



Monitoring veterinary drug residues in milk and milk products- present scenario in India



Processing and public health significance of antibiotic residues in milk and milk products

- Inadequate curdling and improper ripening of cheese during their manufacture.
- Decrease in acid/flavor production during manufacture of fermented dairy products.
- Reduced starter growth during propagation
- Milk from treated cows may contain large number of potential pathogens.
- Failure to respond to therapeutic dose of antibiotics.
- Allergic reactions.

Emerging Global requirements on quality and safety of dairy foods

- Development of codex standards as bench mark for export.
- Harmonization of Indian standards with quality norms recommended by codex.
- Food safety and standard act 2006

Present status on testing of antibiotic and drug residues at farm and processing levels

- No routine test for antibiotic and drug residues available at farm level. Contaminated milk is directly pooled into good quality milk.
- No well defined routine tests available at dairy processing units. Export consignments are normally checked from independent testing labs.

Limitation in using international kits for antibiotic and drug residues

- Highly expensive if applied at farms and processing levels
- Require huge infrastructure installation & skilled manpower
- High degree of false positive / negative results
- Poor sensitivity in detecting majority of antibiotic groups
- Problem in procurement of kits

Current tests available for monitoring drug residues in milk and milk products

- **Microbial inhibitor tests**
- **Test based on enzyme inhibition**
- **Immune receptor test**

