

# Decontamination of the BD FACSAria II: Is the Prepare for Aseptic Sort Procedure Effective?

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# Summary

- Overview
  - BD FACSAria™ II fluidics
  - Prepare for Aseptic Sort (PAS) procedure
- Decontamination of the BD FACSAria II
  - Bacteria
  - Endotoxin
- Tips to limit contamination

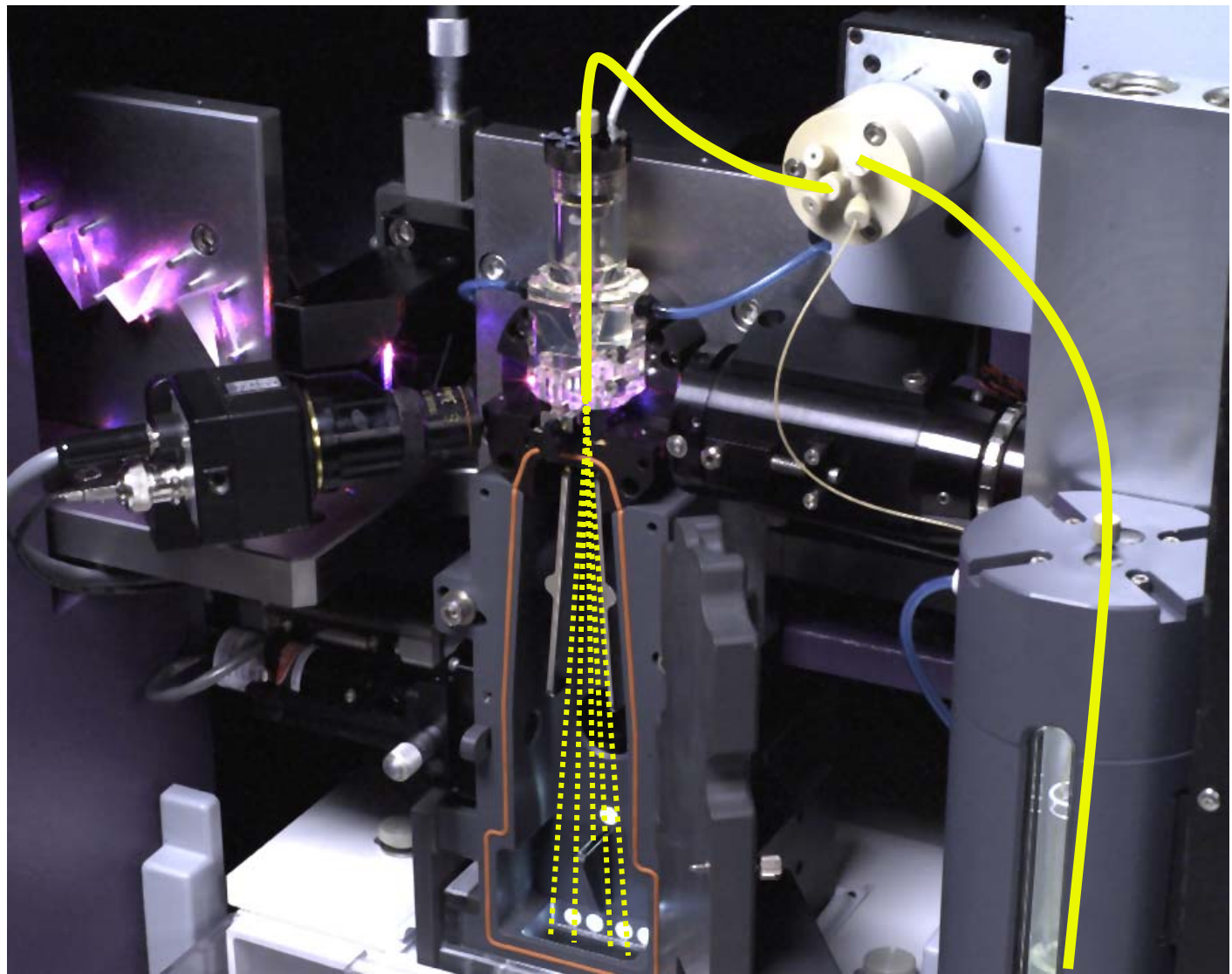


# BD FACSAria II and PAS procedure

- The BD FACSAria system was launched in 2003 and was a paradigm shift for cell-sorter design.
  - Gel-coupled cuvette
  - Octagon detectors
  - Digital electronics
  - Novel fluidic design
    - Automated modes
      - Startup and shutdown
      - Prepare for Aseptic Sort
- The BD FACSAria II system was launched in 2008.



# BD FACS Aria sample path



# Non-pressurized supply

Pump from and to containers

- 10-L tanks
- 20-L cubitainers

Storage of all cleaning fluids  
in 5-L tanks

- Deionized water
- Bleach
- Ethanol



## Voice of the customer

- Customers requested a streamlined fluidics system that was easier to decontaminate

