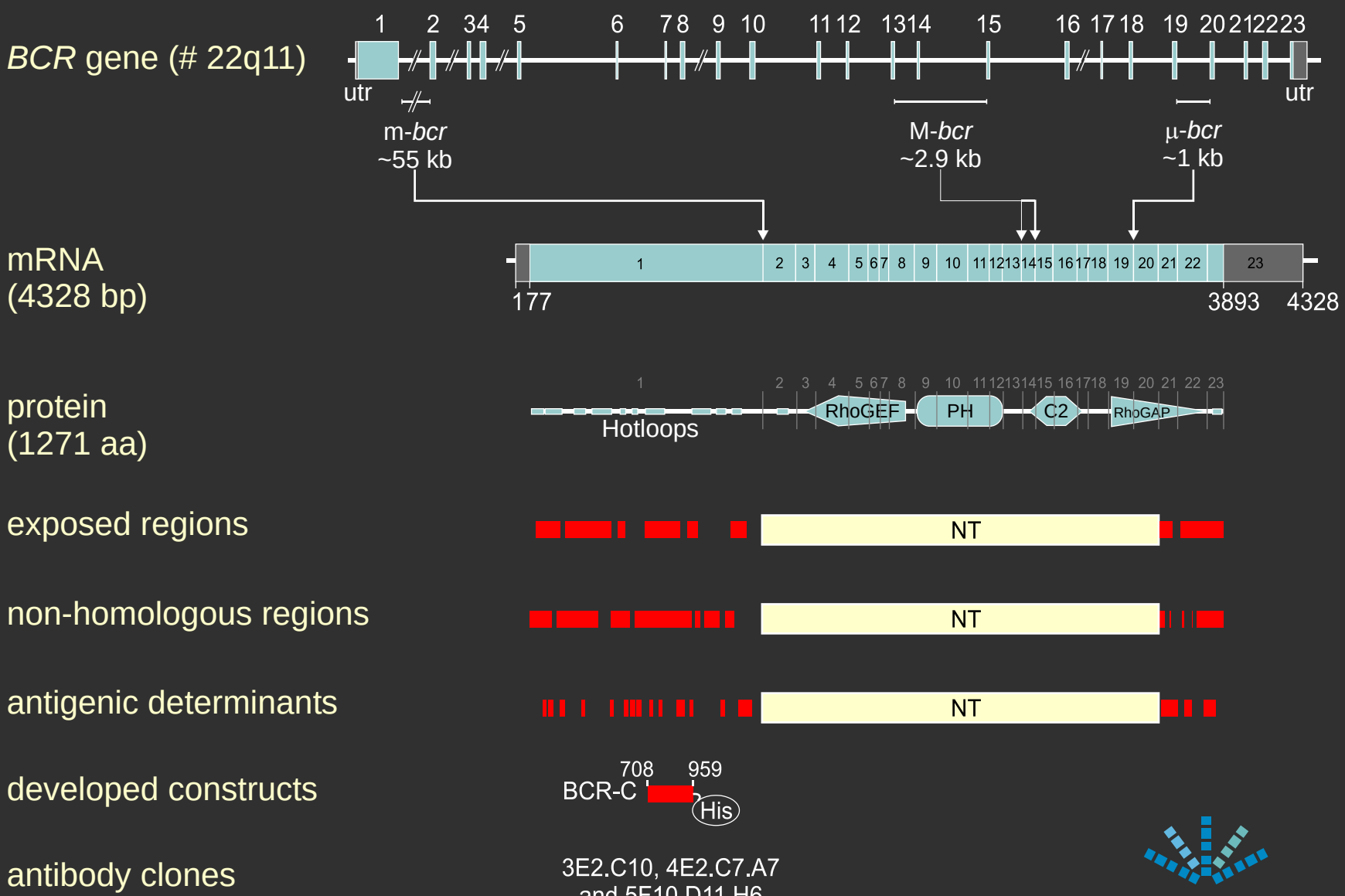
Multiple variants of *BCR-ABL* transcripts caused by multiple different *BCR* breakpoint regions



EuroFlow

F. Weerkamp, et al. Leukemia 2009; 23: 1106-1117.

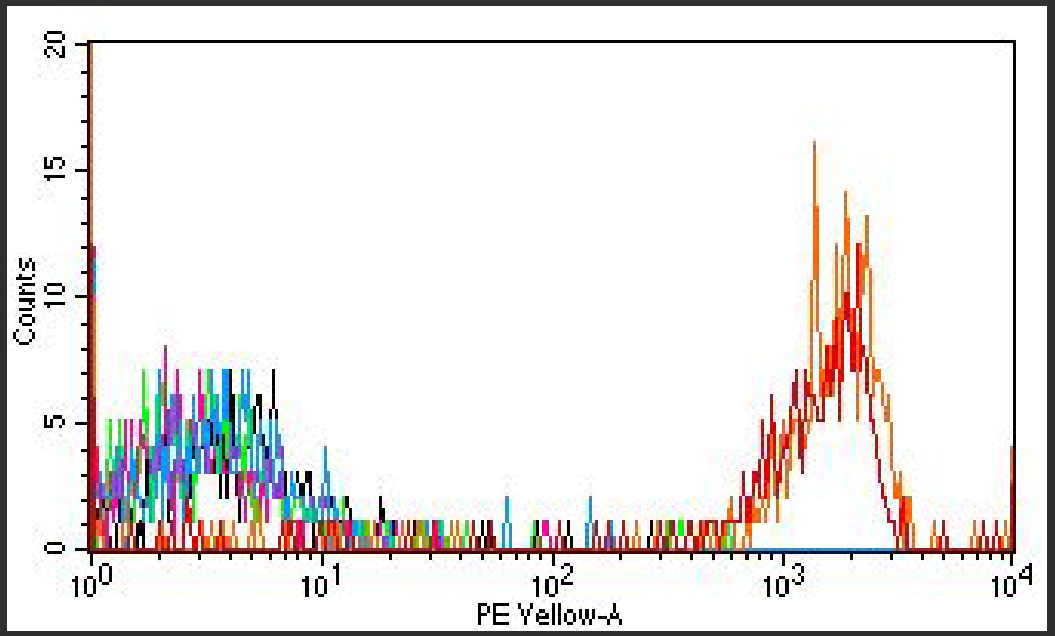
Design of anti-*BCR* antibodies for fusion protein beads



Dept. of Immunology, Erasmus MC, Rotterdam

EuroFlow

Cytometric Bead-array platform for immunobeads: detection of BCR-ABL t(9;22) fusion protein

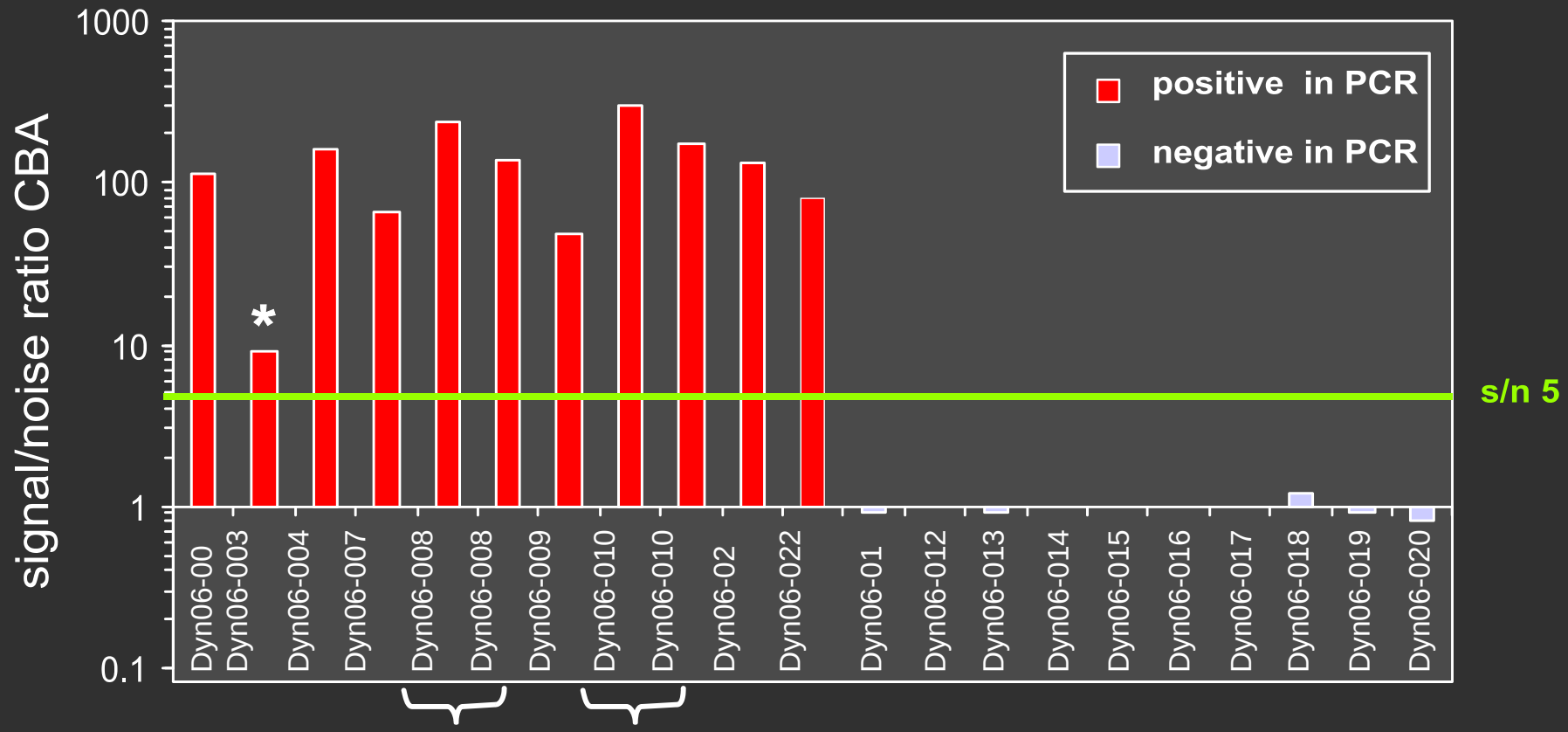


Black: PBMC (neg. control)
Green: 697 (E2-PBX1; neg. control)
Purple: REH (TEL-AML1; neg. control)
Blue: RS4,11 (MLL-AF4; neg. control)
Orange: K562 (BCR-ABL p210)
Red: Lama-84 (BCR-ABL p210)

Catching antibody: anti-ABL
Bead: BD-Flex bead (A7)
Detection antibody: anti-BCR (biotinylated)

EuroFlow

BCR-ABL CBA for precursor B-ALL (diagnosis)



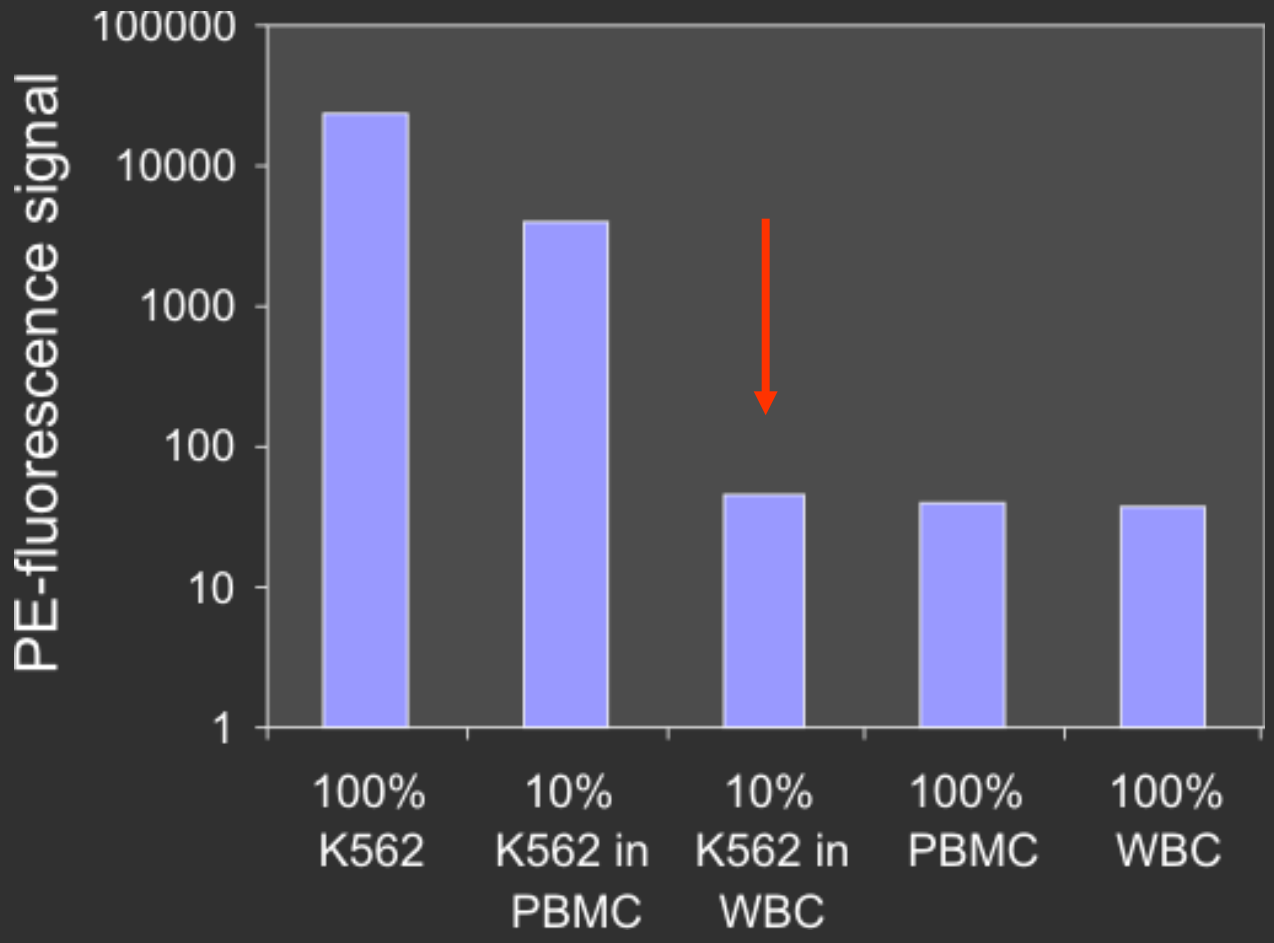
same sample tested in 2 separate experiments

