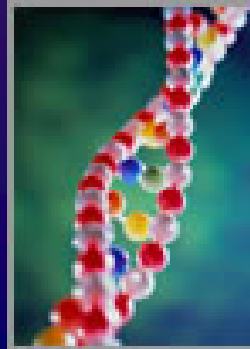


# Quantitative analysis of chimerism after allogeneic hematopoietic stem cell transplantation

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**Centre of molecular biology and gene therapy**  
*Department of Internal Medicine-Hematooncology*  
*Faculty Hospital Brno*



# Chimera

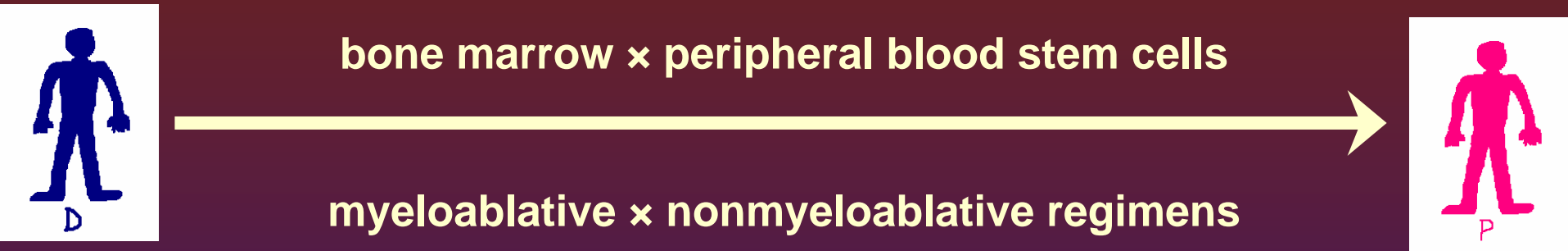


- ❖ Greek Mythology – a fire-breathing female monster with a lion's head, a goat's body, and a serpent's tail
- ❖ Biology – an organism containing a mixture of genetically different tissues, formed by processes such as fusion of early embryos, grafting

# Chimerism

- ❖ co-existence of cells from two different organism (evolved from two different zygotes) in one body
- ❖ the presence of lymphohematopoietic cells of nonhost origin

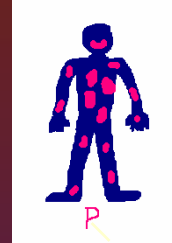
# Allogeneic transplantation



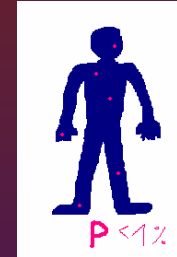
- ❖ used last three decades for treatment of patients with
  - ❖ malignant hematological diseases (leukemia and lymphomas)
  - ❖ non-malignant hematological diseases (severe aplastic anemia, severe combined immunodeficiency or hemoglobinopathies)

# Possible outcomes after transplantation

❖ mixed chimerism (MC)



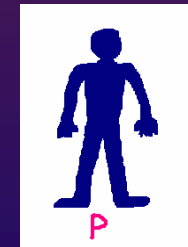
❖ microchimerism  
~MC <1%



❖ split chimerism



❖ complete chimerism (CC)  
(full, donor chimerism)



❖ autologous reconstitution



# Quantification of chimerism

= proportion assessment of the donor- and recipient-derived hematopoiesis



# Purposes of chimerism evaluation and its dynamics in time

- ❖ Prediction of negative events
  - ❖ Graft versus Host Disease (GvHD)
  - ❖ Disease relapse
  - ❖ Graft rejection
- ❖ Support in finding the optimal curative attitude
  - ❖ Modulation of immunosuppression
  - ❖ Donor lymphocyte infusion - DLI

# Techniques for Chimerism

## Assessment I

*(qualitative methods)*

- ❖ Red blood cell phenotyping
  - ❖ Immunoglobulin isotype analysis
  - ❖ Cytogenetics (sex-mismatch)
- ☹ Limitations: limited degree of polymorphism,  
poor sensitivity

# Techniques for Chimerism

## Assessment II

*(quantitative methods)*

- ❖ FISH (Fluorescence in situ Hybridisation)

- ☹ Limitations: limited degree of polymorphism – sex-mismatch pairs only

# Techniques for Chimerism

## Assessment II

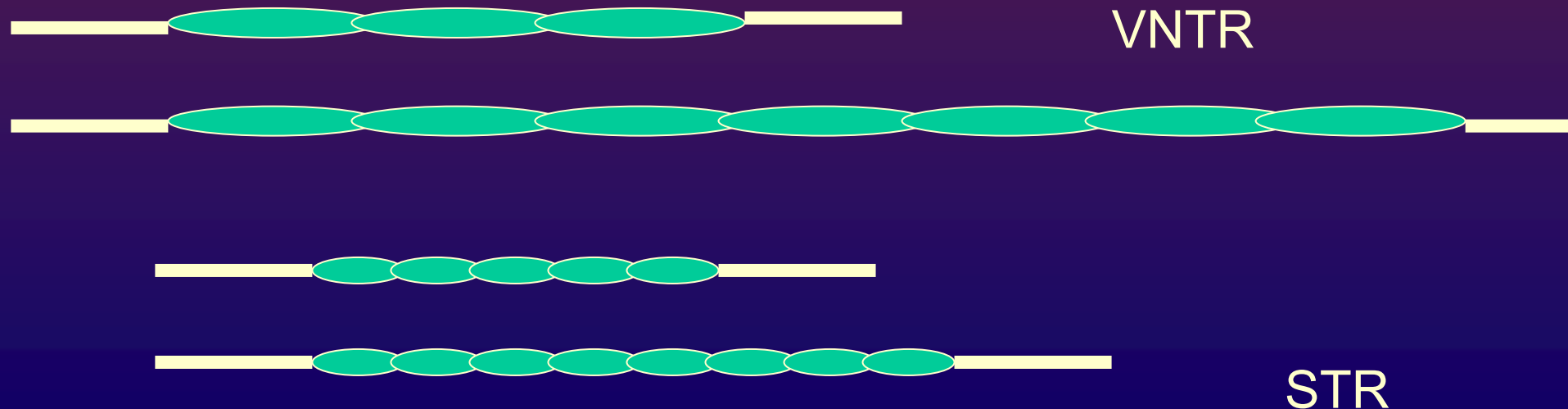
*(quantitative methods)*

- ❖ RFLP (Restriction Fragment Length Polymorphism)
  - ❖ Neutral variations in DNA sequence created by
    - ❖ Either loss or gain of a restriction enzyme cleavage site (biallelic polymorphisms)
    - ❖ insertion or deletion of DNA between restriction sites (multiallelic)
      - ❖ Variable Number of Tandem Repeats (VNTR)
      - ❖ Short Tandem Repeats (STR)

