Proteomics Standards Research Group

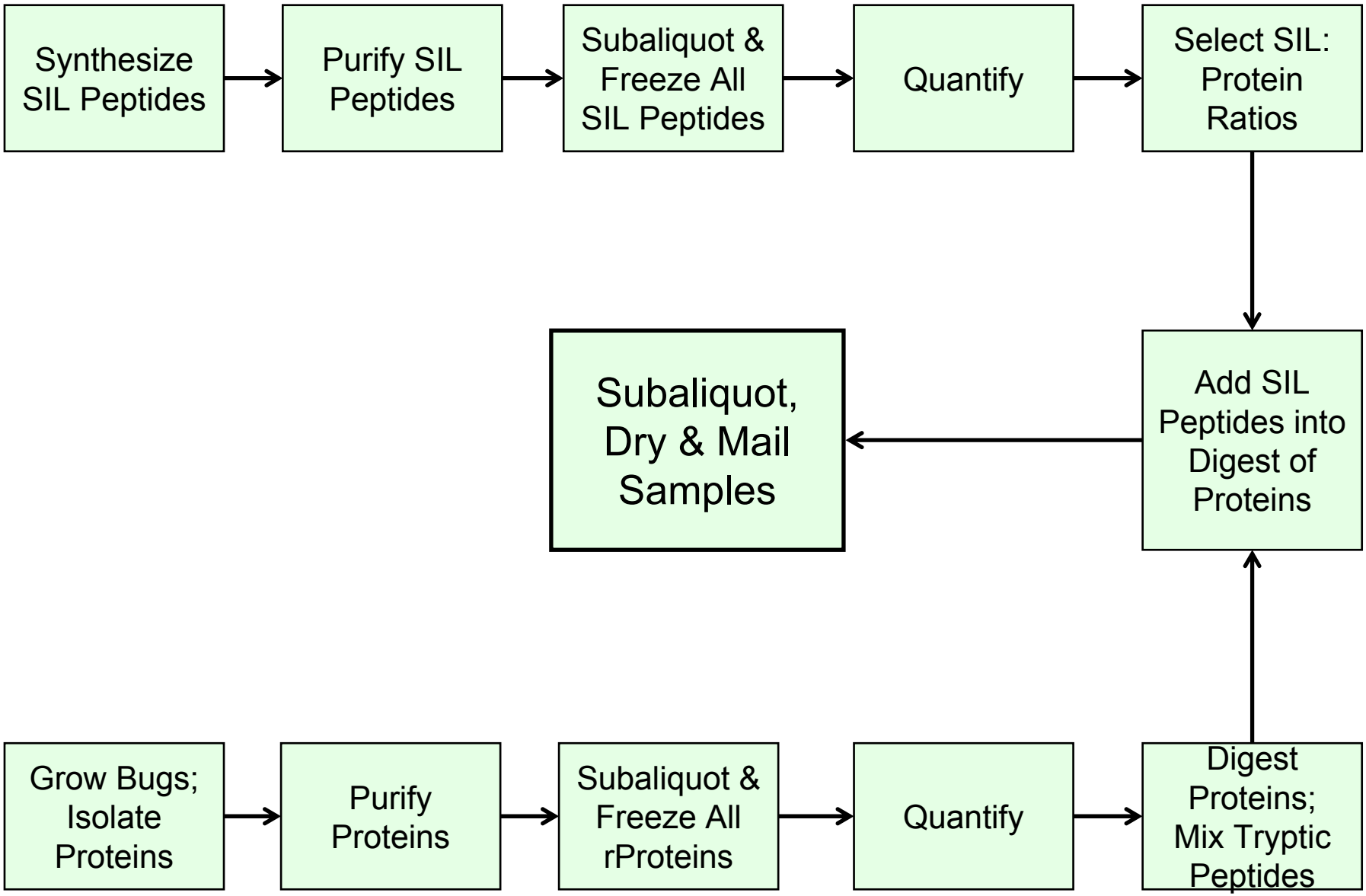
Proteomics Standards Research Group (sPRG)

www.abrf.org/sprg

ABRF-sPRG09: Development of a Quantitative Proteomics Standards

sPRG 2009 Study Goal

- A tube containing sets of Natural and Labeled Peptides in defined ratios and amounts.
- Natural peptides: Derived from trypsin digest of several proteins found in human plasma.
- Stable Isotope Labeled peptides: differing only in mass from the Natural peptides
- Suitable for use in assessing a laboratory's capabilities for absolute quantitative analysis.


