Identification and Characterization of Microorganisms Using Molecular Methods

Michael Waddington
New England PDA
Burlington, MA
February 8, 2006

ACCUGENIX®
IDENTIFY WITH THE LEADER.

Accugenix

- A cGMP compliant service laboratory specializing in bacterial and fungal identification for pharmaceutical manufacturers
- Dedicated to providing the highest quality, fastest turnaround time (same day) and the lowest price
- Allows manufacturers access to the best technology –

  without the time and expense of researching, purchasing, and validating new systems

ACCUGENIX®
IDENTIFY WITH THE LEADER.

Overview of Microbial Identification

ACCUGENIX®
IDENTIFY WITH THE LEADER.

Methods for Identification of Microorganisms

- **Classical Methods**
  - In-house biochemicals
  - Manual phenotypic methods (API Strips, BBL etc.)
  - Automated phenotypic methods (Vitek)
- **Cellular Fatty Acids** (MIDI Sherlock System)
- **Carbon Source Utilization** (Biolog)
- **Genetic** (MicroSeq, Riboprinter)

ACCUGENIX®
IDENTIFY WITH THE LEADER.

Limitations of Identification Methods

- Phenotypic properties (biochemical / carbon source utilization) can be variable, subjective, dependent on growth parameters and health of organism
- Cellular fatty acid profiles change with temperature, age of culture and growth medium
- Some systems require subjective off-line testing such as gram stain, oxidase, coagulase, etc., before determining the appropriate test card
- DNA Sequencing system is not automated, numerous, somewhat complicated lab procedures, and manual interpretation of results requires microbial phylogenetic experience

ACCUGENIX®
IDENTIFY WITH THE LEADER.

A Revolution in Bacterial Taxonomy

G. Fox, et. al. 1980. Science. 209:457-463

- Toward a natural system of classification based on phylogenetics rather than taxonomic characters

- In many cases taxonomic characters are not phylogenetically valid- ie morphological characters such as cell shape, mode of cell division, lack of cell wall……can be misleading

ACCUGENIX®
IDENTIFY WITH THE LEADER.

Road Map to Bergey's

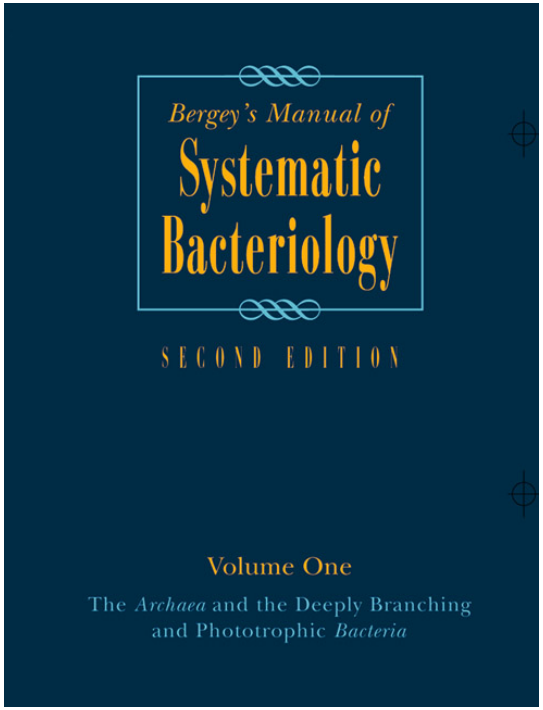
George M. Garrity and John G. Holt

"Bergey's Manual of systematic bacteriology, one of the most comprehensive and authoritative works in the field of bacterial taxonomy, has been extensively revised in the form of a five volume second edition. Since the first edition was published in 1984, the field has undergone explosive growth, with over 2,200 new species and 390 new genera having been described. Numerous taxonomic rearrangements and changes in nomenclature have resulted from more than 850 published new combinations.

These developments, which are attributable to rapid advances in molecular sequencing of highly conserved regions of the procaryotic genome, most notably genes coding for the RNA of the small ribosomal subunit, have led to a natural classification that reflects the evolutionary history of Bacteria and Archaea, and to the development of new, universally applicable methods of identifying these organisms."

ACCUGENIX®
IDENTIFY WITH THE LEADER.

Phylogenetic Taxonomy



- Universal system
- Reproducible from lab to lab – and over time
- Sequence characters are not subjective
- More informative than phenotypic ID
- No specific growth requirements
- Accepted gold standard for taxonomy

ACCUGENIX®
IDENTIFY WITH THE LEADER.

Regulatory Focus

ACCUGENIX®
IDENTIFY WITH THE LEADER.

Pharmaceutical cGMPs for the 21st Century

Guidance for Industry

Sterile Drug Products Produced by Aseptic Processing — Current Good Manufacturing Practice

DRAFT GUIDANCE

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)
Office of Regulatory Affairs (ORA)

September 2004
Pharmaceutical CGMPs

ACCUGENIX®
IDENTIFY WITH THE LEADER.

FDA - Sterile Drug Products Produced by Aseptic Processing

Environmental Monitoring:

"**Genotypic methods have been shown to be more accurate and precise than traditional biochemical and phenotypic techniques. These methods are especially valuable for investigations into failures (e.g., sterility test; media fill contamination).**

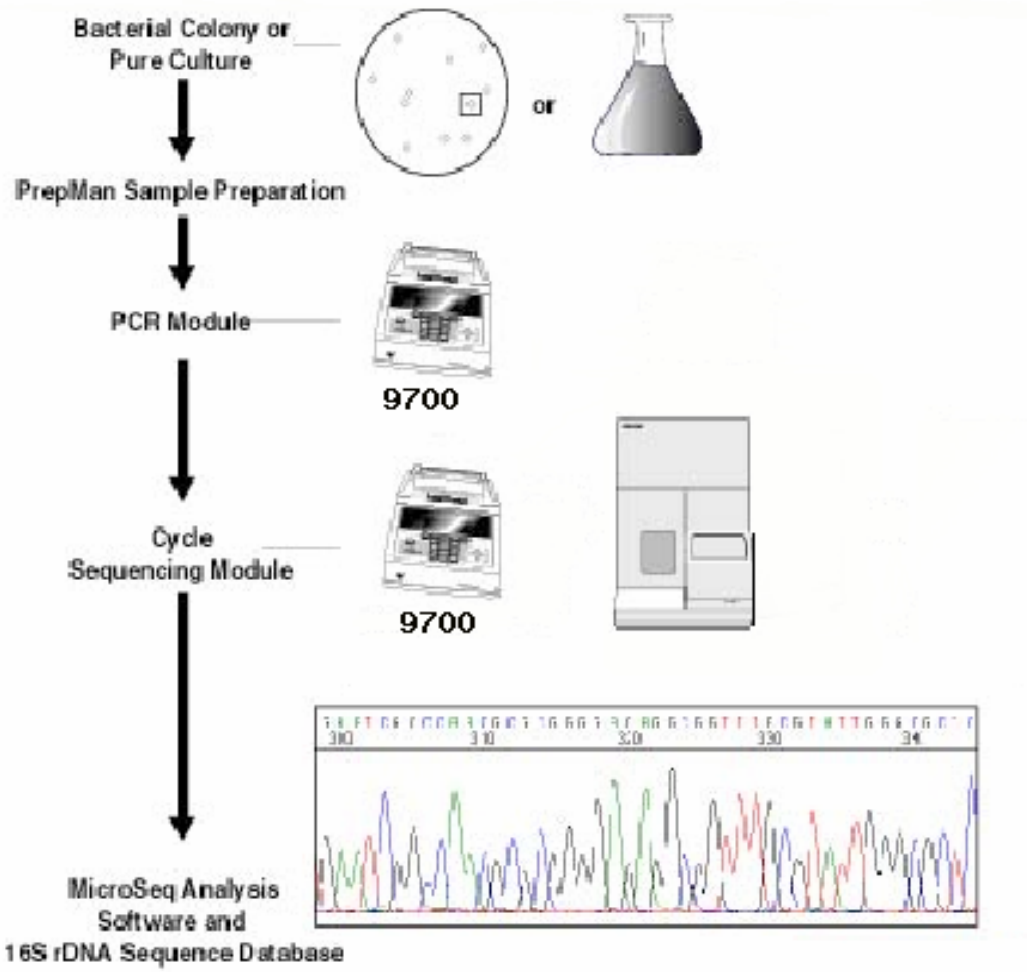
Sterility Testing:

"**Advanced identification methods (e.g., nucleic-acid based) are valuable for investigational purposes. When comparing results from environmental monitoring and sterility positives, both identifications should be performed using the same methodology"**

ACCUGENIX® IDENTIFY WITH THE LEADER.