# Scheme of STR and VNTR Polymorphism

Tandem repetitive unit of DNA

- ❖ VNTR – repetitive sequence 10-50 bp long
- ❖ STR – repetitive sequence 2-6 bp long



# Techniques for Chimerism

## Assessment II

*(quantitative methods)*

❖ RFLP (Restriction Fragment Length Polymorphism)

☹ Limitations: cumbersome, time consuming, requirement of a large amount of DNA

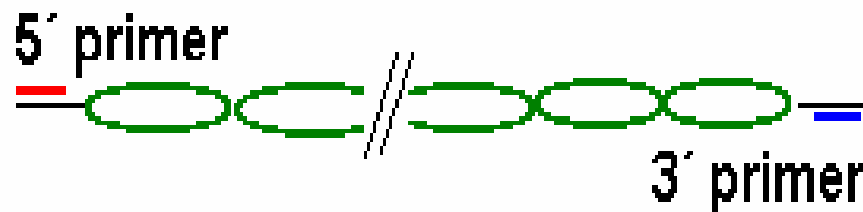
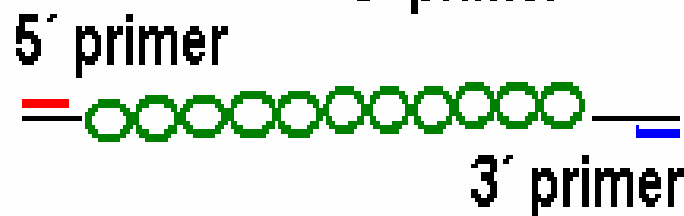
# Polymerase Chain Reaction (PCR)

- ❖ Enhanced sensitivity
- ❖ Analysis possible from a small number of cells

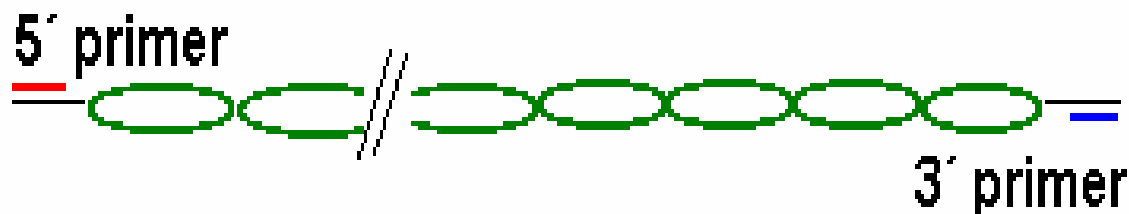
# Polymerase Chain Reaction (PCR)



STR

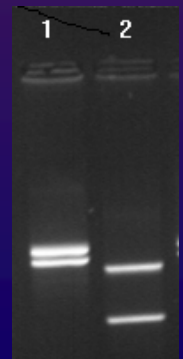


VNTR



# Polymerase Chain Reaction (PCR)

- ❖ primers flank the repeat sequences  $\Rightarrow$  PCR products of different length (determined by the number of repeats in each allele)
- ❖ PCR products electrophoresed on an agarose/polyacrylamide gel and visualised
  - ❖ Ethidiumbromide
  - ❖ Radioactivity



# Fragment Analysis

(Capillary Electrophoresis with Fluorescence Detection)

- ❖ Usage of fluorescently labeled primers (Scharf *et al.* 1995)
  - ❖ greater precision (over PAGE)
  - ❖ easier performance of quantitative analysis
  - ❖ (avoidance of radioactivity)

Genetic analyzer CEQ 8000  
(Beckman Coulter)



# Method

## A. Before transplantation

1. Testing to provide an informative marker
2. Calibration Standard Dilution → Calibration Standard Curve

## B. After transplantation

1. Amplification of the selected locus
2. Assessment of chimerism according to a calibration curve

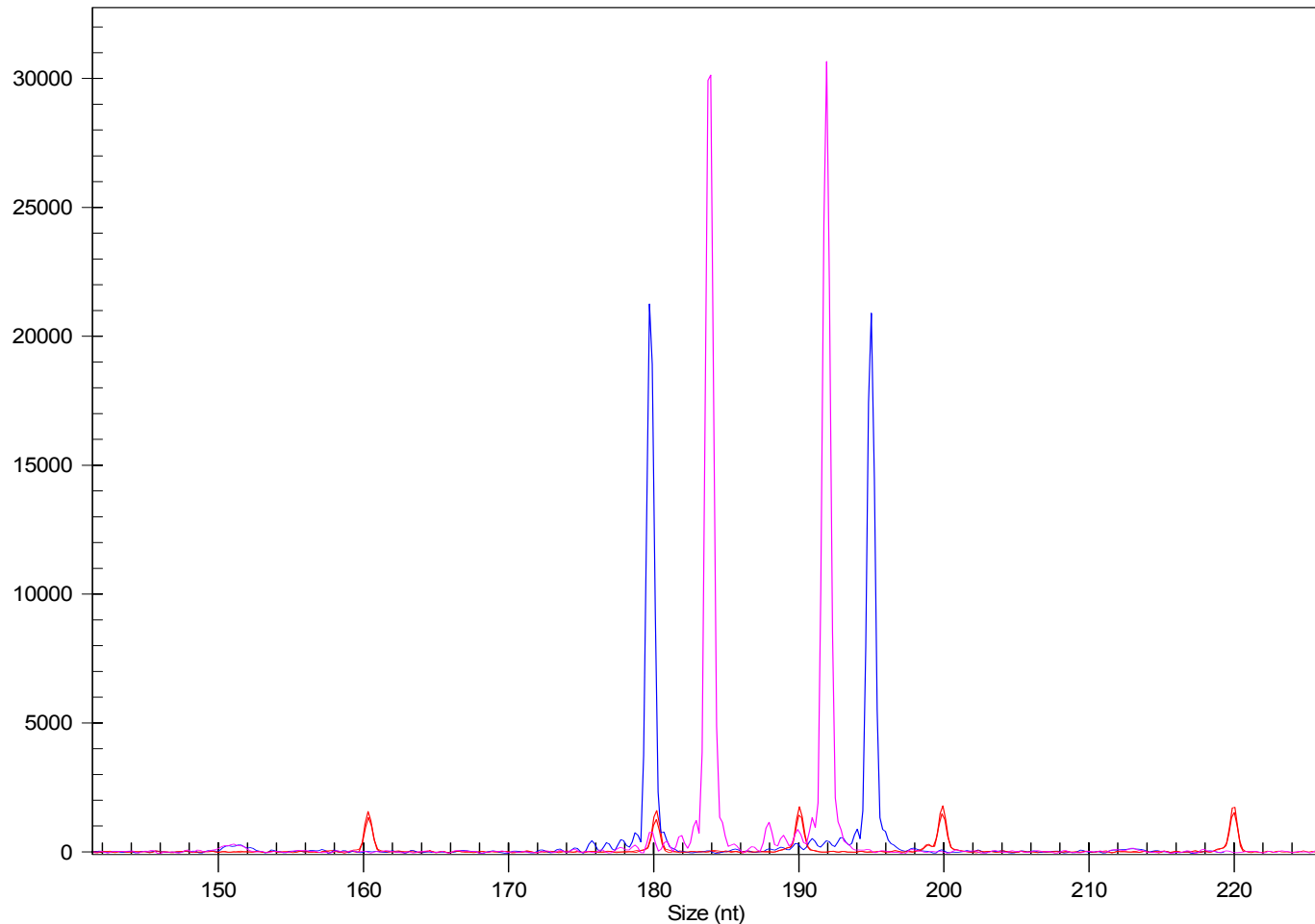
# Testing to provide an informative marker

