

Erasmus MC

University Medical Center Rotterdam



werkgroep "Moleculaire diagnostiek in de Pathologie"
vrijdag 28 januari 2011, Utrecht

Technische aspecten rondom B- en T-cel
clonaliteitsanalyses en Europese richtlijnen

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Afd. Immunologie, Erasmus MC

Moleculaire diagnostiek bij leukemieën en lymfomen

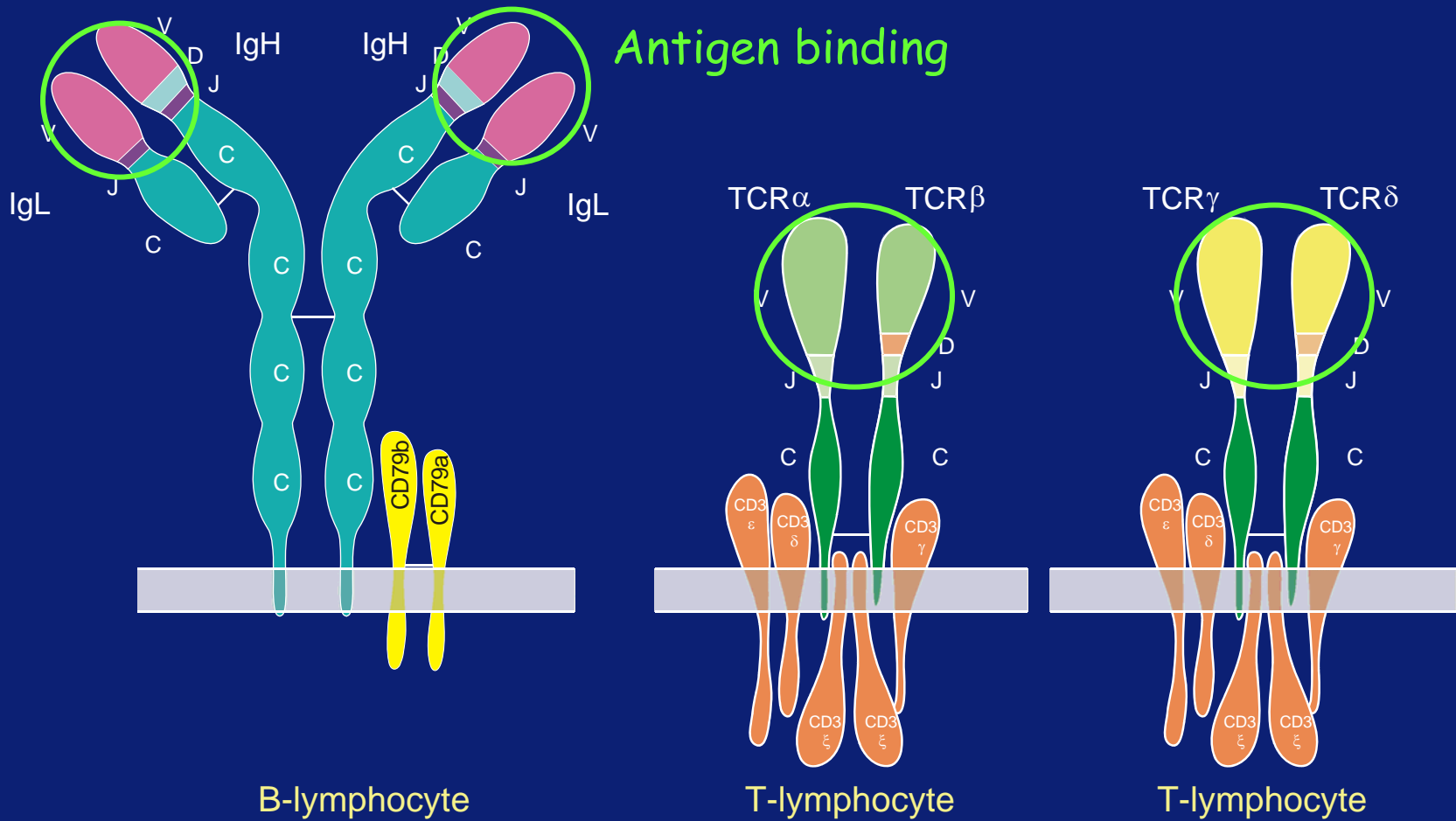
⇒ **Diagnose** : tumor (= kloon van cellen) of reactie op infectie ?

Klonaliteitsanalyse op basis van Ig/TCR herschikkingen

⇒ **Classificatie / prognose** : (klin.) heterogeniteit en verschillen in overleving

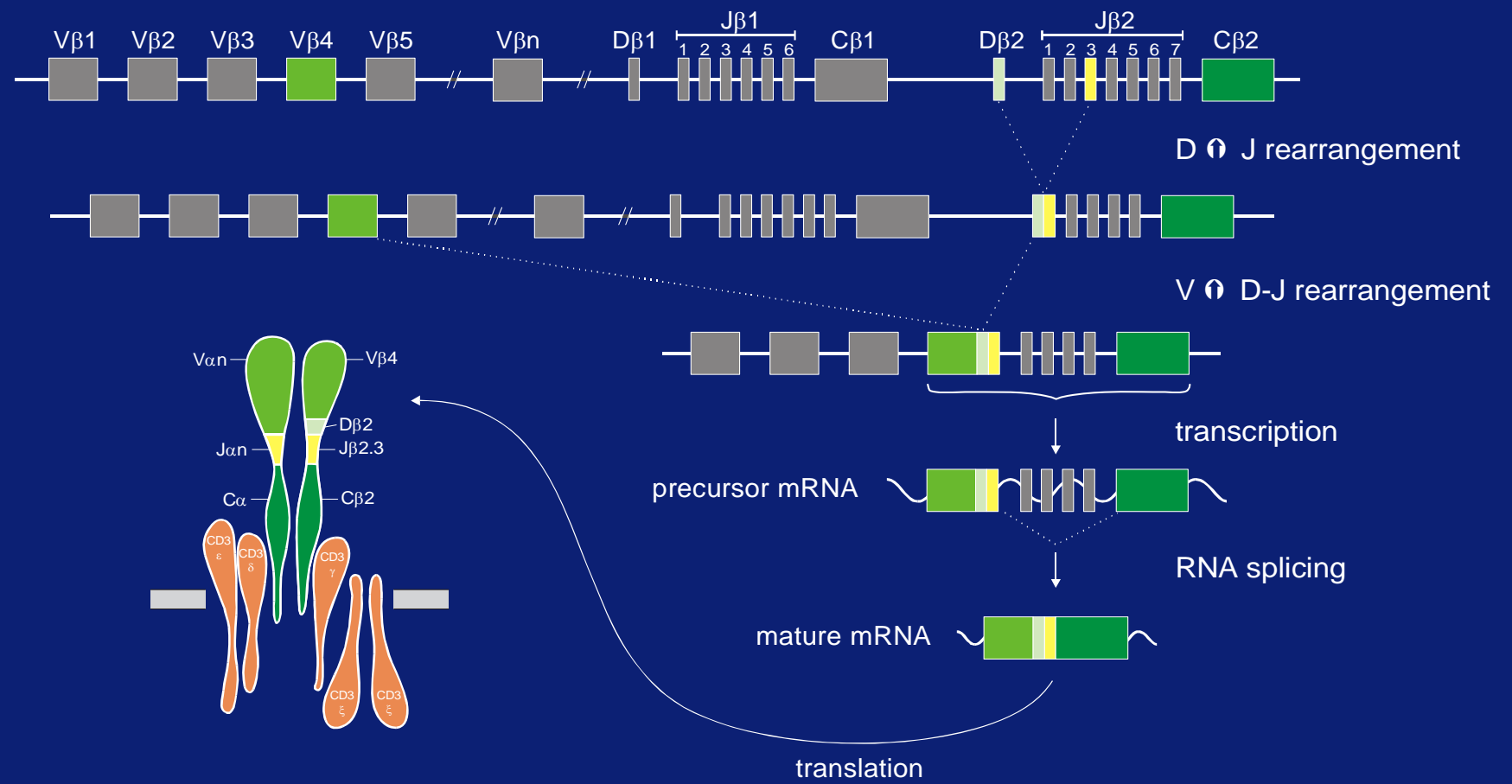
⇒ **MRD analyse** : monitoren van therapie effect

B- en T-celreceptoren

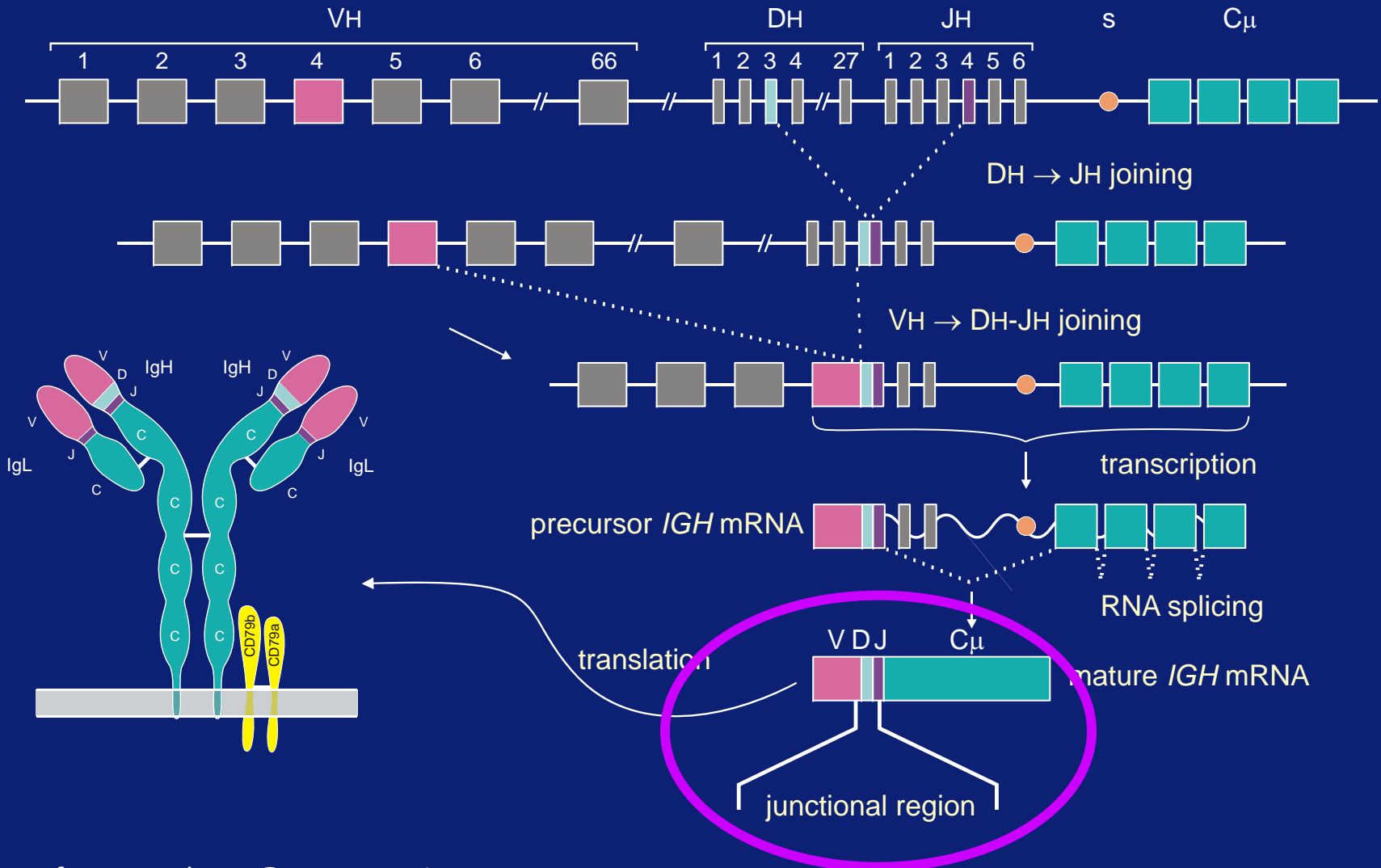


TCRB generschikking

TcR=β gene rearrangement



IGH generschikking



Meer diversiteit → junctional region



VH3-21 (germline)

insertion

DH3-3 (germline)

insertion

JH4-1 (germline)

