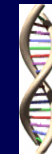


High-Throughput method for the detection of authorized and unauthorized GMO: From Multiplexing to SNPlexing

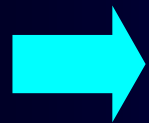
Maher Chaouachi
PhD (to be defend in march 2008)



Paris – France
Feb. 20-22 2008

EUROPEAN BACKGROUND

- Reluctant opinions
- Discrepancy with consumers' attitudes
- Overall request for reliable labeling



A panoply of EU regulations for keeping freedom of choice to consumers

EUROPEAN BACKGROUND

- **Mandatory labeling above a threshold of fortuitous presence of:**
 - ✓ **0.9% for approved GMOs**
 - ✓ **0.5% for unapproved GMOs but with positive safety assessment (temporary)**
- **Obligation (01/18 and 1829/03) made to notifiers to provide sampling plans and quantitative identification method.**
- **Forecasted increase of GMO pressure in EU**
 - ✓ **Growing number and acreages of GMO for food and feed**
 - ✓ **Non-food and non-feed-GMO not having to enter the food supply chains**

WHY DETECTING GMO?

**1- Give freedom of choice
to consumers**



**2- Ability to comply with new EU
regulations**



**3- For Co-existence and Traceability
of GM and non-GM in supply chains**

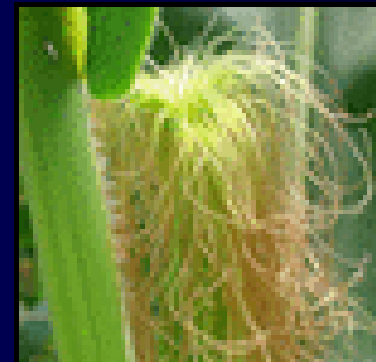


DIAGRAM FOR GMO DETECTION IN ROUTINE ANALYSIS



GMO screening

Negative

GMO detection

Positive

Authorized?

