Diagnosis of Fungemia using Molecular Methods

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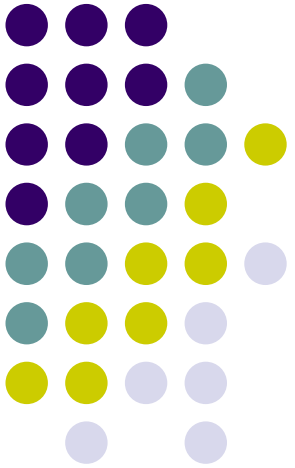
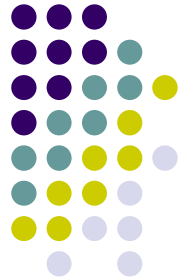


Sepsis Diagnosis

- Blood culture, the gold standard in diagnosis of bacterial and fungal BSI, typically becomes positive 8-36 h after sampling.
- The therapy is based on Gram-stain characteristics.
- A more precise pathogen identification and susceptibility profile is not available until up to 24-48 h (minimum).
- For a significant number of patients with clinically apparent FUNGAL sepsis the blood culture is negative, making difficult the optimal antimicrobial therapy

Molecular Methods for Sepsis Diagnosis



- Advantages on using molecular methods as compared with the traditional culture
  - Phenotypic changes do not affect the results, as it can occure in cultures
  - Species can be accurately identified
  - Short time for diagnosis and identification
  - Most of the molecular methods present sensitivity and specificity higher than culture

Shoham et al. Ther Clin Risk Manag. 2008 Jun;4(3):637-40.
Forrest et al. J Antimicrob Chemother. 2006 Jul;58(1):154-8.

Molecular Methods for Sepsis Diagnosis

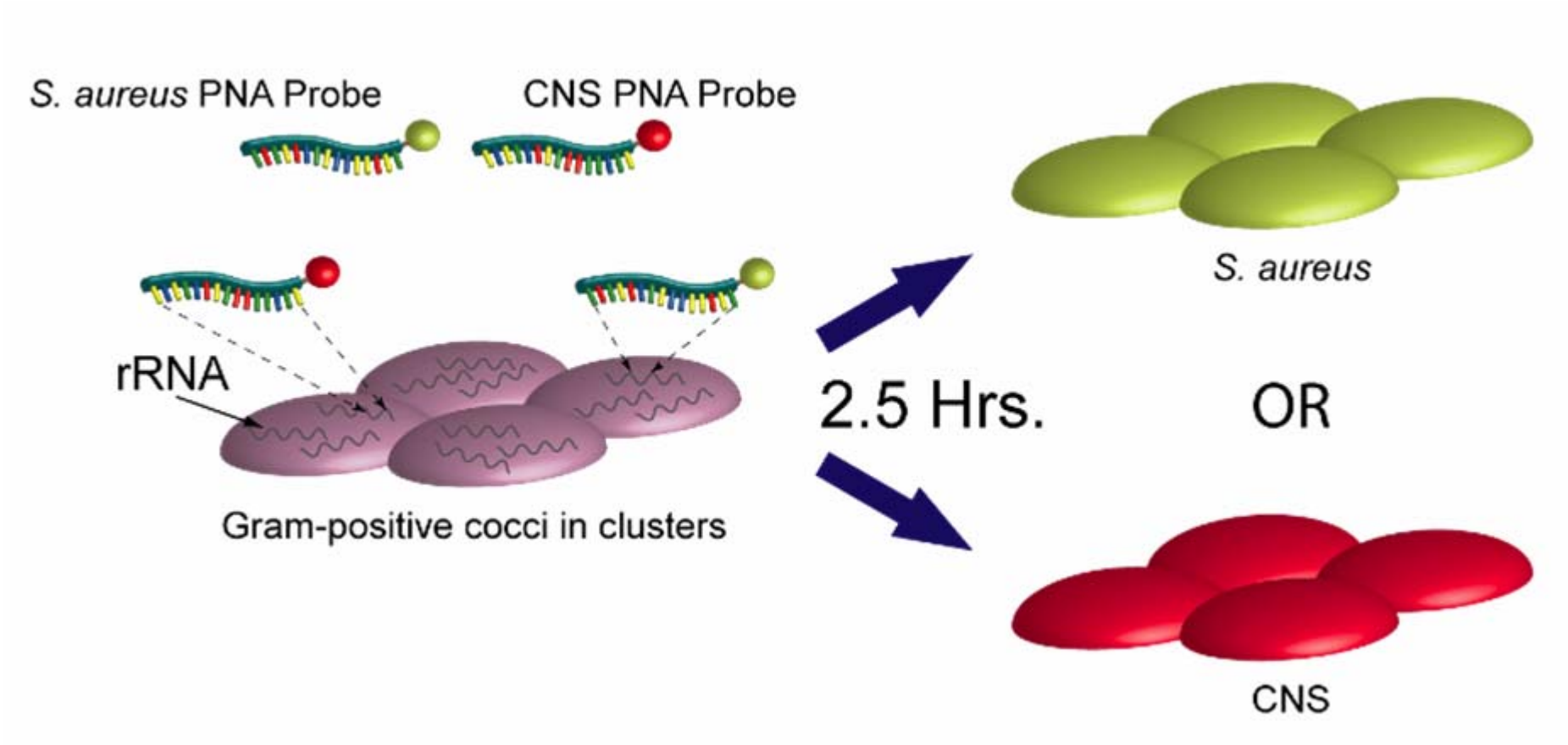
- Method available in Brazil cleared by ANVISA
  - Septi*Fast* (Roche)
- Method submitted to ANVISA clearance
  - PNA-FISH (AdvanDx, USA)
- Methods under validation for clinical samples by the manufacturers
  - Luminex (fungi)
  - Ibis technology (bacteria and fungi)

PNA FISH test

(AdvanDx, Inc.Woburn, MA,USA)

PNA - FISH

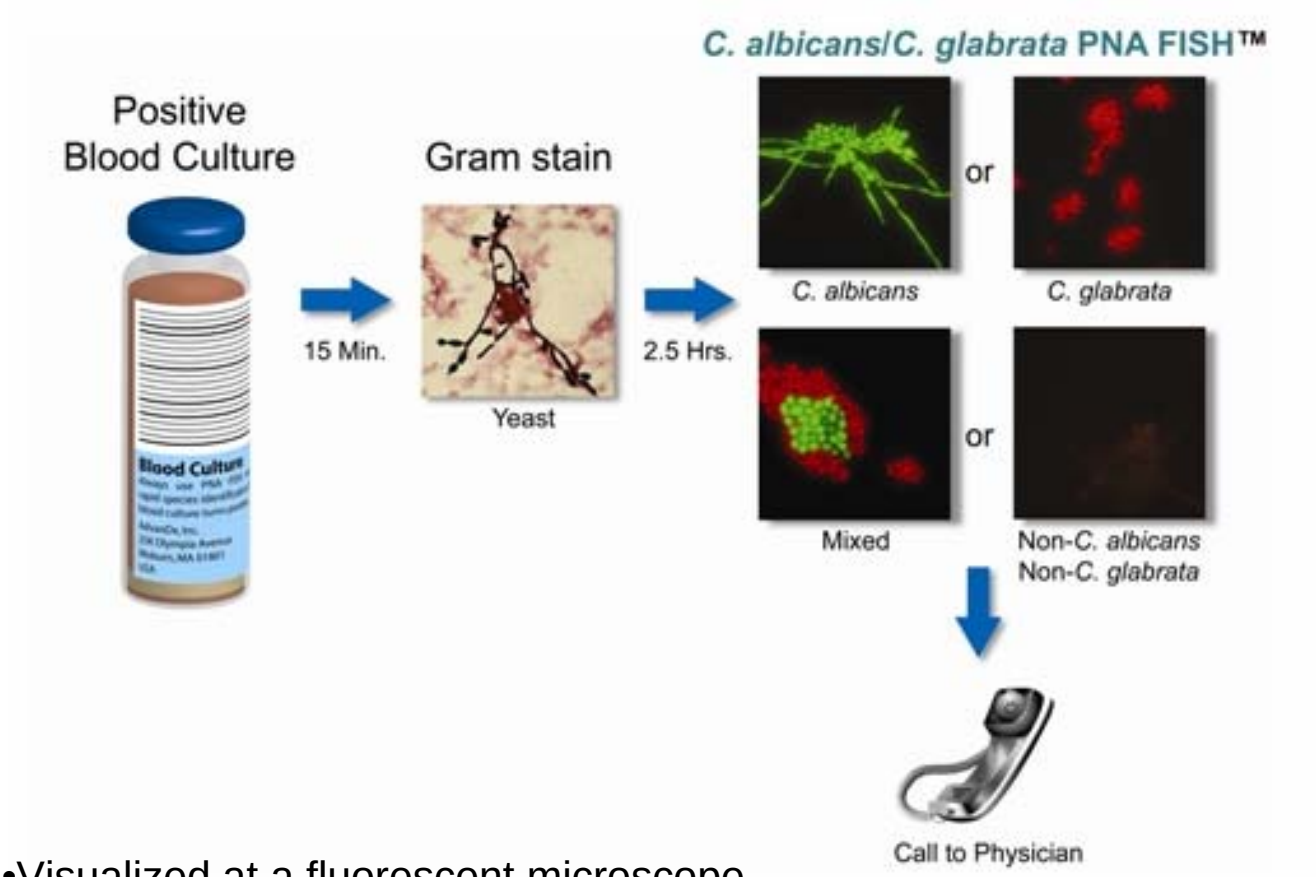
(Peptide Nucleic Acid Fluorescence In Situ Hybridization)



