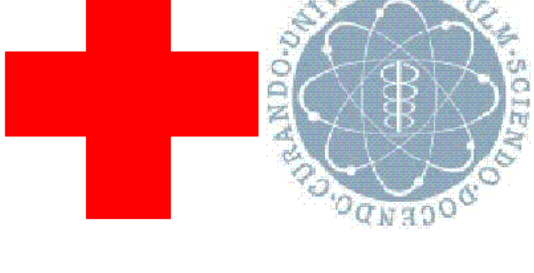
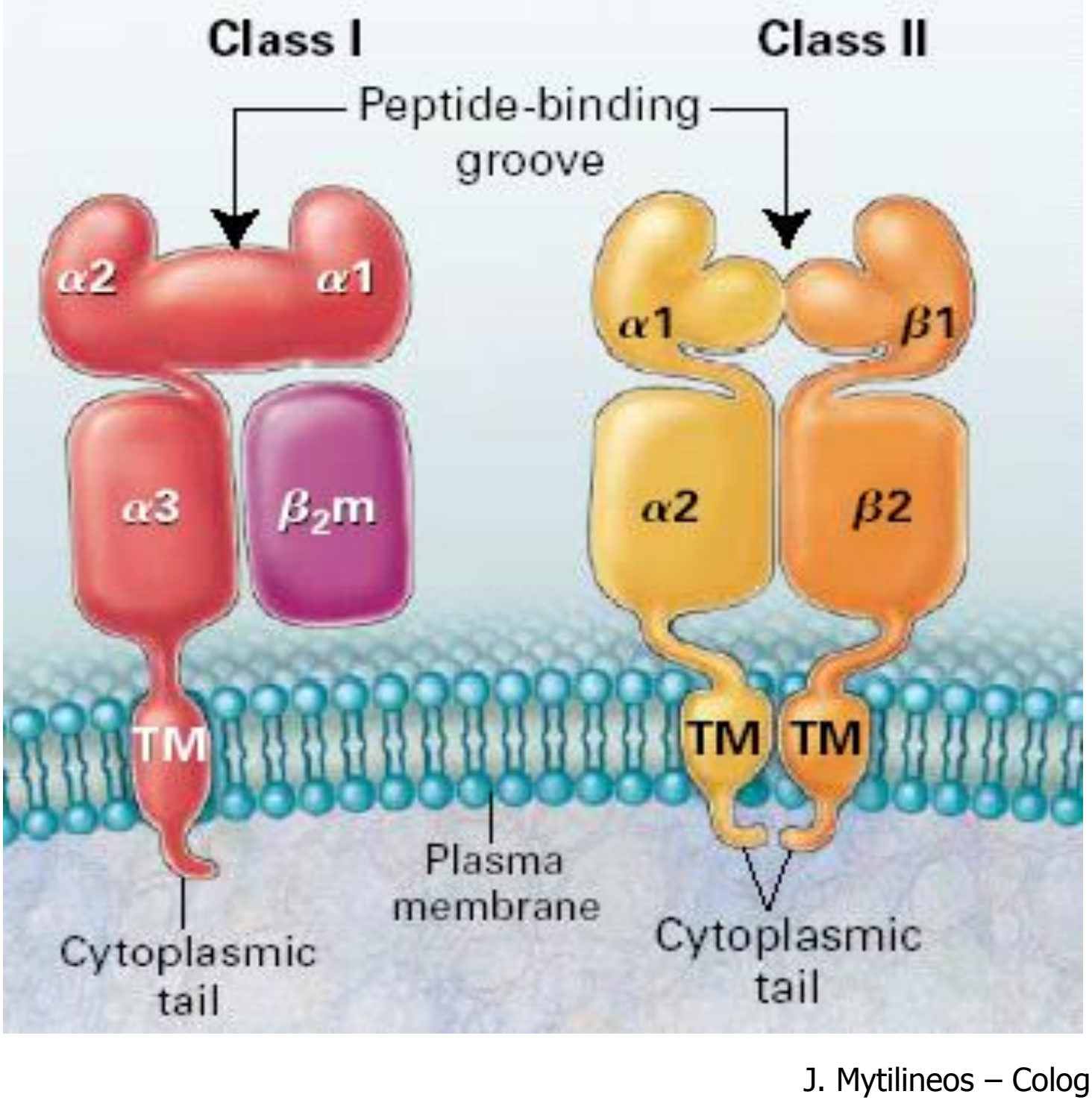
HLA-Typing Strategies

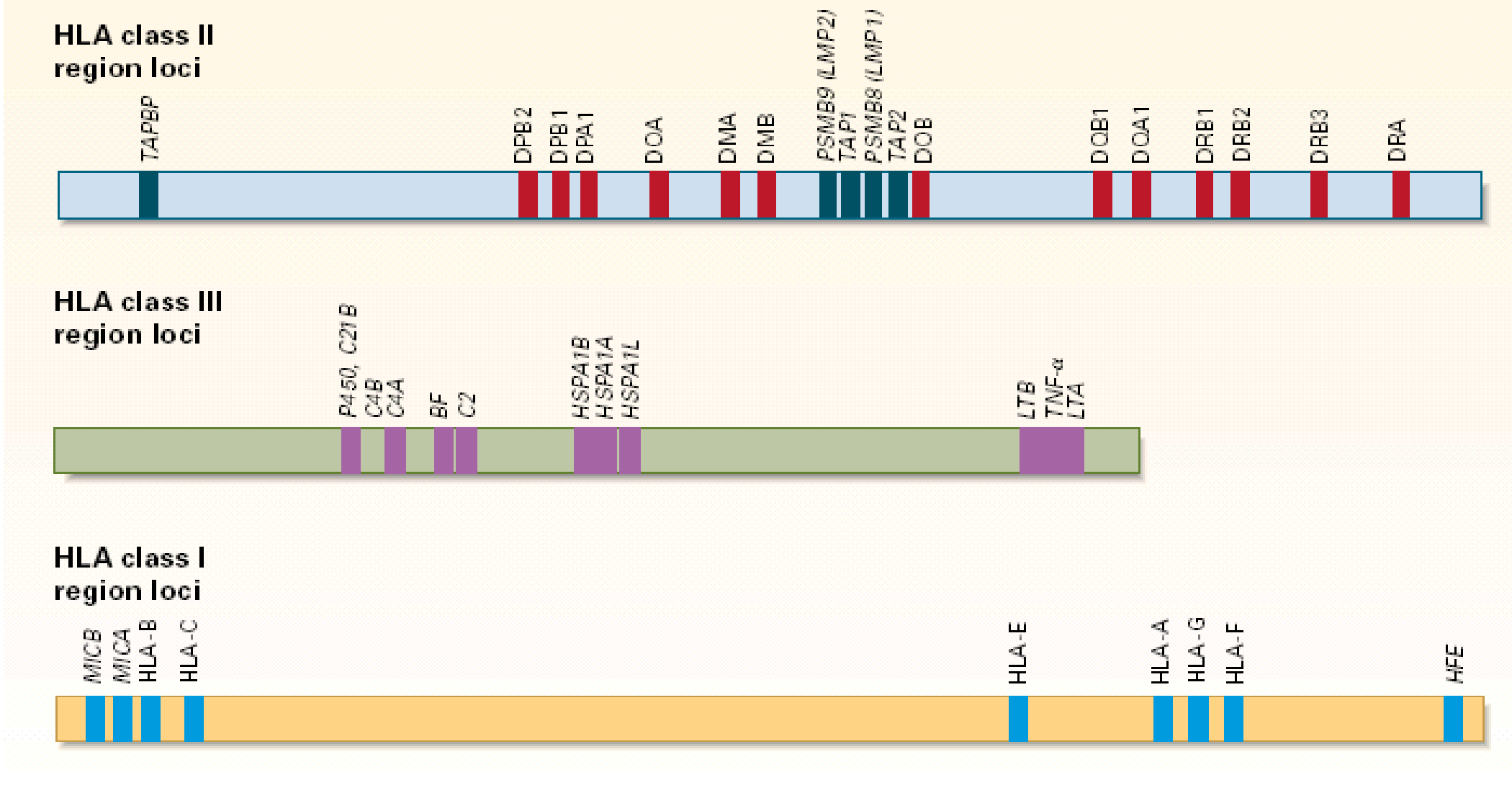
Cologne, 13.5.2017



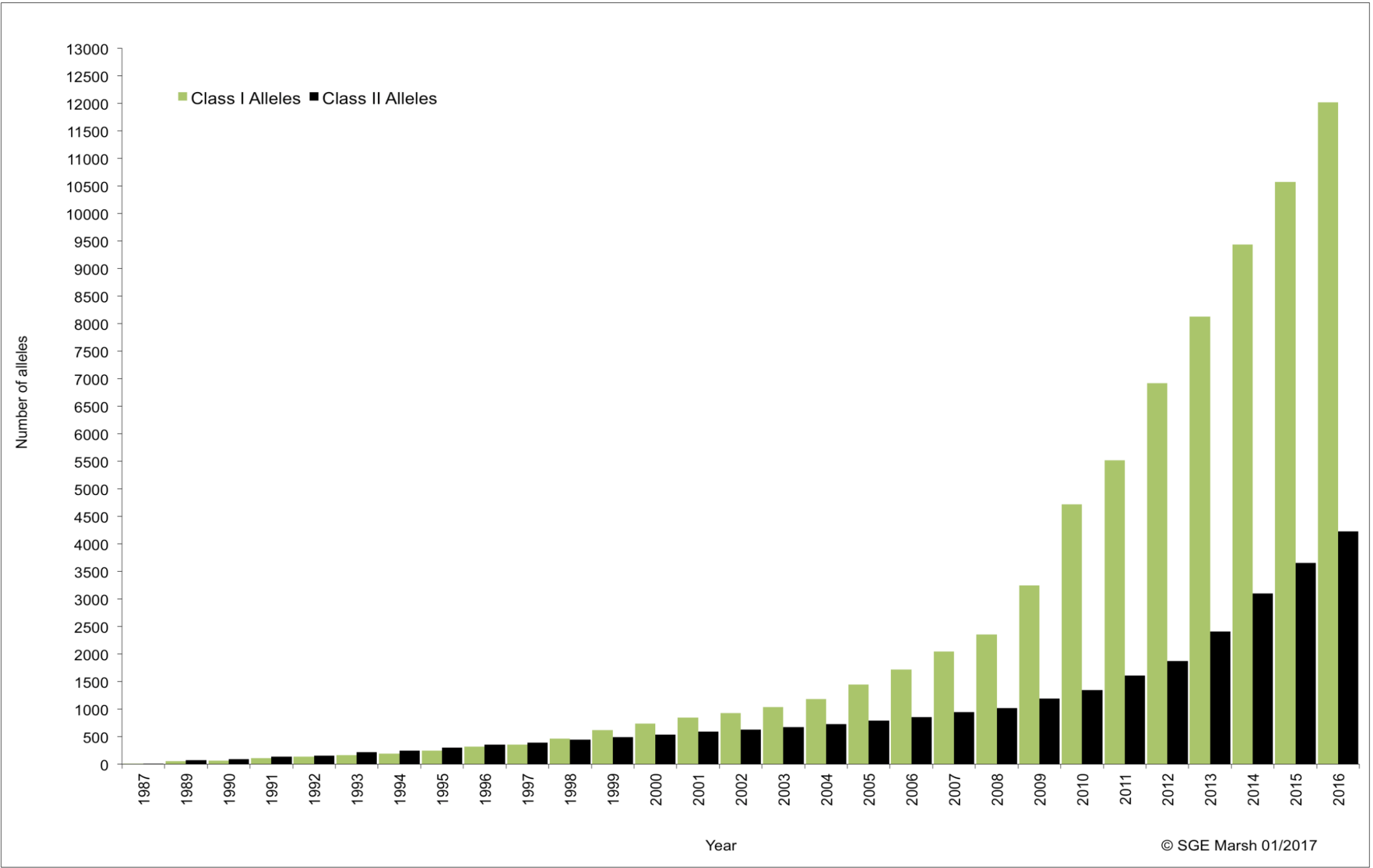
Joannis Mytilineos MD, PhD
Department of Transplantation Immunology
Institute for Clinical Transfusion Medicine and Immunogenetics
German Red Cross Blood Transfusion Service, and
Department of Transfusion Medicine - University Clinic Ulm
Ulm, Germany


Class I
Class II
Peptide-binding groove
α2
α1
α1
β1
α3
β2m
α2
β2
TM
TM
TM
Plasma membrane
Cytoplasmic tail
Cytoplasmic tail

Human MHC


HLA class II region loci
TAPBP
DPB2
DPB1
DPA1
DOA
DMA
DMB
PSMB9 (LMP2)
TAP1
PSMB8 (LMP1)
TAP2
DOB
DQB1
DQA1
DRB1
DRB2
DRB3
DRA
HLA class III region loci
P450, C21B
C4B
C4A
BF
C2
HSPA1B
HSPA1A
HSPA1L
LTB
TNF-α
LTA
HLA class I region loci
MICB
MICA
HLA-B
HLA-C
HLA-E
HLA-A
HLA-G
HLA-F
HFE