Accugenix Process

ACCUGENIX®
IDENTIFY WITH THE LEADER.



ACCUGENIX®
IDENTIFY WITH THE LEADER.

Process

- Sample Accessioning
- DNA Extraction
- PCR
- PCR Cleanup
- Cycle Sequencing
- Cycle Sequencing Cleanup
- Automated Fluorescent DNA Sequencing
- Data Analysis
- Data Interpretation

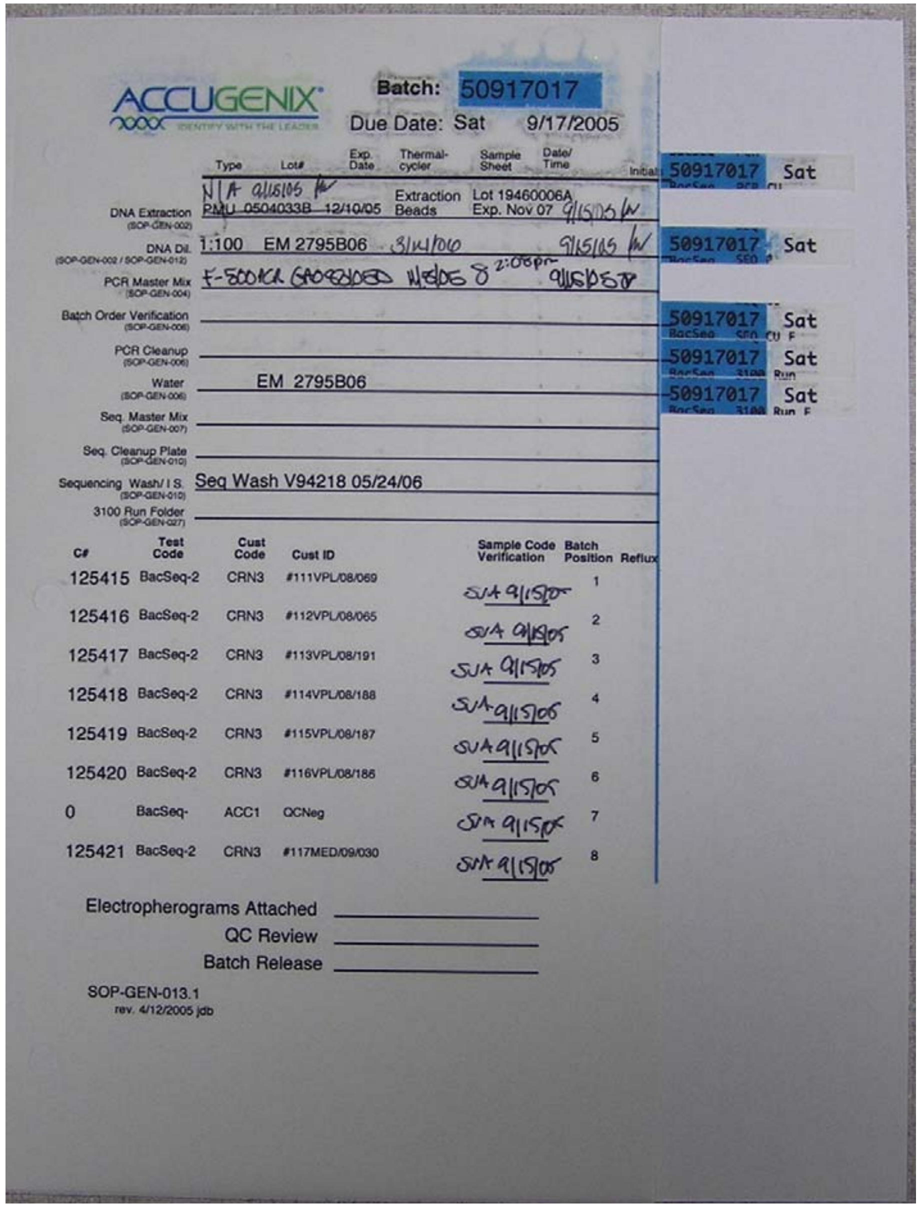
ACCUGENIX®
IDENTIFY WITH THE LEADER.

Sample Accessioning

- Samples are received and checked for damage
- Samples are matched to paper work and check for correct number, match to sample codes, and correctness of requested test code
- Samples are prioritized based on requested Turn Around Time (due date scheduling)
- Samples are accessioned into Master Log and database
- Samples are grouped into batches, and batch records are created

ACCUGENIX®
IDENTIFY WITH THE LEADER.

Sample Accessioning



ACCUGENIX IDENTIFY WITH THE LEADER

Batch: 50917017

Due Date: Sat 9/17/2005

	Type	Lot#	Exp. Date	Thermal-cycler	Sample Sheet	Date/Time	Initials
DNA Extraction (SOP-GEN-002)	PMU	0504033B	12/10/05	Extraction Beads	Lot 19460006A Exp. Nov 07		
DNA Dil. (SOP-GEN-002 / SOP-GEN-012)	1:100	EM 2795B06					
PCR Master Mix (SOP-GEN-004)							
Batch Order Verification (SOP-GEN-006)							
PCR Cleanup (SOP-GEN-006)							
Water (SOP-GEN-006)		EM 2795B06					
Seq. Master Mix (SOP-GEN-007)							
Seq. Cleanup Plate (SOP-GEN-010)							
Sequencing Wash/ I.S. (SOP-GEN-010)	Seq Wash V94218 05/24/06						
3100 Run Folder (SOP-GEN-027)							

C#	Test Code	Cust Code	Cust ID	Sample Code Verification	Batch Position	Reflux
125415	BacSeq-2	CRN3	#111VPL/08/069		1	
125416	BacSeq-2	CRN3	#112VPL/08/065		2	
125417	BacSeq-2	CRN3	#113VPL/08/191		3	
125418	BacSeq-2	CRN3	#114VPL/08/188		4	
125419	BacSeq-2	CRN3	#115VPL/08/187		5	
125420	BacSeq-2	CRN3	#116VPL/08/186		6	
0	BacSeq-	ACC1	QCNeg		7	
125421	BacSeq-2	CRN3	#117MED/09/030		8	