No

Yes

Illegal or tolerant 0.5%

Assay individual ingredients

GMO quantification

No need for labeling

Less than 0.9%

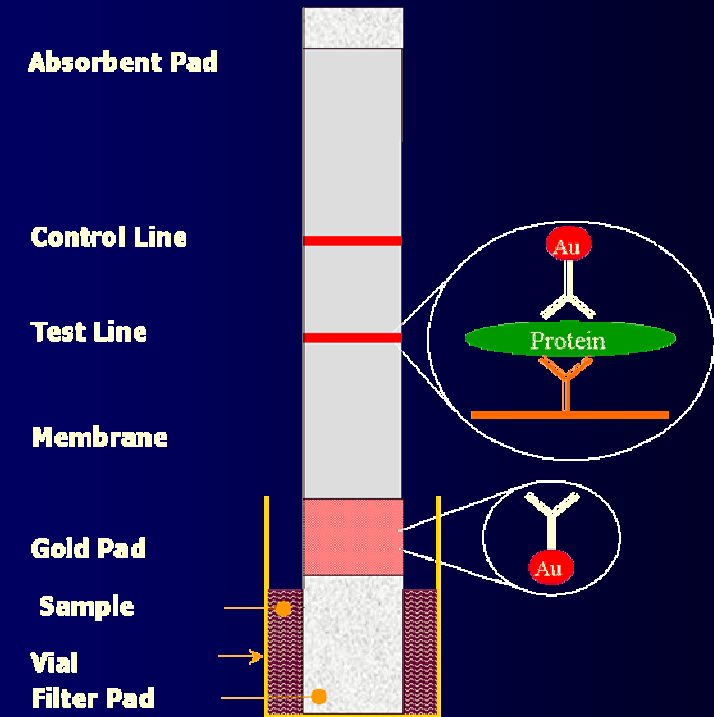
Labeling required

More than 0.9%

GMO identification

Most common detection methods already available

- Bioassay tests
- Protein based (ELISA, Strip tests..)
- DNA based



PCR and real time PCR

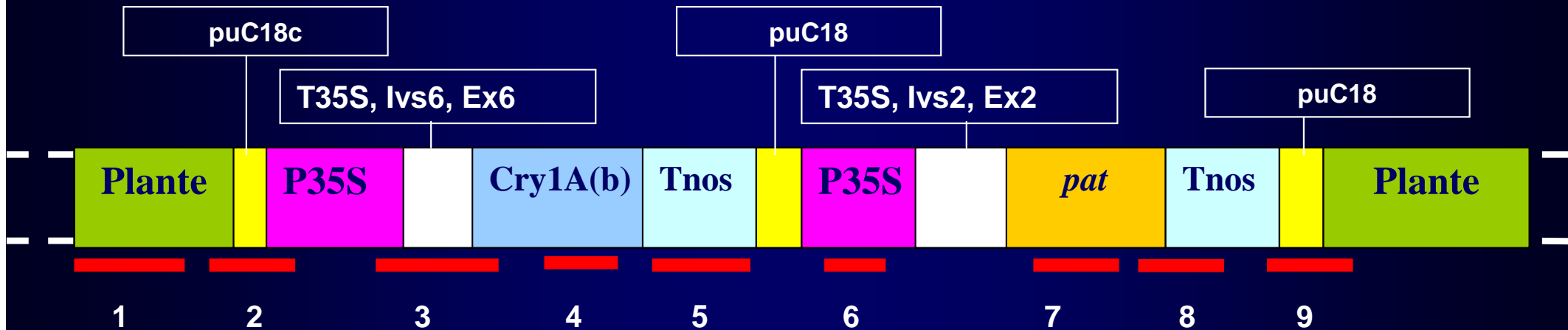


Multiplex GMO detection

- Increase of GMO events
- Alternative to reduce time, cost and analysis

DETECTION OF MANY TARGETS IN ONE TUBE USING ONE SAMPLE

EXAMPLE: event Bt11



Multiplex based methods

- Qualitative PCR detection (nineplex,...)

(Onishi et al., 2005)

- Quantitative RT-PCR (only duplex..)

(Väitilingom et al., 1999; Alary et al., 2002; Terry et al., 2002, Holst-jensen et al. 2006)

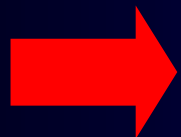
- Alternative methods

- o DNA chips

(Rudi et al, 2003; Peano et al., 2005 ; Leimanis et al., 2006 ; Xu et al., 2007)

