



**eurofins**

GeneScan

# New developments in quantitative PCR testing of GMOs

**eurofins international seminar**

*Feb 16 & 17; Paris*

*Andreas Wurz*

## Company Profile:

- Analytical service
- Analytical Kits
- Custom assay development & research



# PCR analysis of food

Sample preparation and  
homogenisation



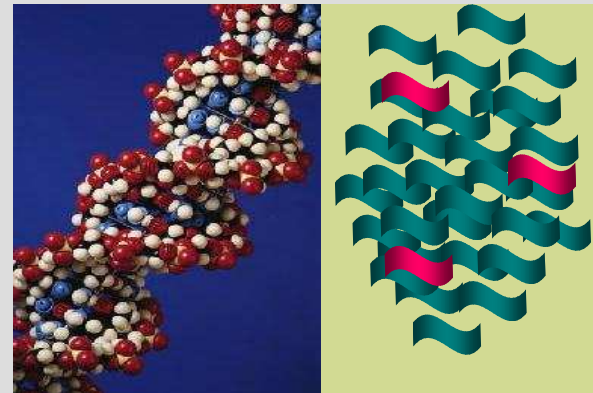
DNA-Extraction



Realtime PCR analysis



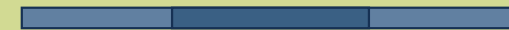
Interpretation of Results



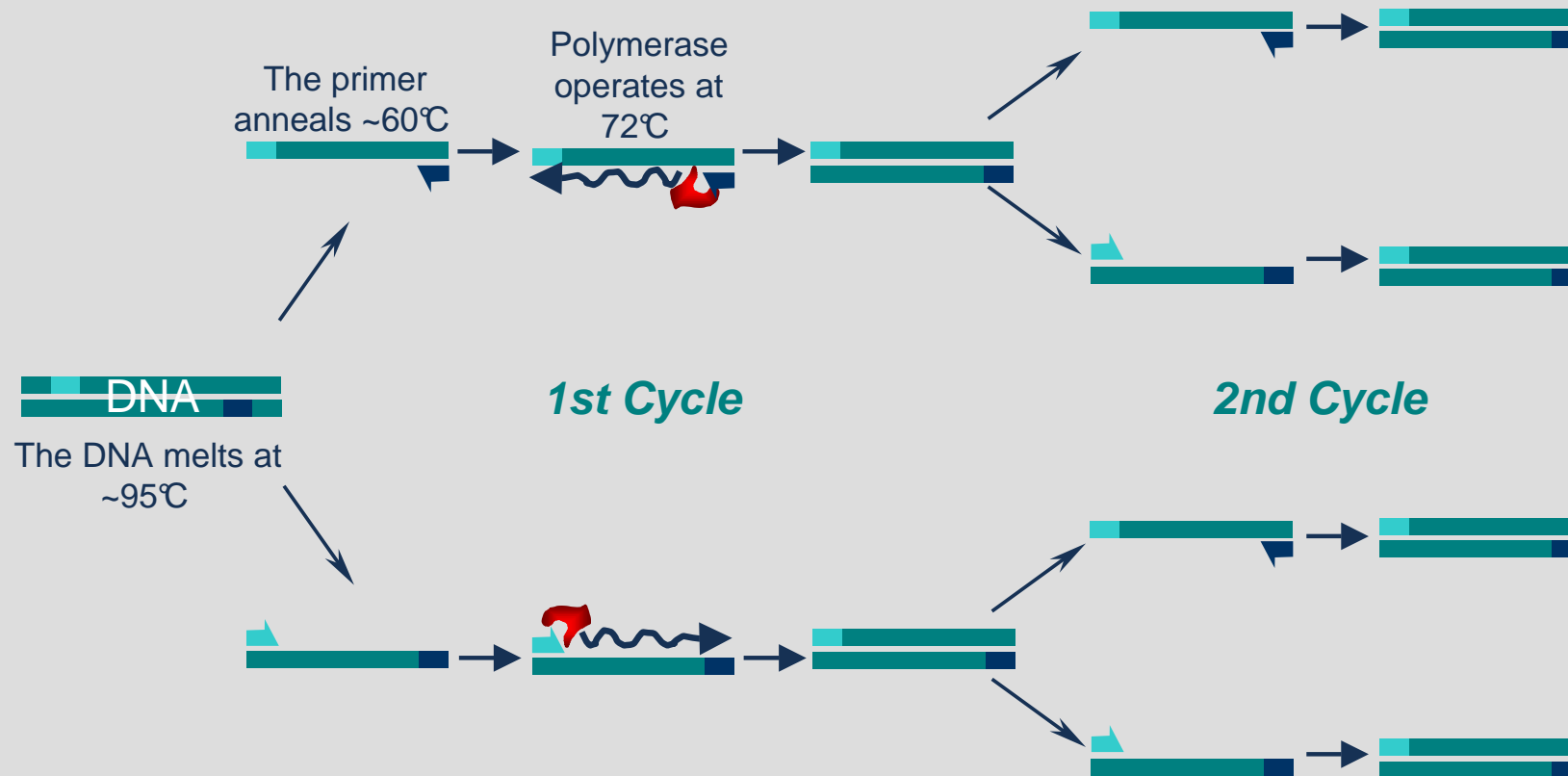
GMO

:

Reference



# PCR- „quick tour“



## Topic

- New options in multiplexing of realtime PCR
- Conclusions and implications for methods and validation

## Recent developments

[Alternative  
RT-principles]

New reagents  
polymerases

Realtime  
PCR

Available dyes/  
labels

Fast detectors/  
multichannel  
machines

Multiplexing  
capabilities

# Lots of Dyes and Filters – Spoilt for Choice ?



## More options, new expectations

### **New FAST PCR detectors for Realtime PCR**

→ higher throughput ? faster results ?

### **New , faster PCR Reagents**

→ higher throughput ? faster results ?

### **384 well PCR blocks**

→ Higher throughput at lower cost ?

### **More detection Channels**

→ Higher multiplexing capabilities ? Better internal controls ? Higher throughput? Need for spectral compensation algorithms? Need for internal calibration?

## But the reality still is:

### Simplex FAM/Tamra based simplex assays

- FAM/Tamra assays can run on all available detectors