* patient with mutation close to a ABL-binding site

Only frozen samples tested

EuroFlow

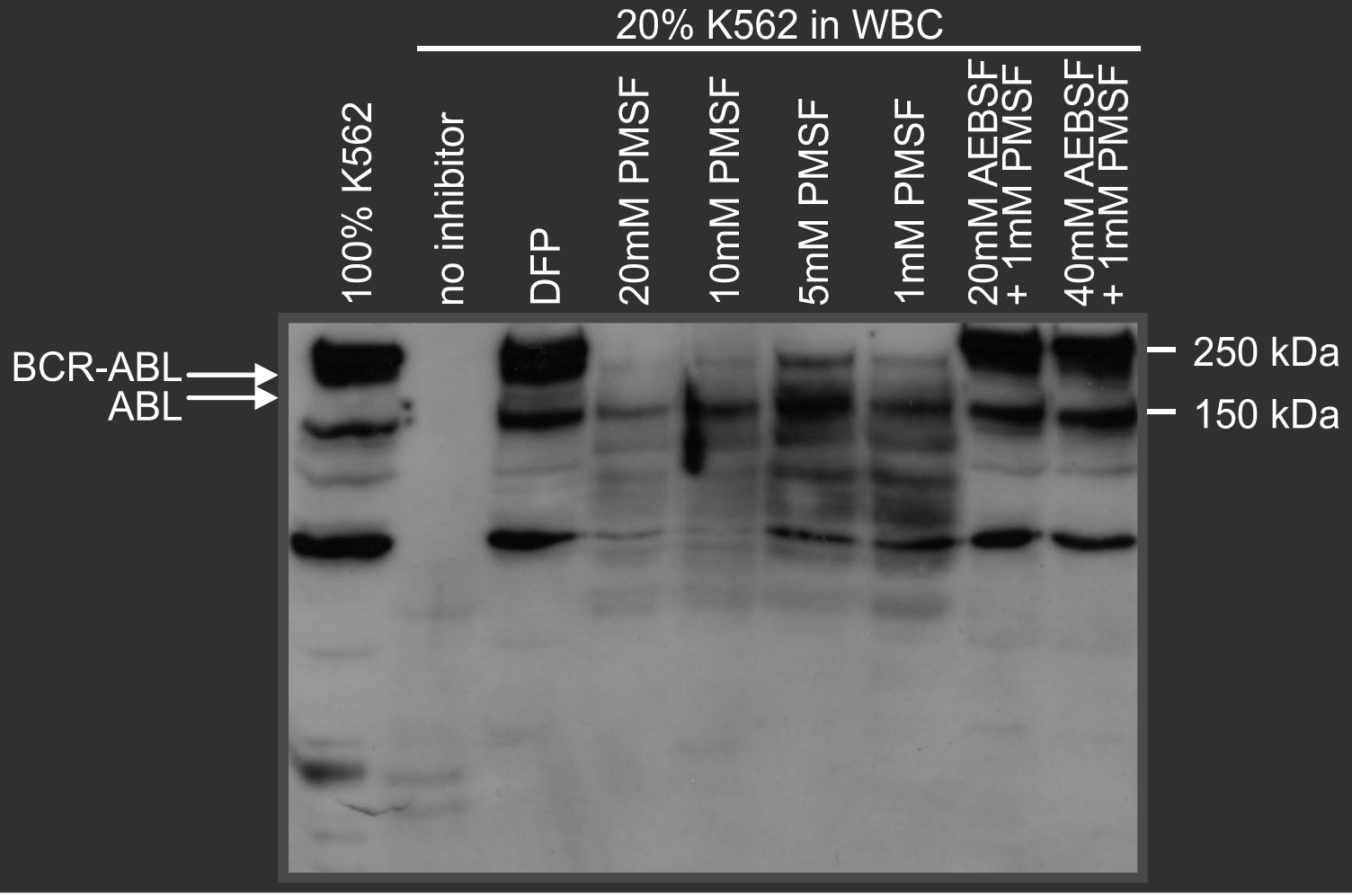
Loss of BCR-ABL signal in the presence of mature myeloid cells, e.g.WBC



EuroFlow

F. Weerkamp, et al. Leukemia 2009; 23: 1106-1117.

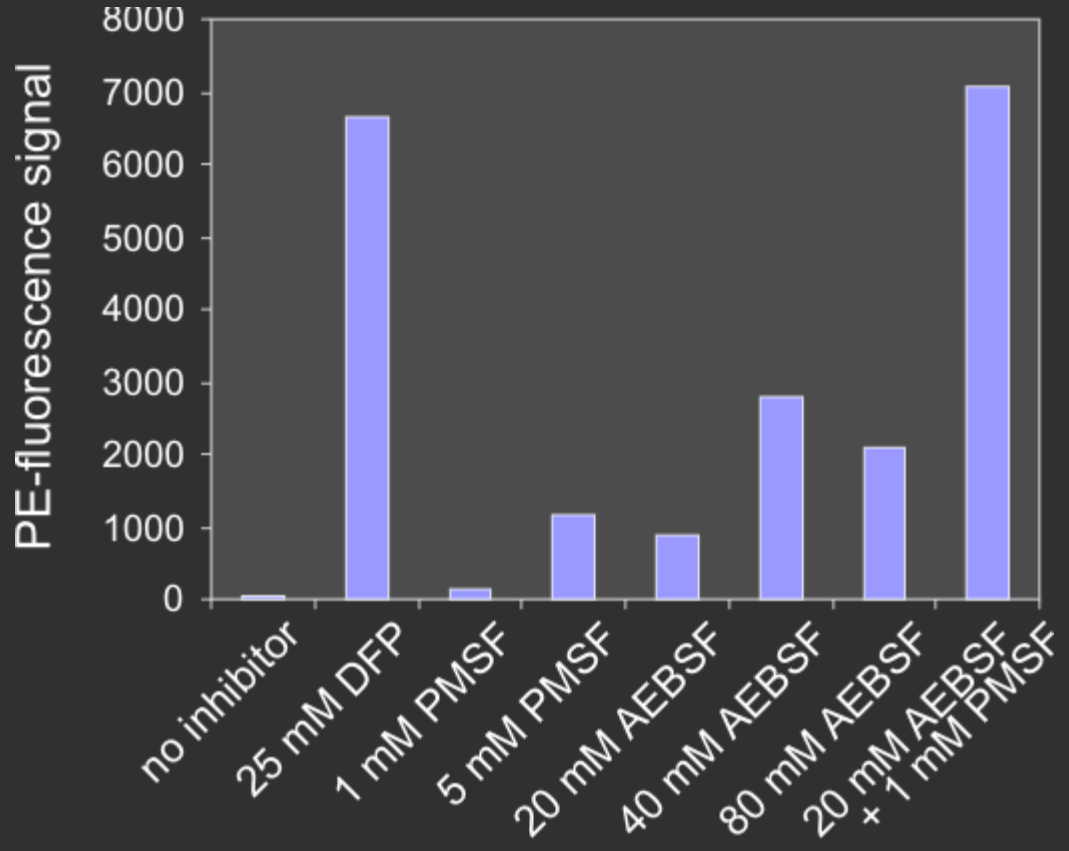
Degradation of BCR-ABL (and other proteins) by protease activity



EuroFlow

F. Weerkamp, et al. Leukemia 2009; 23: 1106-1117.

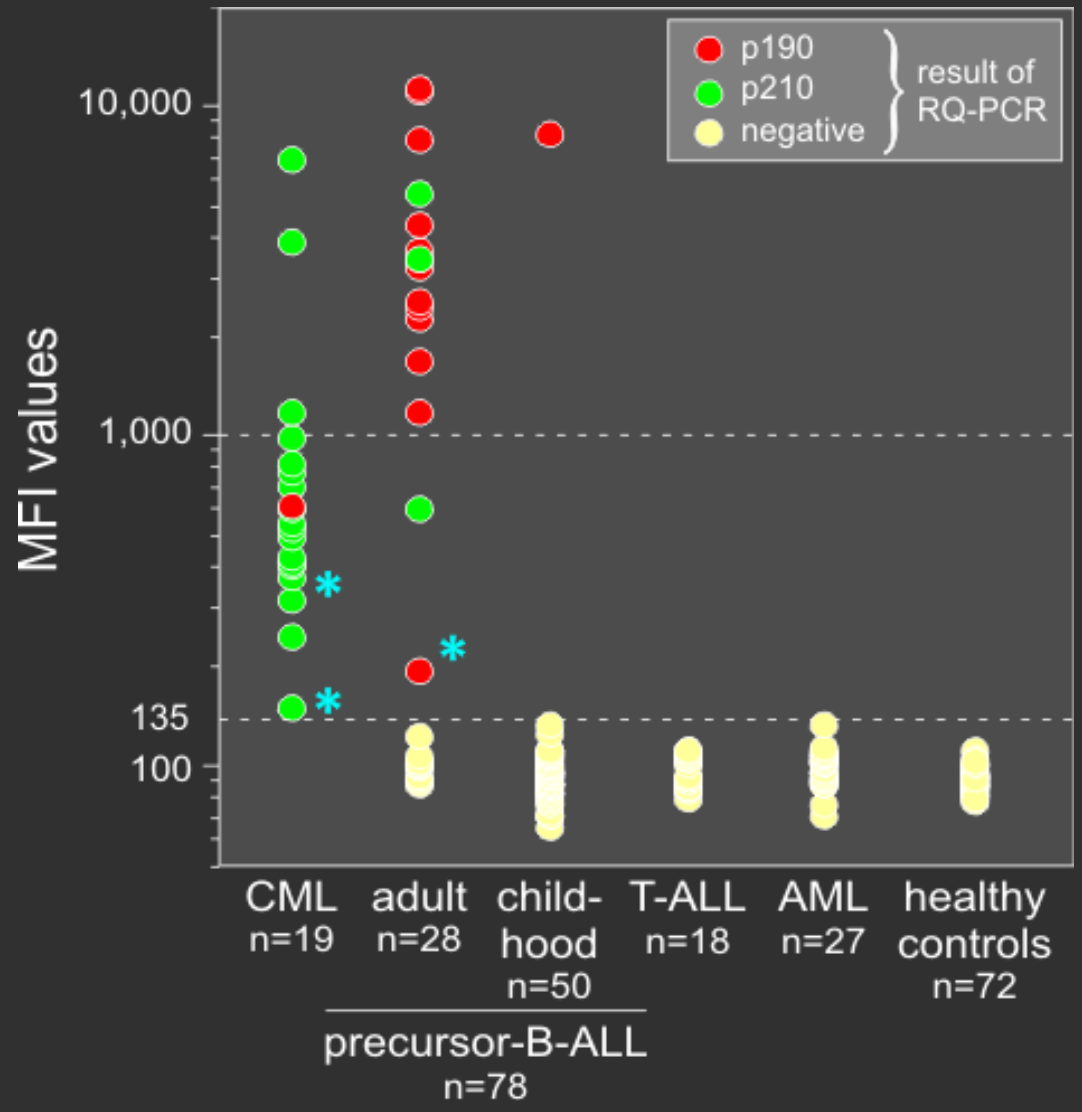
Blocking of protease activity via protease inhibitors