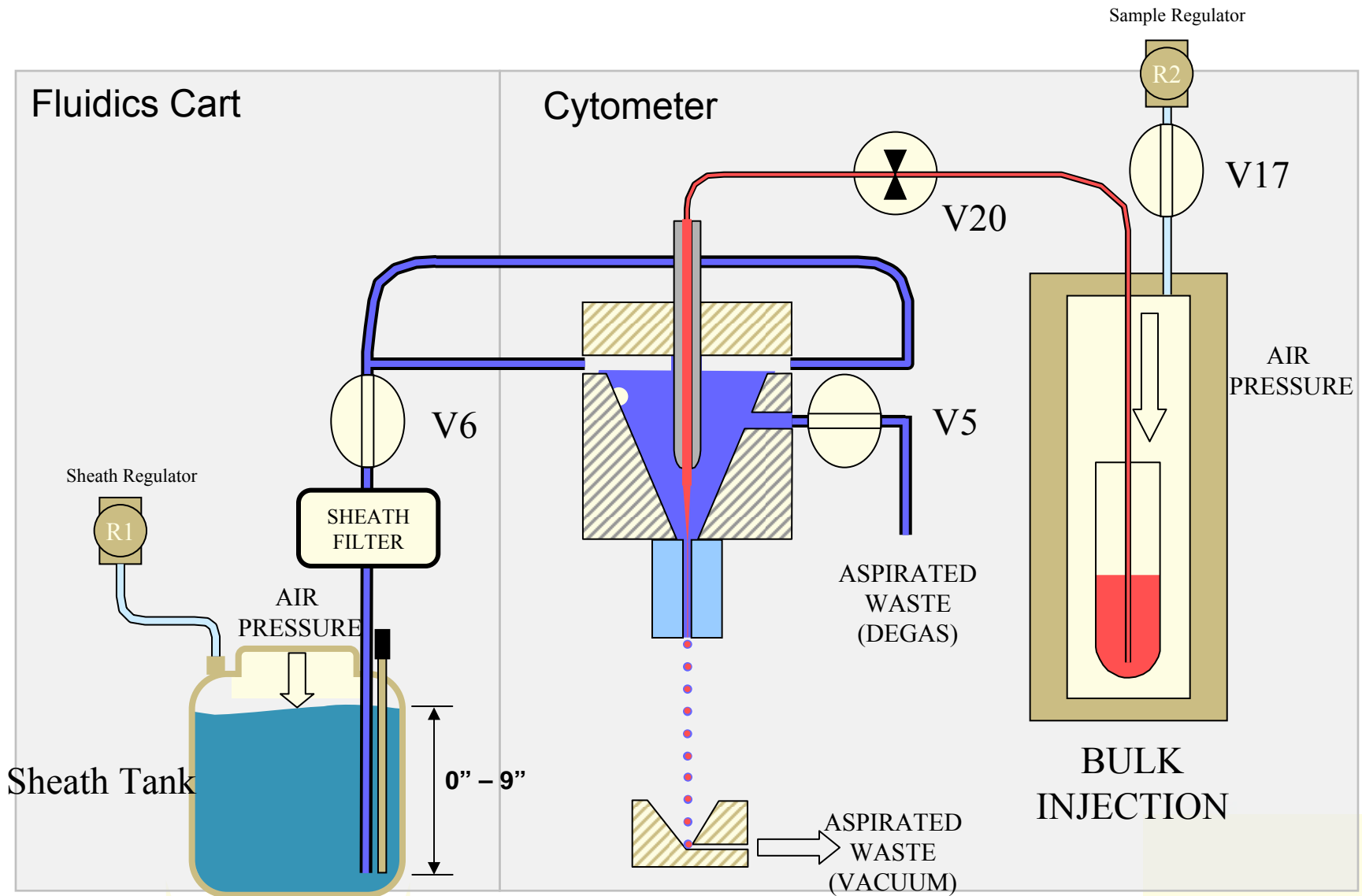
# New pinch valve: Improved design



Sample line

Pinch valve

# The BD FACSAria II fluidics system design





# New wet cart



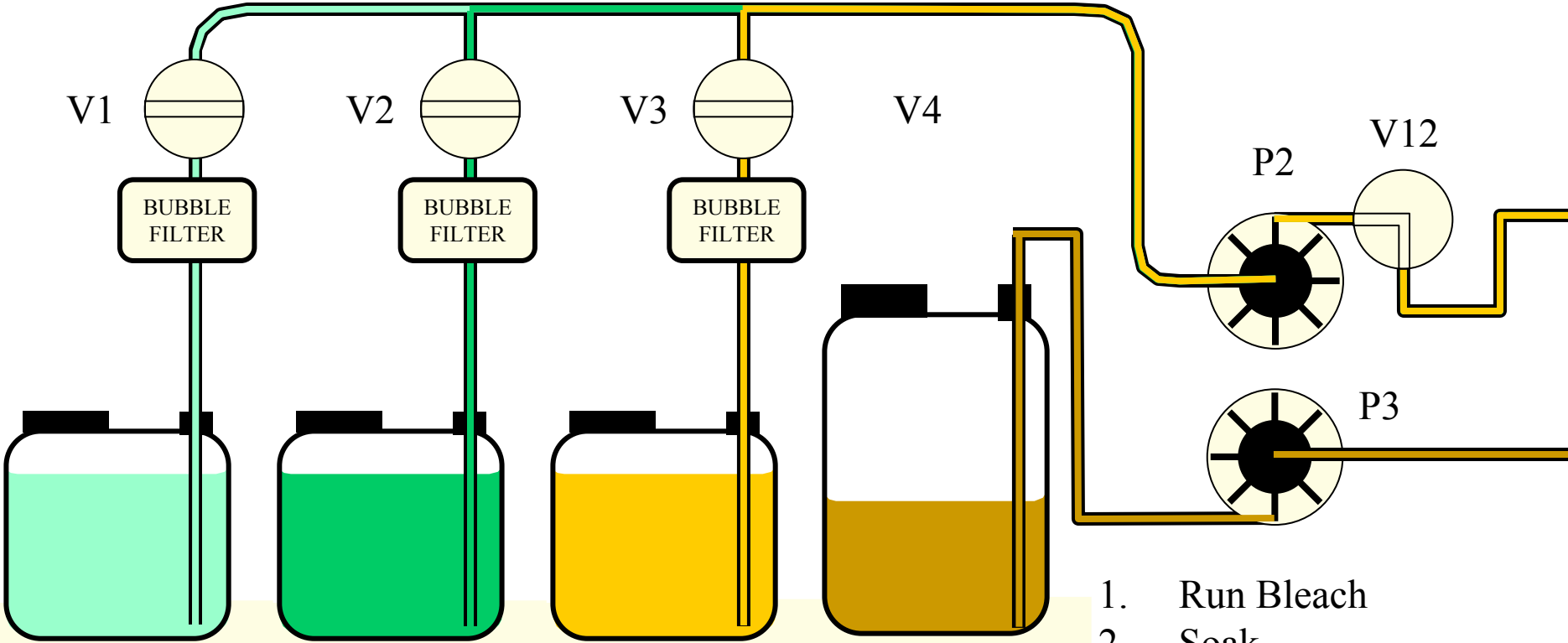
- New 10-L pressurized sheath tank
  - New level sensor allows use of 95% of tank volume
- Pressurized 5-L ethanol shutdown tank
- Filters moved to front for easy access

# PAS procedure

- Wizard in BD FACSDiva™ software leads the cytometer operator through the decontamination of these components:
  - Sheath path
  - Flow cell
  - Sample path
- During the PAS procedure:
  - The sheath umbilical line is connected to the Liquid Out port of the wet cart.
  - The customer is required to autoclave the sheath and DI tanks for optimal decontamination.
- Upon completion of the PAS procedure:
  - The sheath umbilical is reconnected to either the sheath tank or pressurized ethanol tank (for startup or shutdown, respectively).
  - The sheath filter is replaced.