- FAM/Tamra based assays: A wealth of experience does exist



- Only limited experience in official inter-laboratory trials for GMO testing using duplexed assays (Hird et al. 2003)

## When it comes back to food testing ...

### The challenge is ...

- Wide range of GMO contents (%), matrices and products under study
- Different DNA extraction methods per matrix
- Different DNA contents per matrix or batches
  - Variability in impurities e.g. inhibitory substances
  - Variability in DNA concentrations in PCR

### Food routine testing needs ...

- Suitable operating range
- Reliability and **robustness** of the assay
- Standardisation of methods

## Major technical requirements

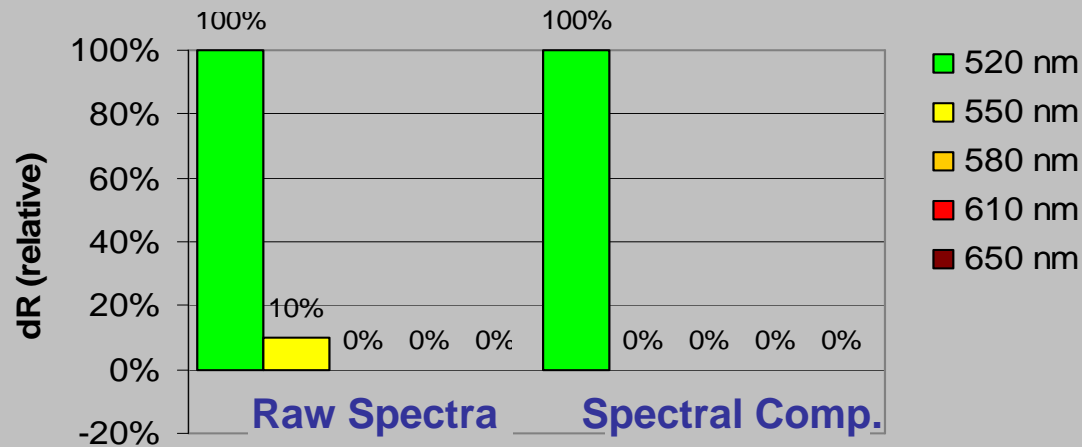
Log linear calibration function for the transgene target at varying contents of the reference target and with a broad range of samples requires:

- **No cross-talk** between dye channels (or if so appropriate robust/reliable compensation algorithm) and robustness against sample derived substances interfering with fluorescence detection
- Quantitative: **No measurable competitive effect** on PCR level in the dynamic range required (e.g. down to 0,1% GMO)
- Qualitative: **No measurable suppression** of positive amplification down to the LOD required (e.g. 0,01%)
- **Robustness** against matrix effects and unintentional fluctuations in assay parameters and component qualities

