# 3 Strategies to Stop a Pervasive High-Throughput Sample Preparation Quality Problem You Likely Didn't Know You Had

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## Introduction

### Background

In all areas of laboratory testing, the clinical laboratory must ensure proper quality measures are in place to reduce the incidence of false positive and false negative results. Clinical testing using liquid chromatography-tandem mass spectrometry (LC-MS/MS) is generally perceived to be more specific than antibody-based detection methods. However, the actual limitations of this relatively new approach to testing are not fully known. The existence of regulations and guidelines for the development of high complexity assays do exist but can only address previously identified quality issues. For example, ion suppression and enhancement is a widely known phenomenon in mass spectrometry, but experiments proposed to address all known aspects of this issue in a robust manner are not standardized or explicitly required by regulatory agencies, resulting in a raised awareness of possible gaps in quality within the rapidly progressing field of clinical mass spectrometry.

An aspect of clinical mass spectrometry that has not been well studied relative to errors is the sample preparation method and associated complexity of increased batch size. The transition from individual vials to 96-well plate based, high-throughput sample preparation methods is one example. During our transition from individual vials to a 96-well based method for various quantitative drug assays, we identified an increased likelihood of falsely elevated results for samples in close proximity to significantly elevated wells at an estimated rate of approximately 4%. To mitigate this quality concern, technologists would manually identify elevated samples above a laboratory-established threshold, pinpoint up to eight surrounding samples for each elevated sample, and determine if any surrounding sample results fit established criteria for contamination due to their proximity to the well with elevated analyte concentration. The manual process required extensive analysis of the data, a working knowledge of laboratory established criteria, and included a time-consuming process of identifying surrounding wells and detection of potential contamination. Although numerous attempts were made to identify and optimize all steps in the workflow to reduce well-to-well contamination, the problem appeared to be most associated with the sample preparation methods, specifically a consequence of the use of commercially available forced evaporation equipment.

### Objectives

We present three independent methods investigated to detect, reduce and eliminate this potentially disastrous quality concern with mass spectrometry testing.

## Results and Discussion

### 1. Description of the "hotspot" quality issue

- "Hotspot" carryover – phenomenon observed in assays using a forced evaporation with commercially available equipment
- During the evaporation stage, a proportional amount of analytes present in an extracted sample can contaminate surrounding wells by being carried with the volatilized solvent (Figure 1)
- Analyte concentration in the "hotspot" well that is considerably higher than the analyte concentration in the surrounding wells has the potential to cause a clinically significant change in quantitation of the samples in the surrounding wells and thus potentially disastrous clinical implications (Figure 2A)
- Experiment design in Figure 2B used to characterize and quantify the hotspot carryover

**FIGURE 1. Cartoon diagram of the "hotspot" carryover phenomenon.**

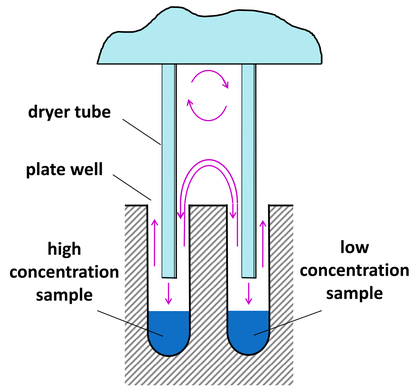



**FIGURE 2. (A) Putative "hotspots" designated as "H" may contaminate surrounding wells "X" during a forced evaporation step. (B) Plate map used for characterization and detection of hotspots with numbered "zones" used in subsequent analysis.**

A

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | | X | H | X | | | | | |
| B | | | | | X | X | X | | | | | |
| C | | | | | | | | | X | X | X | |
| D | | | | | | | | | X | H | X | |
| E | | | X | X | X | | | | X | X | X | |
| F | | | X | H | X | | | | | | | |
| G | | | X | X | X | | | | | | | |
| H | | | | | | | | | | | | |

B

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | 1 | 1 | 1 | B | 4 | 4 | 4 | 7 | 7 | 7 |
| B | | | 1 | H | 1 | B | 4 | H | 4 | 7 | H | 7 |
| C | | | 1 | 1 | 1 | B | 4 | 4 | 4 | 7 | 7 | 7 |
| D | | | 2 | 2 | 2 | B | 5 | 5 | 5 | 8 | 8 | 8 |
| E | | | 2 | H | 2 | B | 5 | H | 5 | 8 | H | 8 |
| F | | | 2 | 2 | 2 | B | 5 | 5 | 5 | 8 | 8 | 8 |
| G | | | 3 | 3 | 3 | B | 6 | 6 | 6 | 9 | 9 | 9 |
| H | | | 3 | H | 3 | B | 6 | H | 6 | 9 | H | 9 |

### 2. Approach #1: Use of a quality control program to identify putative hotspots for reanalysis.

- "HotSpot" software program – to detect, monitor and mitigate the risk of reporting false positive results due to observed well-to-well contamination
- Prior to "HotSpot", monitoring conducted with a laborious, time consuming manual method
- With a manual method, ~4% potential contamination rate observed for 96-well based assays
- The automated tool identified ~2% potential contamination rate with 0.7% falsely elevated results identified with repeat testing prior to reporting

- HotSpot program - written in the statistical language R with graphics generated using the ggplot2 package.
- Raw data exported as a text files from the Waters MassLynx software or ABSciex software packages.
- Two images generated for each data file:
  - Sample plate map with the wells identified as "hotspots" based upon laboratory established criteria Figure 3)
  - Sample interpretation plate map identifying samples potentially affected by hotspot carryover (Figure 4)
- "Fail" flag assigned to all wells where a "corrected" concentration is outside of laboratory established assay tolerances when compared to the original result from the analysis.
- Correction based on the analyte specific carryover characterized in experiments outlined in Figure 2 and takes into account the concentration of the hotspot and the potential resultant carryover.
- Performance of the HotSpot software for over 76,000 clinical samples reported in Table 1.

**FIGURE 3. HotSpot program output with the identified hotspots in red and non-hotspots in blue.**

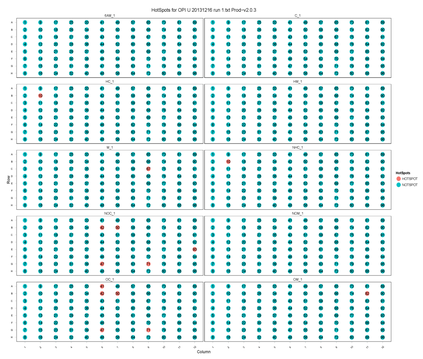


**FIGURE 4. HotSpot program output identifying the potentially contaminated wells with a pass or fail flag indicating which wells require further investigation.**

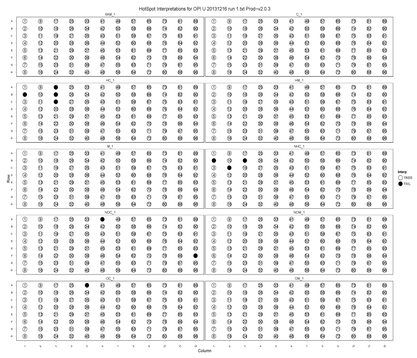


**TABLE 1. HotSpot performance in over 76,000 clinical samples across multiple drug classes.**

| Analyte | Tested Samples | Re-extracted Samples | HotSpot True Positive | HotSpot False Positive | Positive Predicted Value (%) |
|---|---|---|---|---|---|
| Amphetamine | 11,102 | 310 | 27 | 283 | 9 |
| Methamphetamine | | 331 | 17 | 314 | 5 |
| Codeine | 57,020 | 23 | 12 | 11 | 52 |
| Morphine | | 254 | 104 | 150 | 41 |
| Hydrocodone | | 251 | 102 | 149 | 41 |
| Hydromorphone | | 19 | 4 | 15 | 21 |
| Oxycodone | | 513 | 241 | 272 | 47 |
| Methadone | 7497 | 7 | 1 | 6 | 14 |
| EDDP | | 49 | 14 | 35 | 29 |
| THC | 1010 | 5 | 2 | 3 | 40 |
| Total | 76,629 | 1762 | 524 | 1238 | 30 |

### 3. Approach #2: Use of a High End Mass Spectrometer and Reduced Sample Volume.

- "Hotspot" carryover proportional to the analyte concentration in the hotspot sample
- Significant reduction of hotspot contamination for an opioid panel assay achieved by considerable reduction in specimen volume afforded by the use of a high end mass spectrometer.

#### Sample Preparation

- 25 µL of specimen, calibrator, QCs
- 500 µL of deuterium-labeled IS in 0.1 M Na phosphate buffer
- 425 µL of 0.1 M sodium phosphate buffer
- 925 µL sample pipetted onto SPEware PSCX SPE column array
- Wash each column with 1 mL of each of the following:
  - Wash 1 – Nanopure water
  - Wash 2 – 0.1 M acetic acid
  - Wash 3 – methanol
- Dry the columns for 3 min
- Elute samples with 0.5 mL of freshly prepared 75:20:5 (v/v/v) ethyl acetate:isopropanol:ammonium hydroxide
- Evaporate elution solvent with evaporative dryer at 40 ºC

#### LC-MS/MS

- AB Sciex Triple Quad™ 5500 mass spectrometer
- ESI in positive mode (TurbolonSpray interface)
- CTC PAL HTC-xt-DLW autosampler
- Agilent 1260 Infinity Series LC system
- Waters HSS T3 column (2.1 x 50 mm, 1.8 µm)
- MP-A: 5 mM $NH_4$ formate + 0.05% formic acid in water
- MP-B: 0.2% formic acid in methanol
- Flow rate 0.5 mL/min
- Injection volume 5 µL
- Column temperature 28 °C
- Analytical gradient time 4.8 min

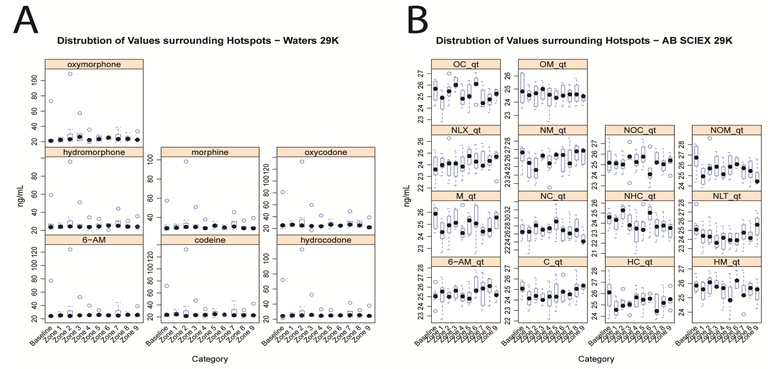
**TABLE 2. Method comparison for the reduced sample volume opioid quantitative method.**

| Analyte | Deming regression | $S_{y/x}$ | R | n |
|---|---|---|---|---|
| Morphine | y = 0.968x + 23.47 | 121.32 | 0.9941 | 106 |
| Morphine ≤400 ng/mL | y = 0.964x + 2.96 | 11.60 | 0.9946 | 53 |
| Oxymorphone | y = 1.011x + 12.25 | 48.20 | 0.9962 | 105 |
| Oxymorphone ≤400 ng/mL | y = 1.130x - 1.57 | 11.89 | 0.9901 | 88 |
| Hydromorphone | y = 1.047x + 0.14 | 85.80 | 0.9893 | 166 |
| Hydromorphone ≤400 ng/mL | y = 0.995x + 3.25 | 11.47 | 0.9900 | 140 |
| Codeine | y = 1.082x + 20.08 | 175.44 | 0.9740 | 81 |
| Codeine ≤400 ng/mL | y = 1.110x - 2.32 | 23.31 | 0.9797 | 48 |
| Oxycodone | y = 1.155x + 29.48 | 189.71 | 0.9879 | 105 |
| Oxycodone ≤400 ng/mL | y = 1.263x - 9.38 | 30.56 | 0.9578 | 37 |
| Hydrocodone | y = 0.963x - 2.57 | 132.26 | 0.9913 | 129 |
| Hydrocodone ≤400 ng/mL | y = 0.942x - 0.02 | 12.54 | 0.9952 | 57 |
| 6-AM | y = 1.184x - 52.89 | 167.16 | 0.9885 | 35 |
| 6-AM ≤400 ng/mL | y = 0.946x - 1.99 | 33.98 | 0.9650 | 17 |

**TABLE 3. Selected opioid assay performance characteristics.**

| Parameter | Concentration (ng/mL) |
|---|---|
| LOD | 5 |
| LOQ | 10 |
| Cutoff | 20 |
| ULOQ | 4,000 |
| AMR | 20 – 4,000 |
| CRR | 20 – 100,000 |

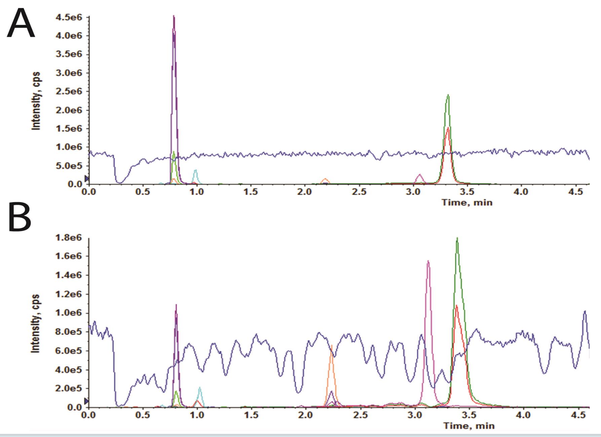
**FIGURE 5. Hotspot characterization comparison between the existing assay on Waters Acquity® TQD (A) and the new assay on AB SCIEX Triple Quad™ 5500 (B). Hotspots were ~29,000 ng/mL of the respective analytes.**




### 4. Approach #3: Use of a Dilute & Shoot Method.

- Simple way to circumvent hotspot carryover
- Eliminates requirement for forced evaporation step in sample preparation
- Considerable quality and reproducibility concerns - vulnerable to matrix effects
- Internal standard does not always adequately compensate for matrix effects
- Thorough investigation of and compensation for matrix effects crucial for method robustness

**FIGURE 6. Unacceptable variation in matrix suppression profiles with dilute and shoot sample preparation. (A) A sample with minimal matrix effects and (B) a sample with considerable matrix effects.**




## Summary and Conclusions

We present an underappreciated quality concern that is most likely a pervasive issue in clinical mass spectrometry based assays that use forced evaporation in 96-well format. Three independent methods were investigated successfully to identify, reduce or eliminate hotspot contamination. Moreover, one if not several of the strategies reported here could be implemented in any clinical mass spectrometry laboratory. At the very least, we strongly encourage each laboratory utilizing a forced evaporation step actively investigate the potential for carryover due to "hotspots" to ensure the delivery of accurate mass spectrometry-based results for the highest level of patient care.