# Next generation sequencing for immunogenetics

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# Purpose of Next Generation Sequencing (NGS) and some cautionary notes

- NGS-systems are designed primarily for biology, metagenomics and plant genomics:
  - High genomic complexity
  - Long ranges of repetitive sequences
  - Multiple overlaying genomes
- NGS is a testing strategy in which:
  - Brute force is applied to generate high volumes reads
  - Massive parallel sequencing is a prerequisite
  - Bioinformatics might distort the data analysed

**Not designed primarily for medical applications**

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# Most developed medical areas for next generation sequencing

- Immunogenetics
- Virology:
  - ultradeep sequencing
  - susceptability
- Immunology:
  - IgH and TCR
- Oncology:
  - Multiple polymorphisms
  - Regions of resistence polymorphisms

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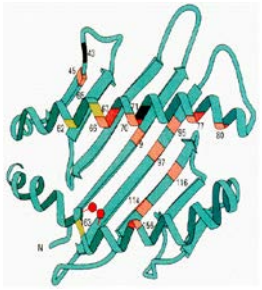
# Why?

- Higher sensitivity than Sanger
- Finding small populations, but high relevance for the patient´s fate (f.i. HIV resistence)
- Detecting multiple polymorphisms

deletions, inversions at once:

- Compound mutations
- In alleles
- Haplotypes



# New alleles – every third day

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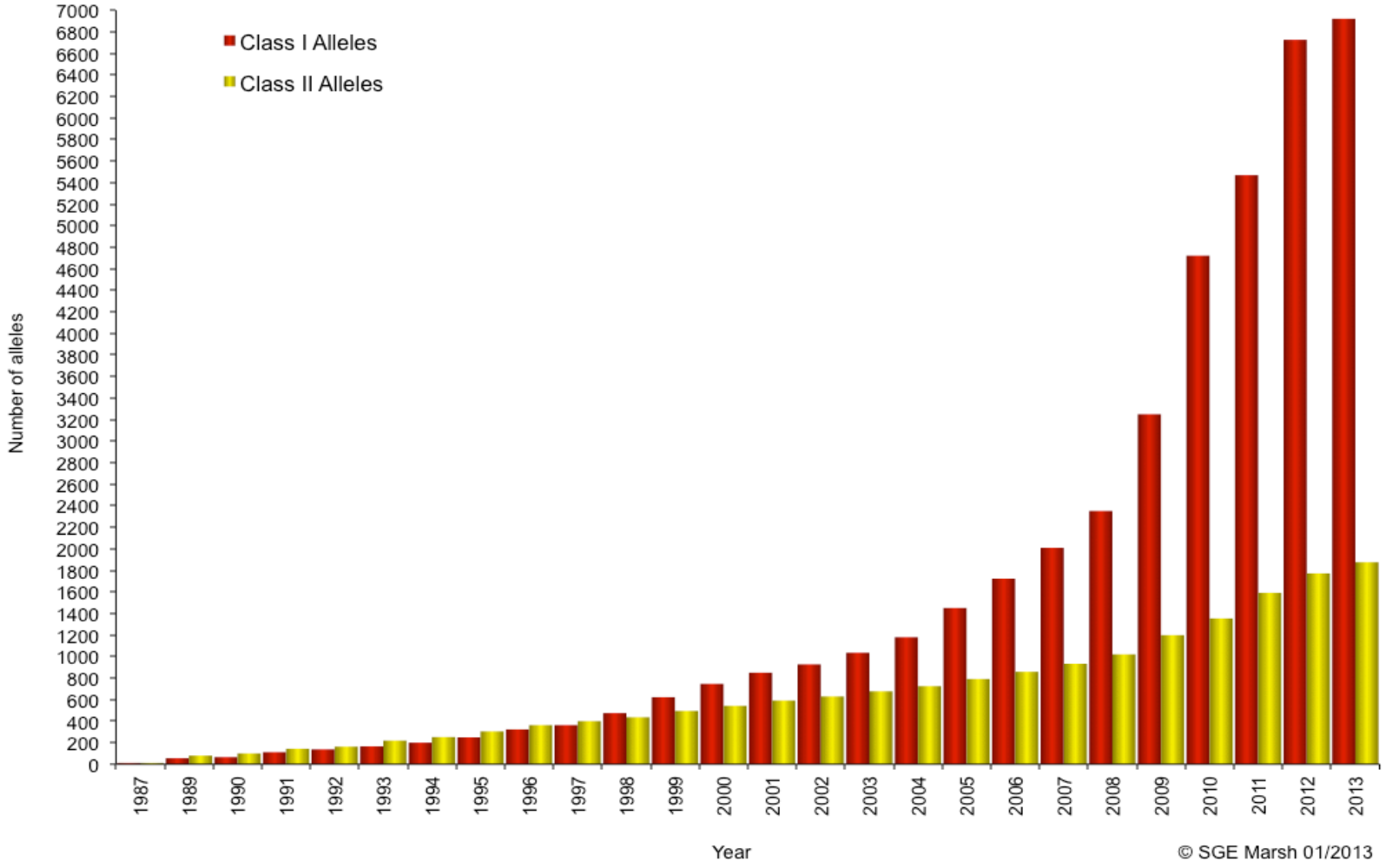

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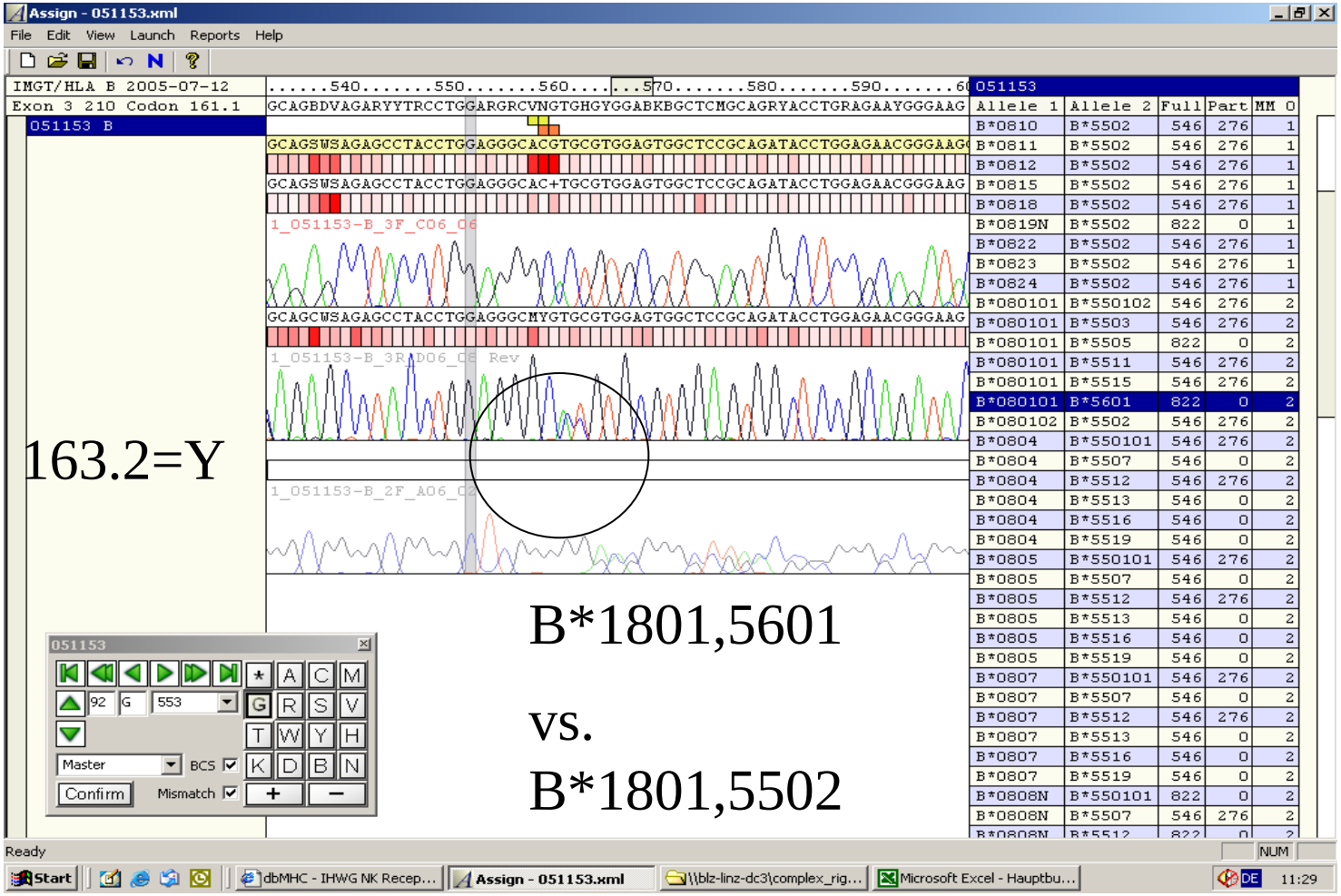
# Sequencing (sequencing-based typing, SBT)



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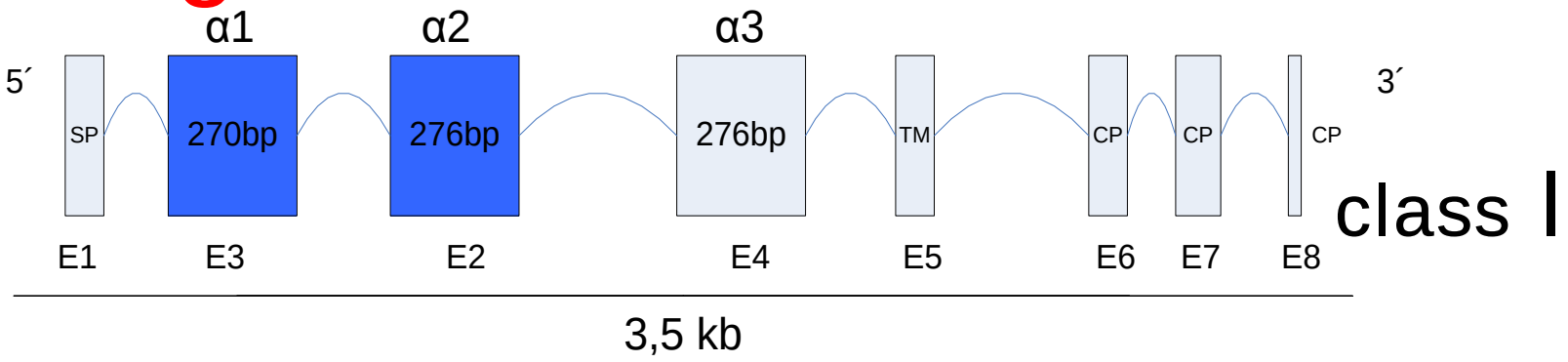
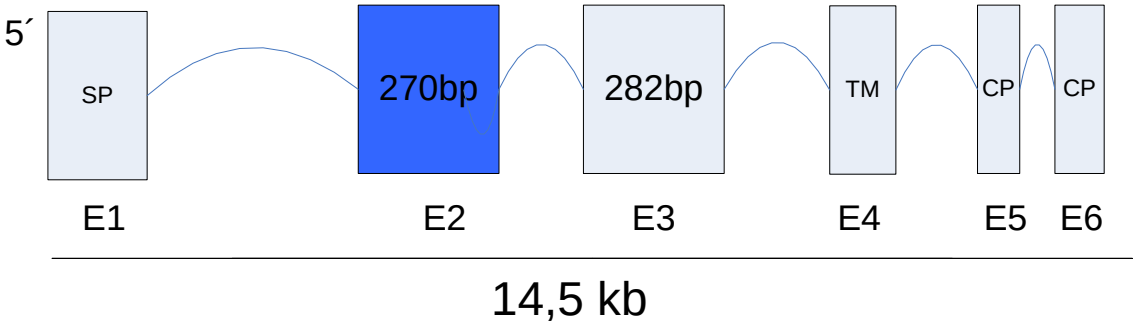
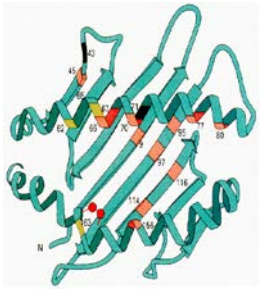
# Regions of interest




class I




class II



# Allele ambiguity

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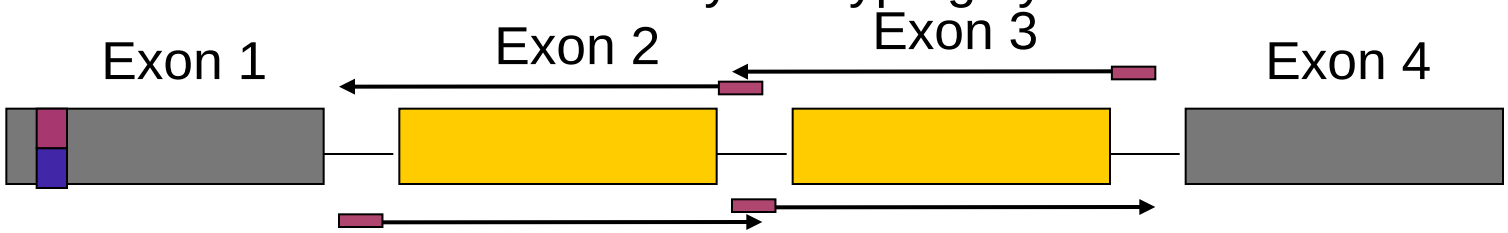
**outlier mutations:** allele ambiguitiy results when polymorphisms that distinguish alleles fall outside of the regions examined by the typing system




Polymorphic positions

Core heterozygous sequence data

example: HLA-B

B*0702, 4402

B*0702, 4419N

# Genotype ambiguities

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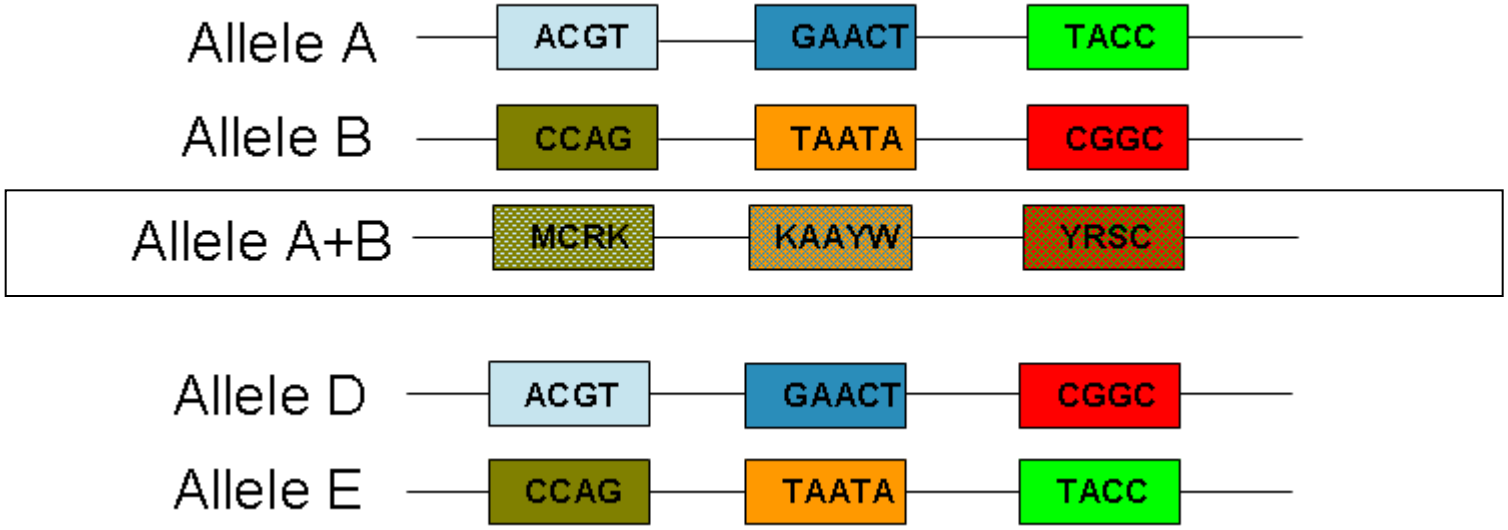
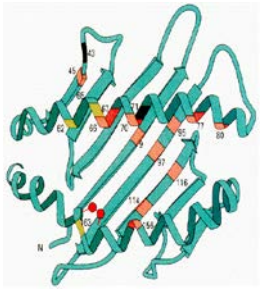
Results from an inability to establish **phase** between closely linked polymorphisms identified by the typing system




