

**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

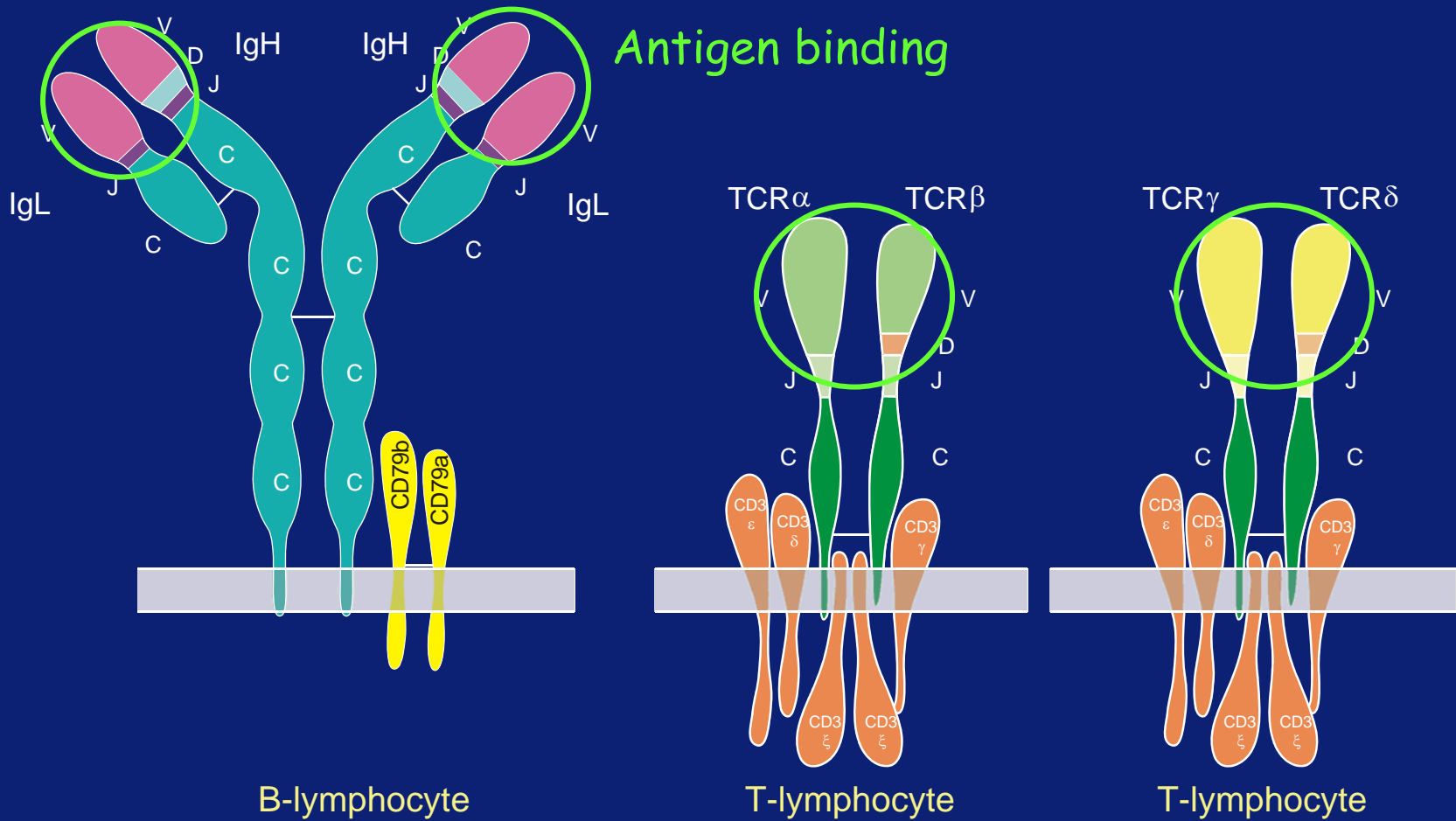
⇒ **Diagnose** : tumor (= kloon van cellen) of reactie op infectie ?

**Klonaliteitsanalyse** op basis van Ig/TCR herschikkingen

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

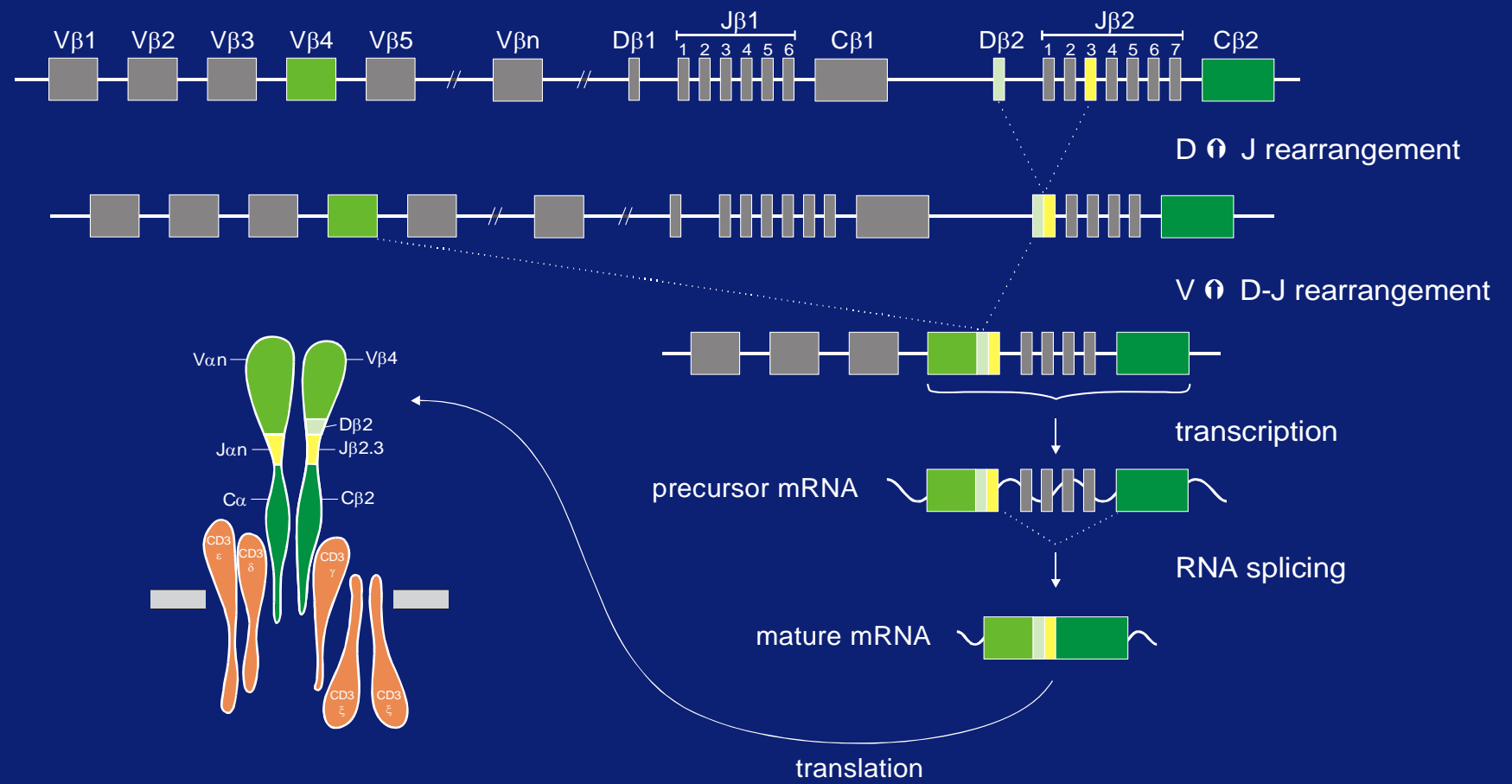
⇒ **MRD analyse** : monitoren van therapie effect