TGTATTACTGTGCGAGA

GTATTACGATTTTGGAGTGGTTATTATACC

ACTACTTTGACTACT

TGTATTACTGT
 TGTATTACTGTGCG
 TGTATTACTGTGC
 TGTATTACTGTGCGAG
 TGTATTACTG
 TGTATTACTGTGCG
 TGTATTACTGTGCGA
 TGTATTACT
 TGTATTACTGTGCG
 TGTATTACTGTGC
 TGTATTACTGTGCGAGA
 TGTATTACTGTG
 TGTATTA
 TGTATTACTGTGC
 TGTATTACTGTGCGA

AGGC
 TATCCGGA
 CCGGACTG
 CTGAGTC
 ACATCGA
 CGT
 CCGG
 GATG
 TTCA
 GGCTAG
 GTCCAG
 CCGGA
 ACGC
 CGTA

CGATTTTGGAGTGGTTATTATA
 TTACGATTTTGGAGTGGTTATTATAC
 TTTTGGAGTGGTTATTATACC
 TATTACGATTTTGGAGTGGTTAT
 CGATTTTGGAGTGGTTATTATA
 TACGATTTTGGAGTGGTTATTAT
 TTACGATTTTGGAGTGGTTATTATACC
 GATTTTGGAGTGGT
 ATTACGATTTTGGAGTGGTTATTATA
 TTTGGAGTGGTTATTATA
 ATTACGATTTTGGAGTGGTTATTATACC
 TATTACGATTTTGGAGTG
 CGATTTTGGAGTGGTTATTATA
 ATTACGATTTTGGAGTGG
 GTATTACGATTTTGGAGTGGTTATTATACC

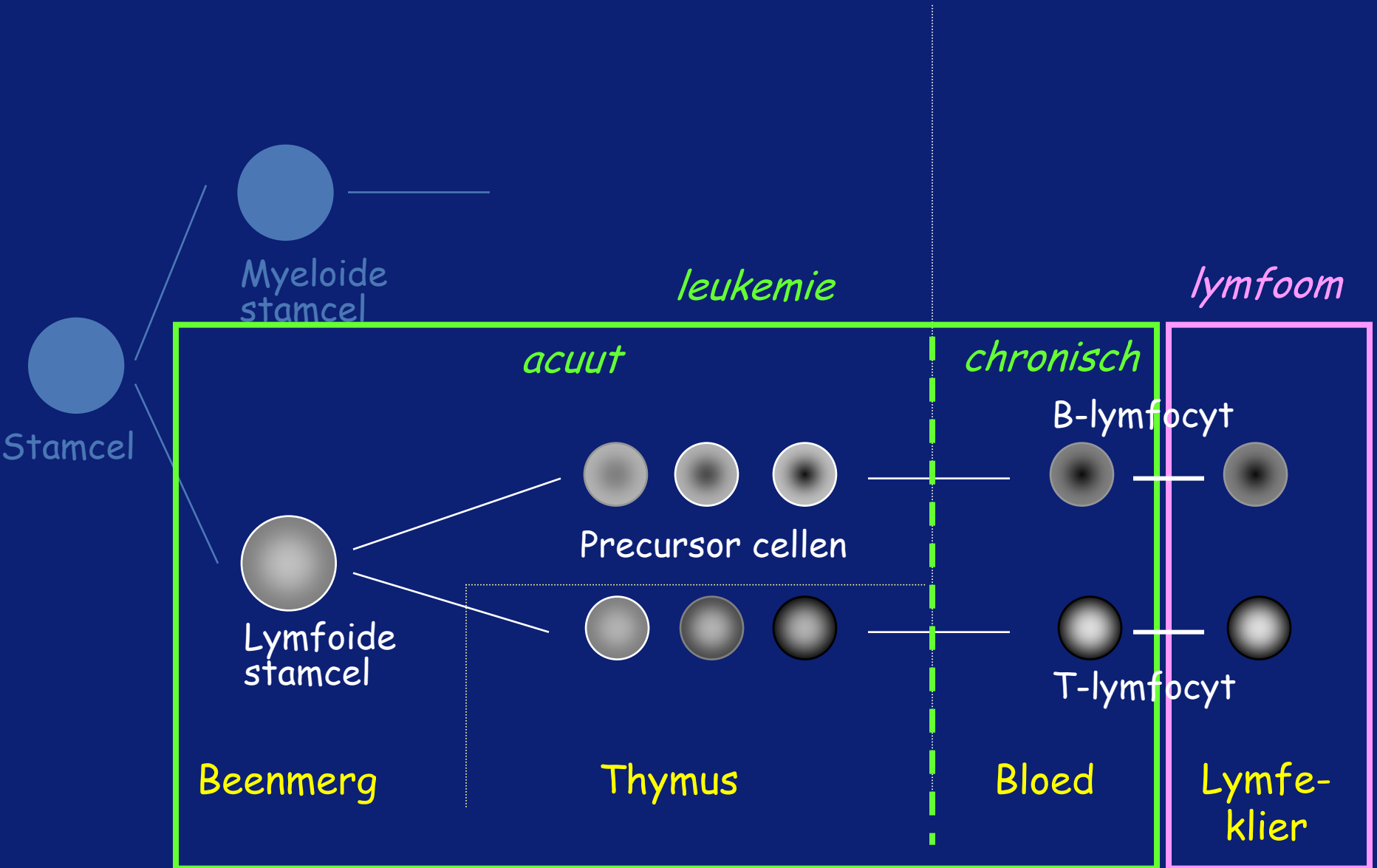
GTCCA
 CGATCG
 GGT
 CGTAGCGTA
 CGTAG
 GGCTAAGG
 CCGAGC
 GGTC
 CGATCGA
 CC
 GTCG
 CCGTAG
 C
 GTACG
 GGCA

TGACTACT
 CTTTGACTACT
 ACTACTTTGACTACT
 TTTGACTACT
 ACTTTGACTACT
 TGACTACT
 TACTTTGACTACT
 ACTACTTTGACTACT
 CTTTGACTACT
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 GACTACT
 CTACTTTGACTACT
 ACT
 TTTGACTACT
 ACTTTGACTACT

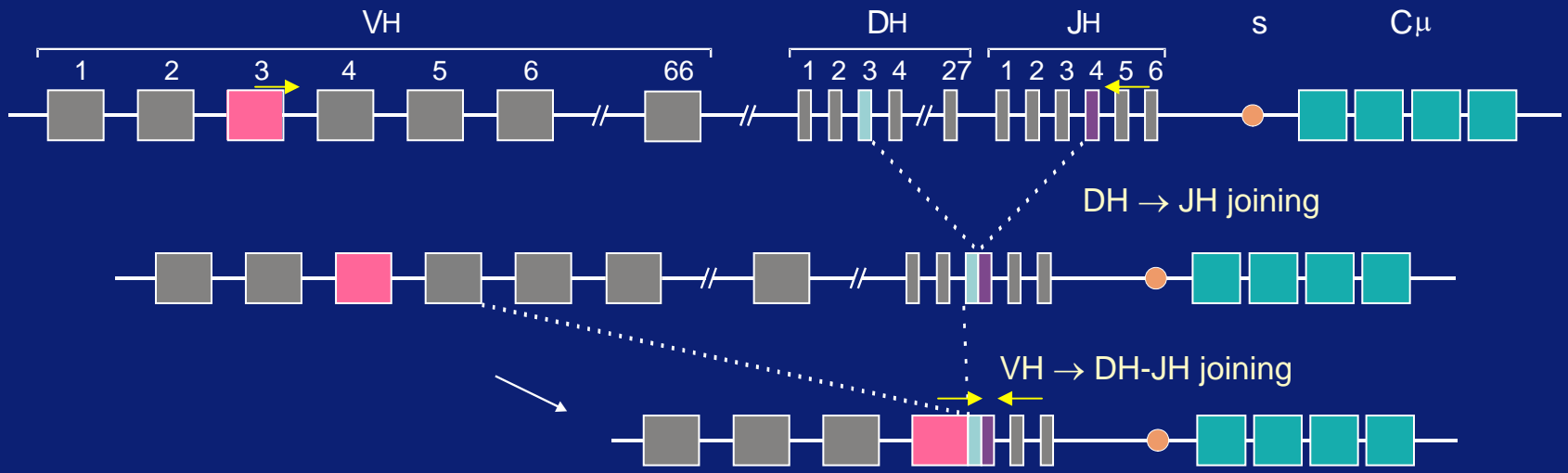
Schatting van potentiële primaire repertoire van humane Ig en TCR moleculen

	IgH	Igκ	Igλ	TCR $\alpha\beta$		TCR $\gamma\delta$	
				TCR α	TCR β	TCR γ	TCR δ
Aantal genen							
- V genen	> 100	> 50	> 40	> 50	> 70	6	6
- D genen	27	-	-	-	2	-	3
- J genen	6	5	4	55	13	5	4
Combinatie diversiteit	> 5 × 10 ⁶			> 5 × 10 ⁶		> 5000	
Junctional region diversiteit	++	±	±	+	++	++	++++
Schatting van totale primaire receptor repertoire	> 10 ¹²			> 10 ¹²		> 10 ¹²	

Leukemieën en lymfomen vs. lymfocyten

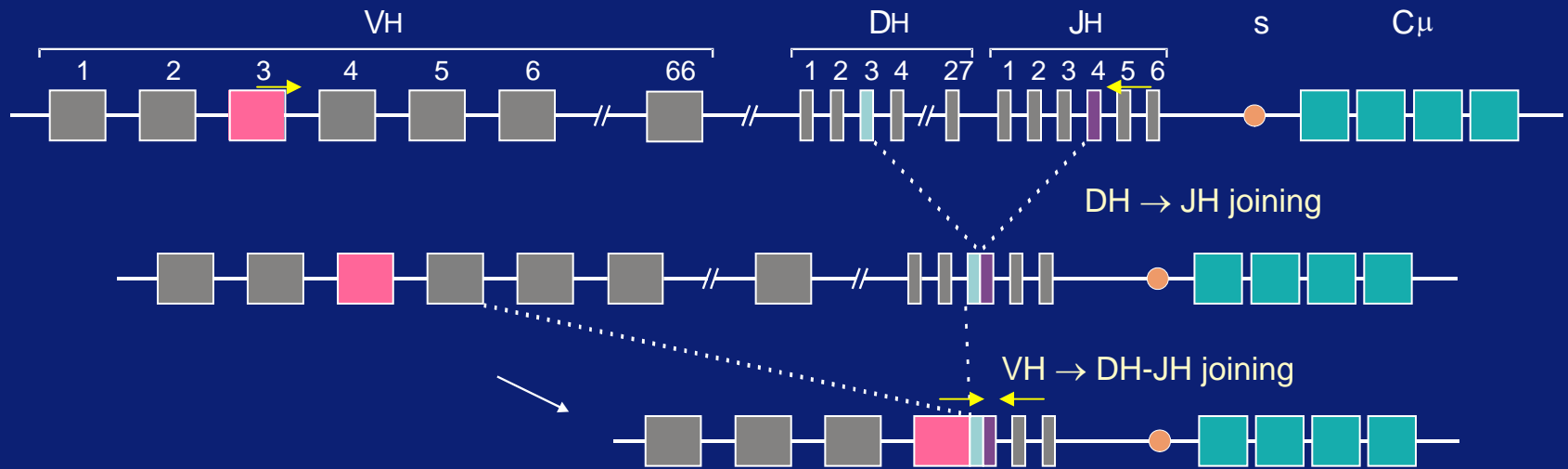


Rationale PCR-gebaseerde analyse



- niet-hemopoietisch : Ig / TCR segmenten **uiteen** \rightarrow geen PCR product

