Supplementary Figure 1. CPTAC Schematic Representation of the Assay Characterization Guidance Document

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https://proteomics.cancer.gov/sites/default/files/CPTAC%20Schematic%20Representation%20of

%20the%20Assay%20Characterization%20Guidance%20Document.pdf).

Assay Development Working Group - Experiments for Assay Characterization

Experiment 1: *Response Curve*

- Development of multipoint response curve (1 blank and a minimum of 6 concentration points).
- Samples prepared in digested matrix background (i.e. plasma, tissue, cells, etc).
- Used for the determination of LOD, LLOQ and linearity.
- Multiple replicates analyzed.

Experiment 2: *Mini-Validation of Repeatability*

- Examines intra- and inter-assay variability.
- Uses the LLOQ from Experiment 1 from which 3 concentrations (Low, Medium and High) are used to assess repeatability.
- 3 replicates processed and measured on 5 different days.

Experiment 3: *Selectivity*

- Examines the response of a peptide in six different biological replicates of the matrix.
- Replicates analyzed with no spike and ½ the Medium and Medium concentrations defined in Experiment 2.

Experiment 4: *Stability*

- Examines the stability of a peptide spiked into a background matrix
- Stability assessed based on peak area variability following:
  - different storage conditions (4C and -70C) over time.
  - freeze-thaw cycles
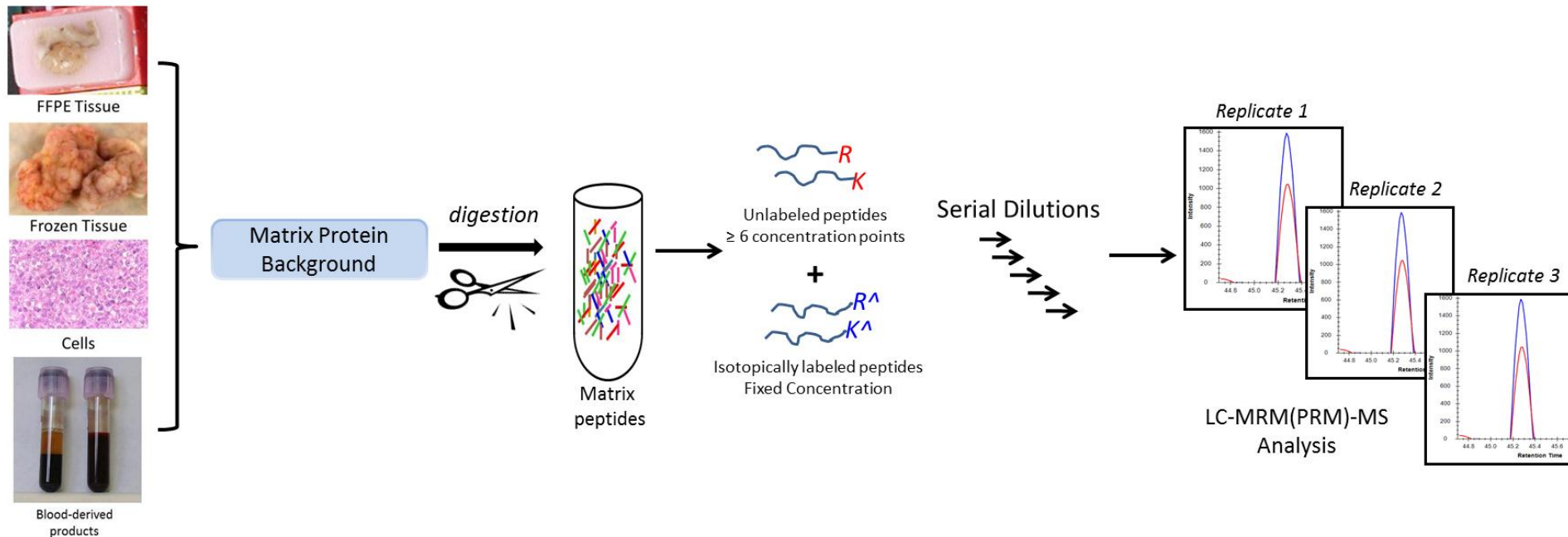- Variability compared to data collected from Experiment 2.

