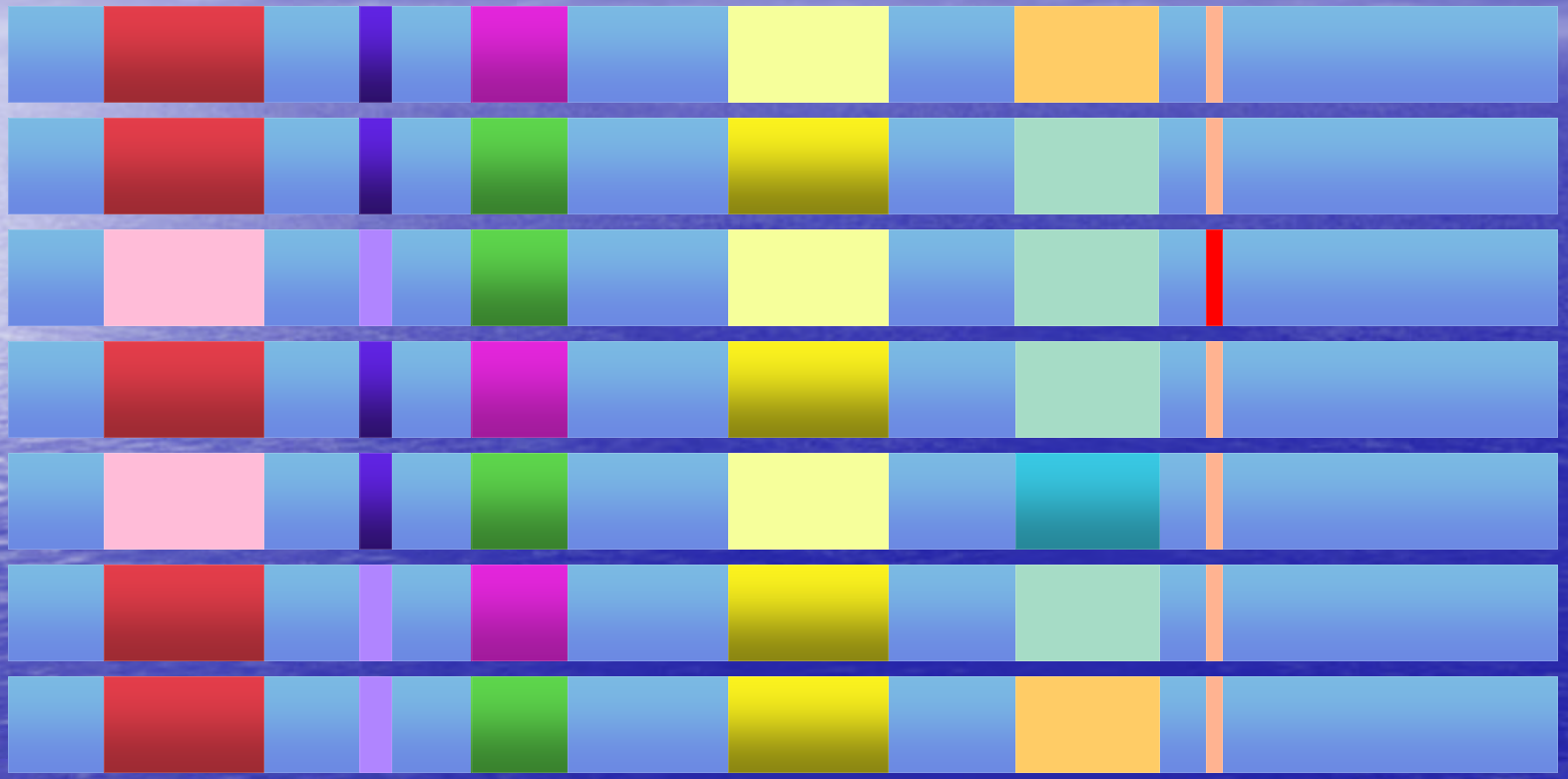