EuroFlow

F. Weerkamp, et al. Leukemia 2009; 23: 1106-1117.

Results of the BCR-ABL RUO testing by the EuroFlow laboratories

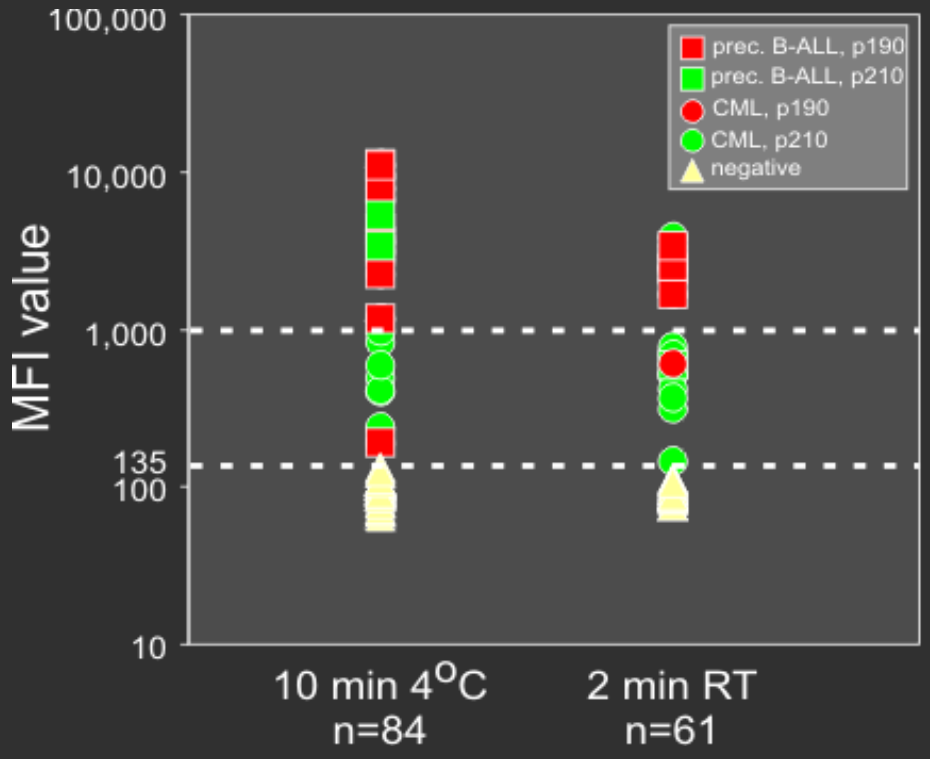


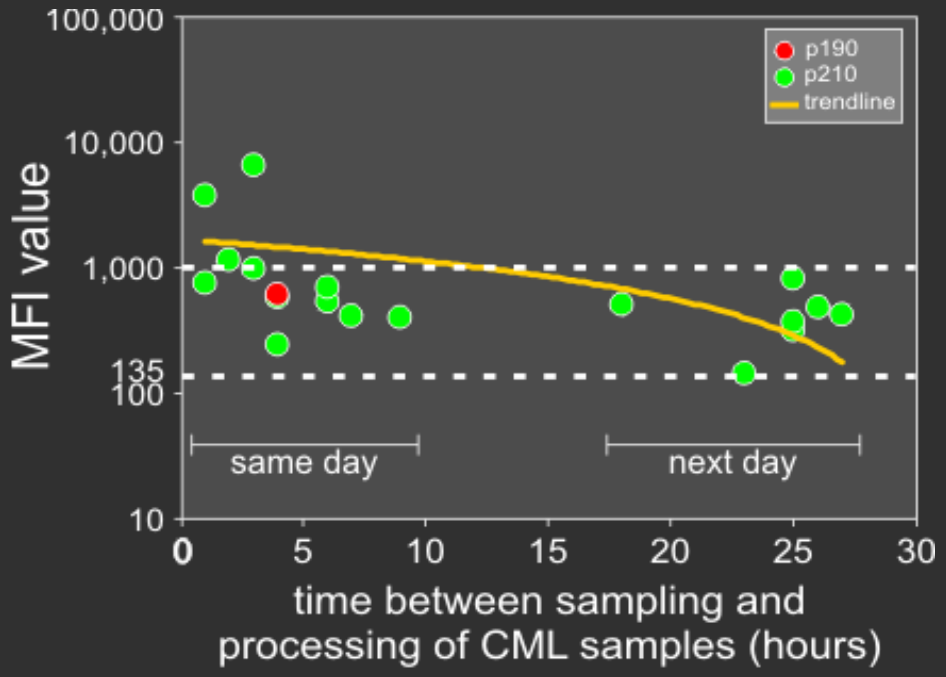
EuroFlow

F. Weerkamp, et al. Leukemia 2009; 23: 1106-1117.

Stability fo BCR-ABL fusion proteins

influence of temperature and transportation-processing time

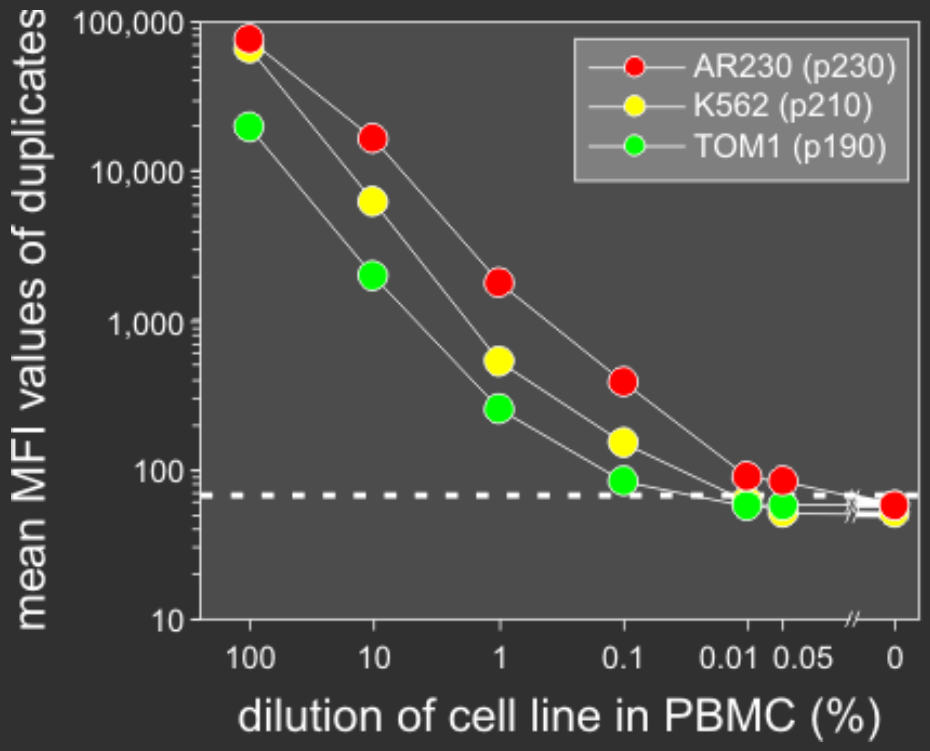


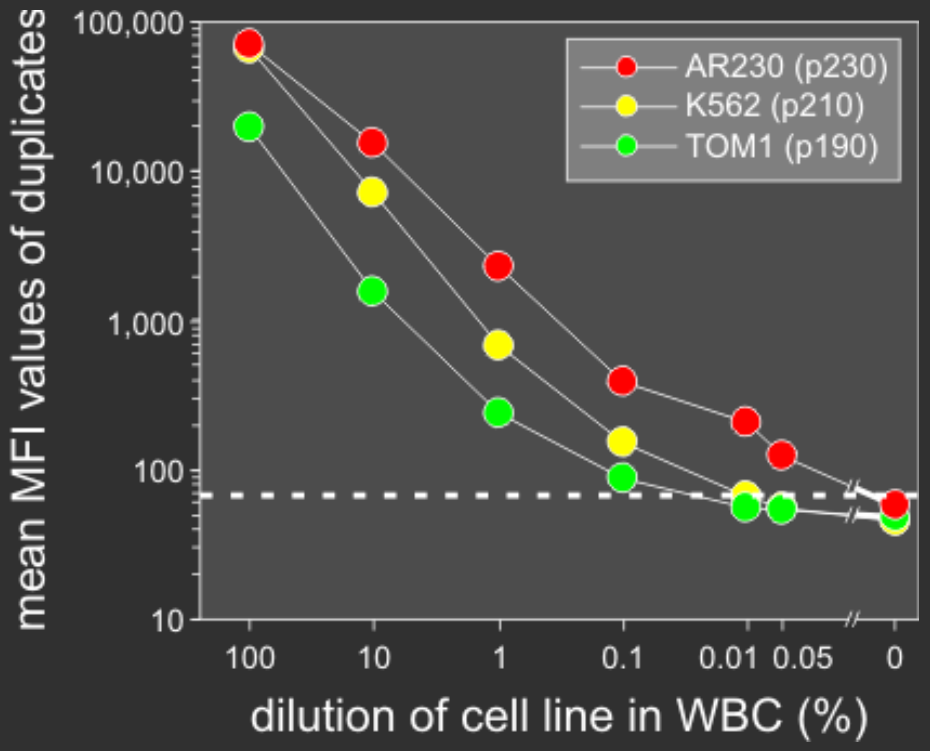


EuroFlow

F. Weerkamp, et al. Leukemia 2009; 23: 1106-1117.

Sensitivity of the BCR-ABL RUO immunobead assay





EuroFlow

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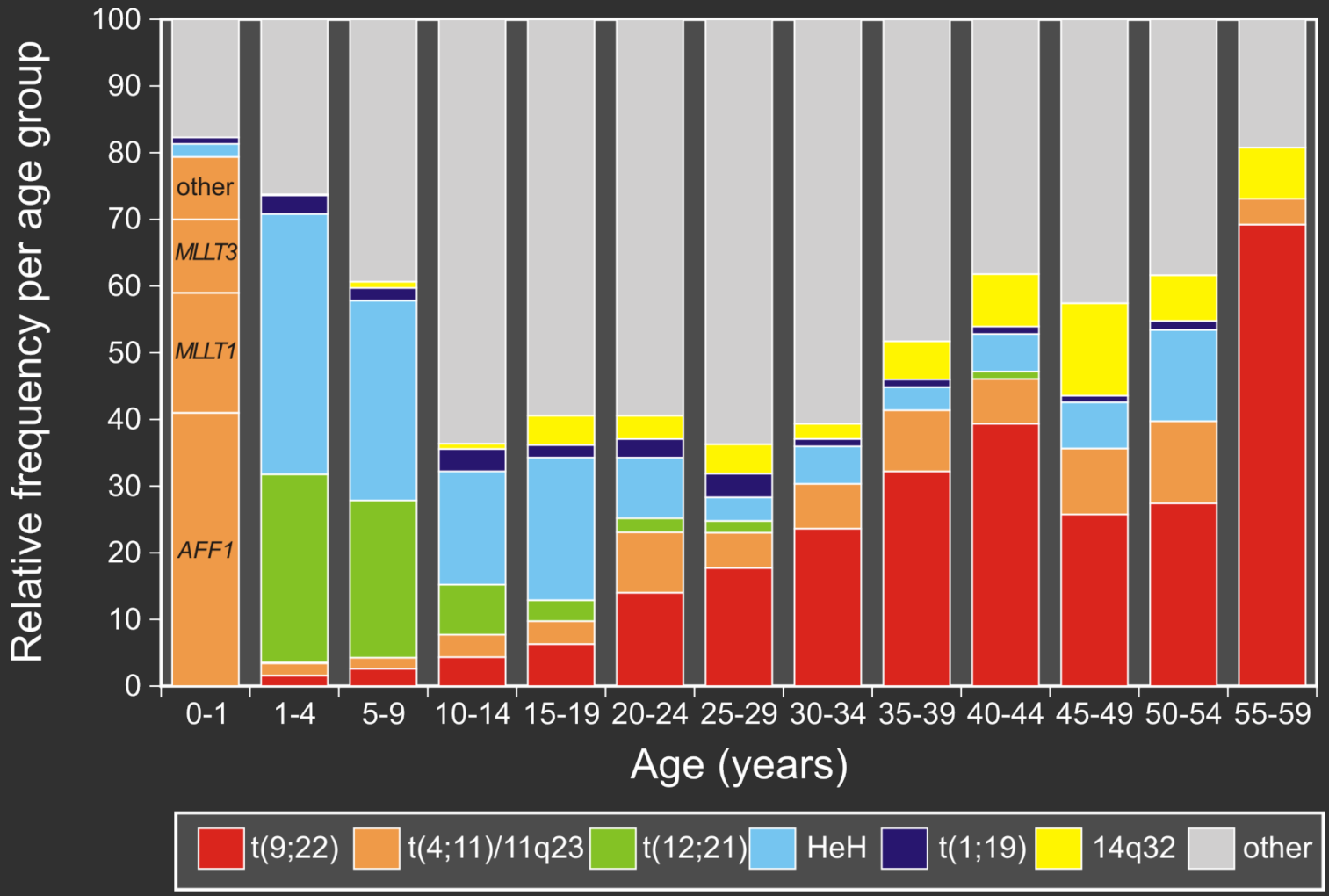

