

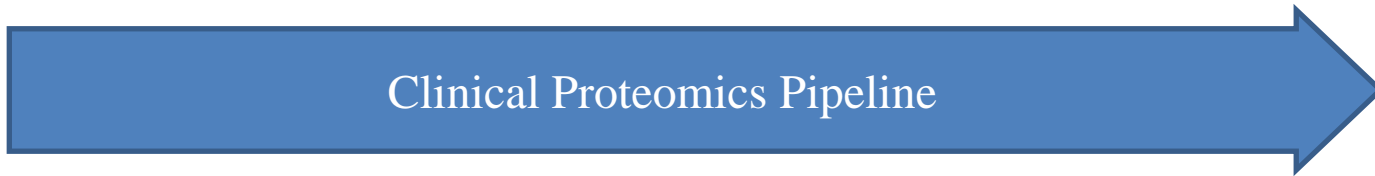
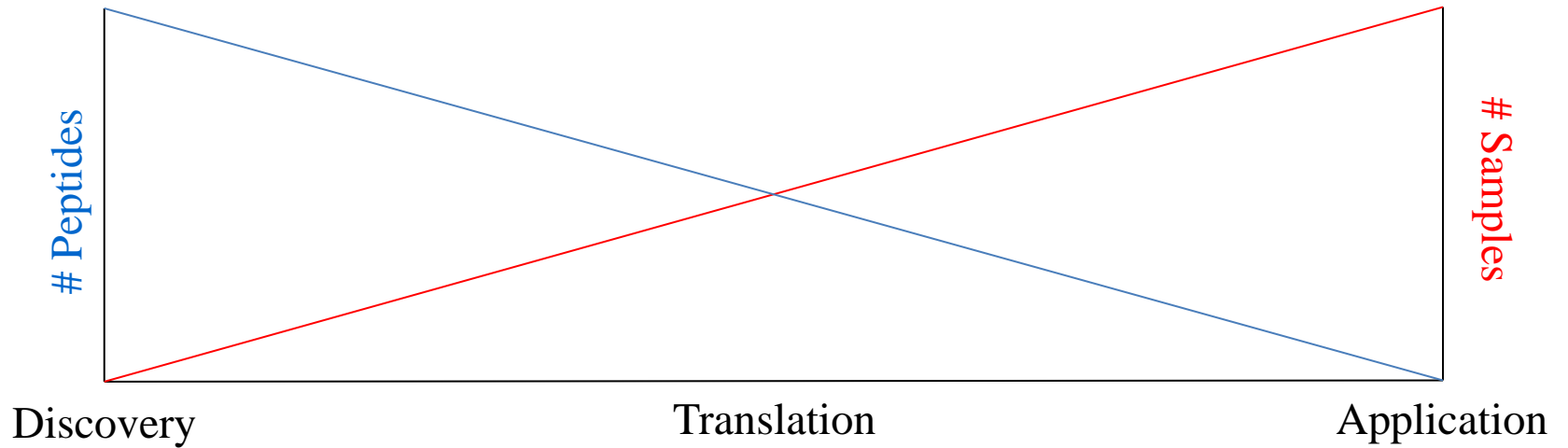
Real-world Application of Skyline in the Development of a Clinically Actionable Protein Measurement

Christopher M. Shuford

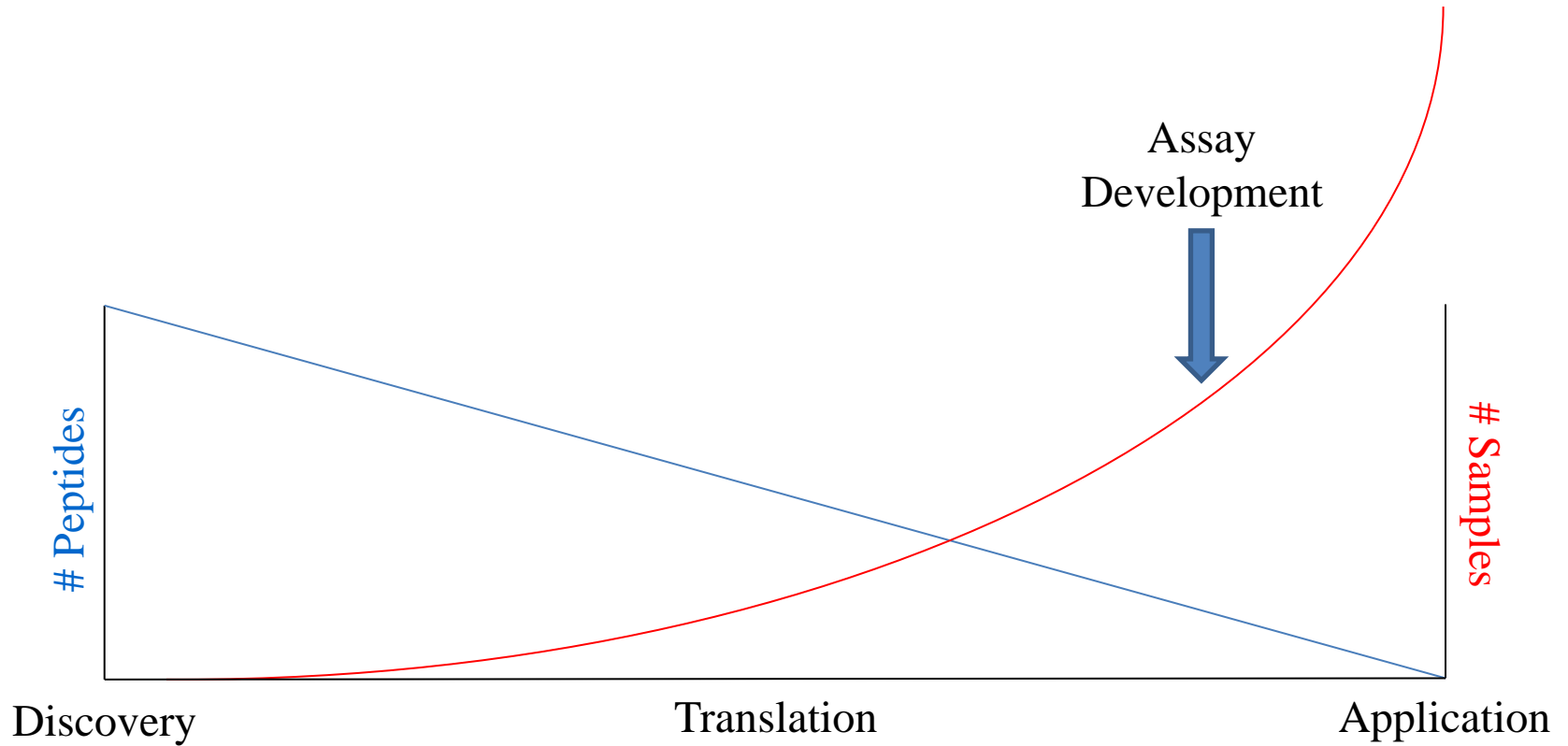
Laboratory Corporation of America® Holdings