- ❖ 8 STR (possible multiplex PCR)
- ❖ 4 VNTR
- ❖ 1 sex determined polymorphism (Amelogenin)
  - ❖ Amplification
  - ❖ Agarose gel electrophoresis – checking the PCR product
  - ❖ Fragment analysis

# Fragment analysis – – sample preparation

- ❖ Adequate portion of the labeled PCR product is mixed with 30  $\mu$ l solution containing SLS (Sample Loading Solution) and size standard (60-400 or 60-600 bp)
- ❖ Vortexing (tubes) or pipeting (plate)
- ❖ Centrifugation
- ❖ Analysis
  - ❖ Denaturation
  - ❖ Electrokinetics injection of the sample
  - ❖ Separation
  - ❖ Evaluation

# Testing to Provide an Informative Marker



# Informativeness

- ❖ presence of at least one unique donor and recipient allele for a marker to be eligible **X** an unique recipient allele distinguishable from the donor allele(s)
  - ❖ correlation with quantitative analysis – standard curve vs. calculation from general formula
- ❖ donor and informative recipient alleles must be separated at least by two repeat units to prevent interference with stutter peaks

# Informativeness

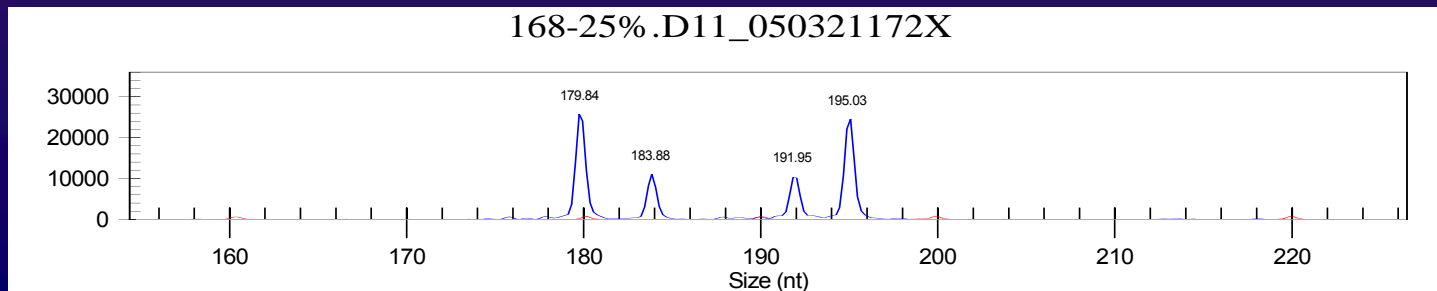
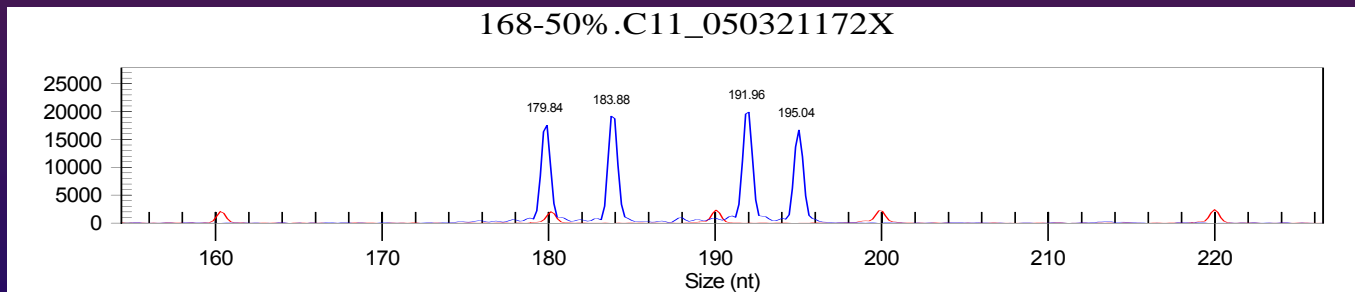
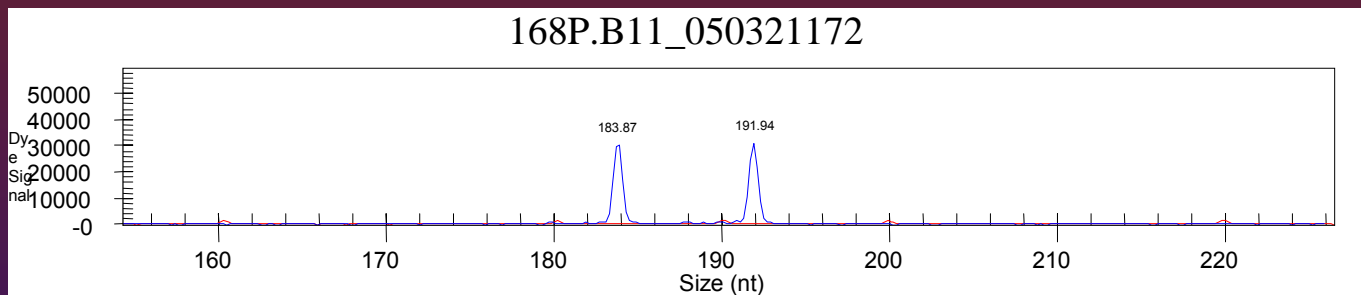
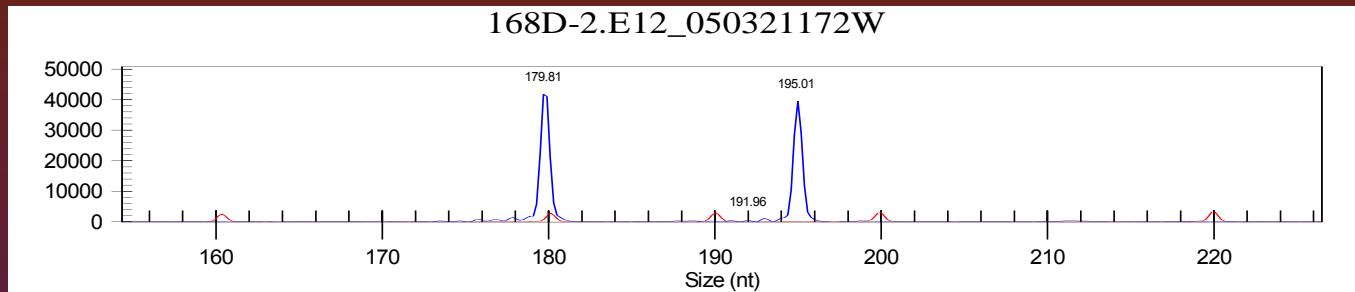
(VNTR vs. STR)

