

Specimen Preparation for Clinical LC-MS Assays

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Clarke Disclosures


- Grant/Research Support: NIH, Thermo Fisher Scientific, Werfen, Roche Diagnostics, BD, Saladax Biomedical, Shimadzu, Radiometer
- Consultant/Advisory Board: Werfen, Radiometer, Roche Diagnostics, Lumira Dx, Saladax Biomedical, Truvian, Thermo Fisher Scientific, HepQuant, Red Abbey Labs
- Equity: None
- Honorarium/Expenses: None
- Intellectual Property/Royalty Income: Johns Hopkins University, Elsevier

Why Focus on Specimen Preparation?

- Pre-analytical part of the process
 - What can we do to the sample to reduce the complexity of what we're putting into the analytical system?
- Cleaner samples = more reliable results
- Increased sensitivity (Better S/N ratio)
- Improve selectivity
- Robustness and Instrumentation
 - Minimize maintenance and cleaning
 - Harmonization of methods between labs



Fit for Purpose

- Definition: Well-equipped or well suited for its designated role or purpose (Oxford English Dictionary)
 - What does that even mean??
- 

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- What does that even mean??
 - Consider clinical need
 - Consider analytical need
- Goal: rational method development based on what we know about our analytical capabilities and what we know about the clinical or research applications

Drivers of Purpose

- How will the result be used by the analyst?
- What is the time frame needed for analysis?
- What are the cost limitations?
- What is the concentration range of the analyte?
- What instrumentation is available?



Optimal vs. Usable

- Optimal = best or most favorable
 - Usable = able or fit to be used
- 

Optimal vs. Usable

- Optimal = best or most favorable
- For us; this means development until we have reached the analytical limits of our capability
 - Analytical purist
 - Metrological perfectionist
- Usable = able or fit to be used
- For us; this means development toward until it meets criteria based on the application
 - Pragmatist
 - Recognizes that further development can take place as needs change

Example

- Tricyclic Antidepressants
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 - Wide linear range from LLOQ to 1000 ng/mL or greater (3-4 orders of magnitude)

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 - Wide linear range from LLOQ to 1000 ng/mL or greater (3-4 orders of magnitude)
- Usable
 - Therapeutic range/target 50-150 ng/mL; risk of arrhythmia at >500 ng/mL
 - LLOQ doesn't need to be lower than 25 ng/mL
 - ULOQ doesn't need to be much more than 500 ng/mL (reliably)

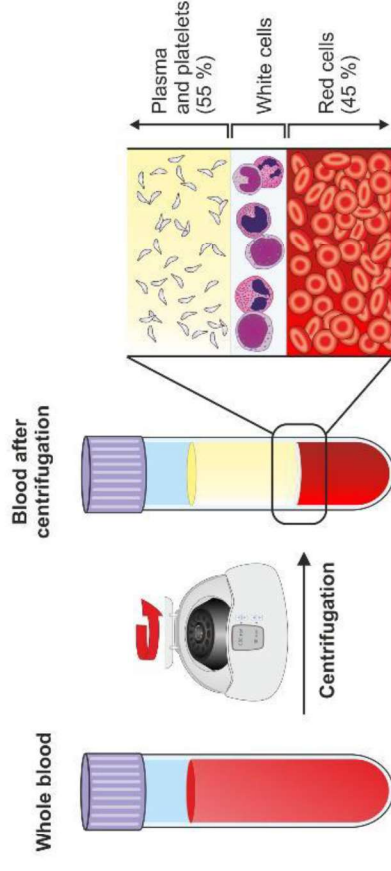
Biological Matrices

- Clinical testing by LC-MS can involve a number of biological matrix types including blood, urine, tissue, hair, body fluids and secretions
 - Most are complex matrices with significant amounts of proteins and potential endogenous interferents
- Proteins can foul or plug HPLC columns and cause significant matrix interferences
 - Removal of protein can remove analyte also due to protein binding
 - Stability can be affected by protein binding (+/-)
- The most common matrices for clinical testing are blood and urine

Blood Samples

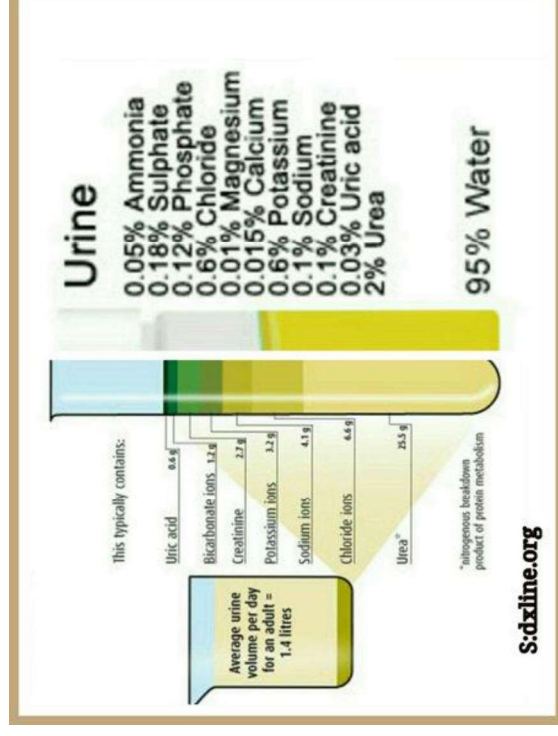
- Most common matrix = blood
 - Significant amount of protein (~75 mg/mL)
 - Lipids and metabolites
 - Cellular components if whole blood is used (~45% cells and ~55% plasma)
- Whole blood is approximately 85% water, while serum and plasma are approximately 93% water

- It's important to consider components of the collection tubes such as anticoagulants or clot accelerators, separator gels, surfactants, and the makeup of the rubber stoppers

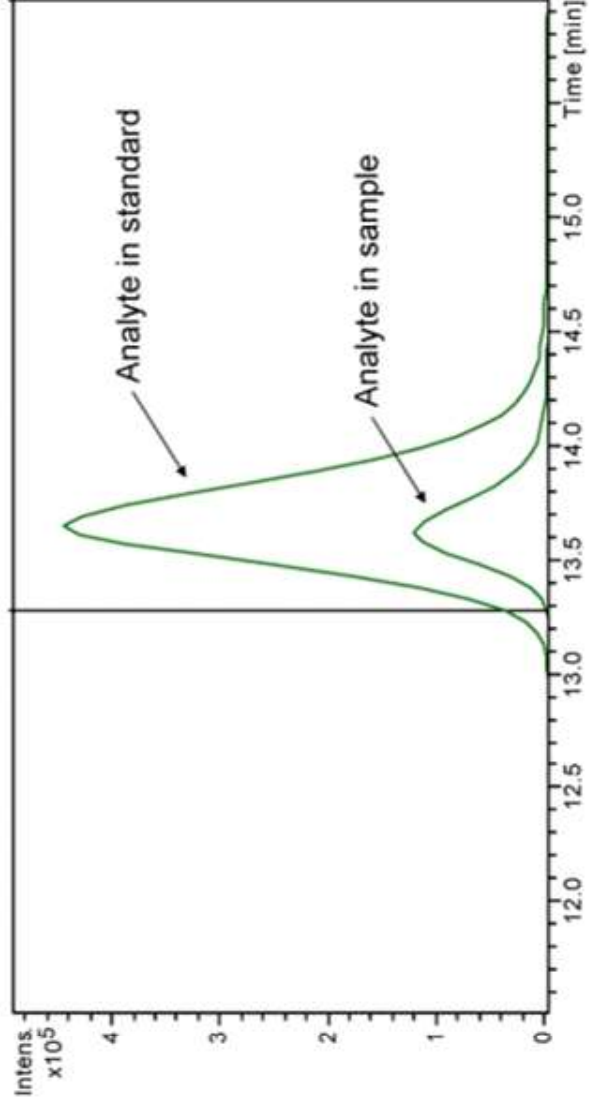


Urine Samples

- Urine is a much simpler matrix typically
 - Much less protein and cells
 - Much greater water content relative to blood
 - Significant variation in pH
- Urine is a non-invasive sample, so available volume is usually much greater
- Still must consider chemical additives to collection vessels, primarily preservatives