Clinical Proteomics

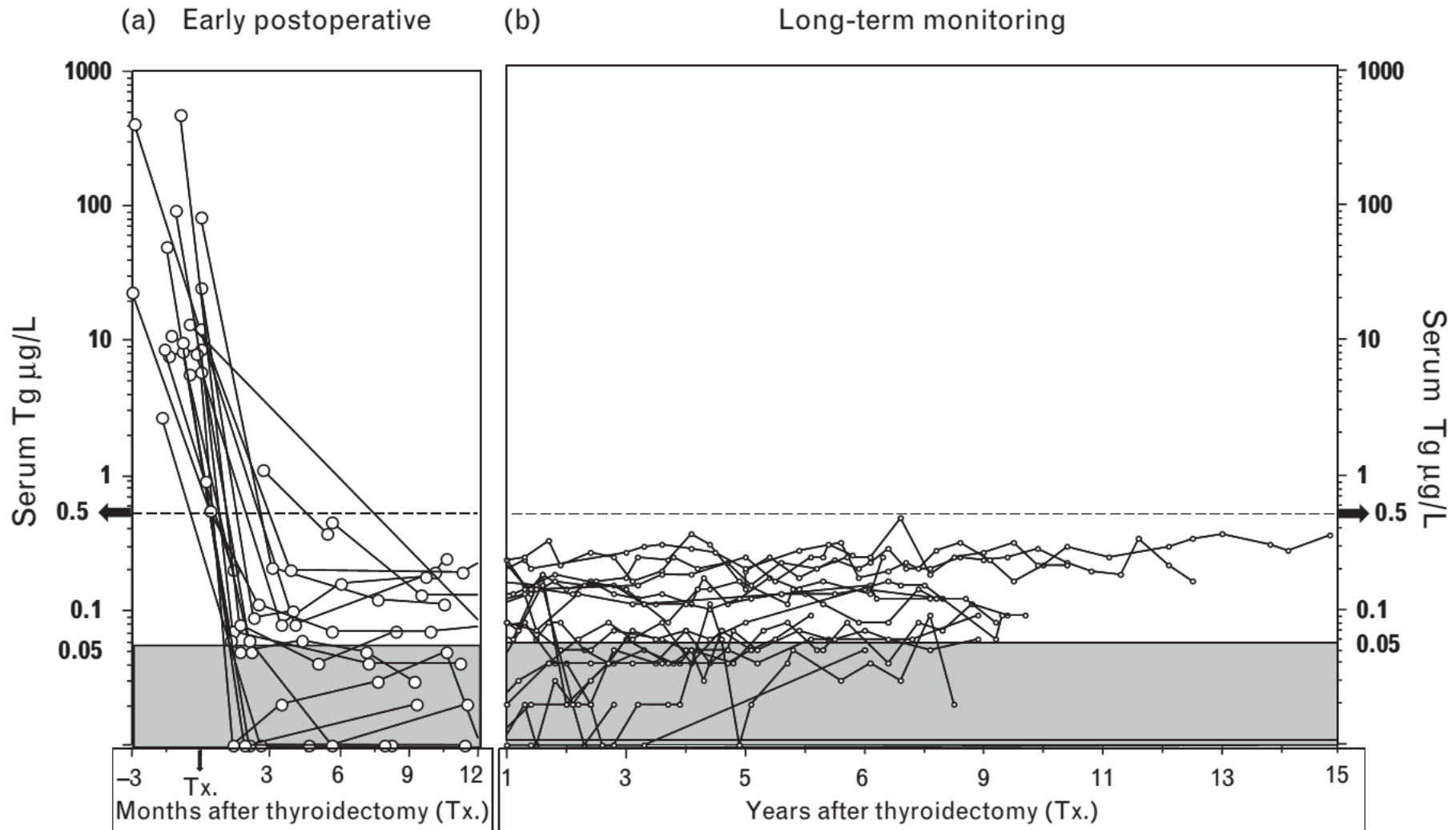


Industrialized Proteomics

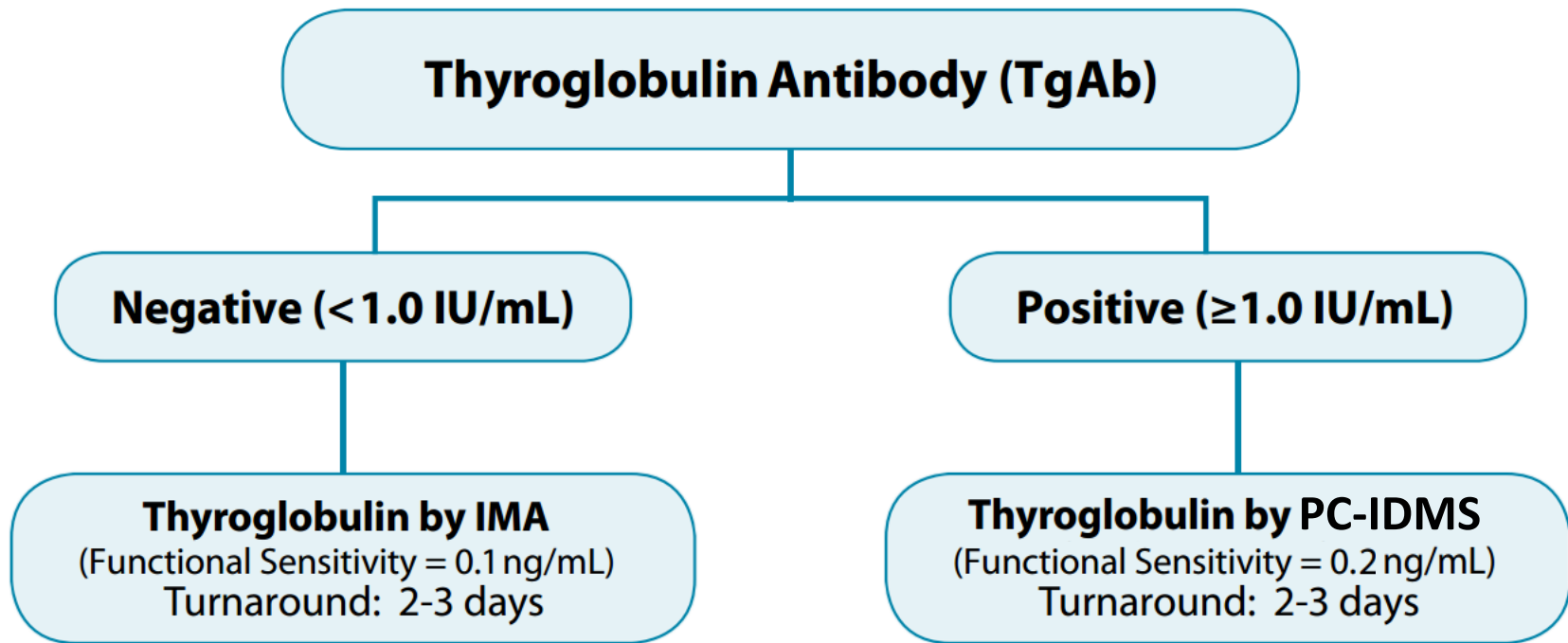


The "Real" Clinical Proteomics Pipeline

Differentiated Thyroid Cancer (DTC)



Immunometric Autoantibody Interference



*200 amol on column target
<math>< 12 \text{ hours tube-to-result}</math>*

In-silico peptide filters

Automated

Semi-Automated

Manual

114 Peptides

0 missed cleavages

6 - 25 aa

70 Peptides

Exclude Cys

Exclude Met

39 Peptides

Specific for Tg

Common among Tg

28 Peptides

Exclude PTMs

Exclude SNPs

Targets

Target	Accession	Position
R.ETAFLK.Q	[38, 43]	
K.QADYVPQCAEDGSFQTVCQNDGR.S	[44, 67]	
R.SCWCVGANGSEVLGSR.Q	[68, 83]	
R.QPGRPVACLSCQLQK.Q	[84, 99]	
R.QLGRPK.R	[152, 157]	
R.LLHGVGDK.S	[170, 177]	
K.SPPQCSAEGEFMPVQCK.F	[178, 194]	
K.FVNTTDMIMFDLVHSYNR.F	[195, 212]	
R.FPDAFVTFSSFQR.R	[213, 225]	
R.FPEVSGYCHCADSQR.E	[227, 242]	
R.FLAVQSVISGR.F	[283, 293]	
R.FTATSFQHPYVPSR.R	[305, 319]	
R.RNGDYQAVCCQTEGPCWCVDAQGK.E	[321, 343]	
K.EMHGT.R	[344, 349]	
R.QQGEPPSCAEGSCASER.Q	[350, 367]	