- ❖ STR – only a limited number of highly polymorphic loci needs to be tested to provide an informative marker
- ❖ preferential amplification of shorter fragments during PCR
  - ❖ VNTR – increased sensitivity, if recipient fragment is shorter
  - ❖ STR – also longer alleles can be used

# Patient-Specific Standard Curve

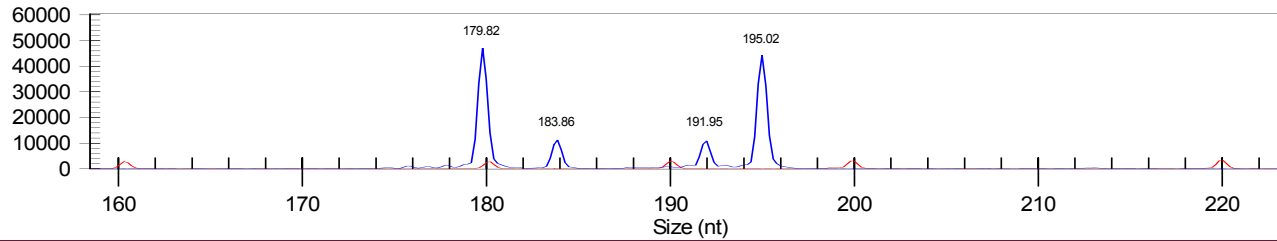
- ❖ equalization of concentration donor and pre-transplant recipient DNA
- ❖ mixing of these DNAs together in proper ratio ( $\approx 50, 25, 15, 10, 5, 3, 2, 1, 0.5\%$  pre-transplant recipient DNA in DNA of donor)
- ❖ amplification  $\rightarrow$  checking the PCR product on agarose gel electrophoresis  $\rightarrow$  fragment analysis