# PAS: Wetcart Liquid Delivery



DI

BLEACH

ETHANOL

WASTE

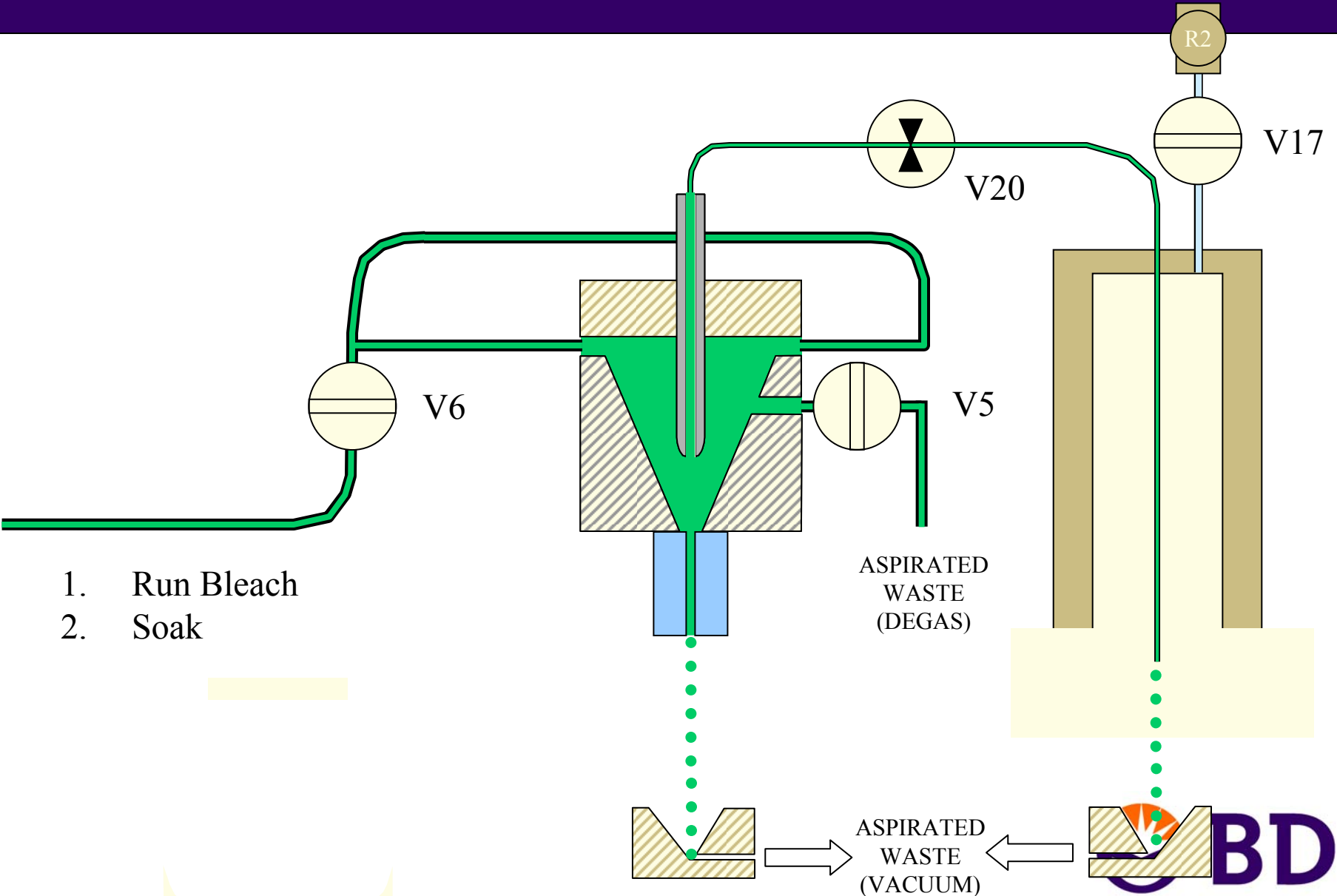
WETCART

- 1. Run Bleach
- 2. Soak
- 3. Rinse with DI
- 4. Soak
- 5. Rinse with Ethanol

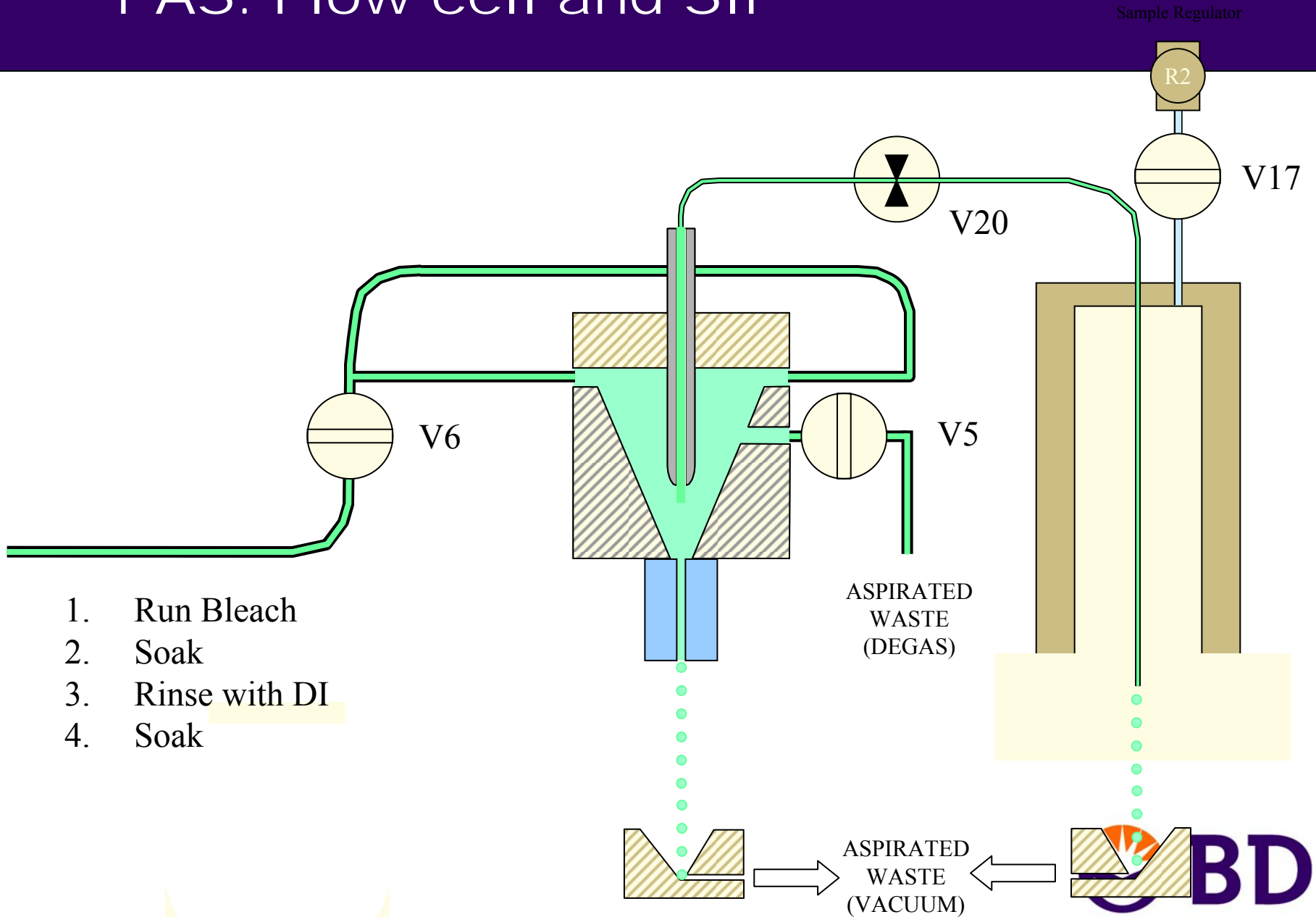


# PAS: Flow cell and SIP

Sample Regulator



# PAS: Flow cell and SIP





# PAS procedure: Cleaning residue

- Residual levels of bleach and ethanol remaining in the fluidics system after a PAS procedure were checked during product development.
- The residual levels were found to be
  - <0.01 parts per million (ppm) of bleach
  - <0.01 ppm of ethanol



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# Application notes

- Two studies performed that investigated the effectiveness of the PAS procedure
  - Decontamination of the BD FACSAria II System Using the Prepare for Aseptic Sort Procedure
  - Reduction in Endotoxin Levels After Performing the Prepare for Aseptic Sort Procedure on the BD FACSAria II Flow Cytometer



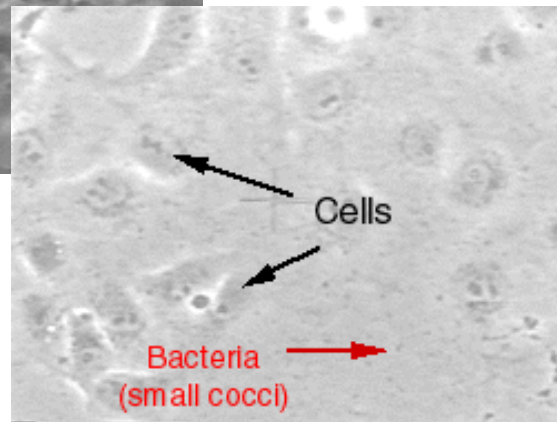
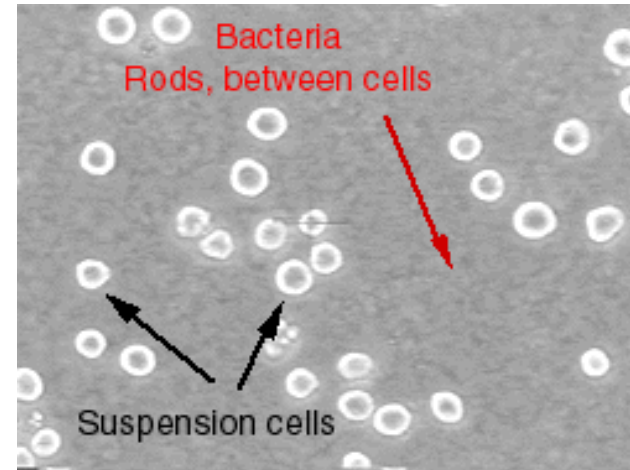
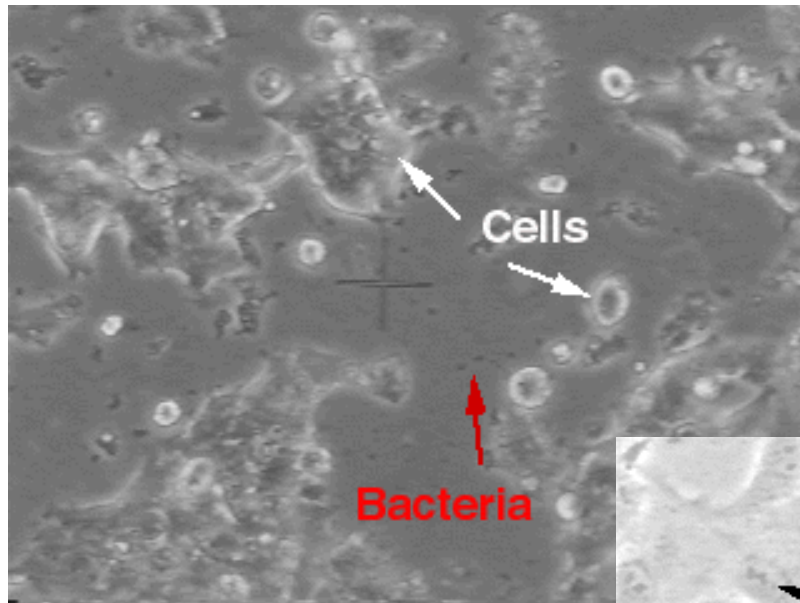
# Background: Cell culture

- Cell culture is a common procedure used in the laboratory.
  - Cells
  - Flask
  - Cell-culture medium
  - Incubator
- Cell culture requires sterility of all components that come in direct contact with the cells.



# Bacterial contamination

- Bacterial contamination of cell cultures can destroy an experiment!



# Bacterial contamination

- Antibiotics can be used to prevent or control contamination, BUT antibiotics can:
  - Be toxic to or affect the metabolism of the cells in culture
  - Result in low level of antibiotic-resistant bacteria that might:
    - Mask the presence of contamination
    - Deplete essential nutrients and other factors required by the cells
    - Cause the the accumulation of bacterial metabolites
- The best remedy is not to have contamination in the first place!