# B- en T-celreceptoren

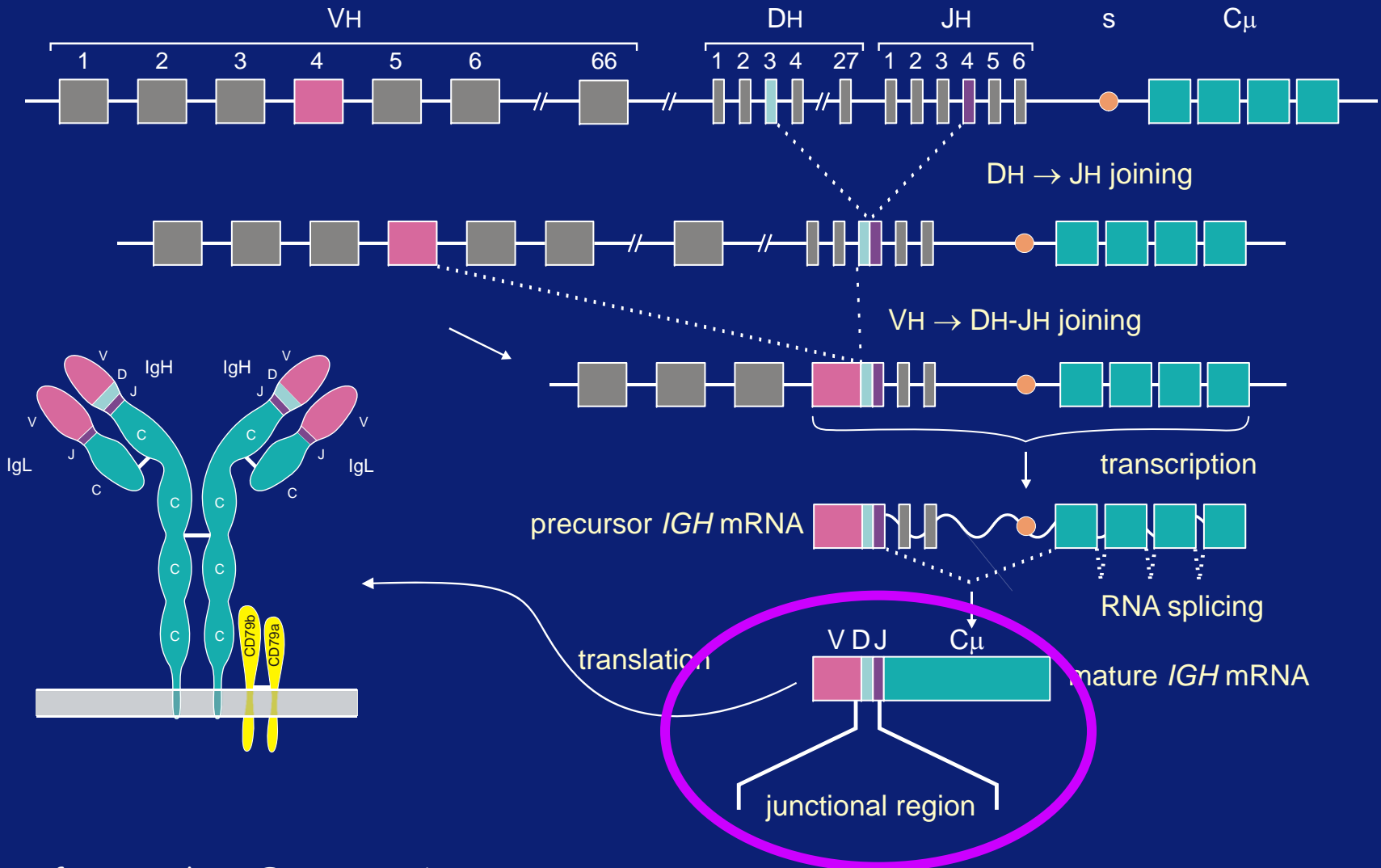


# TCRB generschikking

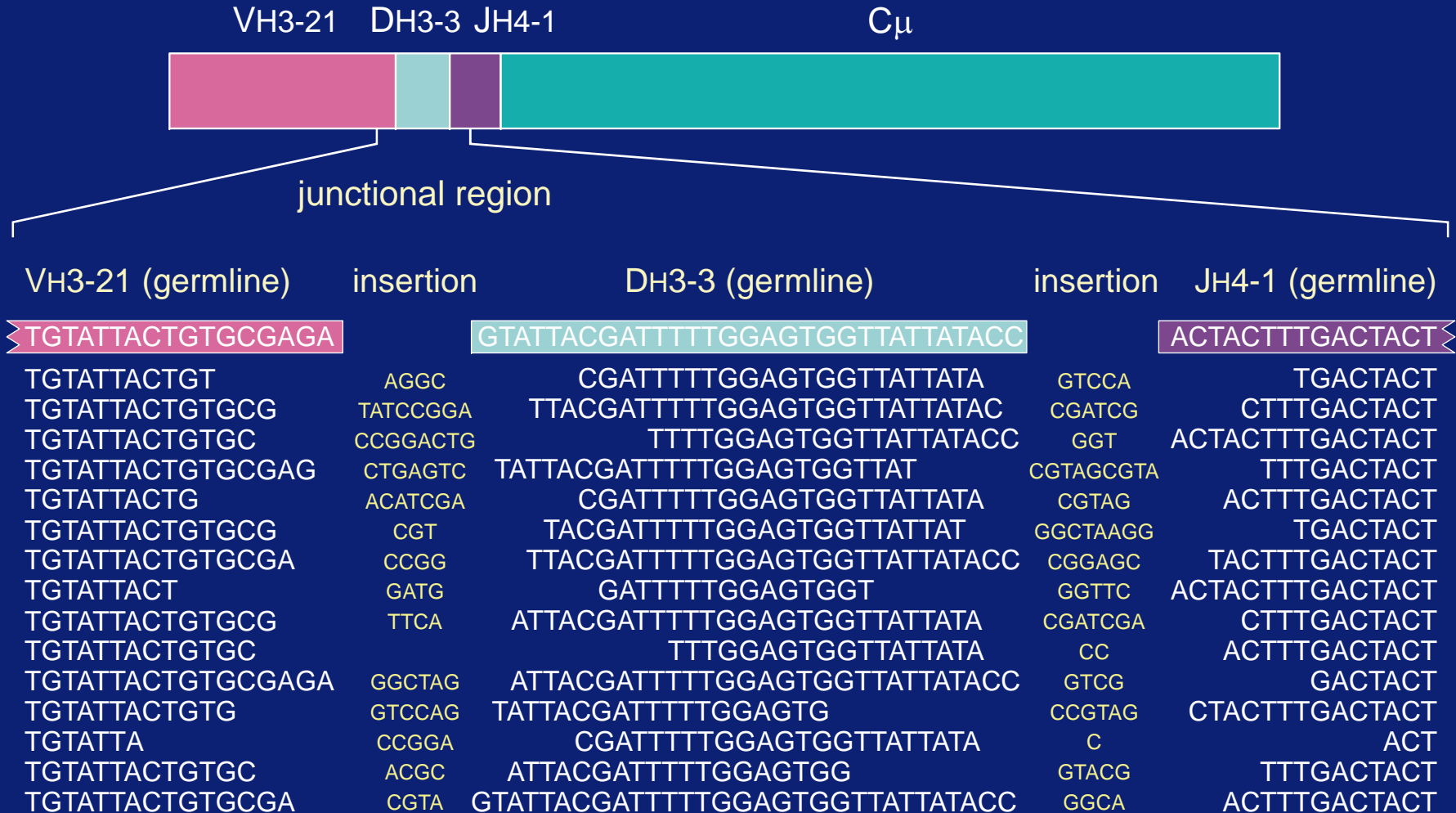
TcR=β gene rearrangement



# IGH generschikking



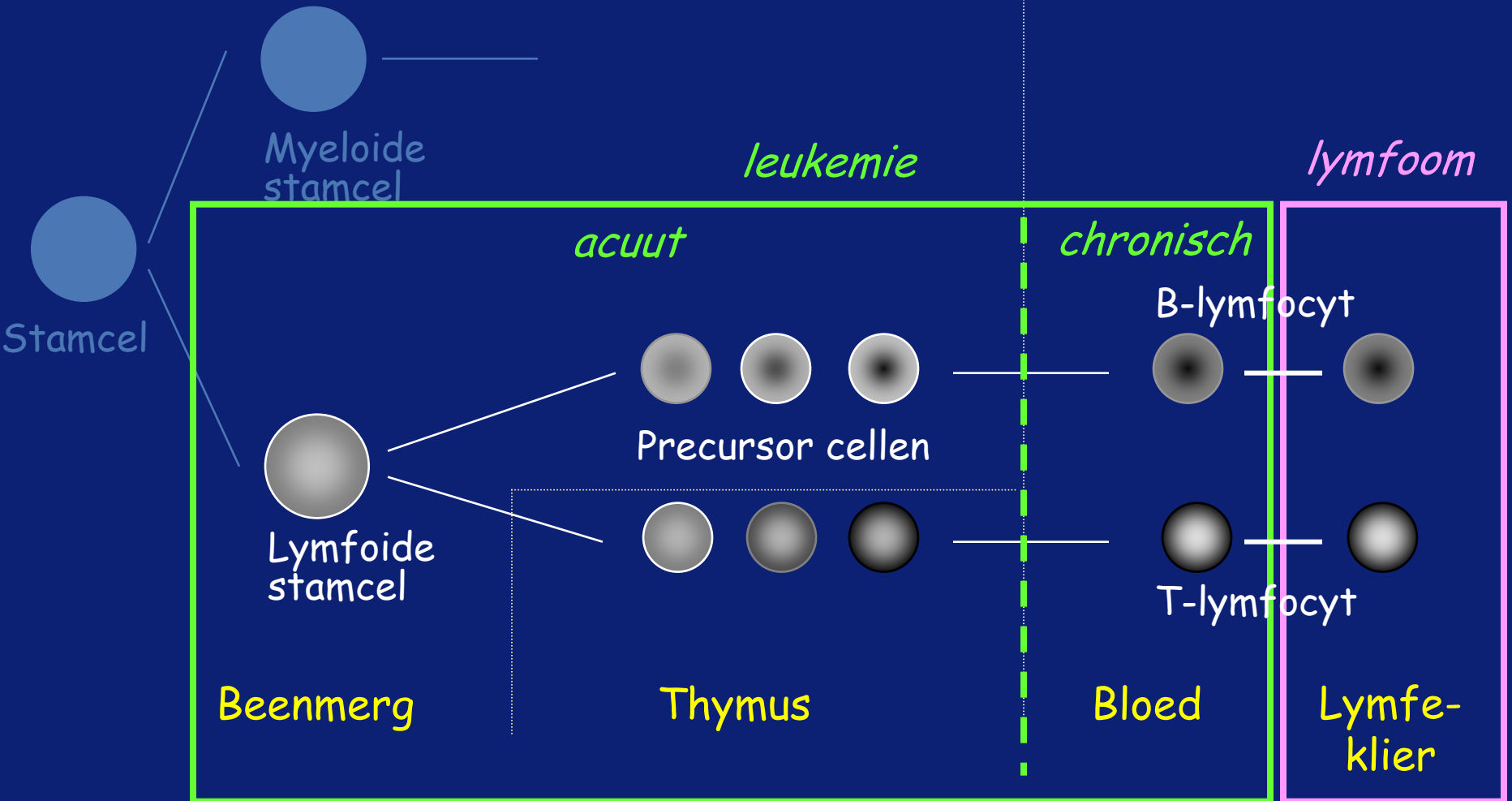
# Meer diversiteit → junctional region



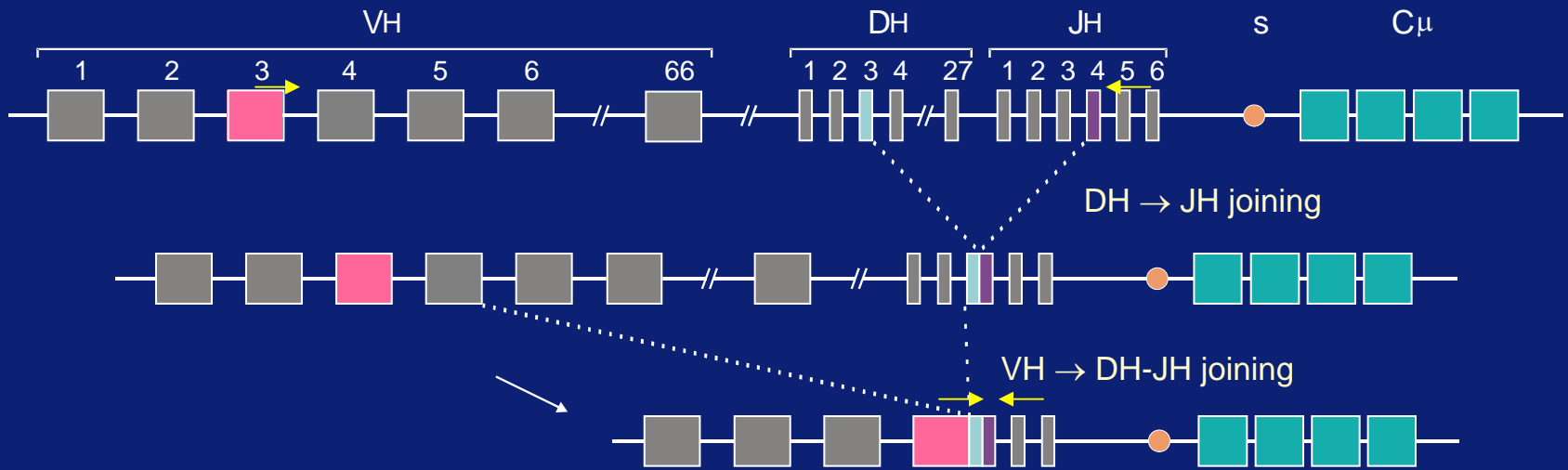
# Schatting van potentiële primaire repertoire van humane Ig en TCR moleculen

