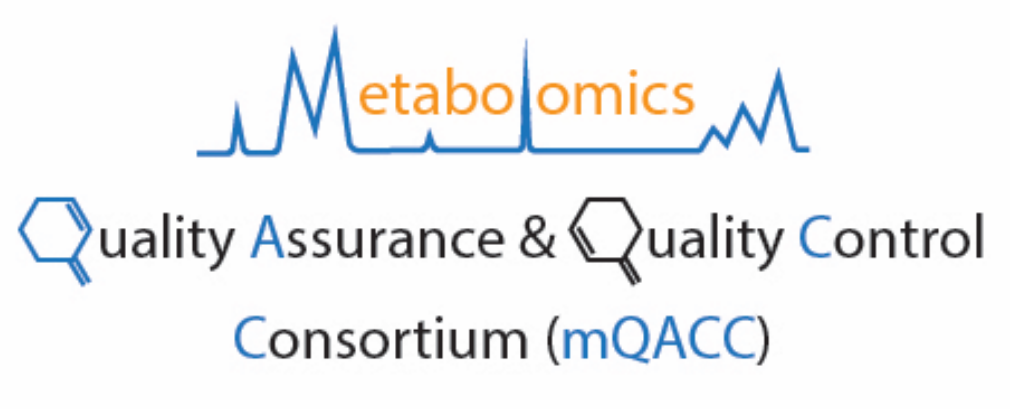
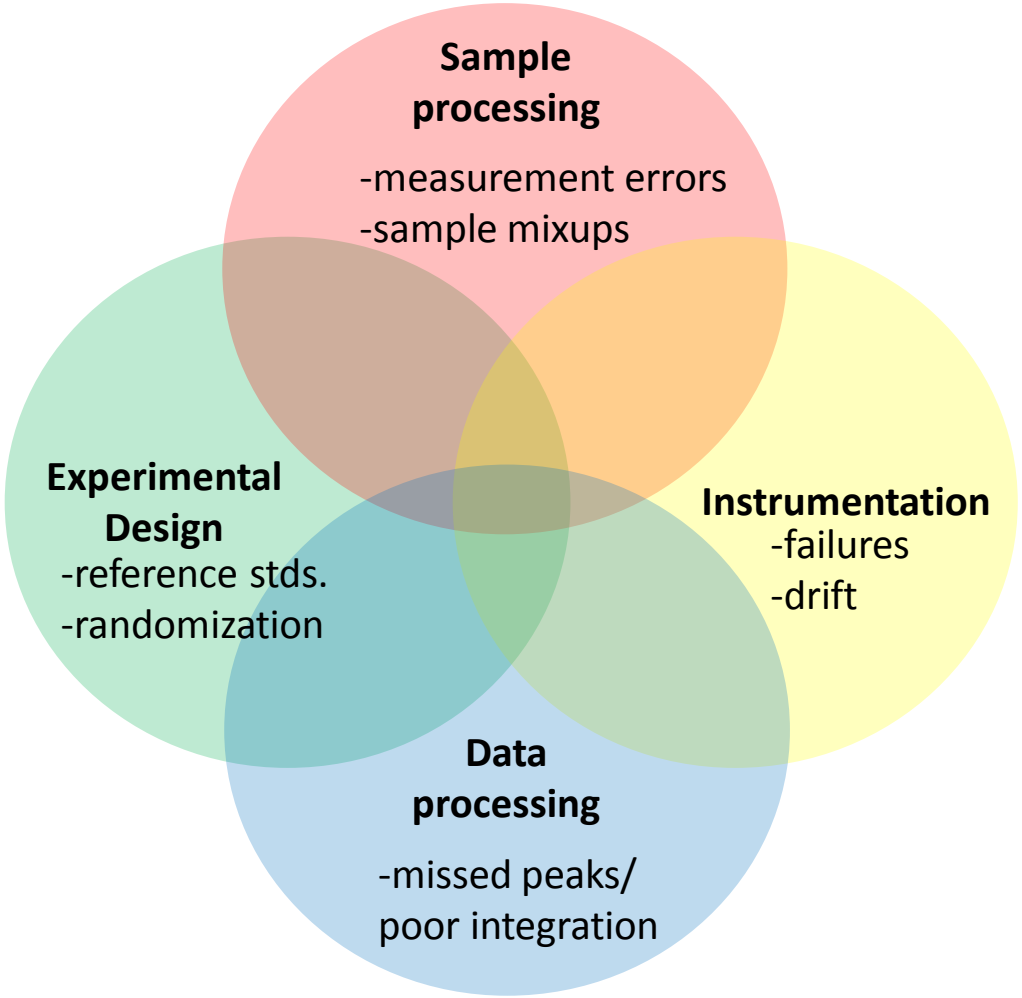


QC in Untargeted Metabolomics

Ping-Ching Hsu
Clary Clish
Dan Bearden

Introduction

- The purpose of quality control (QC) is to monitor the performance of metabolomics workflows against standards to detect problems and inform corrective actions
- Why do we need QC in untargeted metabolomics?
- Metabolomics is a complicated process; variability and problems may come from a number of sources, individually or in combination



Outline

- General QC practices for untargeted metabolomics
  - Study design & QC practices used during data acquisition
  - QC practices used during data processing

- Real life examples
  - Replicates in LC-MS
  - QC in larger studies of human disease
  - Use of test materials (e.g. NIST Standard Reference Materials; SRMs)

Study design and QC during data acquisition

- Column conditioning
  - SOPs for preparing LC columns and evaluating performance
- Randomization of sample analysis order
  - Mitigate systematic bias
- Pooled samples
  - Regular, repeated measures of a representative sample
  - “Real time” review during analysis of large sample numbers
- Blanks
  - Identification of system contaminants and batch-to-batch carryover of biological sample
- Replicates (technical and process)
  - Evaluation of reproducibility
- Internal standards
  - “Real time” review during analysis of large sample numbers
  - Acceptance criteria and triggering repeats
- Reference samples
  - Metabolite standards, long term reference samples, Standard Reference Materials (SRMs)

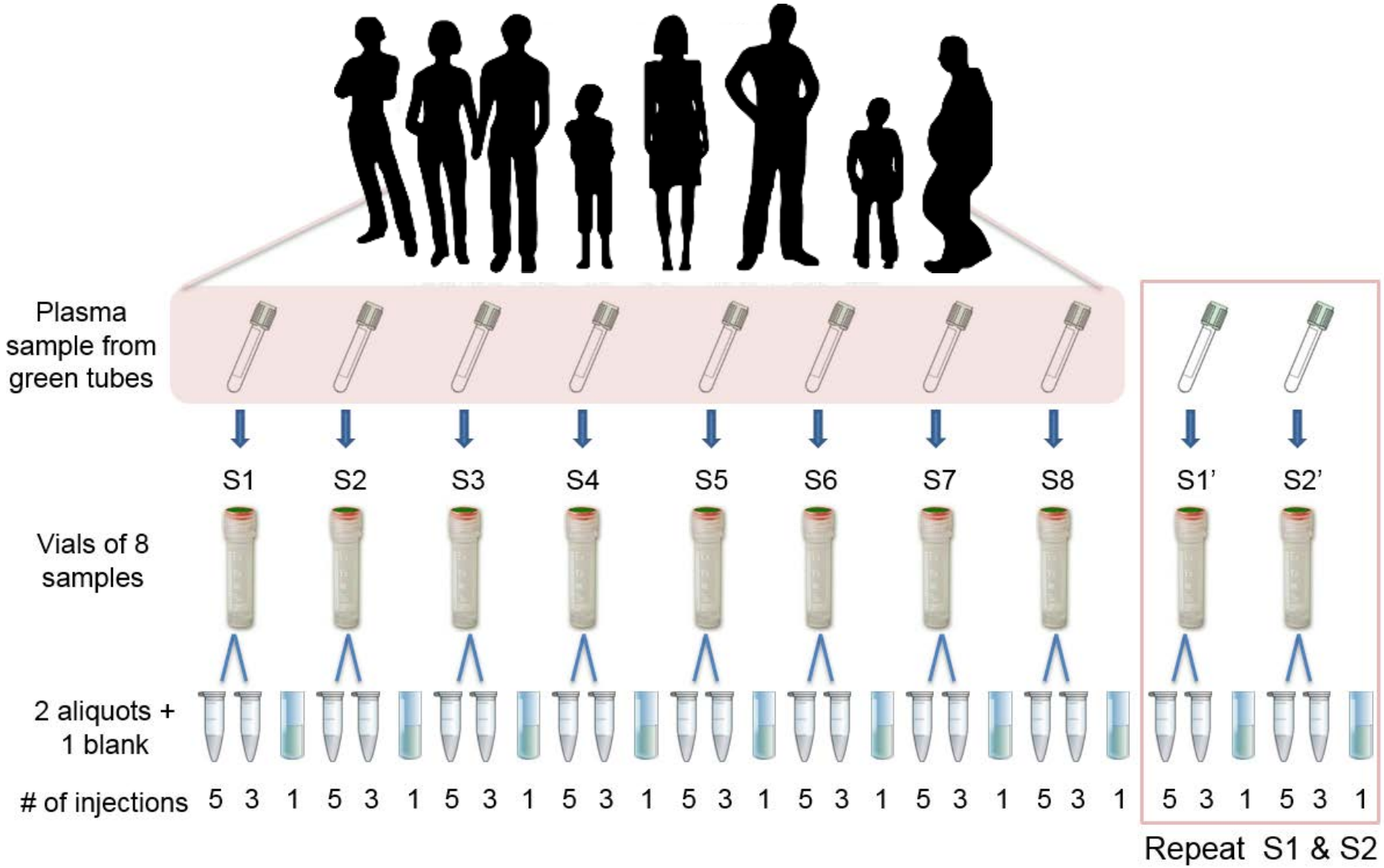
QC during data processing

- Pooled QC samples
  - Overlay of raw data (e.g. TIC) among pooled QCs
  - Evaluation of coefficients of variation for every metabolite
- Review of internal standards among all samples
- Principal component analysis
  - Identification of obvious outliers
  - Confirmation of clustered pooled QCs, replicates, and/or reference samples
  - Batch effects
- Correlation of replicate samples
- Manual review of peaks
  - Confirmation of accurate peak integration (mainly “knowns”)
- Peak filtering and data reduction
  - Redundant ion features, features with many missing values, features above a CV threshold, …