Rationale PCR-gebaseerde analyse



- niet-hemopoietisch : Ig / TCR segmenten **uiteen** \rightarrow geen PCR product
- normal BM, PB, LK : Ig/TCR segmenten **gekoppeld** \rightarrow PCR product

PCR-gebaseerde klonaliteitsanalyse - valkuilen + oplossingen

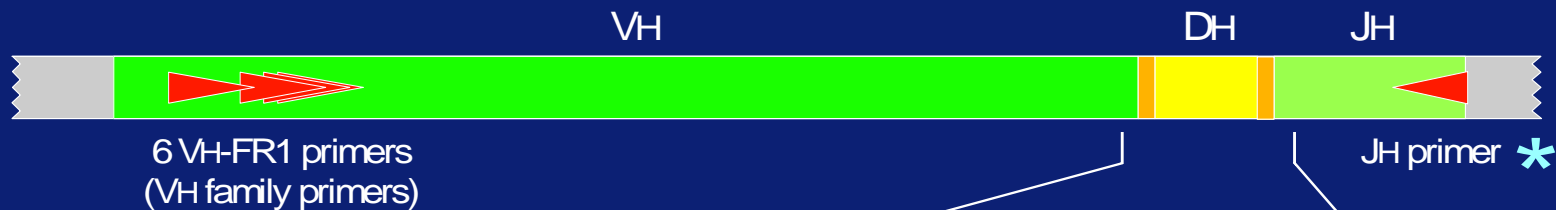
vals-positiviteit

t.g.v. slecht onderscheid tussen monoklonale (identieke) en polyklonale (variabele) PCR producten



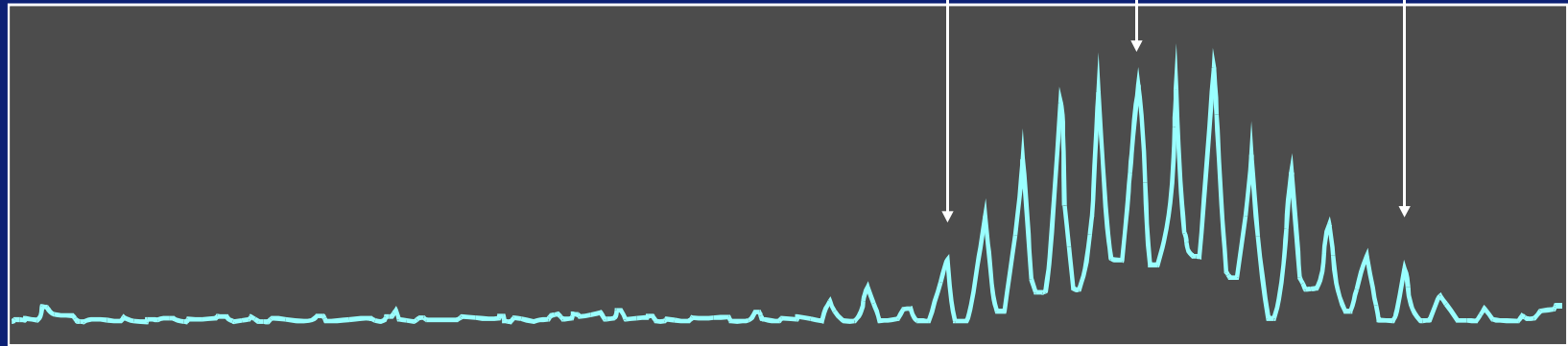
PCR amplificatie gevolgd door *GeneScan* analyse
en/of heteroduplex analyse

Klonaliteitsanalyse : GeneScan



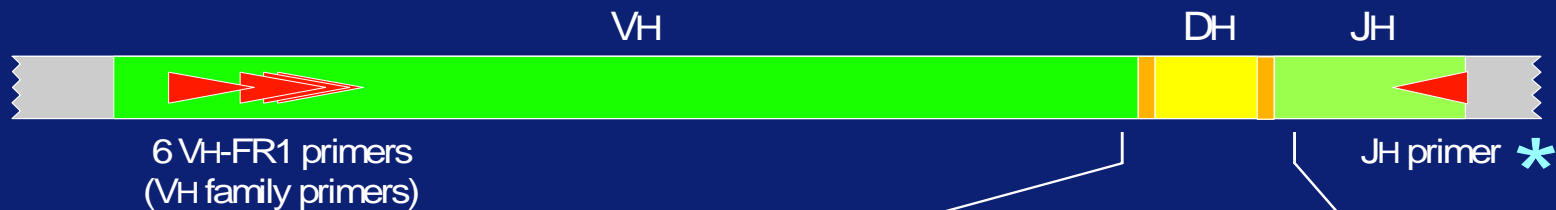
hoeveelheid
fluorescent
product

CTGTGCAAGAG_{CGGG}CTATGGTTCAGGGAGTTAT_{GG}CTACTACGGTATGGACGTCTGG
 CTGTGCAAGAG_{GACGAA}ACAGTAACT_{Gc}CTACTACTACTACGGTATGGACGTCTGG
 CTGTGCAAGAGA_{GAT}AGTATAGCAGCTCGTACAACCTGGTTCGACTCCTGG
 CTGTGCAAGAGA_{TCCGGGc}AGCTCGTTTTGCTTTTGATATCTGG
 CTGTGCAAGAG_{cCTCTCTCCACTGGGATGGGGG}CTACTGG
 CTGTGCAAGAG_{cAGCAGCTCGGCCc}CTTTGACTACTGG
 CTGTGCAAGAG_{GACTTTGG}ATGCTTTTGATATCTGG
 CTGTGCAAGAG_GGTGGGAGCTACTAGACTACTGG
 CTGTGCAAG_GGTAGCTAAACCTTTGACTACTGG
 CTGTGCAAT_{TAT}CTACTTTGACTACTGG



lengte van DNA fragmenten

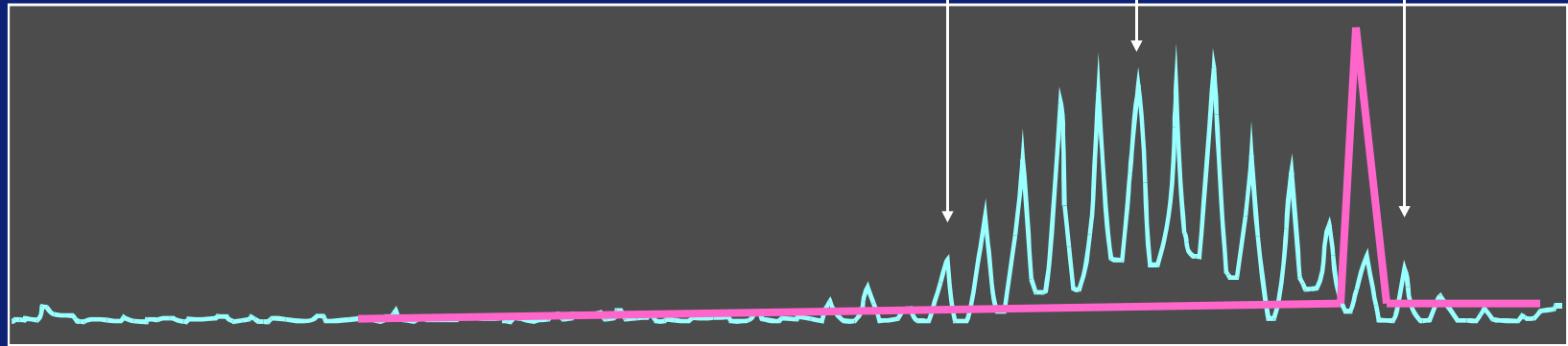
Klonaliteitsanalyse : heterogeen of identiek?



hoeveelheid fluorescent product

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CTGTGCAAGAGCGGGCTATGGTTCAGGGAGTTATGGCTACTACGGTATGGACGTCTGG
CTGTGCAAGAGGACGAAACAGTAACTGcCTACTACTACTACGGTATGGACGTCTGG
CTGTGCAAGAGAGATAGTATAGCAGCTCGTACAACCTGGTTCGACTCCTGG
CTGTGCAAGAGATCCGGGcAGCTCGTTTTGCTTTTGATATCTGG
CTGTGCAAGAGcCTCTCTCCACTGGATGGGGGGCTACTGG
CTGTGCAAGAGcAGCAGCTCGGCCcCTTTGACTACTGG
CTGTGCAAGAGGACTTTGgATGCTTTTGATATCTGG
CTGTGCAAGAGGGTGGGAGCTACTAGACTACTGG
CTGTGCAAGGGTAGCTAAACCTTTGACTACTGG
CTGTGCAATTATCTACTTTGACTACTGG
    
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Heteroduplex analyse : band of smear ?

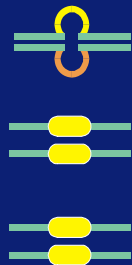
monoclonal cells

monoclonal cells in
polyclonal background

polyclonal cells



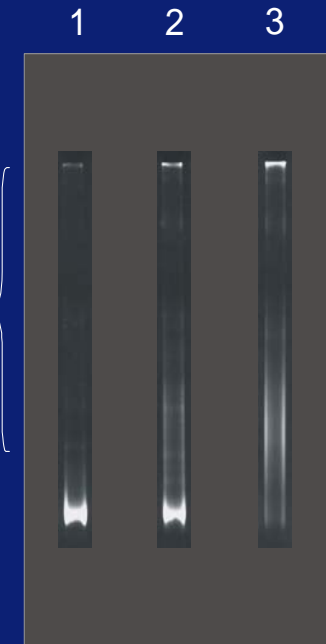
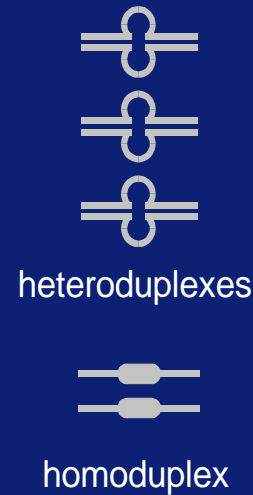
denaturation / renaturation



1

2

3



PCR-gebaseerde klonaliteitsanalyse - valkuilen + oplossingen

vals-positiviteit

t.g.v. slecht onderscheid tussen monoklonale (identieke) en polyklonale (variabele) PCR producten



PCR amplificatie gevolgd door **GeneScan analyse**
en/of **heteroduplex analyse**

vals-negativiteit

t.g.v. slechte aanhechting primers (10-30%)



meerdere primers per reactie (→ multiplexing)