# Calibration: D, R, 50, 25%

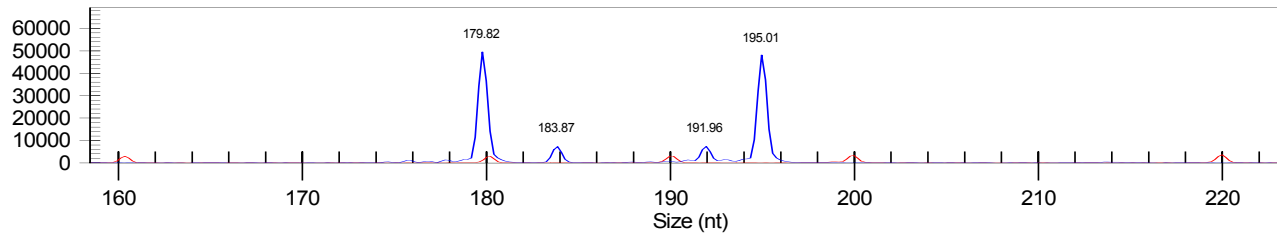


# Calibration : 15-3%

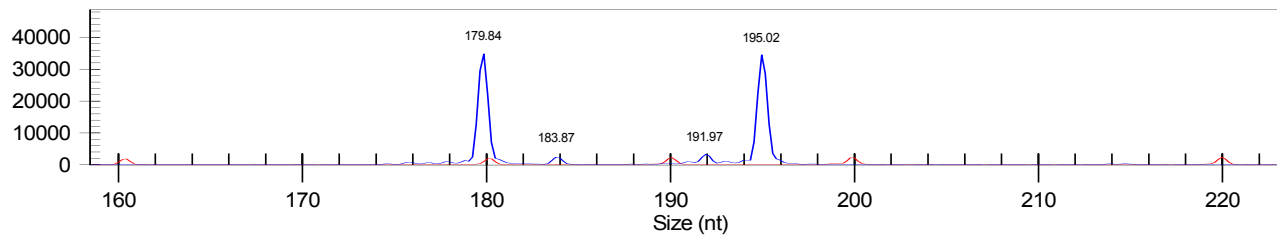
168-15%.E11\_050321172W



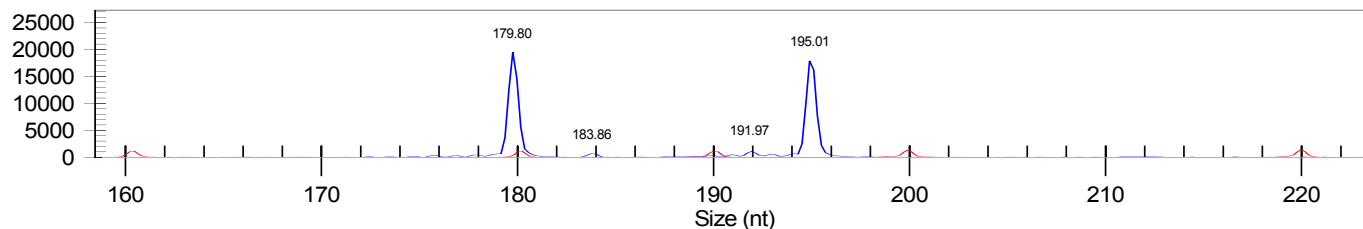
168-10%.F11\_050321172W



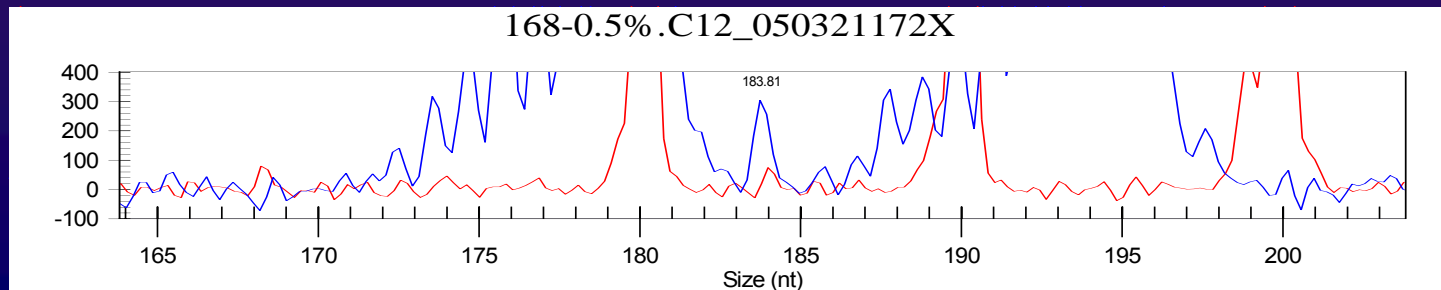
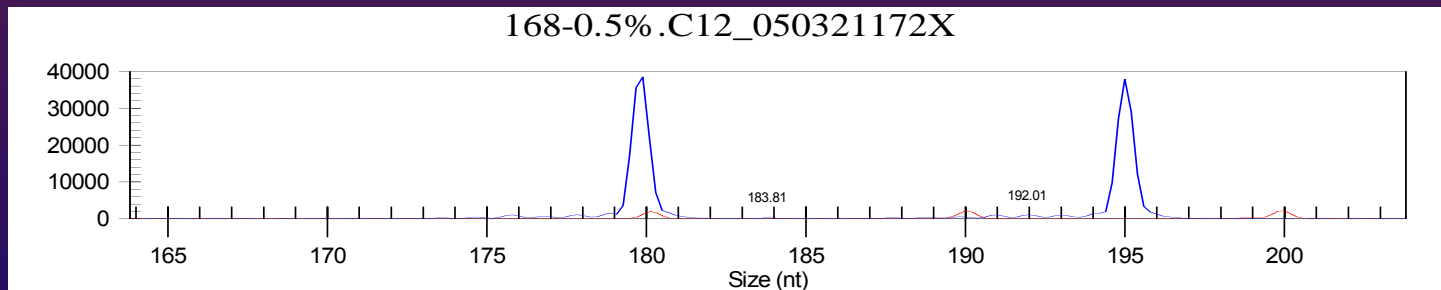
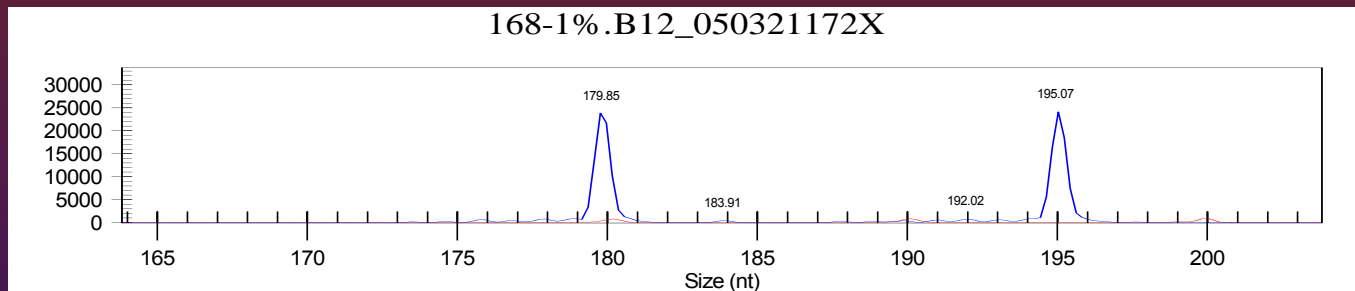
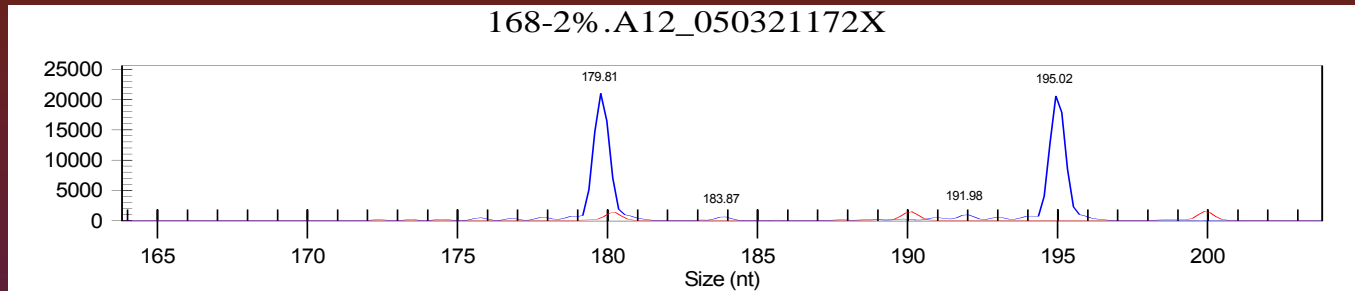
168-5%.G11\_050321172V



168-3%.H11\_050321172V



# Calibration : 2-0,5%



# Calibration – Peak Area

Fragment Analysis - spacek3 \* - [Fragment List]

