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Multiplex Assay Platform Integration: Biobriefcase as a Case Study

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Overview

- Introduction
- System requirements → conceptual design
- Assay implementation on a flow-through platform
- System performance
- Extension of this platform to other assays and applications

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BioBriefcase is a collaboration between Lawrence Livermore and Sandia National Laboratories

Objective – to develop a microfluidics-based bioagent detection system for national security applications

- **Operations -**
  - Autonomous
  - 1-hour analysis time
  - 30 days between servicing
- **Costs -**
  - Inexpensive to own and operate
  - Low reagent consumption
- **Assays -**
  - Multiplex analysis
  - Parallel detection trains
- **Deployment -**
  - Easily deployed and networked
  - Robust for outdoor applications

Funding history:
2003 – DOE/CBNP
2004-2005 – DHS ORD
2006 – DHS HSARPA BAND

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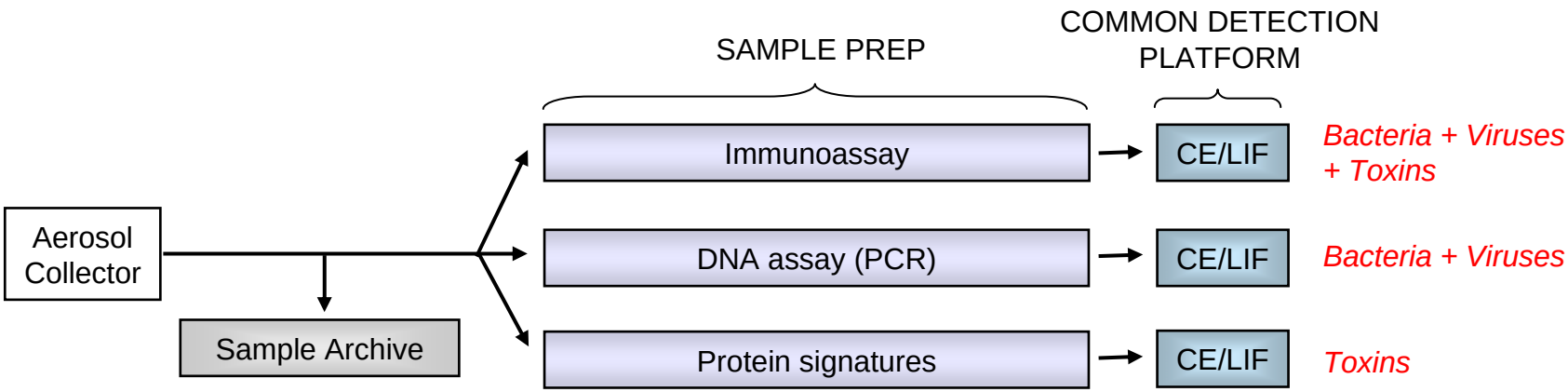
System conceptual design:
Three assay trains to detect the full biothreat spectrum



- Two orthogonal assays for each agent → to achieve low false alarm rate
- Reporter-based assays provide for common capillary electrophoresis detection platform
- LIF-based detection → sensitive detection

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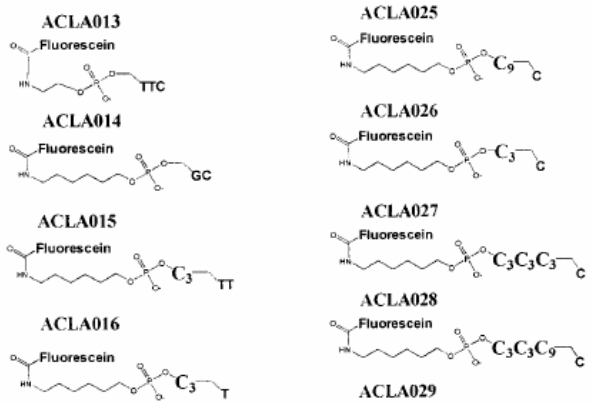
BioBriefcase uses eTag-based assays for both immuno and nucleic acid assays

eTag assays were developed for medical diagnostics by Monogram Biosciences, customized for BioBriefcase for bioagent detection

Reporter Molecules –

- Decouple recognition from detection
- Optimized conditions for each step
- **Enables multiplex detection**
- **Multiple loci and antibodies for a single agent**



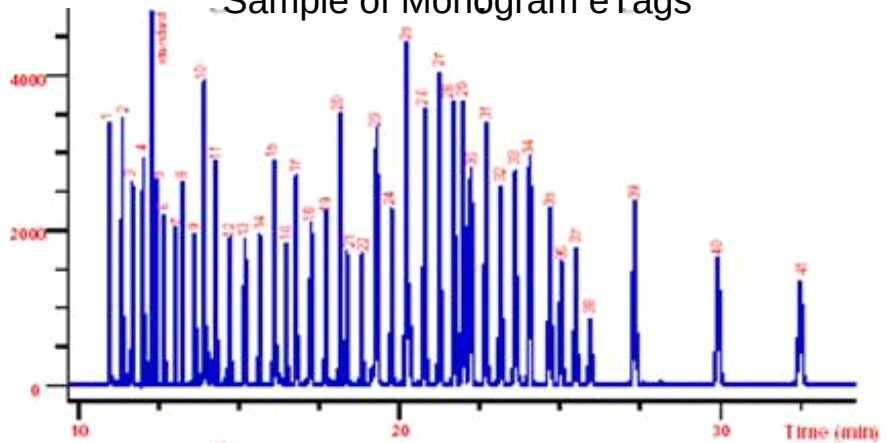
Monogram eTags -

- Organic molecules 500-1000 MW
- Well characterized electrophoretic mobility
- Tagged with same fluorophore

CE Analysis -

- Amenable to analysis on chip
- Rapid separations

Sample of Monogram eTags



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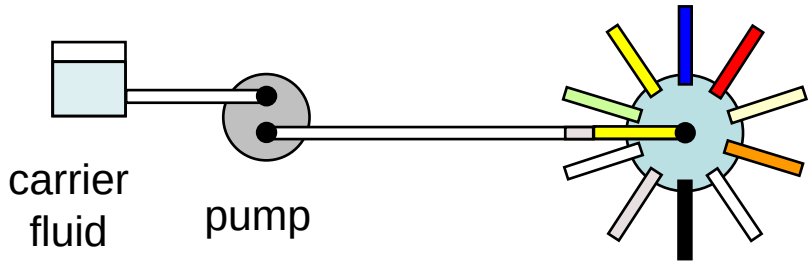
Zone fluidics – Automated approach for manipulating fluids for chemical processes

- Can perform most standard unit operations
- Simple components – reversible pump, multi-port selection valve, versatile control software
- Air bubbles are used to separate reagents and minimize diffusion
- Compatible with microfluidics



Global FIA mini FloPro

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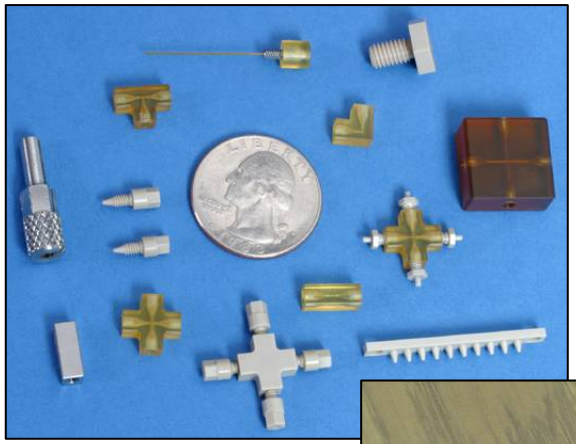
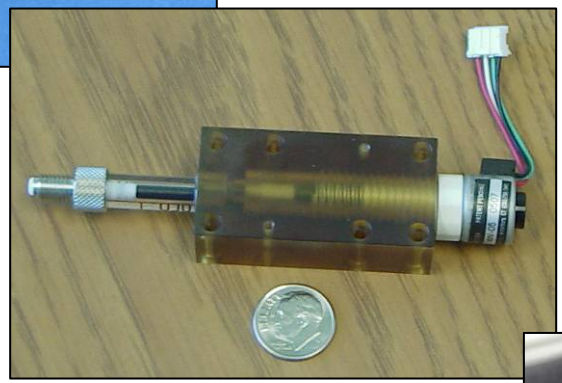
Enabling Technologies - Microfluidics

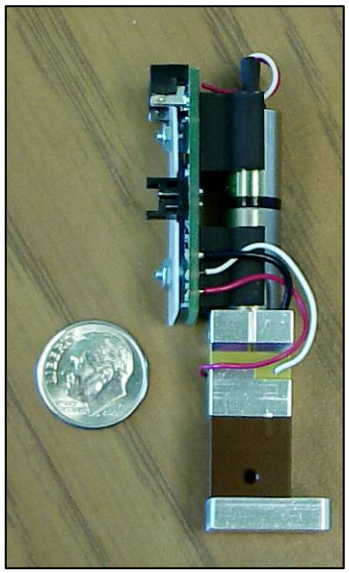


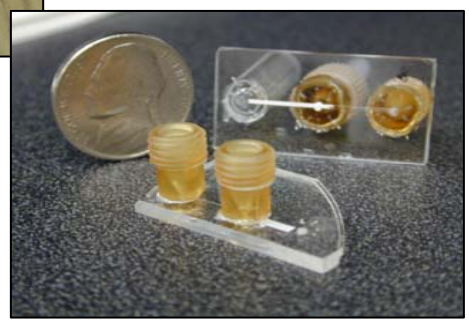
CapTite™ Fluidic Fittings
- One piece design
- Reusable
- Finger or tool tightened
- Up to 10 KSI

Reliable fluid handling in small, autonomous systems



Syringe pumps
- Linear stepper motor
- Controlled fluid metering
- Bidirectional





EK pumps
- no moving parts
- high pressure
- fast
- accurate

Miniature valves
- 10 KSI capability
- Electrically actuated
- Low swept volumes (~10 nl)

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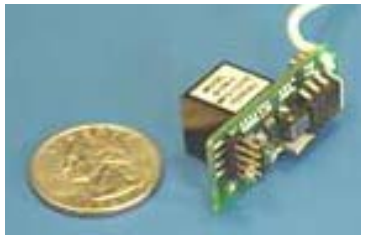
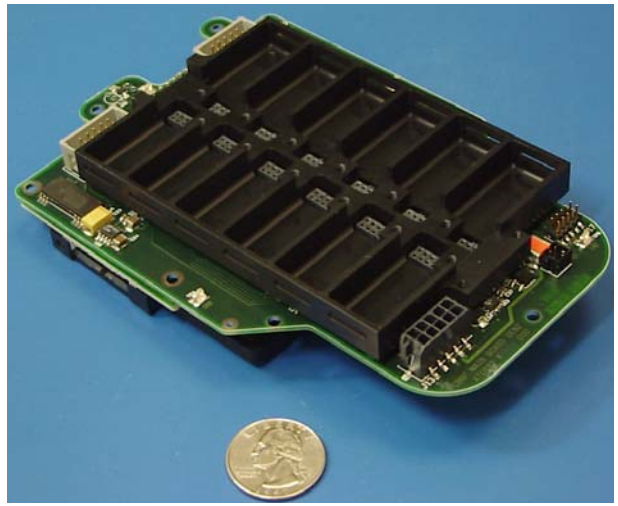
Enabling Technologies - Electronics



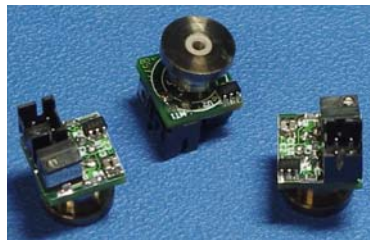
HV Power Supplies

- Programmable
- Outputs to 10 KV



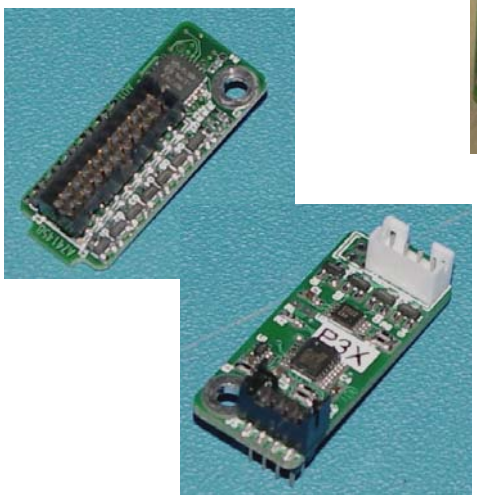
Sensors

- Pressure Transducers
- Photomultiplier



Controller

- Modular backplane
- Computer interface



Component Interfaces

- Stepper motor drive
- Valve control/monitor

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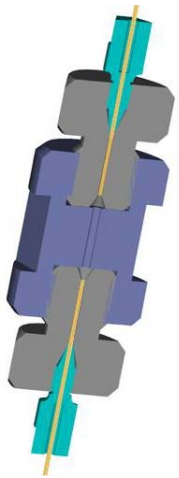
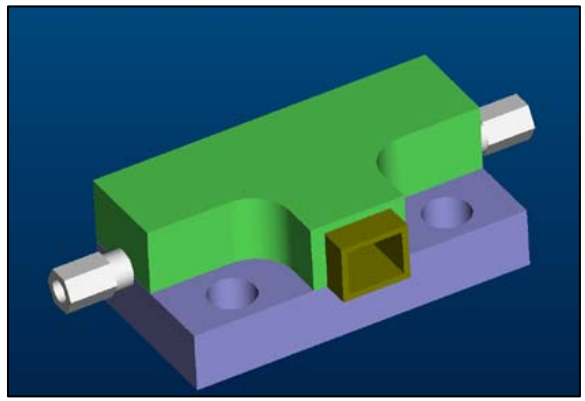
Enabling Technology: Modular Sample Processing

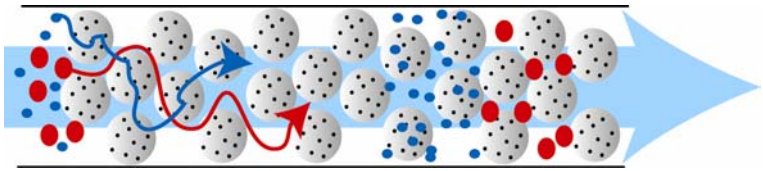
Packed Bed Cartridges

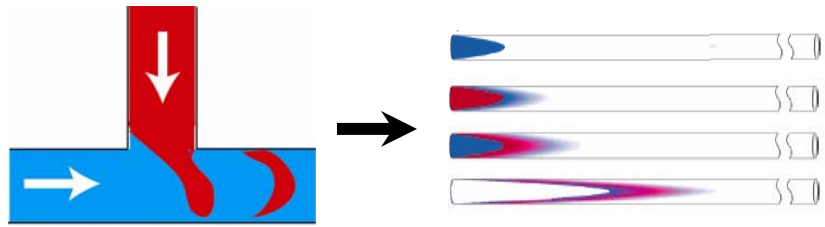
- concentration
- fractionation
- filtering
- sieving
- desalting
- contaminant removal
- digestion
- buffer exchange





Thermo/chemical lysis





In-Capillary Labeling

Multiple technologies provide flexibility to optimize system performance

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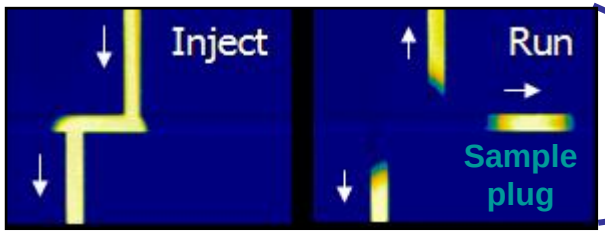
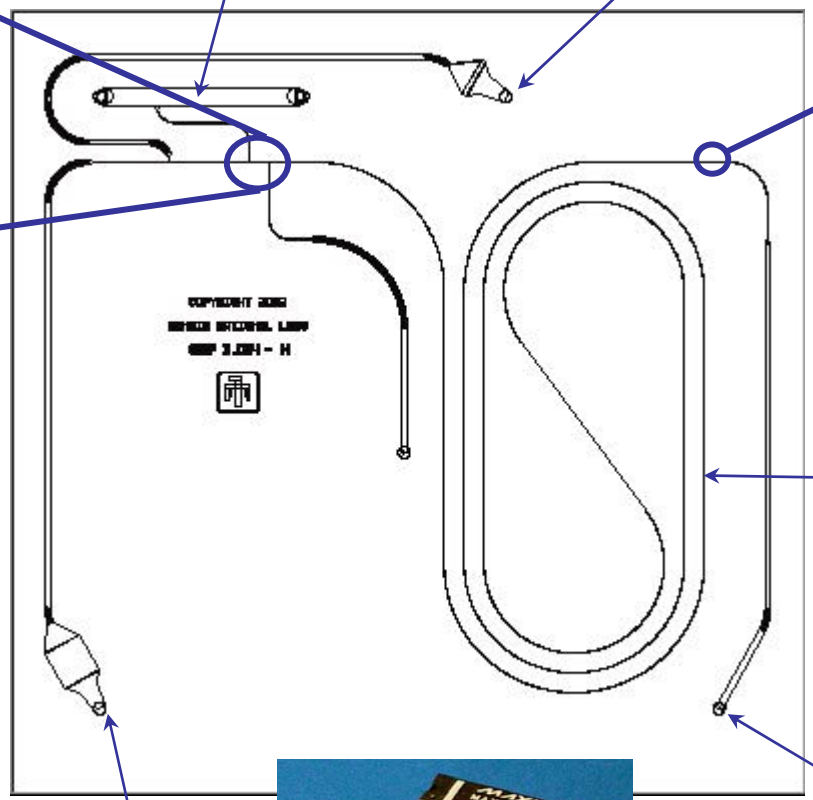
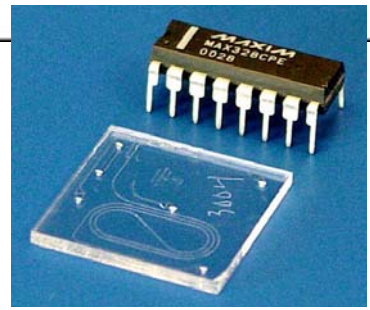
Enabling Technology: Chip-Based Microseparations

Electrokinetic injection





- **Chemical separations is an established analytical technique**
- **On the microscale,**
  - Time required for a separation is dramatically reduced
  - Reagent volumes are minimal
  - Parallel and sequential separations become relatively easy
    - Provide differential selectivity
    - Improve detection verity
    - Lower false alarm rates
- **A single chip can be used for hundreds of separations**