# Aseptic technique

- Techniques used in the laboratory to minimize the chance of contamination
  - Step 1: Make sure everything is sterile to start with
  - Step 2: Make sure everything stays sterile
- Bacteria
  - Approximately 1,000 different species of bacteria present on human skin
  - Estimated that there are  $10^{12}$  bacteria on an average human



# Aseptic technique

- It's all about risk: minimize the chance of contaminants coming in contact with the cells
  - Wear gloves and tie back hair
  - Keep all surfaces as clean as possible
    - Biological safety cabinet
    - Bleach and alcohol
  - Use sterile equipment whenever possible
    - Tissue-culture flasks
    - Pipets
    - Tubes
  - Use sterile reagents whenever possible
    - Media
    - Buffers
    - Sera
  - Reduce clutter

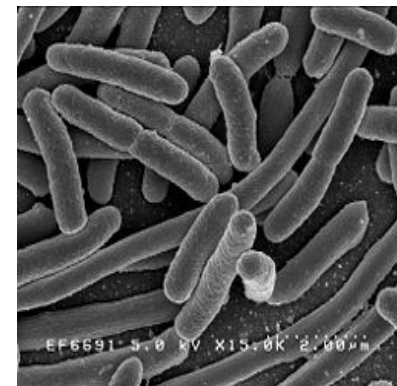
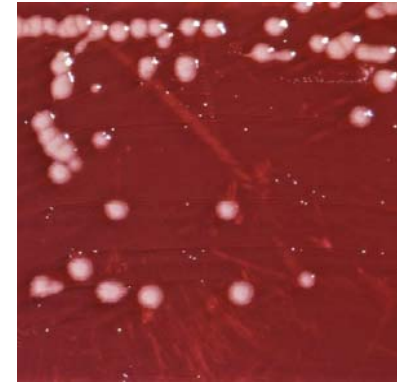
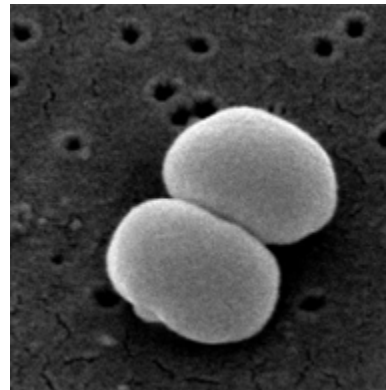
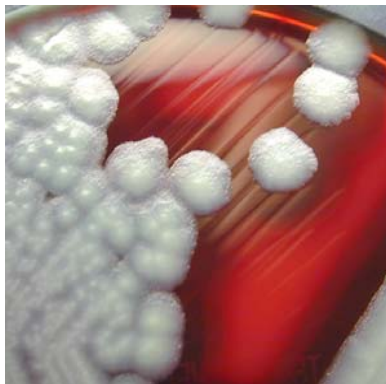


# Cytometers can be very “dirty”

- Used by lots of different operators
- Different operators use different sample types
  - Human blood
  - Non-human samples
  - Cultured cells
  - Bacteria
  - Yeast
- Sometimes not maintained properly

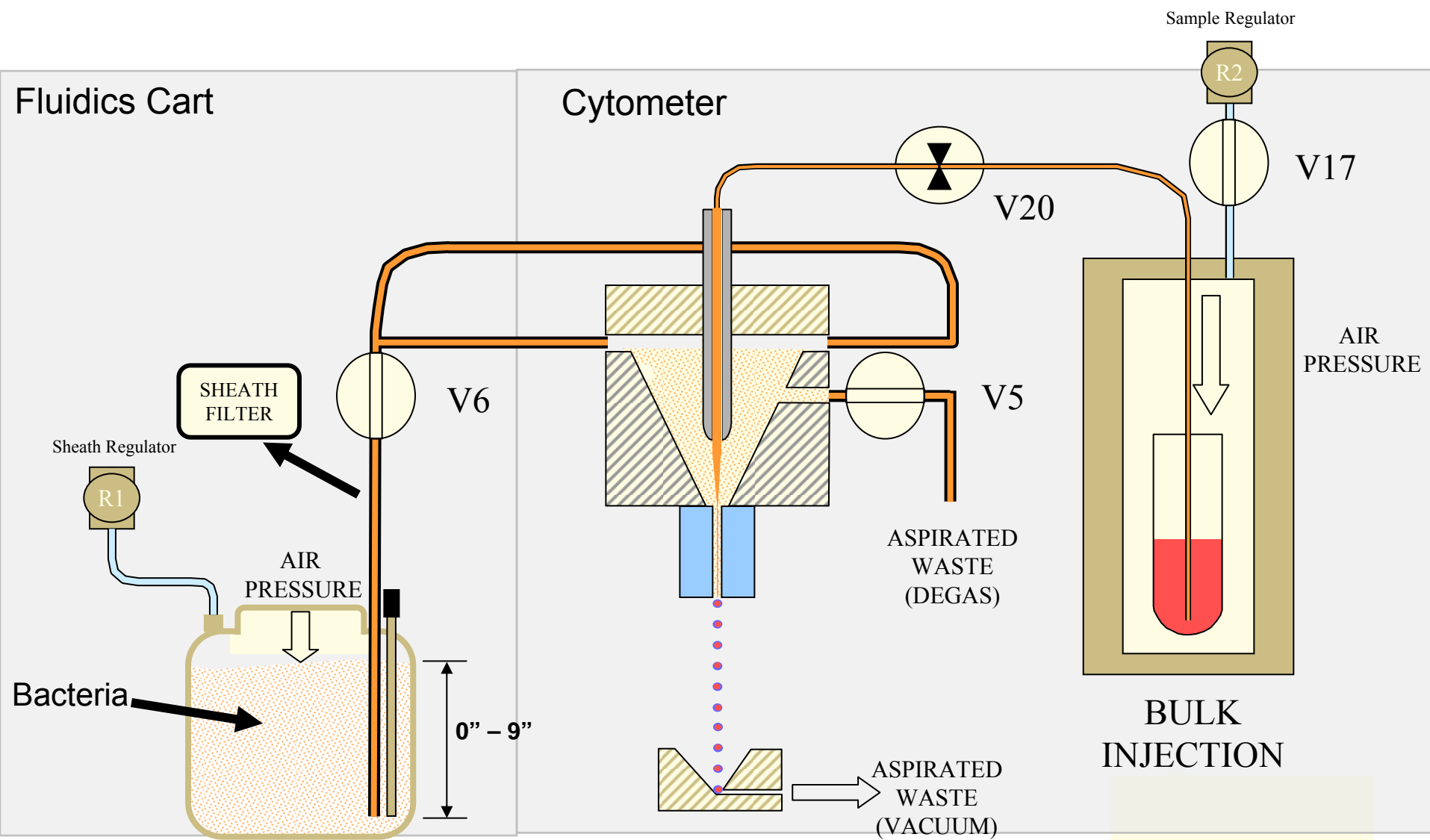
# Application note: Bacterial contamination of the BD FACSAria II

- Log phase cultures of four different kinds of common bacteria
  - *P. aeruginosa*
  - *E. coli*
  - *B. cereus*
  - *S. epidermis*





# Bacterial contamination of the BD FACSAria II



# Method and results

- Day 0: Added bacteria to the sheath tank
- Day 4
  - Sample:  $9 \times 10^5$  CFU/mL
  - PAS procedure
    - Bleach, water, and ethanol through fluidics
    - Autoclaved sheath tank
    - Replaced sheath filter
  - Sample: 8 CFU/mL
- Days 5, 6, 7, and 8
  - Sample: 0 CFU/mL



# Is the PAS procedure effective?

- Yes!
  - This proof of principle experiment showed that:
    - The PAS procedure can be effective at decontaminating a BD FACSAria II contaminated with up to  $9.8 \times 10^5$  CFU/mL
    - The fluidics system remained “bacterium free” for at least 4 days after the PAS procedure was performed



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  - Bacteria
  - **Endotoxin**
- Tips to limit contamination