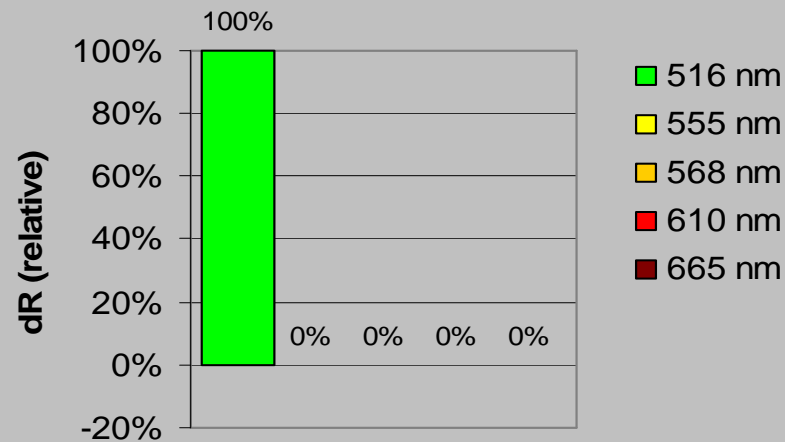
# (1) Cross Talk /channels

## Fam (525 nm)

I



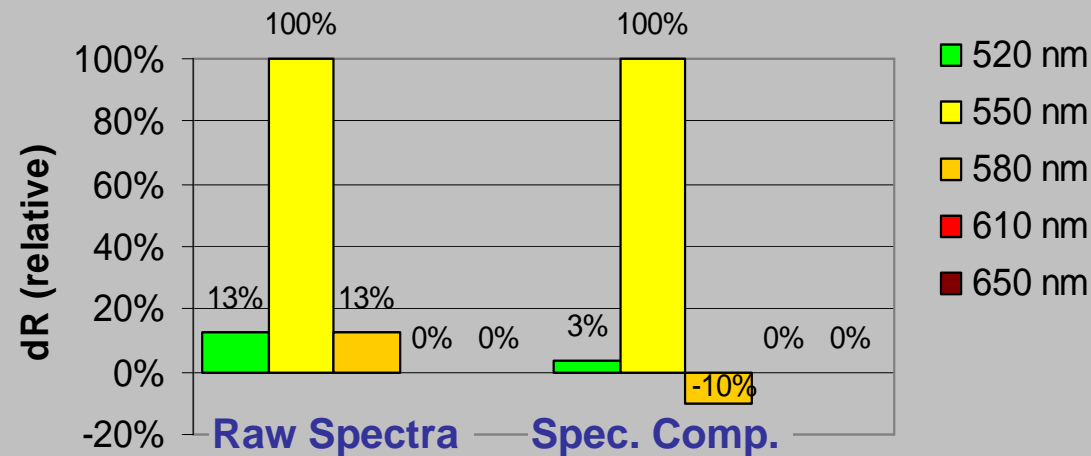
II



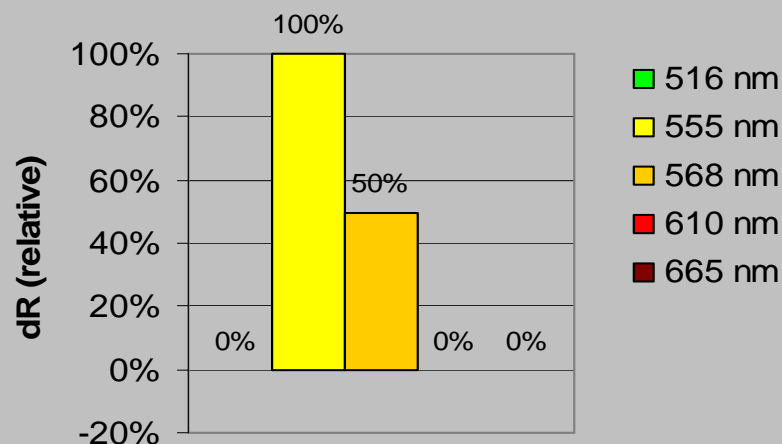
# (1) Cross Talk

## Yakima Yellow (548 nm)

I



II

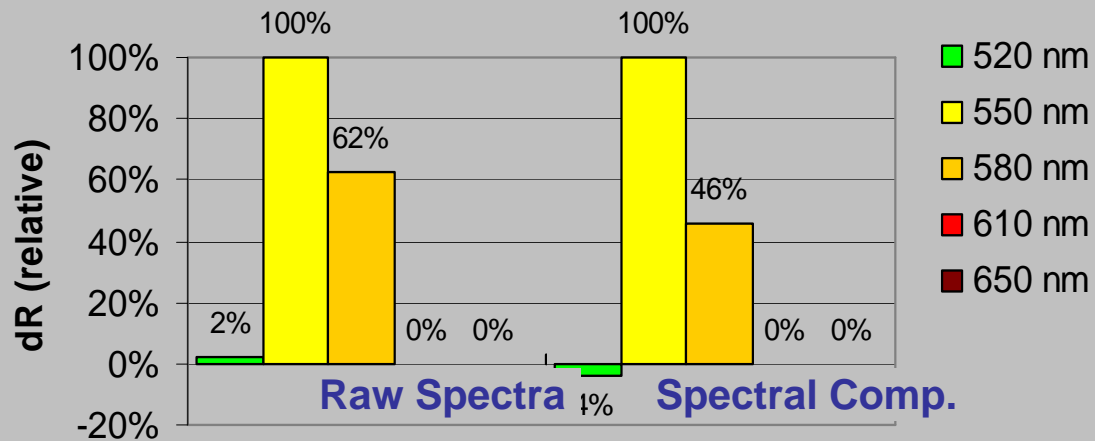


Which channels can be used simultaneously ?  
Spectral compensation ?