A+B=D+E

example: HLA-B
B*0702, 4402
B*0720, 4416
B*0724, 4421



# cis/trans Problems

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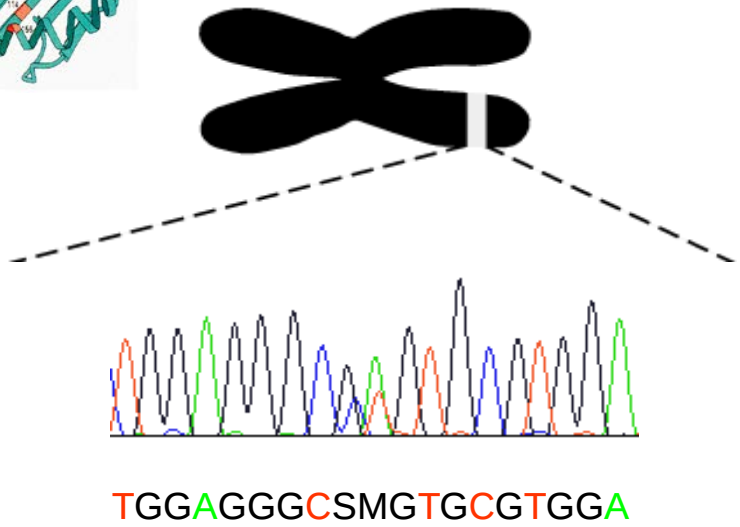

TGGAGGGCSMGTGCGTGGA

TGGAGGGCSMGTGCGTGGA

S = G und C

M = A und T

----------SM----------

----------GA----------

----------CT----------

----------GT----------

----------CA----------

Number of possible linkages = $2^n$

n=2; 4 combinations

n=4; 16 combinations

| IUB Code | K | S | W | M | Y | R |
|---|---|---|---|---|---|---|
| Bases | G,T | G,C | A,T | A,C | C,T | A,G |


without ambiguities?
Hey dumbheads!
How can we get faster and more reliable results?

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# Strategies

- Amplicon-based:
  - Patients and donors prior to transplantation VHRT
  - For registries – IRT („quick and dirty“)
- Genome-based:
  - Transcript-Sequencing

# Strategies

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1 Exon-based typing

**flexibility: high**
**capacity: gentle**
**full allele drop out: rare**
**DNA quality: not critical**
**Scabillity: medium/ high/ ultra high**
**library prep: complex**
**automation: yes**

Shorter amplicons allow the complete coverage in reverse and forward direction !

? Ambiguities library prep


Gabriel C et al: **Rapid high-throughput human leukocyte antigen typing by massively parallel pyrosequencing for high-resolution allele identification**. *Hum Immunol* 2009, **70:**960-964.

Bentley G et al: **High-resolution, high-throughput HLA genotyping by next-generation sequencing**. *Tissue Antigens* 2009**, 74:393-403**.

Erlich RL et al: **Next-generation sequencing for HLA typing of class I loci**. *BMC Genomics* 2011, **12:**42.

Moonsamy PV et al: **High throughput HLA genotyping using 454 sequencing and the Fluidigm Access Array™ System for simplified amplicon library preparation.** TissueAntigens 2013 Mar;81(3):141-9.

Danzer M et al: **Rapid, scalable and highly automated HLA genotyping using next-generation sequencing: A transition from research to diagnostics.** *BMC Genomics 2013, **14**:221*

Grumbt B et al*:* **Diagnostic applications of next generation sequencing in immunogenetics and molecular oncology.** *Transfus Med Hemother. 2013 Jun*

Trachtenberg EA et al: **Next-generation HLA sequencing using the 454 GS FLX system.** *Methods Mol Biol. 2013*

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Holcomb CL et al: **Multi-site study using high-resolution HLA genotyping by next generation sequencing**. *Tissue Antigens* 2011, **77:**206-217.

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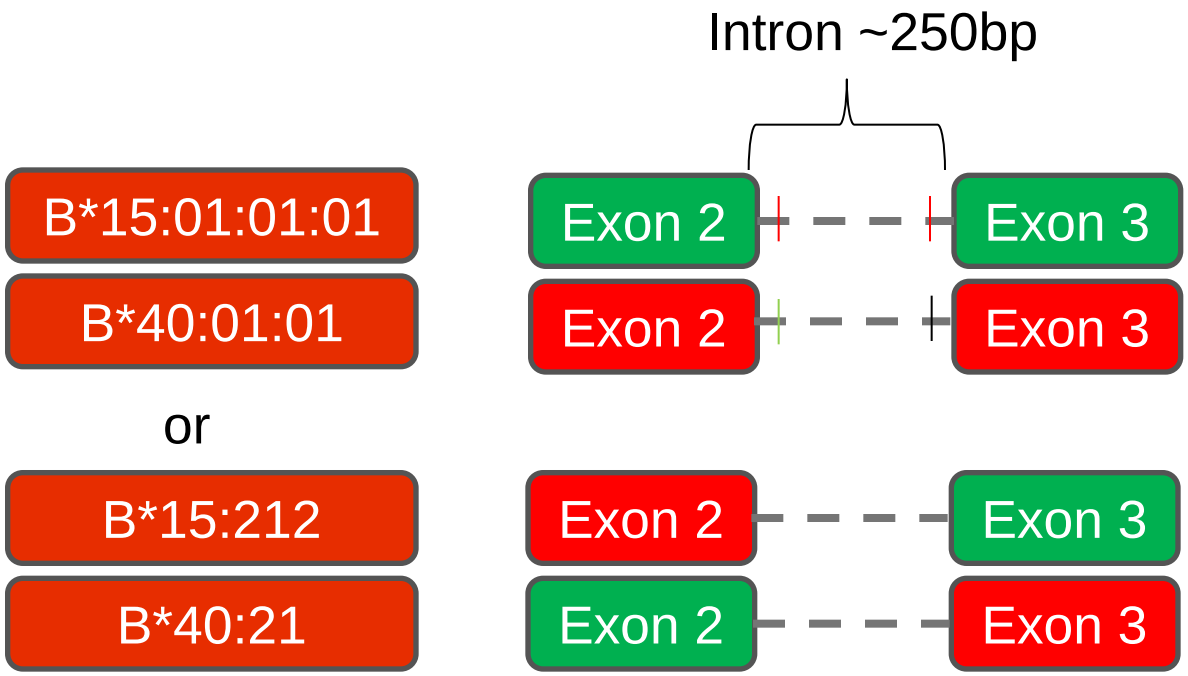
# Amplicons

- HLA -A, -B, -C, -DP, -DQ, -DR
- class I:
  - A: exons 2,3,4
  - B: exons 1,2,3,4
  - C: exons 1,2,3,4,6,7
- class II:
  - DP: exon 2
  - DQ: exons 2,3
  - DR: exons 2,3

# Limitations

## Ambiguities between Exons



# Strategies

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2 cDNA-based typing

flexibility: medium
capacity: ideal
full allele drop out: possible
RNA quality: very critical
Scabillity: : medium/ high
library prep: reduced complexity
automation: yes

? N- alleles
RNA quality


Lank SM et al: **A novel single cDNA amplicon pyrosequencing method for high-throughput, cost-effective sequence-based HLA class I genotyping**. *Hum Immunol* 2010, **71:**1011-1017.

Lank SM et al: **Ultra-high resolution HLA genotyping and allele discovery by highly multiplexed cDNA amplicon pyrosequencing**. *BMC Genomics* 2012, **13:**378.

# Strategies

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3 Full genomic typing

flexibility: low
capacity: max
full allele drop out: possible
DNA quality: critical
Scabillity: ultra high
library prep: complex

? DRB1,
Software issues (phasing)
cinical relevance

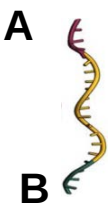

Lind C et al: **Next-generation sequencing: the solution for high-resolution, unambiguous human leukocyte antigen typing**. *Hum Immunol* 2010, **71:**1033-1042.

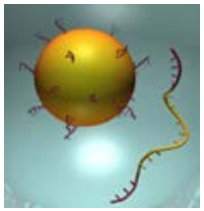
Wang C et al: **High-throughput, high-fidelity HLA genotyping with deep sequencing**. *Proc Natl Acad Sci U S A* 2012, **109:**8676-8681.

Shiina T et al: **Super high resolution for single molecule-sequence-based typing of classical HLA loci at the 8-digit level using next generation sequencers**. *Tissue Antigens* 2012, **80:**305-316.

Hosomichi K et al: **Phase-defined complete sequencing of the HLA genes by next-generation sequencing.** BMC Genomics 2013, **28**;14:355.

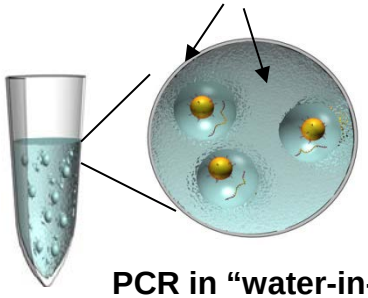
Ozaki Y. et al: **HLA-DRB1, -DRB3, -DRB4 and -DRB5 genotyping at a super-high resolution level by long range PCR and high-throughput sequencing.** *Tissue Antigens* 2014



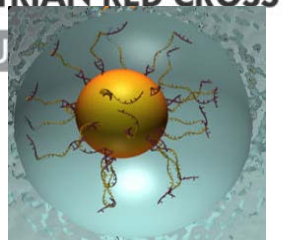



Mix amplicons & capture beads

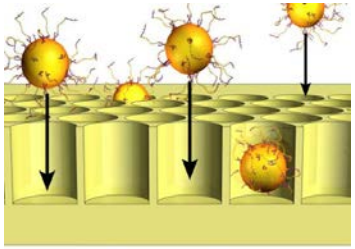
Add PCR Reagents & emulsion oil



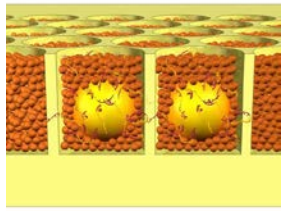
PCR in “water-in-oil” emulsion

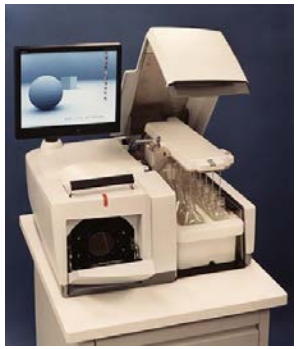


Isolate DNA containing beads



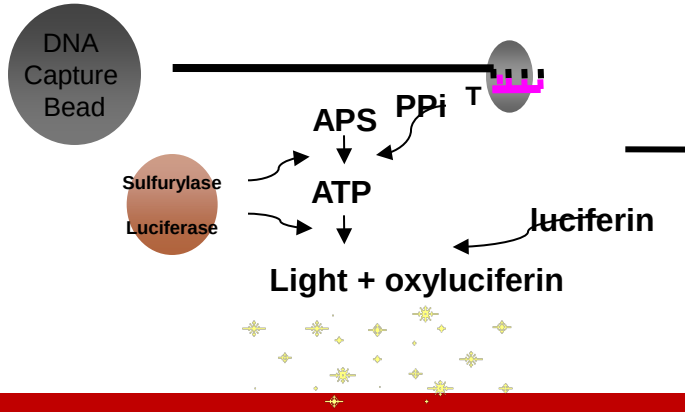
Load Enzyme Beads



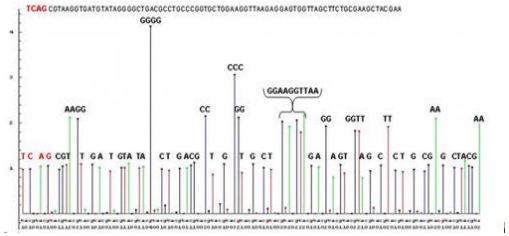


cooled 16Mpixel CCD camera

Pyro-sequence




Read Flowgram



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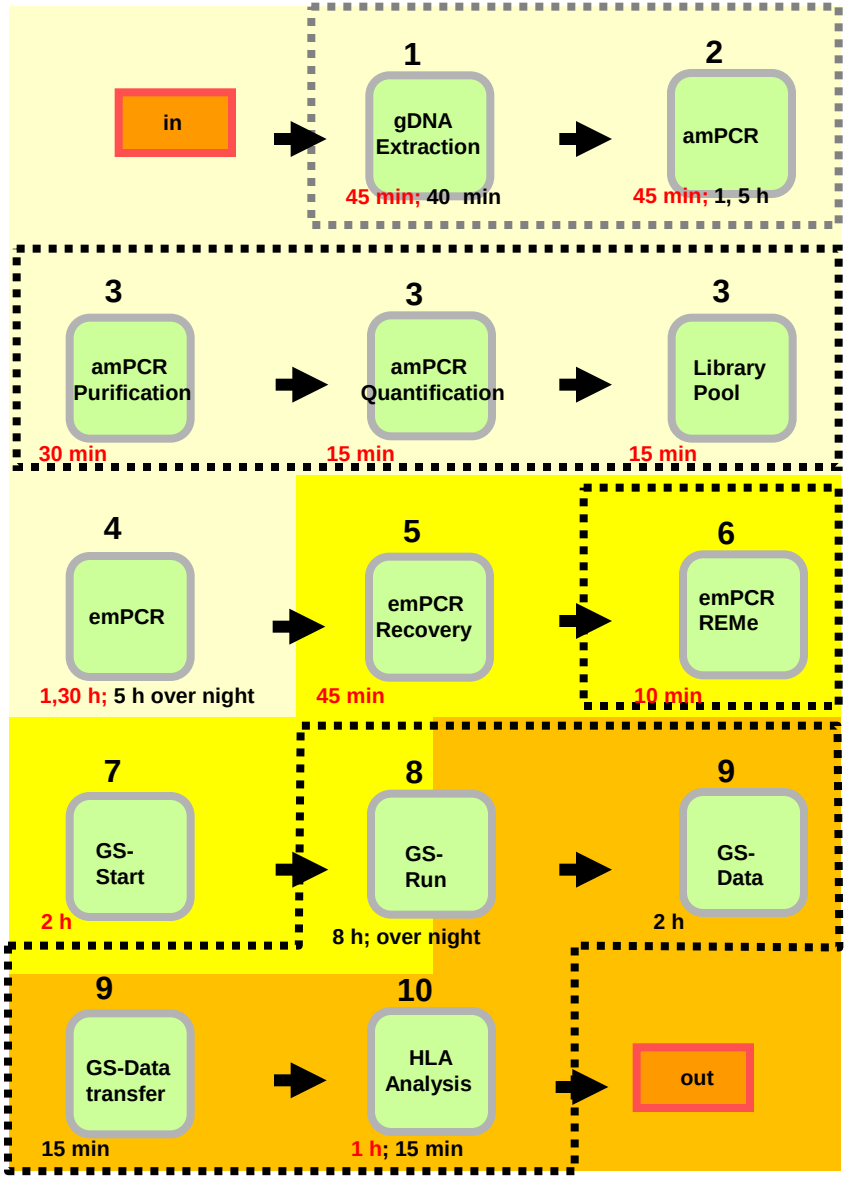


| | | |
|---|---|---|
| Lab 1 and 2<br>DAY 1 | MagNa 96<br>STARlet | Auto Block 1 |
| Lab 3 | STAR | Auto Block 2 |
| Lab 4 and 3<br>DAY 2 | STARlet<br>REM e | Auto Block 3 |
| Lab 5 and 6<br>DAY 3 | Bioinformatics | Auto Block 4 |
| Lab 6 and Office<br>DAY 3 | | |