# Application notes

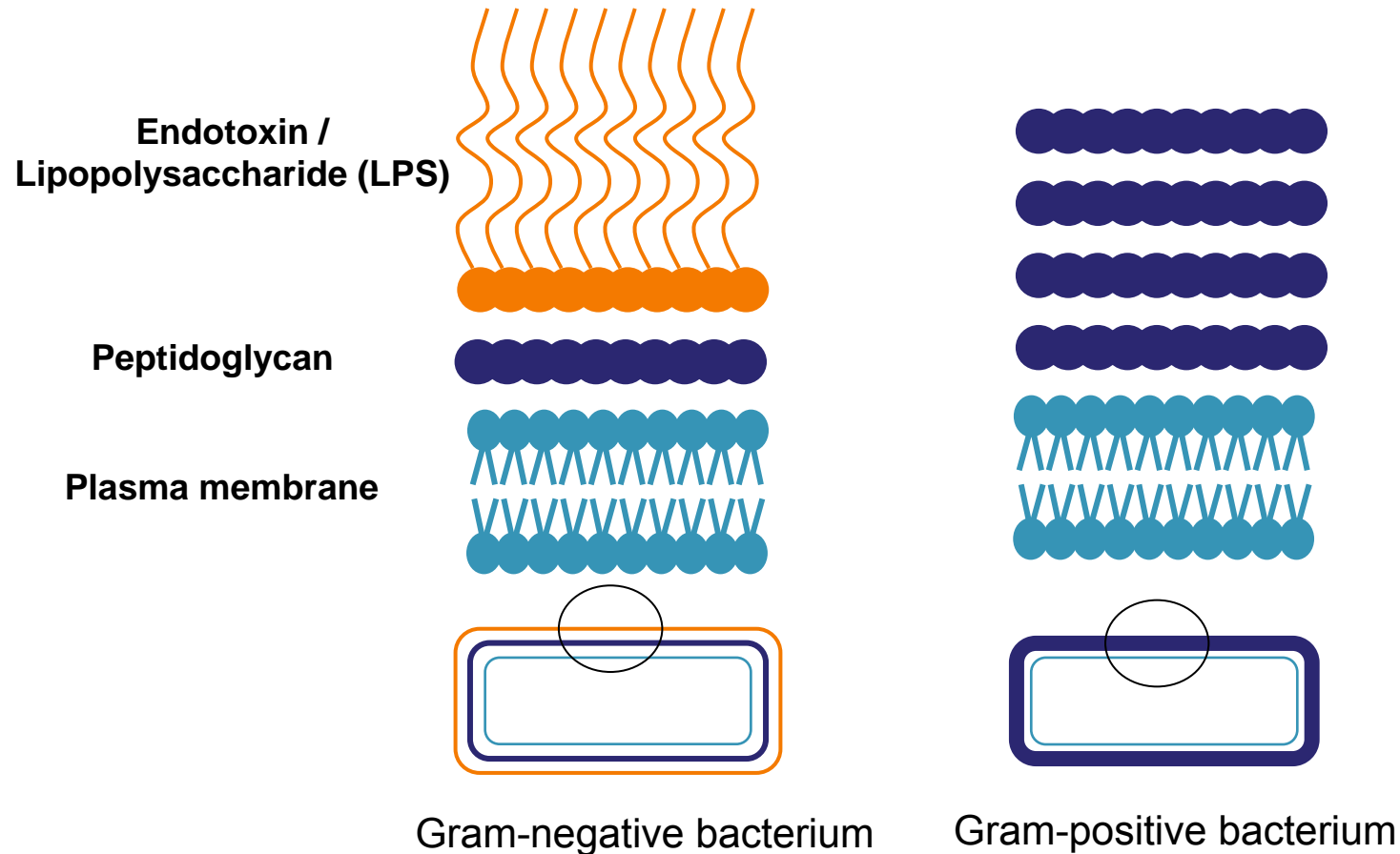
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# Background: Bacteria and the potential for contamination

- **Bacteria**
  - Numerous
  - Ubiquitous
  - Can survive in nutrient-poor conditions
  - Classified into gram positive and gram negative
    - Endotoxin is a component of the cell wall of gram-negative bacteria

# Simplified bacterial cell wall structure



Gram-negative bacterium

Gram-positive bacterium

Endotoxin is released in:

- Large amounts when a gram-negative bacterial cell dies
- Small amounts during gram-negative bacterial cell division

# Biological properties of endotoxin

- Activation of monocytes and macrophages
  - Production of inflammatory mediators
    - Nitric oxide synthase II
    - Cyclo-oxygenase-2
    - Endothelin-1
    - TNF $\alpha$
    - IL-1 $\beta$
    - IL-6
    - Other inflammatory cytokines
  - Used (on purpose) in experiments to induce these cytokine responses for research studies





# Biological properties of endotoxin

- In vivo effects:
  - Pyrogen
  - Toxic shock
  - Tissue injury
  - Death
    - Safe dose for a 20-g mouse: 0.1 Endotoxin Units (EU) administered over a 1-hour period
      - 0.1 EU/mL was used as our target value for this study
- Thought to play a role in:
  - Innate immunity
  - Tolerance
  - Allergy
  - Other immune diseases



# Importance of endotoxin

- Endotoxin has the potential to have an effect on:
  - Any in vitro study that includes monocytes or macrophages as a direct or indirect component
    - PBMCs
    - Whole blood

**Example:** Studies on T-cell activation are often performed using PBMC cultures. The presence of endotoxin might cause non-specific or unintended effects.
  - Any in vivo study of drugs, devices, or biologics
    - Efficacy
    - Safety
    - Toxicity



# Endotoxin testing

- Limulus Amoebocyte Lysate (LAL) kinetic assay
- Many products available that are tested for endotoxin
  - Referred to as:
    - Non-pyrogenic
    - Low endotoxin
    - Endotoxin free
- Testing is the **ONLY** way that you can determine if endotoxin is present.



# Sources of endotoxin

- Anywhere that a gram-negative bacterium has been
  - Surfaces: eg, pipets, tubing, lab bench, tubes
  - Equipment: eg, flow cytometer, centrifuge
  - Samples: eg, cell suspension
  - Cleaning solutions: eg, bleach, ethanol, water
  - Reagents: eg, antibodies, medium, serum
- Can be readily transferred between any of these
- Endotoxin is everywhere!

So...

- The level of endotoxin in a cell preparation can only be as low as the “most contaminated” reagent or surface that the cells in the preparation have come in contact with.