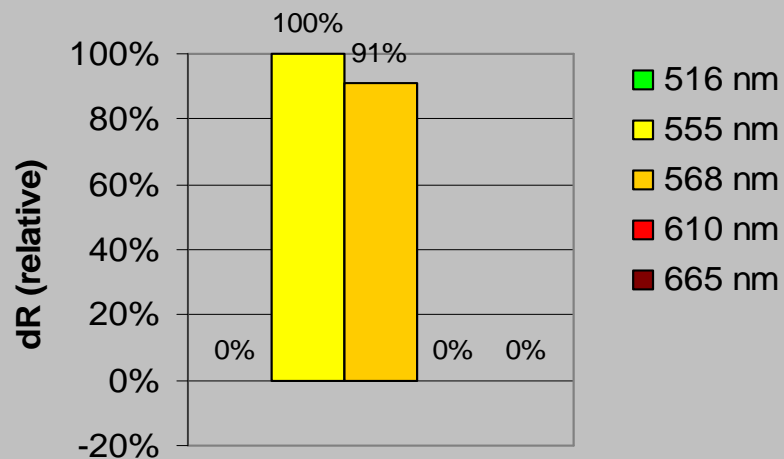
# (1) Cross Talk

I

## Hex (556 nm)



II

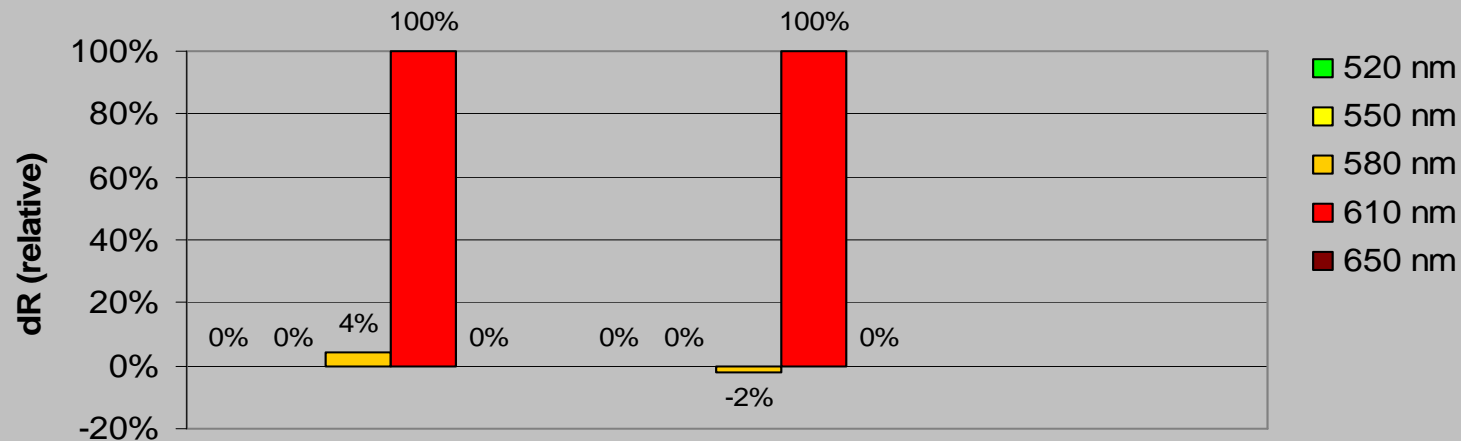


Adjacent channels can't be used with this dye

