Development of Microarray-Based Genotyping for MAS

Joong Hyoun Chin and Enrico F. Mercado

Apr 27, 2007

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

CIMMYT

Generation

CONTENTS

- **Introduction**
- **Workflow and flow of data**
- **Case study of MBG –*xa*5**

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

Why microarray-based genotyping?

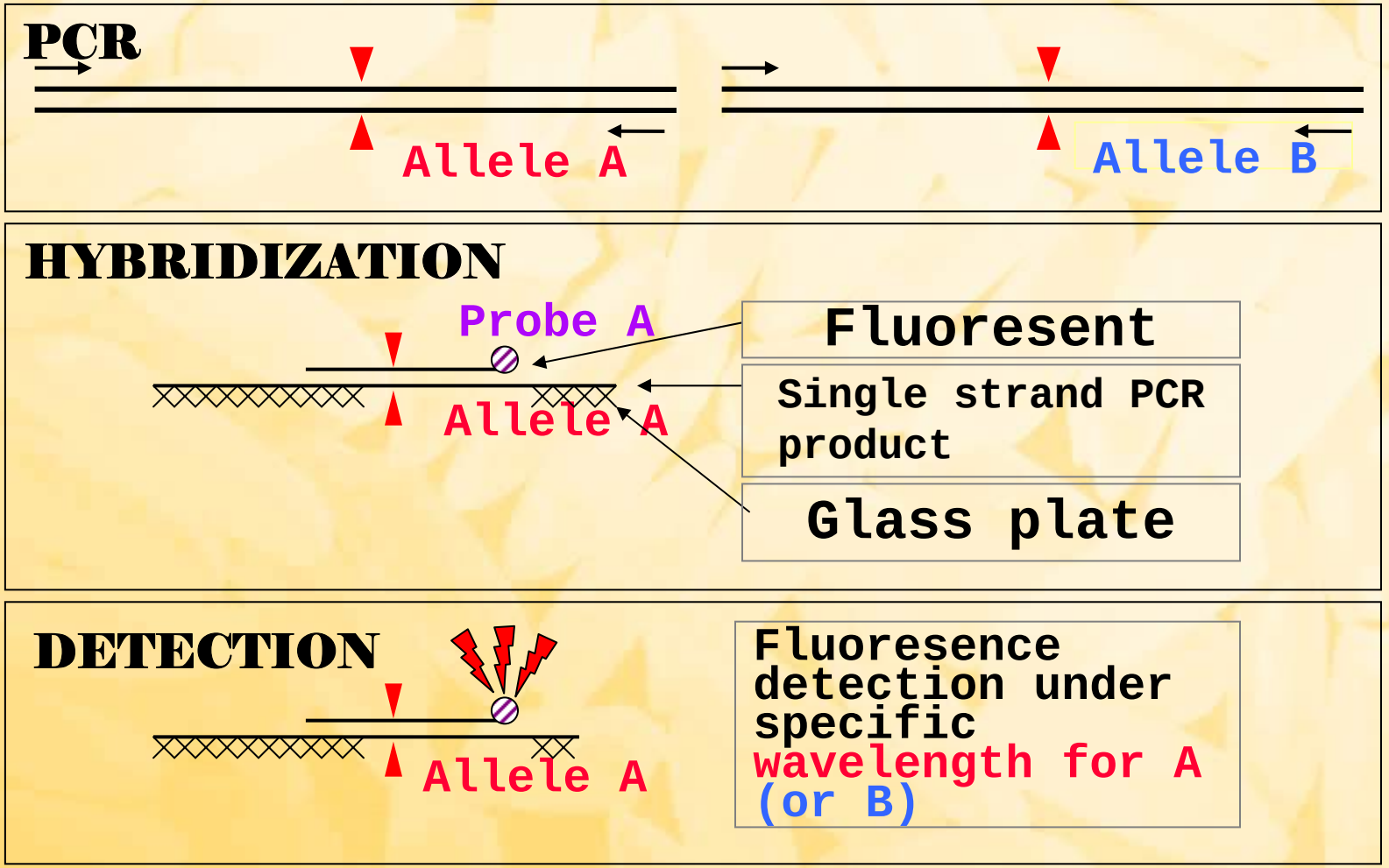
- Based on relatively simple theory
- Commercialized equipment and reagents
- Establishment of high-throughput data acquisition by adequate software
- Efficient foreground selection in two allele system
  (vs. SFP – background and whole genome)

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

Workflow and dataflow of MBG

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

Schematic diagram for MBG



IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

DNA preparation

Collecting leaves

DNA extraction

Quality check (Concentration & purity)

Normalization of DNA quantity

Low/Bad

Spectrophotometer

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

PCR (Polymerase chain reaction)

✓To amplify Gene-specific region

PCR

Purification of PCR products
(NaOAc or column)

Bad /Low

Quality check (Concentration & pu

Spectrophotometer

Transfer D
well p

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

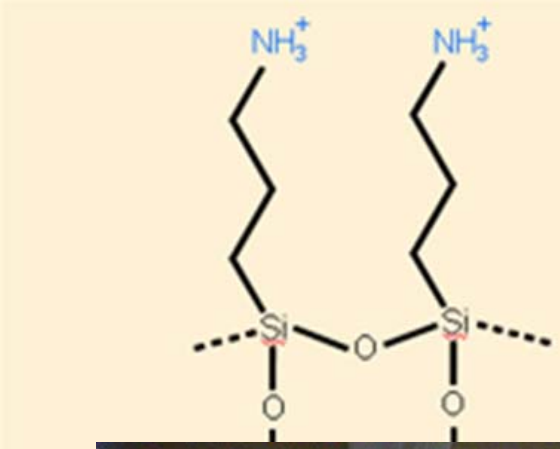
Arraying and printing PCR products

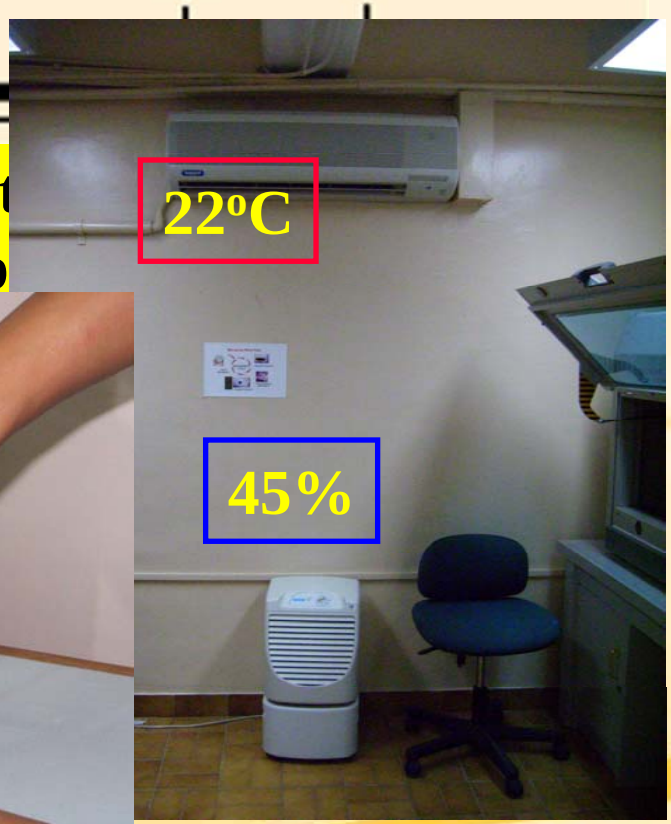
PCR product in 384 well plate

Print on GAPS™ slides

Bad

St





IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

Arraying and printing PCR products

G3 arrayer

✓**In high throughput genotyping system**
-> **Information of array enable errors predictable**

