

# Dot Blot Assay

## INTRODUCTION

One method for identifying desired DNA fragments is through hybridization with a specific probe. This is based on the idea that complementary base pairing between DNA strands only occurs when there is a high level of similarity between the probe sequence and the DNA sequence. This idea is used in Southern and Northern blots, to detect genomic and mRNA sequences. It is possible to adapt this idea to identify specific SNP alleles, using probes designed for this purpose. Allele Specific Hybridization (ASH) aims to use a small, labeled probe to detect one allele of a SNP specifically and then identify that allele using the label on the probe.

Dot blots have been used to detect SNP polymorphisms in rice (Shirasawa, 2006) and polymorphic haplotypes in *Brassica* (Fujimoto, 2003) as well as for other applications in DNA detection. Dot blot hybridization is a very simple way to assay SNP polymorphisms without large investments in equipment. The use of a digoxigenin (DIG) label allows non-radioactive labeling and detection, which improves safety and decreases cost of waste disposal. DIG is a small molecule derived from the *Digitalis* plant (foxglove), and there are antibodies that recognize DIG very specifically. The detection method for the dot blot can either be chromogenic (producing a color change) or chemiluminescent (producing light energy which can be detected using film) depending on the enzyme substrate. The cost of the technology is similar to the use of traditional SSRs. The dot blot method cannot be multiplexed for different loci, but the process can be made more high-throughput by multiplexing the PCR step, and by spotting a high density of genotypes onto the membrane.

## OVERVIEW

Dot blot genotyping begins by PCR amplifying a region of the genomic DNA around the SNP for each plant to be genotyped (PCR target).

Following PCR, the DNA fragments must be attached to a solid support for the detection steps. The PCR product is first denatured by heating, to give single stranded DNA, and then a small amount of the PCR product is spotted onto a positively charged nylon membrane. If detection of both alleles is desired, two identical blots must be made. The single DNA strands are bound to this membrane ready for hybridization.

The two allele specific probes, which have a DIG molecule attached, are used separately on a single blot. They bind specifically to the complementary DNA target sequence, with specificity dependant on several factors. Below are examples of two allele specific probes for a SNP locus, each with a 5' DIG label.

```
A > TATATATATCCTGACTCTCGATCTGGCATACTTAGGTAGCAGCATCAGGAATAATCC
      GCTAGACCGTATGAATCCAT-dig

B > GATATATATCCTGACTCTCGATCTGGCACACTTAGGTAGCAGCATCAGGAATAATCC
      GCTAGACCGTGTGAATCCAT-dig
```

The DIG label is then bound to an antibody. The antibody has an enzyme attached to it, alkaline phosphatase, which changes a detection substrate from colorless to purple, identifying each spot where hybridization occurred. Each spot represents a positive result.

