

Microtiter Plate Assay (PCR-ELISA™) Genotyping for MAS

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Introduction

Microtiter Plate Assay/PCR-ELISA™

- Combination of two established techniques
- Polymerase Chain Reaction (PCR): amplifying the locus
- ELISA: differentiating the allele

Polymerase Chain Reaction (PCR)

- Kary B. Mullis (1993 Nobel Prize for Chemistry)
- Primers + *Taq* polymerase + dNTPs
- 30-35 cycles of denaturation, annealing, extension

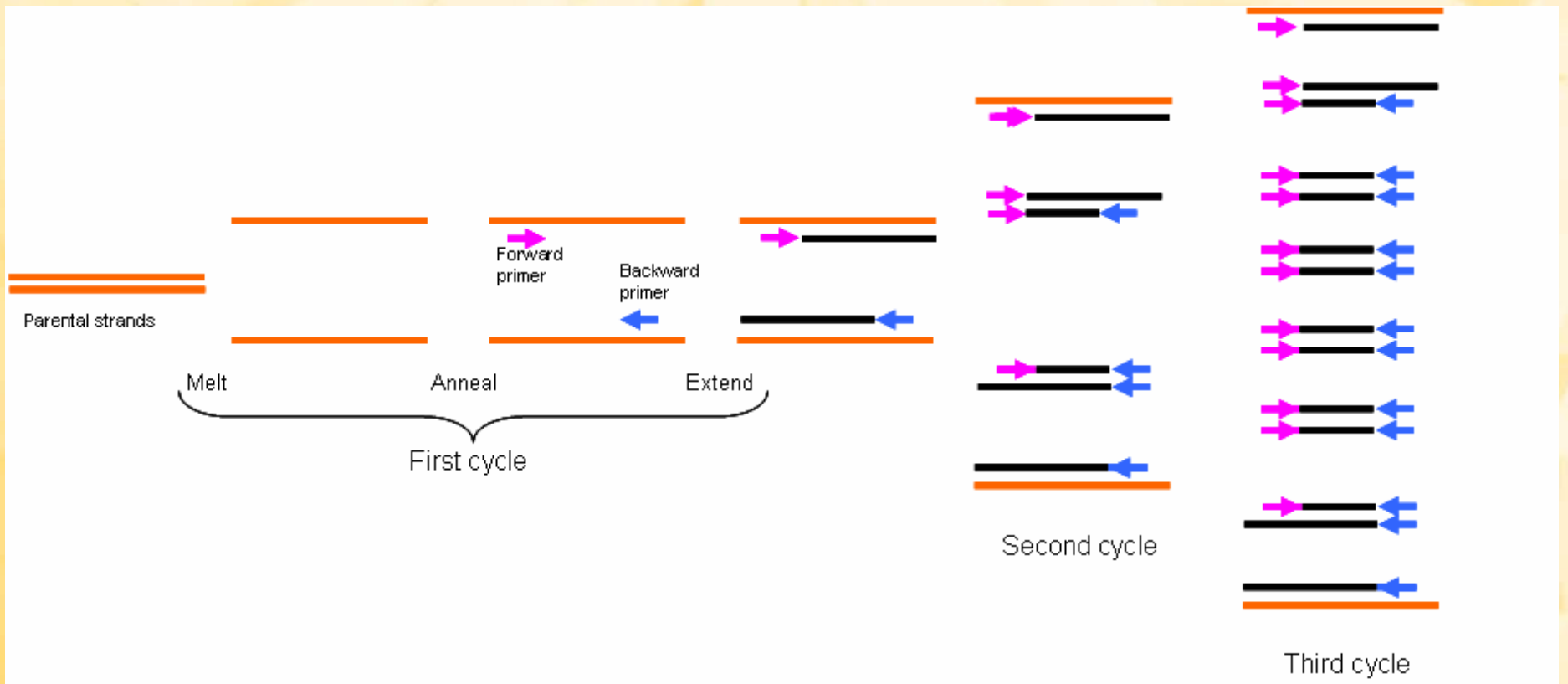


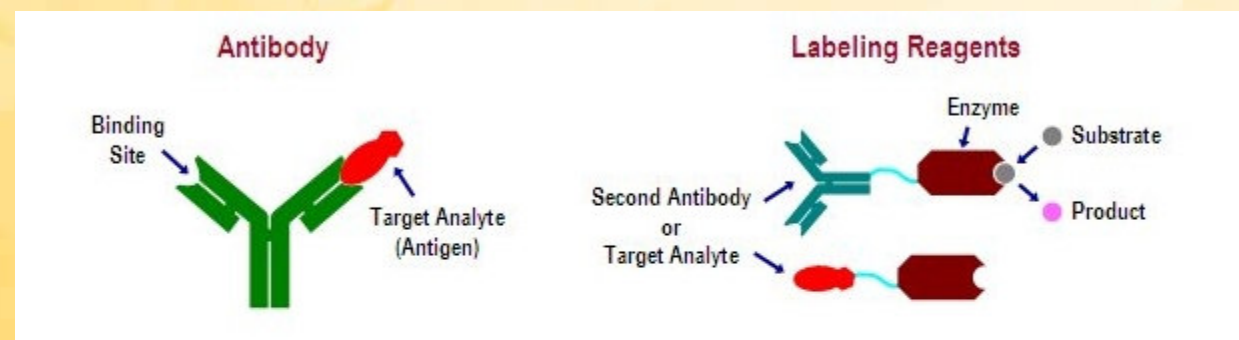
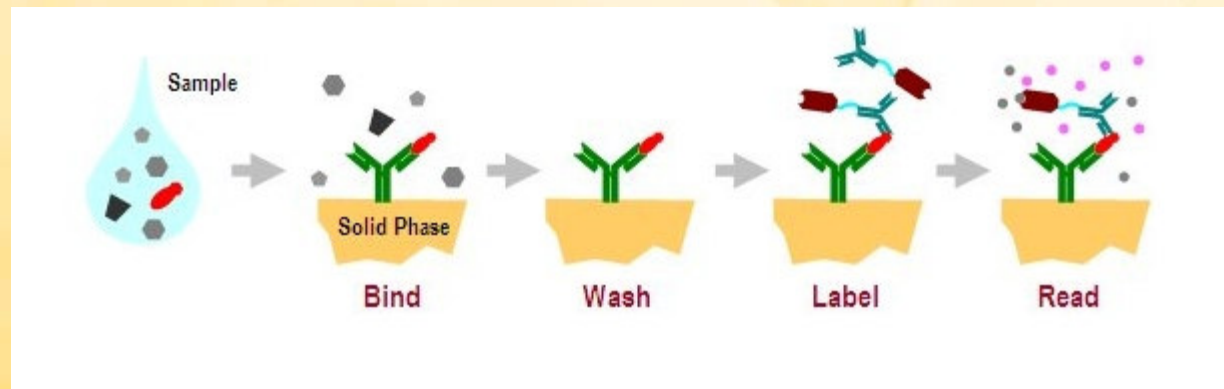
Illustration of polymerase chain reaction

ELISA

- Enzyme-Linked Immunosorbent Assay
- Specificity of antibodies
- Sensitivity of enzyme assays
- Indirect method of detection
- Virology and diagnostic medicine

Principle

ELISA Mechanism (Double Antibody Sandwich)



http://www.biosystemdevelopment.com/site_graphics/elisa.jpg

Combining the two techniques: PCR-ELISA™

- Labeling of products with DIG-11-dUTP during PCR
- Digoxigenin (DIG), a steroid only found in the leaves of *Digitalis purpurea* or *D. lanata* (Foxglove)
- Hybridization with specific biotin-labeled capture probe
- Detected using anti-DIG antibodies