Table. Arraying program in G3 arrayer

Step No.	Step Action	Time (sec)
1	Sonic bath (water)	8
2	Bath 4 (water)	4
3	Bath 3 (70% isopropyl alcohol)	4
4	Heater	4
5	Air dry	8

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

Probe labeling

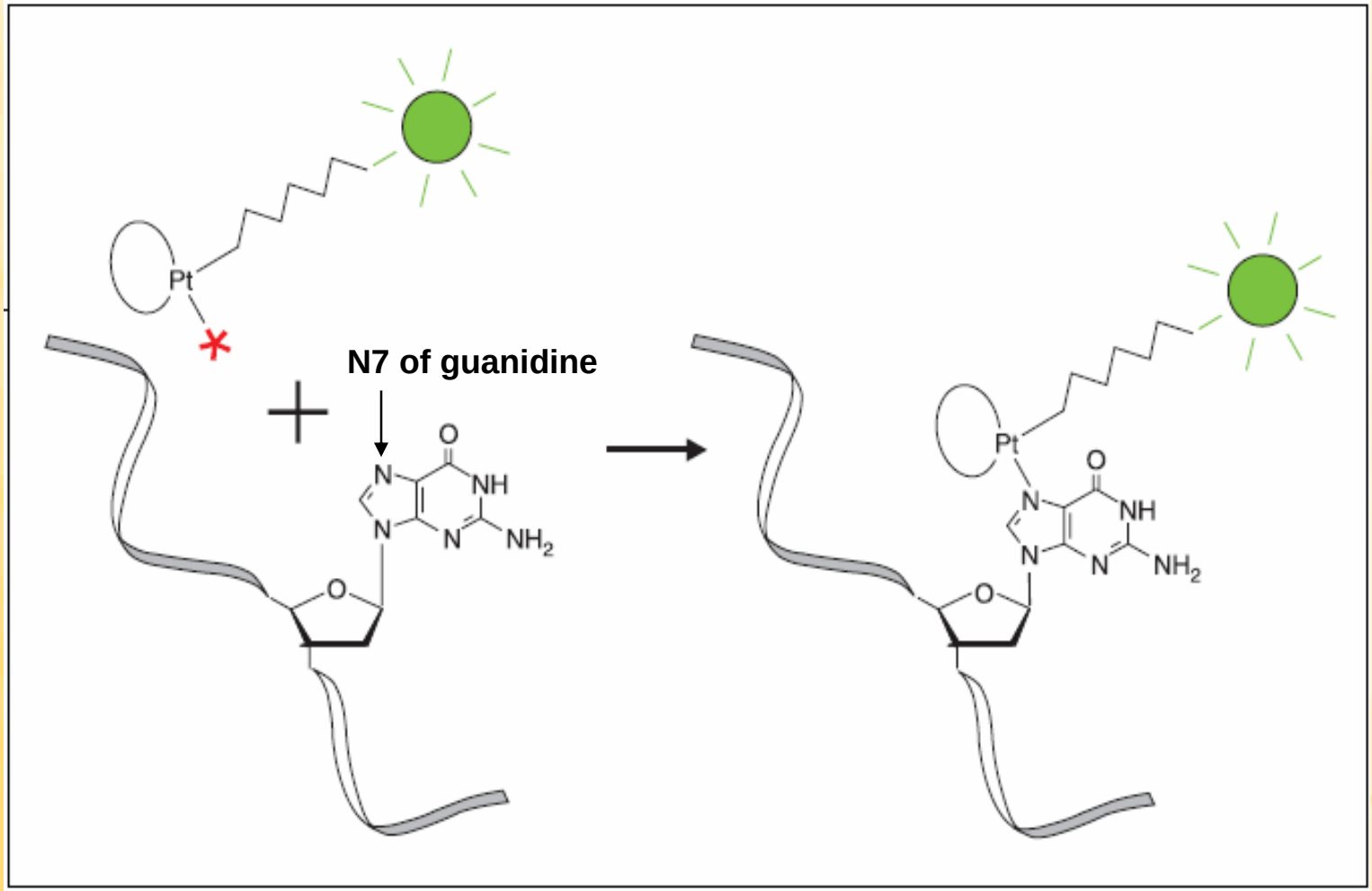
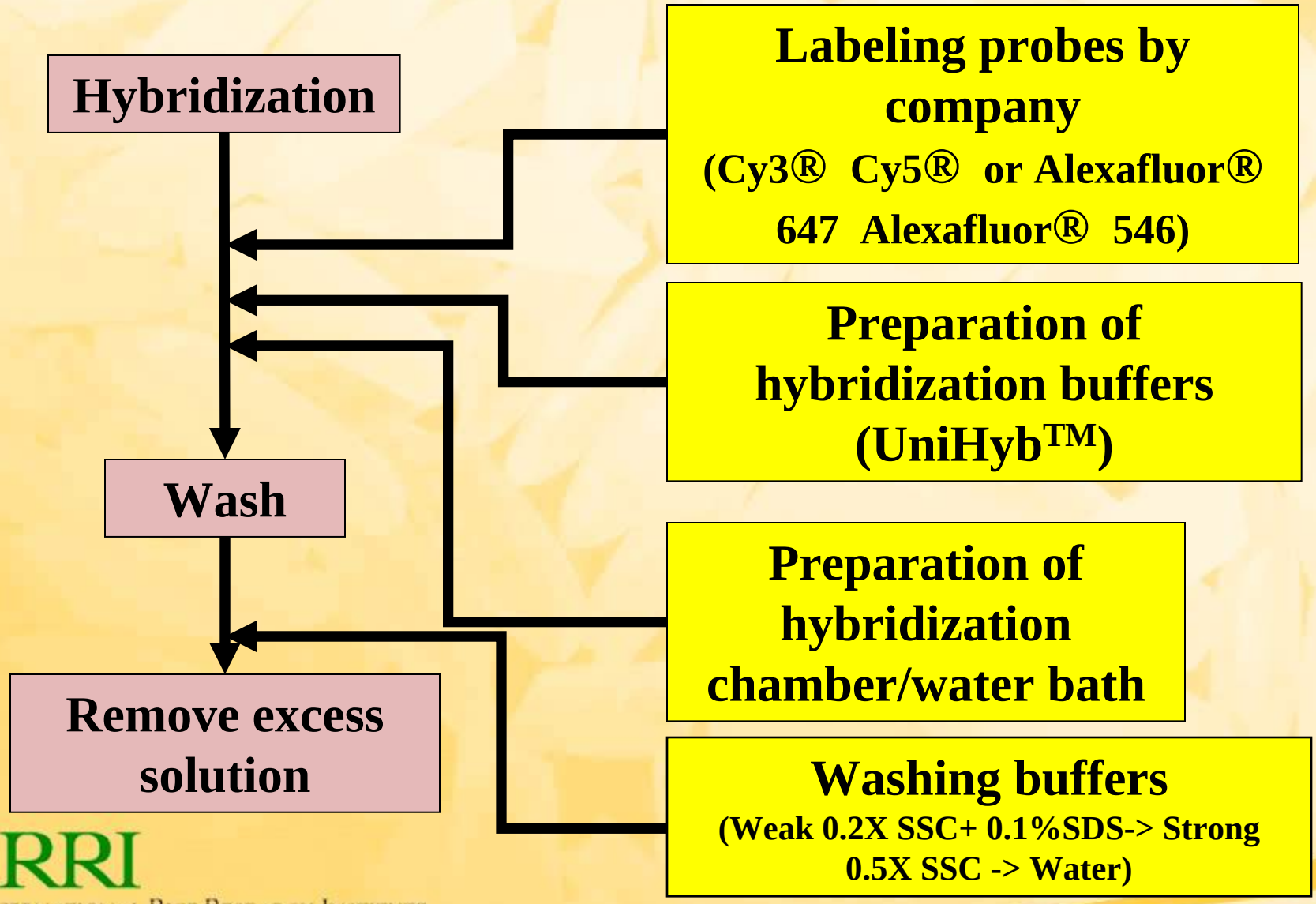