R.QQALSR.L	[368, 373]	
R.LYFGTSGYFSQHDLFSPEK.R	[374, 393]	
R.FATSCPTIK.E	[403, 412]	
R.AIFPSR.G	[445, 450]	
R.LALQFTTNP.K	[455, 464]	
R.LQQNLFQGGK.F	[466, 474]	
K.FLVNVGQFNLSGALGTR.G	[475, 491]	
R.GTFNFSQFFQQLGLASFLNGGR.Q	[492, 513]	
R.QEDLAKPLSVGLDNSNSTGTPEAAK.K	[514, 538]	
R.DLGDVMTVLSQTCQTPER.L	[593, 613]	
K.ELPGRS.V	[644, 649]	
R.CPTDCEK.Q	[657, 663]	
R.SAIGKPK.K	[717, 723]	
K.CPTPCQLQSEQAF.LR	[725, 739]	
R.QVCCNGPPEQVFELYQR.W	[770, 786]	
R.WEAQNK.G	[787, 792]	
K.GQDLTPAK.L	[793, 800]	
R.NCWCVDEAGQELGMR.S	[896, 911]	
K.LPTCPGSCFEAAK.L	[917, 928]	
R.VLQFIR.E	[931, 936]	
R.ETEIEVSNSSR.F	[937, 949]	
R.FPLGESFLVAK.G	[950, 960]	
R.NEDLGLPPLFPPR.E	[966, 978]	
R.EAFAEQFLR.G	[979, 987]	
R.GSDYAIR.L	[988, 994]	
R.LAAQSTLSFYQR.R	[995, 1006]	
R.FSPDSDAGASALLR.S	[1009, 1022]	
K.GGFIPGSLTAR.S	[1055, 1065]	
R.SLQIPQCTTCEK.S	[1066, 1078]	
R.TSGLLSSWK.Q	[1081, 1089]	
R.SQENPSPK.D	[1093, 1100]	
K.DLFVPACLETGEYAR.L	[1101, 1115]	
K.SGVLRS.R	[1153, 1158]	
R.VSPGYVPACR.A	[1160, 1169]	
R.VTGGQAPACESPR.C	[1202, 1213]	
R.QGSWSVFPFGPLICLESGR.W	[1250, 1269]	

