Detección de proteínas de fusión de translocaciones cromosómicas mediante técnica de immuno-beads y citometría de flujo. Estudio de casos

Departamento de Medicina, Centro de Investigación del Cáncer y Servicio de Citometría.
Universidad de Salamanca, Salamanca, España.
EuroFlow™ Consortium

Curso avanzado de Actualización en Onco Hematología. Buenos Aires, Mayo, 30 a Junio, 1, 2011

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**

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

   - relation with **prognosis**
   - relevance of **risk-group definition** in treatment protocols

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)

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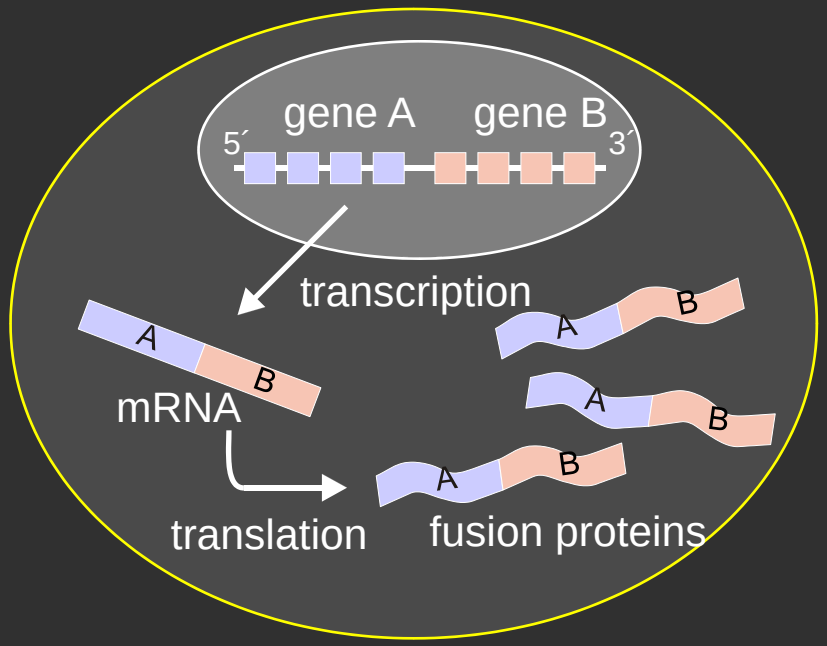
Fusion genes and fusion proteins



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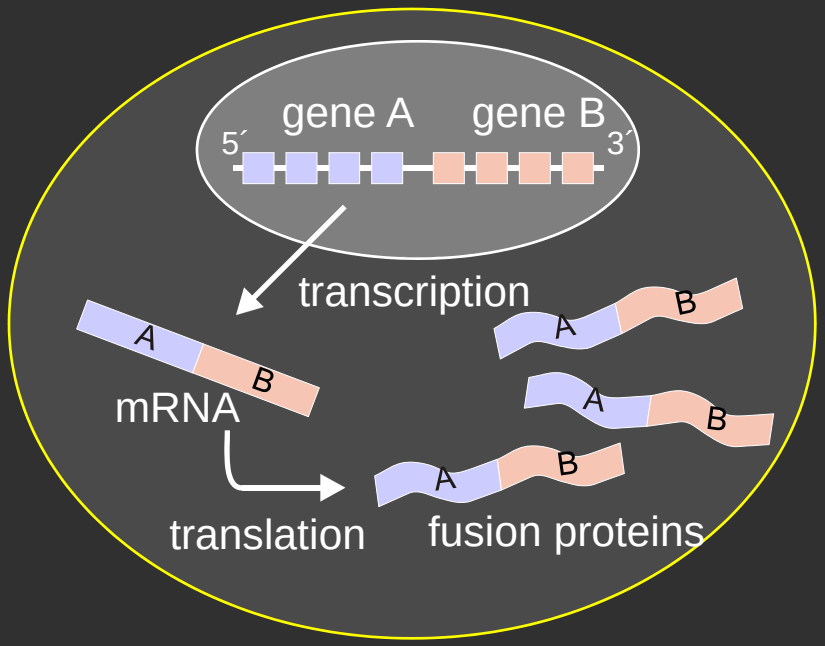

Occurrence of fusion proteins

1. Hematological malignancies
   - acute leukemias: 35-40%
   - CML: >98%
2. Solid tumors

   overall: ~20% of all patients

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CHROMOSOME ABERRATIONS & FUSION GENES IN AL

Chromosome aberration	Fusion genes	Relative frequency per type of acute leukemia				
		Precursor-B-ALL		AML		
		children	adults	children	adults <60 y	adults >60 y
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.

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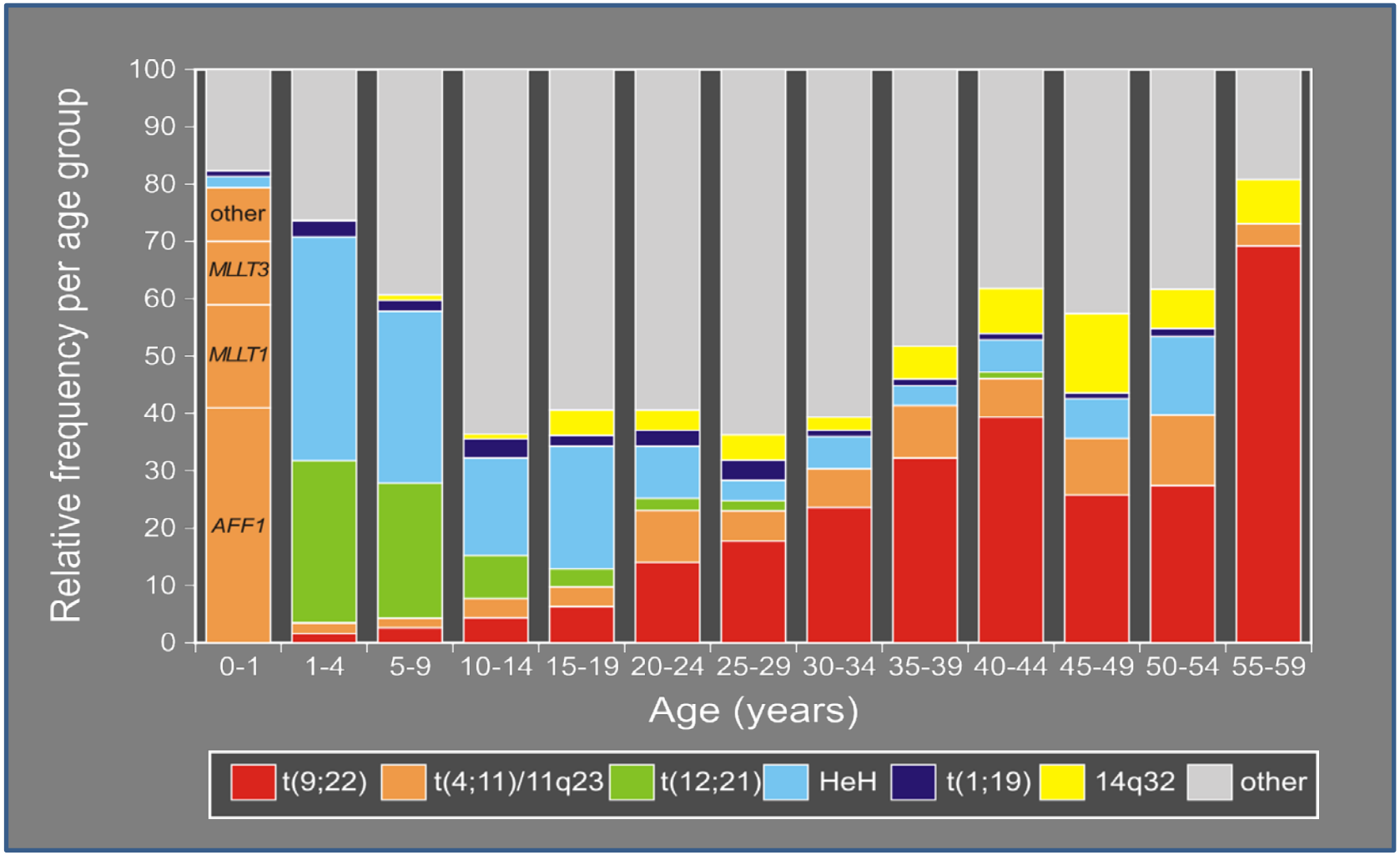
RELATIVE FREQUENCY OF GENETICS ABNORMALITIES IN ALL BY AGE GROUPS



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

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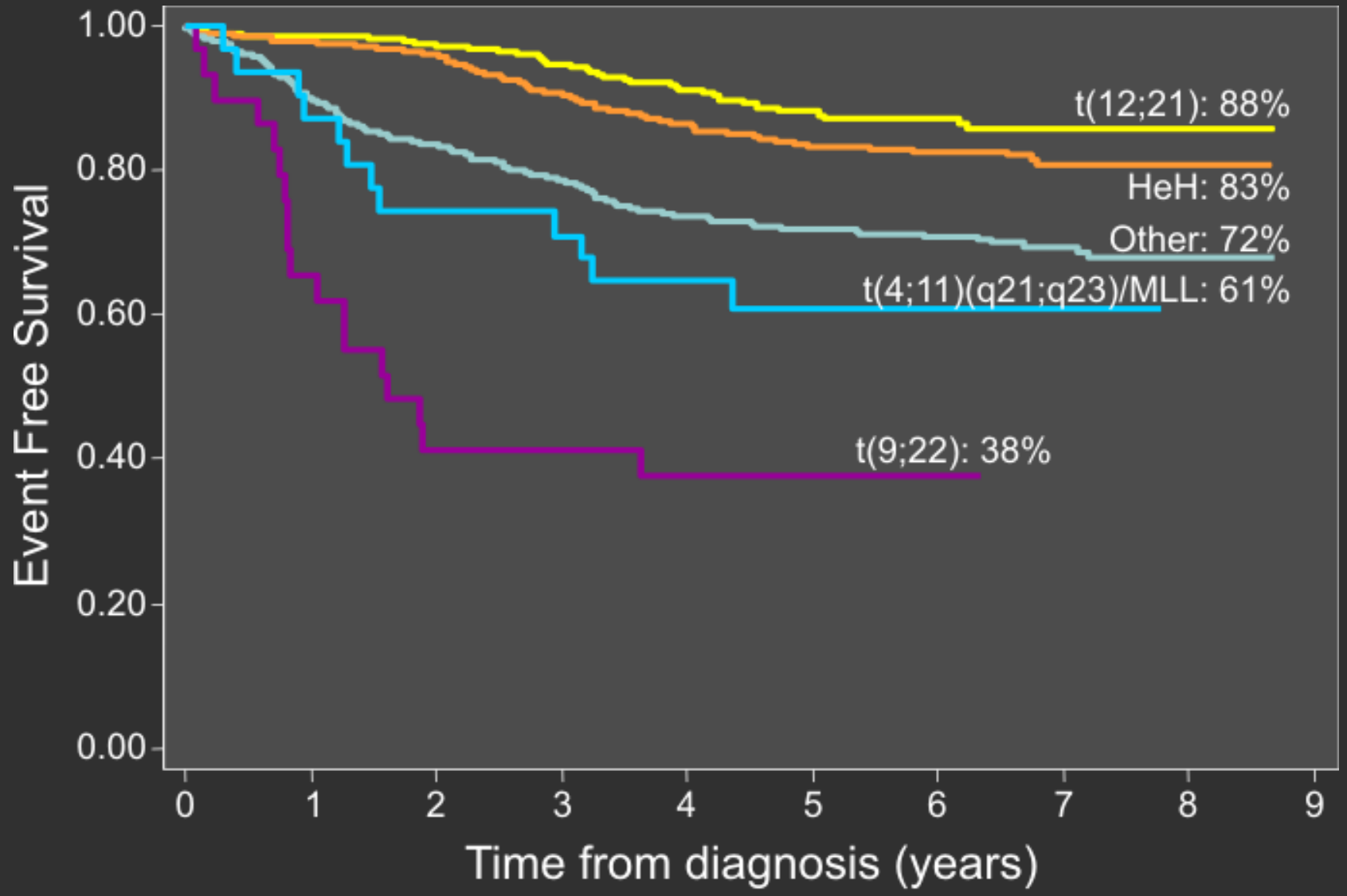
Event-free survival in childhood ALL according to chromosome aberrations