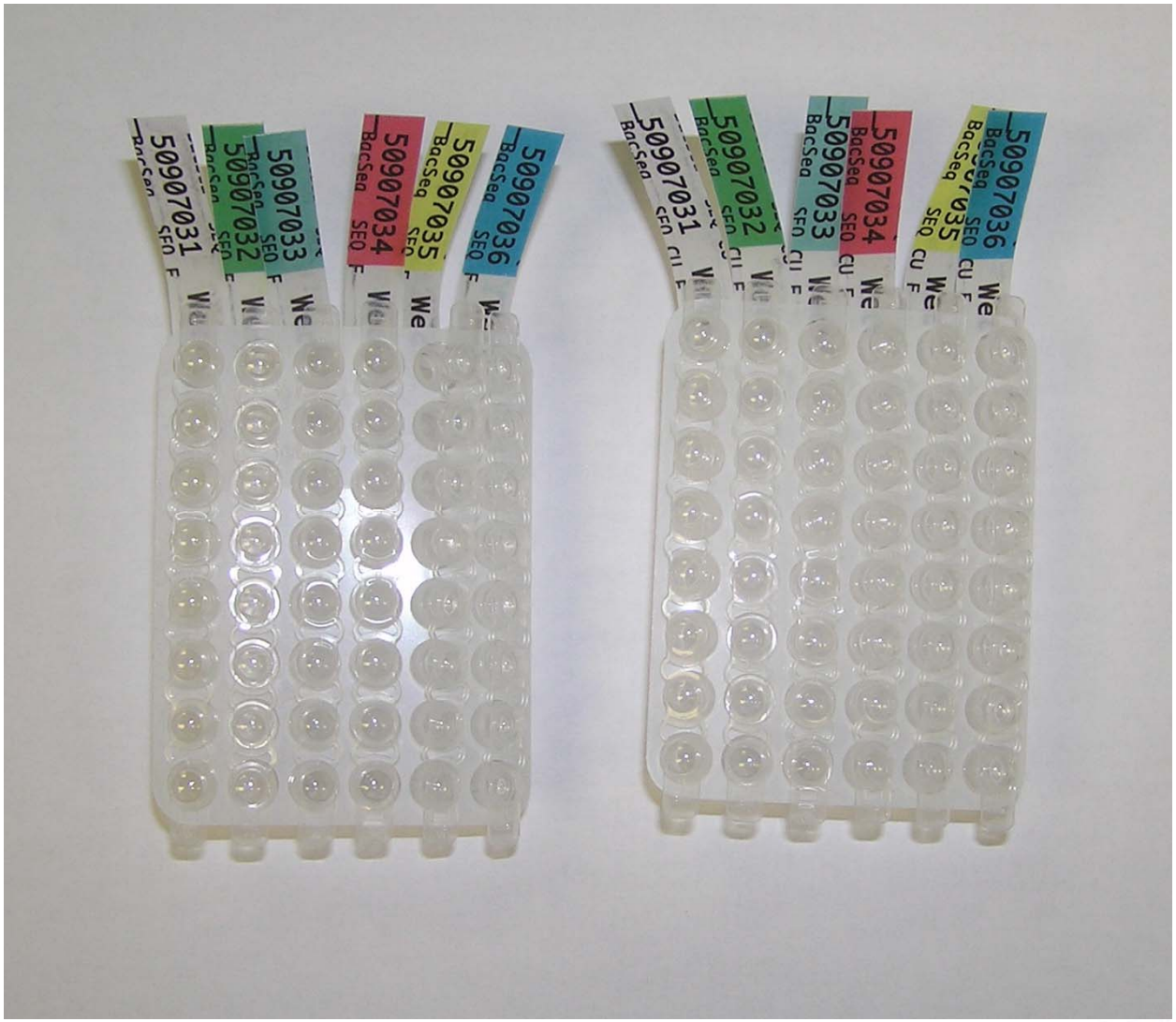
Electropherograms Attached ________

QC Review ________

Batch Release ________

SOP-GEN-013.1
rev. 4/12/2005 jdb

Sample Accessioning



ACCUGENIX®
IDENTIFY WITH THE LEADER.

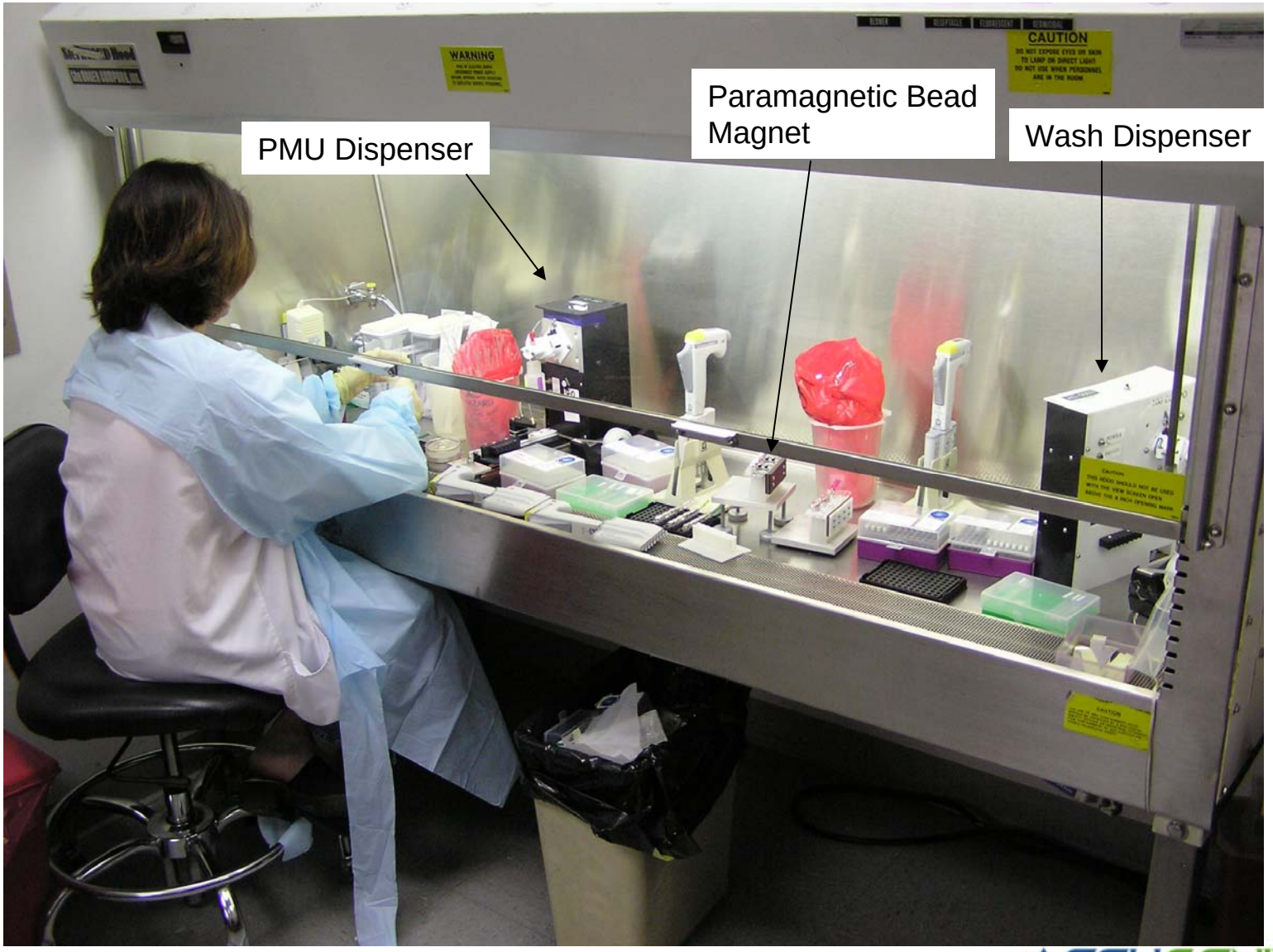
DNA Extraction

- PrepMan Ultra
- Cells
- Paramagnetic Beads

- Fast & easy
- Results in clean DNA
- Lower failure rate, due to elimination of PCR inhibitors PMU alone does not remove

ACCUGENIX®
IDENTIFY WITH THE LEADER.

DNA Extraction



ACCUGENIX®
IDENTIFY WITH THE LEADER.