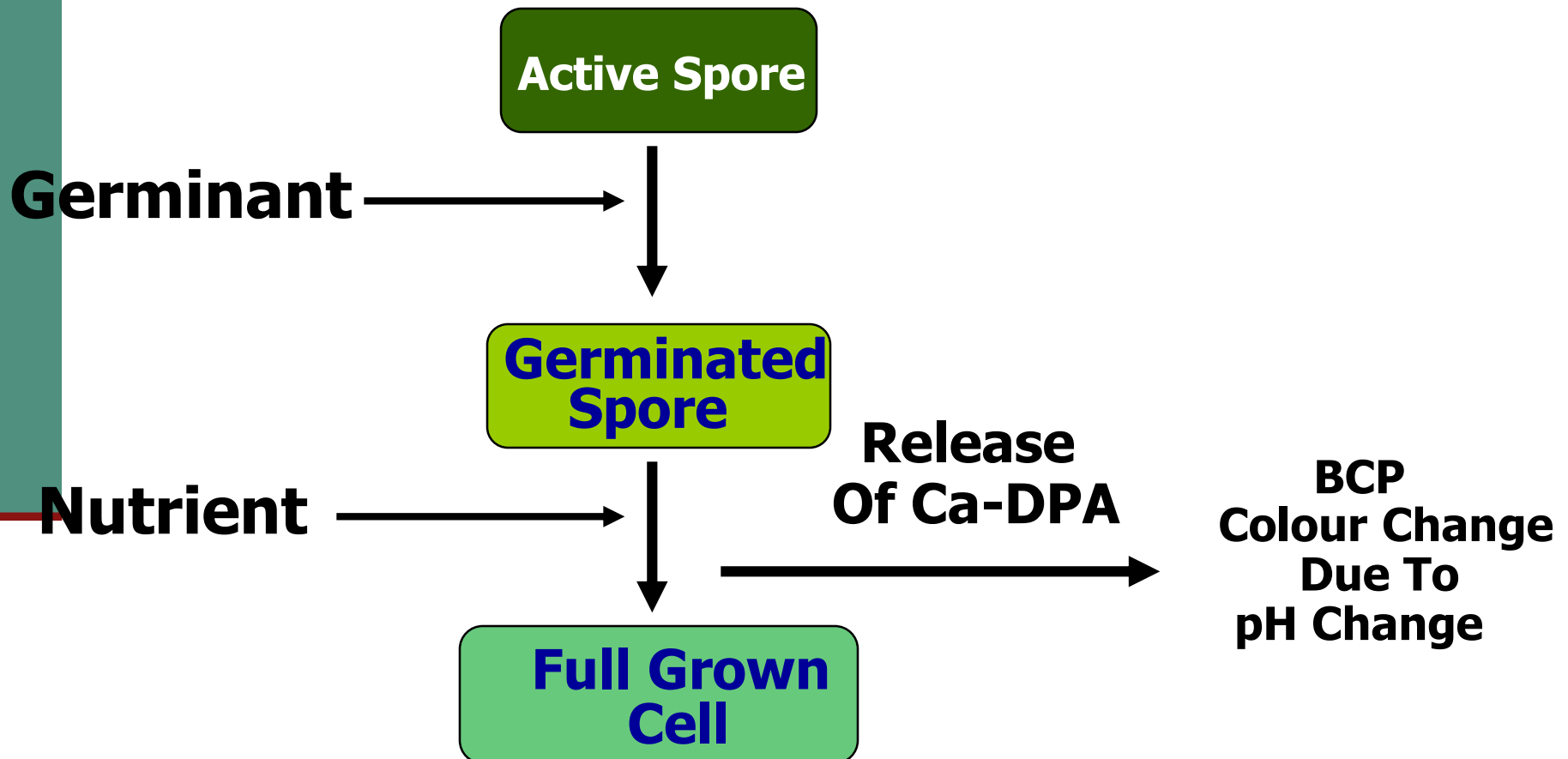
Microbial inhibitor tests

- ✓ The organism grows and can be detected visually either by **opacity of the agar growth medium or by a color change**
- ✓ Involved a **susceptible organism which** fails to grow in the presence of antibiotics
- ✓ tests are sensitive to **Beta-lactam group** and can be made to detect **sulfonamides and other antimicrobials.**
- ✓ Reliable, cost effective but require incubation for several hours.

Microbial based kits available for the detection of antibiotic residues in milk

Name of Microbial Inhibition Assays	Manufacturer	Time per test	Antibiotic tested/cost	Reaction type
Delvotest SP Kit (AOAC approved)	Gist-brocades /DSM Netherland	2. h 30 min-3h	Broad spectrum (Rs 100/- test)	Colour change
Copan test (tablet added into medium)	Copan Italia SpA	2 h 30 min -3 h	Broad spectrum (Rs 100/test)	Colour change
Charm Farm Test –liquid medium	Charm Sciences, USA	2 h 30 min -3 hr	Broad spectrum (Rs 100/-test)	Colour change

Detection of antibiotic residues in milk based on Inhibition principle



SAMPLE PREPERATION

1



Weigh exactly 30 mg of germinant mixture in to a clean test tube

2



Transfer 1.6 ml of milk into the test tube; mix the contents properly to dissolve.

3



Incubate at 100⁰ C for 3 min and cool it down to ambient temperature

TEST PROCEDURE

4



Transfer 25 μ L into kit.

5



Incubate the kits at 64⁰ C
2.30-3.00 hours

6



No color change indicates antibiotic (s) positive,
change in color indicates negative

RESULT

	Antibiotic /groups tested	EU MRL limits (µg/kg)	Level of detection (LOD) (µg/kg)
β-lactams	Penicillin Ampicillin Amoxicillin Cloxacillin Cefalexin Cephazolin	4 4 4 30 100 50	4 4 4 30 100-150 50
Sulphonamides	Sulphamethazine	100	100-150
Aminoglycosides	Gentamicin Neomycin Streptomycin Kanamycin	100 500 200 150	100 500 200 150-200

Antibiotic /groups tested		EU MRL limits (µg/kg)	Level of detection (LOD) (µg/kg)
Tetracycline	Doxycycline	0.0	0.1
	Oxytetracycline	100	100
	Tetracycline	100	100
Macrolides	Erythromycin	40	40
Miscellaneous group	Bacitracin	150	500
	Chloramphenicol	0.1	10
	Lincomycin	150	150
	Trimethoprim	50	50-100
	ofloxacin	75	100
	polymixin	50	100-500