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

CHROMOSOME ABERRATIONS & FUSION GENES IN MPN

Chronic myeloproliferative neoplasms		
Chromosome Aberration		**Disease category**
t(9;22)(q34;q11)	(BCR-ABL1)	Chronic myeloid leukemia, BCR-ABL1+
		Chronic neutrophilic leukaemia (CNL)
Mutated *JAK2* (V617F or exon 12)		Policitemia vera (PV)
Mutated *JAK2* (V617F) W515L/K		Primary myelofibrosis (PMF)
Mutated *JAK2* (V617F) W515L/K		Essential thrombocythaemia (ET)
alt 4q12 t(5;12)(q31-33;p13) t(8;13)(p11;q12)	(PDGFRA / PDGFRB / FGFR1)	Chronic eosinophilic leukaemia, not otherwise specified (CEL, NOS)
c-Kit (D816V)		Mastocytosis
		Myeloproliferative neoplasm, unclassifiable

CHROMOSOME ABERRATIONS & FUSION GENES IN MPN

Myelodisplastic / Myeloproliferative neoplasms

Disease categoy
Chronic myelomonocytic leukemia
atypic Chronic myeloid leukemia, BCR-ABL1 -
Juvenile myelomonocytic leukemia
Myelodisplastic / Myeloproliferative neoplasms, unclasifiable

Absence of BCR-ABL1

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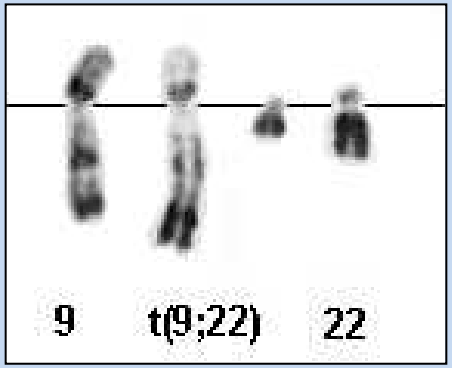
AL: GENOTYPIC-PHENOTYPIC ASSOCIATIONS

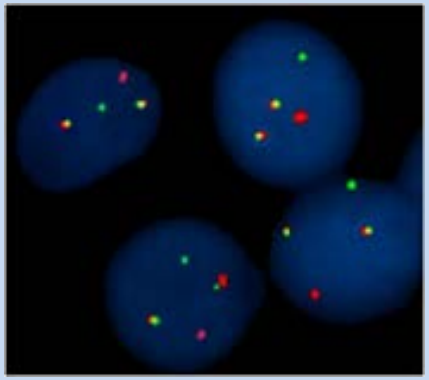
Diagnosis	Genetic lesion	Aberrant immunophenotype
BCP-ALL	t(9;22)	$CD34^{hi}$,$CD10^{+}$,$CD38^{lo}$,$CD13^{lo}$
	t(12;21)	$CD34^{het}$,$CD10^{+}$,$CD20^{-}$,$CD13^{lo}$
	11q23	$CD34^{+}$,$CD10^{-}$,7.1^{+},$CD15^{+}$
AML	t(15;17)	$CD34^{-/+}$,$CD15^{-/lo}$,$CD2^{-/lo}$,$CD13^{het}$
	Inv(16)	MPO^{hi},$CD2^{-/lo}$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

Ortuño F, Orfao A, Cytometry B, 2004

CHROMOSOME ABERRATIONS. Detection techniques



Cytogenetics



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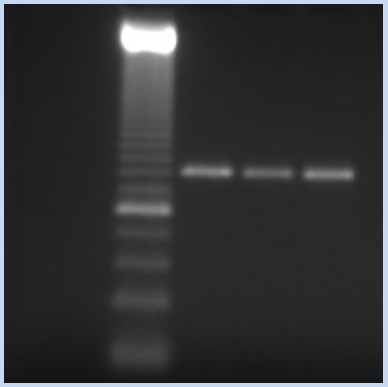
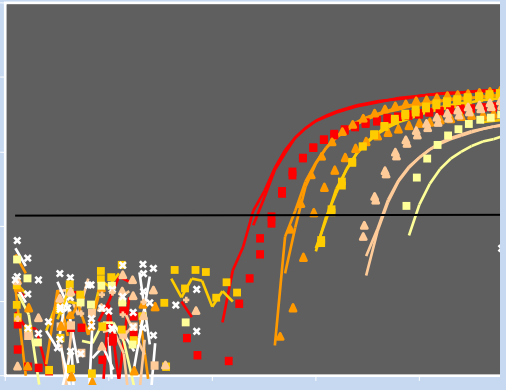


PCR

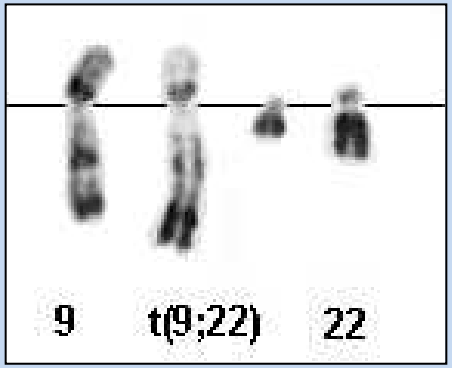


RQ-PCR

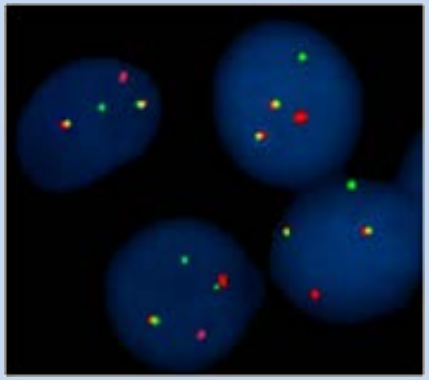
Advantages of molecular techniques:

- 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 !

CHROMOSOME ABERRATIONS. Detection techniques



Cytogenetics



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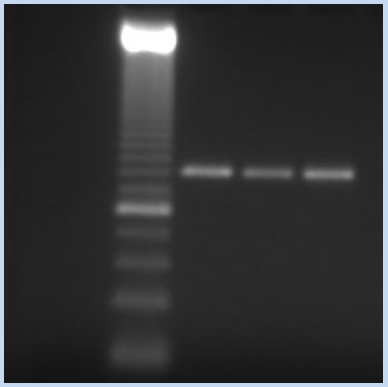
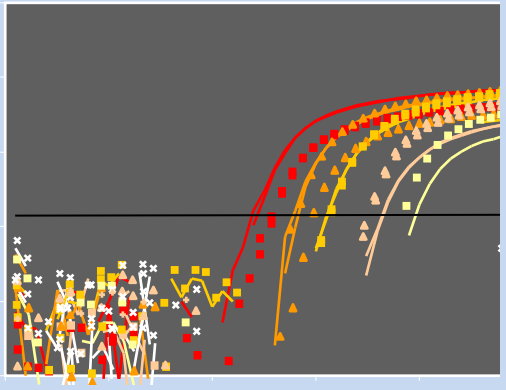


PCR



RQ-PCR

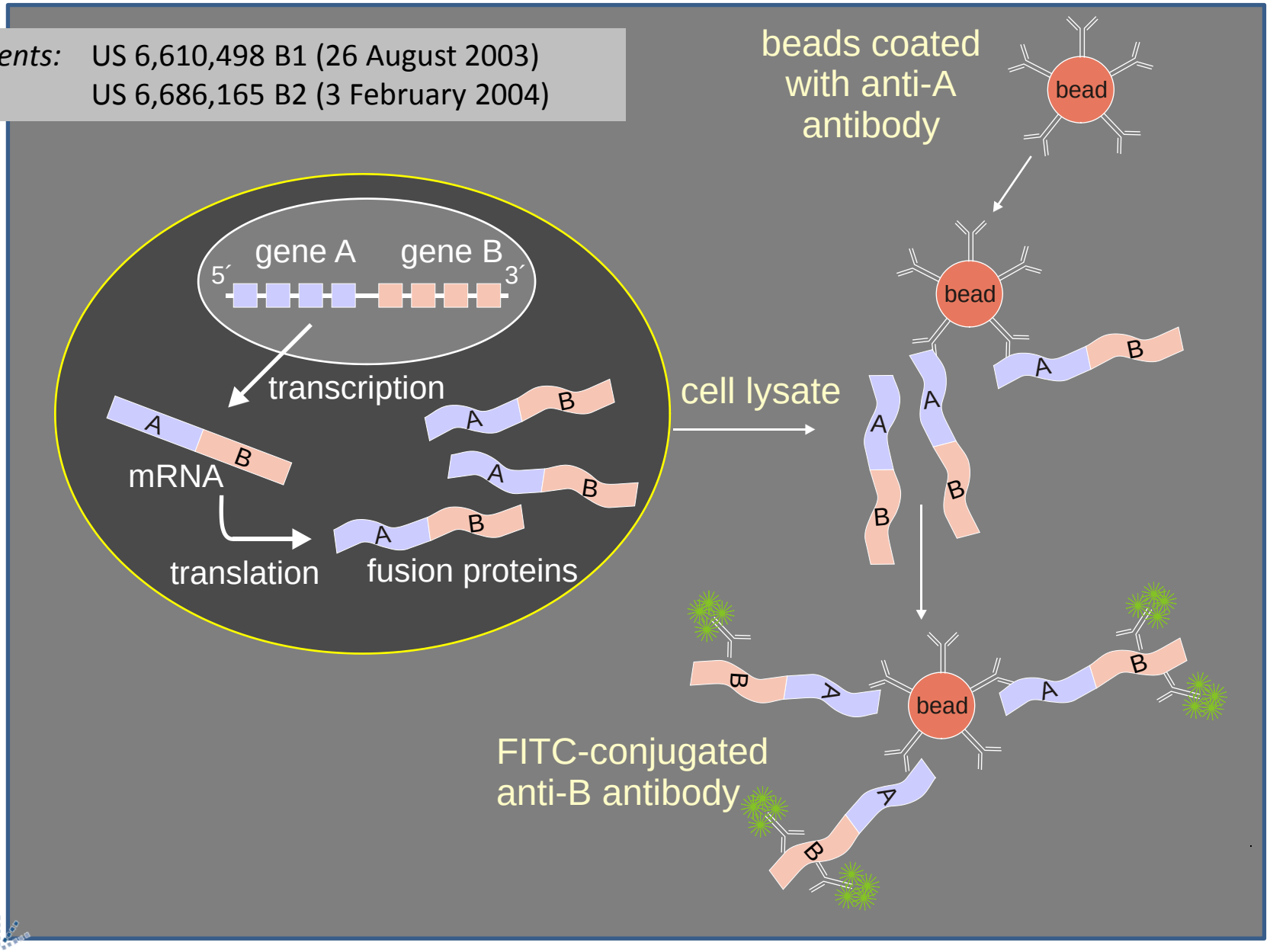
Disadvantages of molecular techniques:

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

→ Faster technique needed: at protein level?

