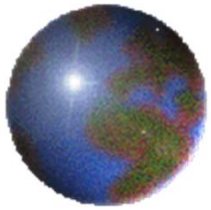
Endotoxin Detection by LAL for Parenteral Products



by

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CR Endosafe Asia Technical Manager

Woo Jung BSC, Inc.

Principal Microbiology Tests

- STERILITY: Detection of live microorganisms

- PYROGEN: Detection of cell-wall fragments from Gram(-) bacteria. History of a product's exposure to GNB

Endotoxins and Pyrogens

- Pyrogens are fever-inducing agents in humans and animals
  - include endotoxin, gram + cell debris, fungi
- Endotoxins are components from the outer membrane of gram-negative bacteria

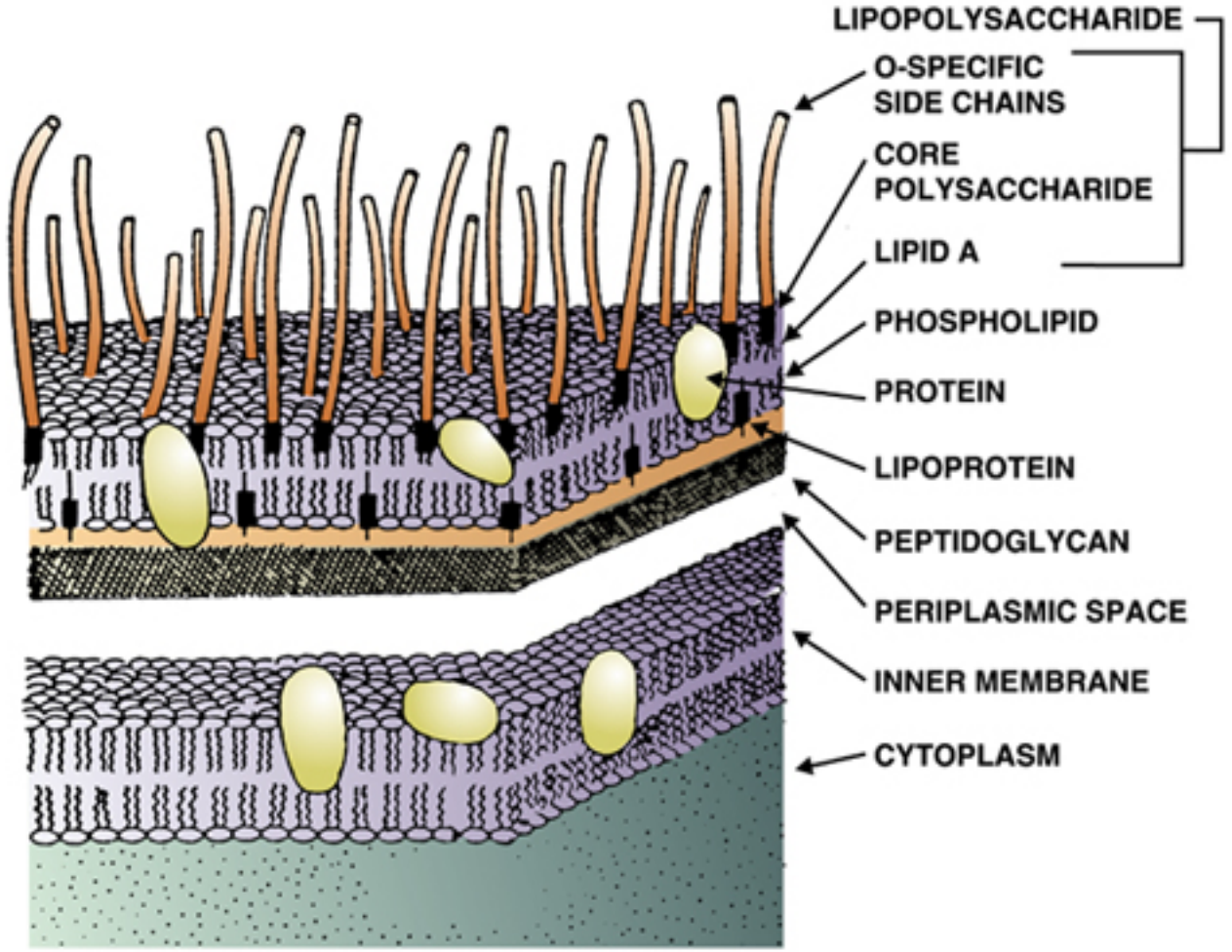


DIAGRAM OF A GRAM-NEGATIVE CELL MEMBRANE

Endotoxin

- Endotoxin - Component from outer membrane of gram-negative bacteria
  - Environmental (unpurified) endotoxin: complex of protein, carbohydrate & lipid
  - Purified endotoxin: lipopolysaccharide molecule of polysaccharide and lipid a, without protein

Purified Endotoxin - Lipopolysaccharide

- Passes through sterilizing membrane filters
- Lipid A region causes LAL reaction and potent pharmacological activities associated with endotoxin
- poorly dispersible in water
- pyrogenicity correlates with LAL assays

Why Test for Endotoxins?

- To protect against adverse reactions (sepsis) in humans and animals
- FDA and USP guidelines require final product testing on all parenterals and medical devices
- To safeguard against diminished effectiveness of a product due to endotoxin

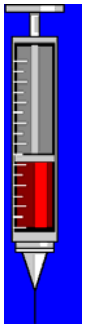
Endotoxicity

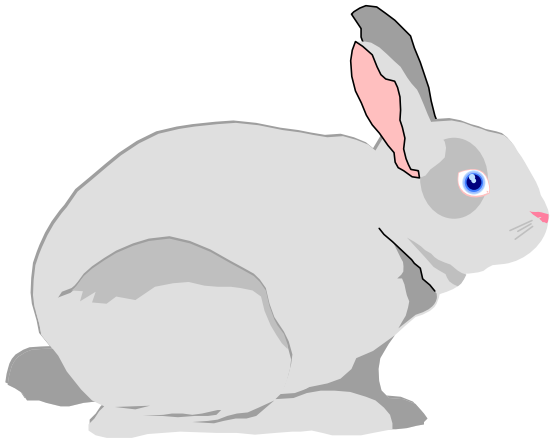
- ENDOTOXIN CAUSES MYELOID TISSUE TO RELEASE INFLAMMATORY MEDIATORS
- CYTOKINES INDUCE A VARIETY OF TISSUE DAMAGE
- SHOCK and MULTIPLE ORGAN DYSFUNCTION MAY OCCUR

How to test for Endotoxins?

- Rabbit Pyrogen Test
  - FD approved in 1941
- LAL / TAL Test
  - gel clot LAL approved in 1973
  - kinetic LAL in 1987
- Cell based assays
  - In Vitro pyrogen assay

Rabbit Pyrogen Test





Measure the change in temperature of 3 rabbits, after intravenous injection of the test solution.

LAL

- Limulus - genus of Horseshoe Crab
- Amoebocyte - blood cells
- Lysate - blood extract

LAL is a FDA regulated biological that reacts chemically in the presence of gram negative cell debris. This reaction can be measured and quantified.