Short time for visualization

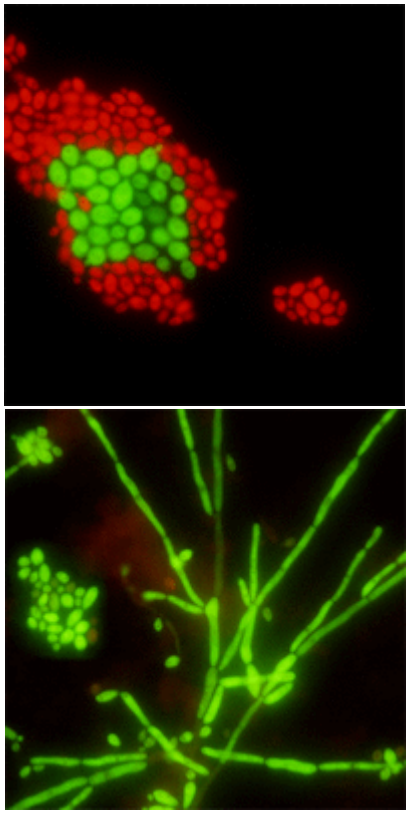
PNA FISH test

(AdvanDx, Inc.Woburn, MA,USA)



- Visualized at a fluorescent microscope
  - Blood culture is tested directly on a slide
  - Species identification