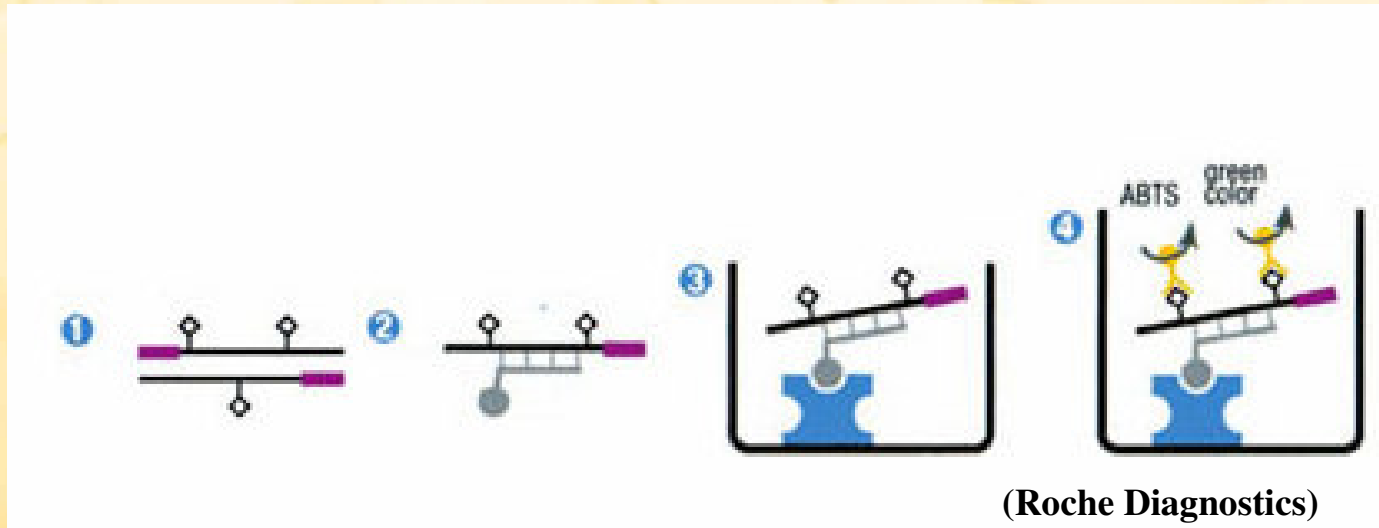


Illustration of PCR-ELISA using anti-DIG-POD antibodies and ABTS for detection

2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid)

PCR-ELISA

- DIG-11-dUTP: antigen for anti-DIG antibody
- Biotin:Streptavidin - allows probe-PCR product immobilization
- Anti-DIG antibody is conjugated to peroxidase (or phosphatase)
- Substrate-enzyme complex is indirect indicator