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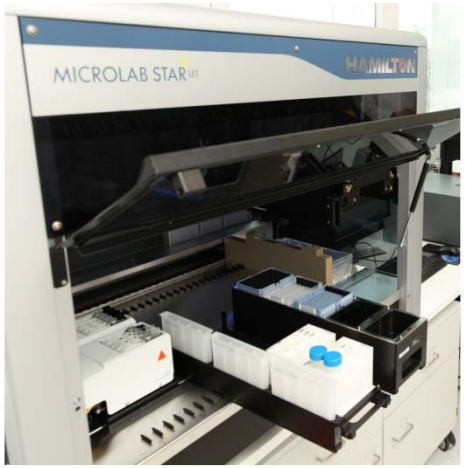
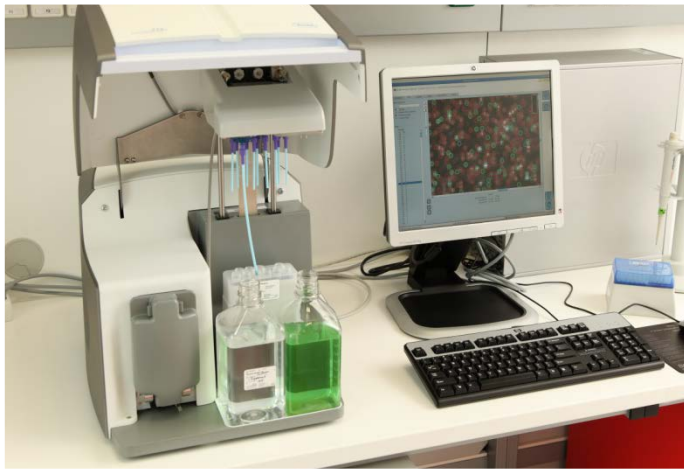



**Hamilton STAR**
**Hamilton STARlet**
**454 REM e**



**GS Junior**
**GS FLX**

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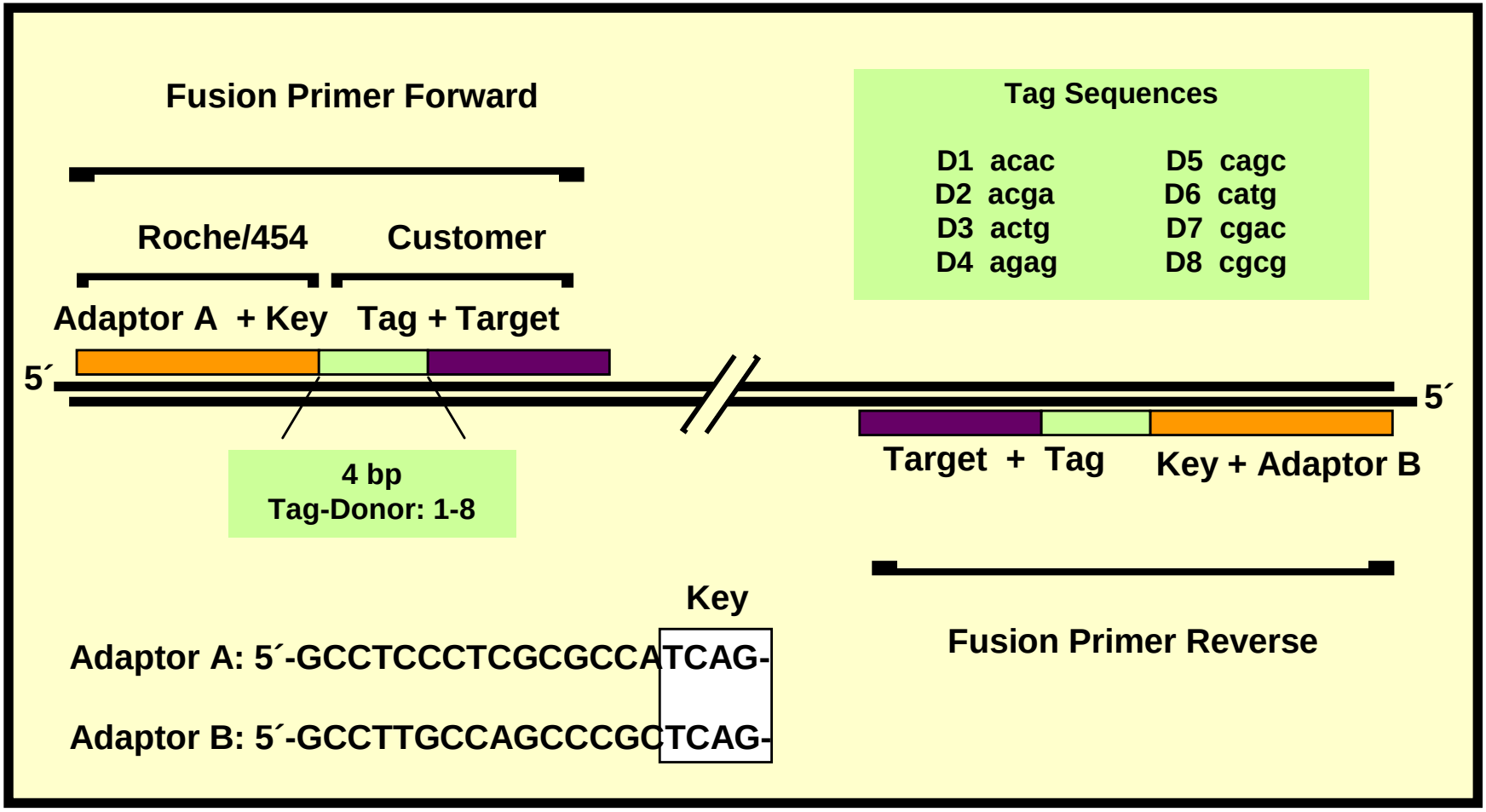
# Composition of primers with individual identification tags



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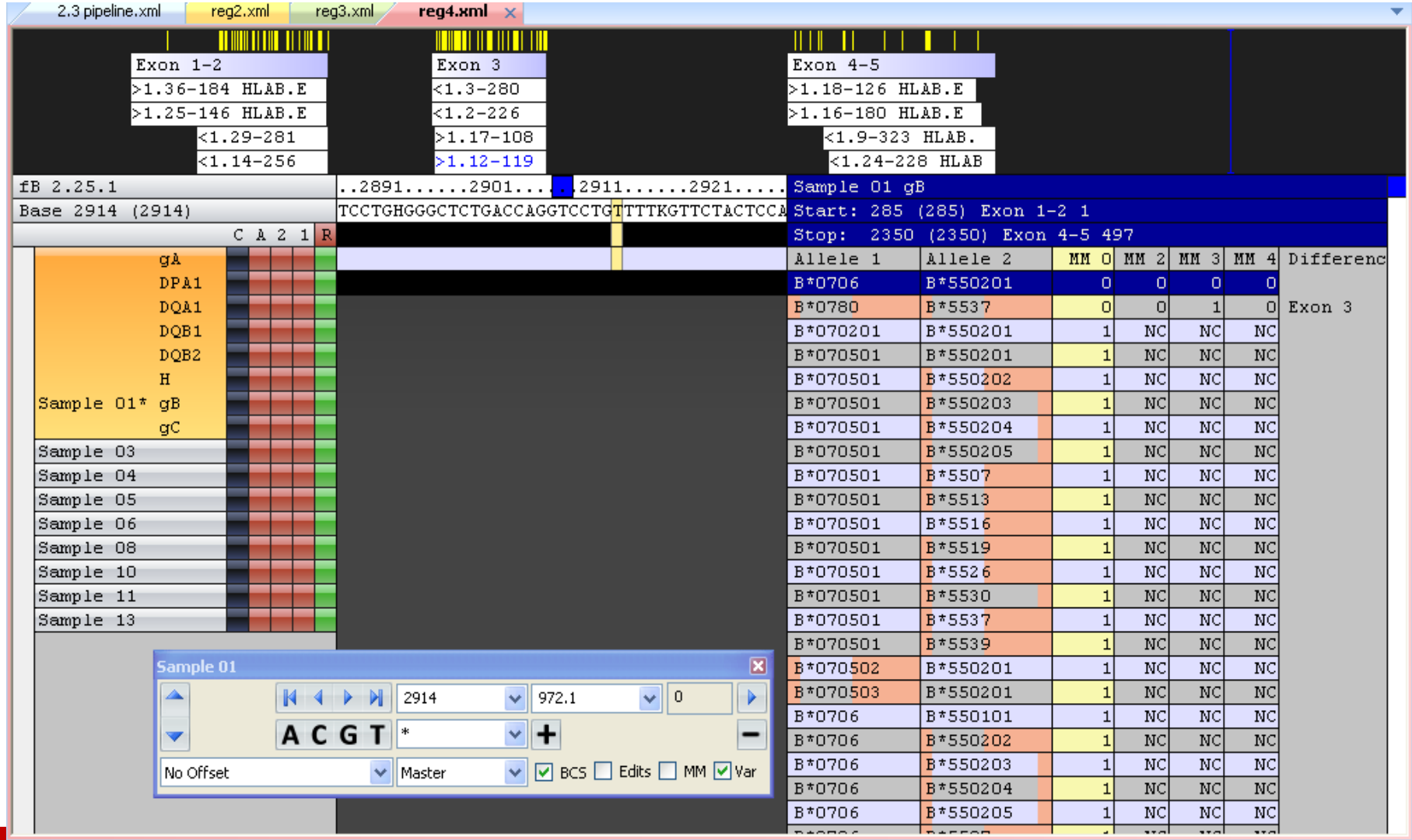
# DBUG HLA-B Ultra High Resolution



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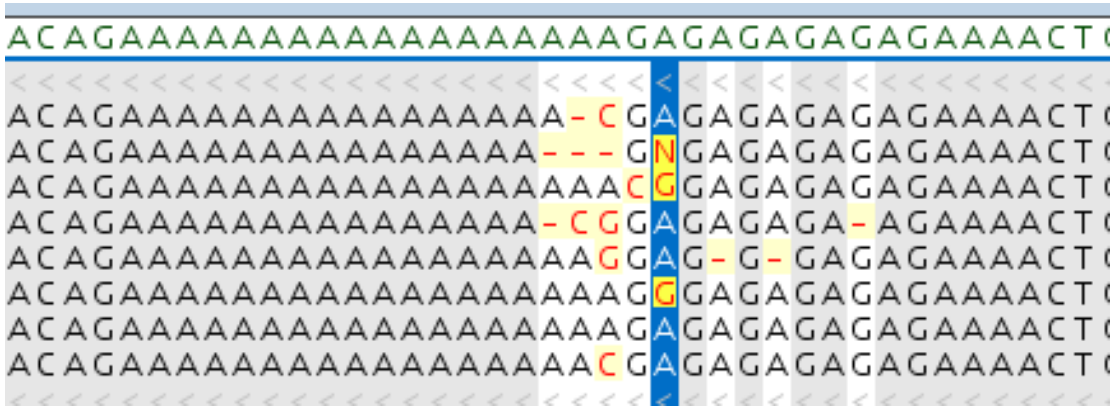
# Sequencing errors

- not just homopolymers, also subsequent regions



- „difficult“ sequence pattern

Blutzentrale Linz

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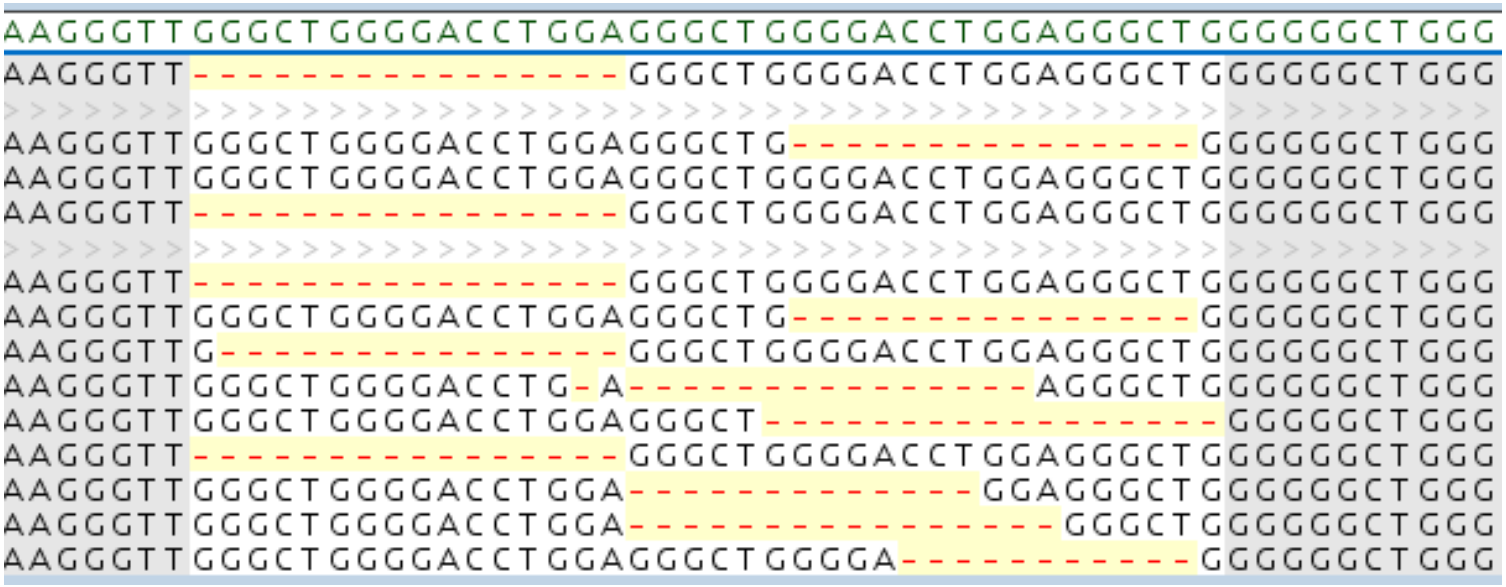
# Alignment problems