LAL Discoveries by Bang and Levin

- Described role of endotoxin in coagulation of Limulus blood
- Prepared Endotoxin - responsive lysate from Amoebocytes

LAL Assay

- LAL is > 100 times more sensitive, specific and accurate
- Economy of LAL permits more testing
- Tolerance of rabbits to endotoxin is a concern for FDA
- LAL activity correlates with pyrogenicity

History of LAL Regulations

1941 Rabbit Pyrogen test included in USP

1973 FDA announced LAL as a biological product

1977 FDA described conditions for use of LAL as end-product test for human biological & medical devices

1980 Federal register drafted guidelines for use of LAL test for end-product testing of human & animal injectable drug products

History of LAL Regulations

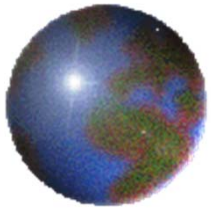
- 1983 Federal Register lists final guidelines on LAL testing including chrom and turb
- 1987 USFDA LAL - Test Guideline
- 1991 Interim Guidance - Kinetic LAL Test
- 1995 USP 23 Bacterial Endotoxin Test
- 1998 European Pharmacopoeia BET
- 2000 Harmonized BET

Other Regulatory Documents

- USP monographs
- Manufacturer's Package Insert
- Internal SOP
- AAMI guidelines
- other Pharmacopoeia

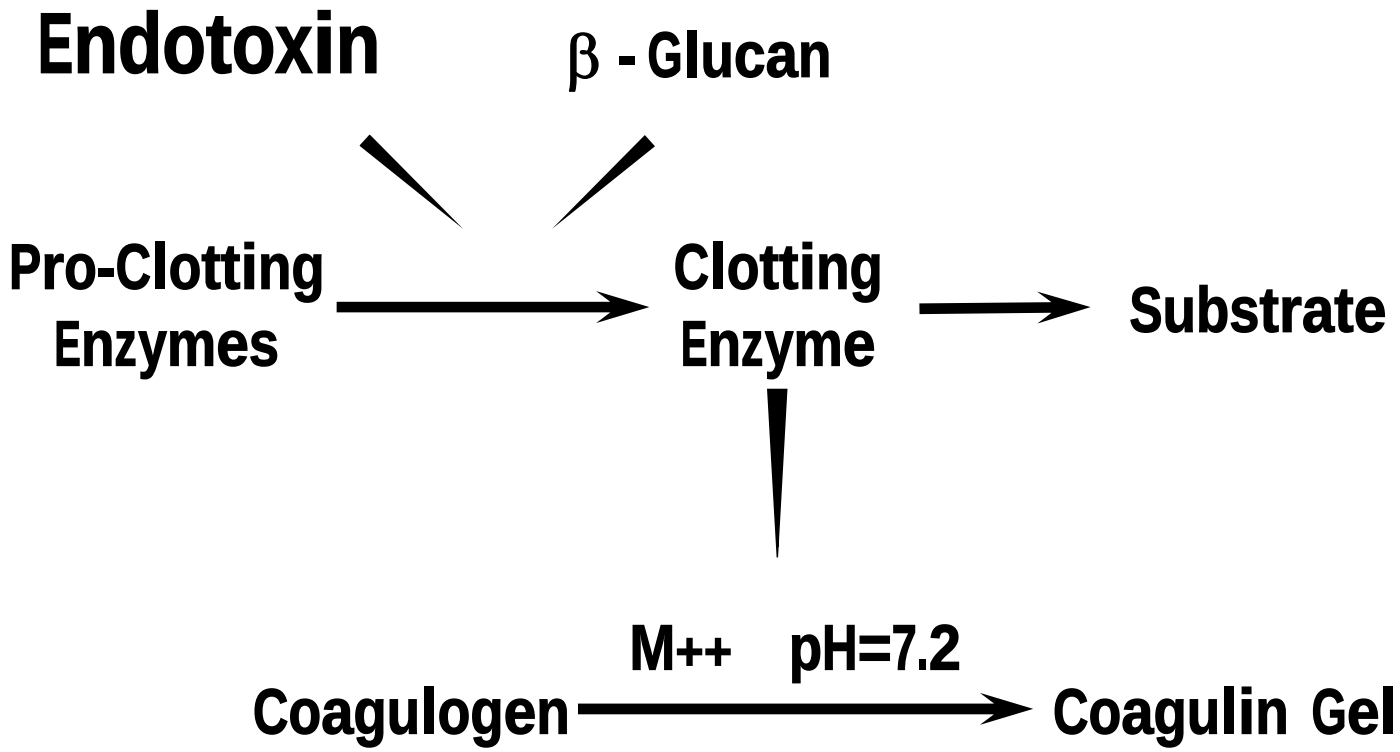
New Harmonized BET

- Effective July 2000
- First Microbiological test harmonized by ICH
- Allows use of endotoxin-specific LAL
  - must test for glucans if suspected in your sample
- Includes kinetic LAL methods



Gel Clot & Kinetics – The Basics

Clotting Reaction For Activated LAL

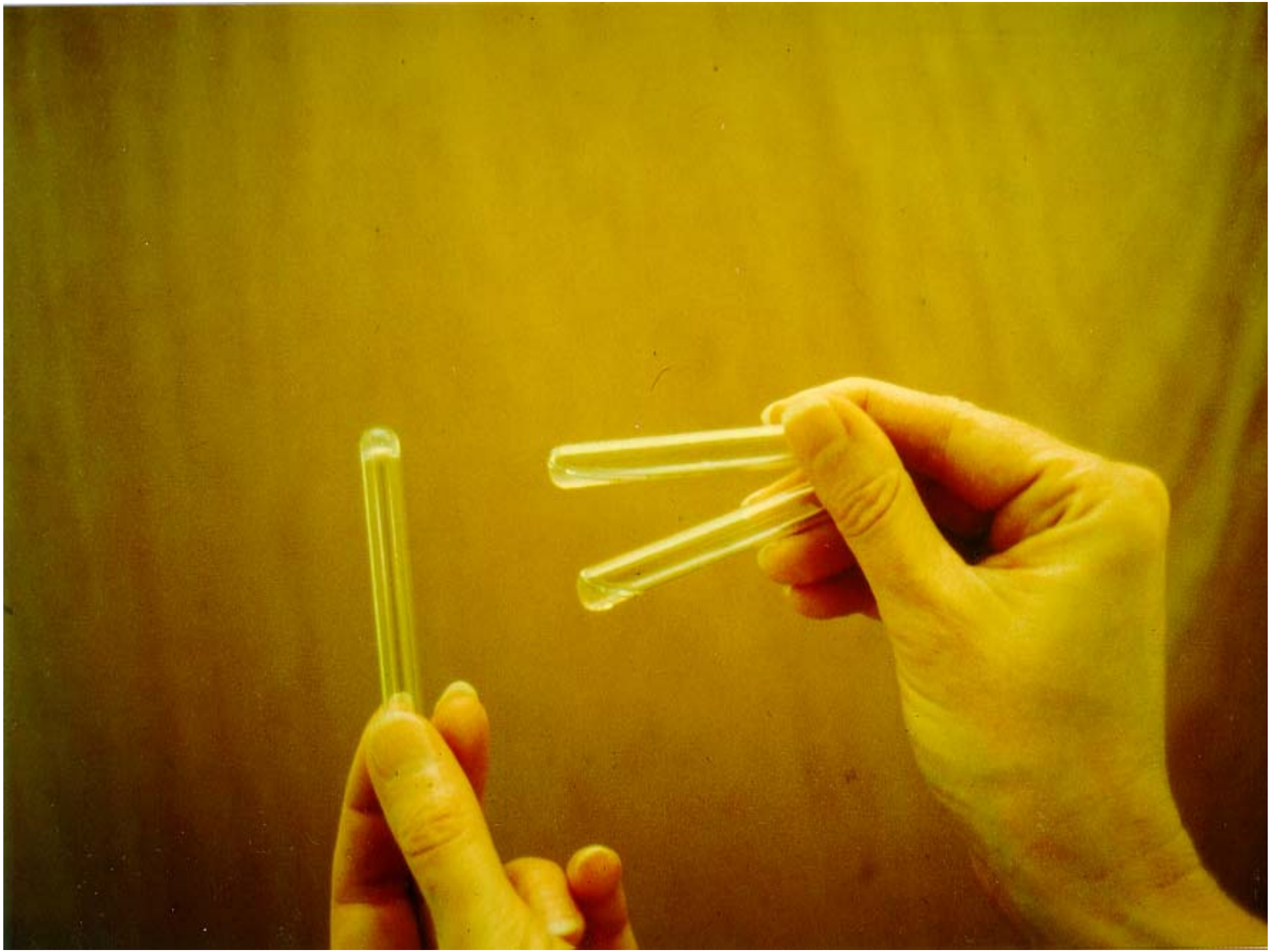


LAL Testing

- Gel Clot(Limit Test)
- Gel Clot(Semi-Quantitative)
- Endpoint Chromogenic
- Endpoint Turbidimetric
- Kinetic Chromogenic
- Kinetic Turbidimetric