Fig. Two techniques of labeling oligonucleotide

Hybridization and washing



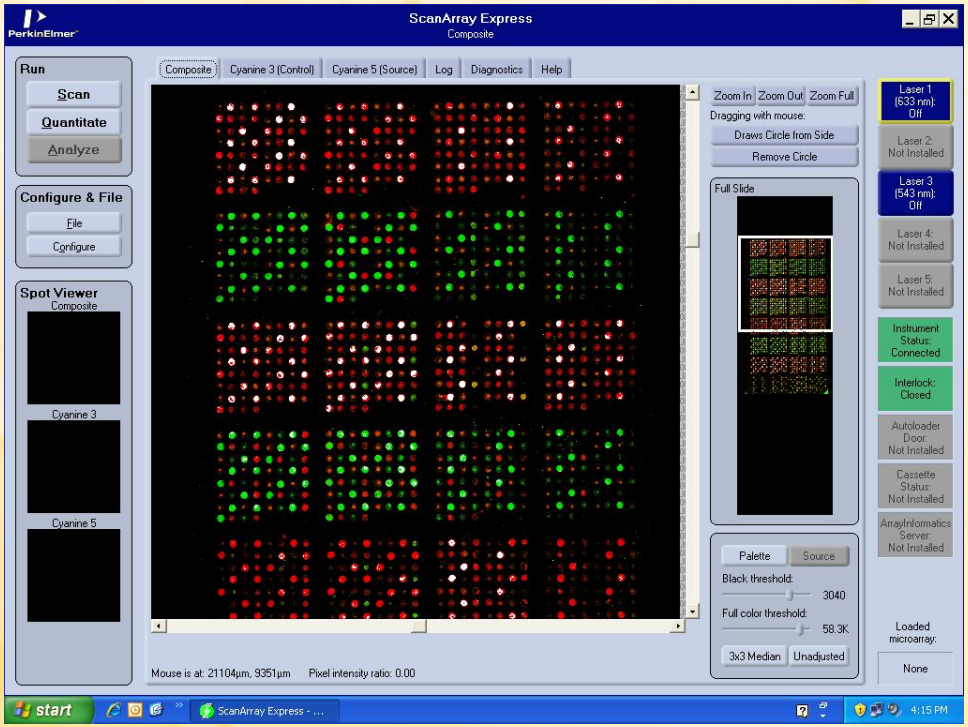
IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

