New multiplexing tools for reliable analysis of GMOs

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Multiplex tools: introduction

- Use of GMOs is in constant progress
  - Increasing presence of GMOs in the market
    - Global culture hectarage, # countries planting GMOs, use of stacked traits
  - Increasing number of authorized GMO events
    - Higher diversity of species, traits and introduced regulatory elements

- The analytical technologies must evolve towards higher throughput, lower cost GMO diagnostics
  - combination of 2 or more assays in one single reaction: Multiplex tools
**Multiplex PCR tools: introduction**

**MULTIPLEX PCR**: combination various PCR assays in one reaction tube

NOT an easy strategy:

- Interaction and competition between the reaction components and products
- The combination of high numbers of reactions is at the expense of sensitivity and uniform amplification of targets

**OLIGOPLEX PCR**: limited # targets

Oligoplex PCR-AGE

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Detection of Bt11 (468 bp); MON810 (280 bp); T25 (177 bp) and GA21 (72 bp) maize GMOs


Real-time PCR

Detection of P35S and GA21 maize

**Multiplex PCR tools: introduction**

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Above a certain degree of multiplexing, novel strategies are required for identification of the amplification products
Multiplex PCR-CGE-SC

**PRINCIPLE**

- Short and similarly sized amplicons
- High and similar amplification efficiency for all targets
- Resolution by size and color in a CGE format

Sample DNA → PCR

Forward specific primers: (HEX), (FAM) or (TET)
Reverse specific primers (not labelled)

Labelled amplicons (one single step) → CGE-SC

Resolution by size and color in a CGE format
Multiplex PCR-CGE-SC

5-plex: 4 event specific  
1 plant species (maize)

6-plex: 5 event specific  
1 plant species (cotton)

- Based on (validated) real-time PCR assays targeting flanking regions
- Limit of detection 0.1% GMO / around 20 copies
- Specific
- Transferable to a second laboratory (validation of 5plex in progress: JRC)
- Used in parallel to routine analyses (food and feed samples)

Multiplex PCR-CGE-SC

9-plex:
- 2 construct
- 6 event specific
- 1 plant species (maize)

To attain more similar amplification efficiencies, a universal 5’sequence is added in all primers

Method subjected to several modifications to make it:
- Semi-quantitative (above / below a limit): limitator technology
- Quantitative

Multiplex PCR-CGE (quantitative approaches)

PRINCIPLE

sample DNA

1st PCR bipartite primers

Ligation

Universal primer

PCR

Labeled universal primer

Labeling

CGE-SC

CGE

CGE

MATFORSK

Co-Extra International Conference 
2-5 June 2009 Paris
With an adequate design, the products of various oligoplex-PCRs can be simultaneously resolved in one single CGE (e.g. 11 targets)

5-plex reaction  6-plex reaction

2 oligoplex PCR reactions in 1 PCR run

1 CGE run
Multiplex PCR-microarray

- The products of various oligoplex-PCRs can be simultaneously identified in one single microarray

PRINCIPLE

Amplification
via 3 different multiplex PCRs with biotinylated primers

Hybridization
of the 3 PCR reactions on 1 DualChip GMO microarray

Detection
with Silverquant® colorimetric detection
Multiplex PCR-microarray

- Validation coordinated by the EU CRL - JRC, in the framework of Co-Extra:
- The method can be considered as fit for purposes of screening
- Detection of different elements at 0.1% concentration of GM and 1% plant with a 95% accuracy rate

Microarray Method for the Screening of EU Approved GMOs by Identification of their Genetic Elements

http://bgmo.jrc.ec.europa.eu/home/docs.htm
Multiplex PCR-microarray

DualChip GMO V2.0: 31 targets in 1 chip

- 12 screening
- 11 event specific
- 7 plant species
- 1 donor organism
- process controls

Leimanis et al. 2006 Plant Molecular Biology 61(1-2):123-139
Hamels et al. 2009 Eur Food Res Technol 228:531-541
NAIMA-microarray

NASba Integrated Multiplex Amplification (NAIMA)