Summary of Gel Clot Test

- Endpoint sought by 180 inversion of sample tube
  - Positive = Firm Gel
  - Negative = Flow down side of tube



Gel Clot Reagent

- In general
  - Lowest reagent cost
  - Simple reporting (+-)
  - Found in all Pharmacopoeia
  - Good stability
  - Simple technique
- Endosafe
  - firm gel
  - buffered lysate
  - better interference resistance
  - .015 EU/ml

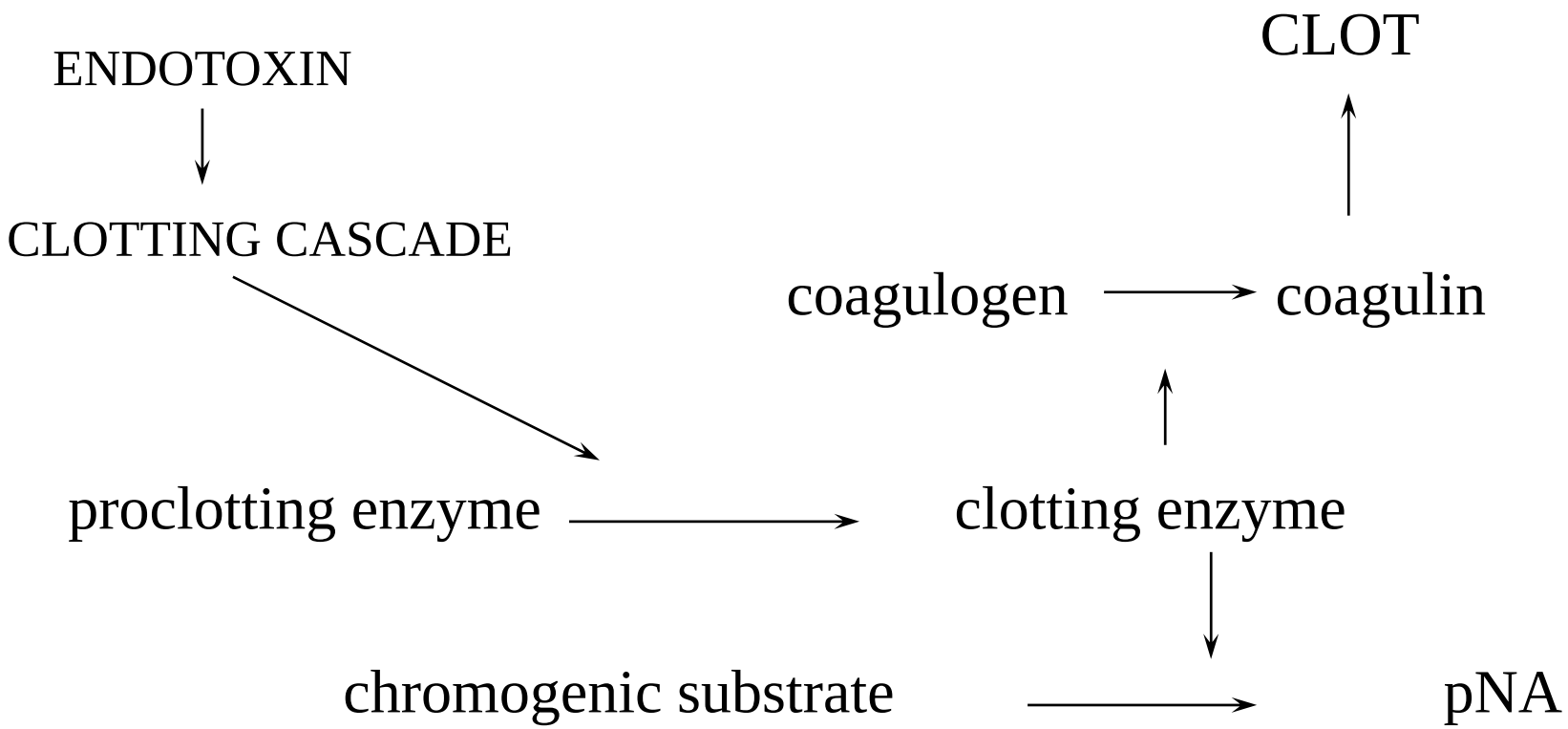
Sources of gel clot test problems

- Use of wrong EU/ng ratio
- use of improper reaction or dilution tubes
- Incubating temperature too hot or too cold
- Improper dilutions
- Improper handling of lysate

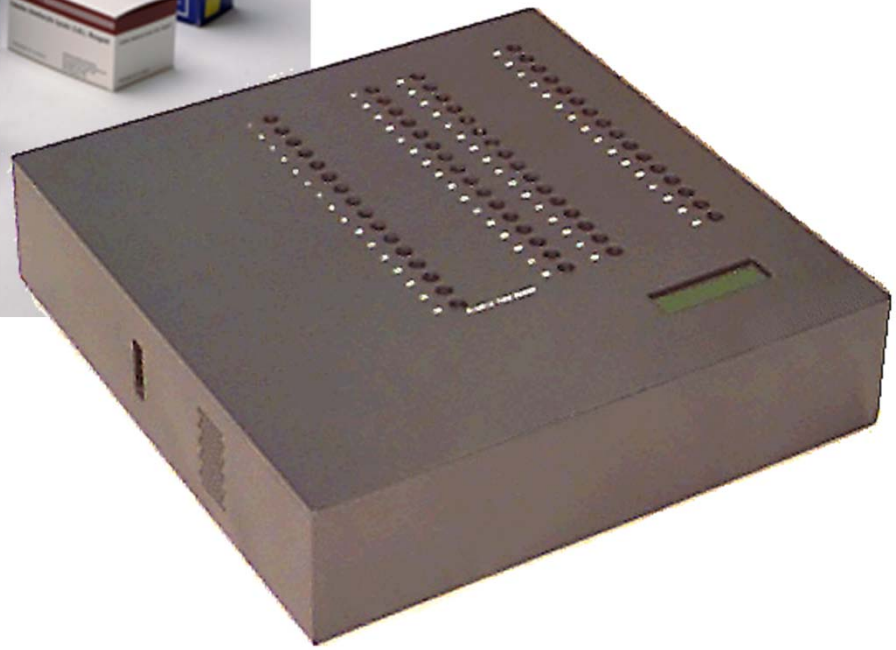
Gel Clot Disadvantages

- Limits test
- Least sensitive
- Most time consuming
- Poor trending options
- Variability, subjectivity