Workflow

IRRI

INTERNATIONAL RICE RESEARCH INSTITUTE

Lab Work Overview

Use *xa5* bacterial blight resistance gene

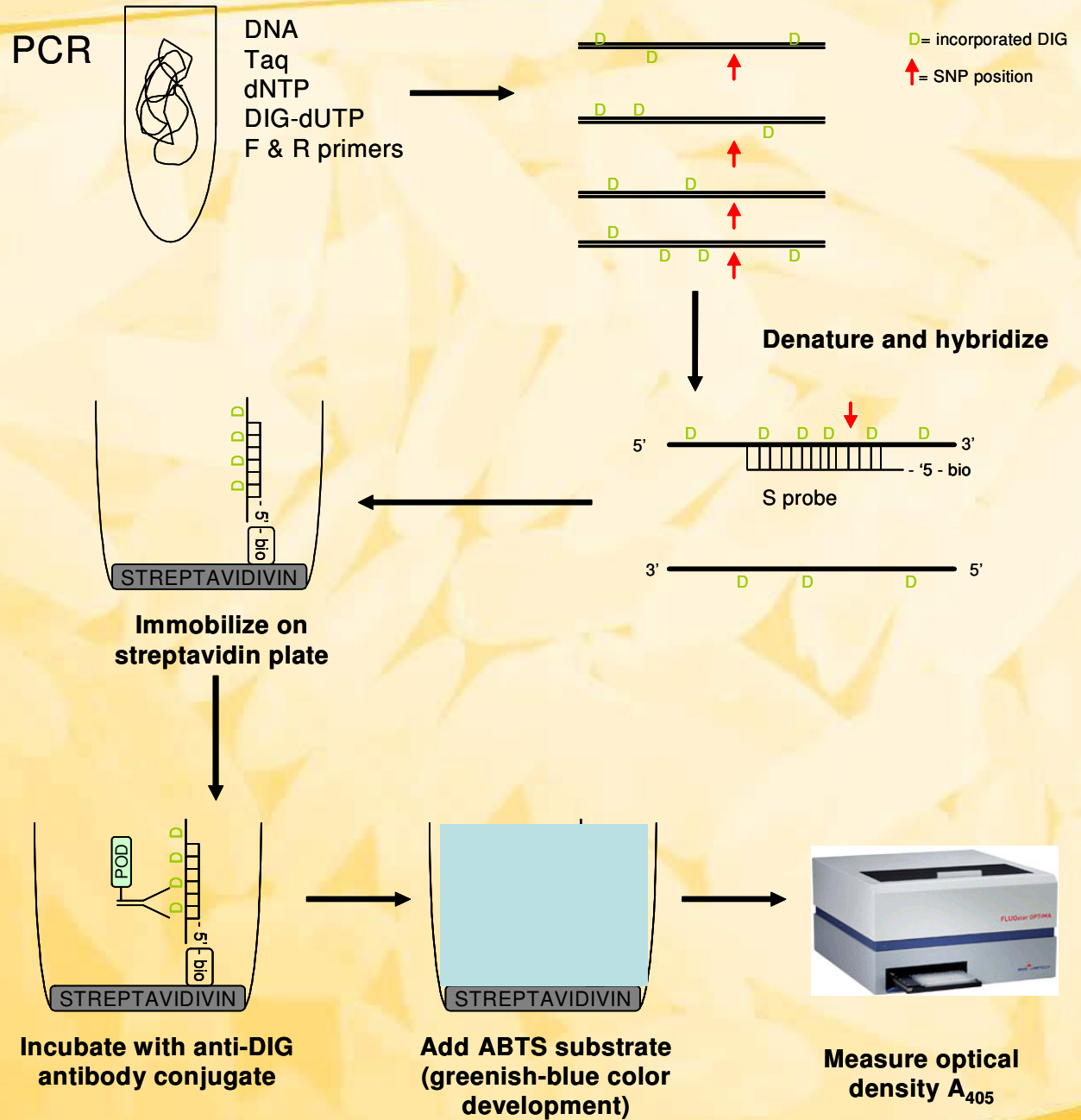
1. Amplify a segment (~275bp) of *xa5* gene (both R and S alleles amplified); Incorporate DIG-dUTP
2. Hybridize a biotin-labeled allele-specific capture probe for the S allele
3. Immobilize probe-PCR product complex to streptavidin coated surface (ELISA plate)
4. Detection of the incorporated DIG using immunoassay

xa5 Probes

Xa5 5' tcagcccgga gctcgccatt caagttcttg **tcca**gtttga taaggatatct 3'
Susceptible Allele

xa5 5' tcagcccgga gctcgccatt caagttcttg **agca**gtttga taaggatatct 3'
Resistant Allele