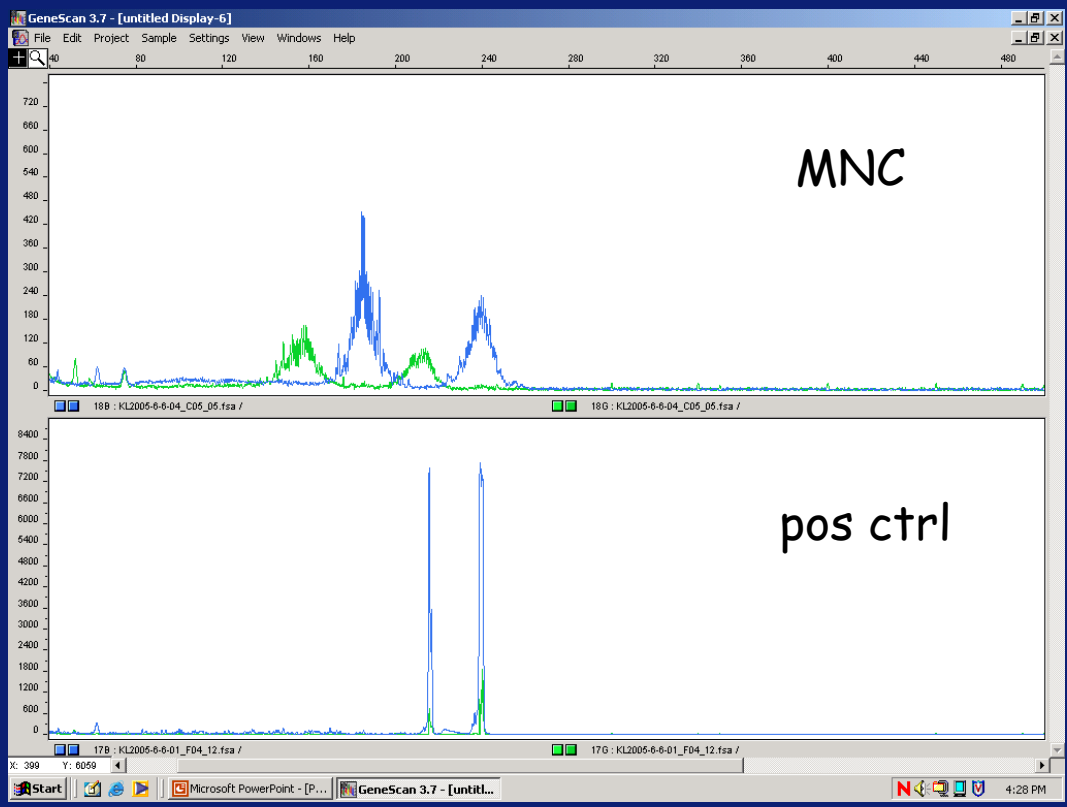
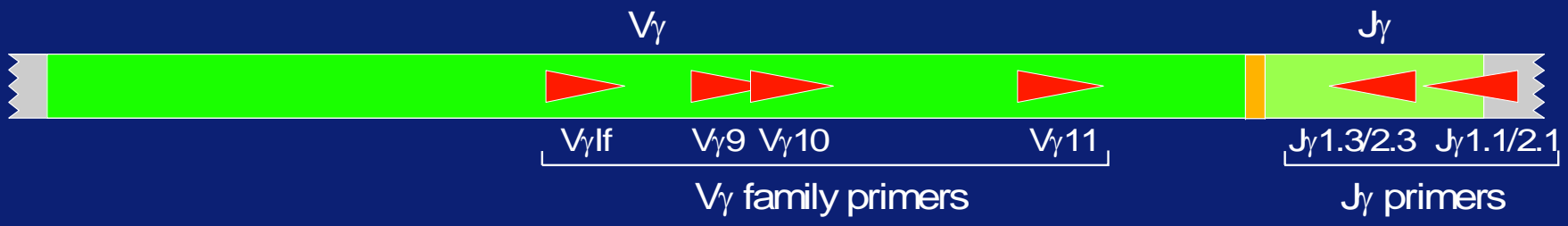


meerdere primersets per target (IGH)



meerdere loci analyseren (e.g., IGH+IGK, TCRB+TCRG) → complementariteit van targets

GeneScan analyse van TCRG genherschikkingen



TCRG tube A

IGH: meerdere primer sets per target



IGH tube A

Primer	Position	Sequence (5' to 3')
VH1-FR1	(1-2) (-252)	GGCCTCAGTGAAGGTCTCCTGCAAG
VH2-FR1	(2-5) (-284)	GTCTGGTCCTACGCTGGTGAAACCC
VH3-FR1	(3-7) (-256)	CTGGGGGGTCCCTGAGACTCTCCTG
VH4-FR1	(4-4) (-256)	CTTCGGAGACCCTGTCCCTCACCTG
VH5-FR1	(5-51) (-255)	CGGGGAGTCTCTGAAGATCTCCTGT
VH6-FR1	(6) (-263)	TCGCAGACCCTCTCACTCACCTGTG

IGH tube B

VH1-FR2	(1-2) (-192)	CTGGGTGCGACAGGCCCTGGACAA
VH2-FR2	(2-5) (-190)	TGGATCCGTCAGCCCCAGGGAAGG
VH3-FR2	(3-7) (-189)	GGTCCGCCAGGCTCCAGGGAA
VH4-FR2	(4-4) (-188)	TGGATCCGCCAGCCCCAGGGAAGG
VH5-FR2	(5-51) (-190)	GGGTGCGCCAGATGCCCGGGAAGG
VH6-FR2	(6) (-194)	TGGATCAGGCAGTCCCCATCGAGAG
VH7-FR2	(7) (-192)	TTGGGTGCGACAGGCCCTGGACAA

IGH tube C

VH1-FR3	(1-2) (-55)	TGGAGCTGAGCAGCCTGAGATCTGA
VH2-FR3	(2-5) (-54)	CAATGACCAACATGGACCCTGTGGA
VH3-FR3	(3-7) (-57)	TCTGCAAATGAACAGCCTGAGAGCC
VH4-FR3	(4-4) (-48)	GAGCTCTGTGACCGCCGCGGACACG
VH5-FR3	(5-51) (-69)	CAGCACCGCCTACCTGCAGTGGAGC
VH6-FR3	(6) (-63)	GTTCTCCCTGCAGCTGAACTCTGTG
VH7-FR3	(7) (-69)	CAGCACGGCATATCTGCAGATCAG

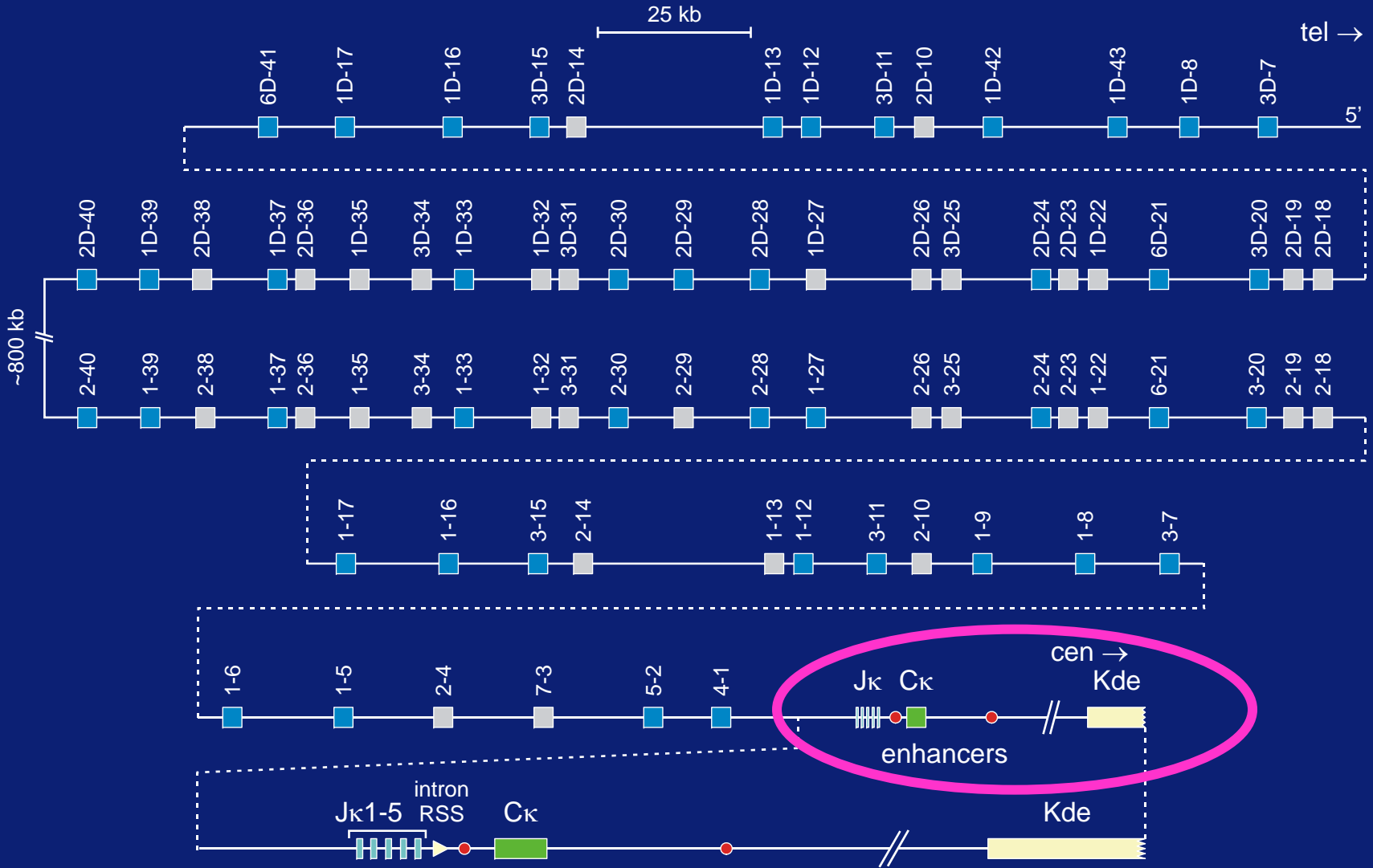
IGH tubes A, B, and C
 3' CCAGTGGCAGAGGAGTCCATTC 5' (+57) JH consensus