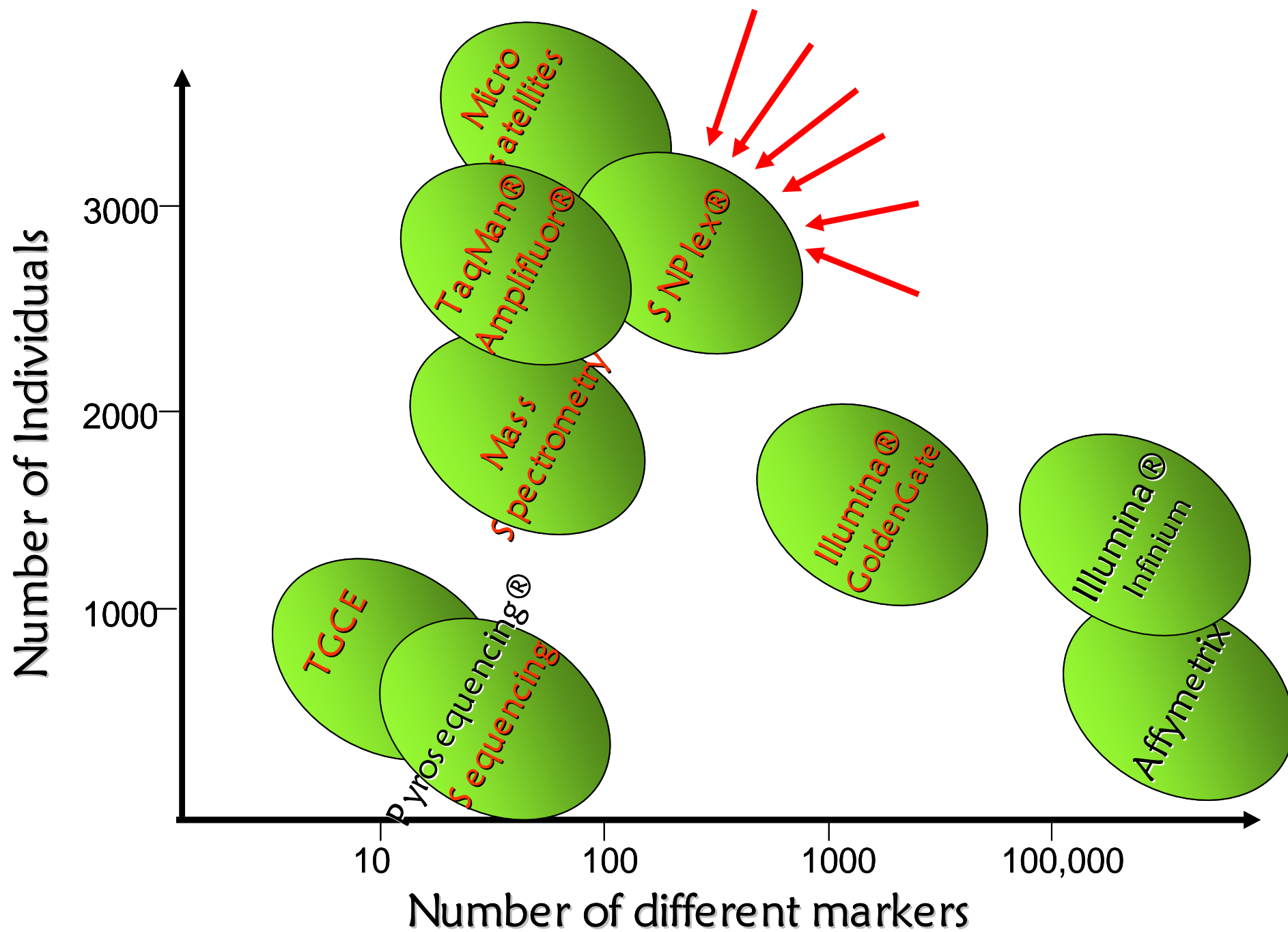
- o Biosensors, Genesensors

(Manelli et al., 2003; Carpini et al., 2004; Meric et al., 2004)



**NEW HIGH-THROUGHPUT DETECTION TECHNOLOGIES
ARE STRONGLY NEEDED**

HIGH-THROUGHPUT SEQUENCE BASED TECHNOLOGIES: - STATE OF ART -



OUR OBJECTIVES

- **Adaptation of the SNPLex technology to the qualitative detection of:**
 - **GMOs (all kind of sequences of the inserts),**
 - **Plant taxa (selection and design of reference systems)**
 - **Donor organisms (CaMV, *Agrobacterium*...)**
- **Reduction of cost and time of analysis**
- **Allow an identification of the GMOs present in complex matrixes**
- **Find a strategy for the detection of unauthorized or unknown GMOs (use of DSS)**

Basic Assay Steps for the SNPLex

Encoding

Generation of genotype specific products through multiplex oligonucleotide ligation reaction (OLA)