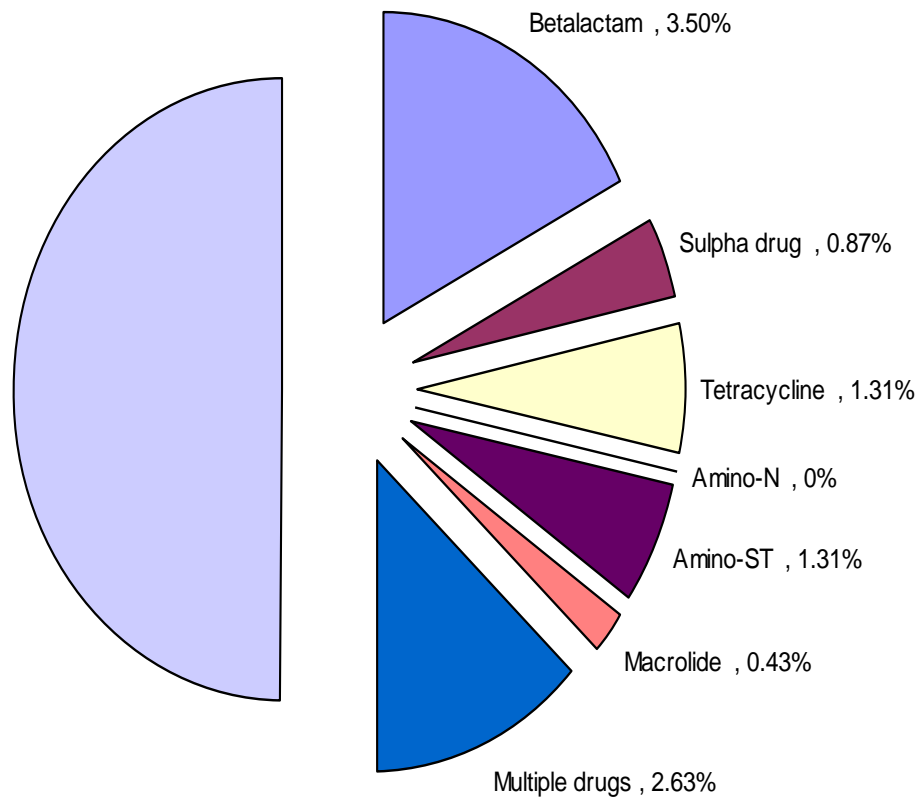
Peer validation

Objective: To study the result of different antibiotics spiked in milk with the kit.

	Antibiotics spiked in milk	Conc. In ppb	Result	EU MRL	Remarks
1	Penicillin	4	positive		
		10	positive	4	Complies to EU MRL
		0.5	negative		
2	Sulphadiazine	5	negative		
		100	positive	100	Complies to EU MRL
		200	positive		
3	Oxytetracyclin	5	negative		
		100	positive	100.00	Complies to EU MRL
		200	positive		
4	DSM Negative milk	0	negative		
5	Milk sample	0	negative		

Comparative screening of raw, heat treated & dried milk for antibiotic residues using broad spectrum Kit & charm assay at \geq MRL codex limits

The comparative analysis on 228 samples of milk and dried product procured from different sources revealed that our lab kit work with false positive results at 0.43% & false negative results at 1.19%. Total Incidence at MRL Level 10.08%



Novelty in patent process

(# ipr/4.9.1.4/0574 /1479/del/2006)

- Cost effective
 - Better sensitivity
 - Semi-quantitative detection
 - Minimum false positive /negative results
 - Validation with AOAC approved system
 - Insensitivity towards detergents / sanitizers
 - Stability of product under storage condition
 - Consistency in color development with in 3.0 hrs
 - Wide spectrum of application for different types of milk
-