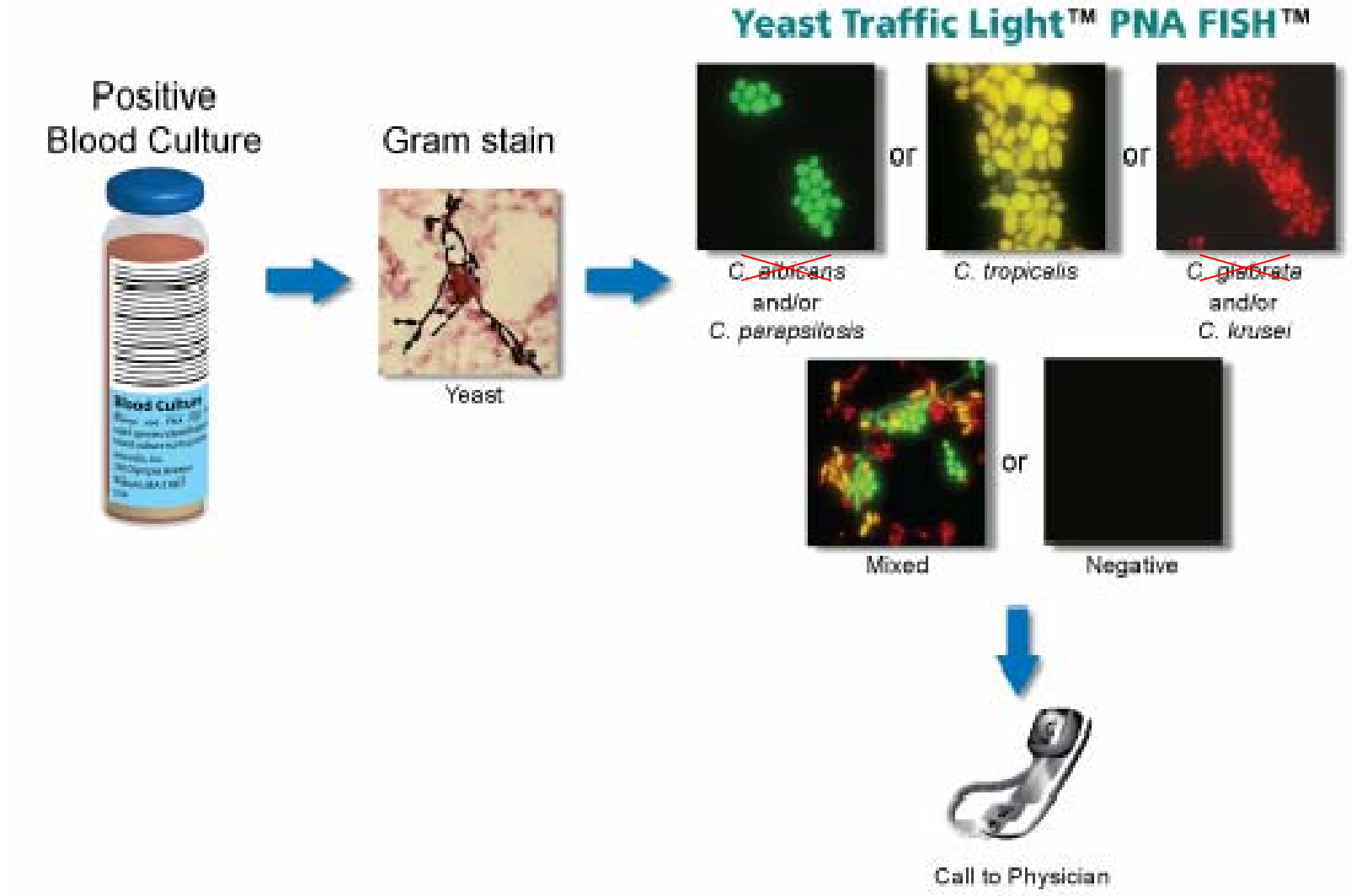
PNA FISH
C. albicans* and *C. glabrata

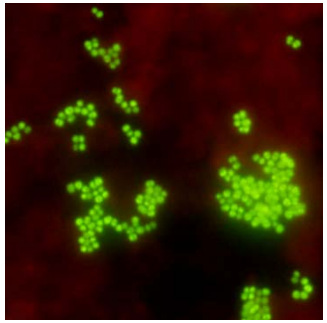
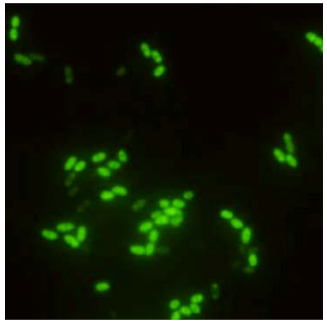
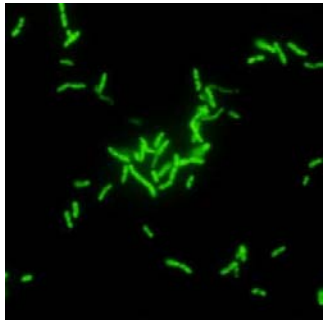
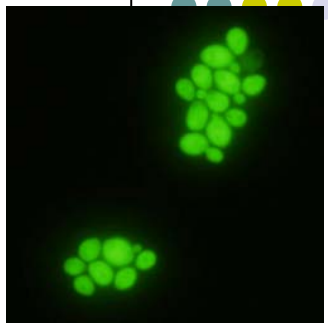
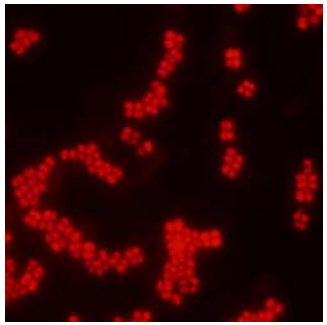
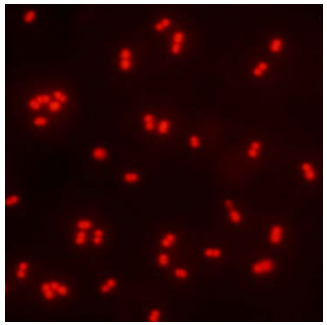
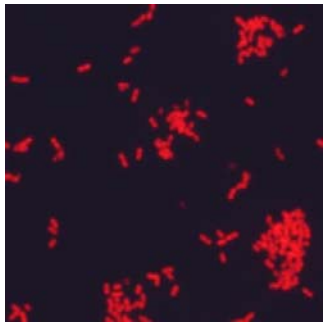
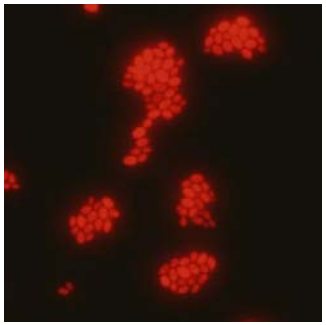
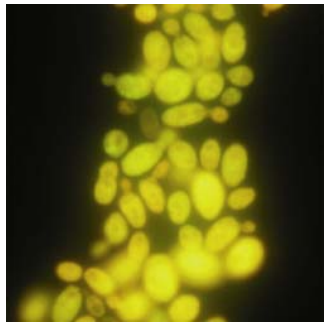
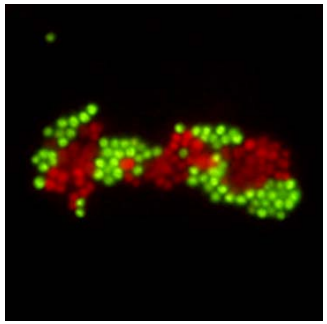
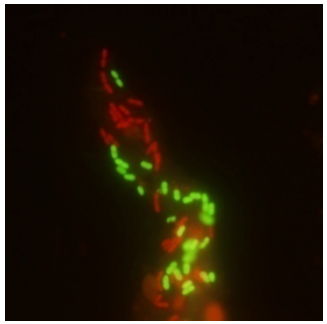
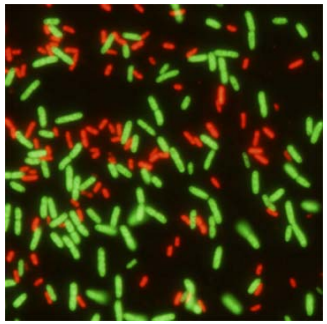
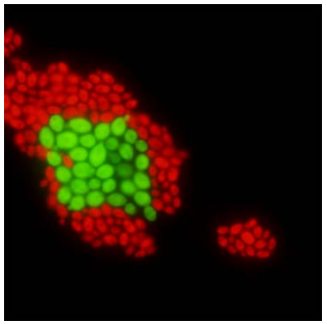
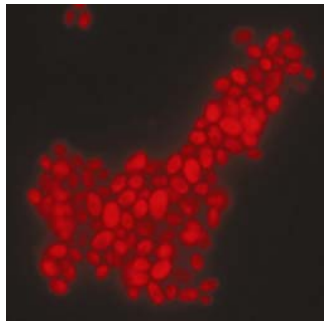


PNA FISH *C. albicans*

PNA FISH test

(AdvanDx, Inc.Woburn, MA,USA)



KT005	KT003	KT007	KT006	KT009
				
S. aureus	***E. faecalis***	***E. coli****	***C. albicans***	***C. albicans/C. parapsilosis***
				
Coag-Negative Staph	**Other enterococci**	***P. aeruginosa****	***C. glabrata***	***C. tropicalis***
				
Mixed – SA/CNS	**Mixed – EF/OE**	**Mixed – EC/PA***	**Mixed – CA/CG**	***C. glabrata/C. krusei***

* Pending FDA clearance

PNA FISH test

(AdvanDx, Inc.Woburn, MA,USA)

JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2008, p. 50–55 Vol. 46, No. 1
0095-1137/08/$08.00+0 doi:10.1128/JCM.01385-07

Multicenter Evaluation of the *Candida albicans*/*Candida glabrata* Peptide Nucleic Acid Fluorescent In Situ Hybridization Method for Simultaneous Dual-Color Identification of *C. albicans* and *C. glabrata* Directly from Blood Culture Bottles▽

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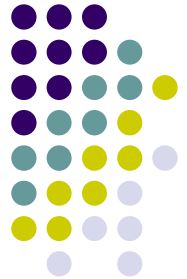
AdvanDx Inc., Woburn, Massachusetts[1]; Columbia University Medical Center, New York, New York[2]; Johns Hopkins Medical Institutes, Baltimore, Maryland[3]; University of Maryland Medical Center, Baltimore, Maryland[4]; University Hospital RWTH Aachen, Germany[5]; Cleveland Clinic, Cleveland, Ohio[6]; and Duke University Medical Center, Durham, North Carolina[7]

PNA FISH test

(AdvanDx, Inc.Woburn, MA,USA)



- Samples testes: 197 yeast-positive blood culture bottles
- 5 clinical laboratories were included in the study
- Comparison of PNA FISH X conventional culture performance

TABLE 4. Performance statistics for the *C. albicans*/*C. glabrata* PNA FISH method at five clinical laboratories

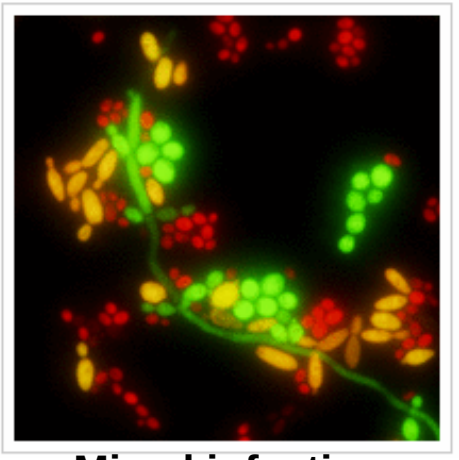
Laboratory	Blood culture system(s)	% (no./total)				
		Sensitivity		PPV	NPV	Specificity
		C. albicans	*C. glabrata*			
A	BacT/Alert, BACTEC	100 (10/10)	100 (5/5)	100 (15/15)	100 (3/3)	100 (3/3)
B	BACTEC	100 (25/25)	100 (5/5)	100 (30/30)	100 (27/27)	100 (27/27)
C	BacT/Alert	92.30 (12/13)	100 (3/3)	100 (5/5)	90.0 (9/10)	100 (10/10)
D	BacT/Alert	100 (17/17)	100 (11/11)	100 (28/28)	100 (25/25)	100 (25/25)
E	BacT/Alert, BACTEC 9240	100 (14/14)	100 (13/13)	100 (27/27)	100 (17/17)	100 (17/17)
Total		98.7 (78/79)	100 (37/37)	100 (115/115)	98.8 (81/82)	100 (82/82)

Shepard et al. 2008. JCM, 46:50-55

PNA FISH test

(AdvanDx, Inc.Woburn, MA,USA)

- Features:
  - Rapid, molecular identification of bacteria and yeast directly from positive blood cultures
  - Results available in 3 hours
  - Identify the 5 *Candida* species most frequent in BSI
  - Mixed infection can be identified
  - Cost equivalent to the convencional culture (in USA)
  - Submitted to ANVISA clearance



