

Microarray “double-hybridization” system using LNA modified probes and magnetic nanobeads

Martin Bunčák

GENERI BIOTECH s.r.o.



GENERI BIOTECH s.r.o.

- Commercial

synthesis of oligonucleotides, paternity testing, molecular services
(cloning, sequencing etc.)

- Research

Several projects mainly focussed on molecular genetics (detection of highly pathogenic bacteria for Czech Ministry of defence, use of oligonucleotides as therapeutics, molecular resistance of cancer cells to cytostatics caused by membrane transporters etc.)

GENERI BIOTECH employs approximately 20 staff, half of which are university graduates. 6 of them are Ph.D.

Approximately one third of employees work in the commercial division and 2/3 in the research division.

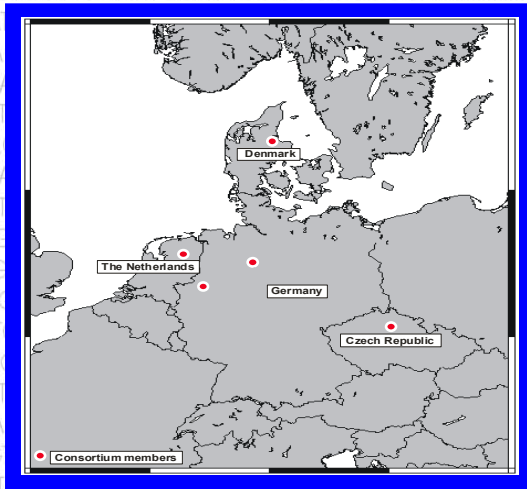




Nano-biotechnical components of an advanced bio-analytical microarray system („GenSensor-Nanoparts“)

COORDINATOR: D. Blohm

University of Bremen, CAG-UFT, Leobener Str. D-28359 Bremen, Tel. +49-421-218-4780, email: dhb@biotec.uni-bremen.de



Consortium

- Centre for Applied Gensensorik (CAG), University of Bremen, Germany (Co-ordinator)
- Exiqon A/S, Denmark
- Generi Biotech s.r.o., Czech Republic
- Miltenyi Biotec GmbH, Germany
- Plant Research International b.v., The Netherlands

Objective

Based on an advanced flow through microarray system (gensensor) a new approach for increasing the sensitivity of a label-free detection system has been proposed to be developed.

Improving of this essential step in the microarray based gene analytical method should lead to a less complex, PCR independent and better quantifiable analytical process, useful for routine diagnostics in the clinic and in the industry.



Microarrays

- **Detection of specific DNA/RNA**

- **Need to use labels**

- **Sample preparation is crucial**

 - **Minute amounts of detected sequence(s) in a plenty of other sequences and/or complex matrix**

The Nanoparts approach

- **Combine the hybridization power of LNA bases modification with the magnetic nanobeads for combined sample isolation, hybridization and detection**
- **Design of a „probe pair“ close together**
 - DNA probes spotted on the array
 - LNA probes bound to magn. nanobeads

Features

- **LNA = Locked Nucleic Acids**

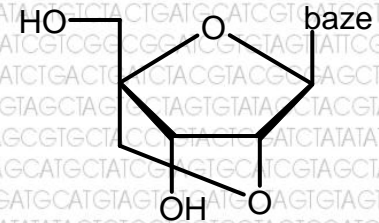
- **Property of Exiqon S/A, Denmark**

- **high affinity RNA mimics with the sugar ring locked in the 3'-endo conformation**
- **Increased Tm (Tm increases by 2 - 8°C per base)**