FCM DETECTION OF FUSION PROTEINS

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



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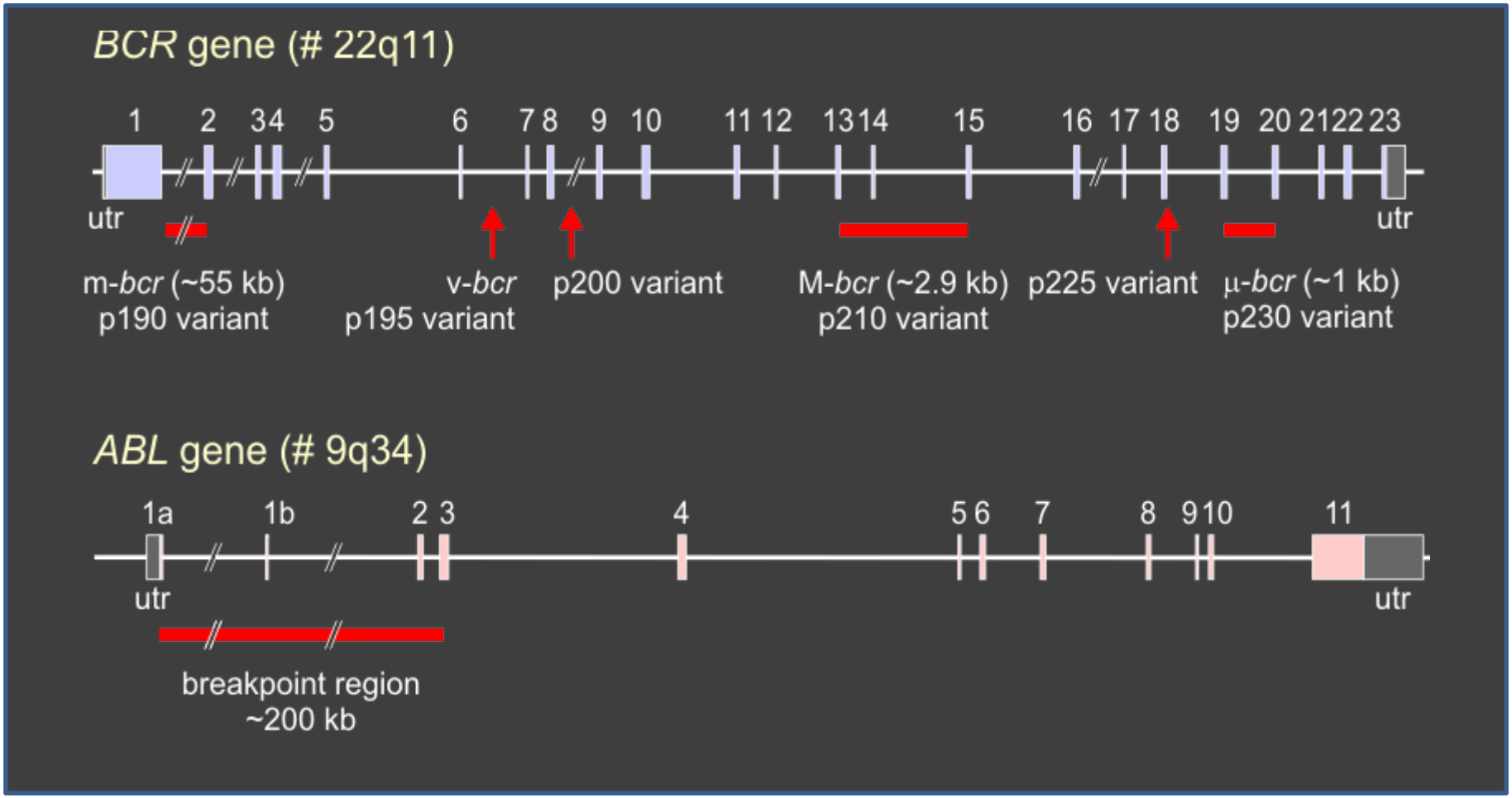
BCR-ABL FUSION PROTEIN

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



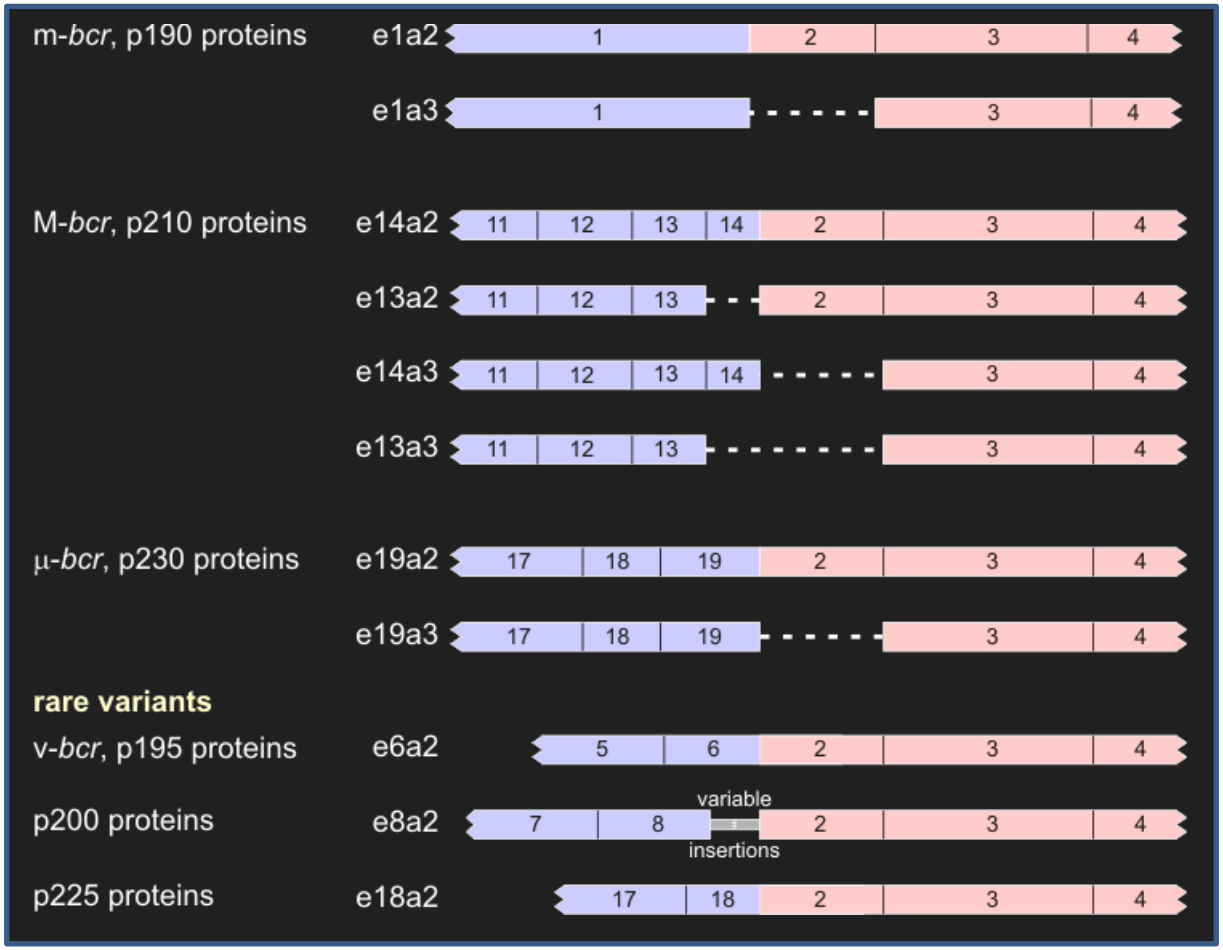
F. Weerkamp, et al. Leukemia 2009; 23: 1106-1117.

EuroFlow

BCR-ABL FUSION PROTEIN

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

Multiple variant *BCR-ABL* transcripts



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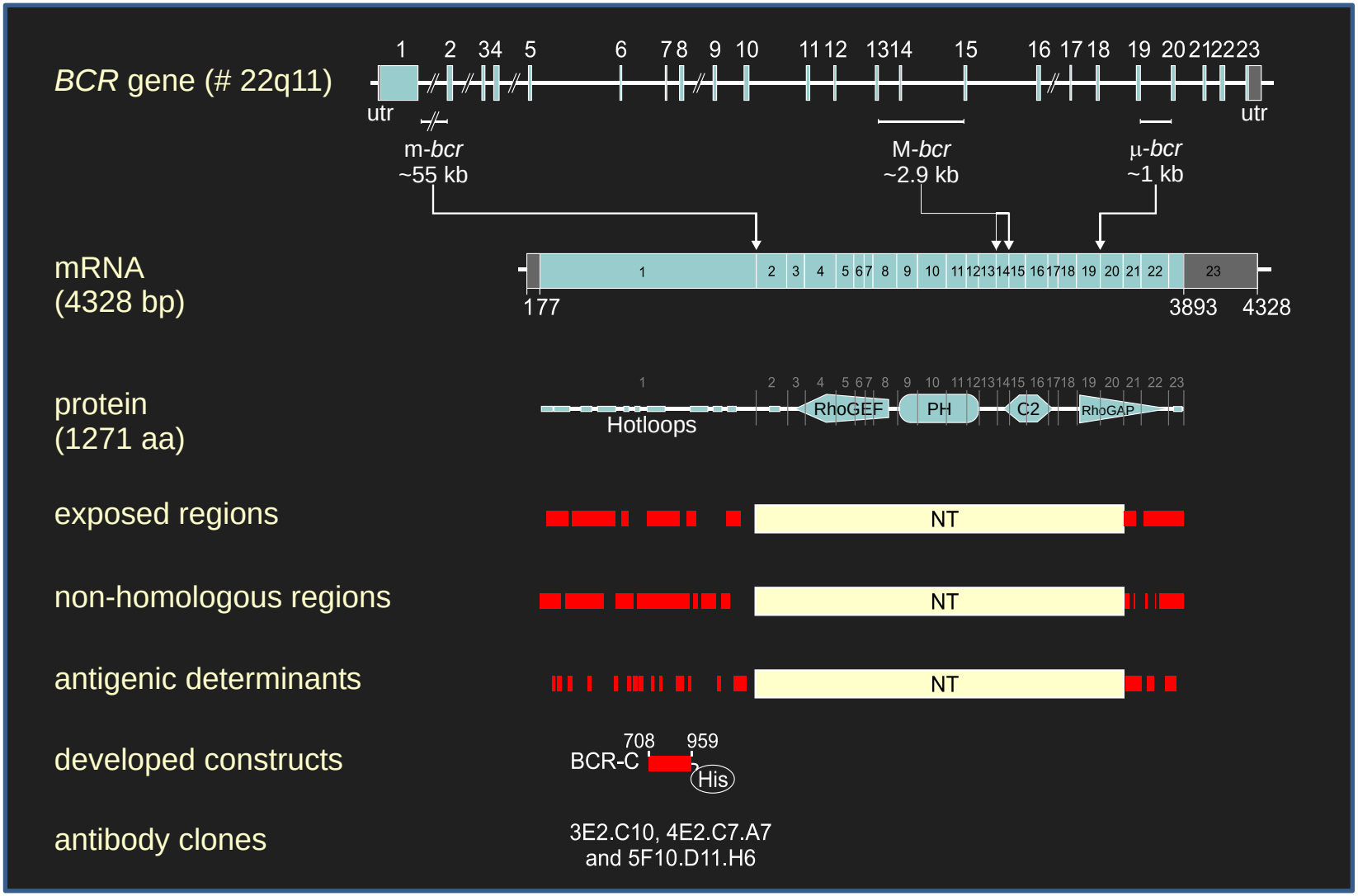
DESIGN OF ANTI-BCR ANTIBODIES FOR BEADS SYSTEM



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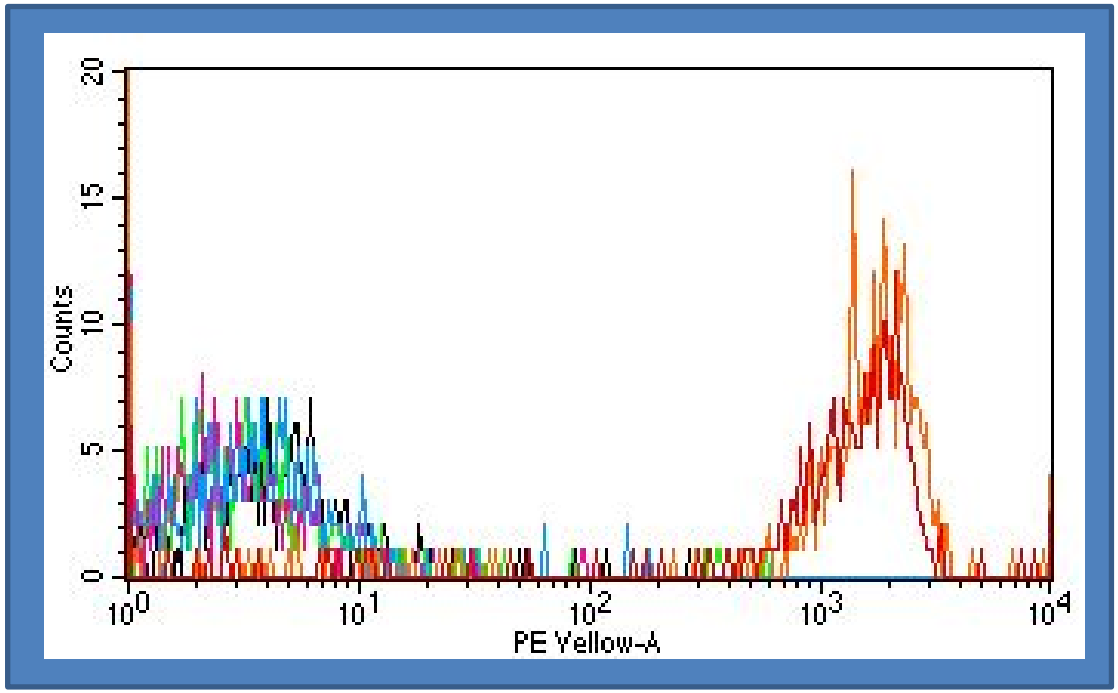