Schematic Diagram



For the Lab Work

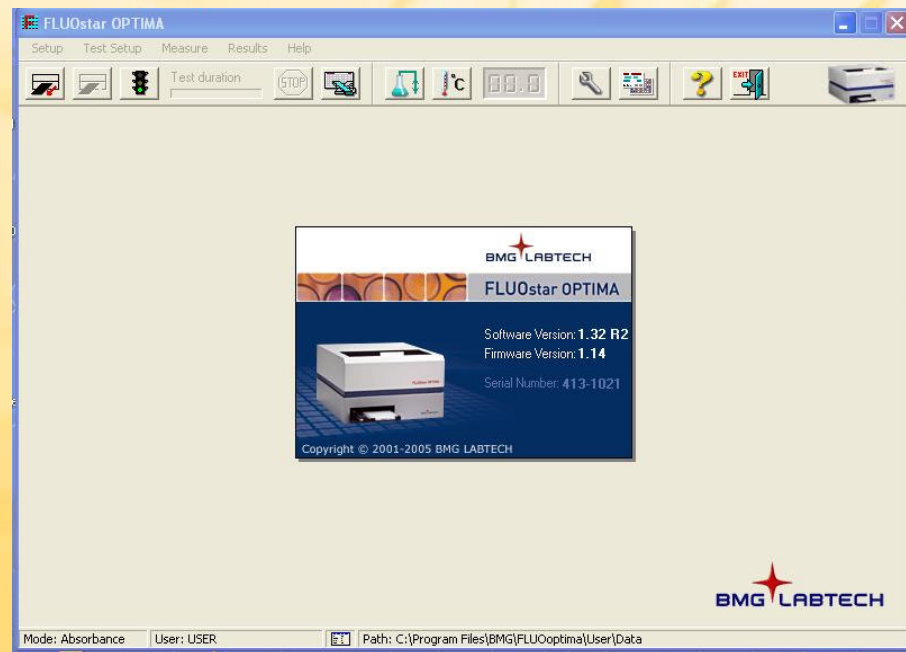
- Four groups
- Will not do PCR and dilution (done before hand)
- 13 samples (+ 2 controls)
- Start with Denaturation then Hybridization/Immobilization
- Detection/Immunoassay
- Data acquisition and Analysis

Data Acquisition & Analysis

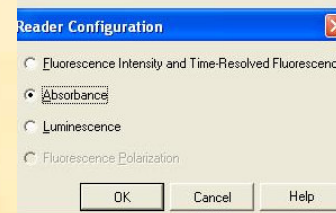
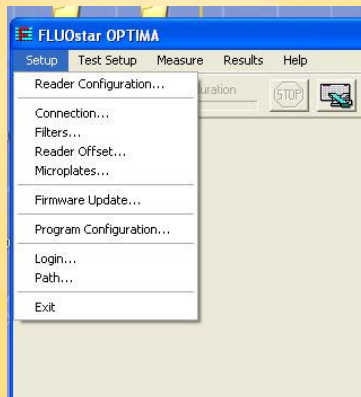
- Use FLUOStar Optima multiplate reader
- Absorbance $A_{405\text{nm}}$
- Use MS Excel (automatically generated by FLUOStar software)

Operation of multiplate reader

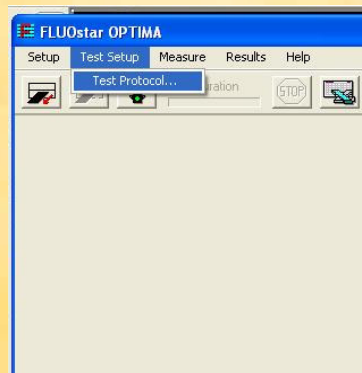
- Launch the Application: BMG LabTech FLUOStar Optima



- Click SETUP and select ABSORBANCE. Click OK. A dialogue box will appear telling you to manual turn the optics to the right position.



- Click TEST SETU P=> TEST PROTOCOL and select (double-click) protocol from the list.

A screenshot of the 'Test Protocols' dialog box. It contains a table with columns for Protocol Name, Microplate, Mode, and Config. The 'ABS TEST- 405' protocol is selected. Below the table are buttons for 'New', 'Edit', 'Copy', 'Export', 'Import', 'Delete', 'Close', and 'Help'.

Protocol Name	Microplate	Mode	Config
DSINGH FRET	GREINER 781091 UCLEAR 384	Plate	Fluorescence
FITC-TMR	BMG LABTECHNOLOGIES 96	Plate	Fluorescence
FRET TODAY	BMG LABTECHNOLOGIES 96	Plate	Fluorescence
FRET-JCHEN	BMG LABTECHNOLOGIES 96	Well	Fluorescence
FURA-2	BMG LABTECHNOLOGIES 96	Well	Fluorescence
JC	BMG LABTECHNOLOGIES 96	Plate	Fluorescence
260 280	BMG LABTECHNOLOGIES 96	Plate	Absorbance
260/280/ICDY	BMG LABTECHNOLOGIES 96	Plate Fly	Absorbance
384	GREINER 781091 UCLEAR 384	Plate	Absorbance
ABS QC	BMG LABTECHNOLOGIES 96	Plate	Absorbance
ABS TEST- 405	CORNING 25880 96	Plate	Absorbance
BRADFORDS	BMG LABTECHNOLOGIES 96	Plate	Absorbance
DNAQUANT_ABSORB-EVE	BMG LABTECHNOLOGIES 96	Plate Fly	Absorbance
ICDY ELISA	BMG LABTECHNOLOGIES 96	Plate	Absorbance