Chip-based microseparations provide powerful analytical capability

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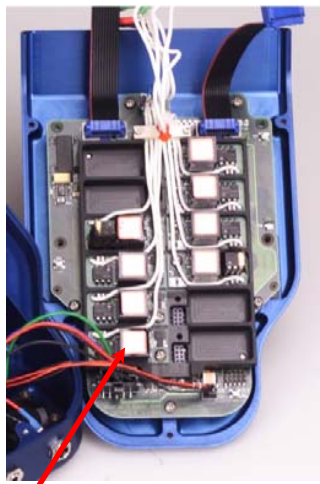
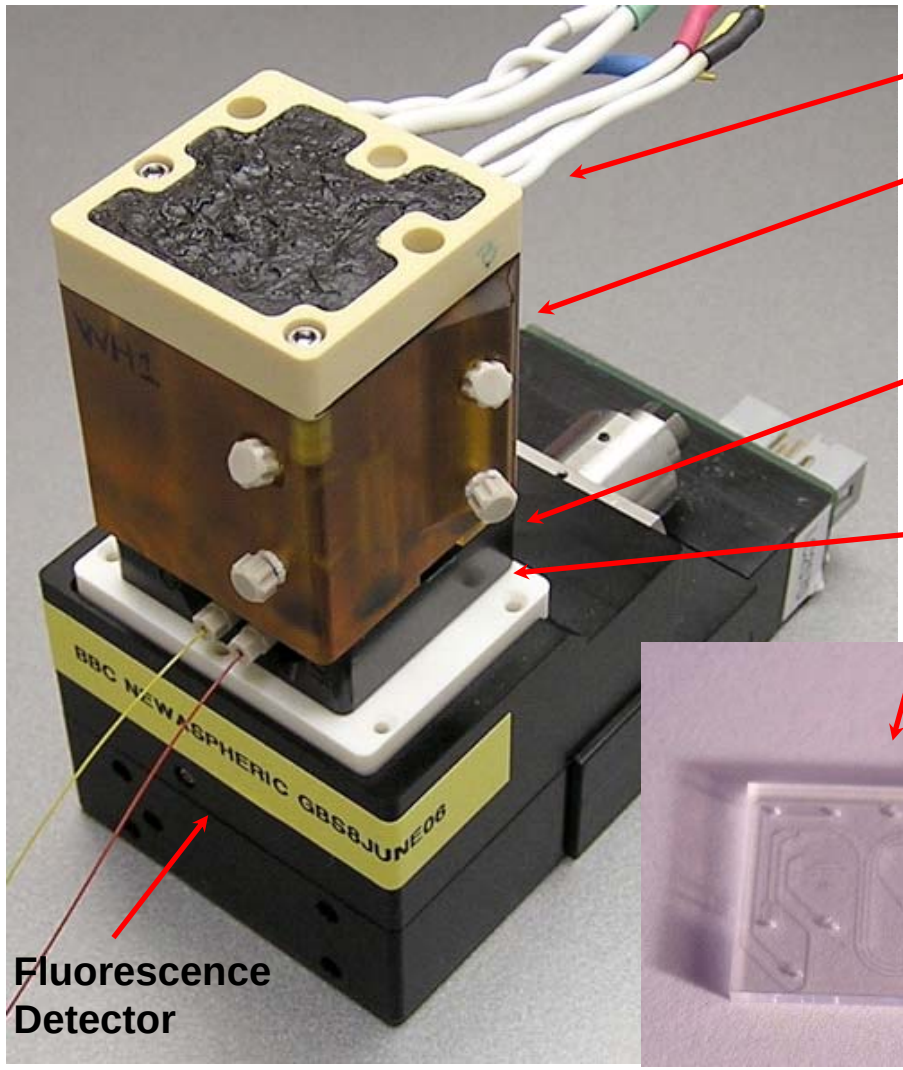
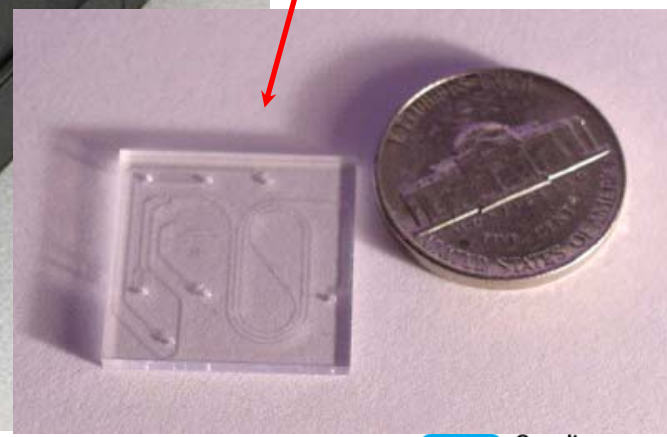
Sandia's µChemLab™ hand-held instrument provides the CE/LIF detection platform for BioBriefcase



High-Voltage
Power Supplies





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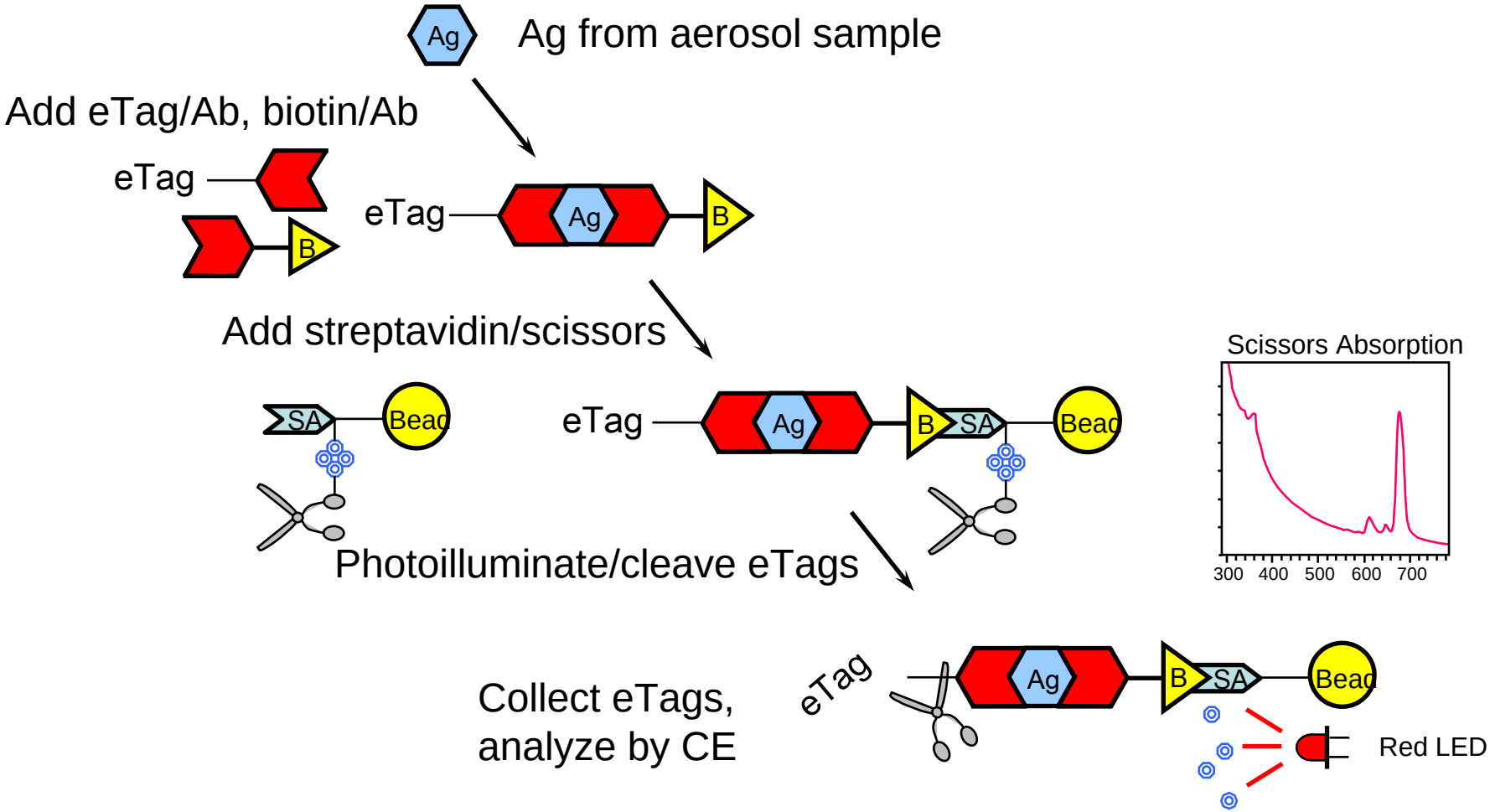
eTag reporter immunoassay for toxins



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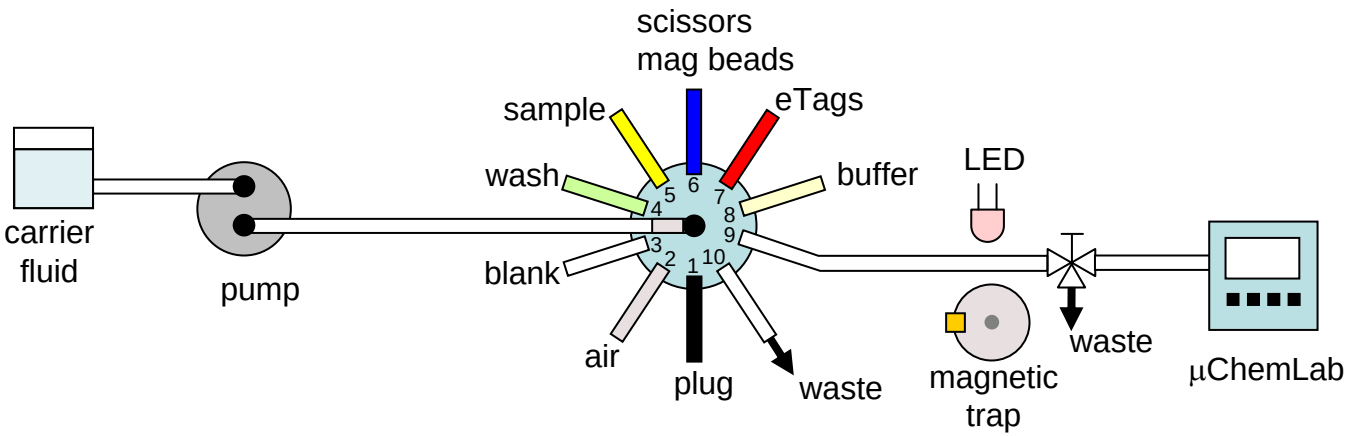
eTag Immunoassay on Mini Flow Pro



Pull up air bubble

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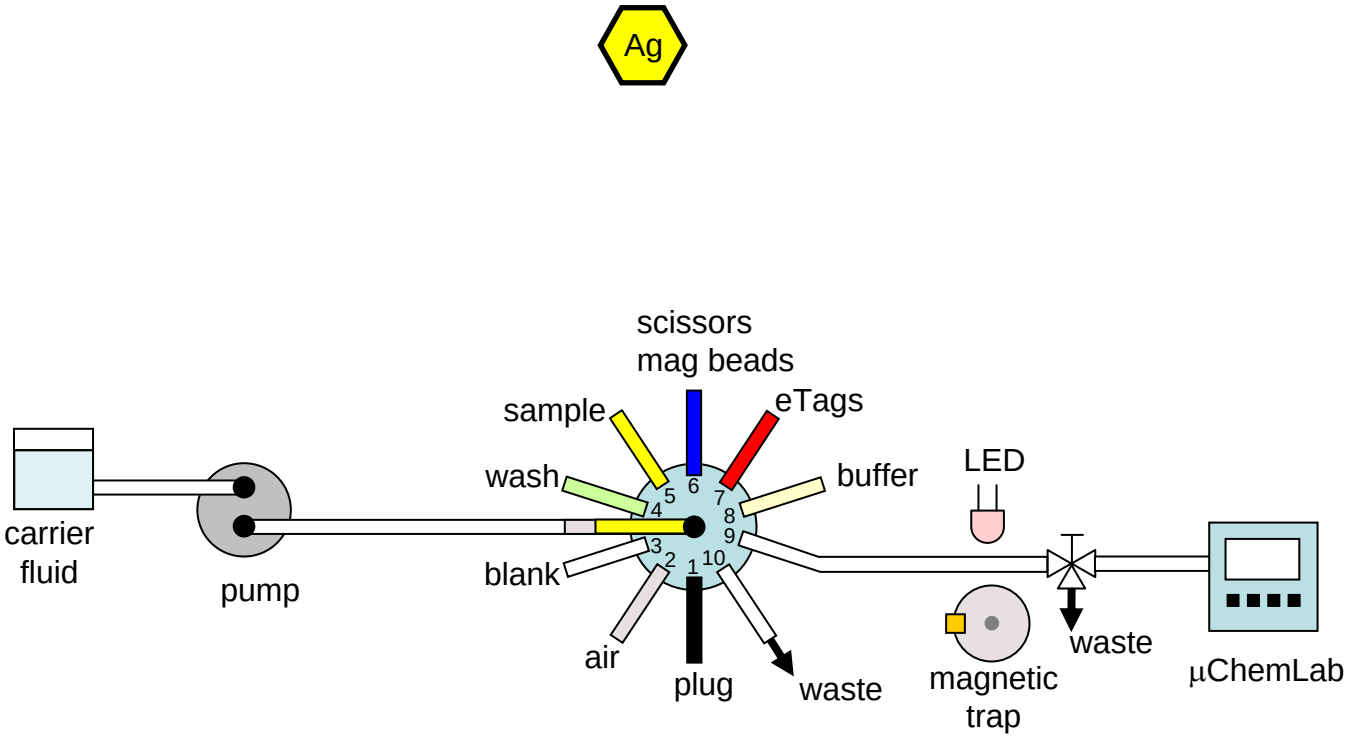
eTag Immunoassay on Mini Flow Pro



Pull up sample plug

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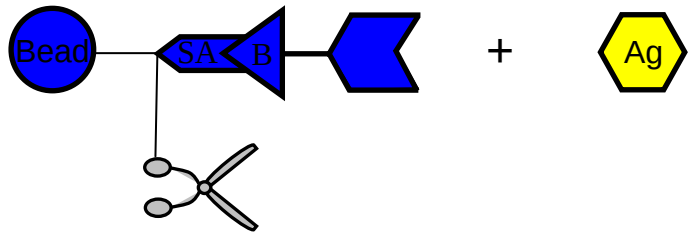
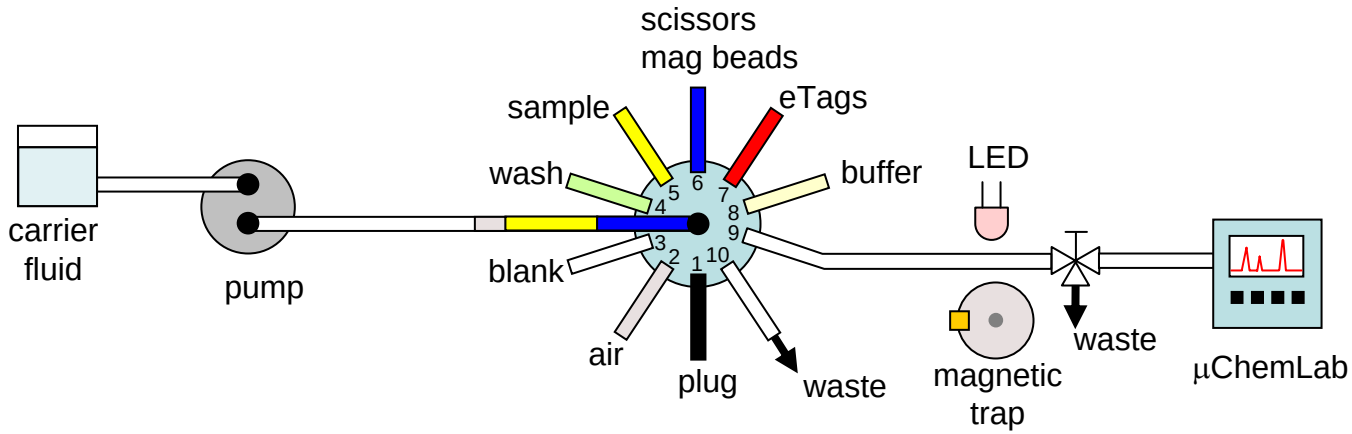
eTag Immunoassay on Mini Flow Pro





Pull up scissors/beads plug

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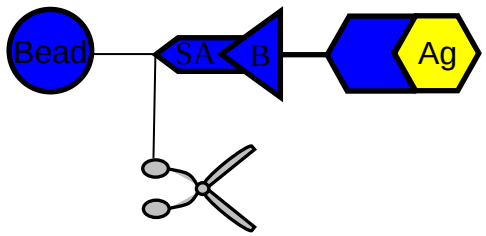
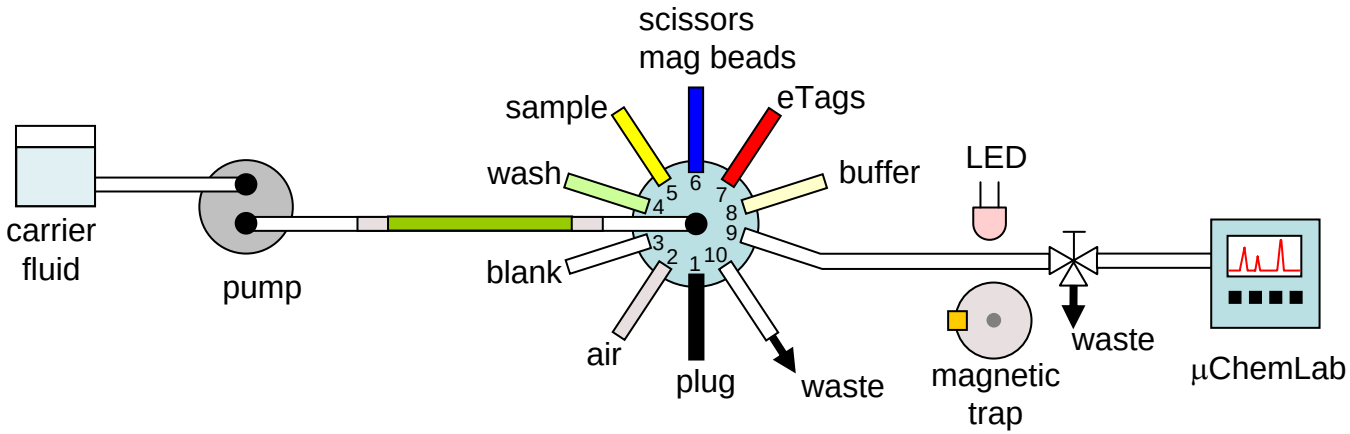
eTag Immunoassay on Mini Flow Pro





Pull up air plug, mix

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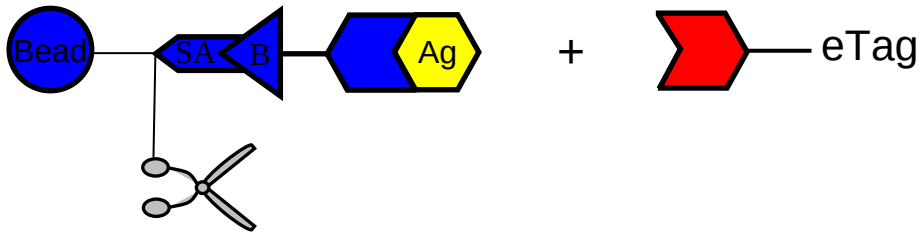
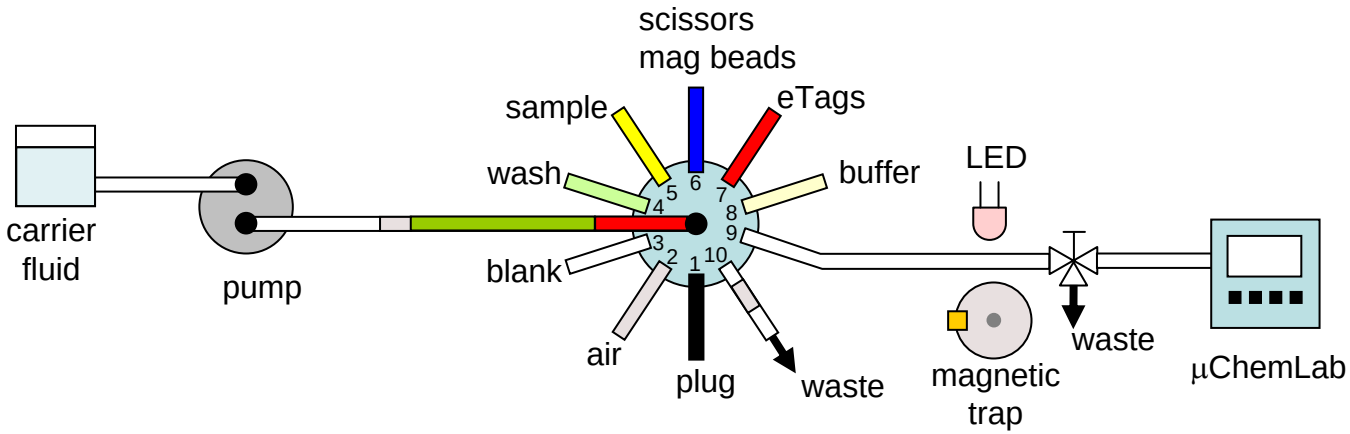
eTag Immunoassay on Mini Flow Pro