- alignments over multiple reads not consistent



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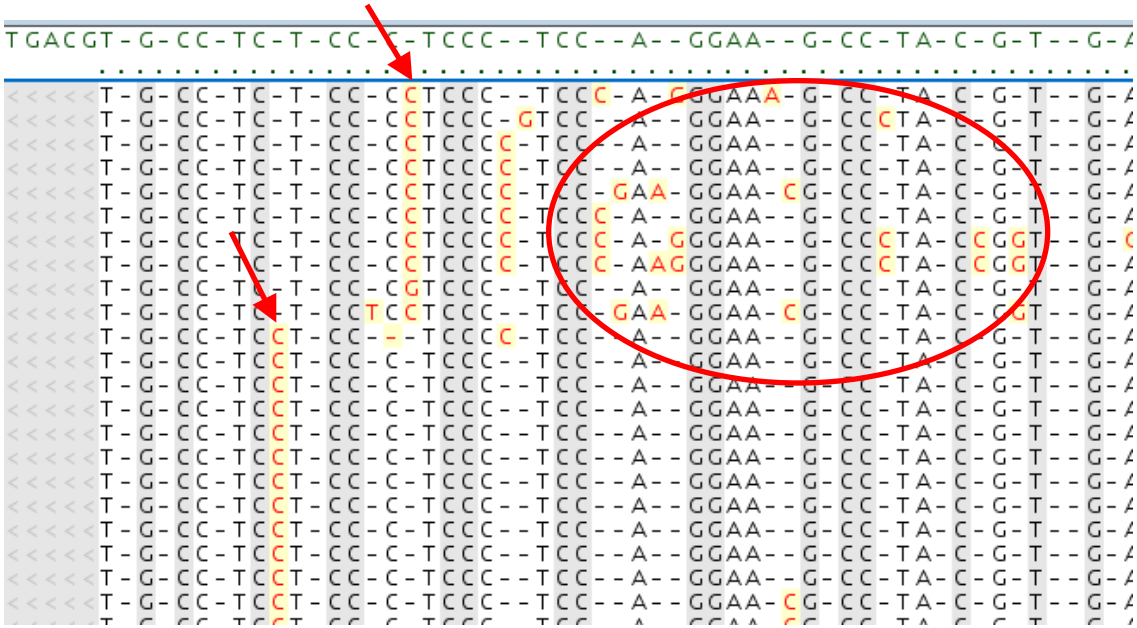
# Alignment problems

- existing variants are not reported
- many single sequencing errors



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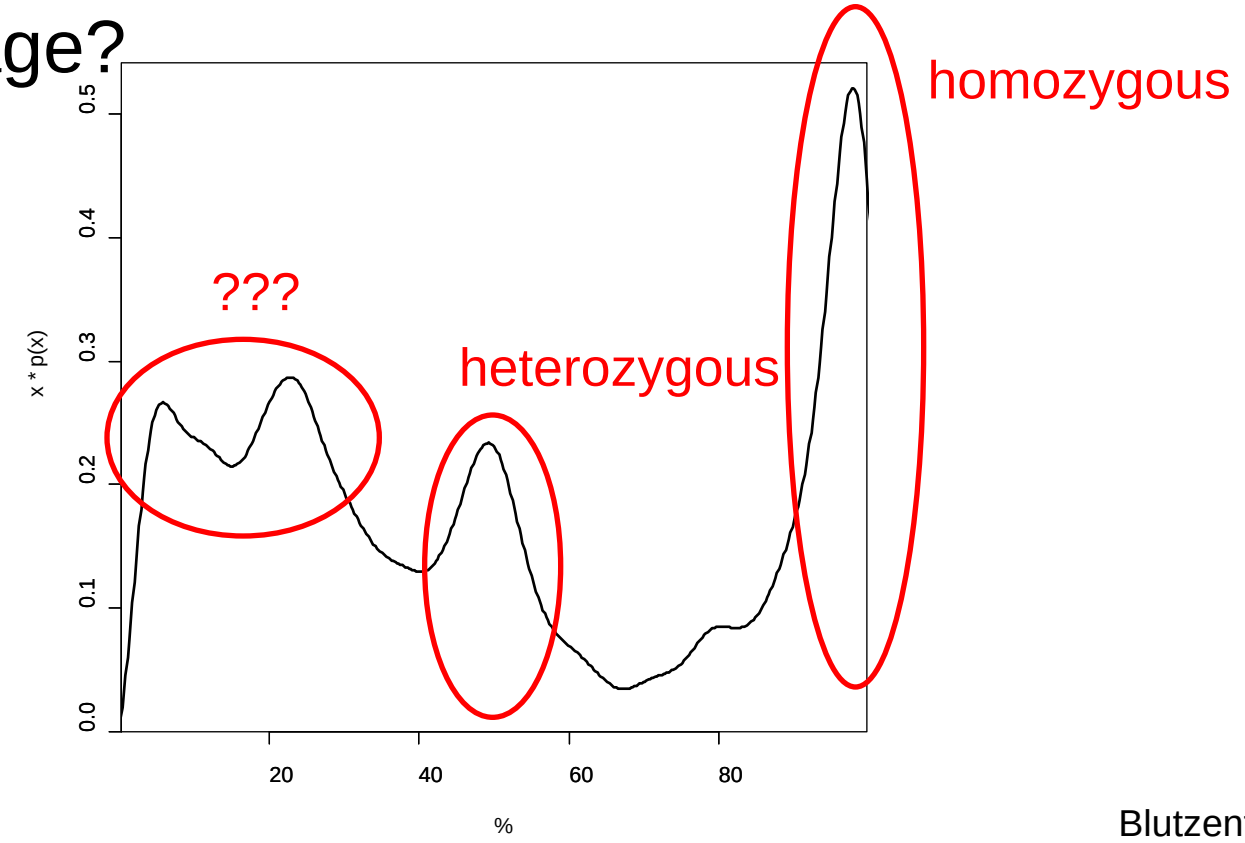
# Variants

- how to interpret variants with low percentage?



# HLA Study Participants *from Europe and U.S.*

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Dr. Wassmuth, DKMS, Dresden

Dr. Klein, SEQIT Kaiserslautern, Germany

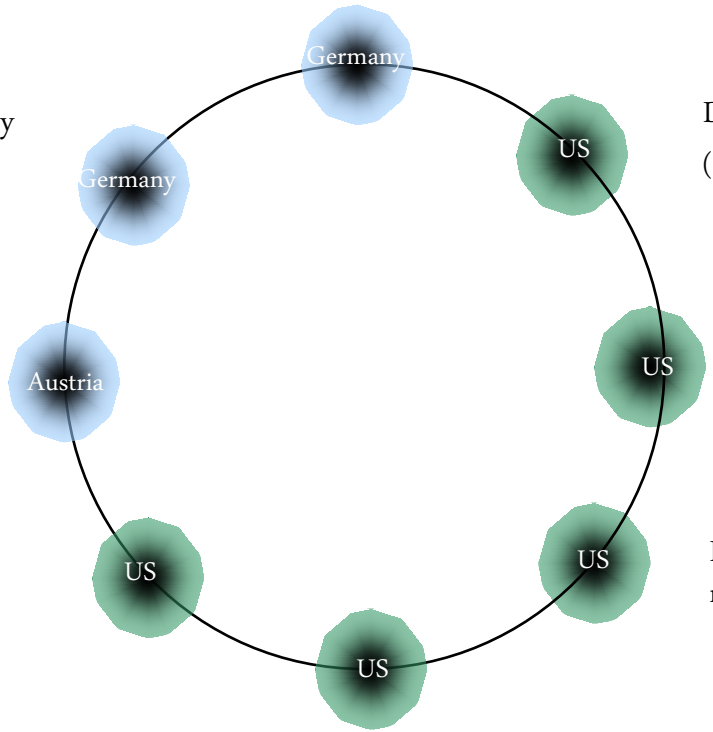
Dr. Tyan, Stanford University (GSFLX at Stanford SGTC)

Dr. Gabriel, Blood Bank, Linz

Dr. Trachtenberg, Childrens Hospital Oakland

*Dr. Simen, 454, Branford*

Dr. Monos, Childrens Hospital Philadelphia (454 run and GS FLX rental)




*Dr. Erlich, RMS, Pleasanton*

The following slides of this study have been kindly provided by Ch Holcomb, Roche

# Genotype Assignment

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***Assignment for 95% of the 2240 genotypes examined***

***Causes for Failure to Assign Genotype***

| Cause | # of Genotypes Omitted | # of Sites Affected |
|---|---|---|
| New allele | 24 (2%) | 8 |
| Plate seal failure in gPCR | 16 | 1 |
| Amplicon not added to pool | 7 | 4 |
| forward sequencing primer not annealed | 4 | 1 |
| Bias against B-2 reverse reads | 6 | 1 |

**3%**

4/8 sites had no genotypes omitted due to procedural/technical issues

# Summary of Agreement and Concordance

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| Locus | % Agreement | % Concordance with Known Variants | | % Req'd Man. Ed. |
|---|---|---|---|---|
| | | Genotype | Allele | |
| HLA-A | 89 | 91 | 94 | 13 |
| HLA-B | 93 | 96 | 98 | 15 |
| HLA-C | 94 | 94 | 97 | 4 |
| DPB1 | 99 | 100 | 100 | 3 |
| DQA1 | 100 | 100 | 100 | 5 |
| DQB1 | 99 | 100 | 100 | 4 |
| DRB1 | 97 | 98 | 99 | 10 |
| DRB3,4,5 | 97 | 98 | 99 | 13 |
| Overall | 96 | 97 | 98 | 8 |

Agreement = Identical ambiguity string obtained

Concordance = Reported genotype/allele in a limited ambiguity string matched submitted

# Conclusions

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- 454 Life Sciences GS FLX System with Conexio ATF software can deliver high-throughput, high resolution sequencing:  20 samples can be typed in a single run for HLA-A, HLA-B, HLA-C, DPB1, DQA1, DQB1, DRB1 and DRB3, 4, 5.
- The genotyping system gives robust and reproducible results
  - 1280 genotypes considered
  - Assignment possible for 95%
  - 97% overall concordance with known genotypes
  - 98% overall concordance with known alleles
  - New alleles were correctly identified by the software as “No matches” with the database, and location of sequence variation is displayed

# GS Junior Performance in our hands

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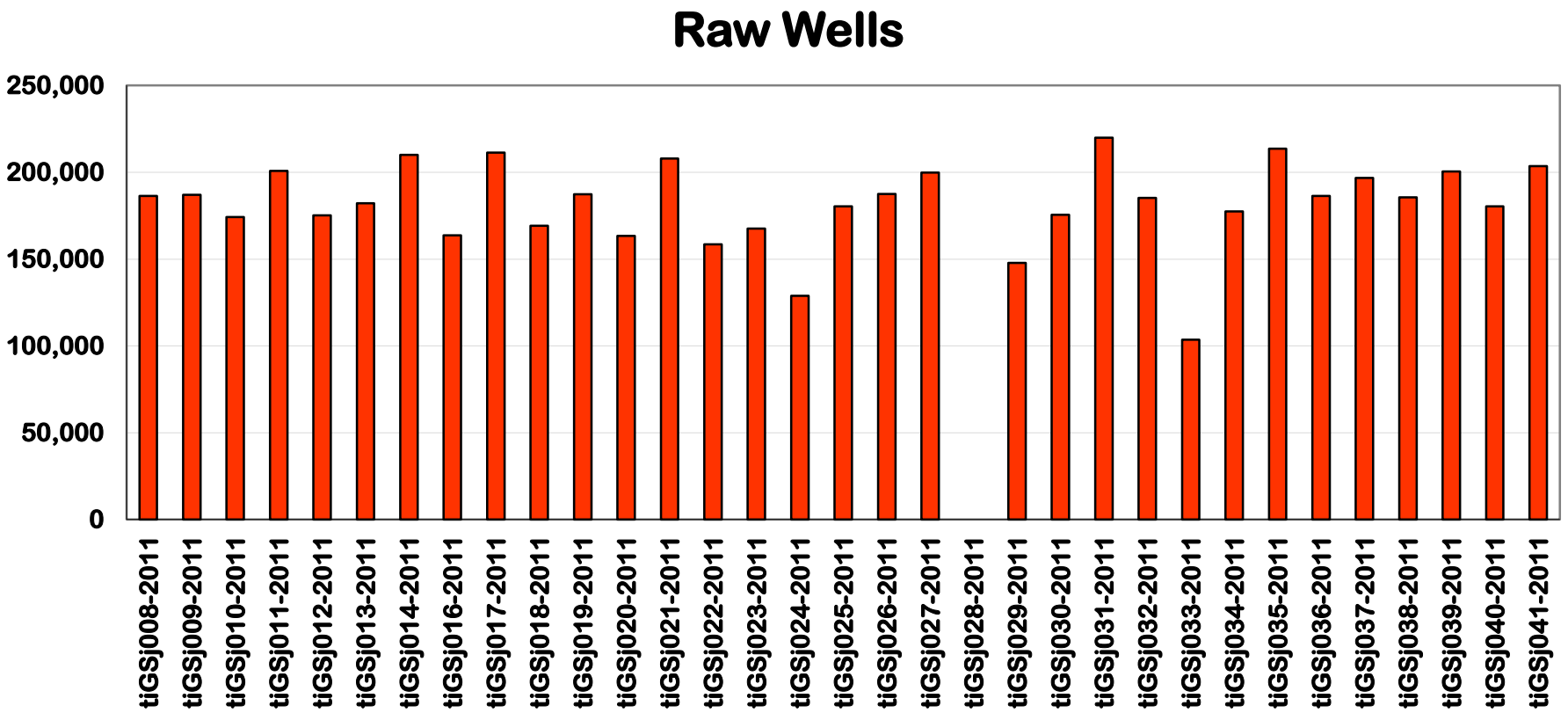