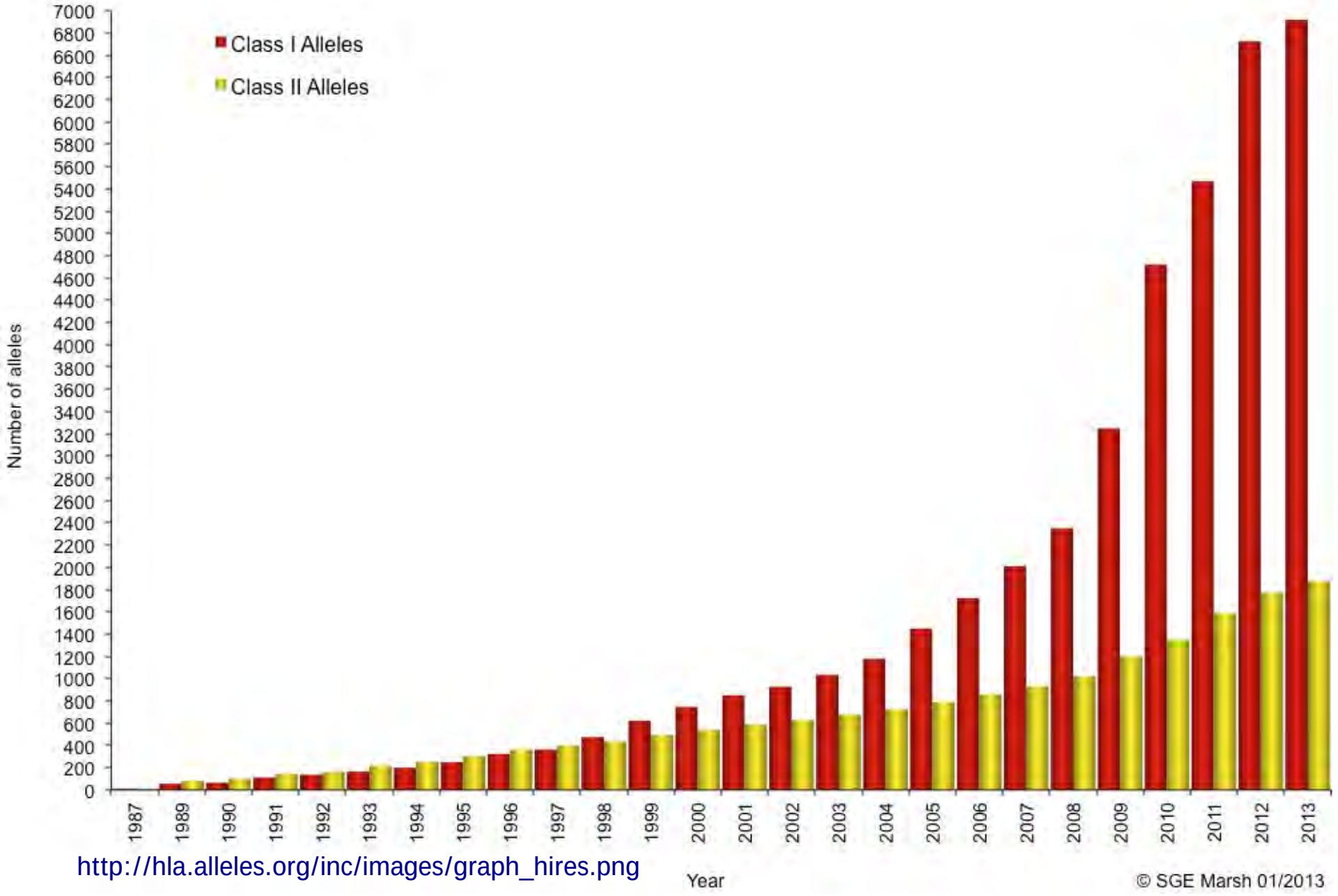
Sequence Based Typing Concepts