- **Magnetic nanobeads**

- **Miltenyi Biotec GmbH, Germany**

- **Diameter of 20nm to 200nm**



workflow

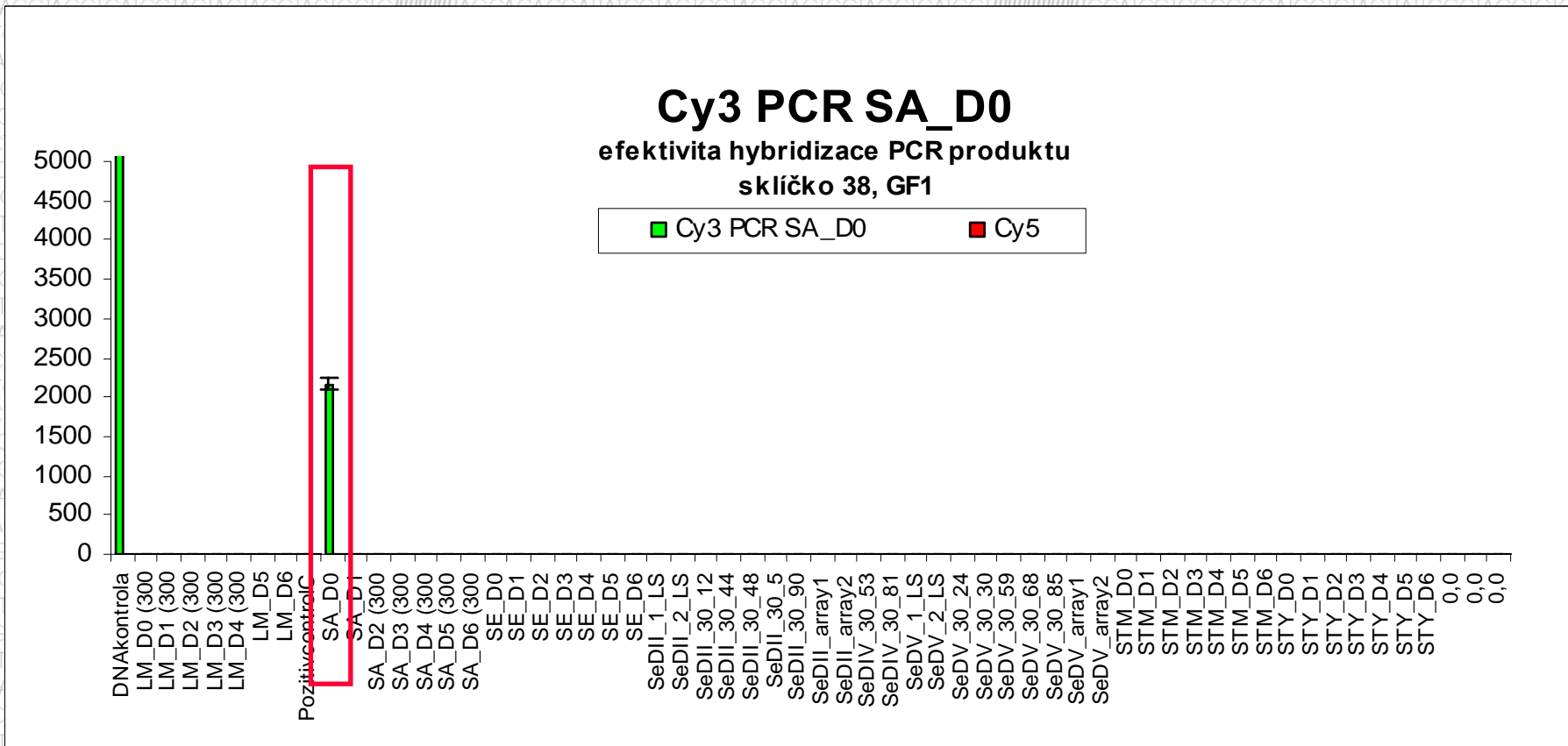
- **In-silico design for selected 5 bacterial species**
(*Salmonella enterica subsp. enterica serovar Typhi Ty2*, *Salmonella typhimurium LT2*, *Staphylococcus epidermidis ATCC 12228*, *Staphylococcus aureus subsp. aureus Mu50*, *Listeria monocytogenes str. 4b*)
- **Use of Cy3 labelled PCR products of „domains“ containing targets for each DNA/LNA probe pair**
- **Positive selection of suitable DNA array probes**
- **Negative selection of LNA probes (cross-hybridizing with array-bound DNA probes)**
- **Positive selection of LNA probes hybridizing to the target**
(„double hybridization“ of Cy3 labelled target PCR with Cy5 labelled LNA probe on the array)
- **„fishing“ experiments (catching of Cy3 labelled PCR product by LNA-magn. nanobeads and detection on the array)**

conditions

- DNA oligos (5`-NH₃ + C18 spacer – GB; LNA – Exiqon)
- Slides from CAG Bremen (non-contact spotting)
- Hybridization 3xSSC/0,1% SDS / 42°C
- ArrayBooster for array hybridization in GeneFrames
- Axon GenePix4000B scanner
- Miltenyi magnetic separator