Annual increase of known HLA-Alleles



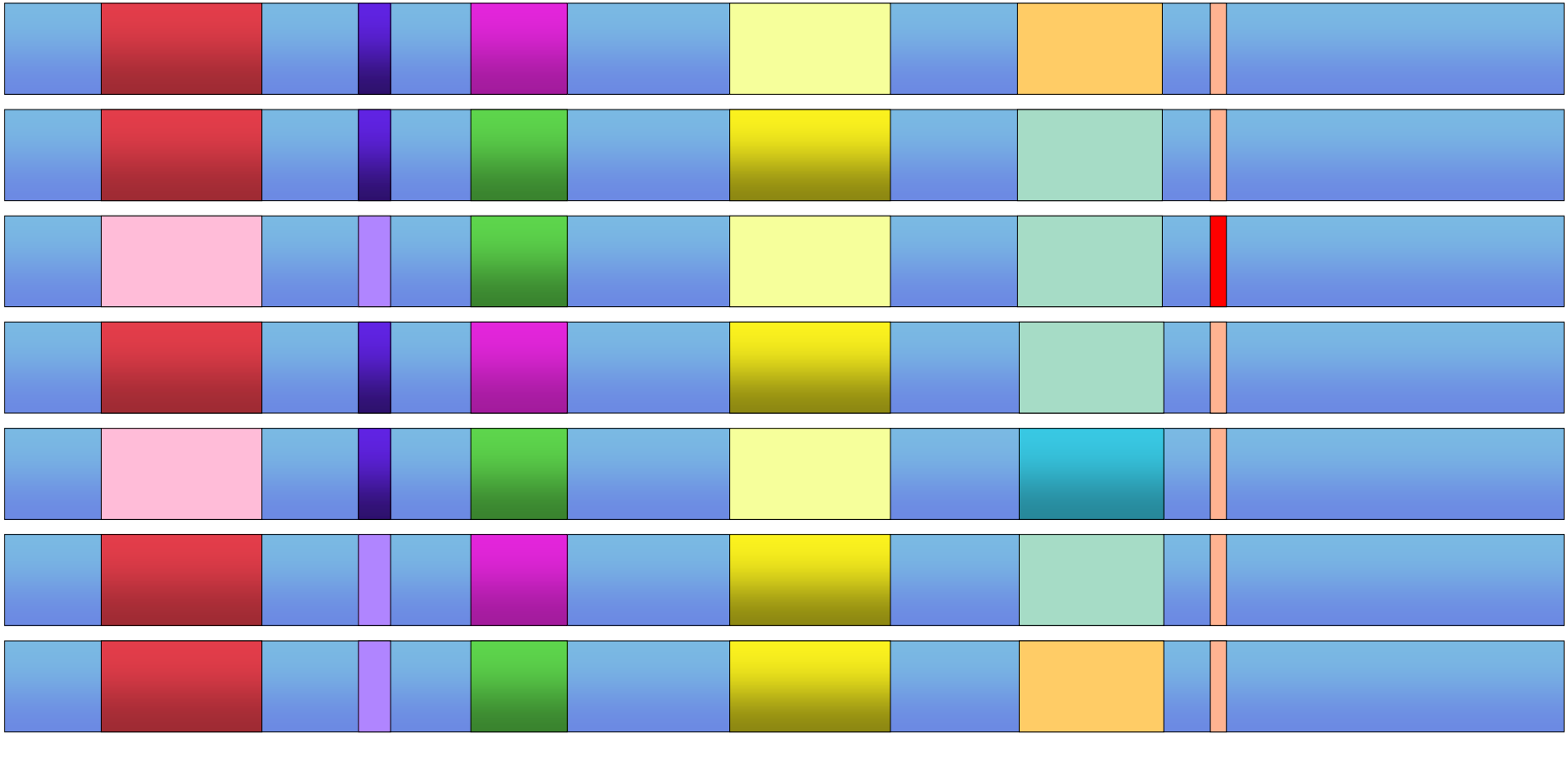
Current Number of recognized HLA-Alleles

Apr 2017/(Dec. 2008) /http://hla.alleles.org/nomenclature/stats.html)

HLA-A	**3.830 (733)**
HLA-B	**4.647 (1.115)**
HLA-Cw	**3.382 (392)**
HLA-DRB	**2.252 (697)**
HLA-DQB1	**1.054 (95)**
HLA-DPB1	**740 (132)**

A total of 16.251 HLA Alleles as of Apr. 2017

HLA alleles possess a “patchwork” pattern of polymorphism



Inheritence of HLA

Mother		
HLA-A:	01:01	29:01
HLA-B:	08:01	35:03
HLA-Cw:	07:01	04:01
HLA-DRB1*:	07:01	03:01
HLA-DQA1*:	02:01	05:01
HLA-DQB1*:	03:03	02:01
HLA-DPB1*:	09:01	04:01
	1	2

Father		
HLA-A:	02:05	03:01
HLA-B:	08:01	40:01
HLA-Cw:	03:03	07:02
HLA-DRB1*:	13:01	08:01
HLA-DQA1*:	01:03	04:01
HLA-DQB1*:	06:03	04:02
HLA-DPB1*:	04:01	15:01
	3	4

Child 1		
HLA-A:	29:01	03:01
HLA-B:	35:03	40:01
HLA-Cw:	04:01	07:02
HLA-DRB1*:	03:01	08:01
HLA-DQA1*:	05:01	04:01
HLA-DQB1*:	02:01	04:02
HLA-DPB1*:	04:01	15:01
	2	4

Child 2		
HLA-A:	01:01	03:01
HLA-B:	08:01	40:01
HLA-Cw:	07:01	07:02
HLA-DRB1*:	07:01	08:01
HLA-DQA1*:	02:01	04:01
HLA-DQB1*:	03:03	04:02
HLA-DPB1*:	09:01	15:01
	1	4

Child 3		
HLA-A:	29:01	03:01
HLA-B:	35:03	40:01
HLA-Cw:	04:01	07:02
HLA-DRB1*:	03:01	08:01
HLA-DQA1*:	05:01	04:01
HLA-DQB1*:	02:01	04:02
HLA-DPB1*:	04:01	15:01
	2	4

Child 4		
HLA-A:	01:01	02:05
HLA-B:	08:01	08:01
HLA-Cw:	07:01	03:03
HLA-DRB1*:	07:01	13:01
HLA-DQA1*:	02:01	01:03
HLA-DQB1*:	03:03	06:03
HLA-DPB1*:	09:01	04:01
	1	3

Why do we type for HLA

- Transplantation
  - Solid Organs
    - Kidney
    - Pancreas
    - Heart
    - Cornea
  - Bone Marrow + HSC
  - HLA-A, B for platelet transfusions
- Disease association
  - B27 with AS
  - DR4 with RA
  - DR3, 4 & DQ2, 8 with Diabetes and coeliac disease
- Recurrent foetal loss
- Paternity testing

Methods for HLA-Typing

- Resolution degree
  - Allelic Resolution = > 2 fields: e.g. A*02:01:01:01
  - High Resolution with G-Codes: e.g. A*02:01:01G
  - High Resolution = 2 fields: e.g. A*02:01
  - Low Resolution = 1 field: e.g. A*02
- Method
  - Serological
  - Molecular
  - Cellular
  - Biochemical

HLA Typing for Donor Registries
Considerations

- Typing Quality – Errors make the Registry unattractive
- Typing Resolution – „Two fields“ currently sufficient
- Number of Loci – The more the better
- Expenses – The cheaper the better
- Data Handling – The lesser the better
- Only 1% of the donors typed will be needed


Collection Set

Classical Methods

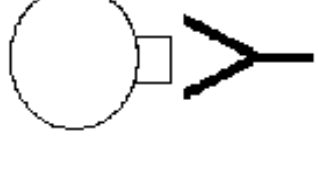
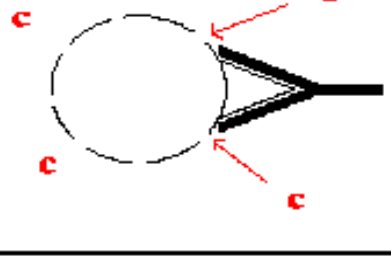
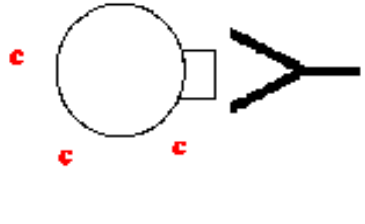
- ☛ Serological Typing (CDC)
  - NIH-Test, LCT, Micro-lympho-cytotoxicity test
- Cellular Tests
  - MLC (Mixed Lymphocyte Culture)
  - PLT (Primed Lymphocyte Typing)
- Biochemical Typing
  - One-dimensional, isoelectric focussing
  - Two-dimensional, isoelectric focussing

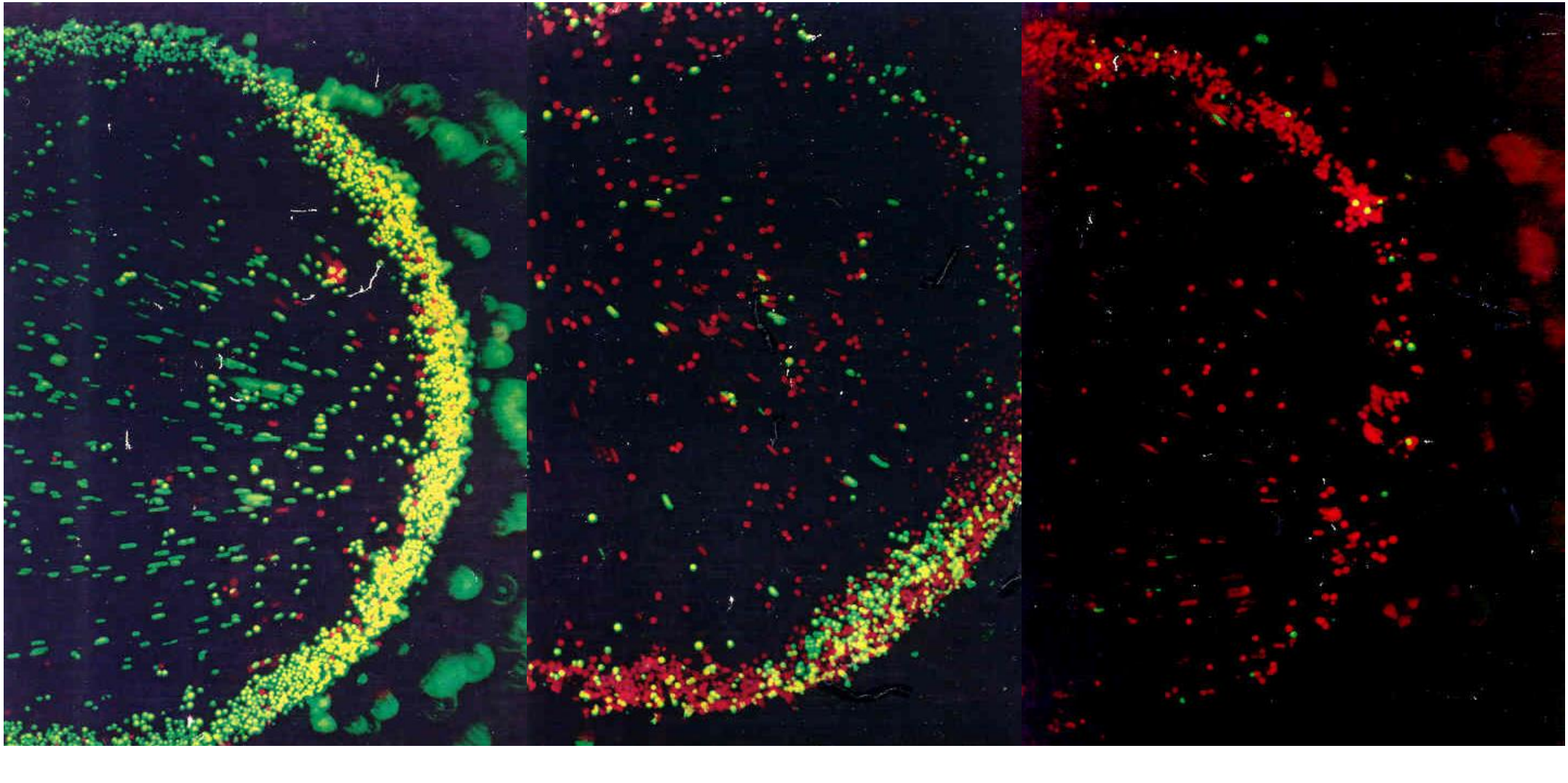
Serology

- Pros:
  - Low implementation costs (no expensive equipment)
  - Cheap and quick

- Cons:
  - Low Resolution
  - Fresh material required!
  - Insufficient quality for HLA-class II Typing

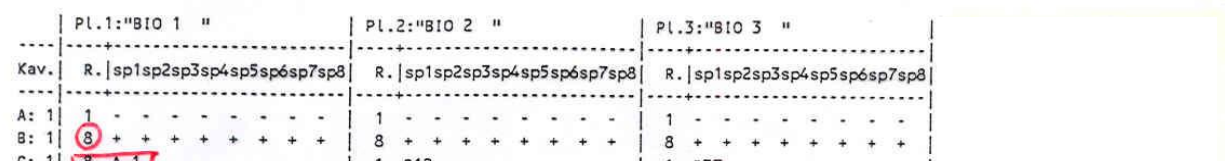
Microlymphocytotoxicity Test

	+ve Reaction	-ve Reaction
HLA antiserum (Ab) + lymphocyte suspension (Ag) (Ag - Ab reaction)		
Complement dependent cell lysis		
Staining with AO/EB/Hb (as seen through microscope)		



SCORING

Percent dead cells	Score	Interpretation
0 -10%	1	negative
11 - 20%	2	possibly negative
21 - 50%	4	weakly positive
51 - 80%	6	positive
81 - 100%	8	strongly positive
	0	not interpretable