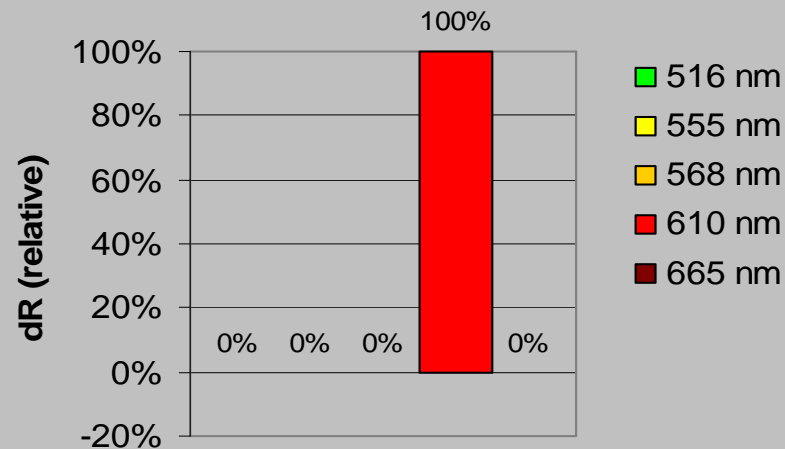
# (1) Cross Talk

## Texas Red (603 nm)

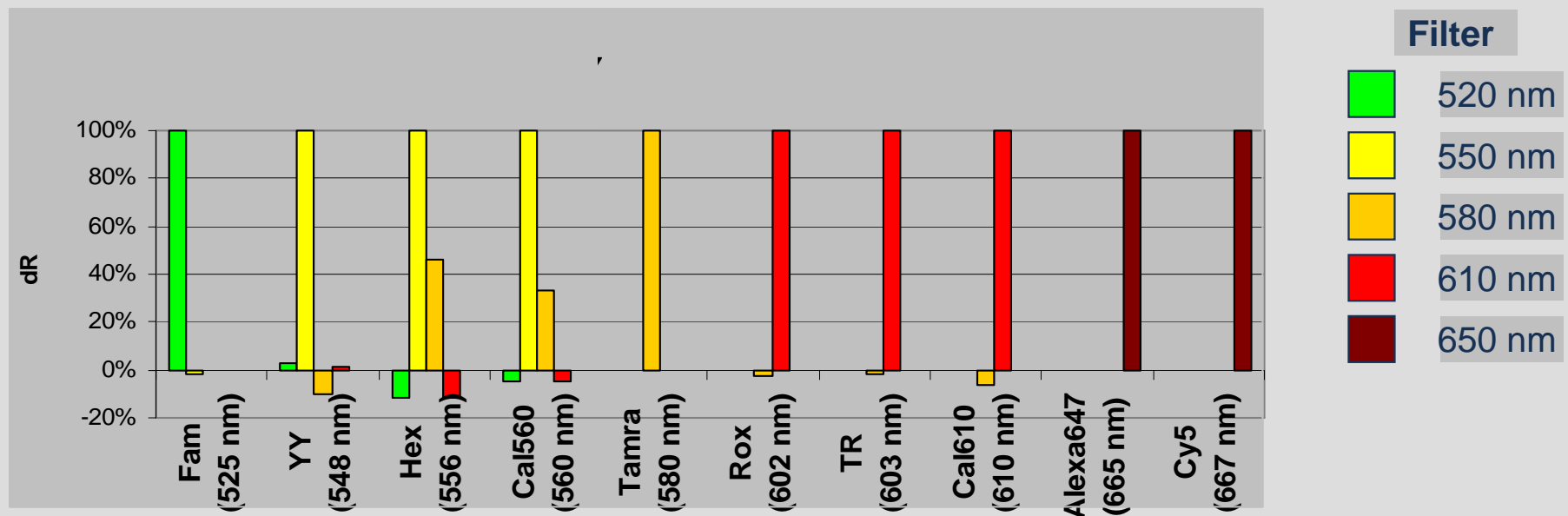
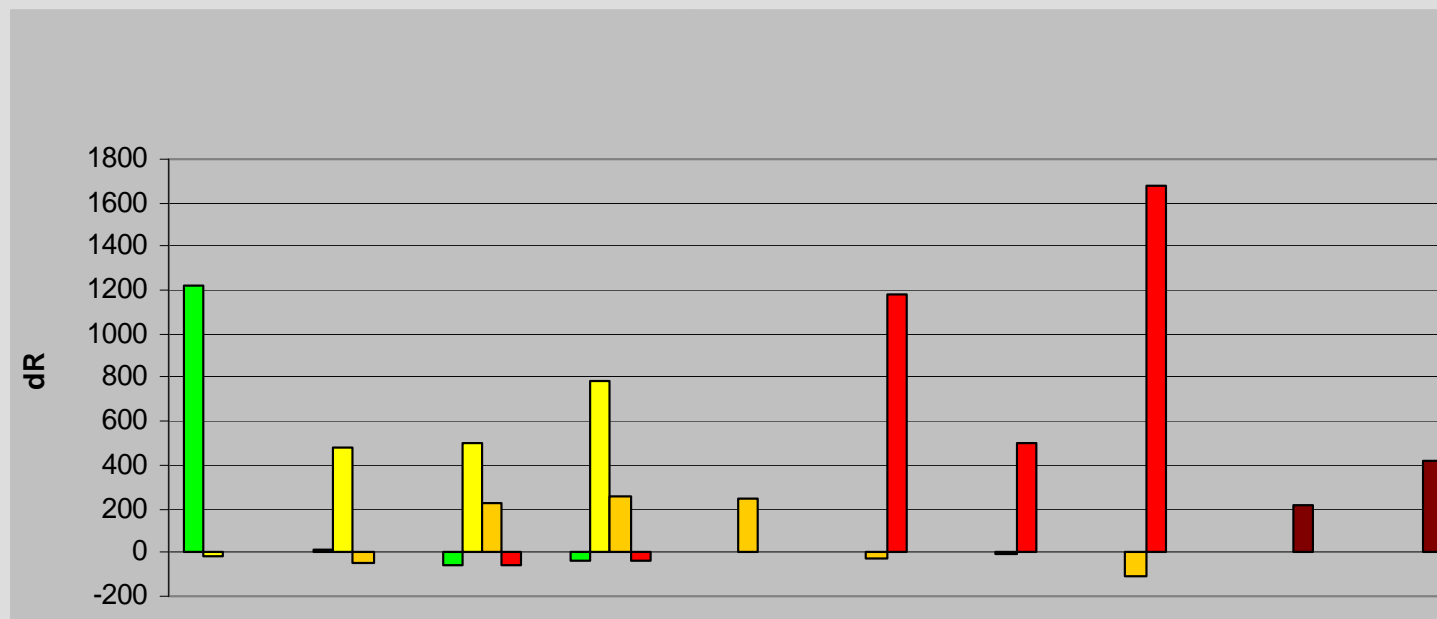
I