Amplification

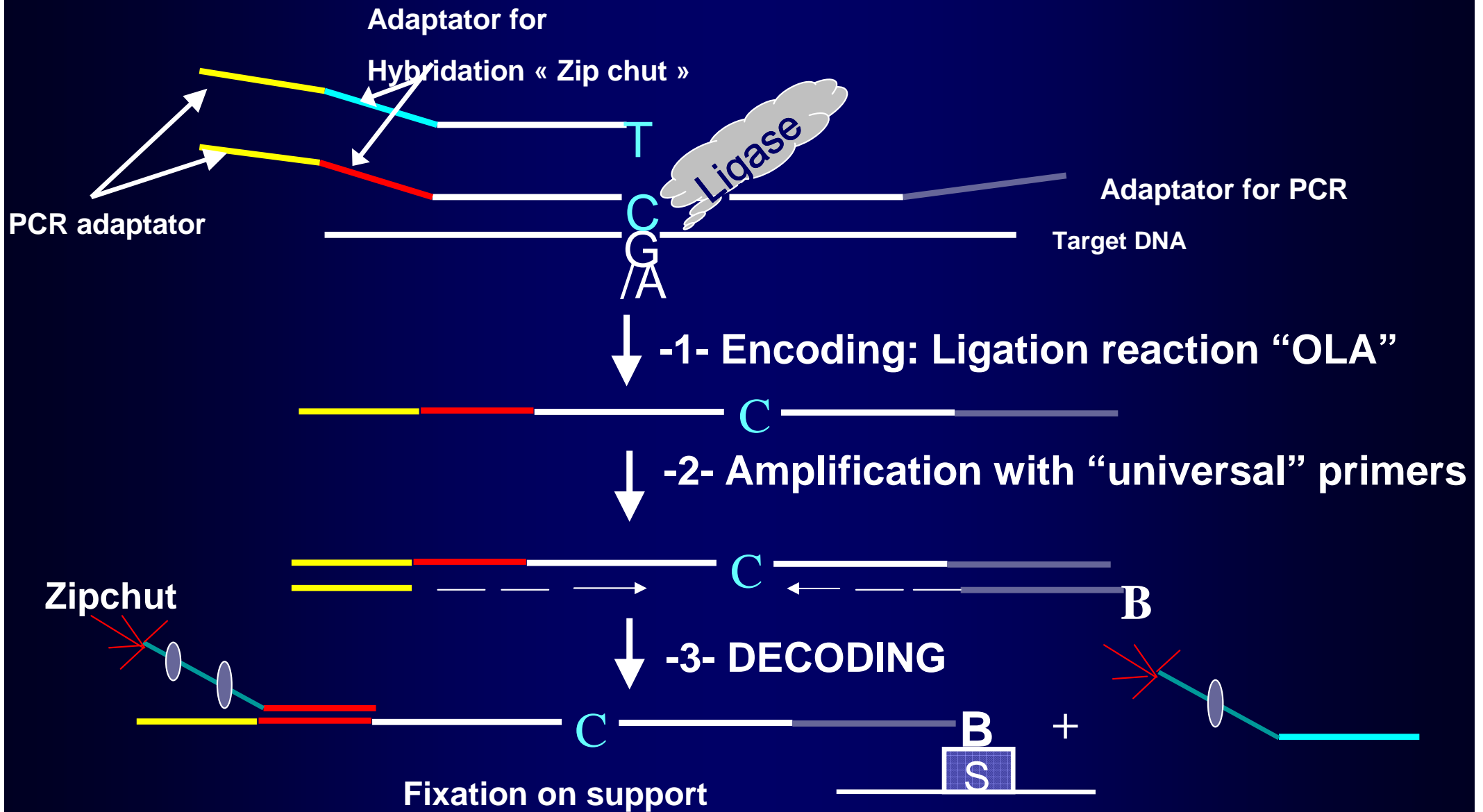
Multiplex PCR with universal primers



Decoding

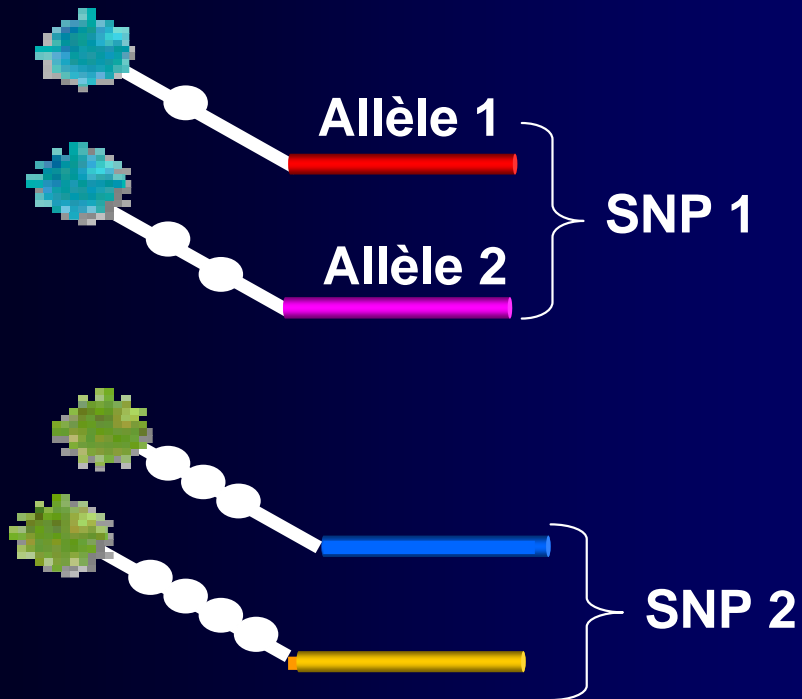
Hybridization of universal ZipChute probes to amplicons and identification of eluted ZipChutes

SNPLex principle



DETECTION WITH THE « ZIPCHUTE » PROBES

Wash and elute
Drag Chutes



Load on CE instrument



Software

« Gene Mapper »