Targets

Target	Accession	Position
R.ETAFLK.Q	[38, 43]	
R.QLGRPK.R	[152, 157]	
R.LLHGVGDK.S	[170, 177]	
R.FPDAFVTFSSFQR.R	[213, 225]	
R.FLAVQSVISGR.F	[283, 293]	
R.QQALSR.L	[368, 373]	
R.LYFGTSGYFSQHDLFSPEK.R	[374, 393]	
R.AIFPSR.G	[445, 450]	
R.LALQFTTNP.K	[455, 464]	
R.LQQNLFQGGK.F	[466, 474]	
K.FLVNVGQFNLSGALGTR.G	[475, 491]	
R.GTFNFSQFFQQLGLASFLNGGR.Q	[492, 513]	
R.QEDLAKPLSVGLDNSNSTGTPEAAK.K	[514, 538]	
K.ELPGRS.V	[644, 649]	
R.SAIGKPK.K	[717, 723]	
R.WEAQNK.G	[787, 792]	
K.GQDLTPAK.L	[793, 800]	
R.VLQFIR.E	[931, 936]	
R.ETEIEVSNSSR.F	[937, 949]	
R.FPLGESFLVAK.G	[950, 960]	
R.NEDLGLPPLFPPR.E	[966, 978]	
R.EAFAEQFLR.G	[979, 987]	
R.GSDYAIR.L	[988, 994]	
R.LAAQSTLSFYQR.R	[995, 1006]	
R.FSPDSDAGASALLR.S	[1009, 1022]	
K.GGFIPGSLTAR.S	[1055, 1065]	
R.TSGLLSSWK.Q	[1081, 1089]	
R.SQENPSPK.D	[1093, 1100]	
K.SGVLRS.R	[1153, 1158]	
R.WESQLPQPR.A	[1270, 1278]	
R.LGVNVTWK.S	[1361, 1368]	
R.LEDIPVASLPDLHDIER.A	[1371, 1387]	
R.FTDLIQSGSFLHLDK.T	[1398, 1414]	
K.TFPAETIR.F	[1415, 1422]	
R.FLQGDHFGTSPR.T	[1423, 1434]	
K.VIFDANAPVAVR.S	[1578, 1589]	
R.DALGNSK.A	[1646, 1652]	
K.ATSFGLR.C	[1653, 1660]	
R.SHGQDSPAVYK.K	[1667, 1678]	
K.QGGSSTTLQK.R	[1680, 1689]	
R.LGDQEFIK.S	[1792, 1799]	
K.SLTPLEGTQDFTNFQVYLWK.D	[1800, 1821]	
K.VILEDK.V	[1954, 1959]	
K.VSLDSWQSLALSSVVDPSIR.H	[2087, 2107]	
R.HFDVAHVSTAATSNFSAVR.D	[2108, 2126]	
R.EEATHYR.K	[2177, 2184]	
R.KPGISLLSYEASVPSPISTHGR.L	[2185, 2207]	
R.SQAIQVTSWK.Q	[2212, 2222]	
K.QVDQFLGVPPYAPPLAER.R	[2223, 2240]	
R.FQAEPLNWTGSDWASKPR.A	[2242, 2260]	
R.GFGDPR.R	[2371, 2377]	
R.VSLAADR.G	[2379, 2385]	
R.GGADVASHILLTAR.A	[2386, 2399]	
R.ATNSQLFR.R	[2400, 2407]	
R.AQQQAIALAK.E	[2428, 2437]	
R.QKPNVNLDAQT.K.L	[2455, 2467]	
K.LLVAQSGPFHYGVIDGHFLR.E	[2468, 2488]	
R.SLWVEVDLLIGSSQDDGLNLR.A	[2498, 2518]	
K.TAFYQALQNSLGGEDSDAR.V	[2536, 2554]	
R.VEAATWYYSLEHSTDDYASFSR.A	[2555, 2577]	
R.ALENATR.D	[2578, 2584]	
R.SGNPNPYEFFSR.K	[2664, 2675]	
K.VPTFATPWDFVPR.A	[2677, 2690]	
R.AGGENYK.E	[2691, 2697]	
K.EFSELLP.NR.Q	[2698, 2706]	
K.TSADGAK.G	[2726, 2732]	
K.GGQSAESEEEELTAGSGLR.E	[2733, 2751]	
R.EDLLSLQEPGSK.T	[2752, 2763]	

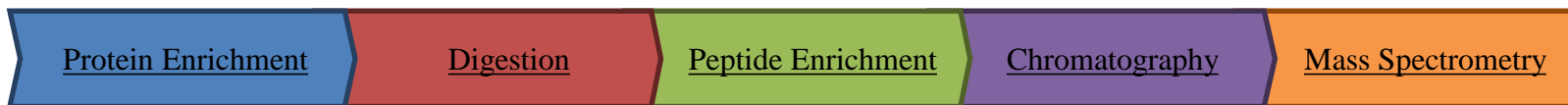
Targets

Target	Accession	Position
R.WESQLPQPR.A	[1270, 1278]	
R.LGVNVTWK.S	[1361, 1368]	
R.LEDIPVASLPDLHDIER.A	[1371, 1387]	
R.FTDLIQSGSFLHLDK.T	[1398, 1414]	
K.TFPAETIR.F	[1415, 1422]	
R.FLQGDHFGTSPR.T	[1423, 1434]	
K.VIFDANAPVAVR.S	[1578, 1589]	
R.DALGNSK.A	[1646, 1652]	
K.ATSFGLR.C	[1653, 1660]	
R.SHGQDSPAVYK.K	[1667, 1678]	
K.QGGSSTTLQK.R	[1680, 1689]	
R.LGDQEFIK.S	[1792, 1799]	
K.SLTPLEGTQDFTNFQVYLWK.D	[1800, 1821]	
K.VILEDK.V	[1954, 1959]	
K.VSLDSWQSLALSSVVDPSIR.H	[2087, 2107]	
R.HFDVAHVSTAATSNFSAVR.D	[2108, 2126]	
R.EEATHYR.K	[2177, 2184]	
R.KPGISLLSYEASVPSPISTHGR.L	[2185, 2207]	
R.SQAIQVTSWK.Q	[2212, 2222]	
K.QVDQFLGVPPYAPPLAER.R	[2223, 2240]	
R.FQAEPLNWTGSDWASKPR.A	[2242, 2260]	
R.GFGDPR.R	[2371, 2377]	
R.VSLAADR.G	[2379, 2385]	
R.GGADVASHILLTAR.A	[2386, 2399]	
R.ATNSQLFR.R	[2400, 2407]	
R.AQQQAIALAK.E	[2428, 2437]	
R.QKPNVNLDAQT.K.L	[2455, 2467]	
K.LLVAQSGPFHYGVIDGHFLR.E	[2468, 2488]	
R.SLWVEVDLLIGSSQDDGLNLR.A	[2498, 2518]	
K.TAFYQALQNSLGGEDSDAR.V	[2536, 2554]	
R.VEAATWYYSLEHSTDDYASFSR.A	[2555, 2577]	
R.ALENATR.D	[2578, 2584]	
R.SGNPNPYEFFSR.K	[2664, 2675]	
K.VPTFATPWDFVPR.A	[2677, 2690]	
R.AGGENYK.E	[2691, 2697]	
K.EFSELLP.NR.Q	[2698, 2706]	
K.TSADGAK.G	[2726, 2732]	
K.GGQSAESEEEELTAGSGLR.E	[2733, 2751]	
R.EDLLSLQEPGSK.T	[2752, 2763]	