Dispense air bubble, pull up eTags plug

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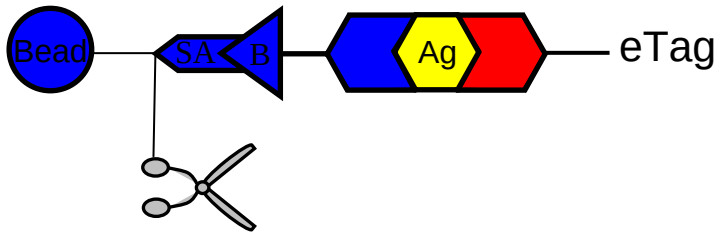
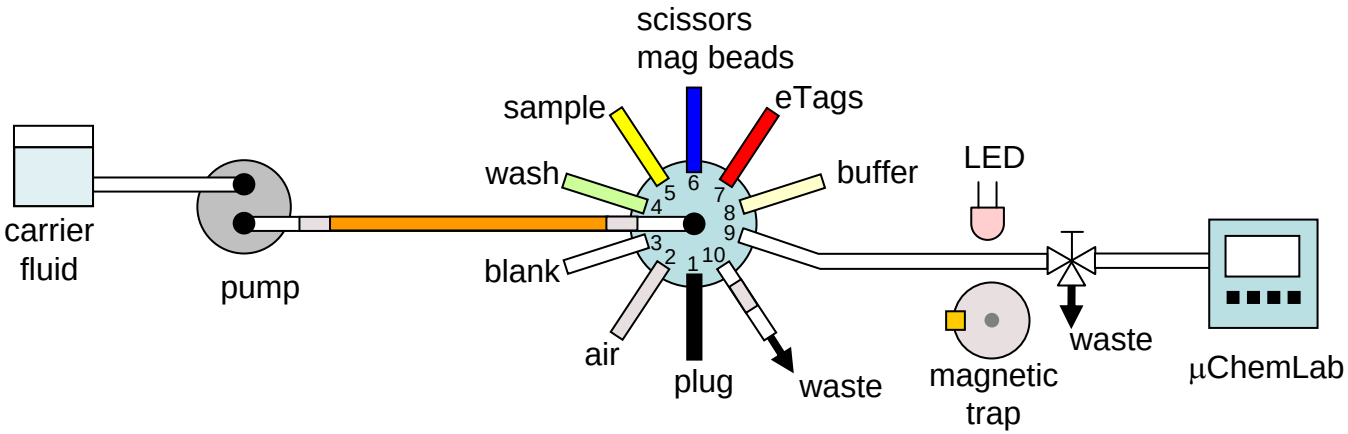
eTag Immunoassay on Mini Flow Pro





Pick up air bubble, mix

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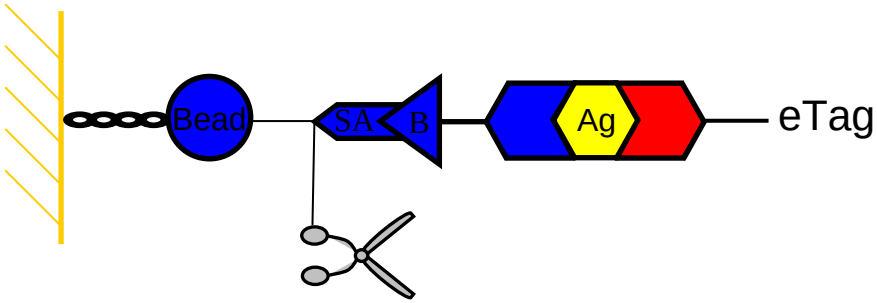
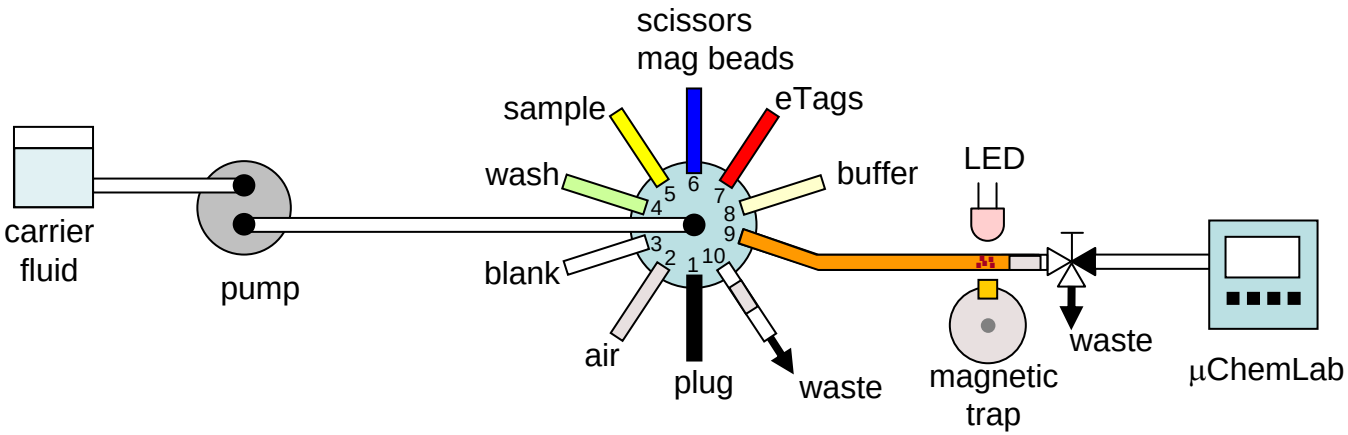
eTag Immunoassay on Mini Flow Pro





Switch magnetic trap on, dispense eTag complex,
Collect magnetic beads in trap

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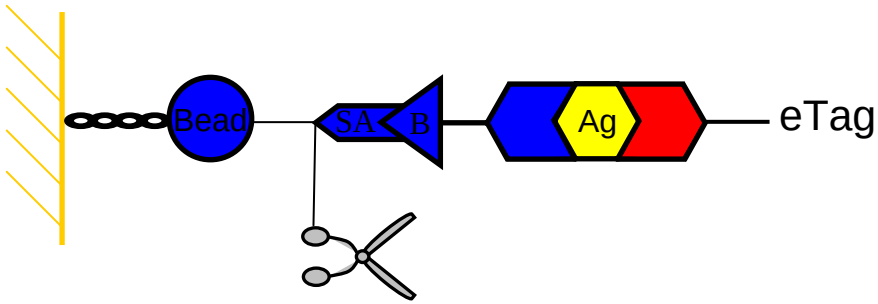
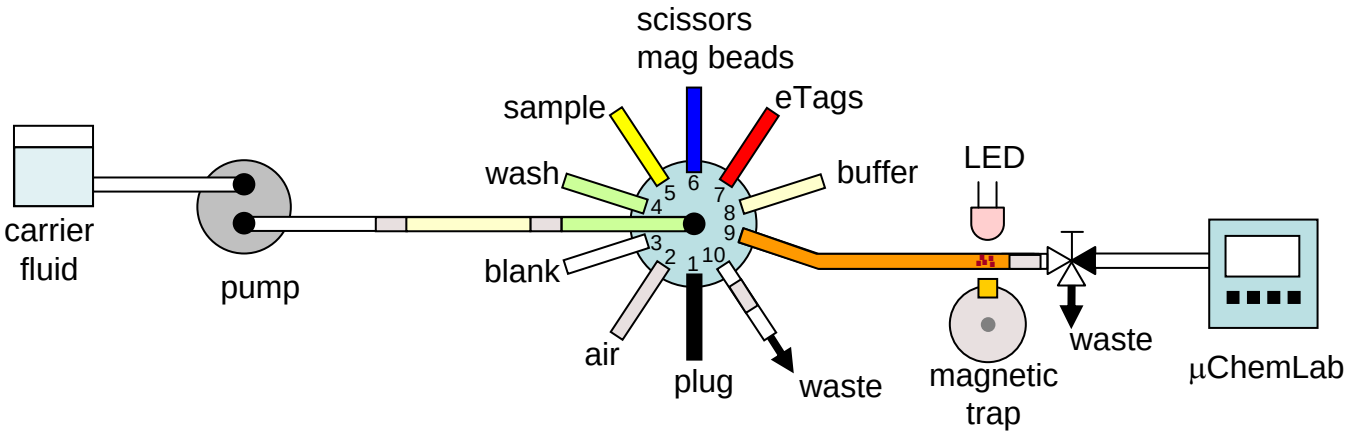
eTag Immunoassay on Mini Flow Pro





Pull up plugs of buffer and wash fluid

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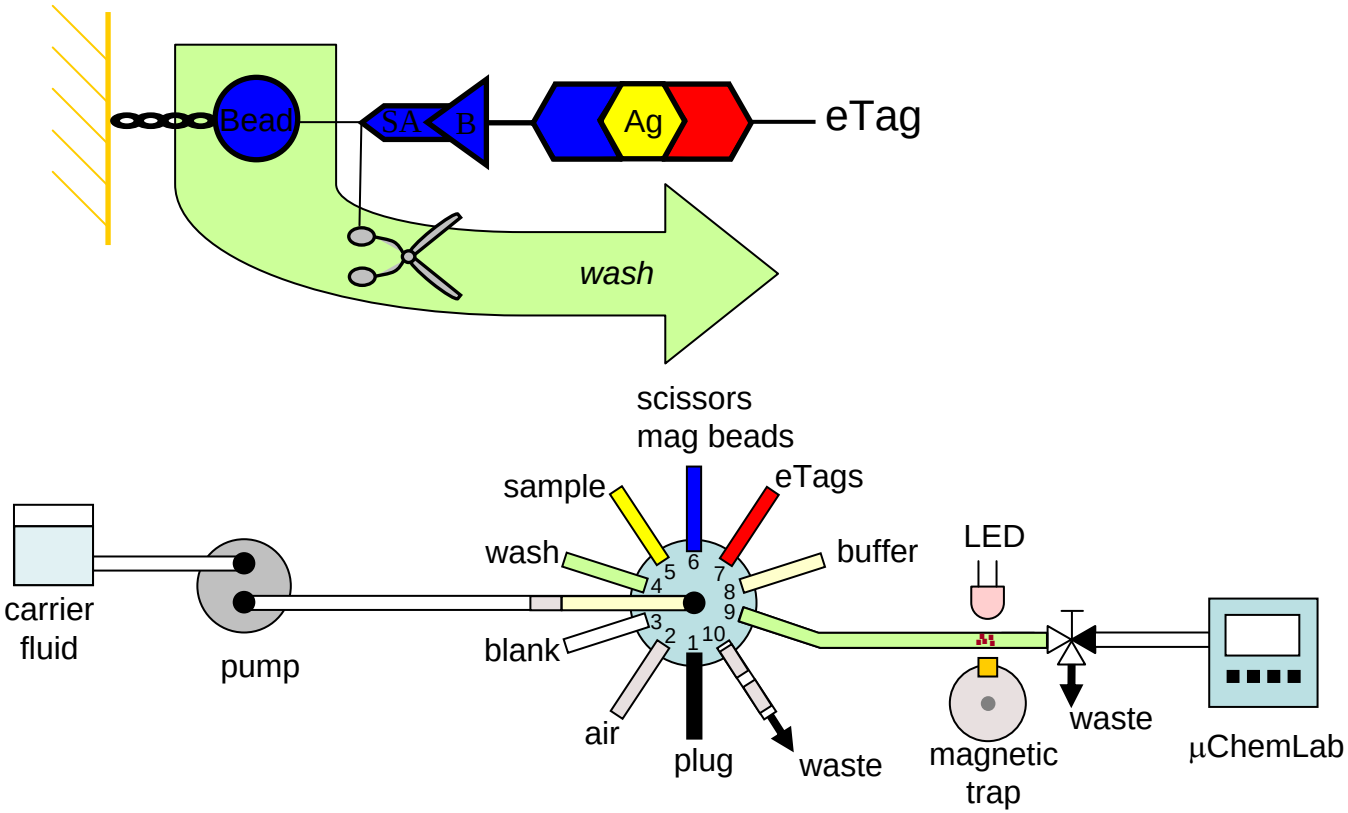
eTag Immunoassay on Mini Flow Pro



Wash beads, send bubble to waste

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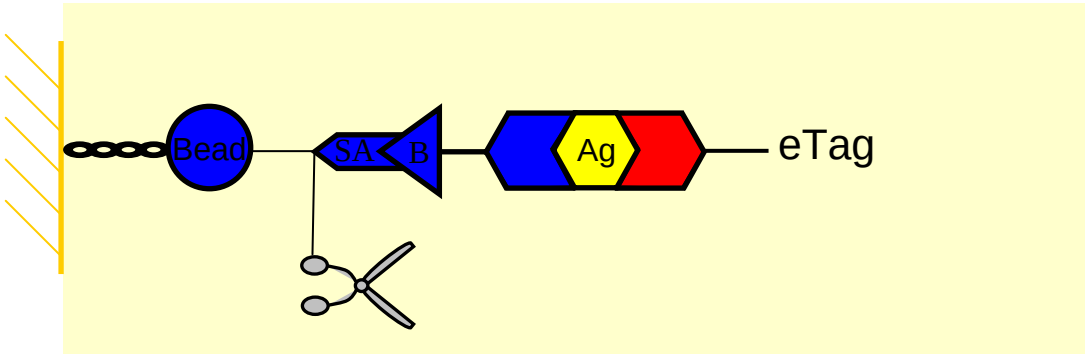
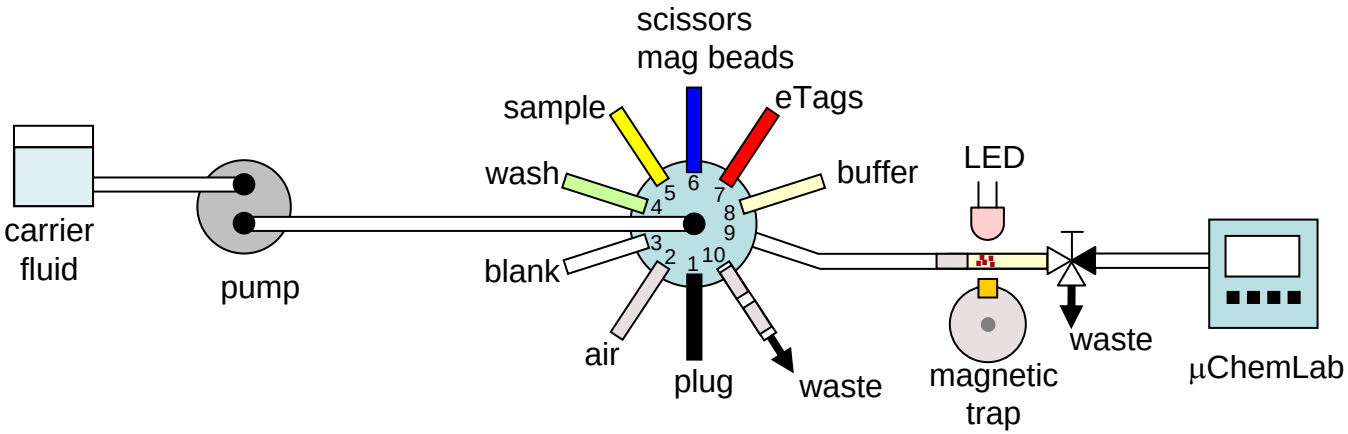
eTag Immunoassay on Mini Flow Pro





Suspend beads in eTag buffer

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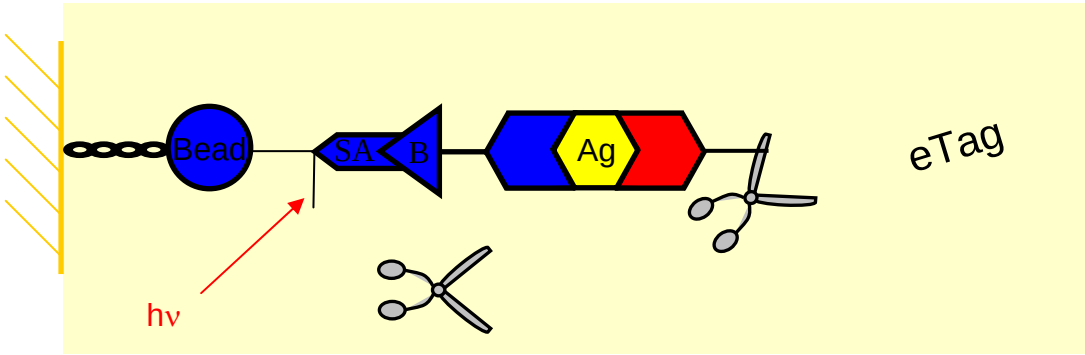
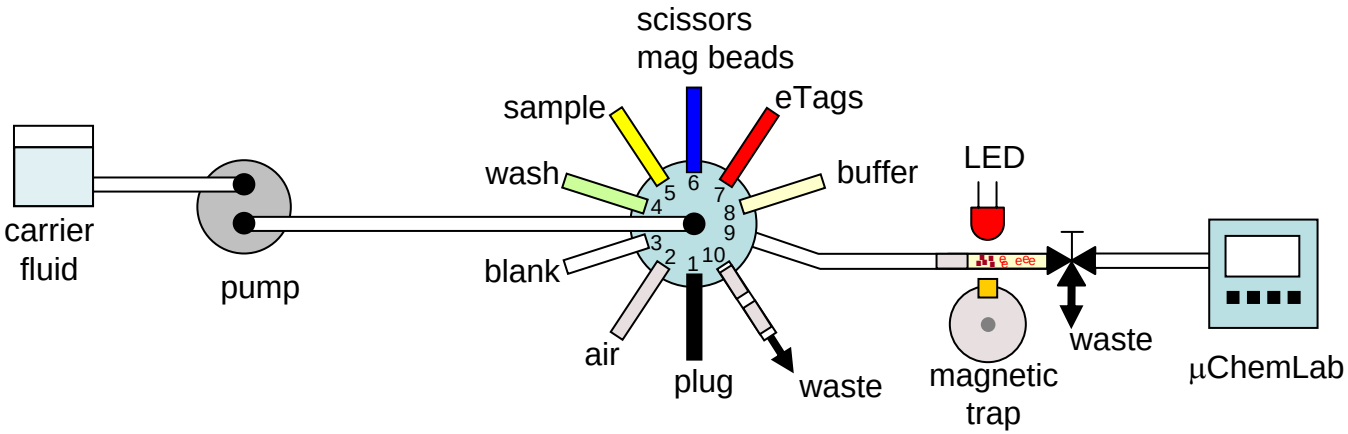
eTag Immunoassay on Mini Flow Pro