Experiment 5: *Reproducible Detection of Endogenous Analyte*

- Representative sample containing endogenous analyte is digested 5 times on each of 5 days.
- Examines intra- and inter-assay variability of the entire assay workflow, including digestion.

Experiment 1: *Response Curve*

KEY POINTS
- Development of multipoint response curve (1 blank and a minimum of 6 concentration points).
- Samples prepared in digested matrix background (i.e. plasma, tissue, cells, etc).
- Used for the determination of LOD, LLOQ and linearity.
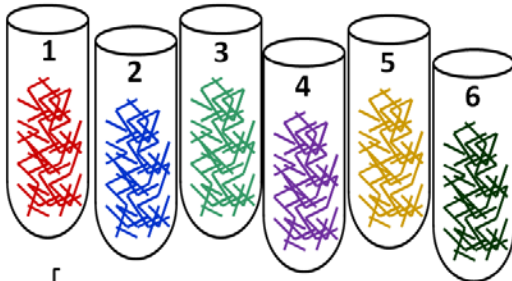- Multiple replicates analyzed.

Experiment 2: *Mini-Validation of Repeatability*

KEY POINTS
- Examines intra- and inter-assay variability.
- Uses the LLOQ from Experiment 1 from which 3 concentrations (Low, Medium and High) are used to assess repeatability.
- 3 replicates processed and measured on 5 different days.

Target Peptides Stock Solution

3X LLOQ
50X LLOQ
80% ULOQ

Matrix Digest

+

+

Isotopically Labeled Peptides Fixed Concentration

LC-MRM(PRM)-MS Analysis

Replicate 1

Replicate 2

Replicate 3

Repeat for 5 Days

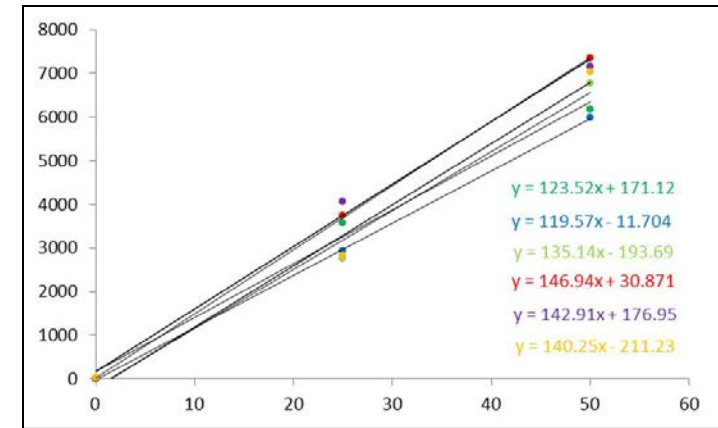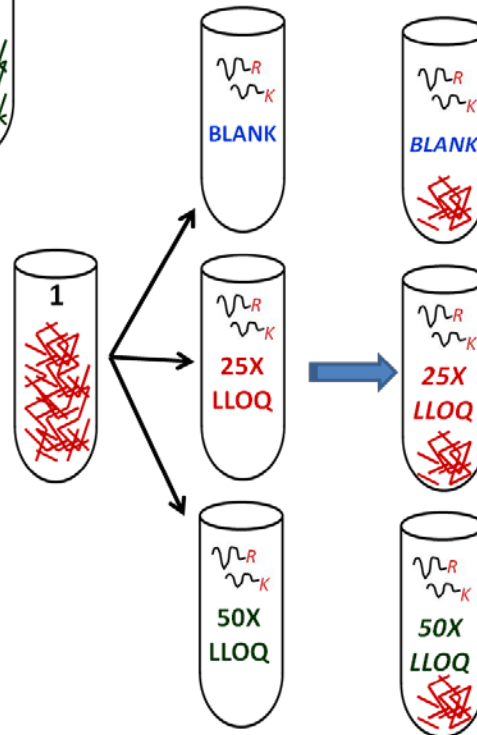|		Replicate			
Day	1	2	3		
1	2156	1989	2095		4.1%
2	2066	1653	2103		12.9%
3	1509	1755	1536		8.4%
4	1862	2132	2454		13.8%
5	1617	1515	1724		6.5%
				Ave	9.1%
	15.1%	13.8%	18.1%	15.7%	
				Total error	18.1%