Kav.	Pl.1:"BIO 1 " R.	sp1sp2sp3sp4sp5sp6sp7sp8	Pl.2:"BIO 2 " R.	sp1sp2sp3sp4sp5sp6sp7sp8	Pl.3:"BIO 3 " R.	sp1sp2sp3sp4sp5sp6sp7sp8
A: 1	1	- - - - - - - -	1	- - - - - - - -	1	- - - - - - - -
B: 1	8	+ + + + + + + +	8	+ + + + + + + +	8	+ + + + + + + +
C: 1	8	A 1	1	B12	1	B37
D: 1	8	A 1	1	B44	1	B37
E: 1	8	A 1A36	1	B44	1	B37
F: 1	8	A 1A36	1	B44	1	B40B13
F: 2	8	A 2	1	B45	1	B40B13B47
E: 2	8	A 2	1	B45	1	B40
D: 2	8	A 2	1	B13	1	B60
C: 2	8	A 2A28	1	B13	1	B40B13B41B47B48
B: 2	1	A 3	1	B14	1	B40B 7B42B48
A: 2	1	A 3	1	B14	1	B40B13B47B48
A: 3	1	A 3	1	B64B16B42	1	B60B48
B: 3	1	A 9	1	B65B 8B59	1	B60B48
C: 3	1	A23	1	B15B49B13	1	B60B48
D:	1	A23	1	B15	1	B61B 7B42B48B55
E: 3	1	A24 +++A24+++	1	B62B75B77	1	B48
F: 3	1	A24	1	B62B75	1	B41B42
F: 4	1	A10A43	4	B75B35	1	B41
E: 4	1	A25	1	B77B 5B49B53	1	B41
D: 4	1	A25	1	B77 B63	1	B42
C: 4	1	A26A66	1	B63	1	B42
B: 4	1	A26A66	1	B76	1	B46B77
A: 4	1	A26	1	B16	1	B46
A: 5	1	A34 A66	1	B16	1	B47B27
B: 5	1	A34	1	B38	1	B47B27
C: 5	1	A66A11	1	B38	1	B59B 5
D: 5	1	A11	1	B39B67	1	B59B 8
E: 5	1	A11	1	B39	8	B70B21B15B53B46B56
F: 5	1	A28	8	B17B63	1	B70B12B21B15B56B41
F: 6	1	A28	8	B17	1	B70B62B75
E: 6	1	A69	6	B57B15B49B46B52	1	B71B56
D: 6	1	A29	6	B57B15	1	B73
C: 6	1	A29A43	2	B57B15B46	1	B73
B: 6	1	A30A31	1	B57	1	C 1
A: 6	1	A30A31	1	B18	1	C 1
A:	1	A30	1	B18	1	C 2
B: 7	1	A30	1	B18	1	C 2
C: 7	1	A31A30	2	B21B45	1	C 3
D: 7	1	A32A31A30A33A74	1	B21	1	C 3
E: 7	1	A32A25 A69	1	B49B52	6	C 3
F: 7	1	A32A25	1	B49	8	C 4
F: 8	1	A33	1	B50B62B75B70	8	C 4
E: 8	1	A33	1	B22C 7	8	C 4
D: 8	1	A33	1	B22B67B42	1	C 5
C: 8	1	A33A34A28	1	B22	1	C 5
B: 8	1	A43	1	B54	1	C 5
A: 8	1	A74A29A31A32A33A43	1	B54B55	8	C 6
A: 9	1	A74A33A32A29A31A30A34	1	B55	8	C 6
B: 9	1	B 5	1	B55B56	8	C 4C 6
C: 9	1	B51	1	B56	1	C 7
D: 9	1	B51	1	B27	1	C 7
E: 9	1	B52B44	1	B27	1	C 7
F: 9	1	B52 B49	1	B27	1	C 8 C 3
F:10	1	B 7	8	B35B53B 5	8	B 4B50
E:10	1	B 7	4	B35B53	8	B 4
D:10	1	B 7B27	6	B35B53	8	B 4
C:10	1	B 8	8	B35B75	8	B 6
B:10	1	B 8	1	B53B 5	8	B 6
A:10	1	B 8B59	1	B53B 5	8	B 6

HLA/A 1 . 2

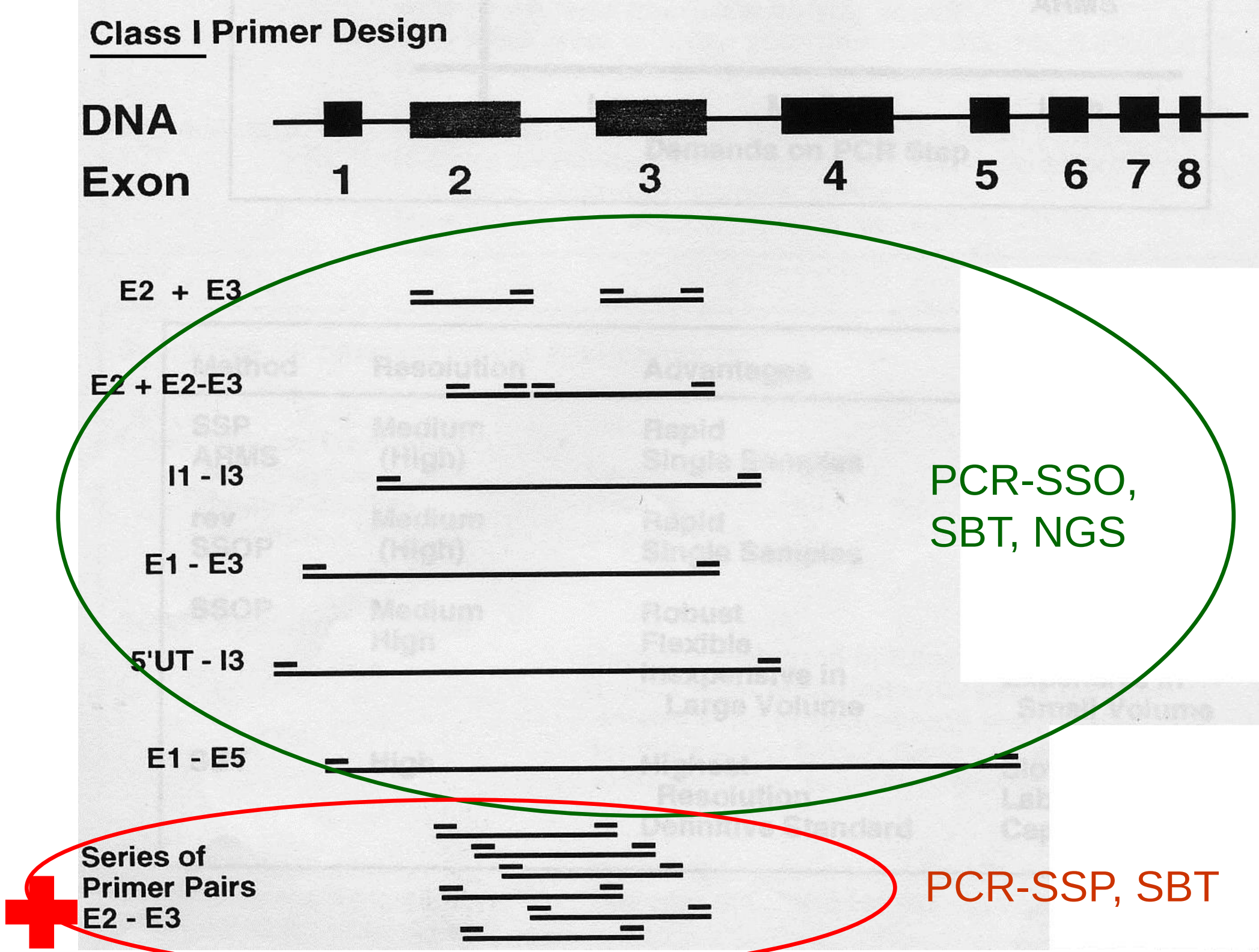
HLA/B 35 . 57

HLA/C 4 . 6

HLA/DR 7 . 13

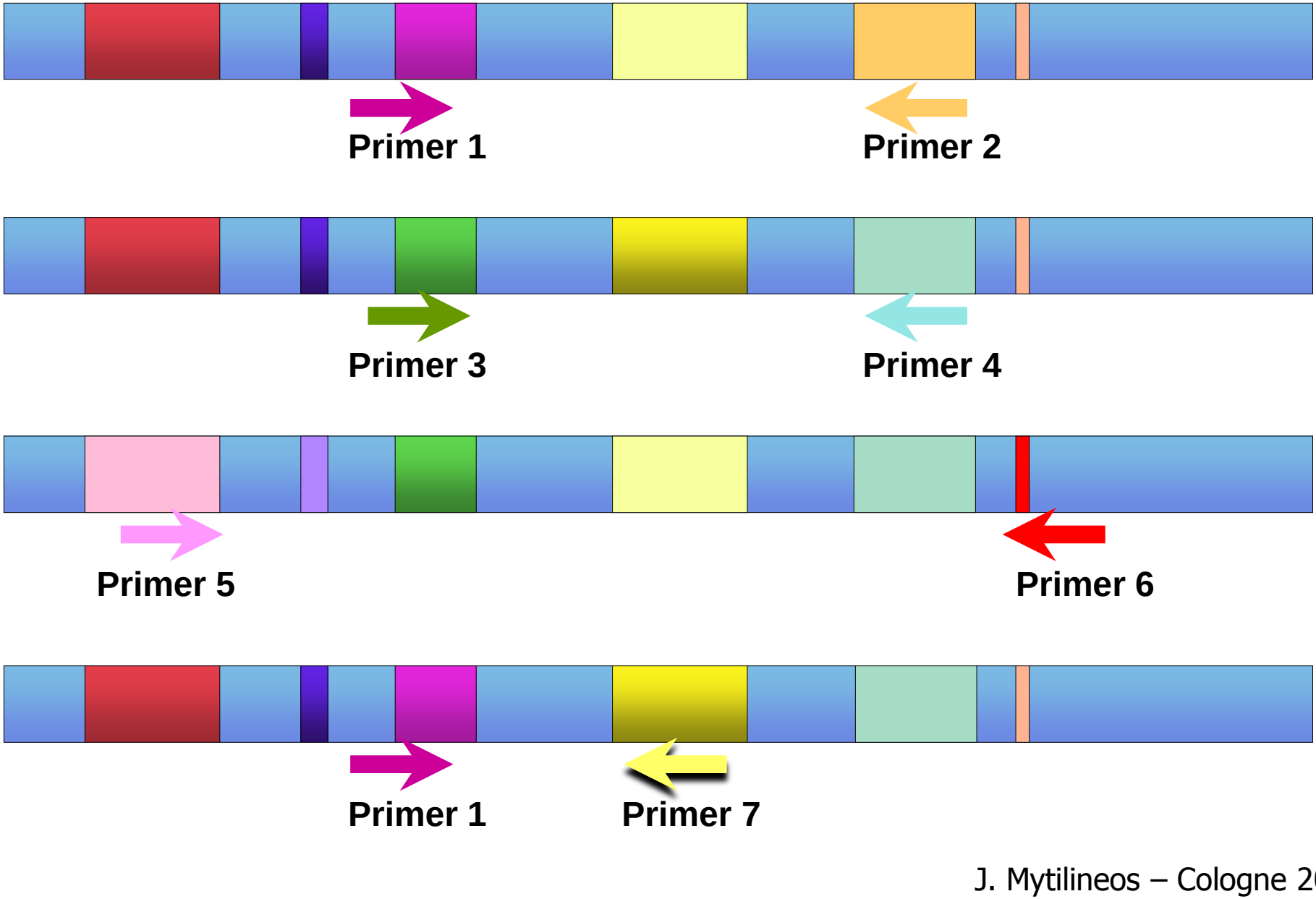
Molecular Methods for HLA-Typing

- **PCR-SSP**
- **PCR-SSO**
  - **Luminex**
- **SBT – Sanger**
- **Next Generation Sequencing (NGS)**
  - Amplicon Based
  - Whole Gene


Class I Primer Design
DNA
Exon
1
2
3
4
5
6
7
8
E2 + E3
E2 + E2-E3
I1 - I3
E1 - E3
5'UT - I3
E1 - E5
PCR-SSO,
SBT, NGS
Series of
Primer Pairs
E2 - E3
PCR-SSP, SBT

PCR-SSP

Sequence Specific Primer Design



PCR-SSP - Workflow

DNA-Isolation

↓

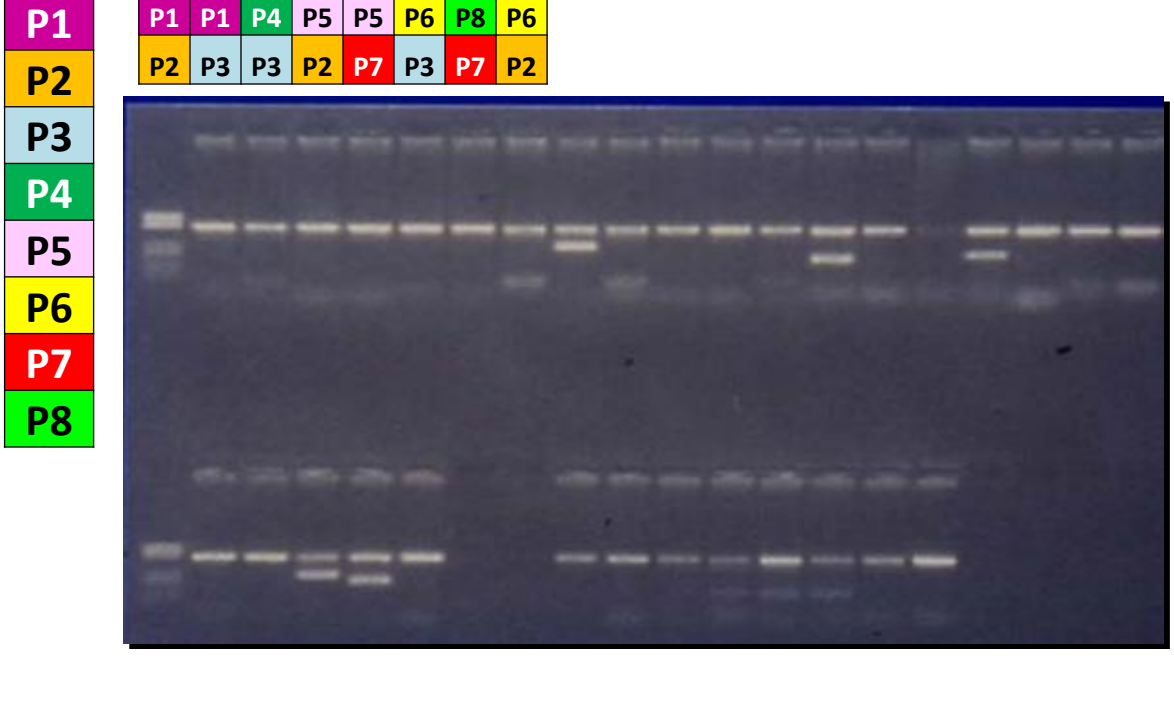
PCR with sequence-specific Primer-combinations