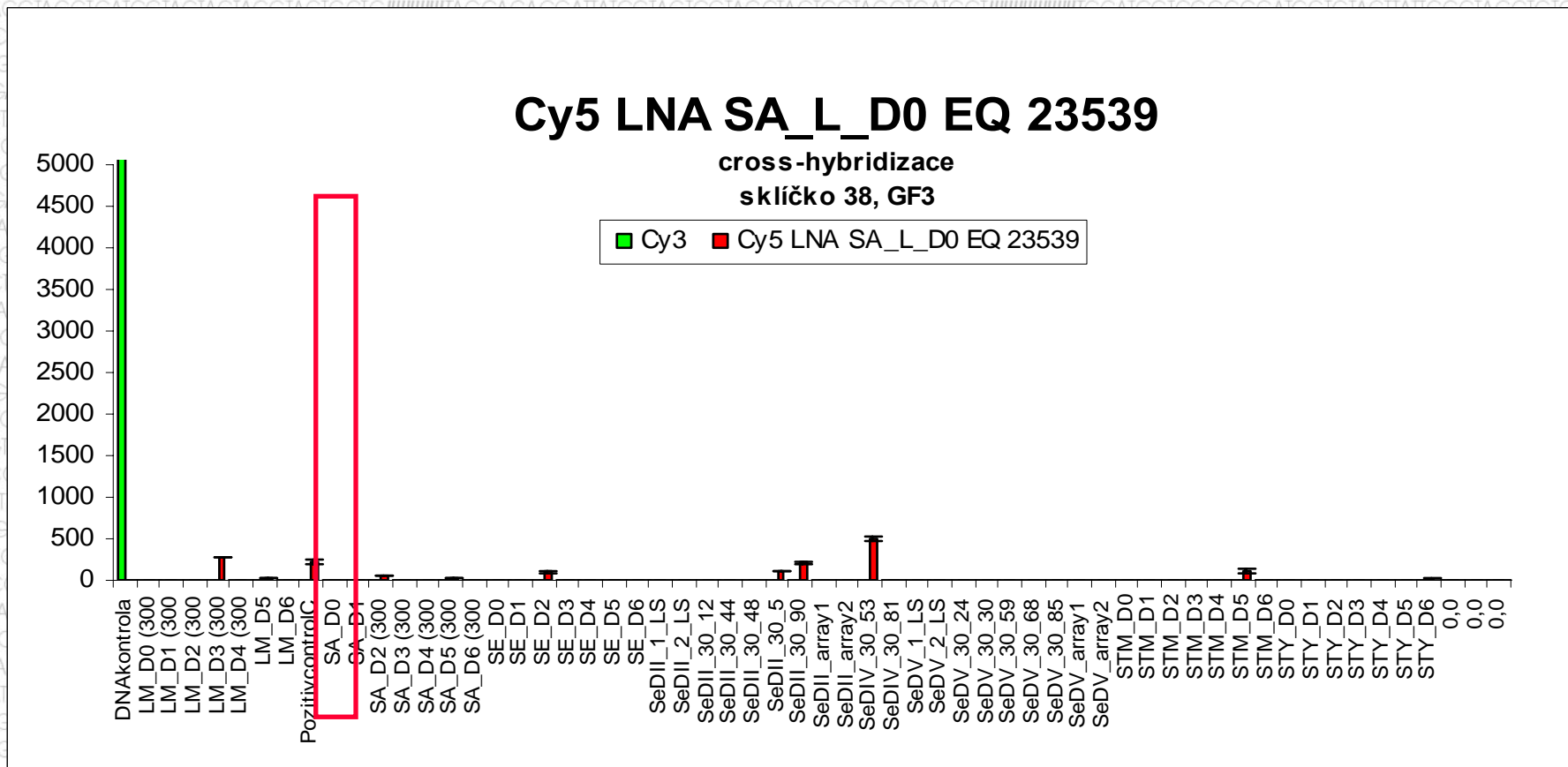
Example SA_D0

DNA probe hybridization



Example SA_D0

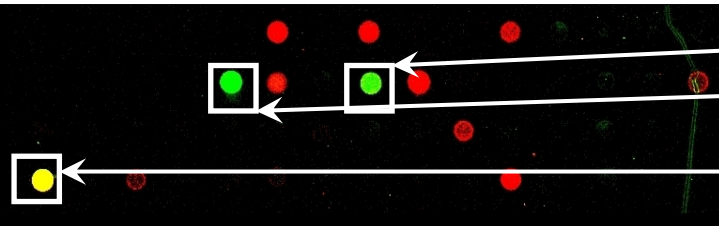
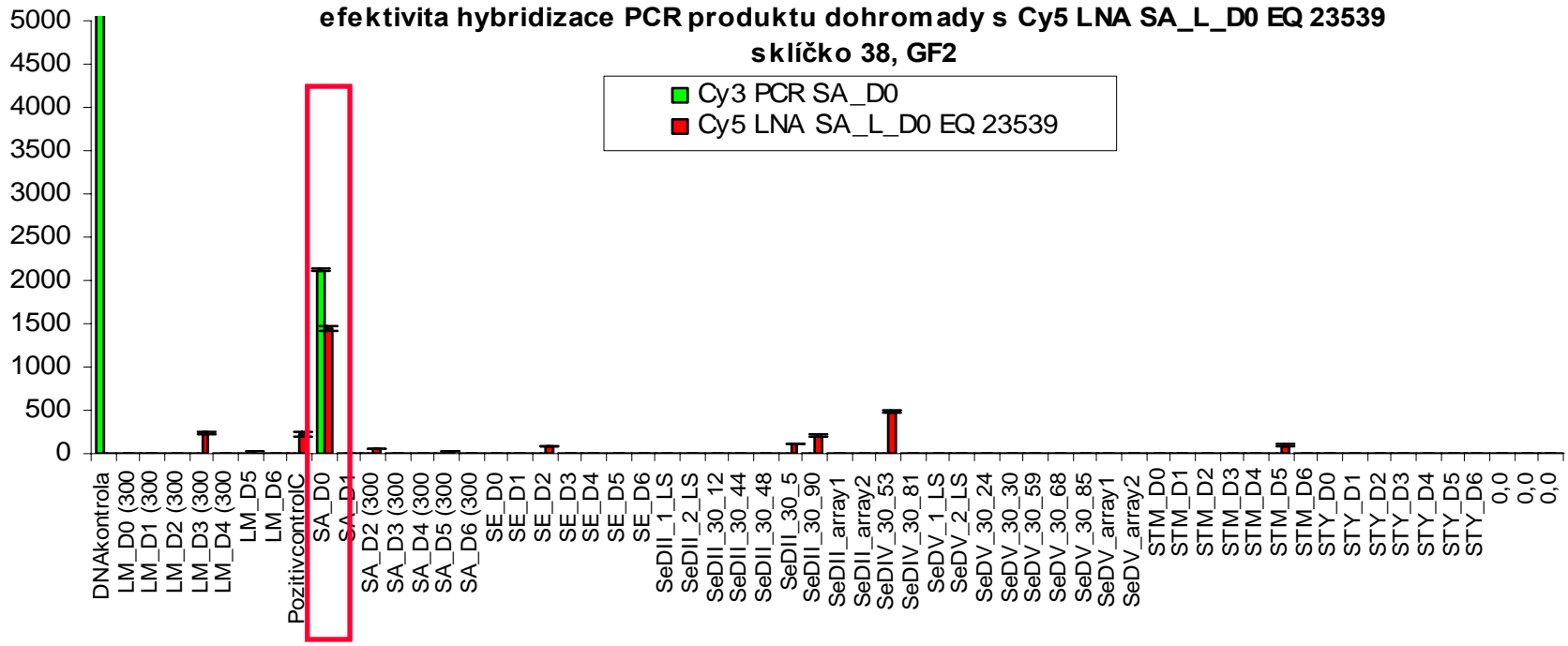
LNA probe cross-hybridization



Example SA_D0 – „double“ hybridization

Cy3 PCR SA_D0

efektivita hybridizace PCR produktu dohromady s Cy5 LNA SA_L_D0 EQ 23539
sklíčko 38, GF2



Positive controls

THE sweet spot!



