Considerations for Sample Prep and Method Development

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- **Nothing to disclose**

Learning Objectives

After this presentation you should be able to:

1. **List advantages and disadvantages of the common sample preparation techniques used for clinical mass spectrometry (MS)**
2. **Create a due diligence plan to select sample prep automation for clinical MS**
3. **Describe pitfalls and solutions encountered when using automated liquid handlers for clinical MS sample preparation**

Common Sample Prep Options

1. Dilution (Urine)
2. Protein Crash or Ultrafiltration (Serum) / Phospholipid removal (PPT)
3. Liquid-Liquid extraction (LLE) & Supported Liquid Extraction (SLE)
4. Solid Phase extraction (SPE) – offline & online
5. Other less common options, not listed

Comparing Sample Prep Techniques

Differentiator	SPE	PPT	LLE	Online
Charged Analyte Recovery	1	2	4	3
Neutral Analyte Recovery	2	4	1	2
Highly Polar Analyte Recovery	1	2	4	3
Thermally Labile Analyte Recovery	3	2	4	1
Generic Protocol	2	1	4	2
Assay Ruggedness	3	4	1	2
Matrix effects	2	4	1	2
Selectivity	2*	4	1	3
Sample Concentration	2	4	1	3
96-well plate prep	1	3	4	2
Automatable	2	3	4	1
Simplicity	3	1	2	4
Method development speed	4	1	3	2
Cost (cheapest)	4	1	2	3
Sample Type Variance	3	2	1	4
Prepartion time (fastest)	4	2	3	1
Sample Volume (limited)	3	2	3	1
Extract direct injection	3	2	4	1
* = ion exchange SPE mode	Rank ordered 1=best, 4=worst			

*table from Russell Grant, Ph.D. – MSACL Quant. MS Development & Validation short course, with permission

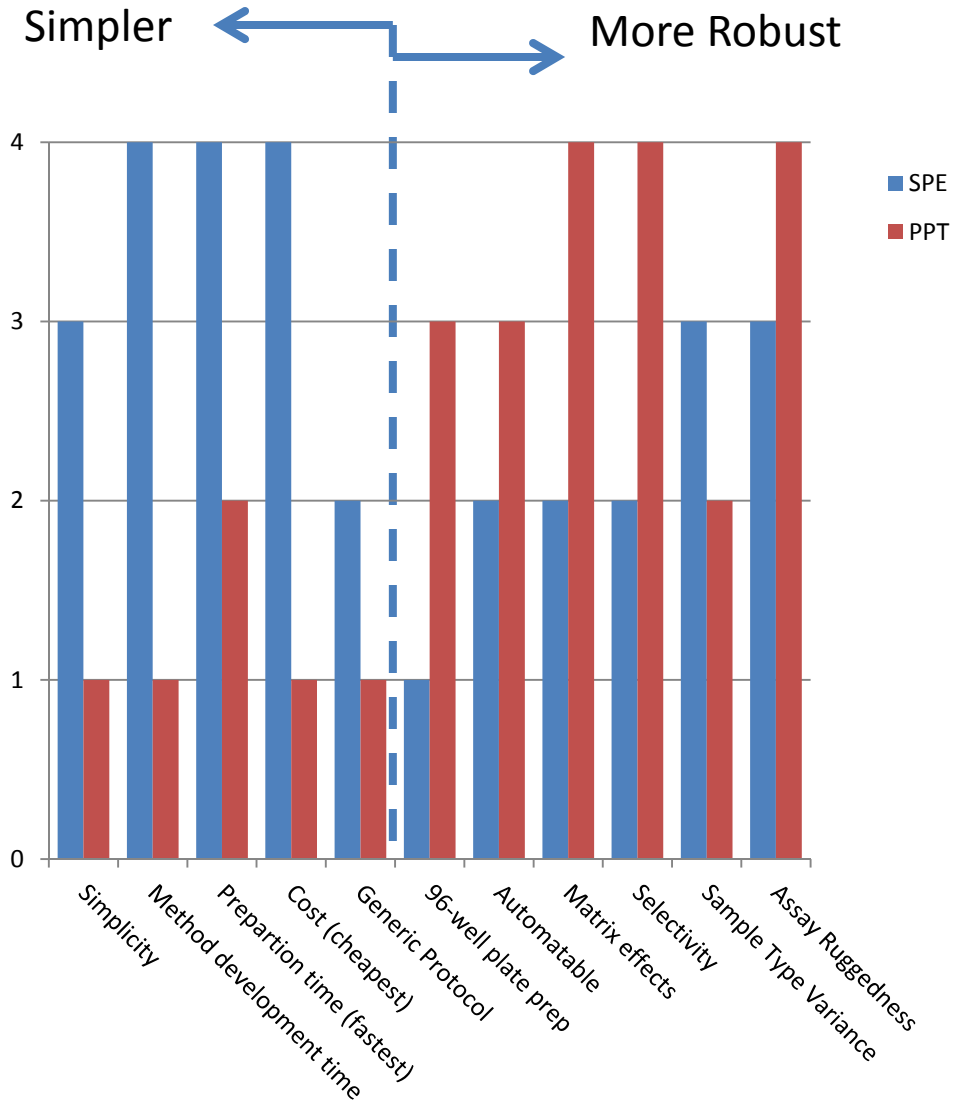
What Extraction mode to use?

- **Scientific approach*:**
  - **Nature of the molecule is 1st decision point (charged, neutral, highly polar, pKa)**
  - **2nd decision point - concentrate or dilute to achieve desired LLOQ & robustness?**
- **Compromise with reality approach:**
  - **Expertise/FTEs available for development & production (comfort zones)?**
  - **Accessory equipment & capitol $ available to "get cleaner," scale up, automate?**

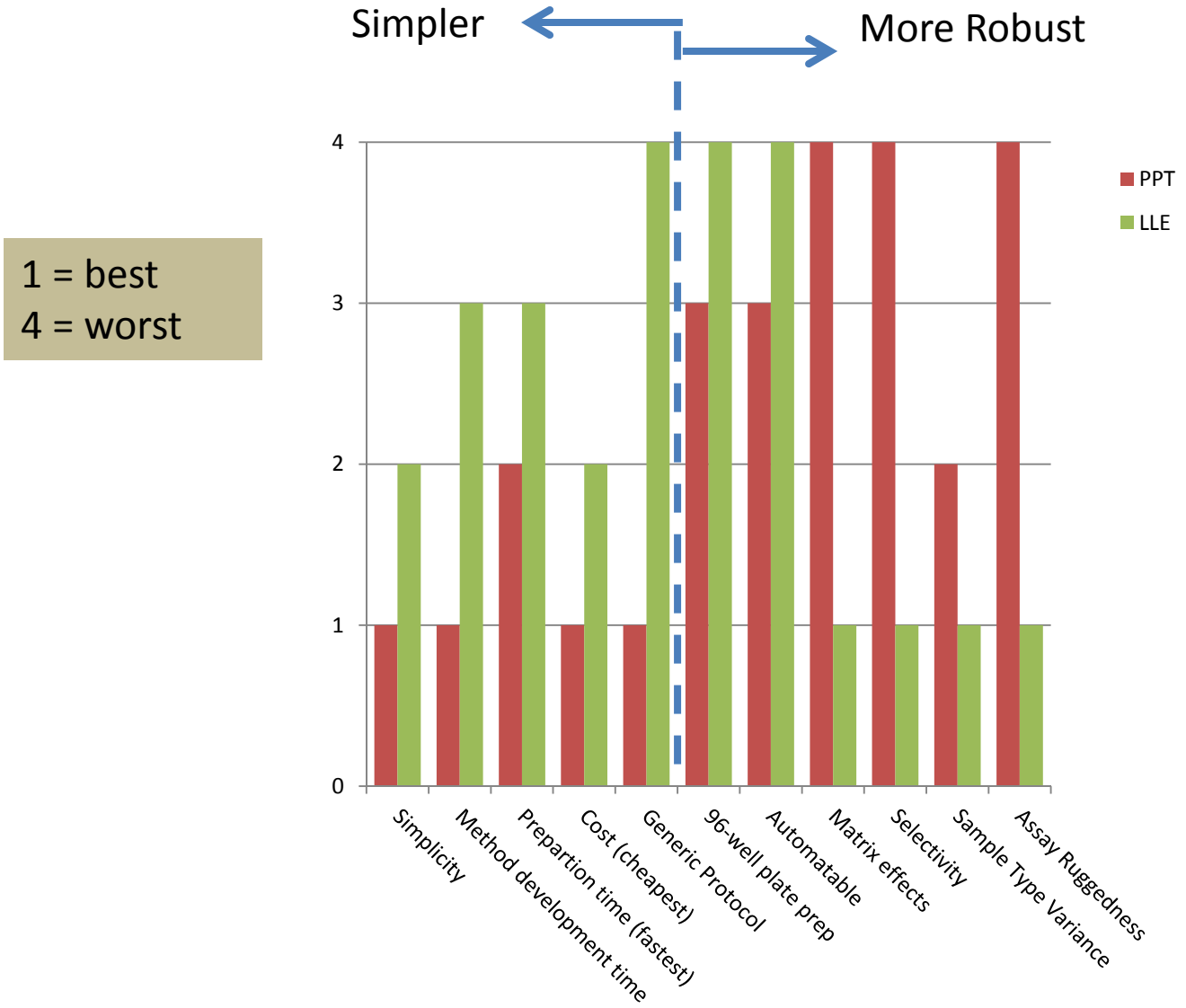
*Russell Grant, Ph.D. – MSACL Quant. MS Development & Validation short course, with permission

Simpler to More Robust progression

1 = best
4 = worst



Simpler to More Robust progression



But dilution &/or protein crash works just fine in my lab…….

- Workload
- Test menu/instrument
- Criteria used for data review & robustness?
- How to quantify instrument down time & troubleshooting time (technical & service support)?
- All dilution & PPT protocols are not equal – the details matter

Qualifiers – the details* matter

- **SLE (supported liquid extraction) easier to automate than LLE → similar robustness** (but sample volume limitations – able to concentrate x4)
- **C18 SPE <<< selective than mixed mode SPE (C18 + cation/anion exchange)** (need a charged analyte)
- **PPT combined with phospholipid removal plates can be very robust** (but precipitation in the plate doesn't always work)
- **All PPT protocols are not the same** (two stage MeOH/ZnSO4 + Acetonitrile w 1% formic acid had better linearity, long term precision and was "cleaner" [less cleaning of MSMS] than Acetonitrile alone)

* Take Russell Grant's Quant MS Development & Validation short course – MSACL, March 2014!!

Lessons learned the hard way

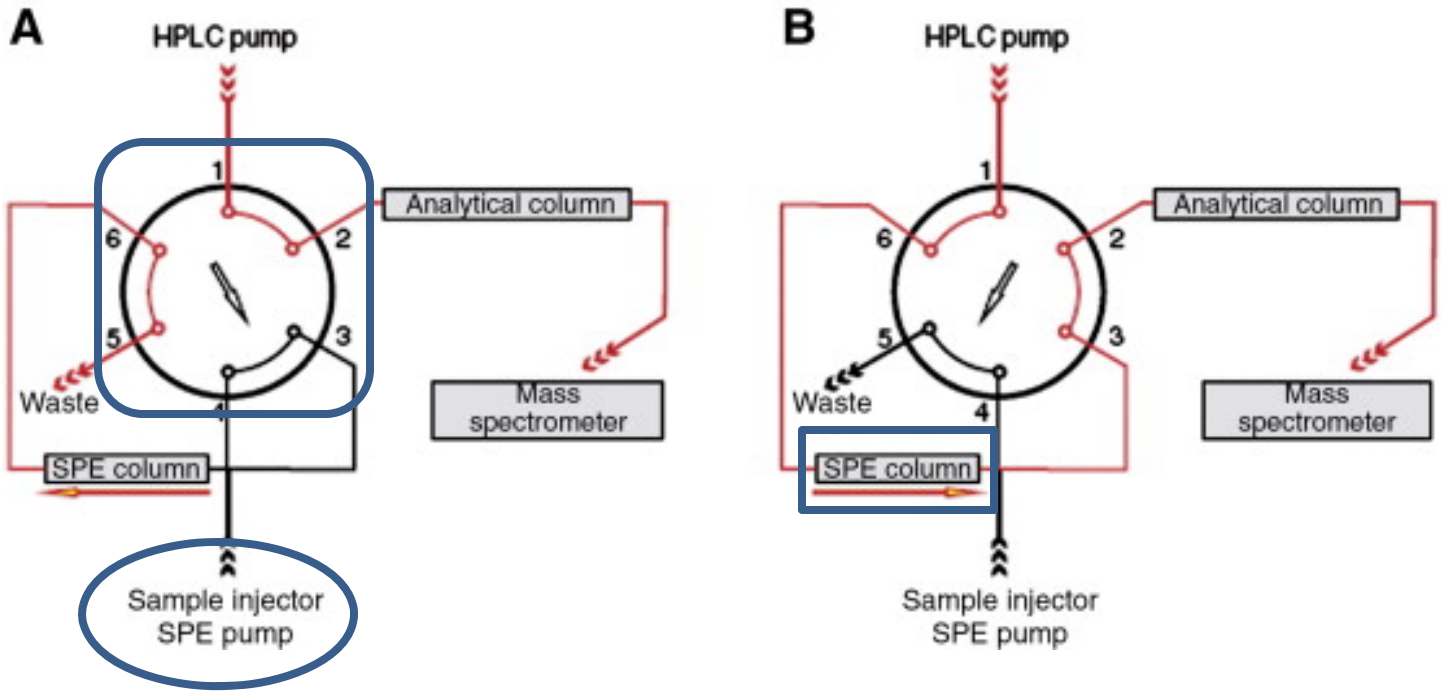
- **Do qualitative (post-column infusion with injection of multiple patient extracts) AND quantitative (no matrix & pre- & post-extraction spikes with matrix) testing for ion suppression** (getting pre- & post-spikes correct, accurate & precise – NOT SO EASY!)

- **Challenge with numerous patient samples EARLY in development** (avoid the sample prep that is beautiful with calibrators and QC but, oops, doesn't work with patient samples)

- **Don't ignore outliers** (1 failure in 10 samples translates to 10 repeats/day with a batch of 100 and 250 repeats/day with batches of 2,500! How will you resolve MRM ratio failures, I.S. low recovery failures, interfering peaks, etc.?)

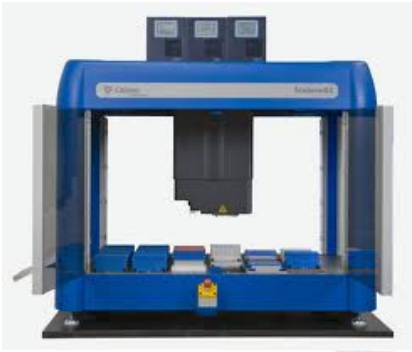
Automation

Online Extraction - concept



Automated Liquid Handling

- 1, 2, 4, 8 and/or 96 pipetting channels
- Washable/reusable vs disposable tips
- Accessories for extraction (shaker, heater, cooler, vacuum module, evaporator)
- 96 well plates and tubes
- Barcoding & interfacing capabilities

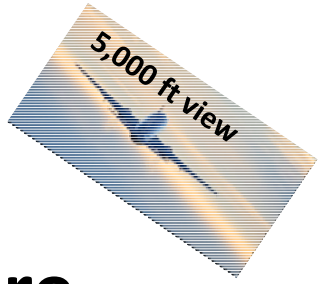


TECAN
Freedom EVO MCA 150

Choices for Sample Prep Automation

- Online extraction
  - Vendor "complete" solutions
    - Thermo Scientific/Cohesive Technologies - TLX Turbo-Flow online SPE extraction
    - Spark-Holland/Symbiosis – dual channel cartridge online SPE
    - Gerstel MPS Workstation with in-tip dispersive SPE
  - User-developed online SPE
    - Most LC-MSMS vendors have packages/software - extraction pump(s), switching valves, installation, application notes
- Automated Liquid Handling (ALH) – many vendors & options, useful also for tube to plate with online or off-the-deck extraction (e.g. positive pressure SPE)

Comparing Online vs ALH (Automated Liquid Handling) if you can only have one



Parameter	Online	ALH
CLS Hands on time/sample	Less	More
LC sophistication needed	More	Less
Capitol Cost (online vendor complete solution)	More~	Less
Capitol Cost (adding online home brew to existing LC)	Less~	More
Flexibility (options for different types of extraction)	Less	More

Considerations for Online Extraction- ROI & support

- Do you have a single, high volume assay  or multiple, lower volume assays  ?
- In-house troubleshooting, repair?
- Realistic throughput estimate - extraction vs LC time, parallel or serial?
- Vendor support for method development?

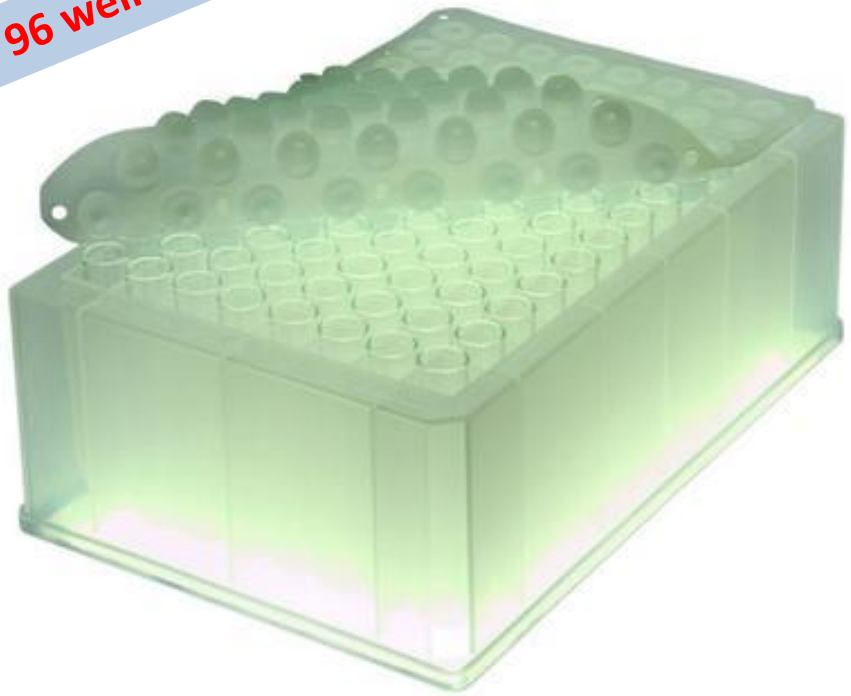
Considerations for Online Extraction- Analytical Issues

- **Limited sample volume (entire aspirated sample to analytical column)?** 
- **Analyte thermally labile (no dry down needed)?** 
- **# of solvents, solvent types for wash & regeneration of SPE column (solvent select valves, miscibility, *CARRYOVER*)?**
- **Elute (strong solvent) from the extraction column and retain downstream on the analytical column (weaker solvent) – how to**

Considerations for Online Extraction - Process

- How to do LC gradient development, SST, validate recovery & ion suppression (can you inject with bypass of the extraction column)?
- Sample and internal standard still have to be mixed before the sample is put into the LC-MS/MS (glucuronide/sulfate hydrolysis?)
- Evaluate cycle times of extraction & LC run with care
  - Are two extraction lines "multiplexed" to one LC?
  - are more than one extraction + LCs lines "multiplexed" to one MSMS?
  - Will the LC be waiting for the extraction or the extraction waiting for the LC?
- Compatible with all MSMS vendors (customers in production)?

Single biggest improvement in productivity – 96 well format



Considerations for Automated Liquid Handling – ROI & Support

- **Disposable tips vs Washable** (consumable cost vs carryover risk, less throughput)
- **# of pipettor channels** (>$ = >throughput, 8 channel for tube to plate transfer, 96 channel for "plate stamping")**?**
- **Barcode reading** (plates & tubes) **& aliquot/primary tube automated feed** (barcode heights, formats?)
- **Deck space** (# of SBS footprint positions – tips, plates, shaker, vacuum, etc.)
- **Redundancy** (service support)**?**
- **Application support** (programming)**?**

Considerations for Automated Liquid Handling – Analytical Issues

- **Minimum and maximum pipettable volumes** (and with what precision)**?**
- **Pipetting of difficult liquids precisely & w/o damaging components?** (hexane, dichoromethane, acids, bases)
- **Liquid level sense & clot detection?**
- **Reagent delivery channel(s)** - how many solvents**?**
- **Hard to effectively MIX a column of liquid(s) in a tall, narrow, plastic well**

Considerations for Automated Liquid Handling – Process

- # of plates/tubes/tips that can be stored on the deck (stackable?)?
- Off deck labware storage with auto-transfer to the deck?
- Moving plates around the deck (gripper)?
- Span capability (X, Y, Z independence of pipettor channels – for different tube sizes, racks, plates)?
- File In/Out capability (interfacing to LIS/middleware)?

Automated Liquid Handling – Case Histories

Thanks to my colleagues who did the work for liquid handling cases

- Julia Drees, PhD, Scientific Director – TPMG Kaiser Regional Laboratories, NCal
- Tony DaSilva, MS, Automation/IT Applications Engineer - TPMG Kaiser Regional Laboratories, NCal
- Bret Martin – Applications Programmer, Hamilton Scientific

Case 1 -To touch off or not to touch off – that is the question

Task

- Pipette serum and internal standard (I.S.) to a 96-well plate for a 25-OH Vitamin D assay by LC-MSMS.
- Desired intra-assay precision (within plate) = <5% CV

Constraints

- Sample + I.S. volume must = 75 µL
- 50 µL of serum needed for LLOQ – so I.S. must be 25 µL
- Conical bottom, 1 mL, polypropylene plate

Development History

Original Protocol:

- **50 µL I.S. dispensed into plate**
- **50 µL serum added, mix with tips**
- **Intra-assay precision 4-6%**

Revised Protocol:

- **2 precipitation reagents instead of 1 (more robust)**
- **Total volume had to stay the same**
- **I.S. volume must be decreased to 25 µL**

Plate Stamping – 96 head

1. Transfer 50 µL (now 25 µL) I.S. from reservoir to plate (8 tips, pick up & dispense 12 times)

8 channel pipet on ALH_1

I.S. reservoir

2. Transfer serum from tubes to plate (8x12 tips) & mix w tips

8 channel pipet on ALH_1

Tube to Plate

3. Move plate to ALH_2 w 96 channel head & add two precipitating reagents (mix w tips)

4. Transfer precipitate to hybrid filtration plate on vacuum module to remove protein & phospholipids

Problem - %CV ↑ to 6-9% when I.S. volume ↓ from 50 µL to 25 µL

- Re-measure bottom of plate dimensions w calipers & adjust height of dispense – not fixed
- Lower height of dispense (with only 25 µL not touching off on bottom of plate?) – not fixed
- Slow speed of dispense – not fixed
- Pause after dispense – not fixed
- Blow out of residual volume in tips – not fixed

Solution

- Dispense serum 1st
- Dispense I.S. 2nd - touch off to serum (liquid) instead of questionable touch off to (dry) plate
- Intra-assay % CV decreased to 2-4%
- Trade offs
  - touch off to serum requires new tip each well
  - increased tip cost (from 8 to 96 tips)
  - tube to plate time increased 15-27 min
- Lesson learned – small changes can make a big difference in liquid handling precision

Case 2 – Missing filtrates

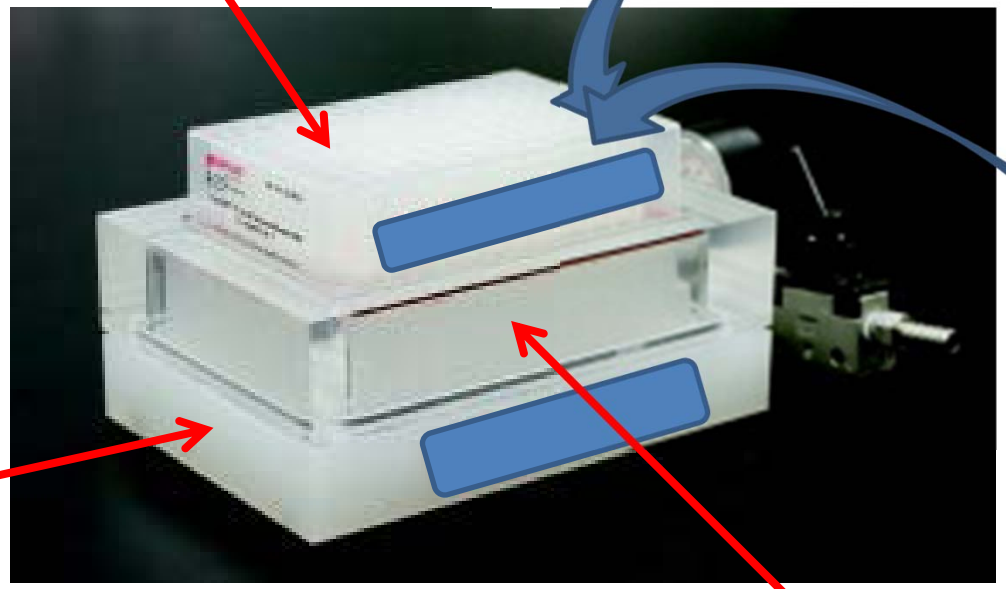
Task

- **– Precipitate serum + I.S. in a hybrid (protein & phospholipid removal) filtration plate – transfer filtrate to collection plate (vacuum)**

Constraints

- **– Maintain (sample + I.S.) volume to precipitating reagent volume at 1:3**
- **– Total volume in hybrid filtration plate ≤ 450 µL**
- **– Don’t move plates off deck during the process to maintain viable work flow for 25 plates/day**

Hybrid filtration plate on top of vacuum module



vacuum module

Collection plate inside vacuum module

1. Transfer serum + I.S. to hybrid filtration plate with 96 head

2. Transfer precipitating reagent to hybrid filtration plate with 96 head & mix with tips

3. Apply vacuum to move filtrate to collection plate

Problem

- First few plates look good
- Then for a few wells in every plate – no filtrate transferred to collection plate
- All other wells in those plates have expected recovery

?

Investigation

- **Call vendor and complain – onsite support**
- **Change of plate lot? - not fixed**
- **Breakthrough of filtration membrane in surrounding wells → inadequate vacuum for problem wells? - unable to prove or disprove**
- **Clogged membrane in problem wells – why OK before & not now?**

Eureka!

- **Frozen aliquots of pooled serum in use** → **switch to fresh patient samples**
- **Vendor validated with frozen rat serum**
- **Hypothesis**
  - **– frozen serum precipitate ≠ fresh serum precipitate**
  - **– difference in size of particulate clogging filter?**

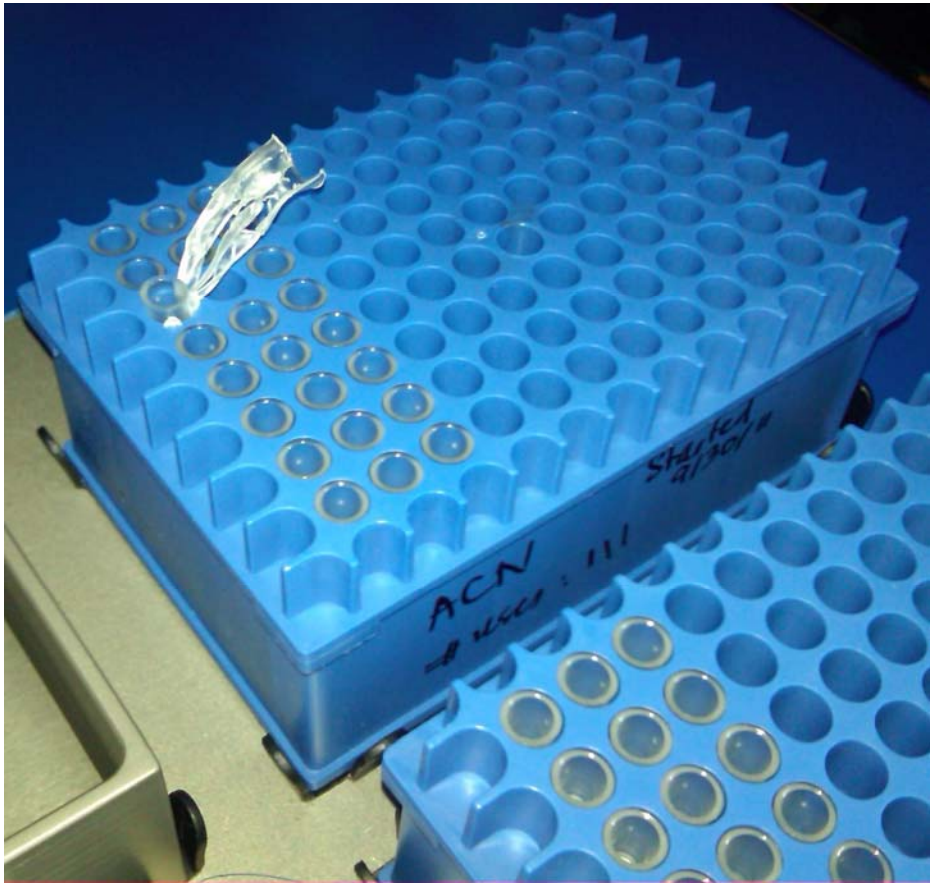
Solution - crash, wait, then "sip" & transfer

- **Crash in plain, conical bottom, 1 mL, plate INSTEAD of crashing INSIDE hybrid filtration plate**
- **Wait 5 min for heavier clumps to settle**
- **"Sip" upper layer of milky white precipitate (avoid heavy clumps at bottom) with 96 head and transfer to hybrid filtration plate** (apply vacuum)
- **Plate could stay on deck – no off deck centrifugation required**
- **Recovery was acceptable**

Lessons learned

- Give the low-tech solution a try
- Extraction automation is about surface chemistry:
  - characteristics of different plastics
  - surface tension, flow & density of liquids
  - micro/macro-architecture of particulates, media, filters
- Positive pressure is more reliable than vacuum
- Mixing can be a challenge
- Pay attention to the millimeters (teaching the robot)
- Don’t give up & be creative – the details matter!

Case 3 – static cling of tips to pipet head (tips not shucked)



Solution

- **Purchase tips *without* plastic film wrapping of individual tip boxes**
- **Only re-use reagent tips a few times (static builds up with re-use)**
- **Static seemed to vary with humidity**
- **Anti-static devices were not too helpful**

In conclusion…

- **Attention to trivial detail & persistence was the key to success**
- **Automation made possible extraction & LC-MSMS batch submission of 2,500 samples/day by two CLS in 4-5 hrs (LC-MSMS ran for 20 hrs)**
- **Long term (18 mos) precision across 3 instruments (6 streams) - 4-6% CV**

Thanks for your attention