↓

Electrophoresis

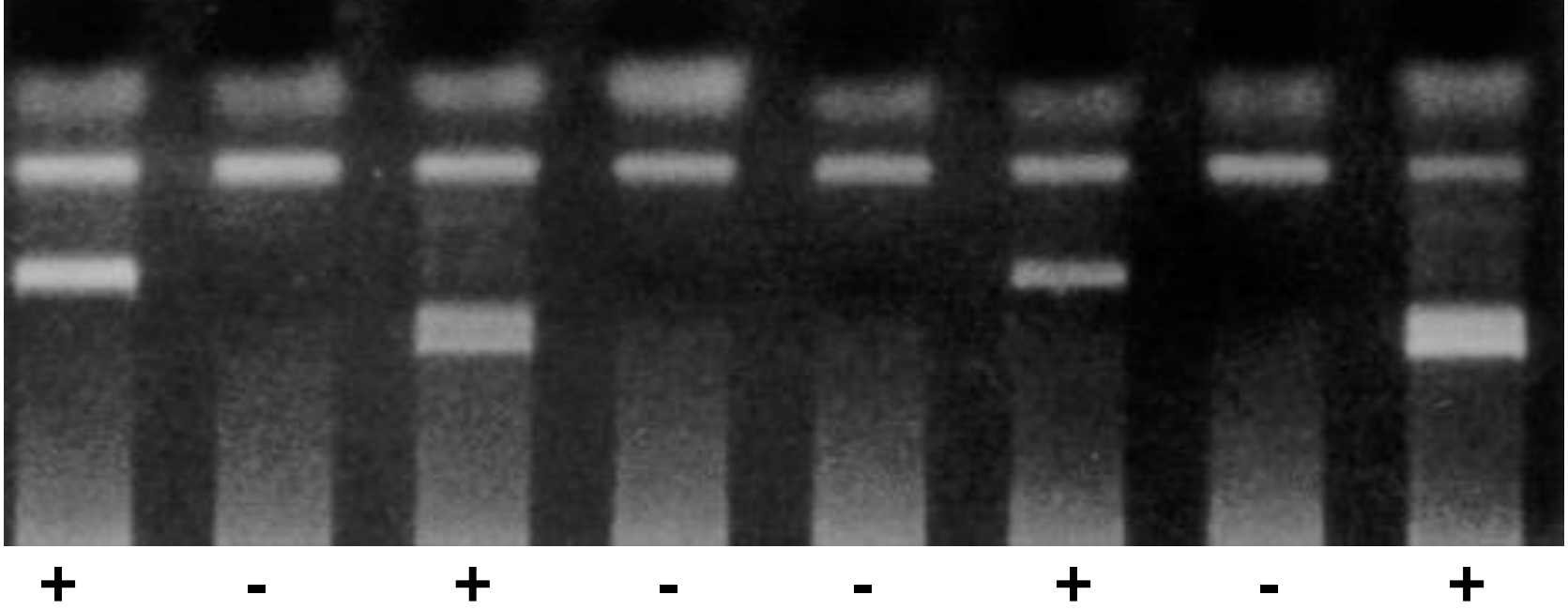
↓

Reading and Interpretation



Example of a simple PCR-SSP-Typing

	P1	P1	P4	P5	P5	P6	P8	P6
	P2	P3	P3	P2	P7	P3	P7	P2
Mix Nr.	1	2	3	4	5	6	7	8



	+	-	+	-	-	+	-	+
DQB1*	05:01	06	05/06	02	0301	03:02	03:03	03/04

Typing Result: DQB1*03:02, 05:01

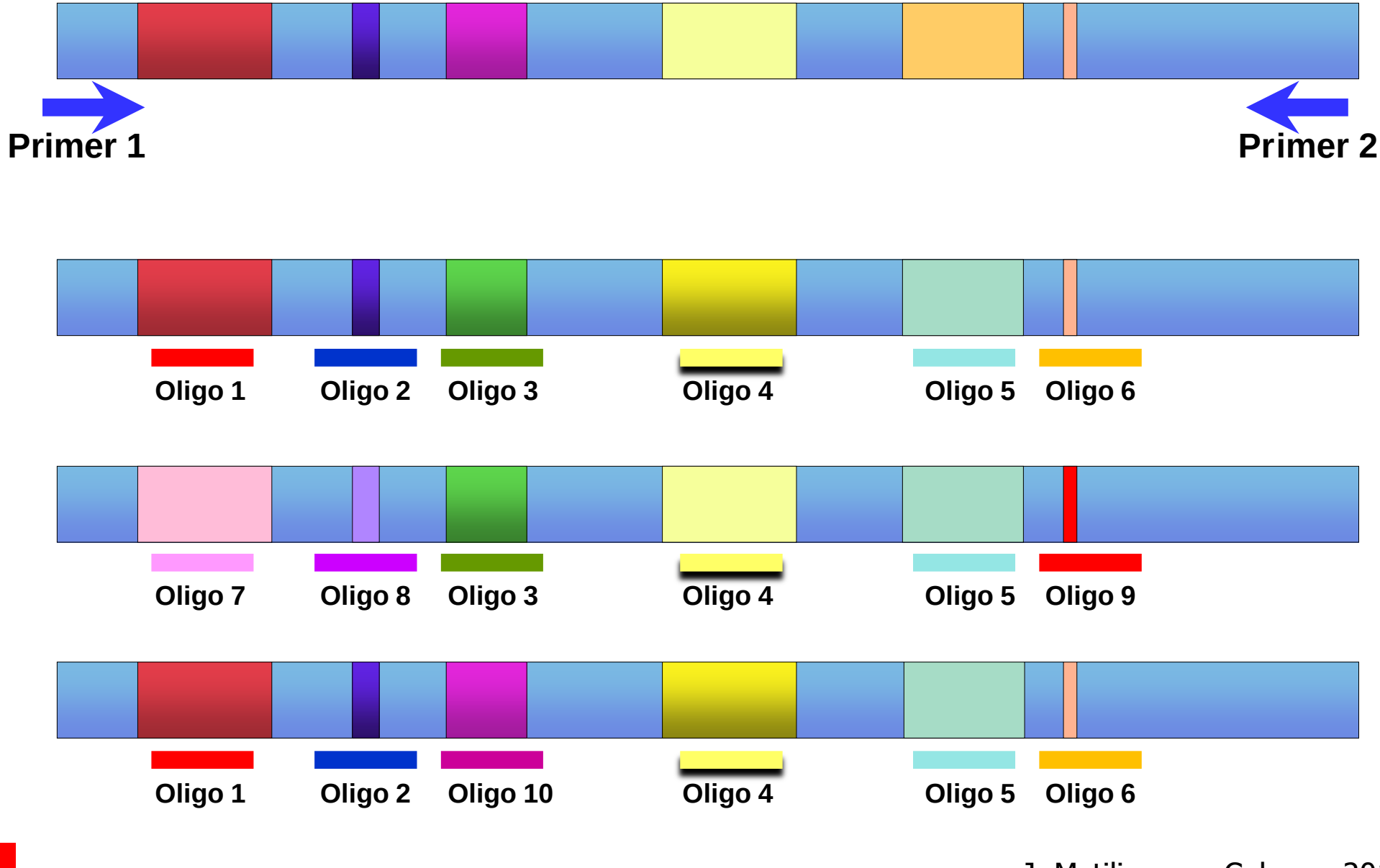
PCR-SSP

- Pros:
  - Low implementation costs (no expensive equipment)
  - Straight forward interpretation of results
  - Quickest molecular method (<3 hours)

- Cons:
  - High consumption of DNA (in particular for high res)
  - Limited resolution in view of the increas. number of alleles
  - Constant need for primer updating – too cumbersome
  - Only low throughput possible

PCR-SSO

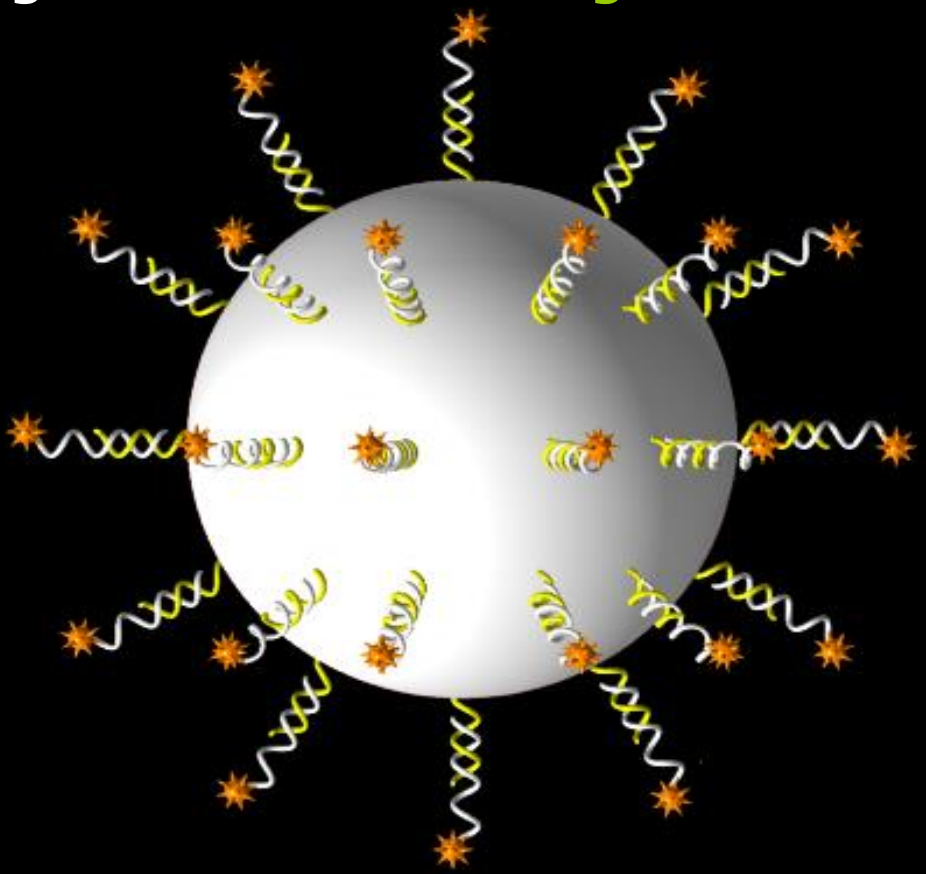
Sequence Specific Oligonucleotides Design