Black: PBMC (neg. control)
Green: 697 (E2-PBX1; neg. control)
Purple: REH (TEL-AML1; neg. control)
Blue: RS4,11 (MLL-AF4; neg. control)
Orange: K562 (BCR-ABL p210)
Red: Lama-84 (BCR-ABL p210)

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

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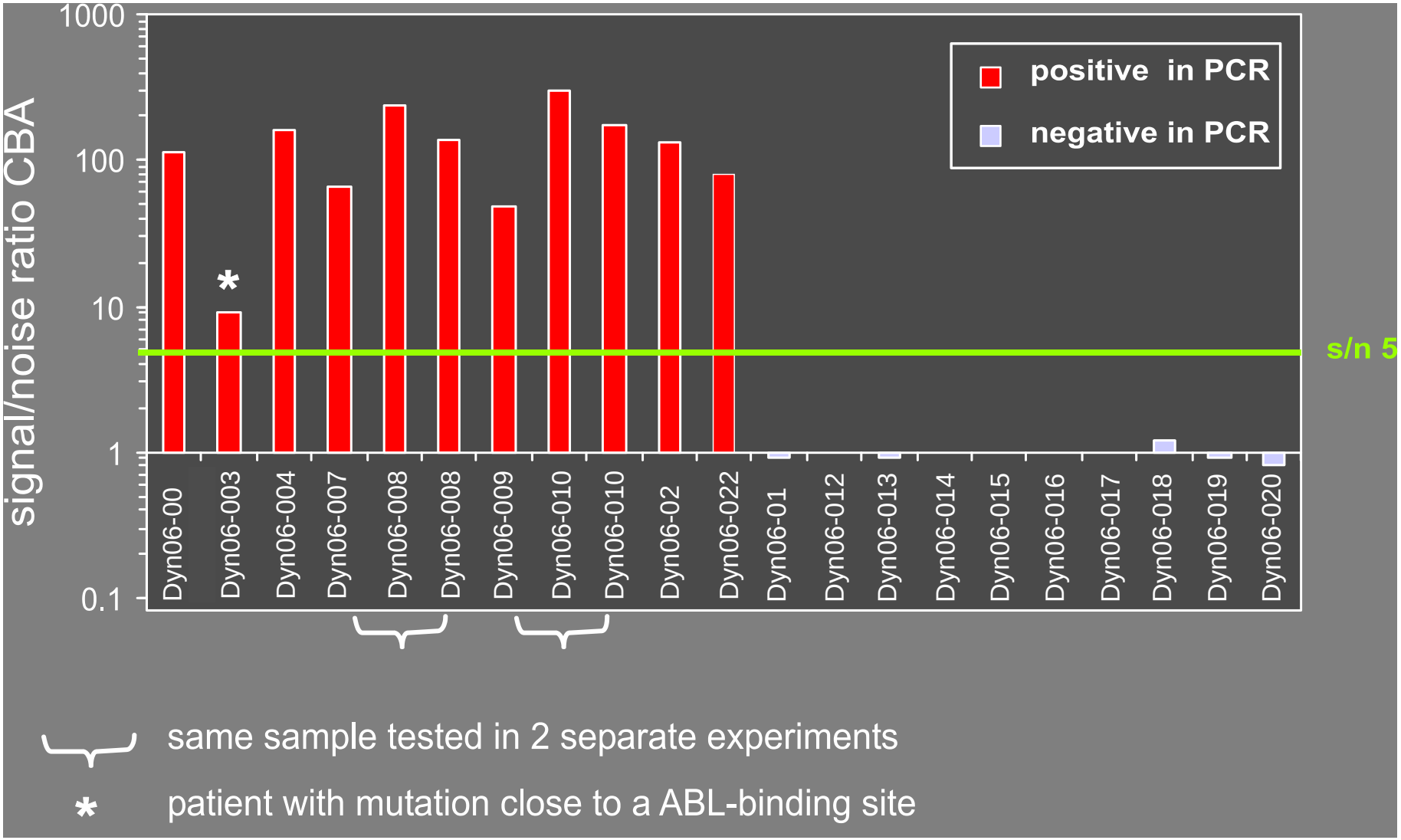
CBA FOR BCR-ABL DETECTION IN BCP-ALL SAMPLES



Only frozen samples tested

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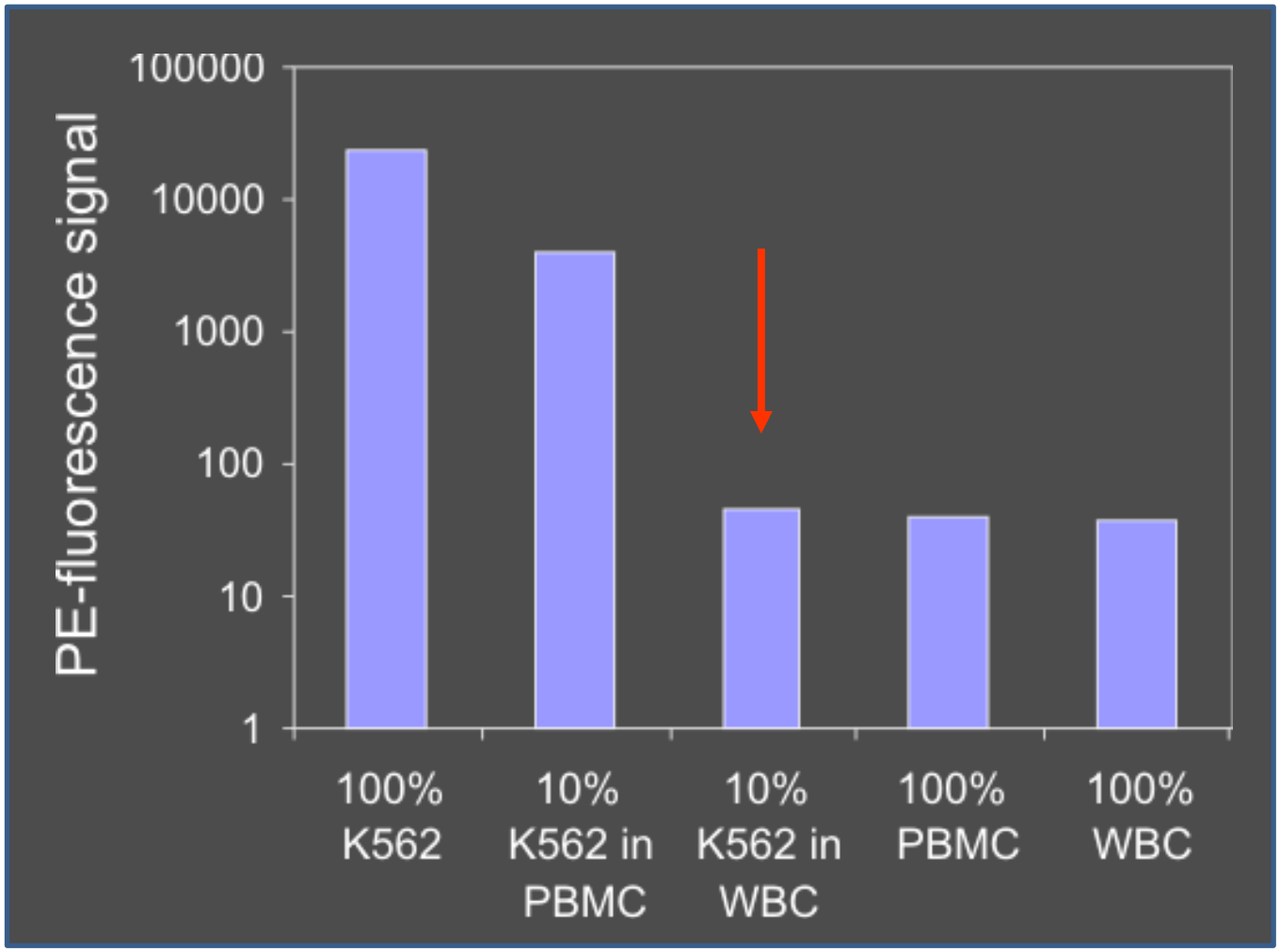
CBA FOR BCR-ABL DETECTION IN WBC



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

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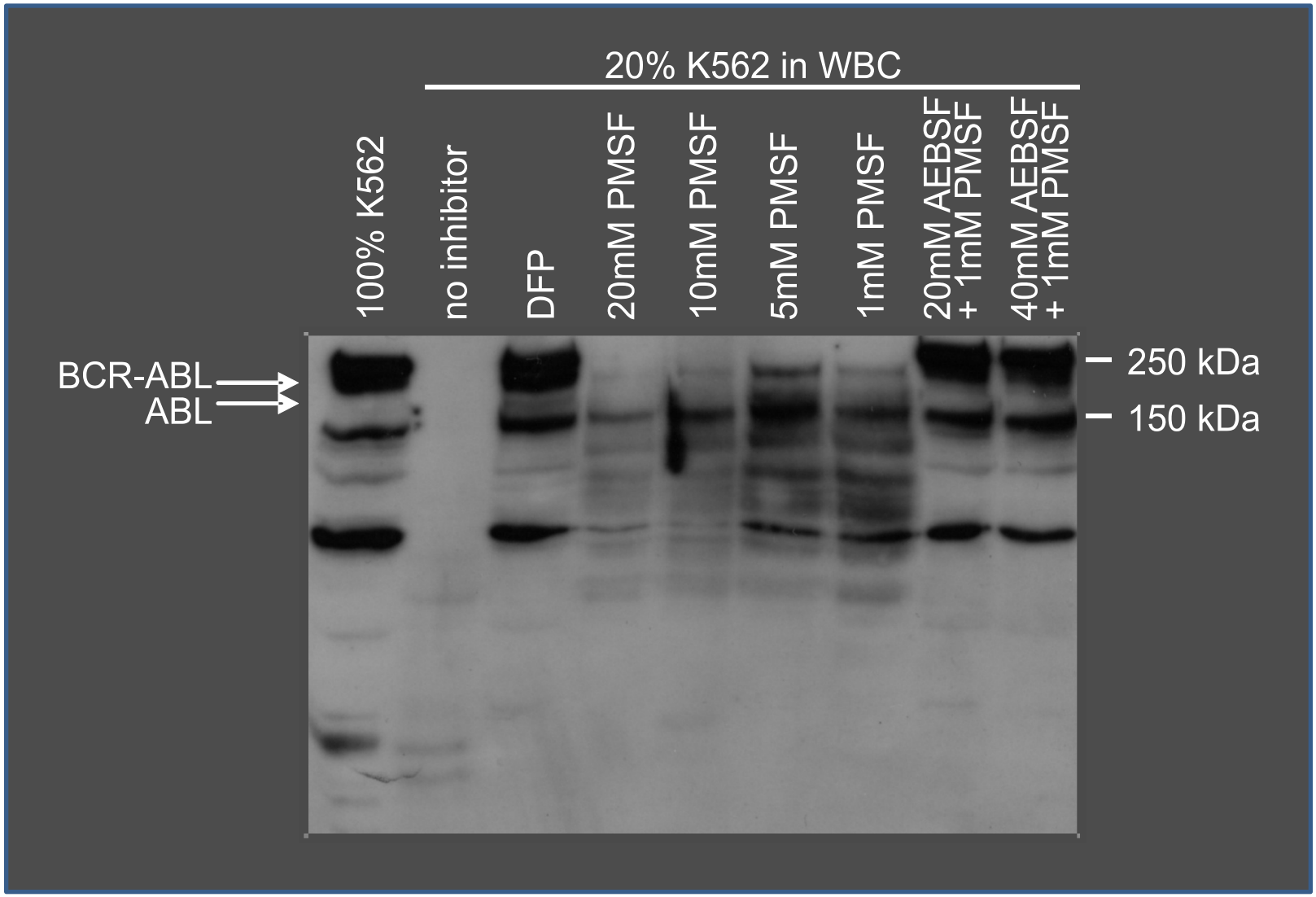
PROTEASE ACTIVITY / BCR-ABL DEGRADATION