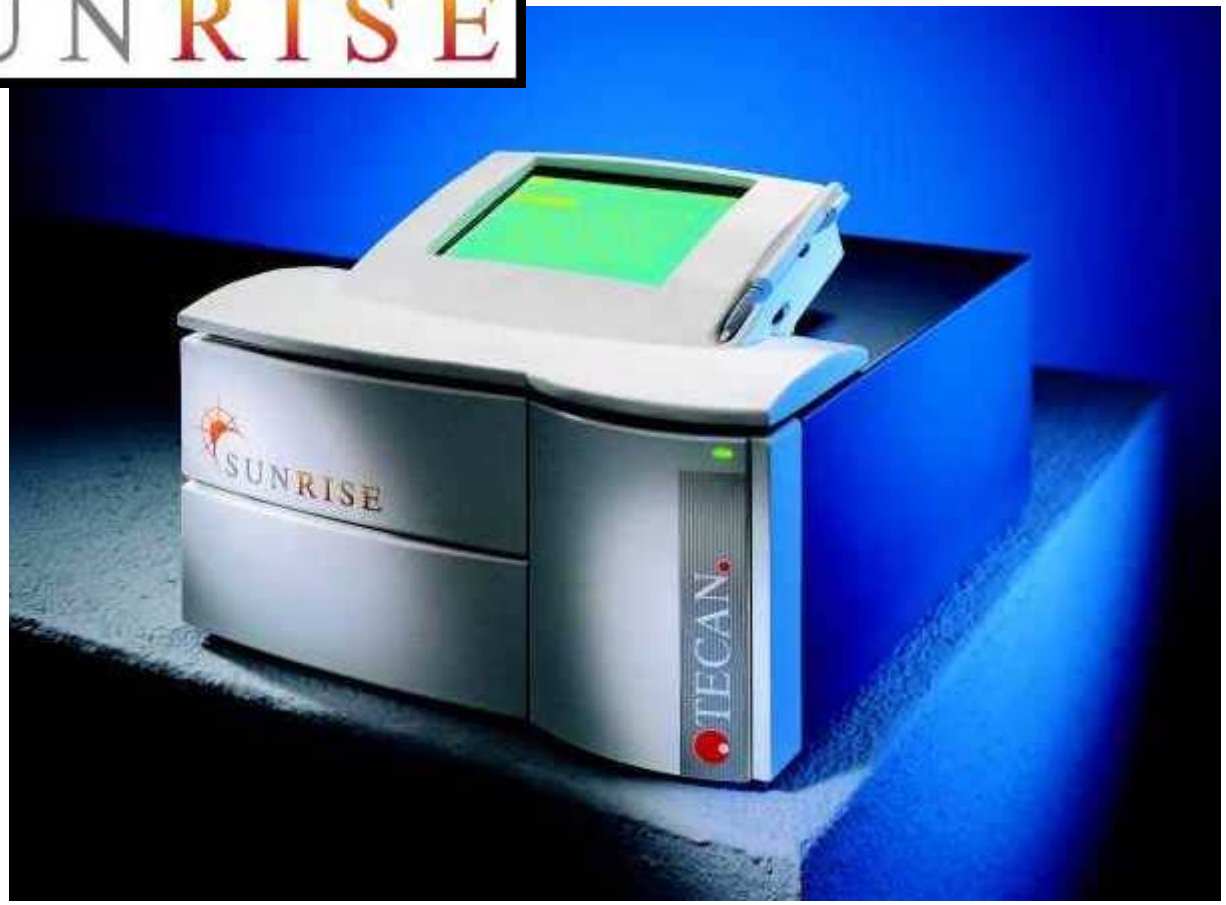
KINETIC LAL TESTING




EndoScan-V



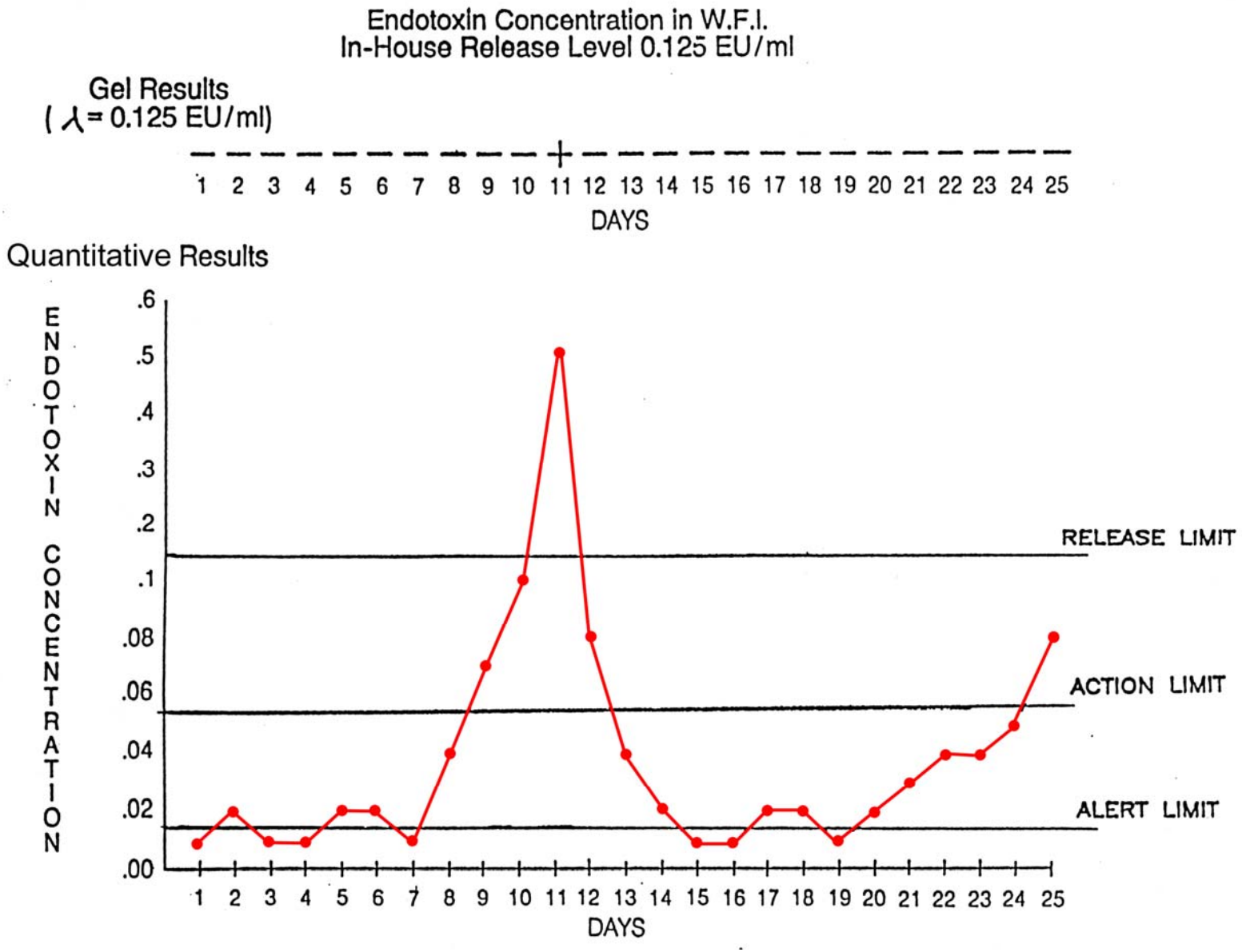
SUNRISE



Microplate Absorbance Readers

Kinetic LAL Advantages

- Quantitative results
- Higher throughput (96 wells)
- Lower sensitivity (to .001)
- Wider standard curve range
- Clear concise reporting
- Allows for data trending