ROBOTIC PLATFORM NEEDED



➔ **REDUCE OF TIME AND COST OF EXPERIMENTS**

SNPlex adaptation to GMO analysis: Definition of 'panels'

- **Panel** = group of “signature sequences” to be looked at in one experiment
- Need for exact **sequences** :
 - search existing tests
 - PCR and sequencing
 - obtained 79 different sequences
 - submit to ABI software for analysis
- **Capacity**: 1536 samples (one experiment)

Panel 1 test : 47 targets in a tube

8

13

9

14

Reference genes	Screening	Construct specific	Edge fragment
Maize (<i>adh1</i>) Potato (<i>UGPase</i>) Cotton (<i>Sad</i>) Wheat (<i>Wax</i>) Rice (<i>ppi-PPF</i>) Rapeseed (<i>BngACC8</i>) Sugarbeet (<i>Gln synthase</i>) Tomato (<i>LAT52</i>)	NPTII NITRILASE Cry1AC TNOS CP4EPSPS mEPSPS P35S PFMV PNOS PAT BAR Barstare T35S	IVS6/P35S P35S/BAR P35S/PAT Tnos/Pract Cry9C/P35S OTP/mEPSPS Cry1A(b)/Hsp70 P35S/CP4EPSPS Pract/EPSPS	Bt10 (Tnos/plant) Bt176 (bar/plant) Mon863 (P35S/plant) T25 (P35S/plant) Bt11(Tnos/plant) CBH351(Tnos/plant1) Cotton531(Cry/plant) Mon810 (P35S/plant) TC1507 (PAT/plant) Cotton1445 (Pnos/plant) T25 (PUC18/plant) CBH351 (Tnos/plant) Tq7RF (PG/TNOS) Soybean RRS (P35S/plant)
Controls	<p>3</p>		
Cauliflower mosaic virus Agrobacterium tumefaciens Bacillus thuringiensis			

MATERIAL TESTED: 192 samples at one time

- 3 CULTIVARS OF 17 DIFFERENT TAXA

- **GMO EVENTS AND VARIETIES:** maize (4 varieties MON810, T25, NK603, GA21, event176 (*Garonna et Pactol*), Bt11, Bt10, Starlink, DAS59122, TC1507), rapeseed: (MS1, MS8, RF1, RF2, RF3, TOPAS19/2, OXY235, T45, GT73), Soja RRS, tomato (NCIMB40015, NCIMB40134, TG7TF), sugarbeet: (GTSB77, T120-7), cotton (event531, 1445, LLcotton25) and rice (LLRICE62)

- **CRM (CERTIFIED REFERENCE MATERIAL):** events: MON810, RRS, TC1507, Bt11, Bt176, GA21, NK603, MON863, MON810xMON863)

- **DIFFERENT PERCENTAGES FOR SENSITIVITY TEST:** (tomato TG7TF, rapeseed Topas19/2, maize T25, maize Bt10, colza MS1,2 et 8, rapeseed RF1,2 et 3, rice (event LLRICE62), cotton (event531, event1445, LLcotton25) and sugarbeet (RR et LL)

- **DNA FROM DONOR ORGANISMS:** (*CaMV* (2 strains *Ji et Cabss*), 2 strains *Agrobacterium tumifaciens* and 2 strains *Bacillus thuringiensis*).