Hybridization and washing

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

Data acquisition and analysis

Scanning



Visual analysis

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

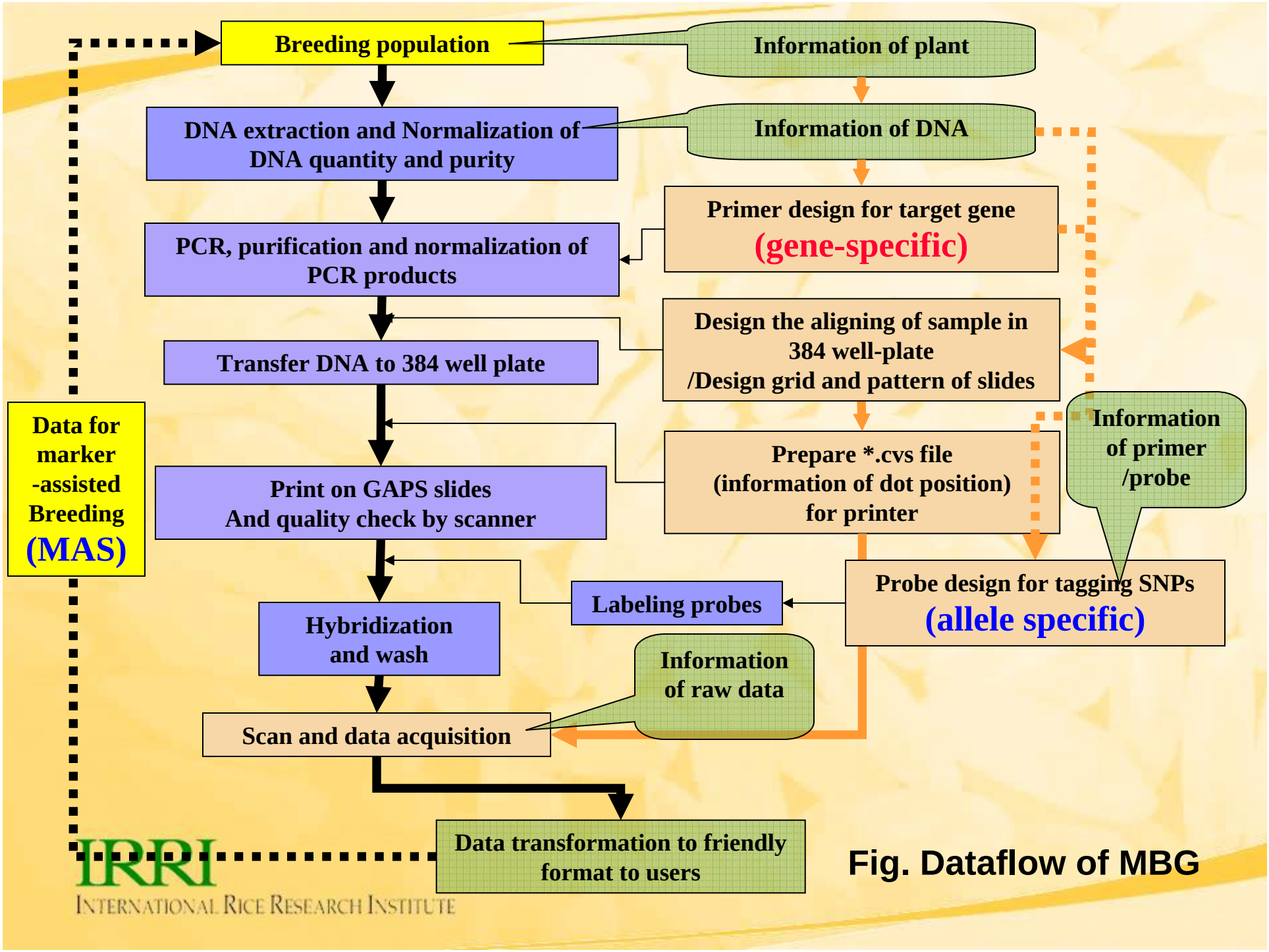


Fig. Dataflow of MBG

Case study for MBG (*xa5*)

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

Primer and probe designing

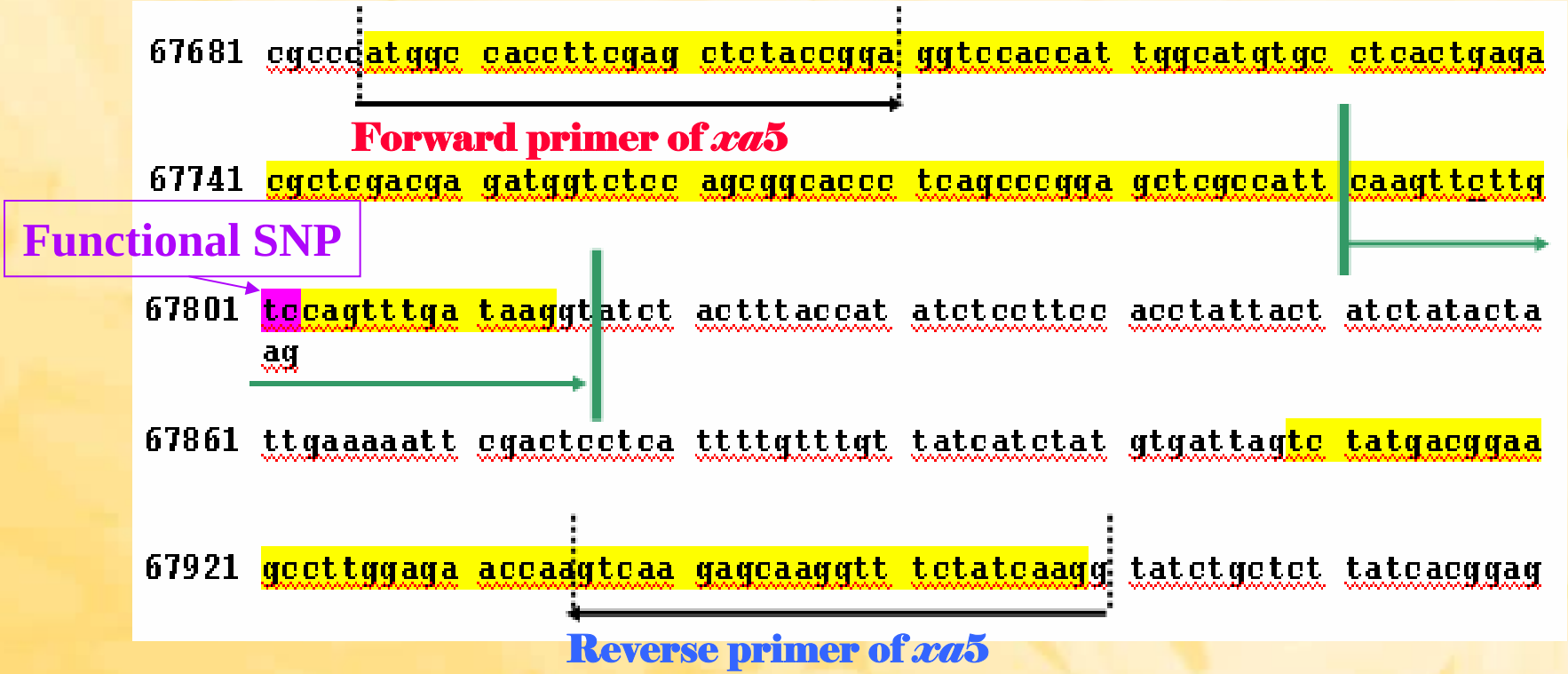


Fig. Primer and probe designing for *xa*5 gene

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

DNA samples

- Standard set of 96 DNA samples (IRBB NILs and some mega varieties) of rice accessions prepared for validation using *xa5.*

- Control accessions with and without *xa5* gene, popular breeding varieties, near isogenic lines (NILs) for four bacterial blight resistance genes and lines from NARES partners.

- Amplified segment of *xa5* gene containing the two functional SNPs using MBG primers

- PCR products were purified by acetate precipitation.

<Positive and Negative>

- Positive control
- Negative control
- Tolerance lines
- Susceptible lines
- Hetero check

<For MAS>

- Fastprep quality DNA
- Purified DNA

<Dilution factor>

- Minimum amount of PCR product

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

Table. Isogenic lines for bacterial blight in IRRI

Plate number	Genotype	BB genes known
1	IRBB1	Xa1
2	IRBB3	Xa3
3	IRBB4	Xa4
4	IRBB5	xa5
5	IRBB7	Xa7
6	IRBB8	xa8
7	IRBB11	Xa11
8	IRBB13	xa13
9	IRBB14	Xa14
10	IRBB21	Xa21
11	IRBB51	Xa4+xa13
12	IRBB52	Xa4+Xa21
13	IRBB53	Xa5+xa13
14	IRBB54	xa5+Xa21
15	IRBB55	xa13+Xa21
16	IRBB56	Xa4+xa5+xa13
17	IRBB57	Xa4+xa5+Xa21
18	IRBB58	Xa4+xa13+Xa21
19	IRBB59	xa5+xa13+Xa21
20	IRBB60	Xa4+xa5+xa13+Xa21
21	IRBB61	Xa4+xa5+Xa7
22	IRBB62	Xa4+Xa7+Xa21
23	IRBB63	xa5+Xa7+xa13
24	IRBB64	Xa4+xa5+Xa7+Xa21
25	IRBB65	Xa4+Xa7+xa13+Xa21
26	IRBB66	Xa4+xa5+Xa7+xa13+Xa21