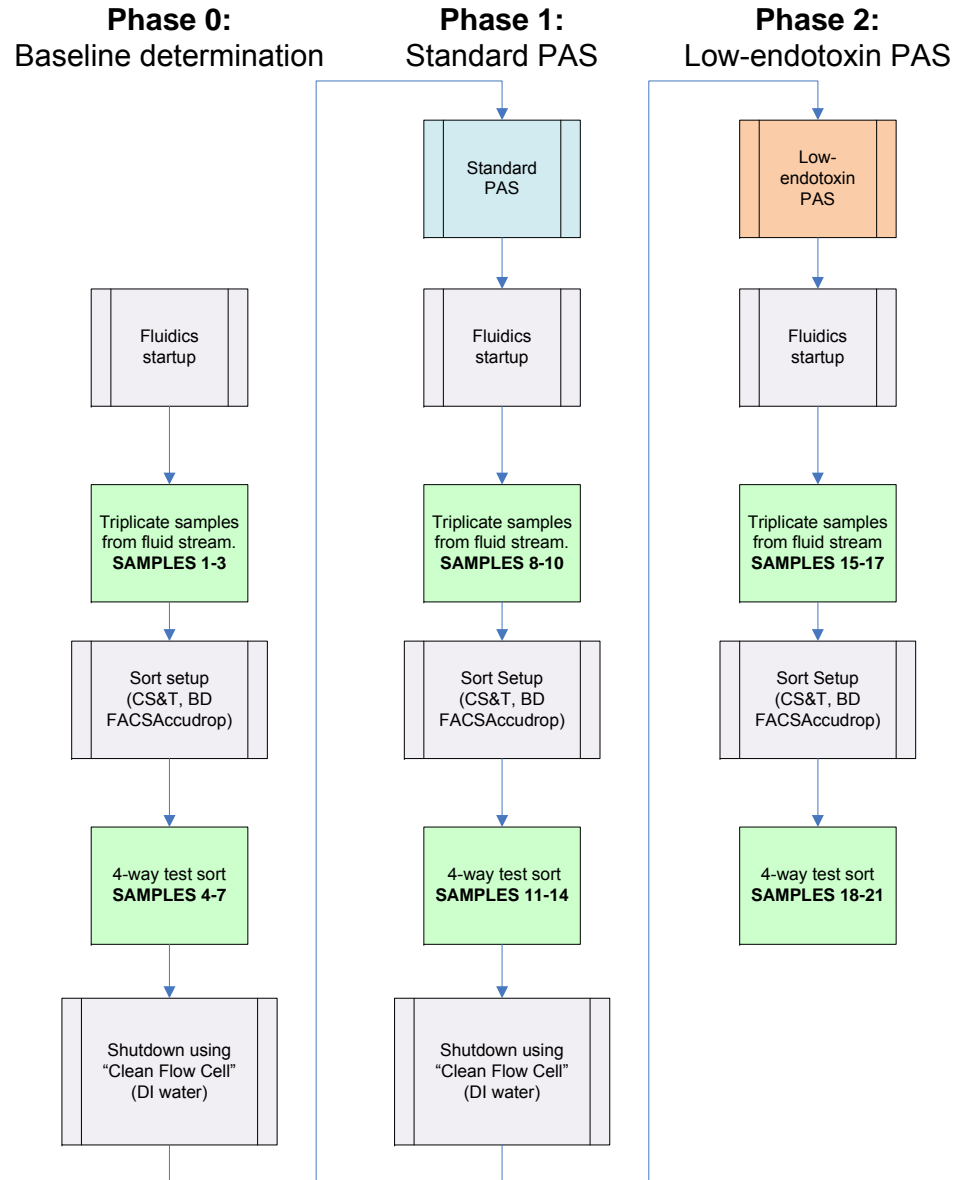


# Application note study plan

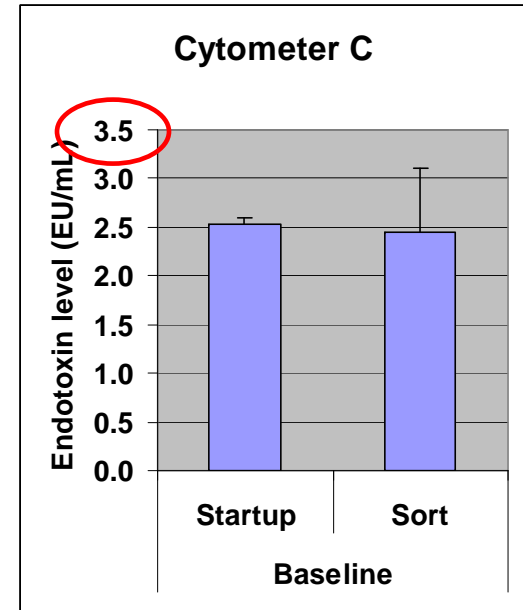
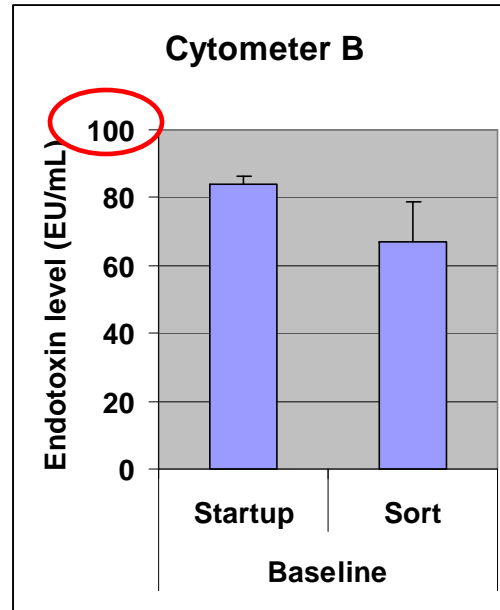
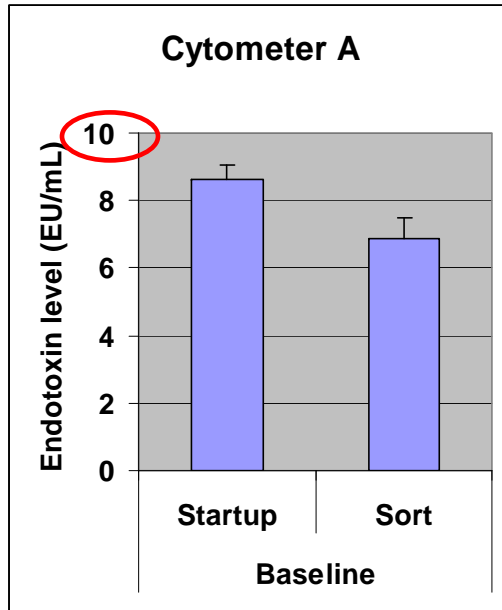
- Three-phase approach
  - Baseline
  - Standard PAS
  - “Low-endotoxin” PAS
- Three BD FACSAria II flow cytometers
  - Triplicate samples when machine turned on
  - Quadruplicate samples after “mock” sort
  - All samples were collected prior to endotoxin testing (blind study)
- Samples sent to an outside laboratory with experience in cGMP testing for endotoxin
  - Testing in triplicate over a dilution range



# Study plan



# Baseline endotoxin levels

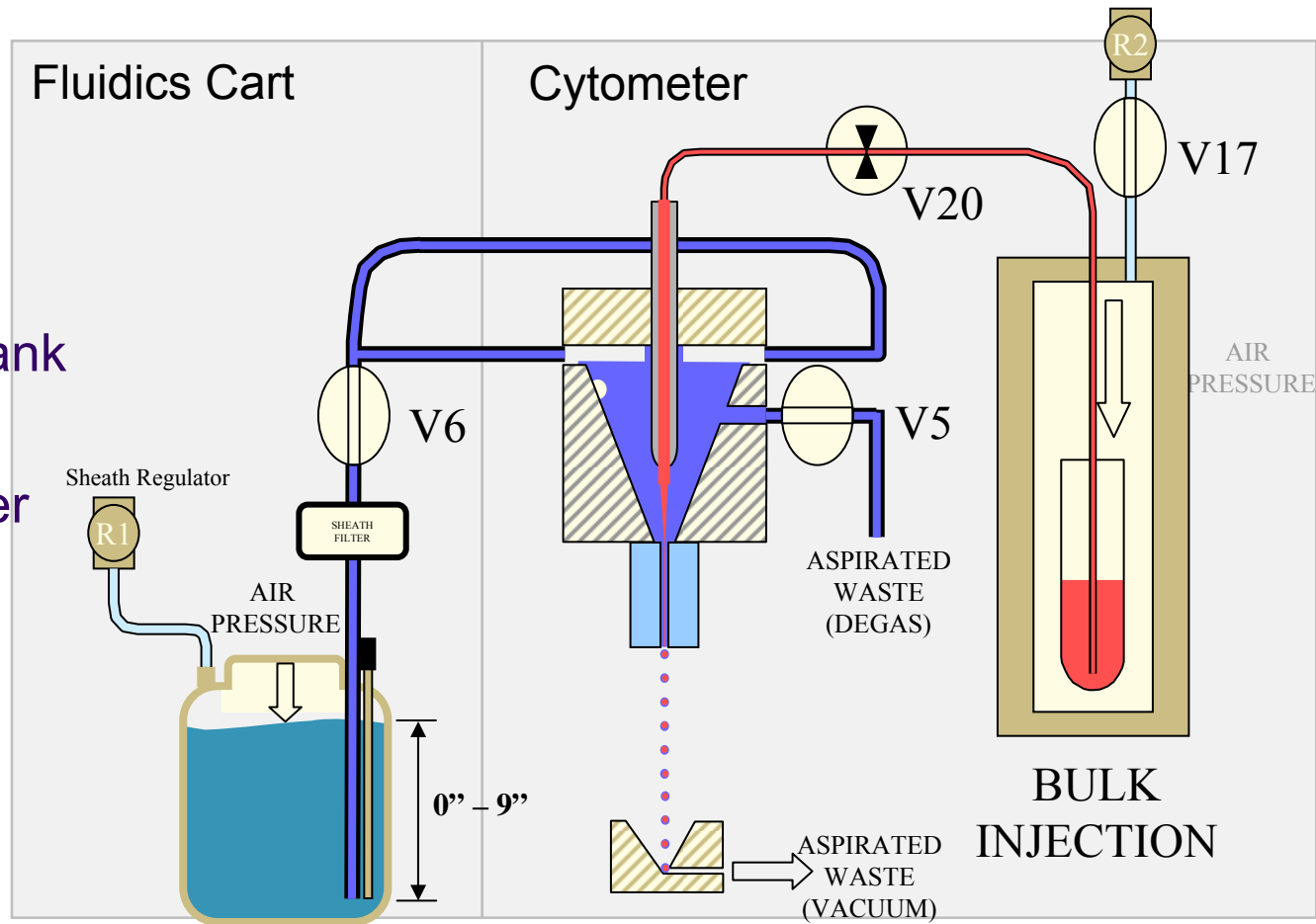


- The level of endotoxin present in a BD FACSAria II is extremely variable
- None of the baseline levels are  $<0.1$  EU/mL (target)