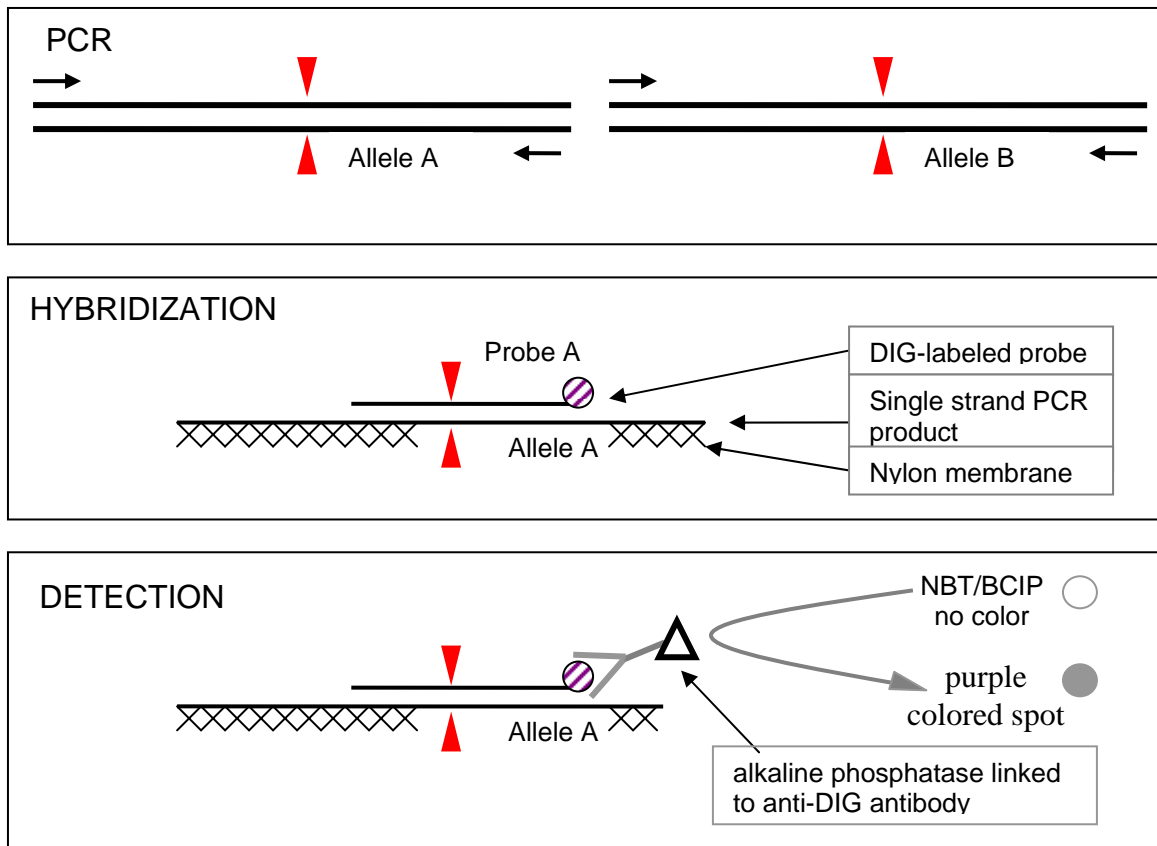
## ASSAY DESIGN

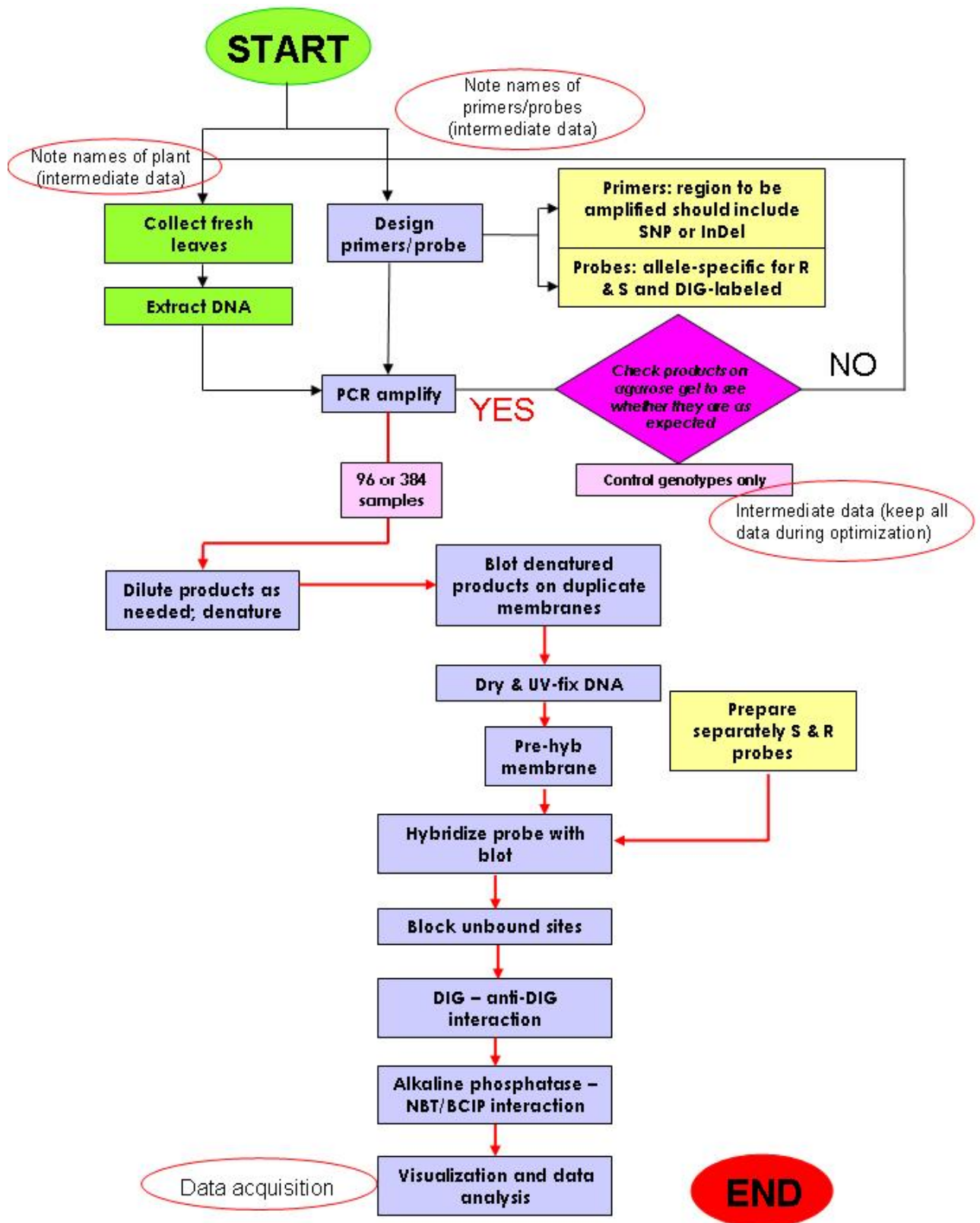
**PCR targets:** The amplified region around the SNP can be from 300 bp up to 1.2 kb long. The sequences should not be shorter than 300 bp as the products need to be denatured, and smaller products will renature quickly. The presence of single stranded DNA is essential for good hybridization. With long products, it is important to avoid other sites where the probe might bind (cross-hybridization).

**Probes:** The probe specificity depends on the difference in melting temperature between a perfectly matched probe and one with a mismatch. To maximize this difference, the probes must be short when detecting a 1 or 2 base SNP. Probes of 17 – 20 bp work well, and the polymorphism must be placed at the center of the probe.

**Hybridization conditions:** The hybridization temperature depends on the length of the probe and the components of hybridization buffer. For 18 and 20 bp probes a hybridization temperature of 42 – 45 °C is used with the Roche DIG EasyHyb buffer. For the homemade buffer included in this manual, the temperature is higher, 55 – 58 °C.

## SCHEMATIC DIAGRAM





## FLOW CHART FOR DOT BLOT ASSAY

Version 3.1 - 4/3/2007