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

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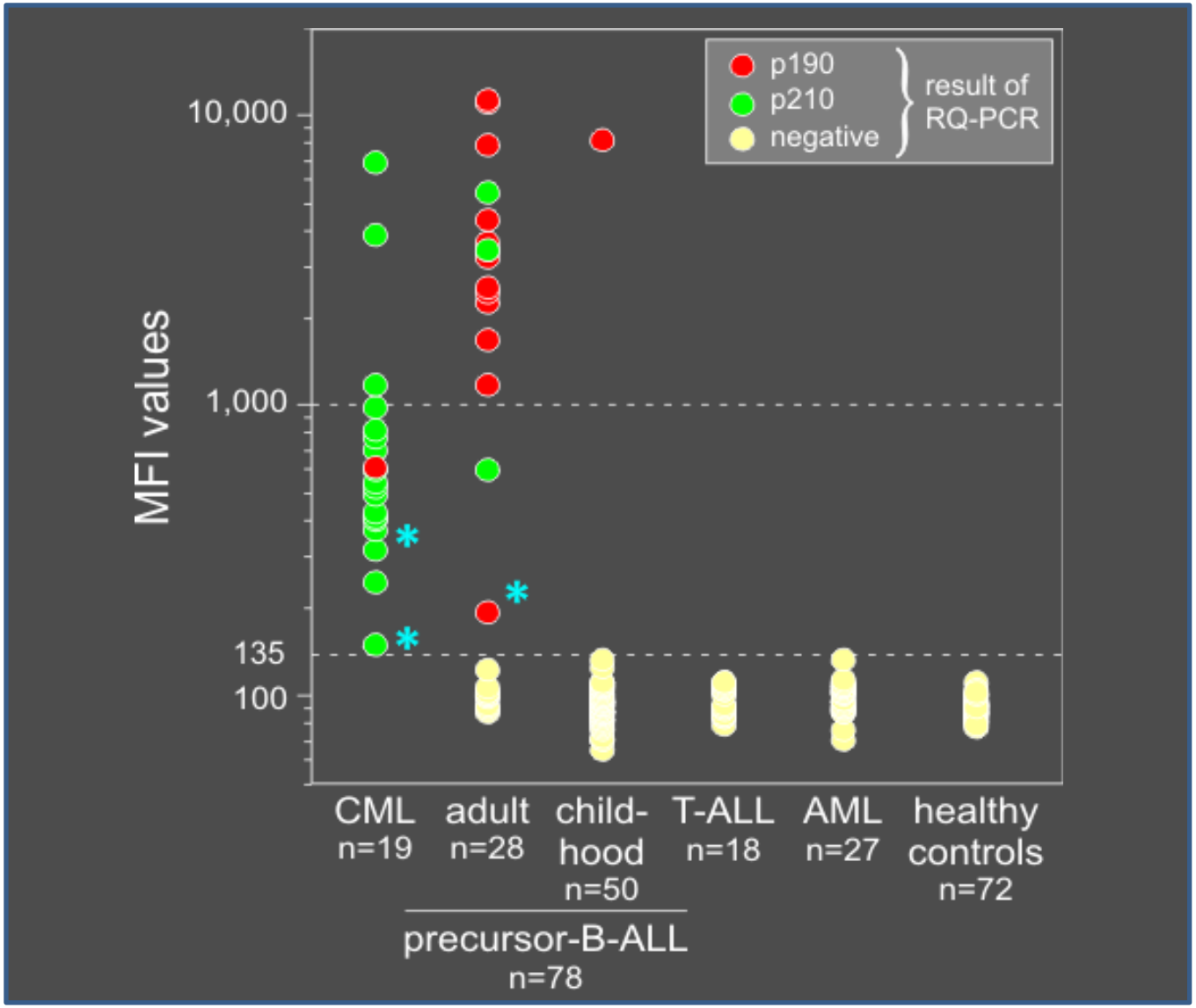
BCR-ABL RUO TESTING BY EUROFLOW™ LABORATORIES



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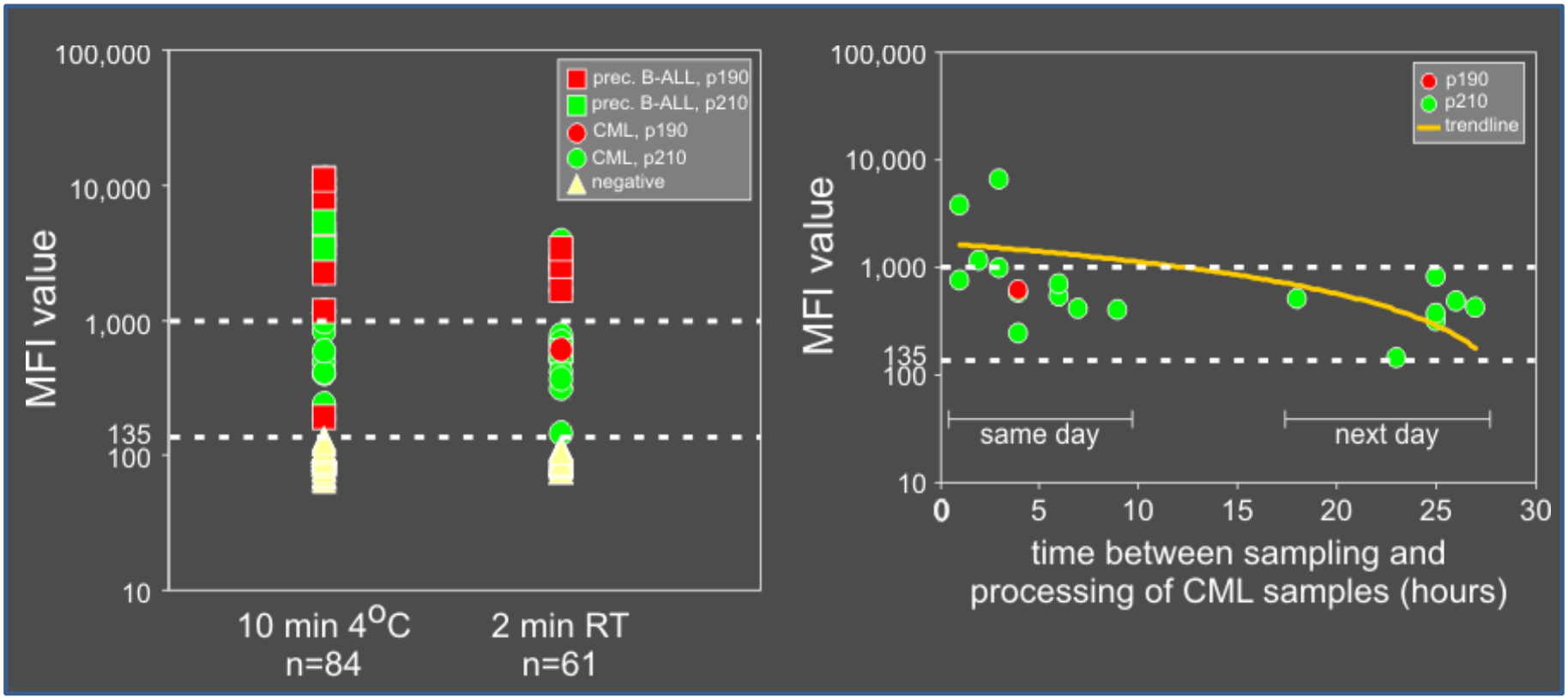
BCR-ABL RUO TESTING. TEMPERATURE AND TIME EFFECT



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

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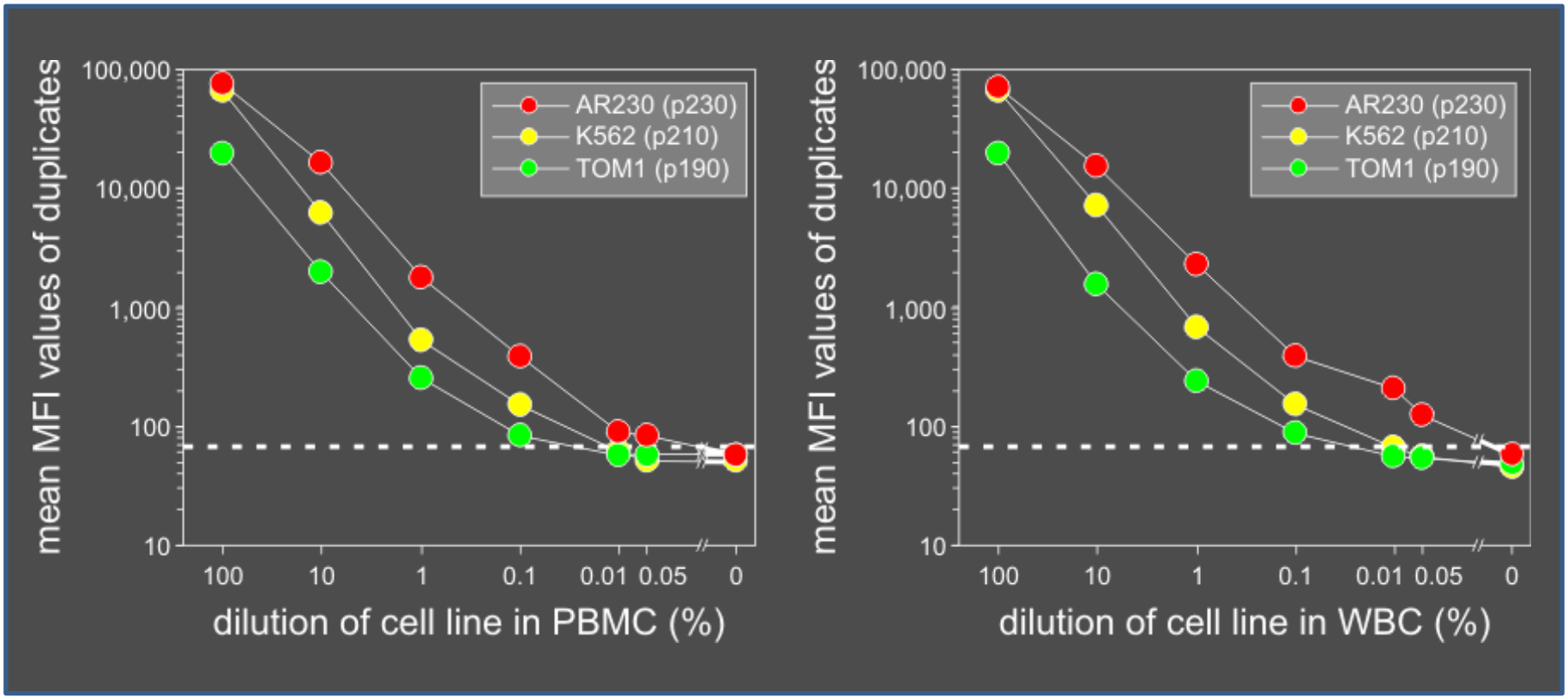
BCR-ABL RUO TESTING. SENSIBILITY



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

BCR-ABL RUO KIT: CONCLUSIONS (Leukemia, 2009)

.- High sensitivity & specificity:

- Absolute concordance with PCR resuls:
  12/41 BCP-ALL (all adults)
  12/12 CML
  0/29 AML & T-ALL

.- Amount of bcr/abl protein:

- Heterogeneous pattern with two groups of positive cases:
  - High expression: IMF ≥ 2.000
  - Lower expression: IMF ≥ 500 y < 2.000
- MFI of negative samples: < 150

.- Different pattern of expression in BCP-ALL vs CML:

BCP-ALL: 83% (10/12) displayed high expression
CML: 75% (9/12) showed dim/intermediate expression*