Experiment 3: *Selectivity*

KEY POINTS
- Examines the response of a peptide in six different biological replicates of the matrix.
- Replicates analyzed with no spike and ½ the Medium and Medium concentrations defined in Experiment 2.
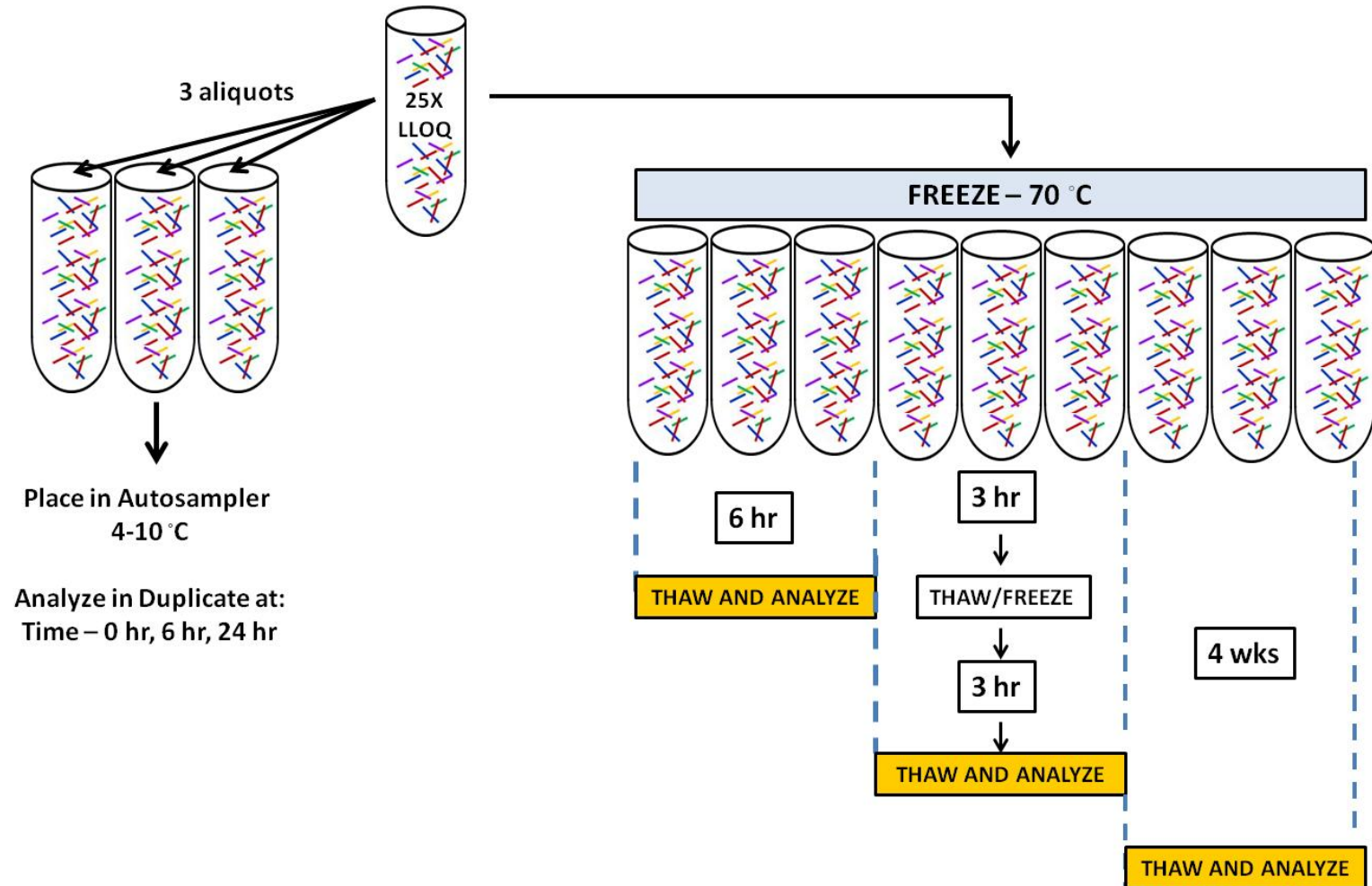
Experiment 4: *Stability*