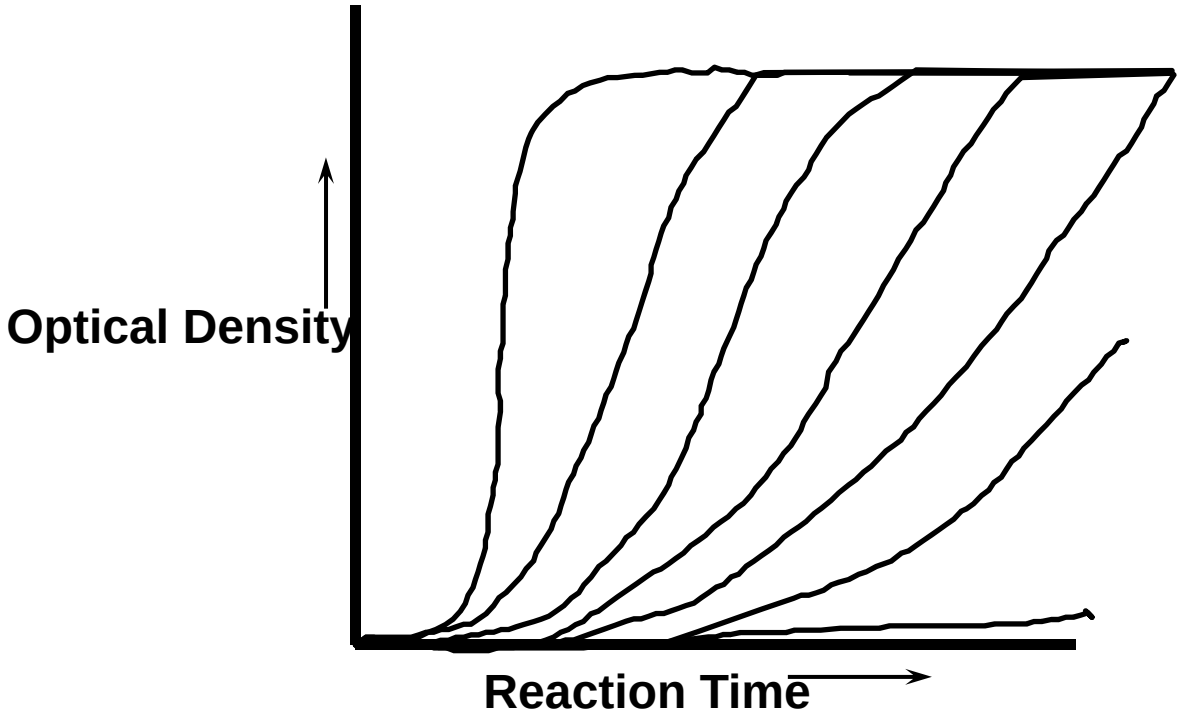

Endotoxin Concentration in W.F.I.
In-House Release Level 0.125 EU/ml
Gel Results
(λ = 0.125 EU/ml)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
DAYS
Quantitative Results
ENDOTOXIN CONCENTRATION
.6
.5
.4
.3
.2
.1
.08
.06
.04
.02
.00
RELEASE LIMIT
ACTION LIMIT
ALERT LIMIT
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
DAYS

FUNCTION: Kinetic LAL System

- MEASURE CHANGES IN OPTICAL DENSITY (OD)
- RELATES CHANGES IN OD TO ENDOTOXIN CONCENTRATION
- AUTOMATED CALCULATIONS
- REPORTS RESULTS of ANALYSIS

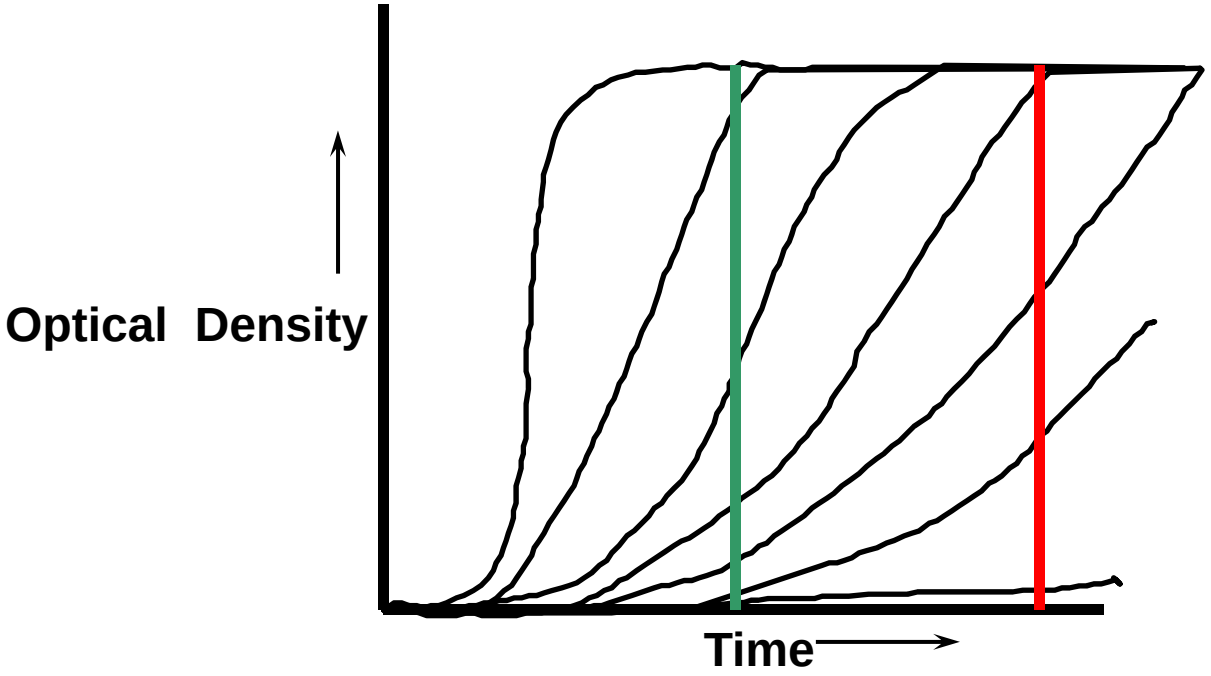
Principles of Quantitation

Change in O.D. over Time



Quantitative LAL Test Results

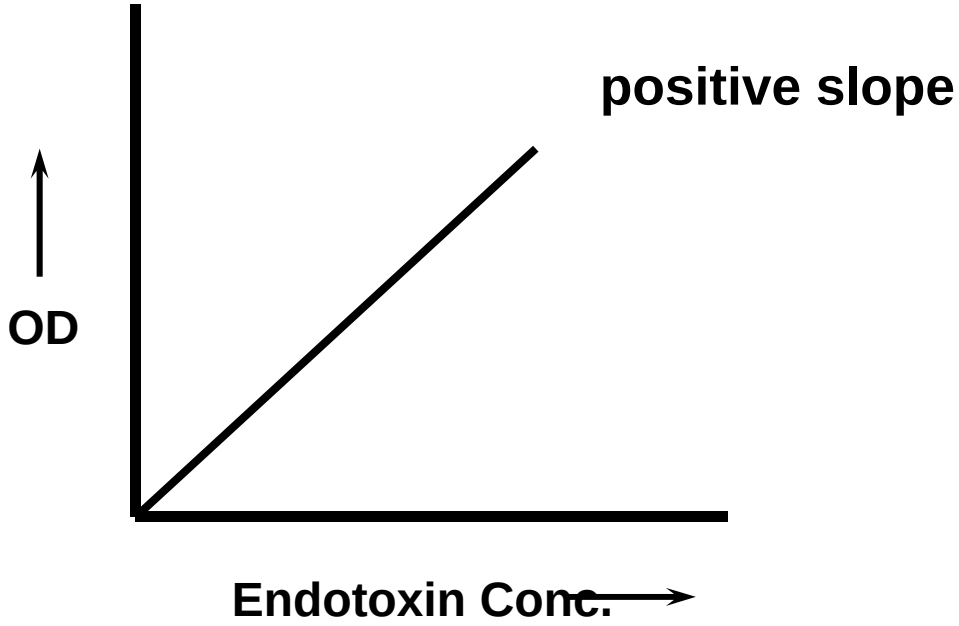
Endpoint: “Fixed Time”