	IgH	Igκ	Igλ	TCR $\alpha\beta$		TCR $\gamma\delta$	
				TCR $\alpha$	TCR $\beta$	TCR $\gamma$	TCR $\delta$
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 <sup>6</sup>			> 5 × 10 <sup>6</sup>		> 5000	
Junctional region diversiteit	++	±	±	+	++	++	++++
Schatting van totale primaire receptor repertoire	> 10 <sup>12</sup>			> 10 <sup>12</sup>		> 10 <sup>12</sup>	

# Leukemieën en lymfomen vs. lymfocyten

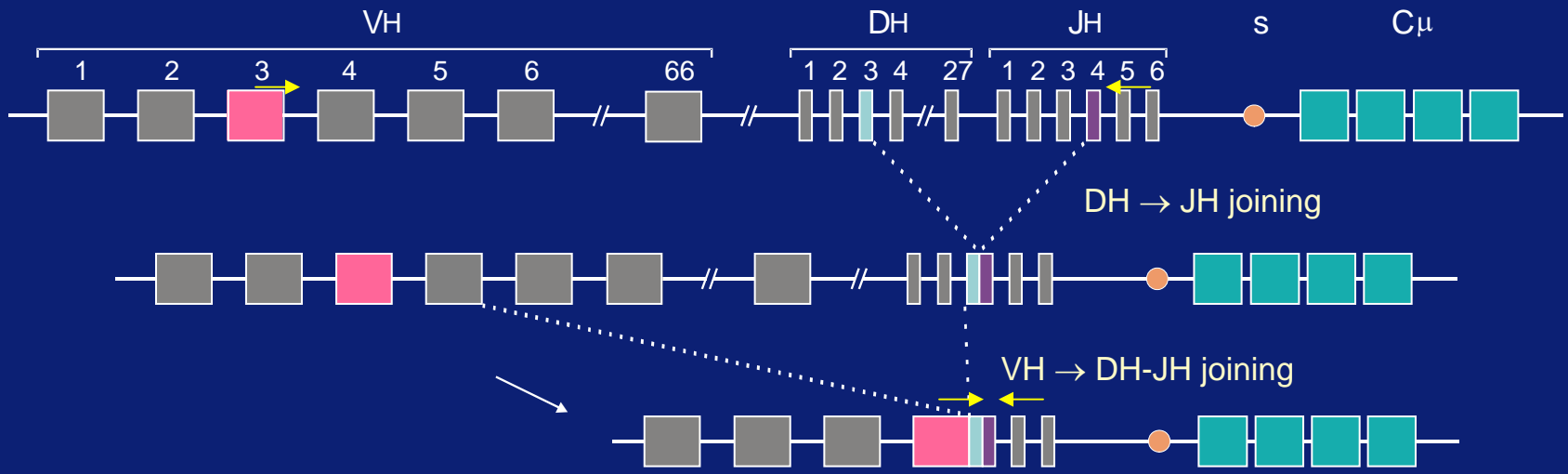


# Rationale PCR-gebaseerde analyse



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

# Rationale PCR-gebaseerde analyse



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

# PCR-gebaseerde klonaliteitsanalyse - valkuilen + oplossingen

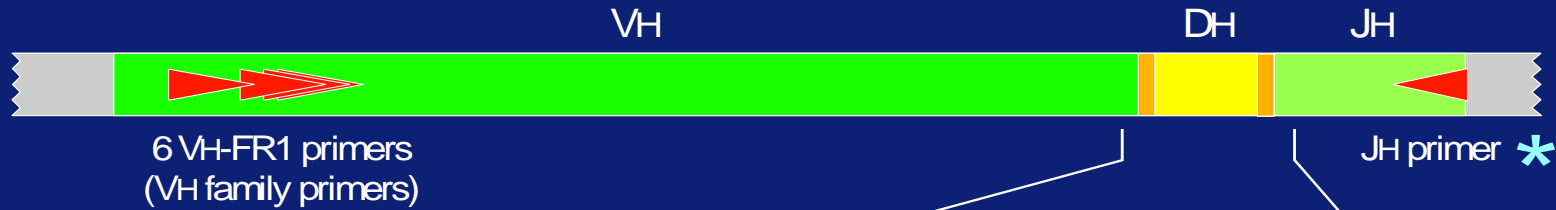
## vals-positiviteit

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



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

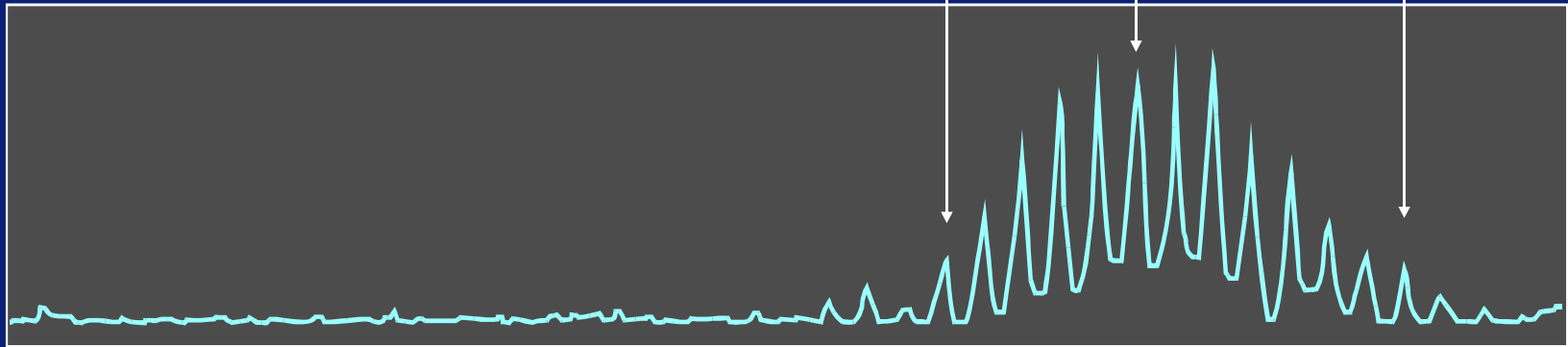
# Klonaliteitsanalyse : GeneScan



hoeveelheid  
fluorescent  
product

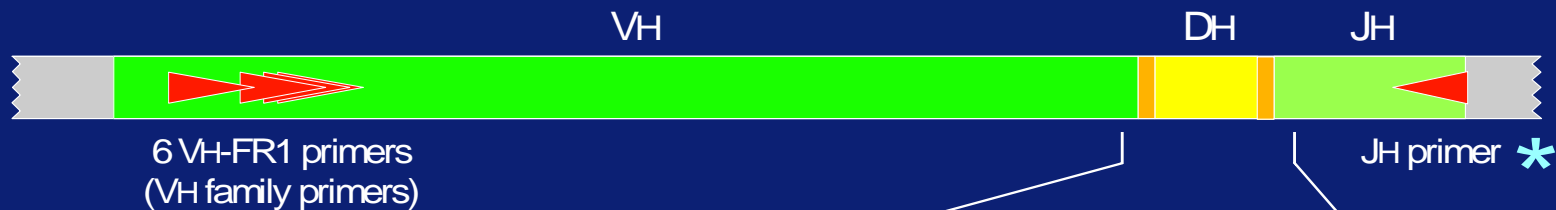
```

CTGTGCAAGAGCGGGCTATGGTTCAGGGAGTTATGGCTACTACGGTATGGACGTCTGG
CTGTGCAAGAGGACGAAACAGTAACTGcCTACTACTACTACGGTATGGACGTCTGG
CTGTGCAAGAGAGATAGTATAGCAGCTCGTACAACCTGGTTCGACTCCTGG
CTGTGCAAGAGATCCGGGcAGCTCGTTTTGCTTTTGATATCTGG
CTGTGCAAGAGcctctctccactgggatgggggctactgg
CTGTGCAAGAGcagcagctcggcccccctttgactactgg
CTGTGCAAGAGgactttggatgcttttgatatctgg
CTGTGCAAGAGggtgggagctactagactactgg
CTGTGCAAGggtagctaaacctttgactactgg
CTGTGCAATTATCTACTTTGACTACTGG
    
```



lengte van DNA fragmenten

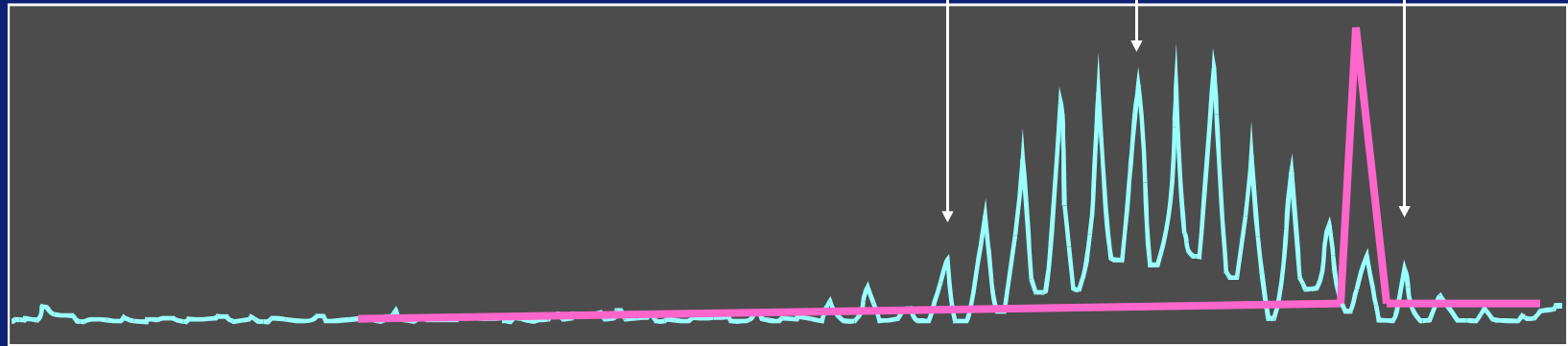
# Klonaliteitsanalyse : heterogeen of identiek?