Patenting and Licensing of Technology

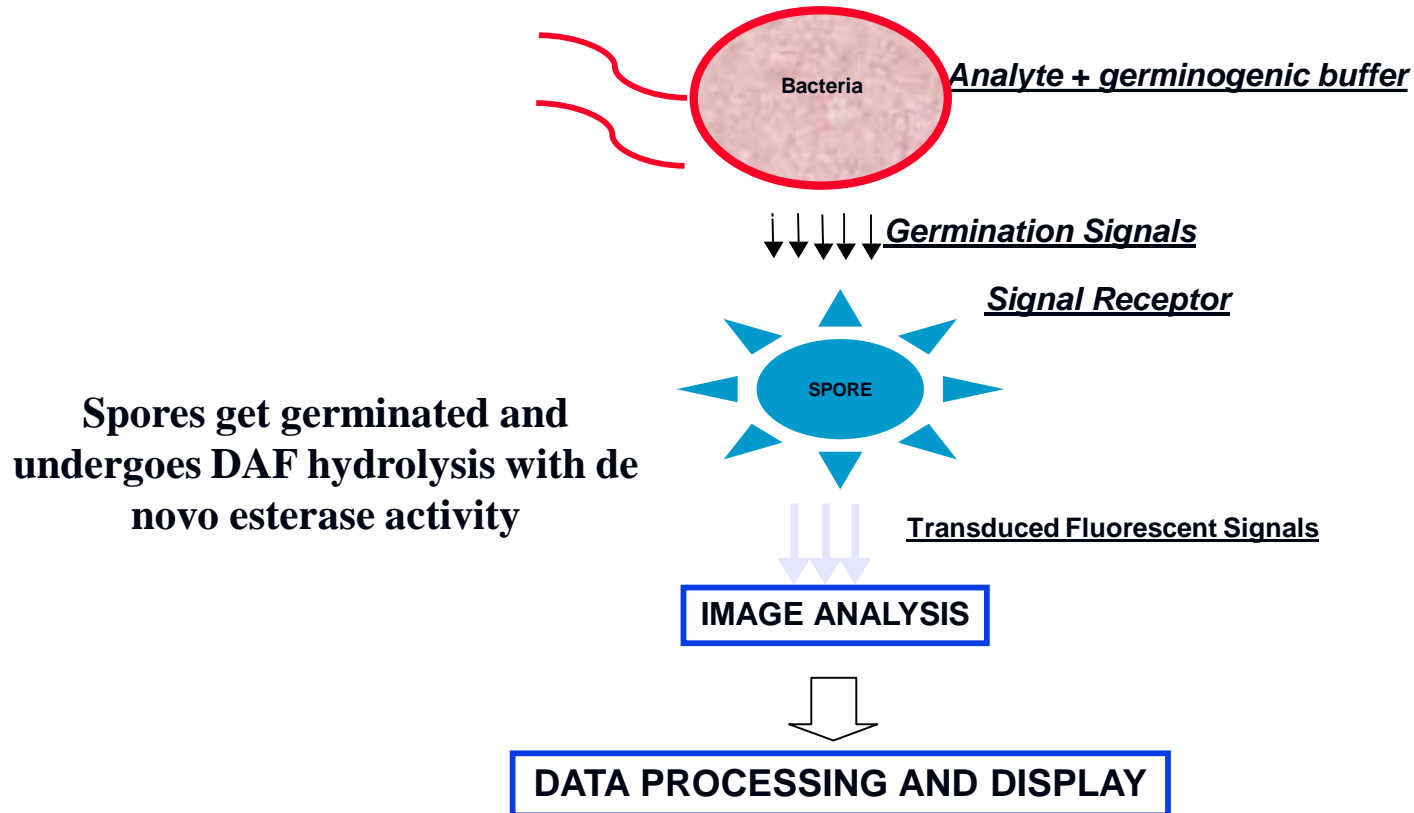
- Development of analytical process for detection of antibiotic residues in milk using bacterial spores as biosensor (patent # ipr/4.9.1.4/0574/1479/del/2006)
- The broad spectrum kit was commercialized to **M/S Neugen Diagnostics India PVT. Ltd.** with non-exclusive license fee of Rs. 2.50 lakhs, royalty 2.0% & license period for 7 years .