Mixed infection

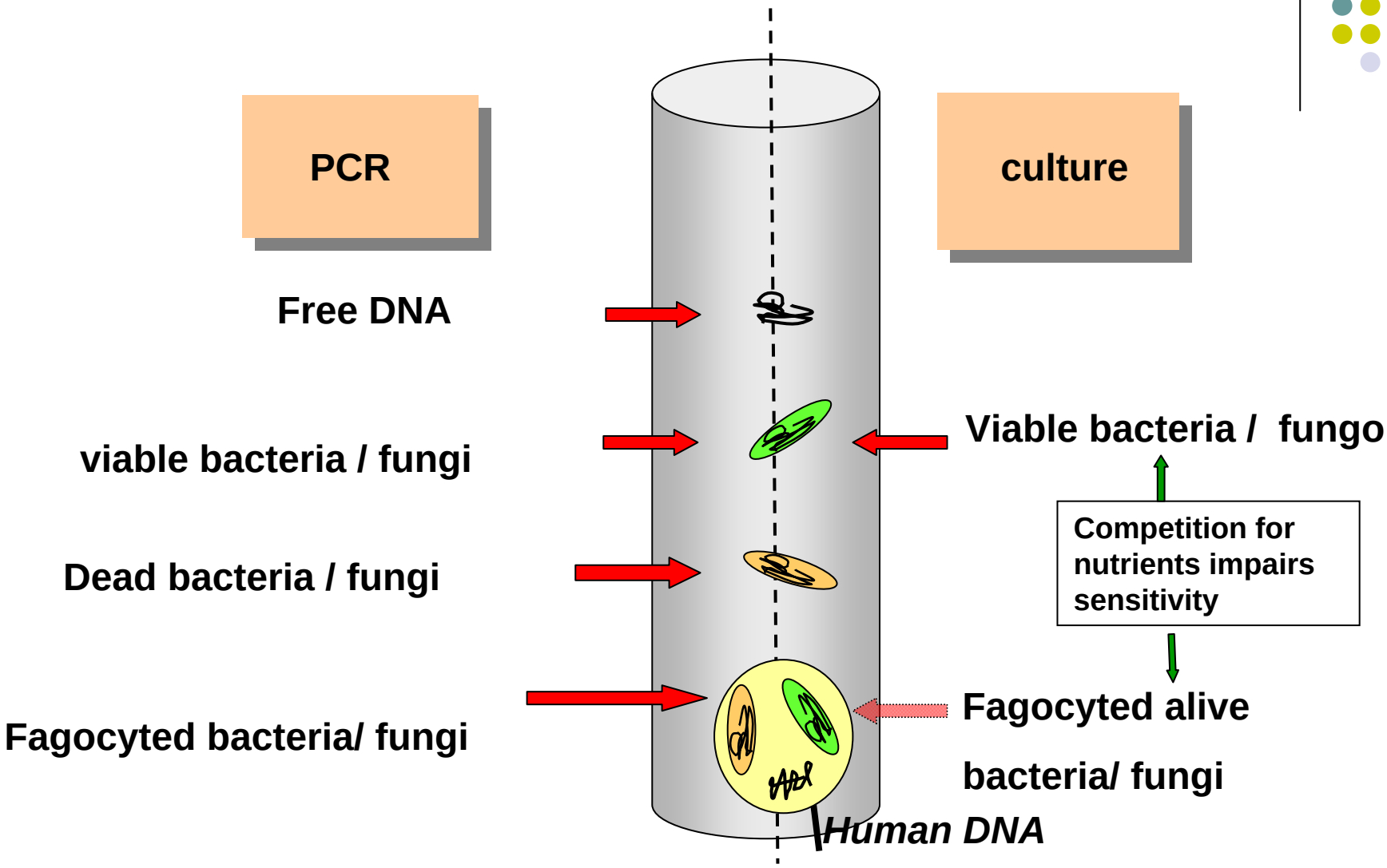
LightCycler® Septi*Fast* Test (Roche)

Sepsis Diagnosis

- LightCycler® Septi*Fast* Test (Roche) is a commercial method based on Real Time PCR methodology
- Allow the laboratory to detect and to identify the 25 bacteria and fungi species most important in bloodstream samples
- These species are responsible for 90% of all bloodstream infections
- Performed with whole blood specimens (K-EDTA)
- The Sepf*Fast* test requires specifically the LightCycler 2.0 instrument

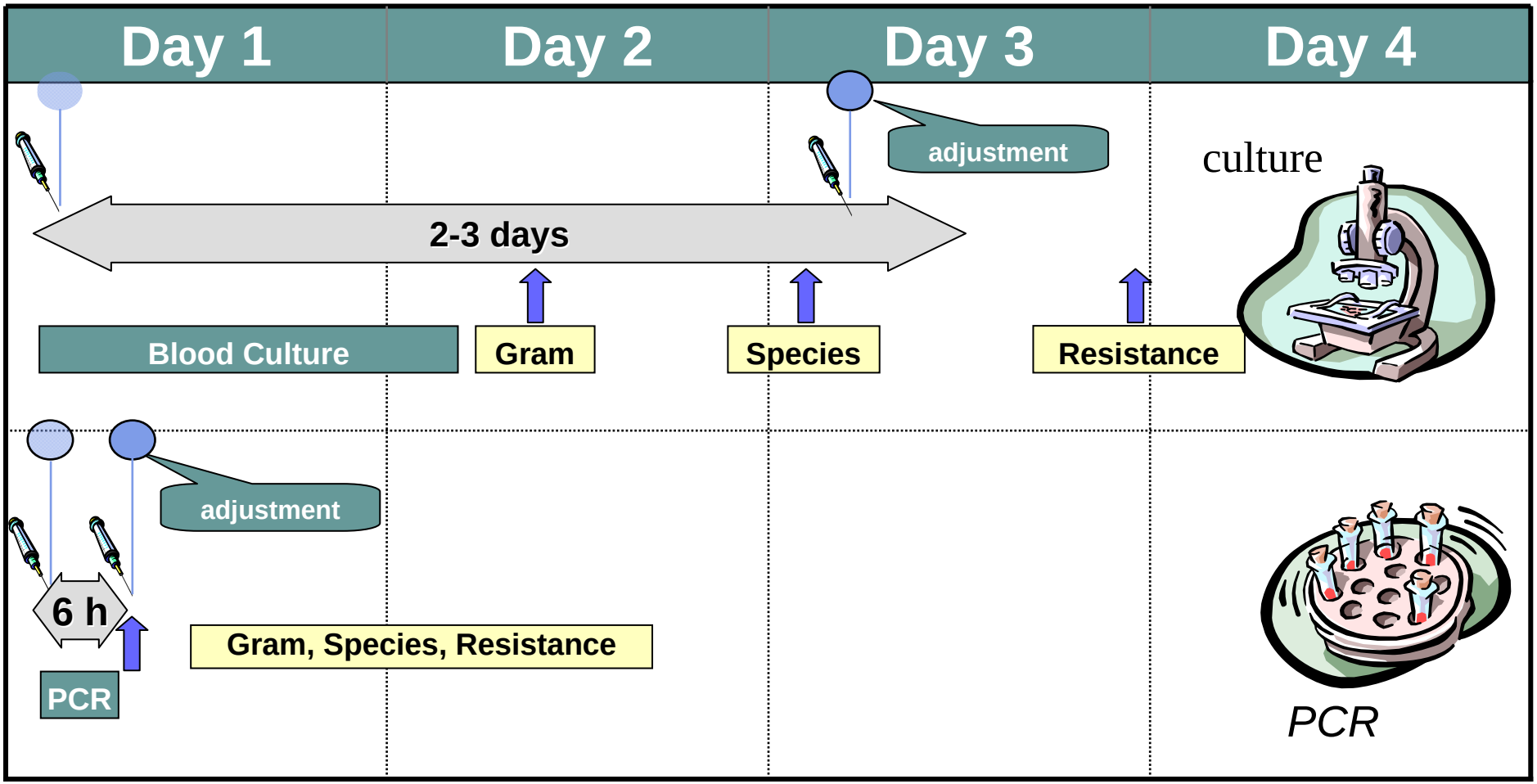
LightCycler 2.0

Comparing PCR X CULTURE



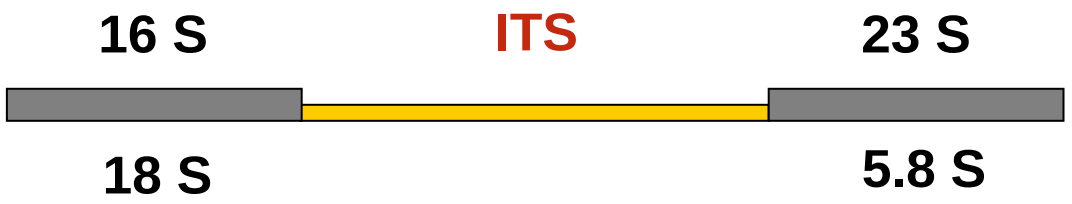
PCR presents more chances to detect pathogens than the traditional culture