II



# Dye comparison



## (1) Important for making a dye choice is

- Dyes that have good „efficiency“ - high  $\Delta R$
- Optimise match of dyes with the filters of the detector (excitation filter and emission filter) to avoid cross-talk
- Availability of appropriate filter sets
- Availability of high quality label/probe synthesis
- Dark quenchers (instead of TAMRA)

### **But:**

The ideal dye combination is platform dependent

The ideal dye combination is dependent on multiplexing degree (2, 3, 4, or even morefold?) and if ROX shall be used as internal reference for normalisation

## Technical challenges for assay development:

### Example: Triplex system detecting

- GMO (e.g. RRS transgene)
- Reference target (e.g. lectin le1 gene)
- IPC (heterologous artificial internal positive control)

### Example: Challenging sample

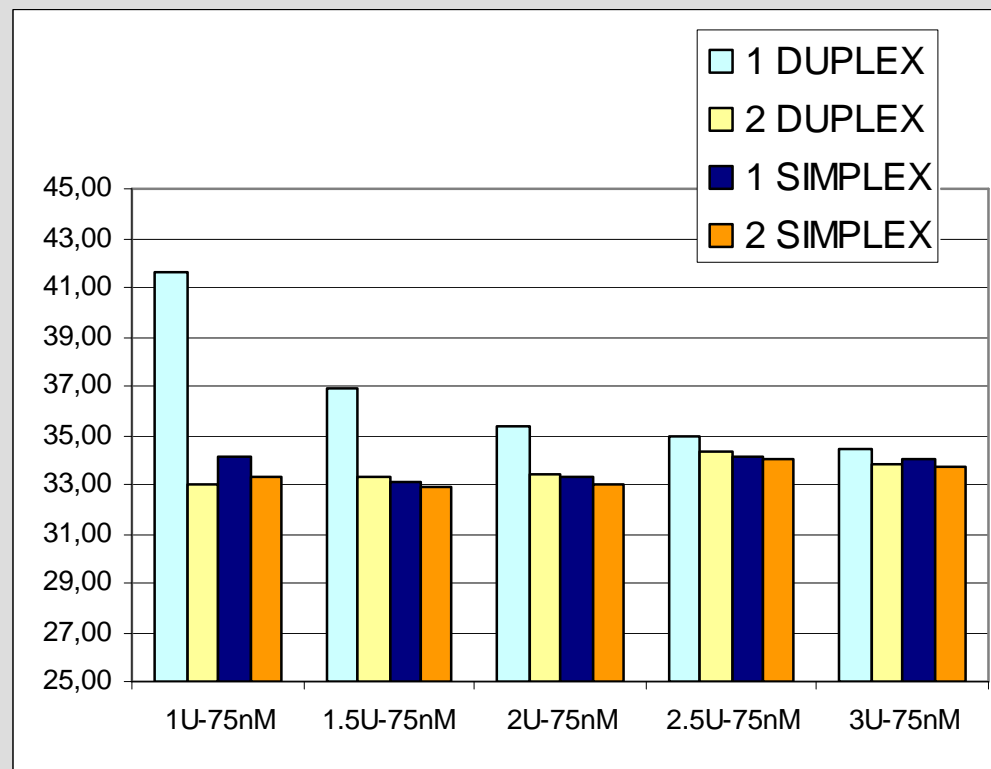
|              | High DNA<br>10% GMO | <b>High DNA<br/>0,1% GMO</b> | Low DNA<br>100% GMO | Low DNA<br>1% GMO |