## PROTOCOL

### I. PCR amplification of sample DNA using designed primers (This section will not be performed during the workshop proper)

#### Xa21 amplification from rice DNA

Materials – see Appendix 7 for primer and product info

- DNA (50 ng/μl)
- Xa21 Forward and Reverse primers (50 ng/μl)
- 10X PCR Buffer: 100mM Tris-HCl (pH 8.3), 500mM KCl, 15mM MgCl<sub>2</sub>, and 0.1% gelatin
- dNTPs (2mM)
- Taq polymerase (5units/μl)
- Sterile distilled water (sdwater)
- Micropipets, tips, tubes
- PCR machine

#### PCR cocktail

<b>component</b>	<b>μl</b>
sdwater	12.8
10X PCR Buffer	2.0
2mM dNTPs	2.0
50 ng/μl Xa21 Forward primer	1.0
50 ng/μl Xa21 Reverse primer	1.0
Taq polymerase (5units/ μl)	0.2
	<hr/>
	19.0
DNA (50ng/ μl)	1.0
	<hr/>
	20.0

#### PCR Profile

Step	Temperature (°C)	Time
1	94	4 min
2	94	1 min
3	55	1 min
4	72	2 min
5	34 more times to Step 2	
6	72	8 min
7	4	
8	END	

During optimization of the assay, check your products on gels to ensure even amounts of product made, and to estimate the amount of PCR product to be spotted.