Close valve, turn on LED, release eTags

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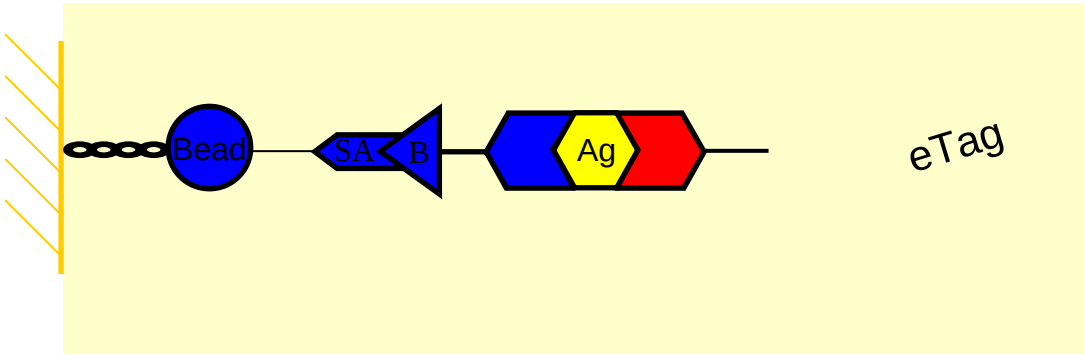
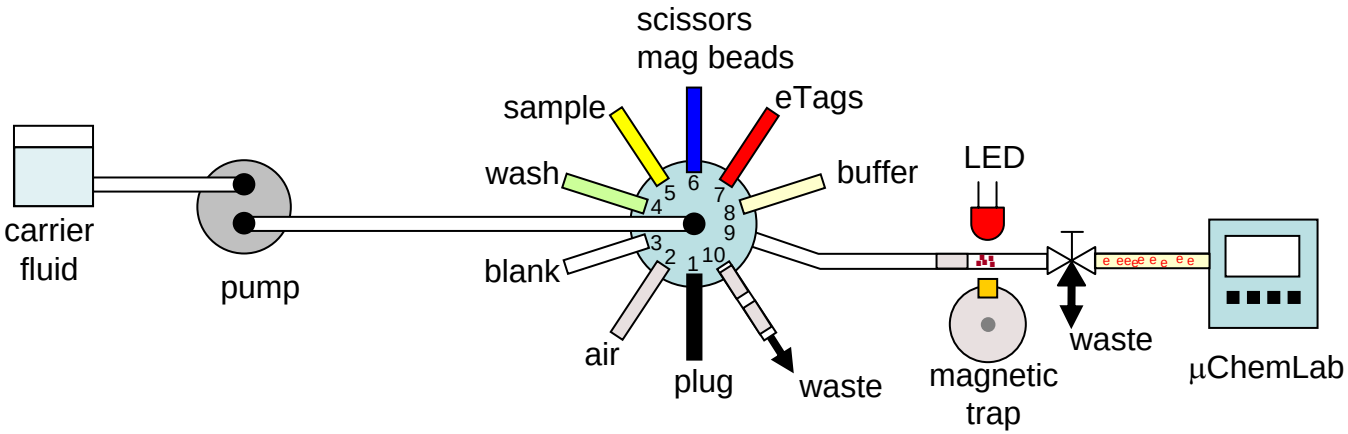
eTag Immunoassay on Mini Flow Pro





Open valve, send eTags to µChemLab

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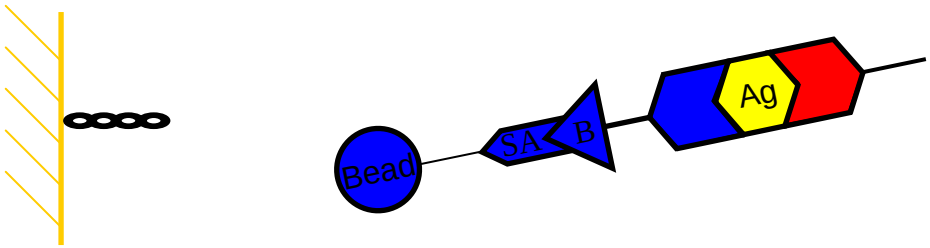
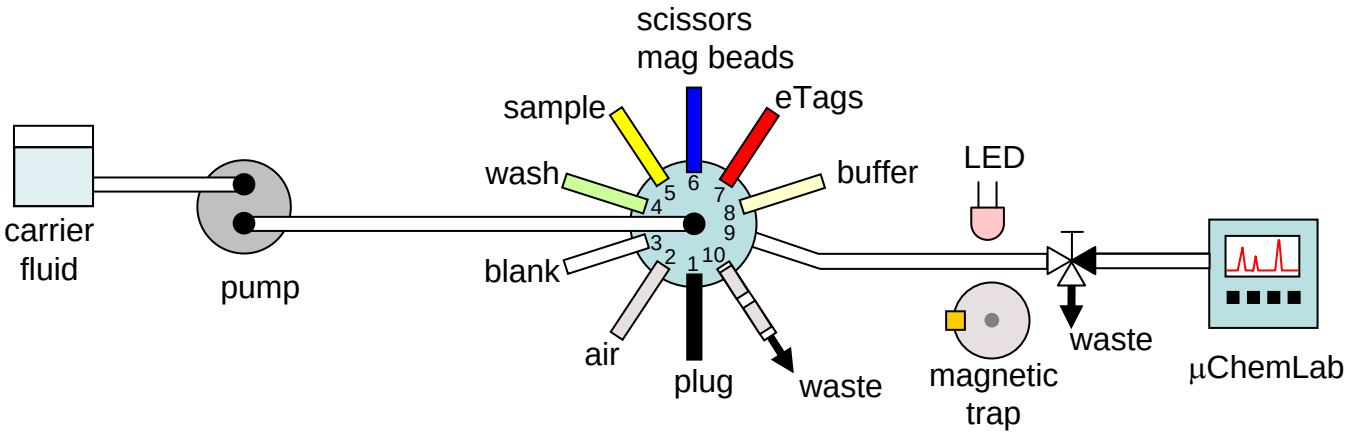
eTag Immunoassay on Mini Flow Pro





Perform analysis, release beads, send to waste

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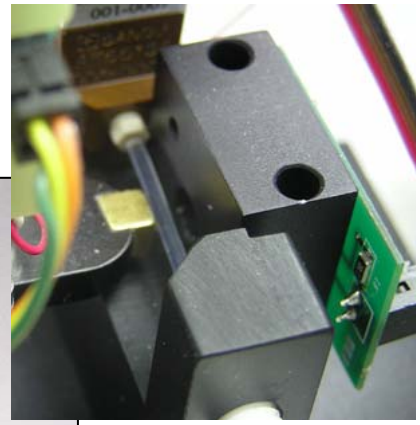
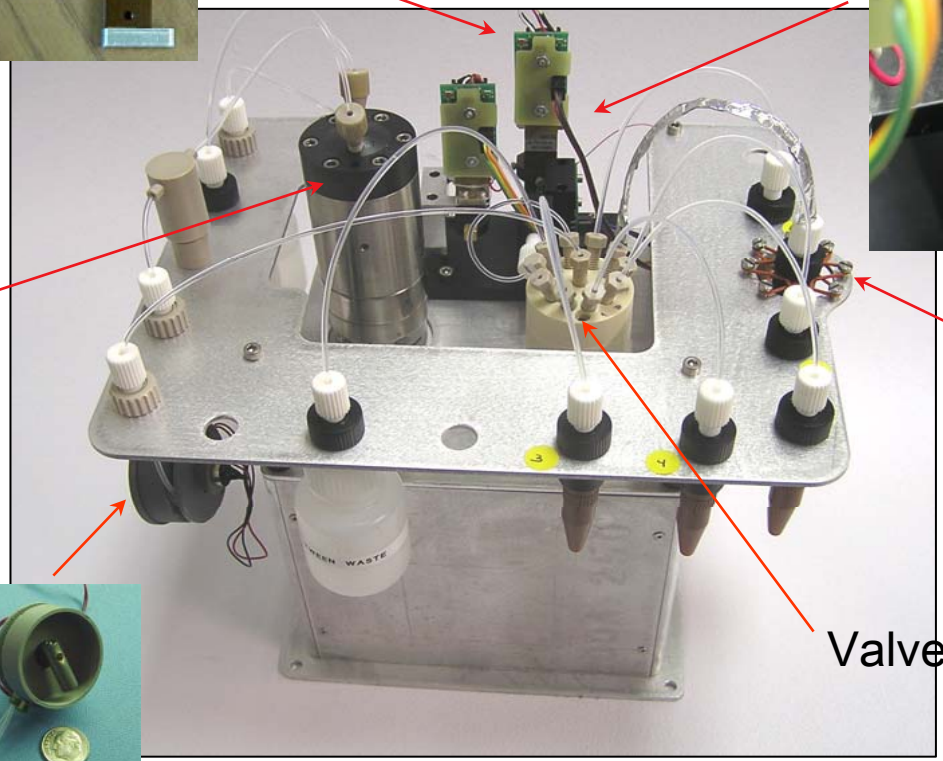
Integrated hardware can perform automated bead-based immunoassay

3 way valve
< 50 nl dead volume



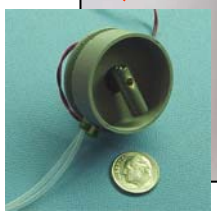
Magnetic bead trap and eTag release

Pump



Bead reagent mixer

Tube bead mixer



Valve

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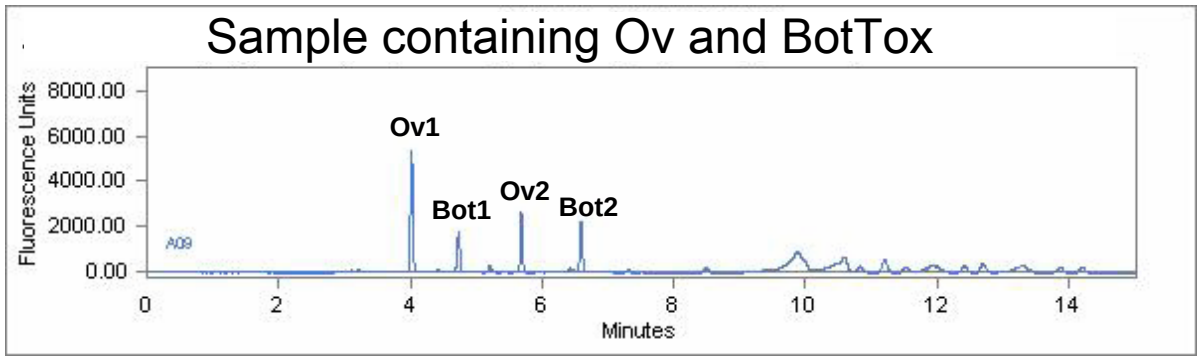
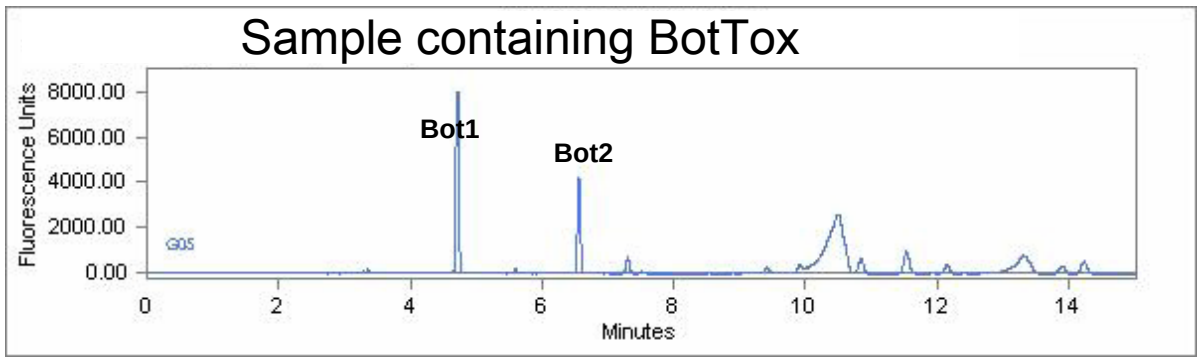
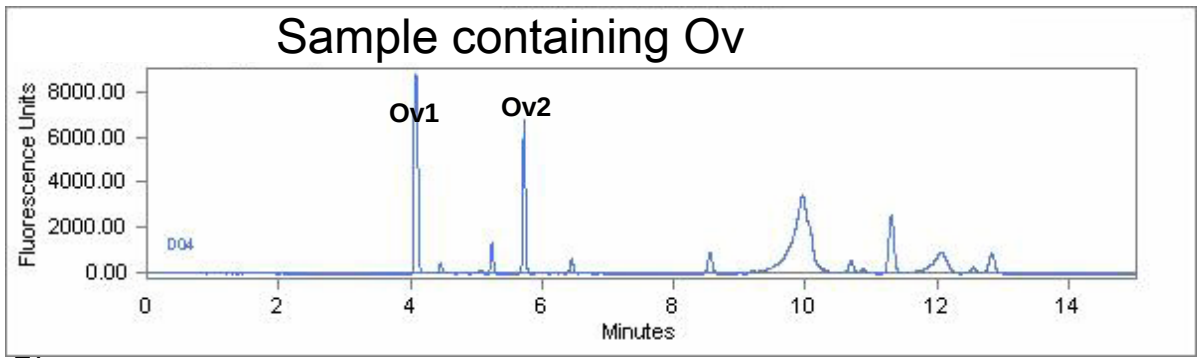
Developed two antibody approach for immunoassay to lower false alarm rates







Antigen:
125 ng/ml
ovalbumin &
botulinum toxoid A

Antibody:
Ra Pro1-Ov1
Sh Pro12-Ov2
Ra Pro11-Bot1
Gt Pro13-Bot2

eTags:
Photo-cleavable

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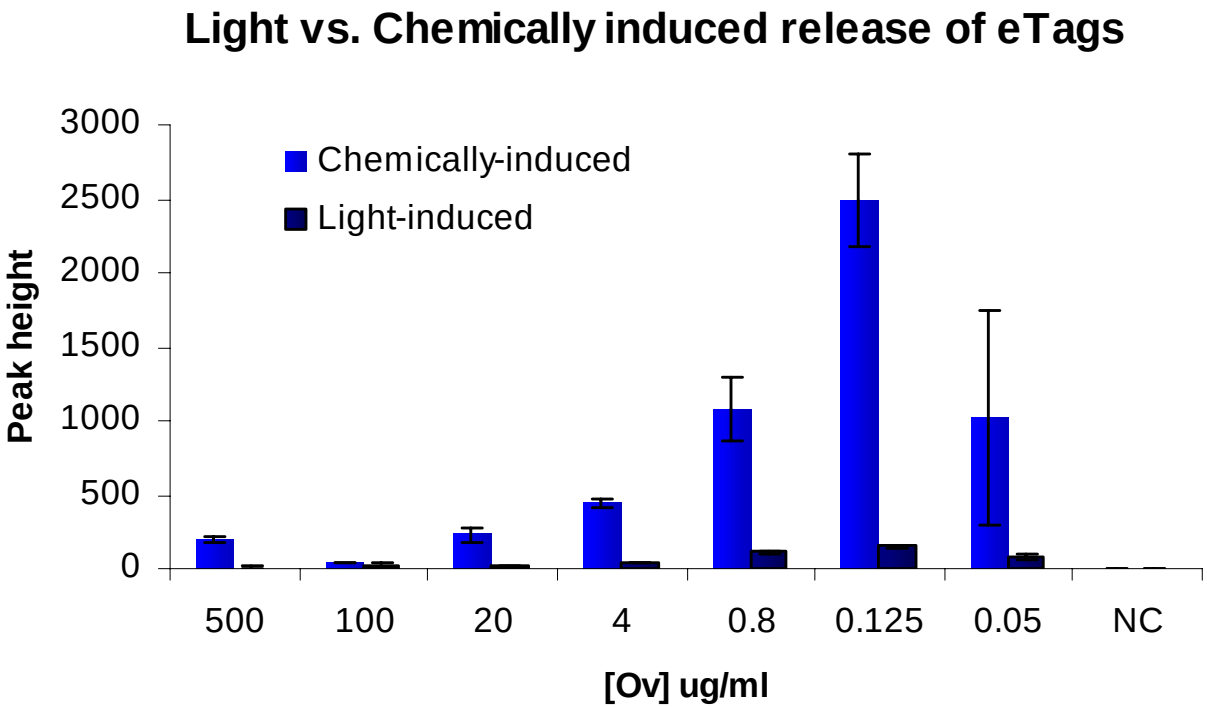
Chemically-cleavable eTags give improved immunoassay signal intensity over photo-cleavable



Introduction of chemically cleavable eTags increased signal intensity by 50 fold and decreased cost per assay

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Effect of Interferent on Assay Performance

Suspicious powder	Sample		Suspicious powder	Sample	
	Assay Buffer	Ovalbumin		Assay Buffer	Ovalbumin
SP1 Spackling powder	Negative (<1000 FU)	Saturated (>10000 FU)	SP9 Dipel	Negative	Saturated
SP2 Baking soda	Negative	Saturated	SP10 Chalk ($MgCO_3$)	Negative	Saturated
SP3 Instant nonfat dried milk	Negative	Saturated	SP11 Foot powder	Negative	Saturated
SP4 Talcum powder	Negative	Saturated	SP12 Ajax cleaner with bleach	Negative	Saturated
SP5 Flour	Negative	Saturated	SP13 Dairy creamer	Negative	Saturated
SP6 Salt	Negative	Saturated	SP14 Kaolin	Negative	Saturated
SP7 Yeast	Negative	Saturated	SP15 Bentonite	Negative	Saturated
SP8 Powdered sugar	Negative	Saturated	SP16 Aerosil R812S	Negative	Saturated

- 16 interferents (suspicious powder) were tested.
- None of the interferent made detectable difference on the assay performance.

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Effect of Interferent on Assay Performance

Interferent Reference Material	Sample		Interferent Reference Material	Sample	
	Assay Buffer	Ovalbumin		Assay Buffer	Ovalbumin
IRM 1 Green signal smoke	Negative (<1000 FU)	Saturated (>10000 FU)	IRM 10 Clay soil	Negative	Saturated
IRM 2 Vero cell supernatant	Negative	Saturated	IRM 11 Sage pollen	Negative	Saturated
IRM 3 Loamy Soil	Negative	Saturated	IRM 12 Burning Fog Oil	Negative	Saturated
IRM 4 Yellow signal smoke	Negative	Saturated	IRM 13 Burning rubber	Negative	Saturated
IRM 5 BSA fraction V	Negative	Saturated	IRM 14 HC smoke / Gunshot	Negative	Saturated
IRM 6 Water	Negative	Saturated	IRM 15 Sandy Soil	Negative	Saturated
IRM 7 Burning vegetation	Negative	Saturated	IRM 16 Violet signal smoke	Negative	Saturated
IRM 8 Burning Diesel	Negative	Saturated	IRM 17 Red signal smoke	Negative	Saturated
IRM 9 Aspergillus niger	Negative	Saturated	IRM 18 Malathion	Negative	Saturated

- 18 Interferent Reference Materials were tested.
- None of the interferent made detectable difference on the assay performance.