File View Fragments Analysis Reports Window Help

D1 D2 D3 D4

Study Explorer

- Study
  - AFLPs
  - Binnings
  - Peak Ratios

Fragment List

RN	est frag size (nt)	pk area (rfuxmm)	pk height (rfu)	loc
168-3%:H11_050321172V	80	8439	19528	
168D:A11_050321172X	80	10056	21522	
168-31D:H12_050321172V	81	7911	17391	
168D-2:E12_050321172X	81	20744	43859	
168-2%:A12_050321172X	81	9650	20935	
168-14D-2:F12_050321172W	82	17921	38140	
168-10%:F11_050321172W	82	22374	49587	
168-15%:E11_050321172W	82	21735	47066	
168-14D:D12_050321172W	82	24222	51834	
168-28D:G12_050321172V	83	16362	35518	
168-0.5%:C12_050321172X	83	18689	39428	
168-5%:G11_050321172V	84	16351	35032	
168-25%:D11_050321172X	84	12583	26552	
168-50%:C11_050321172X	84	8948	18021	
168-1%:B12_050321172X	84	11206	24380	
168-0.5%:C12_050321172X	D4 No	183.81	158	323
168-14D-2:F12_050321172W	D4 No	183.85	9105	19392
168-14D:D12_050321172W	D4 No	183.85	13313	28428
168-31D:H12_050321172V	D4 No	183.86	767	1688
168-3%:H11_050321172V	D4 No	183.86	268	637
168-28D:G12_050321172V	D4 No	183.86	640	1465
168-15%:E11_050321172W	D4 No	183.86	5311	11309
168-5%:G11_050321172V	D4 No	183.87	1028	2292
168-10%:F11_050321172W	D4 No	183.87	3241	7194
168P:B11_050321172X	D4 No	183.87	14881	31563
168-2%:A12_050321172X	D4 No	183.87	208	551
168-25%:D11_050321172X	D4 No	183.88	5148	10993
168-50%:C11_050321172X	D4 No	183.88	9804	20019
168-1%:B12_050321172X	D4 No	183.91	123	342
168-14D-2:F12_050321172W	D4 No	191.92	8390	17545
168-14D:D12_050321172W	D4 No	191.93	13060	27707
168-31D:H12_050321172V	D4 No	191.94	768	1756

Exclusion Filter Set

New Fragment Filter Set 5

ID	Name	Operator	Values
1	dye	Not Equal	D4
2	pk height (rfu)	<	4000
3	<select>		

Analyses Data Reports

Ready

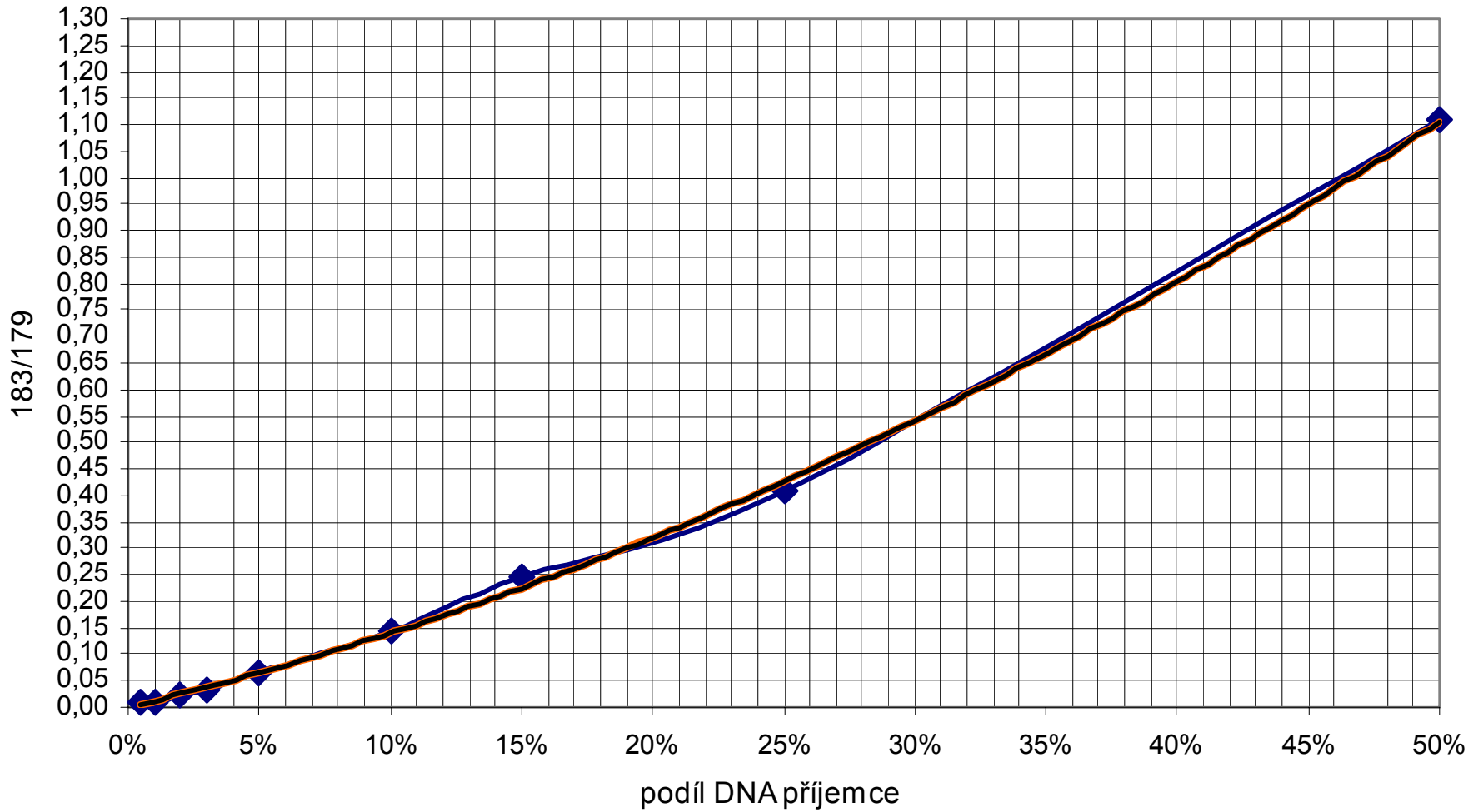
Start Main Menu - Versi... Data Manager Fragment Anal... Microsoft Word 11:41 PM

est frag size (nt)	pk area (rfuxmm)	pk height (rfu)	
179,83	18689	39428	
183,81	158	323	
195,04	17177	37800	
179,85	11206	24380	
183,91	123	342	
195,07	10560	24064	
179,82	22374	49587	
183,87	3241	7194	
195,01	20929	48056	
179,82	24222	51834	
183,85	13313	28428	
195,01	23294	50097	
179,82	17921	38140	
183,85	9105	19392	
195,01	16612	36318	
179,82	21735	47066	
No	183,86	5311	11309
No	195,02	20251	43948
No	179,81	9650	20935
No	183,87	208	551
No	195,02	9407	20748
No	179,84	12583	26552
No	183,88	5148	10993