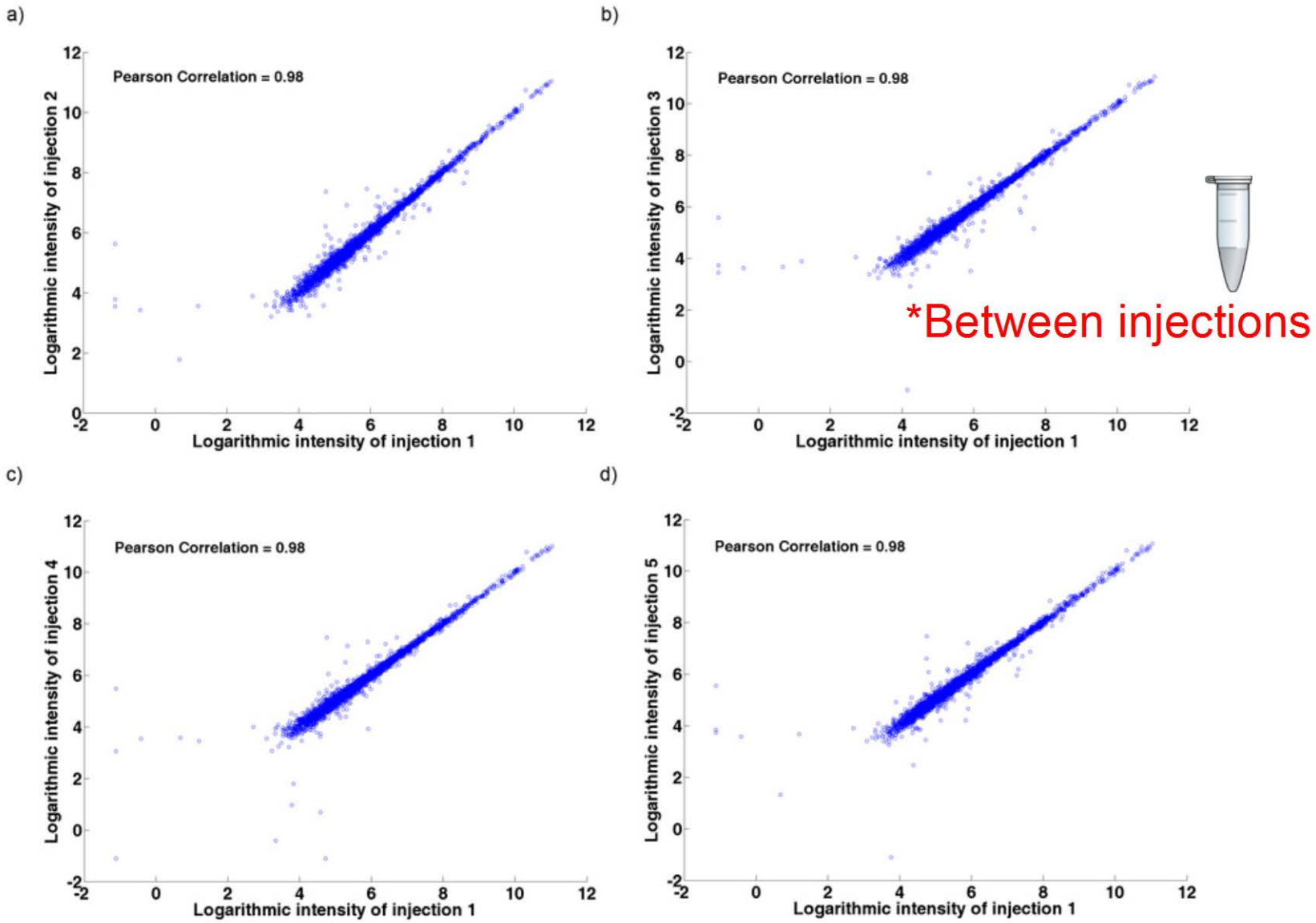
Example 1:
Replicates in LC-MS

Ping-Ching Hsu

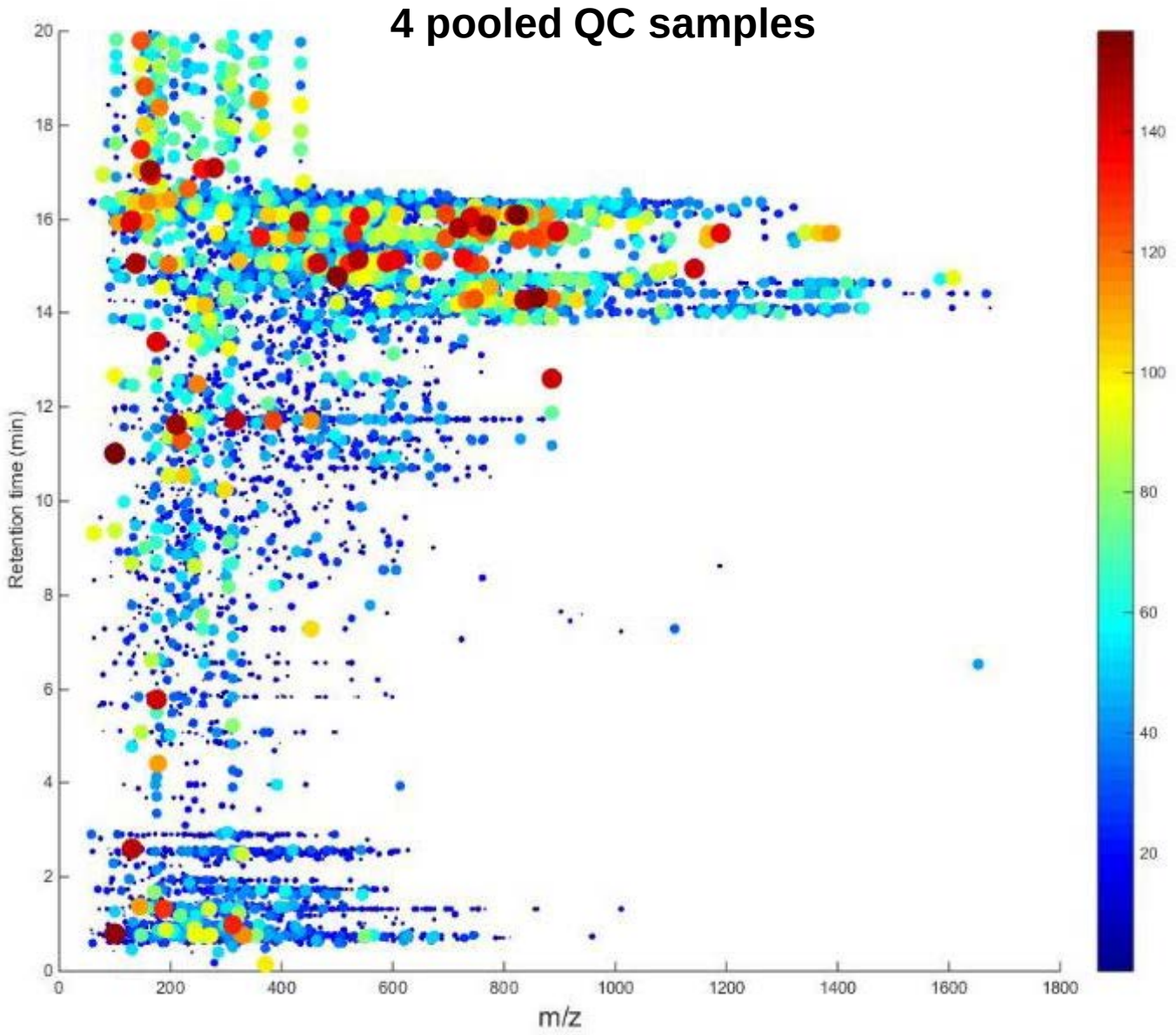
Experimental design used to test the reproducibility of UPLC-QTOF-MS