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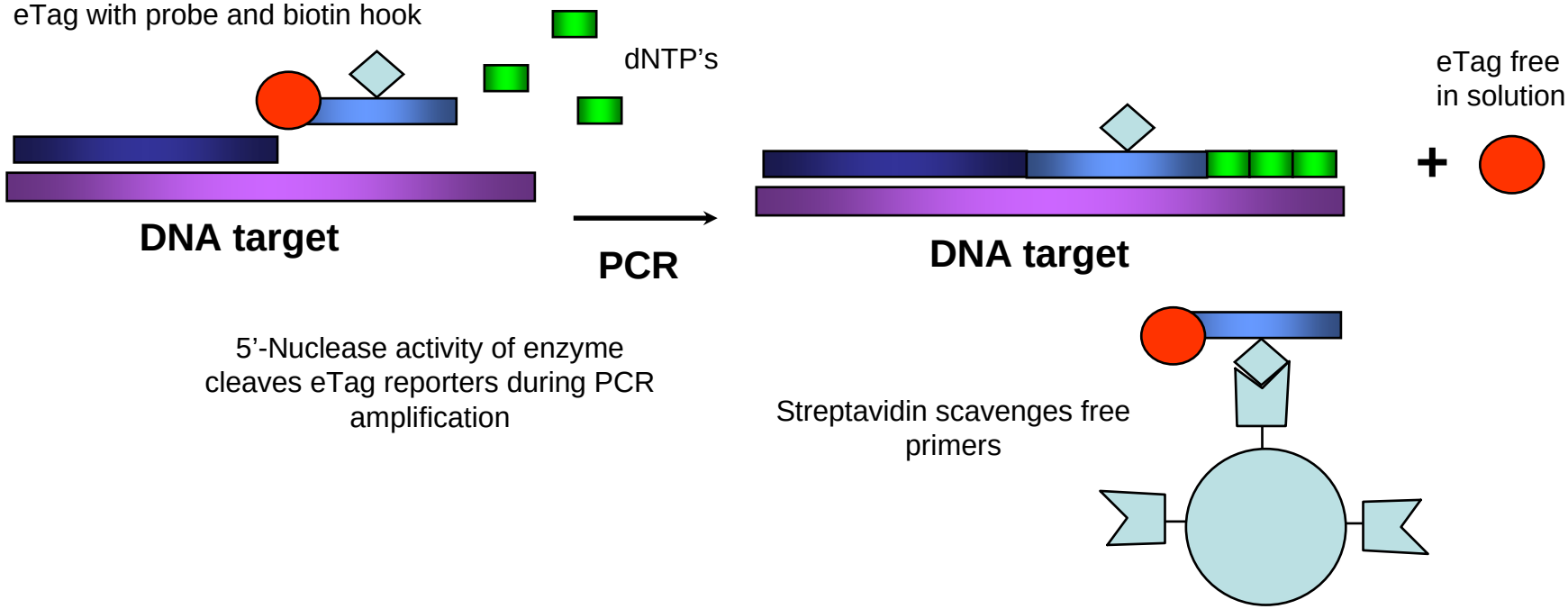
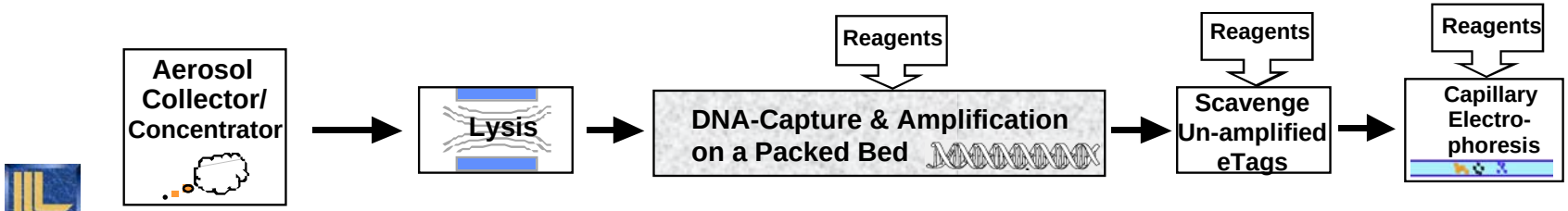
eTag reporter nucleic acid assay for bacteria and viruses





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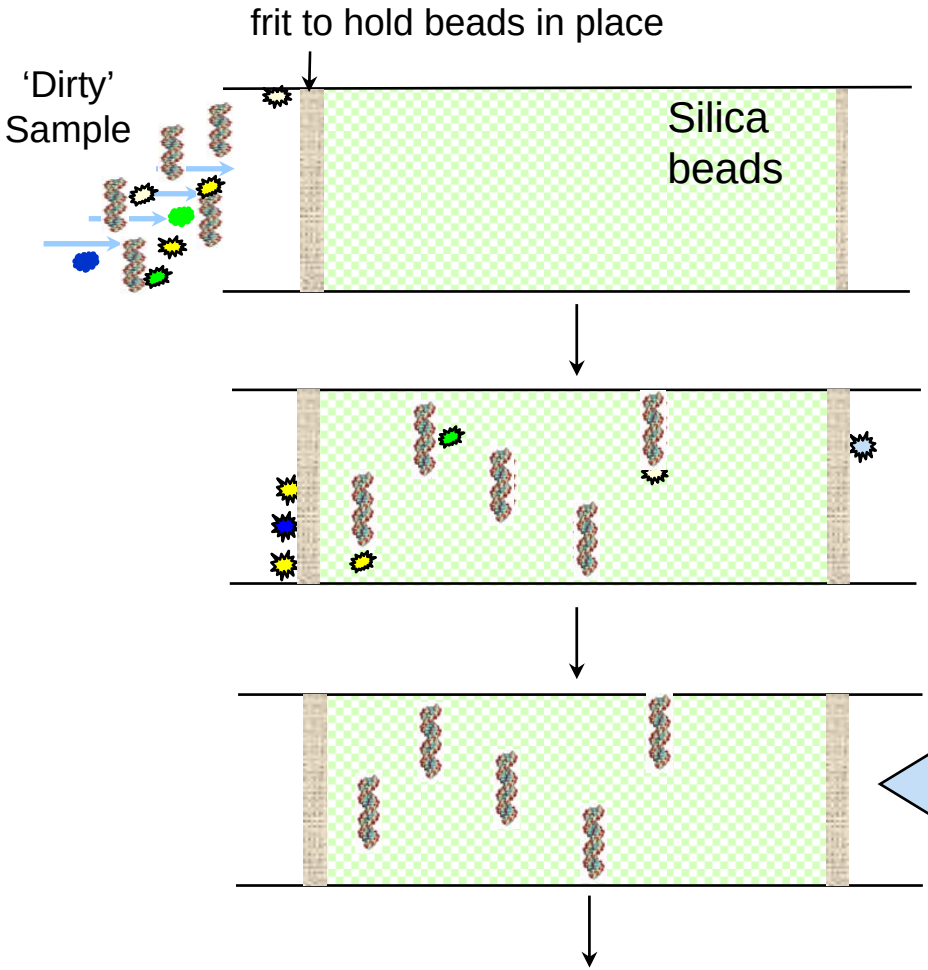
DNA purification and concentration on a packed bed minimizes losses, removes inhibitors



Dirty DNA sample is mixed with chaotropic salt and passed through a packed bed of silica beads

DNA binds to silica beads

Inhibitors are washed away

Standard columns elute DNA at this step, resulting in ~60% of DNA remaining bound to beads at low DNA masses

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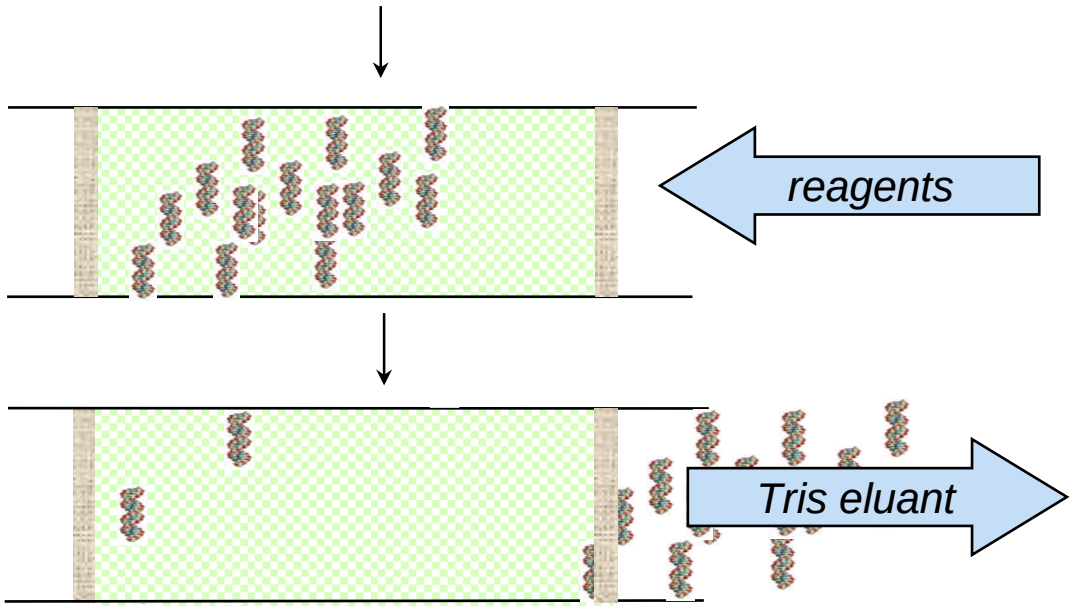
Amplifying DNA bound to silica beads improves sensitivity



Amplification occurs on bound DNA; DNA is not eluted from surface, reducing losses

Amplified DNA is eluted for analysis

	% successful PCR	
Amount of DNA (ng)	**Within column**	**Eluted from column**
2	**100**	**100**
0.5	**100**	**100**
0.05	**100**	**20**
0.02	**100**	**0**

LLNL patent filed April 2006

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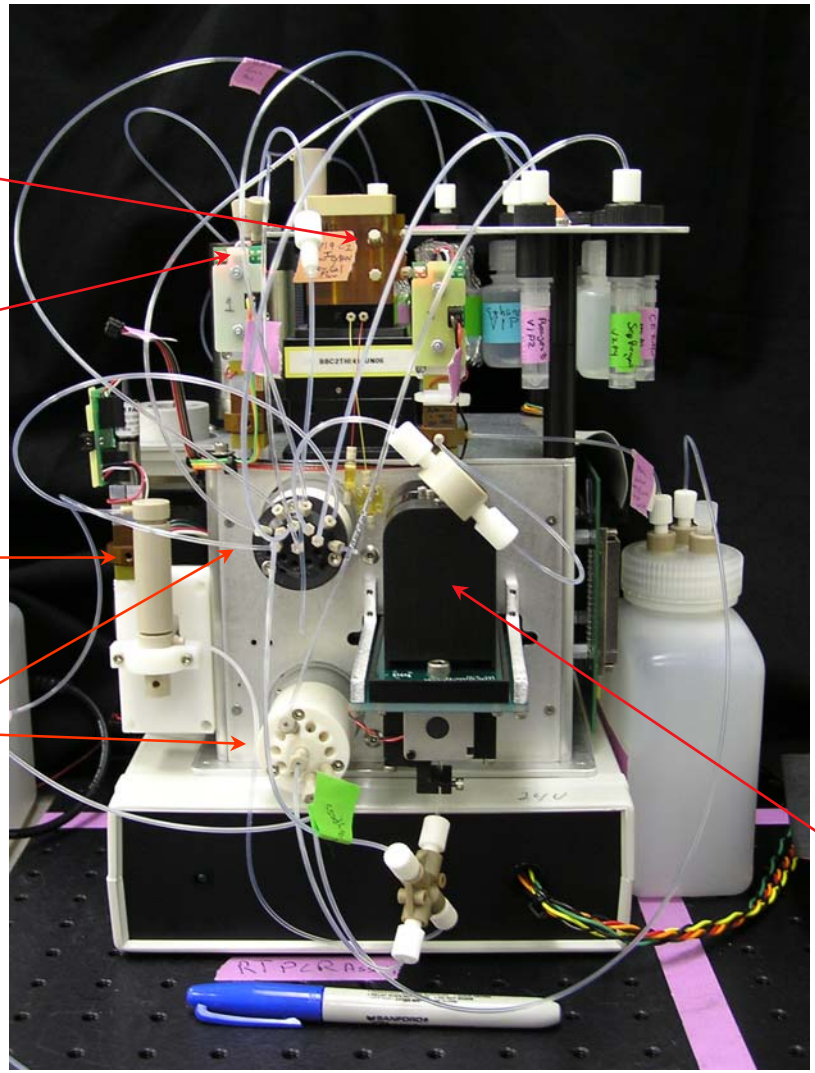
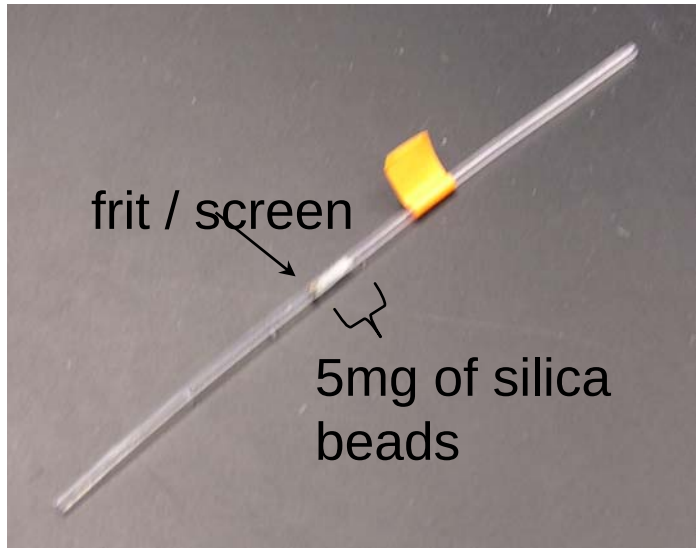
Integrated hardware can perform automated PCR and RT-PCR analyses





PCR packed bed

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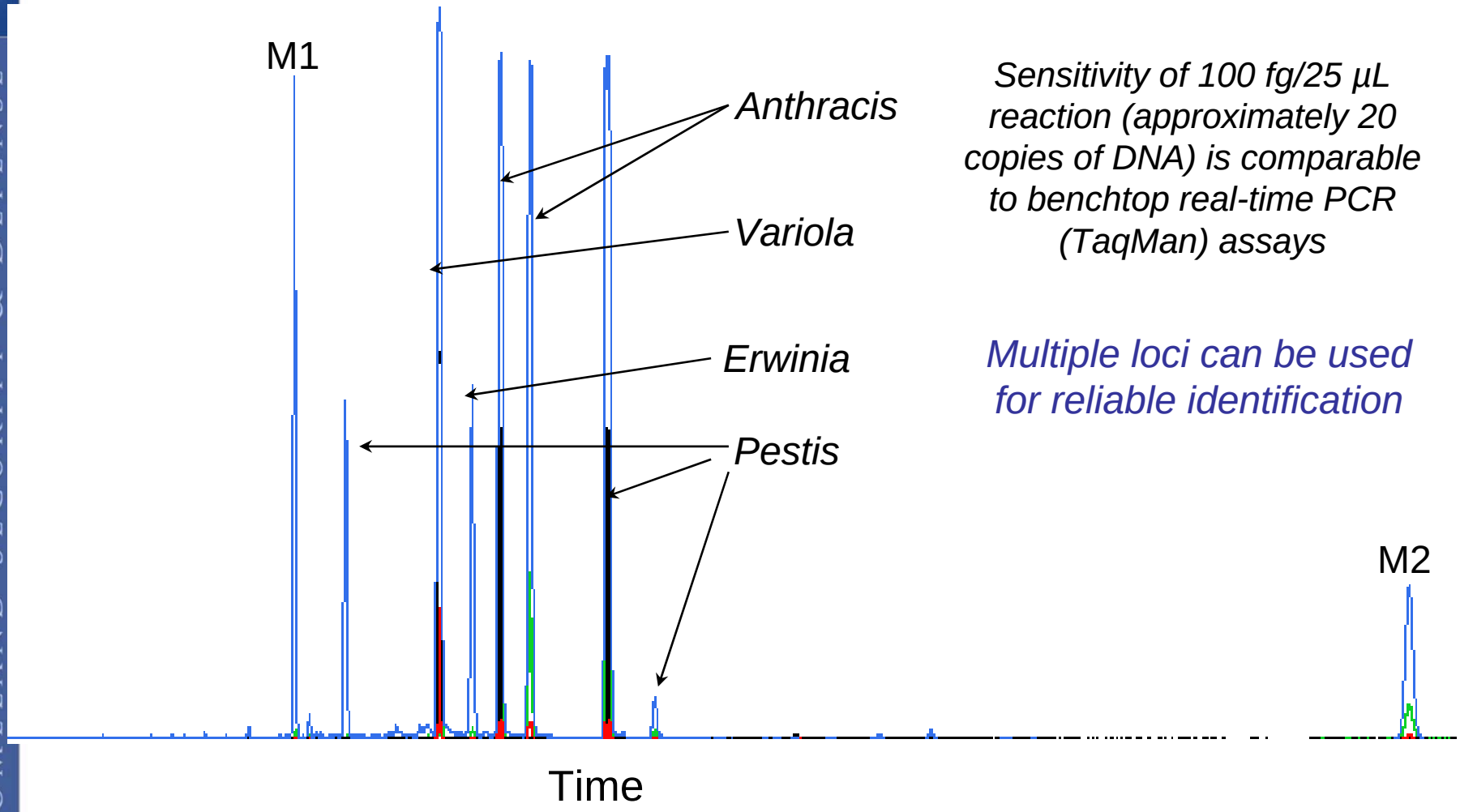
Multiplex nucleic acid eTag assay works in flow-through thermal cycler



Sensitivity of 100 fg/25 µL reaction (approximately 20 copies of DNA) is comparable to benchtop real-time PCR (TaqMan) assays

Multiple loci can be used for reliable identification

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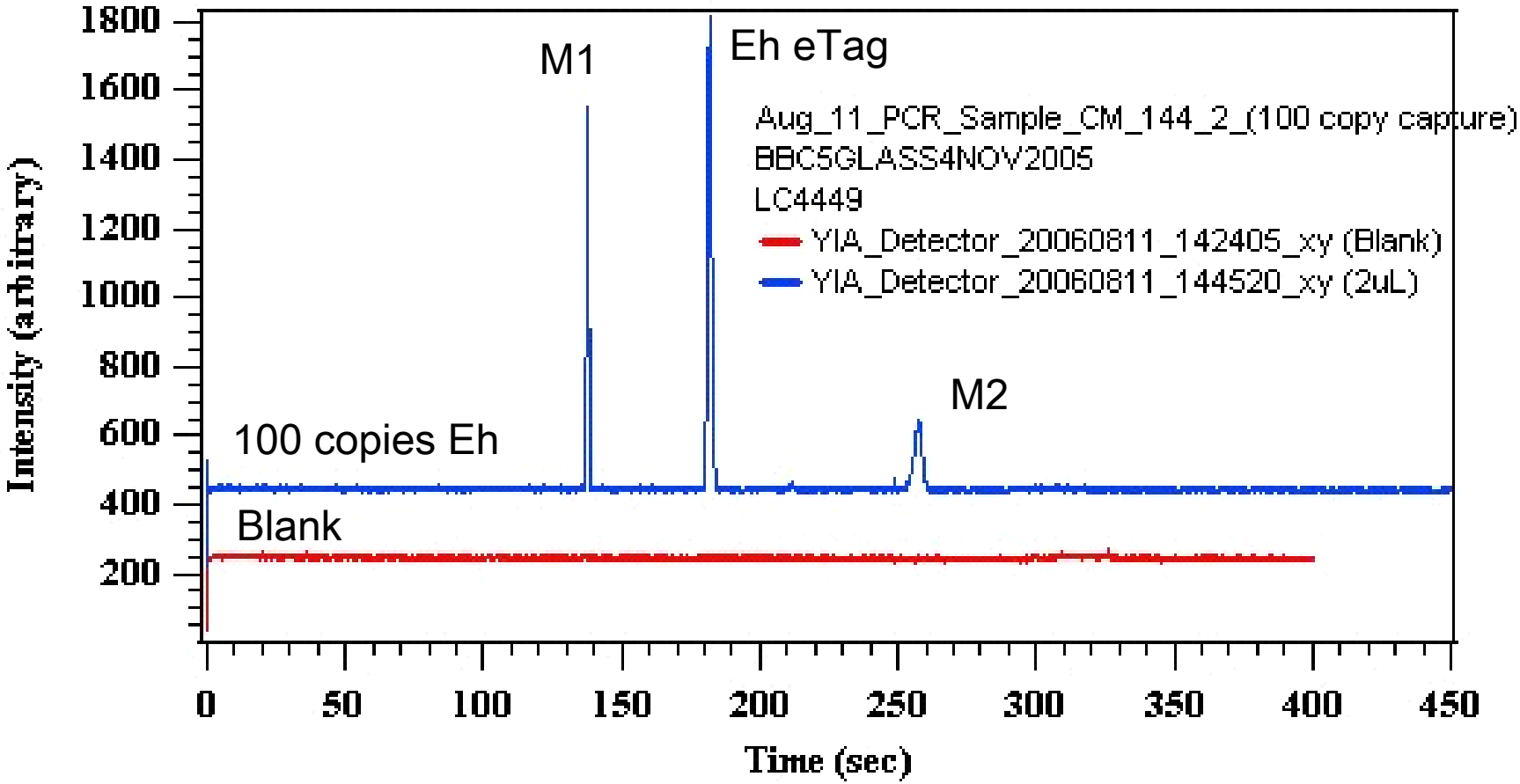
100 copies of *Erwinia herbicola* DNA captured & amplified on automated flow-through platform then analyzed on CE detector