Table. 384 well formatting for arraying for MBG

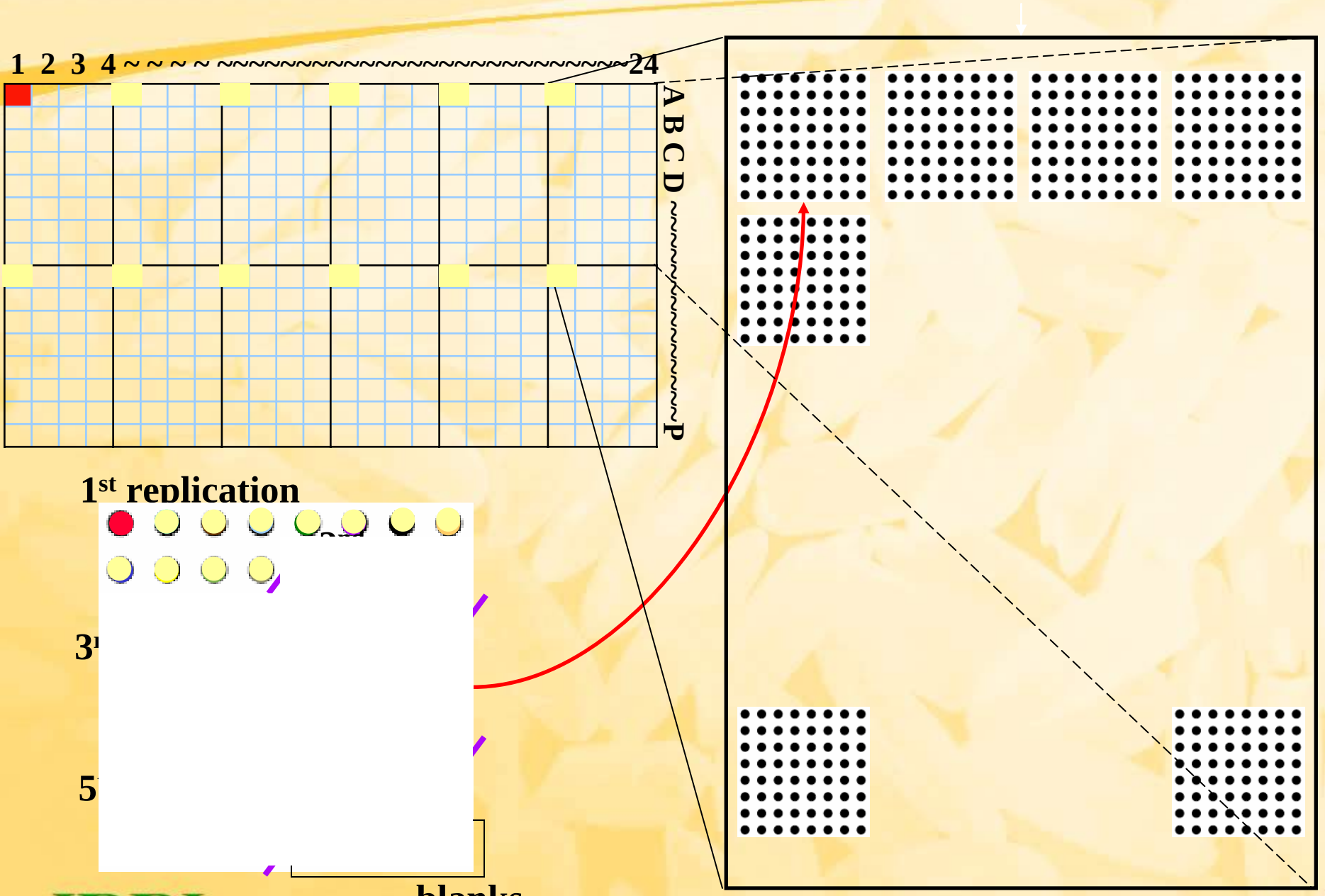
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	IR24												R26 (2uM)						R26 (0.2 uM)					
B	IRBB5												S26						S26					
C	IRBB21												R50						R50					
D	IRBB5												S50						S50					
E	IR24 (1/10 DILUTION)												R26						R26					
F	IRBB5 (1/10 DILUTION)												S50						S50					
G	IRBB24 (UNPURIFIED)												R50						R50					
H	IRBB5 (UNPURIFIED)												CONTROL (R26)						CONTROL (R50)					
I	R26 (2uM)						R26 (0.2 uM)						Set of 96 lines (Real population for MAS)											
J	S26						S26																	
K	R50						R50																	
L	S50						S50																	
M	R26						R26																	
N	S50						S50																	
O	R50						R50																	
P	CONTROL (R26)						CONTROL (R50)																	

R26 = 26 mer complementary oligo nucleotide for resistant *xa*5 allele
S26 = 26 mer complementary oligo nucleotide for susceptible *Xa*5 allele
R50 = 50 mer complementary oligo nucleotide for resistant *xa*5 allele
S50 = 50 mer complementary oligo nucleotide for susceptible *Xa*5 allele
CONTROL = R probe (26 mer and 50 mer) that is not complementary. So no hybridization with S allele should occur.

Arraying and printing

- PCR products were printed on Corning GAPS slides using a GeneTac G3 High Density Arrayer.
- Synthetic oligonucleotides complementary to the *xa5* detector probe were also printed to serve as positive control.
- Negative control
- After printing, PCR products spotted were fixed under UV light.