# Calibration curve – Charts

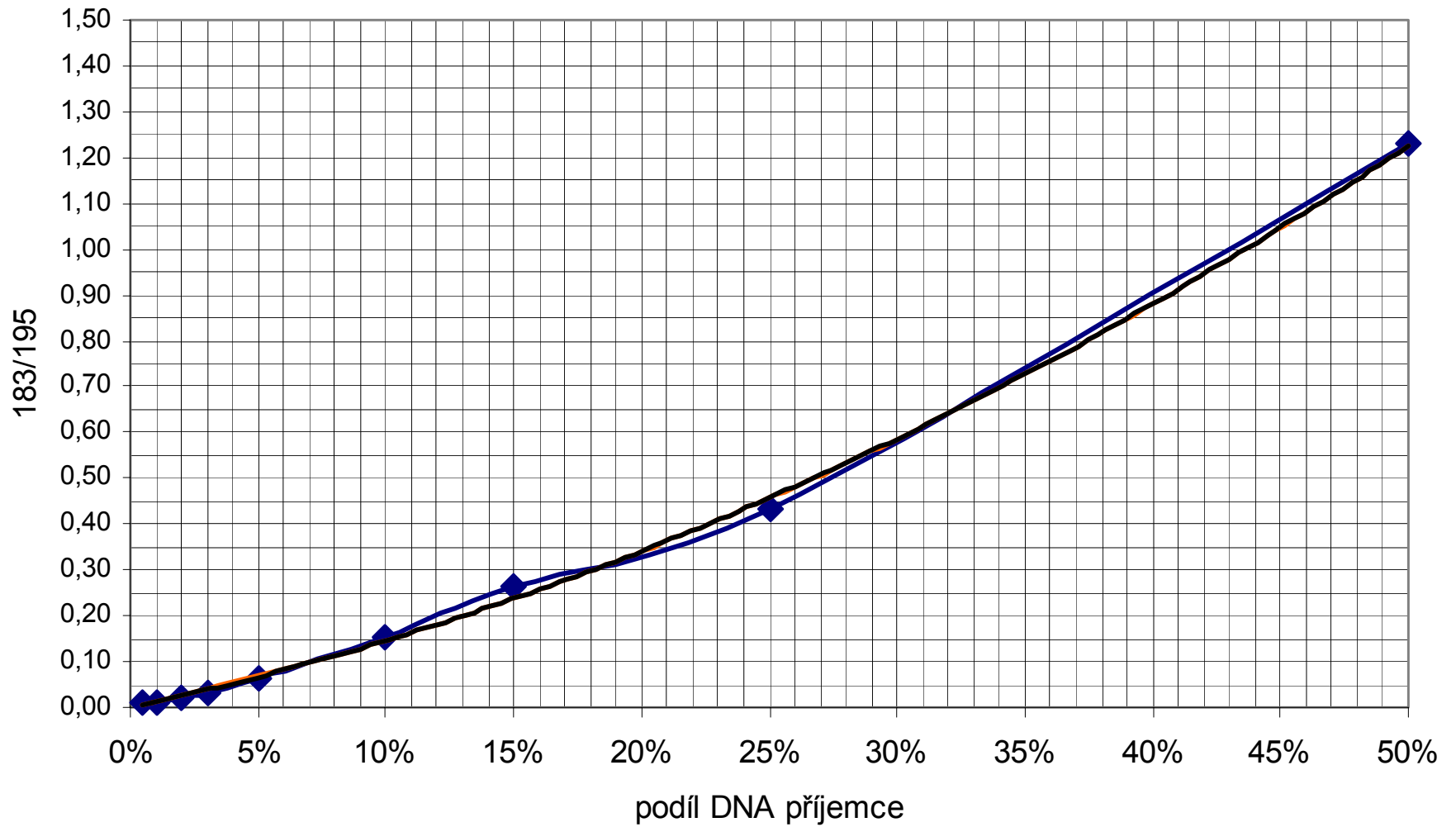
$$y = 2,0311x^2 + 1,1959x$$
$$R^2 = 0,9993$$