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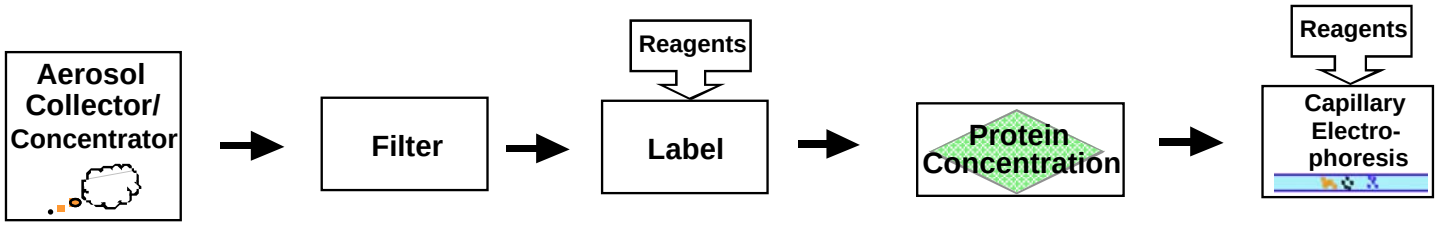
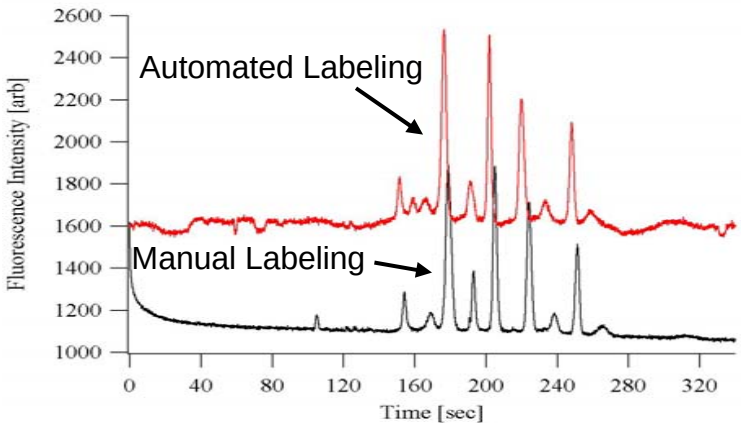
Protein signatures train utilizes custom hardware developed through the µChemLab program

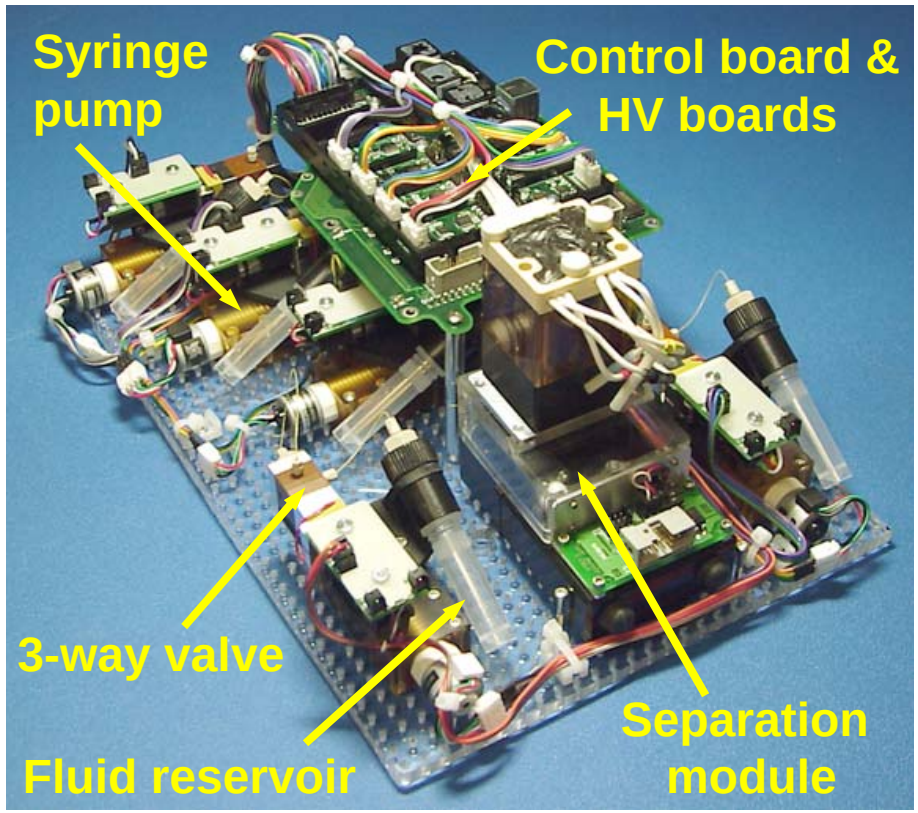


Automated fluorescent labeling

- Meter dye and sample at 1:9 flowrate
- Stop flow and mix 1 minute for labeling
- Meter labeled solution into µChemLab

CE analysis of protein standards





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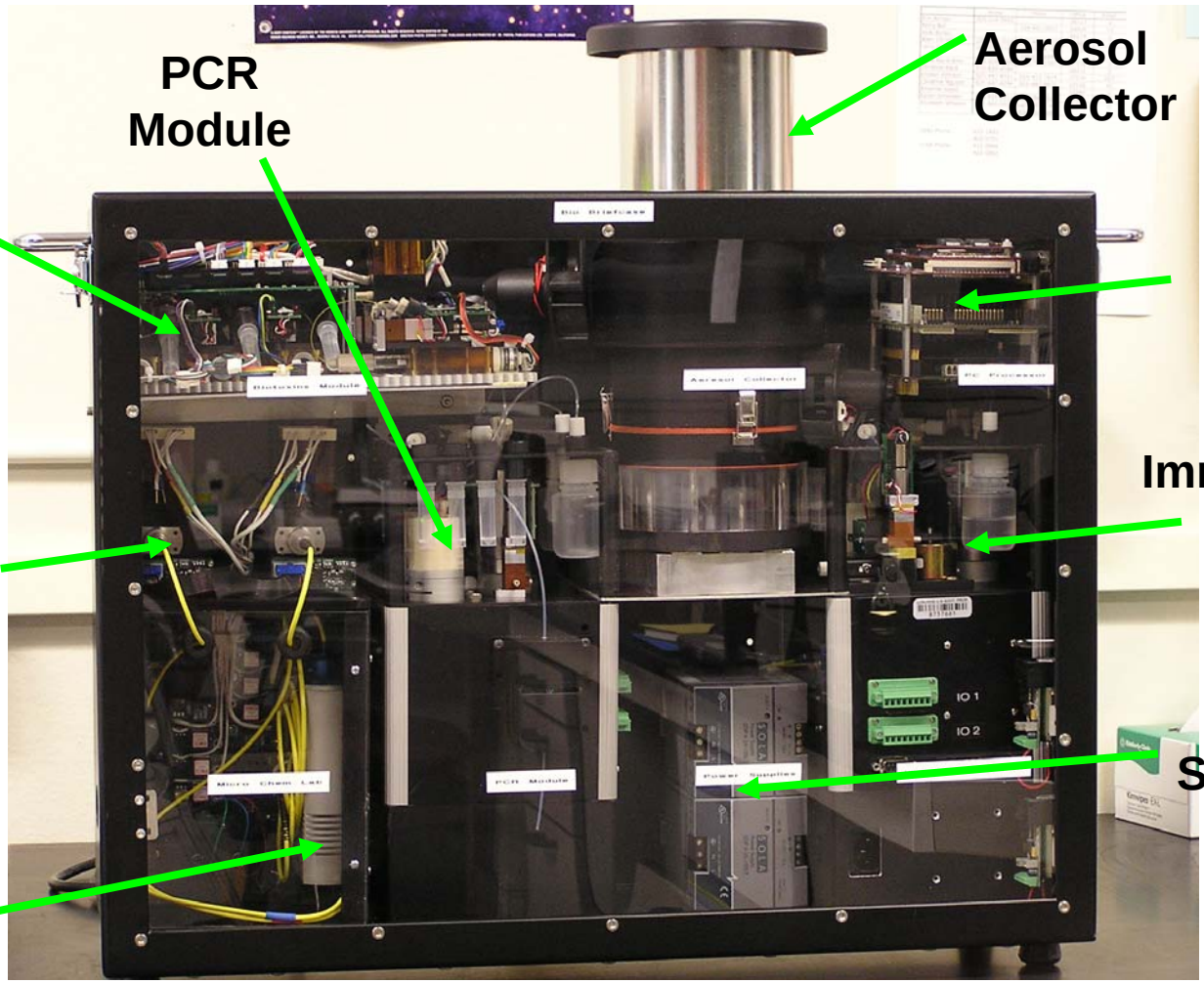
BioBriefcase laboratory components tested at Univ. of Nevada Las Vegas in early 2006



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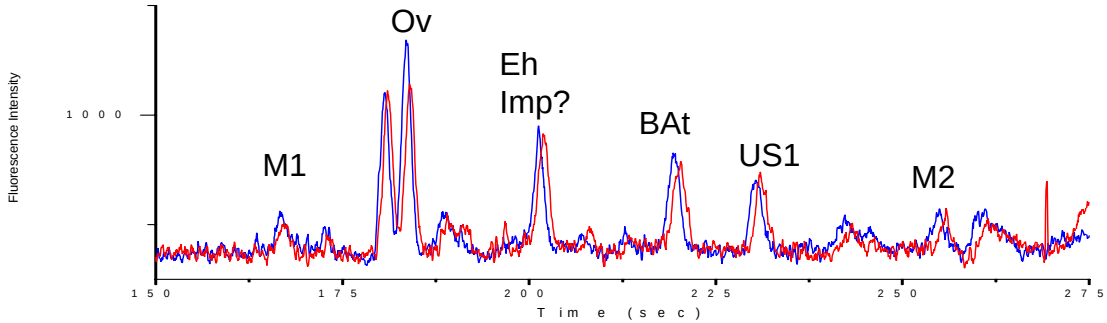
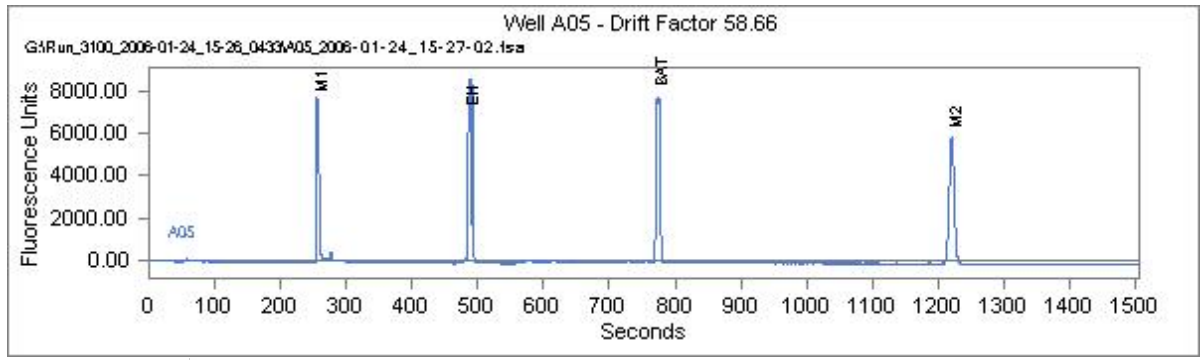
UNLV Results (January 2006) – Eh, Bg, and Ov were released, collected and analyzed using fully or partially integrated BioBriefcase modules

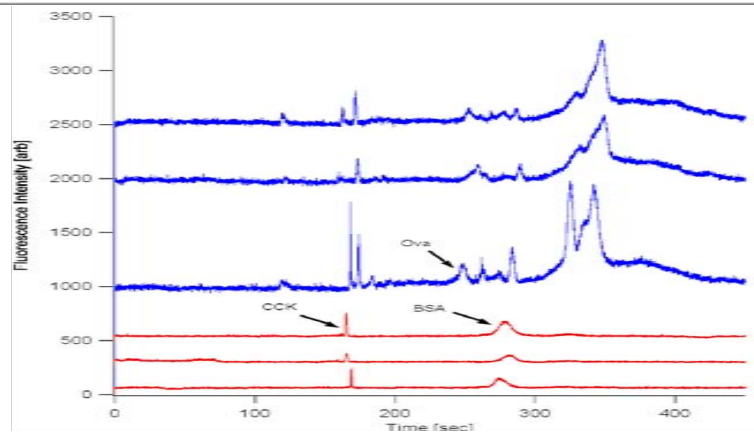
eTag Immunoassay



eTag PCR assay



Protein Signatures assay



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DHS Bioagent Autonomous Networked Detector program performance goals

- **Performance Targets**
  - Continuous, fully autonomous operation
  - 3 hour sampling window, 1 hour assay
  - **Broad agent coverage** > 20 agents
  - **High sensitivity** – Limit of detection of 100 organisms (10 ng toxin)
  - **Low single agent false positive rate** of 10^{-7} with a goal of 10^{-8}
- **Cost of Ownership**
  - Unit acquisition cost of $25K per unit or less (quant. > 1000's)
  - Operating costs per unit of $10K per year or less inclusive
- **System Requirements**
  - Preserve samples for 5 days for confirmation and forensics
  - Robust wireless, autonomous remote operation
  - Maintenance interval exceeding 1 month
  - Operation in full range of outdoor environments
  - Modest packaging and logistical requirements (i.e. 2 ft^3)

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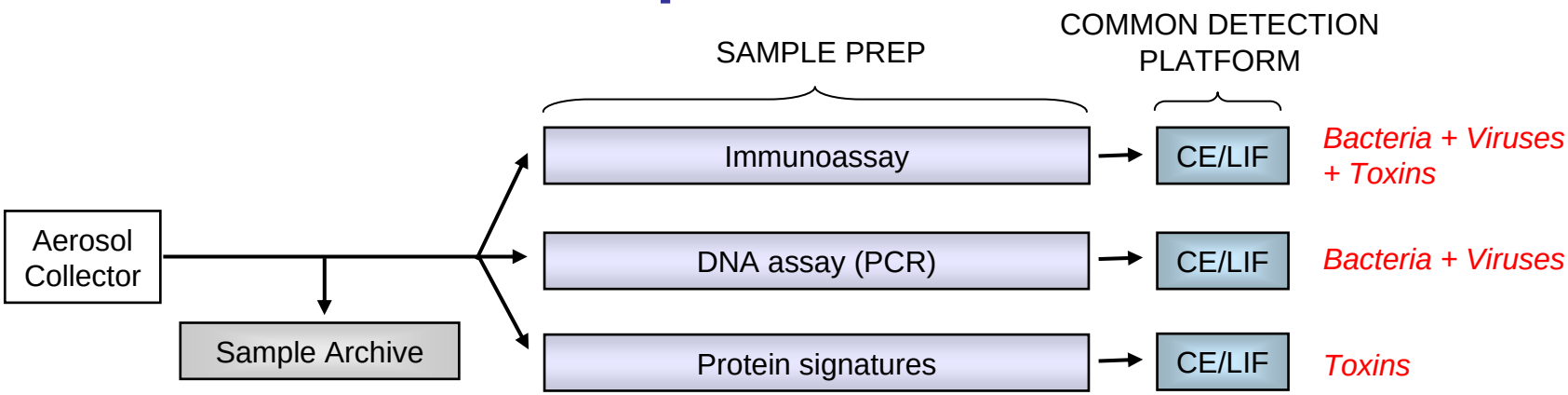
System conceptual design: Three assay trains to detect the full biothreat spectrum



- Problems with this approach when migrated to the BAND program:
  - Multiplexing demands for IA and PCR trains were difficult to meet using one train for each
    - 20 agents x 2-5 reagents per agent + controls suggested up to 100-plex required per assay train
    - Detection of RNA viruses introduced need for RT-PCR
  - Sensitivity of immunoassay train was not sufficient for bacteria and viruses

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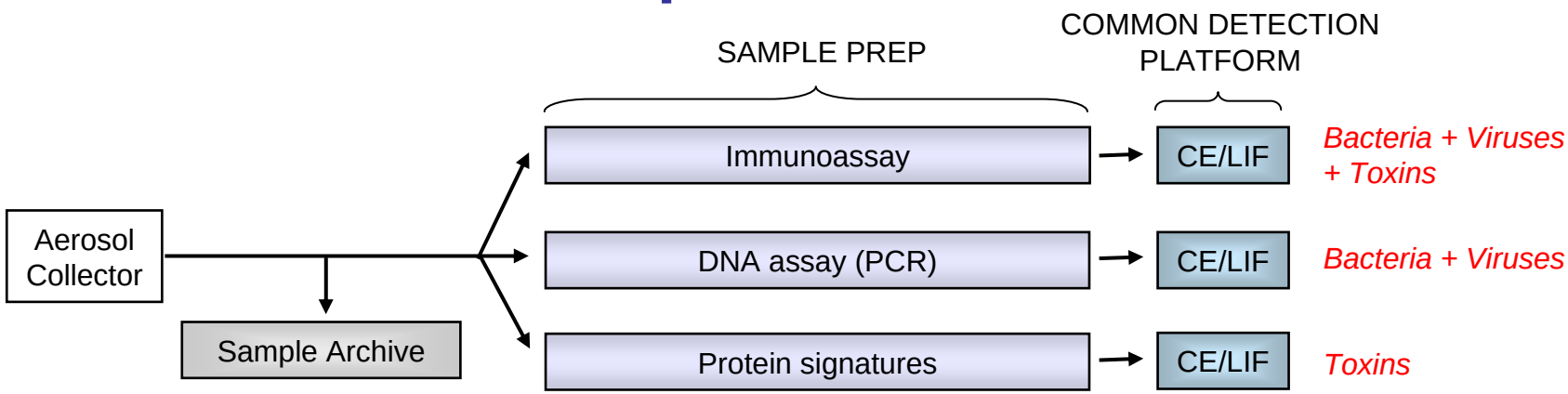
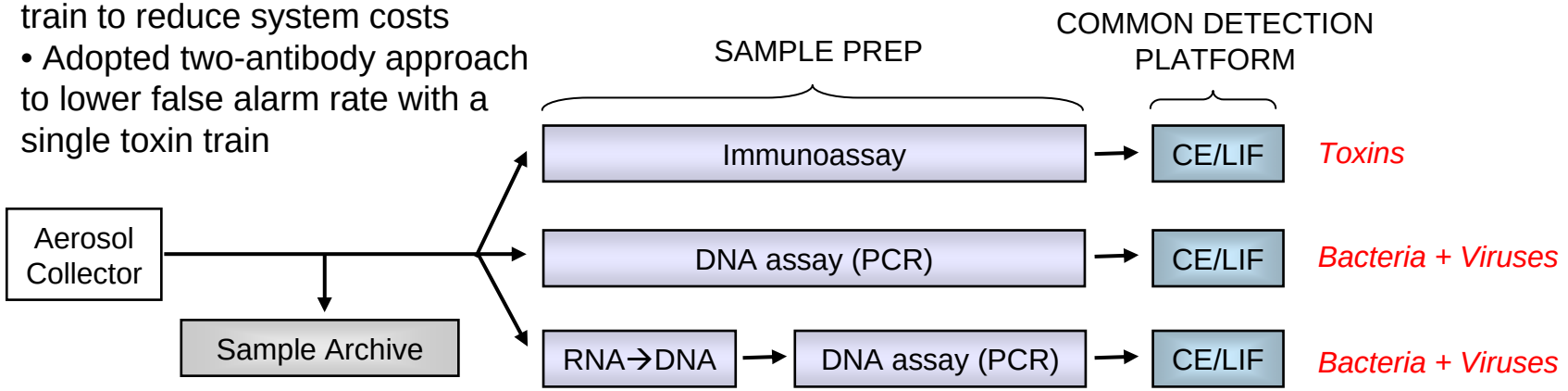
System conceptual design: Three assay trains to detect the full biothreat spectrum