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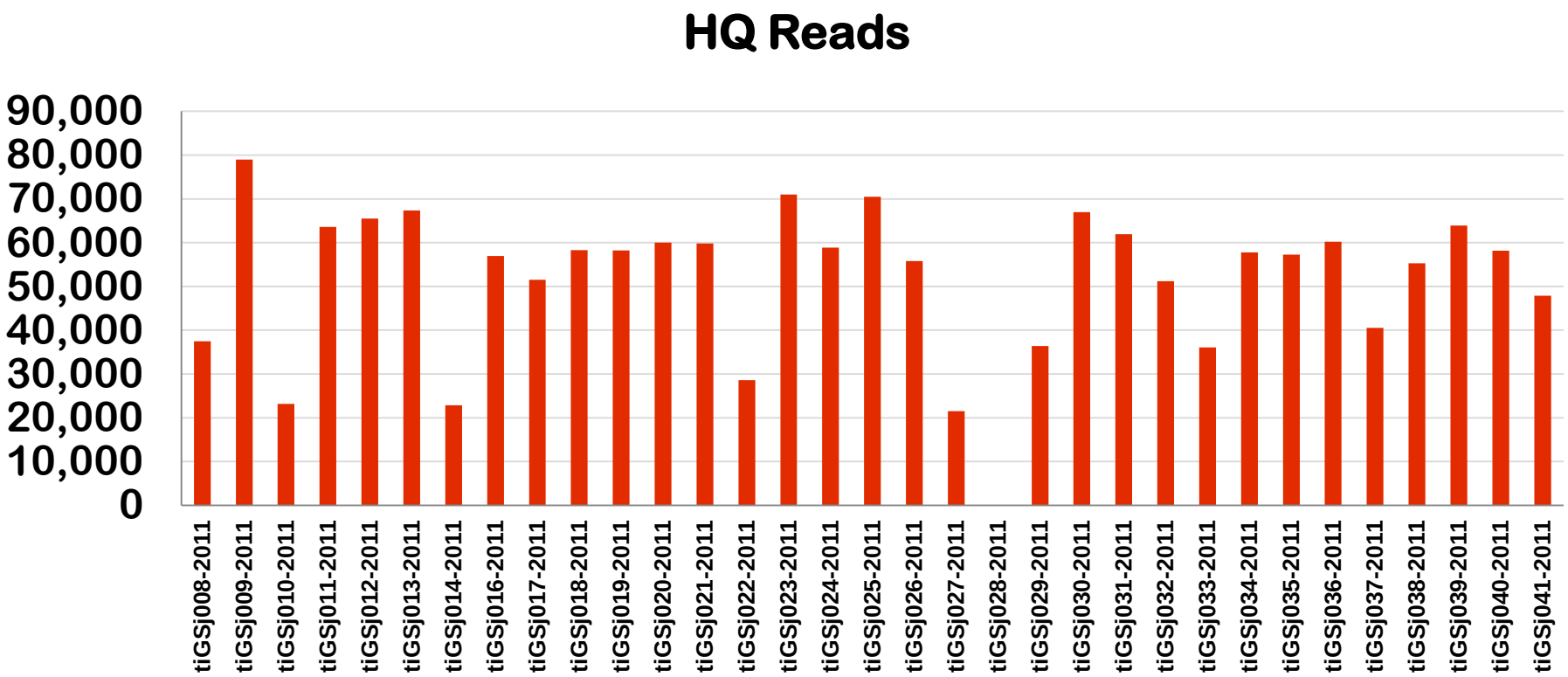
# GS Junior Performance in our hands



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# GS Junior Sequencing Performance - Validation Study.

| Parameter | Avg. | S.D. | CV (%) |
|---|---|---|---|
| Raw Wells [wells] | 182565 | 32665 | 18 |
| HQ Reads per Run [reads] | 66078 | 16821 | 25 |
| Median Read Length [bp] | 425.33 | 24.55 | 6 |
| QC (400bp, 98%) [%] | 77.14 | 4.03 | 5 |

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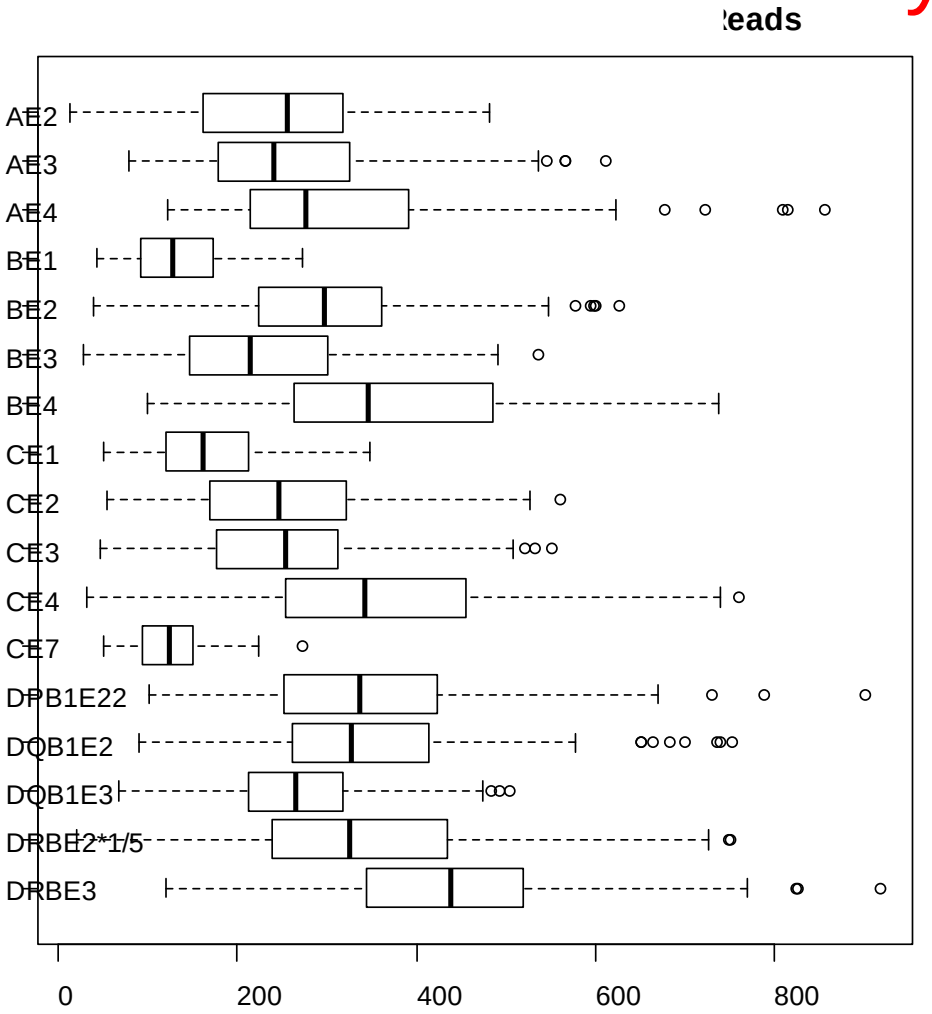
# Raw 454 reads – Validation Study.



# Raw 454 reads vs used ATF reads – Validation Study

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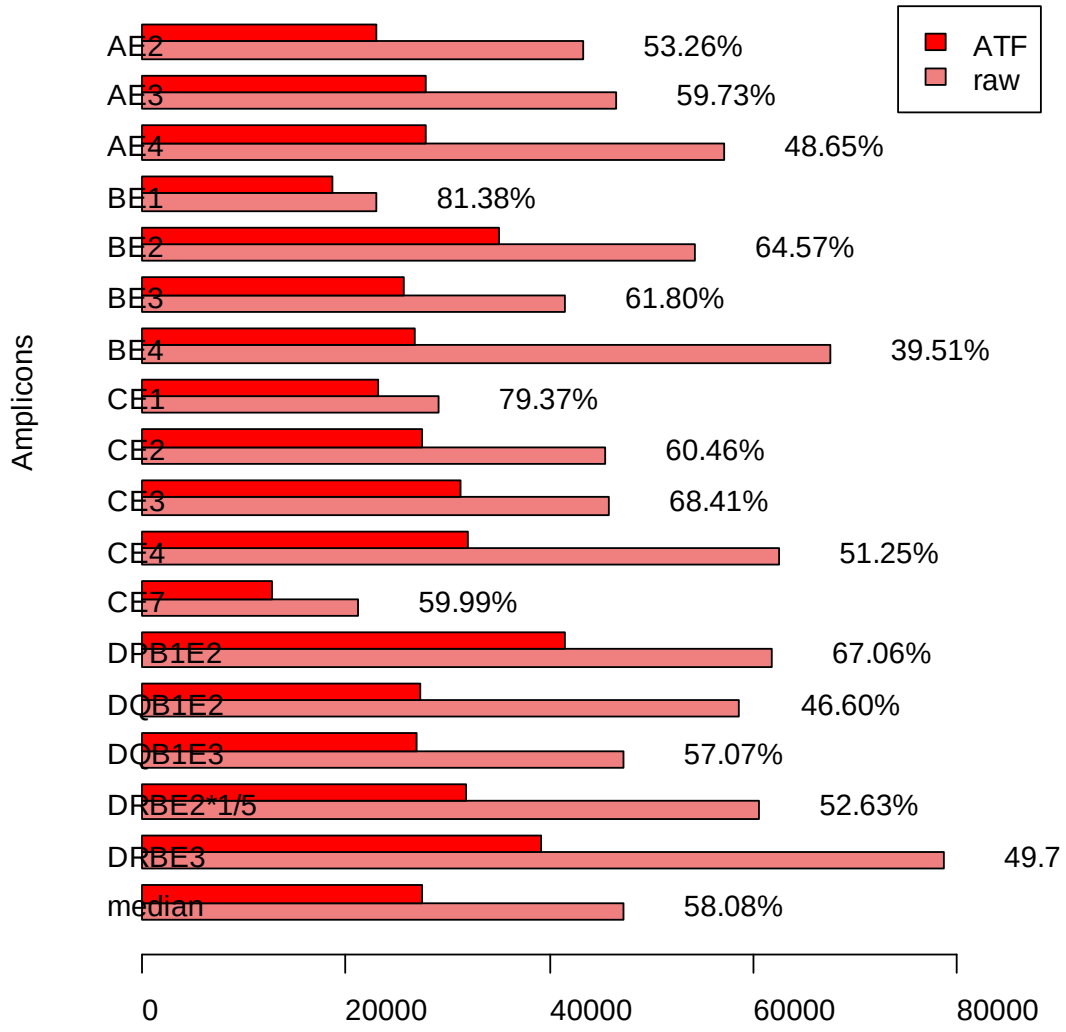


**Reasons:**

**Recognized Sequencing Errors**

**(Filter settings, Coverage)**

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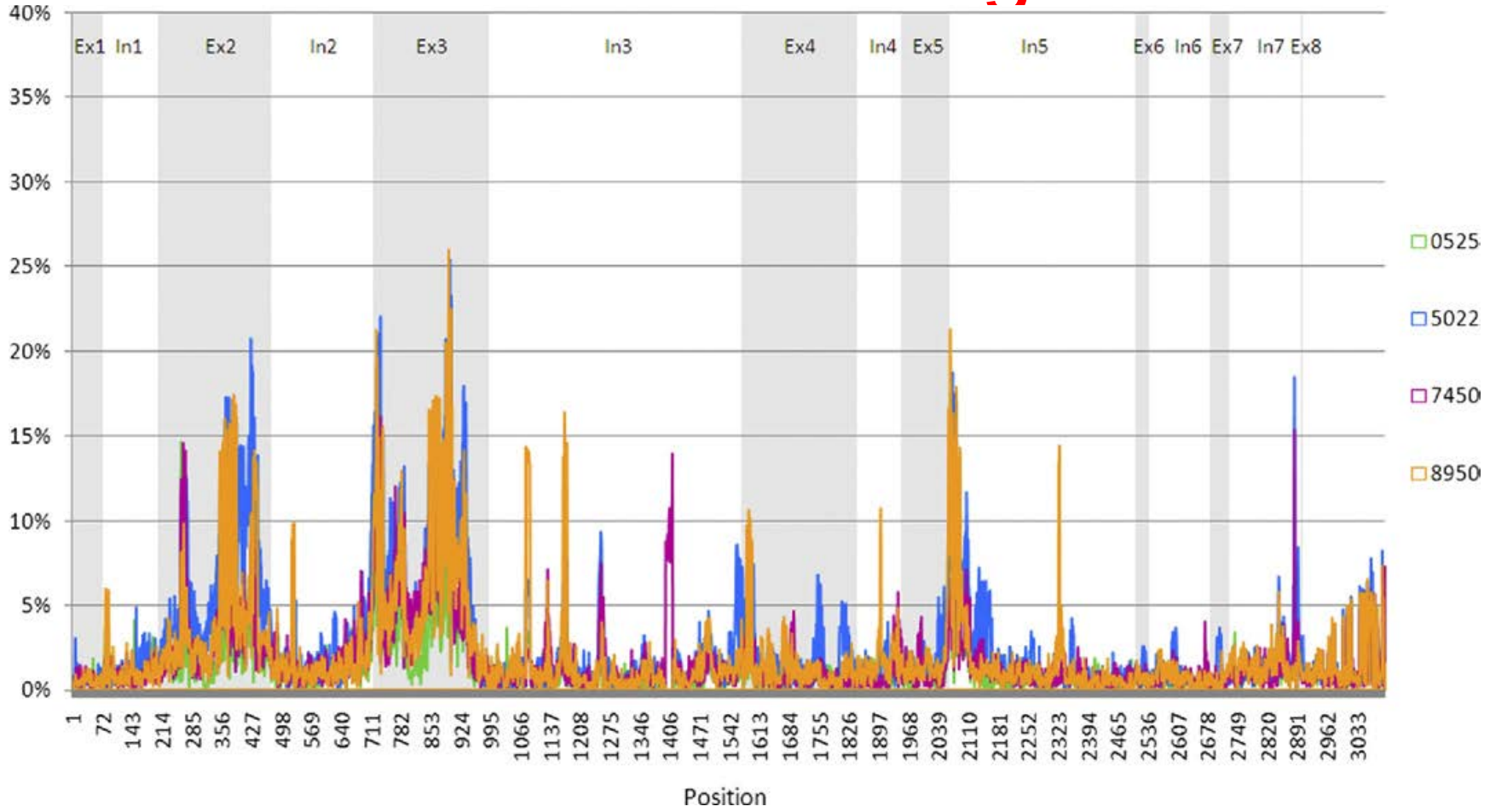
# Errors accounted to region




Lind et al Human Immunology 17, 1033 ff.

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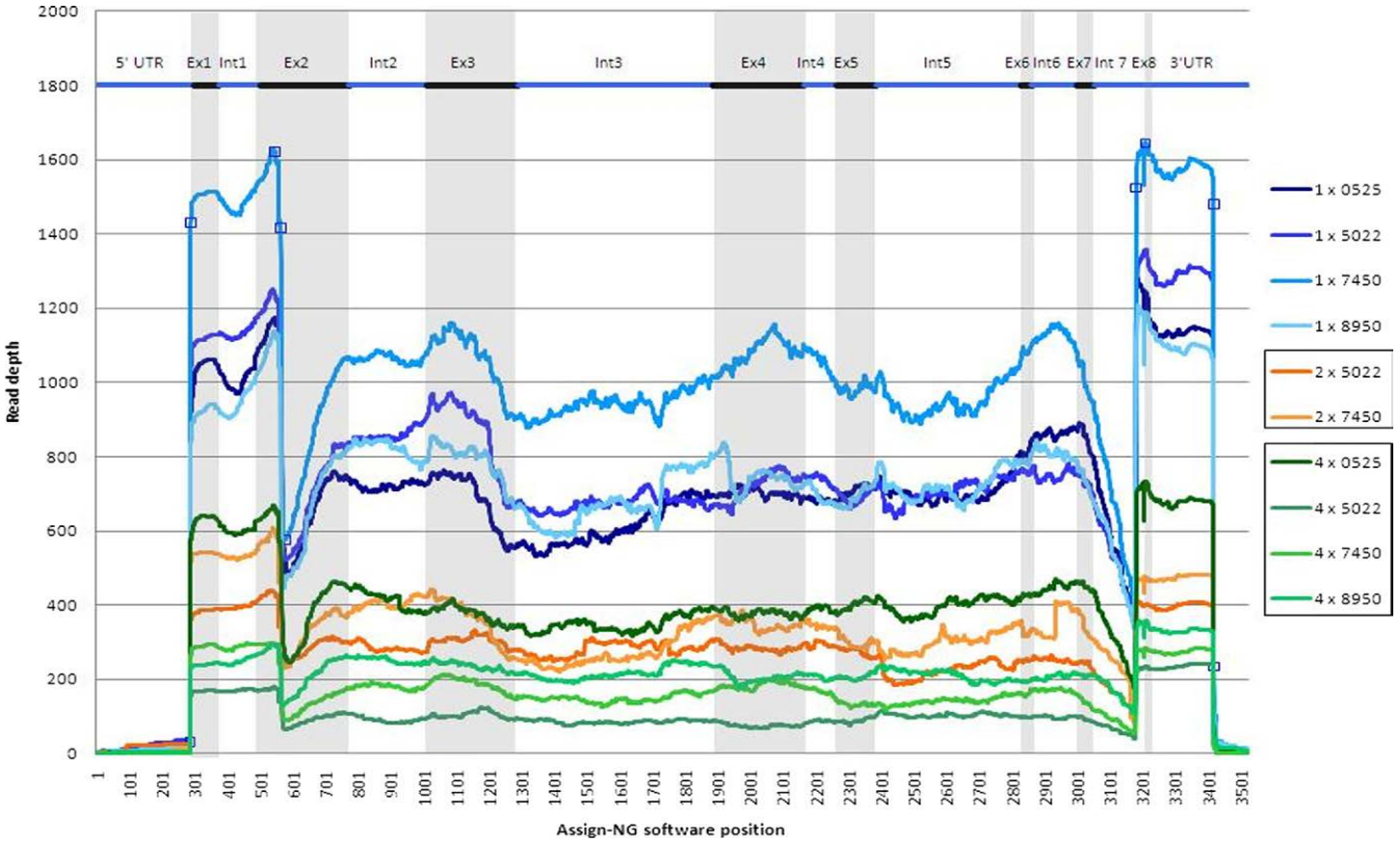
# Depth of base calls varies at each position




Lind et al Human Immunology 17, 1033 ff.