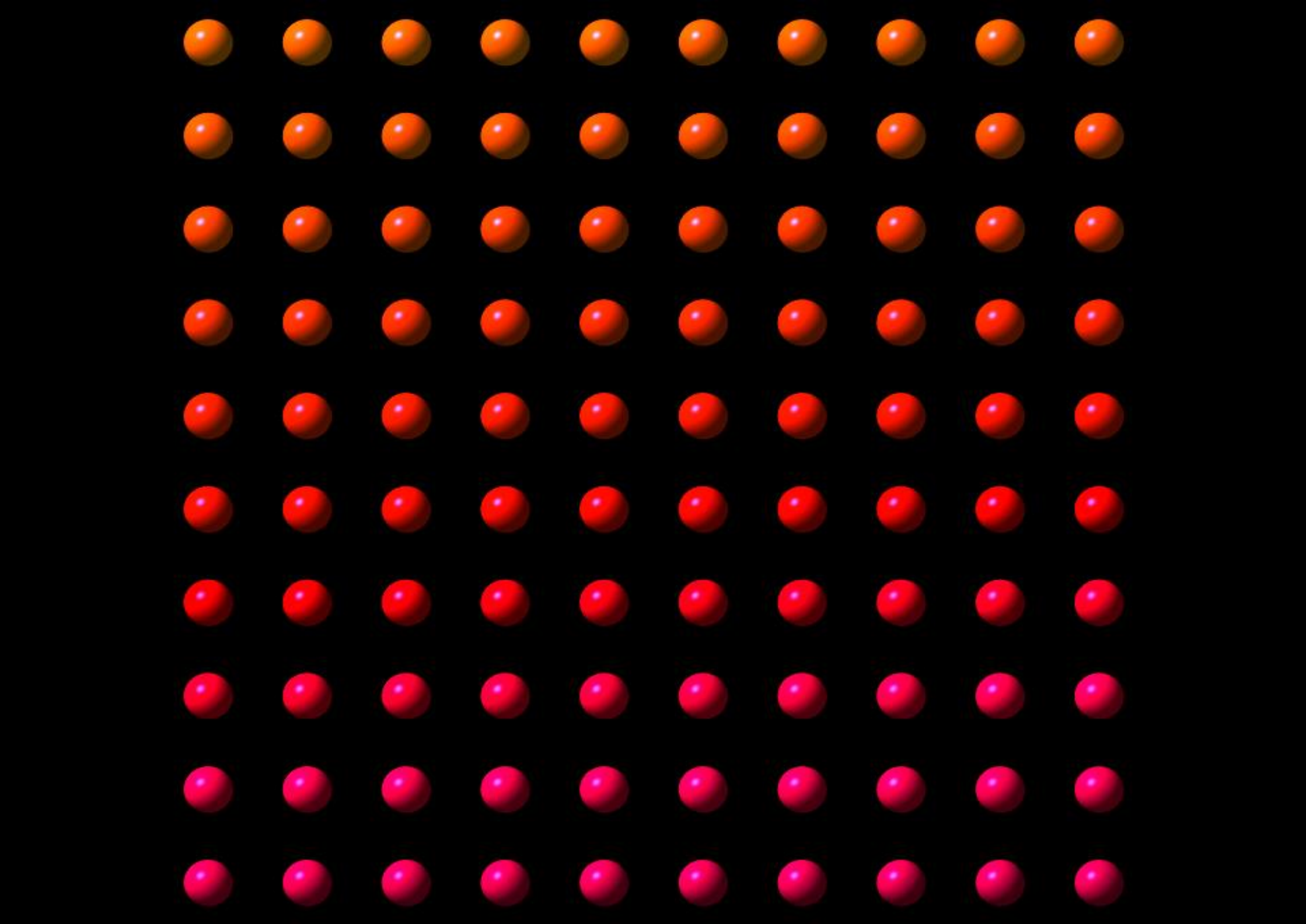


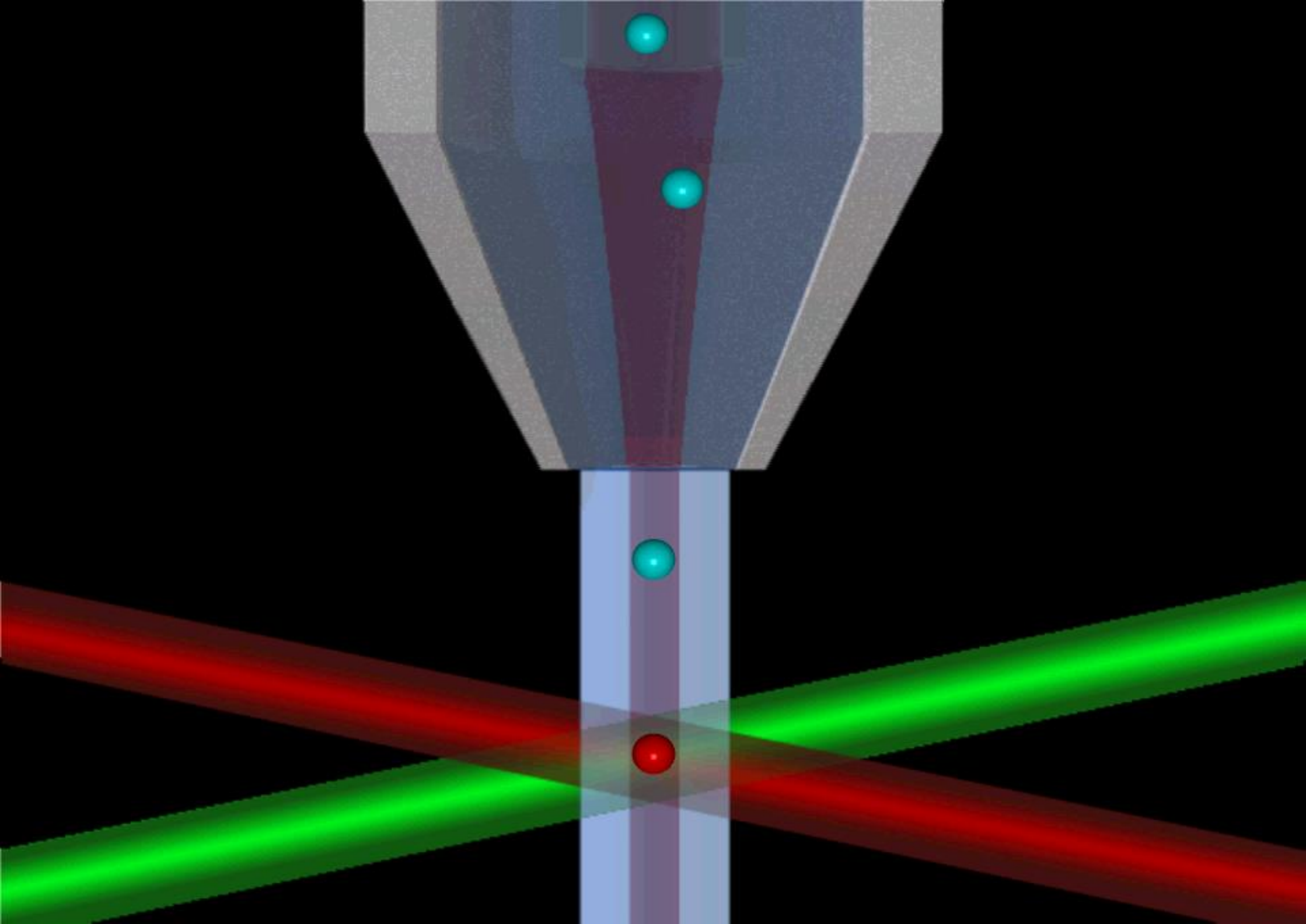
Example of an allele specific hybridization reaction:

Specific bead set is coupled with a specific oligonucleotide to the surface.

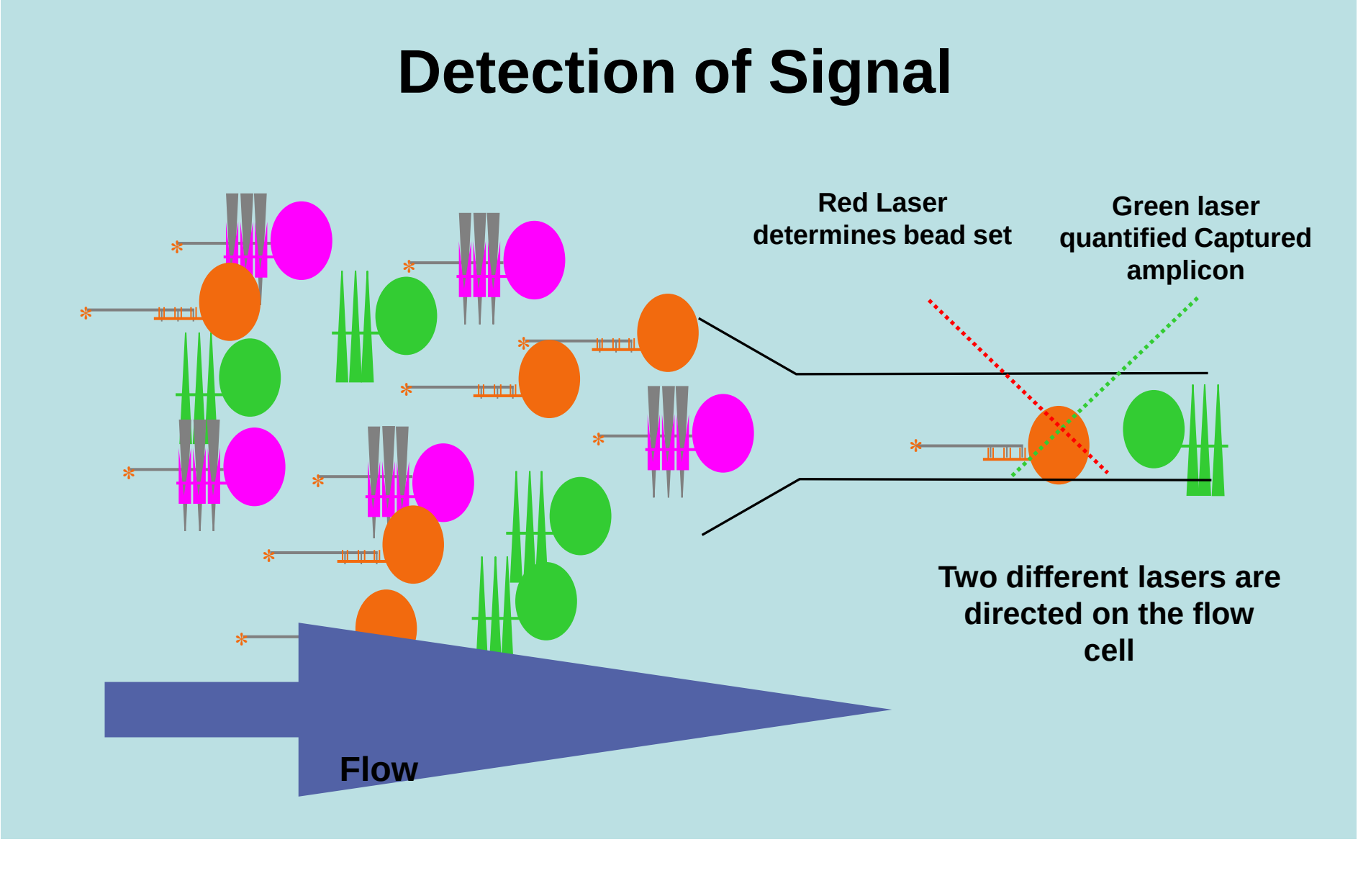
To develop the biological reaction an orange fluorescent labeled PCR product is used







Sample will be taken up by the instrument and will pass the lasers



One Lambda - HLATools - Data Source=ULM-TRAPLA-15\ONELAMBDA;Initial Catalog=LTI;Integrated Security=SSPI;

LABType

LABType Home

Lambda Explorer

LABType Interactive

Patient Explorer

Lambda Reporter

LABScreen

20051107_DQ
20051107_DR
141544
141545
141546
141547
141548
141549
141550
141551
141552
141553
141554
141555
141556
141557
141559
141560
141561
141562
249641
249642
249643
249644
249645
249646
249648
249649
259650
249651
249652
259653
249654
249655
259041
141496
259147
259148
259149
259154
259155
259156
259151

FalseReactions

FalseReactions : 16 FP (2 items)

Allele1	Allele2
DRB1*07APT	DRB1*1339
DRB1*07APT	DRB1*1331

FalseReactions : 61 FP (1 item)

Allele1	Allele2
DRB1*0708	DRB1*13XX2

FalseReactions : 70 FP (1 item)

Allele1	Allele2
DRB1*070102	DRB1*13XX2

FalseReactions : 75 FN (1 item)

Allele1	Allele2
DRB1*0704	DRB1*13XX2

< Collapse

Matched
All Alleles
Type/Subtype
Closest Rxns
Specificity

DNA

DRB1*0701 DRB1*1302

Print

Serological

DR7 DR13

Restore

Locked

Filter: Europe

-Comments-

No False Reactions
** 1 Matched Reaction Pair **
(DRB1*07APT & DRB1*13XX2) --> (DRB1*070101, DRB1*0703, DRB1*0705, DRB1*0707, DRB1*0709 & DRB1*130201, DRB1*130202)
4 Close Reactions