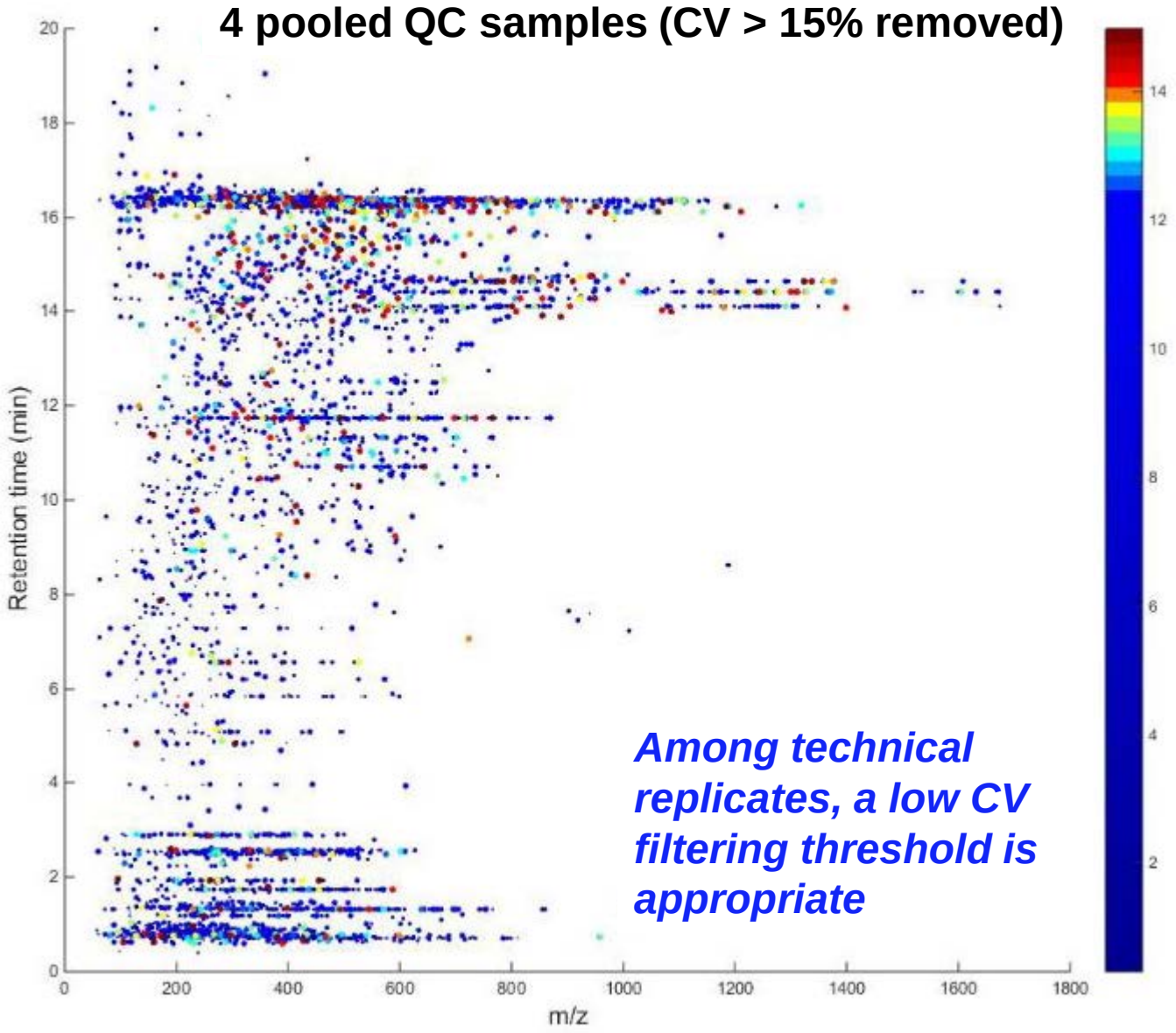
Evaluation of the variation due to the measurement noise



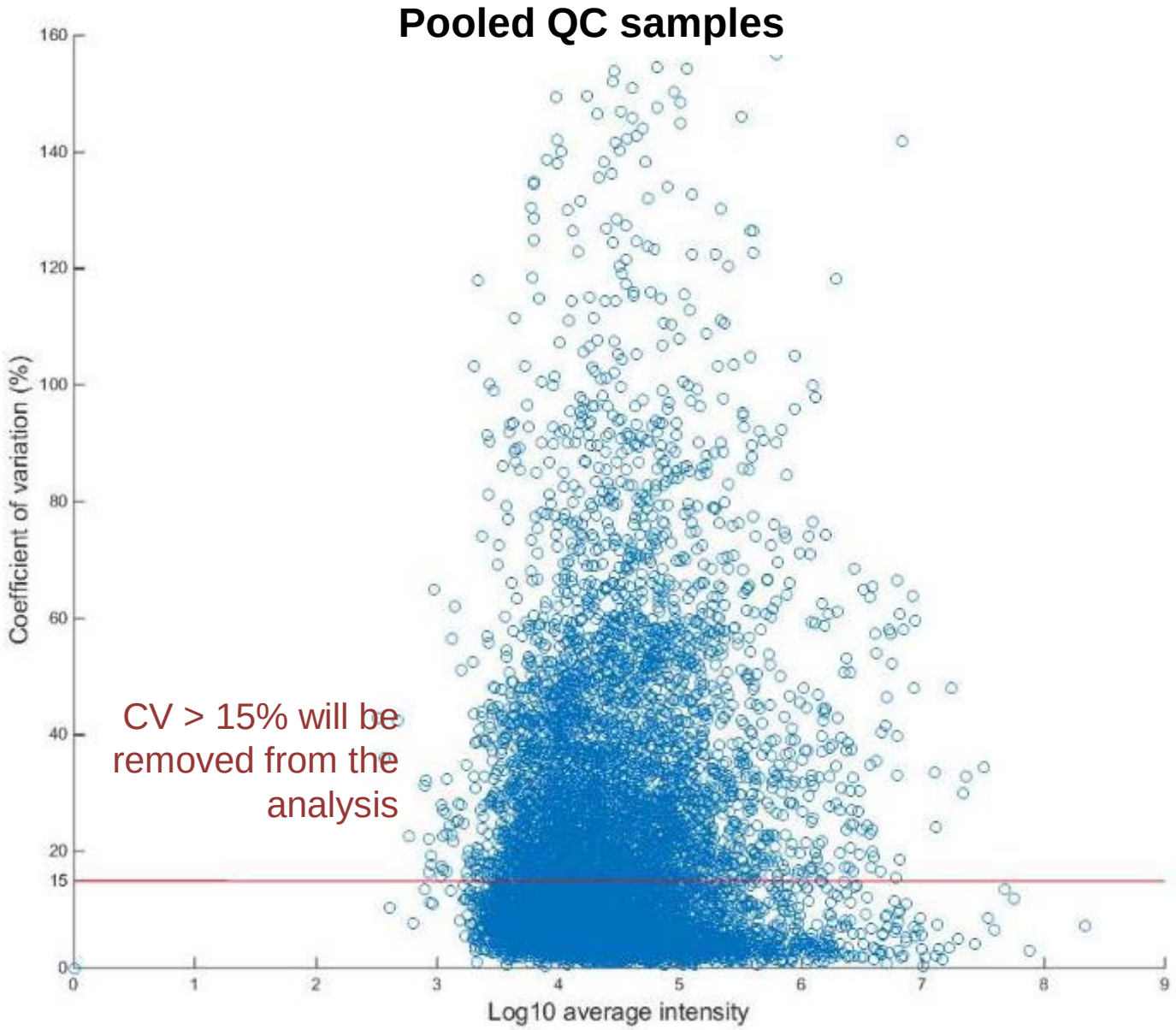
Distribution of the estimated measurement error in CV(%)



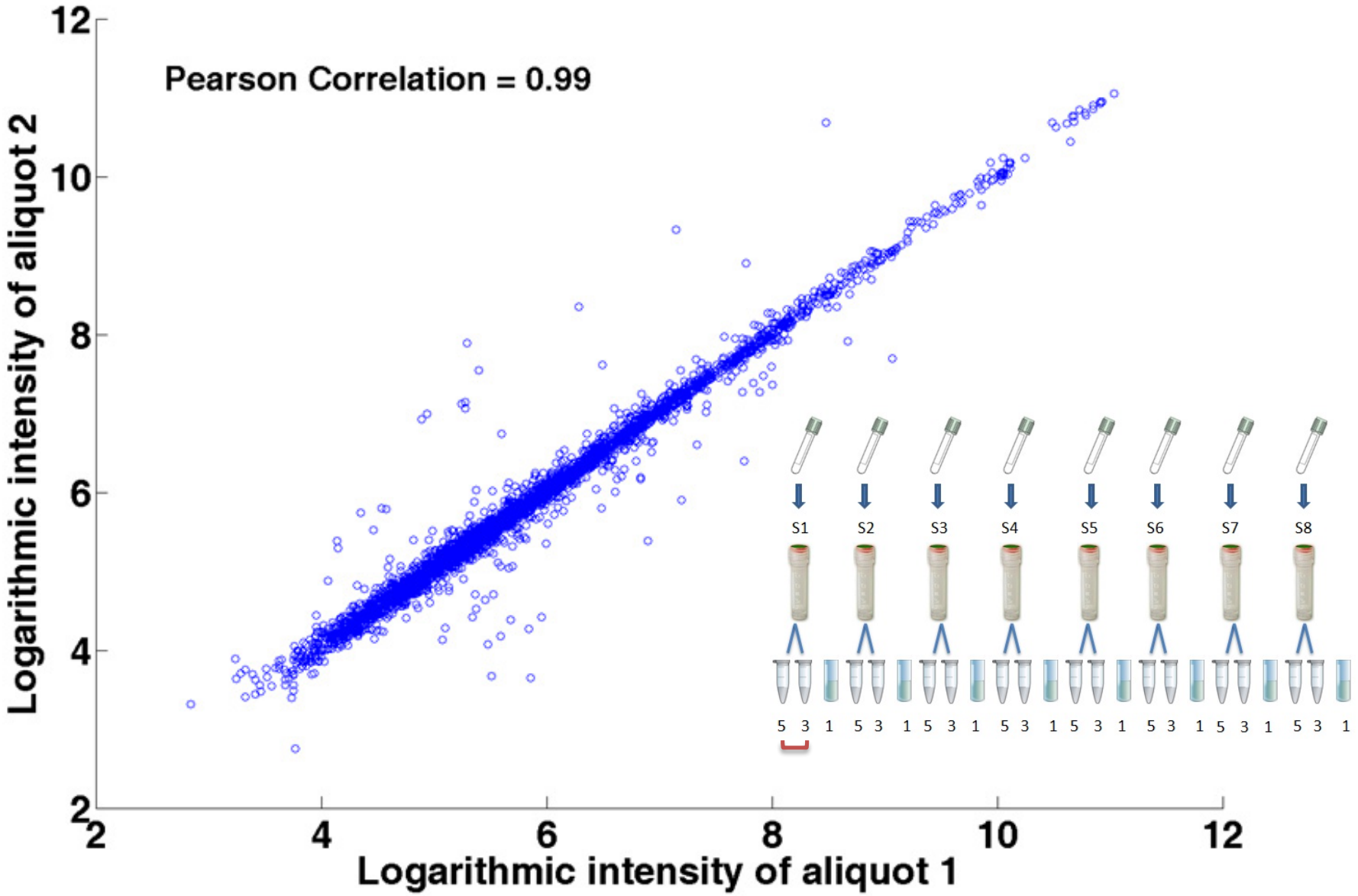
Filtering out features based on a CV threshold