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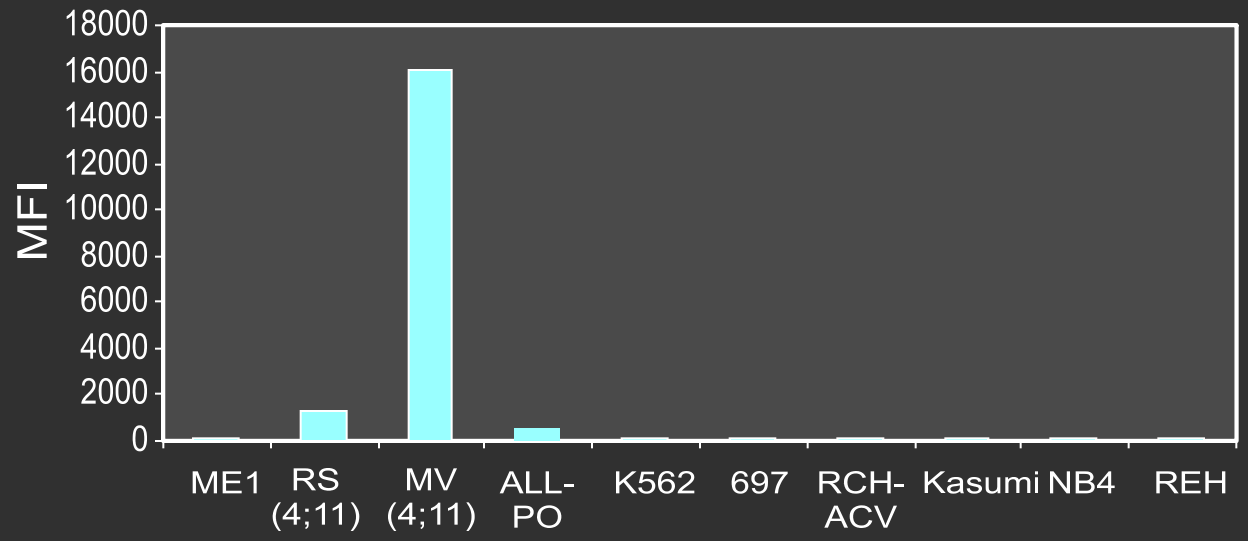
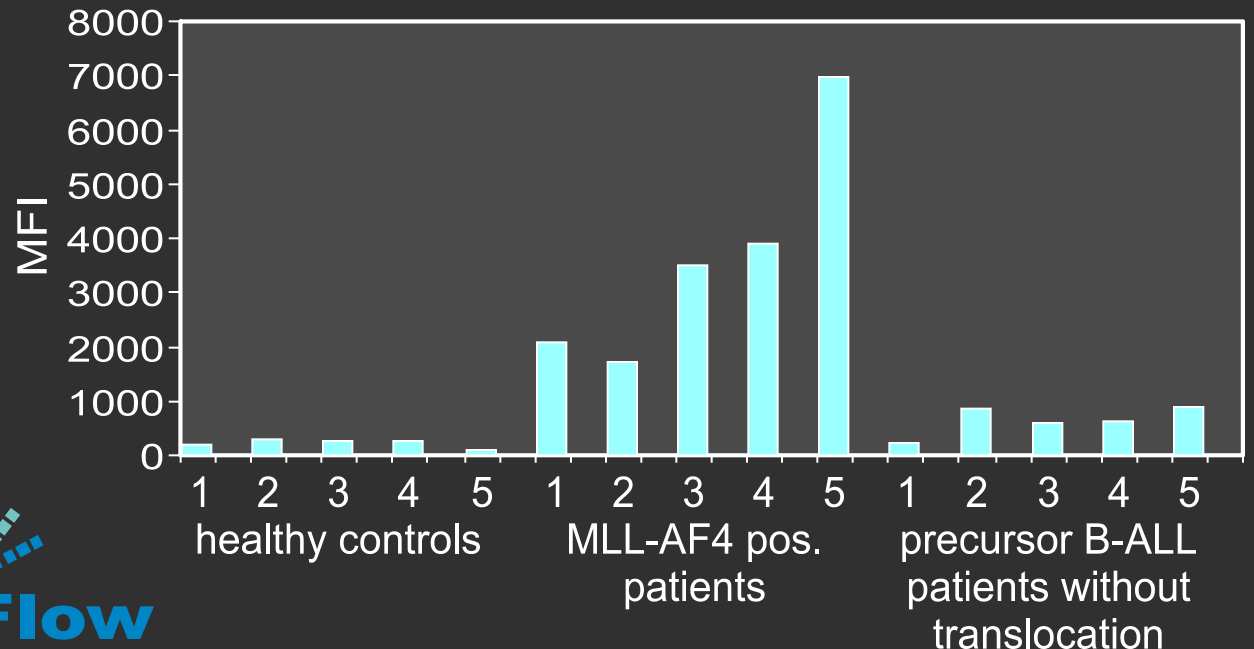
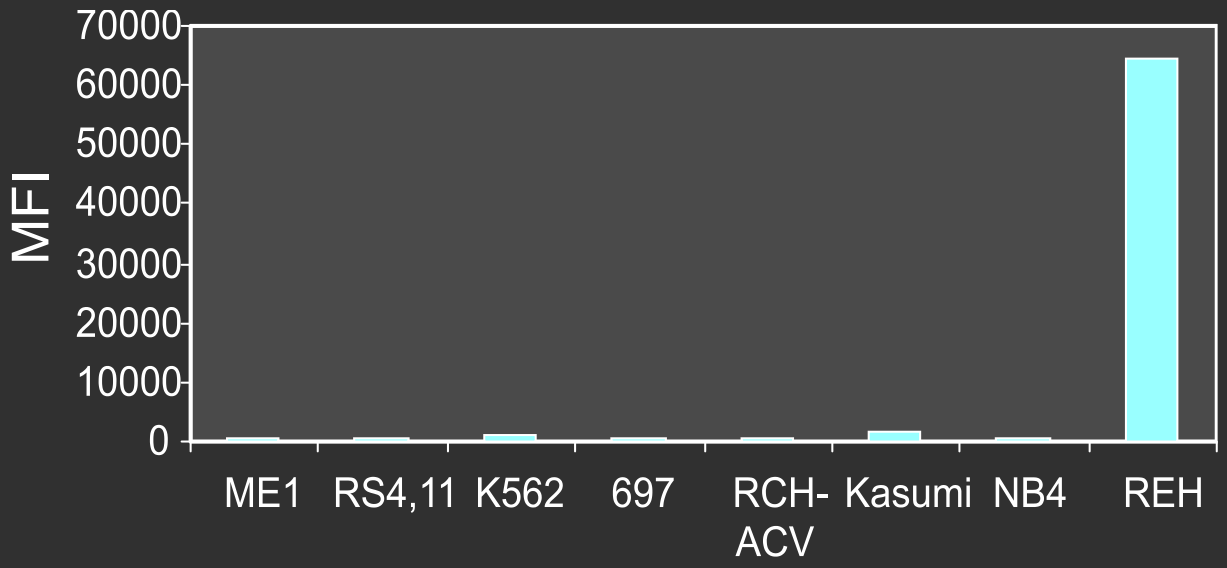
Flow cytometric MLL-AF4 immunobead assay

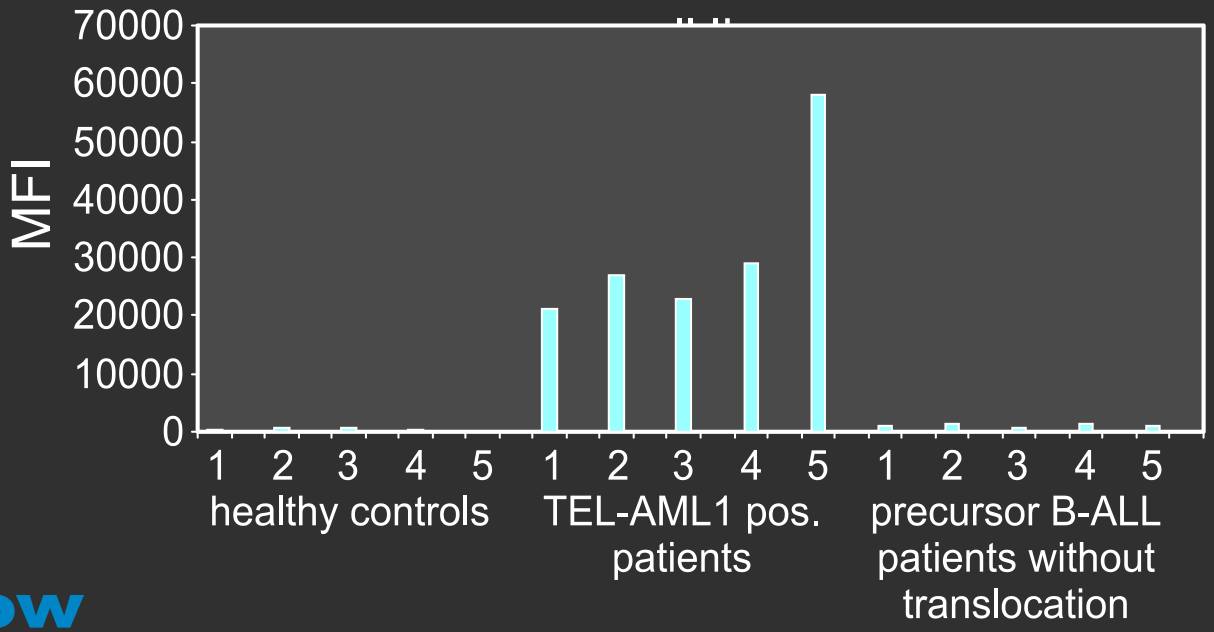




EuroFlow

Flow cytometric TEL-AML1 immunobead assay





EuroFlow

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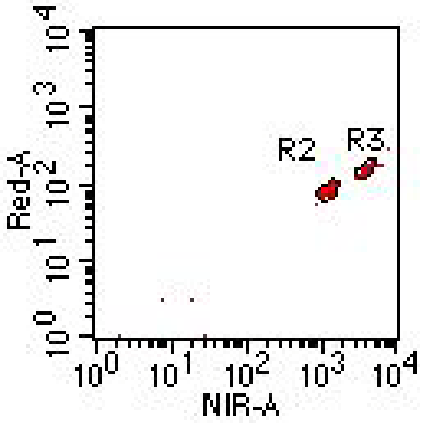
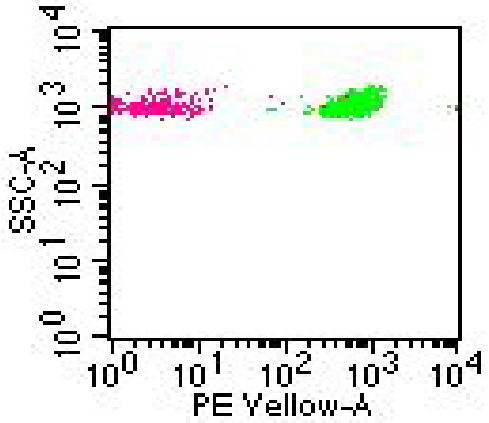
SIMULTANEOUS DETECTION OF BCR-ABL & E2A-PBX FUSION PROTEINS