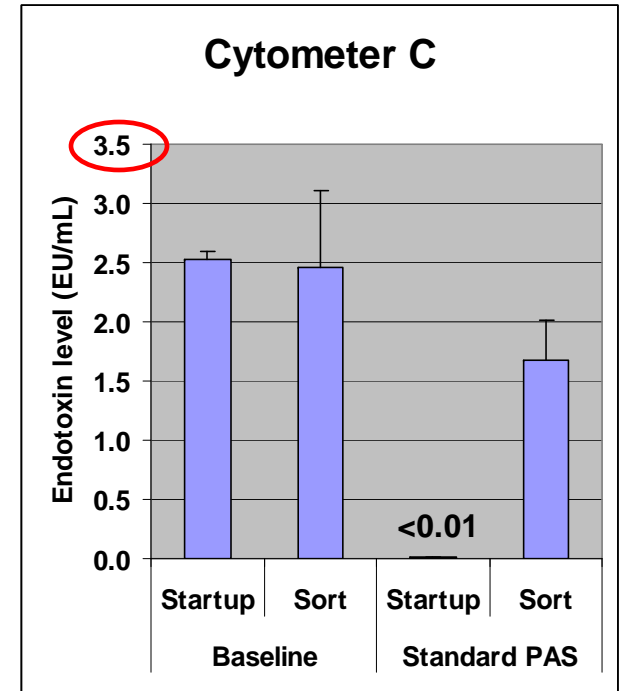
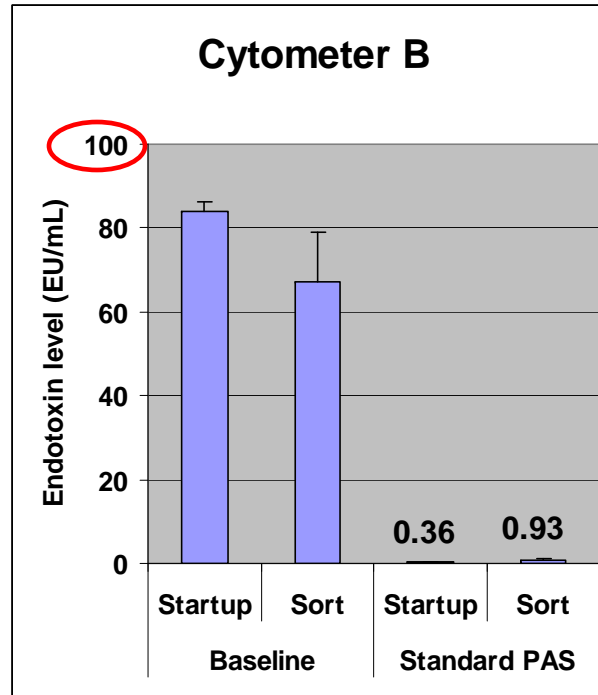
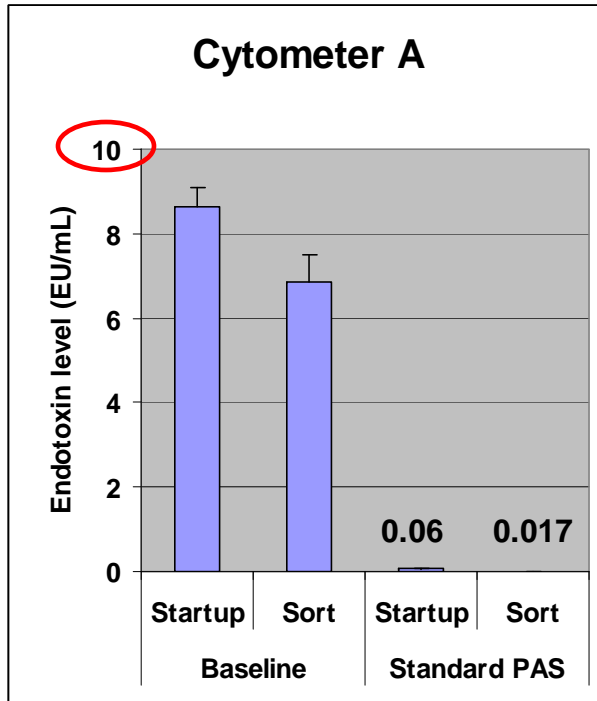


# PAS procedure

- Fluidics
  - Bleach soak
  - Water soak
  - Ethanol soak
- Autoclave sheath tank
- New sheath fluid
- Replace sheath filter



# Endotoxin levels after a standard PAS procedure



- The standard PAS procedure is highly effective at reducing endotoxin levels.
- The reduction in endotoxin is influenced by the starting level.
- Two of three instruments achieved the target value of <math><0.1</math> EU/mL.
- In two of three instruments, the endotoxin level rose after a “mock sort”.

# Low-endotoxin PAS procedure

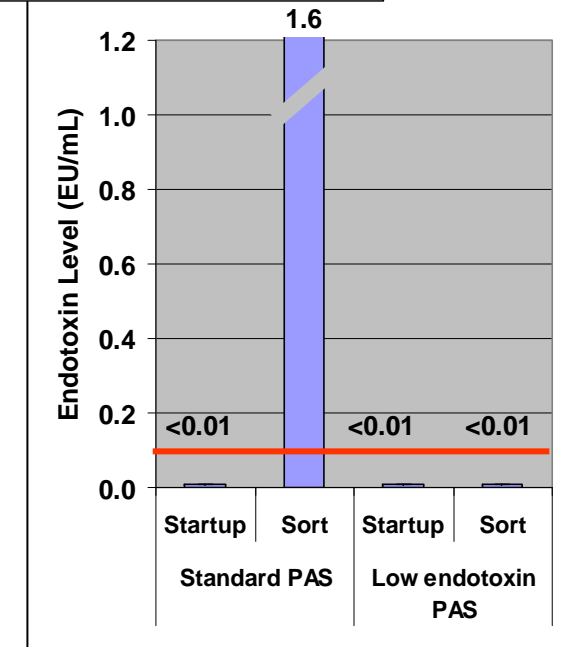
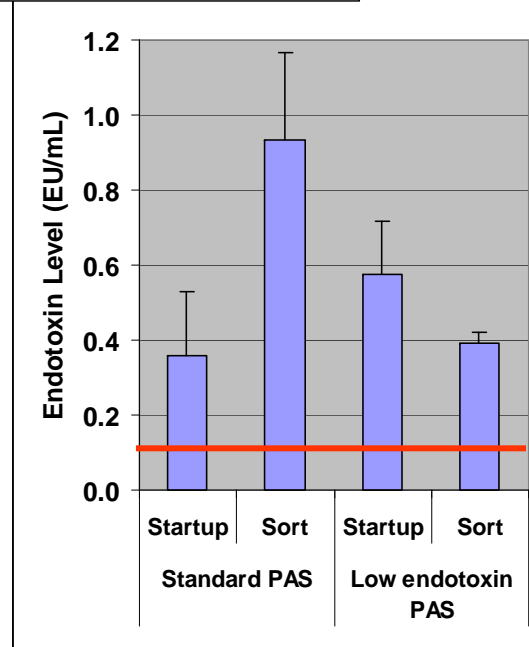
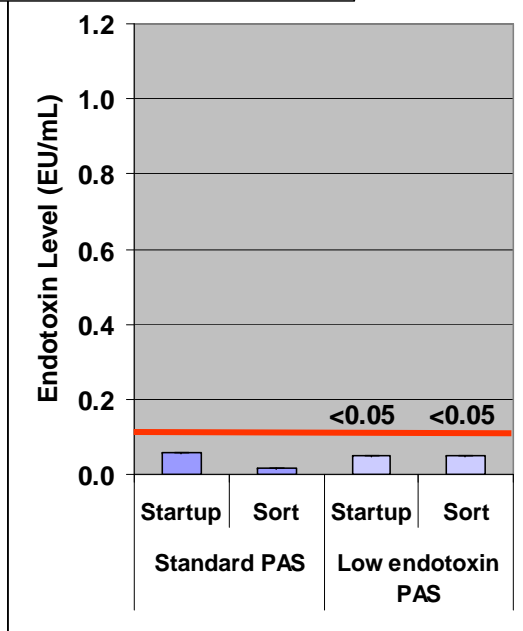
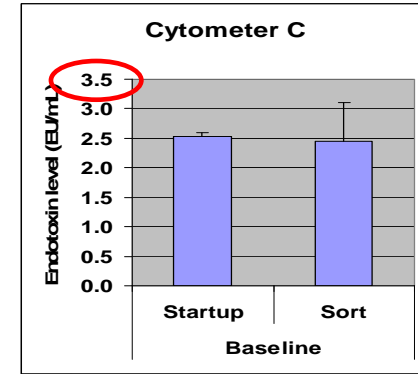
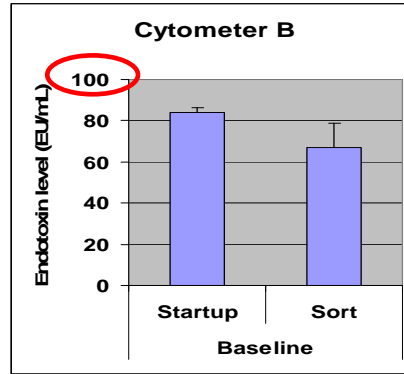
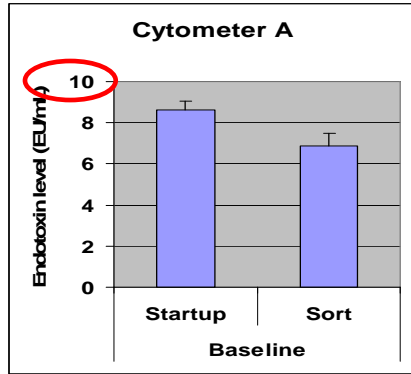
Item	Phase 1 (Standard PAS)	Phase 2 (Low-endotoxin PAS)
Sheath	Replaced: Low-endotoxin and sterile IVD reagent	Replaced: Low-endotoxin and sterile IVD reagent
Ethanol	As found	Replaced: Low-endotoxin and sterile USP reagent
Bleach	As found	Replaced: Sterile USP reagent
DI (water)	Replaced: Low-endotoxin and sterile reagent	Replaced: Low- endotoxin and sterile USP reagent
CS&T beads	General lab stock	New (unopened) vial
BD FACS™ Accudrop beads	General lab stock	New (unopened) vial
12 x 75-mm tubes	Sterile, non-pyrogenic	Sterile, non-pyrogenic
Sheath tank	Autoclaved	Cleaned (Contrad) and autoclaved
Sheath filter	Replaced	Replaced
Fluid containers (DI water, bleach, and ethanol)	As found	Cleaned and autoclaved
DI water probe	Decontaminated in bleach	Decontaminated in sterile USP bleach
Bleach and ethanol probes	As found	Decontaminated in sterile USP bleach
Fluidics cart filters	As found	Replaced
Collection device	As found	Cleaned and decontaminated
Accessible cytometer components	As found	Cleaned