#### Opaque2 amplification from maize DNA

Materials – see Appendix 7 for primer and product info

- DNA (approx 10 ng/μl)
- O2SNP3F1 Forward and O2SNP3R2 Reverse primers (10 μM)
- 10X PCR Buffer (without MgCl<sub>2</sub>)

- 25 mM MgCl<sub>2</sub>
- dNTPs (2.5 mM each)
- Taq polymerase (5 units/μl)
- Sterile distilled water (sdwater)
- Micropipets, tips, tubes
- PCR machine

#### PCR cocktail

<b>component</b>	<b>μl</b>
sdwater	7.2
10X PCR Buffer	2.0
MgCl <sub>2</sub> - final [2 mM]	1.6
2.5 mM dNTPs	2.0
O2SNP3F1 primer	1.0
O2SNP3R2 primer	1.0
Taq polymerase (5units/ μl)	0.2
	<hr/>
	15.0
DNA (10 ng/ μl)	5.0
	<hr/>
	20.0

#### PCR Profile

Step	Temperature (°C)	Time
1	94	3 min
2	94	45 sec
3	58.5	1 min
4	72	1 min
5	34 more times to Step 2	
6	72	3 min
7	10	
8	END	

During optimization of the assay, check your products on gels to ensure that even amounts of product is made across samples, and to estimate the amount of PCR product to be spotted.

## II. Blotting of PCR products

### Materials

- PCR products
- sdwater
- PCR machine
- Ice
- Centrifuge (microplate plate rotor needed if samples are in microplate)
- 2 μl micropipet if available
- Multichannel pipet, 2-20 μl volume (optional)
- Tips
- Positively charged nylon membrane, 1 blot/probe (for a 96-sample format – 11.0 x 7.5 cm)
- Forceps
- UV-crosslinker or oven

## Procedure

1. Dilute PCR products 1:20 for Xa21, 1:10 for O2 (determined empirically) with water, in 0.5ml tubes or PCR plate, mix well.

*After denaturation, volume may be decreased so make extra volume.*

2. Denature PCR product for 5 min at 95°C (use PCR machine). Transfer immediately to ice, cool quickly for 2 min and spin down.
3. Mark blot (using pencil) with probe to be used. Blot 2 µL of diluted PCR product on positively charged nylon membrane. Make duplicate blots for each allele. Do not let the tip touch the membrane. Air dry.

*Make sure the tips do not touch the membrane. This will give consistently shaped spots.  
If you have access to a 0.5ul multichannel, adjust dilution and use smaller volumes.*

*A spotting guide can be useful for large blots, for example a plexiglass guide for the pipette, or placing the membranes over a printed guide and using a light box to show where to spot.*

4. Fix DNA to the membrane using a UV-cross linker. (preset 0 = 0.120 kjoule, or 'autocrosslink' on 'Stratalinker'). The membrane can be stored dry between sheets of filter paper at room temp.