Synthesize SIL Peptides
Purify SIL Peptides
Subaliquot & Freeze All SIL Peptides
Quantify
Select SIL: Protein Ratios
Add SIL Peptides into Digest of Proteins
Subaliquot, Dry & Mail Samples
Grow Bugs; Isolate Proteins
Purify Proteins
Subaliquot & Freeze All rProteins
Quantify
Digest Proteins; Mix Tryptic Peptides

sPRG2009 Study

- One tube containing purified recombinant proteins in specified amounts which were then:
- Reduced, Alkylated and Trypsin Digested and
- Spiked with selected Stable Isotope Labeled peptides in specified amounts corresponding to natural trypsin peptides.
- Aliquotted, dried and sent to requesters.
- Requesters determine the relative ratios of Natural to Heavy peptides
- Sufficient material will be provided so that participants can perform a number of replicate analyses .
- Participants will be invited to use the LC-MS proteomics workflow of choice to determine the absolute concentration of constituent proteins.
- Participants will be able to report their results via a web-based survey tool.

sPRG2009 Study

- Proteins:
  - Recombinant human proteins
  - Derived from the constituents found in Sigma's Universal Proteomics Standard
  - Based on the sPRG2006 Study sample

Tryptic Peptides Corresponding to the Proteins

- At least 3 Peptides per protein
- Meeting a Number of Criteria

Peptide Considerations: Synthetic & Natural

- Label: C-Terminal K, [$^{13}C_6{}^{15}N_2$] or R, [$^{13}C_6{}^{15}N_4$].
- Length: 8 < N < 20 amino acid residues
- Avoid Possible Imide Sites: [DN].[GASN]
- Avoid DKP Formation: C-terminal peptide with G or P at C2 position.
- Avoid Multiple Amino Acid Repeats: e.g., XXX
- Avoid Overly Hydrophobic Sequences: multiple aliphatic aa's e.g., A, L, I, V
- Minimize Incomplete Side Chain Removal: tBu and Trt

Peptide Considerations: Natural & Synthetic

- No Acid Labile Residues: [DP]
- No Oxidation Sensitive Residues: Met, Trp_n
- No N-terminal Gln Residues (→pyroGlu)
- No Pro-Pro; cis-trans isomers in chromatography
- Chromatography:
  - Retained by C18 column and
  - Eluted between 2-75% MeCN buffer

Peptide Considerations: Natural

- Enzymatic Digestion: Has to Be Very Good
  - Every Missed Cleavage Affects the Quantitation
  - Every Semi-Tryptic Cleavage Affects the Quantitation
- No N- or C-terminal KK, KR, RK, or RR cleavages; no KP or RP cleavages
- No known PTMs or consensus recognition sequences (e.g., N-glycosylation)
- Chromatography: Peptides retained by C18 column and eluted in a reasonable percentage of MeCN buffer

Real World Consideration

- If you want to detect the protein, you've got to pick a peptide or two…
- For our study: Minimum of 3 proteotypic peptides per protein

Testing Pilot Sample

- 5 Proteins: Digested Individually then pooled in 3 different amounts
- Spiked with corresponding SIL peptides all at the same level
- Dried and sent to sPRG members to test for ratios

Results for Pilot Study

- Ratios off!
- Back to Drawing Board with Clock Ticking

Plan for Prototype 1

- Test Four Digest Conditions
  - Pick best
- Repurify Proteins
  - Subaliquot and Store at -80C until used.
- Quantitate Proteins and SIL Peptides:
  - AAA in triplicate
- Perform Digest on Protein Pool and Test
- Formulate Digest with SIL Peptides
- Test Prototype 1 in sPRG Labs

Quantitation

- Amino Acid Analyses (AAA)
- Proteins and SIL peptides
- In Triplicate
- In Two Labs

Testing of Digest Conditions

- Protein Mixture (based on BCA analysis):
  - ALBU: 140 pmol
  - SYHC: 88 pmol
  - NQO2: 70 pmol
  - PRDX1: 88 pmol
  - UBIQ: 147 pmol
- Instrumentation: Michrom BioResources Paradigm MS4 HPLC; Thermo Fisher LTQ-FT. MS analyses: data-dependent acquisition of the eight most abundant ions in the survey scan; precursors were detected in the FT, fragmentation was conducted in the LTQ.
- Data analyses: Mascot database search against the Swiss-Prot database (human) using 10-ppm mass accuracy for precursor ions and 0.5-Da accuracy for product ions; Scaffold (Proteome Software) was used for processing of the Mascot search results.