hoeveelheid fluorescent product

```

CTGTGCAAGAGCGGGCTATGGTTCAGGGAGTTATGGCTACTACGGTATGGACGTCTGG
CTGTGCAAGAGGACGAAACAGTAACTGcCTACTACTACTACGGTATGGACGTCTGG
CTGTGCAAGAGAGATAGTATAGCAGCTCGTACAACCTGGTTCGACTCCTGG
CTGTGCAAGAGATCCGGGcAGCTCGTTTTGCTTTTGATATCTGG
CTGTGCAAGAGcCTCTCTCCACTGGATGGGGGGCTACTGG
CTGTGCAAGAGcAGCAGCTCGGCCcCTTTGACTACTGG
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CTGTGCAAGAGGGTGGGAGCTACTAGACTACTGG
CTGTGCAAGGGTAGCTAAACCTTTGACTACTGG
CTGTGCAATTATCTACTTTGACTACTGG
    
```



# Heteroduplex analyse : band of smear ?

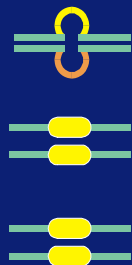
monoclonal cells

monoclonal cells in  
polyclonal background

polyclonal cells



denaturation / renaturation



1

2

3

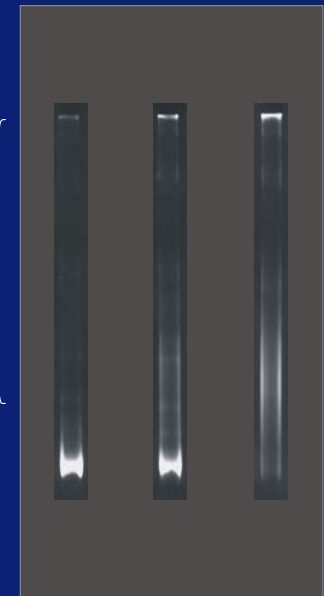


heteroduplexes



homoduplex

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)

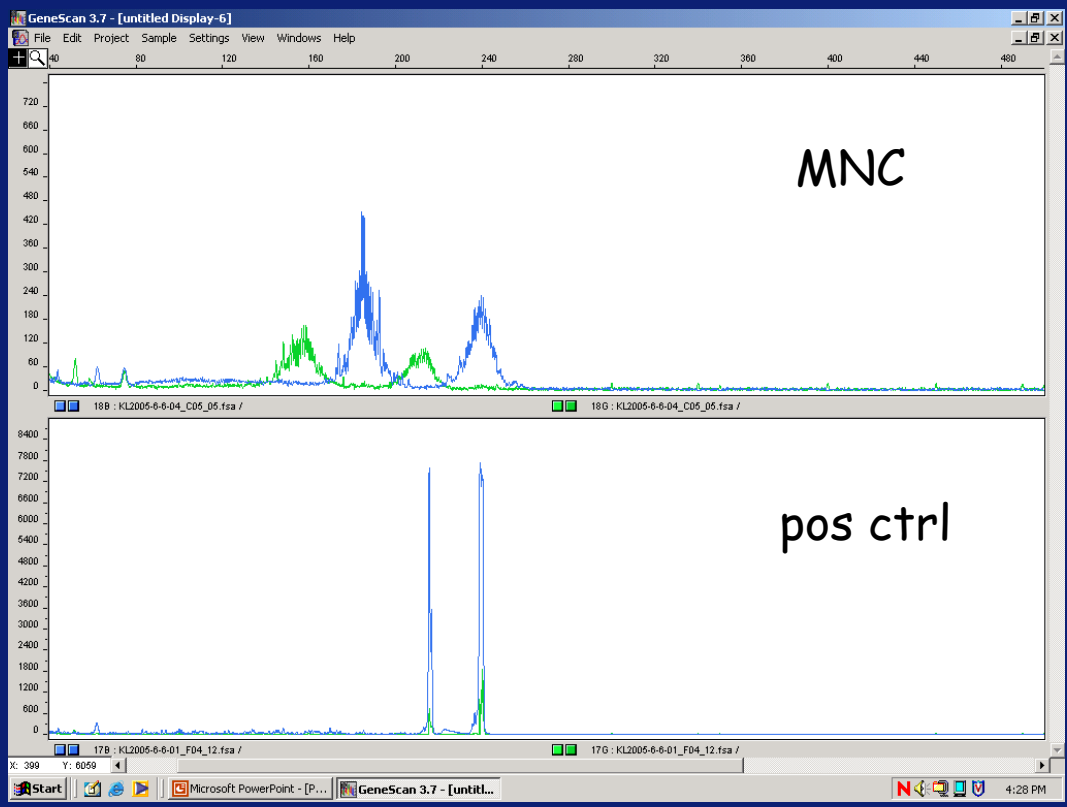
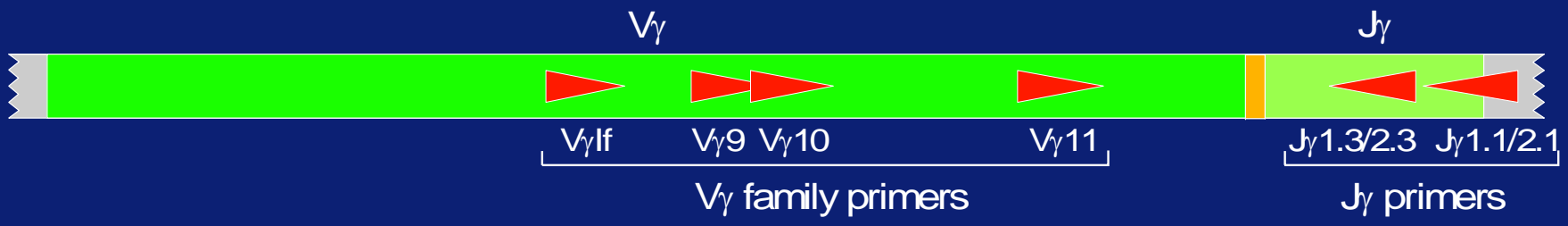


**meerdere primersets** per target (IGH)



**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

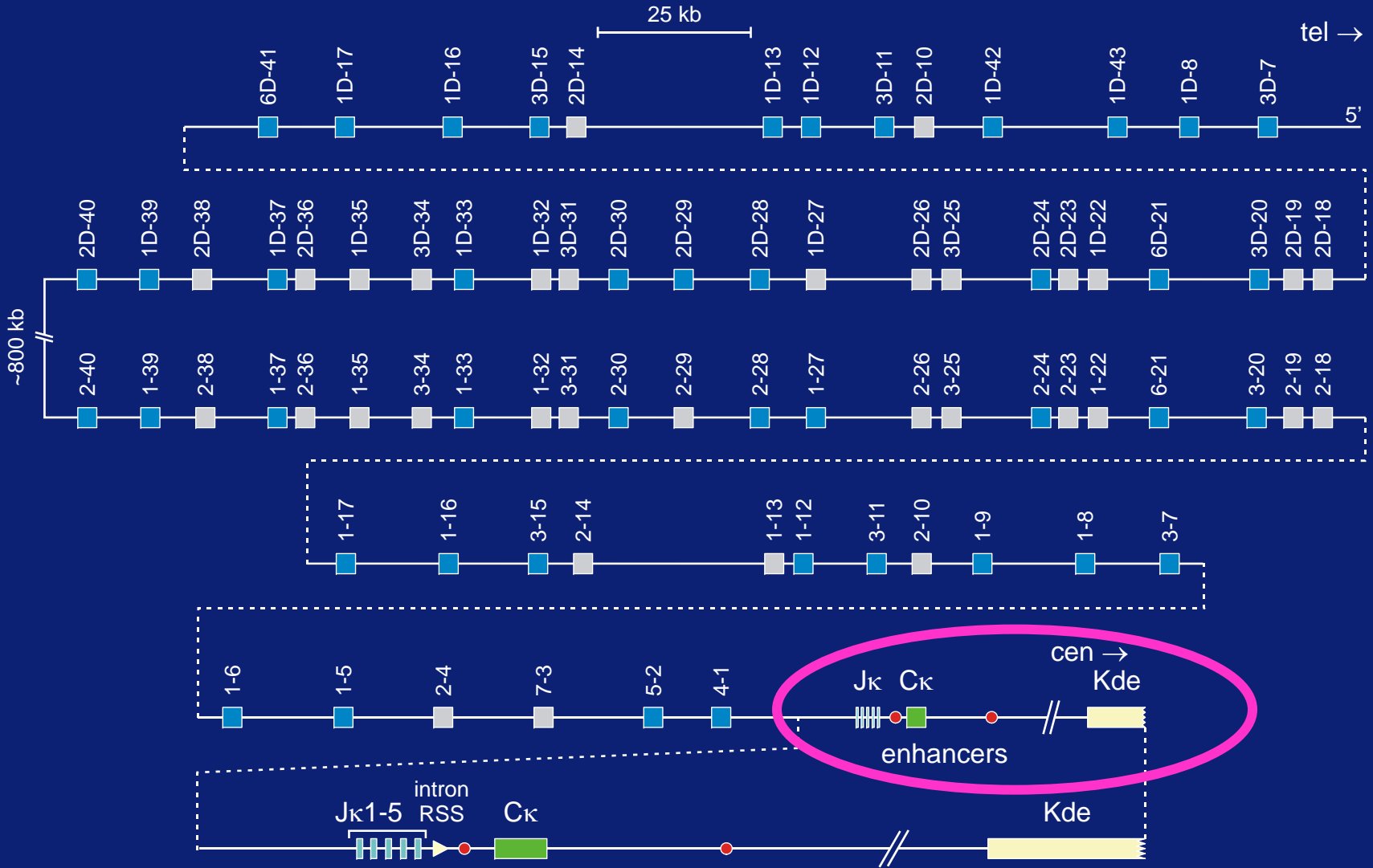
IGH tubes A, B, and C  
 3' CCAGTGGCAGAGGAGTCCATTC 5' (+57) JH consensus