PCR provides early diagnosis



LightCycler ® Septi*Fast* test

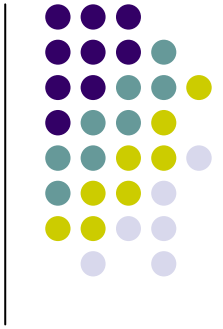
- Test based on multiplex Real Time PCR
- Internal Transcribed Spacer Region (ITS) of ribosomal gene is the probe target
- ITS has been widely used for identification because:
  - Micoorganisms genome possess multi copies of this region
  - It is a variable region that allows species identification



Barry, T., Glennon, C.M., Dunican, L.K., Gannon, F., 1991. The 16s/23s ribosomal spacer region as a target for DNA probes to identify Eubacteria. *PCR Methods Appl* 1, 149.

Gurtler, V., Stanisich, V.A., 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. *Microbiology* 142 (Pt 1), 3-16.

Septi*Fast* Master List (SML)



Gram (-)

- *Escherichia coli*
- *Klebsiella (pneumoniae/oxytoca)*
- *Serratia marcescens*
- *Enterobacter (cloacae / aerog.)*
- *Proteus mirabilis*
- *Pseudomonas aeruginosa*
- *Acinetobacter baumannii*
- *Stenotrophomonas maltophilia*

Gram (+)

- *Staphylococcus aureus*
- *CoNS* [1]
- *Strep. pneumoniae*
- *Streptococcus* spp. [2]
- *Enterococcus faecium*
- *Enterococcus faecalis*

Fungi

- *Candida albicans*
- *Candida tropicalis*
- *Candida parapsilosis*
- *Candida glabrata*
- *Candida krusei*
- *Aspergillus fumigatus*

- [1] *ConS* coagulase negative *Staphylococci* (including e.g., *S. epidermidis, S. haemolyticus*)
- [2] *Streptococcus* species (including e.g., *S. pyogenes, S. agalactiae, S. mitis*)

LightCycler® Septi*Fast* Test (Roche)

Med Microbiol Immunol
DOI 10.1007/s00430-007-0063-0

ORIGINAL INVESTIGATION

A multiplex real-time PCR assay for rapid detection and differentiation of 25 bacterial and fungal pathogens from whole blood samples

Lutz Eric Lehmann · Klaus-Peter Hunfeld · Thomas Emrich · Gerd Haberhausen · Heimo Wissing · Andreas Hoeft · Frank Stüber

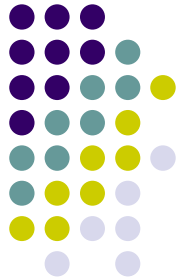
Received: 29 June 2007

Septi*Fast* Test validation

LightCycler® Septi*Fast* Test (Roche)

- **Assay workflow**
  - 1. Specimen preparation by mecanical lysis and purification of DNA
  - 2. Real-time PCR amplification of target DNA in three parallel reactions (Gram +, Gram -, fungi)
  - 3. Automated identification of species using specific software

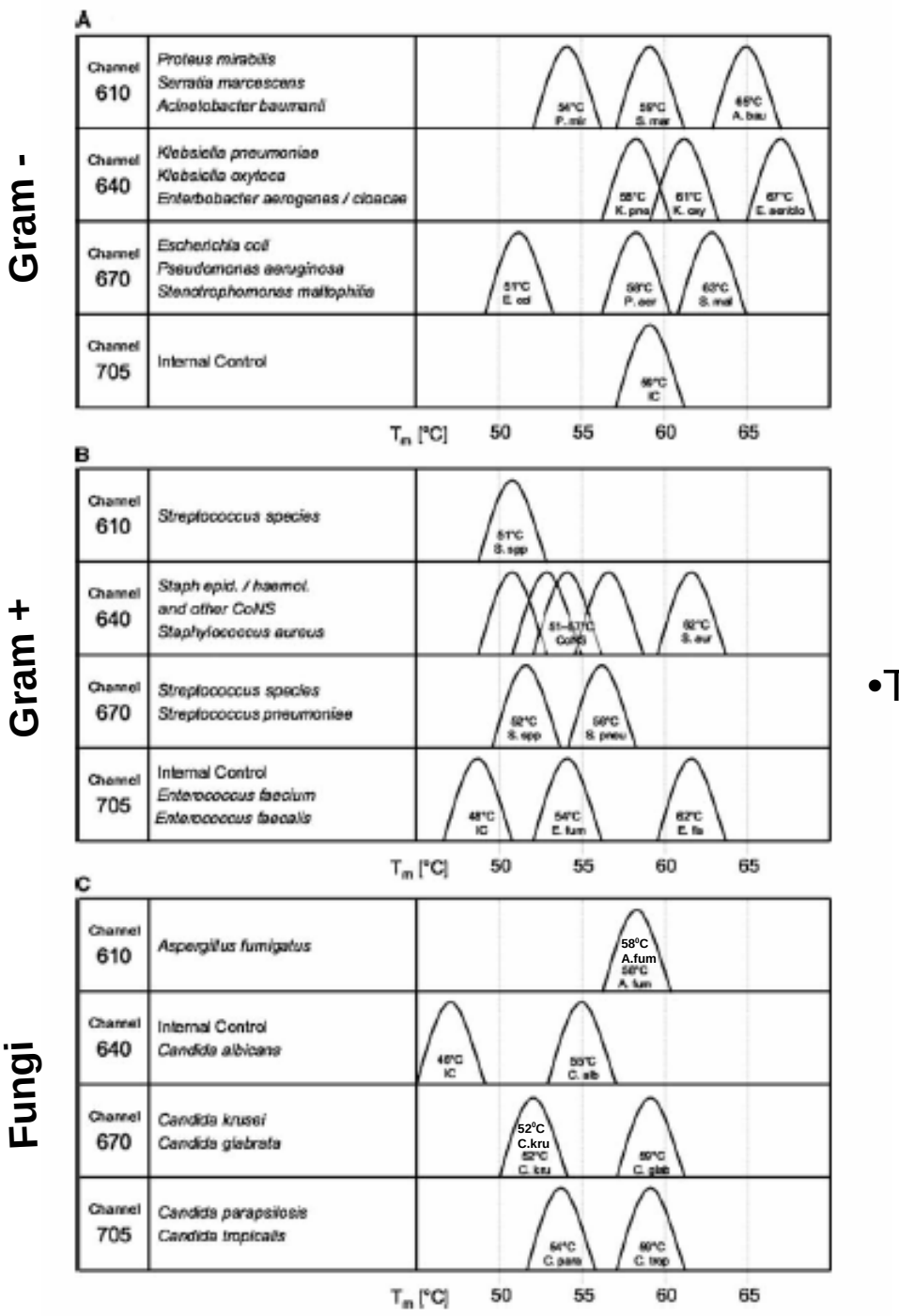
LightCycler® Septi*Fast* Test (Roche)



- During PCR reaction, specific products are detected using dye-labled hybridization probes by fluorescence measurement