Detection of Aflatoxins M1 in milk based on Inhibition principle

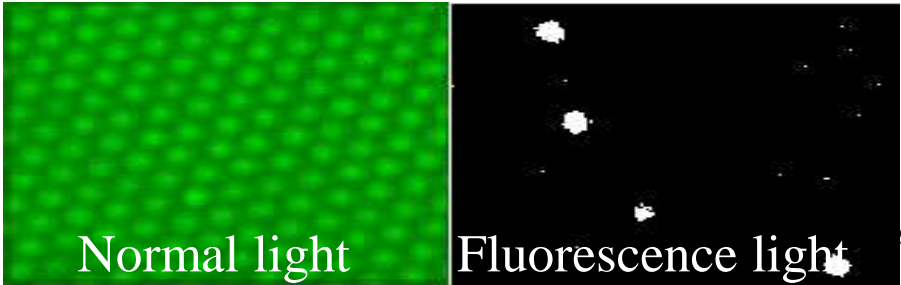
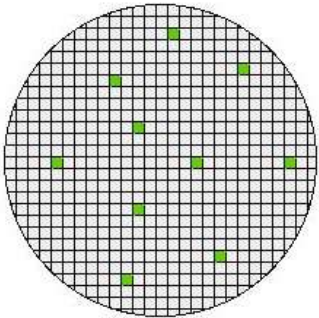
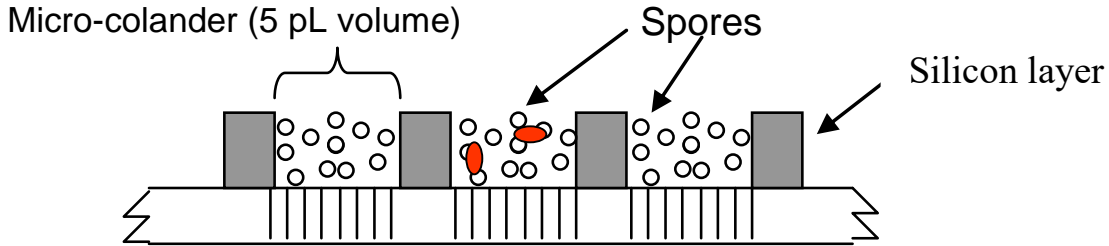
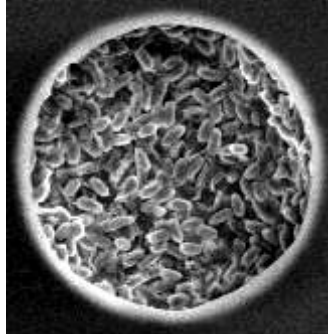
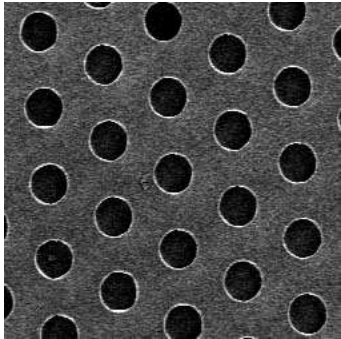
Six strains of *Bacillus megaterium* procured from IMTECH were screened for aflatoxin M1 sensitivity at different conc. One strain was found positive with zone of inhibition