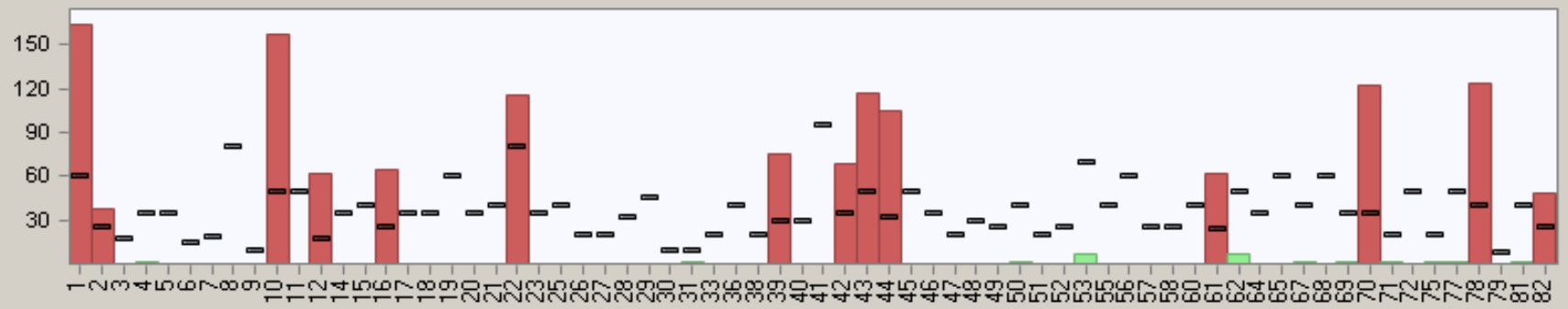
Previous Accept Next

Sample ID: 141547
Date: 11/9/2005

Birth: 1/1/1901
Race: Unk
Patient: 141547

Assignment Patient

DRB1*1302 / DRB1*1502



Assignments Bead Analysis Rxn Grid Raw Data

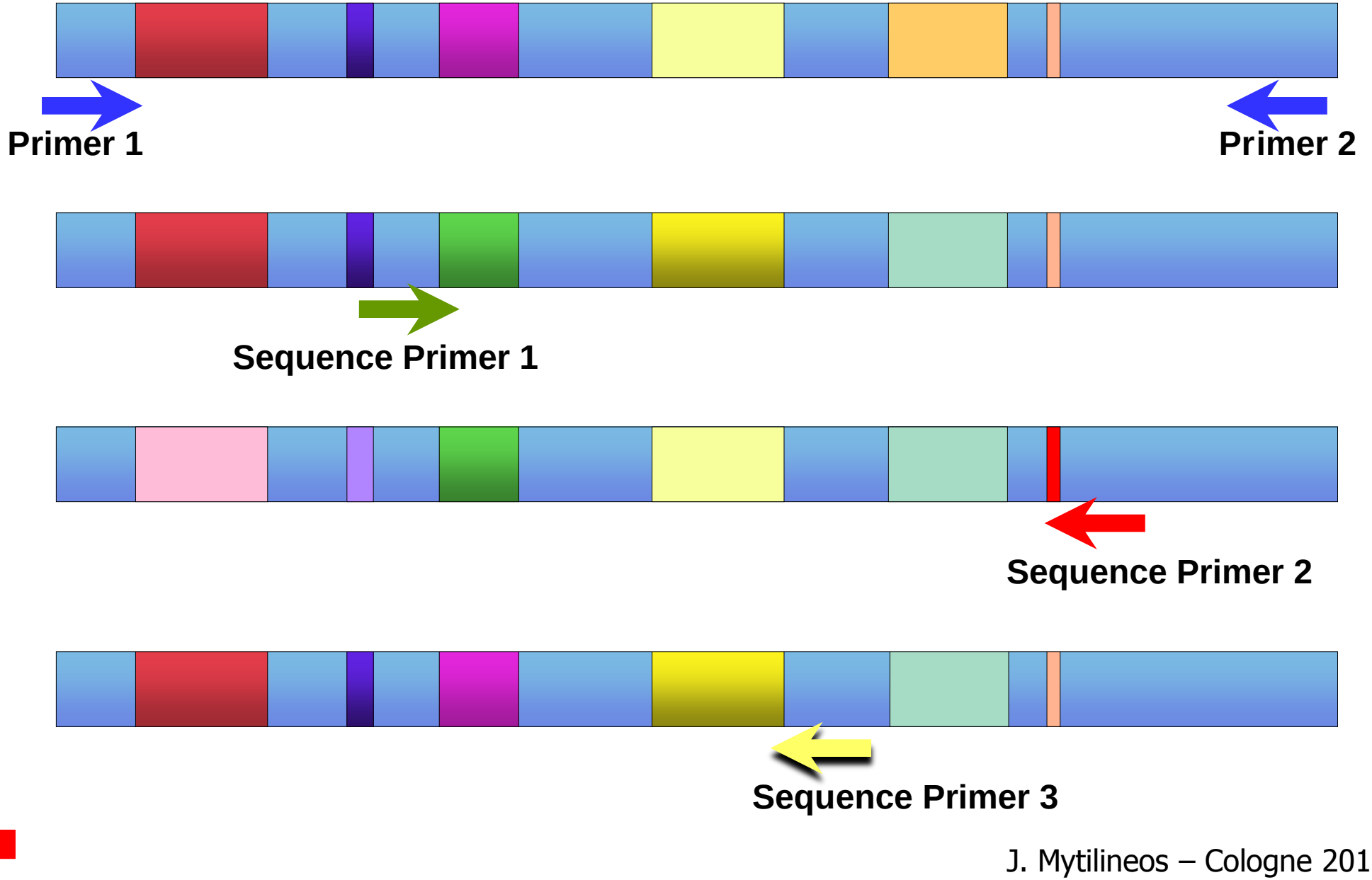
PCR-SSO (Luminex)

- Pros:
  - Simultaneous Hybridisation of one PCR product with >100 oligonucleotides in one single tube (liquid chip)
  - High throughput with minimal staff requirments
  - Very few DNA material required

- Cons:
  - Reagent costs
  - Constant need for probe updating – too cumbersome
  - Limited resolution in view of the increas. number of alleles

SBT

Sequence Based Typing



Why Sequencing Approaches

```
A*01:001:  AACGCCGATCCGTTACGCTAG
  SSO:      ???GCC?????????????TAG
  SSP:      ???GCC?????????????TAG
  SBT:      AACGCCGATCCGTTACGCTAG
```

Some years later….

```
A*01:040:  AACGCCGACAGGTTACGCTAG
```

SBT Steps

1. DNA-Isolation

2. Locus +/- Allele-group specific Amplification (PCR)

3. PCR Control

4. Purification of Amplification Product

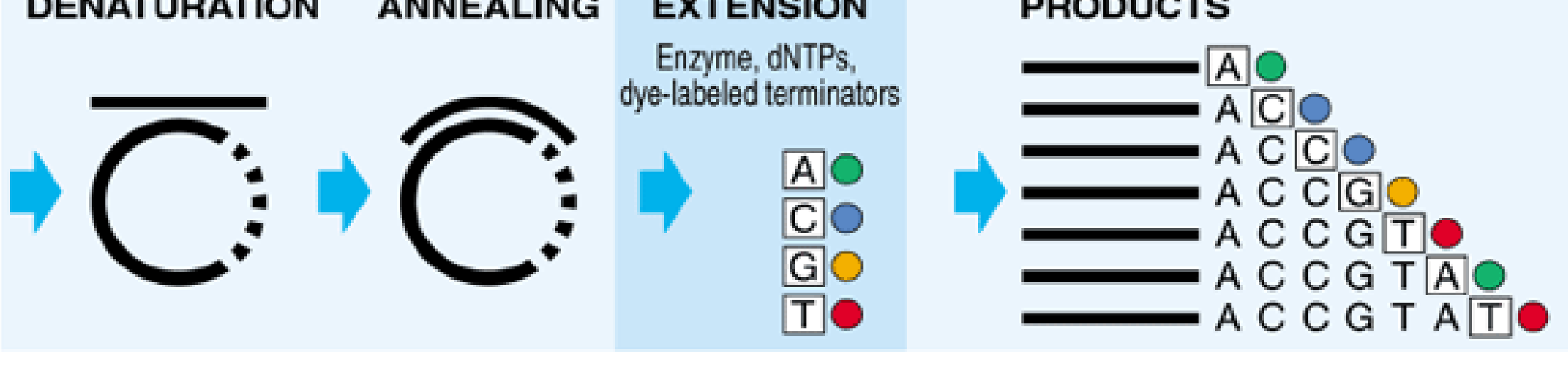
5. Cycle Sequencing

6. Purification of the „Cycle Seq“ Products

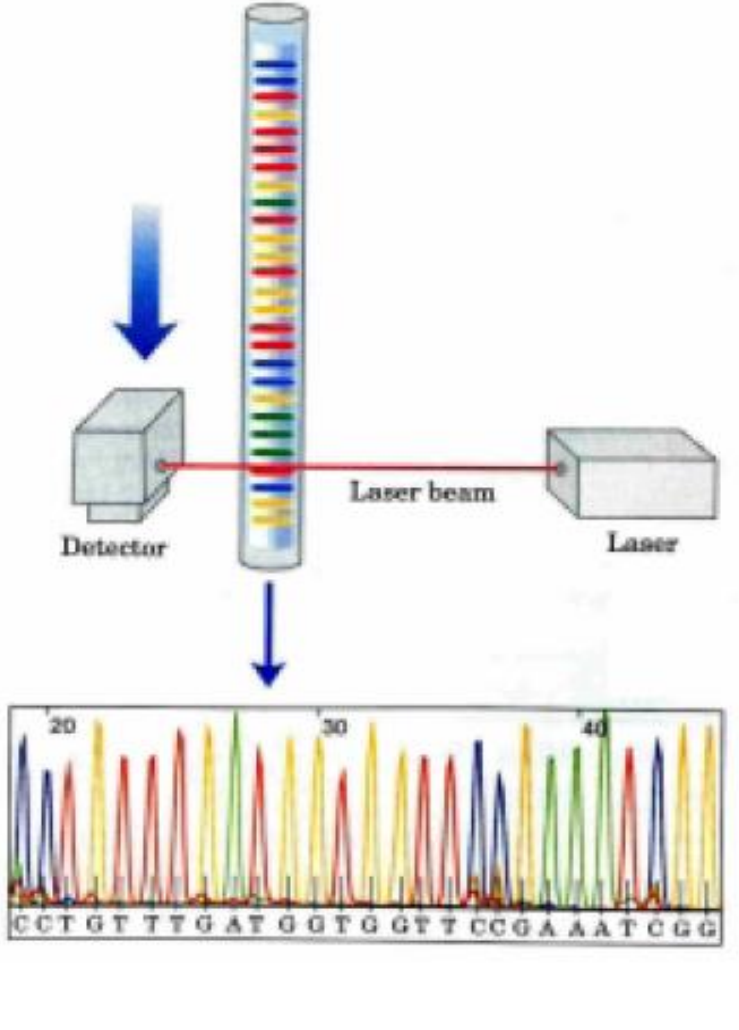
7. Separation of the „Cycle Seq“ Fragments in an automatic Sequencer

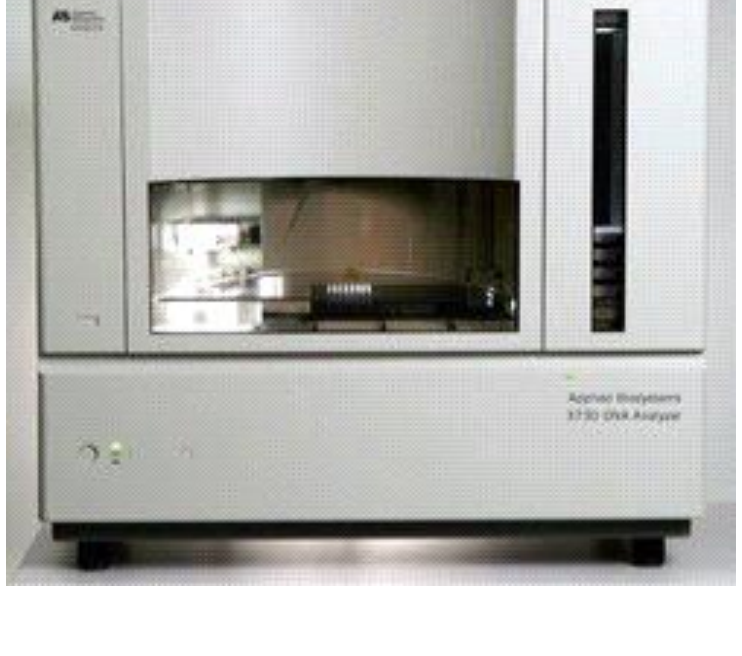
8. Interpretation and Evaluation of the Sequences

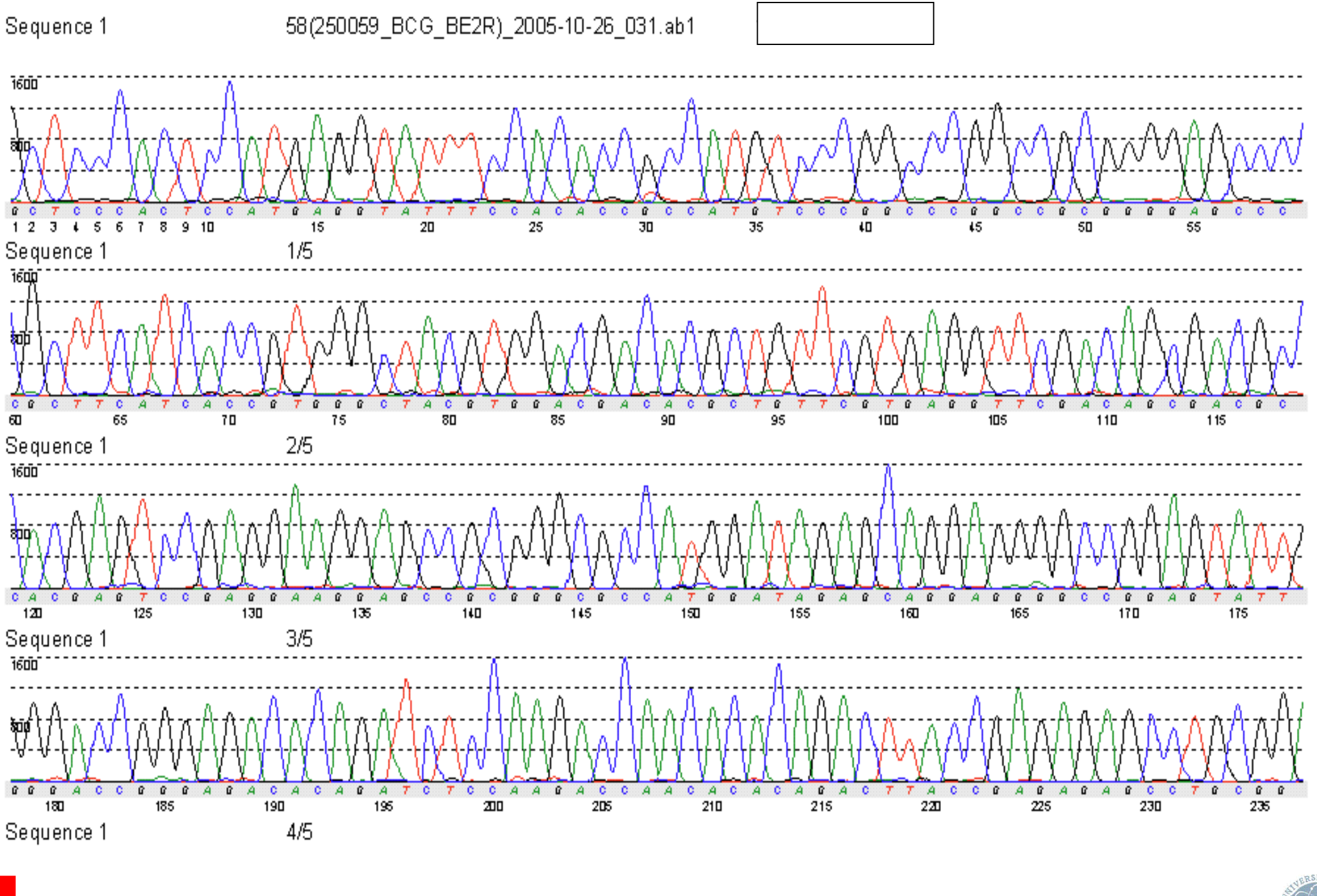
5. SBT – Cycle Seq Step



7. SBT – Separation of the Cycle Seq fragments






Sequence 1
58(250059_BCG_BE2R)_2005-10-26_031.ab1
Sequence 1
1/5
Sequence 1
2/5
Sequence 1
3/5
Sequence 1
4/5

SBT

- Pros:
  - High Resolution approach
  - Direct reading of sequence information
  - Very few DNA material required

- Cons:
  - Costs for reagents and equipment
  - Rather time consuming and complex if more than exons 2 and 3 need to be considered
  - Limited resolution in view of the steadily increasing number of alleles (no cis-trans-separation -> Ambiguities)

Problem: Ambiguities

Allele 1: AcgTTAAggTagcgcATcTgAcccAATCTT

Allele 2: AcgCTAAggTagcgcATcTgAgggTTACTT

Sequencing-Result: AcgYTAAggTagcgcATcTgASSSWWWCTT

Allele 3: AcgTTAAggTagcgcATcTgAgggAATCTT

Allele 4: AcgCTAAggTagcgcATcTgAcccTTACTT

Sequencing-Result: AcgYTAAggTagcgcATcTgASSSWWWCTT

Final Result: Allele 1 + Allele 2 or
Allele 3 + Allele 4
} = Ambiguities

NGS

- Principle:
  - Massive Parallel Sequencing
  - Different Plattforms and Technologies
    (Illumina, PGM, PacBio, Nanopore etc)

- Resolution: High, Maximum

Why NGS?