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE



IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

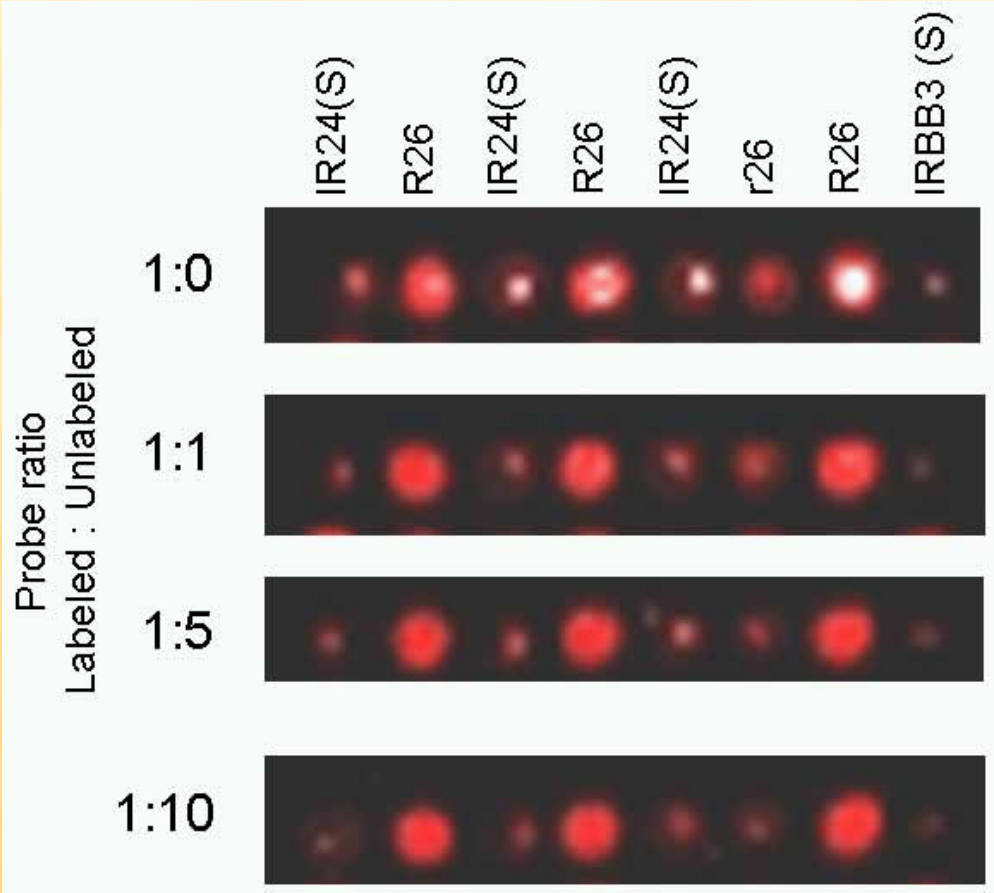
Fig. Aligning positions of array with those of 384-well plate

Hybridization and washing

- Fluorescently labeled 26-mer probe specific to S allele was hybridized with the PCR products printed on the slides at 65°C for 16 hours.

- Competitive hybridization was also tested using unlabeled probes (26-mer) which were specific to the R allele.

- Water treatment for 5 min. enhanced the quality of data.



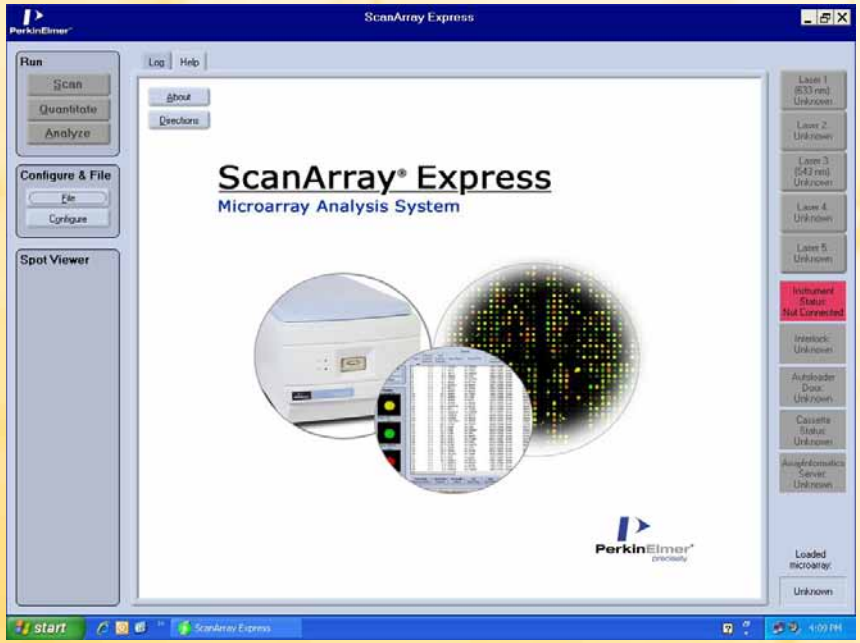
(By reference of Shirasawa et al, 2006)

E. Mercado, B. Collard, D. Skinner

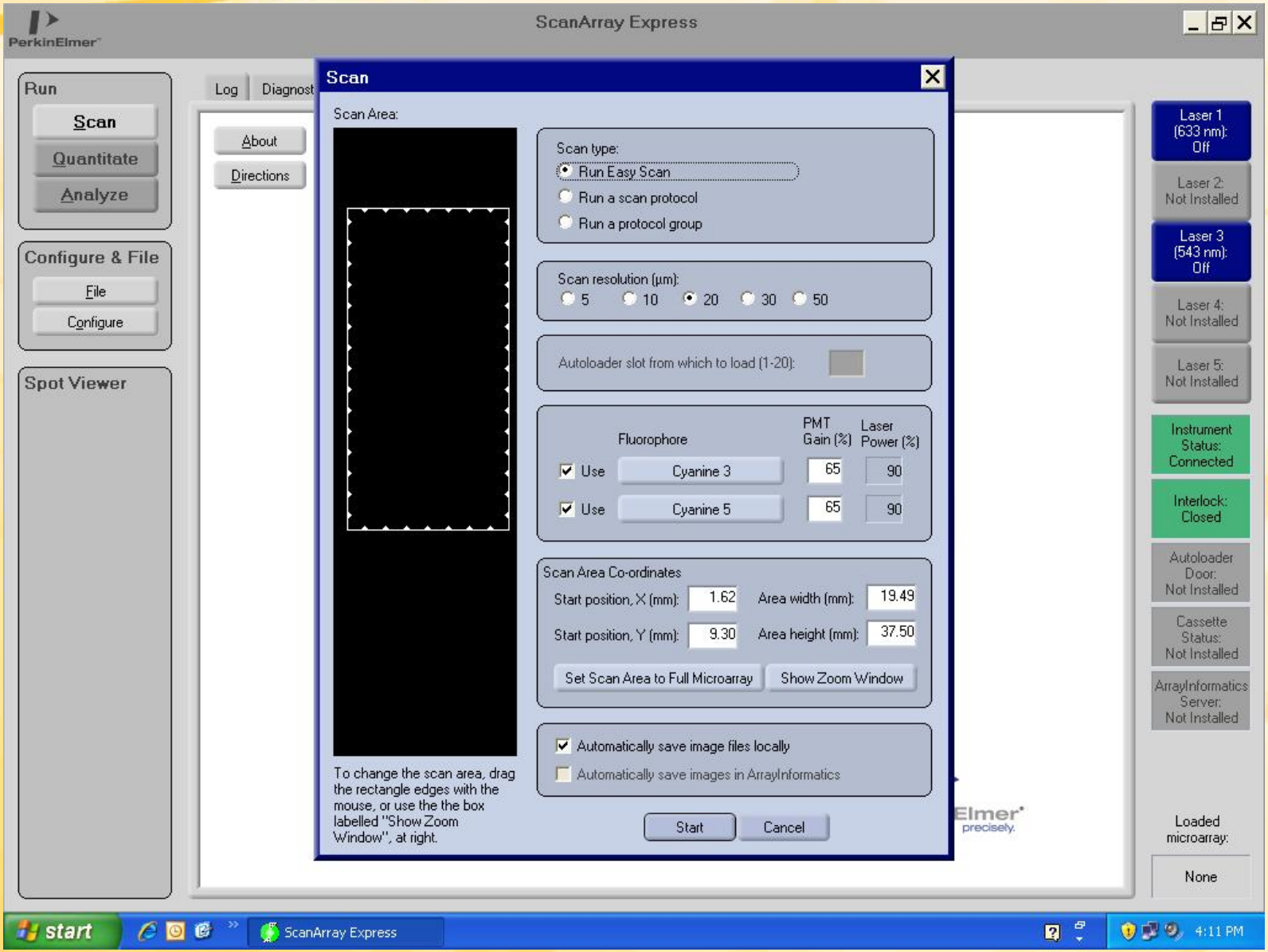
Fig. Competitive hybridization using labeled R and unlabeled S probes at two-, five- and ten-fold volumes decreased intensities of unspecific spots