Bacterial spores as biosensor for targeting hygiene indicator organisms : Operating principle



Schematic of Bio-Chip



Development of bacterial spore based operating system

Action Plan

Milk sample is processed & bacteria are separated



The bacterial suspension is mixed with the germinogenic substrate followed by micro spotting on bio-Chip



BioChip is incubated at 37°C



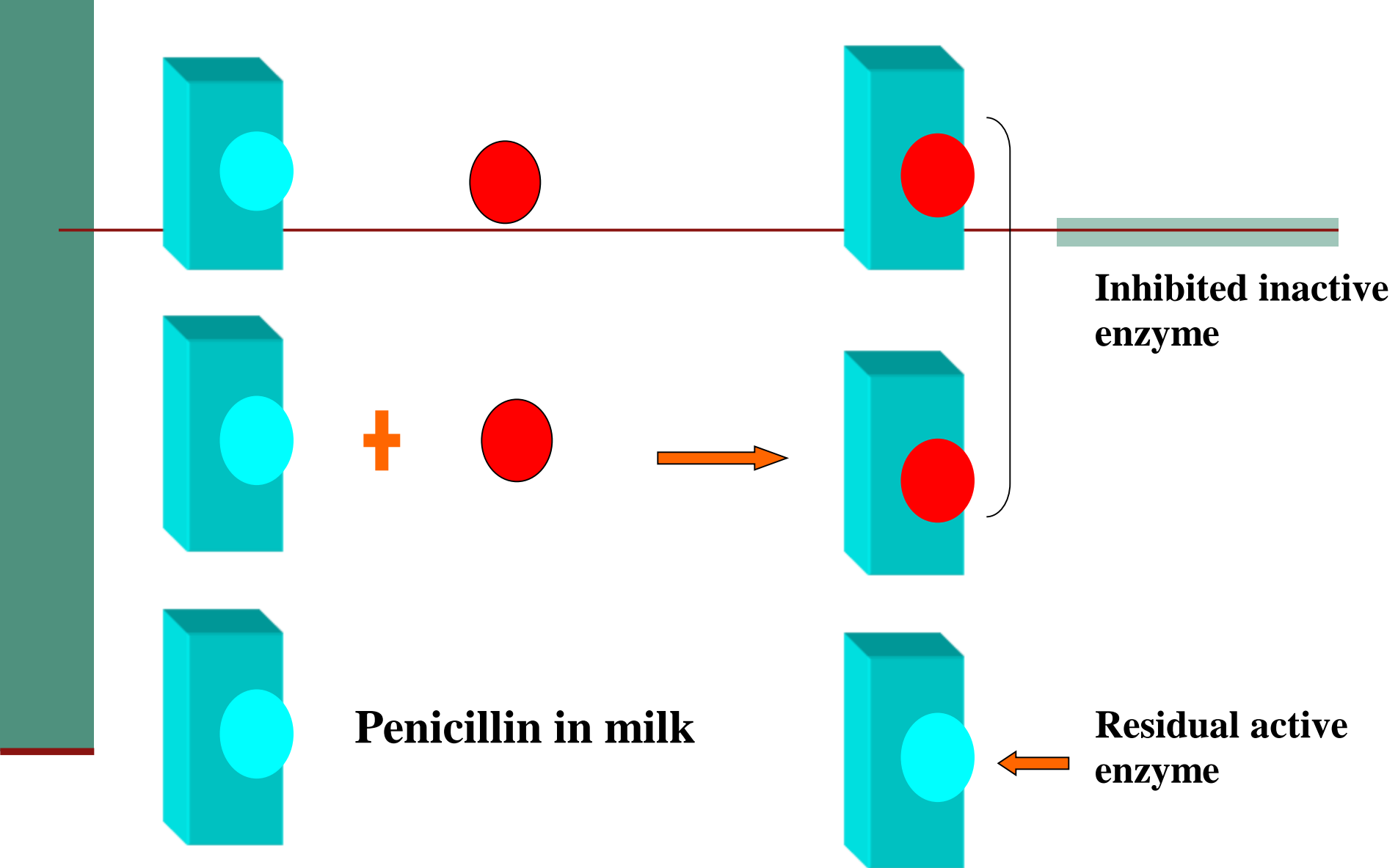
Fluorescent images of BioChip are captured at time intervals