BIOMED-2 multiplex PCR - complementariteit van Ig targets

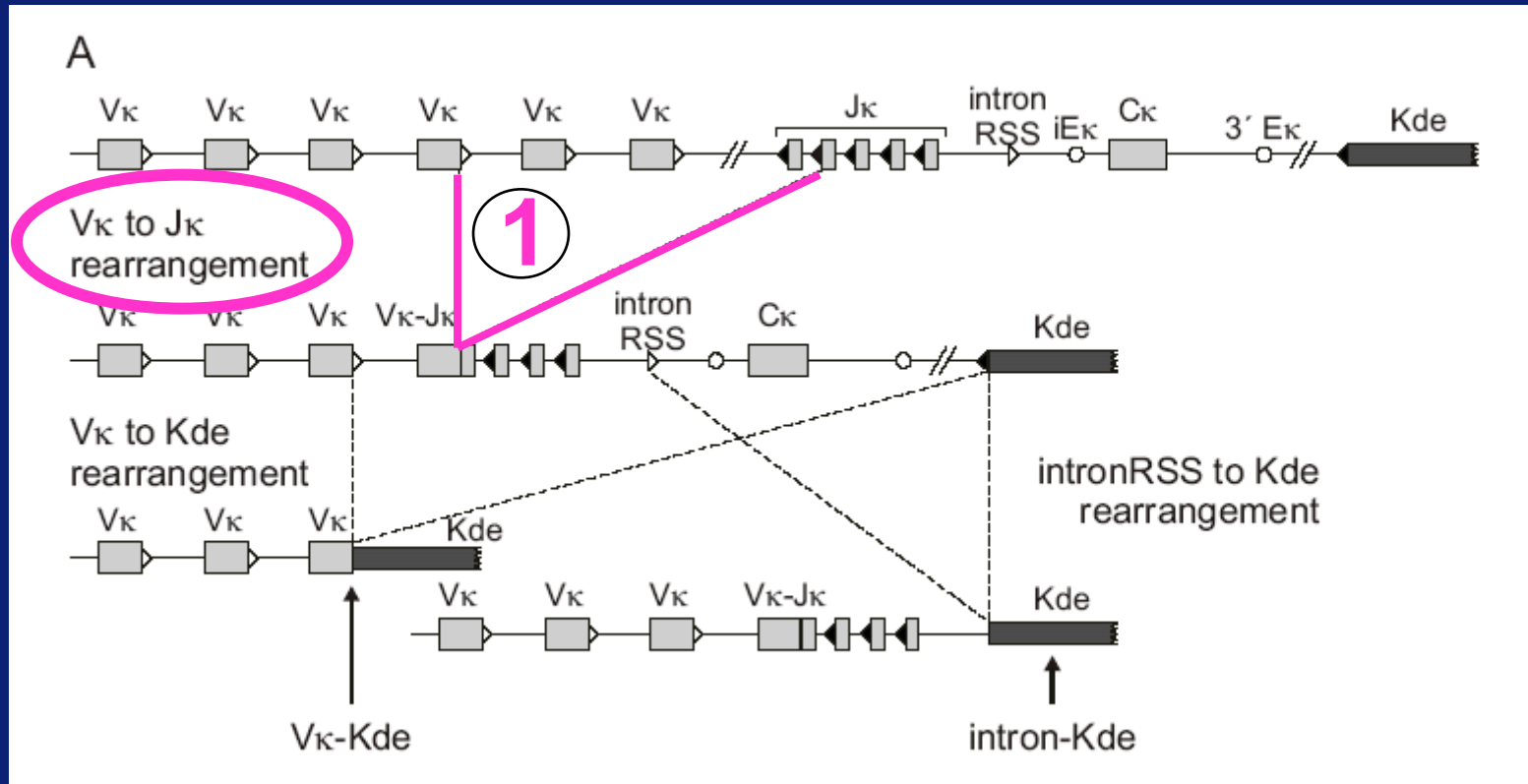
DLBCL (n=116)		FCL (n=109)	
- FR1	67 %	- FR1	73 %
- FR2	57 %	- FR2	76 %
- FR3	47 %	- FR3	52 %
- alle FR	77 %	- alle FR	84 %
- IGH + <i>IGK</i>	91 %	- IGH + <i>IGK</i>	100 %

~10 % zonder klonale Ig targets

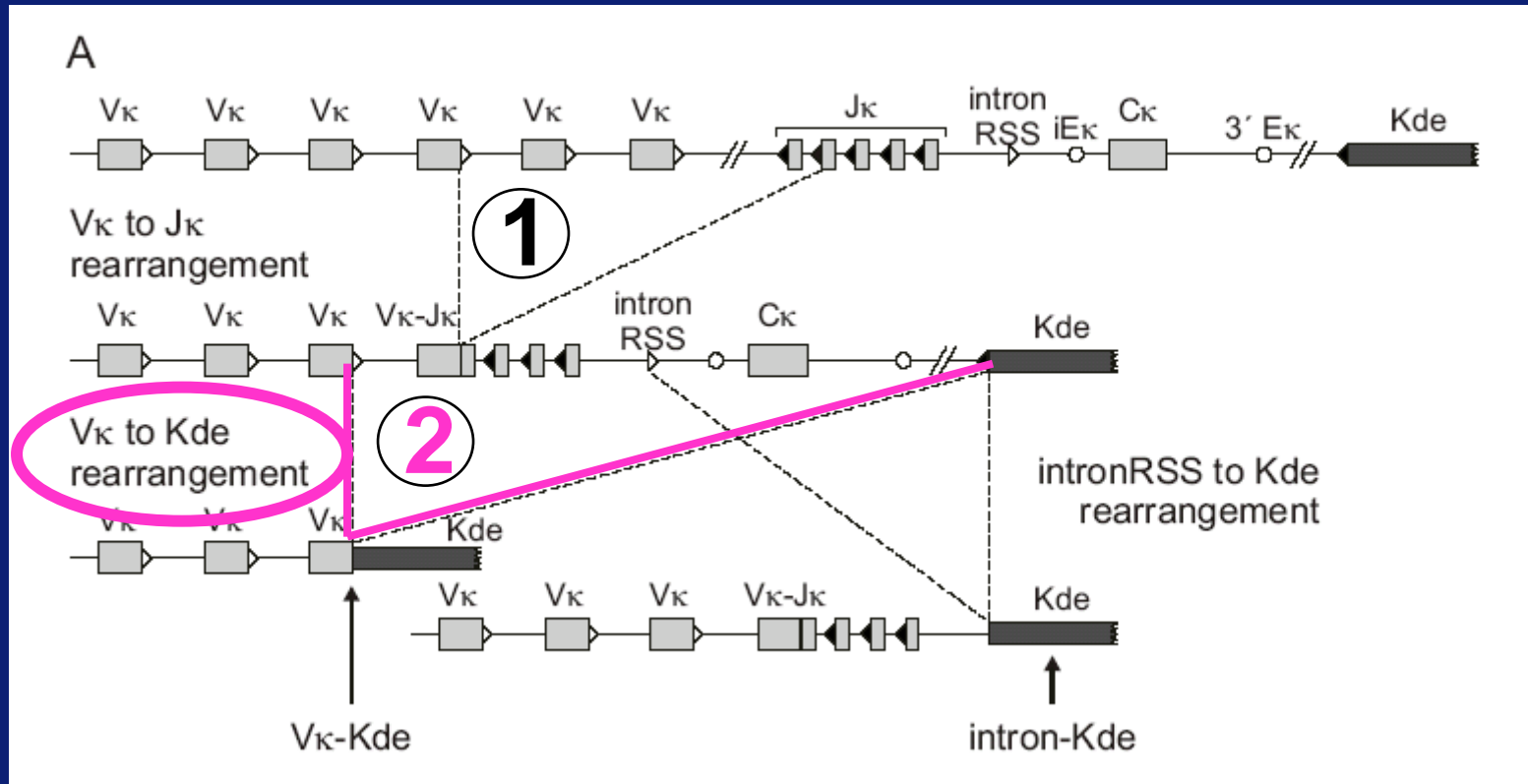
Human IGHK locus (#2p11.2)