„fishing“ experiments

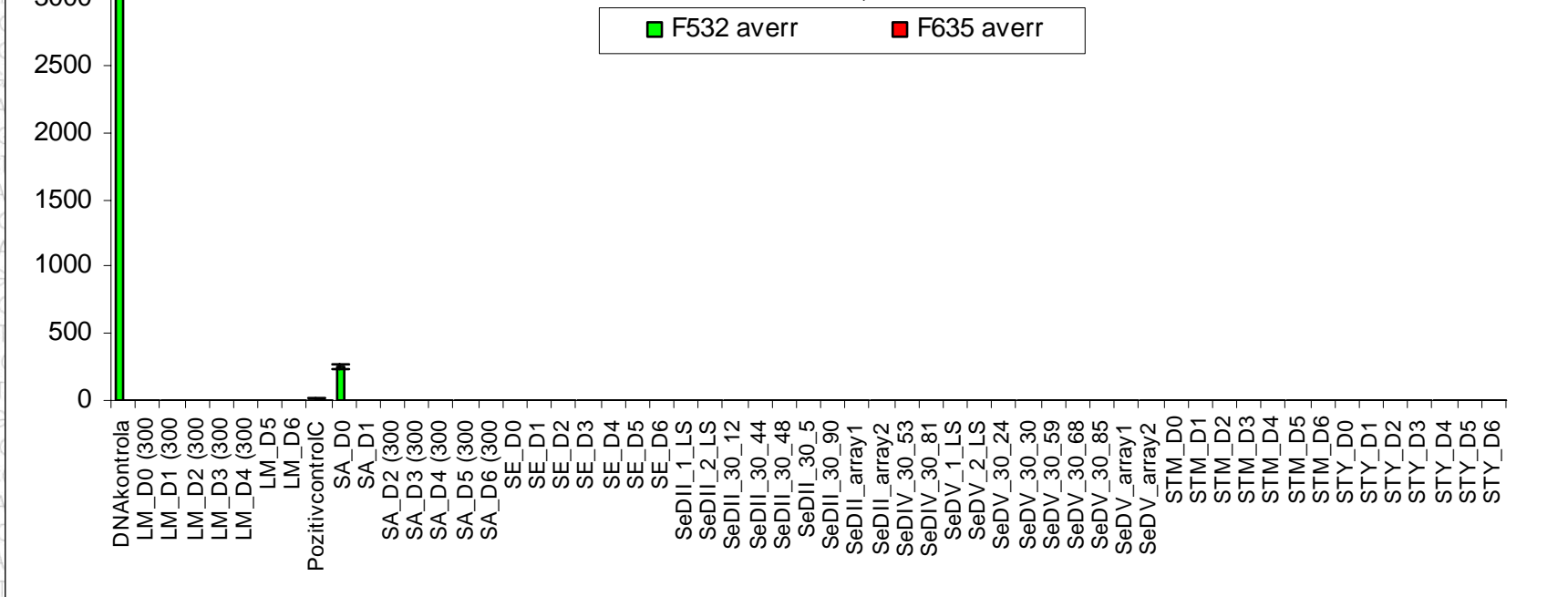
- magn. nanobead with LNA probe + Cy3 labeled specific PCR product + unspecific Cy3 labeled PCR products
- Hybridization (same conditions as for array hybridization)
- Magnetic separation
- Array hybridization
- Detection

Fishing:

1. with magnetic separation
 - Low salt WASH (0,1xSSC)
 - High salt WASH (3xSSC)
2. Without separation at all

domain SA_D0, low salt wash

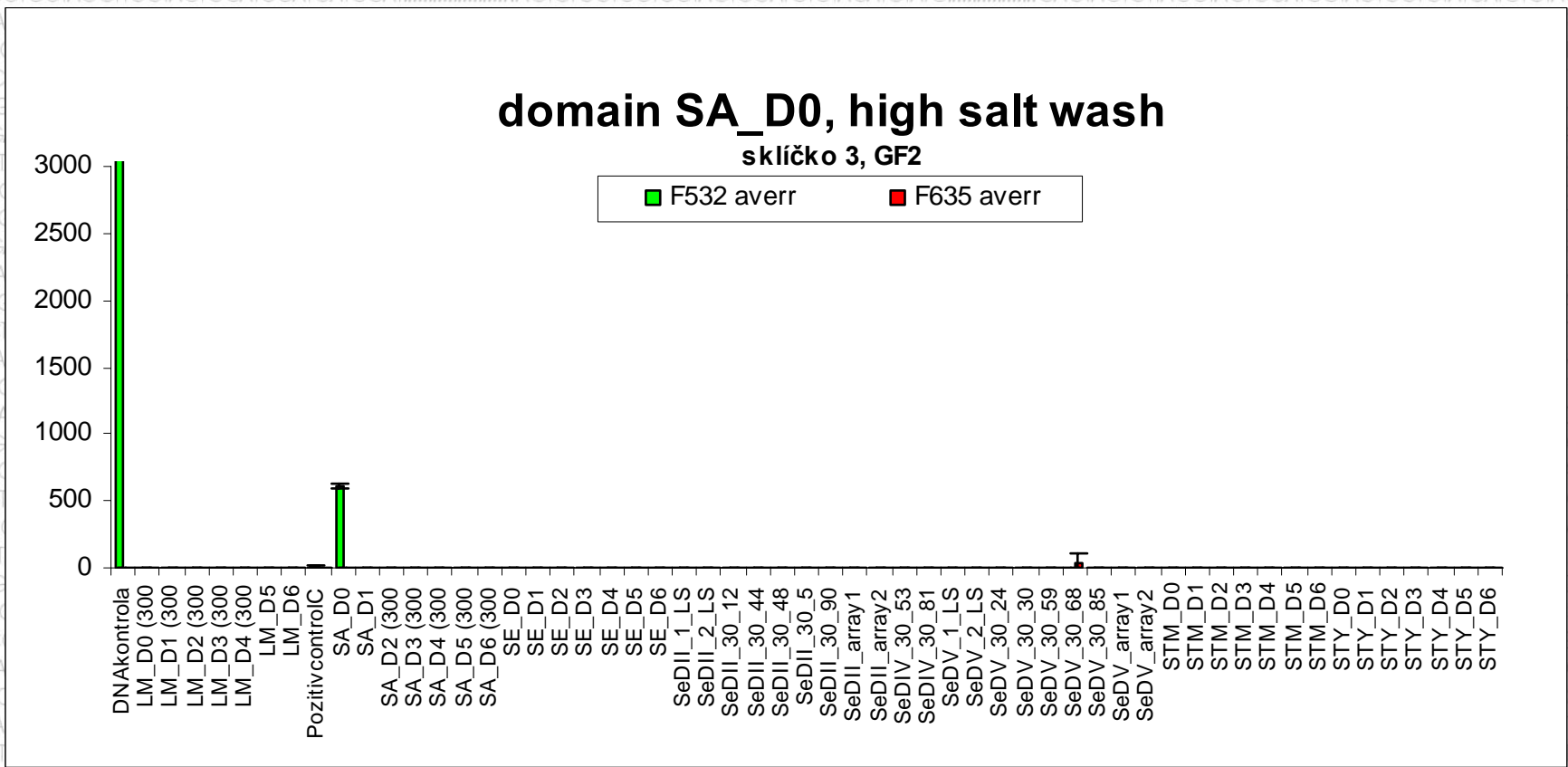
sklíčko 3, GF1



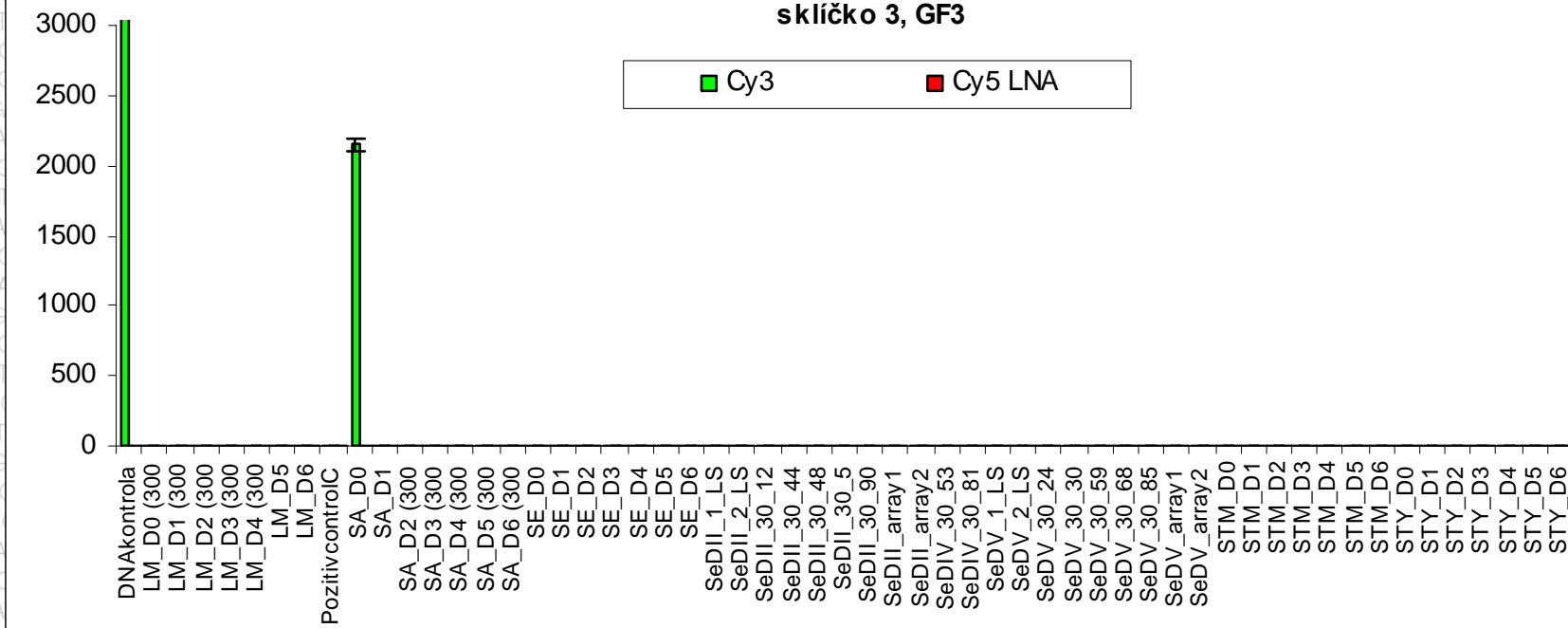
domain SA_D0, high salt wash

sklíčko 3, GF2

■ F532 averr ■ F635 averr



domain SA_D0, without wash sklíčko 3, GF3



Design and optimization „hit rate“

Microorganism	DNA/LNA probes designed	„domains“ cloned	DNA/LNA pairs selected + with the best signal
<i>Staphylococcus epidermidis</i>	16/8* + 6/6	2* + 4	4 + 1
<i>Staphylococcus aureus</i>	6/6	5	4 + 1
<i>Salmonella typhi</i>	6/6	4	2 + 1
<i>Salmonella typhimurium</i>	6/6	6	2 + 1
<i>Listeria monocytogenes</i>	6/6	6	3 + 2

* - exploratory design and optimizations

Conclusion

- in-silico design not optimal
- no significant correlation of any design parameter with functionality yet
- need to test more sequences
- more than twice as difficult as the design for DNA array probes is
- generally the „double hybridization“ concept works fine
- fishing with LNA-modified magn. nanobeads is working but the hybridization conditions need to be improved

Support

Šárka Holasová and Antonín Libra

Genensor-Nanoparts Consortium members

- **prof. D. Blohm, CAG Bremen, Germany**
- **S. Peters, PRI, Netherlands**
- **U. Bohnebeck, CAG/BIBIS Bremen, Germany**
- **F. Meyerjuergens, CAG Bremen, Germany**
- **P.S. Nielsen, Exiqon, Denmark**
- **S. Krauthäuser and I. Johnston, Miltenyi Biotec, Germany**

