Research and Development on Human Biospecimen Integrity

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BRN Symposium

March 29, 2011

PROGRAM OVERVIEW

CAPRION PROTEOMICS

Overall goal:

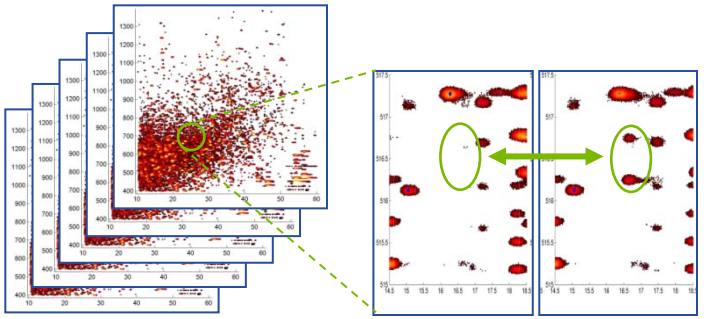
- Best practices for proteomics analysis of blood-derived biospecimens
  - Collection, manipulation and storage of samples
    - Guidelines to follow
    - Sample quality assessment assay
    - Tools to facilitate each step

MS-BASED BIOMARKER DISCOVERY PROCESS

CAPRION PROTEOMICS

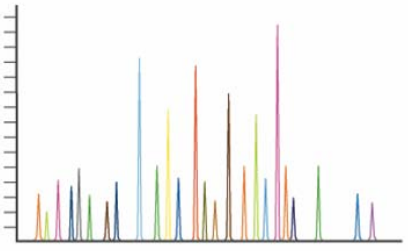
Biomarker Discovery

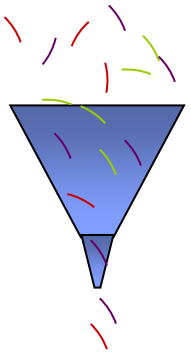
- Label-free, gel-free quantitative mass spectrometry
- Non-hypothesis based discovery approach
- Profile 1000's of proteins in 100's samples
- Identify differentially expressed proteins as candidate biomarkers



Multiplexed Assays

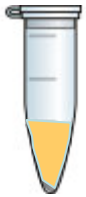
- Quantify 1 to 700 proteins in a single "MRM" assay
- Proteins from mass spec, literature, transcriptomics, etc.
- Rapid assay development
- Ab-free or ab enrichment
- Synthetic labeled standards for absolute quantification
- Profile candidate markers in 1,000's samples





BIOMARKER DISCOVERY PROCESS: PROTEOMIC EXPRESSION PROFILING

CAPRION PROTEOMICS

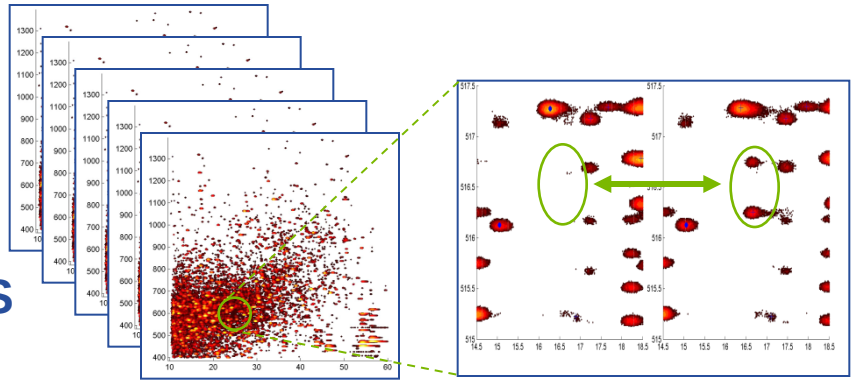


CSF
Plasma
Serum
Urine
Other

Depletion of abundant proteins

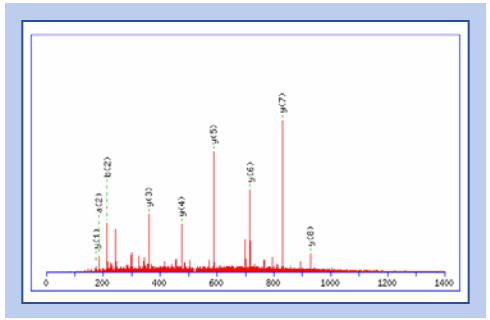
Digestion to peptides

LC-MS



Informatics-based
Quantitative Peptide Expression Profiling

Statistical Analysis



Targeted Sequencing

>gi|41327732|ref|NP_958439.1| epidermal growth factor receptor isoform b [Homo sapiens]
MRPSGTAGAALLALLAALCPASRALEEKK**VCQGTSNK**L
TQLGTFEDHFLSLQRMFNNCEVVLGNLEITYVQRNYDL
SFLKTIQEVAGYVLIALNTVERIPLENLQIIRGNMYYE
NSYALAVLSNYDANKTGLKELPMR**NLQEILHGAVR**FSN
NPALCNVESIQWRDIVSSDFLSNMSMDFQNHLGSCQKC
DPSCPNGSCWGAGEENCQKLTK**IICAQQCSGR**CRGKSP
SDCCHNQCAAGCTGPRESDCLVCRKFRDEAT……..