- Enter the appropriate microplate and filter.

The screenshot shows the 'Plate Mode (Absorbance)' software window. The 'Test name' is 'ABS TEST-405' and the 'Microplate' is 'CORNING 25880 96'. The 'General Settings' section includes 'Positioning delay' (0.5 s), 'Flying mode' (unchecked), and 'No. of kinetic windows' (1). The 'Kinetic Window 1' section includes 'Ng. of cycles' (1), 'Measurement start time' (0.0 s), 'No. of flashes per well and cycle' (20), and 'Cycle time' (50 s). The 'Filter Settings' section includes 'No. of multichromatics' (1), 'Excitation filter' (A-405), 'Emission filter' (empty), and 'Gain' (945). The 'Well Scanning' section is set to 'None'. The 'Pause before cycle' is set to 0 seconds. The window has tabs for 'Basic Parameters', 'Layout', 'Concentrations / Volumes / Shaking', and 'Injection Timing'. Buttons for 'Check timing', 'OK', 'Cancel', and 'Help' are visible at the bottom.

- Click the LAYOUT tab and input your layout.

Plate Mode (Absorbance)

Basic Parameters | Layout | Concentrations / Volumes / Shaking | Injection Timing

Content:
 Sample

Groups:
 On

Index:
Start value: 40
 Constant
 Increase

Replicates:
Number: 1
 Horizontal
 Vertical

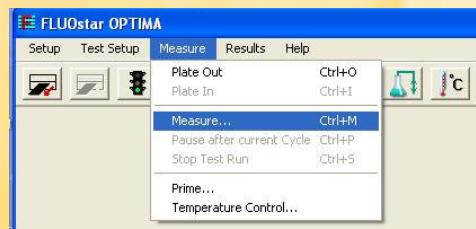
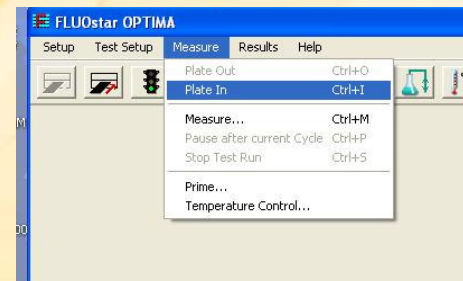
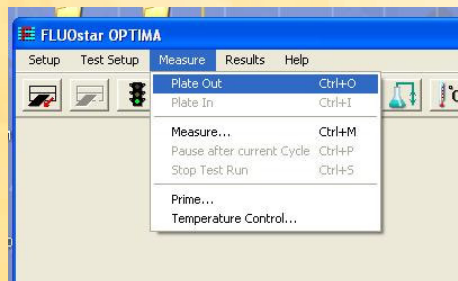
Reading direction:

96	1	2	3	4	5	6	7	8	9	10	11	12
A				X16	X17	X18						
B	N	N	N	X19	X20	X21						
C	P	P	P	X22	X23	X24						
D	X1	X2	X3	X25	X26	X27						
E	X4	X5	X6	X28	X29	X30						
F	X7	X8	X9	X31	X32	X33						
G	X10	X11	X12	X34	X35	X36						
H	X13	X14	X15	X37	X38	X39						