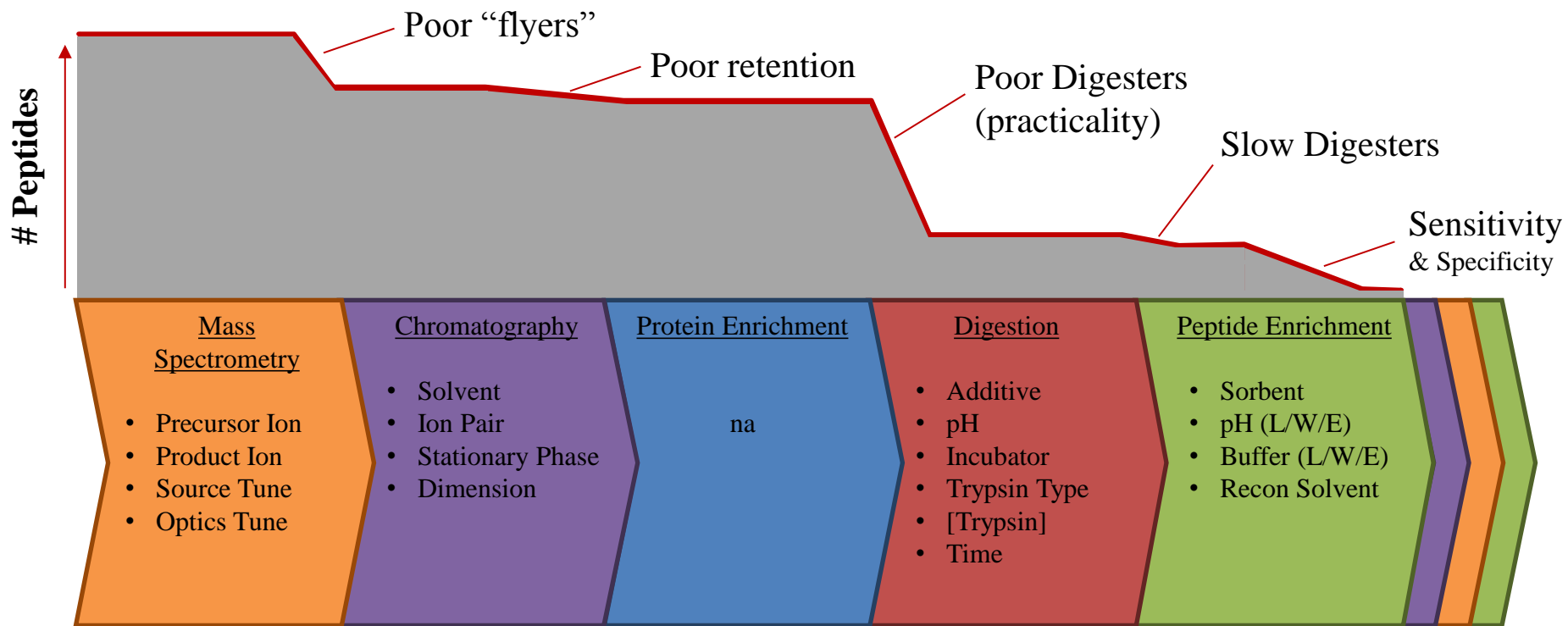
Targets

Target	Accession	Position
R.WESQLPQPR.A	[1270, 1278]	
R.LEDIPVASLPDLHDIER.A	[1371, 1387]	
R.FTDLIQSGSFLHLDK.T	[1398, 1414]	
K.TFPAETIR.F	[1415, 1422]	
R.FLQGDHFGTSPR.T	[1423, 1434]	
K.VIFDANAPVAVR.S	[1578, 1589]	
R.DALGNSK.A	[1646, 1652]	
K.ATSFGLR.C	[1653, 1660]	
R.SHGQDSPAVYK.K	[1667, 1678]	
K.QGGSSTTLQK.R	[1680, 1689]	
R.LGDQEFIK.S	[1792, 1799]	
K.SLTPLEGTQDFTNFQVYLWK.D	[1800, 1821]	
K.VILEDK.V	[1954, 1959]	
R.HFDVAHVSTAATSNFSAVR.D	[2108, 2126]	
R.EEATHYR.K	[2177, 2184]	
R.KPGISLLSYEASVPSPISTHGR.L	[2185, 2207]	
R.SQAIQVTSWK.Q	[2212, 2222]	
R.VSLAADR.G	[2379, 2385]	
R.GGADVASHILLTAR.A	[2386, 2399]	
R.ATNSQLFR.R	[2400, 2407]	
R.AQQQAIALAK.E	[2428, 2437]	
K.TAFYQALQNSLGGEDSDAR.V	[2536, 2554]	
R.SGNPNPYEFFSR.K	[2664, 2675]	
K.VPTFATPWDFVPR.A	[2677, 2690]	
R.AGGENYK.E	[2691, 2697]	
K.EFSELLP.NR.Q	[2698, 2706]	
K.TSADGAK.G	[2726, 2732]	
R.EDLLSLQEPGSK.T	[2752, 2763]	