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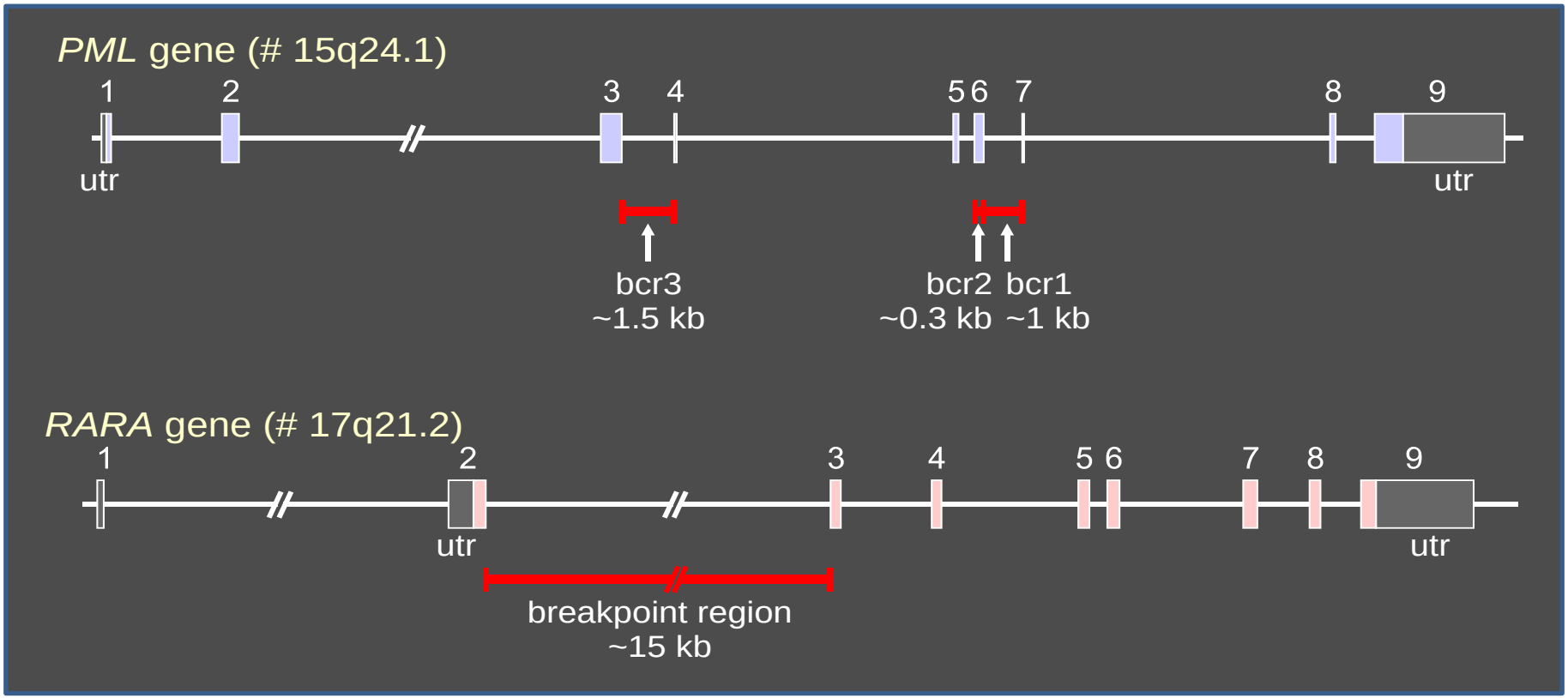
Breakpoint regions in t(15;17)(q24;q21) with PML and RARA genes



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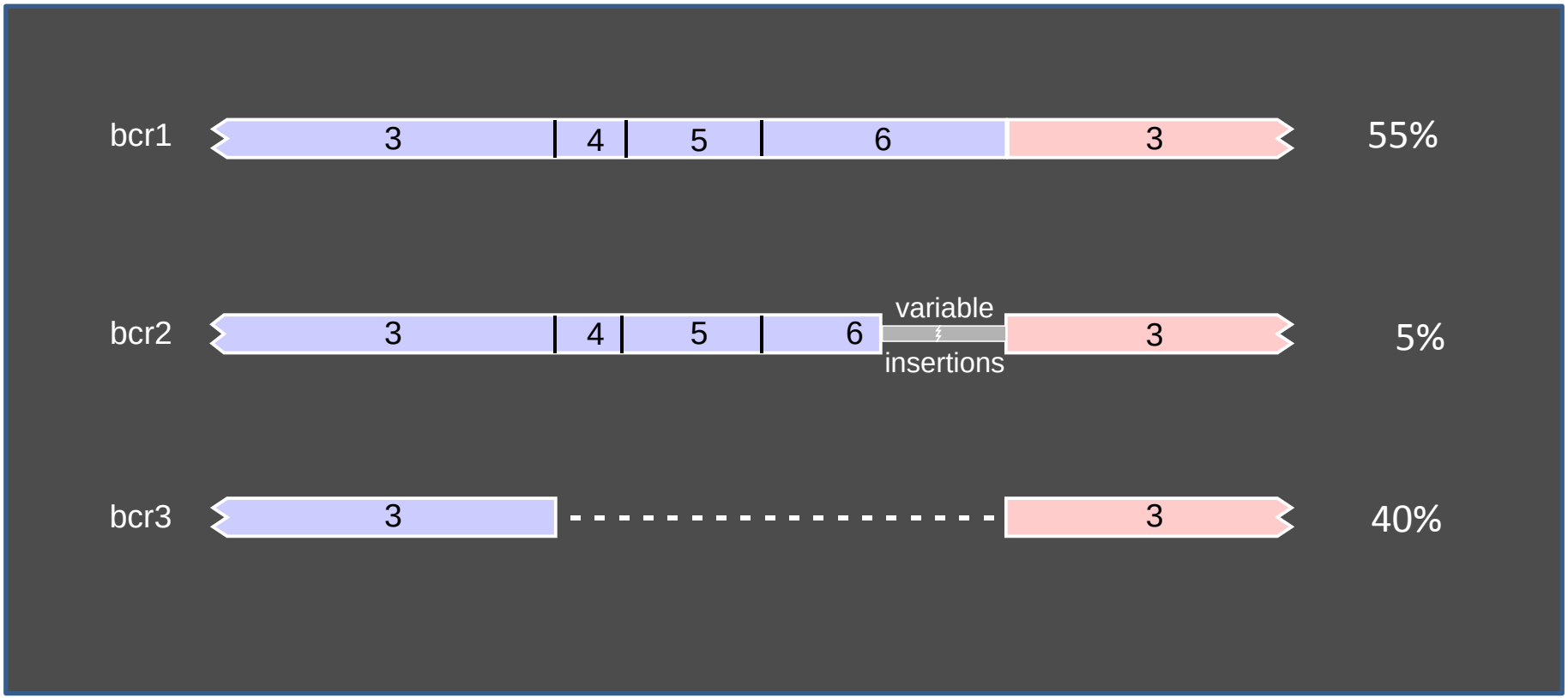
Breakpoint regions in t(15;17)(q24;q21) with PML and RARA genes



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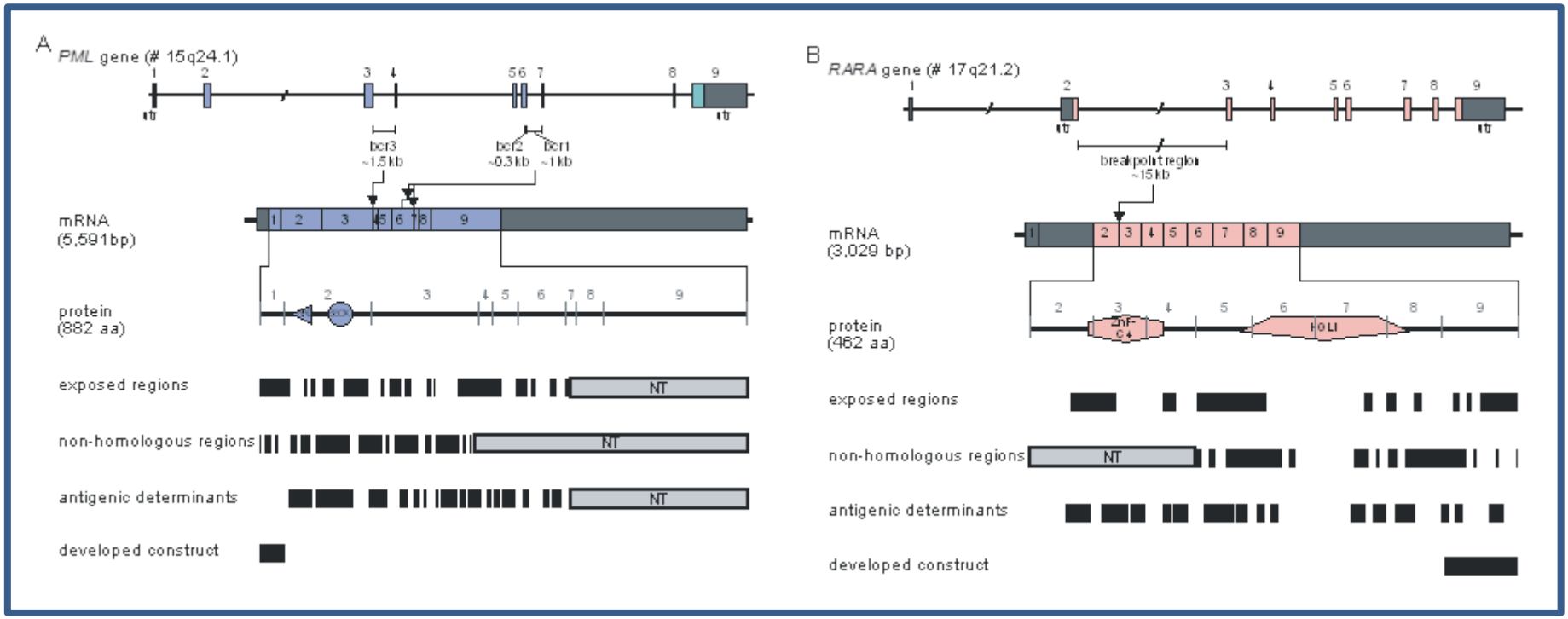
PML-RARA DESIGN OF ANTIBODIES FOR BEADS SYSTEM

PML

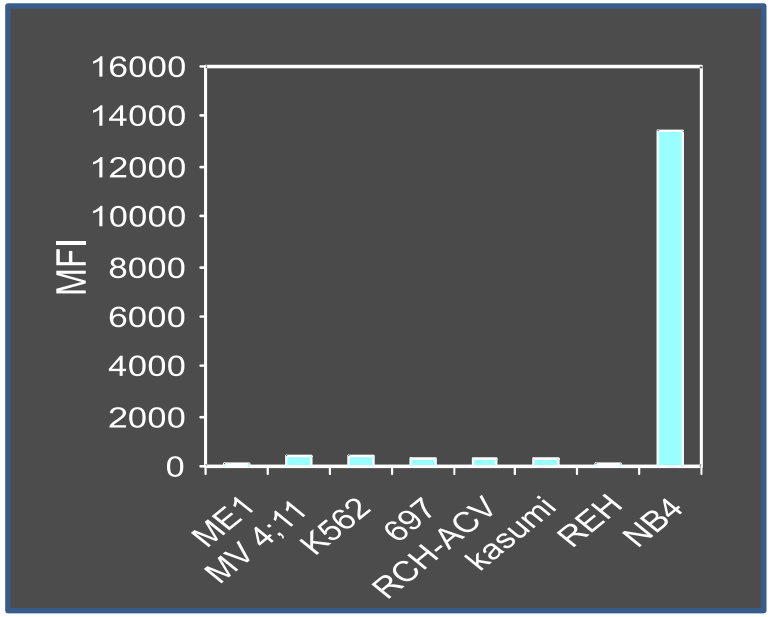
RARA



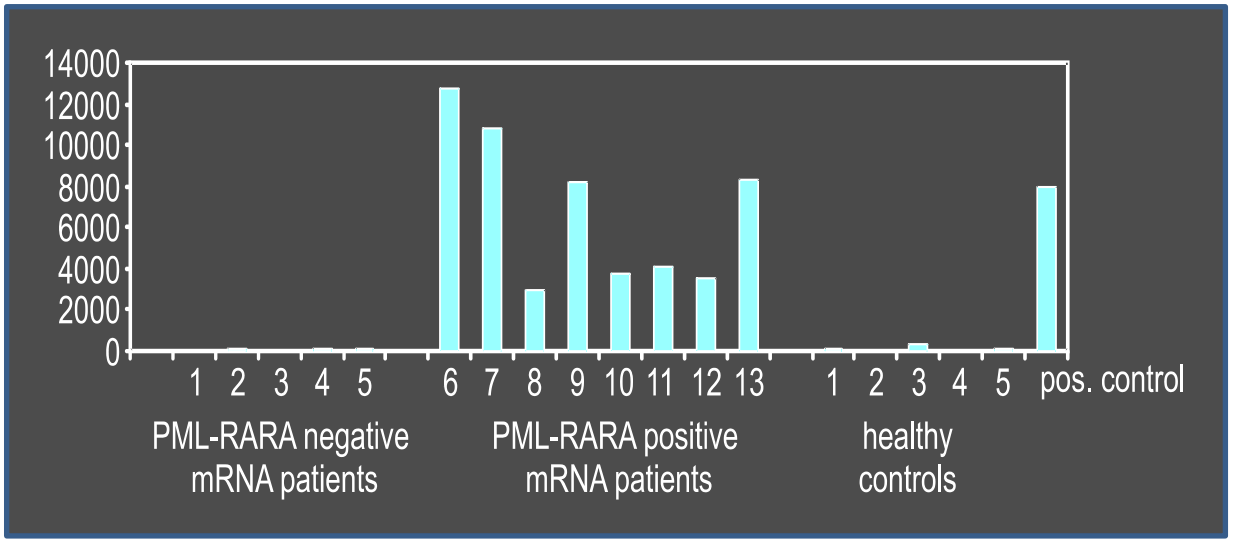
E.H.A Dekking, et al. Submitted to Leukemia 2011