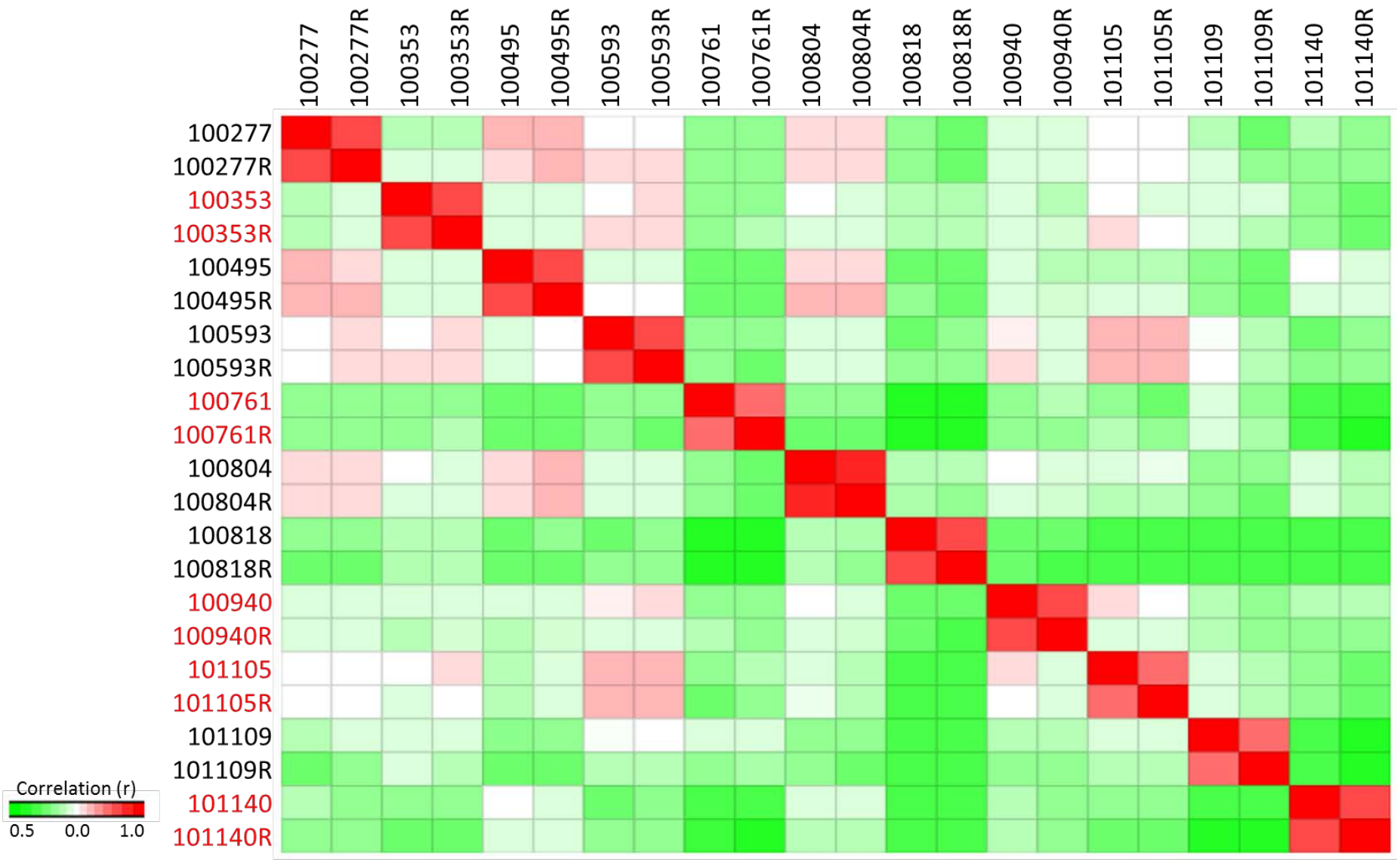
Scatter plot of the estimated measurement noise in CV (%)



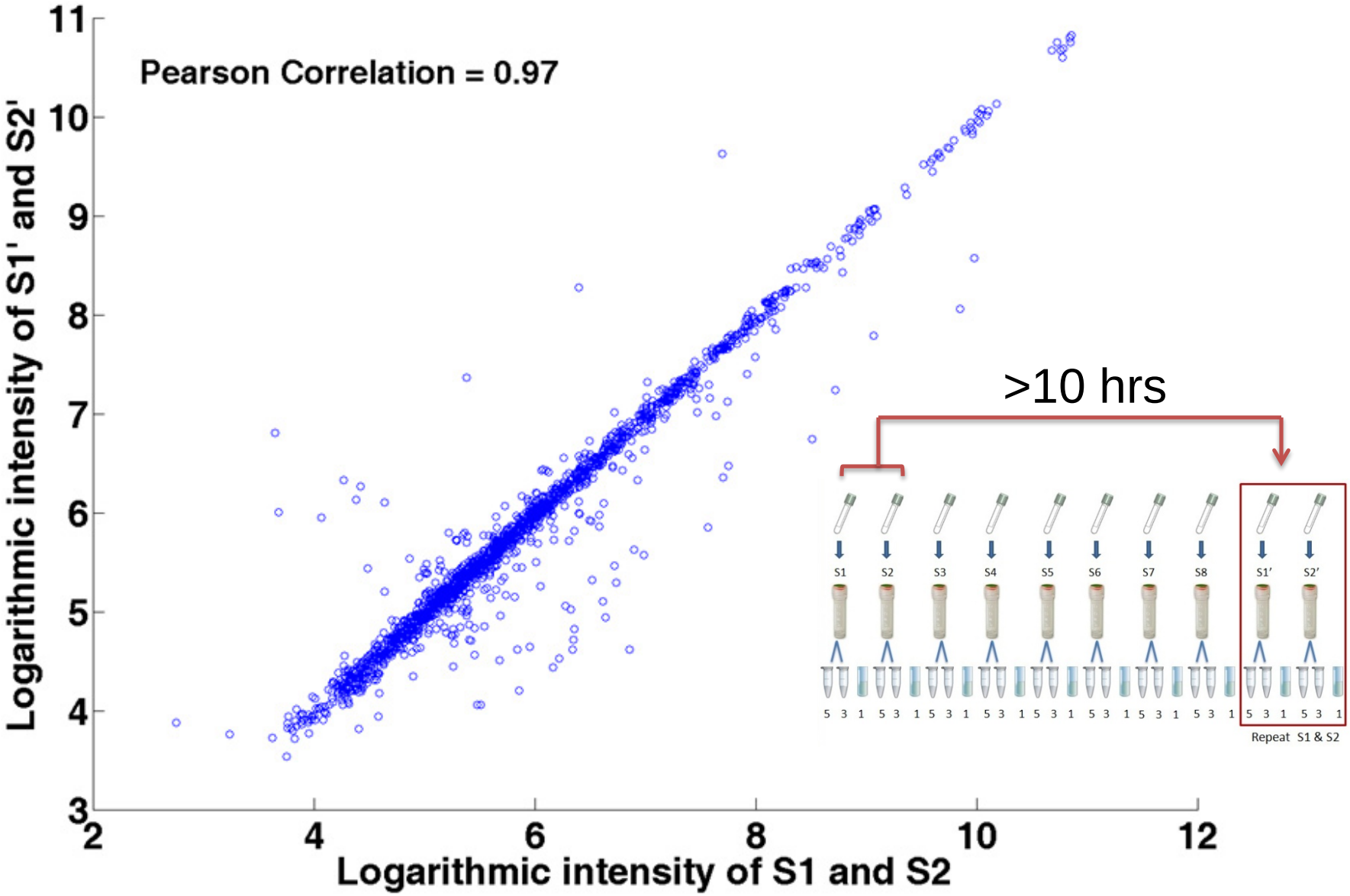
Evaluation of the variation due to sample preparation