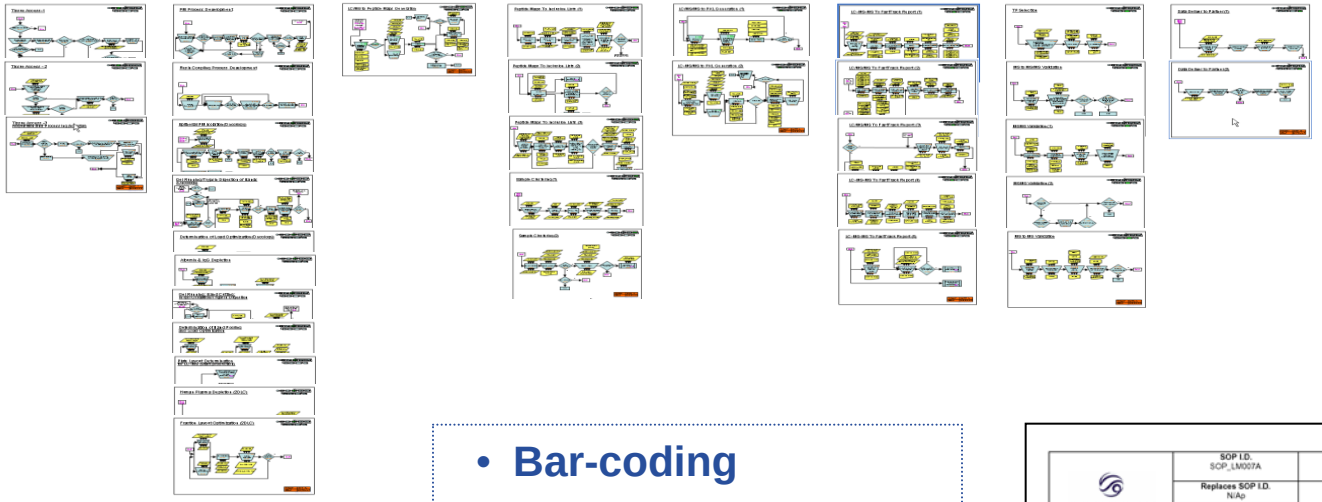
Protein Identification

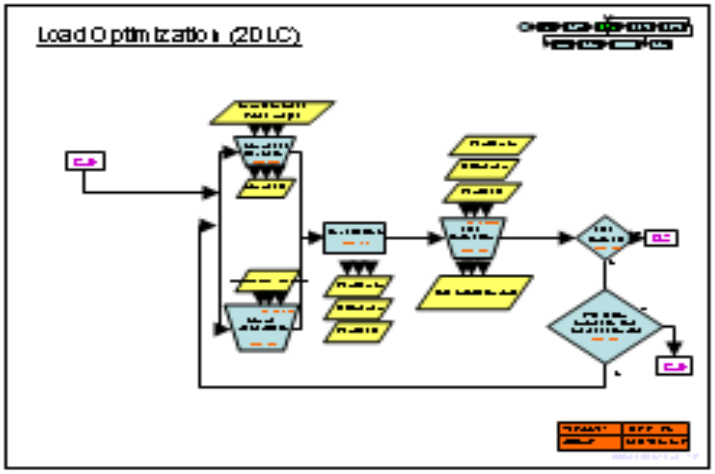
QA ENVIRONMENT AND PROCESS ARE ESSENTIAL TO REDUCING VARIABILITY

CAPRION PROTEOMICS

Gated Processes

Sample Collection | Sample Prep | LC-MS | Peptide Profiling | MSMS | Protein ID | Platform Auditing | Delivery

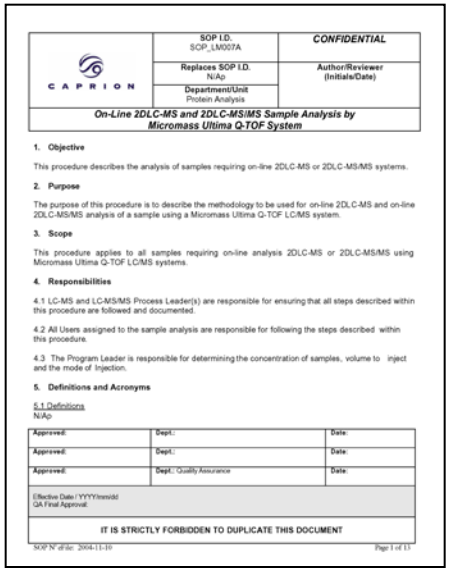




Process Control

- **Bar-coding**
- **Electronic Data Management (LIMS)**
- **Standard Operating Procedures**
- **Locked freezers**
- **Locked raw data**
- **Automatic backup**

Chain of Custody



CAPRION	SOP I.D. SOP_LM007A	CONFIDENTIAL
	Replaces SOP I.D. N/Ap	Author/Reviewer (Initials/Date)
	Department/Unit Protein Analysis	

On-Line 2DLC-MS and 2DLC-MS/MS Sample Analysis by Micromass Ultima Q-TOF System

1. **Objective**

This procedure describes the analysis of samples requiring on-line 2DLC-MS or 2DLC-MS/MS systems.

2. **Purpose**

The purpose of this procedure is to describe the methodology to be used for on-line 2DLC-MS and on-line 2DLC-MS/MS analysis of a sample using a Micromass Ultima Q-TOF LC/MS system.

3. **Scope**

This procedure applies to all samples requiring on-line analysis 2DLC-MS or 2DLC-MS/MS using Micromass Ultima Q-TOF LC/MS systems.

4. **Responsibilities**

4.1 LC-MS and LC-MS/MS Process Leader(s) are responsible for ensuring that all steps described within this procedure are followed and documented.

4.2 All Users assigned to the sample analysis are responsible for following the steps described within this procedure.

4.3 The Program Leader is responsible for determining the concentration of samples, volume to inject and the mode of Injection.

5. **Definitions and Acronyms**

5.1 Definitions

N/Ap

Approved:	Dept.:	Date:
Approved:	Dept.:	Date:
Approved:	Dept.: Quality Assurance	Date:
Effective Date / YYYY/mm/dd QA Final Approval:		

IT IS STRICTLY FORBIDDEN TO DUPLICATE THIS DOCUMENT

SOP N° d'ordre: 2004-11-10 Page 1 of 13

SOPs

PROBLEM:

CAPRION PROTEOMICS

No clear guidelines for the collection, processing, storage and analysis of plasma/serum for proteomics analysis

- Serum or plasma
- EDTA or heparin (for plasma)
- Added protease inhibitors (commercial tubes or DIY)

- Acceptable processing times and temperatures (before and after spin)

- Freezer temperature and storage time

- Multiple freeze-thaw cycles

- High abundance protein depletion

SAMPLE COLLECTION AND STORAGE VARIABLES: FULL STUDY

CAPRION PROTEOMICS

1. **Type of collection tube**
   - BD serum SST tube with gel and clot activator (red/grey top)
   - BD heparin tube (green top)
   - BD K_2-EDTA tube (lavender top)
   - Becton Dickinson (BD) P100
   - BD K_2-EDTA tube (lavender top) with protease inhibitor cocktail added at the time of pipetting separated plasma

2. **Variation in the 2 key bench times**
   - Before centrifugation
   - After centrifugation but before pipetting and freezing
   - Up to 4 days
   - 20ºC or 37ºC

3. **Number of freeze-thaw cycles**

4. **Length of time in -20ºC or -80ºC storage**

5. **Cancer patients and age and gender-matched controls**
   - Prostate and breast cancer

DESIGNING A WELL CONTROLLED STUDY

CAPRION PROTEOMICS

- Well defined and documented study design
- Protocols and SOPs for each step
- Bar coding of all tubes
- Chain of custody
- LIMS for information storage and sample tracking
- Well maintained and documented instrumentation
- Freezers on alarm with remote email alert
- Tool for managing blood collection, processing and storage
  - Interactive prompter and timer
  - Allows analysis of time taken for each step and variability
  - Allows documentation and comparison of process at multiple centers in clinical trial

ORACLE-BASED LIMS (NAUTILUS)