# BIOMED-2 multiplex PCR - complementariteit van Ig targets

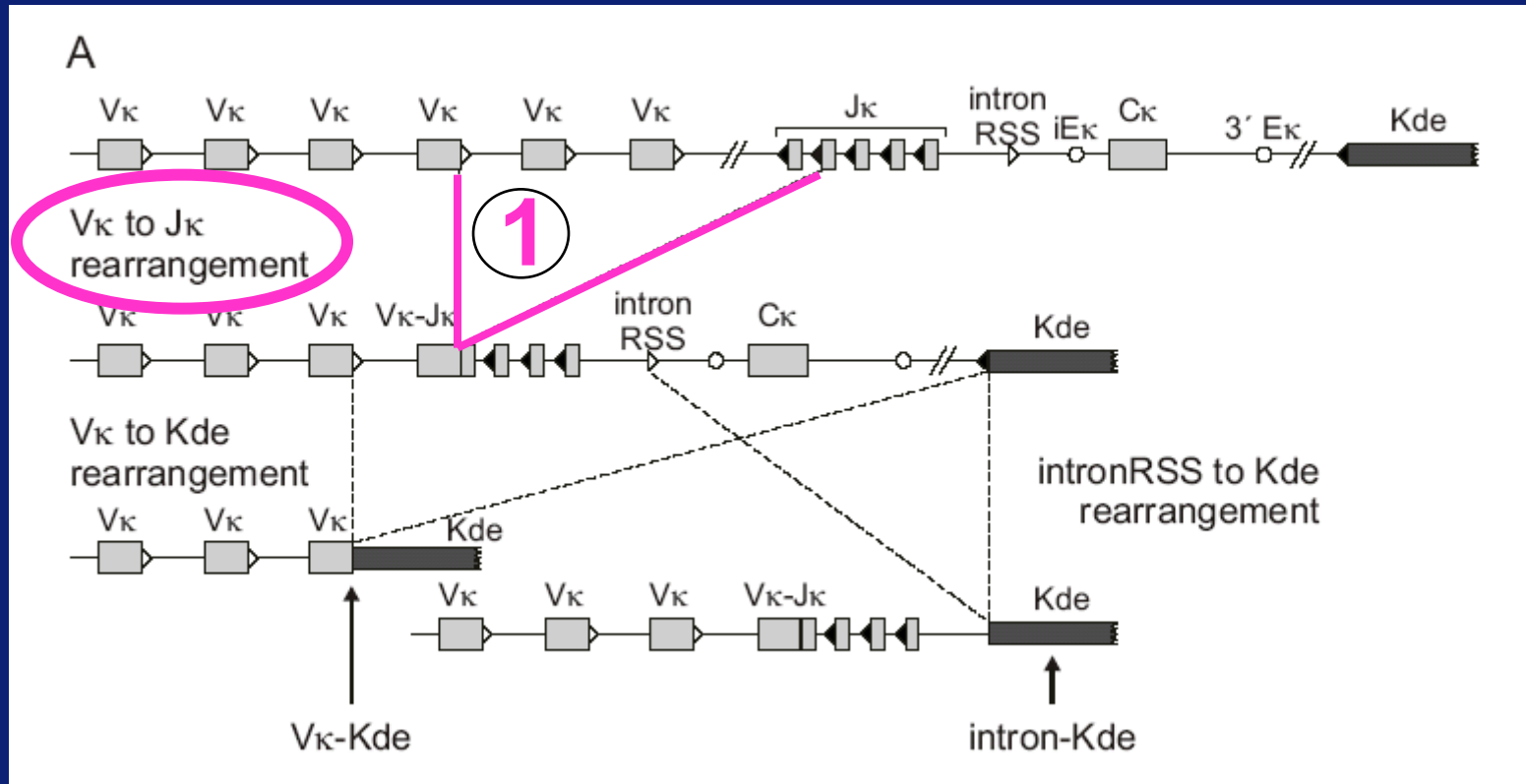
DLBCL (n=116)		FCL (n=109)	
- FR1	67 %	- FR1	73 %
- FR2	57 %	- FR2	76 %
- FR3	47 %	- FR3	52 %
- alle FR	77 %	- alle FR	84 %
- IGH + <i>IGK</i>	91 %	- IGH + <i>IGK</i>	100 %

~10 % zonder klonale Ig targets

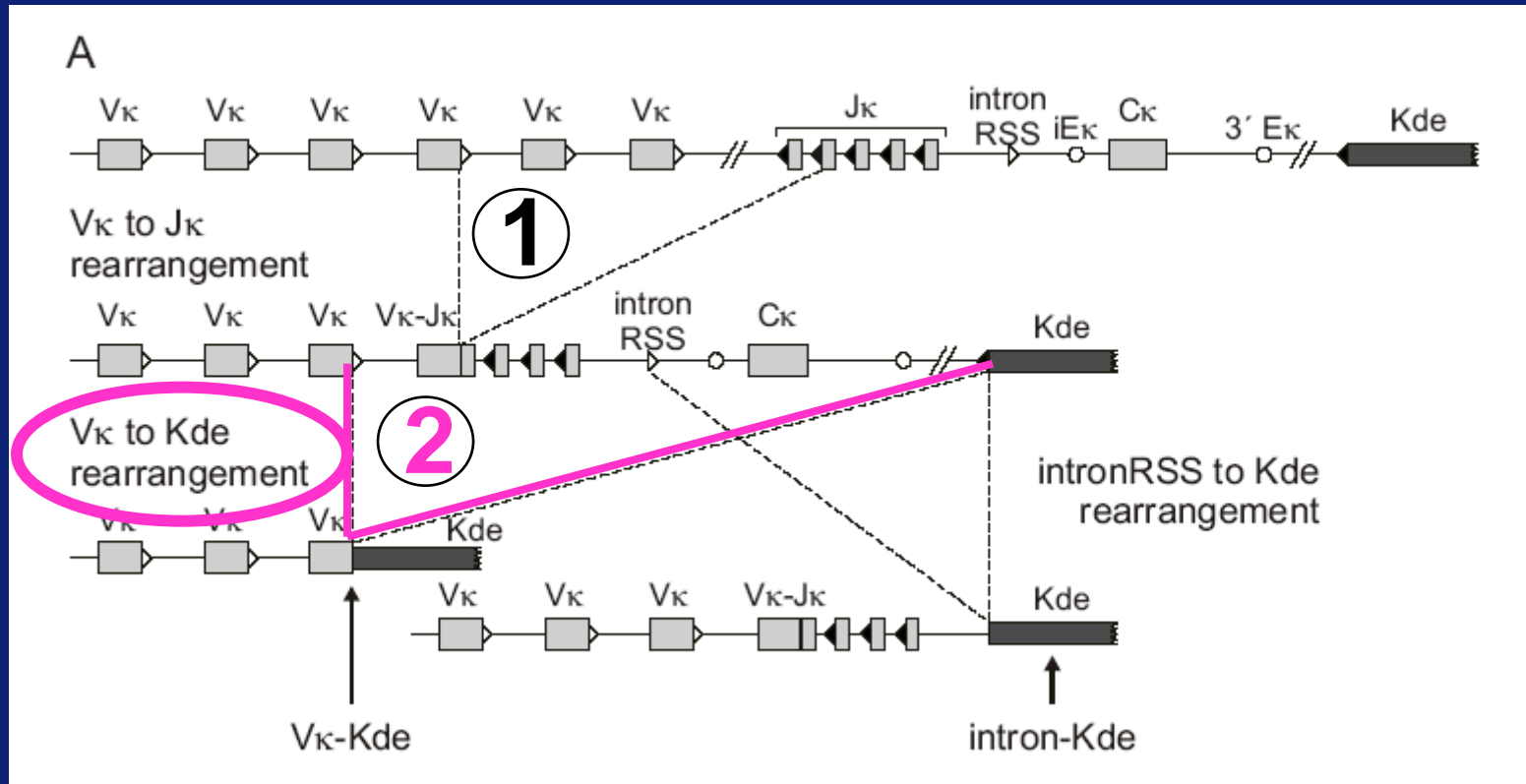
# Human IGH locus (#2p11.2)