PCR

- Bacterial & Fungal Master Mixes
  - DNA Polymerase
  - dNTP's
  - Primers
- Extracted genomic DNA

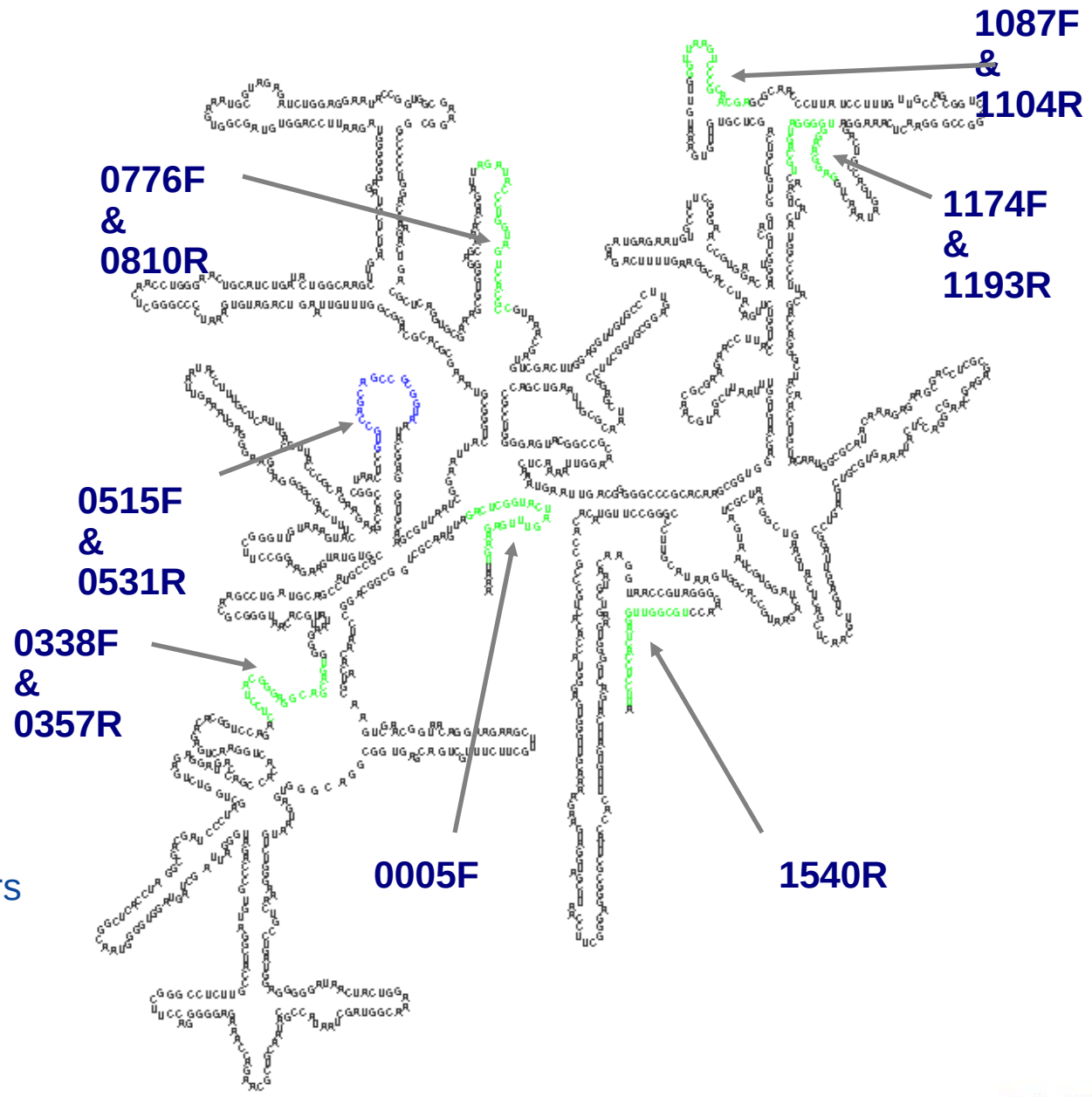
- Reactions set-up in a Laminar Flow Hood in a dedicated laboratory to minimize contamination of amplified products

- Dedicated equipment and materials

- Technicians wear disposable gowns while working on PCR set-up

ACCUGENIX®
IDENTIFY WITH THE LEADER.