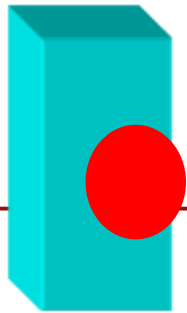
Digital images are processed to yield number of bacteria in the sample

Commercial Test method based on enzyme inhibition (UCB Bio-products, Belgium)

- ❖ **Principle: Beta lactam antibiotics prevent bacterial multiplication by inhibiting the activity of the enzyme DD – Carboxy-peptidase .**
- ❖ **DD–carboxy-peptidase activity liberates D -alanine from an enzyme substrate which is visualized by a color change occurs.**
- ❖ **Produces a pink color when a sample contains no antibiotics whilst a yellow color is interpreted as positive.**



**Active DD-
Carboxypeptidase**



+

Substrate



No reaction



+

Substrate



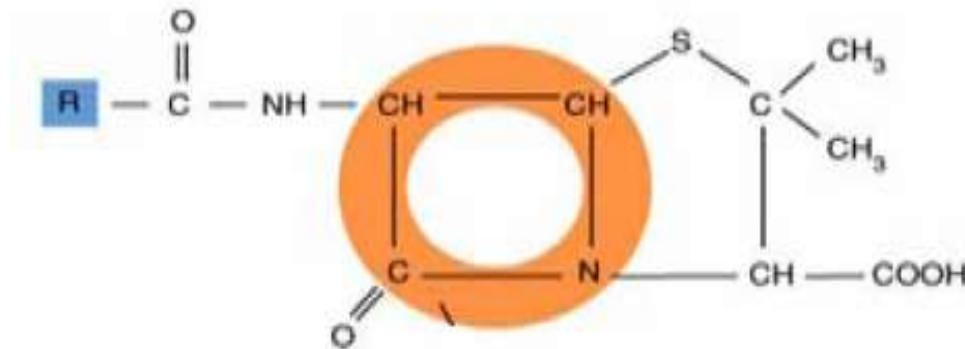
**Liberation of
D-alanine**



+ other
reagent

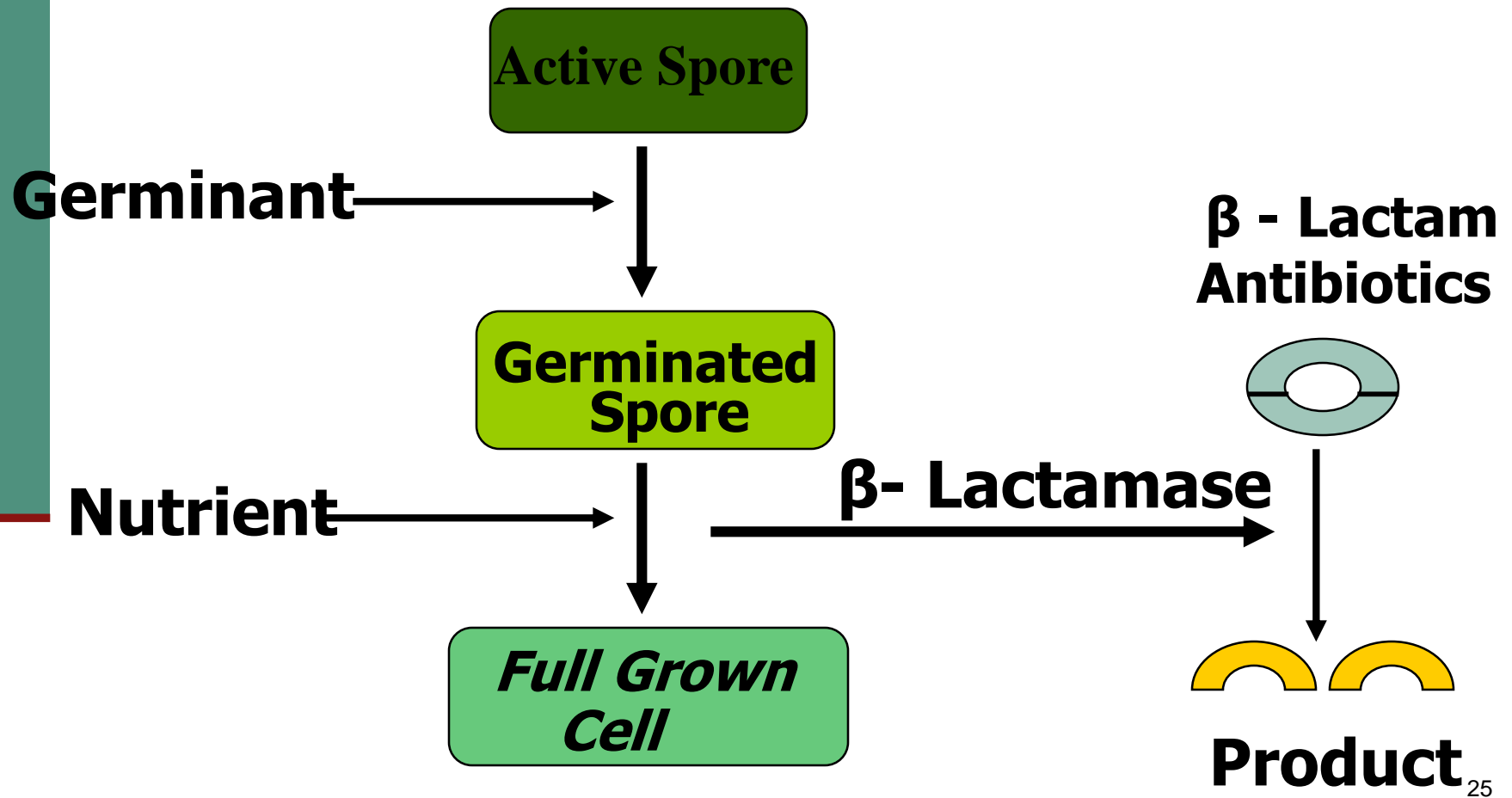
Pink colour

Development of β -lactam kit based on Iodometric assay principle



Iodometric method based on starch + iodine mixture

Development of β -lactam kit based on Iodometric assay principle



Development of kit prototype & working test procedure

SAMPLE PREPERATION

1



Weigh exactly 200mg of dextrose and 230 mg of whey powder into a clean test tube

2



Transfer 1.6 ml of milk into the test tube; mix the contents properly to dissolve.

3



Incubate at 100 ° C for 3 min and cool it down to ambient temperature

TEST PROCEDURE

4



Transfer 25 µ L into kit.

5



Incubate the kits at 37° C till complete germination takes place.

6



Transfer 3mg of Penicillin -G in 100µL of phosphate buffer and, reincubate the kits at 37 ° C for another one hour.

RESULT

7



Add 20µL of Starch and 75µL of Iodine solution into each kit.

8



Observe for the color change from blue to yellow.