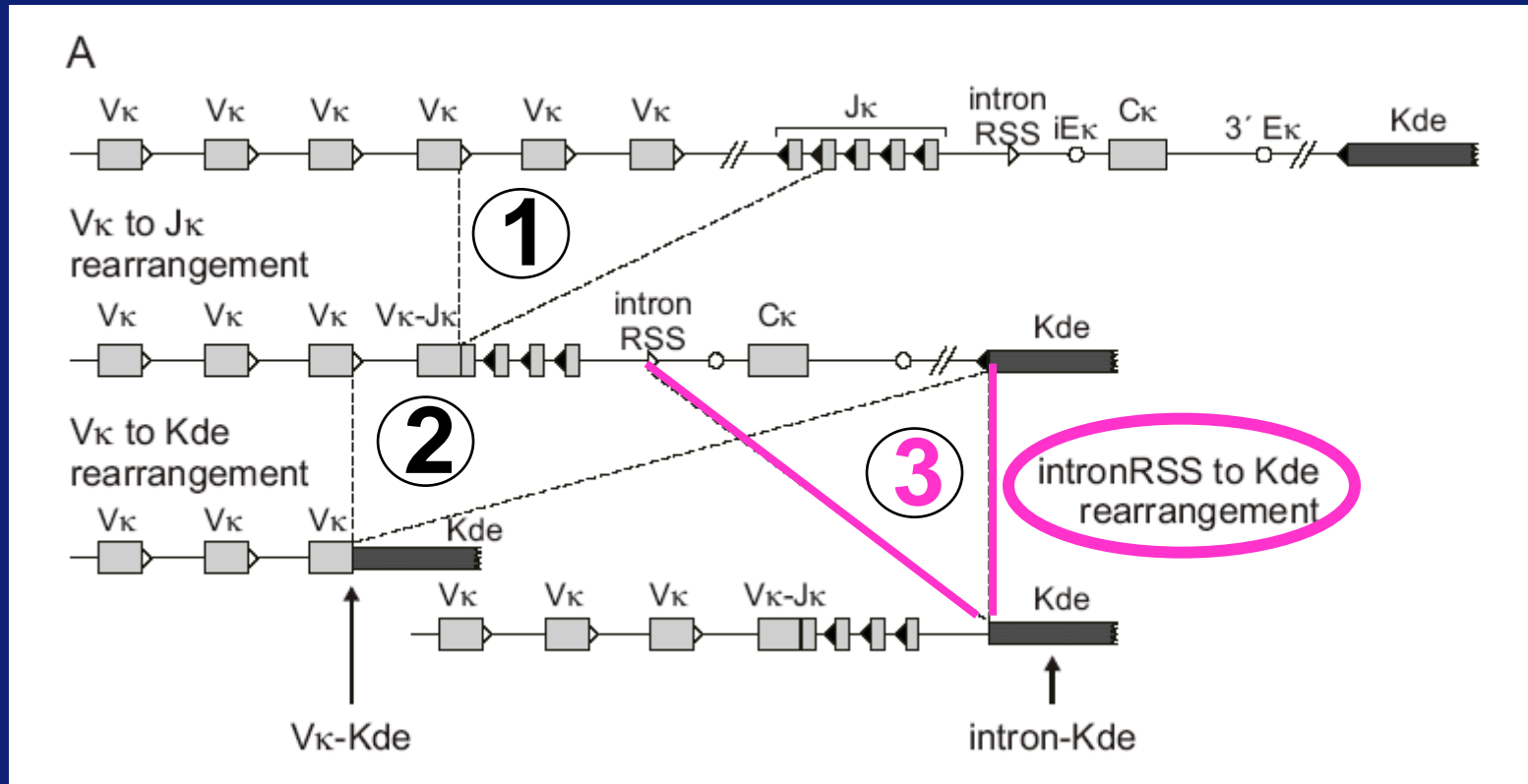
# IGK locus : V-J en Kde herschikkingen



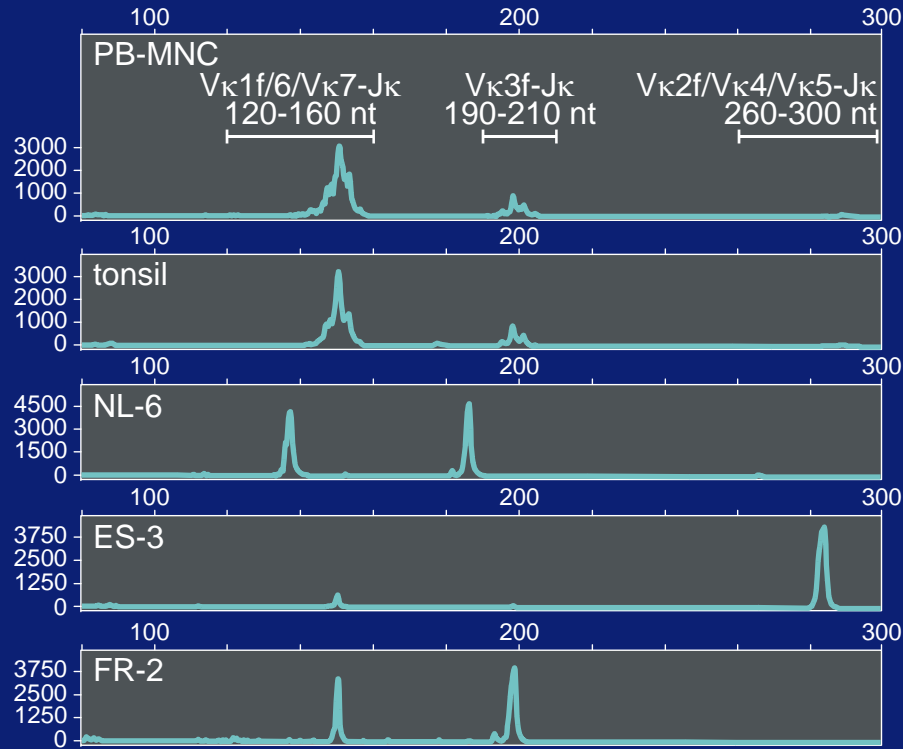
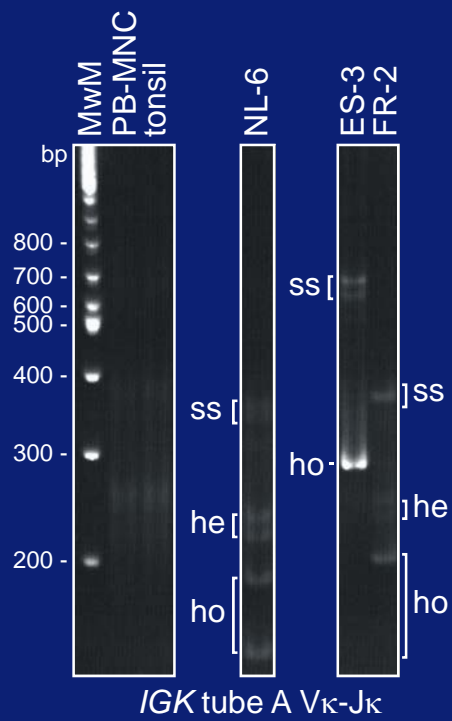
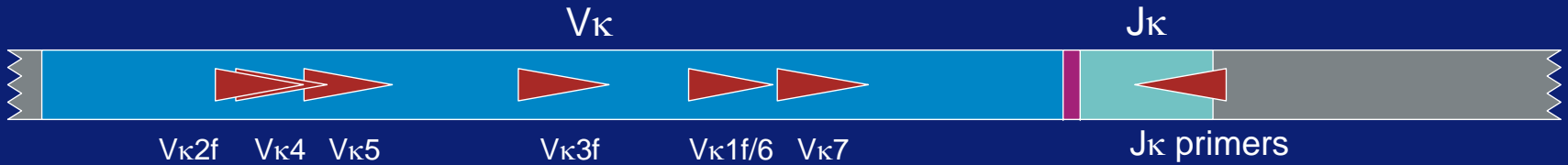
# IGK locus : V-J en Kde herschikkingen



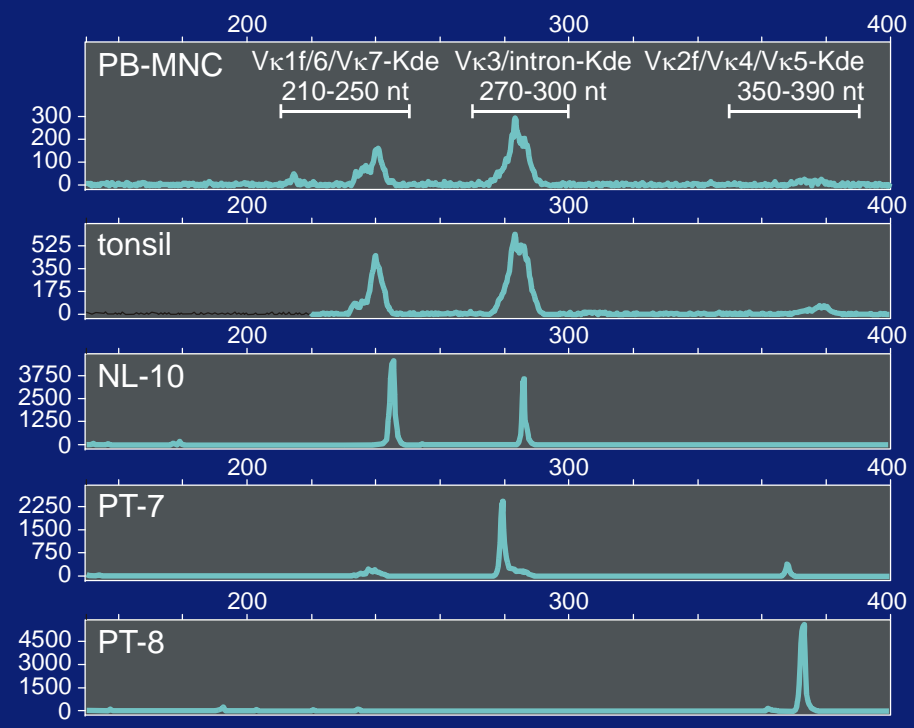
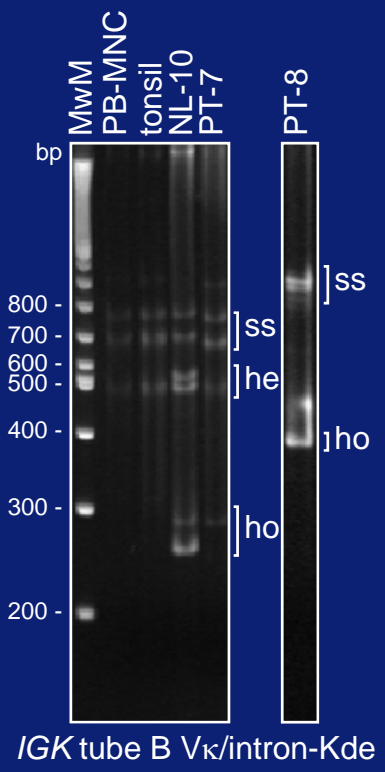
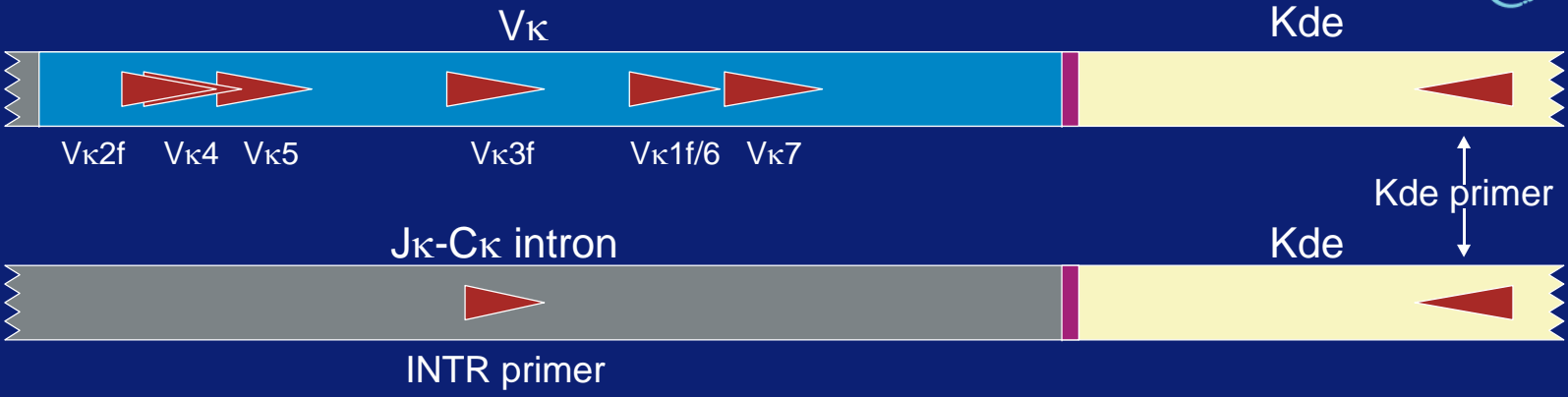
# IGK locus : V-J en Kde herschikkingen



# 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
GTGGTCTAAGTGCAACATCCATTC	(+53)	Jβ1.1
CTGGTCCAATTGGCAACATCCATTC	(+53)	Jβ1.2
TTCAACCGAGTGACAACATCCATTC	(+55)	Jβ1.3
CTTGGGTCGAGAGACAGAACCCATAC	(+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
AGTGGCACGATCCATTCTTCC	(+59)	Jβ2.1
ACTGTCACGAGCCATTGCCCC	(+58)	Jβ2.3
AGAGTCACGACCCATTGACC	(+59)	Jβ2.4
CACGAGCCACACGCGC	(+57)	Jβ2.5