Standard Curve:

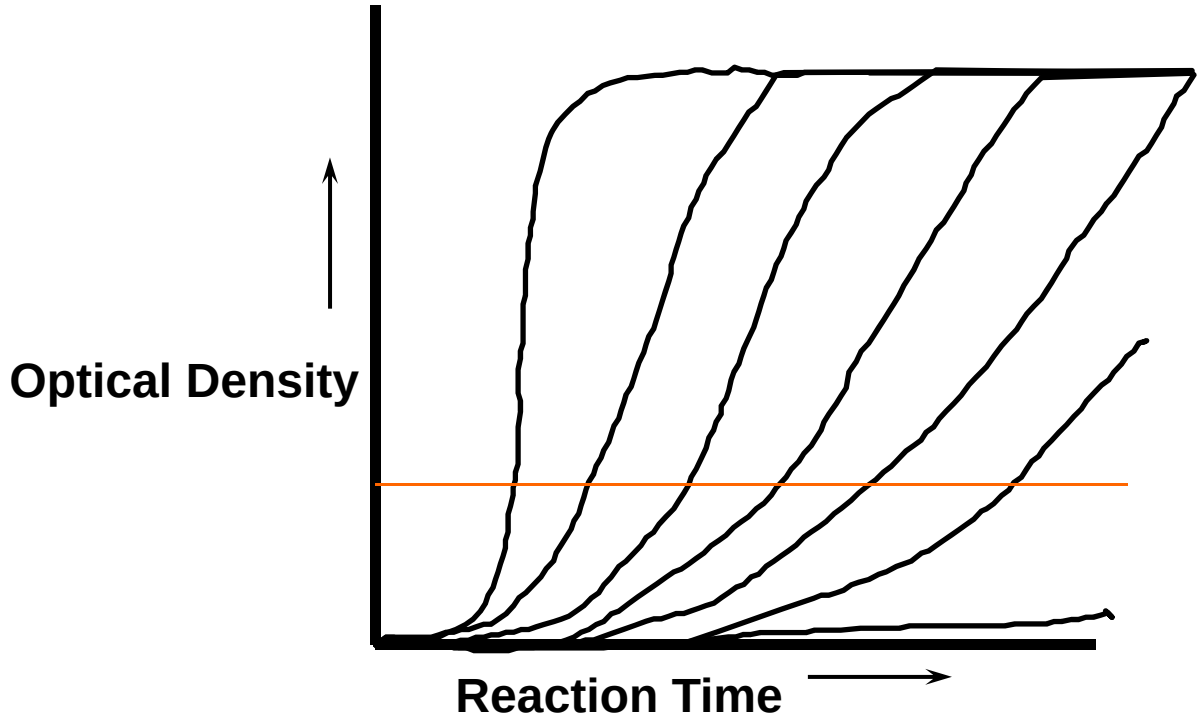
Endpoint Test

Beer's Law: Absorb. vs. Conc.



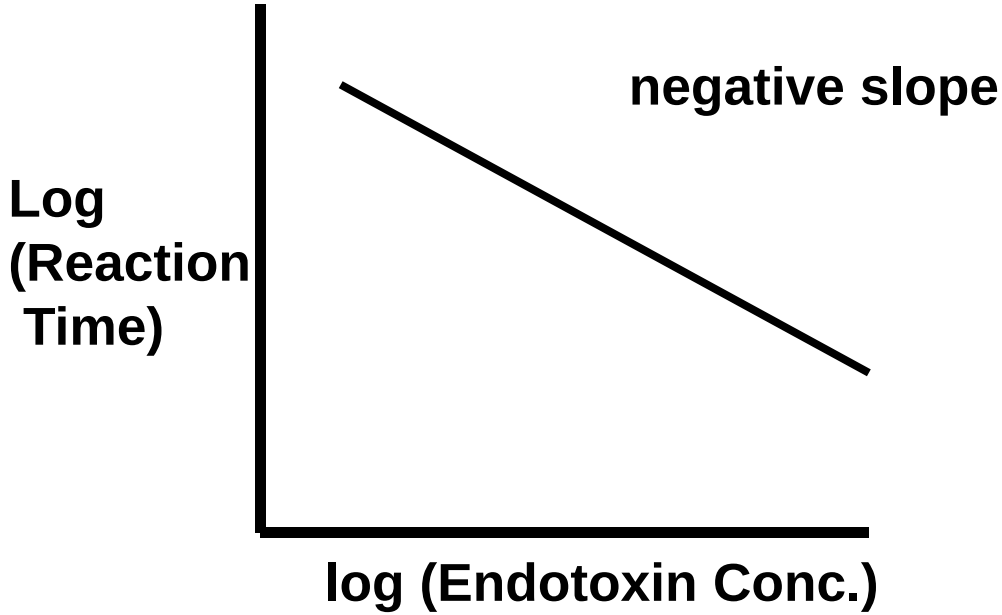
Quantitative LAL Results

"Fixed" Optical Density

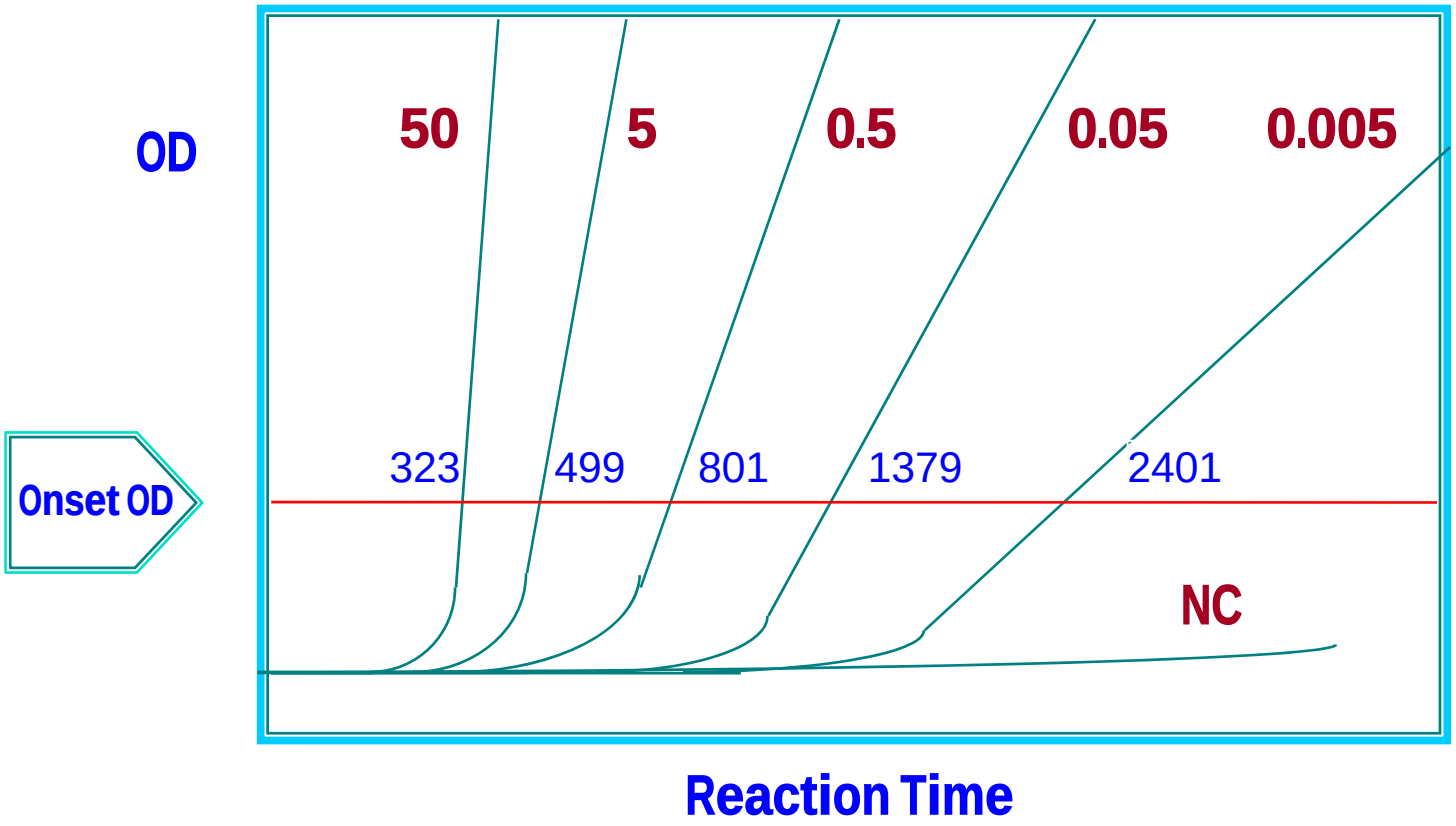


Standard Curve:

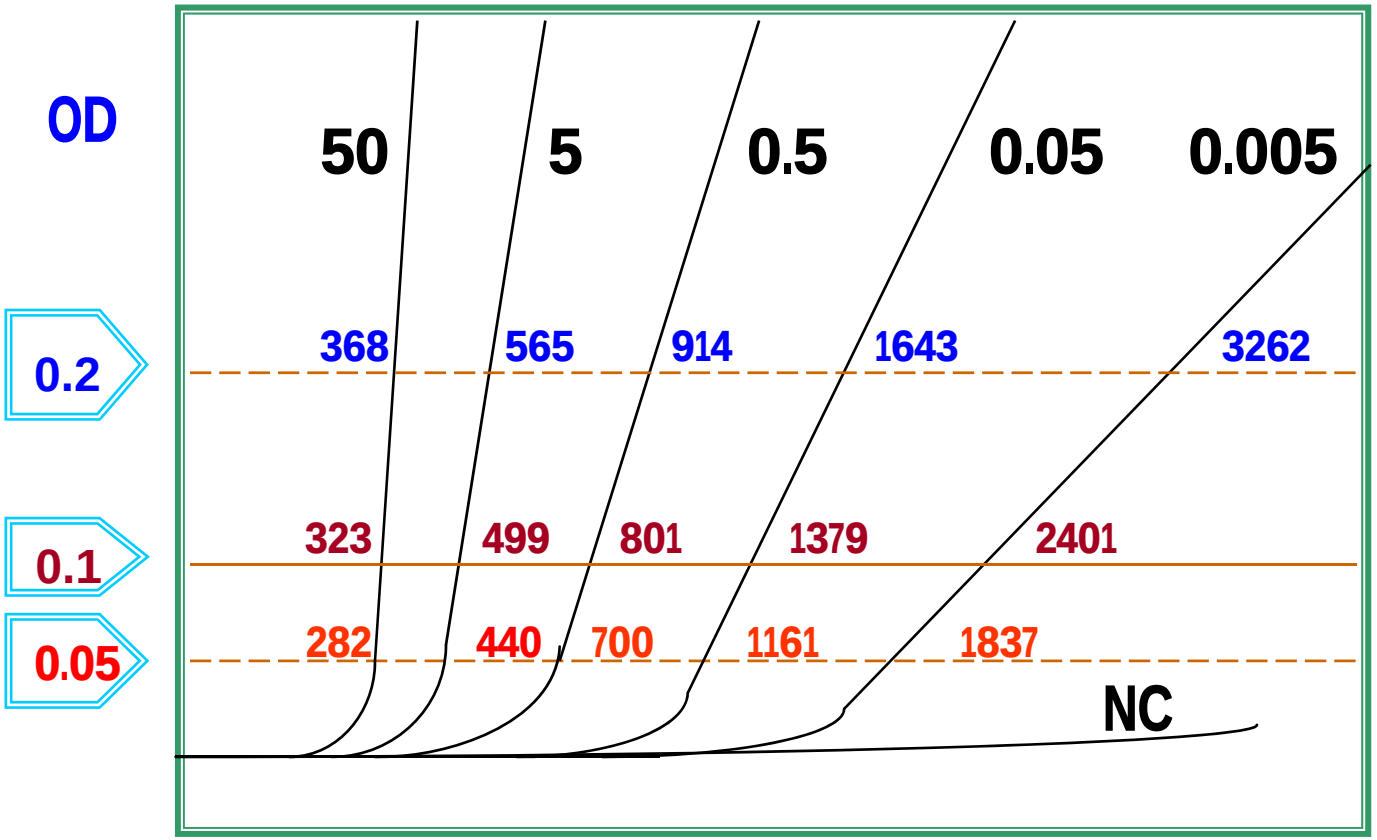
Kinetic Test



Optical Signals from LPS Interaction with Kinetic L



Optical Signals from LPS Interaction with Kinetic LAL



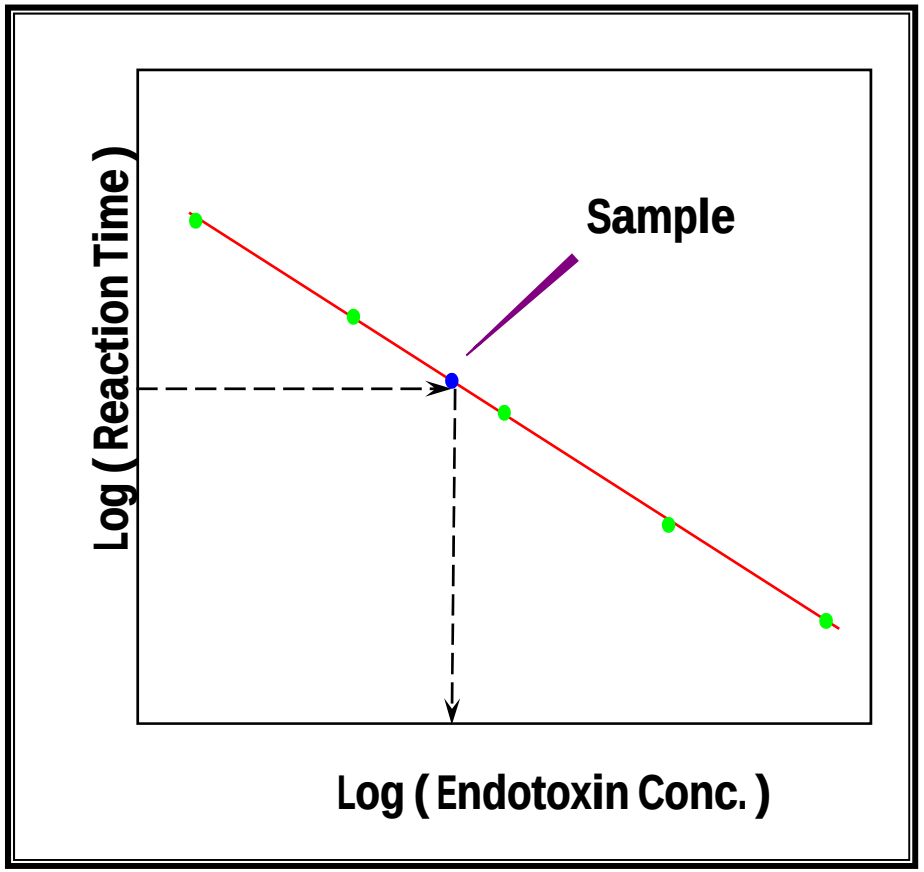
How to Quantify the Amount of Endotoxin in the Sample

A standard curve is constructed by plotting

the log of onset time

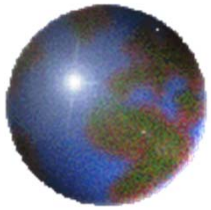
versus

the log of concentration



Summary of Kinetic Assay

- The amount of endotoxin present is inversely proportional to the time of reaction
  - the more endotoxin present, the quicker the well or tube will react (change color or become turbid)
- A standard curve is generated based on how long each of the standards take to reach an onset OD
- Unknowns are then compared to the standard curve to generate a endotoxin values



Selecting Kinetic LAL Reagents

Turbidimetric or Chromogenic?