# Summary Typing Results – Validation Study

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.

| Locus | Total | successful | Insufficient Read counts | wrong | No Sanger | successfull Typings [%] | Concordance Rate [%] |
|---|---|---|---|---|---|---|---|
| A | 173 | 167 | 5 | 0 | 1 | 97,09% | 100,00% |
| B | 173 | 172 | 1 | 0 | 0 | 99,42% | 100,00% |
| C | 172 | 166 | 4 | 0 | 2 | 97,65% | 100,00% |
| DRB1 | 172 | 169 | 2 | 0 | 1 | 98,83% | 100,00% |
| DRB3 | 115 | 91 | 20 | 0 | 4 | 81,98% | 100,00% |
| DRB4 | 77 | 73 | 1 | 0 | 3 | 98,65% | 100,00% |
| DRB5 | 53 | 52 | 0 | 0 | 1 | 100,00% | 100,00% |
| DQB1 | 162 | 158 | 0 | 0 | 4 | 100,00% | 100,00% |
| DPB1 | 30 | 29 | 1 | 0 | 0 | 96,67% | 100,00% |
| all | 1127 | 1077 | 34 | 0 | 16 | 96,94% | **100,00%** |

# High throughput genotyping

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- no full exploitation of sequencing capacity with 26 patients
- according to the manufacturer‘s promotion up to 500 patients possible - in theory
- in HLA typing sequencing of 48 patients (à 14 amplicons) per run is performed
- simple amendment of further amplicons for blood group genotyping (ABO, etc.)

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# NGS HLA useability

- Variation in the selection of primers and exons – there is no standard
- NGS is useful but only under certain conditions:
  - High throughput
  - Automation
  - Full exploitation of bioinformatics

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# Conclusions

- Primers from Sanger SBT to NGS are not transferable. Some need new design
- Clonal sequencing by NGS enables
  - resolution of cis/trans linkage of SNPs
  - clear identification of duplications
  - *de novo* alterations can be identified easily
- NGS suitable for immunogenetics in very high resolution mode

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# SEQUENCING IGH AND TCR

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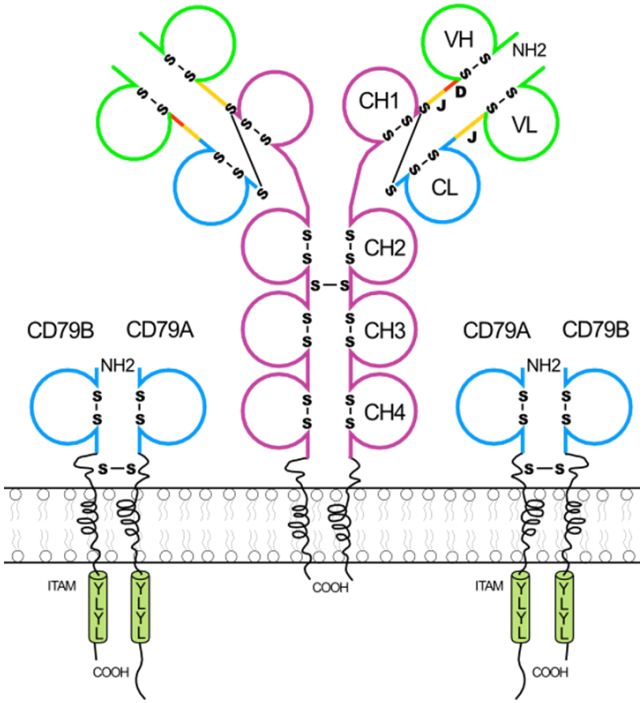
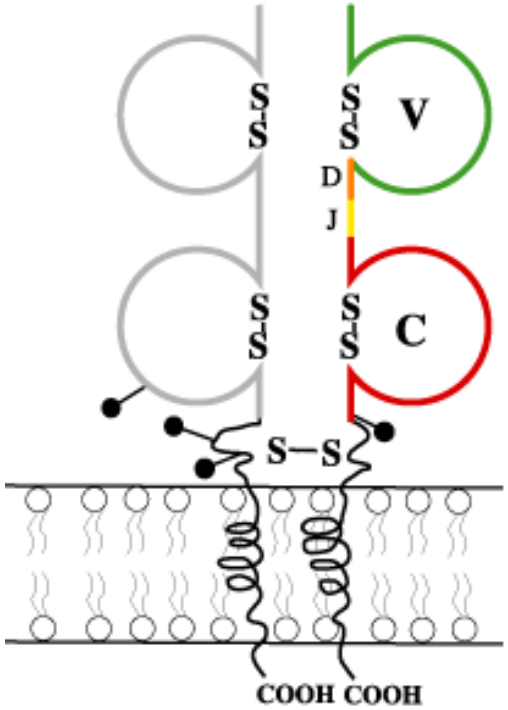
# Structure




BCR




TCR

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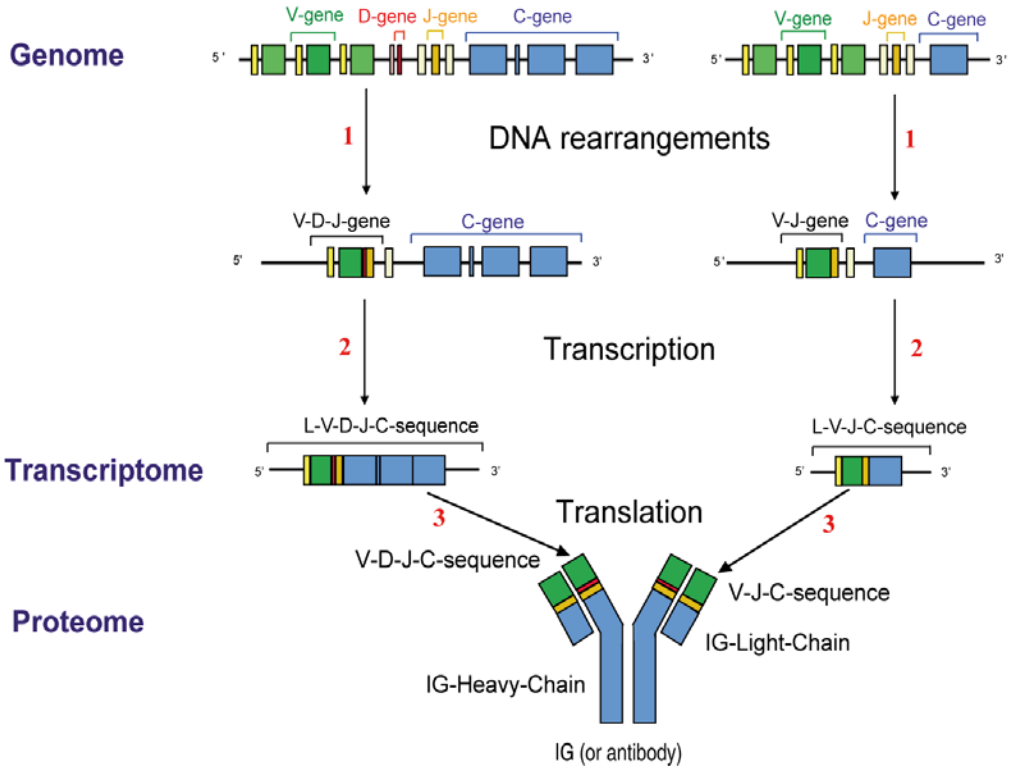
# Receptor-recombination




ca. $10^{12}$ variants of receptors

One specific receptor per cell

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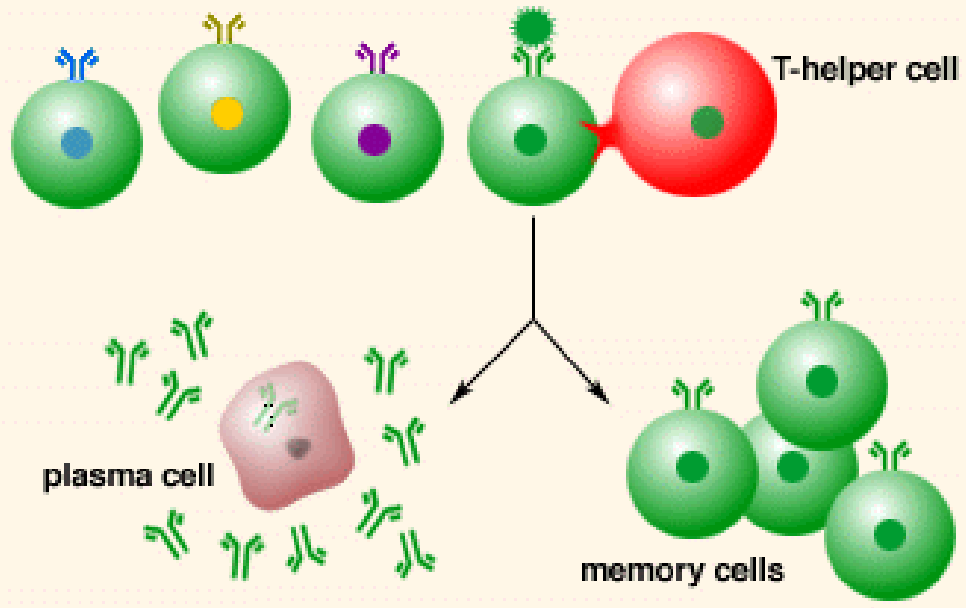
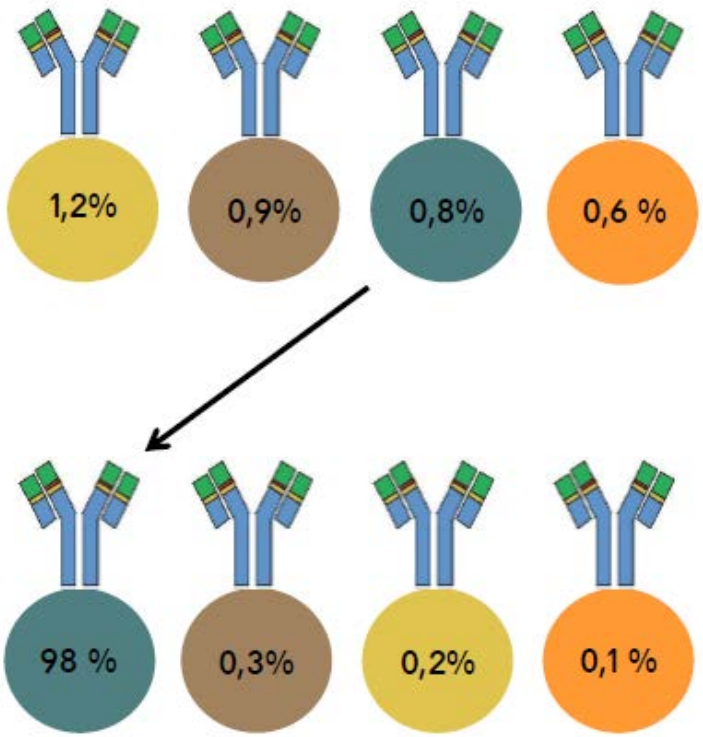
# Clonal selection and proliferation




Cells with high affinity to epitope proliferate

# Clonal Repertoire




Clonal development of the BCR/TCR repertoire by directed or undirected proliferation

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# METHODS

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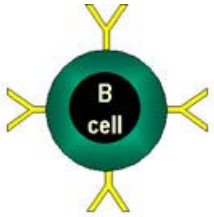
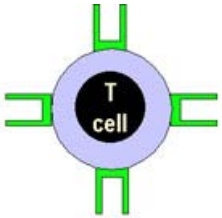
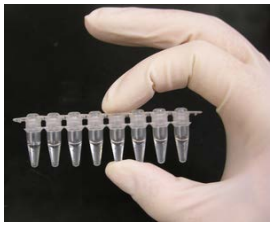
# Workflow



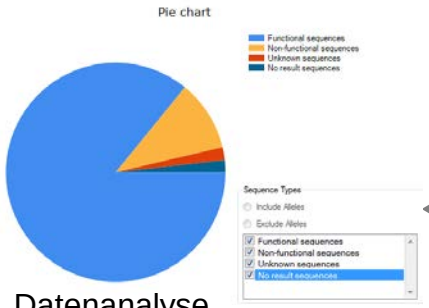

B- und T-Zell Isolation

DNA-
Extraktion



NGS Sample
Preparation

NGS




Datenanalyse

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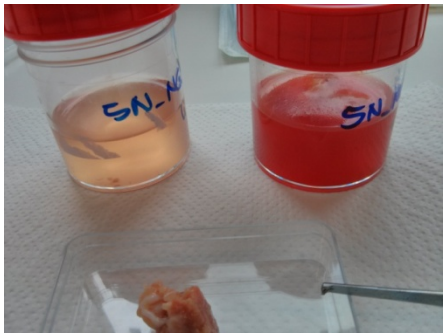
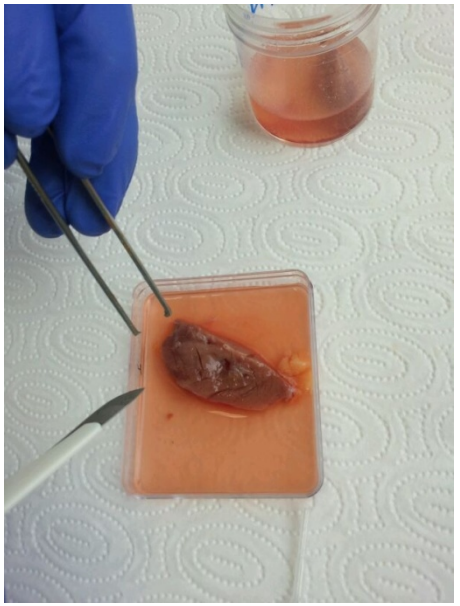
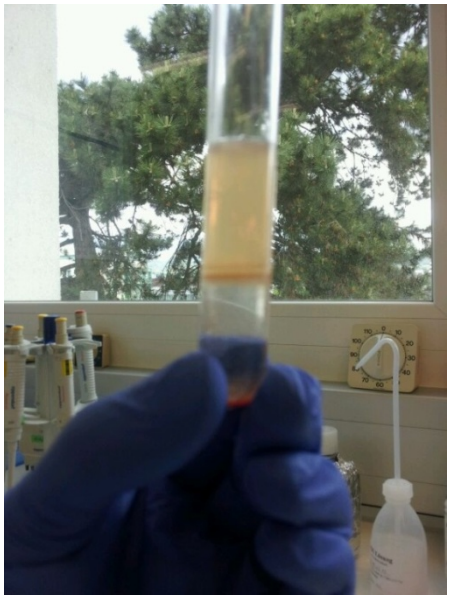
# Material

- Blood
- Urine
- Kidney tissue







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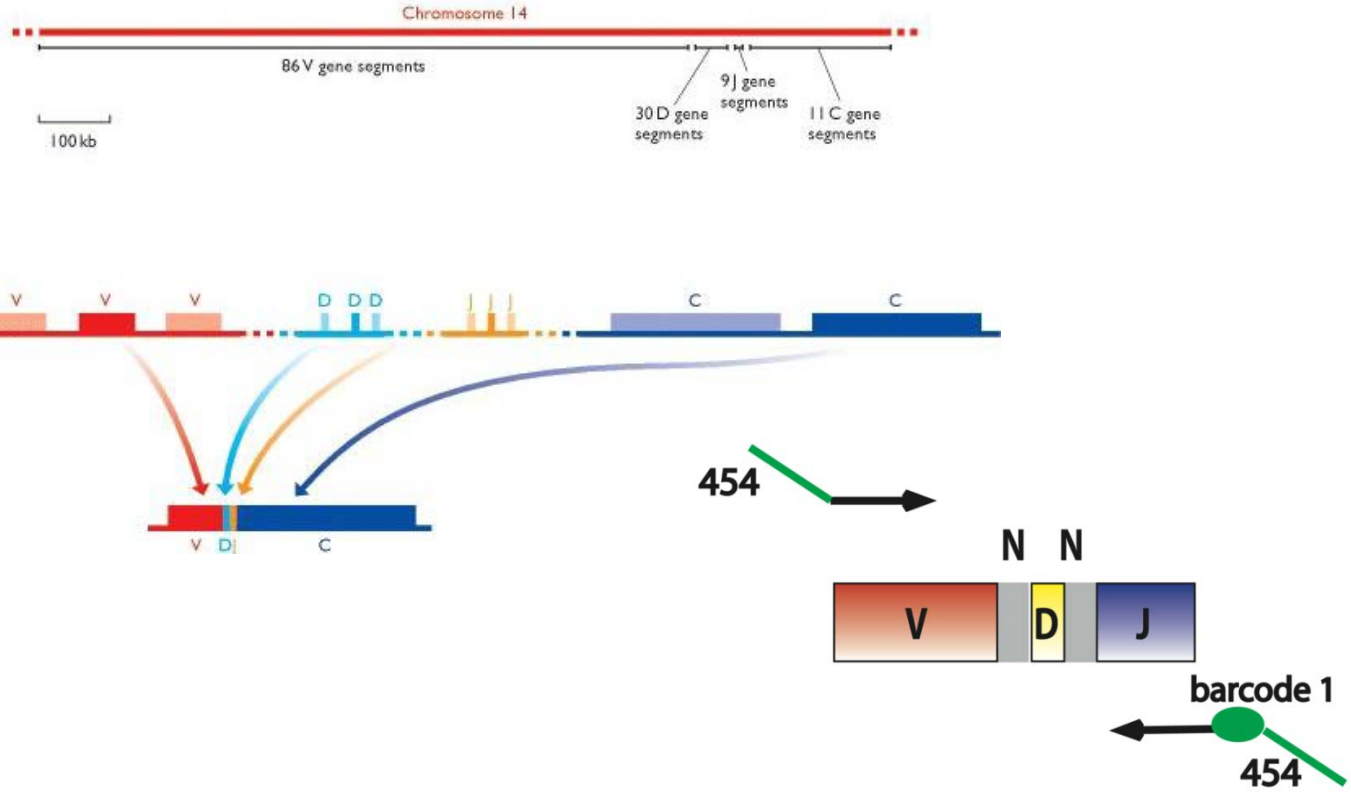
# Amplicons of the recombinant IGH and TCRB Loci

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# NGS Sequencing

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GSj

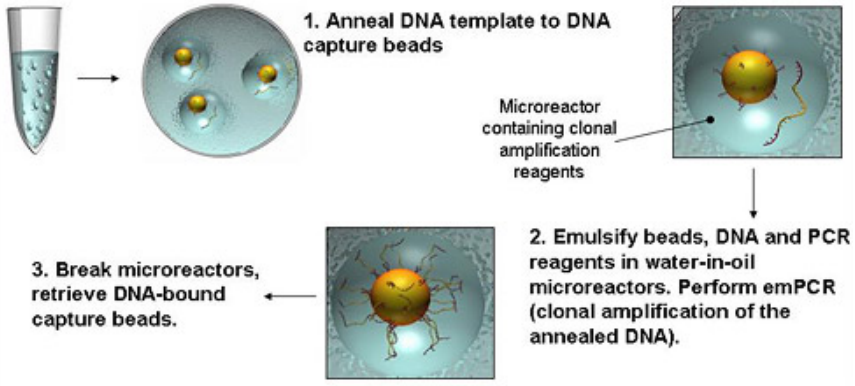
100,000 reads
Länge: 400bp

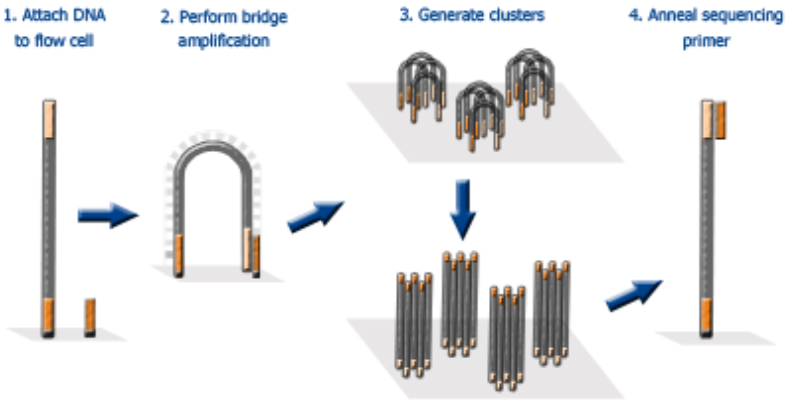
GS FLX(+)

700,000 reads
Länge: 400-700bp

MiSeq

15,000,000 reads
Länge: 250bp

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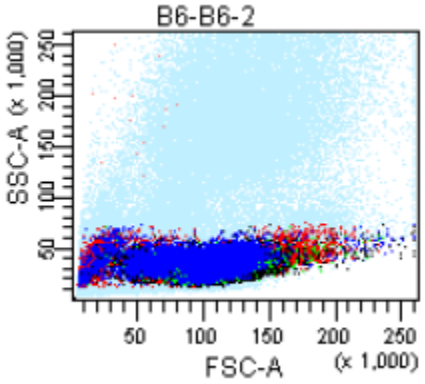
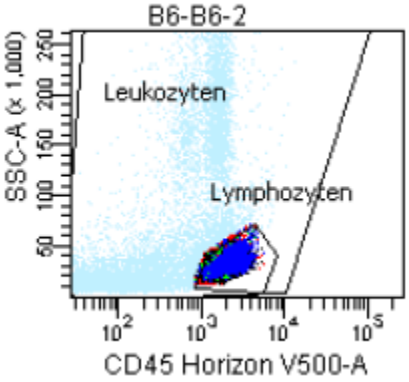
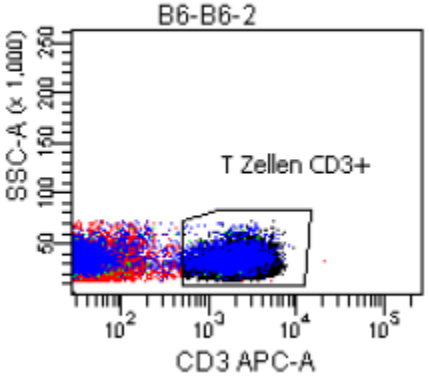
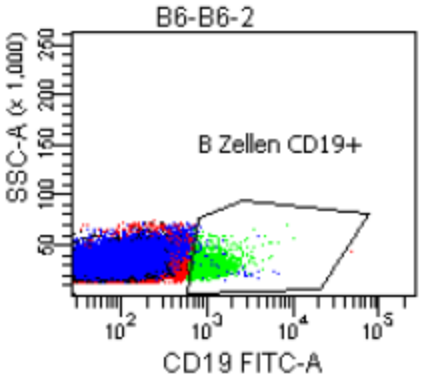
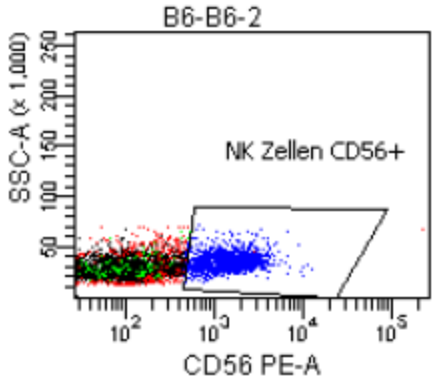
# FACS Blood




Tube: B6-2

| Population | #Events | %Parent | %Total |
|---|---|---|---|
| All Events | 300,000 | #### | 100.0 |
| Lymphozyten | 61,790 | 20.6 | 20.6 |
| T Zellen CD3+ | 35,282 | 57.1 | 11.8 |
| B Zellen CD19+ | 1,900 | 3.1 | 0.6 |
| NK Zellen CD56+ | 16,699 | 27.0 | 5.6 |
| Leukozyten | 299,880 | 100.0 | 100.0 |

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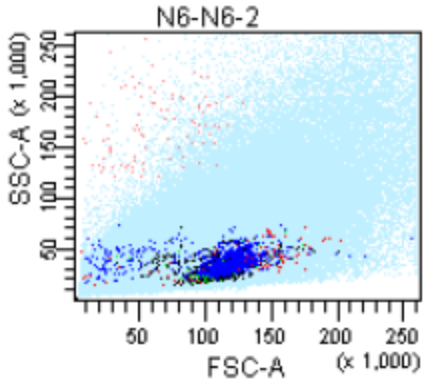
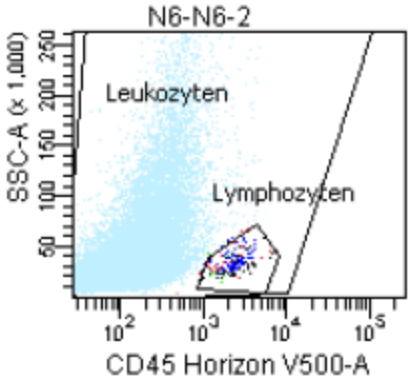
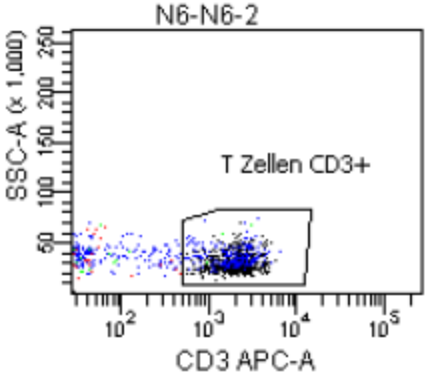
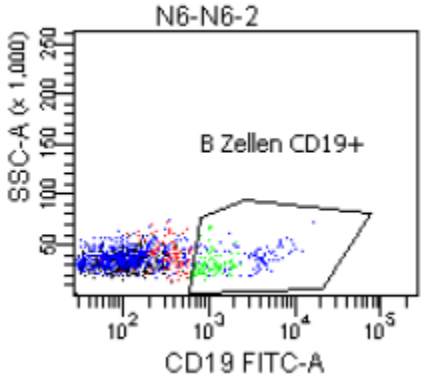
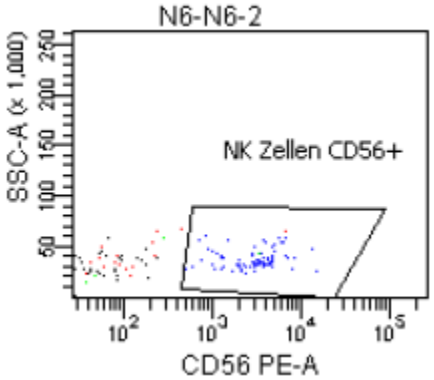
# FACS tissue




Tube: N6-2

| Population | #Events | %Parent | %Total |
|---|---|---|---|
| All Events | 300,000 | #### | 100.0 |
| Lymphozyten | 1,846 | 0.6 | 0.6 |
| T Zellen CD3+ | 835 | 45.2 | 0.3 |
| B Zellen CD19+ | 188 | 10.2 | 0.1 |
| NK Zellen CD56+ | 949 | 51.4 | 0.3 |
| Leukozyten | 299,828 | 99.9 | 99.9 |

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# CDR3 distribution of healthy volunteers

| Sequence | Count | Percentage |
|---|---|---|
| ACWSSDTLNSTGP | 325 | 1,55405728 |
| ASS*GEQSAPA | 275 | 1,31497155 |
| ASSETVDYGYT | 182 | 0,87027208 |
| ATEGYGYT | 120 | 0,57380577 |
| ASSFLRGGYGYT | 109 | 0,5212069 |
| ASNQNTGAPYEQY | 101 | 0,48295319 |
| ASSHRDRGRSLKL | 85 | 0,40644575 |
| ASSYGRQAISPS | 78 | 0,37297375 |
| ASSPGLTVRSYEQY | 78 | 0,37297375 |
| ASRQSYGYT | 77 | 0,36819203 |
| TSS | 74 | 0,35384689 |

TRB

| Sequence | Count | Percentage |
|---|---|---|
| ARVFRY | 311 | 0,140360695 |
| ARGIDY | 261 | 0,117794667 |
| AR | 213 | 0,09613128 |
| ARCYGMDV | 203 | 0,091618074 |
| ARGGNSDY | 196 | 0,088458831 |
| ARWHDNIPADY | 175 | 0,078981099 |
| ARDVGTLERWYYFDY | 164 | 0,074016572 |
| ASTYYGMDV | 155 | 0,069954687 |
| AASSG | 155 | 0,069954687 |
| ARGYYGMDV | 147 | 0,066344123 |
| ARALDVDY | 140 | 0,063184879 |

IGH

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# Patient 1

- Nephrectomy due to Haydronephrosis
- Kidneybiopsy: chronic interstitial nephritis with tubulitis and atrophy of tubules, as a response to chronic pyelonephritis
- Tissue sample: 2x3cm

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# CDR3 9N TCRB vs 9B TCRB

9N

| Sequence | Count | Percentage |
|---|---|---|
| ASSPYTTGRKLF | 12299 | 18,6249716 |
| ASSKEYRGAGGYT | 9588 | 14,519573 |
| ASSPDRGGNQPQH | 6352 | 9,61914136 |
| ASSYSPTMNTEAF | 3527 | 5,34110699 |
| ASSYGGDGYT | 1904 | 2,88331945 |
| ASSATLEGAGYGYT | 835 | 1,26448096 |
| ATLNYGYT | 599 | 0,90709472 |
| ASSSTA*GHNQPQH | 595 | 0,90103733 |
| ASSSILLRTLLRAV | 486 | 0,73597335 |

9B

| Sequence | Count | Percentage |
|---|---|---|
| ASSKEYRGAGGYT | 6170 | 4,79562254 |
| ASSYSPTMNTEAF | 5997 | 4,66115857 |
| ASSPYTTGRKLF | 5753 | 4,47150996 |
| ASSYGGDGYT | 5510 | 4,2826386 |
| ASSATLEGAGYGYT | 3311 | 2,5734694 |
| ASSPDRGGNQPQH | 847 | 0,65832938 |
| ASSPRDS*TLKL | 670 | 0,52075642 |
| ASS*GC*QGMAT | 622 | 0,4834485 |
| ASSEGVGTFSAPLH | 552 | 0,42904111 |

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# CDR3 9N IGH vs 9B IGH

9N

| Sequence | Count | Percentage |
|---|---|---|
| ASVMGPLLWFGKSQHRYYFDY | 22536 | 35,4256072 |
| MKRC*RSSD*SSYGP*L | 382 | 0,60048731 |
| AREVGRRPPEAWEVWT | 337 | 0,52974927 |
| ASVMGPLLWFGKSQHRYYFDS | 257 | 0,40399277 |
| ASVMGPLLWFGKSQHTYYFDY | 253 | 0,39770494 |
| ATSLI | 174 | 0,2735204 |
| AKRVGAYSPFEY | 169 | 0,26566061 |
| TKTFSRLVKYFCRD*L | 161 | 0,25308496 |
| F*LAAGAY*FDC | 136 | 0,21378606 |

9B

| Sequence | Count | Percentage |
|---|---|---|
| ASVMGPLLWFGKSQHRYYFDY | 2164 | 6,69948299 |
| AKRVGAYSPFEY | 1587 | 4,91316058 |
| IAVVTAFRLDVSDI | 1168 | 3,61598712 |
| FYYDSGCLAGST | 1018 | 3,15160521 |
| AKRVGASSPFEY | 227 | 0,70276462 |
| GRDSVETGGLT | 138 | 0,42723136 |
| ARV*TTMRVVGRFGGLT | 108 | 0,33435497 |
| ASVMGPLLWFGESQRRYYFDF | 102 | 0,3157797 |
| ARDWGIQLWLLGGMDV | 101 | 0,31268382 |

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# CLL patient

| Sequence | Count | Percentage |
|---|---|---|
| ARAGINWGPYFDC | 17701 | 95,5158645 |
| ARAGINWAHTLT | 79 | 0,42628966 |
| AKGLTTTGTTPDY | 65 | 0,35074466 |
| ARAGINWGPYFD | 48 | 0,25901144 |
| VKGLTTTGTTPDY | 41 | 0,22123894 |
| ARAGINWGPYLDC | 24 | 0,12950572 |
| ARAGINWGSYFDC | 20 | 0,10792143 |
| ARAGINWSPYFDC | 17 | 0,09173322 |
| ARVGINWGPYFDC | 16 | 0,08633715 |

IGH

| Sequence | Count | Percentage |
|---|---|---|
| ASSRGRGYEQY | 2124 | 3,54035404 |
| ASSIIGNQPQH | 1496 | 2,49358269 |
| ASVQVTMAT | 942 | 1,57015702 |
| ASSRGQSYGYT | 584 | 0,97343068 |
| ASSRGQNYGYT | 578 | 0,96342968 |
| ASREDGRLRAV | 472 | 0,78674534 |
| ASSRGQGYEQY | 444 | 0,74007401 |
| ASSRGLSYEQY | 438 | 0,73007301 |
| ASSRGGSYEQY | 381 | 0,63506351 |

TRB

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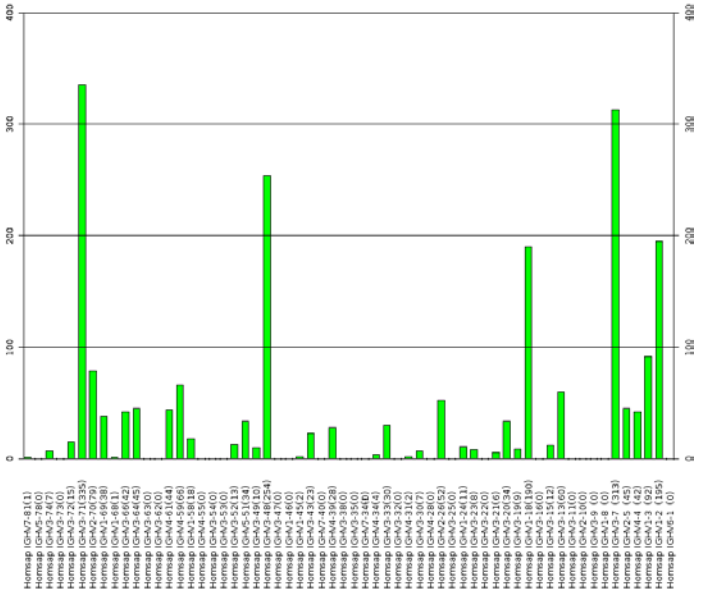
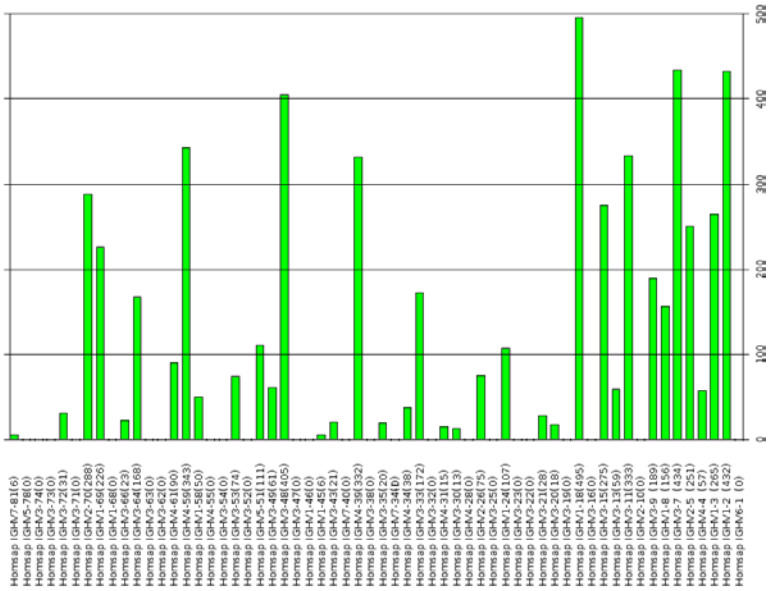
# V-Elements of different patients

Proband 1



Proband 2



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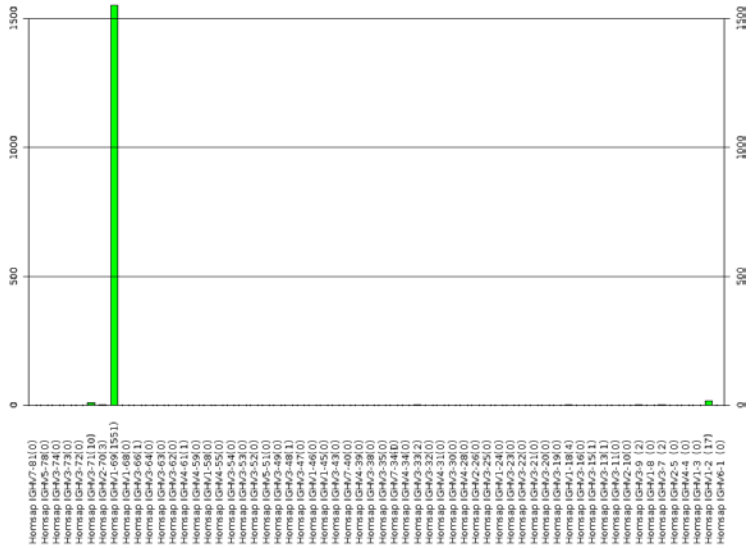
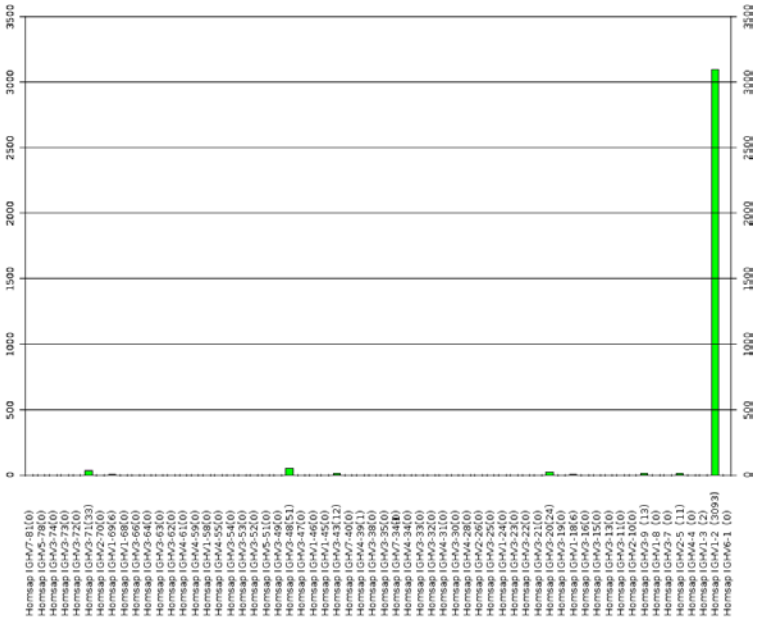
# V-Elements Lymphoma

Patient 1



Patient 2



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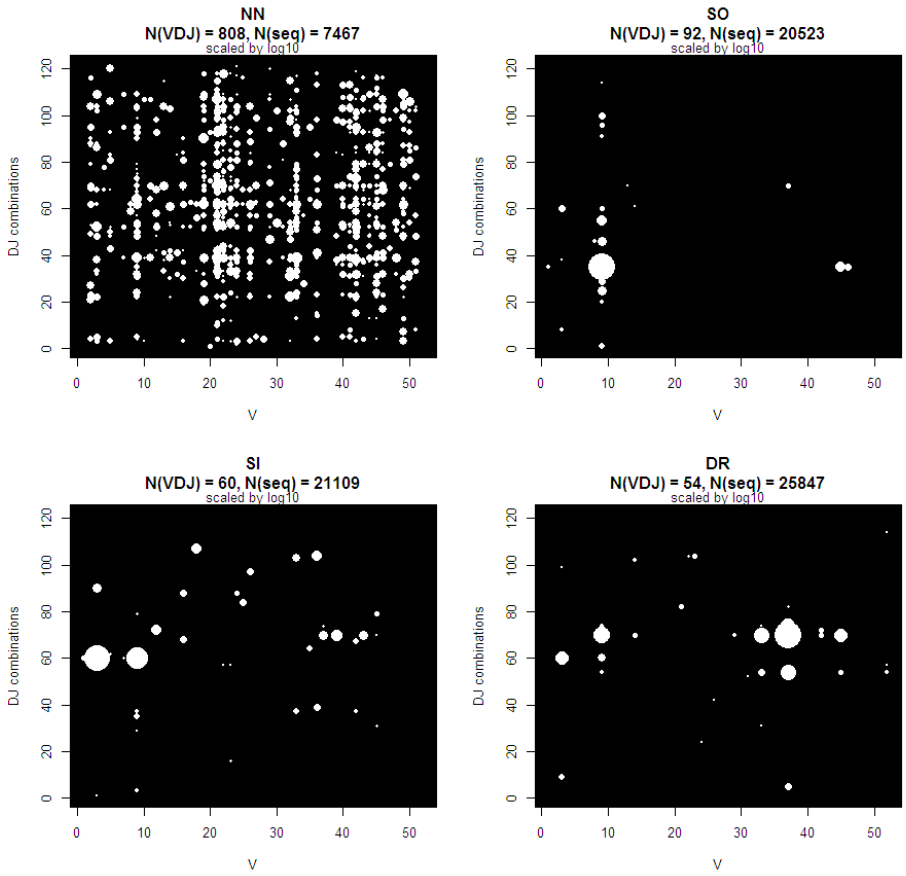
# V(D)J recombination: Spectra IgH



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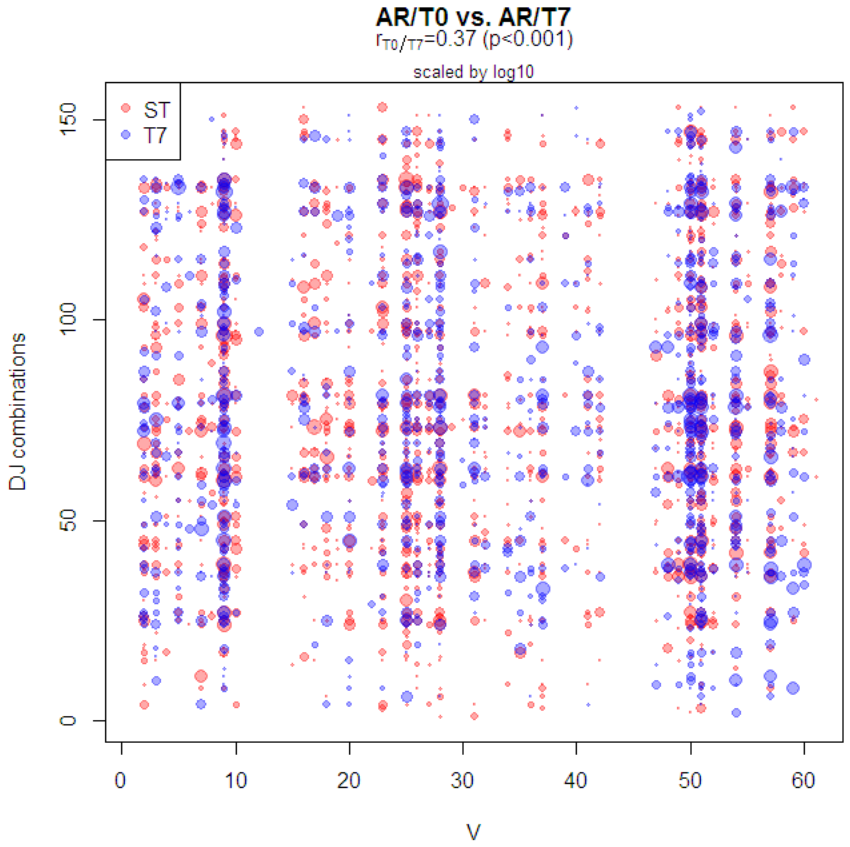
# 2 V(D)J spectra same patient at different times



# Working with NGS is sometimes slippery

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You need a really clear vision what you want

# And sometimes serenity

# Team Genomics


Johannes Pröll, Christa Hackl, Katja Hofer, Steffi Stabentheiner, Martin Danzer, Norbert Niklas