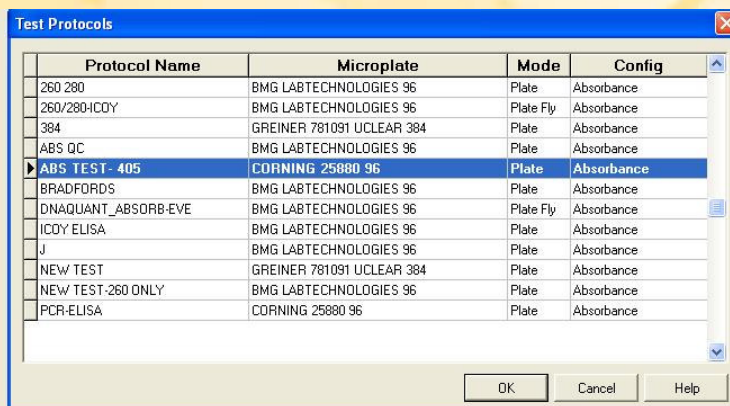
Check timing

OK Cancel Help

- Click OK. Click MEASURE and select PLATE OUT. Place microplate on tray and click MEASURE => PLATE IN. Click MEASURE => MEASURE.

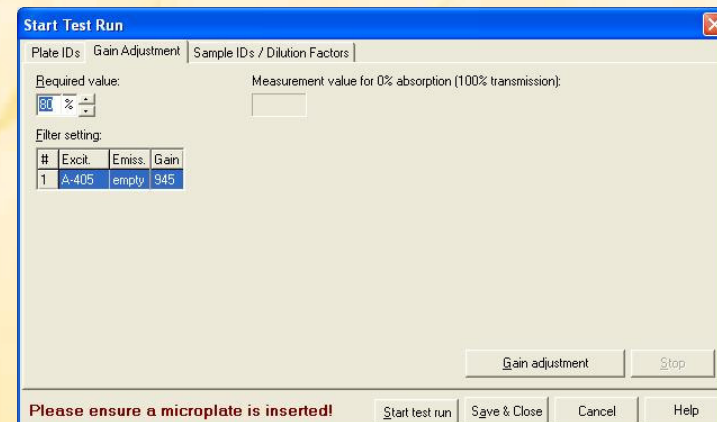
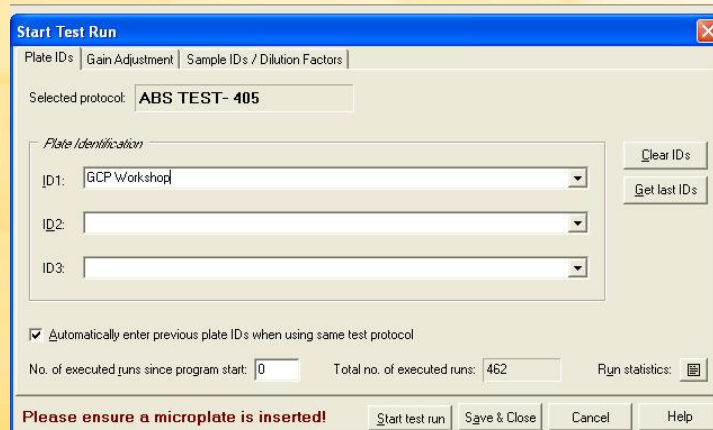


- Select (double click) protocol from list.



Protocol Name	Microplate	Mode	Config
260 280	BMG LABTECHNOLOGIES 96	Plate	Absorbance
260/280-ICOY	BMG LABTECHNOLOGIES 96	Plate Fly	Absorbance
384	GREINER 781091 UCLEAR 384	Plate	Absorbance
ABS QC	BMG LABTECHNOLOGIES 96	Plate	Absorbance
ABS TEST- 405	CORNING 25880 96	Plate	Absorbance
BRADFORDS	BMG LABTECHNOLOGIES 96	Plate	Absorbance
DNAQUANT_ABSORB-EVE	BMG LABTECHNOLOGIES 96	Plate Fly	Absorbance
ICOY ELISA	BMG LABTECHNOLOGIES 96	Plate	Absorbance
J	BMG LABTECHNOLOGIES 96	Plate	Absorbance
NEW TEST	GREINER 781091 UCLEAR 384	Plate	Absorbance
NEW TEST-260 ONLY	BMG LABTECHNOLOGIES 96	Plate	Absorbance
PCR-ELISA	CORNING 25880 96	Plate	Absorbance

- Enter name for experiment. Click GAIN ADJUSTMENT tab. Click GAIN ADJUSTMENT BUTTON at the bottom of the window.