*0.120 kjoule is optimal dosage for DNA attachment and hybridization signal sensitivity.  
An alternative to fixing DNA with UV light is to bake the membrane either at 120°C for 30 min or 80°C for 2 hours.*

## **III. Prehybridization and Hybridization**

### Materials

- 2 units Incubator shaker - set to 42/45°C (**Xa21/O2**) and 60/55°C (at least one incubator required)
- Hybridization buffer (Roche DIG Easy Hyb), pre-warmed at 42/45°C
- labeled blots (with target DNA fixed)
- 3 containers, preferably just a little bigger than blots (heat resistant, can be tightly sealed)
- 10 µM Oligo probe stocks:
  - **Xa21**: SNPDB\_513-530\_Xa21R (resistant probe) and SNPDB\_513-530\_Xa21S (susceptible probe)
  - **O2**: O2SNP2pAT2 (donor *opaque2* probe) and O2SNP2pTC2 (normal probe)
- micropipets
- PCR machine
- 15 ml and 50 ml sterile disposable tubes
- Low Stringency Buffer: 2x SSC + 0.1% SDS for next day
- High Stringency Buffer: 0.5x SSC + 0.1% SDS, preheated to correct wash temperature (60/55°C) for next day

### NOTES

*Before the start of prehybridization, hybridization, and washing steps, there are a few things that need to be prepared (can be done ahead of experiment schedule):*

- *Determine the appropriate hybridization temperature ( $T_{hyb}$ ) according to the characteristics of your probe, target DNA, and hybridization buffer (See Appendix 8).*
- *Determine how much hybridization buffer you will need (for both prehybridization and hybridization).*
- *Determine the correct wash temperature for the High Stringency wash. Use the guidelines in Appendix 8.*

- Pre-warm hybridization buffer in sealed container with appropriate  $T_{hyb}$ .
- Preheat the High Stringency buffer to the correct wash temperature.
- Recommended reagent volumes are good for a pair of blots.

### Procedure

1. Pre-hybridize blots together at  $T_{hyb} = 42^{\circ}\text{C}$  using 20 ml of pre-warmed DIG-Easy Hyb buffer in small container for at least 30 minutes with shaking at 50 rpm.

*Make sure there are no bubbles. Seal container tightly to prevent hyb buffer from releasing  $\text{NH}_4$  which changes pH of incubation.*

2. Prepare the probe:
  - Dig-labeled oligo probes should be resuspended in TE pH 8 to a stock solution of 200  $\mu\text{M}$ .
  - Prepare a 50  $\mu\text{l}$  working stock of 10  $\mu\text{M}$  in water and store at  $-20^{\circ}\text{C}$ . Prepare several tubes and store frozen, to avoid several thaw cycles and contamination.

*Digoxigenin is light sensitive. Protect from light by wrapping some aluminum foil around the tube.*

3. Denature probe at  $68^{\circ}\text{C}$  for 10 minutes in PCR machine or water bath. Chill probe quickly on ice for 2-5 minutes.
4. Immediately add 10  $\mu\text{l}$  of the probe to 10 ml prewarmed hyb buffer in hybridization container. Mix the hybridization solution thoroughly. Do the same for the other probe in a separate container.

*Use  $x \mu\text{l}$  of 10  $\mu\text{M}$  probe in  $x \text{ ml}$  of hyb buffer.*

5. Remove blots from prehyb container and quickly transfer one blot to each hybridization container. Refreeze and reuse prehyb buffer up to 3 times.

*Do not allow the blot to dry at any time during prehybridization, hybridization and detection procedures to prevent high background. Remove prehyb container from oven only when the hyb solution is ready. Avoid adding the unmixed probe-hyb solutionsolution directly to the blot to prevent the probe from unequally hybridizing in one area of the blot.*

6. Hybridize with shaking at  $T_{hyb}$  for at least 6 hours or up to overnight (16 hours).

*At this point, put high stringency buffer into  $60^{\circ}\text{C}$  incubator shaker.*

*Do NOT discard the hyb/probe solution! It can be used 3-5 times. Store covered in a 50 ml tube at  $-20^{\circ}\text{C}$  and label with the number of uses. Before next use heat hyb/probe solution to  $65^{\circ}\text{C}$  to denature.*

## **IV. Washing detection and visualization of probe-target hybrids**

### Materials

- 40 ml Low Stringency Buffer: 2x SSC + 0.1% SDS
- 40 ml High Stringency Buffer: 0.5x SSC + 0.1% SDS, preheated to correct wash temperature ( $60/50^{\circ}\text{C}$ )
- Roche Blocking Reagent
- 60 ml Washing Buffer: 0.1 M Maleic acid, 0.15 M NaCl, pH 7.5; 0.3% (v/v) Tween 20