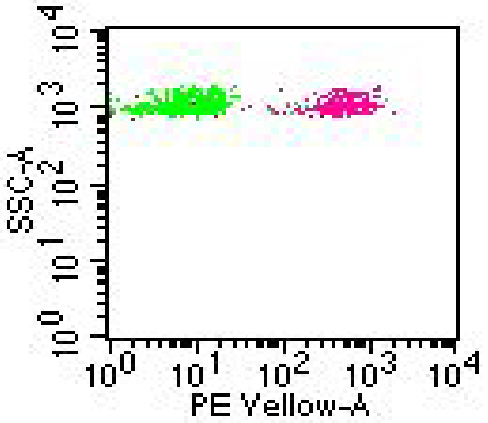
R2 = green = BCR beads

R3 = pink = E2A beads

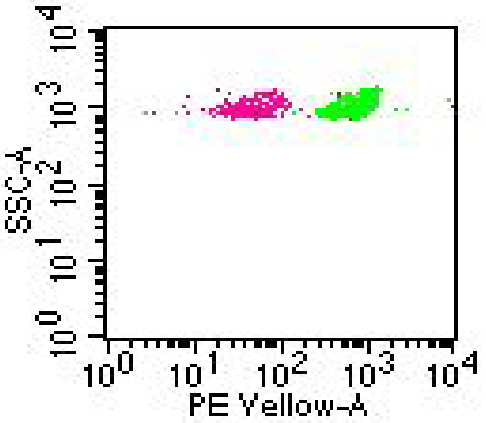
100% K562



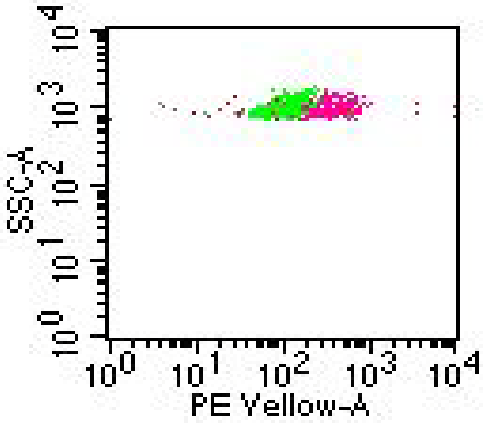
100% 697



10% 697 K562



10% K562 697



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WHO CLASSIFICATION OF AML

AML with recurrent genetic abnormalities

- AML with t(8;21)(q22;q22); RUNX1-RUNX1T1
- AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
- APL with t(15;17)(q22;q12); PML-RARA
- AML with t(9;11)(p22;q23); MLLT3-MLL
- AML with t(6;9)(p23;q34); DEK-NUP214
- AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
- AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1
- AML with mutated NPM1
- AML with mutated CEBPA

AML with myelodysplasia-related changes

AML NOS

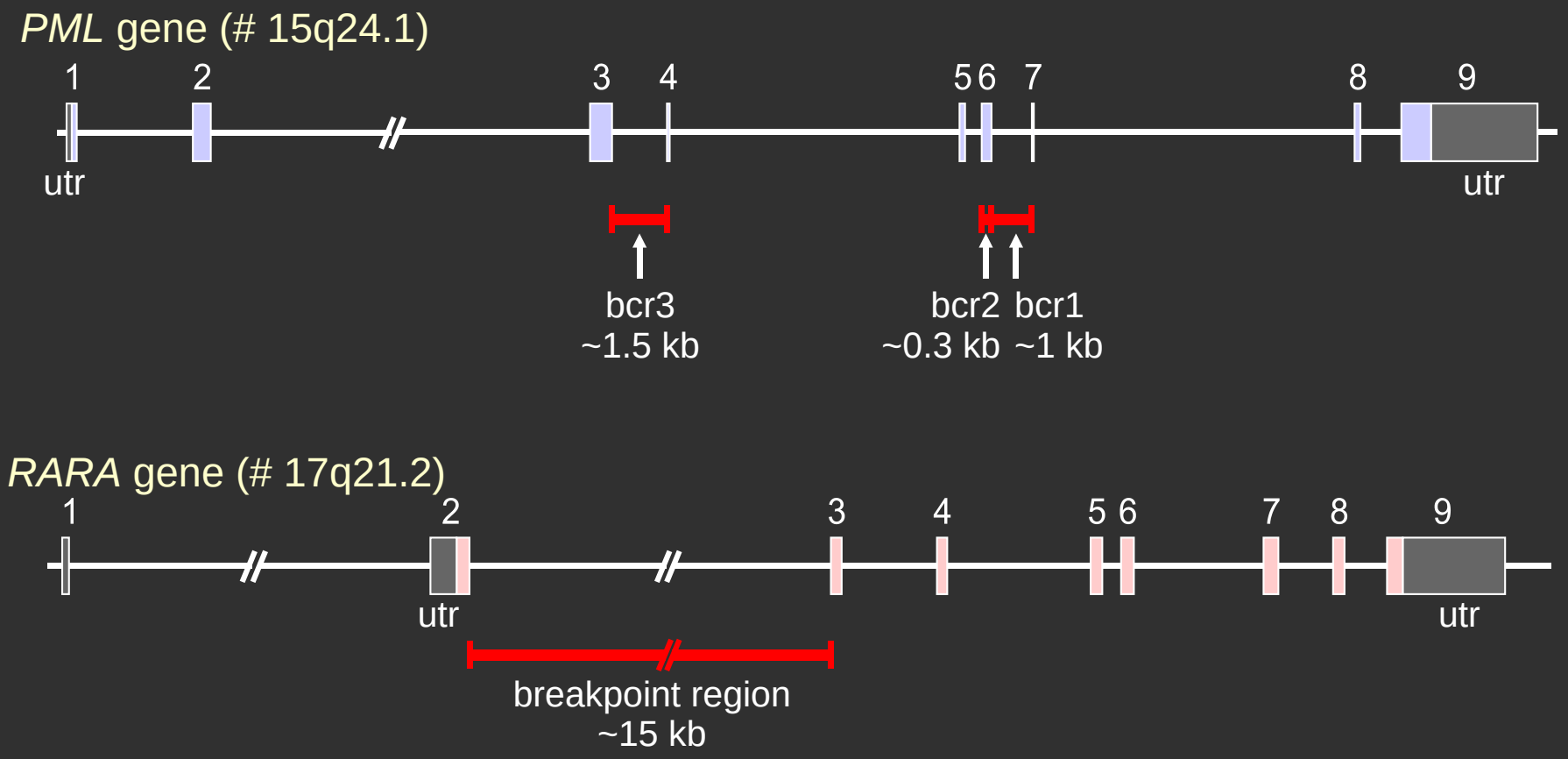
Therapy-related myeloid neoplasms

Myeloid sarcoma

Myeloid proliferations related to Down syndrome

Blastic plasmacytoid dendritic cell neoplasm

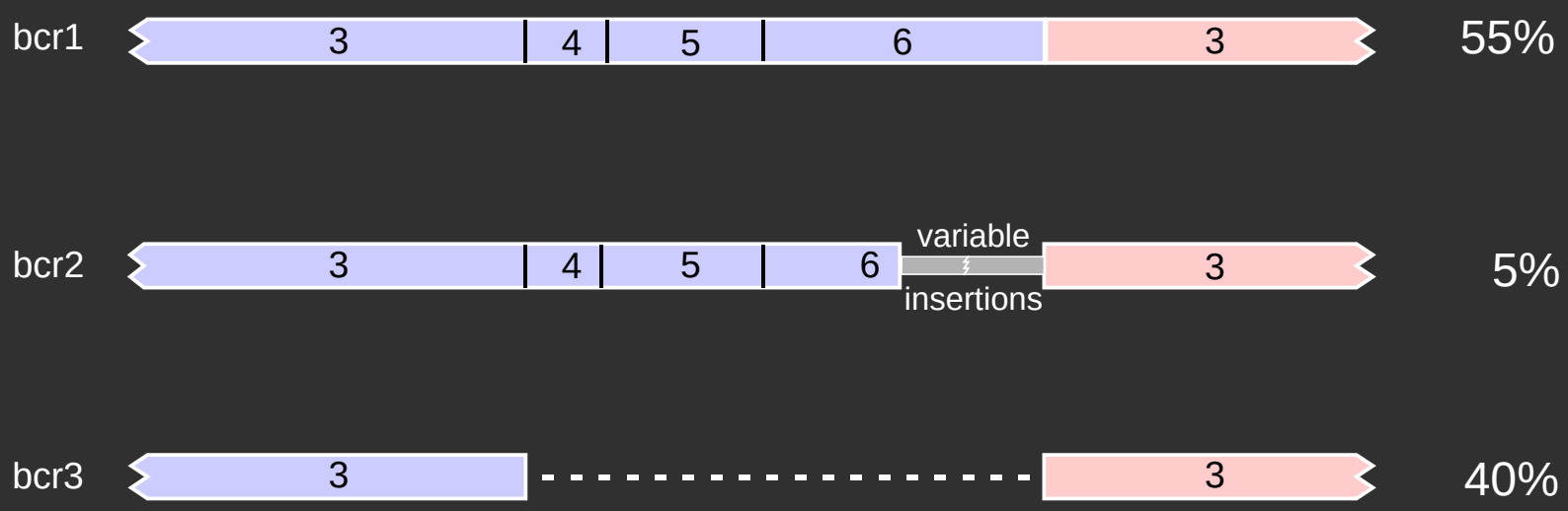
Breakpoint regions in t(15;17)(q24;q21) with *PML* and *RARA* genes



EuroFlow

Dekking et al, Leukemia, 2012

Variants of *PML-RARA* transcripts caused by different *PML* breakpoint regions



EuroFlow

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Bead-based flow cytometric assay for detection of fusion proteins