Selection of Kinetic LAL Reagent

- cost of reagent
- unique interference problems
- analysis time
- sensitivity

Kinetic Lysate Options

- Turbidimetric
  - least expensive, 2-log range down to .01
- KTA^2
  - mid-priced, 3-log range down to .005, better interference resistance
- Chromogenic
  - most expensive, 4-log range (50-.005), best interference resistance

Kinetic Chromogenic Reagent

- In general
  - 4 log standard curve
  - Low sensitivity (.005)
  - Fast reaction
  - Best for biologicals
  - Most expensive lysate
- Endosafe
  - Fastest (.005 in 50 min)
  - Less expensive
  - Flexible packaging
  - Best linearity
  - Best stability

KINETIC CHROMOGENIC (KCA)

- ENDOCHROME-K, LAL
  - REHYDRATE VIAL WITH 3.2 - 3.4 mL LRW
  - SET WAVE LENGTH of READER to 405 nm.
  - SET ONSET OD to 0.1 OD UNITS
  - SET UP 2 to 4 LOG RANGE
  - EXPECT INCUBATION TIME < 40 MINUTES

Kinetic Turbidimetric Reagent

- In General
  - Price similar to gel
  - Linear over two logs
  - Stable
  - .05 in about 50 minutes
  - Trend analysis
- Endosafe KTA
  - Linear over wider range
  - Better spike recovery
  - products other than water
  - Also true gel clot
  - No reconstitution buffer

KINETIC TURBIDIMETRIC (KTA)

- ENDOSAFE KTA LAL
  - REHYDRATE VIAL WITH 5.2 mL OF LRW
  - SET WAVE LENGTH OF READER TO 340 nm.
  - SET ONSET OD to 0.05 OD units
  - SET UP 2 or 3-LOG RANGE
  - EXPECT INCUBATION TIME <45 MINUTES

KTA^2 Turbidimetric Lysate

- 1st turb lysate to run at .005 EU/ml
- Faster than existing turbs (.005 in < 1 hour)
- Interference resistance of chromogenic lysate
- Priced closer to conventional turb
- Can be used with polynomial regression

Glucans ((1-3)-B-D-glucan)

- Materials that cause false positive in LAL by activating Factor-G pathway
- Non-pyrogenic
- Sources include cellulosic membranes and fungal or yeast extract
- Reacts like endotoxin in gel clot
- Causes enhancement in kinetic methods

LAL and Glucans

- No current LAL on the market is totally unreactive to glucans
- Reactivity tied to extraction method
- Variability between lots
- Best way to test today is to use glucan blocker

LAL - Test Interference

- Inhibition:

  Recovery < Expected Amount

- Enhancement:

  Recovery > Expected Amount

- Non-interference:

  Recovery = Expected Amount

Product Screen (Inh / Enh)

- Calculate EL, MVD, MVC
- Make sample dilutions between undiluted and MVD
- Screen these dilutions to determine non-interfering test concentration
- Once a non-interfering sample dilution is determined perform validation on three lots of this product

Product Screen (Inh / Enh)

- Measure and document pH of sample + LAL
  - If no pH adjustment is made then, the validation data serves as documentation for routine testing, and pH measurements will not have to be performed routinely
  - If pH adjustments are made then routine testing should include pH measurement and documentation.

LAL Compatibility

- A test sample is compatible with LAL reagent if the positive product control (ppc) is recovered.
  - in gel-clot, at least 2-lambda is recovered
  - in kinetic LAL, 100 ±50% of a known amount of CSE is recovered relative to a standard curve.

Scope of Interference Problem

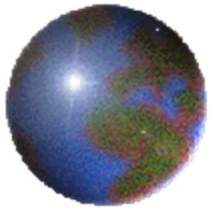
- Only 25% of pharmaceuticals are testable undiluted
- Inhibition is usually solved by dilution
- Devices are seldom inhibitory, unless coated

Eliminating Causes Of Interference

PROBLEM	SOLUTION
pH	Buffered LAL Reagent
Endotoxin loss	In-plate spiking
Mg depletion	Permissible Dilution
LAL-RM	Endotoxin-Specific buffer

Current Trends in LAL Testing

- Continued move toward kinetic testing
  - over 55% of all LAL tests performed today
- Compliant software
- Database trending
- Robotics
- In-vitro pyrogen test



LAL Applications

LAL Applications

- Pharmaceuticals
- Medical Devices
- Water testing
- Dialysis testing
- Cell culture

Acceptance of LAL by Pharmaceutical Industry

- LAL IS > 100 TIMES MORE SENSITIVE, SPECIFIC and ACCURATE
- ECONOMY of LAL PERMITS MORE TESTING
- TOLERANCE of RABBITS to ENDOTOXIN = CONCERN for FDA

Baxter Report on Non-Endotoxin Pyrogens

- 143,196 LAL and 28,410 RABBIT TESTS
- ALL PYROGENIC TESTS GAVE POSITIVE LAL RESULT
- NO FALSE-POSITIVE LAL TESTS
- NOT ALL POSITIVE LAL TESTS WERE PYROGENIC in RABBITS
- RABBIT WAS OFTEN UNRELIABLE

Improved Quality of U. S. Pharmaceuticals

- 1974 FDA STUDY FOUND 45% of ALBUMIN PYROGENIC
- REGULATED In-PROCESS TESTING ELIMINATED PYROGENIC ALBUMIN
- PYROGENS REMOVED from INFLUENZA VACCINES
- LAL REQUIRED for SPINAL-FLUID DRUGS
- NO OUTBREAKS of FEVER SINCE 1980

LAL Application - Medical Devices

- 1987 FDA Guideline
- Extraction test
- Endotoxin Limit -
  - 0.05 EU/ml up to 20 EU/device
- Turbidimetric most cost effective method for testing

Medical Devices Requiring Pyrogen(Endotoxin) Testing

- DEVICES that PENETRATE SKIN
- MEDICAL IMPLANTS
- FLUID PATHWAYS for IV Medications
- NOT APPLICABLE to:
  GLOVES, ORTHOPEDIC PRODUCTS, WOUND DRESSINGS

Official Role for LAL Testing in Device Quality

- Comparison Study revealed LAL advantages over Pyrogen (rabbit) Test
- DEVICE INDUSTRY SWITCHED TO LAL TESTING BY 1987
- DEVICES with NON-PYROGENIC LABEL REQUIRE LAL TEST

LAL Application - Water system

- Routine monitoring
- Turbidimetric is the lysate of choice
- Trending allows you to set action and alert limits

Sources of Endotoxin in Water System

- COLONIZATION OF SOFTENERS, CARBON BEDS and DI UNITS by G(-) RODS
- BIOFILM on RO MEMBRANES, WATER PIPES, and in STORAGE TANKS

LAL Application - Dialysis

- Testing of dialysis water and concentrate
- Testing performed in clinics, hospitals, and reference labs
- Gel clot is method of choice because of low volume of tests
- New AMMI limit on water for dialysis is 2 EU/ml

LAL Application - cell culture

- Effects of endotoxin very greatly depending on cell line.
- in vitro fertilization adversely affected
- Classified broadly as a parenteral product
  - Endotoxin testing by BET is imminent