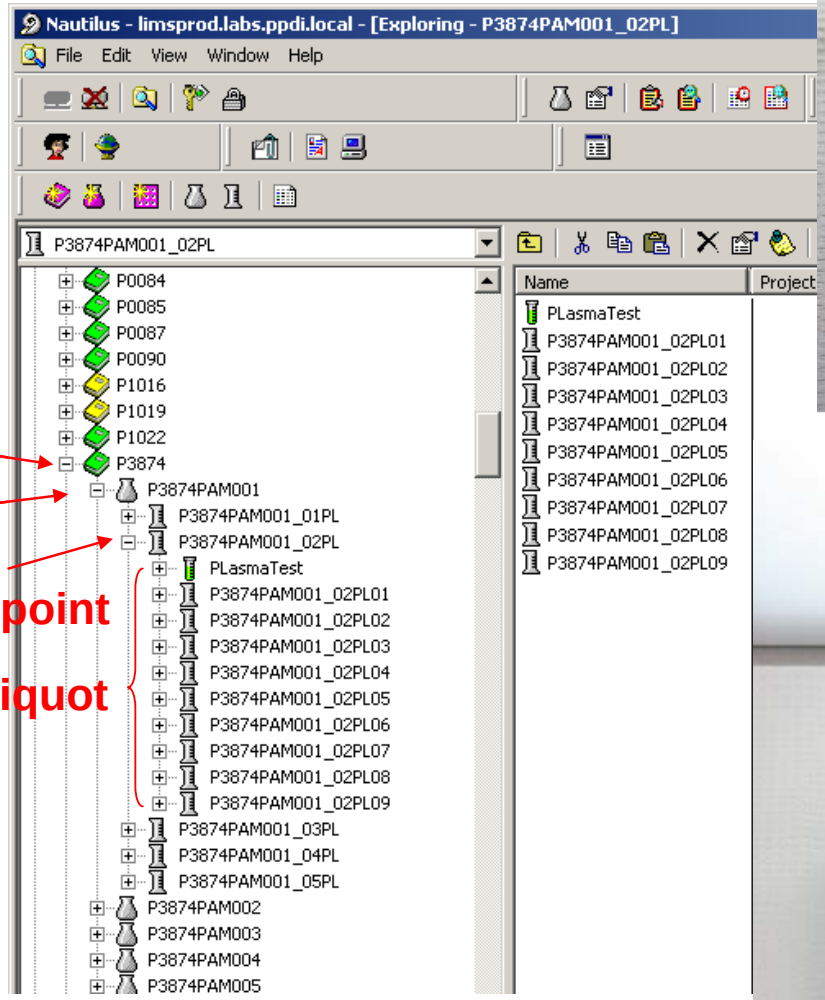
CAPRION PROTEOMICS

Study

Subject

Draw/time-point

Frozen aliquot



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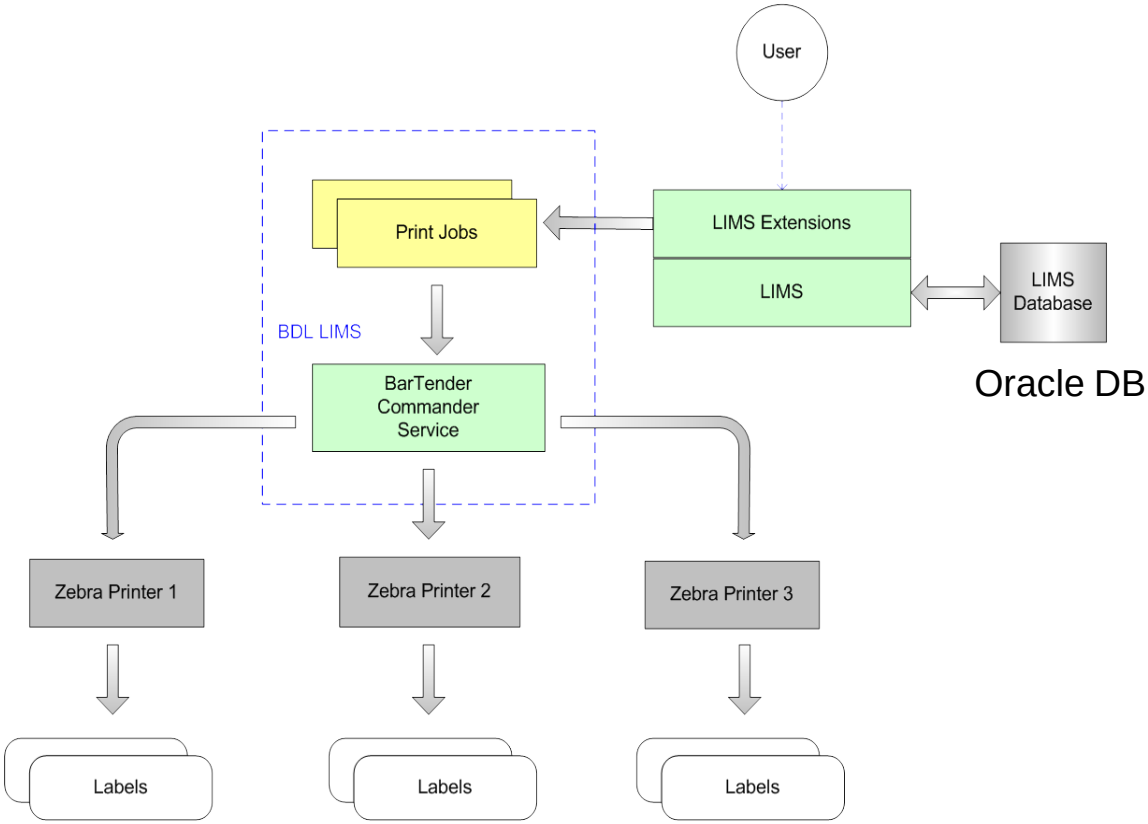
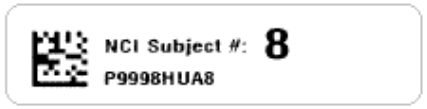
LIMS Barcode Label System v2.0



Oracle DB

Two-dimensional barcodes

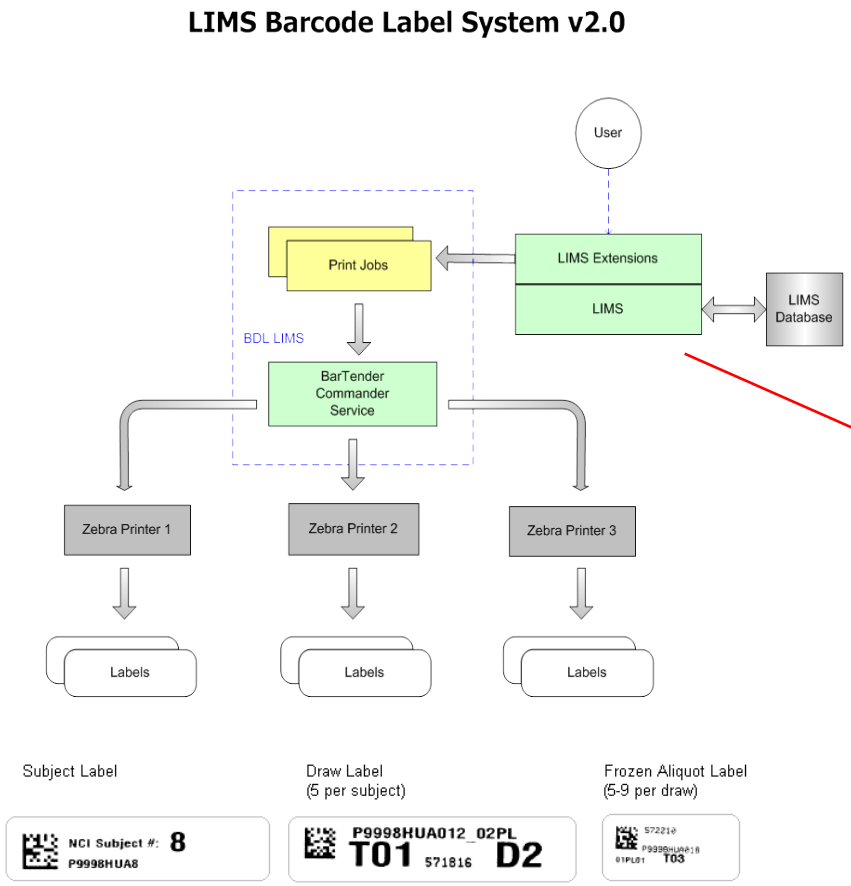
Subject Label



Draw Label
(5 per subject)