Patents: US 6,610,498 B1 (26 August 2003)
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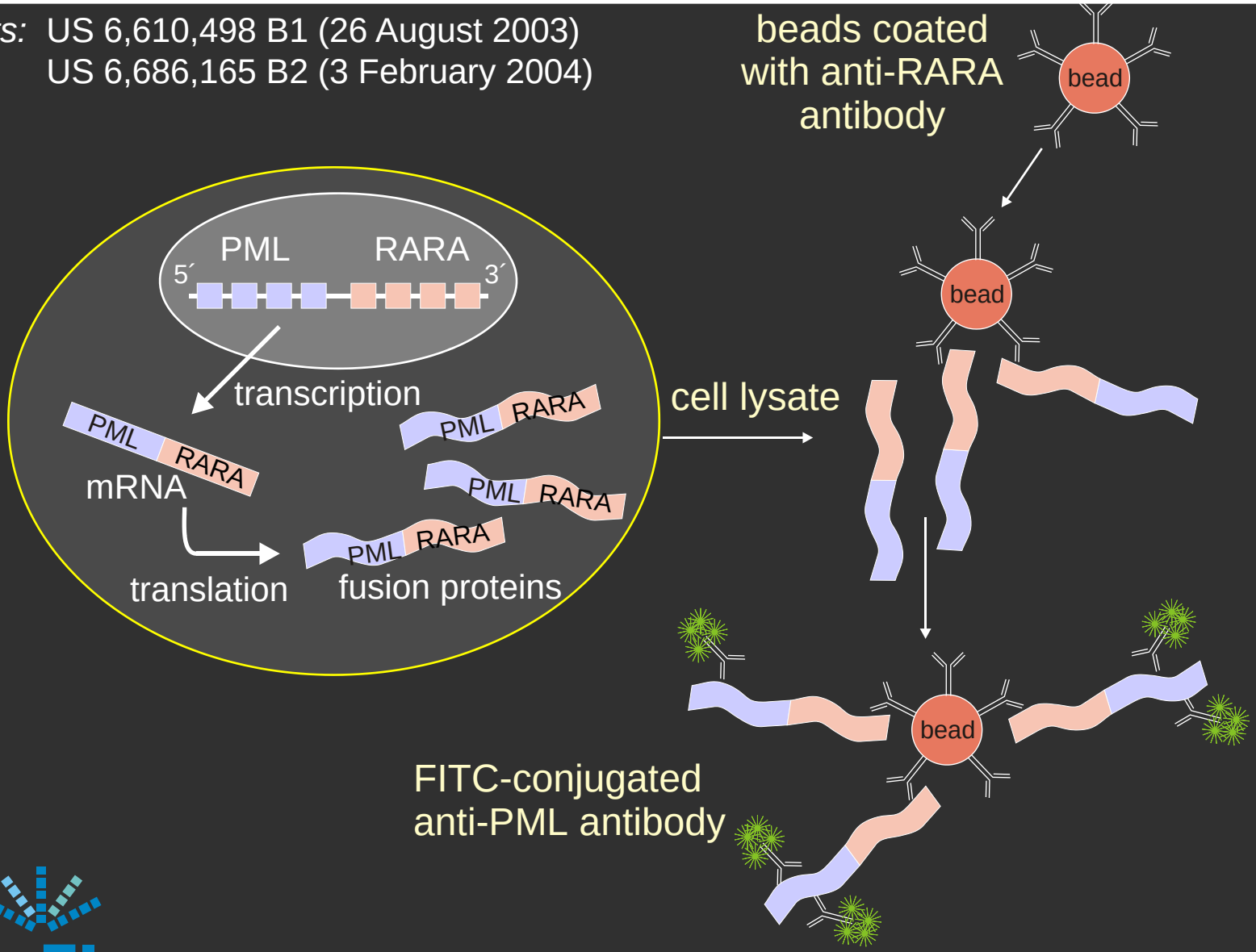
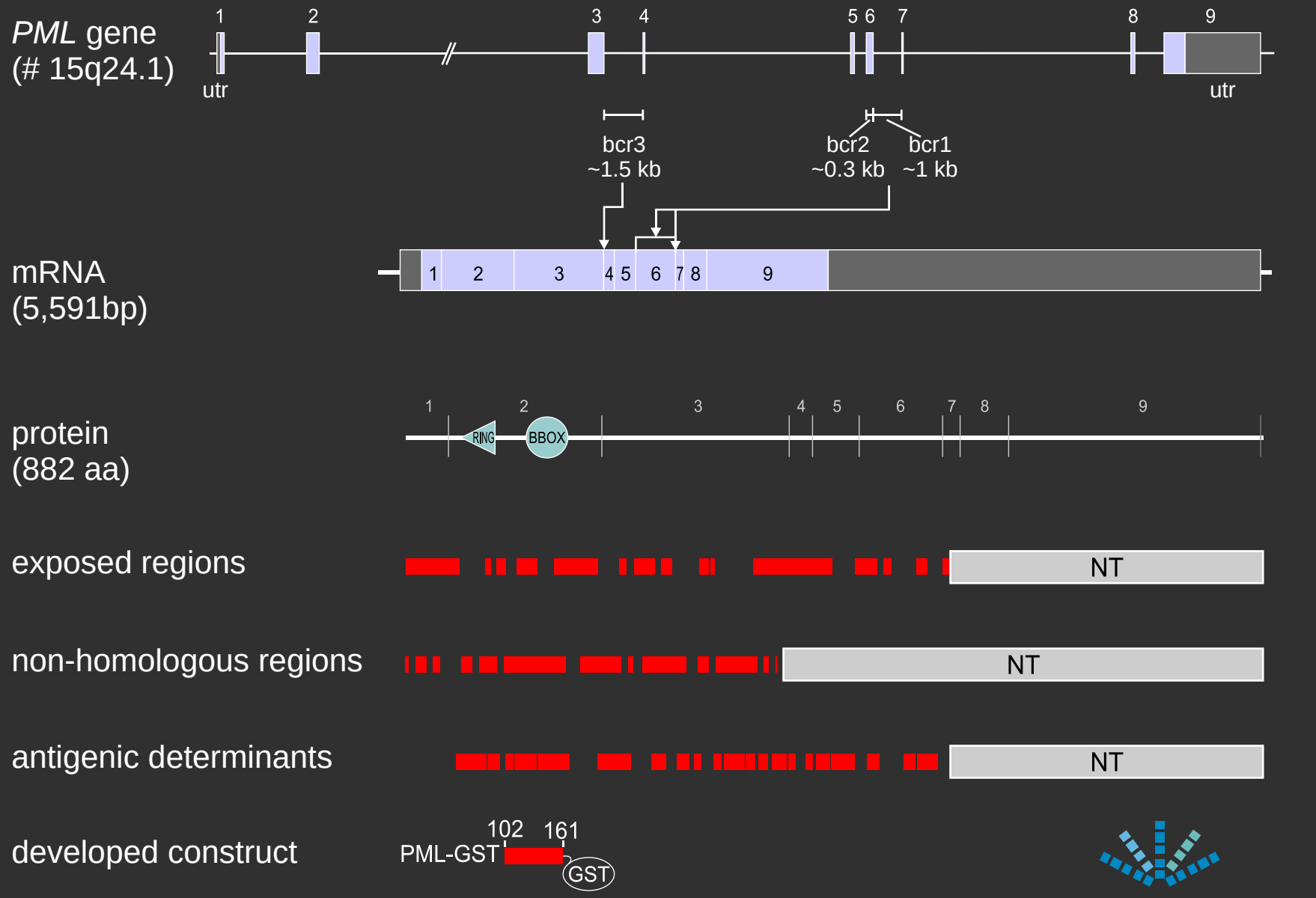


EuroFlow

Dept. of Immunology, Erasmus MC, Rotterdam

Design of anti-*PML* antibodies for fusion protein beads



Dekking et al, Leukemia, 2012

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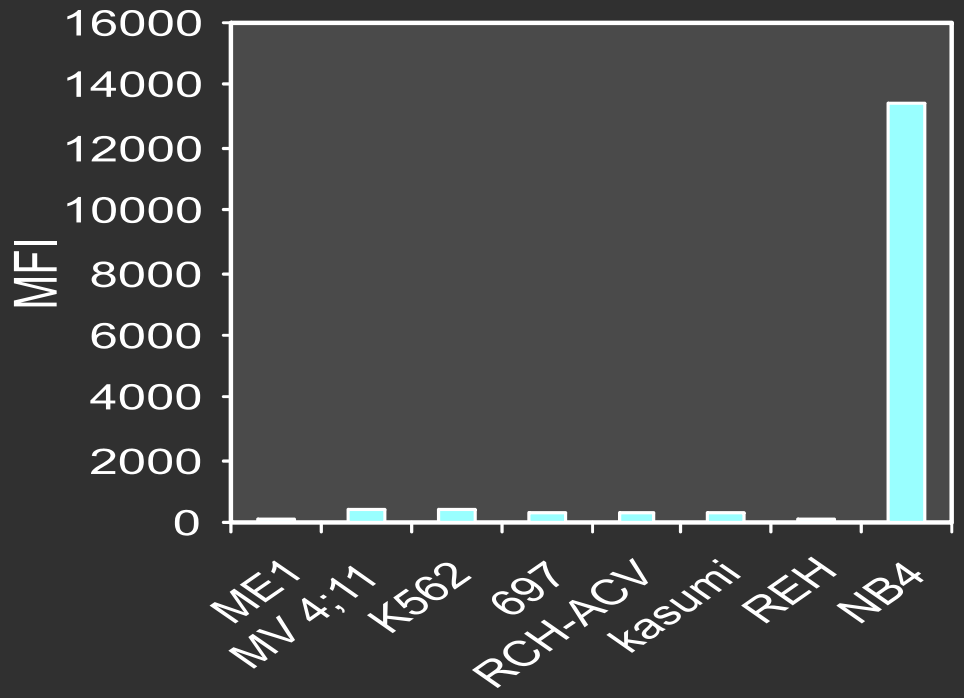
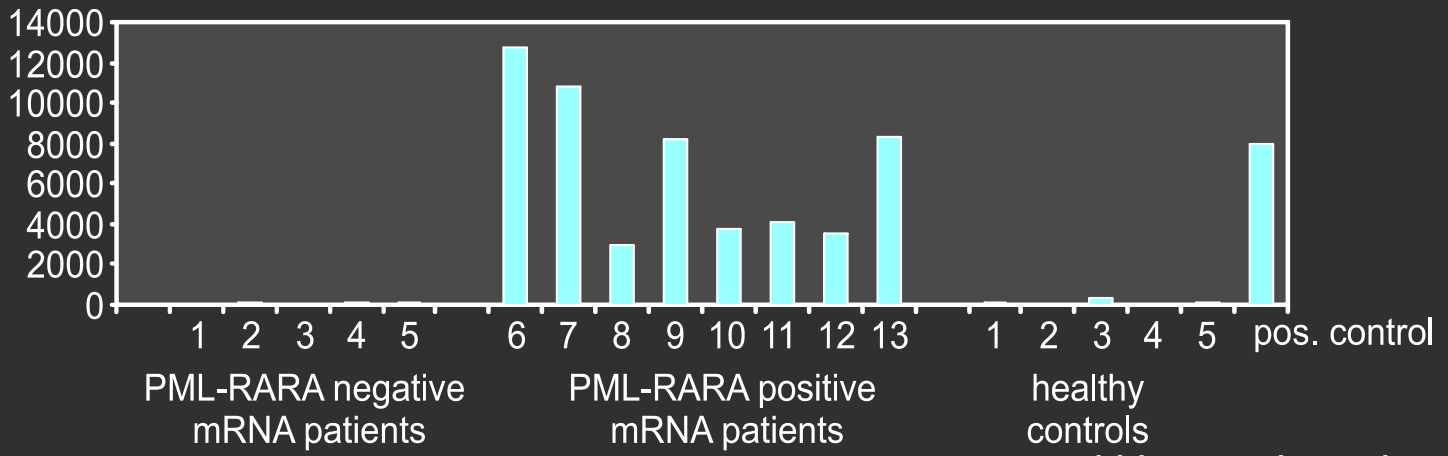
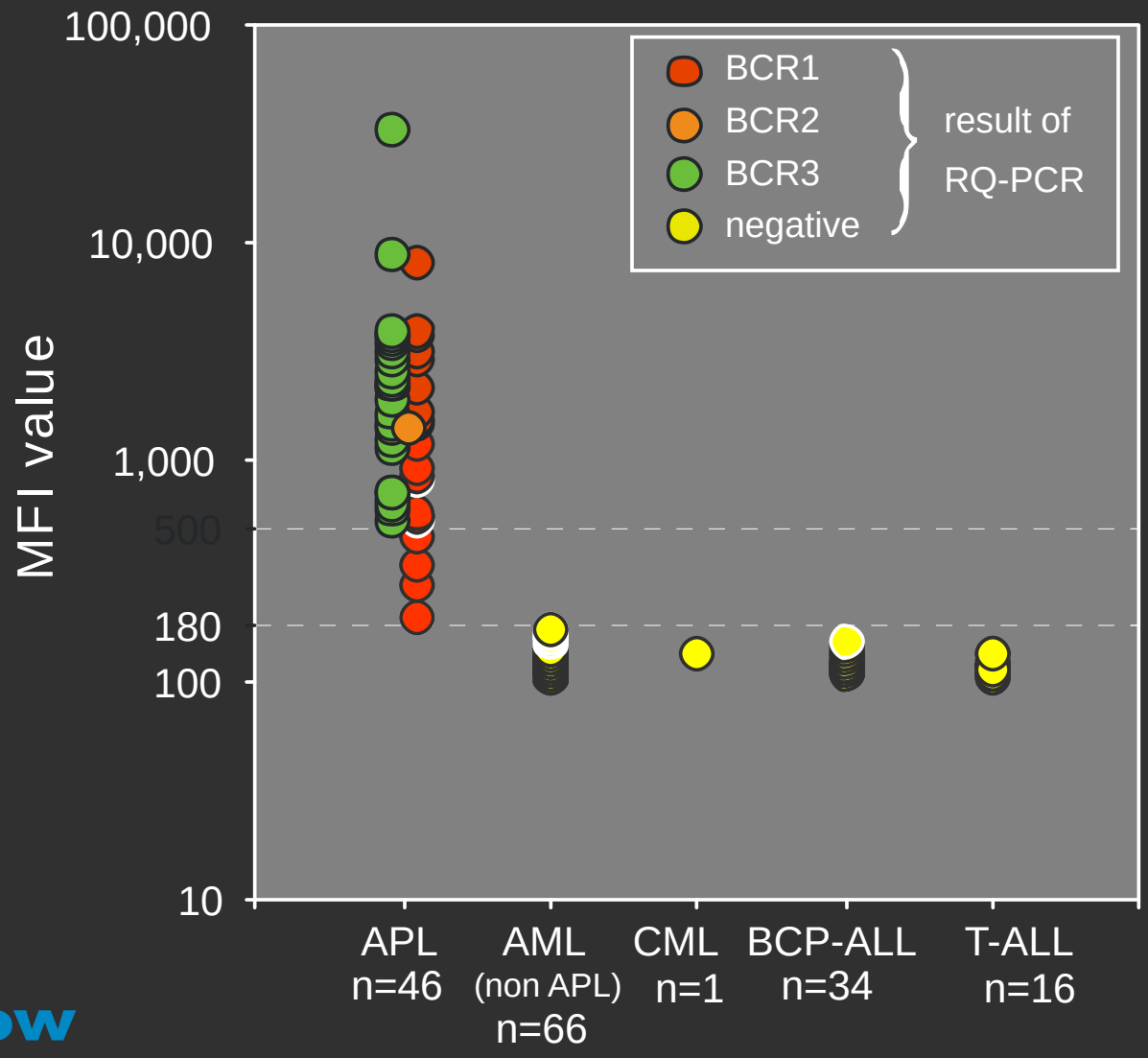
Flow cytometric PML-RARA immunobead assay