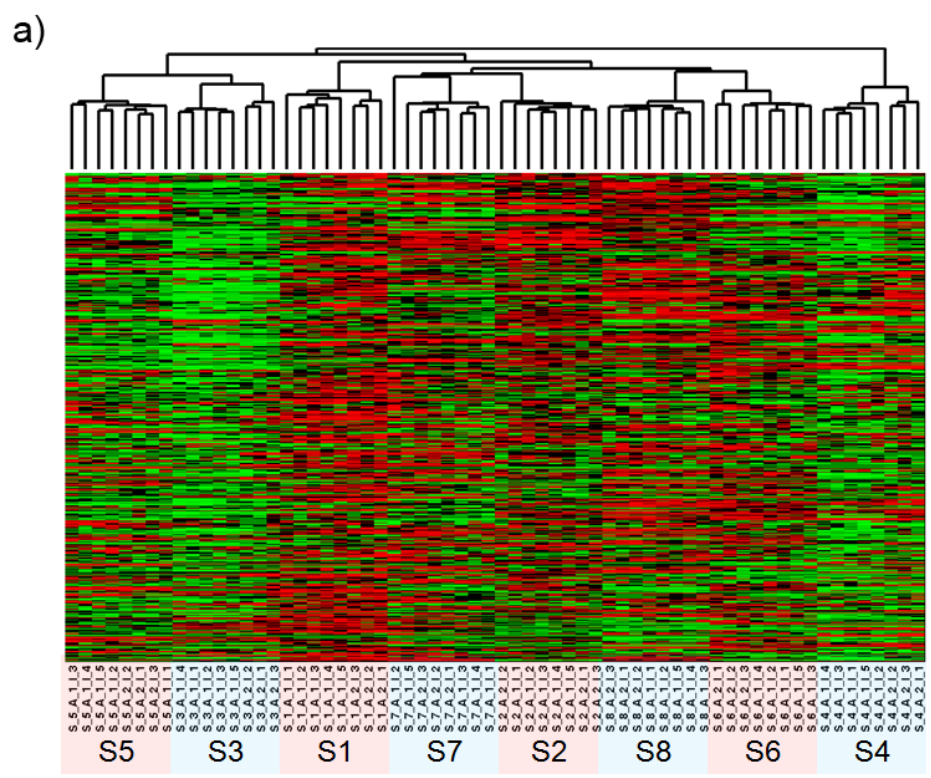
Correlation of replicates

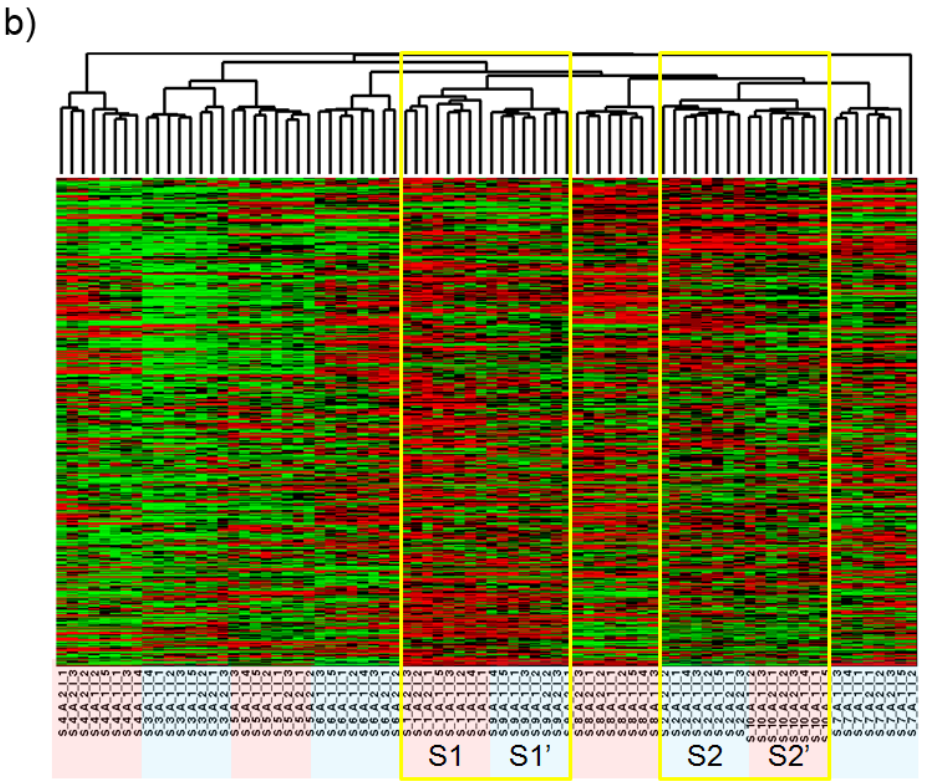


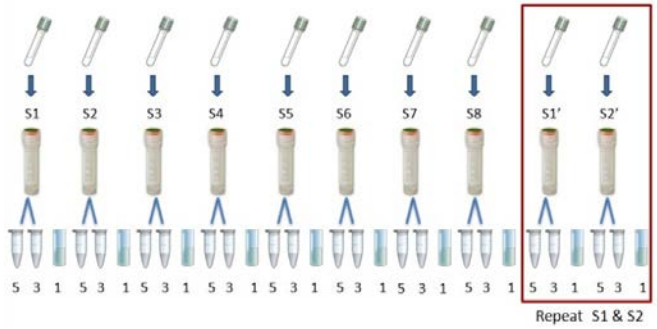
Evaluation of the effects on run time, measurement error & sample preparation variation



Hierarchical clustering of all metabolites with and without analytical replicates



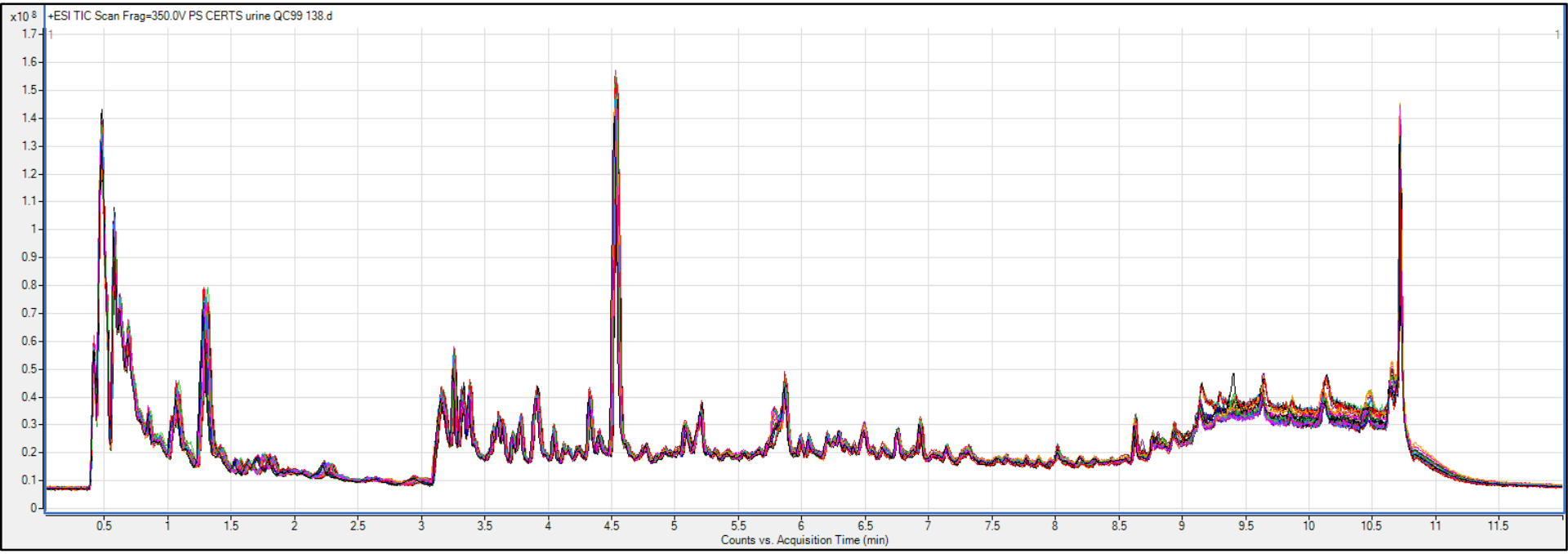




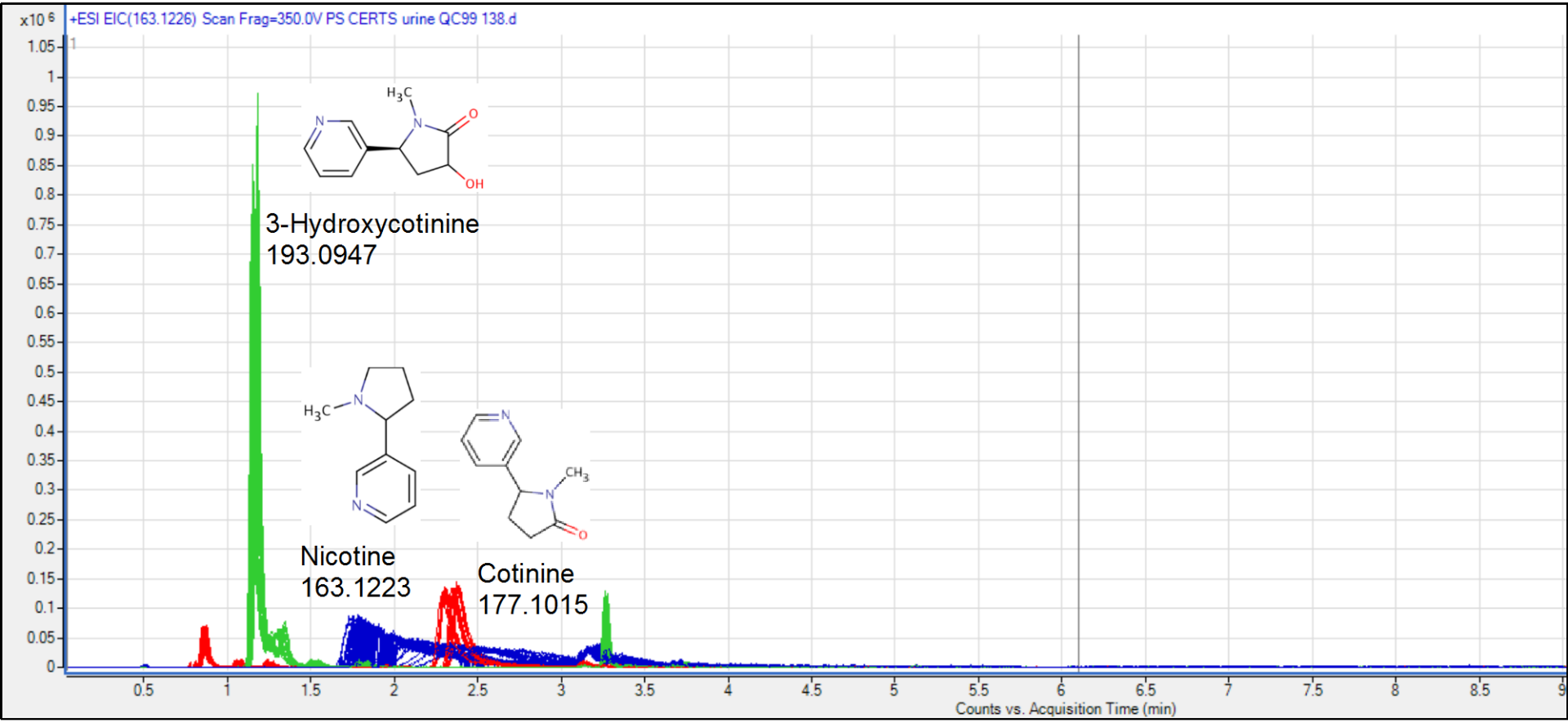
Summary of the measured variation in human plasma samples

	Mean of CV (%)	Median of CV (%)
Technical variation	7.2-8.8	5.7-7.2
Experimental variation	7.2-12.3	4.6-8.7
Biological	22.0-22.3	17.2-18.2

Visual QC: 31 TICs overlaid



Overlay of 3 nicotine metabolites among 31 QCs



Filtered out from the analysis ➡

	Mass	CV(%)
Nicotine	163.1226	22.33
Cotinine	177.1023	2.91
3-hydroxycotinine	193.0974	3.28

Summary Example 1

- It's important to pilot methods using technical and processing replicates in order to understand analytical performance
  - This type of pilot may be done before engaging in large studies
- Inclusion of technical and/or processing replicates may be feasible for smaller studies
- Measurement variation and sample preparation variation are generally low when samples are measured consecutively
  - Therefore, a low CV threshold may be applied to filter signals from replicate data

Example 2:
QC in larger studies of human disease

Clary Clish

Challenges associated with applying nontargeted methods to discover early indicators of disease in humans

- Metabolic dysregulation may be very modest early in disease
  - E.g. metabolite levels may differ by only 10% between incident cases and controls
  - Large sample numbers are needed for statistical power
- Funds tend to need to be applied to increase biological ”n’s” rather than cover cost of technical “r’s”
  - Replicates are generally out
- It’s often necessary to analyze samples over multiple LC columns and over periods of months
  - Risk of complications due to batch effects are high
- Data must be standardized across batches
- Small differences in measured retention times and MS mass calibration complicates “aligning” nontargeted features among batches

QC approach for large, nontargeted LC-MS-based studies

Reference mixtures analyzed before and after to assure system performance

Internal standard(s) added in first step of sample extraction

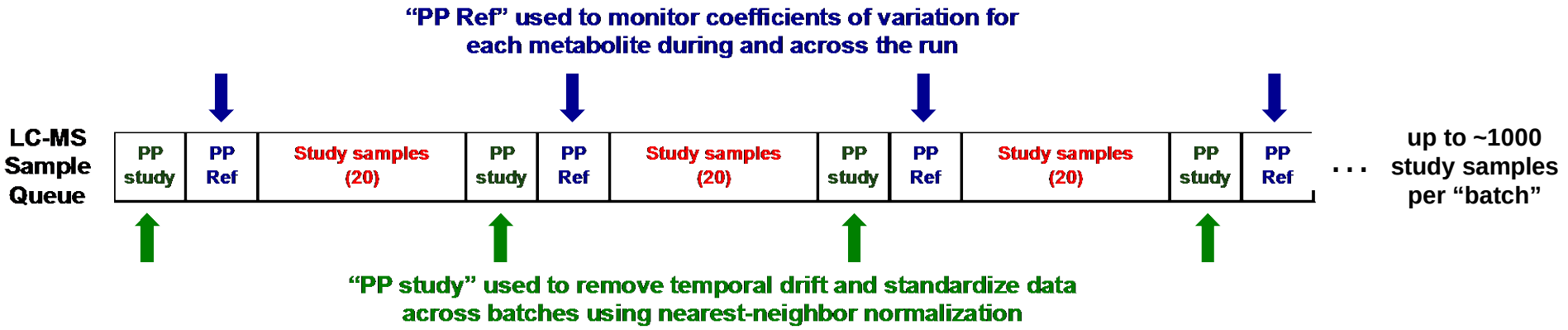
- monitored during analyses
- may be used to standardize data

Pooled study sample: analyzed every 20 study samples

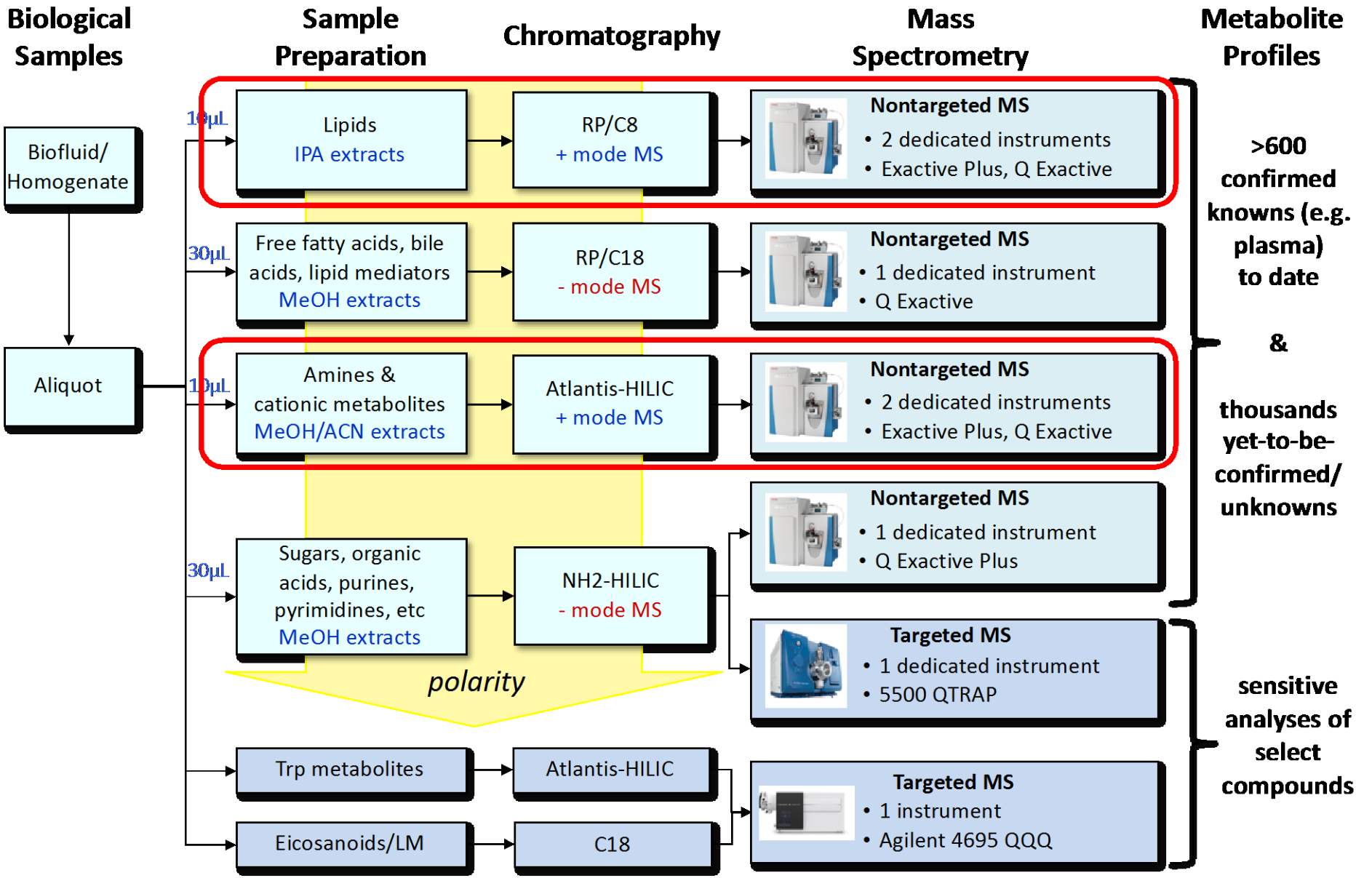
- used to standardize data across datasets

Second pooled reference sample, analyzed every 20 study samples

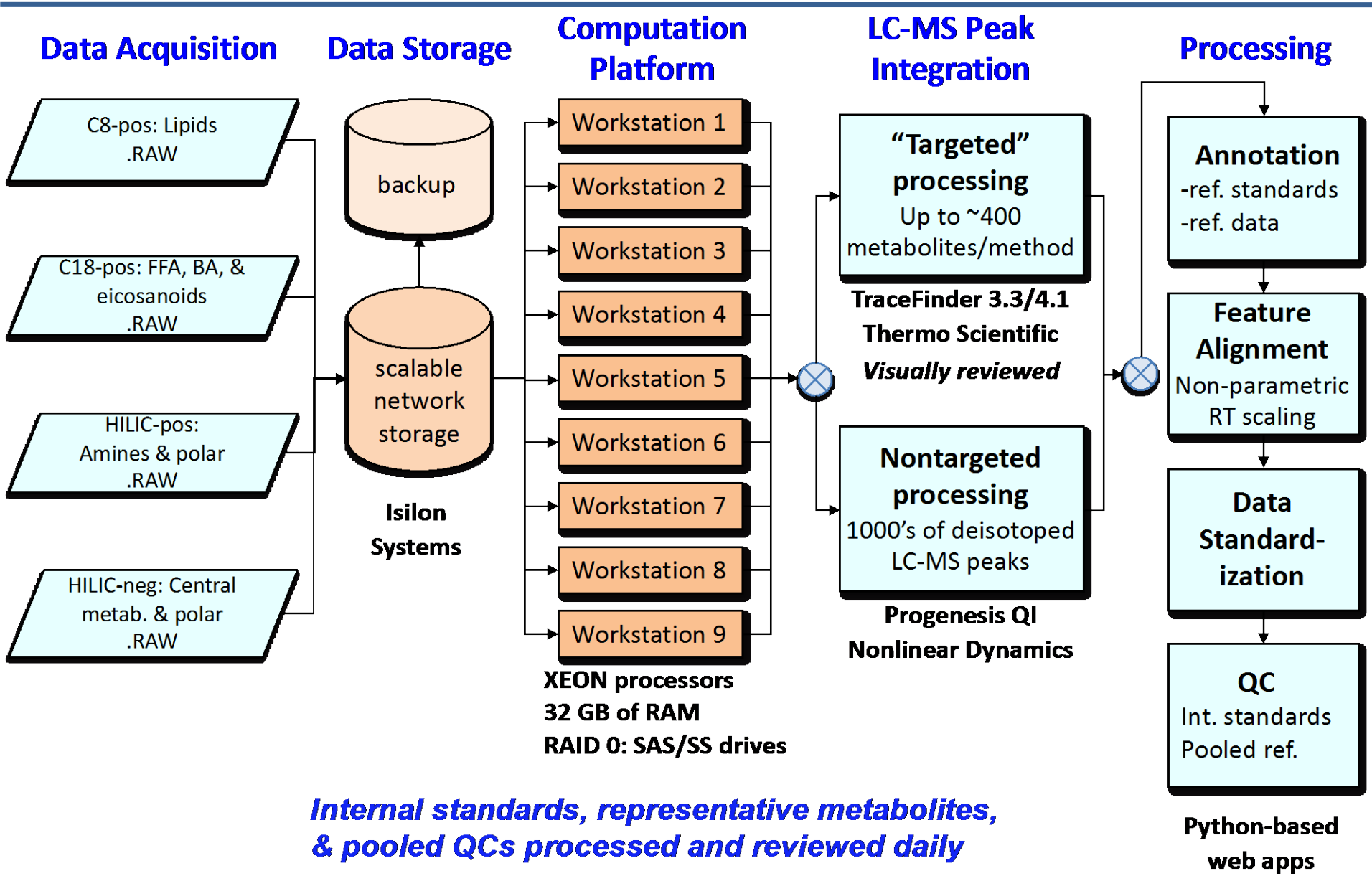
- used to assess: overall reproducibility & impact of standardization procedures
- we typically use the pooled study sample



E.g. Pilot study: 2000 human plasma samples from TOPMed

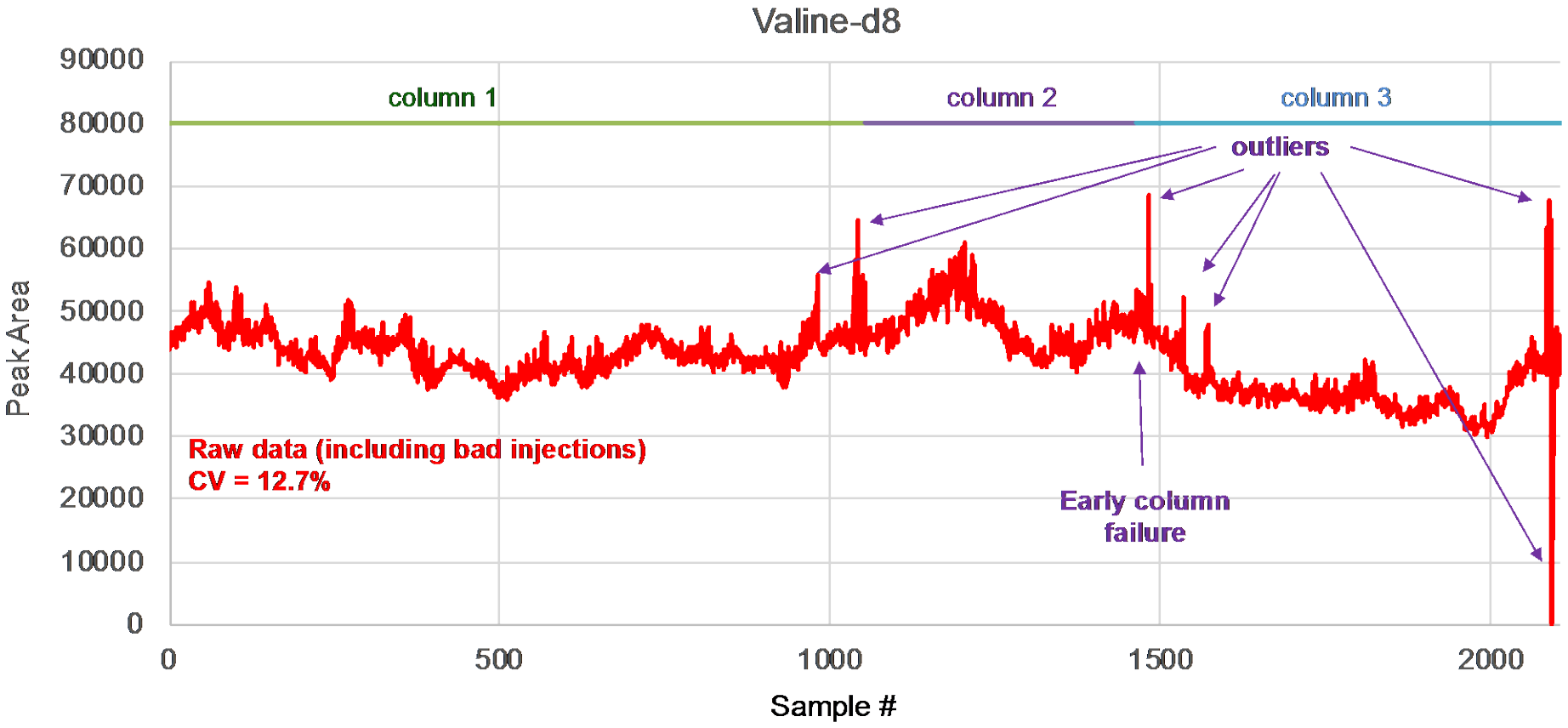


Nontargeted LC-MS metabolomics data processing workflow



- Analysis plan:
  - 1000 samples/column x 2
  - 10% pooled QC samples
- Analyze samples nearly continuously for 1.5-2 months/method

Problems illuminated by QC:
- Injection problems
- Instrument noise and drift
- Failure during second HIL-pos column
- Samples flagged for re-analysis



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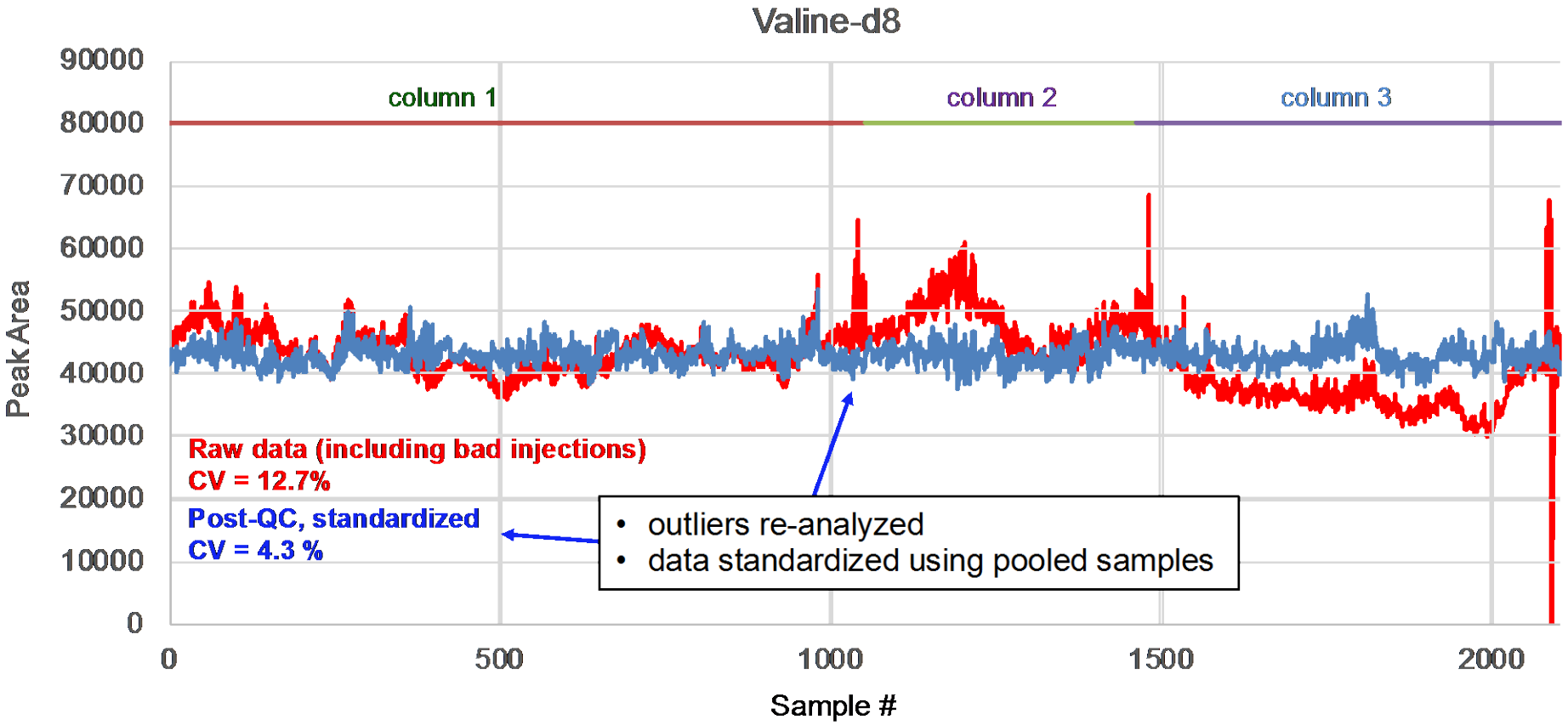


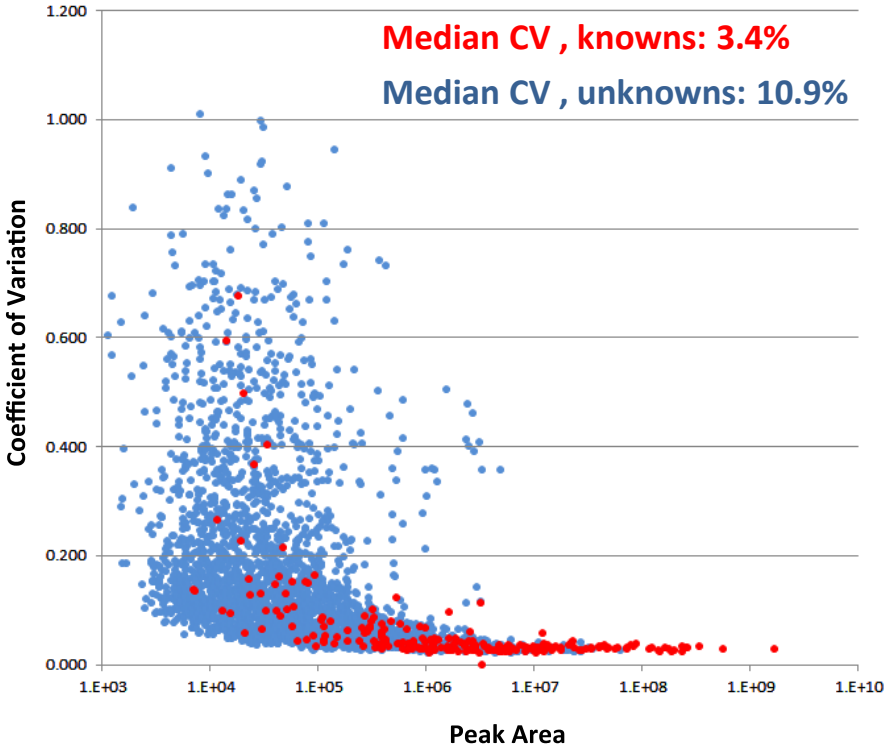
Evaluating reproducibility of pooled QC samples: Pilot study of 2000 TOPMed plasma samples

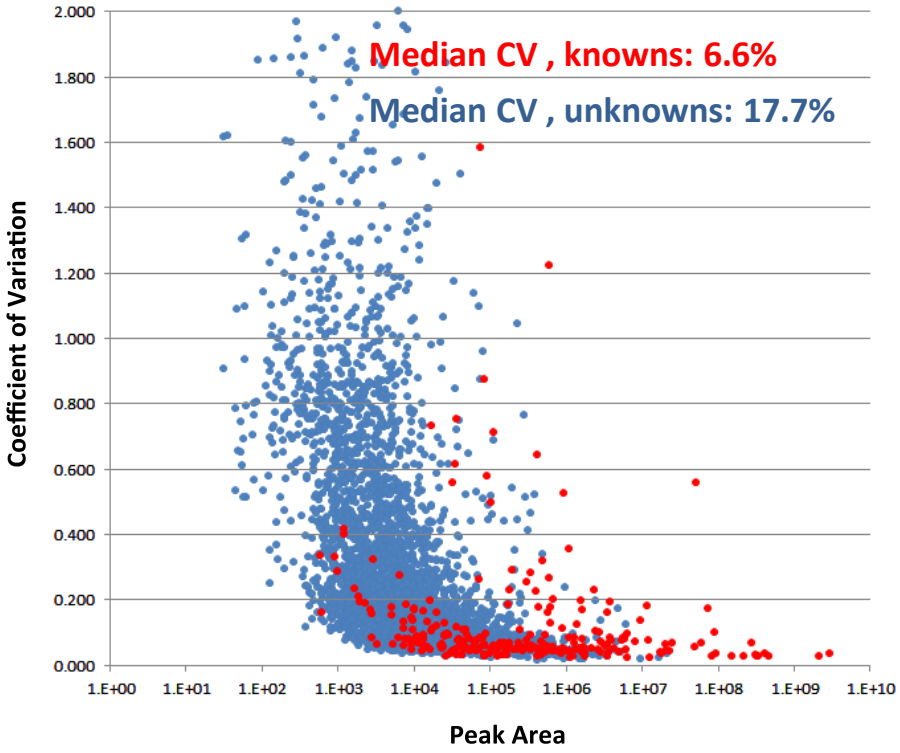
C8-pos

- Nontargeted analysis of lipids
- 2 columns; ~1.5 months
- 228 lipids of known ID
- 2662 unknowns aligned between two columns
- n = 98 pooled QC samples

HILIC-pos

- Nontargeted analysis of polar metabolites
- 3 columns; 2 months
- 253 confirmed knowns
- 3966 unknowns aligned across three columns
- n = 104 pooled QC samples





Do nontargeted methods really measure thousands of unique metabolites in a single analysis?

- No
- Why all the peaks then?
  - Metabolites may form multiple, different ion adducts in the MS ionization source, e.g. $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[M+NH_4]^+$, etc.
  - Molecules may fragment during the ionization process to yield additional product ions
  - dimers, trimers, etc. may form in the MS ionization source
  - many contaminants from both solvents and consumables are measured
  - some data processing algorithms do not “de-isotope” the data (e.g. ^{13}C isotopologue peaks)
  - noise
- Data may be “cleaned” by evaluating correlations among co-eluting peaks and selecting the dominant ion (e.g. $[M+H]^+$)
- However, a multiplicity of ions can sometimes be helpful for ID

Summary Example 2

- It’s generally cost prohibitive to analyze replicates of biological samples in large studies
- Periodic analysis of pooled samples enables both standardization of data between batches and evaluation of measurement reproducibility for all signals
- Daily monitoring of QC data is essential for early detection of problems
- See posters P-349 example of application to a 7000+ sample study and poster P-318 for details on the processing workflow

Sample 3 slides removed per NCI copyright requirements

Concluding remarks

- Untargeted metabolomics presents unique challenges for QC
  - Methods measure both knowns and unknowns
  - Internal standards are not immediately available for unknowns (by definition)
  - A single metabolite can give rise to a number of redundant features
  - It is often difficult to distinguish contaminants from actual metabolites

- Untargeted metabolomics QC procedures are often customized for specific analytical techniques and experimental designs, but there are common elements:
  - Randomization of sample analysis order to avoid systematic bias
  - Internal standards for real time and post-acquisition QC
  - Pooled reference standards, also for for real time and post-acquisition QC
  - Inclusion of reference samples, such as NIST SRM