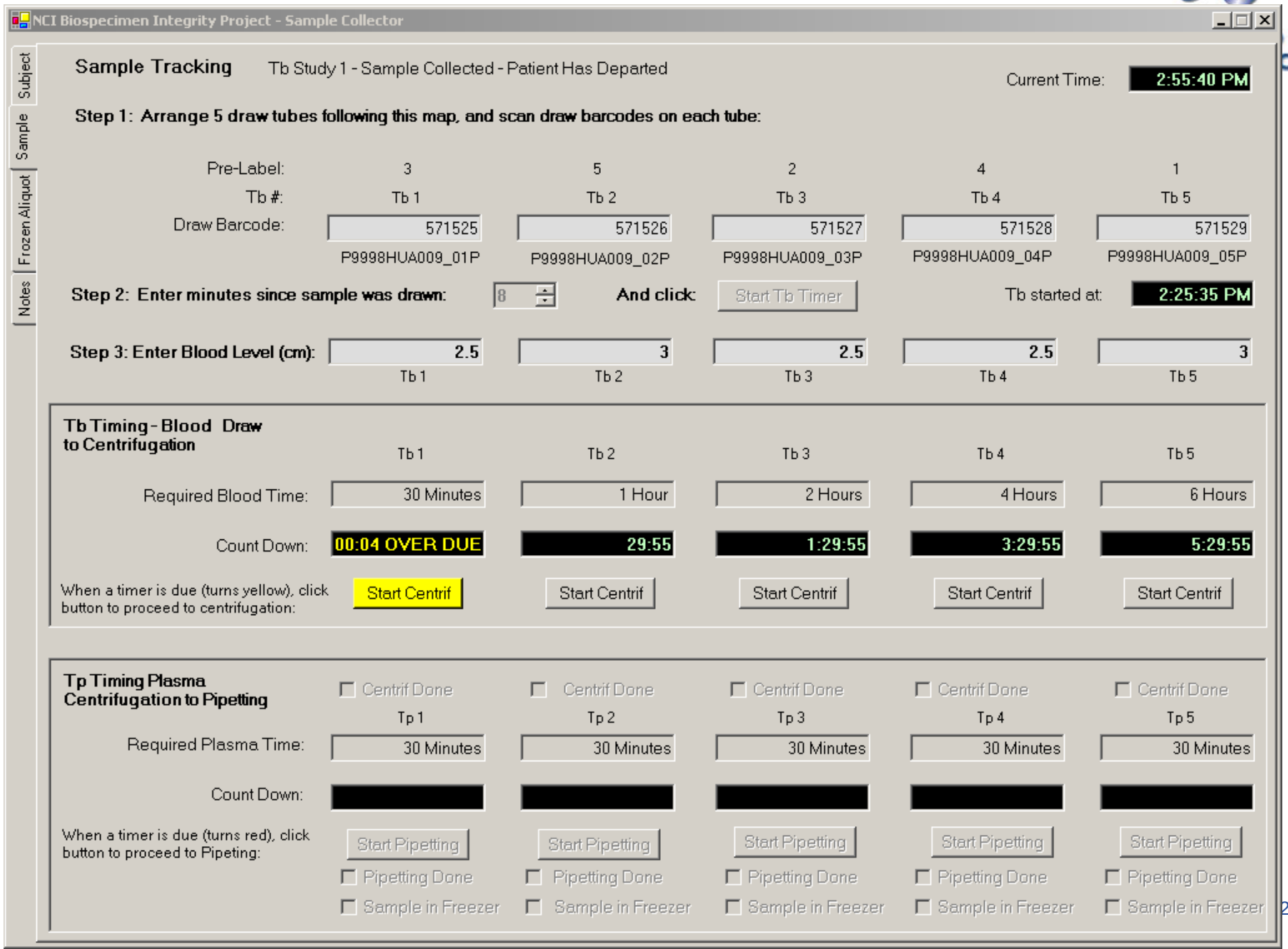
Frozen Aliquot Label
(5-9 per draw)