16S rRNA Structure & Primer Locations

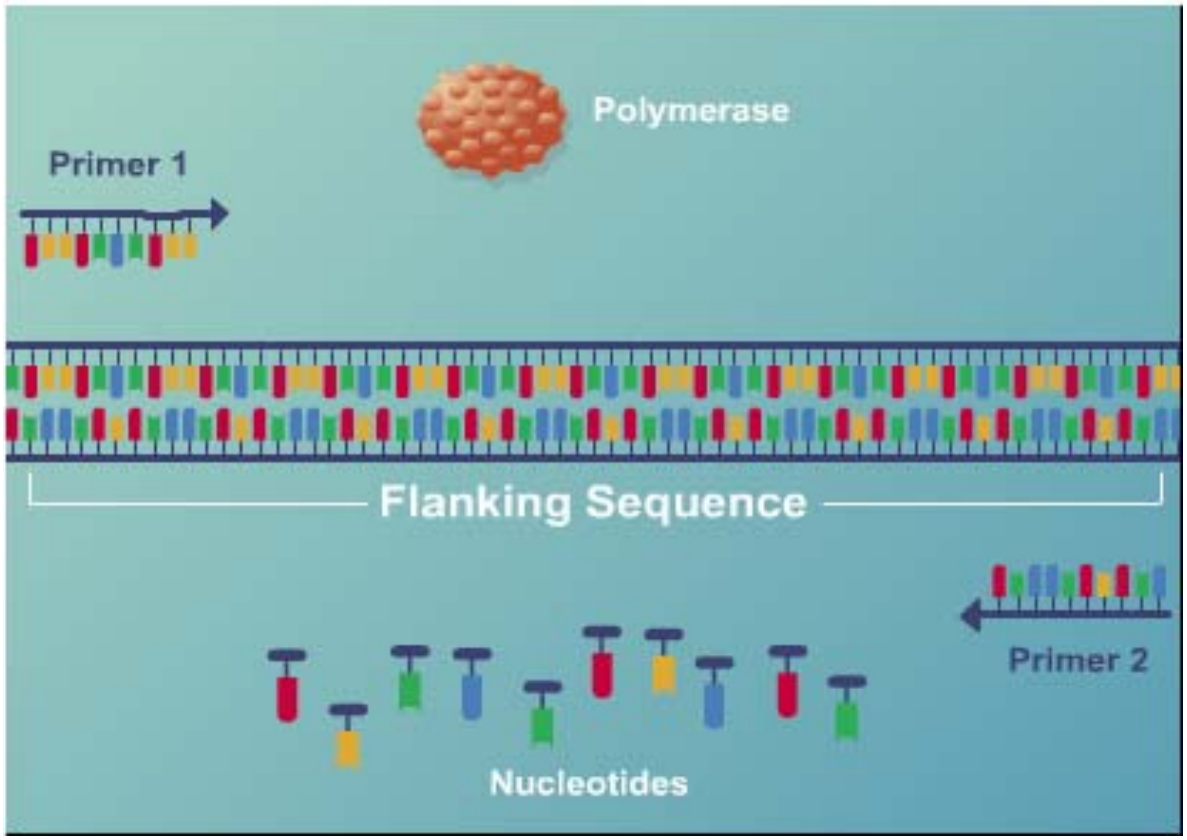


Full Gene
- 0005F
- 1540R
- 12 Seq Primers

500bp
- 0005F
- 0531R
- 2 Seq Primers

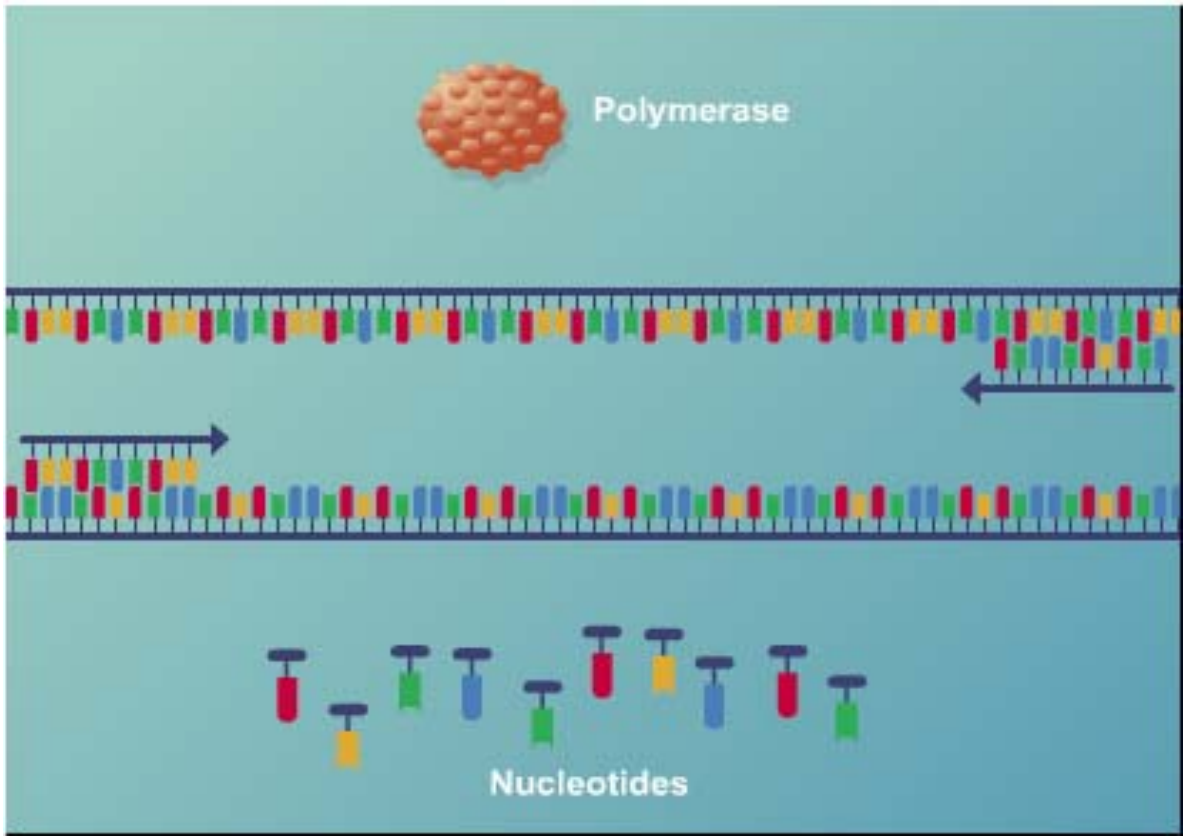
ACCUGENIX®
IDENTIFY WITH THE LEADER.

PCR



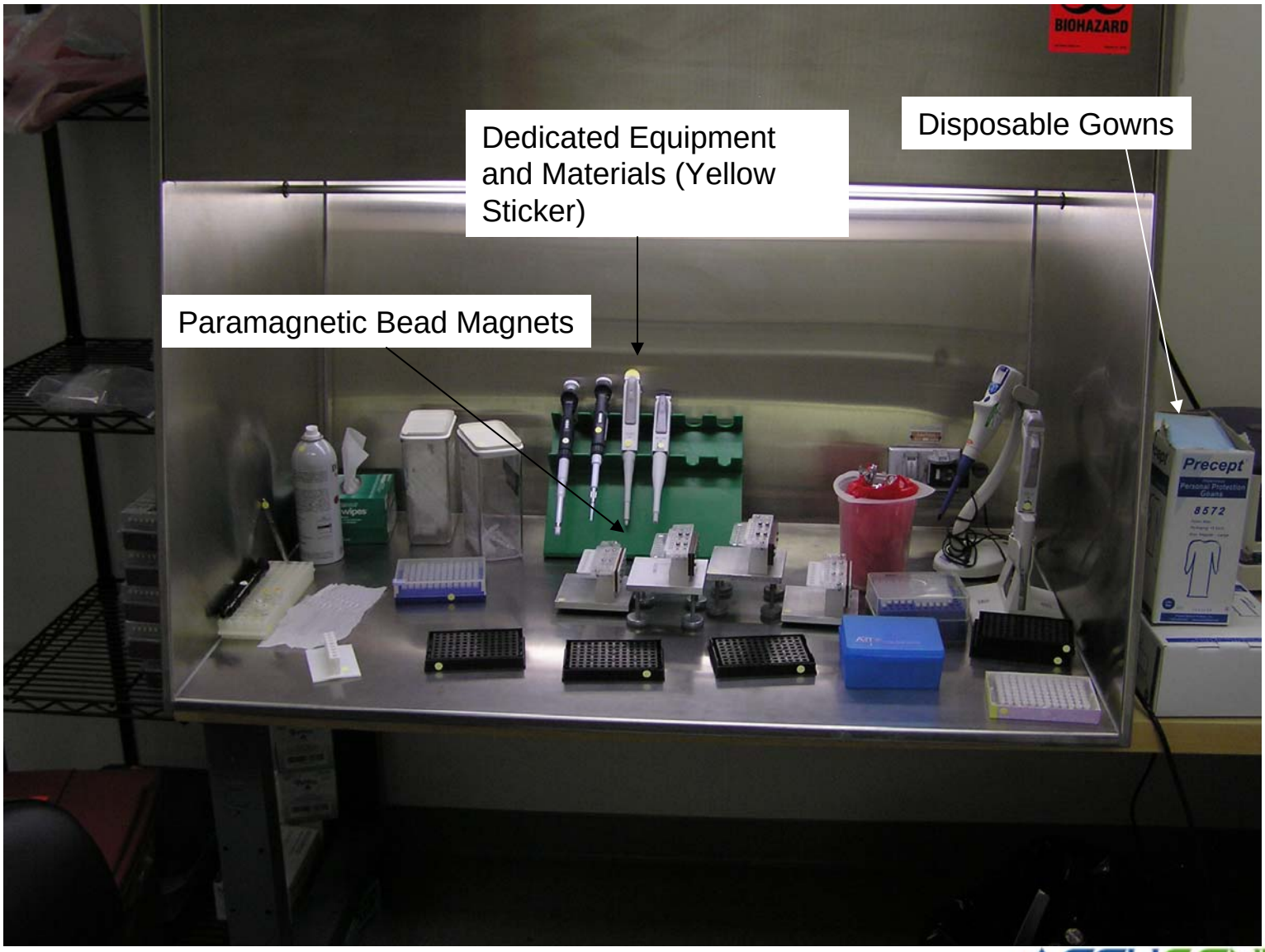
ACCUGENIX®
IDENTIFY WITH THE LEADER.

PCR



ACCUGENIX®
IDENTIFY WITH THE LEADER.

PCR



ACCUGENIX®
IDENTIFY WITH THE LEADER.

PCR Clean-up

- Exo-SAP
  - Enzymatic clean-up step
  - Exonuclease removes excess primers from PCR step
  - Shrimp Alkaline Phosphatase (SAP) removes terminal phosphate from excess dNTP's
- Exo-SAP clean-up results in less amplicon loss than filtration or size exclusion steps

ACCUGENIX®
IDENTIFY WITH THE LEADER.