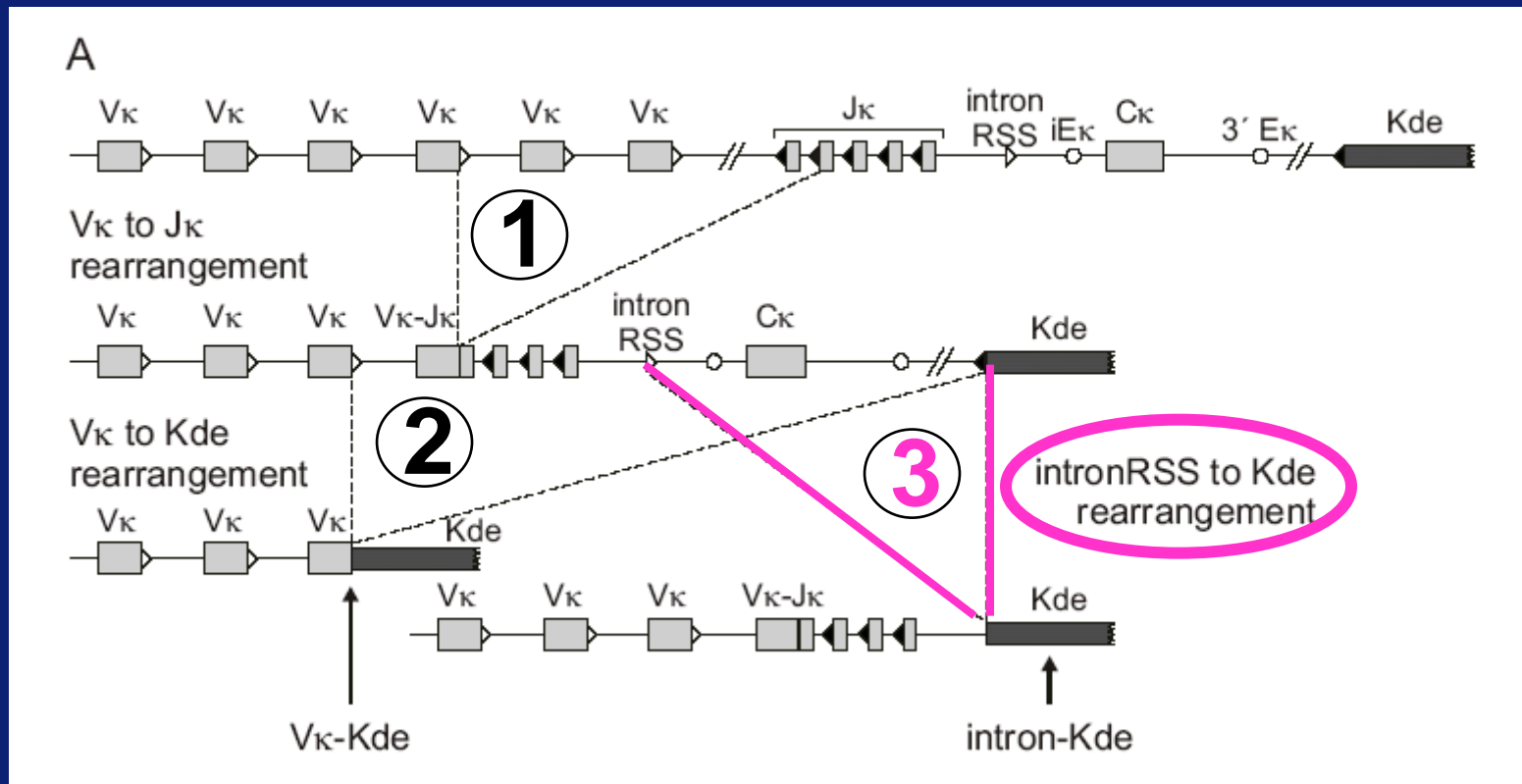
IGK locus : V-J en Kde herschikkingen



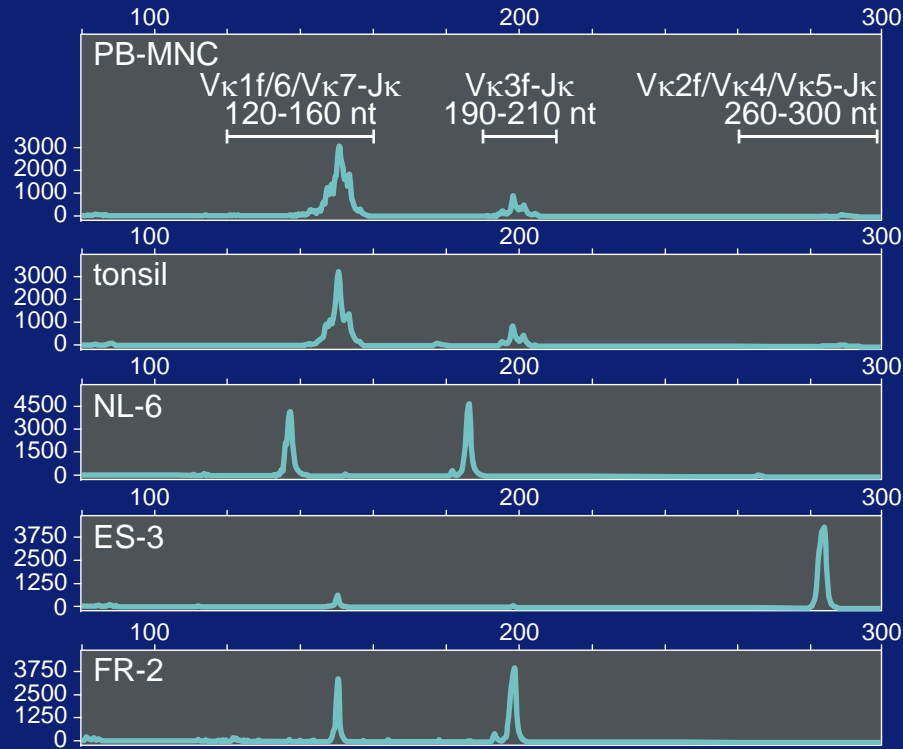
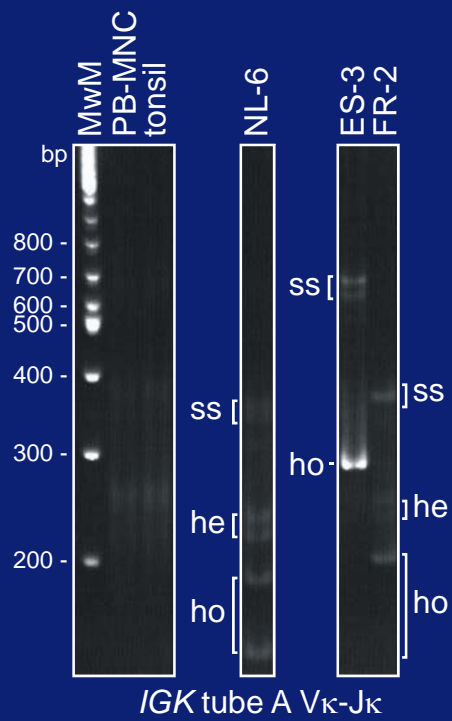
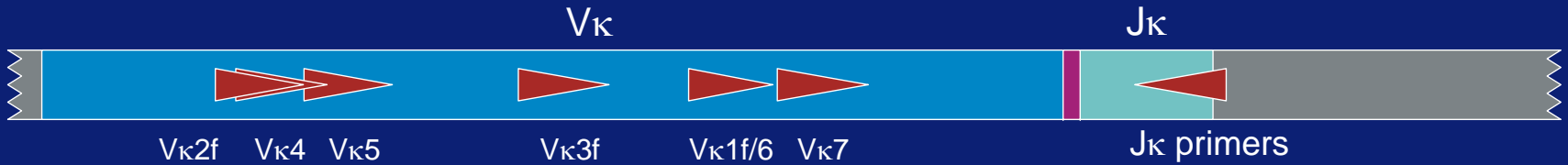
IGK locus : V-J en Kde herschikkingen



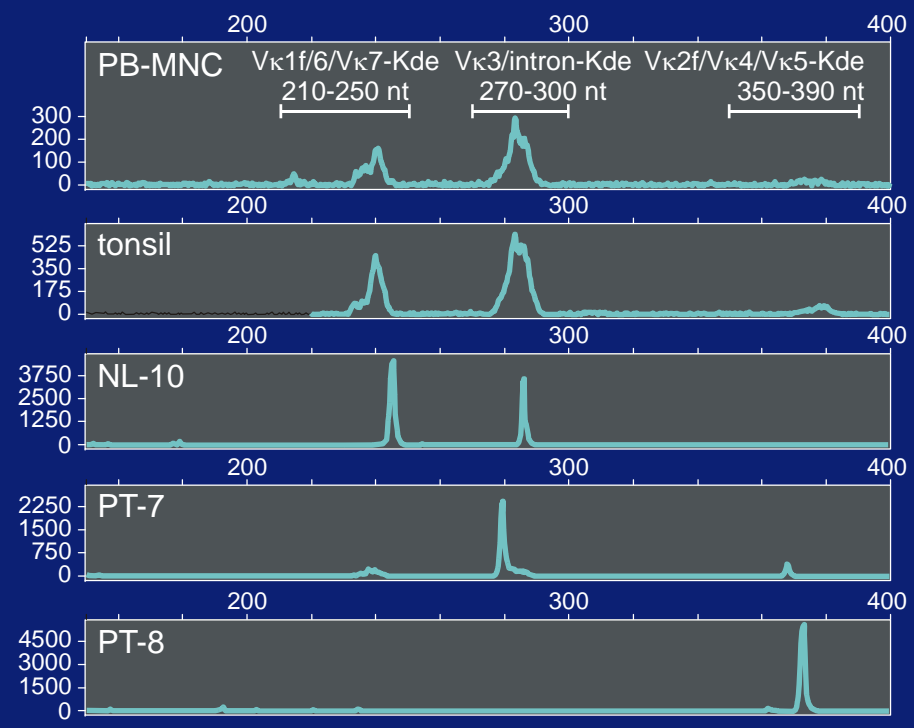
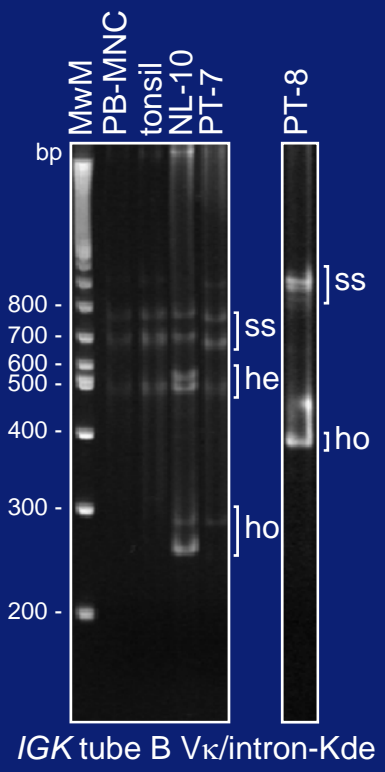
IGK locus : V-J en Kde herschikkingen



BIOMED-2 multiplex IGH tube A: Vκ-Jκ



BIOMED-2 multiplex IGH tube B: Vκ/intron-Kde



Aantal IGK recombinaties in 1 kloon

IGK configuratie	tube A (V-J)	tube B (Kde)	totaal
G / G	-	-	0
V-J / G	1	-	1
V-J / V-J	2	-	2
V-J / V-Kde	1	1	2
V-J + intr-Kde / G	1	1	2
V-Kde / V-Kde	-	2	2
V-J + intr-Kde / V-Kde	1	2	3
V-J+intr-Kde / V-J+intr-Kde	2	2	4

BIOMED-2 multiplex PCR - complementariteit van TCR targets

perifeer T-NHL NOS (n=45, na review)		AILT (n= 36)	
- TCRB	98 %	- TCRB	89 %
- TCRG	93 %	- TCRG	92 %
- TCRB + TCRG	100 %	- TCRB + TCRG	94 %

→ **TCRB** : goed target, toegevoegde waarde in combinatie met TCRG

BIOMED-2 : TCRB analyse



Vβ family primers

Jβ primers

TCRB tubes A and B ↓

TCRB tubes A and C: Jβ A primers

Vβ	Offset	Sequence
Vβ2	(-204)	AACTATGTTTTGGTATCGTCA
Vβ4	(-201)	CACGATGTTCTGGTACCGTCAGCA
Vβ1/5	(-197)	CAGTGTGCTCTGGTACCAACAG
Vβ6a/11	(-201)	AACCCCTTTATTGGTACCGACA
Vβ6b/25	(-201)	ATCCCTTTTTTGGTACCAACAG
Vβ6c	(-201)	AACCCCTTTATTGGTATCAACAG
Vβ7	(-198)	CGCTATGTATTGGTACAAGCA
Vβ8a	(-201)	CTCCCGTTTTCTGGTACAGACAGAC
Vβ9	(-198)	CGCTATGTATTGGTATAAACAG
Vβ10	(-201)	TTATGTTTACTGGTATCGTAAGAAGC
Vβ11	(-198)	CAAAATGTACTGGTATCAACAA
Vβ3/12a/13a/15	(-198)	ATACATGTACTGGTATCGACAAGAC
Vβ13b	(-198)	GGCCATGTACTGGTATAGACAAG
Vβ12b/13c/14	(-198)	GTATATGTCTGGTATCGACAAGA
Vβ16	(-201)	TAACCTTTATTGGTATCGACGTGT
Vβ17	(-198)	GGCCATGTACTGGTACCGACA
Vβ18	(-201)	TCATGTTTACTGGTATCGGCAG
Vβ19	(-201)	TTATGTTTATTGGTATCAACAGAATCA
Vβ20	(-???)	CAACCTATACTGGTACCGACA
Vβ21	(-201)	TACCCTTTACTGGTACCGGCAG
Vβ22	(-201)	ATACTTCTATTGGTACAGACAAATCT
Vβ8b/23	(-201)	CACGGTCTACTGGTACCAGCA
Vβ24	(-197)	CGTCATGTACTGGTACCAGCA

Sequence	Offset	Jβ primer
GTGGTCTAAGTGCAACATCCATTC	(+53)	Jβ1.1
CTGGTCCAATTGGCAACATCCATTC	(+53)	Jβ1.2
TTCAACCGAGTGACAACATCCATTC	(+55)	Jβ1.3
CTTGGGTCGAGAGACAGAACCATAAC	(+56)	Jβ1.4
CTGAGCTGAGAGGTAGGATCCATTC	(+55)	Jβ1.5
GTCCGAGTGACACTGTCCATAC	(+58)	Jβ1.6
TCCGACTGGCATGACCCATTC	(+56)	Jβ2.2
TCCGACTGGCAGACCCGCTC	(+58)	Jβ2.6
GTCCGAGTGCCAATGTCCATTC	(+52)	Jβ2.7

TCRB tubes B and C: Jβ B primers

Sequence	Offset	Jβ primer
AGTGGCACGATCCATTCTTCC	(+59)	Jβ2.1
ACTGTCACGAGCCATTGCCCC	(+58)	Jβ2.3
AGAGTCACGACCCATTGACC	(+59)	Jβ2.4
CACGAGCCACACGCGC	(+57)	Jβ2.5