- After completion of amplification, melting curve analysis is performed to further prove specificity of products

- Emitted fluorescence is measured in one of four detection channels of the LightCycler 2.0 instrument

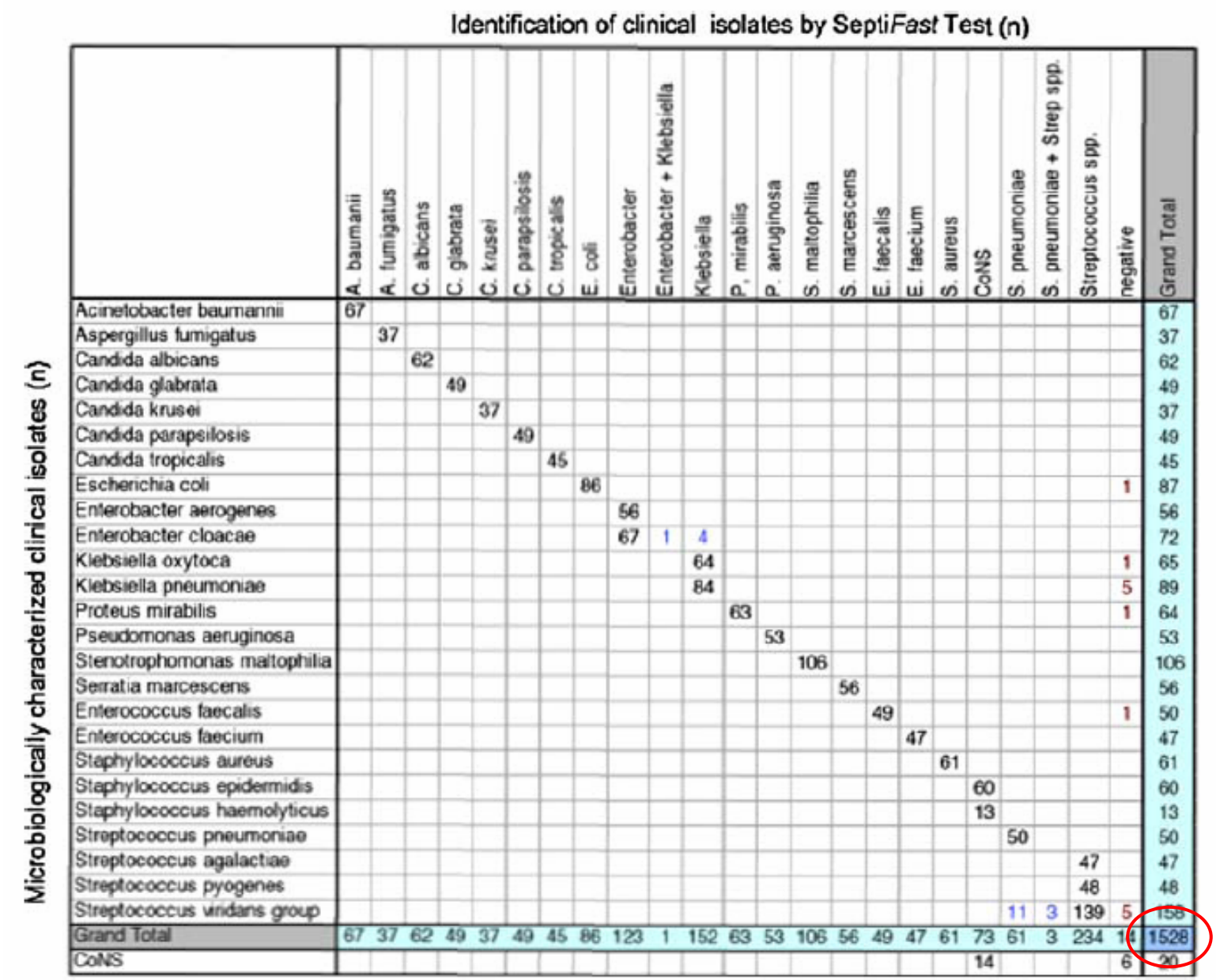


Melting Curves and detection channels for all micoorganisms and internal controls

- The melting temperature depends upon:
  - Fragment lengh
  - Composition of sequence

Mixed infection can be detected

Lehman et al. 2007. Med Microbiol Immunol



Identification of clinical isolates by Septi*Fast* Test (n)

Microbiologically characterized clinical isolates (n)

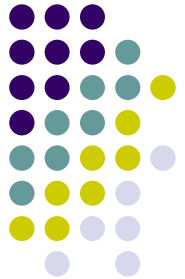
	A. baumannii	A. fumigatus	C. albicans	C. glabrata	C. krusei	C. parapsilosis	C. tropicalis	E. coli	Enterobacter	Enterobacter + Klebsiella	Klebsiella	P. mirabilis	P. aeruginosa	S. maltophilia	S. marcescens	E. faecalis	E. faecium	S. aureus	CoNS	S. pneumoniae	S. pneumoniae + Strep spp.	Streptococcus spp.	negative	Grand Total
Acinetobacter baumannii	67																							67
Aspergillus fumigatus		37																						37
Candida albicans			62																					62
Candida glabrata				49																				49
Candida krusei					37																			37
Candida parapsilosis						49																		49
Candida tropicalis							45																	45
Escherichia coli								86															1	87
Enterobacter aerogenes									56															56
Enterobacter cloacae									67	1	4													72
Klebsiella oxytoca											64												1	65
Klebsiella pneumoniae											84												5	89
Proteus mirabilis												63											1	64
Pseudomonas aeruginosa													53											53
Stenotrophomonas maltophilia														106										106
Serratia marcescens															56									56
Enterococcus faecalis																49							1	50
Enterococcus faecium																	47							47
Staphylococcus aureus																		61						61
Staphylococcus epidermidis																			60					60
Staphylococcus haemolyticus																			13					13
Streptococcus pneumoniae																				50				50
Streptococcus agalactiae																						47		47
Streptococcus pyogenes																						48		48
Streptococcus viridans group																				11	3	139	5	158
Grand Total	67	37	62	49	37	49	45	86	123	1	152	63	53	106	56	49	47	61	73	61	3	234	14	1528
CoNS																			14				6	20

Comparison

Phenotypical method
X
Septi*Fast* Test

Lehman et al. 2007. Med Microbiol Immunol

LightCycler® Septi*Fast* Test (Roche)



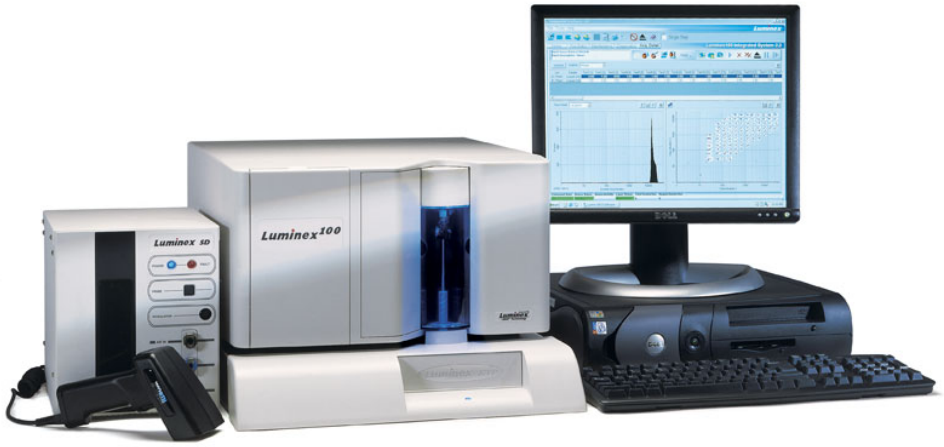
- Advantages of LightCycler® Septi*Fast* Test
  - Short time for diagnosis (<6h)
  - Performed directly form whole blood specimens (not require pre-incubation of blood culture)
  - Identify 90% of micoorganisms responsible for sepsis
  - Widely used in Europe (~40 centers)
  - USA - not cleared by FDA yet
  - Brazil: cleared by ANVISA for diagnosis use
  - Two Brazilian centers have the LightCycler® Septi*Fast* Test
  - Cost: LightCycler 2.0 = US$ 50.000 /Each test ~ R$ 650-700