|--------------|---------------------|------------------------------|---------------------|-------------------|
| GMO Targets  | 100 00              | <b>100</b>                   | 100                 | 1                 |
| Ref. Targets | 100 000             | <b>100 000</b>               | 100                 | 100               |
| IPC Targets  | 50                  | <b>50</b>                    | 50                  | 50                |

## (2) Reaction Conditions

Reference target amplification shall not interfere with GMO target amplification: Optimisation of reaction conditions.

*Amplification of the GMO target at 100 copies at high ("1" - 0,1% GMO) and low ("2" - 100% GMO) excess reference target concentrations:*

**Competitive effects** in dependence of polymerase concentration



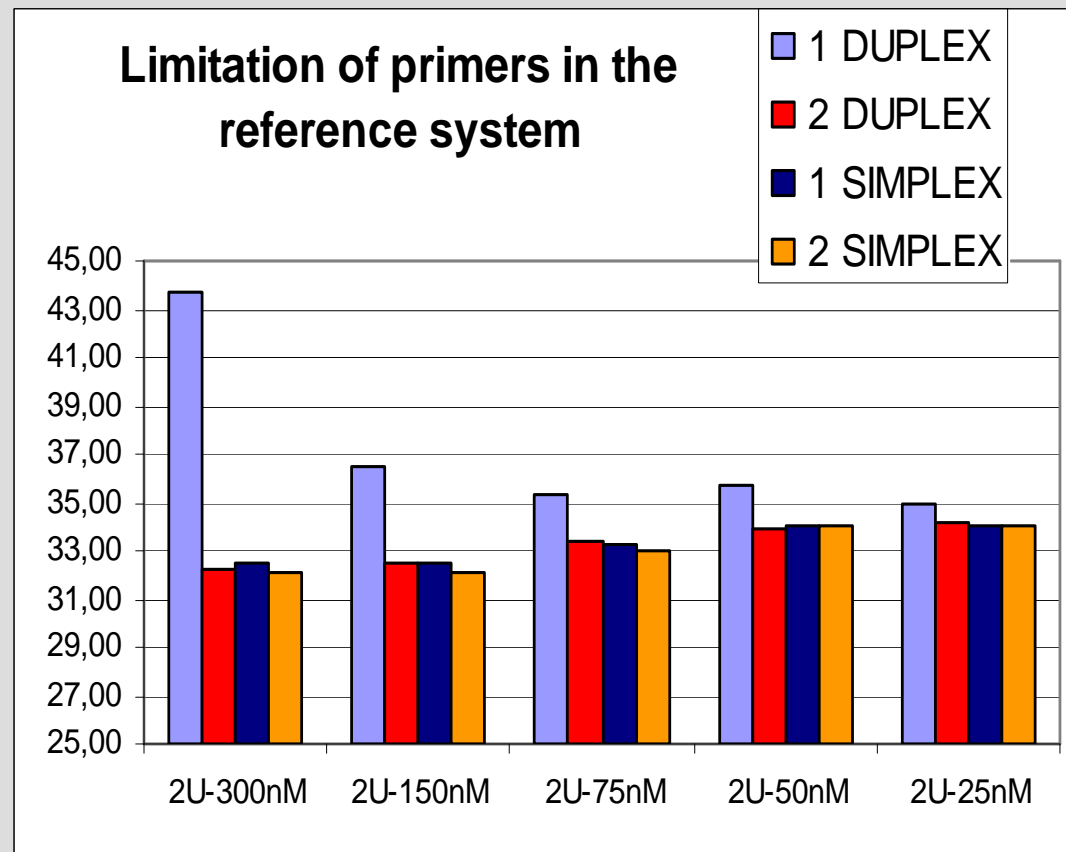
Optimising  
polymerase  
concentration

## (2) Reaction Conditions

Optimisation of reaction conditions: Effect on transgene detection.