Dekking et al, Leukemia, 2012

Results of PML-RARA fusion protein detection using the immunobead assay



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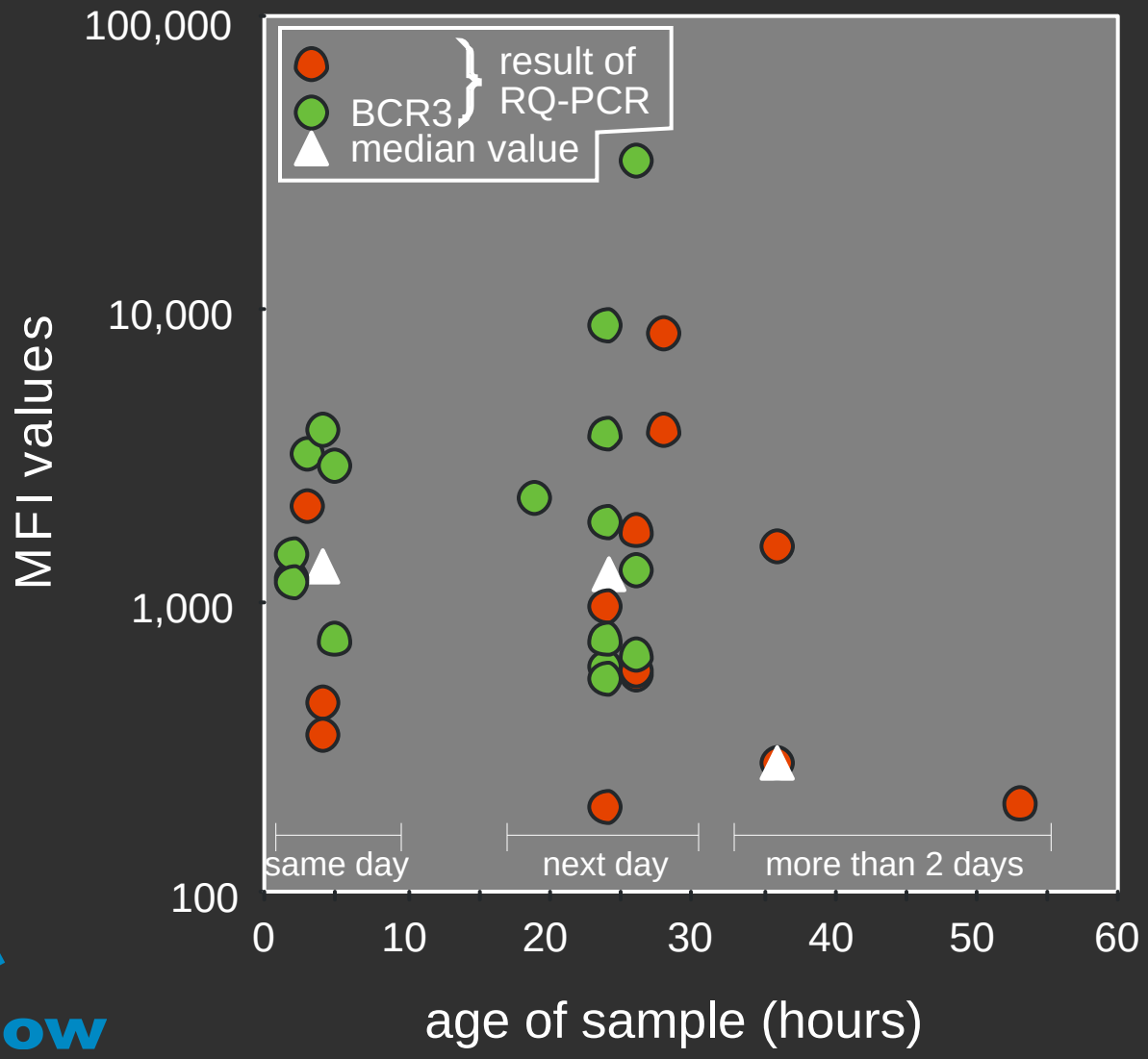
Dekking et al, Leukemia, 2012

Level of PML-RARA fusion protein expression in APL versus time lapse in sample processing



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Dekking et al, Leukemia, 2012

At this moment the technical developments for 7 well-defined fusion proteins have (virtually) been completed:

CML:	– BCR-ABL	: completed	RUO kit launched and published	
precursor-B-ALL:	– BCR-ABL	: completed	Precursor-B-ALL tube Multiplex tube close to completion	
	– TEL-AML	: completed		
	– E2A-PBX1	: completed		
	– MLL-AF4	: completed		
AML:	– AML1-ETO	: completed	Core-factor tube Multiplex tube: prototype completed	
	– CBFB-MYH11	: completed		
	– PML-RARA	: completed	prototype testing completed	

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Advantages of the immunobead system vs classical molecular techniques for fusion gene detection:

- Easy and reliable technique for fusion protein detection
- Independent of breakpoint position in the involved genes
- Multiplex possibilities by use of differential labeling of beads
- Fast technique: provides results within several hours
- No need for special laboratory facilities (only routine flow cytometer)
- Can be run in parallel with standard immunophenotyping (saves technician time!)
- The danger of protease activity requires integrity checking via an ubiquitous (protease sensitive) “household protein”

Conclusion: The immunobead technique can contribute to fast and easy diagnosis and classification of leukemias & other malignancies. If sufficient sensitivity is reached, MRD diagnostics becomes possible as well.

Examples of malignancies with chromosome aberrations that result in fusion proteins

Malignancy	Chromosome aberration	Fusion protein
Chronic myeloid leukemia	t(9;22)(q34;q11)	BCR-ABL
Lymphoma	t(2;5)(p23;q35)	NPM-ALK
Prostate cancer	t(21;21)(q22.3;q22.2)	TMPRSS2-ERG
Ewing sarcoma	t(11;22)(q24;q12)	EWSR1-FLI1
Papillary renal cell carcinoma	t(X;1)(p11;q23)	PRCC-TFE3
Follicular thyroid carcinoma	t(2;3)(q13;p25)	PAX8-PPARG
Fibromyxoid soft tissue sarcoma	t(7;16)(q33;p11)	FUS-CREB3L2
Endometrial stromal carcinoma	t(7;17)(p15;q11)	JAZF1-SUZ12
Soft tissue chondrosarcoma	t(9;22)(q31;q12)	EWSR1-NR4A3
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	EWSR1-WT1
Poorly differentiated carcinoma affecting midline structures	t(15;19)(q14;p13)	BRD4-NUT

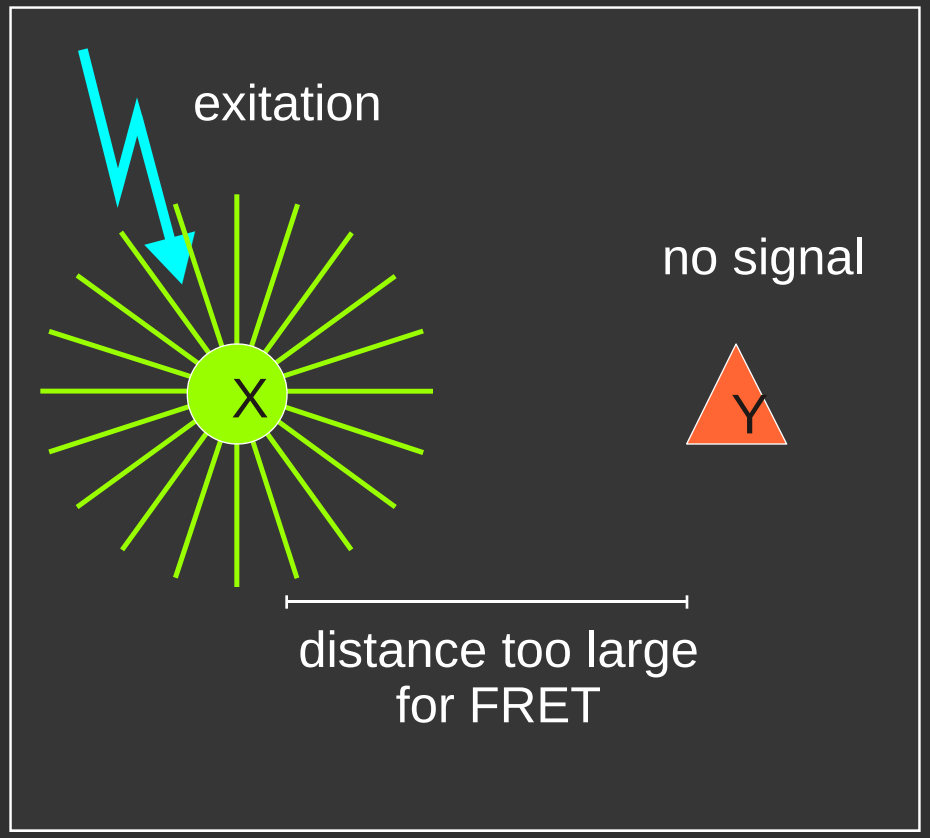
New developments in medicine induce extensive patient monitoring

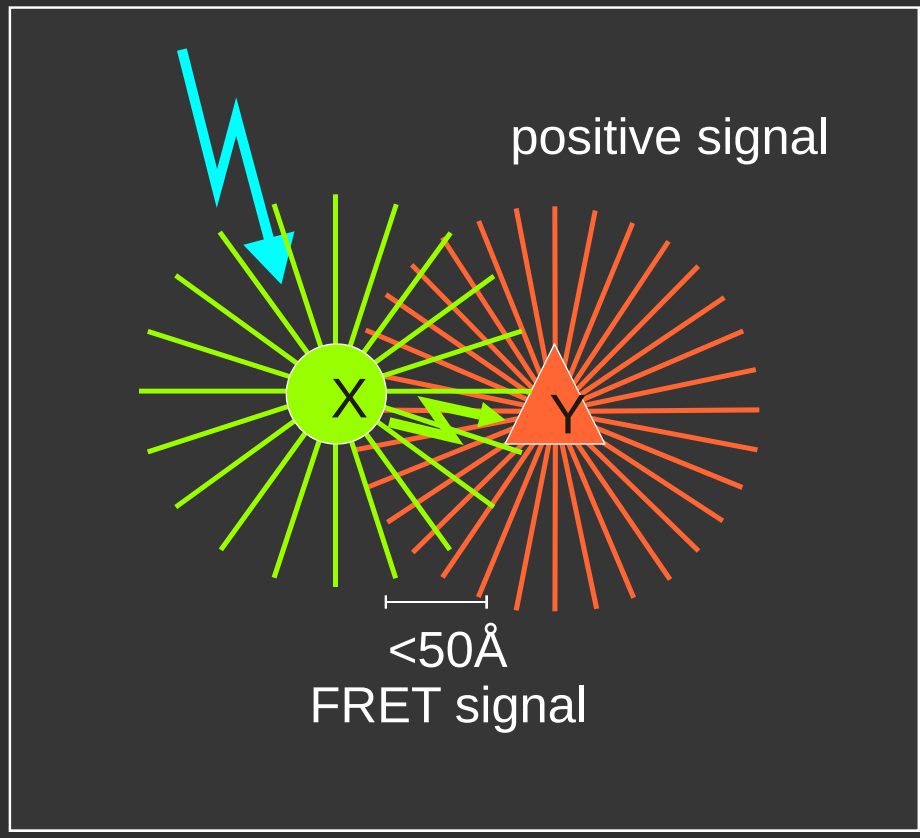
Changes in diagnostic strategies

- Many new therapeutics are being developed, particularly small molecules (e.g. TKI) and humanized antibodies.
- The vast majority of the new therapeutics block or manipulate specific targets. This leads to disease control with significant increase of QOF (not true cure).
- Prediction of therapy resistance will become increasingly important .

CONSEQUENCE: Monitoring, monitoring, monitoring!