KEY POINTS

- Examines the stability of a peptide spiked into a background matrix
- Stability assessed based on peak area variability following:
  - different storage conditions (4C and -70C) over time.
  - freeze-thaw cycles
- Variability compared to data collected from Experiment 2.

Experiment 5: *Reproducible Detection of Endogenous Analyte*

KEY POINTS
- Representative sample containing endogenous analyte is digested 5 times on each of 5 days.
- Examines intra- and inter-assay variability of the entire assay workflow, including digestion.
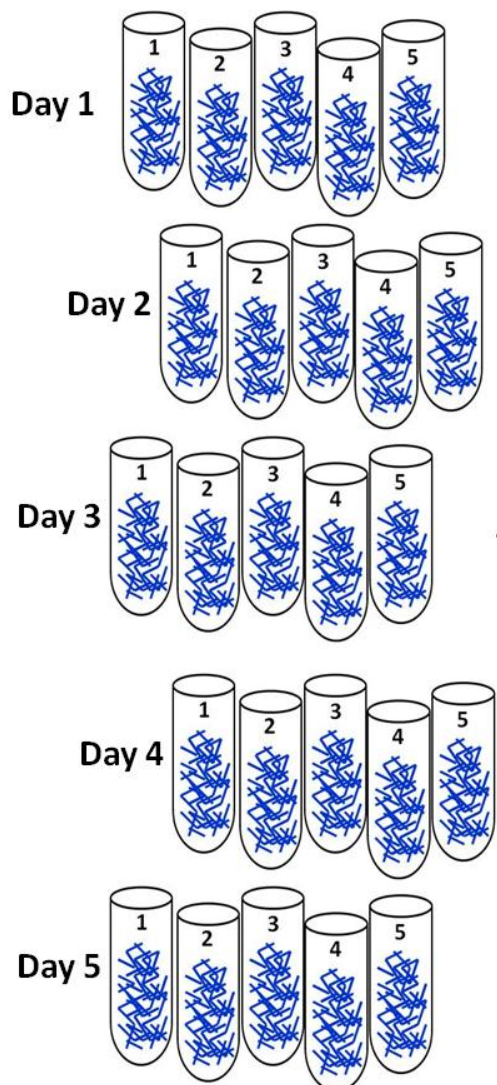
LC-MRM(PRM)-MS
Analysis

Day	Replicate 1	2	3	4	5			
1	19396	17334	17325	15488	16573			8.3%
2	24686	21386	21000	23260	22226			6.6%
3	22121	26532	22050	20064	26730			12.7%
4	17280	18572	20295	14404	21384			14.8%
5	21881	19859	21450	16840	22113			10.7%
							Ave	10.6%
	13.4%	17.2%	9.0%	20.1%	16.5%		13.2%	

Total error   17.0%

Sample 1