- Two PCR trains provide for lower multiplexing per train
- Dropped protein signatures train to reduce system costs
- Adopted two-antibody approach to lower false alarm rate with a single toxin train



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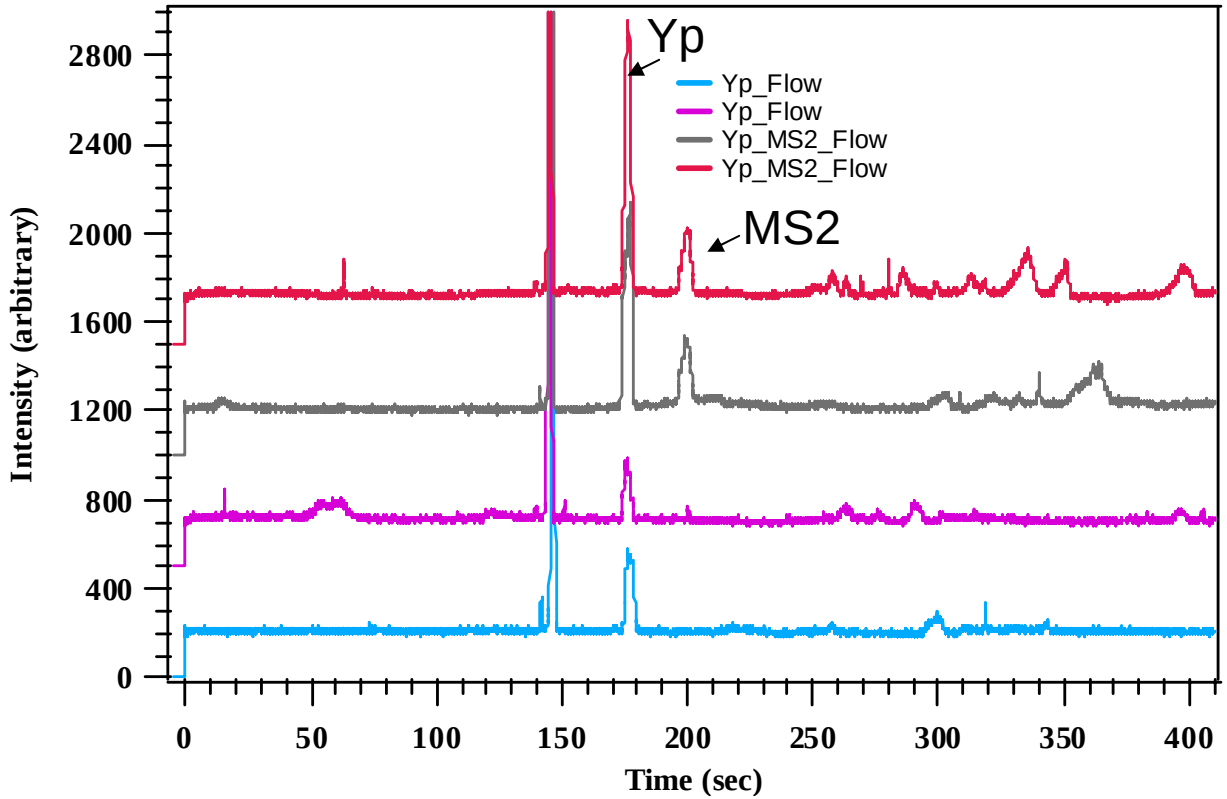
Addition of reverse-transcriptase step prior to PCR amplification allows detection of RNA viruses

4x10^4 cfu Yp
10 copies MS2



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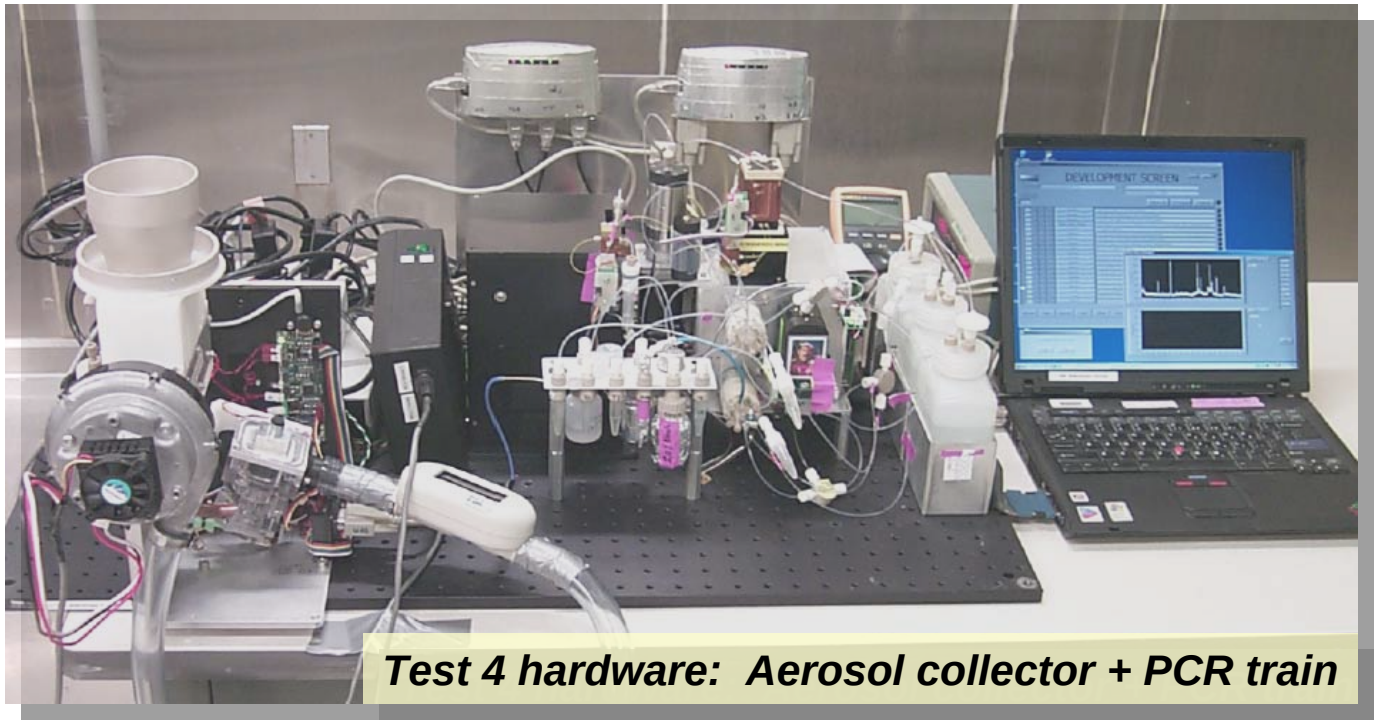
BAND Phase II testing (at ECBC)

- Test 2 – Aerosol collector performance
- Test 3 – Analyzer only (no aerosol collector), spores
- Test 4 – Aerosol collector + analyzer, spores
- Test 5 – Analyzer only for larger test suite, including toxins, vegetative bacteria and RNA viruses



Test 4 hardware: Aerosol collector + PCR train

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Testing conducted at US Army Edgewood Chemical and Biological Center

- **Laboratory testing (20 blind samples) –**
  - Liquid sample (400 µl) was drawn automatically into the instrument and analyzed
  - Data analysis software generated the detection calls (positive or negative for *B. anthracis*)
- **Aerosol chamber testing (18 blind samples) –**
  - Aerosolized sample was produced using an ink-jet aerosol generator directly into the BBC aerosol collector
  - Collection continued for 5 additional minutes, then the collection bowl fluid was pumped into the instrument and analyzed (as above)
- **LLNL and SNL staff performed initial instrument set-up and system maintenance**
- **ECBC personnel performed all system operations associated with the testing protocols**

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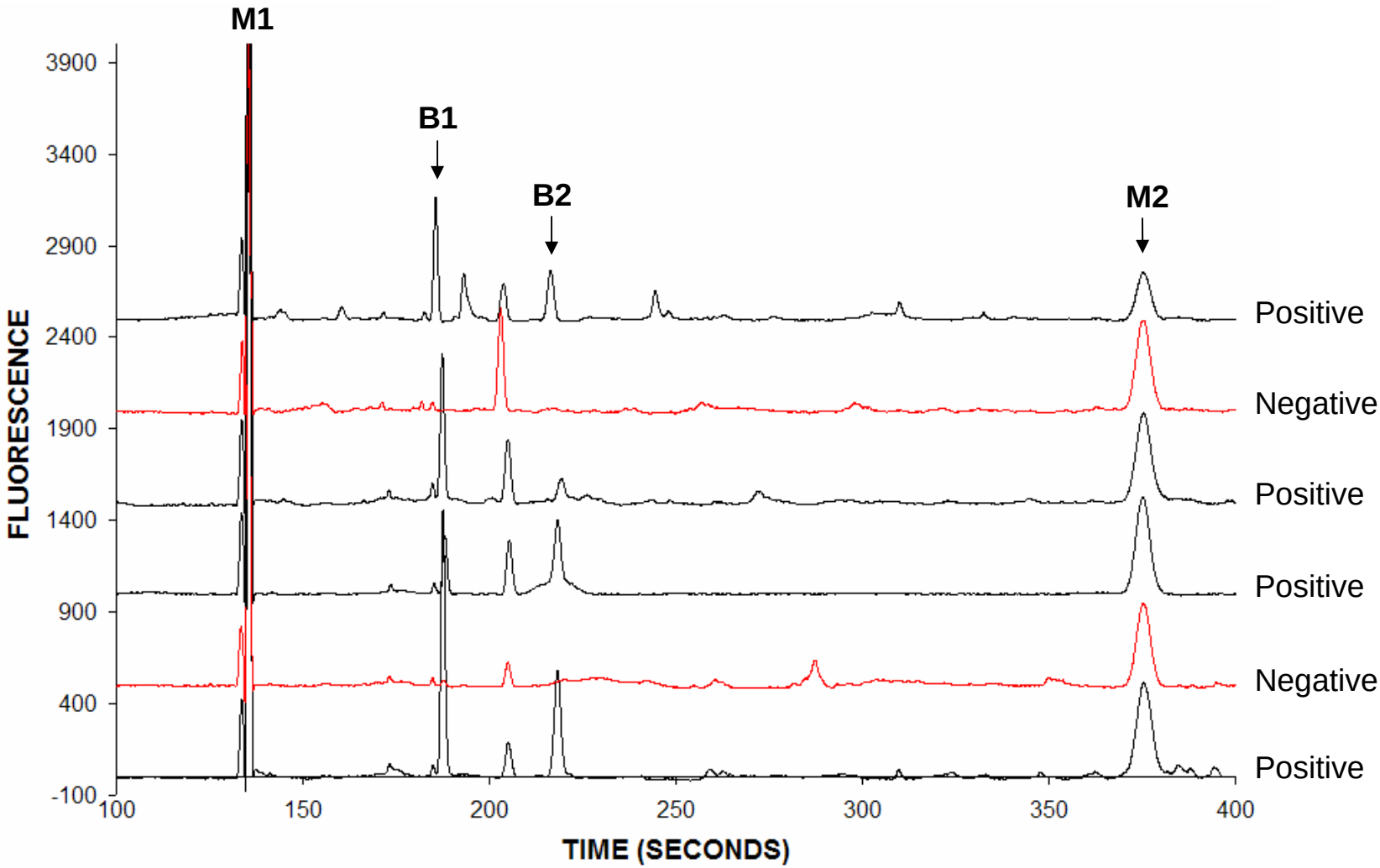
Aerosol chamber testing results for 6 sequential samples correctly identified using Biobriefcase



Samples contained two eTag reporters for B. anthracis: B1 and B2. M1 and M2 are electrophoretic markers used to correlate migration times with database species.

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Summary of testing results

- **Correctly identified 33 of 38 samples (87%) over the two series of blind challenge tests**
- **Reasons for incorrect calls were immediately identified and correctable**
  - False negatives: Mechanical failure (PCR heater) and reagent lot issue
  - False positives: Random noise peak assigned as B1 peak
    - Signal averaging, use of B2 give correct call
- **Ambient temperature variations shift CE peak times; accounted for using internal standards**
  - Independent study of temperature effects indicates a minimum of two internal standards are required for accurate peak calling
  - Fielded instrument will be environmentally controlled

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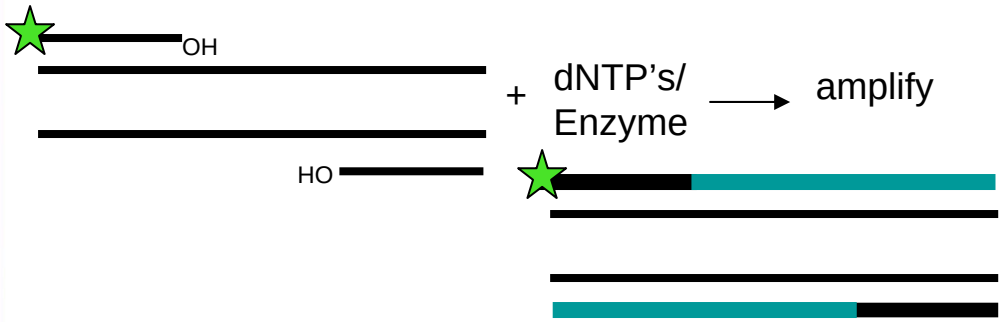
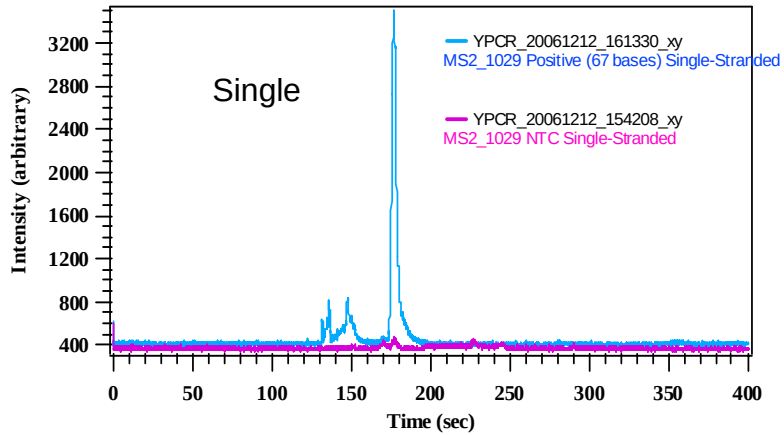
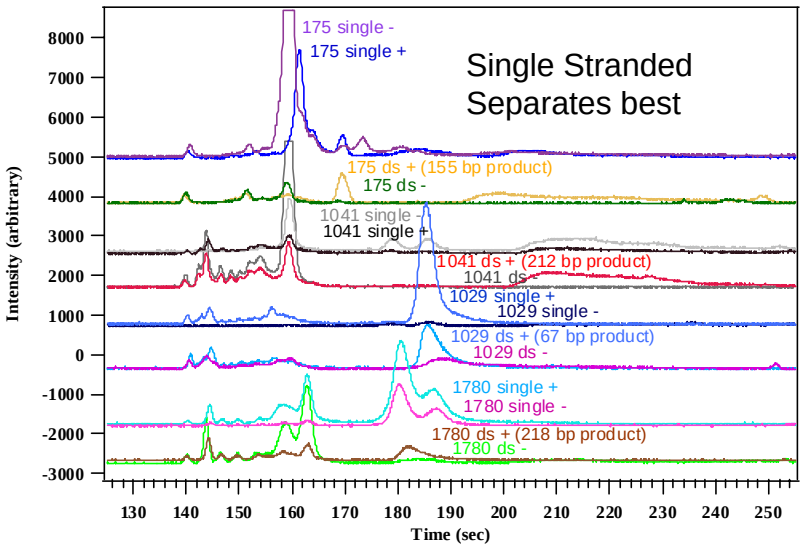
We adapted the same PCR platform to perform an alternative PCR assay to Monogram eTags

Amplification using single fluorescently-labeled Primer







Advantages

- Not dependent on sole source provider
- Significant decrease in cost per assay
- No changes made to present CE matrix
- Separation PCR product size dependent

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Collaborated with Ribomed Biotechnologies Inc. to develop a food sensor under the DHS FBADS program

Abscription – Abortive Transcription

RIBOMED

Abortive Promoter Cassette (APC)

+

Abscriptase® Enzyme (RNAP)

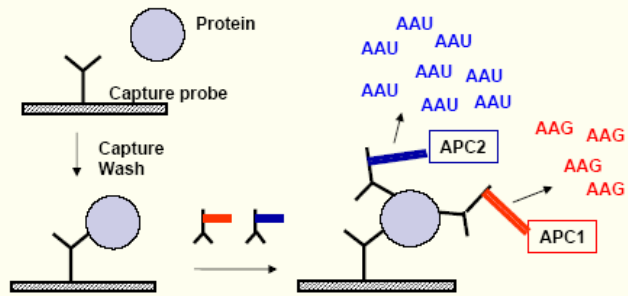
+

Ribologs® (Ribonucleotide Analogs: Initiators & Terminators)

Reiterative Abscription®

Detectable Abscripts®

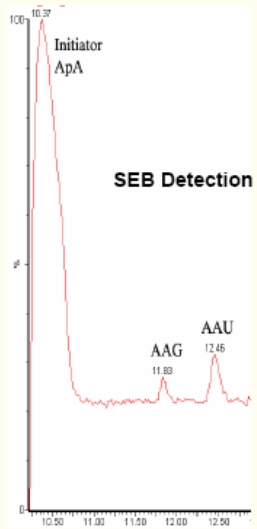
(Thousands per target per minute for single Abscriptase® binding event)



The ratios of the products produced gives a specific and reproducible fingerprint for that target.