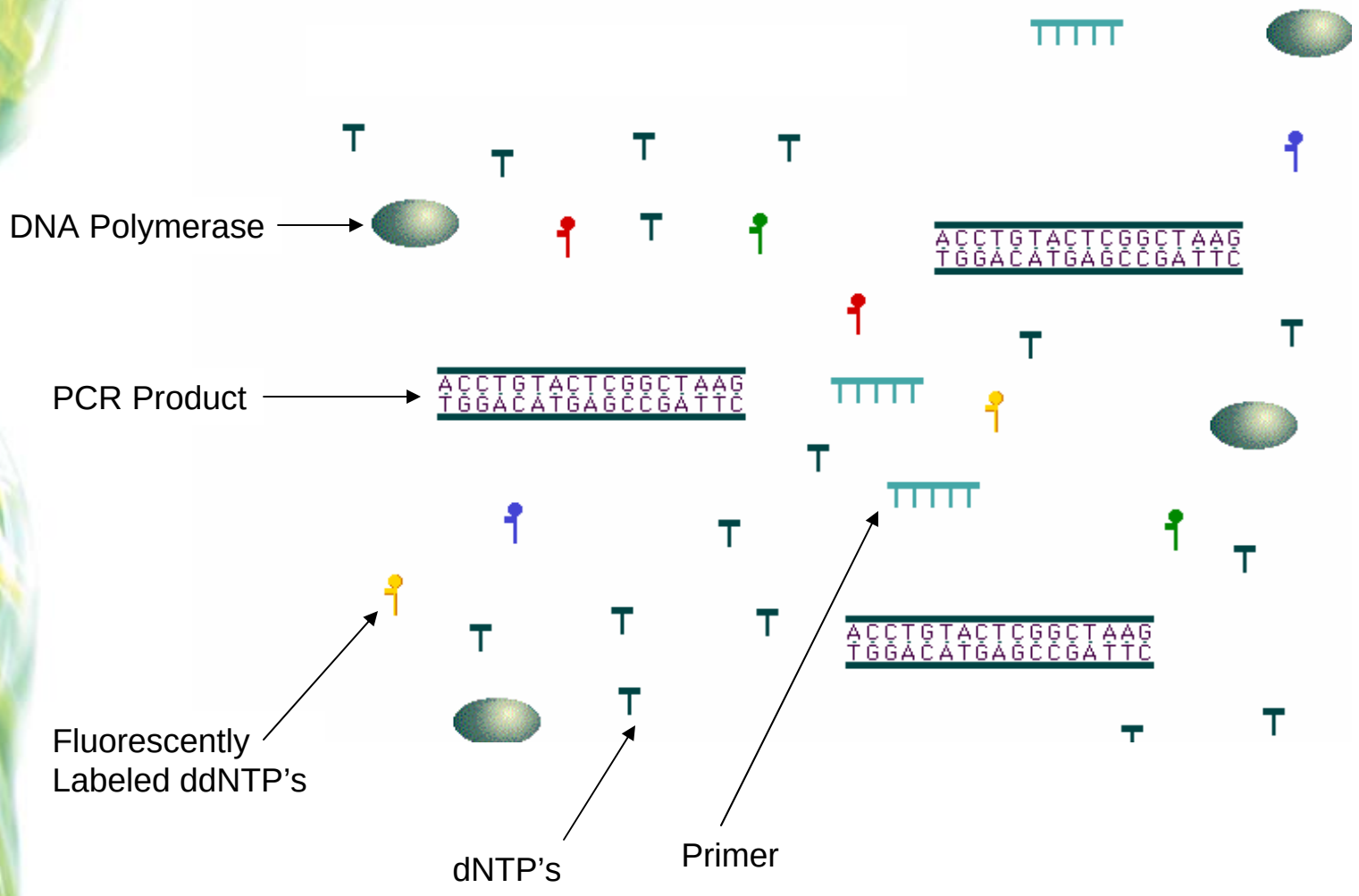
Cycle Sequencing

- Bacterial and Fungal Master Mixes using latest ABI fluorescent dye chemistry
  - DNA Polymerase
  - dNTP's
  - Fluorescently labeled, ddNTP's
  - 1 Primer per reaction
- Purified PCR product

- Generation of extension products, which are used to determine DNA sequence of PCR product

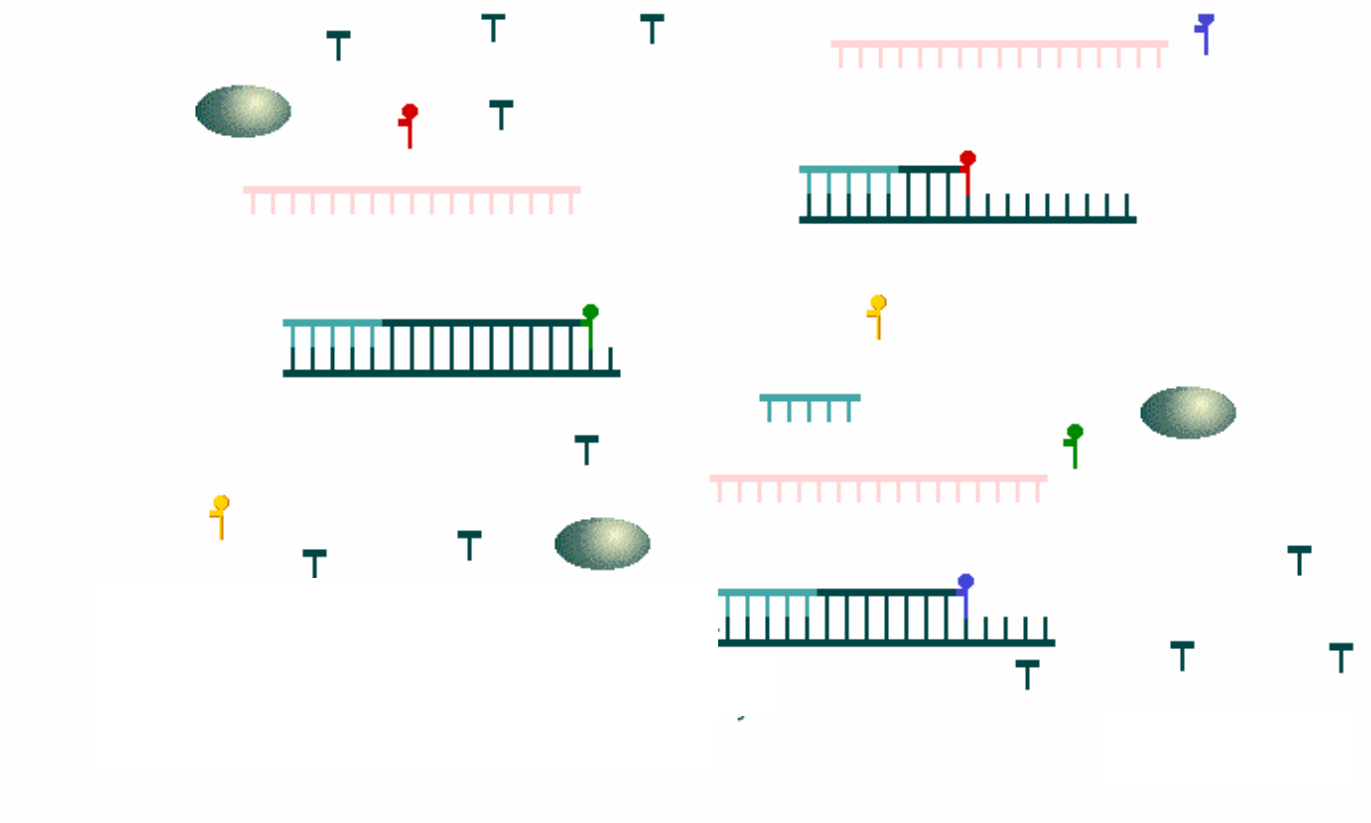
ACCUGENIX®
IDENTIFY WITH THE LEADER.

Cycle Sequencing



ACCUGENIX®
IDENTIFY WITH THE LEADER.

Cycle Sequencing



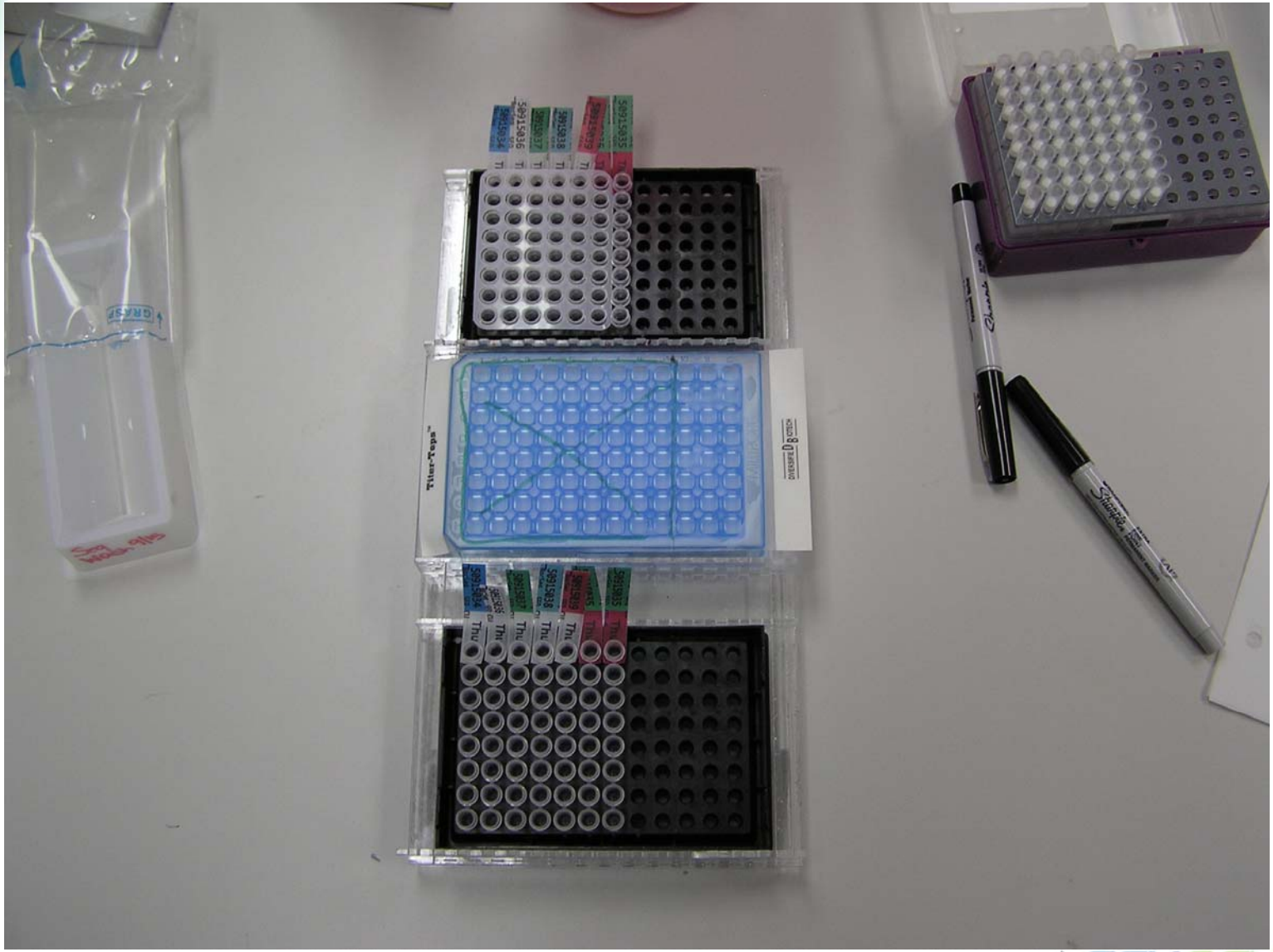
ACCUGENIX®
IDENTIFY WITH THE LEADER.

Cycle Sequencing Clean-up

- Extension products are purifies using size based filters
- Filters allow unincorporated primers, dNTP's, and ddNTP's to pass through
- Extension products remain on the filter
- Extension products are washed with water, then resuspended in Injection Solution (low conductivity)

ACCUGENIX®
IDENTIFY WITH THE LEADER.

Cycle Sequencing Clean-up



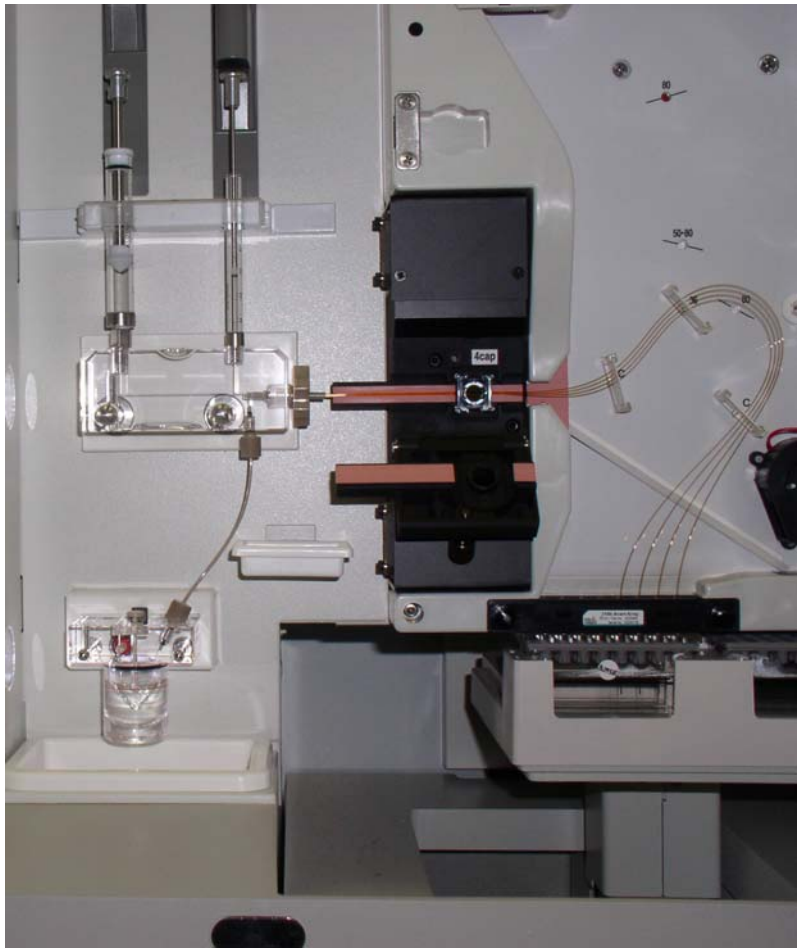
ACCUGENIX®
IDENTIFY WITH THE LEADER.

Automated Fluorescent DNA Sequencing

- Size separation of extension products from Cycle Sequencing reaction
  - ABI 3100 - 16 channels
  - Run time of 1 hour
- Sequencer automatically injects, separates, and detects extension products
- Generates Electropherogram of Sequence data

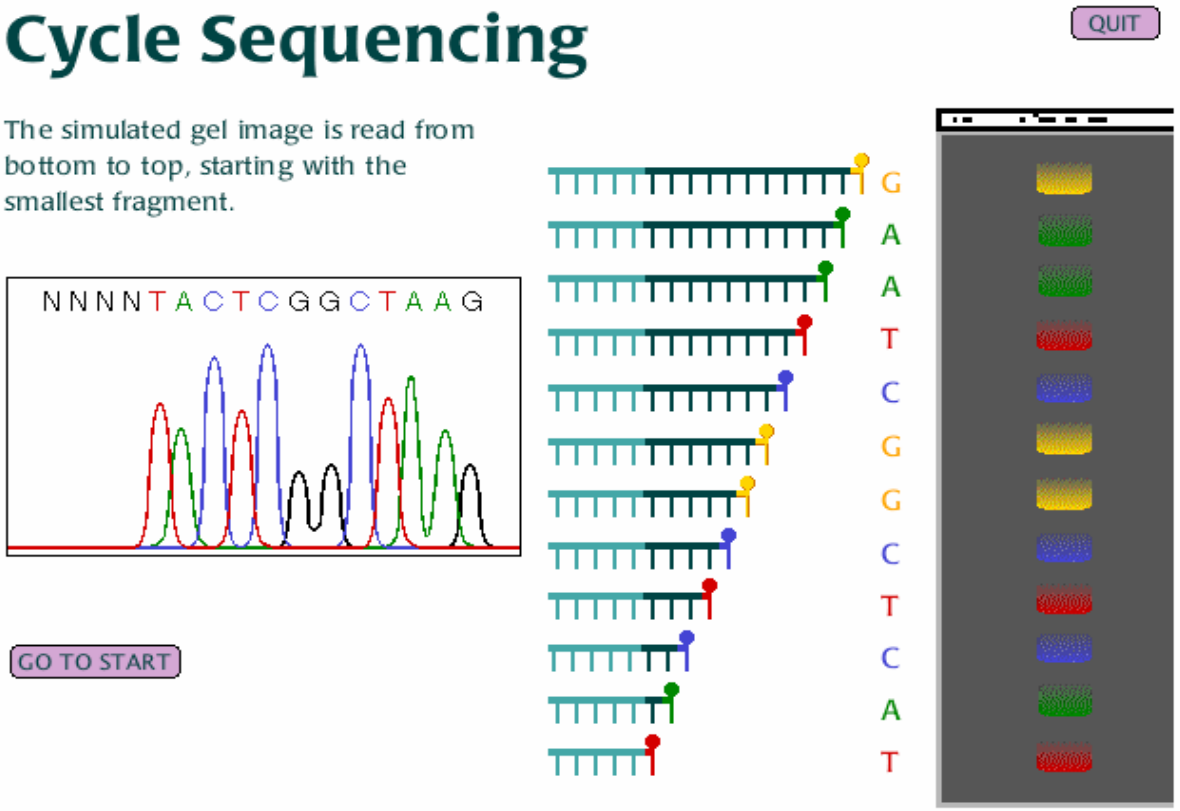
ACCUGENIX®
IDENTIFY WITH THE LEADER.

ABI 3100 Genetic Analyzer



ACCUGENIX®
IDENTIFY WITH THE LEADER.

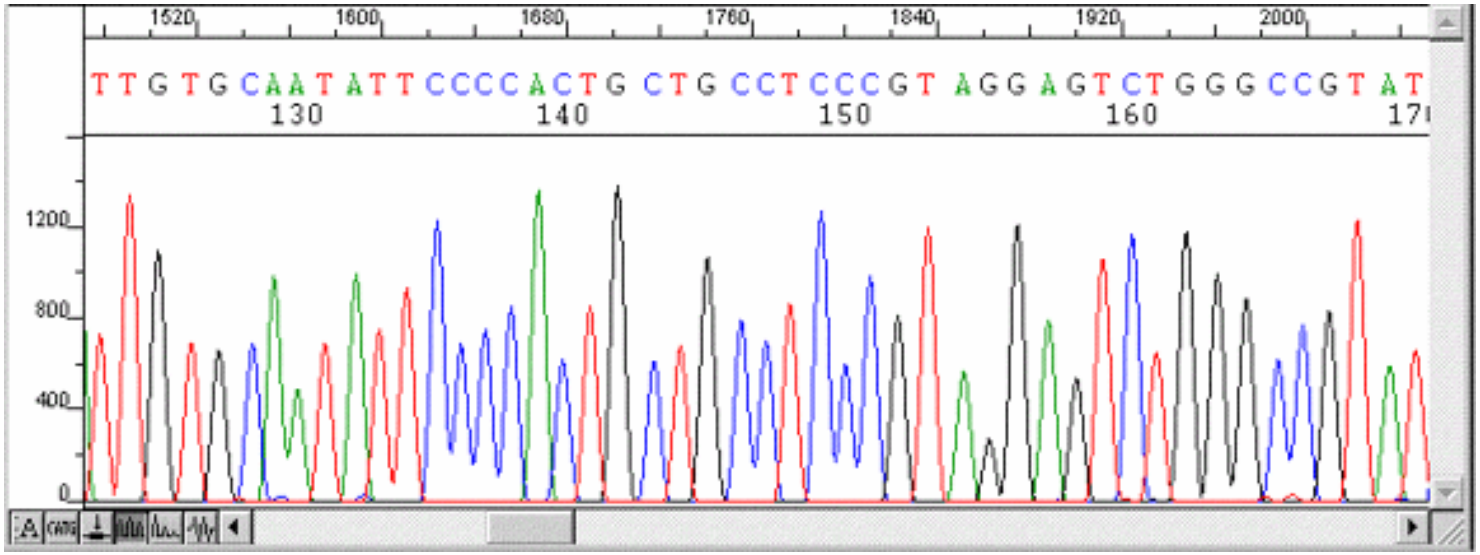
Automated Fluorescent DNA Sequencing



ACCUGENIX®
IDENTIFY WITH THE LEADER.

Automated Fluorescent DNA Sequencing

Analyzed Data (Electropherogram)



ACCUGENIX®
IDENTIFY WITH THE LEADER.

Identification Report - Bacterial 500bp

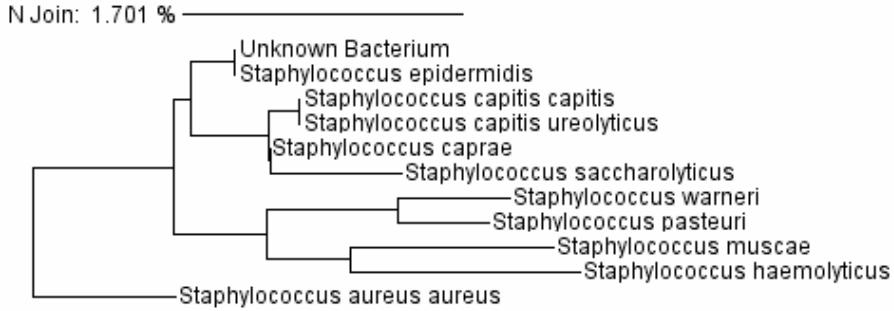
Customer: Example Report

Sample: Unknown Bacterium

Date: Friday, November 8, 2002 11:34 AM

Alignment: 535 Unknown Bacterium
0.00 % 535 Staphylococcus epidermidis
0.75 % 535 Staphylococcus caprae
0.93 % 535 Staphylococcus capitis ureolyticus
0.93 % 535 Staphylococcus capitis capitis
1.54 % 535 Staphylococcus saccharolyticus
2.24 % 535 Staphylococcus warneri
2.24 % 535 Staphylococcus aureus aureus
2.24 % 535 Staphylococcus muscae
2.43 % 535 Staphylococcus pasteuri
2.62 % 535 Staphylococcus haemolyticus

Neighbor Joining Tree



MicroSeq™ Database Search Result

Identification: Staphylococcus epidermidis

Confidence Level: Species

Reviewed _____