$$y = 2,0302x^2 + 1,1964x - 5E-05$$
$$R^2 = 0,9993$$



$$y = 2,4789x^2 + 1,211x$$
$$R^2 = 0,9989$$

$$y = 2,5047x^2 + 1,1955x + 0,0015$$
$$R^2 = 0,9989$$



# Method

## A. Before transplantation

1. Testing to provide an informative marker
2. Calibration Standard Dilution → Calibration Standard Curve

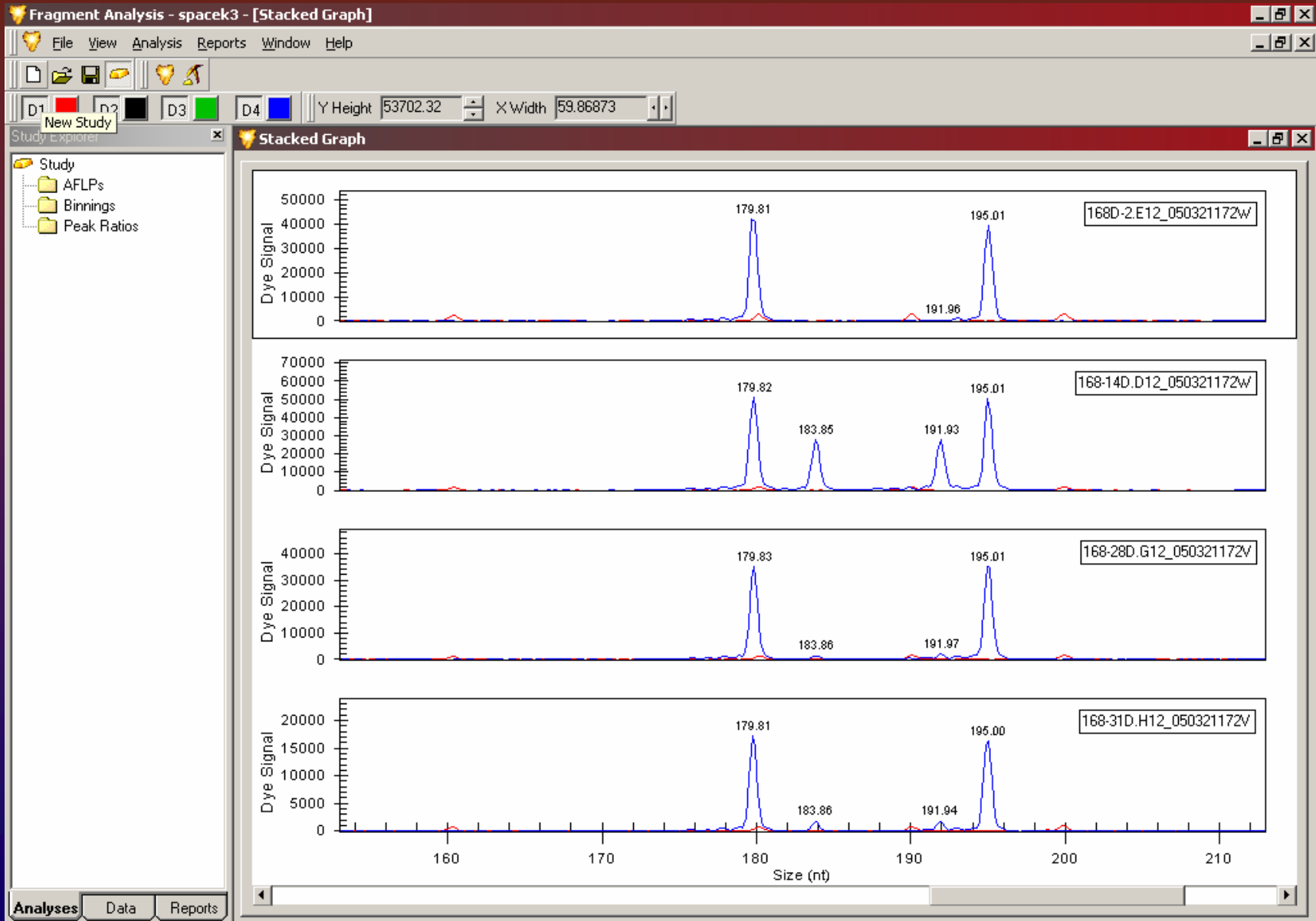
## B. After transplantation

1. Amplification of the selected locus
2. Assessment of chimerism according to a calibration curve

# B. After transplantation

- ❖ Amplification of the selected locus
  - ❖ Singleplex PCR (higher sensitivity)
  - ❖ Parallel amplification of
    - ❖ donor DNA (negative control)
    - ❖ 1-2 previous samples (variability of PCR)
- ❖ amplification → checking the PCR product on agarose gel electrophoresis → fragment analysis

# Sample after Transplantation



# Peak area

Fragment Analysis - spacek3 \* - [Fragment List]

File View Fragments Analysis Reports Window Help

D1 D2 D3 D4

Study Explorer

- Study
  - AFLPs
  - Binnings
  - Peak Ratios

Fragment List

Fragment	RN	dye	size std	est frag size (nt)	pk area (rfuxmm)	pk height (rfu)	loc
168-0.5%.C12_050321172X		D4	No	179.83	18689	39428	
168-0.5%.C12_050321172X		D4	No	183.81	158	323	
168-0.5%.C12_050321172X		D4	No	195.04	17177	37800	
168-1%.B12_050321172X		D4	No	179.85	11206	24380	
168-1%.B12_050321172X		D4	No	183.91	123	342	
168-1%.B12_050321172X		D4	No	195.07	10560	24064	
168-10%.F11_050321172w		D4	No	179.82	22374	49587	
168-10%.F11_050321172w		D4	No	183.87	3241	7194	
168-10%.F11_050321172w		D4	No	195.01	20929	48056	
168-14D.D12_050321172w		D4	No	179.82	24222	51834	
168-14D.D12_05032				183.85	13313	28428	
168-14D.D12_05032				195.01	23294	50097	
168-14D-2.F12_0503				179.82	17921	38140	
168-14D-2.F12_0503				183.85	9105	19392	
168-14D-2.F12_0503				195.01	16612	36318	
168-15%.E11_05032				179.82	21735	47066	
168-15%.E11_05032				183.86	5311	11309	
168-15%.E11_05032				195.02	20251	43948	
168-2%.A12_050321				179.81	9650	20935	
168-2%.A12_050321				183.87	208	551	
168-2%.A12_050321				195.02	9407	20748	
168-25%.D11_05032				179.84	12583	26552	
168-25%.D11_05032					8	10993	
168-25%.D11_05032					9	24972	
168-28D.G12_050321172V		D4	No			35518	
168-28D.G12_050321172V		D4	No	183.86	640	1465	
168-28D.G12_050321172V		D4	No	195.01	16838	36887	
168-3%.H11_050321172V		D4	No	179.80	8439	19528	
168-3%.H11_050321172V		D4	No	183.86	268	637	
168-3%.H11_050321172V		D4	No	195.01	8017	18187	
168-31D.H12_050321172V		D4	No	179.81	7911	17391	
168-31D.H12_050321172V		D4	No	183.86	767	1688	

Exclusion Filter Set

New Fragment Filter Set 5 Apply ...

ID	Name	Operator	Value(s)
1	dye	Not Equal	D4
2	pk height (rfu)	<	4000
3	<select>		

Show Excluded

Analyses Data Reports

Ready

Start Main Menu - Versi... Data Manager Fragment Anal... Microsoft Word 11:42 PM

# Quantitative analysis

The degree of mixed chimerism is carried out:

- ❖ Relative to a patient-specific standard curve established from serial dilutions of pre-transplant recipient DNA in donor DNA

OR

- ❖ By direct measurement of the signal ratio of one recipient-specific allele to the sum of signals of the donor and recipient alleles
  - ❖ Both a recipient- and donor- specific allele requirement
  - ❖ Assumption that all alleles amplify with the same efficiency

# Sensitivity

- ❖ reported average sensitivity in detecting the minor populations ranges from 1/0.5-5% (Lion T., Leukemia 2003 17(1):252-4, own observations)
  - ~ size of the informative recipient allele(s)
  - ~ allelic constellation, numbers of alleles co-amplified
  - ~ type of polymorphism
  - ~ amount of template
    - 50-100 ng DNA ( $\sim 7,5 \times 10^3$  –  $15 \times 10^3$  diploid human cells)  
⇒ detection in the range of 1% (ie about 100 cells)
  - specific white blood cell fractions – generally one or two logs higher than chimerism analysis in whole blood

# Reproducibility

- ❖ Multiple capillary electrophoresis of the same PCR specimens – variation 5%
- ❖ Re-amplification of DNA samples – differences less than 10%
  - ❖ Parallel amplification of
    - ❖ donor DNA (negative control)
    - ❖ 1-2 previous samples (variable PCR)
- ❖ Higher variability around/under 1%

Absence of standardization ⇒ „Debate Round Table“,  
Leukemia, 1999

# Arteficial peaks

## ❖ Stutter peaks

- ❖ Results of polymerase slippage during the amplification of microsatellite loci (STR)
- ❖ Typically migrate at a distance of one or more repeat unit in front/back of the specific (parent) peak
- ❖ May interfere with specific allelic peaks  $\Rightarrow$  donor and informative recipient alleles must be separated at least by two repeat units

## ❖ +A/-A peaks

- ❖ Result of *Taq* polymerase activity

# Summary

- ❖ Accurate quantitative analysis of chimerism kinetics can permit early differentiation between the absence of engraftment and a delay in engraftment as well as early detection of patients with a high risk of GvHD or those liable to relaps
- ❖ Fragment analysis is a good tool to achieve it

**The End**

**Thanks for Attention**