- High specificity
- Low false positives

FOUO



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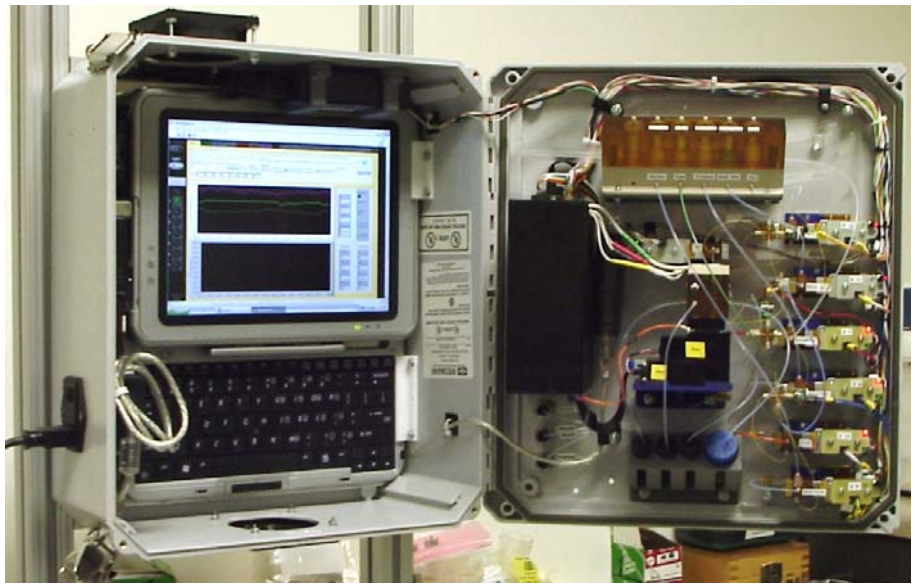
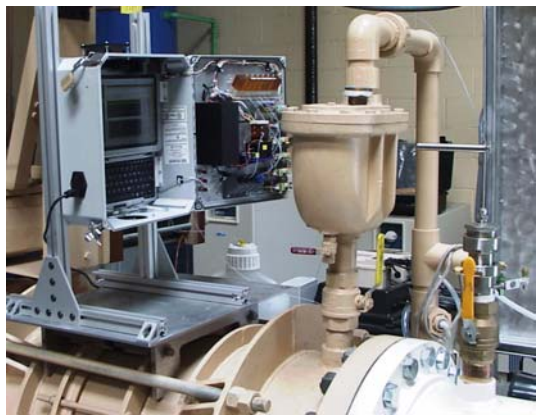
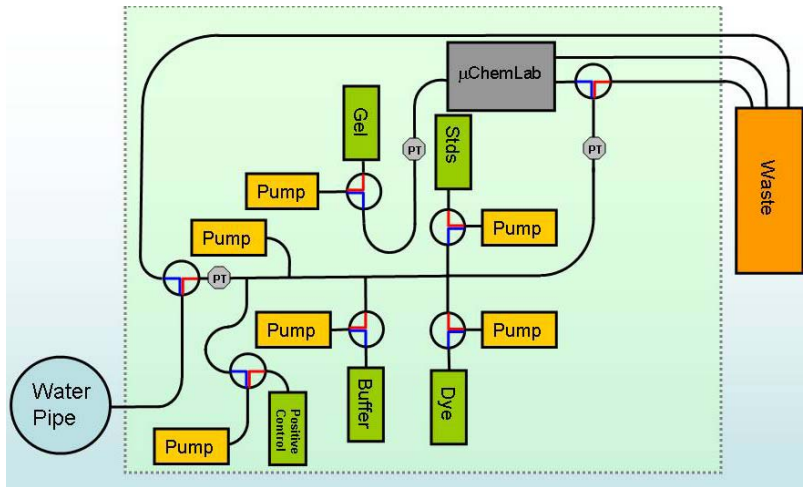
Integrated Biodetection Platforms – Unattended Water Sensor





- **Continuous water monitoring for utilities**
  - 30 day unattended operation
  - Analysis every 30 minutes
  - Detects biotoxins
- **Funded through CRADA with Tenix and CH2MHill**
- **Plan to expand to other pathogens**



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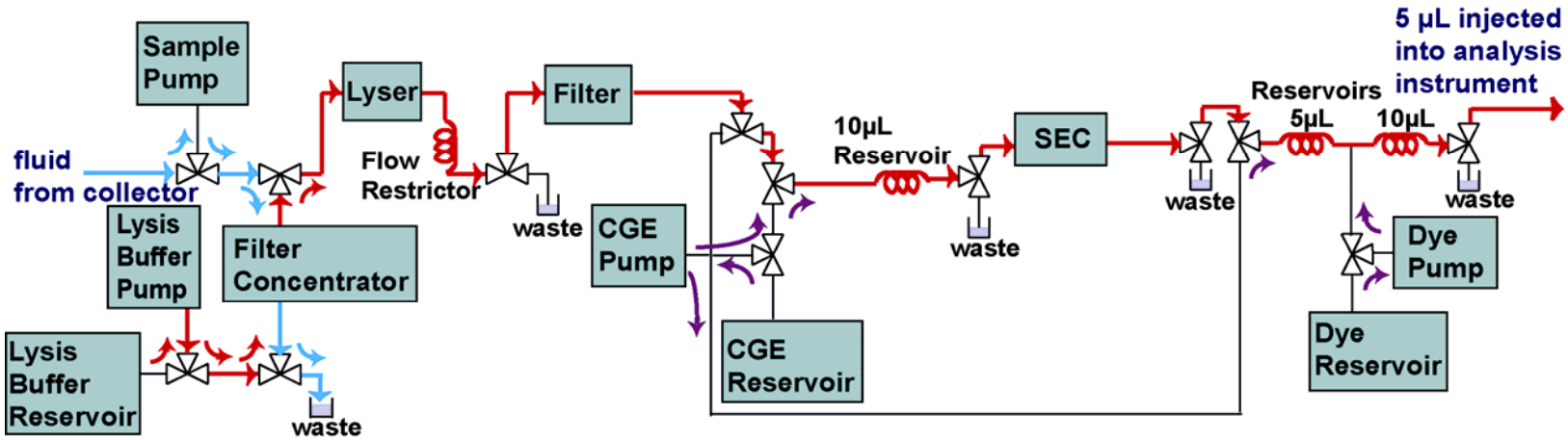
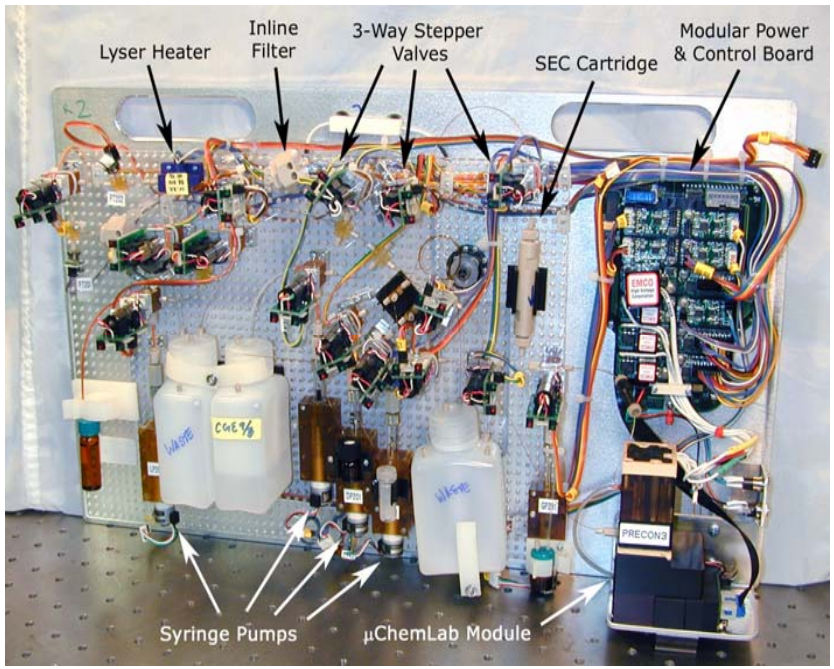
Integrated Biodetection Platforms – Multiple Pathogen Detector for DoD





- **Aerosol point detection**
- **Funded through TTP, then DTO.50 for JCBPDS**
- **Prototype built and tested**
  - Detects toxins, viruses, spores, and vegetative cells
  - 8 hour autonomous operation
  - Rapid response (minutes)
  - Minimal reagents

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Advantages of Abscription

RIBOMED

- **Low False Positives**
  - RiboRNA beat RT-PCR
  - RiboPRO beat Elisa
- **Robust, Heat-stable, Blood-resistant**
- **Isothermal, Rapid, Linear**
- **Adaptable to different detection formats**
- **Works with degraded nucleic acids (25-40 nt)**
- **No Target Amplification**
- **Product insensitive to nucleases**

MS2 RNA	Specificity#
RiboRNA	0.93
RT-PCR	0.16

SEB Toxin	Specificity *
RiboPRO	0.997
ELISA	0.87

Assay was optimized for RT
* 1 APC/Target

- **High Level Multiplexing**
  - Multiple targets/sample
  - Multiple signals/target

FOUO

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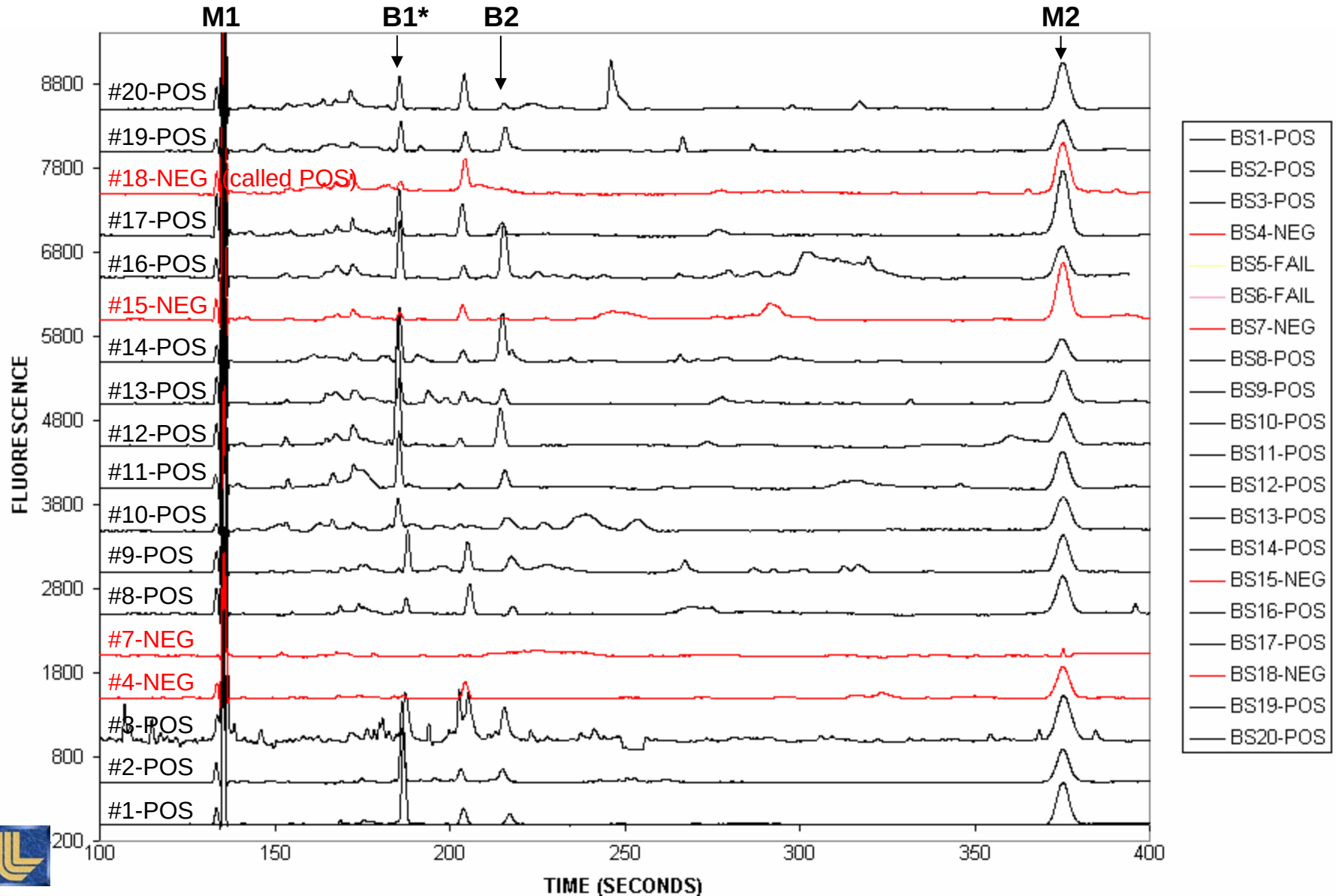
Summary of BAND Test 3 results



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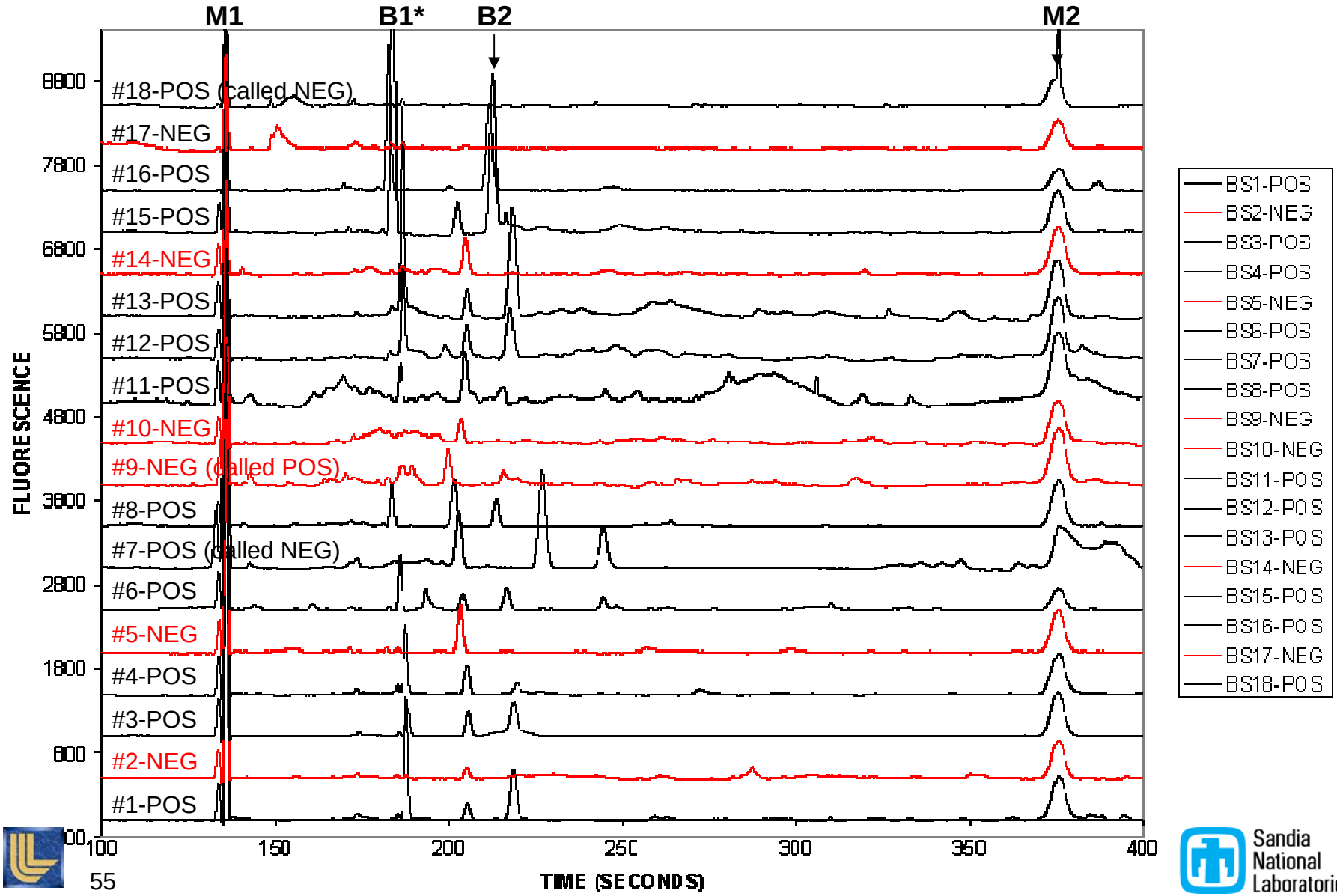
Summary of BAND Test 4 results



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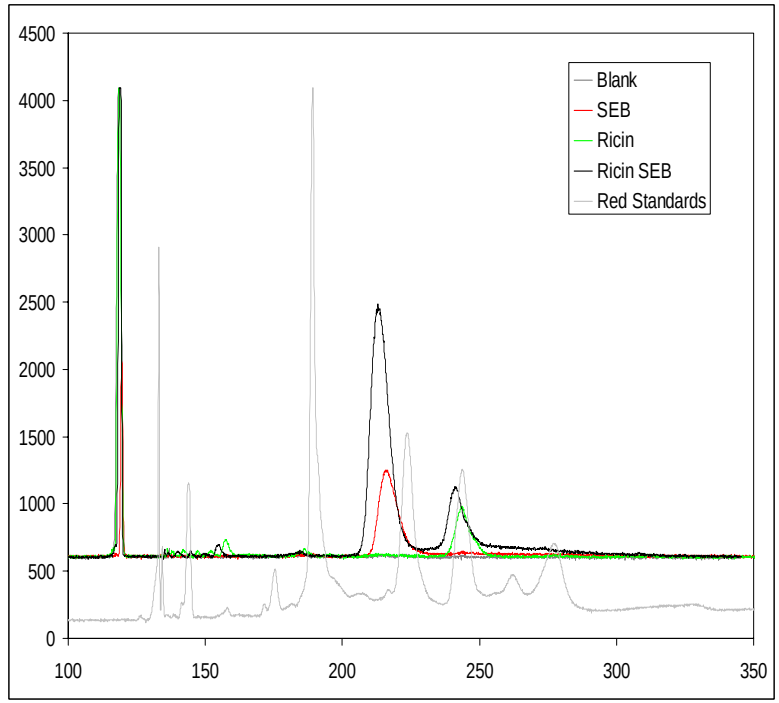
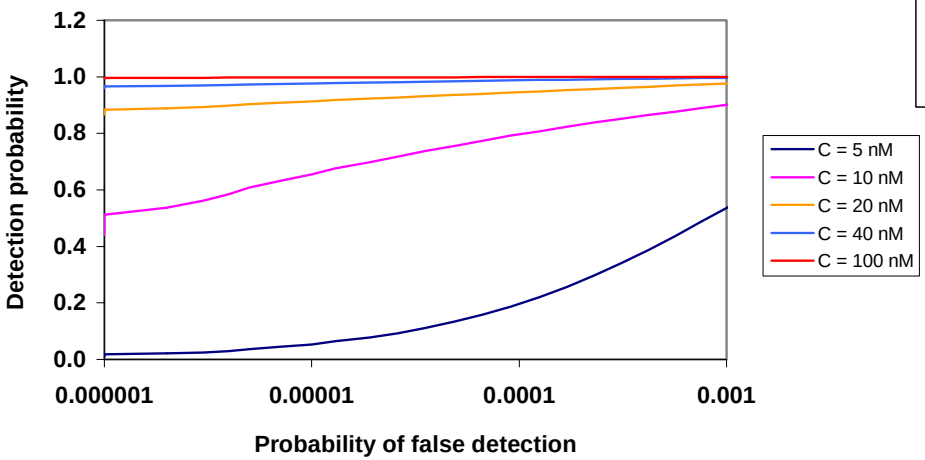
Unattended Water Sensor

- **Analyses:**
  - ✓ Biotoxin signatures
  - – Nitrifying bacteria
  - – Algal toxins
  - – E. Coli



Preliminary UWS Receiver Operating Characteristic Curves for Ricin



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Integrated Biodetection Platforms

System	Application	Sponsor	Drivers	TRL
µChemLab	First responders	DOE	Size, response time	6
UWS	Water monitoring	Tenix / CH2MHill	Reliability, unattended operation	6
BBC	Aerosol monitoring	DHS	Cost, sensitivity, high verity	5
DTO.50	Aerosol point detection	DoD	Portability, response time	4

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TRLs for DTO.50 Prototype Subsystems

Capability	TRL	Comments
Sample collection	6	MiniSass demonstrated on BBC
iDEP Concentration and sorting	3	Other concentration methods are TRL 5
Valves	6	Valves tested 1.2 million cycles
pumps	5	Could use larger commercial pump with TRL 6
Lysis and labeling	4-5	Demonstrated on laboratory prototype
Chip design and fabrication	7	Procured from commercial suppliers
2-color detection module	5-6	Need work on robustness and manufacturability
Separation method	6	Applies to standard CGE
Fast CGE method	3	Required for very rapid response
IEF	3-4	Labeling method is 3. Needed for improved selectivity
Electronics	6	Same as UWS
System integration	5	All components have been integrated, but not in the compact package that is desired.
Environmental robustness	4	Limited data on temperature, vibration, etc
Control software	6	UWS and BBC have demonstrated control software
Data analysis software	6	Same as UWS and BBC
Identification software	4	Need large data set to validate methods
Proteomic characterization	4	Need more data on effects of growth stage

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