Two triplexes:
- 2 screening
- 1 plant species (maize)
- 1 screening
- 1 event specific
- 1 plant species (maize)

- detection and quantification 0.1% to 100% GMO
- food and feed samples

6-plex under evaluation:
- 4 screening
- 1 event specific
- 1 plant species (maize)

Morisset et al. 2008 Food Res Techn 227:1287-97
Morisset et al. 2008 Nucl Acids Res 36(18):e118
NAIMA-microarray

**PRINCIPLE**

1. **Sample DNA**
2. **NAIMA**
3. **Tailed and Universal Primers**
4. **SS RNA Products**
5. **Labeling**
   - **Dendrimers (15 Oyster Dyes/Molecule)**
   - **Signal Amplification (= Sensitive)**
6. **Array**
7. **Detection and Quantification**

**PRINCIPLE**

1. **Poly (A) Tailing**
2. **Ligation**
3. **Array Hybridization**

**Co-Extra International Conference**
**2-5 June 2009 Paris**
High level of multiplex

Adaptation of high-grade multiplex approaches to GMO analysis

**PRINCIPLE**

**ligation** (after hybridization to transgene target)

**PCR** (universal primers)

**detection**
High level of multiplex: SNPlex

Adaptation of SNPlexing detection method to GMO analysis

1. Oligonucleotide Ligation assay (OLA) reaction
   - PCR amplification with universal primers
   - Oligonucleotide Ligation assay (OLA) reaction
     - universal primer
     - adapter for hybridization
     - GMO sequence
     - universal primer

2. PCR amplification with universal primers
   - Biotin

3. Decoding (Specific ZipChute hybridization)
   - ZipChute probe
   - C
   - B

4. ZipChute ‘reading’ by CE instrument and Gene mapper program
High level of multiplex: SNPlex

- 2 panels with 47 and 48 targets in one sample
- Specific detection of up to 46 GMO targets in one sample
  - 13-18 screening
  - 10-6 construct
  - 13-9 event specific
  - 8-12 plant species
- Adequate LOD
- Transferability to other labs not easy (robots needed)

High level of multiplex: Padlock probe ligation-microarray

How does it work?

- **Padlock probes (PLP)**
- **Genomic DNA (gDNA)**

**Hybridisation, ligation**
- **A**, match
- **B**, match
- **C**, match
- **No match**

**Specific PLP-gDNA interactions**
- **Exponential amplification in PCR**
  - \(10^9\) x
- **No amplification in PCR**

**Universal microarray**
- **Non-ligated probes** (d, e, f)
  - ‘negative’ gDNA

High level of multiplex: 22 probes designed so far

- 9 screening elements
- 2 construct
- 5 event specific
- 6 plant species

(maximum level so far: 20 probes with 13 targets. OK)

Detection down to 0.1% GM (~100 copies)

Successful transfer to a second lab (NIB)

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HMG<sup>EN</sup> + + + + + + +
Bar<sup>EL</sup> + + + - - -
P-CaMV 35S<sup>EL</sup> + + + + - -
Cry1Ab<sup>EL</sup> + + + + - -
Cry1F<sup>EL</sup> + + + + - -
Cry3Bb<sup>EL</sup> + + + + - +/-
Pat<sup>EL</sup> + + + + - -
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Transfer results
Multiplex tools

- In general, multiplex tools are foreseen as qualitative, screening approach.
- Multiplex PCR assays can target:
  - Transgenic elements, specific constructs or event specific flanking sequences
  - Plant species specific genes
  - Donor organisms (of transgenic elements, e.g. CaMV)
- If needed, subsequent quantization of the positive targets

Sample DNA

Analyze with the appropriate multiplex tools

Quantify positive targets with validated, real-time PCR assays (1plex)
Multiplex methods do have a high diagnostic potential; which has not yet been fully exploited in the GMO field (Co-Extra is about to finish...)

Coupling a high multiplexing level with cost-effectiveness and simplicity is not evident: challenge

Transferability and validation of multiplex assays: still work to be done

Quantitative aspects
Thank-you!