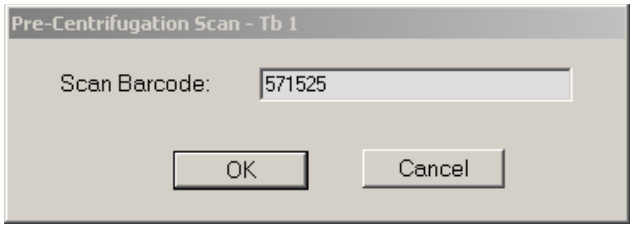
PC TABLET FOR PROCESS CONTROL



PC Tablet

PC TABLET PROCESS CONTROL SCREEN



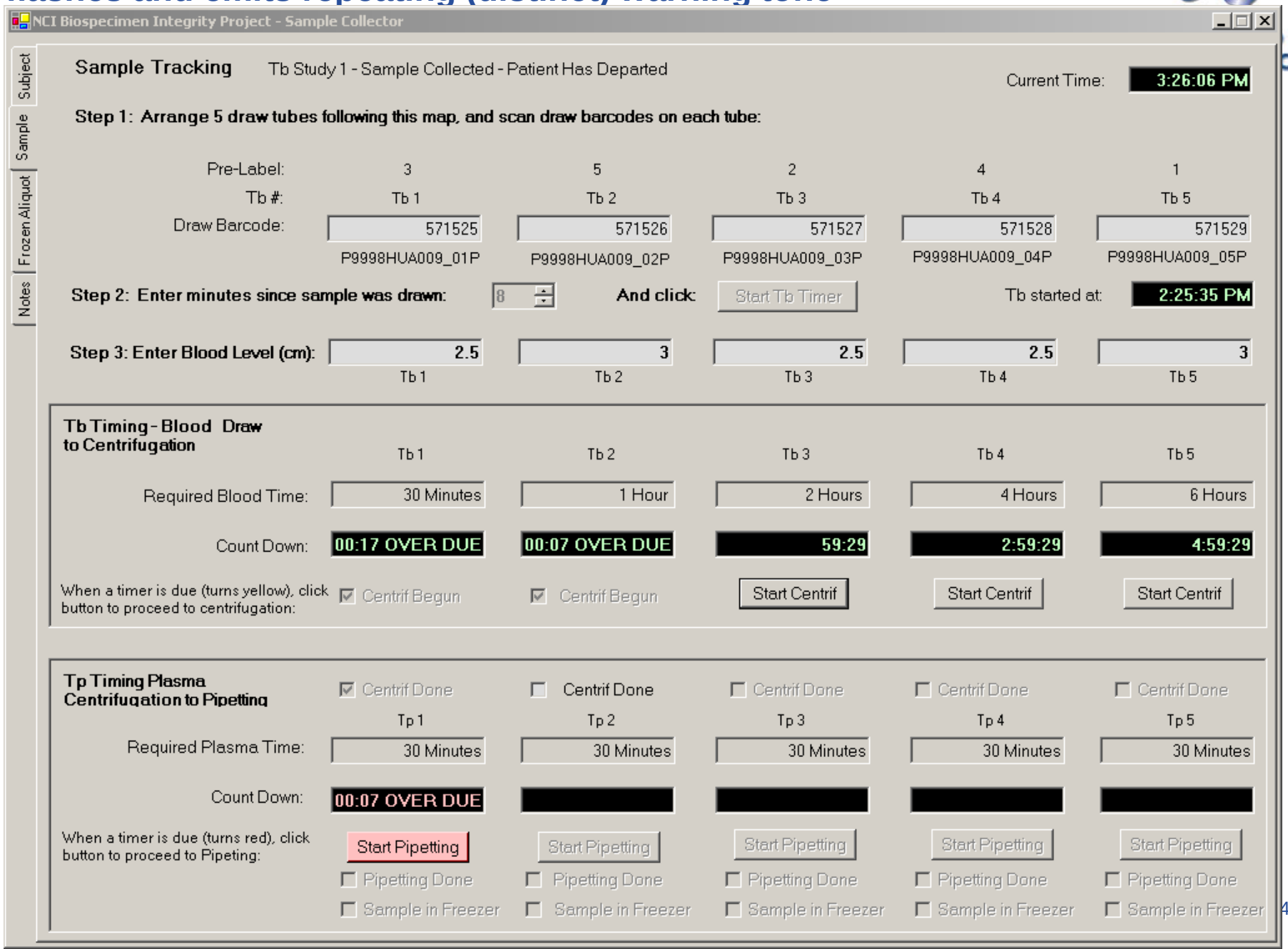


Click “Start Centrif” button,

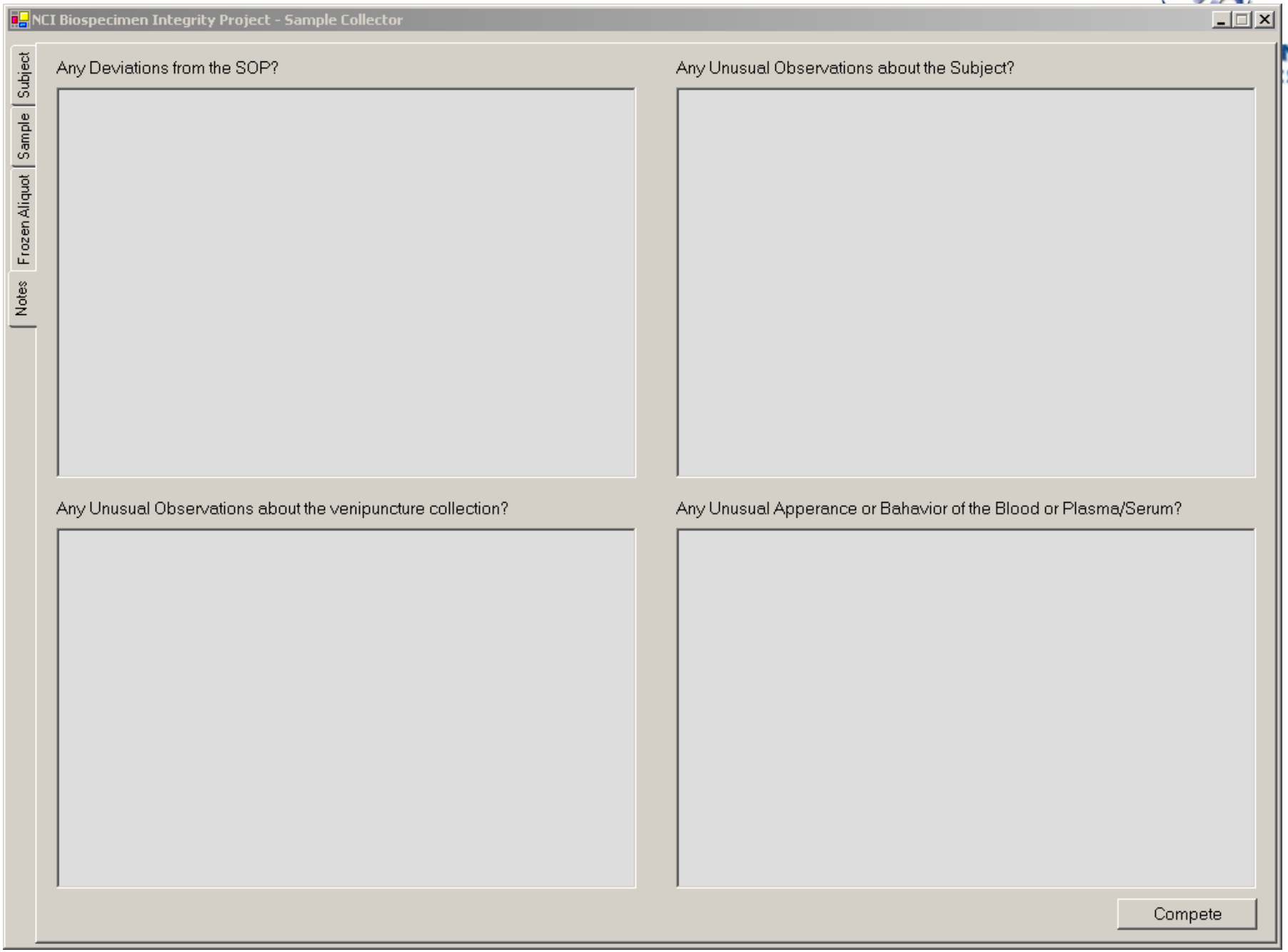
“Pre-Centrifugation Scan” pops up,

requests to scan barcode on Vacutainer tube

Sample Page – Tp 1 time due, timer and "Start Pippeting" button turned red, flashes and emits repeating (distinct) warning tone