9



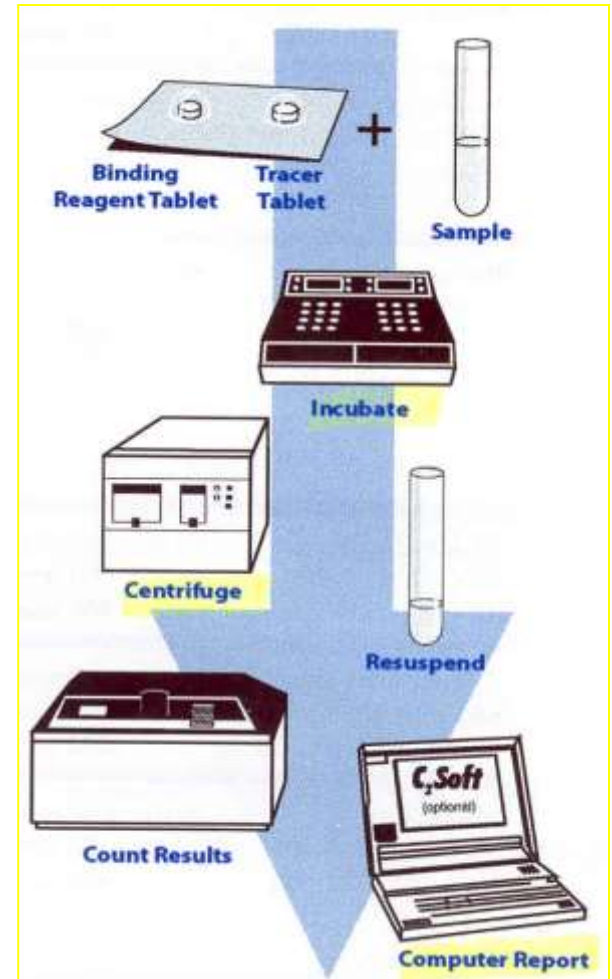
- ❖ Yellow indicates presence
- ❖ Blue indicates absence

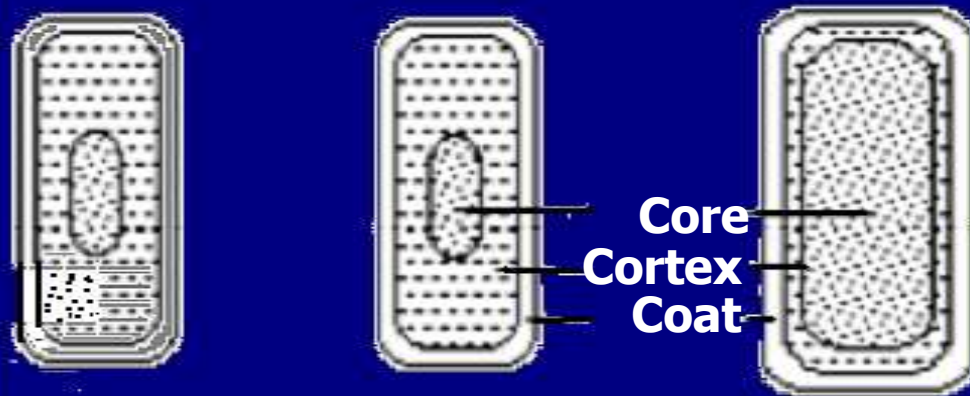
Commercially available immune receptor test

- Rapid test based on ‘immune receptor’ principle
- variation of the well-established ELISA test
- Specific target antibiotic group is captured by immobilized antibodies, or by a broader-spectrum receptor such as a bacteria cell.
- Most test involved a competitive principle .Antibiotics in the sample competes with an internal antibiotic standard for the immune receptor.
- The antibody-antibiotic complex is then usually linked to an enzyme that catalyses a color or fluorescence reaction

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- A comparison of the intensity of the test reaction with that of a control determines whether the sample is positive or negative.
 - Because of their competitive principle, a low intensity usually ‘positive’ whilst a high intensity is regarded as ‘negative’ immune receptor test
 - can be made quantitative but are generally used to provide a pass/fail result.
 - More expensive than microbial inhibitor tests but only detect substances that react immuno-logically with the immobilized receptor and they provide a result in less than 10 min.

Detection of specific group of beta lactam antibiotics using Charm 6602 system





Activation

Germination

Outgrowth

Sporulation



**Dormant
Spore**



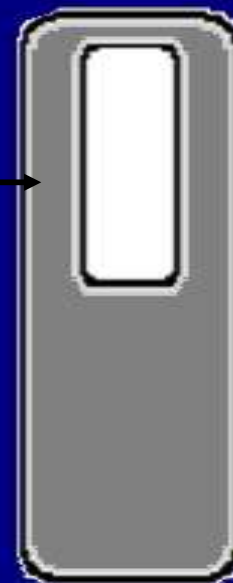
**Activate
Spore**



**Germinated
Endospore**



**Vegetative
Cell**



**Sporulated
Cell**