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

Scanning and data acquisition



IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE



IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

	1 2 3 4 5 6	7 8 9 10 11 12	13 14 15 16 17 18	19 20 21 22 23 24
A	IR24		R26 (2uM)	R26 (0.2 uM)
B	IRBB5		S26	S26
C	IRBB21		R50	R50
D	IRBB5		S50	S50
E	IR24 (1/10 DILUTION)		R26	R26
F	IRBB5 (1/10 DILUTION)		S50	S50
G	IRBB24 (UNPURIFIED)		R50	R50
H	IRBB5 (UNPURIFIED)		CONTROL (R26)	CONTROL (R50)
I	R26 (2uM)	R26 (0.2 uM)	Set of 96 lines (Real population for MAS)	
J	S26	S26		
K	R50	R50		
L	S50	S50		
M	R26	R26		
N	S50	S50		
O	R50	R50		
P	CONTROL (R26)	CONTROL (R50)		

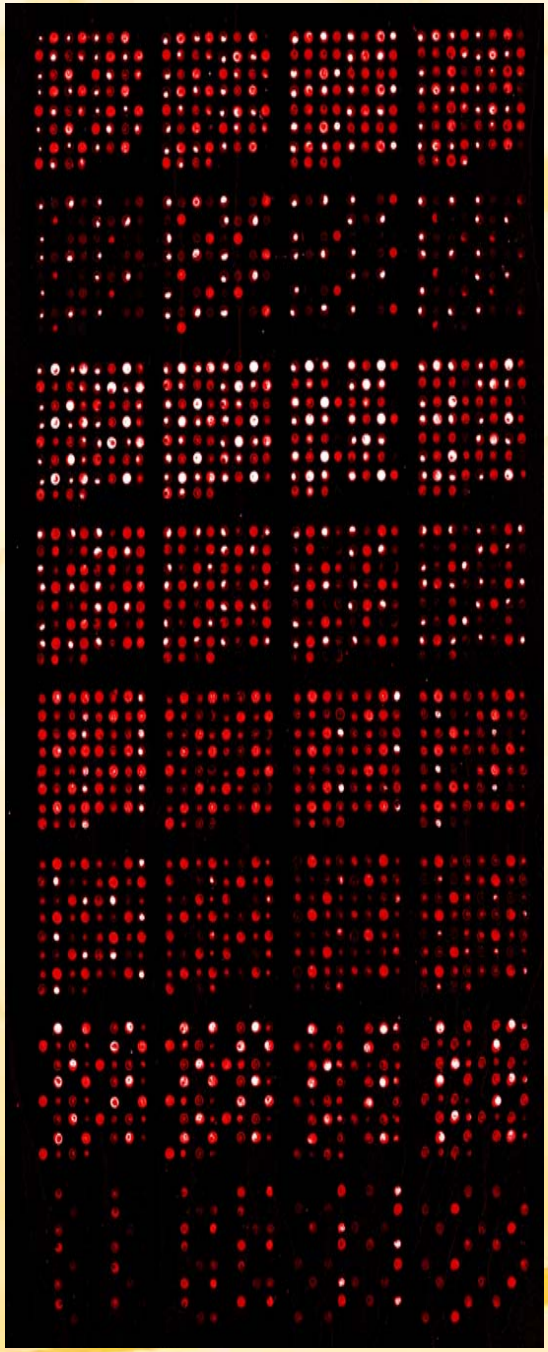
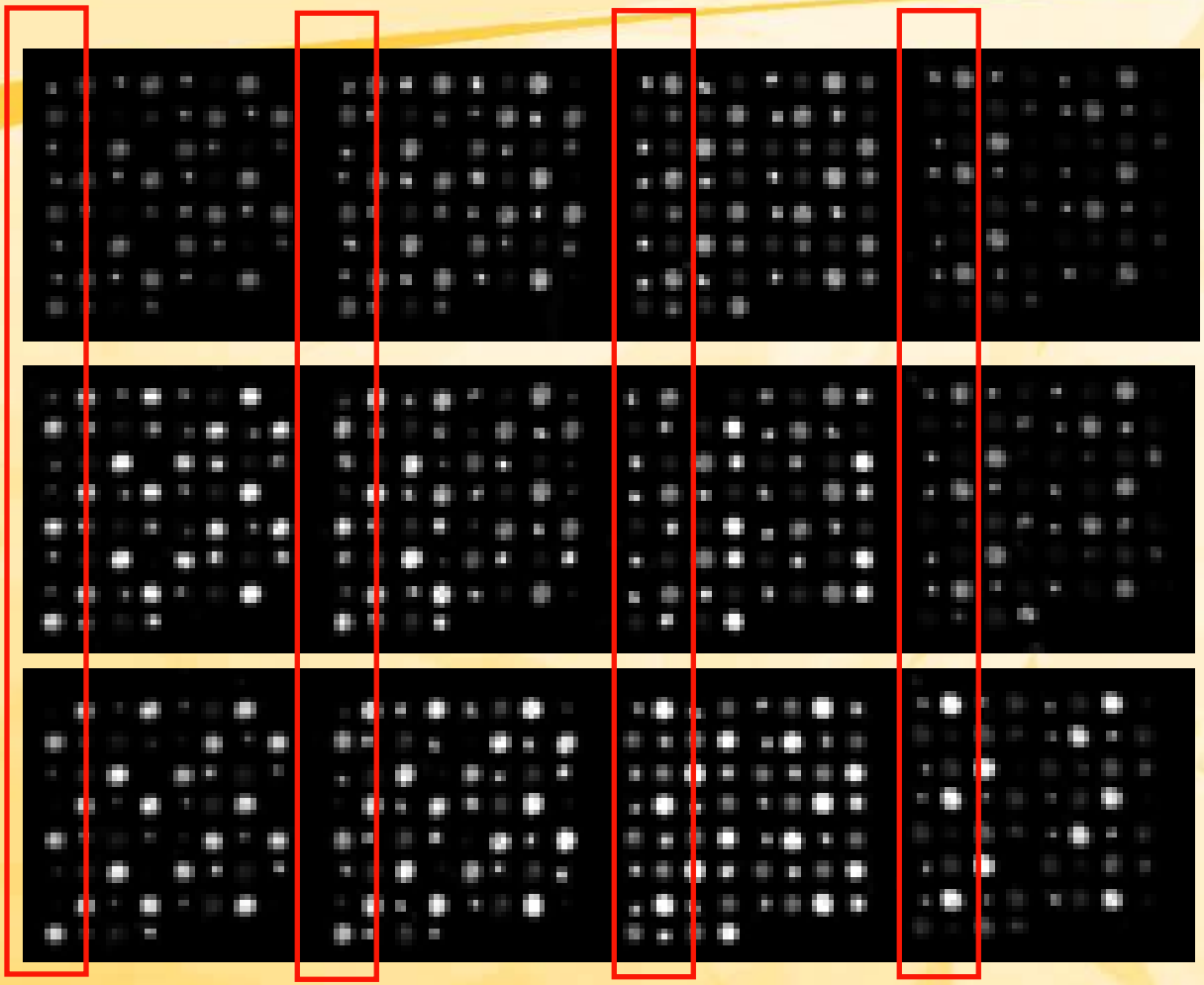