- Reduction of Expenses due to the massive parallel testing feasible by the use Multiplex Identifier Sequences (MID)
- Higher Resolution and avoidance of Ambiguities.
- Higher Throughput
- More Information (more Loci) per Test Run
  - HLA-Loci
  - Other Genes (ABO, KIR, CCR5 etc)

NGS Workflow Overview

1. DNA-Isolation

2. Locus specific Amplification (PCR)

3. PCR Control – Quantification steps

4.Library Preparation (differs dramaticaly between platforms)

5. Quantification - Purification

6. Pooling

7. Sequencing

7. Interpretation and Evaluation of the Sequences

Current NGS Platforms

- ThermoFisher – Ion Torrent (PGM)
- Illumina – Miseq
- Pacific Biosciences RS
- Oxford Nanopore - Minion

Thermofisher - PGM

Illumina - Miseq

Pacific Biosciences - RS

Minion - Nanopore

NGS Typing Approaches

Exon-based typing (selected exonic/intronic sequences)

= Amplicon based

cDNA-based typing (selected exons can be included)

= „Shot gun“ based

Full genomic typing

= „Shot gun“ based

Full length Amplification (5'UTR to 3'UTR) of : HLA-A, HLA-B, HLA-C (~3kb)
HLA-DQA1 (~7kb)
HLA-DQB1 (~7kb)

Partial Amplification of: HLA-DRB1 amplicon ~5 kb (gene ~15kb)
HLA-DPB1 amplicon ~7kb (gene ~12kb)

NGS – Important Molecules

(for Whole Gene and Amplicon Based Approaches)

- Adapter
- Reverse Adapter
- MID (Multiplex Identifier = Molecular Barcode
- Target Specific Primer (= Locus specific)
- Individual Sequence (Targeted Region)

NGS – Library Preparation

= Ligation of double-stranded DNA adaptors to the ends of the DNA template, that are used throughout the process to amplify, capture, and sequence the DNA.

Two strategies for library preparation depending on the size of the DNA region to be sequenced

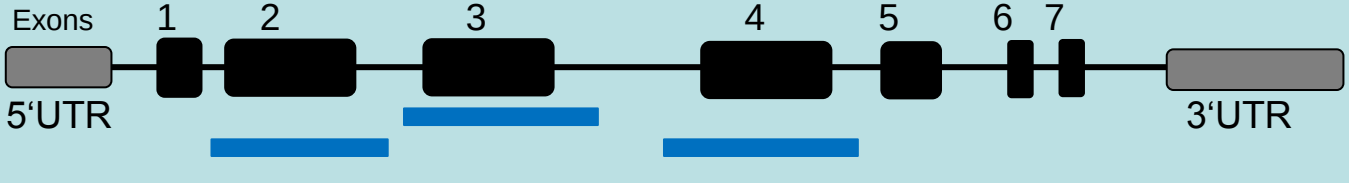
Whole Gene (Long range PCR)

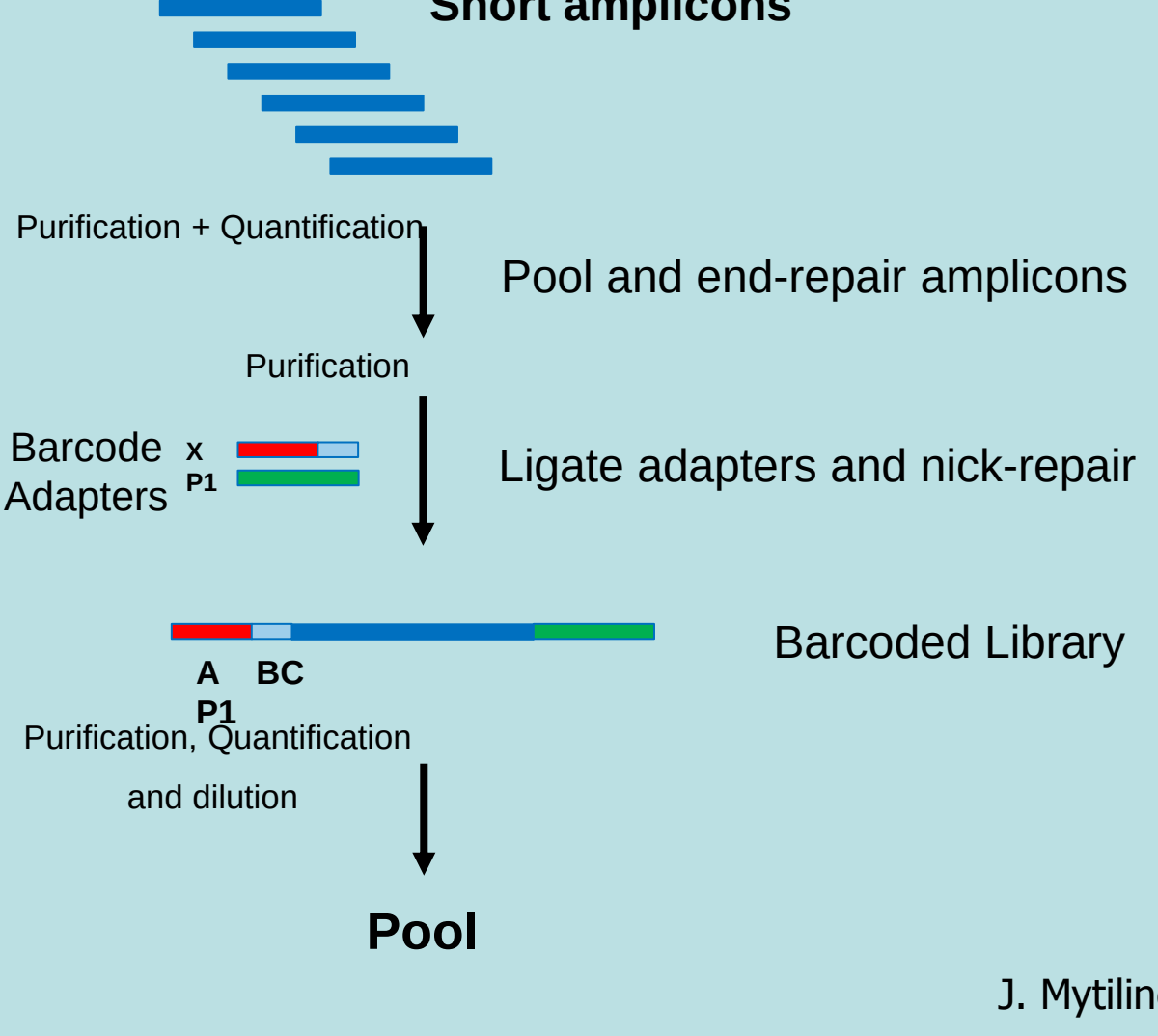
- for targeted regions >500 bases in length
- the starting DNA template (genomic DNA or large PCR amplicons) must be fragmented prior to adaptor ligation.

Amplicon based (Short PCR)

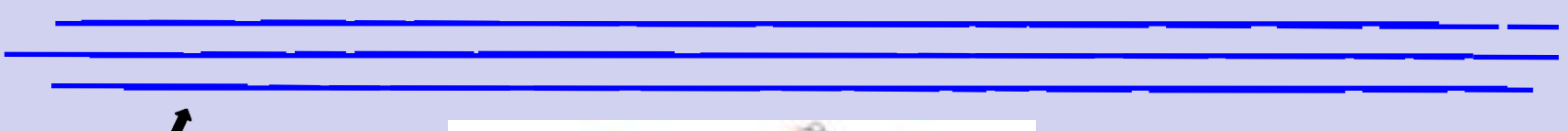
- for targeted regions <500 bases in length
- does not require fragmentation prior to adapter ligation

NGS – Amplicon Based Workflow





Shotgun Genome Sequencing



Complete genome copies

->Fragmented genome chunks

Shotgun Genome Sequencing

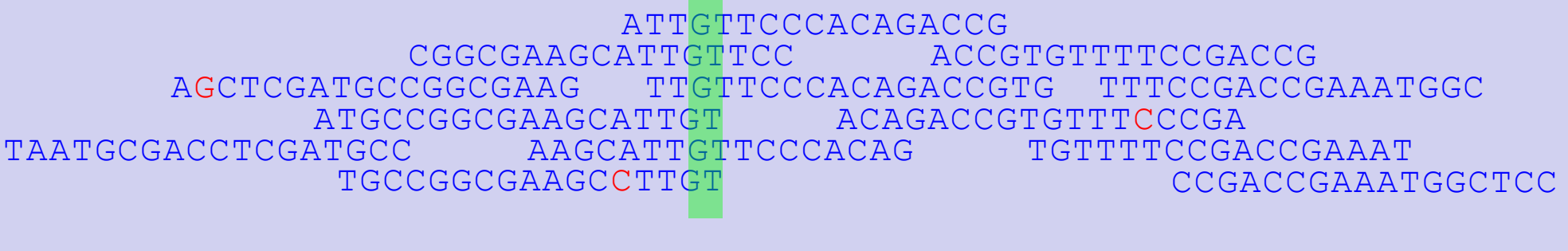
Fragmented genome chunks

NOT REALLY DONE BY DUCK HUNTERS
Hydroshearing, sonication, enzymatic shearing

Assembly

Consensus:

TAATGCGACCTCGATGCCGGCGAAGCATTGTTCCCACAGACCGTGTTTTCCGACCGAAATGGCTCC



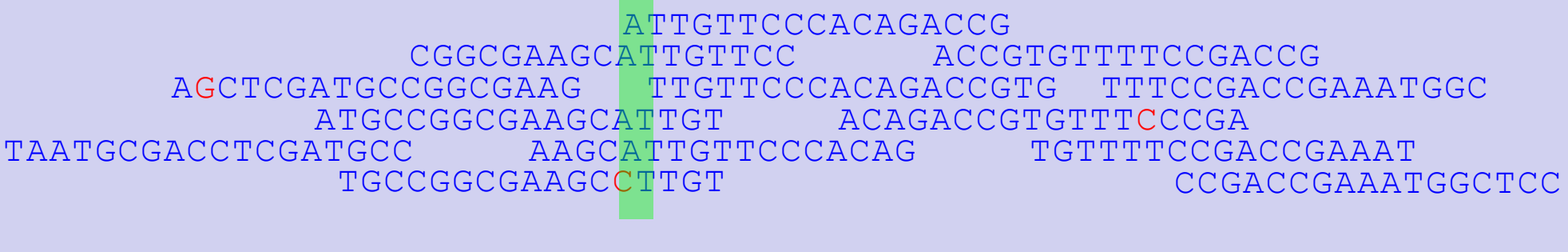
6x coverage
100% identity

Coverage: # of reads underlying the consensus

Assembly

Consensus:

TAATGCGACCTCGATGCCGGCGAAGCATTGTTCCCACAGACCGTGTTTTCCGACCGAAATGGCTCC



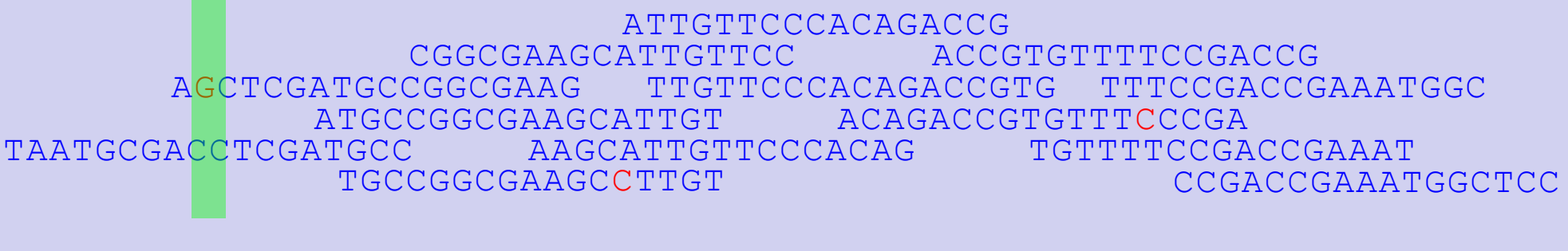
5x coverage
80% identity

Coverage: # of reads underlying the consensus

Assembly

Consensus:

TAATGCGACCTCGATGCCGGCGAAGCATTGTTCCCACAGACCGTGTTTTCCGACCGAAATGGCTCC



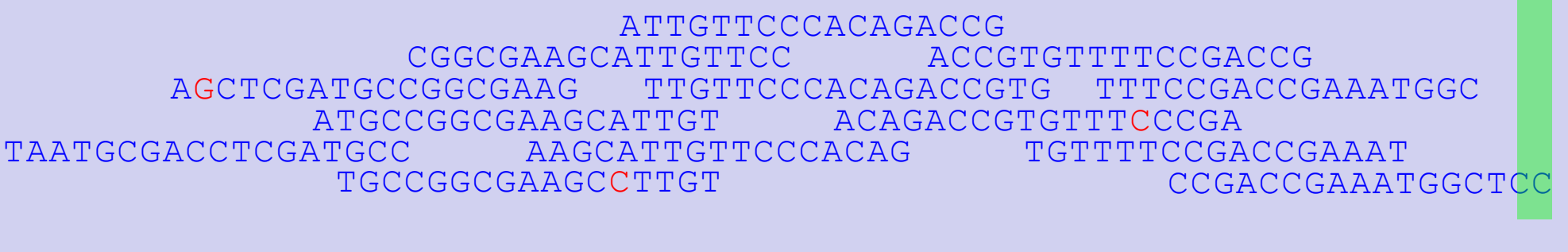
2x coverage
50% identity

Coverage: # of reads underlying the consensus

Assembly

Consensus:

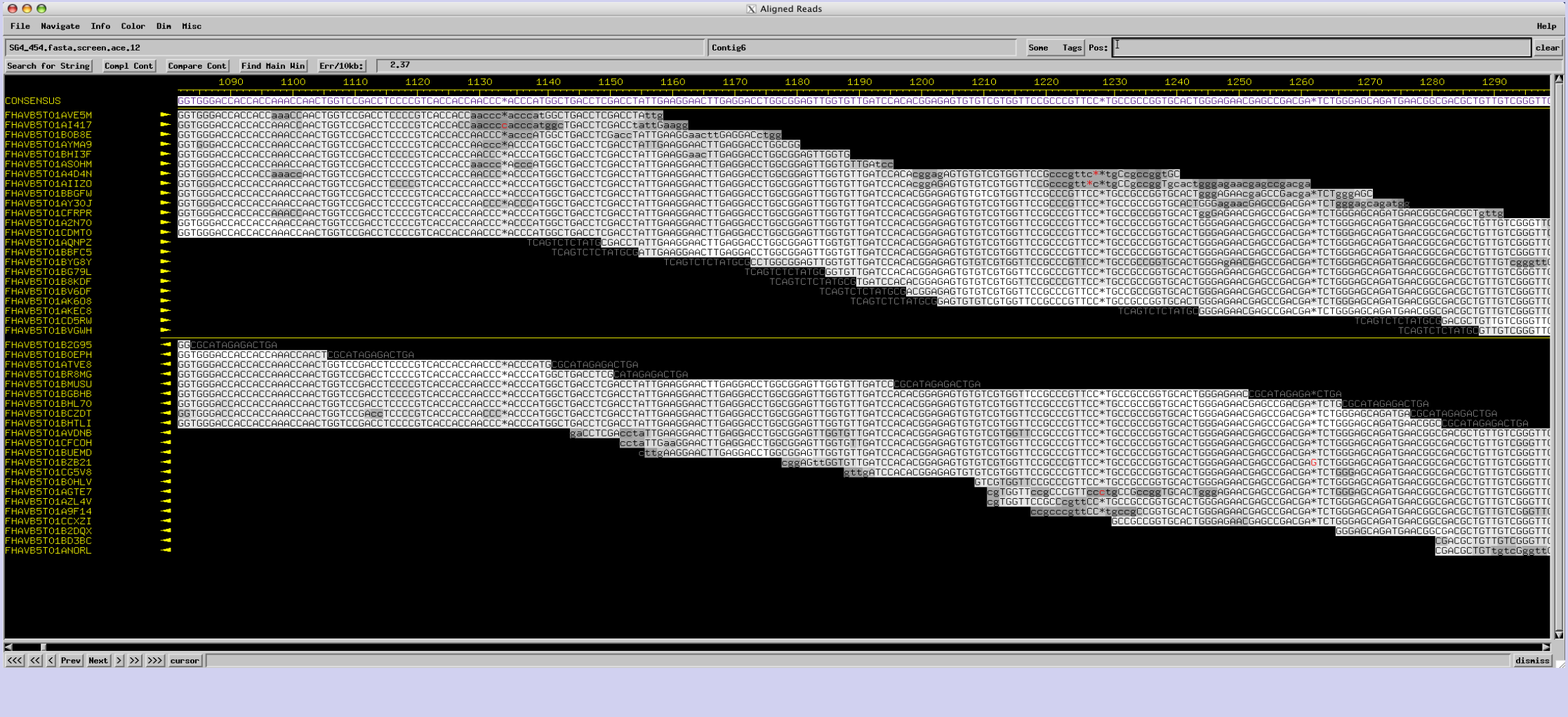
TAATGCGACCTCGATGCCGGCGAAGCATTGTTCCCACAGACCGTGTTTTCCGACCGAAATGGCTCC



1x coverage

Coverage: # of reads underlying the consensus

Assembly


Aligned Reads
File Navigate Info Color Dim Misc
Help
SG4_454.fasta.screen.ace.12
Contig6
Some Tags Pos:
clear
Search for String
Compl Cont
Compare Cont
Find Main Win
Err/10kb:
2.37
1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200 1210 1220 1230 1240 1250 1260 1270 1280 1290
CONSENSUS
FHAVB5T01AVE5M
FHAVB5T01AI417
FHAVB5T01BOB8E
FHAVB5T01AYMA9
FHAVB5T01BHI3F
FHAVB5T01ASOHM
FHAVB5T01A4D4N
FHAVB5T01AIIZO
FHAVB5T01BBGFW
FHAVB5T01AY3OJ
FHAVB5T01CFRPR
FHAVB5T01A2N7O
FHAVB5T01CDMTO
FHAVB5T01AQNPZ
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FHAVB5T01BG79L
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FHAVB5T01AK6O8
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FHAVB5T01A9F14
FHAVB5T01CCXZI
FHAVB5T01B2DQX
FHAVB5T01BD3BC
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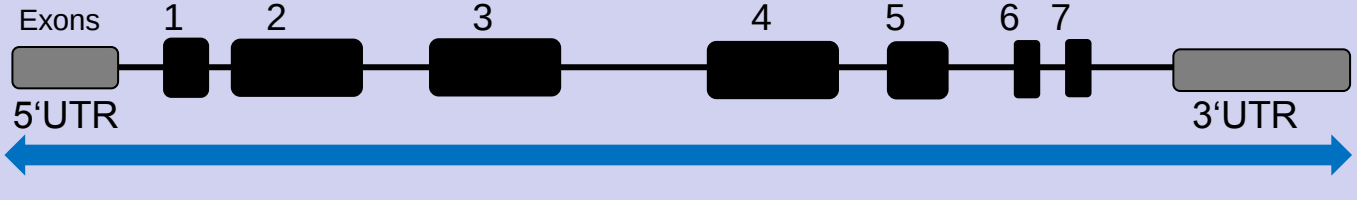
dismiss

NGS – Whole Gene Workflow - Library



Purification + Quantification

Fragmentation

Purification

Barcode Adapters x P1

Ligate adapters and nick-repair

Size-select

Purification

A BC P1

Barcoded Library

Purification, Quantification and dilution

Current NGS Platforms

- ThermoFisher – Ion Torrent (PGM)
- Illumina – Miseq
- Pacific Biosciences RS
- Oxford Nanopore - Minion

Thermofisher - PGM

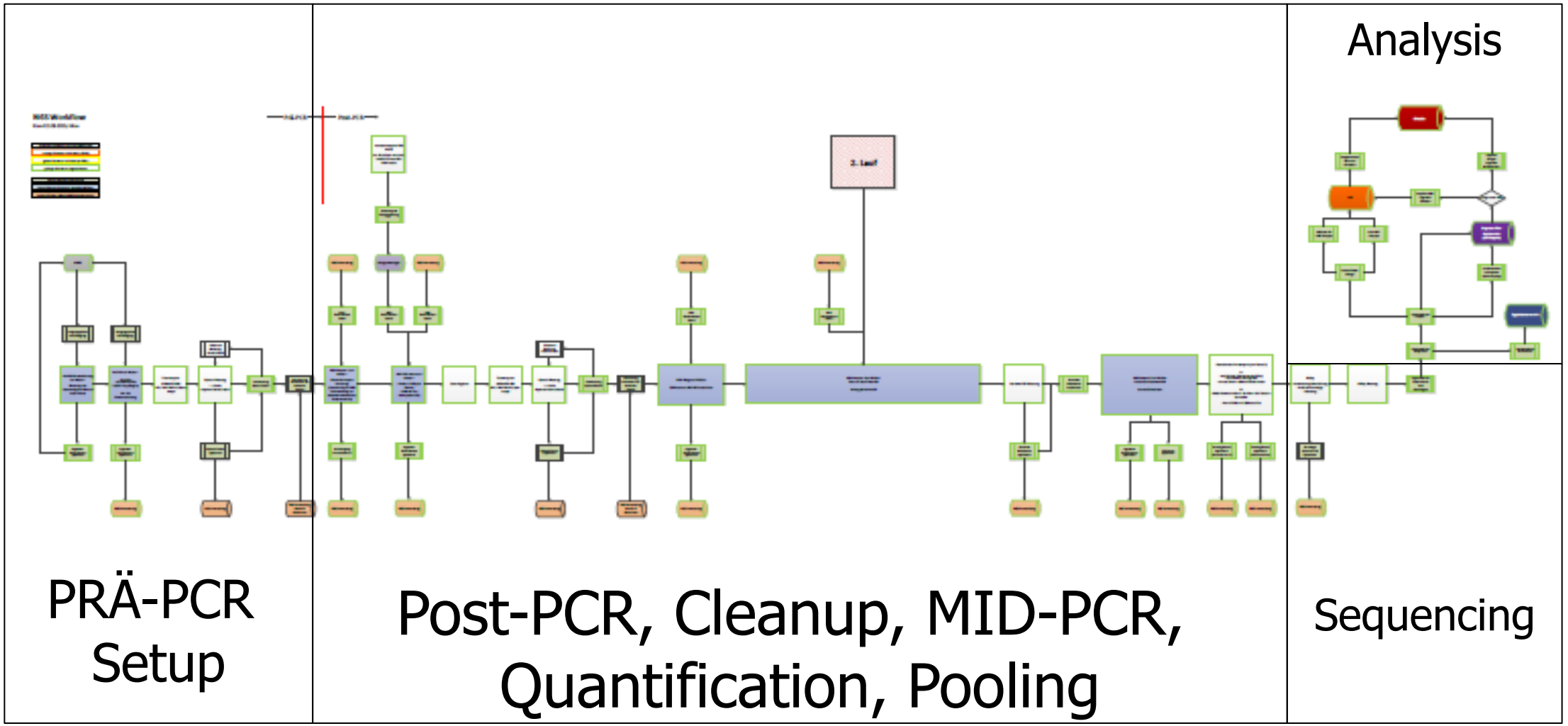
Illumina - Miseq

Pacific Biosciences - RS

Minion - Nanopore

NGS – Sequence Analysis

NGS
Workflow, Automation and Data Processing



NGS – Critical Points

- Fragment length of products to be sequenced – *Platform depended*
- Sequence „Depth“ – *Number of reads per position*
- Sequence „Coverage“ – *reliable sequences per gene area*
- Error Rate of Sequence Reading – *Platform depended*
- Complexity of Library Preparation - *Automation*
- Turn Around Time - *Platform and Approach depended*
- Throughput – *Approach and platform depended*
- Expenses – *Approach depended*
- Data Overload

NGS

- Pros:
  - Maximum Resolution possible
  - Cis-Trans Information provided (less ambiguities)
  - Very few DNA material required
  - Parallel Testing of large number of Material (= low cost)

- Cons:
  - Costs for reagents and equipment
  - Complex Procedures – Automation mandatory
  - Time consuming – TAT significantly longer
  - Large data volumes – Data storage and processing logistics required

HLA Typing for Donor Registries
Considerations

- Typing Quality – Errors make the Registry unattractive
- Typing Resolution – „Two fields“ currently sufficient
- Number of Loci – The more the better
- Expenses – The cheaper the better
- Data Handling – The lesser the better
- Only 1% of the donors typed will be needed

HLA Typing for Donor Registries

Recommendation from today‘s point of view

- NGS – exon based for typing at registration
- ABCDRDQDP at „two field“ level

In order to keep costs reasonably low

And have a reasonably high level of quality and information in an acceptable time frame

HLA-Typising in Future?

Deutsches Rotes Kreuz
Blutspendedienst Baden-Württemberg - Hessen gemeinnützige Gesellschaft mbH

Nanopore Technology

Deutsches Rotes Kreuz
Blutspendedienst Baden-Württemberg - Hessen gemeinnützige Gesellschaft mbH


meinestadt.de