Dβ1 primer

Dβ2 primer

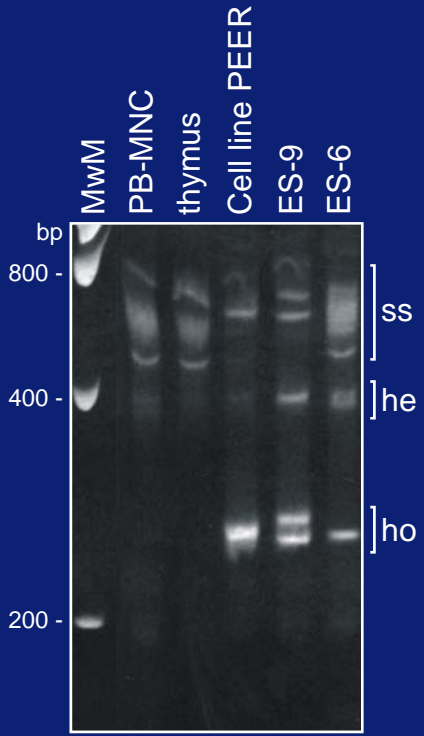
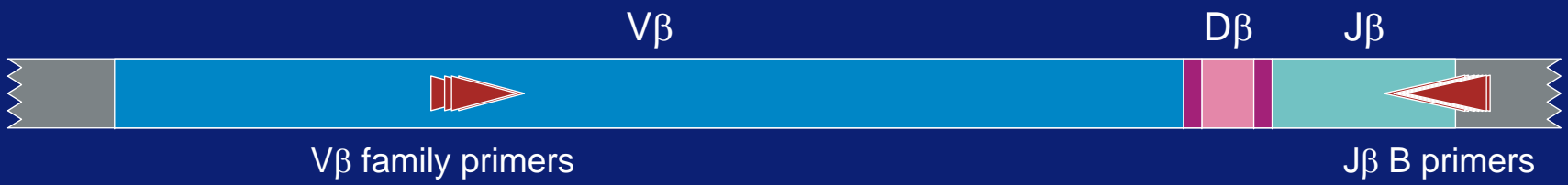
Jβ primers

TCRB tube C

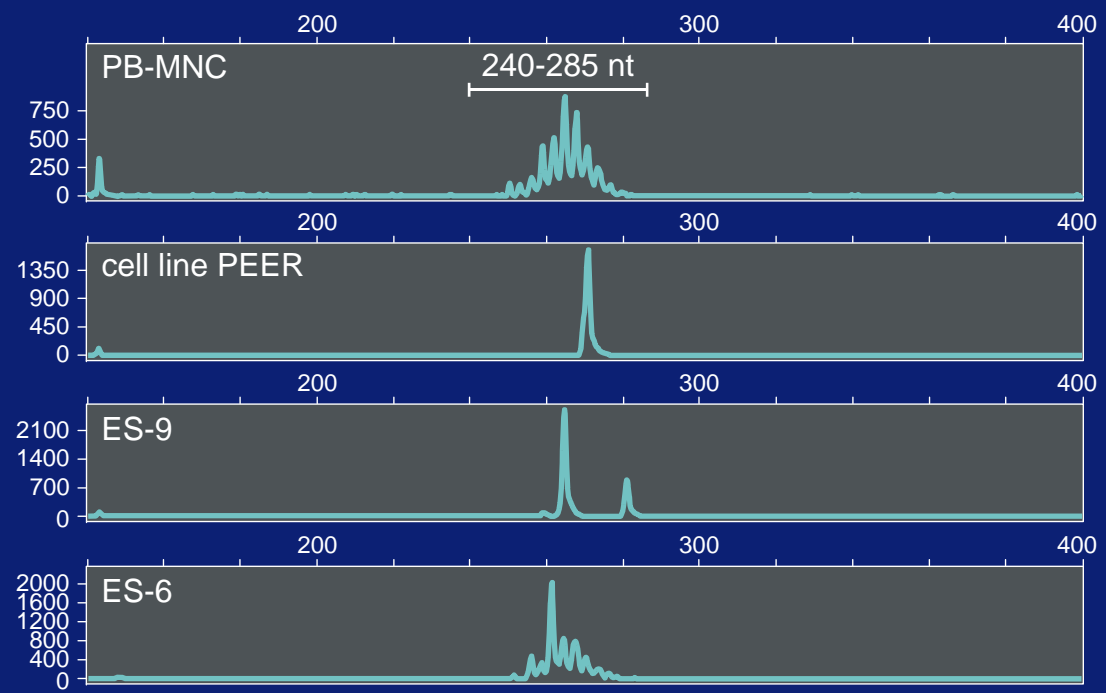
Dβ1 (-252) 5' GCCAAACAGCCTTACAAAGAC 3'

Dβ2 (-137) 5' TTTCCAAGCCCCACACAGTC 3'

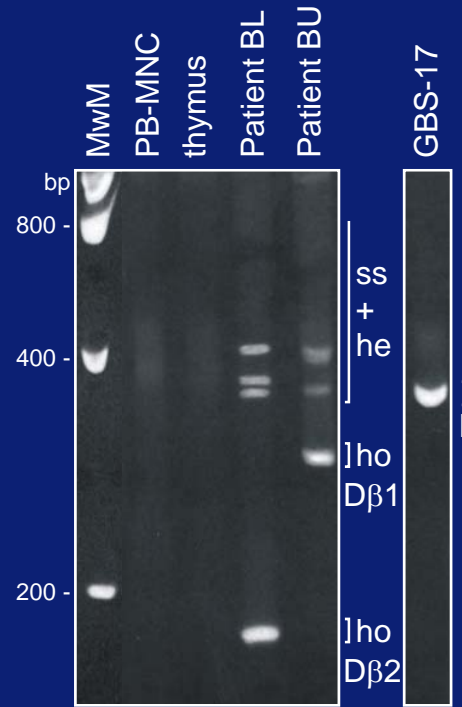
# BIOMED-2 multiplex TCRB tube B: V $\beta$ -J $\beta$



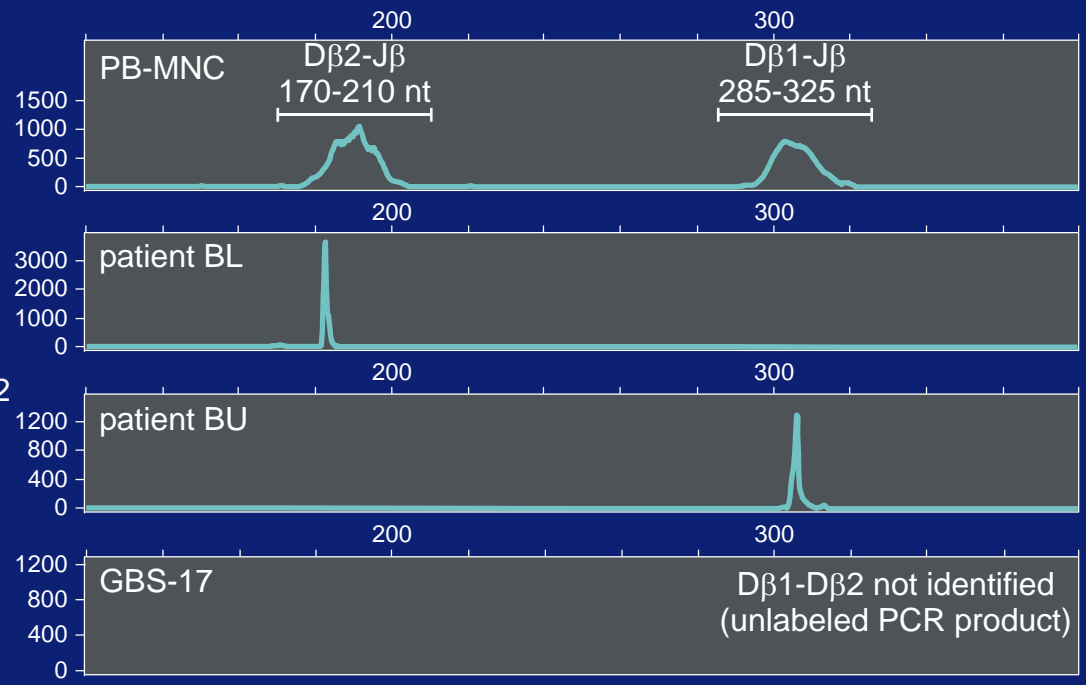
TCRB tube B V $\beta$ -J $\beta$



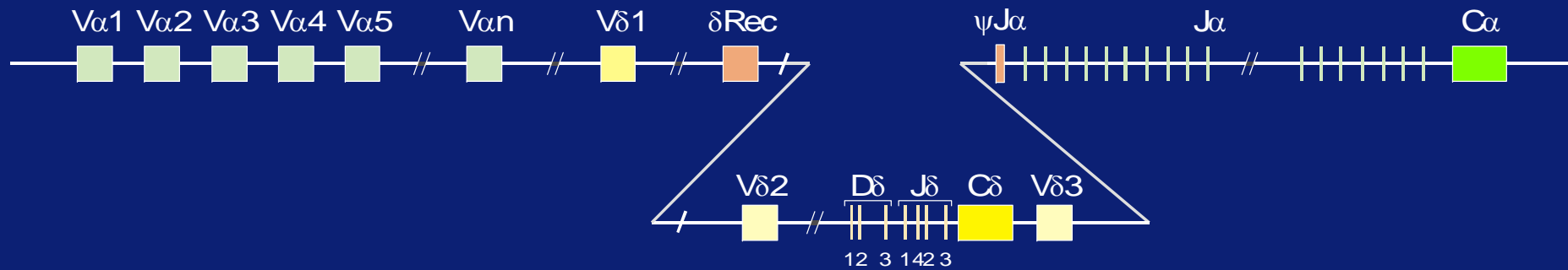
# BIOMED-2 multiplex TCRB tube C: Db-Jb



TCRB tube C Dβ-Jβ



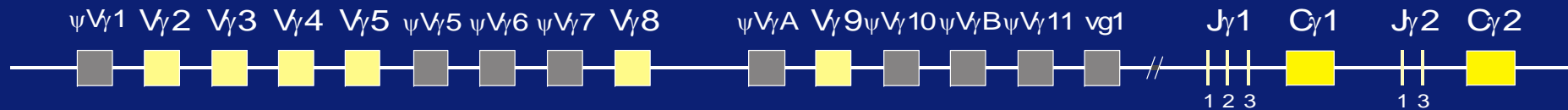
### TCR- $\alpha$ and TCR- $\delta$ gene complex