- Click START TEST RUN. Software will acquire data and will produce an MS Excel file.

Analysis

From the MS Excel file generated, compute threshold value using the formula below:

- Threshold value, $TV = (\text{mean of negative controls}) + (3 \text{ SD of negative controls})$
- Consider samples:
 - a. negative if OD reading $< TV$
 - b. positive if OD reading $> 2 TV$
 - c. indeterminate if OD reading falls between the values of TV and $2TV$

Positive Control

Sus

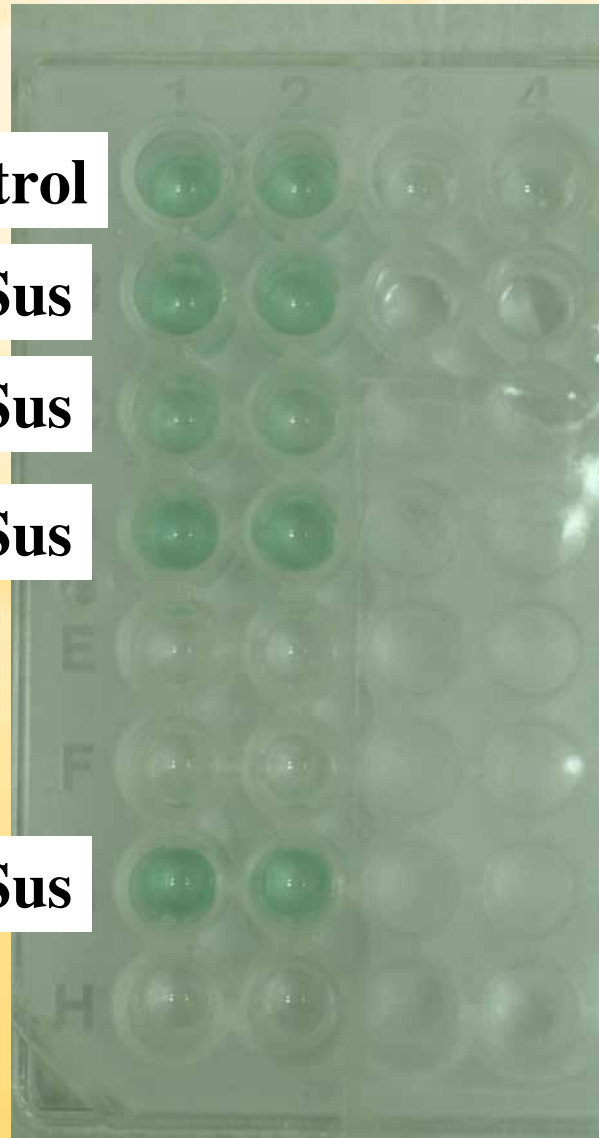
Sus

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Negative Control

Blank



Advantages & Disadvantages

Advantages

1. PCR step is not allele-specific (only one pair used for both alleles).
2. Needed: PCR machine, hybridization oven (incubator-shaker), commercially labeled primers, ELISA plate reader
3. Allows SNP markers to be used in low tech labs.
4. Assay time: PCR duration + ~3.5hr

Disadvantages

1. Sample handling: liquid transfers too tedious/source of contamination
2. Cost of antibodies and or kit (can be reduced by increasing 96 samples to 384)
3. Automation only in hub labs (auto-loader/gripper, plate washer, etc.)

Cost

- Roche kit: 192 samples (2x96) ~\$240
- Excludes cost of primers and probes
- Primers: ~\$1 per base
- Probe: ~\$40 biotin labeling

Investigate use of home made buffers and washes (and even home made strep coating)

Increase Ab dilutions

Trouble Shooting

**Instruction manual
PCR ELISA (DIG Detection)
Roche Cat. No. 11636111910**