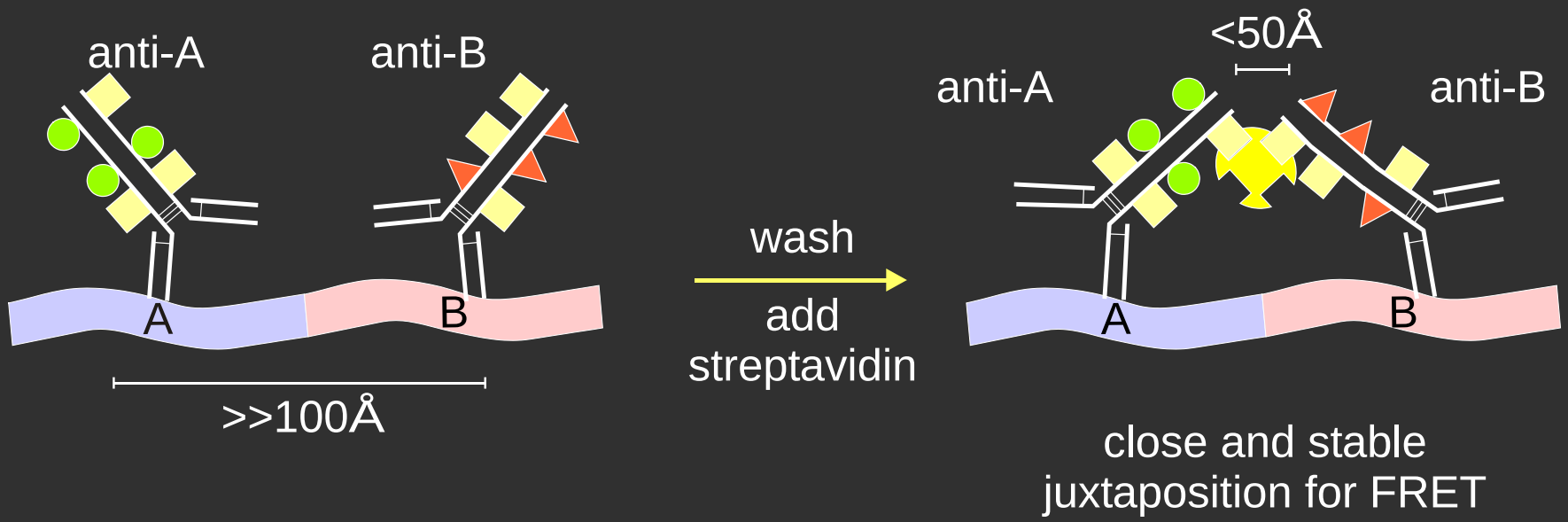
Fluorescence resonance energy transfer (FRET)





Van Dongen JJM, van der Velden VHJ, Orfao A, European Patent, 2007

FRET-mediated detection of A-B fusion proteins



= FRET fluorochrome X

= FRET fluorochrome Y

= biotin

= streptavidin

Van Dongen JJM, van der Velden VHJ, Orfao A, European Patent, 2007

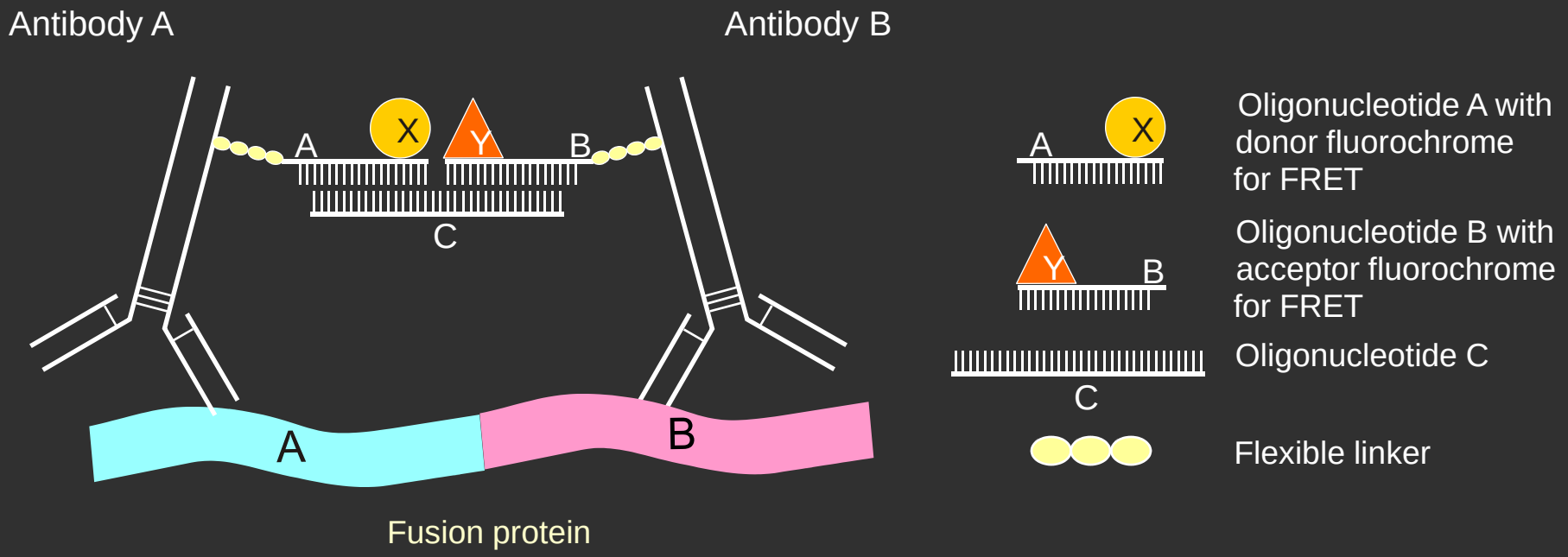
Flow cytometric detection of intracellular fusion proteins

If successful:

- Intracellular staining of fusion proteins allow analysis at the single cell level;
- Quantitative detection of fusion proteins at the single cell level;
- Easy and fast detection of fusion proteins in routine multicolor flow cytometry: Improved possibilities for sensitive MRD diagnostics (<10e4);
- Possibilities for early detection of mutation-induced therapy resistance, e.g. TKI resistance in CML
- Possibilities for studying minor (pre)leukemic cell populations.

FRET mediated detection of intracellular fusion proteins
Usage of PNA oligonucleotides for close and stable linkage

FRET fluorochromes linked to PNA molecules: oligonucleotide C links oligonucleotides A and B so that the FRET fluorochromes act as a tandem dye



Van Dongen JJM, van der Velden VHJ, Orfao A, European Patent, 2007

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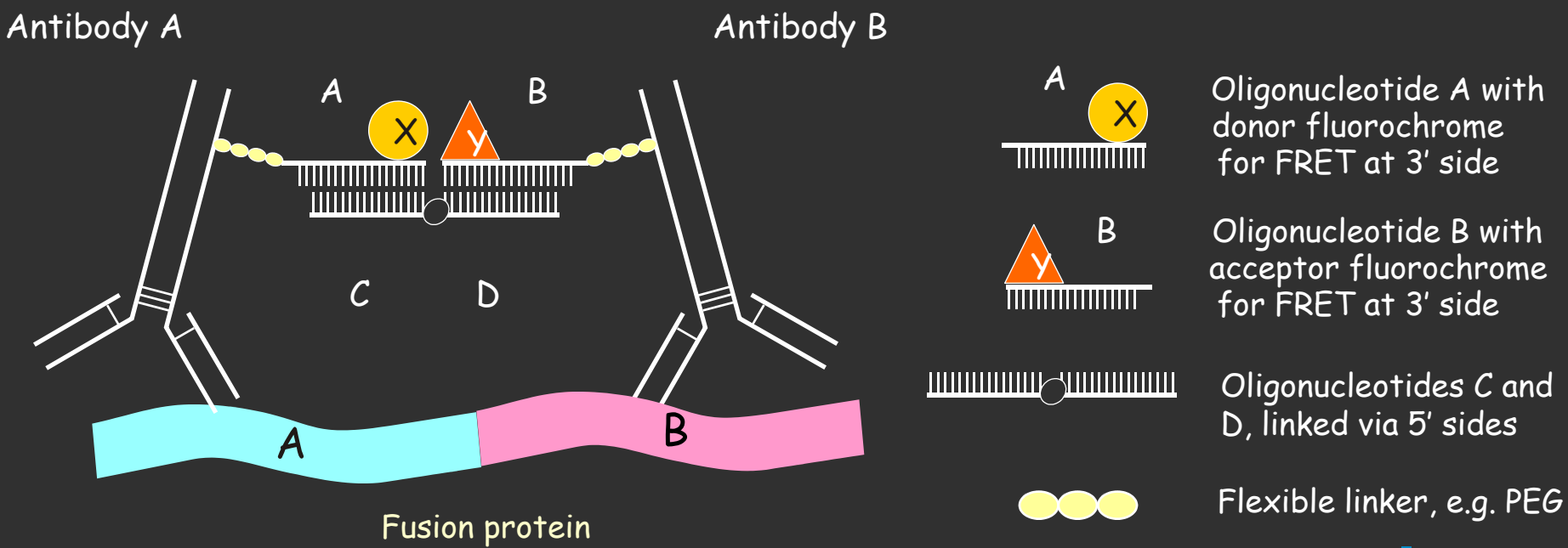
Detection of intracellular fusion proteins by FRET: PNA oligonucleotides for close & stable linkage

FRET fluorochromes linked to PNA molecules: oligonucleotides C and D are coupled via their 5' sides and link oligonucleotides A and B so that the two FRET fluorochromes act as a tandem dye



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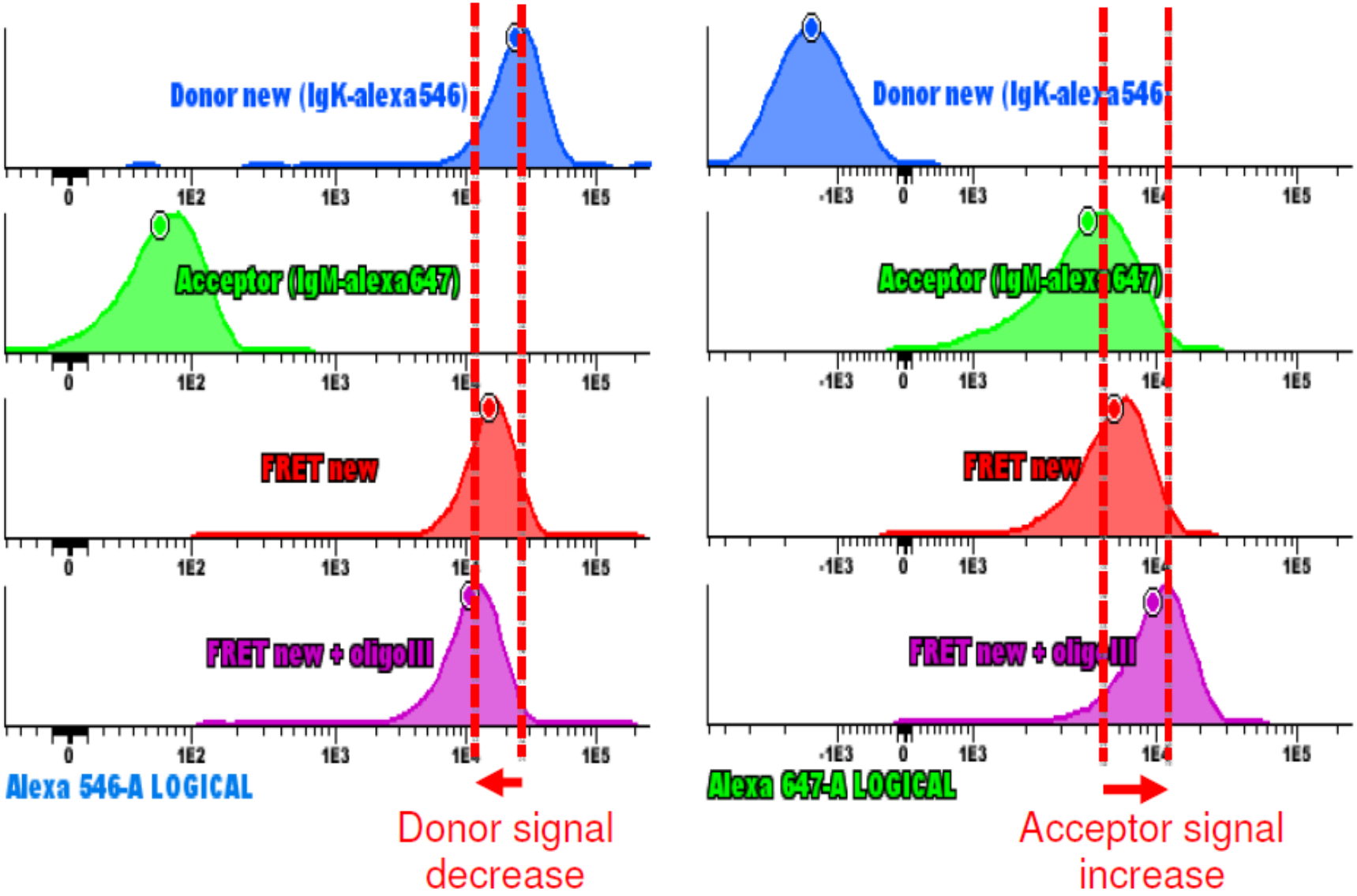
FRET DETECTION OF IgM/K PROTEIN COMPLEXES ON THE B-CELL SURFACE MEMBRANE USING THE PNA SYSTEM



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EuroFlow consortium aims at innovation in flow cytometry

THANK YOU

THANKS TO

The USAL Team