Empirical Peptide Filters

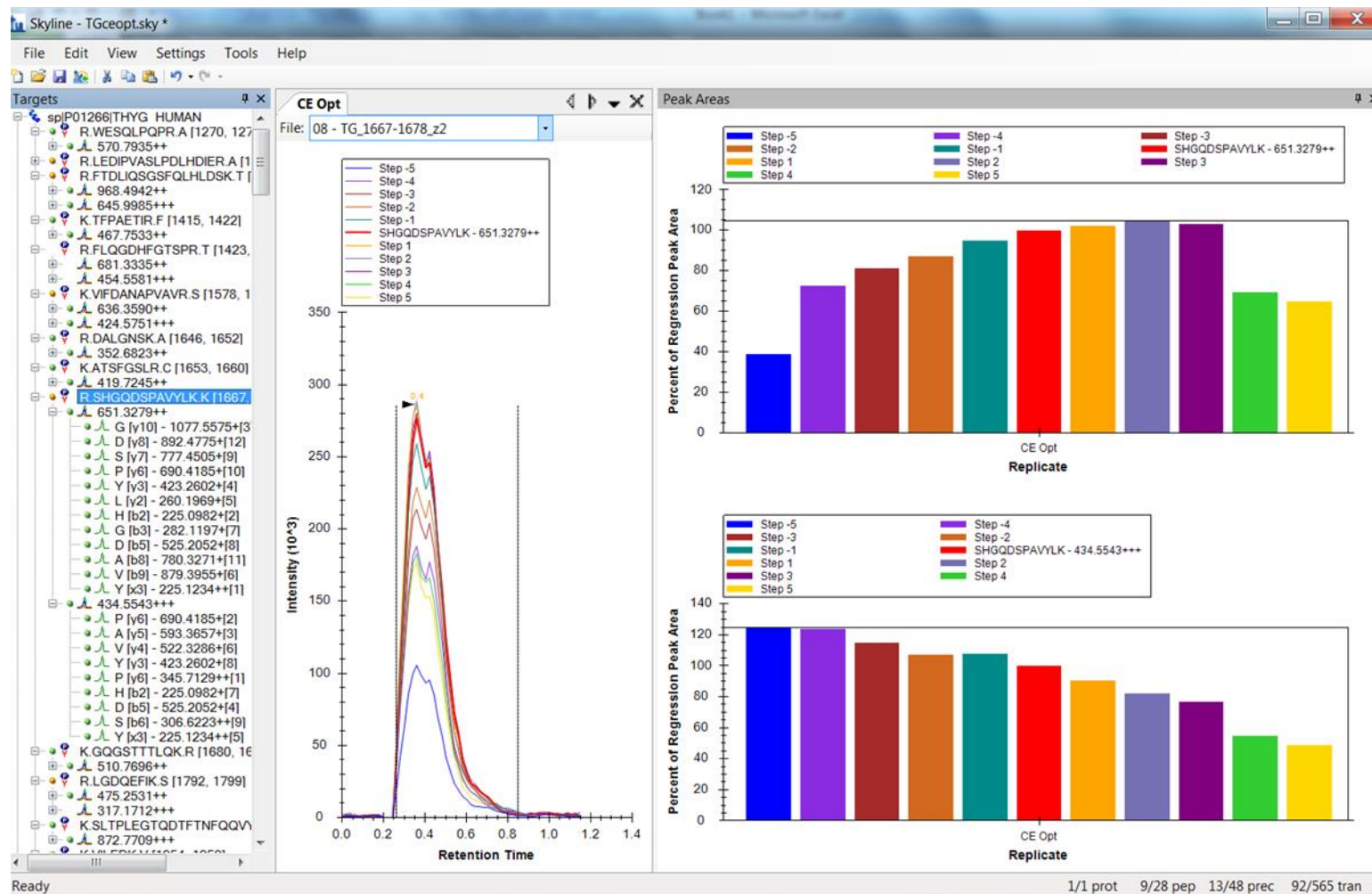


Assay Workflow



Development Workflow

Automated Tuning via Flow-Injection



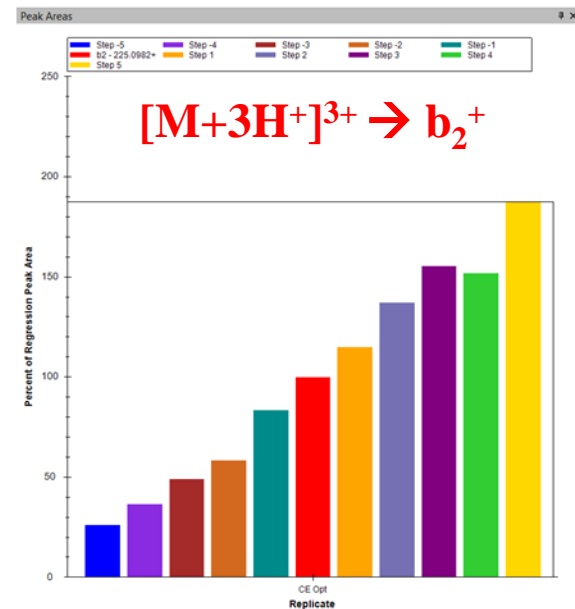
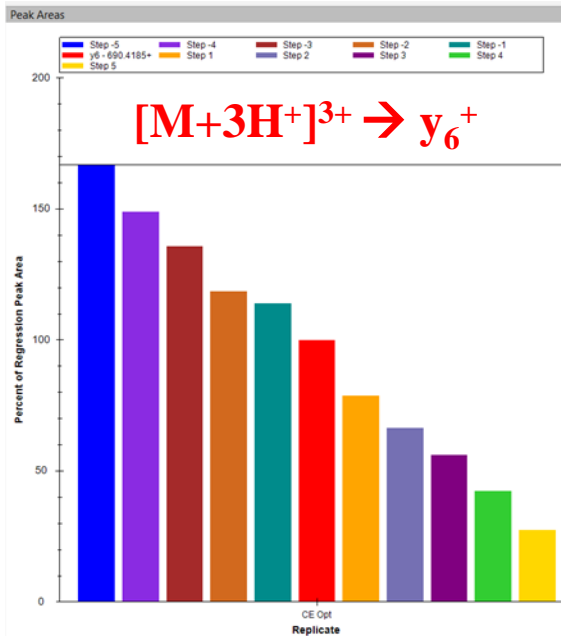
[M+2H]²⁺

[M+3H]³⁺

Flow injection with 70/30/0.1, H₂O/ACN/FA

1 precursor ion optimized per injection (29 peptides ~ 40 injections ~ 60 minutes)

Automated Tuning via Flow-Injection



Transition Settings

Prediction Filter Library Instrument Full-Scan

Precursor mass: Monoisotopic

Product ion mass: Monoisotopic

Collision energy: ABI

Declustering potential: ABI

Optimization library: None

Use optimization values when present

Optimize by:

- Precursor
- Precursor
- Transition

OK Cancel

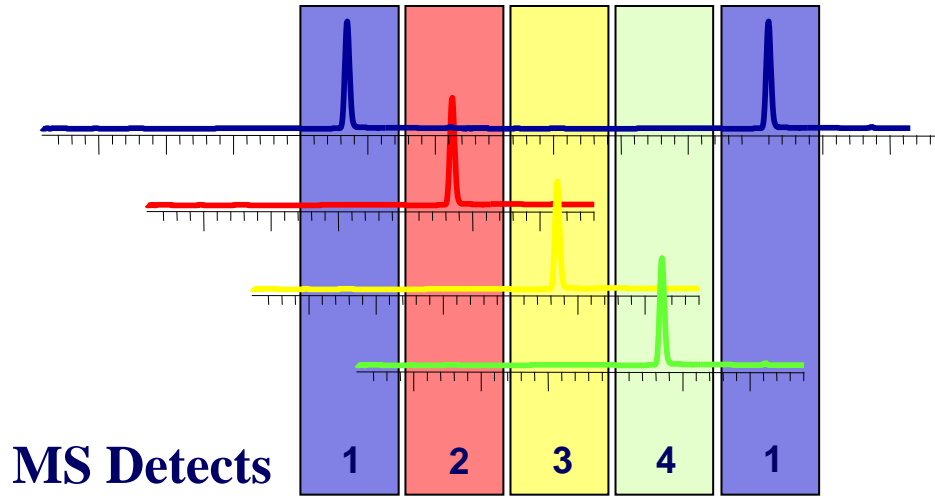
Solvent Chemistry

96 chemistries x 26 peptides = 2496 injections

Effect Relative to ACN:MeOH ($\pm\%$)

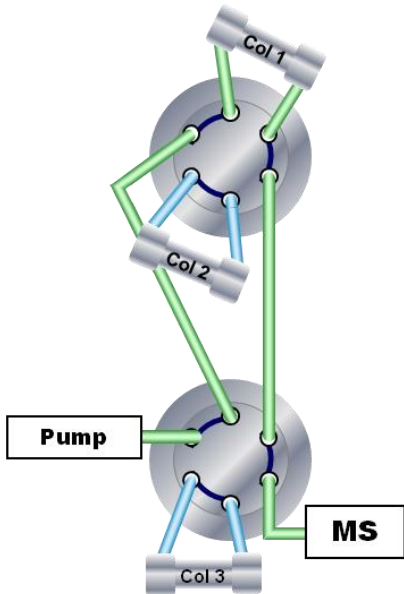
	Median	Peptide A	Peptide B	Peptide C	Peptide D	Peptide E	Peptide F	Peptide G	Peptide H	Peptide I	Peptide J	Peptide K	Peptide L
MeOH	23.3	8.8	-15.0	-72.9	19.4	31.1	26.4	37.6	26.9	39.6	29.5	-50.0	11.4
ACN	-23.3	-8.8	15.0	72.9	-19.4	-31.1	-26.4	-37.6	-26.9	-39.6	-29.5	50.0	-11.4
HOAc	24.5	13.9	87.7	88.8	13.3	36.0	3.9	24.5	97.7	2.5	11.7	62.8	14.3
FA	-0.7	-0.7	32.8	65.8	34.8	39.9	1.7	48.5	92.3	22.6	-0.2	47.5	9.5
TFA	-58.9	-51.3	-19.5	4.2	-70.8	-63.2	-75.8	-66.0	-40.4	-63.3	-72.4	40.5	-59.1
TFA-FIX	-62.6	-62.6	-22.7	17.3	-85.7	-70.0	-72.0	-72.0	-32.8	-52.2	-81.7	244.9	-80.9
NH4Ac	-13.8	-15.7	23.7	13.2	-29.1	-3.9	-44.3	-9.3	18.0	-30.5	-29.3	73.1	-11.4
NH4FA	-9.1	-21.9	14.2	65.4	-5.8	-7.2	-21.2	-9.3	43.3	-5.7	-29.6	88.4	-40.8
	Peptide M	Peptide N	Peptide O	Peptide P	Peptide Q	Peptide R	Peptide S	Peptide T	Peptide U	Peptide V	Peptide W	Peptide X	Peptide Y
MeOH	15.7	39.3	23.3	24.4	38.0	28.0	38.9	-39.7	-19.7	25.1	-6.1	-8.2	8.9
ACN	-15.7	-39.3	-23.3	-24.4	-38.0	-28.0	-38.9	39.7	19.7	-25.1	6.1	8.2	-8.9
HOAc	49.2	91.2	107.9	33.8	58.9	58.7	28.7	-52.4	-9.5	-50.3	0.0	-43.6	-17.0
FA	-27.4	-5.1	-12.5	-24.3	-28.9	-20.7	-34.0	-35.8	-0.2	-43.4	-11.8	-45.8	-34.3
TFA	-56.6	-39.7	-53.1	-57.5	-52.1	-55.6	-29.1	-79.8	-66.1	-89.0	-59.9	-83.5	-58.9
TFA-FIX	-25.1	-17.0	6.2	-14.6	-17.3	-11.9	-5.4	-80.0	-83.9	-77.5	-73.8	-77.3	-73.2
NH4Ac	-17.4	5.2	15.2	-13.8	-6.8	-5.3	-3.3	-55.9	-29.5	-64.7	-25.5	-62.4	-42.3
NH4FA	-5.3	19.8	20.7	-10.9	-4.4	-0.8	-9.1	-55.8	-28.8	-59.6	-35.1	-57.7	-42.6

Column Screening

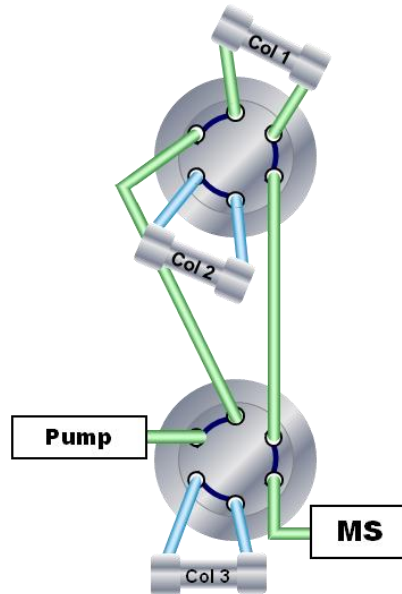


ARIA™ Transcend TLX-4
(4 × 2D-LC Systems → 1 MS)