Matrix Effects

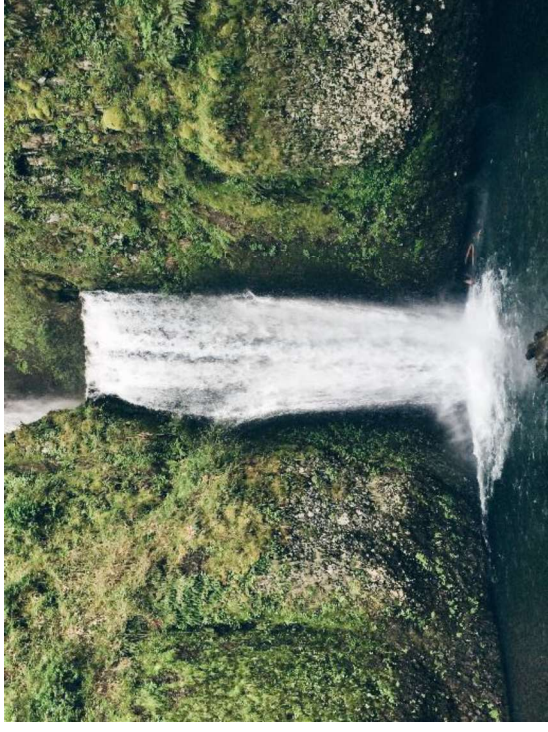


Potential Causes for Matrix Effects

- Sample interferents can compete for excess charge (e.g. protons) in the ionization process
- Sample interferents can slow down the evaporation process, limiting the extent of desolvation or ion desorption
- During desolvation, sample interferents can compete for limited space on the droplet surface for ion desorption
- Ionization enhancement can also occur if interferents lower the surface tension of the droplet leading to enhanced desolvation and ion formation
- **In any case, if matrix effects vary significantly between calibrators, control material, and patients the reliability of the assay will be diminished**

Dilute & Shoot

- Simple premise: dilute out complex matrix and inject
 - Reduces the amount of proteins, etc. introduced to the column
 - Quick and effective (though dirty) approach
- Works best for less complex matrices
- Used most commonly in urine toxicology testing



What Can Go Wrong (or not)?

- Dilute & Shoot works best for less complex matrices (i.e. urine or CSF)
- Particulates or higher than expected protein can foul the column or cause significant matrix effects
- Amount of dilution dependent on expected concentration of analyte and matrix components
- If D&S is a good fit, can be a low-effort, high-throughput option for processing

Protein Precipitation (PPT)

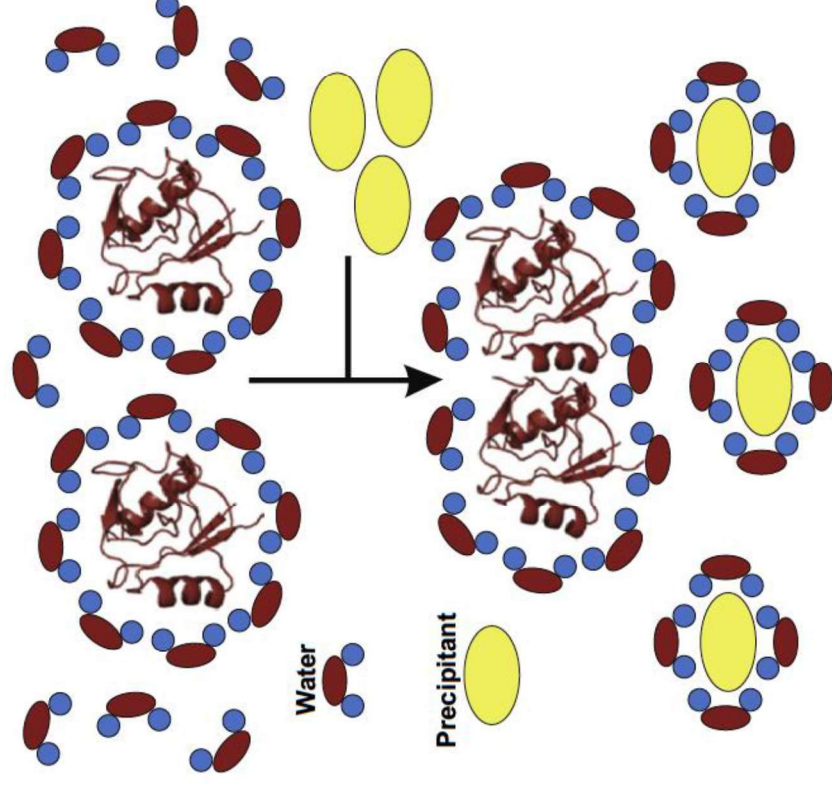


Figure 4.1 The basic principle of the protein precipitation mechanism.

Proteomic Profiling and Analytical Chemistry. <http://dx.doi.org/10.1016/B978-0-444-63688-1.00004-5>
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What is Happening during PPT?

- Water solvates proteins via hydrophilic surface residues
 - Minimizes interaction between proteins
- PPT mechanism is addition of component to disrupt the solvation potential of the solvent, and lower the solubility of the solute
- Considerations: ratio of solvent to sample, pH, polarity, mixing, centrifugation, storage temperature

Important Considerations for Protein Precipitation

- Ratio of precipitating solvent to samples
- Choice of precipitating solvent
- Time from preparation to analysis
- Storage conditions of processed samples prior to analysis
- Addition of internal standard
 - In precipitation solvent or separately added
 - Must consider protein binding and need for equilibration

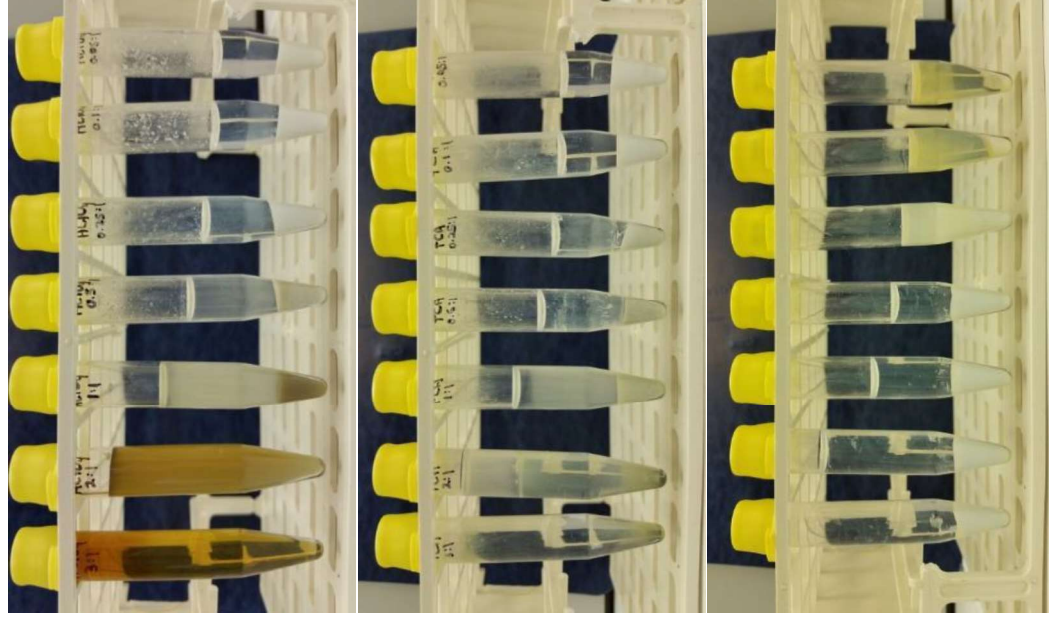
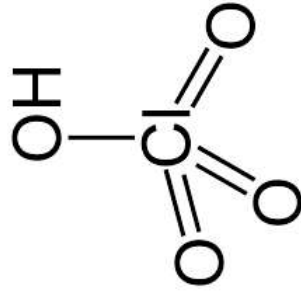
Organic Solvents



Acetonitrile	Methanol
3:1	3:1
2:1	2:1
1:1	1:1

Reducing Dilution Effect using Strong Acid

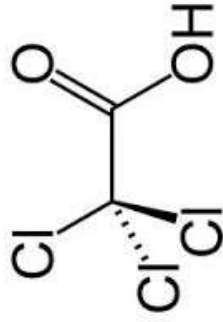
Precipitant: Sample v/v ratio, 3:1, 2:1, 1:1, 0.5:1, 0.25:1, 0.1:1, 0.05:1



70% aq Perchloric Acid

6.1N Trichloroacetic Acid

10% aq Trichloroacetic Acid

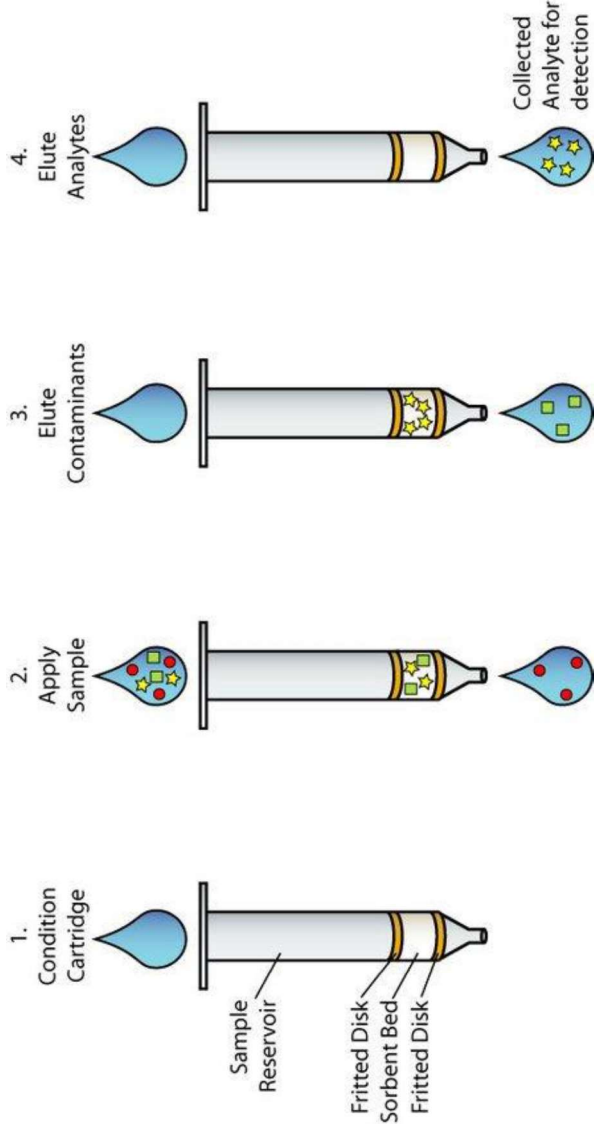


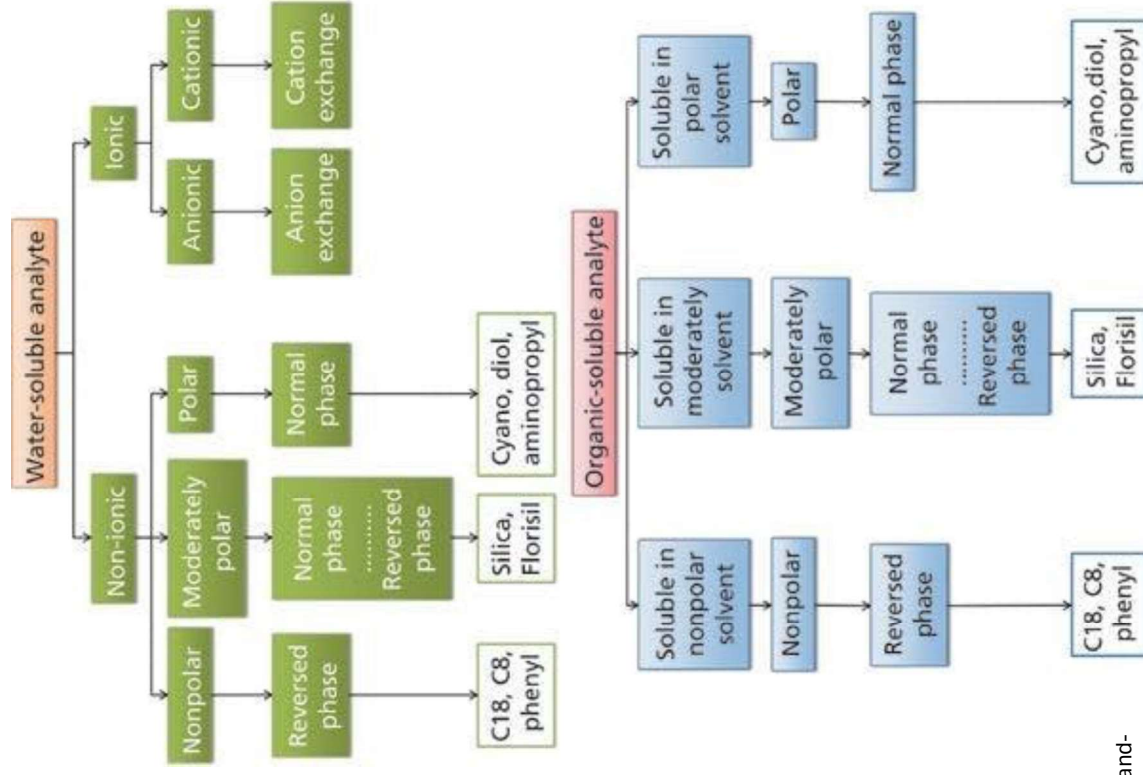
General Guidance for Protein Precipitation

- **Guidance:**
 - With organic solvent, start with 3:1 ratio (*J Chrom B* **2003** Mar 5;785(2):263)
 - For strong acids, less is usually more
 - Vigorous mixing to promote agglutination of protein (3-5 min @ high speed)
 - Maximize centrifuge speed and time (>10 min) for pelleting
 - Use caution when combining internal standard and PPT solution; must verify impact
- **Evaluate:**
 - Time for mixing and centrifugation
 - Optimal solvent for precipitation and compatibility with method
 - Impact of storage time and conditions on specimen quality
 - Impact of storage time and conditions on analyte stability

Solid-Phase Extraction (SPE)

- Sample cleanup based on chromatographic principle (competitive binding between solid phase and liquid phase)
- Solid-phase extraction sorbents can be categorized based on the support material:
 - Nonpolar
 - Polar
 - Ion Exchange
 - Mixed-Mode



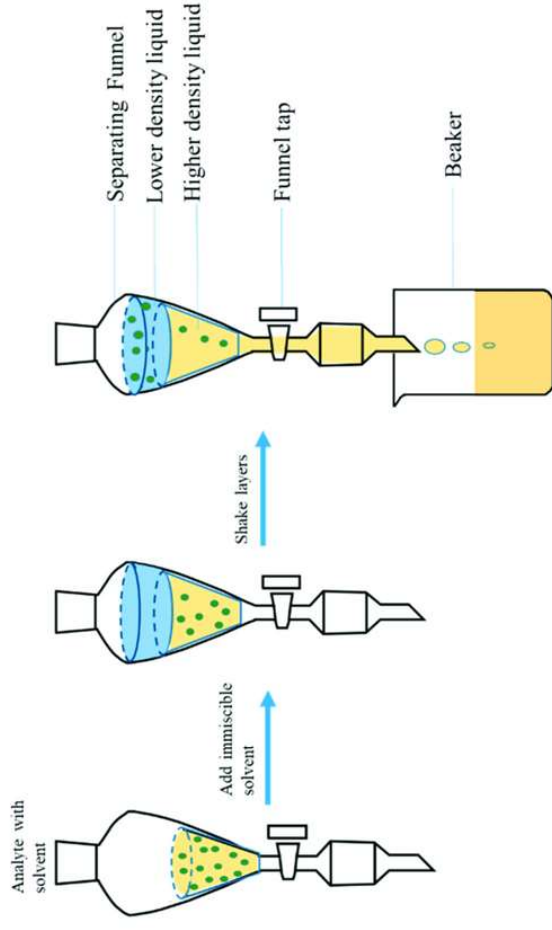


Important Considerations for SPE

- Critical to optimize based on pKa of the analyte of interest, both for loading and elution
- During optimization of SPE, experimentation with different solvent strengths is a key component for tuning the approach
- Sorbent bed should be conditioned using water-miscible organic solvent to optimize functional group interaction
- Consider vacuum manifold vs. positive pressure manifold for elution step
- Determine whether dry down step for solvent exchange or analyte enrichment is desirable
- Consider washes between loading and elution to improve selectivity

Liquid-Liquid Extraction (LLE)

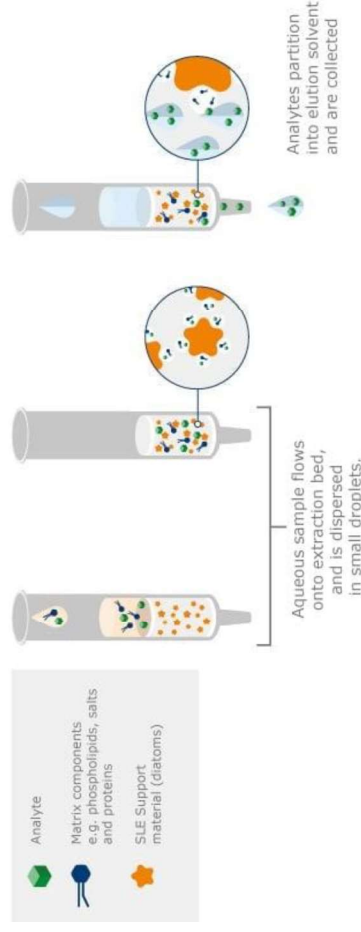
- Separation based on immiscibility of liquids
 - Typically an aqueous biological fluid and an organic solvent
- The mixture is agitated, and the liquids separate based on density
- Analytes are distributed based on their affinity for one of the phases
 - e.g. non-polar analytes will partition into the organic phase



Important Considerations for LLE

- Separation has limited selectivity as it is based solely on miscibility and analyte partitioning
- Solvents must be carefully selected, as incomplete partitioning will lead to suboptimal extraction
- LLE may not remove interferences responsible for matrix effects due to co-partitioning
- Based on the mechanism of agitation and separation, LLE is difficult to automate
- High phospholipid content may cause emulsions which lead to incomplete partitioning; this can be mitigated by addition of salts to the mixture

Supported Liquid Extraction (SLE)



- Support consists of porous diatomaceous earth particles
- Aqueous sample is added to the column (can be pre-treated)
- Water adsorbs to the particles and is dispersed as small droplets
- An immiscible organic solvent is added; as it flows through, analytes partition into the solvent and are extracted
- The eluent is collected for further analysis
- Same principle as LLE, but more compatible with automation

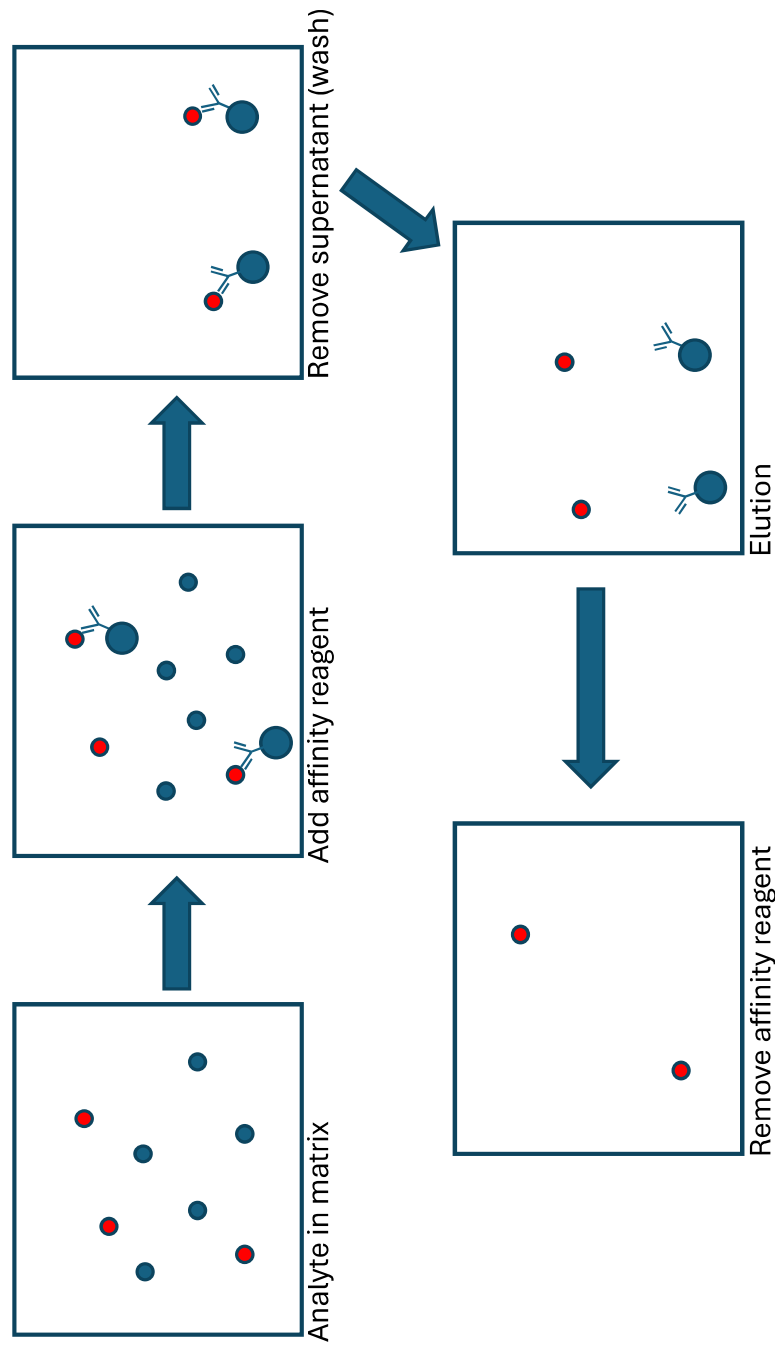
What Kind of Extraction to Use?

- Extraction-based approaches typically give cleaner samples than dilution or protein precipitation-based approaches
 - Prolongs column life
 - Yields less downtime and maintenance for mass spectrometers
- Can reduce matrix effects such as ion suppression
- LLE and SLE are simple approaches based on solvent miscibility
 - SLE more amenable to automation & doesn't require agitation
- SPE method can be optimized to complement the LC modality in the LC-MS assay
- In terms of "cleanliness": SPE > SLE > LLE

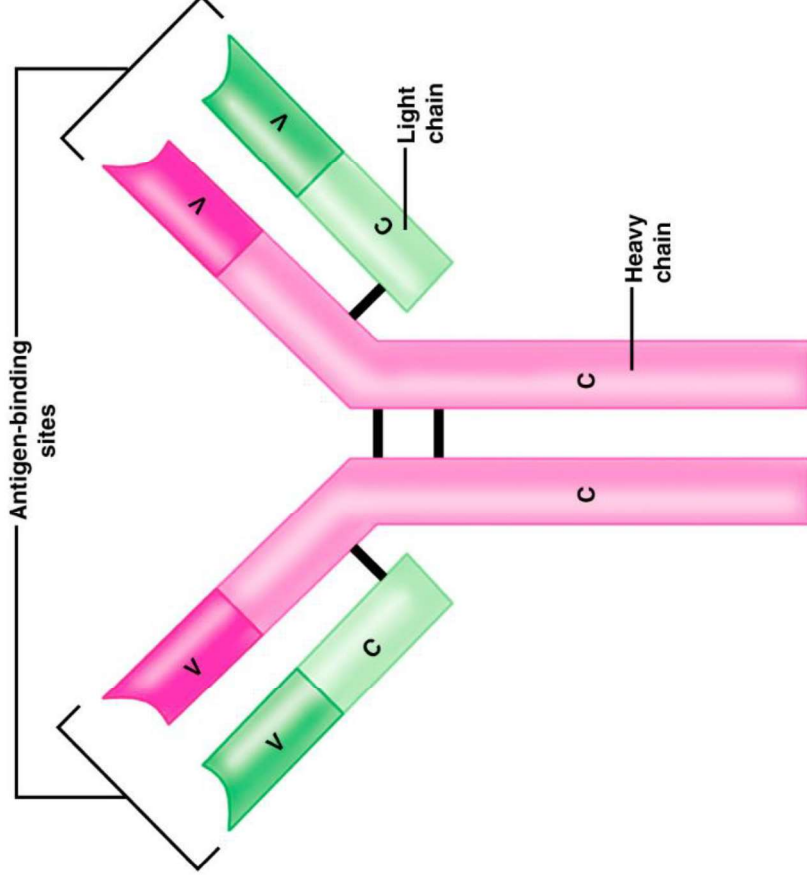
Affinity Preparation

- Different from preparation based on physiochemical characteristics (e.g. polarity)
- Reduction of complexity of sample based on functional groups or structural epitopes
- Multiple approaches
 - Chemical affinity
 - Immunoaffinity
 - Alternate approaches (e.g. molecular imprinting)
- More expensive and optimization is more complex, but improved selectivity may be worth it in some cases

Affinity Purification Workflow

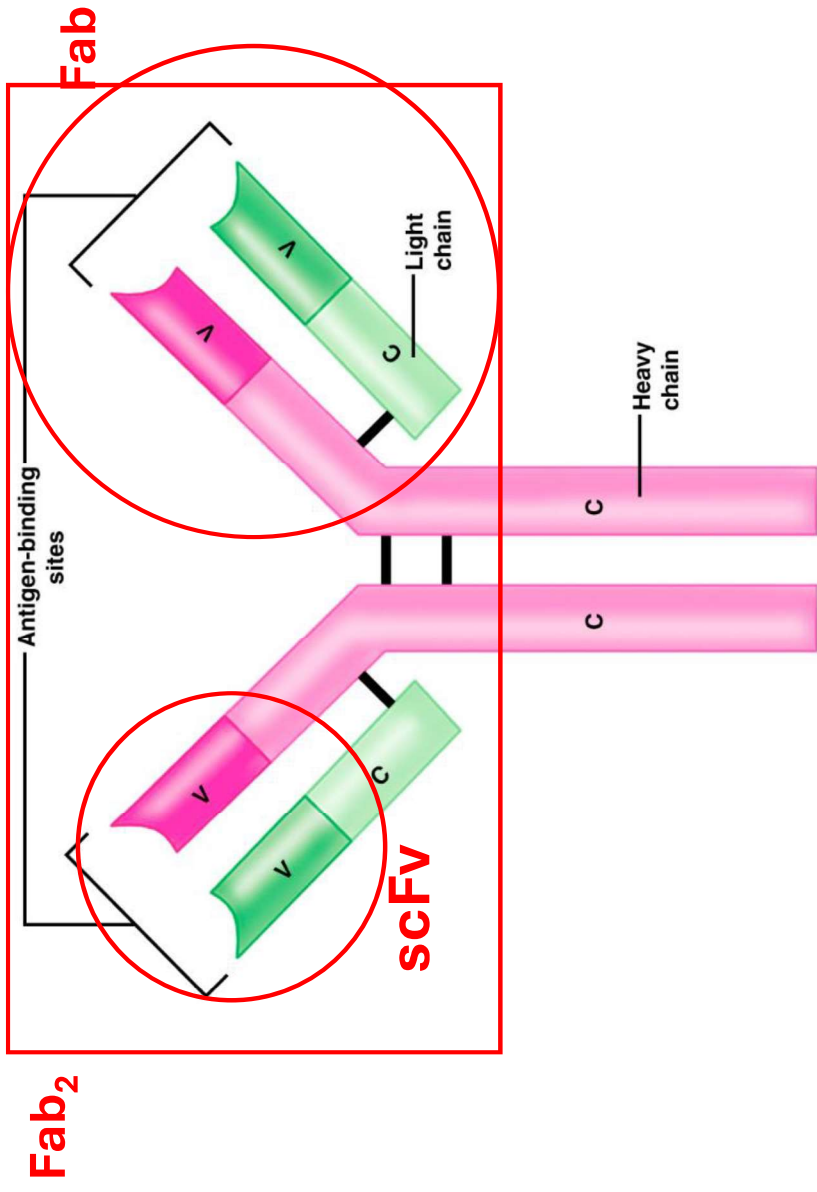


Immunoaffinity Enrichment



<http://blog.immunoreagents.com/portfolio/what-is-an-antibody/>

Antibody Fragments



<http://blog.immunoreagents.com/portfolio/what-is-an-antibody/>

Important Considerations for Immunoaffinity Preparation

- Specificity and Non-Specific Binding must be assessed
- Impact of monoclonal vs. polyclonal antibodies on assay performance
- Optimization of pH and temperature
- Optimization of wash and elution steps
- Volume of elution and dilution effects

Why Use Immunoaffinity Preparation?

- Some immunoassays for peptides and proteins are quite good (well-established analytes)
- In some cases immunoassays and LC-MS assays are not sensitive enough
- One approach is to use immunoaffinity pulldown (immunoprecipitation) to enrich the sample for the analyte of interest
- Isolation of small molecular with an affinity reagent; wash and elute; then analyze eluent with LC-MS
- Antibodies can be used to extract proteins of interest from the sample matrix, or to isolate individual peptides

Quantification of 1 α ,25-Dihydroxy Vitamin D by Immunoextraction and Liquid Chromatography–Tandem Mass Spectrometry

Frederick G. Strathmann,¹ Thomas J. Laha,¹ and Andrew N. Hoofnagle^{1,2*}

Clinical Chemistry 57:9
1279–1285 (2011)

Quantification of Thyroglobulin, a Low-Abundance Serum Protein, by Immunoaffinity Peptide Enrichment and Tandem Mass Spectrometry

Andrew N. Hoofnagle,^{1*} Jessica O. Becker,¹ Mark H. Wener,¹ and Jay W. Heinecke²

Clinical Chemistry 54:11
1796–1804 (2008)

Measurement of Thyroglobulin by Liquid Chromatography–Tandem Mass Spectrometry in Serum and Plasma in the Presence of Antithyroglobulin Autoantibodies

Mark M. Kushnir,^{1,2,*} Alan L. Rockwood,^{1,2} William L. Roberts,[†] Dev Abraham,³
Andrew N. Hoofnagle,⁴ and A. Wayne Meikle^{1,2,3}

Clinical Chemistry 59:6
982–990 (2013)



Why automate??



Why automate??

- **Process Improvement**
 - Standardization of assays
 - Traceability from primary specimen to result
- **Personnel Considerations**
 - Declining workforce numbers in clinical laboratories
 - Repetitive motion injury
- **Economics**
 - Labor and service are a significant contributor toward assay cost
 - Must reduce ratio of human interaction to number of patient results

Considerations for Off-Line Automation

- What type of tube is going on to the system?
 - Parent tube or aliquot?
 - Is it bar coded?
- Are you going tube-to-tube or tube-to-plate (transfer)?
- Are you going plate-to-plate (stamping)?
- Will the system just be moving liquid, or is the processing on-platform?
- How much human interaction will be needed in the workflow?

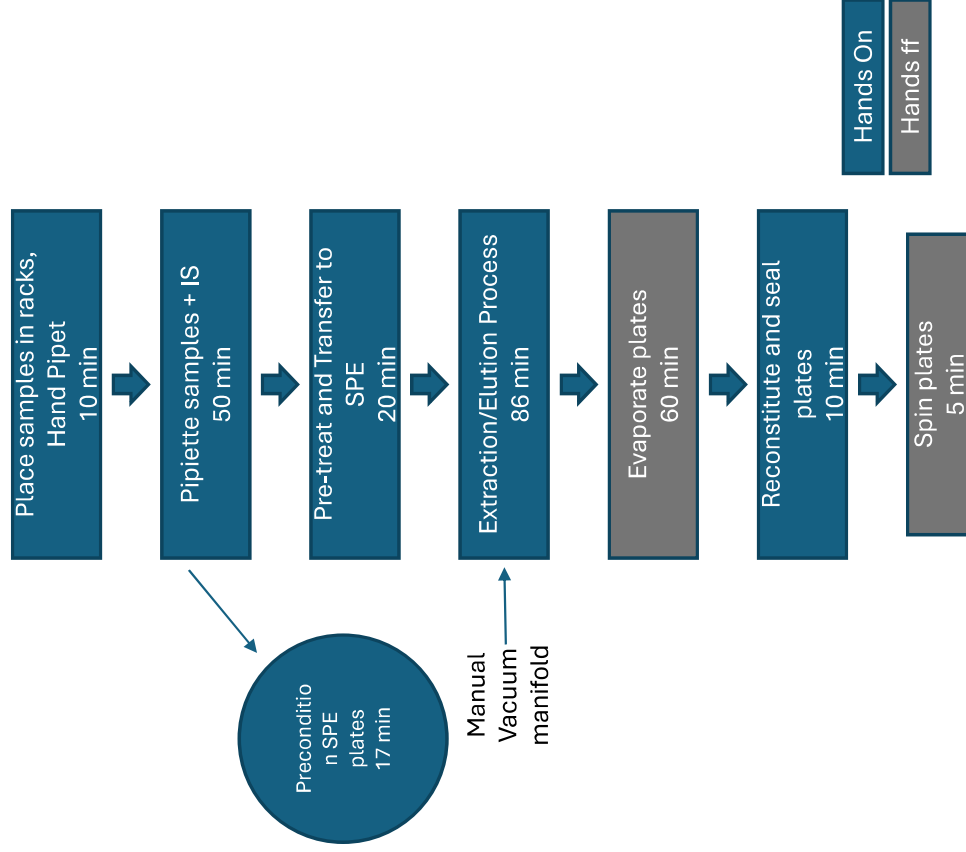
Robotic Liquid Handlers and Processors



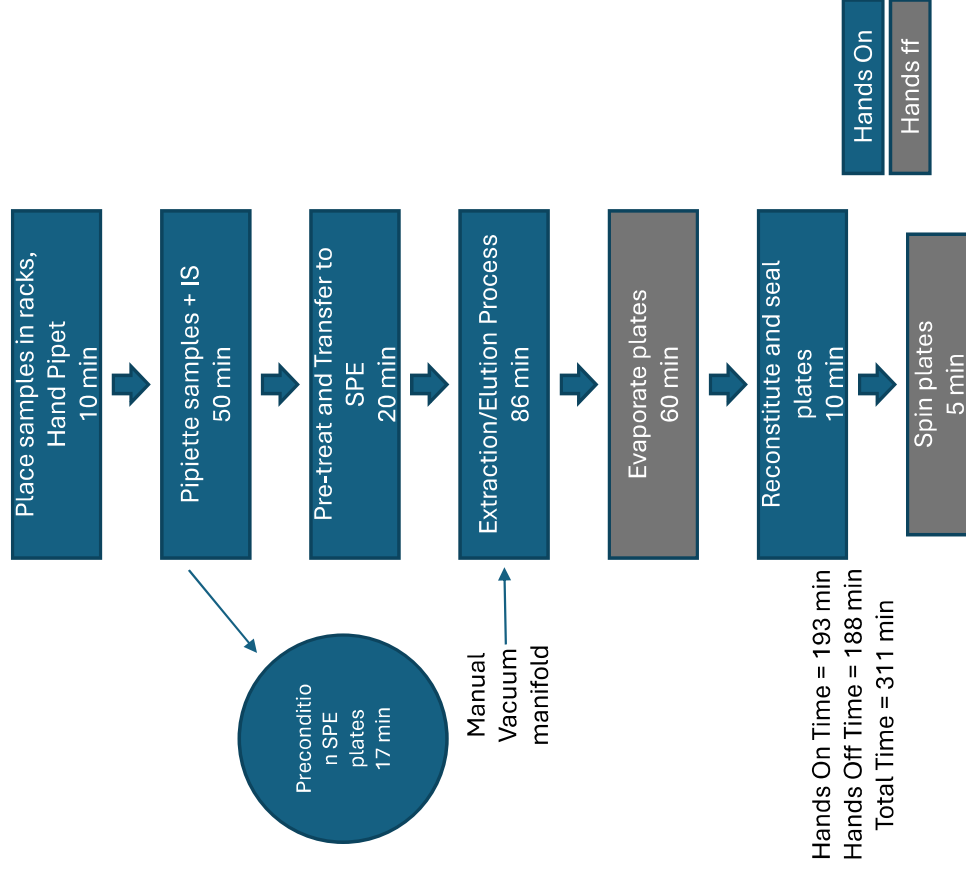
Important Considerations for Automated Liquid Handling

- **Sample Integrity**
 - Is bubble detection possible? Is there debris present in the sample? What happens if there is insufficient volume? Will clots be detected and/or disrupt pipetting?
- **Sample Aspiration**
 - Does your system provide liquid-level tracking? How does speed impact the aspiration performance? Is a post-aspiration delay necessary for optimal performance?
- **Sample Dispensing**
 - Is there an issue with excess liquid external to the tip? How does the speed impact performance for dispensing liquid? Is air displacement adequate, or is positive displacement needed?
- **Liquid Class**
 - Type of liquid can influence any of the above factors – viscous and volatile liquids require extra attention and care in optimization. Does the liquid for your analysis impact analyte adsorption to the tips or vessels?

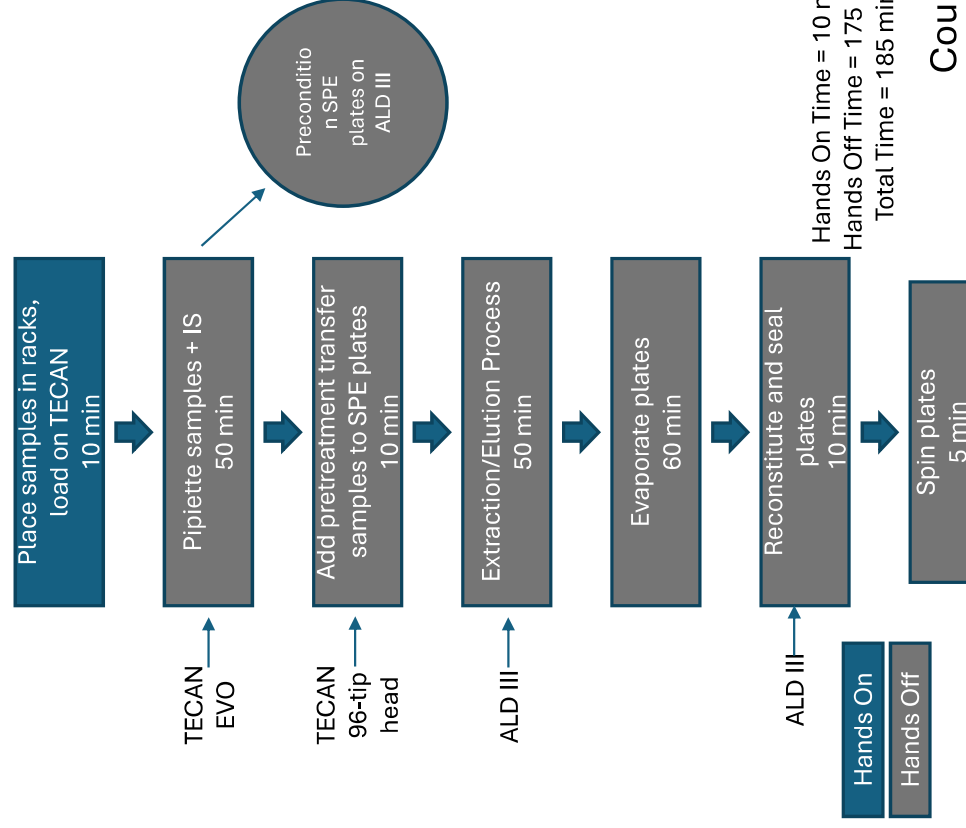
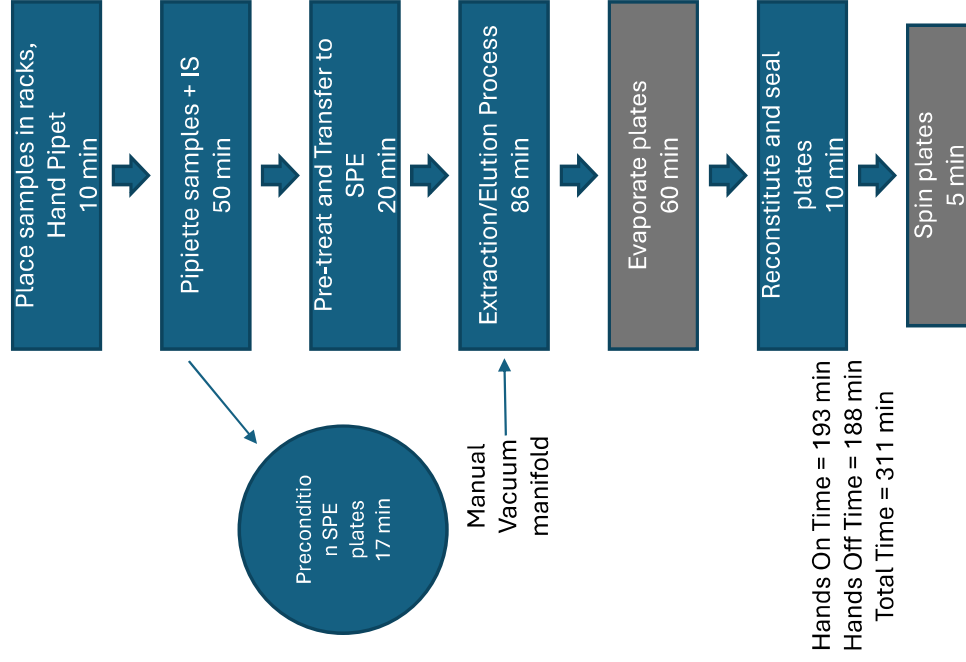
Hands on vs. Hands off



Hands on vs. Hands off



Hands on vs. Hands off



Courtesy Russ Grant

Why Dried or “Micro” samples?

- Reduced sample volume makes less-invasive sample collection feasible
- Enabling of capillary fingerstick samples = bridge to POC testing by mass spectrometry
- Smaller sample volumes make more time points feasible
- For animal studies (clinical research) enables studies without sacrificing the animal (at least not always)
 - Fewer animals = lower cost

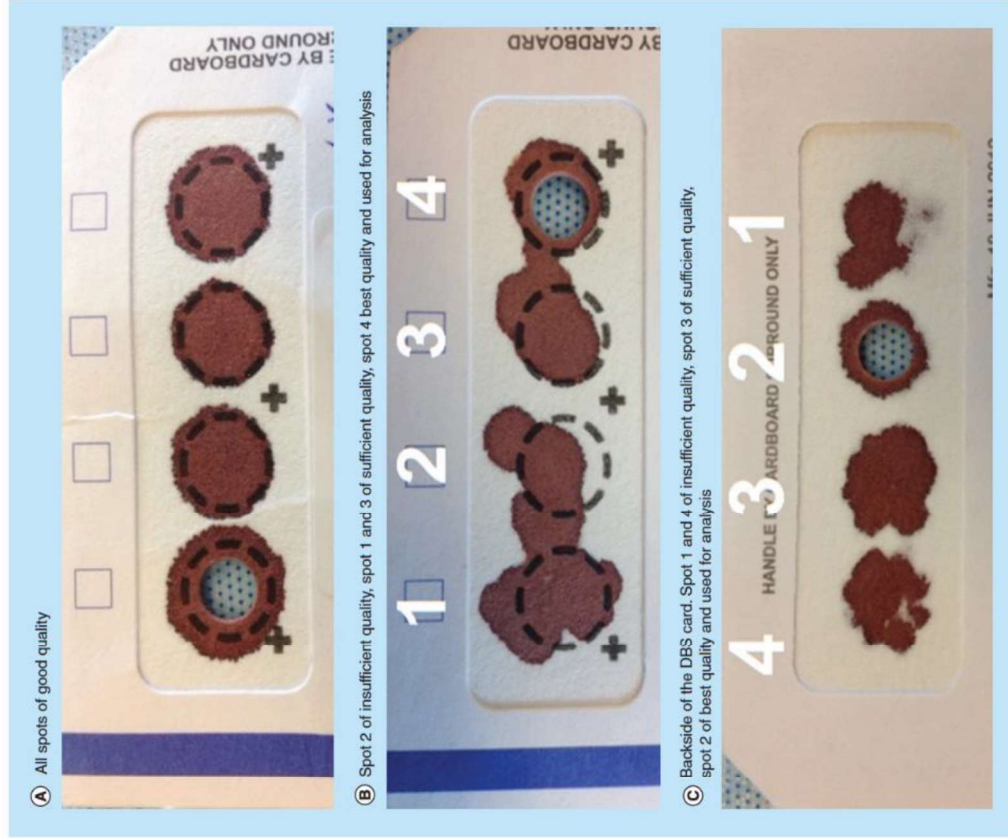


Figure 1. Example of quality of the samples used for analysis. (A) All spots of good quality. (B) Spot 2 of insufficient quality, spot 1 and 3 of sufficient quality, spot 4 best quality and used for analysis. (C) Backside of the DBS card. Spot 1 and 4 of insufficient quality, spot 3 of sufficient quality, spot 2 of best quality and used for

Challenges particular to DBS

- HCT impacts the spread of blood on the paper; variance in concentration of spots
 - Also impacts homogeneity and matrix effects
- Potential for external contamination
- Blood-to-plasma ratio important consideration
- If not room temperature stable; can take up more space in the freezer
- Mostly manual and labor-intensive process

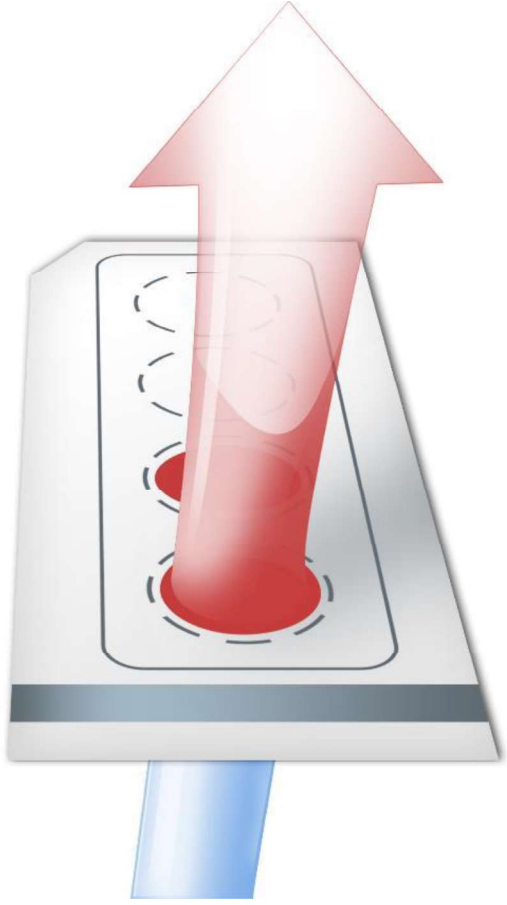
On-Line DBS




Thermo Scientific™ Transcend™ DSX-1 Solution

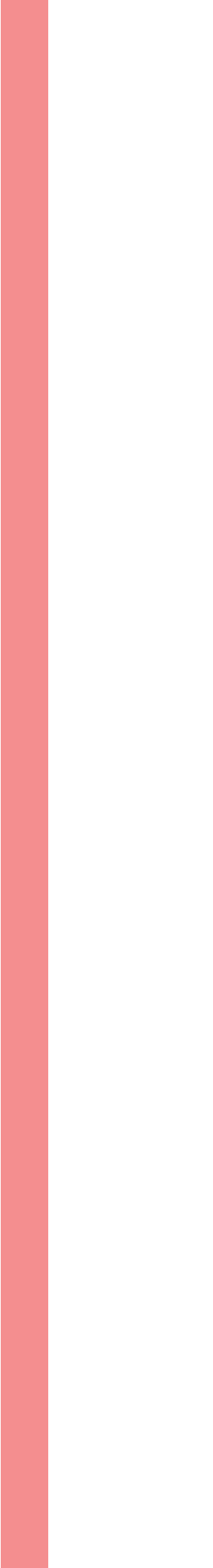
Images courtesy of Thermo Fisher Scientific

On-Line DBS





Challenges with DBSA

- HCT is still an issue – must deal with it in some form or fashion
 - Required in-line cleanup; either SPE or turboblow chromatography
 - Optimizing clamp size relative to required assay criteria
- 

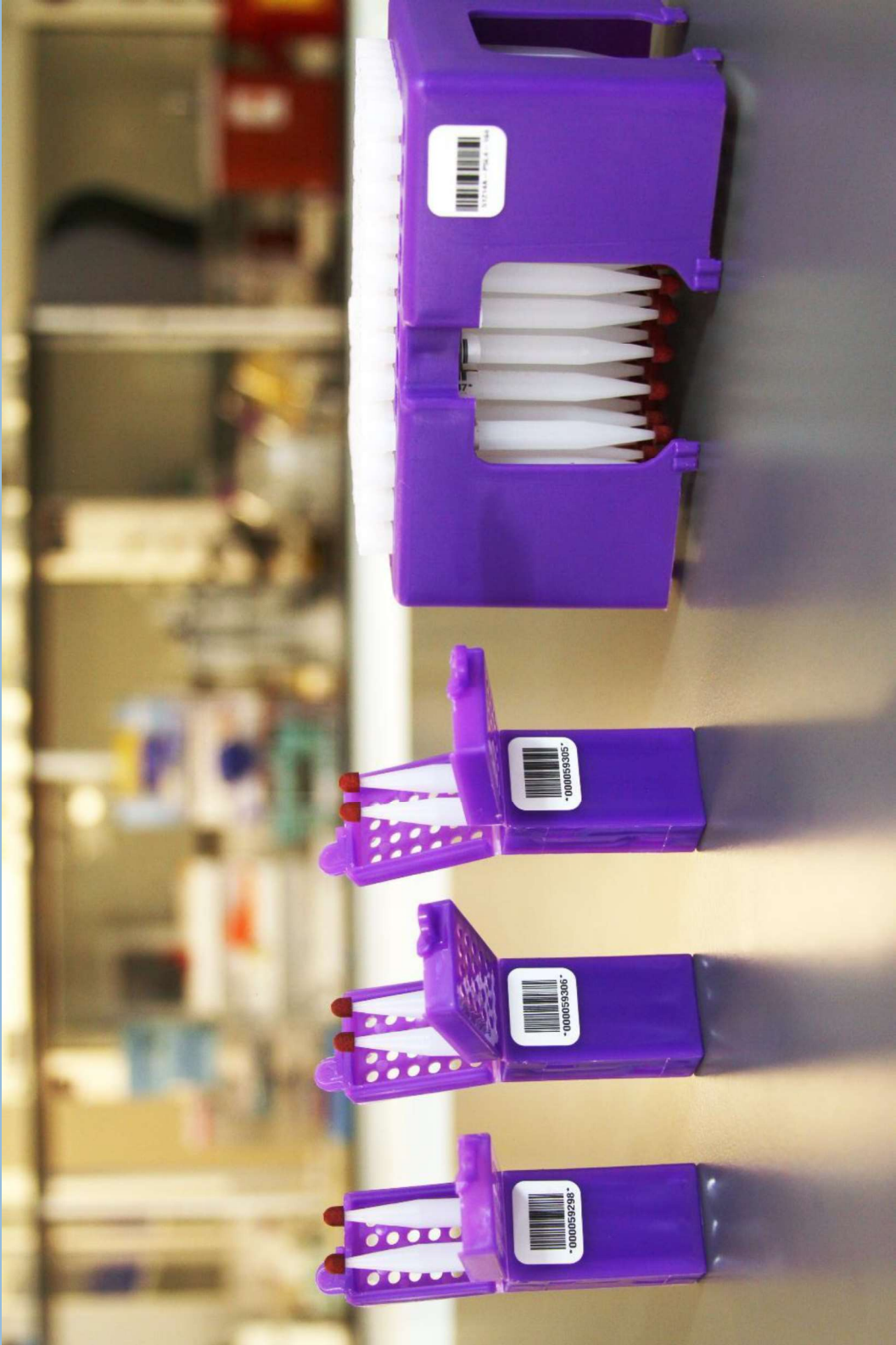


Image courtesy of Neoteryx

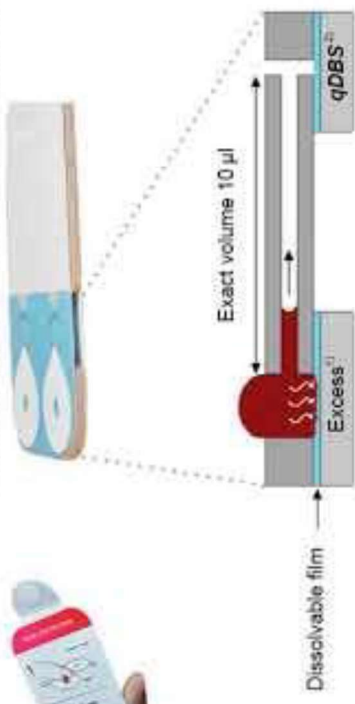
Capitainer



 Capitainer



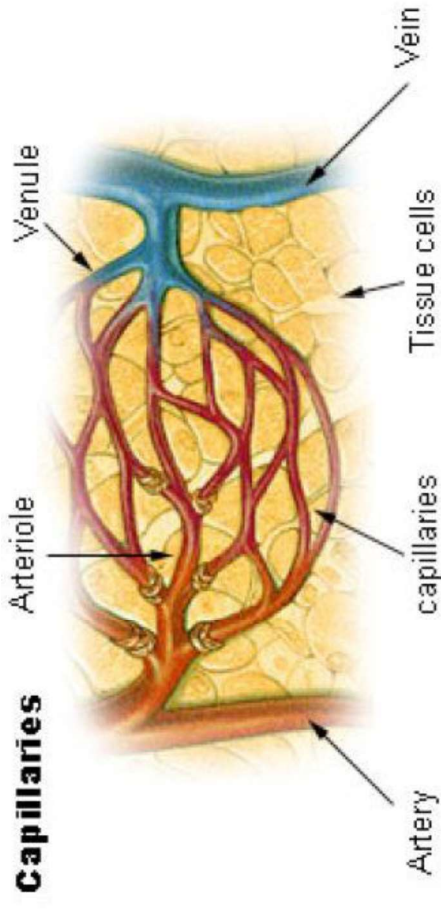
Capitainer qDBS - microfluidic technology



Notes: 1) Minimum amount of blood used is 10 µl (paper instruction page 2/3)
2) 10 µl blood required in Capitainer qDBS paper after 4000rpm grade 2225

Capillary Sampling

- Typically obtained from fingerstick or heel stick
 - We occasionally get inquiries about earlobe sampling
- Appealing sample type due to ease of collection, compatibility with home collection, no need for phlebotomy
- Important to consider many challenges with the sample type:
 - Contraindications: PVD, edema, poor circulation
 - Preanalytical: inconsistent blood volume, dilution with ECF, analyte variance between sample types, more painful than expected, hemolysis



Final Thoughts

- Sample preparation is a critical step for high-performing clinical LC-MS assays
- A cleaner sample will lead to improved assay performance and minimize downtime for analytical systems
- There is a wide array of specimen preparation methods available, ranging from simple and broad (dilute & shoot) to complex and focused (immunoaffinity)
- Important to define desired performance and match specimen prep approach to the analytical methodology and performance needs

QUESTIONS??