### TCR- $\beta$ gene complex



### TCR- $\gamma$ gene complex



# Aantal TCRB recombinaties in 1 kloon

Erasmus MC



<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

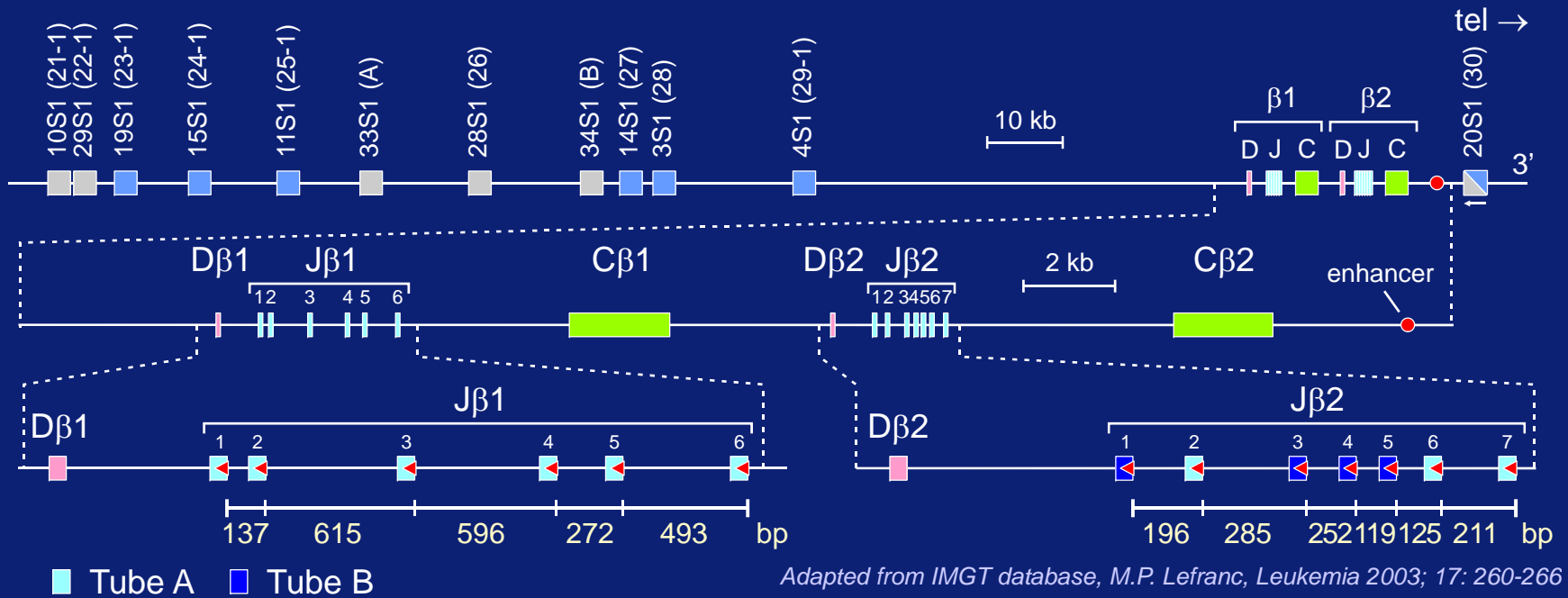
<i>Pitfall</i>	<i>Phenomenon</i>	<i>Solution / action</i>
bands / peaks <u>just</u> outside size range	CDR3 regions / junctions outside <b>5-95% size range</b> interval	accept as true rearrangement product; in case of doubt , sequence for confirmation
<b>undersized</b> bands / peaks	<b>internal deletion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

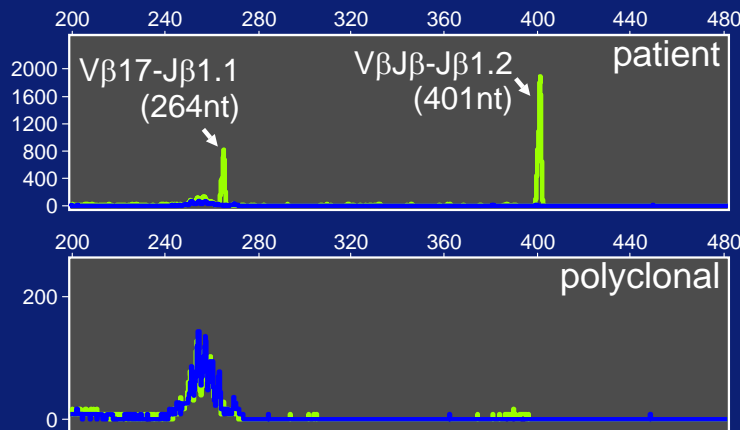
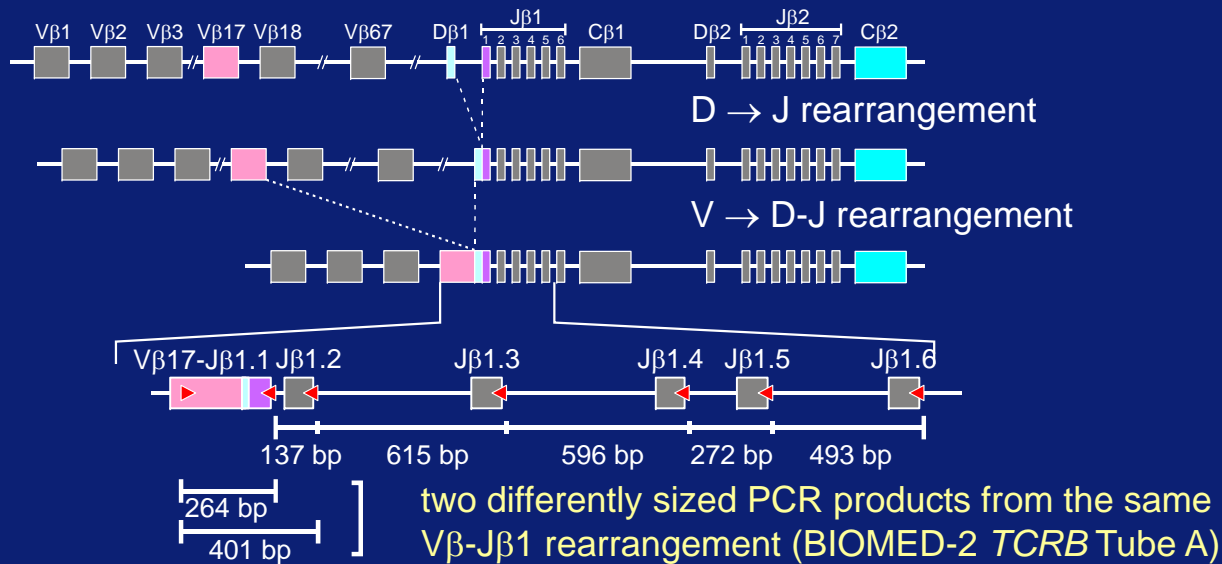
Vargas, *Leukemia Res* 2008;32:335

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

# TCRB locus : J genen dicht bij elkaar



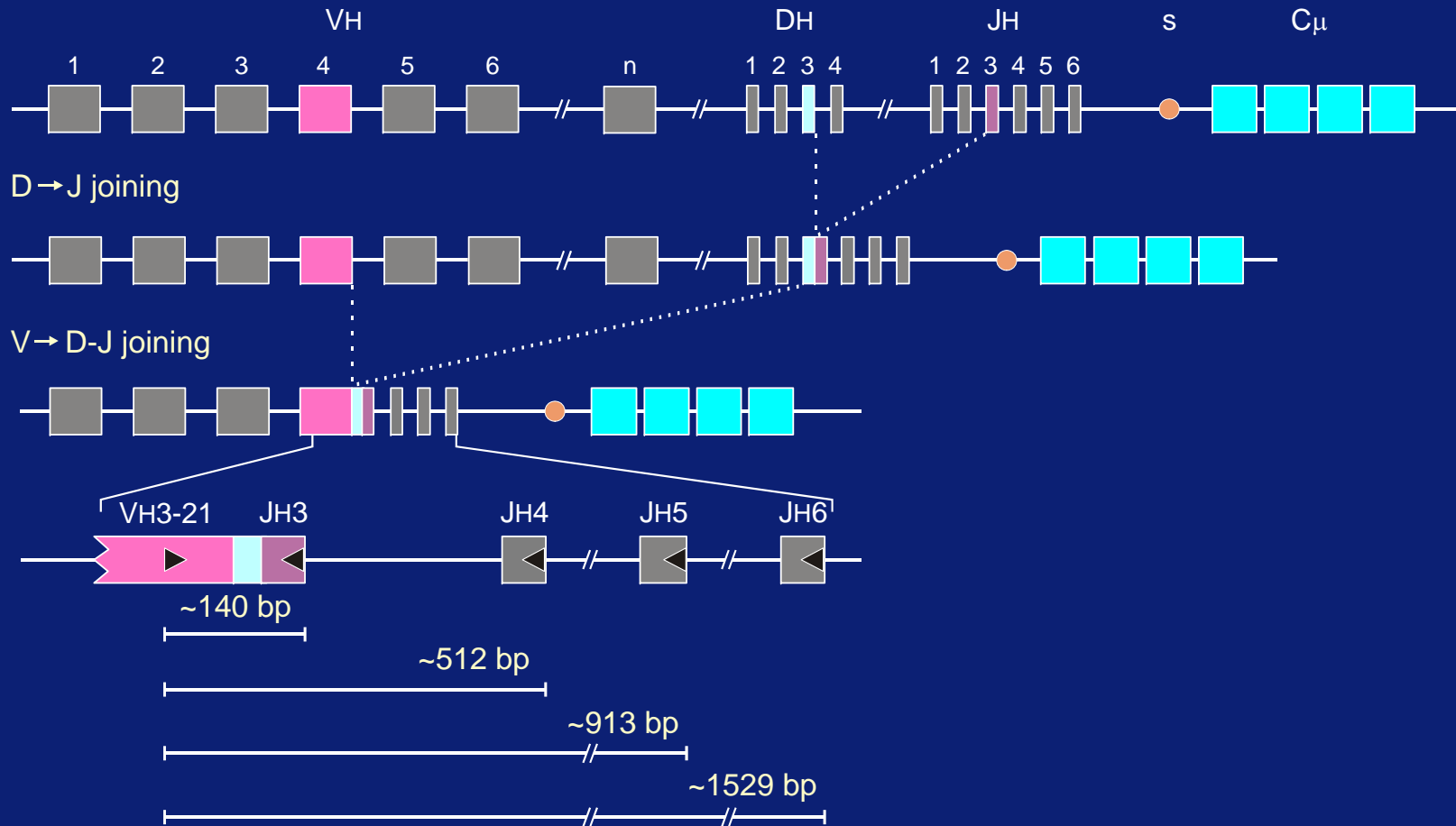
# TCRB : twee producten van dezelfde herschikking



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