FCM IMMUNOBEADS DETECTION OF PML-RARA

Cell Lines



Patients' samples



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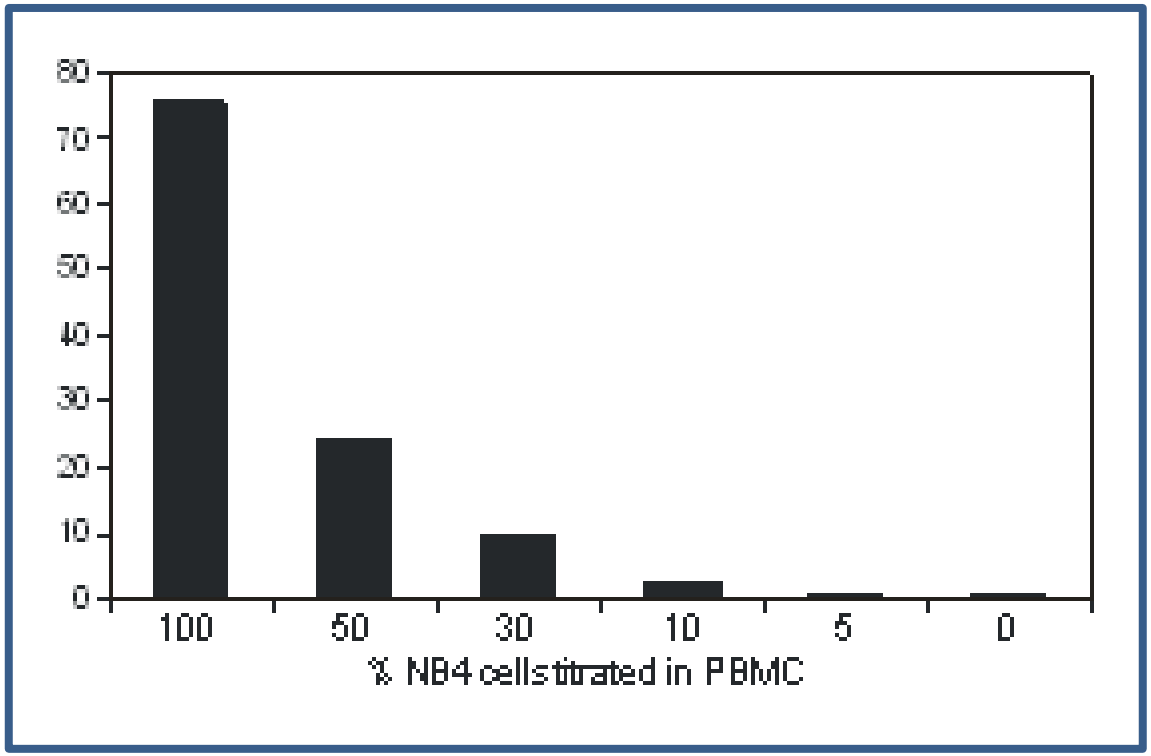
FCM IMMUNOBEADS DETECTION OF PML-RARA

Dilution experiments



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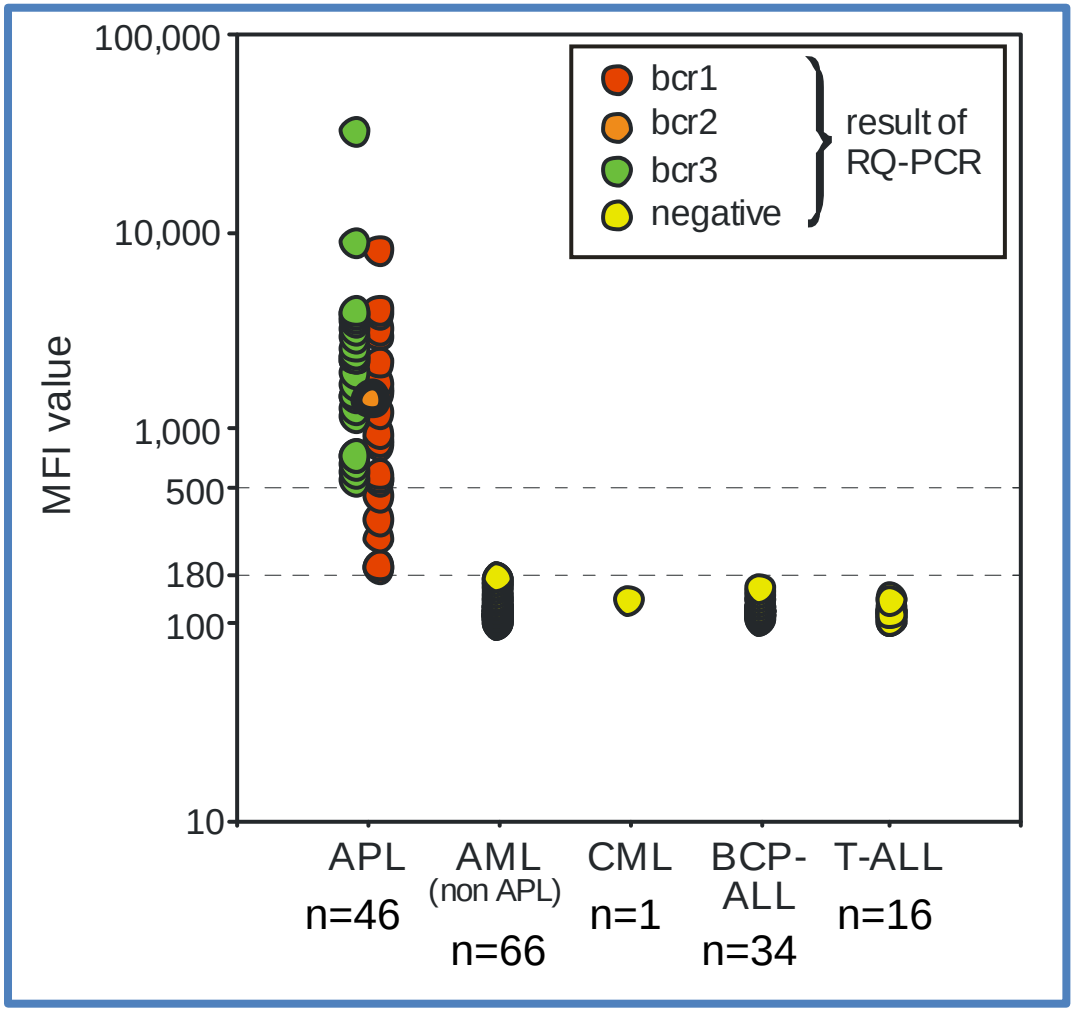
FCM IMMUNOBEADS DETECTION OF PML-RARA

Results in patient's samples. n=163



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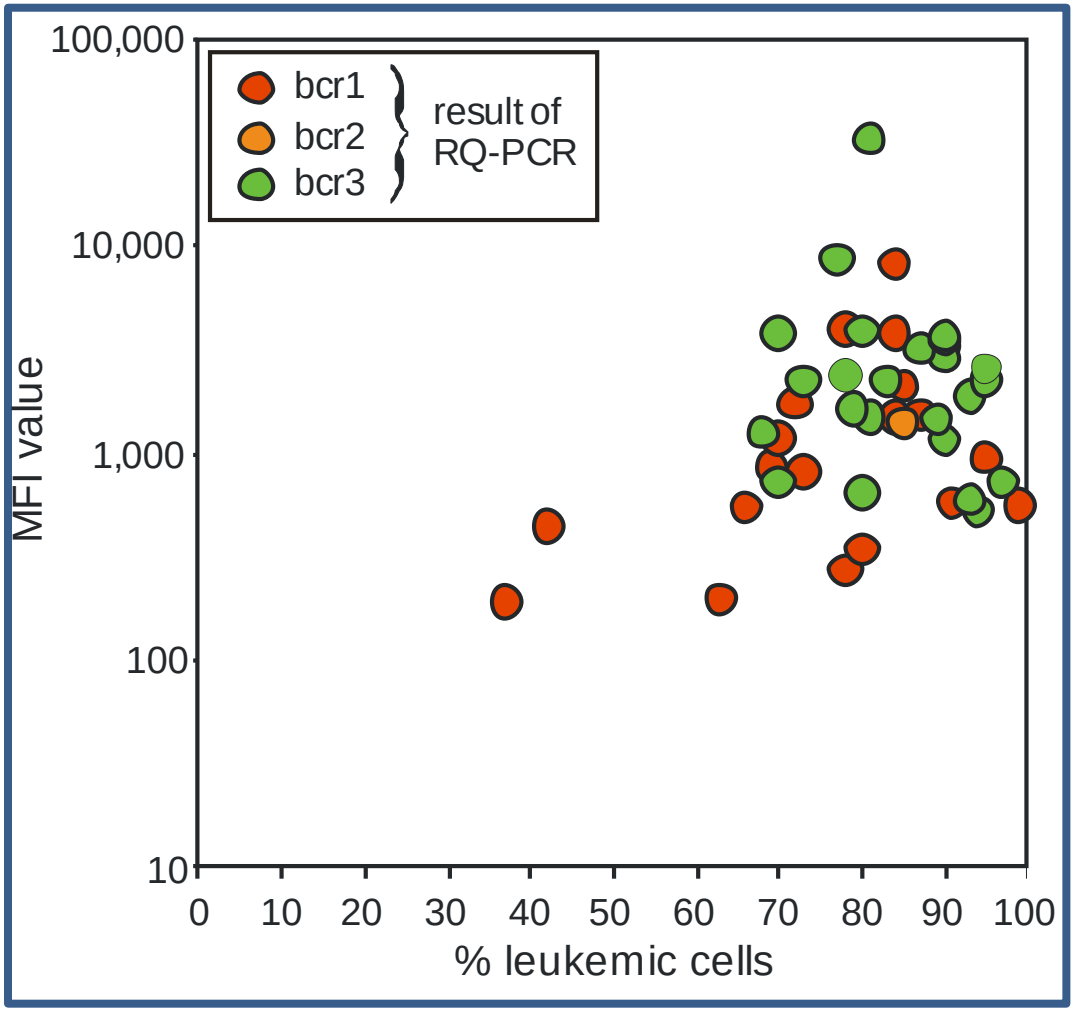

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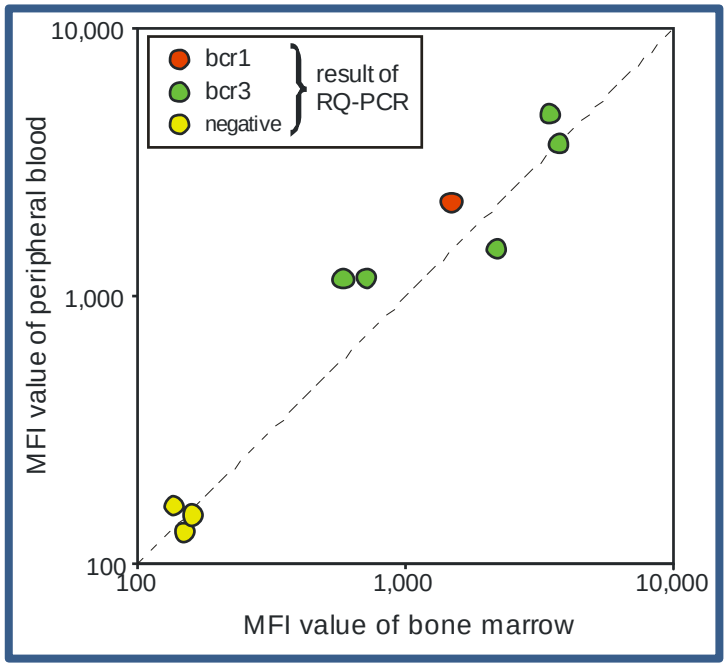
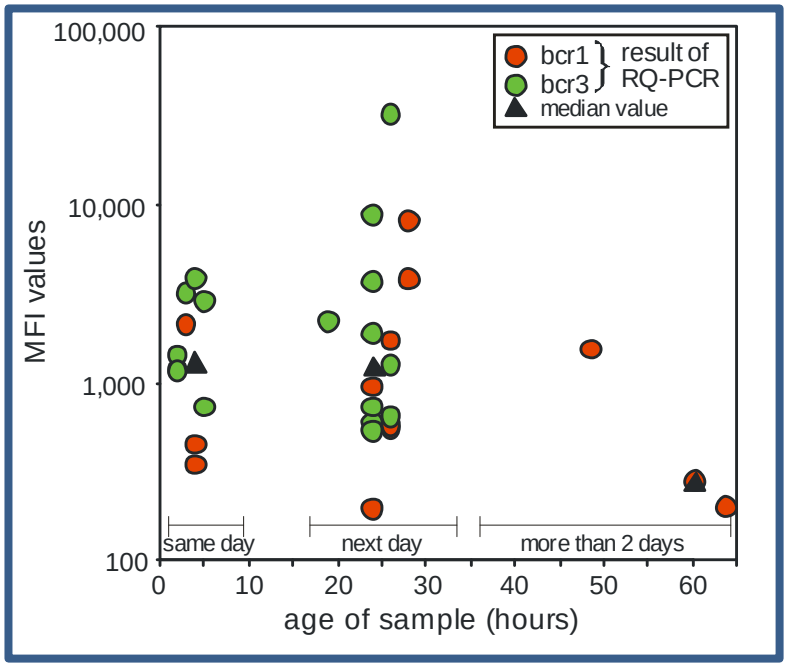
FCM IMMUNOBEADS DETECTION OF PML-RARA

Results in patient's samples.