New technologies are coming for fungal infection diagnosis

Luminex®

xMAP Technology

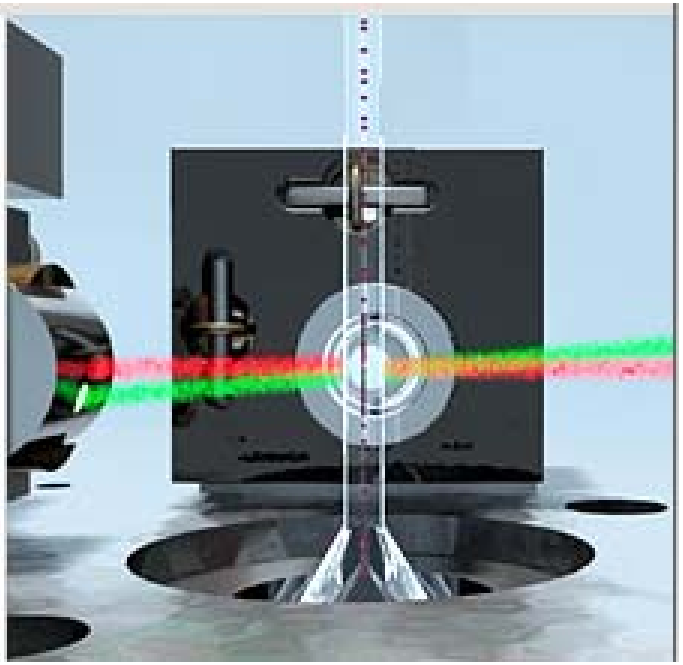
(Multianalyte Profile System)



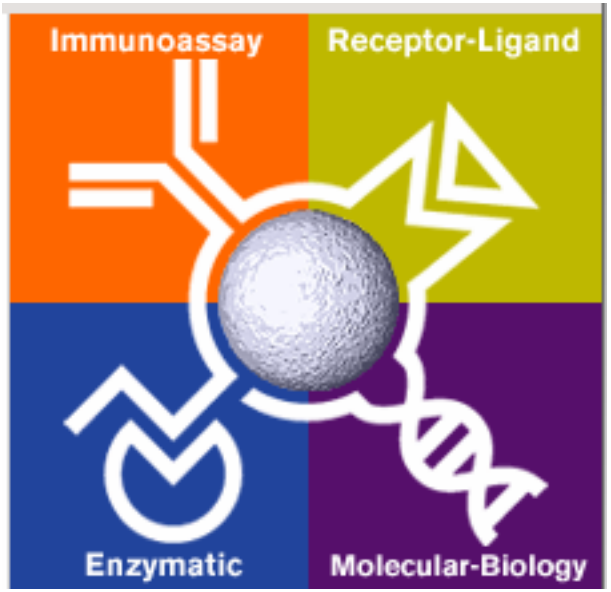
THE POWER INSIDE Luminex | xMAP® TECHNOLOGY Because you want to know more

xMAP Technology

(Multianalyte Profile System)



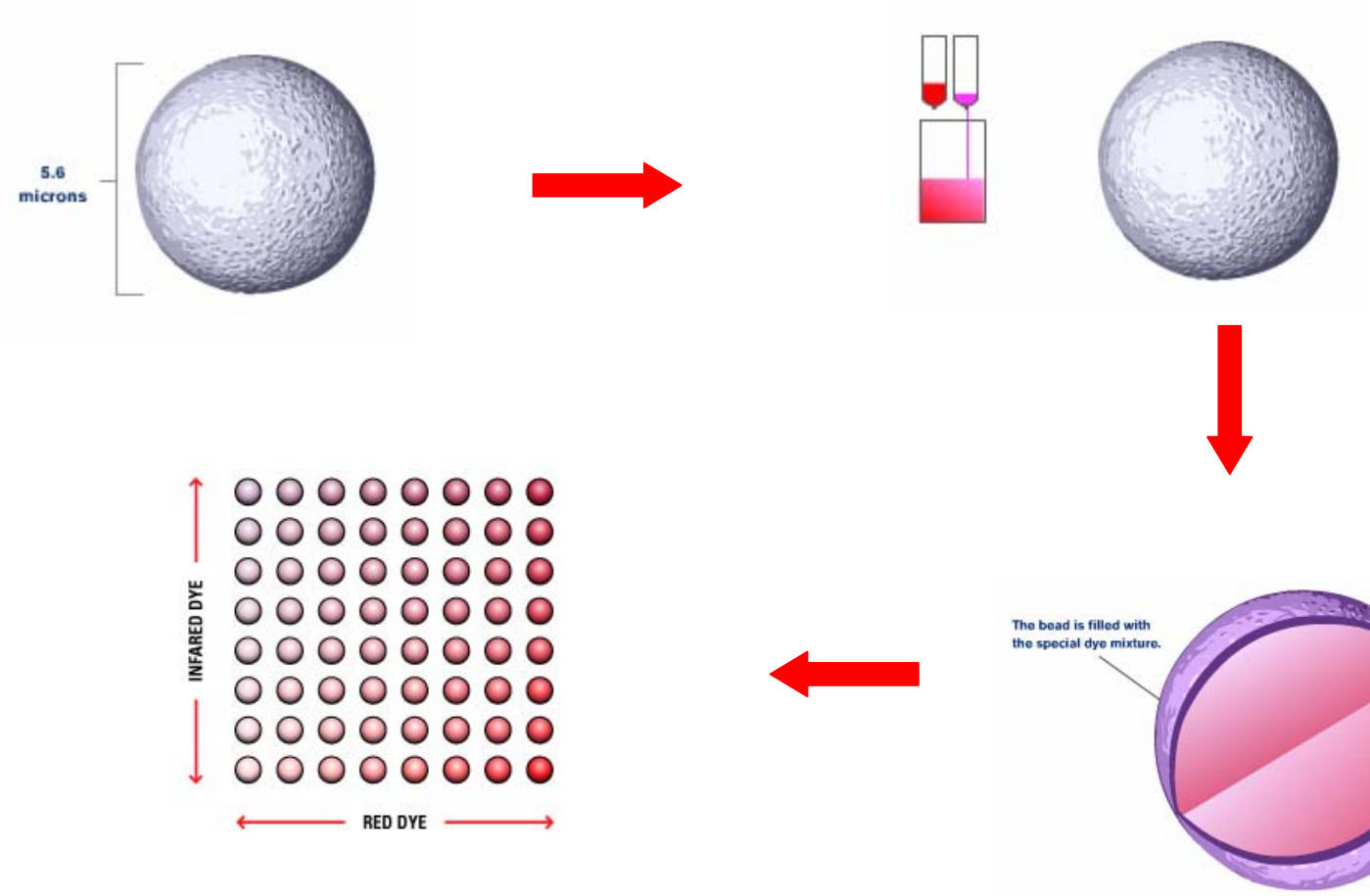
Flow cytometric technology



Applications overview

Luminex®

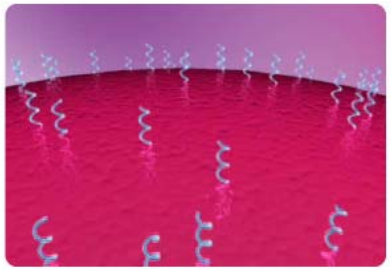
xMAP Technology



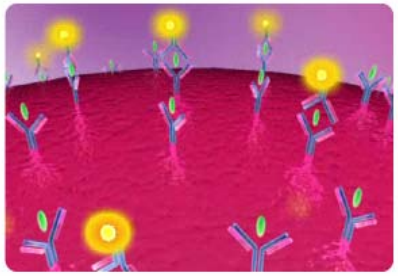
- Microspheres are dyed to create 100 distinct colors
- Each microsphere has ‘spectral address’ based on red/infrared content