RESULTS SNPLex : Specificity, panel 1

SCREENING	REFERENCE GENES	EDGE FRAGMENT	CONSTRUCT SPECIFIC
NPTII (8/9)	Maize (<i>adh1</i>) 16/16	Bt10 (Tnos/Plant) (2/2)	
NITRILASE (1/1)	Potato (<i>UGPase</i>) 2/3	Bt176 (bar/Plant) (1/1)	<u>IVS6P35s</u> (0/1)
Cry1AC (1/1)	Cotton (<i>sad</i>) 6/6	Mon863 (P35S/Plant) (2/2)	P35S/BAR (1/2)
TNOS (15/19)	Wheat (<i>wax</i>) 3/3	T25 (P35S/Plant) (1/1)	<u>P35S/PAT</u> (18/1)
CP4EPSRRS (2/2)	Rice(<i>ppi-PPF</i>) 4/4	Bt11 (Tnos/Plant) (1/1)	Tnos/Pract (1/1)
<u>GA21mEPSPS</u> (13/1)	Rapeseed (<i>ACCg8</i>) 12/12	CBH351 (Tnos/Plant)2 (1/1)	Cry9C/P35S (1/1)
P35S (7/18)	Sugarbeet (<i>Gln synthase</i>) 5/7	Coton531 (Cry1Ac/Plant) (2/2)	OTP/mESPGA21 (1/1)
PFMV (2/3)	Tomato (<i>LAT52</i>) 6/6	Mon810 (P35S/Plant) (2/4)	<u>Cry1A (b)/hsp70</u> (0/4)
PNOS (4/4)	Controls	<u>TC1507(PAT/Plant)</u> (0/1)	P35S/CP4EPSPS (3/2)
PAT (7/7)	Cauliflower mosaic virus (2/2)	<u>Coton1445(P35S/Plant)</u> (0/1)	Pract/EPSPS (1/1)
BAR (6/6)	Agrobacterium tumifaciens (1/2)	T25 (PUC18/Plant) (1/1)	
Barstare (3/3)	Bacillus thuringiensis (2/2)	CBH-351 (Tnos/Plant) (1/1)	
T35S (5/6)		Tq7RF (PG/TNOS) (1/1)	
		<u>Soybean RRS (P35/Plant)</u> (13/1)	

RESULTS PANEL 1

- **Panel 1: 4 probes inactive and 2 unspecific**
- **Redundant targets such as in construct specific (event GA21), edge fragment (event CBH351)**

 **Design of second panel for 48 targets**

Panel 2 : 48 targets in a tube

12

18

6

9

Reference genes	Screening	Construct specific	Edge fragment
Alfalfa (<i>acc</i>) Maize (<i>adh1</i>) Potato (<i>UGPase</i>) Cotton (<i>Sad</i>) Wheat (<i>Wax</i>) Barley (γ - <i>Hordein</i>) Rice (<i>ppi-PPF</i>) Rapeseed (<i>BngACC8</i>) Sugarbeet (<i>Gln synthase</i>) Tomato (<i>LAT52</i>) Sunflower (<i>hel</i>) Soybean (<i>lec</i>)	Cry1A(b) (3seq) NPTII NITRILASE Cry1AC TNOS CP4EPSPS (2seq) Cry 9C P35S PFMV PNOS	P35S/BAR P35S/PAT Cry9C/P35S P35S/CP4EPSPS Tnos/Nitrilase PG/Tnos	Bt10 (Tnos/plant) Mon863 (P35S/Plant) T25 (P35S/Plant) Bt11(Tnos/Plant) CBH351(Tnos/Plant) Coton531 (Cry1A(c)/Plant) Mon810 (Cry1A(b)/Plant) Cotton1445 (P35S/Plant) Soybean RRS (P35/Plant)
Controls Cauliflower mosaic virus Agrobacterium tumefaciens Bacillus thuringiensis	PAT BAR Barstare Barnase T35S		