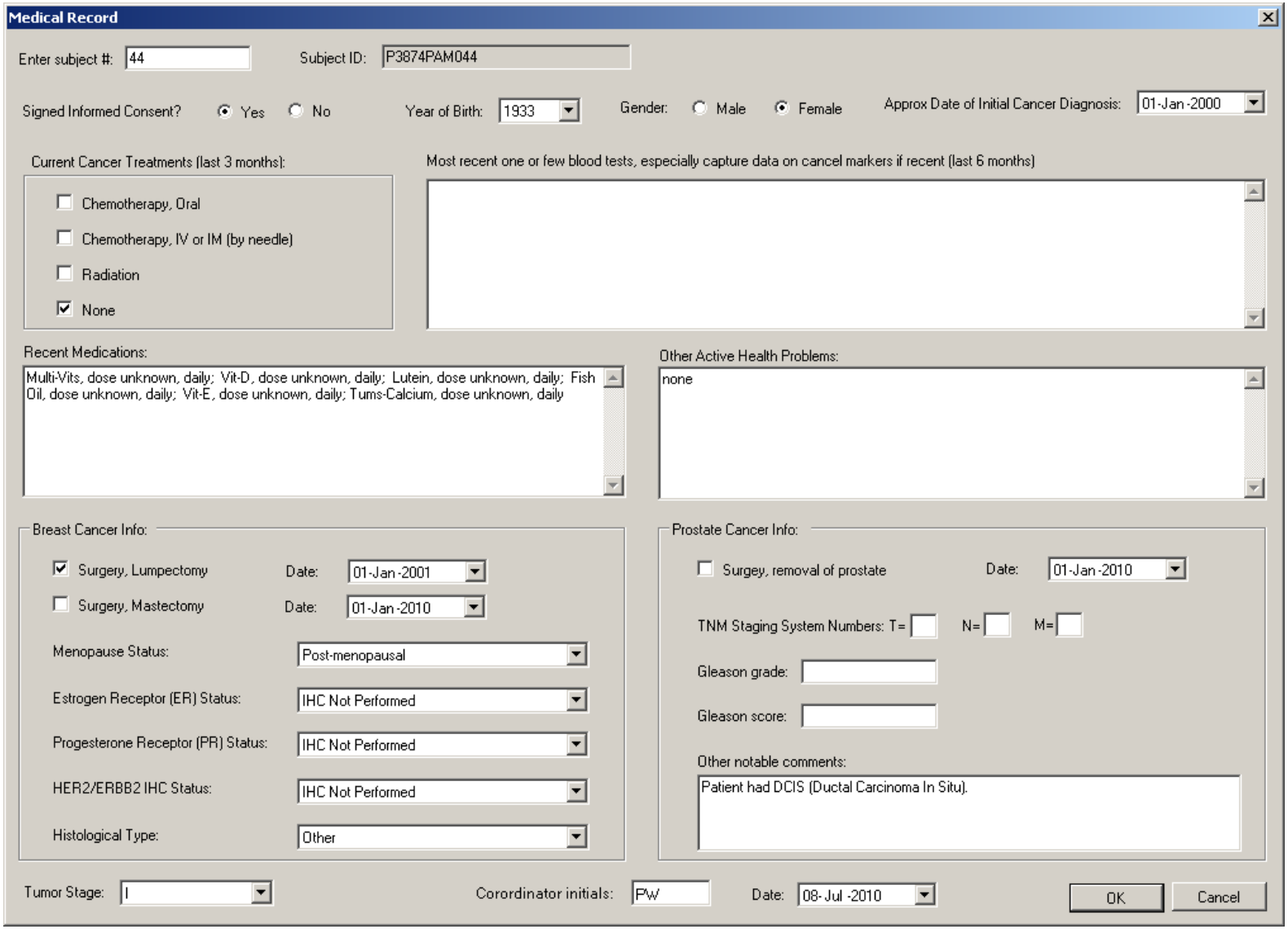


“Deviation Report” Page



LIMS METADATA STORAGE (ELECTRONIC MEDICAL RECORD)

CAPRION PROTEOMICS



Preliminary Biospecimen Analysis

PRELIMINARY BIOSPECIMEN ANALYSIS

CAPRION PROTEOMICS

Scope of preliminary study:

- Proteomic analysis on plasma from cancer patients
  - 3 tube types:
    - Heparin, EDTA, EDTA/PI
  - 3 incubation times at RT prior to centrifugation
    - 0.5, 4 and 24 h
  - n = 10

Analysis performed:

- Samples depleted of high abundance plasma proteins with Agilent MARS-14 column
- Protein digested with trypsin and analyzed by LC-MS
- Peak alignment and matching performed with Rosetta's Elucidator software
- Differential expression analysis
- Peptides sequenced and clustered

PEPTIDE POPULATION DIFFERS BETWEEN HEPARIN AND EDTA PLASMA

CAPRION PROTEOMICS

Heparin vs. EDTA

- Total components: 6,600
- Heparin-specific components: 5,000
- EDTA+/-PI-specific components: 5,700
- Only approx. 4,000 (61%) components shared between heparin and EDTA
- 83% proteins are shared between EDTA and heparin
- Total number of proteins detected is almost the same

EDTA +/- Protease Inhibitors

- 140/5700 (2.5%) components differ > 2 fold between EDTA +/- PI
- Adding protease inhibitors increases number of proteins detectable from 135 to 137

FEW CHANGES SEEN OVER TIME

CAPRION PROTEOMICS

Comparison ID	Description	Differentially expressed peptides	Differentially expressed proteins
1	[Heparin] 0.5 hr vs 4 hr	0	0
2	[Heparin] 0.5 hr vs 24 hr	2	1
4	[EDTA] 0.5 hr vs 4 hr	3	3
5	[EDTA] 0.5 hr vs 24 hr	11	4
7	[EDTA+PI] 0.5 hr vs 4 hr	4	4
8	[EDTA+PI] 0.5 hr vs 24 hr	12	5

Selection criteria:

- Fold-change ≥ 2;
- p-value ≤ 0.05;
- q-value ≤ 0.05

Results:

- Small effects of incubation time
- Only slightly more changes with EDTA than heparin
- Small effect of adding protease inhibitors

IMPACT OF SMALLER CHANGES ON CALCULATING DIFFERENTIALLY EXPRESSED PEPTIDES

CAPRION PROTEOMICS

Comparison_ID	Description	DI > 2 pvalue <= 0.05 qvalue <= 0.05	DI > 1.5 pvalue <= 0.05 qvalue <= 0.05
1	[Heparin] 0.5 hr vs 4 hr	0	1
2	[Heparin] 0.5 hr vs 24 hr	3	3
3	[Heparin] 4 hr vs 24 hr	2	3
4	[EDTA] 0.5 hr vs 4 hr	3	11
5	[EDTA] 0.5 hr vs 24 hr	21	27
6	[EDTA] 4 hr vs 24 hr	20	29
7	[EDTA+PI] 0.5 hr vs 4 hr	7	12
8	[EDTA+PI] 0.5 hr vs 24 hr	26	33
9	[EDTA+PI] 4 hr vs 24 hr	21	30
10	[0.5 hr] Heparin vs EDTA	2,606	3,569
11	[0.5 hr] Heparin vs EDTA+PI	2,897	3,804
12	[0.5 hr] EDTA vs EDTA+PI	141	346
13	[4 hr] Heparin vs EDTA	2,472	3,408
14	[4 hr] Heparin vs EDTA+PI	2,785	3,656
15	[4 hr] EDTA vs EDTA+PI	77	178
16	[24 hr] Heparin vs EDTA	2,729	3,642
17	[24 hr] Heparin vs EDTA+PI	2,846	3,765
18	[24 hr] EDTA vs EDTA+PI	40	76
19	0.5 hr vs 4 hr	2	7
20	0.5 hr vs 24 hr	19	21
21	4 hr vs 24 hr	20	24
22	Heparin vs EDTA	2,732	3,778
23	Heparin vs EDTA+PI	2,967	3,971
24	EDTA vs EDTA+PI	140	368
		3,386	4,498

OXIDIZED PEPTIDES ARE NOT SELECTIVELY DISTRIBUTED

CAPRION PROTEOMICS

Differentially expressed components / peptides / proteins						
(DI > 2 | pvalue <= 0.05 | qvalue <= 0.05)						
			#Peptides with oxidation PTM		#Proteins with oxidation PTM	
Comparison ID	Description	#Components	Upregulated	Downregulated	Upregulated	Downregulated
19	0.5 hr vs 4 hr	2	0	0	0	0
20	0.5 hr vs 24 hr	19	0	0	0	0
21	4 hr vs 24 hr	20	0	0	0	0
22	Heparin vs EDTA	2,732	13	34	10	6
23	Heparin vs EDTA+PI	2,967	11	35	8	7
24	EDTA vs EDTA+PI	140	0	0	0	0
		3,386	17	36	13	8

SEMI-TRYPTIC AND NON-TRYPTIC PEPTIDES ACROSS THREE STUDIES

CAPRION PROTEOMICS

	NCI	NIAID site 1	NIAID site 2
# Sequenced Peptides	3,158	1,527	2,593
# (Fully) Non-tryptic	11	17	1
# Semi-tryptic	506	521	377
# Tryptic	2,641	989	2,215
% semi or non-tryptic peptides	16%	35%	15%

CONCLUSIONS OF PRELIMINARY STUDY

CAPRION PROTEOMICS

- Large number of components detectable in only heparin or EDTA tubes
- 14% more components detectable in EDTA tubes
- Very few components changed in concentration in any tube type after 24h at RT
- Little effect of protease inhibitor cocktail in EDTA tubes after 24h at RT
- The median CV for normalized peptide intensities within each group is very low at 5-7% (mainly processing related)
- Non-normalized raw intensity median CVs ~ 32-40% (processing and biological variability)
- 16% of sequenced peptides were cleaved at a site other than trypsin, suggesting some degradation
  - Similar to one study (well managed samples)
  - Better than a second study (older sample set, multiple freeze-thaws)

QUALITY ASSESSMENT ASSAYS

CAPRION PROTEOMICS

- Multiplexed ELISA assays: Luminex
- Multiplexed MRM mass spec assays

INDEPENDENT ASSESSMENT OF PROTEIN CONCENTRATION AND VARIATION

CAPRION PROTEOMICS

Lower abundance protein panel:

- IL6
- CA125
- CA19-9
- MUC1
- PSA
- PRL
- LEP
- OPN
- MIF
- AFP
- CEA

Higher abundance protein panel:

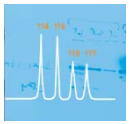
- VTN
- ECM1
- F13A
- VDP
- AT3
- CFH
- FCN3
- LUM

* This group will also be measured by LC-MS

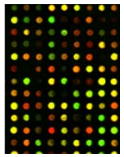
MULTIPLEXED MRM ASSAY DEVELOPMENT STRATEGY

CAPRION PROTEOMICS

1. Literature



2. Proteomics

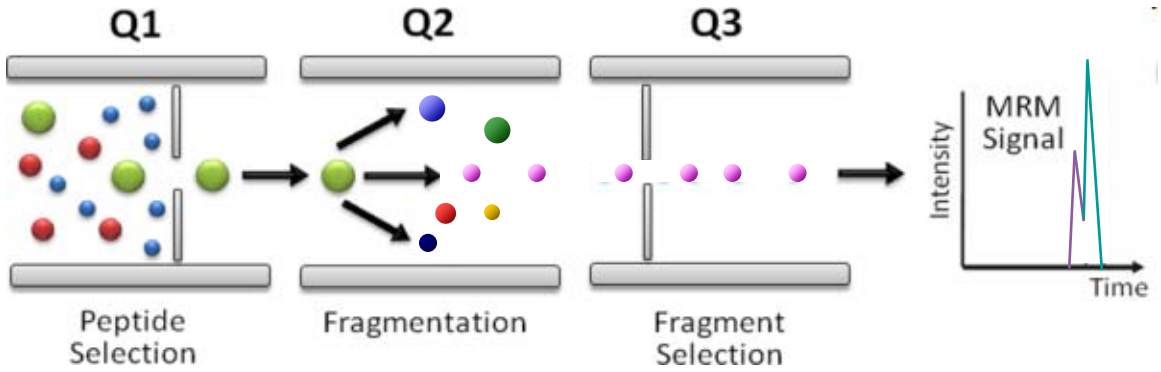


3. Transcript Profiling

Protein Sequence

MSAIQAAWPSGTECIAKYNFHGTAEQD
LPFCK**GDVLTIVAVTK**DPNWYKAKNKV
GREGIIPANYVQKREGVKAGTKLSLMP
WFHGKITREQAER**LLYPPETGLFLVR**E
STNYPGDYTLCVSCDGKVEHYRIMYHA
SKL**SIDEEVYFENLK**MQLVEHYTSDAD
GLCTRLIKPKVMEGTVAAQDEFYRSGW
ALNMKELKLLQTIGK**GEFGDVMLGDYR**
GNKVAVKCIKNDATA…

Candidate markers for MRM assay development can come from proteomics or other sources, including the literature