Fig. Microarray results using *xa*5 R probe labeled by Alexaflor 546
– The first trial (16 hrs, 60C) with competitive hybridization

(By reference of Ji et al, 2004)

E. Mercado and J. Chin

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE



(a) No washing with water

(b) Washing 3 min.

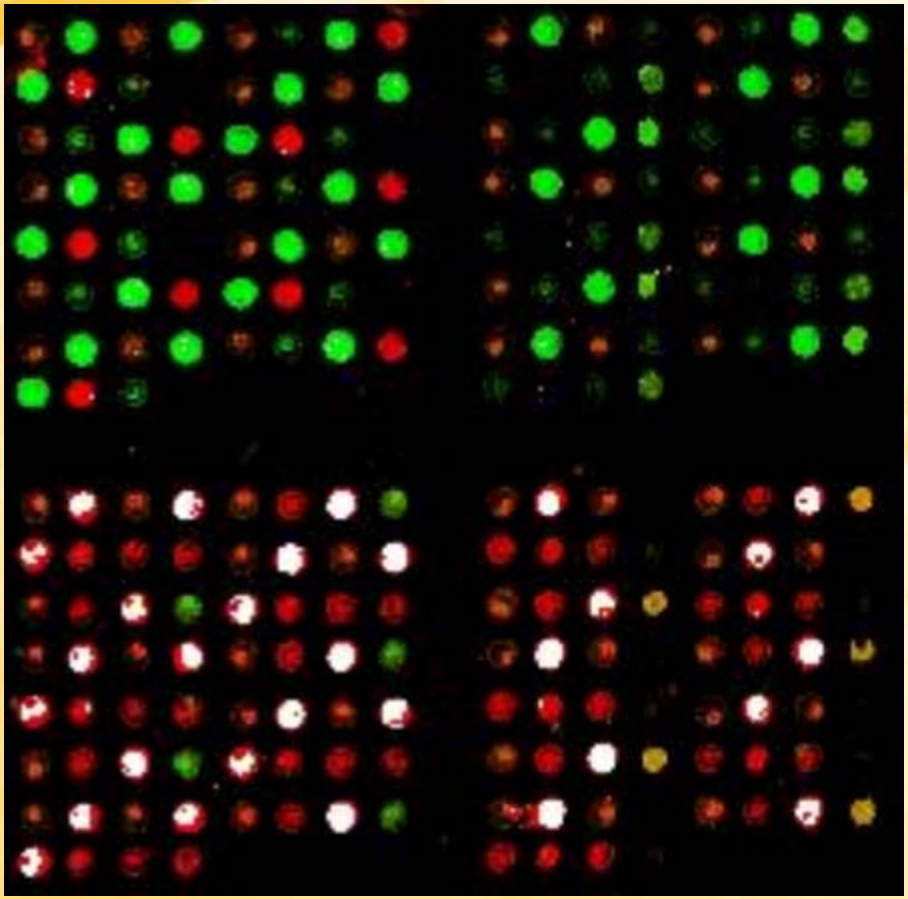
(c) Washing 5 min.

E. Mercado and J. Chin

(By reference of Ji et al, 2004)

Fig. Results of second trial
(Water washing treatment, 1:5 competitive hybridization)

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE



Green spots : Cy3 channel
Alexafluor 647
***Xa5* allele (Susceptible)**

Red spots : Cy5 channel
Alexafluor 546
***xa5* allele (Resistance)**

Yellow spots
***xa5* + *Xa5* allele (Hetero)**

E. Mercado and J. Chin

Fig. Overlay figure of results using *xa5* R and S probes labeled by Alexaflor 546 and Alexafluor 647 (partly)
– 16 hrs, 60C with competitive hybridization (1:5)

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

Troubleshooting

- Low specificity between *a* and *b* allele?
  - ✓ Shorter probe
  - ✓ Chaperon concept (Iwasaki et al., 2002)
  - ✓ Other functional SNP
  - ✓ Normalization of activity of labled probe
- Different value of replicated spots?
  - ✓ Normalization of concentration of DNA and/or PCR products
  - ✓ Check arrayer pinset and contamination
  - ✓ Level of volume of PCR products in 384-well plate
  - ✓ Automatic dispensing

Cost estimation of MBG

Table. Comparison between MBG and gel-based system (Polyacryl amide 100-well vertical gel system) for partners

	Microarray	Hub-lab of MBG	PCR GEL-based
COST (/96 samples)	18 USD	<18 USD	14 USD
COST (/9,600 samples)	~1,900 USD	<1,500 USD	~1,500 USD
EQUIPMENT	HIGH	0	LOW
Infra-structure	LOW	0	HIGH
Time & labor (samples/person/day) without PCR	~12,000	~ 0	~600
Knowledge	MEDIUM	Shuttle training	BASIC

Summary and Conclusion

1. Microarray-based genotyping (MBG) is useful for detect single nucleotide polymorphism (SNP).

2. Readily available software for data management enable MBG to be more convenient to data acquisition.

3. Every SNP probe may have different critical conditions for better performance, which must be optimized (applicability only to hub-lab).

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

Advantages and disadvantages

Advantages

1. Simple concept
2. Relatively cheap for large number of samples
3. Predictable error
4. Supporting software

Disadvantages

1. False hybridization
2. Too sensitive image detection
3. Cost of initializing

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

ACKNOWLEDGEMENT

- Former developers
  - D.Skinner and B.Collard
- E. Mercado
- M. Bernardo
- and… C. Vera Cruz

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE