



Medical Devices and LAL Testing

Associates of Cape Cod, Inc.

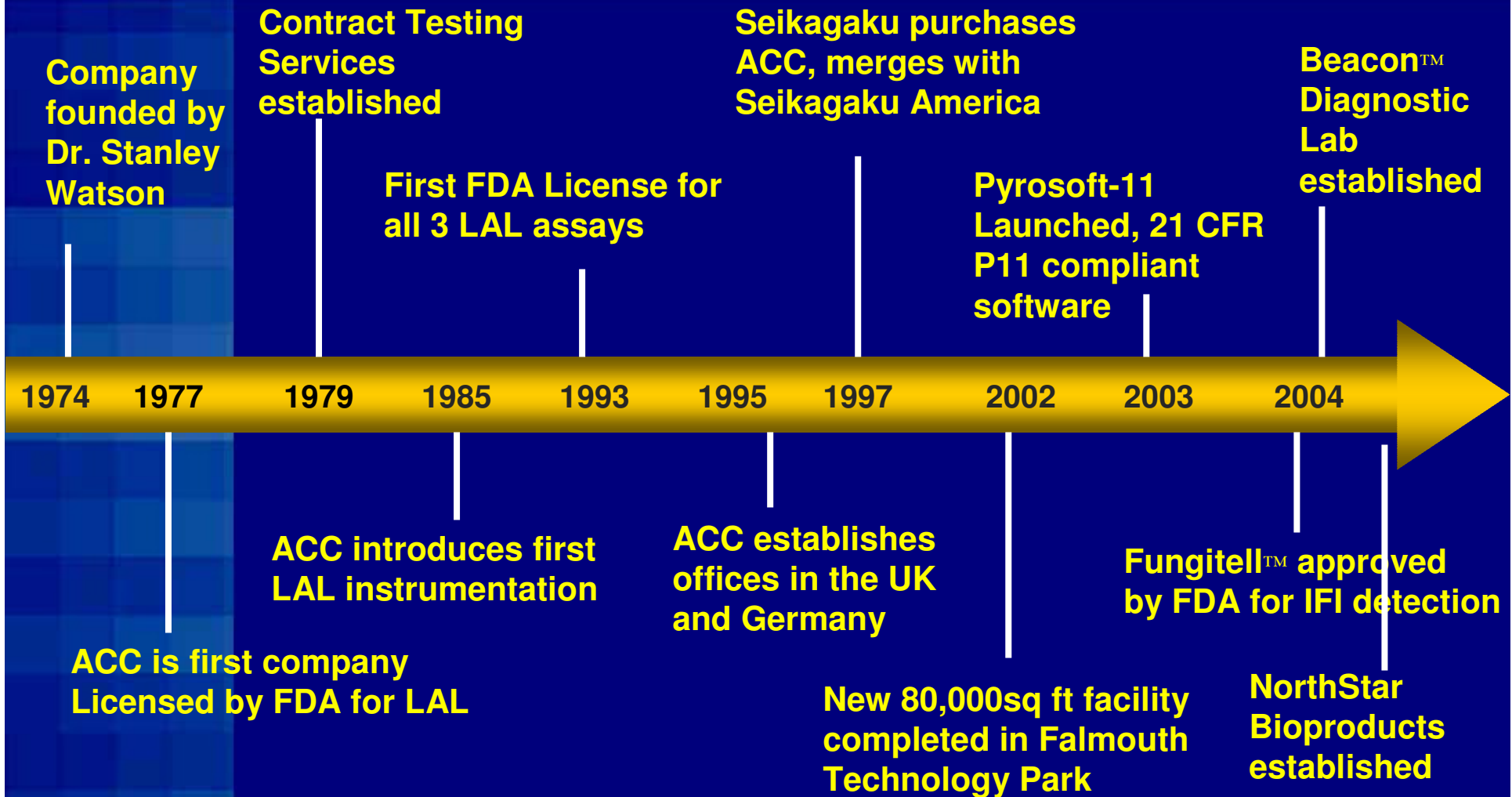
Laurie Fife

Associates of Cape Cod, Inc.

- Falmouth, Massachusetts
- FDA Licensed Manufacturer, License #700, ISO 13485 Certified
- 80,000 square foot state-of-the-art manufacturing building including:
 - Class 100,000, Class 10,000 and Class 100 workspace
 - Research and Development
 - Contract Testing Services
 - Customer Service
 - Technical Service
 - Corporate Offices



ACC History

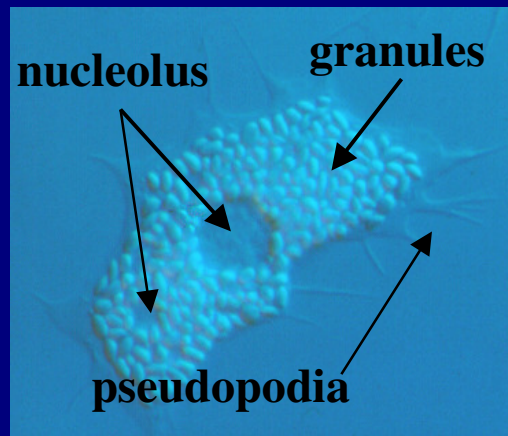


Limulus Amebocyte Lysate

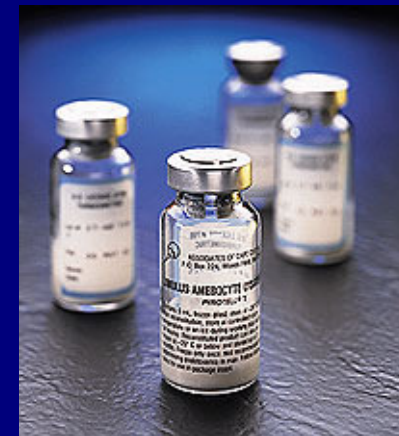
- LAL
- An extract that forms a clot in the presence of endotoxin



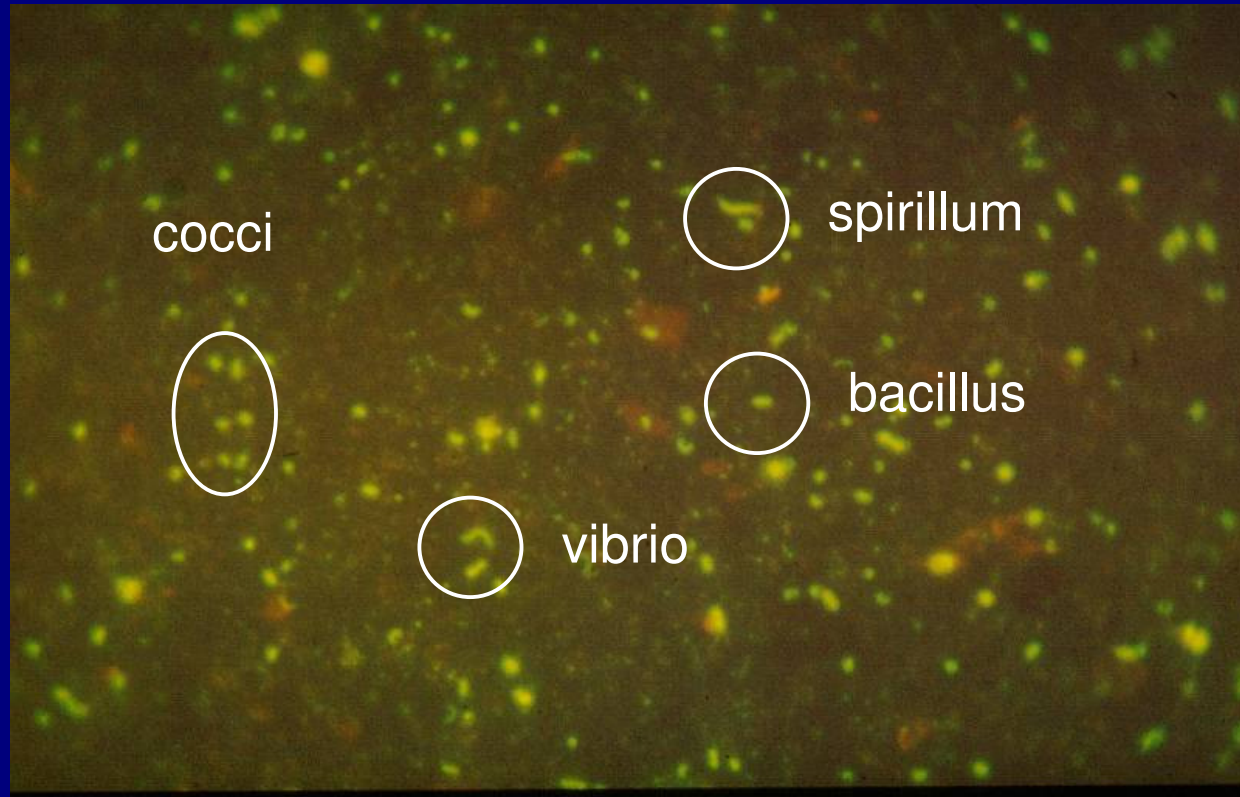
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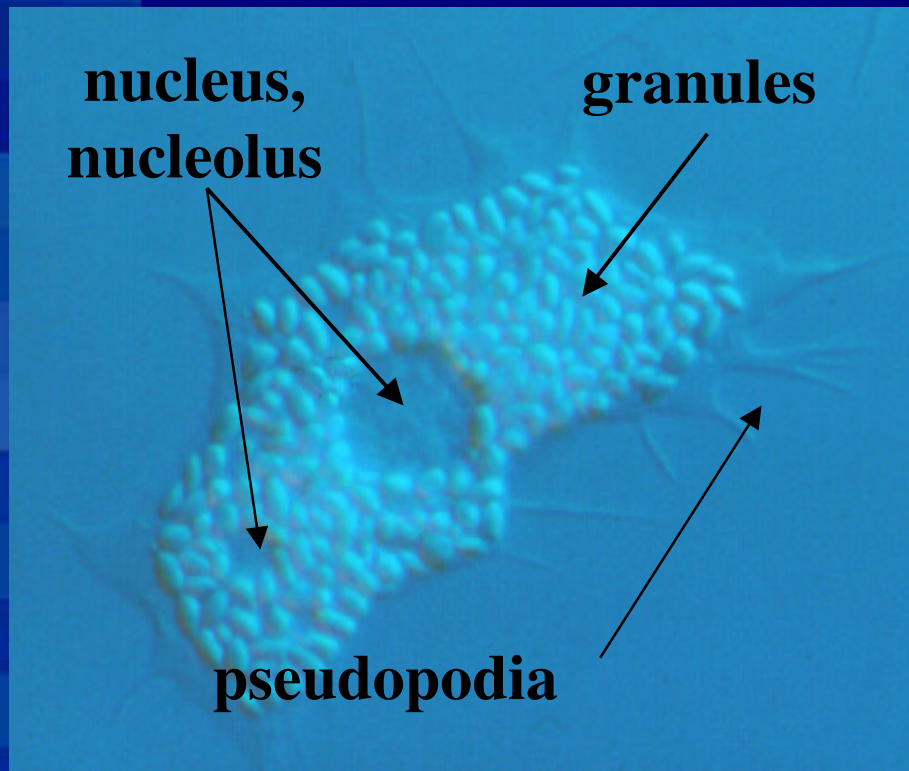


Ocean Environment



1 billion gram negative bacteria/mL
(seawater sample stained with Acridine
Orange)

Host Defense Mechanism



- Granules contain clotting factor/ enzymes
- The crab can survive having most of its blood clot

Host Defense Mechanism

- Gram-negative bacteria or endotoxin causes blood cells (amebocytes) to release proteins into the plasma (hemolymph) causing the blood to coagulate
- Coagulation kills bacteria and neutralizes endotoxin

History and Discovery of LAL

- The earliest work on horseshoe crab blood was carried out in the late 1800's
- Bang described a bacterial disease of the horseshoe crab (1953)
- Levin and Bang demonstrated clotting with endotoxin *in vitro* (1960's)

Levin and Bang's Impact

- Strong clinical interest
- Mixed Clinical results
- Pharmaceutical companies became interested

LAL and USP Pyrogen Test

- Mascoli and Weary (1979) compared ~28,000 rabbit tests to ~143,000 LAL tests

LAL: 37 samples had unacceptable levels of endotoxin

Rabbit pyrogen test only detected endotoxin in 4 samples

And no LAL false negatives

LAL and USP Pyrogen Test

- LAL more sensitive
Use of live animals (*in vivo*) causes variability in test results
- LAL detects sub-pyrogenic amounts of endotoxin

Manufacture of LAL

- Collect animals
- Extract blood
- Centrifuge blood
- Wash cells
- Lyse cells
- Extract proteins

Horseshoe Crab Collection



- Handling with care maximizes survival rate

ACC Manufacturing



ACC Manufacturing



Manufacture of LAL

- QC release
 - Determine sensitivity
 - 6-hour test for quality/negative background
 - pH
 - Test for sterility
 - Test for moisture

Manufacture of LAL

- Labeling
- Submission to and release by the FDA
- Product sales

FDA Licensed Products

LAL (*Limulus Amebocyte Lysate*)

METHOD	TRADENAME
Gel Clot	Pyrotell [®] Multitest
	Pyrotell [®] STV
Turbidimetric	Pyrotell [®] -T
Chromogenic	Pyrochrome [®]
	Chromo-LAL



Endotoxins



Definitions

Pyrogen

- A substance that produces a fever when injected into blood or cerebrospinal fluid

Endotoxin

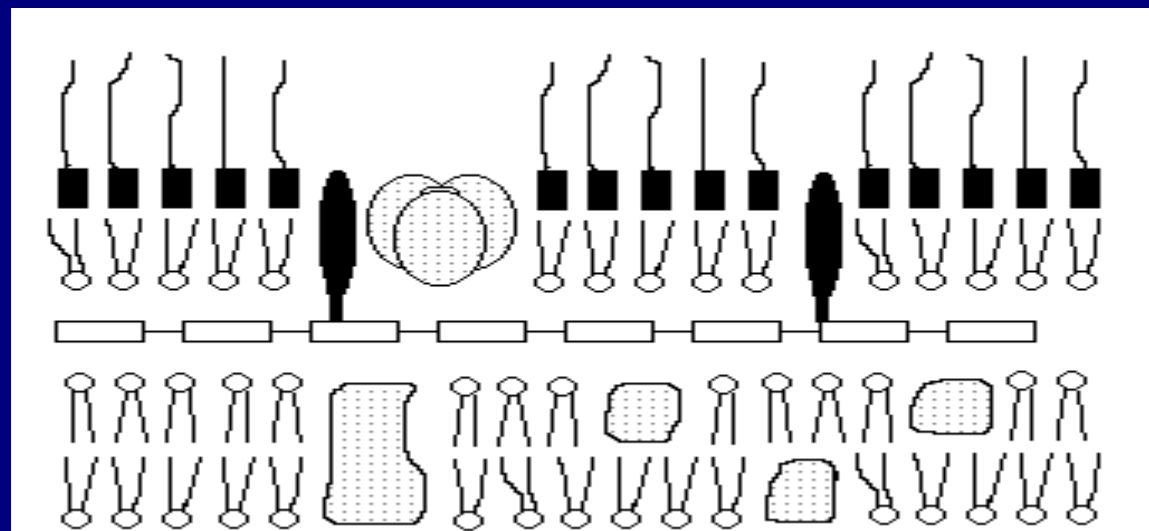
- A pyrogen in the cell wall of Gram-negative bacteria

Lipopolysaccharide (LPS)

- Chemically purified endotoxin

What is Endotoxin?

- A lipo-polysaccharide structural component of gram (-) bacterial cell wall
- An Immunomodulator that can cause:
fever, inflammation, headaches, nausea, chills, vomiting, hypotension, lung toxicity, etc... & death.



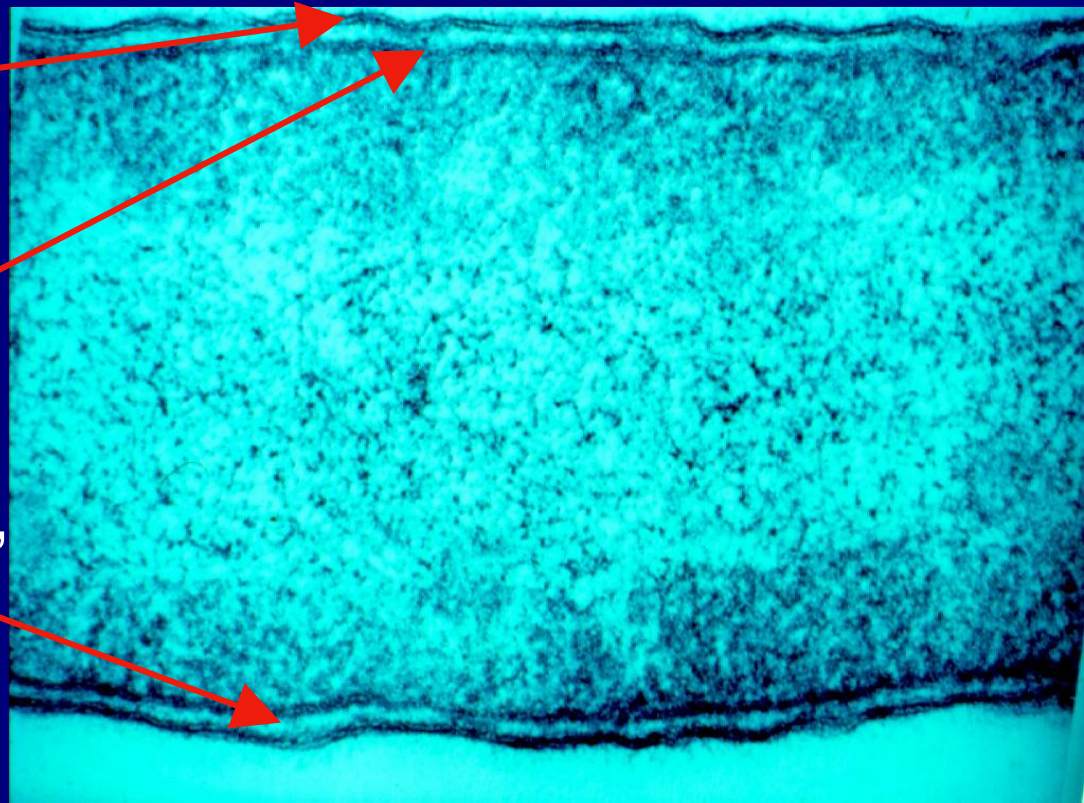
Bacteria

Electron micrograph of Gram-negative rod

Outer
Membrane

Inner
Membrane

“Double-track”



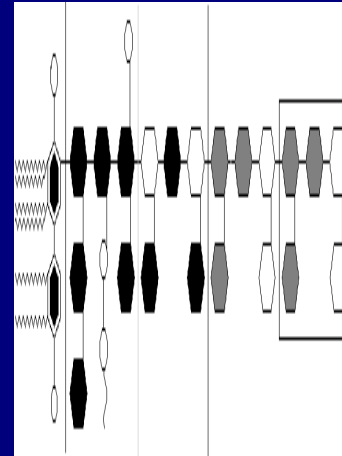
LPS Properties

- Polysaccharide
 - Hydrophilic
- Lipid A
 - Hydrophobic
- Negatively charged
 - Non-specific adsorption
 - Hydrophobic interaction
- Hydrophobic interactions form micelles, etc. stabilized by charge neutralization

Endotoxin

Depyrogenation: *the removal or destruction of pyrogens, particularly endotoxin*

- Dry heat
- Base hydrolysis
- Oxidation



Lipid A

Physical Depyrogenation

Dry Heat

- 170°C for 2 hours (sterilization)
No endotoxin destruction
- 180°C for 3 hours (depyrogenation)
Endotoxin destruction
- 250°C for 30 minutes
Endotoxin destruction

Always Remember

- Sterile
Doesn't mean depyrogenated
- Pyrogen-free
Doesn't mean no pyrogens
- Dry Heat
Most effective depyrogenation

Endotoxin

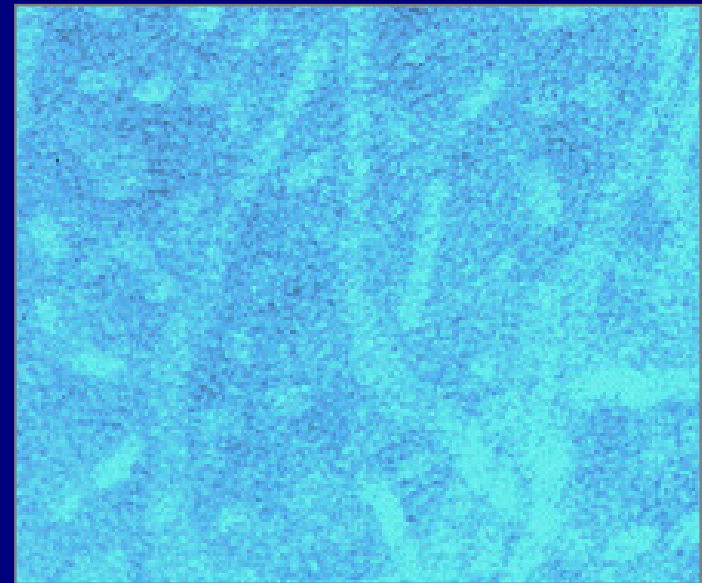
Various sized aggregates (particles)

Chelating agents

Acid or base

Surfactants

Charged/hydrophobic
proteins



Endotoxin

Biological Activity:

(in blood or cerebrospinal fluid)

- Fever
- Headache
- Chills
- Nausea
- Vomiting
- Hypotension
- Lung toxicity
- Abortion
- Death

Endotoxin

Human Threshold Pyrogenic Doses

- Greiseman and Hornick (1969)
Pyrogenicity of different organisms

<u>Organism</u>	<u>Pyrogenic Dose (ng/kg)</u>
<i>Salmonella typhosa</i>	0.1 - 0.14
<i>Escherichia coli</i>	1.0
<i>Pseudomonas sp.</i>	50 - 70

Endotoxin

- Endotoxin Unit (EU) is a unit of biological activity
- 1 EU = 1 IU (International Unit)
- EU is defined for the reference standard endotoxin (RSE) and must be measured for any other endotoxin

Endotoxin Limits

- Parenteral drugs:
 - 5 Endotoxin Units/kg body weight/hour
 - 350 EU/adult/hour
- Intrathecal drugs:
 - 0.2 EU/kg/hr
 - 14 EU/person/hour

Endotoxin Limits

Medical devices

- 20 EU/unit or 200 EU/unit (worst case) if device rinses are pooled for testing

Large volume parenterals

- (700mL/person/hr) = 0.5 EU/mL

USP Water for Injection

- 0.25 EU/mL



Limulus Amebocyte Lysate (LAL) Test Methods

Limulus Amebocyte Lysate

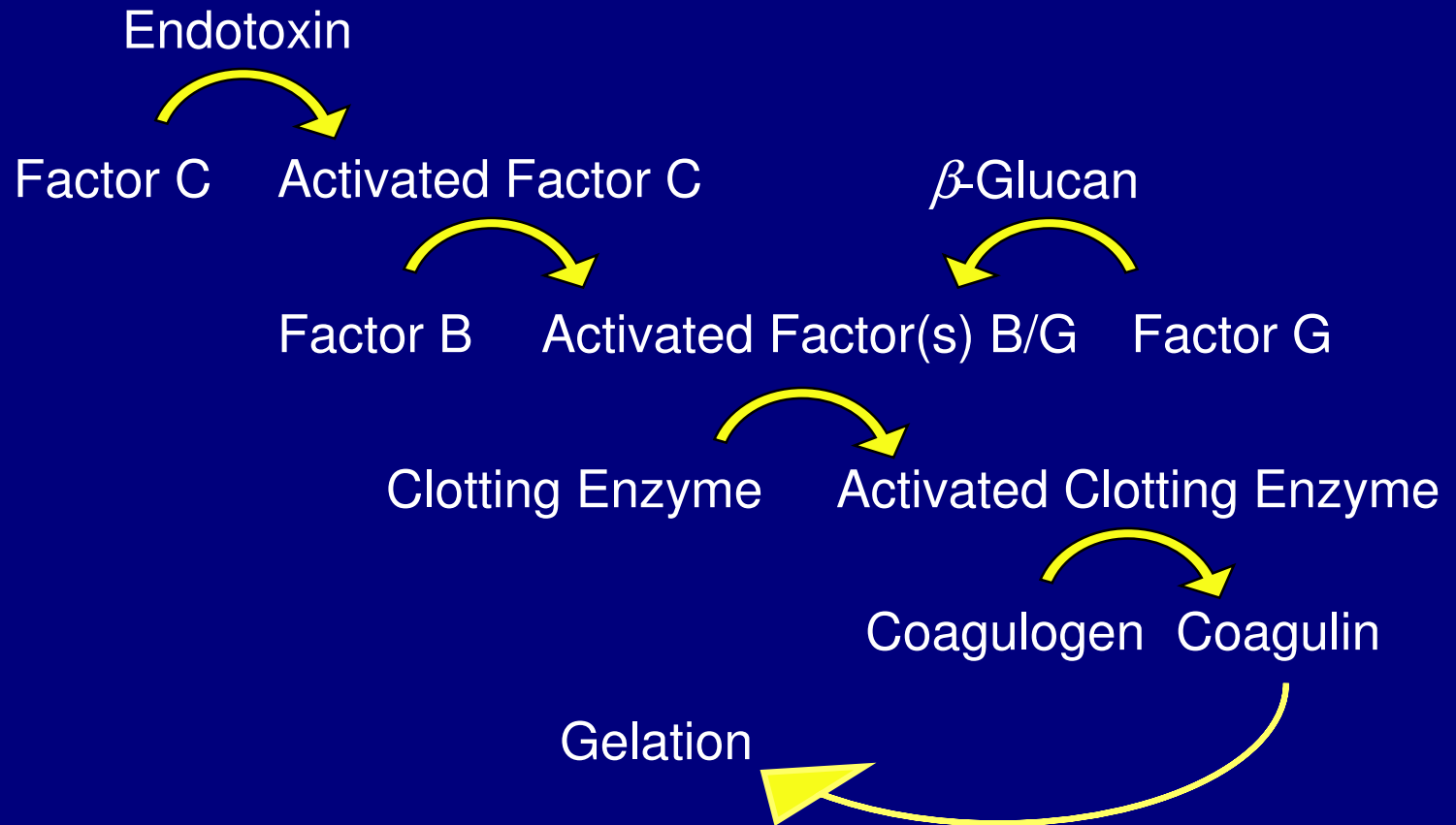


- Extract from blood cells of *Limulus polyphemus*
- Clots in the presence of endotoxin *in vitro*

Limulus Amebocyte Lysate

- Lyophilized blood cell lysate
- Complex mixture of proteins
 - Four involved in the clotting cascade
- Engineered to increase natural sensitivity
 - Formulated with salts, fillers, and buffers for each specific method

Biochemical Cascade

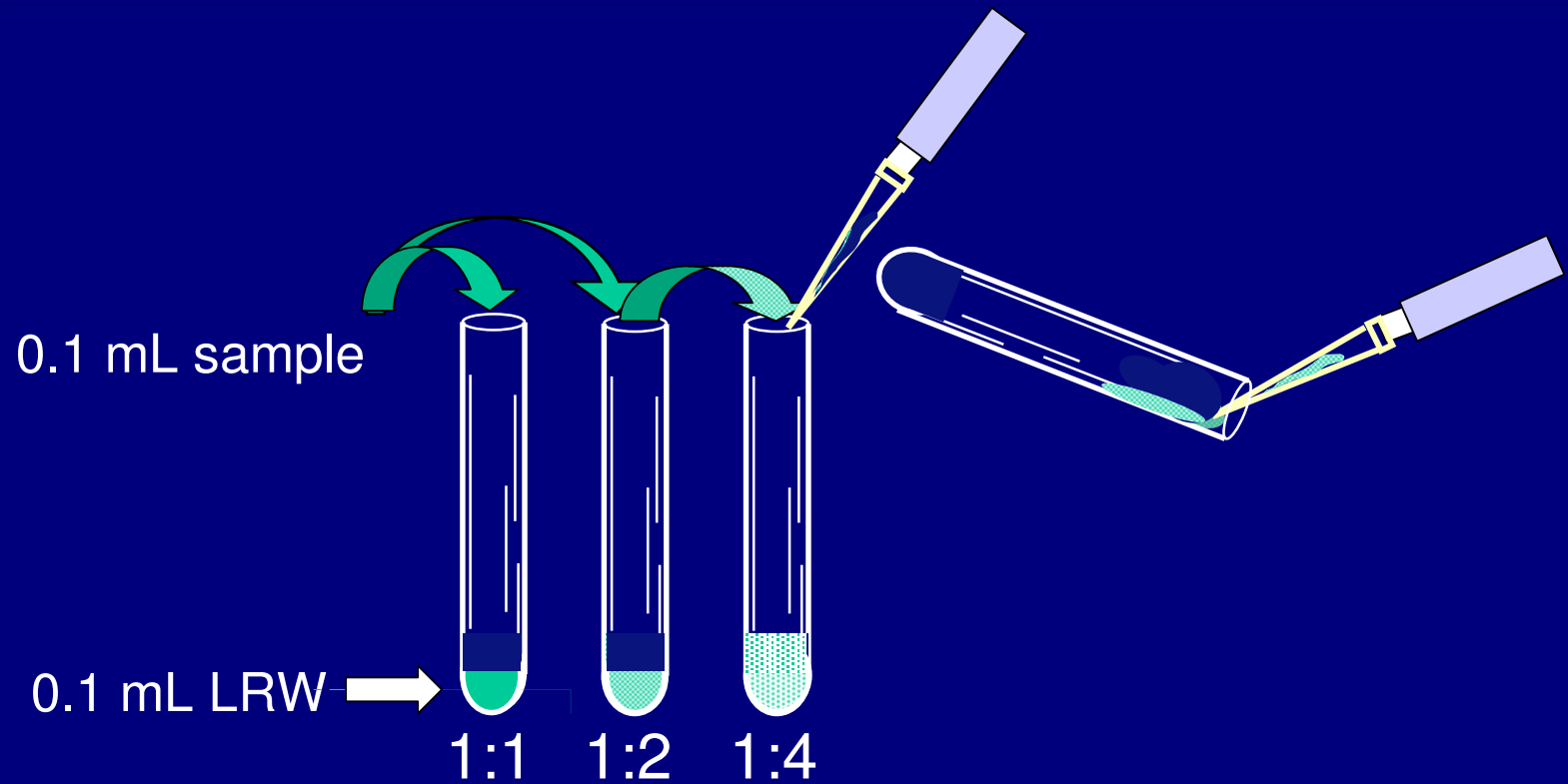


LAL Test

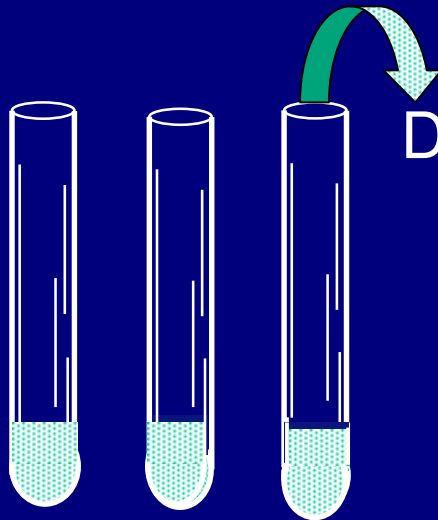
- Gel Clot
- Turbidimetric
- Chromogenic



Setting up the test



Setting up the test

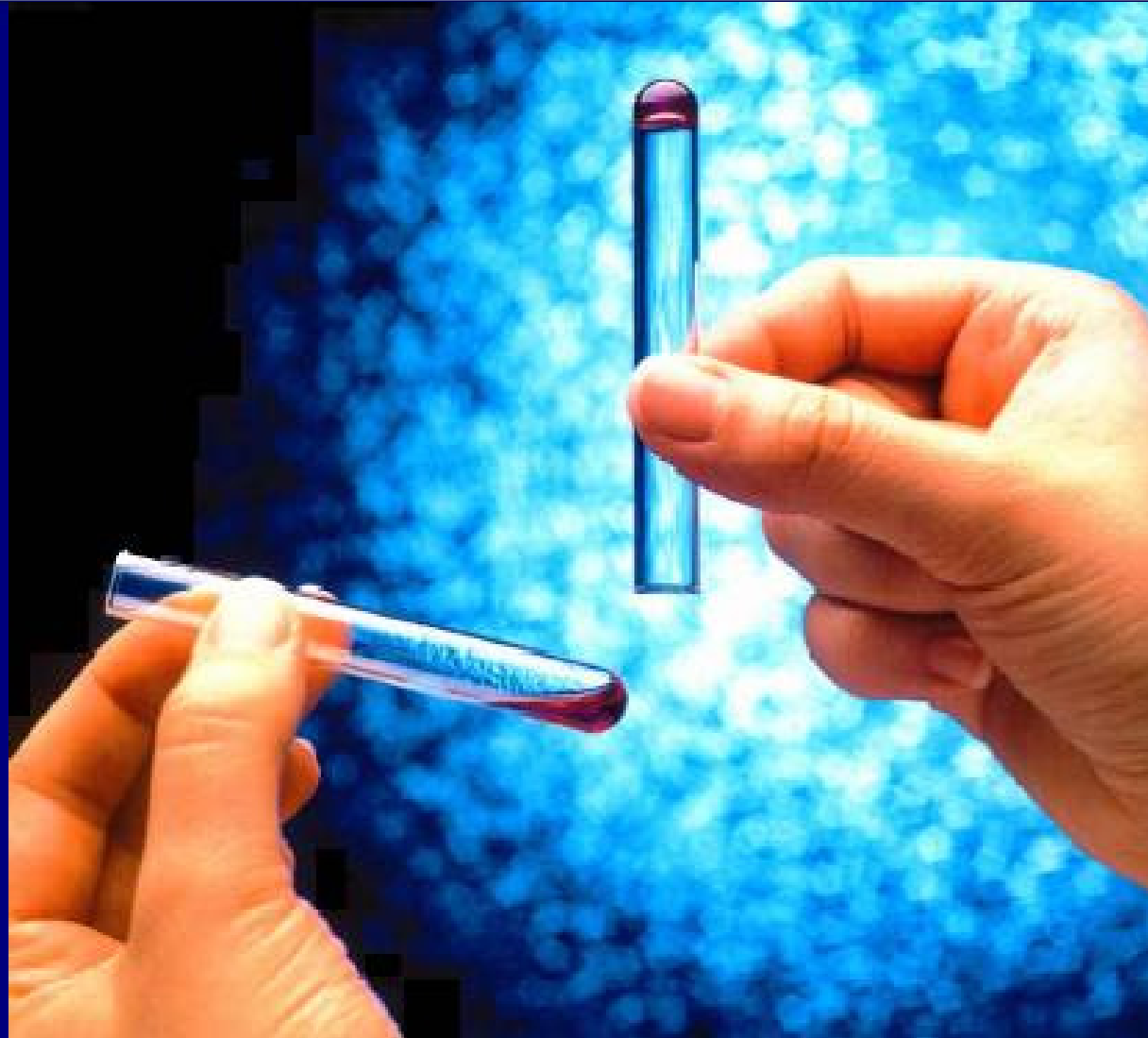


Discard 0.1 mL to maintain equal volumes

Test volume is 0.1 mL
Add 0.1 mL LAL reagent

Mix thoroughly
Incubate immediately

Reading the Gel-Clot Test



LAL Method: Gel Clot

- Low equipment cost
- USP compendial method
- Resilient
- Fewer “outliers”

Photometric Methods



- Chromogenic
 $\lambda = 0.005 \text{ EU/mL}$

- Turbidimetric
 $\lambda = 0.001 \text{ EU/mL}$ in
tube reader
 $\lambda = 0.005 \text{ EU/mL}$ in
plate reader

Two Testing Methods

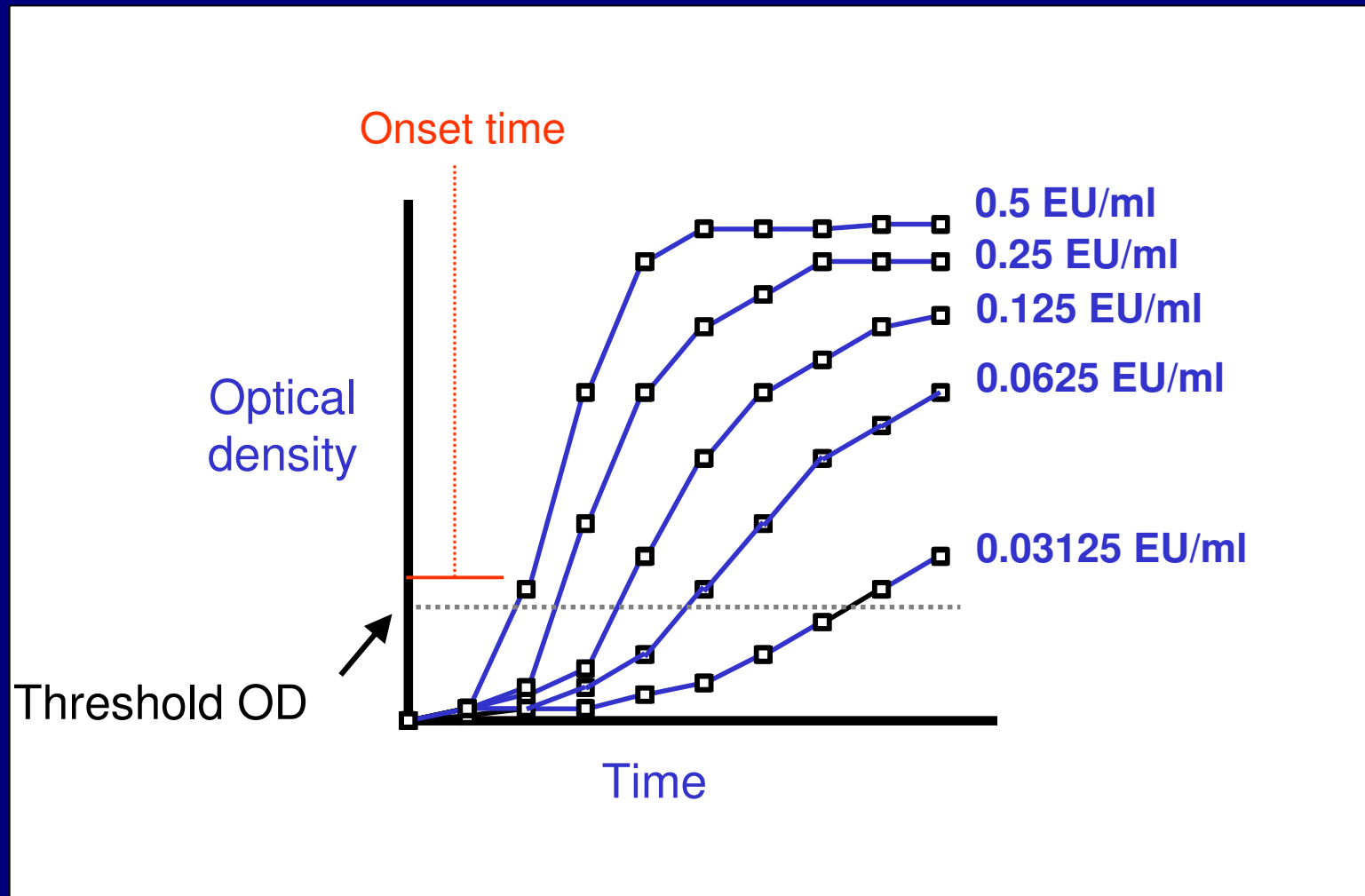
- Kinetic

An LAL test that gathers data in short intervals throughout a variable incubation period

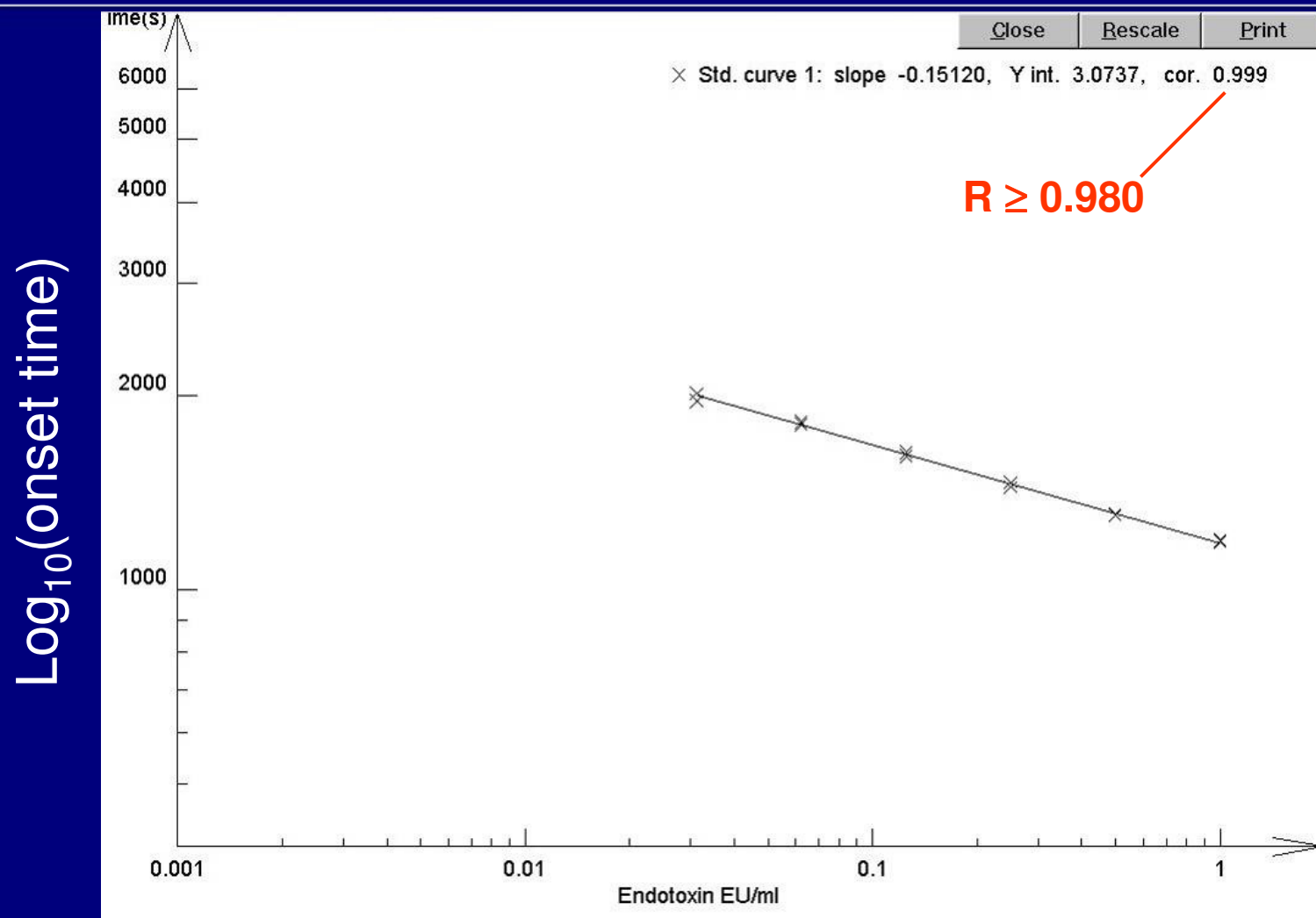
- Endpoint

An LAL test that is read once at the end of a set incubation period

Kinetic Method



Standard Curve (Kinetic Test)



$\text{Log}_{10}(\text{endotoxin concentration})$

Endpoint Method

- Absorbance (optical density) measured after fixed incubation period
- Standard curve
OD vs. endotoxin concentration
- Limitations
Limited range
More technician time involved

Turbidimetric Methods

- Coagulin molecules coalesce and cause increasing turbidity
- The rate of increase in turbidity is a function of endotoxin concentration

LAL Method: Turbidimetric

- Most sensitive
0.001 EU/mL
- Broad range
10 - 0.001 EU/mL
- Temperature control and timing
- Plate reader or tube reader option

Pyros Kinetix™



- 96 wells
- Small volume

$\lambda = 0.001$ EU/mL in tube reader

Chromogenic Reaction

- Color intensity indicates endotoxin levels
- Synthetic peptide
Amino acid sequence mimics natural coagulogen:



- Intact synthetic substrate is colorless

The Chromogenic Reaction

- Clotting enzyme recognizes synthetic substrate amino acid sequence and cleaves
- Paranitroaniline (pNA) released



- Free chromophore is yellow

Kinetic Chromogenic Method

- The rate of increase in optical density is a function of endotoxin concentration
- Onset times are calculated using an arbitrary threshold OD
- Standard curve
 $\log_{10}(\text{onset time})$ vs. $\log_{10}(\text{endotoxin concentration})$

ELX808 Microplate Reader



- $\lambda = 0.005$ EU/mL
- 4 Zone Temperature control



Select a Method: Chromogenic?

- Kinetic:

Sensitivity

0.005 EU/mL

Broad range

50 – 0.005 EU/mL

Rapid assay set-up

Instrumentation flexible

Use in other assays

BET Overview

- LAL Test Validation
 - Confirmation of labeled sensitivity
 - Qualification of technician/laboratory
 - Determine potency of control standard endotoxin (CSE)
 - Inhibition/Enhancement

BET Overview

- Inhibition/enhancement test
 - Does product affect test sensitivity?
 - Compare sensitivity in water to the sensitivity in sample
 - Sample can be extract, rinse, or flush

BET Overview

- Routine Test of a Validated Product
 - Release Test

$\lambda = 0.125$ EU/mL

	1:16	PPC
Repl. 1	-	+
Repl. 2	-	+

0.25	-Ctrl
+	-
+	-

- Sample preparation same as validated conditions
- Use validated dilution or dilution up to the MVD

Medical Device Challenge

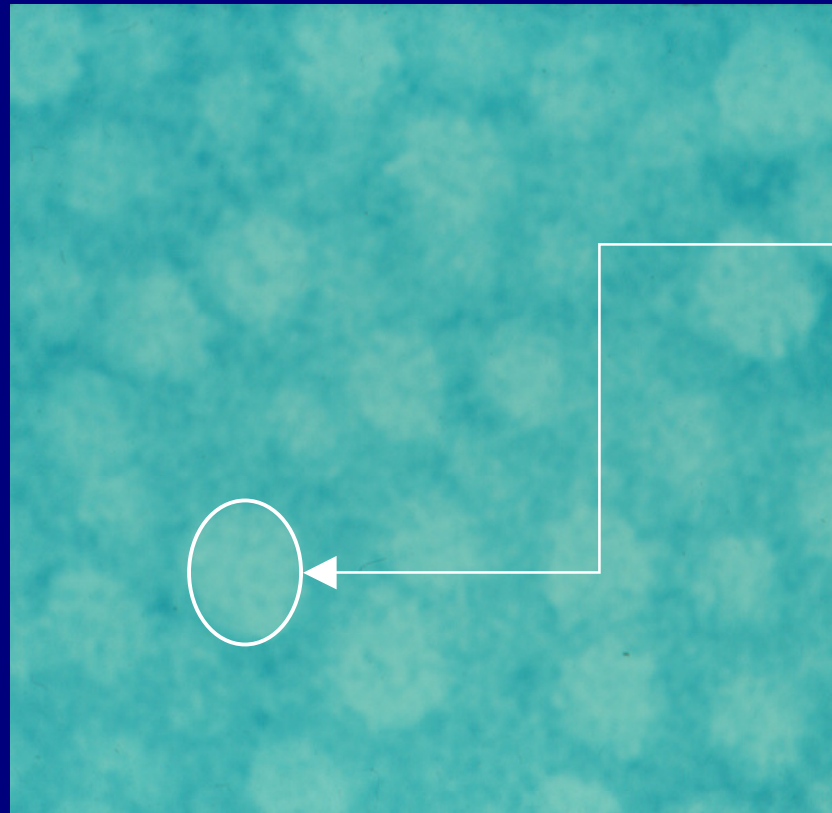
- LAL tests
 - Aqueous samples
- Device
 - Solid, gauze, gel, liquid, etc.
- Sample
 - Extraction
 - Rinse
 - Flush



LPS Properties

- Aggregated
 - MW in millions
 - Salt solutions
 - Divalent cations
 - Acid pH
- Disaggregated:
 - Monomers MW 2,500 - 25,000 Da
 - Surfactants
 - Chelating agents
 - Basic pH
 - Proteins, antibodies

LPS Properties



**Polymyxin B
molecules
neutralize LPS**

LPS Properties

Structural implications:

- Affinity for device surfaces
- Difficult to remove
 - Unlikely to wash off unless rinse solution has greater affinity
- Surface shear useful
 - Agitation

Define Test Sample

- FDA sampling protocol
 - Lot sizes <30, test 2 units
 - Lot sizes <100, test 3 units
 - Lot sizes >100, test 3%
 - Maximum of 10 units
- USP sampling protocol
 - Not less than 3 and not greater than 10 units
- Grouped by common chemical formulation

ANSI/AAMI ST72:2002

Define Test Sample

- Select devices with greatest exposure to tissue/body fluid
 - Non-pyrogenic fluid pathway
 - Non-pyrogenic device
- Test entire unit?
 - Kit?
 - Disassembled
 - Cut pieces
- Endotoxin is not evenly dispersed
 - Localized contamination

Define Extraction Protocol

- Extraction of critical surfaces
- Rinse
- Flush
- Define protocol and composition of extraction fluid
 - Validate fluid, if not LRW

Extraction Fluids

- LRW
 - Least interference
 - Standard solution for LAL test

- Saline
 - LAL interference
 - Least likely to recover endotoxin

Extraction Fluids

- Surfactant/Detergent; <0.1%
 - LAL interference
 - Better endotoxin recovery
- Human Serum Albumin; <0.1%
 - Minimal LAL interference
 - Better endotoxin recovery

Qualify Materials

- Materials contacting device should be
 - Free of detectable endotoxins
 - Sterile
 - Free of interfering substances
- Examples of materials
 - Containers, Manostat tubing, syringes, forceps
 - Aluminum foil, Parafilm strips, gloves(?)

Recommended Rinse Protocols

- Derived from pyrogen tests
- *FDA guideline*: Extract for one hour with fluid at room temperature ($>18^{\circ}\text{C}$) or for 15 minutes with fluid at 37°C
- *USP*: Initiate extraction with fluid at 37°C and extract for one hour at room temperature

Perform Preliminary Tests

- Measure endotoxin in sample
 - Use different sampling protocols
 - Test enough lots to establish level of uniformity of contamination within & between lots
- Inhibition/enhancement test
 - Gel-clot: sample dilution free of endogenous endotoxin
 - Photometric methods: do not require “clean” sample

Limits for Medical Devices

Endotoxin limit in the extract

$$= \frac{K \times N}{V}$$

K = tolerance limit expressed per device unit

N = number of samples pooled

V = total volume of pooled extracts

Limits for Medical Devices

- FDA guideline
 - Maximum 10 units/lot
 - Example:
 - 10 units, non-intrathecal
 - Extract 40 mL fluid/unit

$$\frac{K \times N}{V}$$

$$\begin{aligned} \text{Limit} &= \frac{(20 \text{ EU/unit}) \times (10 \text{ units})}{400 \text{ mL}} \\ &= 0.5 \text{ EU/mL} \end{aligned}$$

Limits for Medical Devices

Another example: (non-intrathecal)

- 10 units
- Extract 1.0 mL fluid/unit

$$\begin{aligned}\text{Limit} &= \frac{(20 \text{ EU/unit}) \times (10 \text{ units})}{10 \text{ mL}} \\ &= 20 \text{ EU/mL}\end{aligned}$$

Limits for Medical Devices

- 5 units
- Extract 20 mL fluid/unit
- Intrathecal Device

$$\begin{aligned}\text{Limit} &= \frac{(2.15 \text{ EU/unit}^*) \times (5 \text{ units})}{100 \text{ mL}} \\ &= 0.1 \text{ EU/mL}\end{aligned}$$

* FDA guideline states 2.4 EU/unit

Strategies

- Concentrate endotoxin in sample
 - At MVD?
 - Large extraction volume?
 - Interference?
- Ultrafiltration/diafiltration
 - Concentrates endotoxin and/or removes low MW interfering substances
 - Filter membrane, cartridge interference
 - Solutes may affect endotoxin MW

Inhibition/Enhancement Test

- Compare LAL sensitivities
 - LRW standard series
 - Sample standard series
- USP
 - Four replicates sample standard series
 - Two replicates water standard series

Inhibition/Enhancement Test

- Endpoints must each be within twofold of the labeled sensitivity of the LAL reagent

2λ	λ	$\lambda/2$	$\lambda/4$	LRW
+	-	-	-	-
+	+	-	-	-
+	+	-	-	-
+	+	+	-	-

Inhibition/Enhancement Test

CSE (EU/mL) in LRW

2λ	λ	$\lambda/2$	$\lambda/4$	- ctrl
+	+	-	-	-
+	+	-	-	-

CSE (EU/mL) in sample

2λ	λ	$\lambda/2$	$\lambda/4$	- ctrl
+	-	-	-	-
+	-	-	-	-
+	-	-	-	-
+	-	-	-	-

Is this test valid?

Slight inhibition

Valid test

Extraction Validation

Reference:

- Bacterial Endotoxin Testing: A Report on the Methods, Background, Data, and Regulatory History of Extraction Recovery Efficiency (2004)

Extraction Validation

Confirm that the extraction validation works

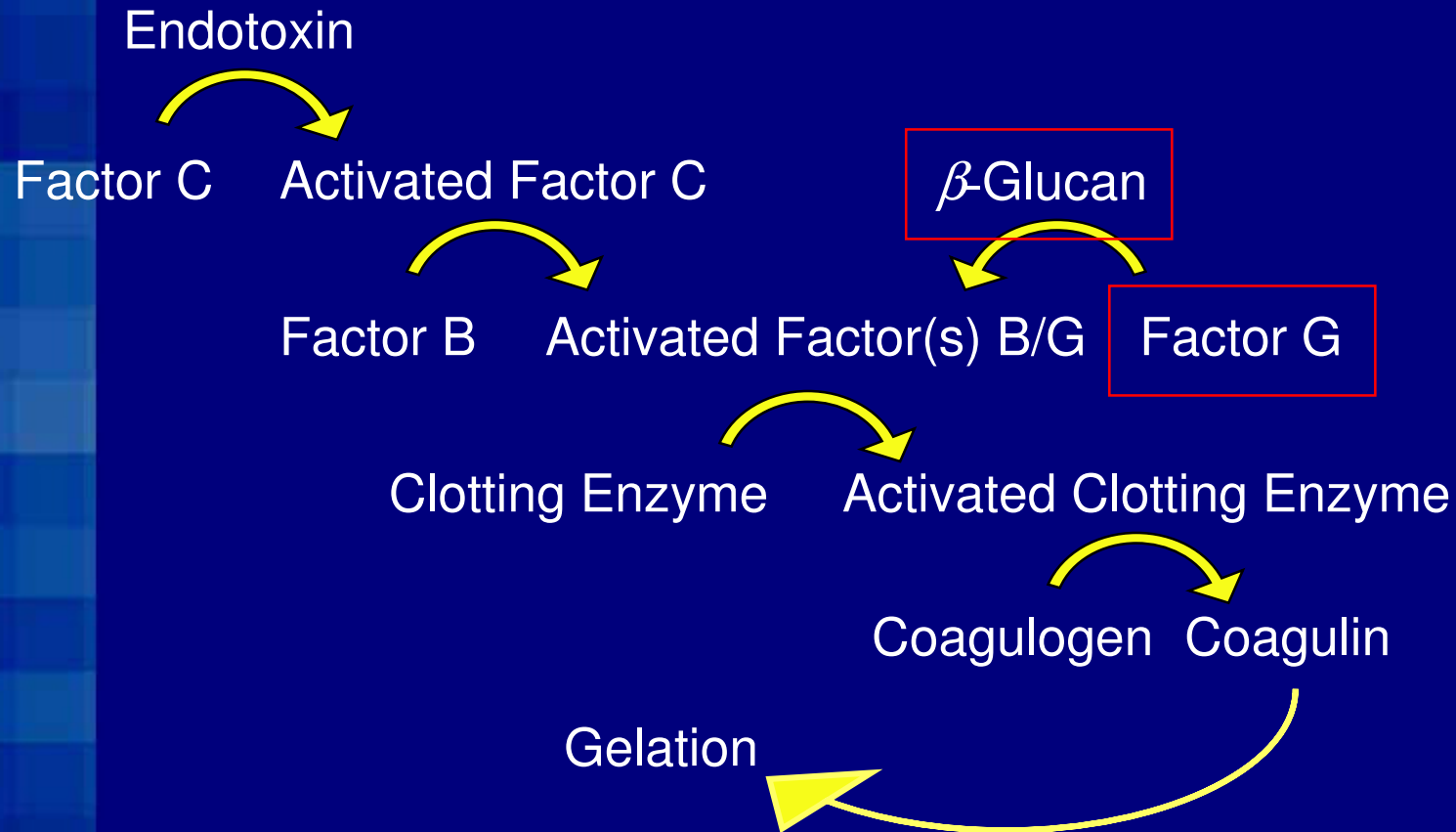
- Challenge device with known amount of endotoxin
- Perform extraction protocol
- Perform LAL test
- Calculate recovery

- If recovery is low, modify limits and/or protocol

Enhancement

- Beta-glucans (polysaccharides with β -linkages sugar molecules)
 - fungi, algae: 1,3-beta-D-glucans
 - cellulose: 1,4-beta-D-glucans
- LAL-Reactive Material (LRM) described in hemodialyzer rinses
 - Cellulose monoacetate, cellulose diacetate, saponified cellulose acetate

Beta-Glucans



Beta-Glucans

- Non-pyrogenic
- Synergistic with endotoxin
 - Low and high molecular weights
 - Variable structure
- Some structures are biologically active immunomodifiers

GlucateLL®

- (1→3)-β-D-glucan quantification (pg)
- Chromogenic method
- Verify false positives

Glucashield™

- LAL insensitive to glucans
- 1,000 ng /mL Pachyman
- Buffer



Conclusion

- 1977 FDA approved LAL test for release of medical devices
- Most devices are very straightforward
 - Water rinses
 - No interference
 - Endotoxin recovered from challenge

Conclusion

- Knowledge of product composition and endotoxin characteristics provides an edge
- Problem Samples
 - Antibacterial coatings
 - Collagens
 - Substances with a high affinity for endotoxin
- Preliminary characterization

References

- Manufacturer's product insert(s)
- USP Bacterial Endotoxins Test (BET) <85>
- “Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices,” FDA, 1987
<http://www.fda.gov/CBER/gdIns/lal.pdf>

References

- ANSI/AAMI ST72:2002
- Transfusion and Infusion Assemblies and Similar Medical Devices, <161> USP
- Bacterial Endotoxin Testing: A Report on the Methods, Background, Data, and Regulatory History of Extraction Recovery Efficiency (2004)



2008 Hong Kong

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