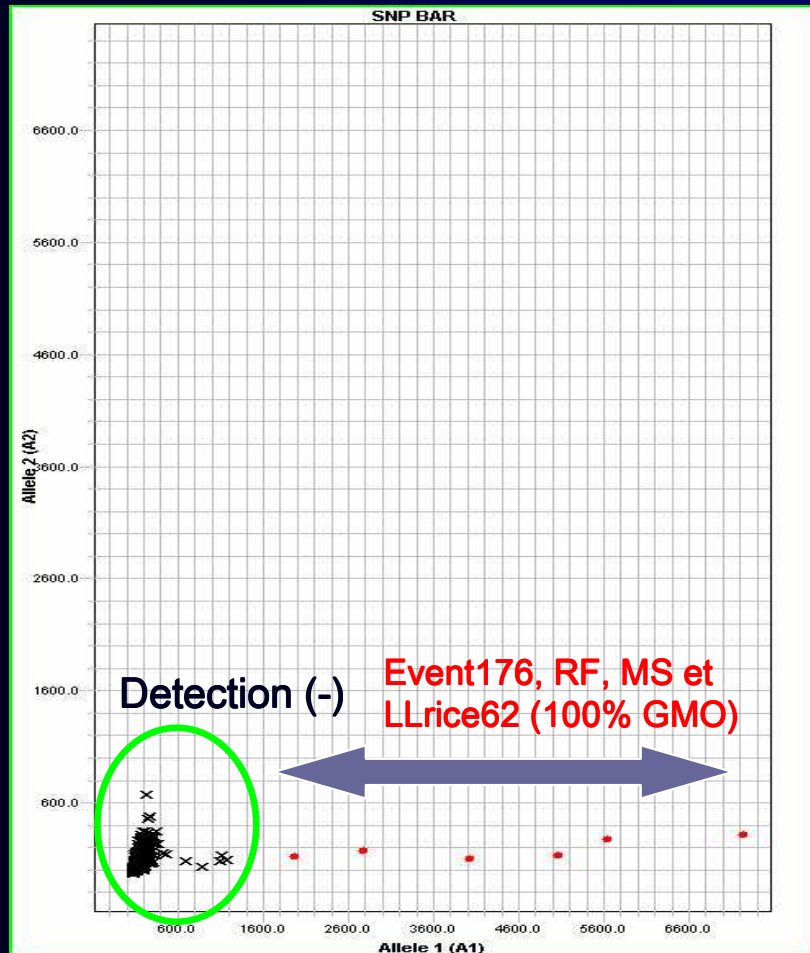
3

RESULTS SNPLex : Specificity, panel 2

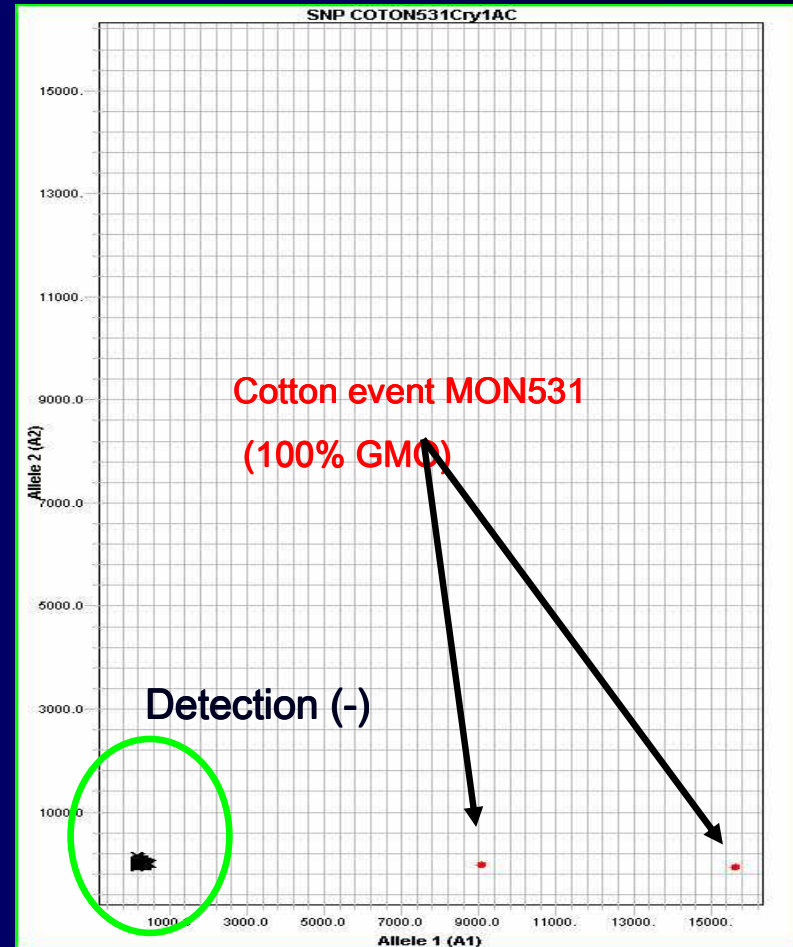
SCREENING	REFERENCE GENES	EDGE FRAGMENT	CONSTRUCT SPECIFIC
NPTII (8/9)	Maize (<i>adh1</i>) 16/16	Bt10 (Tnos/Plant)(2/2)	
NITRILASE (1/1)	Potato (<i>UGPase</i>) 3/3	Mon863 (P35S/G) (2/2)	P35S/BAR (1/2)
Cry1A(C) (1/1)	Cotton (<i>sad</i>) 6/6	T25 (P35S/Plant) (1/1)	P35S/PAT (1/1)
Cry9C (1/1)	Wheat (<i>wax</i>) 3/3	Bt11(Tnos/Plant) (1/1)	Cry9C/P35S (1/1)
TNOS (15/19)	Rice(<i>ppi-PPF</i>) 4/4	CBH351(Tnos/Plant) (1/1)	P35S/CP4EPSPS (2/2)
CP4EPSPSRRS1 (2/2)	Rapeseed (<i>BgnACC8</i>) 12/12	Coton531 (Cry1Ac/Plant) (2/2)	Tnos/Nitrilase (2/2)
<u>CP4EPSPSRRS1(20/2)</u>	Sugarbeet (<i>Gln synthase</i>) 5/7	Mon810 (Cry1A(b)/Plant) (2/2)	<u>PG/Tnos (30/1)</u>
3 Cry1A(b) (1/2)	Tomato (<i>LAT52</i>) 6/6	Coton1445 (P35S/plant)_(1/1)	
P35S (18/18)	Barley (<i>γ-hordein</i>) (3/6)	Soybean RR (P35/Plant) (1/1)	
PFMV (2/3)	Sunflower (<i>hel</i>) (3/3)		
PNOS (4/4)	Alfalfa (<i>acc</i>) (3/3)		
PAT (7/7)	Soybean (<i>Lec</i>) (3/3)		
BAR (6/6)	Controls		
Barstare (3/3)	Cauliflower mosaic virus (2/2)		
Barnase (2/2)	Agrobacterium tumefaciens (1/2)		
T35S (5/6)	Bacillus thuringiensis (2/2)		