- 30 ml Blocking Buffer: 0.1M Maleic acid, 0.15 M NaCl, pH 7.5; 0.5% blocking reagent
- Antibody solution: Centrifuge Anti-DIG-AP for 5 min at 10,000 rpm and pipet 2  $\mu$ l carefully from the surface. Add into 10 ml of Blocking Buffer (1:5000 dilution) in 50 ml tube and mix thoroughly.
- 50 ml Detection buffer: 0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20°C)
- NBT/BCIP detection solution: add 150  $\mu$ l Roche NBT/BCIP stock solution to 7.5 ml Detection buffer
- TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0
- Sterile graduated cylinders or graduated tubes (15ml and 50 ml) for measuring reagents

### Procedure

1. Before beginning washes, make Washing Buffer and Blocking Buffer (this needs to cool to room temp before use!)
2. Wash blots twice with 20ml low stringency buffer for 5 minutes each at room temperature with shaking. Blots can be combined into one container at this point.

*Perform all washes at 50 rpm/gentle shaking*

3. Wash twice with 20 ml high stringency buffer for 15 minutes each at 60°C with shaking.
4. Wash blot with 20 ml Washing Buffer for 2 minutes at room temperature with shaking at 50 rpm.
5. Block with 20 ml Blocking Buffer for 30 minutes at room temperature with shaking.

*Blocking can last to 3 hours without affecting results*

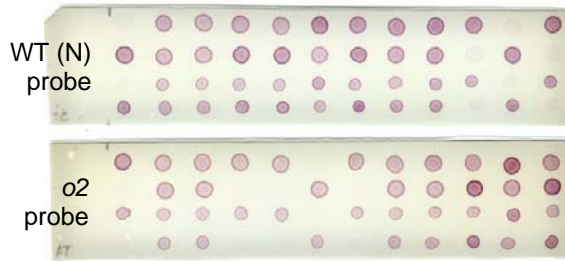
6. Discard Blocking buffer and add Antibody solution. Incubate 30 minutes with shaking at room temperature.
7. Wash twice (15 minutes each) with 20 ml Washing Buffer at room temperature with shaking.
8. Equilibrate (soak) membrane for 3 minutes in 20 ml Detection buffer with shaking.
9. Discard detection buffer. Distribute the NBT/BCIP detection solution over the blot making sure that all it is well covered.

*For multiple blots, process blots separately.*

10. Incubate in the dark without shaking. Check colour development every 20 minutes. When control spots have reached good intensity and background is low, stop the colour reaction by replacing the solution with TE and incubating for at least 5 min.
11. Scan or photocopy the blots immediately to preserve data, and store dried blots in dark. Score the blots: single spot on one blot = homozygous for that allele; spots on both blots = heterozygous.

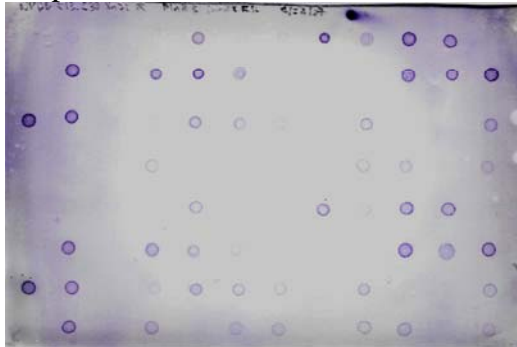
*If you want to make sure you are getting true, and not false, positives, you can also perform competitive hybridization as an alternative (see Appendix 9) to the dot blot process described above.*

Sample blots



Maize QPM inbred lines probed with the WT (normal) and o2 probes.

*Xa21* R probe



Rice bacterial blight breeding lines probed with *Xa21* R and S probes.

*Xa21* S probe