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

HLA alleles possess a “patchwork” pattern of polymorphism



Annual Increase of the Number of HLA Antigens/Alleles



http://hla.alleles.org/inc/images/graph_hires.png

Which Method to use for High Res Class I Typing? – Requirements:

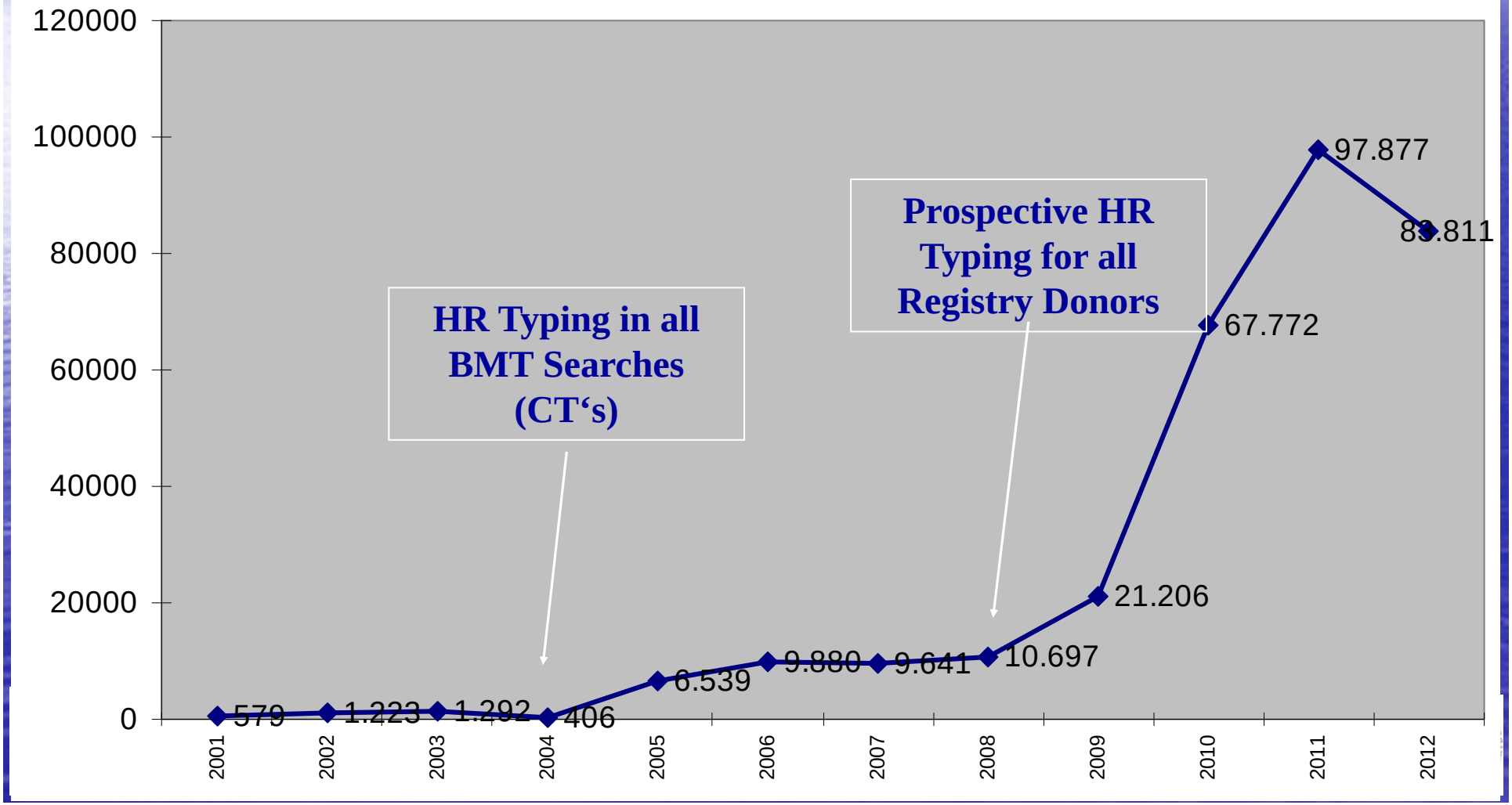
- Simple, and uniform processing of all incoming samples – regardless whether a „previous typing“ is available
- Quality Requirements (EFI/ASHI) to follow
- Highest possible resolution on the „first run“
- Financially „affordable“

Which Method to use for High Res Class I Typing?

Further Requirements:

- Two lines:
  - Day to Day Patient oriented Routine Work
    - Patients and Relatives (if high res needed)
    - Verification Typing for MUDs and Patients
    - High Res specific requests for registry samples
  - Prospective high Res ABCDRB1 Typing of New Bone Marrow Donor registrations

HLA-Class I High Res in Ulm
(Single Loci)



Why SBT?

- Information obtained by PCR-SSP and PCR-SSO is based on certain polymorphic positions within the relevant exons and do not consider the entire sequence of these exons
- The steadily increasing number of alleles makes the validity of SSP or SSO typings short lived
- SBT utilizes the entire sequence of the relevant exons and therefore achieves the highest possible degree of resolution

High Resolution Typing Techniques

```
A*0101: AACGCCGATCCGTTACGCTAG
  SSO:  ???CGC????????????TAG
  SSP:  ???CGC????????????TAG
  SBT:  AACGCCGATCCGTTACGCTAG
```

High Resolution Typing Techniques

```
A*0101: AACGCCGATCCGTTACGCTAG
  SSO:  ???GCC??????????TAG
  SSP:  ???GCC??????????TAG
  SBT:  AACGCCGATCCGTTACGCTAG
```

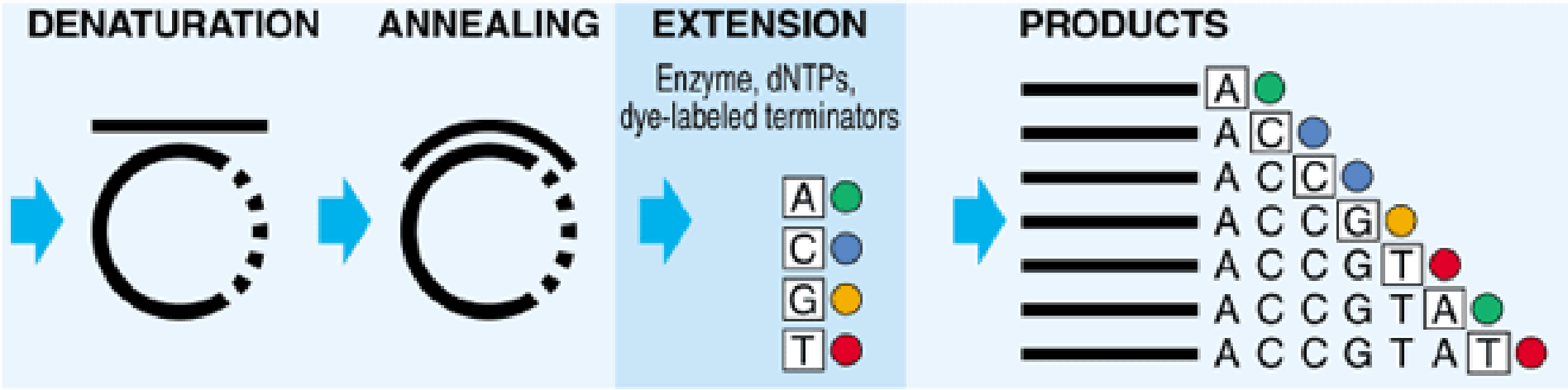
Some years later….

```
A*0140: AACGCCGACAGGTTACGCTAG
```

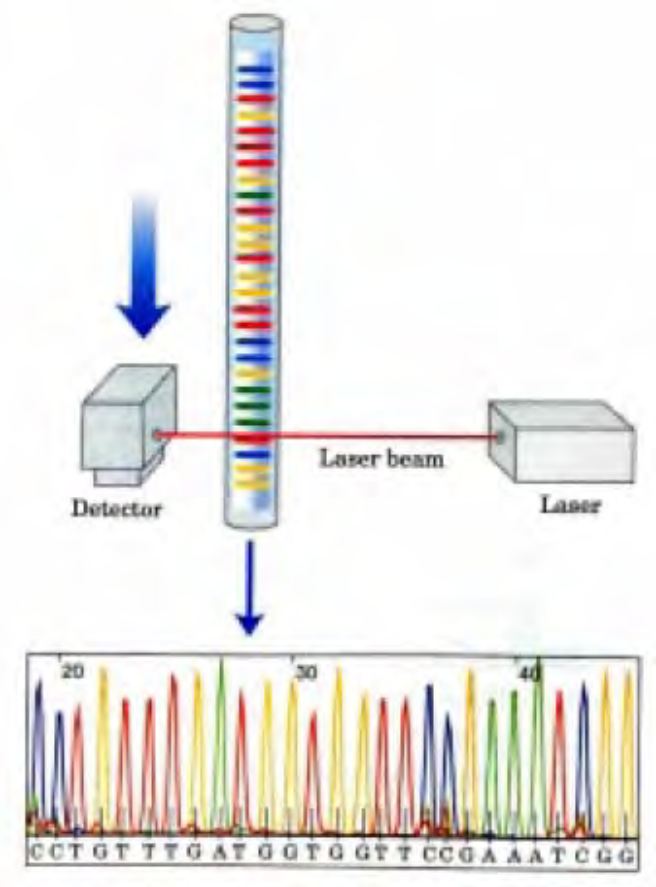
SBT Steps

1. DNA-Isolation
2. Locus-specific Amplification (PCR)
3. PCR Monitoring
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 raw data

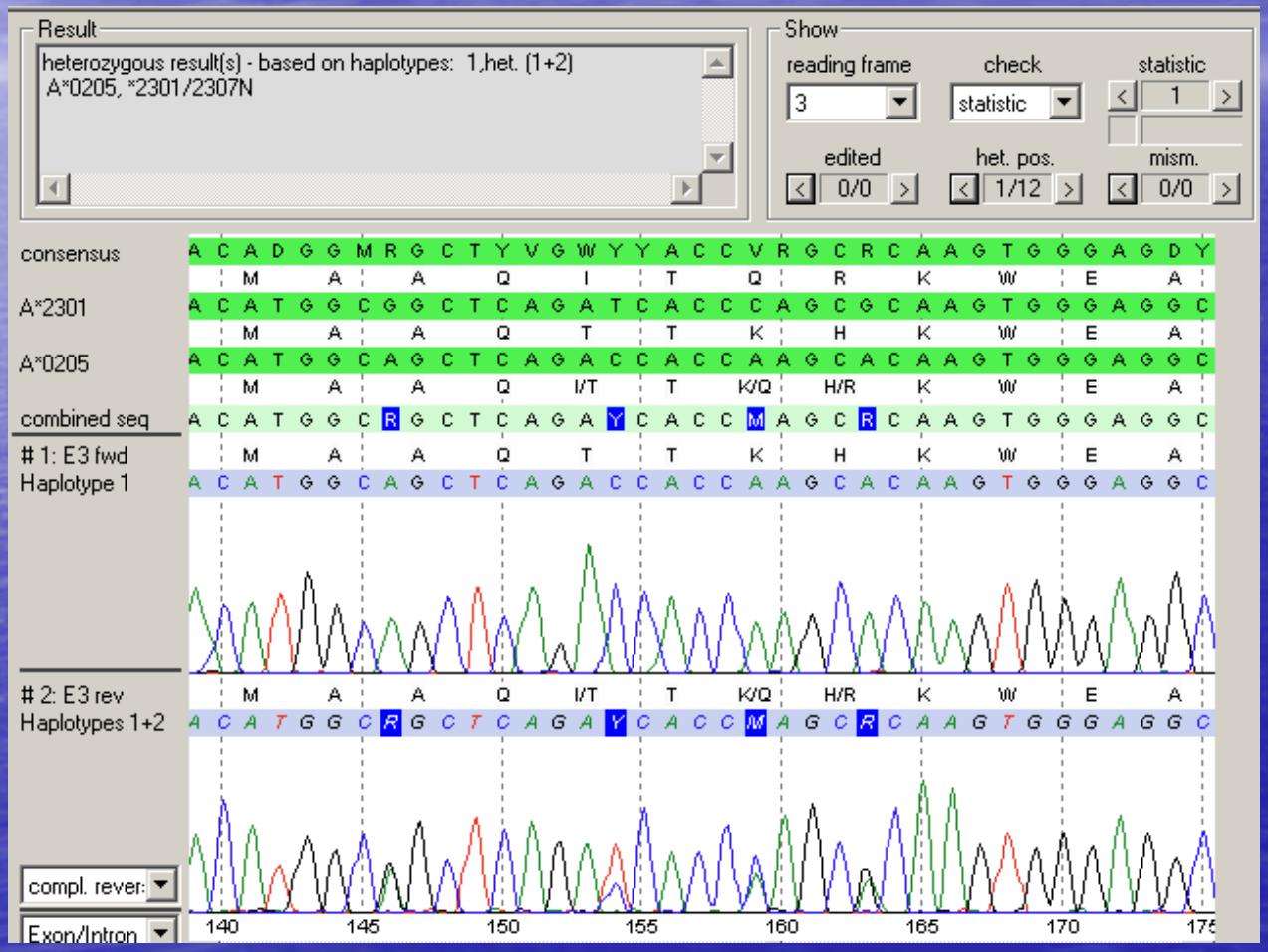
5. SBT – Cycle Seq Step



7. SBT – Separation of the Cycle Seq fragments



8. SBT – Analysis of the raw data



Equipment

- Thermocycler
- Agarose Gel Electrophoresis set
- Seqencing Device
  - ABI 310 (1 Capillary)
  - ABI 3100 (16 Capillaries)
  - ABI 3730 (48 Capillaries)

- Pipeting Devices, Centrifuges etc.

Facilities

PRE

1. DNA-Isolation

2. Locus-specific Amplification (PCR)

POST

3. PCR Monitoring

4. Purification of Amplification product

PRE

5. Cycle Sequencing

POST

6. Purification of the „Cycle Seq“ products

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

8. Interpretation and evaluation of the raw data

Problem: Ambiguities

Allele 1: AcgTTAAggTagcgcATcTgAcccAATCTT

Allele 2: AcgCTAAggTagcgcATcTgAgggTTACTT

Combi Sequence: AcgYTAAggTagcgcATcTgASSSWWWCTT

Allele 3: AcgTTAAggTagcgcATcTgAgggAATCTT

Allele 4: AcgCTAAggTagcgcATcTgAcccTTACTT

Combi Sequence: AcgYTAAggTagcgcATcTgASSSWWWCTT

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

Problem: Ambiguities

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

Expected genotype ambiguities

based on allele frequencies

- HLA-A: 62 %
- HLA-B: 58 %
- HLA-C: 58 %
- HLA-DPB1: 56 %
- HLA-DQB1: 25 %
- HLA-DRB1: 60 %

Sequencing Strategy

- generic
- group specific
- group specific with knowledge of previousy performed low res typing

Sequencing Strategy

- **generic**
  - only 1 PCR per Locus
  - allways heterozygous Sequences
    - 4 Sequencing reactions per Locus (1 forward and 1 reverse Sequence per exon)
  - many Ambiguities

	generic
Costs	
Labour	
Interpretation	
Time	
Resolution	

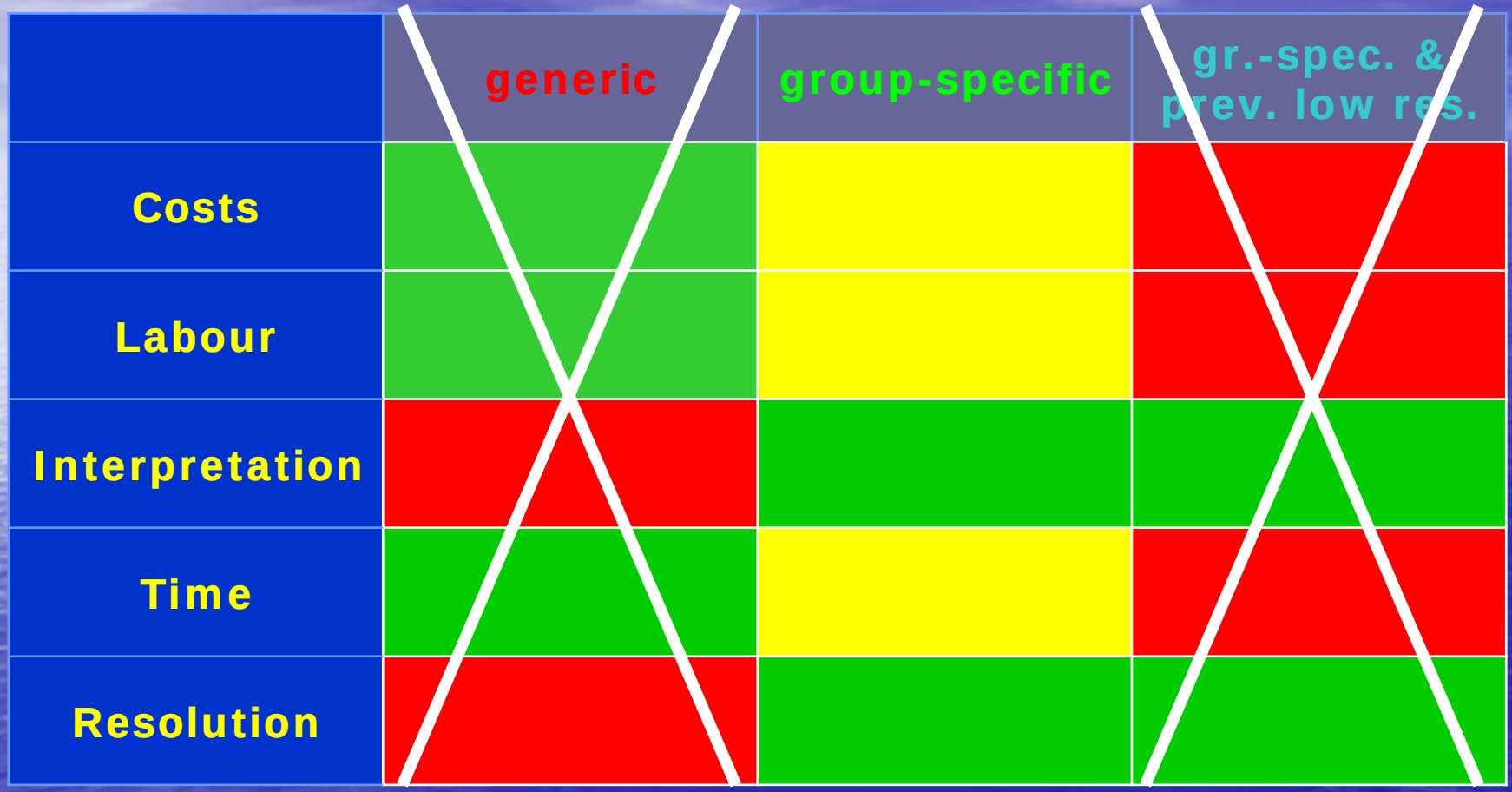
Sequencing Strategy

- **group specific (SSP+SBT)**
  - PCR-Reactions: 2+
  - heterozygous / homozygous Sequences
    - 4-6 Sequencing reactions per Locus
    - heterozygous Sequences fw and rv
    - homozygous Sequences in one direction only
    - less PCR's → more Sequencing Reactions required
    - more PCR's → less Sequencing Reactions required
  - fewer Ambiguities

	generic	group-specific
Costs		
Labour		
Interpretation		
Time		
Resolution		

Sequencing Strategy

- **group specific & knowledge of previous low res result**
  - **PCR-Reactions: 2**
  - **homozygous Sequences**
    - **2-4 Sequencing reactions per Locus**
    - **homozygous Sequences in one direction only**
  - **fewer Ambiguities**


generic
group-specific
gr.-spec. & prev. low res.
Costs
Labour
Interpretation
Time
Resolution

Reagents

- commercial kits
  - Protrans: S1-S4
  - Life Technologies
  - Atria / Abbott
  - Genome Diagnostics
  - BAG
  - and others....
- or home made reagents

Evaluation Software

- commercial packages
  - Sequence Pilot
  - Assign
  - Sequence Engine
  - Score
  - ……

Critical Aspects to be considered for achieving high throughput HLA-high res typing by automated SBT

- Sample registration
- PCR-Robotic station
  - DNA-Isolation
  - PCR-setup
  - PCR-Monitoring
  - Purification of PCR product
  - Cycle-Seq Pipetting
  - Purification of Cycle-Seq products
- Automated analysis

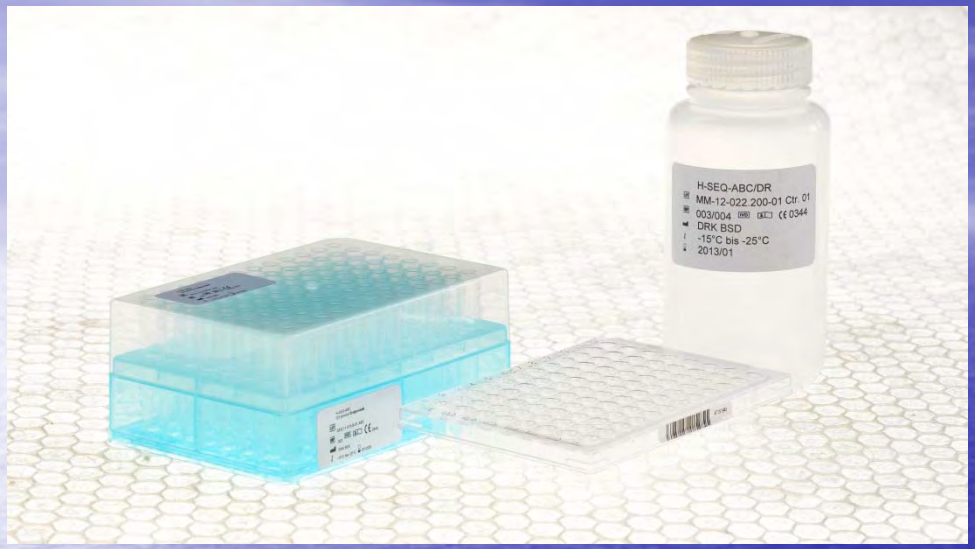
Our Strategy (IKT Ulm)

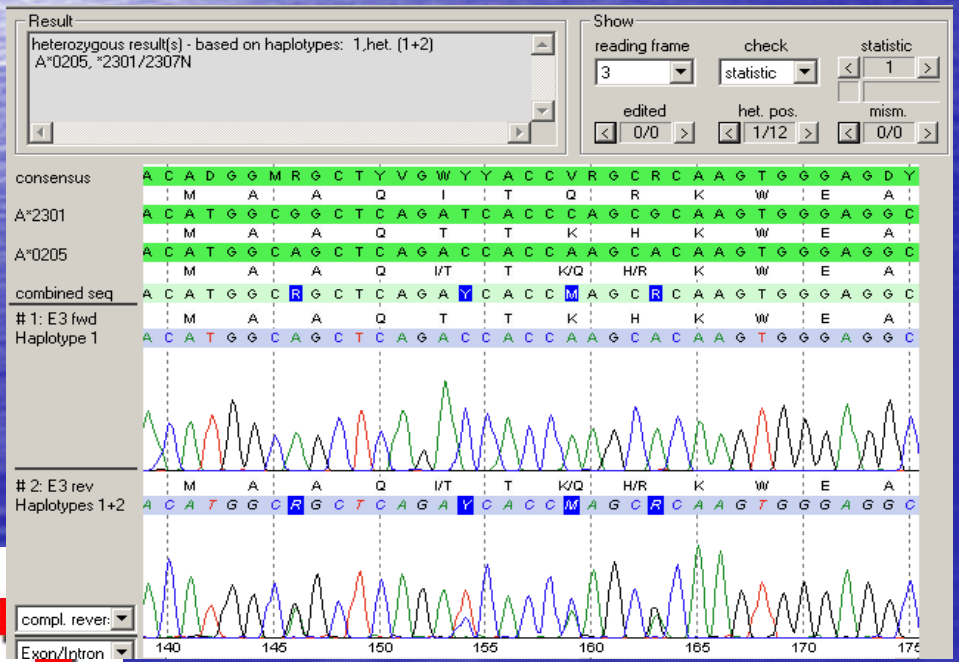
H-Seq ABC (CE-certified) + H-Seq DRB1

96-well Tray format (12 x 8 Stripes)

- 8 PCR‘s per HLA-A, -B, -Cw
  - A-Locus: A-generic, A2, A24
  - B-Locus: B-CG, B-TA, B7, B44
  - C-Locus: generic
- 8 PCR‘s per HLA-DRB1 typing
- 11 Typings (ABC or DRB1) per PCR Tray (+1 Stripe as neg. contr.)
- Pre-pipetted PCR Primers
- identical PCR-Conditions for all Loci

SBT with own Reagents





KEMA

CERTIFICATE

Number: 2110512CE01

CE

CE MARKING OF CONFORMITY
IN VITRO DIAGNOSTIC MEDICAL DEVICES

Issued to:

DRK-Blutspendedienst Baden-Wuerttemberg-Hessen gGmbH
Sandhofstrasse 1
60528 Frankfurt Am Main
Germany

For the product category (Annex II List B):

HLA sequencing kits

KEMA Quality grants the right to use the EC Notified Body Identification Number illustrated below to accompany the CE Marking of Conformity on the products concerned conforming to the required Technical Documentation and meeting the provisions of the EC-Directive which apply to them:

0344

Documents, that form the basis of this certificate:

Certification Notice 2110512CN, initially dated June 3, 2008
Addendum, initially dated October 28, 2009

KEMA Quality hereby declares that the above mentioned manufacturer fulfils the relevant provisions of 'Besluit in-vitro diagnostica', the Dutch transposition of the Directive 98/79/EC of October 27, 1998 concerning In vitro diagnostic medical devices, including all subsequent amendments, and that for the above mentioned product category the Conformity Assessment Procedure Annex IV for class Annex II List B products, is executed by the Manufacturer in accordance with the provisions of the Council Directive 98/79/EC of October 27, 1998. The necessary information and the reference to the relevant documentation, of the products concerned and the assessments performed, are stated in the Certification Notice which forms an integrative part of this certificate.

This certificate is valid until: October 28, 2012
Issued for the first time: June 3, 2008
Reissued: October 28, 2009

KEMA Quality B.V.

drs. G.J. Zoetbrood
Managing Director

M. McCann
Certification Manager

KEMA Medical
KEMA Quality B.V. Utrechtseweg 310, 6812 AR Arnhem P.O. Box 5185, 6802 ED Arnhem, The Netherlands
T +31 26 356 2000 F +31 26 352 5800 www.kemaquality.com Company registration 09085396

Our Strategy (IKT Ulm)

- PCR-Monitoring
  - Qiaxel- Machines (automated)
- Purification of PCR Product
  - AMPure (Agencourt) (Automated – magentic)
- Cycle-Sequencing setup (automated)
- Purification of Cycle-Seq
  - Clean Seq (automated - magnetic)
- Interpretation & Evaluation
  - Sequence-Pilot (JSI)

Our Strategy (IKT Ulm)

- Supplemental Testing (if needed)
  - homozygous Results → PCR-SSO (Luminex)
  - S4 (Protrans)
  - SSP‘s for NULL exclusion

Thank you for your attention!