Results: specificity (panel 2)

«bar» probe



«Cry1Ac» probe



RESULTS: sensitivity (LODa)

- **Samples:** All the available GMO events were used
- Dilution series ranged from 2 $\mu\text{g}/\mu\text{l}$ to 0.01 $\text{ng}/\mu\text{l}$ of the input DNA (in duplicate)



10ng < LODa < 15ng

RESULTS: sensitivity (LODr)

Samples: Artificial mixtures (GMO/ballast non-GMO DNA) were used

Percentages: CRM (5% to 0.1%) and artificial mixtures (10% to 0.01%)



0.4 % <LODr < 0.8%

The sensitivity observed is acceptable to monitor the reliability of the current European 0.9% labelling system

Advantages / drawbacks of SNIPlex

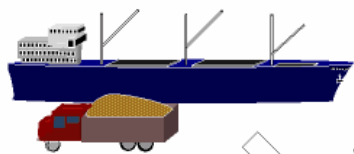
- **High-throughput qualitative detection**
- **Up to 48 targets detectable in a single reaction (foreseen 96 targets)**
- **A huge capacity of detection (4 x 384 wells microplates)**
- **Useful in control plan by multiple attributes (increase sensitivity)**
- **Design of probes by company (Applied Biosystems)**

IN CONCLUSION THIS DEVELOPED SNPLex ASSAY IS:

- SPECIFIC**
- SENSITIVE**
- REPEATBLE**
- REPRODUCTBLE**
- FLEXIBLE**
- RELATIVELY FAST**
- COST-EFFECTIVE**

FUTURE DIRECTIONS

- **How to validate the method?**
- **How it can be useful for the routine analysis?**
- **Use of control plans by attributes (enlargement of sample number and decreasing sample size)**
- **Adaptation of other technologies such as ILLUMINA (*Veracode* or *GoldenGate*) to increase the level of multiplexing to 1536 sequence to be detected in one tube**



Step

Transfer performance criteria

1. SAMPLING

Lot → lotsample

Analyte ratio, particle size

2. SUBSAMPLING

Lotsample → laboratory sample

Analyte ratio, particle size

3. SUBSAMPLING

Laboratory sample → test portion

Analyte ratio, quality (length and structural integrity) and quantity, stability and particle size.

4. ANALYTE EXTRACTION

Test portion → Analyte suspension

Analyte quality, quantity and purity

5. GENERATION OF TEST RESULT

Analyte suspension → test result

AMOUNT OF BLUK SAMPLE

NUMBER OF SUBSAMPLES

NUMBER OF LAB. SAMPLES

NUMBER OF SUBSAMPLES

HIGH-THROUGHPUT ANALYSIS

(Holst-Jensen et al. 2004)

Thanks for your attention