# Low-endotoxin supplies

Item	Product name	Product description	Endotoxin level (from manufacturer's C of A)
Sheath	DPBS w/o Ca and Mg, 10X, (Mediatech) (Diluted 1:10 in WFI – see below)	Sterile IVD	<0.005 EU/mL
Ethanol	STER-AHOL® 70% WFI, Veltek Associates, Inc (VAI)	Sterile, USP, low endotoxin	<0.03 EU/mL
Bleach	HYPO-CHLOR® (VAI) (Diluted 1:10 in WFI – see below)	Sterile, USP	Not tested
DI water	HyPure™ WFI quality water (Hyclone®)	Sterile, USP	<0.01 EU/mL
12 x 75-mm tubes	12 x 75-mm tubes	Sterile, non-pyrogenic	≤0.5 EU/mL
Isopropanol	DECON-AHOL® 70% WFI (VAI)	Sterile, USP low endotoxin	<0.03 EU/mL
Sterile cleaning cloths	Gamma wipes	Sterile	Not tested
Collection and storage tubes for samples	Test tubes, (15 mL)	Sterile, non-pyrogenic	<20 EU/tube



# Endotoxin levels after low-endotoxin PAS procedure



# Endotoxin levels after low-endotoxin PAS procedure

Instrument	Average Endotoxin levels (EU/mL)					
	Phase 0 (Baseline)		Phase 1 (Standard PAS)		Phase 2 (Low endotoxin PAS)	
	Startup <sup>1</sup>	Test Sort <sup>2</sup>	Startup	Test Sort	Startup	Test Sort
<b>A</b>	8.64 ±0.43	6.86 ±0.62	0.06 ±0.00	0.02 ±0.00	<0.05	<0.05
<b>B</b>	83.94 ±2.23	67.22 ±11.78	0.36 ±0.17	0.93 ±0.23	0.58 ±0.14	0.39 ±0.03
<b>C</b>	2.53 ±0.07	2.46 ±0.66	<0.01	1.67 ±0.34	<0.01	<0.01

- Cytometer A maintained the already low endotoxin levels.
- Cytometers B and C showed an additional reduction in endotoxin.
- Two out of three cytometers achieved the target value of <0.1 EU/mL.

1 Mean and SD of triplicate samples

2 Mean and SD of quadruplicate samples



# Discussion: Baseline

- Baseline endotoxin levels of different cytometers are extremely variable.
  - 2.5–83.4 EU/mL
- Probably related to:
  - How often the cytometer is used
  - How well it is maintained
- Leaving the cytometer turned on with sheath fluid in the lines can result in:
  - Growth of bacteria (and other organisms) in the fluid lines
  - Deposition of endotoxin



# Discussion: Standard PAS procedure

- This procedure can be highly effective at removing:
  - Bacteria:  $9 \times 10^5$  CFU down to 0 CFU/mL
  - Endotoxin:  $<0.1$  EU/mL (2 out of 3)
- When setting up for and performing a sort, it is possible to:
  - Maintain low-endotoxin status
  - Increase (re-contaminate) endotoxin status
    - Cytometer B: From 0.36 to 0.93 EU/mL
    - Cytometer C: From  $<0.01$  to 1.67 EU/mL





# Discussion: Low-endotoxin PAS procedure

- Is highly effective at achieving low-endotoxin status
- It is possible to achieve the target value of  $<0.1$  EU/mL (2 out of 3)
- When using extra precautions and diligence when setting up and performing a sort, it is possible to:
  - Maintain extremely low-endotoxin status
    - Cytometer A:  $<0.05$  EU/mL
    - Cytometer C:  $<0.01$  EU/mL
  - Further reduce endotoxin levels
    - Cytometer B: 0.58 to 0.39 EU/mL



# Overall conclusion

- The PAS procedure can be *extremely* efficient at reducing:
  - Bacterial contamination
  - Endotoxin load
- *This procedure must be validated by customers with their own reagents and instruments and may vary under different conditions.*
- The BD FACSAria II is an RUO instrument
- *BD makes no claims of completely eliminating bacteria or endotoxin.*



# Overview

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  - Prepare for Aseptic Sort (PAS) procedure
- Decontamination of the BD FACSAria II
  - Bacteria
  - Endotoxin
- **Tips to limit contamination**



# Tips for limiting bacteria and endotoxin contamination of the BD FACSAria II

- Maintain and clean the cytometer.
- Limit skin contact with the fluidics system.
- Use good quality reagents.
- If you use a central DI water system, make sure it is maintained regularly.
- DO NOT top off fluid containers.
- Use freshly prepared bleach.
  - 10% Clorox® (0.5% sodium hypochlorite) in water is not stable and can lose effectiveness due to oxidation and reaction with impurities in water.
  - BD™ FACSClean solution is made from high quality reagents and is known to be stable.



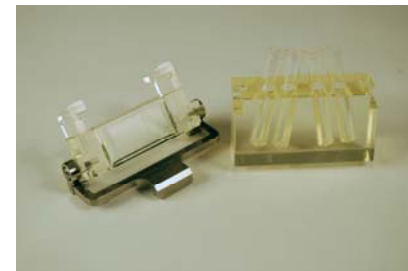
# Tips for limiting bacteria contamination during a sort

- Use a well maintained cytometer.
- Perform the PAS procedure.
- Use aseptic technique and sterile reagents whenever possible.
  - Sheath
  - Staining sample
  - Ancillary reagents
  - Collecting sorted cells



# Tips for limiting endotoxin contamination during a sort

- Limit bacterial contamination (previous slide).
- Always use aseptic technique.
- Use low-endotoxin reagents whenever possible.
  - Antibodies (NA/LE)
  - Serum (often has high endotoxin)
  - Ancillary reagents
  - Cleaning reagents (BD FACSClean solution, water, and ethanol)
- Use low-pyrogen plastic ware (BD Falcon™ products).
- Check C of As.
- Be diligent in the PAS procedure and instrument cleaning.
  - Surface cleaning
    - Reagents
    - Cleaning cloths
  - Fluidic tanks
  - Take special care with the tube holder



# Thank you

- Copies of the application notes can be found on the BD Biosciences website
- [www.bdbiosciences.com](http://www.bdbiosciences.com)
  - Navigate to the BD FACSAria II page
  - Click **More Documents**
- Related BD products
  - No azide/low endotoxin (NA/LE) antibodies
  - BD Falcon products are certified non-pyrogenic