Dβ1 primer

Dβ2 primer

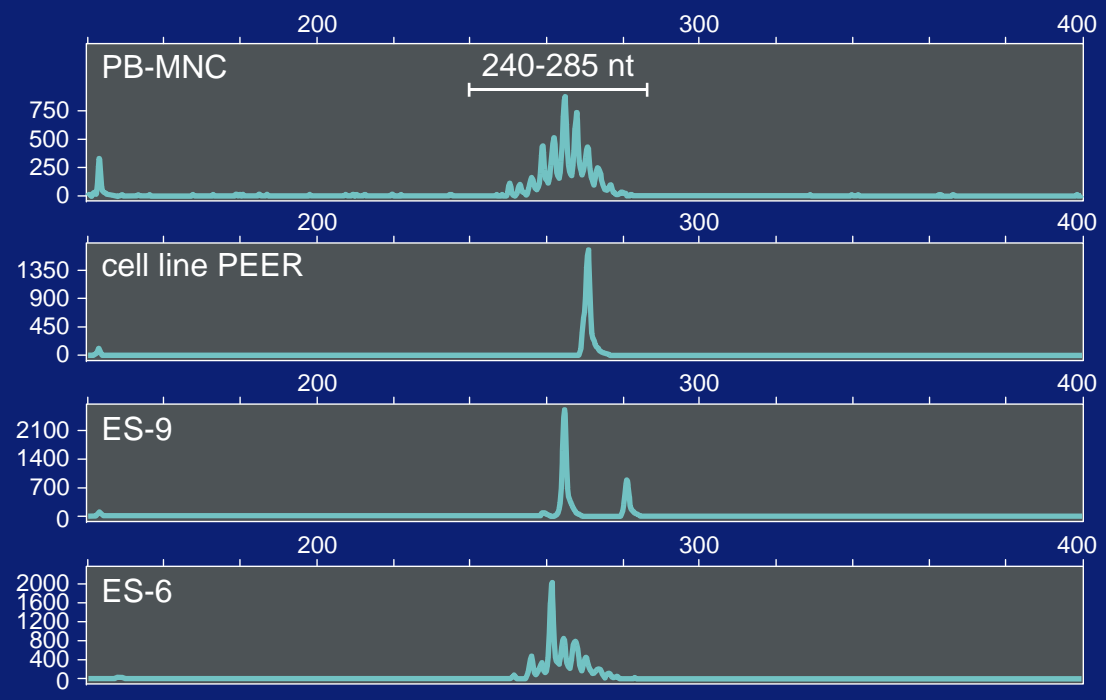
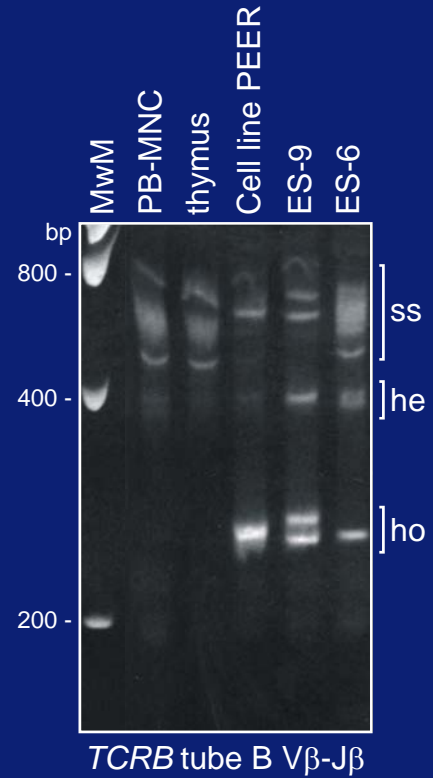
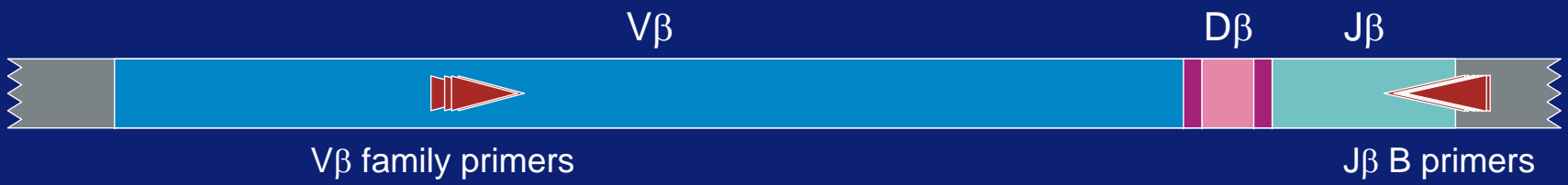
Jβ primers

TCRB tube C

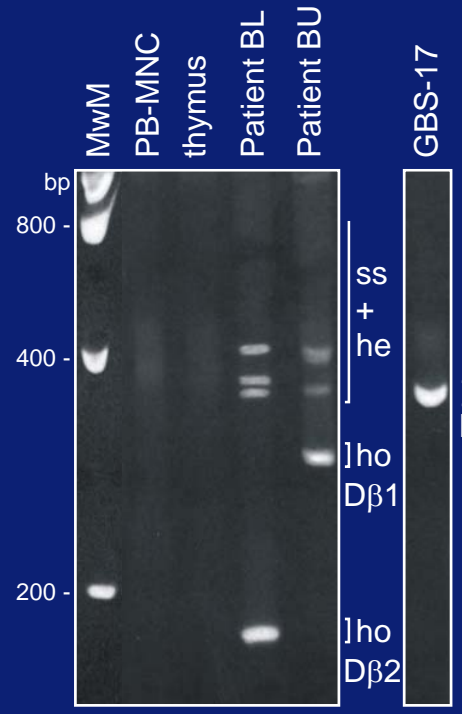
Dβ1 (-252) 5' GCGAAACAGCCTTACAAAGAC 3'

Dβ2 (-137) 5' TTTCCAAGCCCCACACAGTC 3'

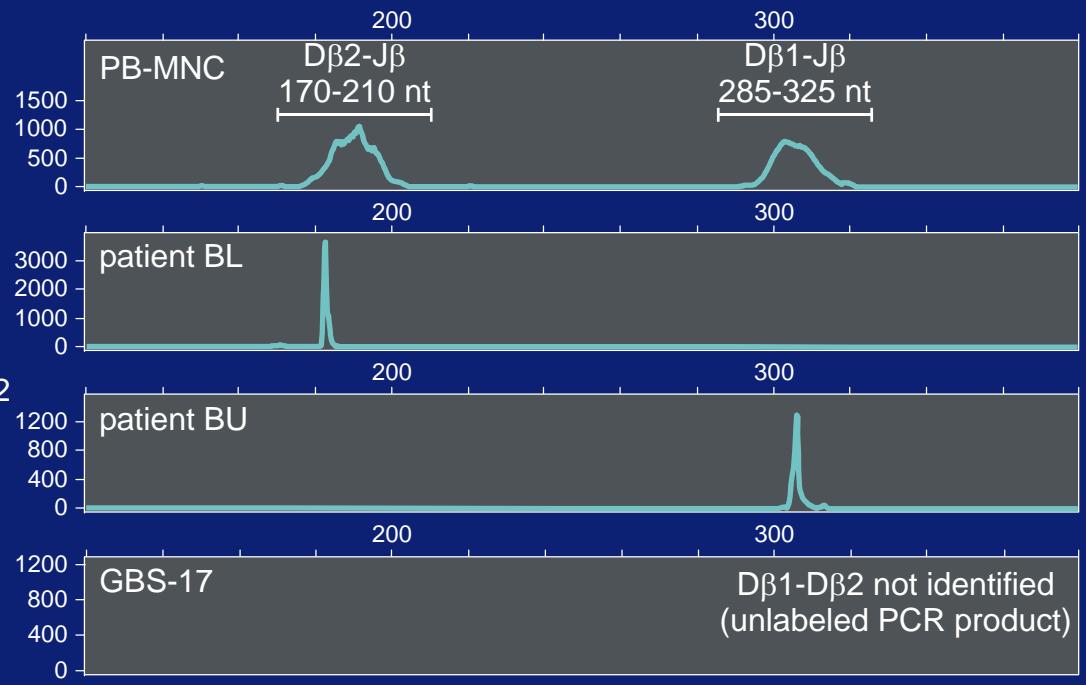
BIOMED-2 multiplex TCRB tube B: V β -J β



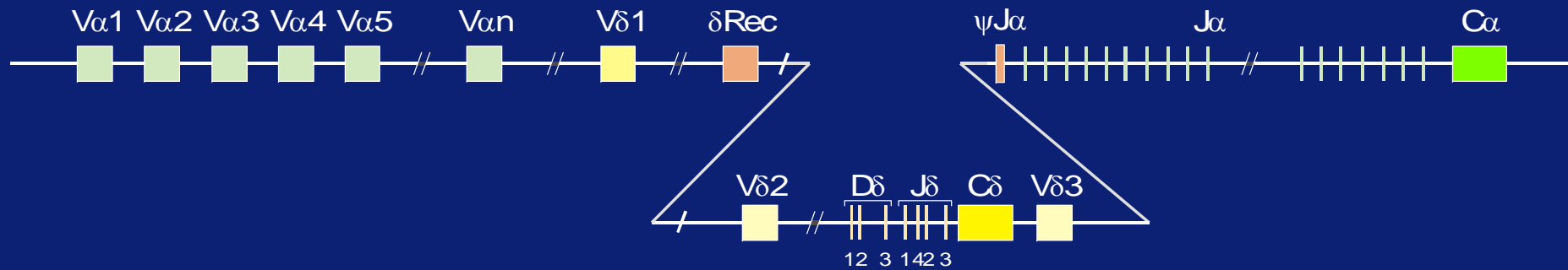
BIOMED-2 multiplex TCRB tube C: Db-Jb



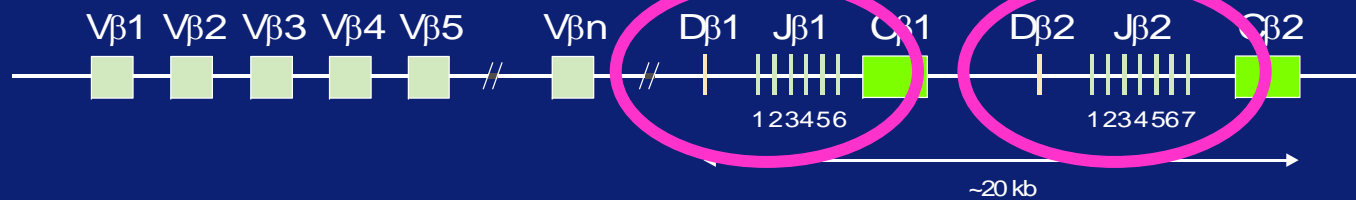
TCRB tube C Dβ-Jβ



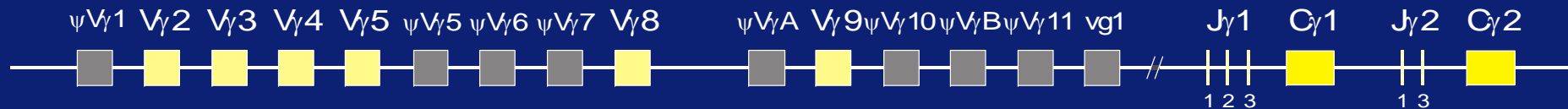
TCR- α and TCR- δ gene complex



TCR- β gene complex



TCR- γ gene complex



Aantal TCRB recombinaties in 1 kloon

<i>TCRB</i> configuratie	tube A + B (V-J)	tube C (D-J)	totaal
<i>G / G</i>	-	-	0
<i>D-J / G</i>	-	1	1
<i>V-J / G</i>	1	-	1
<i>V-J / V-J</i>	2	-	2
<i>D-J1 + D-J2 / G</i>	-	2	2
<i>V-J1 + D-J2 / G</i>	1	1	2
<i>V-J1 + D-J2 / V-J</i>	2	1	3
<i>V-J1 + D-J2 / D-J1 + D-J2</i>	1	3	4
<i>V-J1 + D-J2 / V-J1 + D-J2</i>	2	2	4