BM versus PB



Time effect



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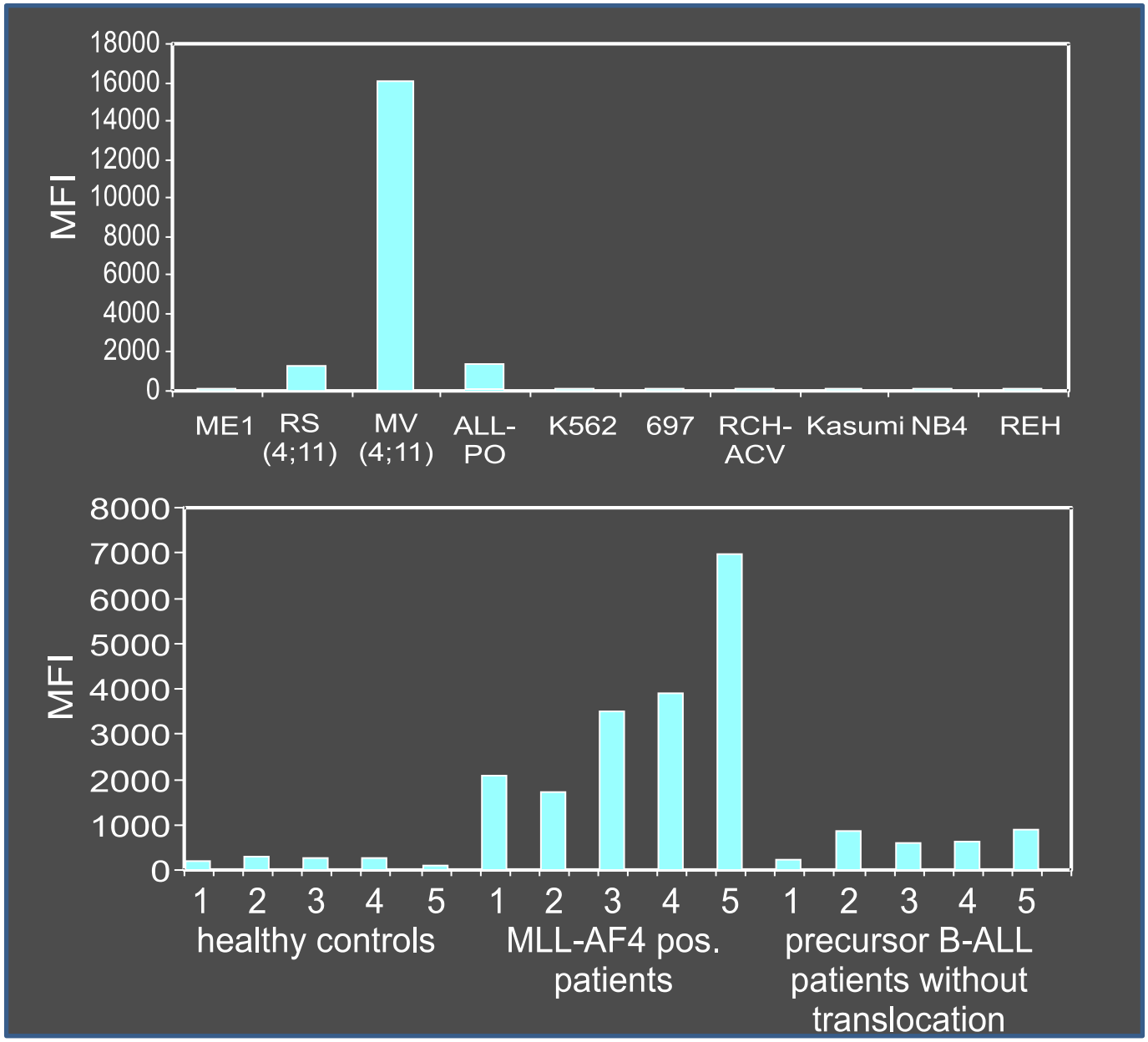
E.H.A Dekking, et al. Submitted to Leukemia 2011

FCM MLL-AF4 IMMUNOBEADS



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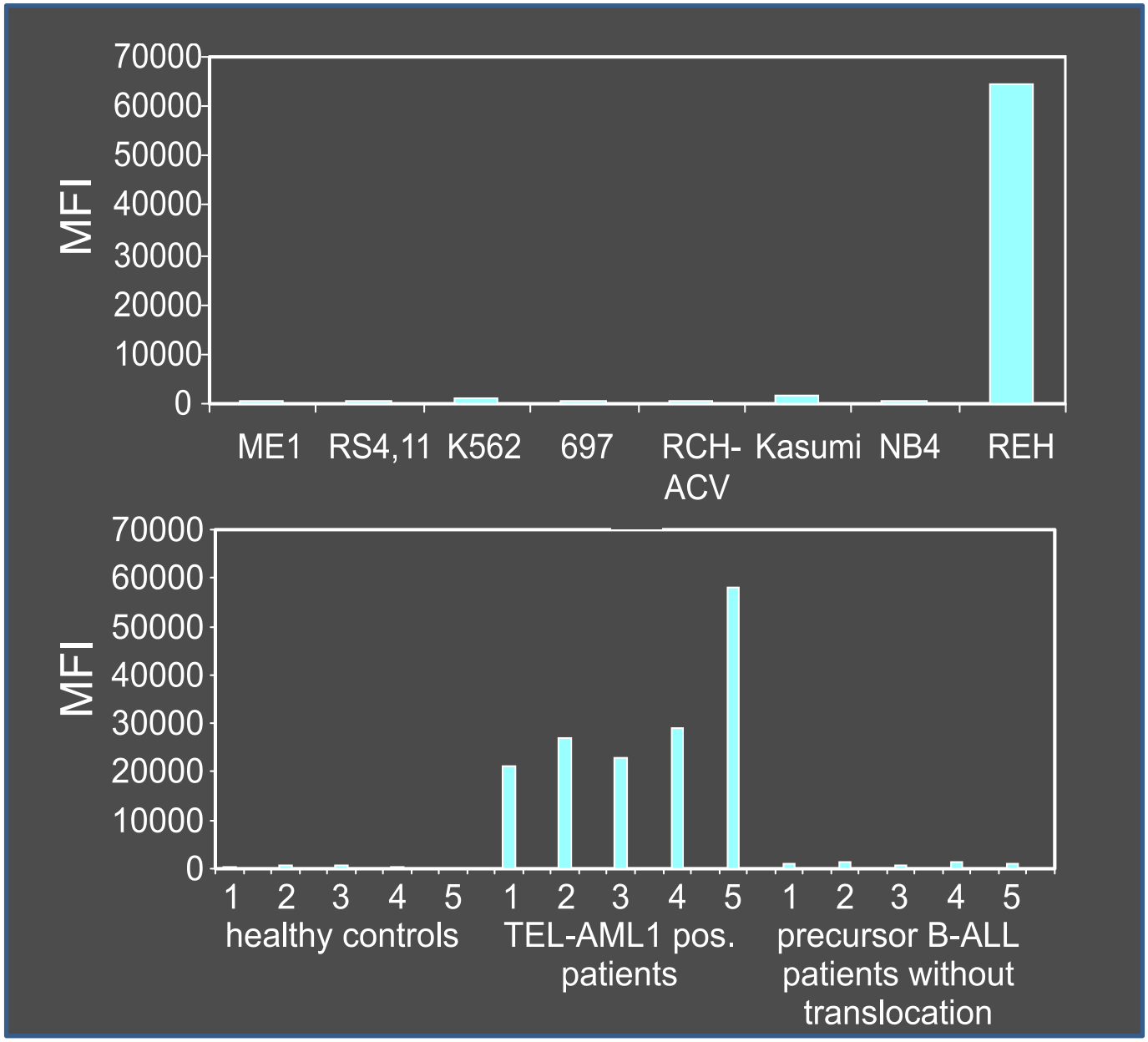
FCM TEL-AML1 IMMUNOBEADS



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CHROMOSOME ABERRATIONS & FUSION GENES IN AL

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.

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

- ***CML*:**
  - BCR-ABL : completed — Published / RUO kit launched
- ***precursor-B-ALL:***
  - BCR-ABL : completed
  - TEL-AML : completed
  - E2A-PBX1 : completed
  - MLL-AF4 : completed

  } **Precursor-B-ALL tube**
  Multiplex tube close to completion
- ***AML:***
  - AML1-ETO : completed
  - CBFB-MYH11 : completed

  } **Core-factor tube**
  Multiplex tube: prototype completed
  - PML-RARA : completed — Submitted / RUO kit in preparation

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dynomics

BD

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From invention through development and production to final diagnostic testing

CBA vs classical molecular techniques

-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!)

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

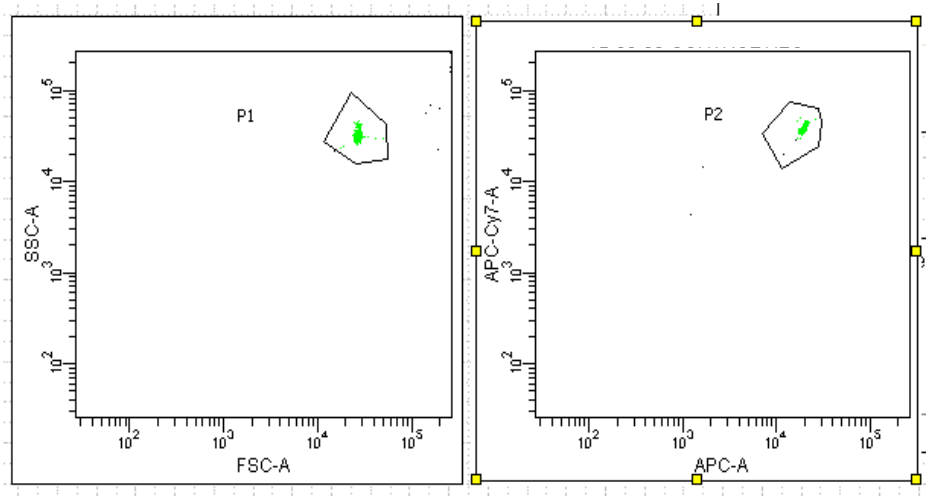
Thanks!!

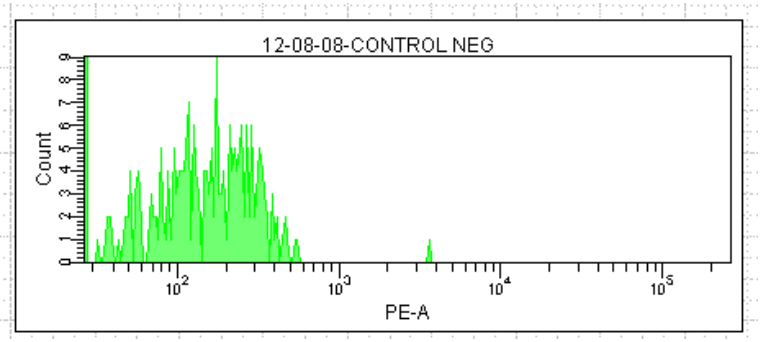
FCM DETECTION OF FUSION PROTEINS

Case Samples

FCM DETECTION OF FUSION PROTEINS

Settings





-FSC and SSC detectors:	30,000 ± 2,000 (each)
-APC channel	20,000 ± 2,000
-APCCy7 channel	40,000 ± 2,000
-PE detector	100 ± 5.