DNA "1": 100 / 100 000 (GMO:REF)

DNA "2": 100 / 100 (GMO:REF)



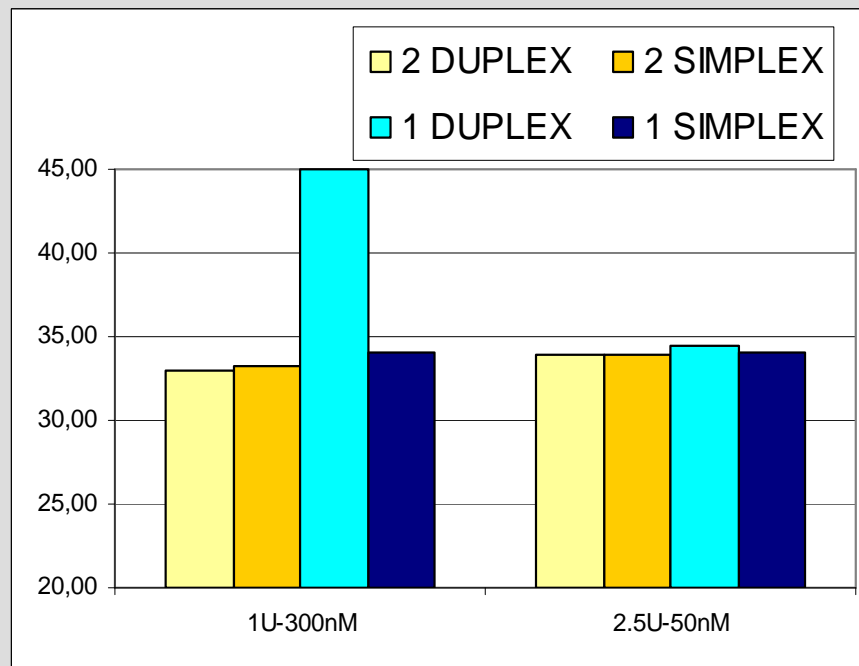
Need of primer limitation

## (2) Reaction Conditions

Reference target amplification shall not interfere with GMO target amplification: Optimisation of reaction conditions.

*DNA "1": 100 / 100000 (GMO:REF)*

*DNA "2": 100 / 100 (GMO:REF)*



before

optimised

### (3) Further factors to be assessed

Which other reaction parameters can improve „**non-suppression**“ of GMO target amplification ?

- Reaction conditions (polymerase, magnesium, additives, buffer, dNTP's)
- Cycling conditions (cycling profile)
- Dye combination
- Reaction volume

What are the optimum IPC conditions to ensure sensitive control of inhibition at no influence of measurement ?

- Do partial inhibitory effects influence the quantification result ?
- What IPC conditions need to be established to reliably reveal such inhibitory effects
- Optimal balance between stability and “non-interference” with detection

→ Work as part of the “Co-Extra” EU-project

## Summary

Experience so far shows that **quantitative** multiplexed assays need very careful optimisation to ensure that high copy number reference target values do not impact the GMO specific target detection.

**Quality control of components** will be crucial and more challenging as with simplex assays to ensure stable performance of any such assay. Suitable quality control measures and controls need to be established.

Assay performance will be very dependent on component quality and detector type: This means that **standardisation** of assays might have to include detector and certain critical reagent supplies

Validation of multiplexed will have to emphasize on assessment of sensitivity/ **competitive effects**: Does a high reference target copy number suppress the amplification of transgene targets within the operating range defined?

# Conclusions

## Multiplexing offers attractive options as e.g. ...

- Higher throughput
- Improved internal controls
- Potential multiplexing of up to 5 or more-fold will be key for the future increasing number of events to be detected cost-effectively

## However ...

- It calls for more thorough in-house validation
- It makes standardisation of assays difficult
- Requires careful design, optimisation and tight quality control for PCR reagents for each single application

**Thank you !**

Andreas Wurz  
GeneScan Analytics, Freiburg