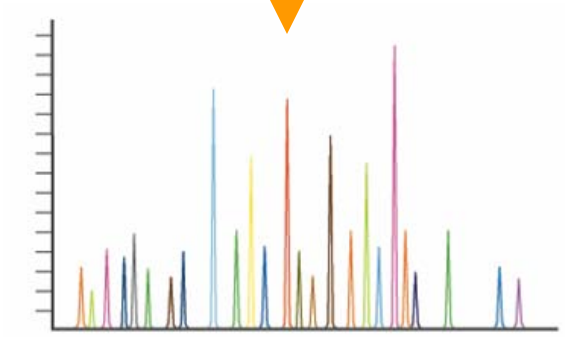


Double selection improves signal/noise and reduces interference

MRM-MS ASSAYS: VERIFY AND VALIDATE CANDIDATE BIOMARKERS

CAPRION PROTEOMICS

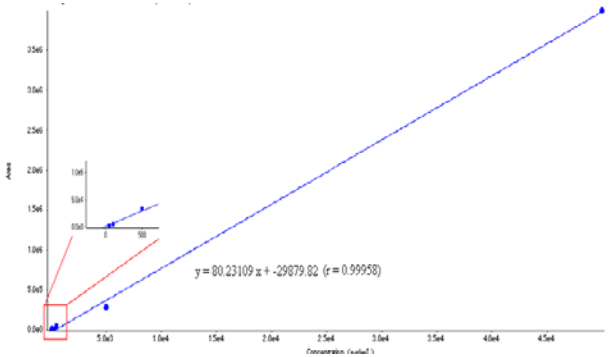
Candidate Biomarkers



Multiplexed MRM assay
1-700 candidate biomarkers

Biomarker validation funnel

Validated biomarkers for MRM or ELISA assay



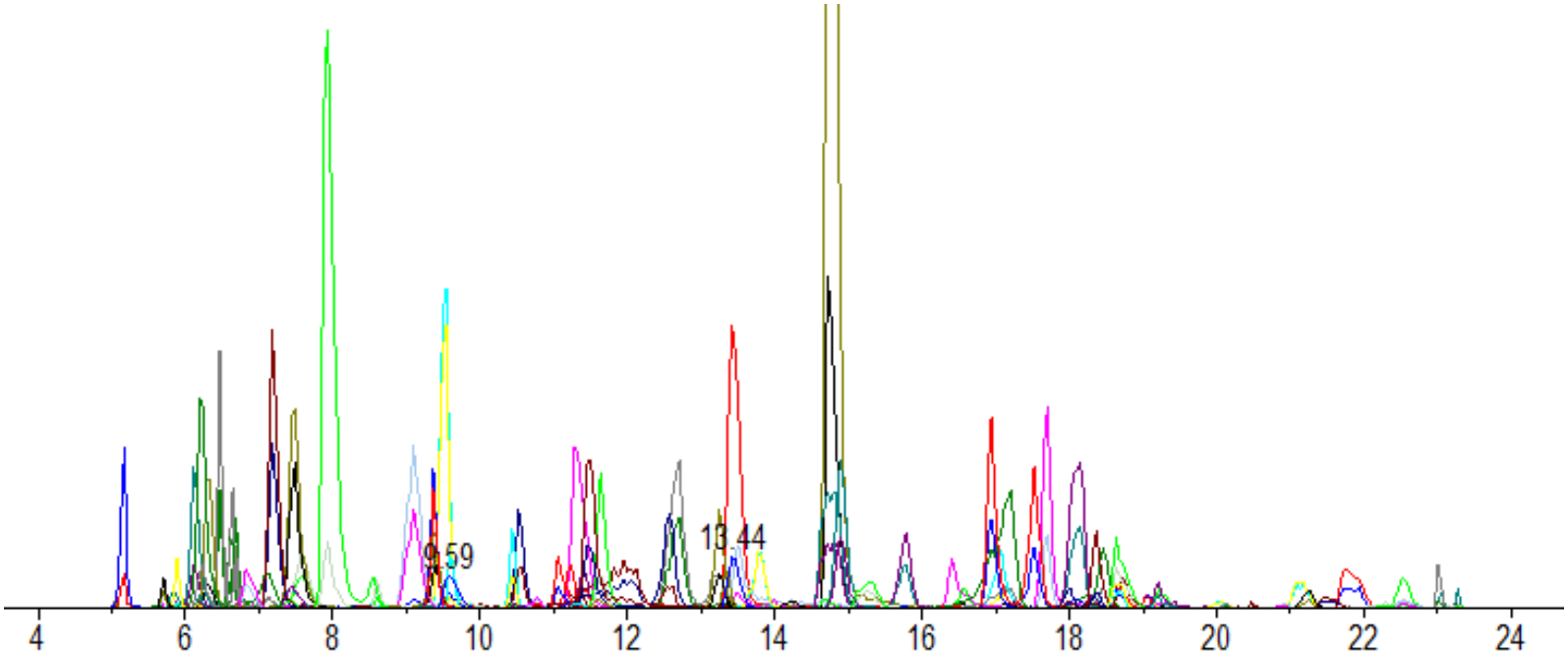
Linear and quantitative

- Rapid assay development
- Multiplexed (up to 700 biomarkers)
- Confirms biomarker ID
- Confirms differential expression
- Determines absolute abundance
- Can be validated for regulatory compliance

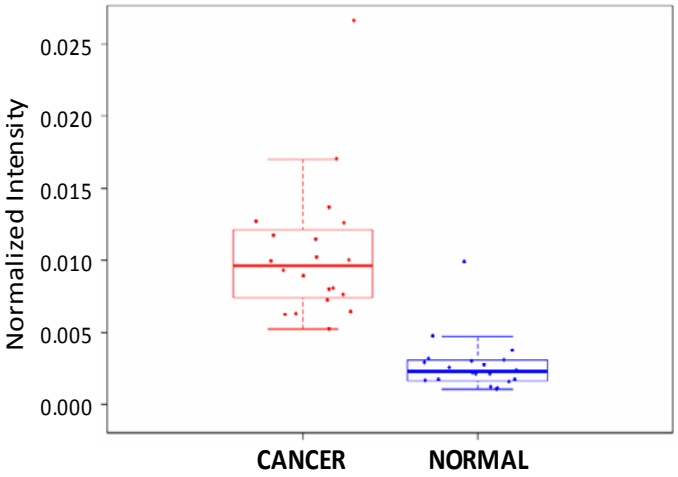
EXAMPLE OF MRM ASSAY DEVELOPED FROM LIST OF 90 LUNG CANCER Dx CANDIDATES

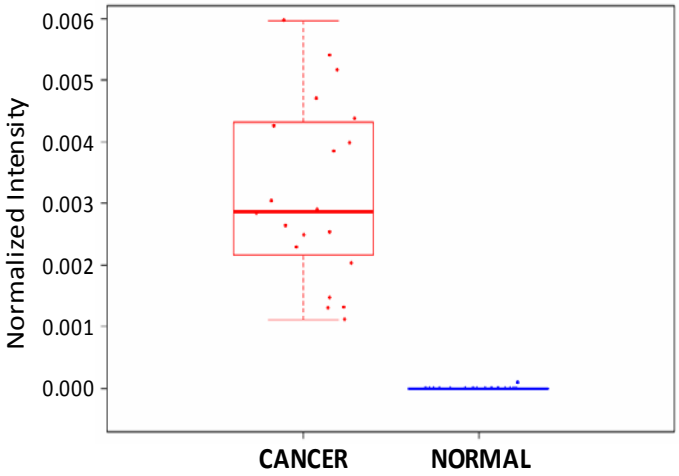
CAPRION PROTEOMICS

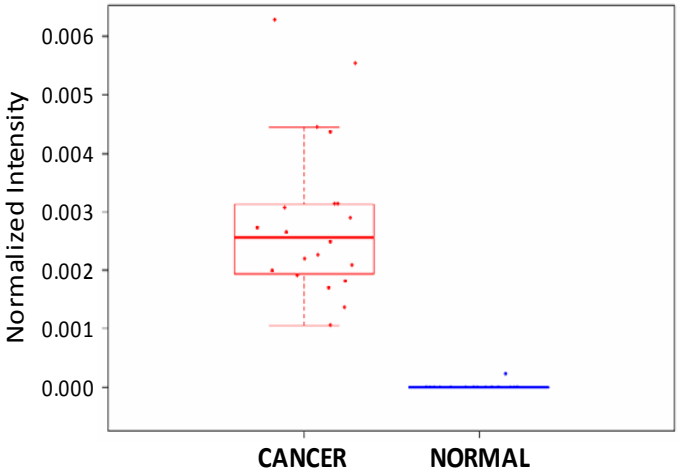
- Predict best 5 peptides/protein, synthesize
- Determine empirically the best 2, monitor 2 transitions each
- Pre-verification study with plasma from 20 cancer and 20 control subjects
- 64 of the targeted proteins (71%) successfully detected in un-spiked plasma samples (cancer and controls)

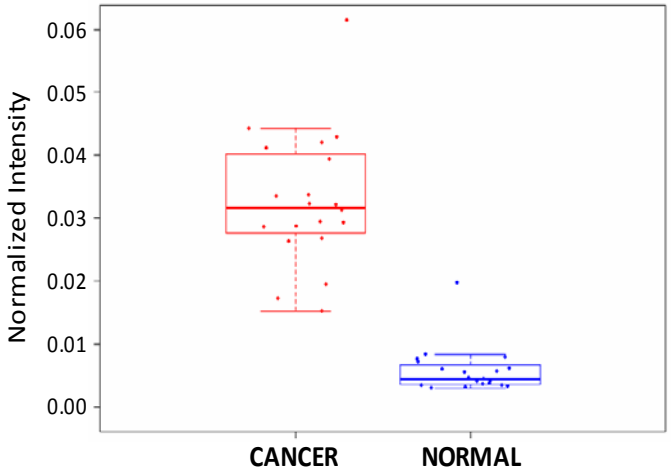


CANDIDATE CLASSIFIERS IDENTIFIED









Current study: Larger set of proteins (700 candidate markers) and more samples (~400) from multiple sources