Luminex

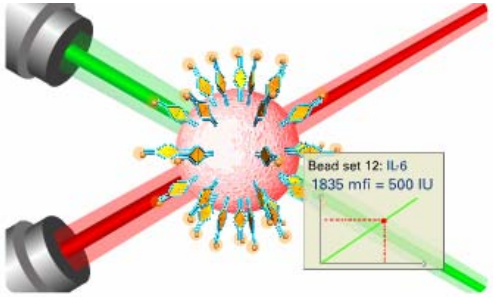
xMAP Technology



- Microspheres are coated with oligonucleotide
- Mix of microspheres coated with different oligonucleotides
- Sample is added to microspheres



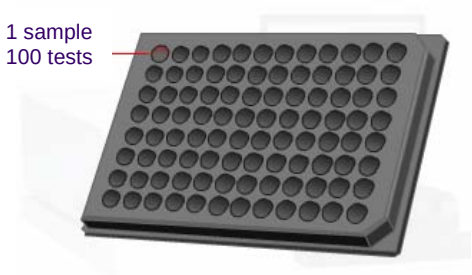
- Analyte is captured to microspheres
- Fluorescent reporter tag added

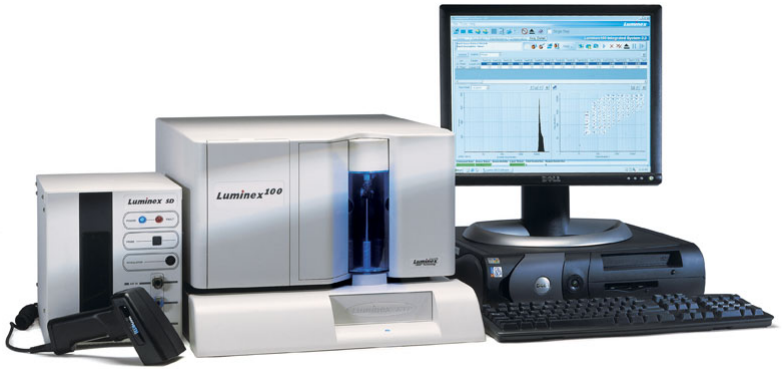


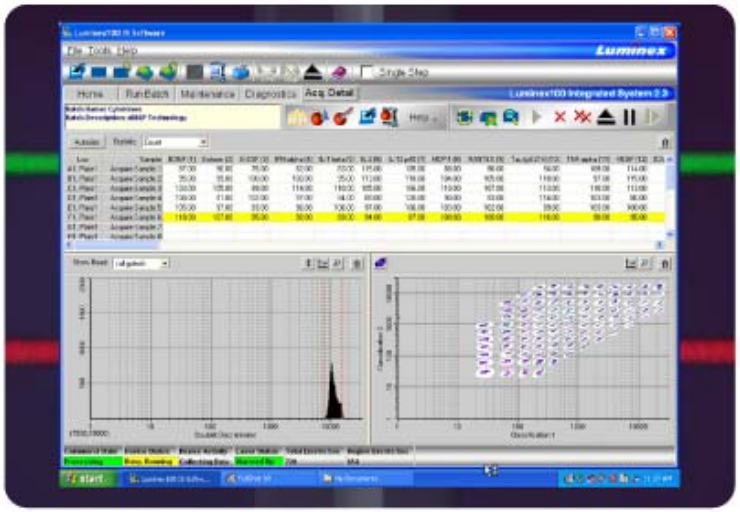
- Lasers excite fluorescent dyes- red laser for bead classification and green for assay result

Luminex

xMAP Technology





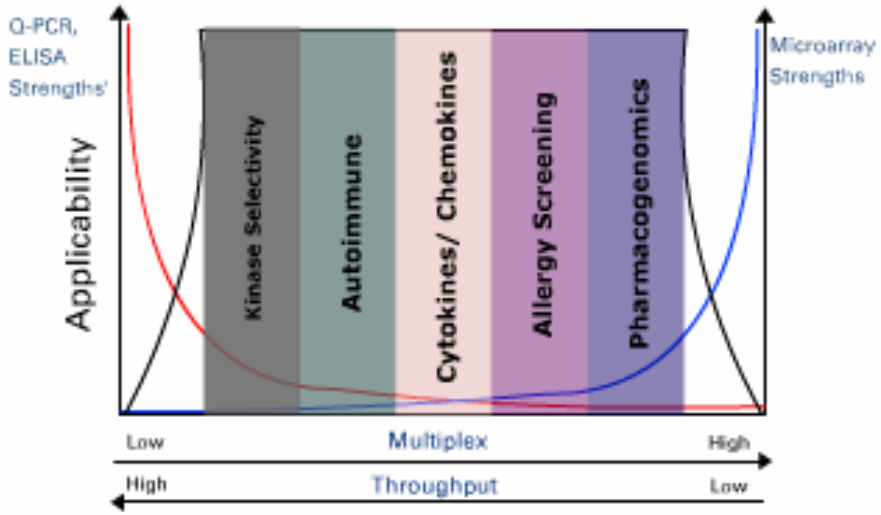


- Software reports results in real-time
- One plate reading time = 1 hour
- Assay time = **4 hours**

Luminex®

xMAP Technology

Commercial tests available

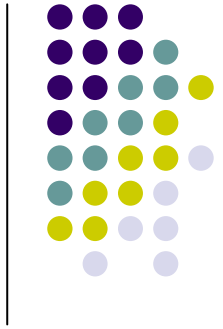


Advantages

- Rapid (4 h)
- Sensitive (1blastoconidia/4ul)
- Specific (>90%)
- Flexibility to home-brew

Luminex

xMAP Technology



- **Fungi aplications (in house methodology):**
  - *Candida* (Das et al., 2006. FEMS Immunol Med Microbiol. 46;244-250. (CDC))
    - Identify 6 *Candida* species: *C. albicans, C. tropicalis, C. glabrata, C. parapsilosis, C. krusei, C. dubliniensis*
  - *Aspergillus* (Etienne et al., 2009. JCM, 47:1096-1100)
    - Identify 6 relevant species: *A. fumigatus, A. flavus, A. niger, A. terreus, A. ustus, A. versicolor.*
  - *Trichosporon* (Diaz & Fell, 2004. JCM, 42:3696)
    - Identify 33 *Trichosporon* species
  - *Fusarium* (O'Donnell et al.2007. JCM,45:2235-2248)
    - Identify 6 medically important species complex, 10 most important species
    - 157 clinical isolates tested

Luminex

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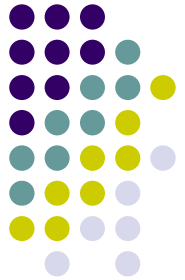


- Cost
  - Equipment: U$ 60,000
  - Price: U$50-U$60/ test (commercial kits)

- Test limitation:
  - Diagnosis limited to the number of probes tested

Ibis Technology

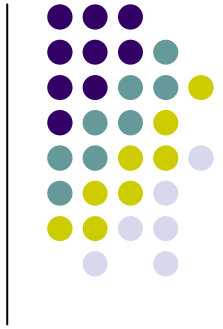
Ibis Biosciences, a subsidiary of Abbott Molecular, Inc.

Ibis Technology

Ibis Technology

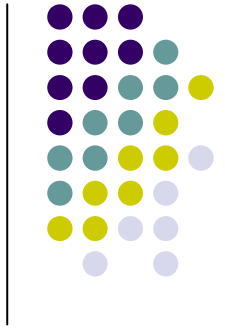
- IBIS Biosciences developed the **T6000™ Biosensor System** to identify infectious disease agents based on weighing DNA
- Based on PCR/ESI-MS (PCR Electrospray Ionization Mass Spectrometry)
- Identify organism by comparing base composition to database of >700,000 entries
- Results available in 6-8 hours
- For research use only

Future Perspective



- **To develop and standardize molecular methodologies that address:**

  - Rapid and sensitive identification of pathogens without culture
  - Detects co-infections with bacteria, viruses, and fungi in different clinical specimens
  - High resolution genotyping, drug resistance, virulence



Thank you