Technische pitfalls in Ig/TCR analyse - productgroottes

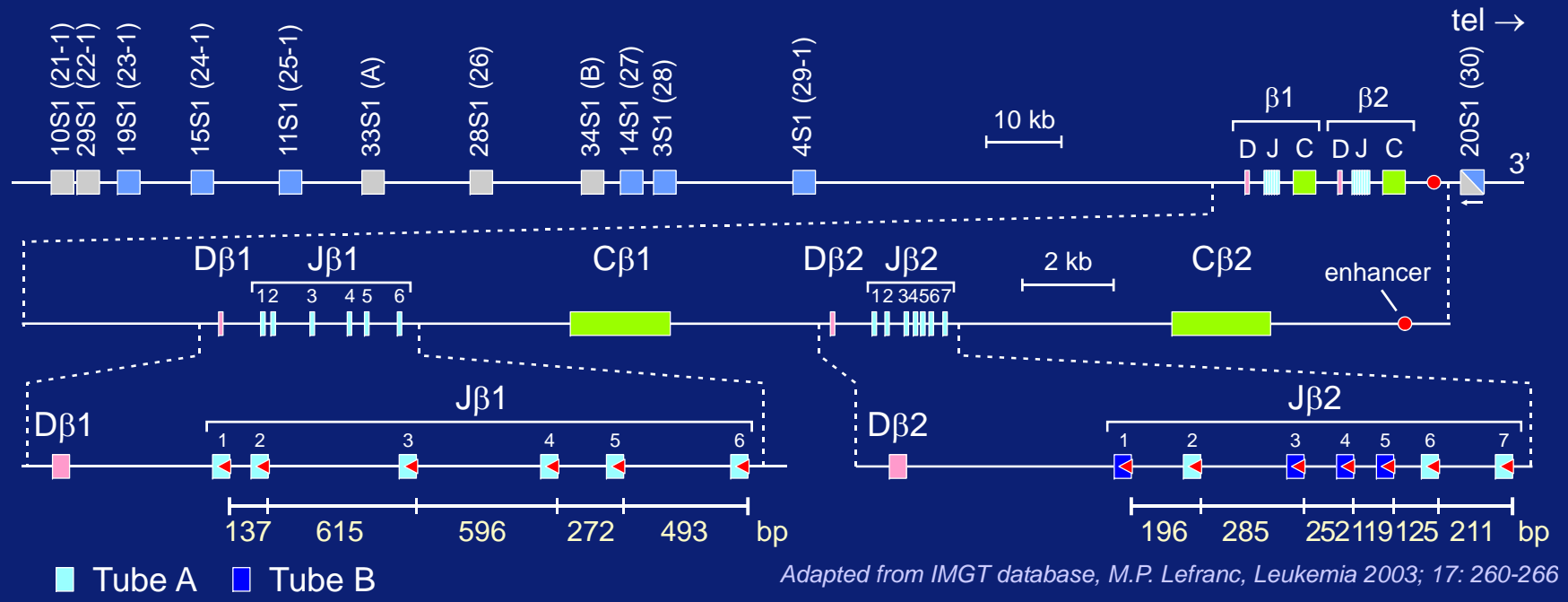
<i>Pitfall</i>	<i>Phenomenon</i>	<i>Solution / action</i>
bands / peaks <u>just</u> outside size range	CDR3 regions / junctions outside 5-95% size range interval	accept as true rearrangement product; in case of doubt , sequence for confirmation
undersized bands / peaks	internal deletion in e.g. V gene (due to e.g. SHM)	potential rearrangement product; final proof by sequencing
oversized bands / peaks	extended amplification from downstream J (due to e.g. SHM in actual J gene)	potential rearrangement product; final proof by sequencing

Langerak, *Leukemia Res* 2008;32:203

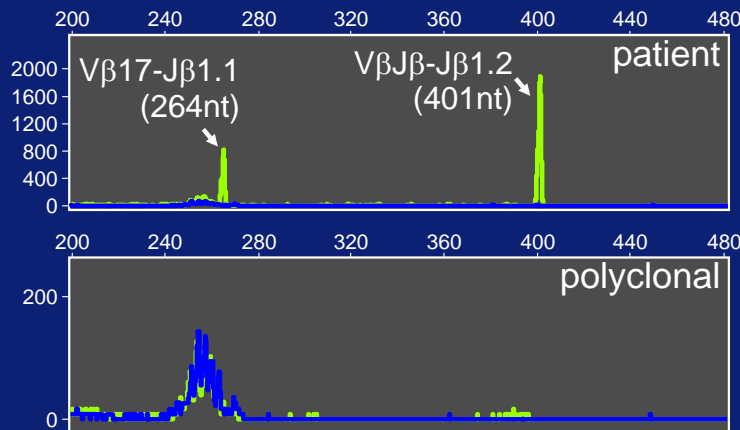
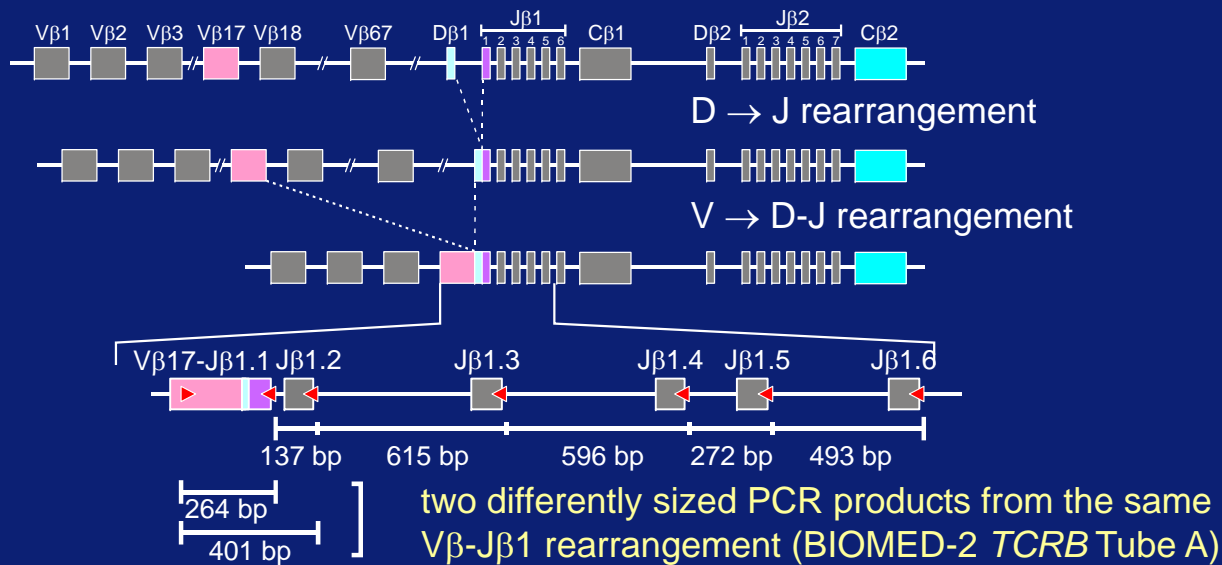
Vargas, *Leukemia Res* 2008;32:335

Langerak, *Expert Opin Med Diagn* 2007;1:451

TCRB locus : J genes dicht bij elkaar



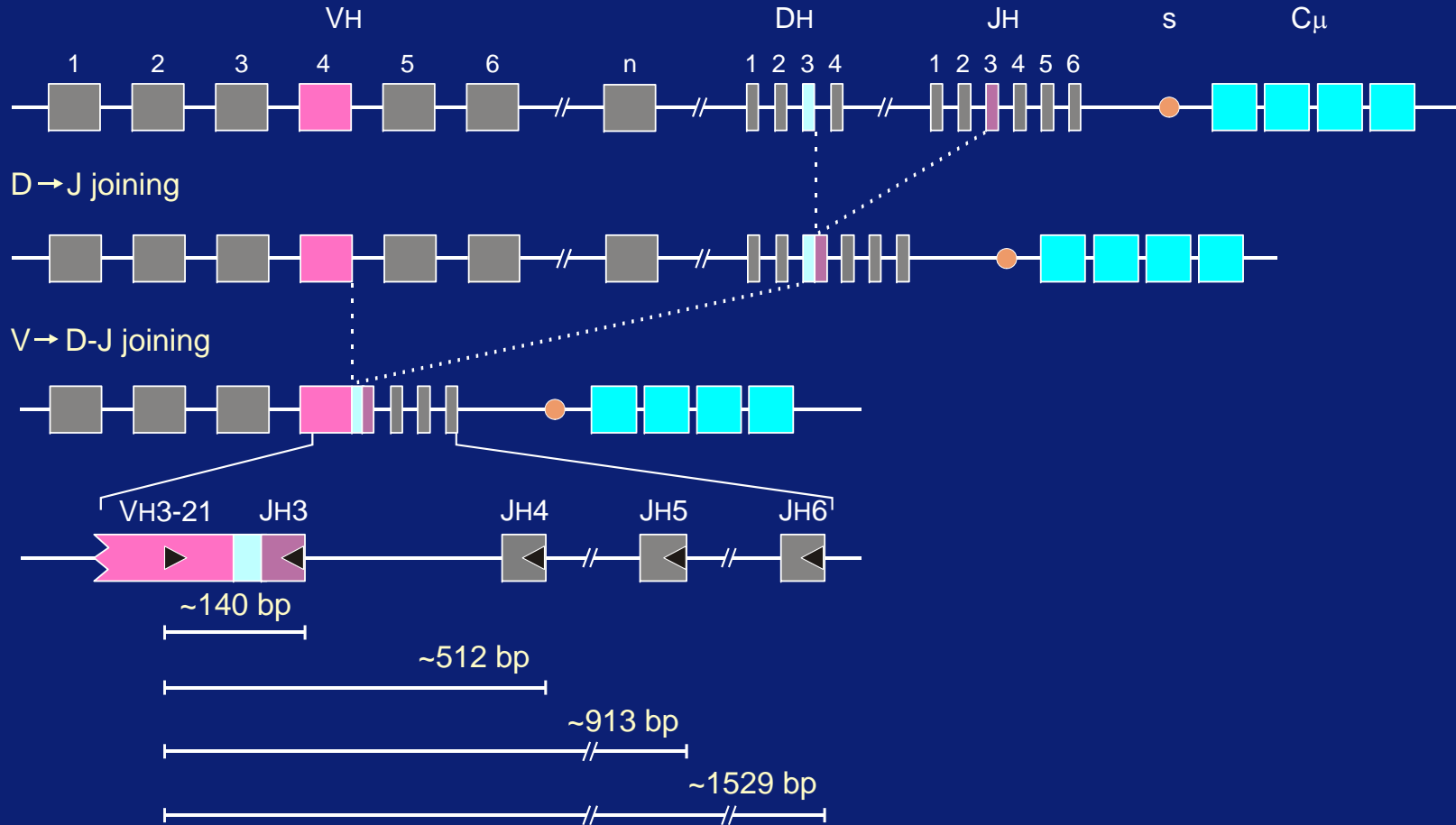
TCRB : twee producten van dezelfde herschikking



Langerak, *Leukemia Res* 2008;32:203

Langerak, *Expert Opin Med Diagn* 2007;1:451

IGH locus : verlengde PCR producten



Biologische pitfalls in Ig / TCR analyse

<i>Pitfall</i>	<i>Phenomenon</i>	<i>Solution / action</i>
oligoclonal T- / (B)-cell repertoire in PB of especially elderly individuals	incomplete immune system, due to e.g. immunosenescence	repeat PCR in triplicate or quadruplicate (same or related tissue) → compare patterns for consistency and compare with primary process (staging)
oligo- / monoclonality in histologically reactive lesion	exaggerated immune response with dominant specificity, presence of large germinal centers	1.repeat PCR in triplicate or quadruplicate (same or related tissue) → compare patterns for consistency 2.(re)evaluate histopathology
selective amplification and pseudoclonality, due to low level of specific template	few T/B cells in sample	repeat PCR in triplicate or quadruplicate (same or related tissue) → compare patterns for consistency

clonaliteitsanalyse =
herkenning van moleculair patroon

“moleculaire morfologie”

→ “common language” om patronen te scoren