Testing of Digest Conditions

	Number of assigned spectra (number of unique spectra)				
Method	ALBU	SYHC	NQO2	PRDX1	UBIQ
Trypsin, no denaturant	518 (100)	230 (48)	161 (36)	171 (30)	30 (6)
Trypsin/Lys-C, urea (8 M)	318 (68)	147 (39)	101 (25)	110 (27)	23 (8)
Trypsin, TFE (40%)	375 (77)	132 (33)	92 (25)	107 (19)	62 (9)
Trypsin, urea (6 M)	401 (75)	155 (42)	102 (25)	112 (24)	34 (8)

The Winning Digest Protocol

- Reconstitute the sample in 20 uL of 2 mM TCEP, 50 mM NH_4HCO_3, 1 mM $CaCl_2$, pH 8.6 buffer.
- 15 minutes at 60°C in the shaker. Cool to 20°C. Add 5 uL of the iodoacetamide stock. 30 min @ 20°C & shaking/dark.
- Add 4.5 uL of the TCEP stock to the tube. 30 min @ 37°C & shaking/dark.
- Add 18 uL of H_2O, and 4 uL of the reconstituted trypsin (15:1 S:E by weight). 16 hrs @ 37°C in the shaker.
- Add another 4 uL of the reconstituted trypsin 6 hrs @ 37°C in the shaker. Final volume is ~55 uL, 20 mM AmBic buffer, 5 mM TCEP-acetamide, < 0.5 mM unreacted TCEP.
- Bring to the volume to 83.3 uL by adding 28.3 uL of 1% formic acid.

Prototype 1 Preparation

- Amounts of Protein Added was based on AAA concentrations.
  - Quantities: from 10 pmol to 0.1 pmol per tube.
- Digested with “Trypsin, no denaturant” protocol.
- Analyses of digests of the five proteins provided insight into the tryptic peptides that were most reliably detected.

Proteins in Prototype-1

	2+ (*m/z*)	
	Unlabeled	**Labeled**
Serum Albumin (ALBU_HUMAN) , 66.4 kDa		
K.DLGEENFK.A	476.2	480.2
K.LVNEVTEFAK.T	575.3	579.3
K.SLHTLFGDK.L	509.3	513.3
K.AEFAEVSK.L	440.7	444.7
Histidyl - tRNA synthetase (SYHC_HUMAN) , 57.4 kDa		
K.DQGGELLSLR.Y	544.3	549.3
K.GLAPEVADR.I	464.2	469.3
R.AALEELVK.L	436.8	440.8
R.IFSIVEQR.L	496.3	501.3
Ribosyldihydronicotinamide dehydrogenase (NQO2_HUMAN) . 25.8 kDa		
K.NVAVDELSR.Q	501.8	506.8
R.SLASDITDEQK.K	603.8	607.8
R.EADLVI FQFPLYWFSVPAILK.G	1248.7	1252.7
Peroxiredoxin - 1 (PRDX1_HUMAN) 22.1 kDa		
K.DISLSDYK.G	470.7	474.7
K.ADEGISFR.G	447.7	452.7
R.GLFIIDDK.G	460.8	464.8
R.LVQAFQFTDK.H	598.8	602.8
Ubiquitin (UBIQ_HUMAN) 9.4 kDa		
R.TLSDYNIQK.E	541.3	545.3
K.ESTLHLVLR.L	534.3	539.3
K.TITLEVEPSDTIENVK.A	894.5	898.5

Prototype 1 Preparation

- Added corresponding SIL peptides to achieve a 1:1 ratio with the original concentration of each protein in the mixture.
- Aliquotted into vials and dry by vacuum centrifugation.
- Sent to sPRG labs for testing.

Conclusions

- Quantitative Analysis of Proteins Using SIL peptides:
  - Optimize the digestion protocol to achieve maximum sequence coverage and reproducibility
  - Select multiple representative proteolytic peptides that are reproducibly generated for each protein
  - Validate the purity/quantity of the SIL peptides

- Analysis of Prototype-1, the sPRG is now ready to prepare and distribute the 2009 study sample to requestors.

- Presentation of results at the 2009 ASMS.

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- For More Details, Please visit our Poster:
  - sPRG Poster RG5 S1
  - “ABRF-sPRG09: Development of a Quantitative Proteomics Standards”