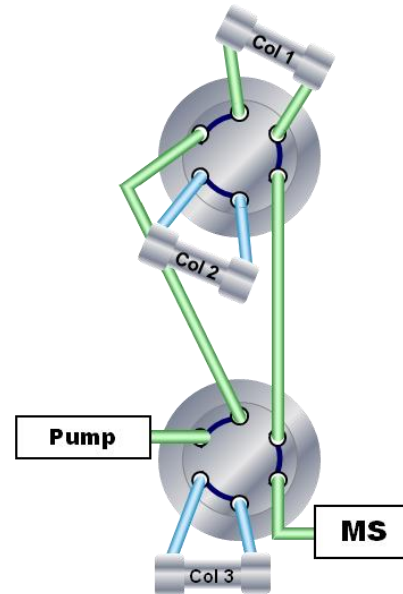
Mobile Phase 1



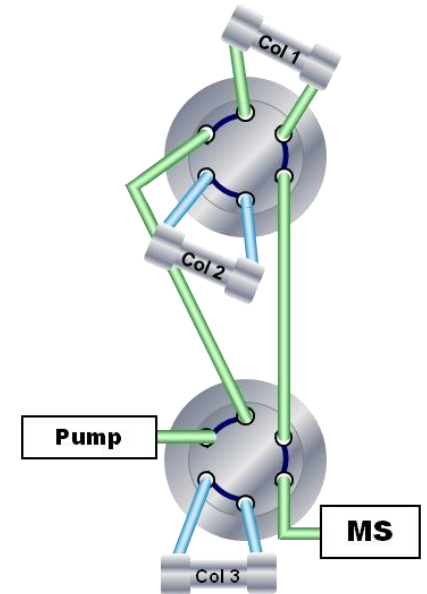
Mobile Phase 2



Mobile Phase 3



Mobile Phase 4



Column Screening

Export Method

Instrument type: **AB SCIEX QTRAP** [OK] [Cancel]

Single method
 One method per protein
 Multiple methods Ignore proteins

Max transitions per sample injection: 22

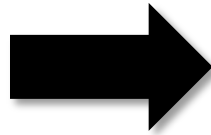
Methods: 11

Optimizing: None

Method type: Standard Dwell time (ms): 10

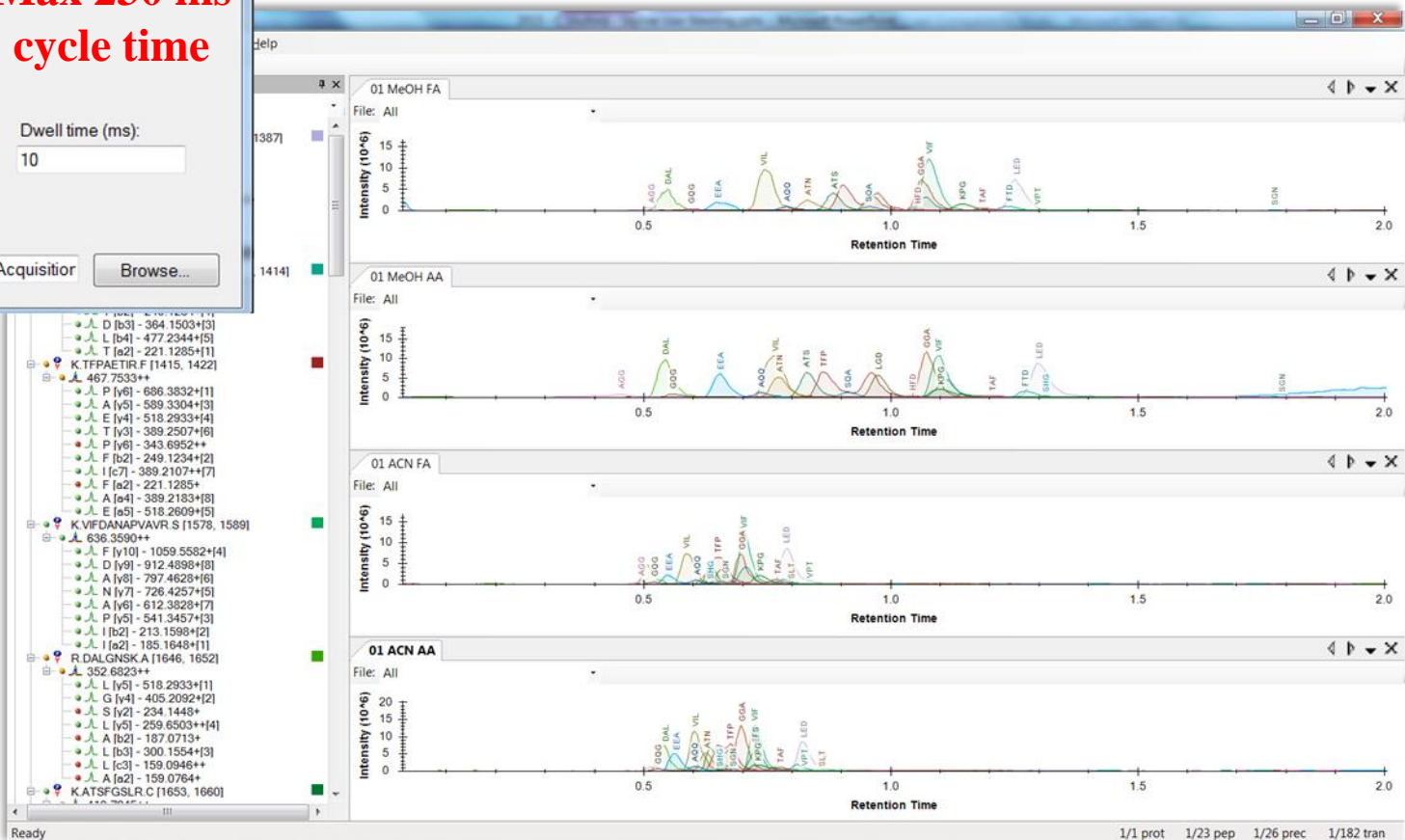
Template file: C:\Analyst Data\Projects\THYG\Acquisitor [Browse...]

**Max 230 ms
cycle time**



12 columns
× 11 injections/column
× 4 mobile phases/column

528 injections → 20 hours → 1104 chromatograms



Column Screening

Tier 1 Column Screen

12 Columns (Multimodal)

4 Mobile Phase

(ACN-FA, ACN-AA, MeOH-FA, MeOH-AA)

528 injections → 1004 Chromatograms

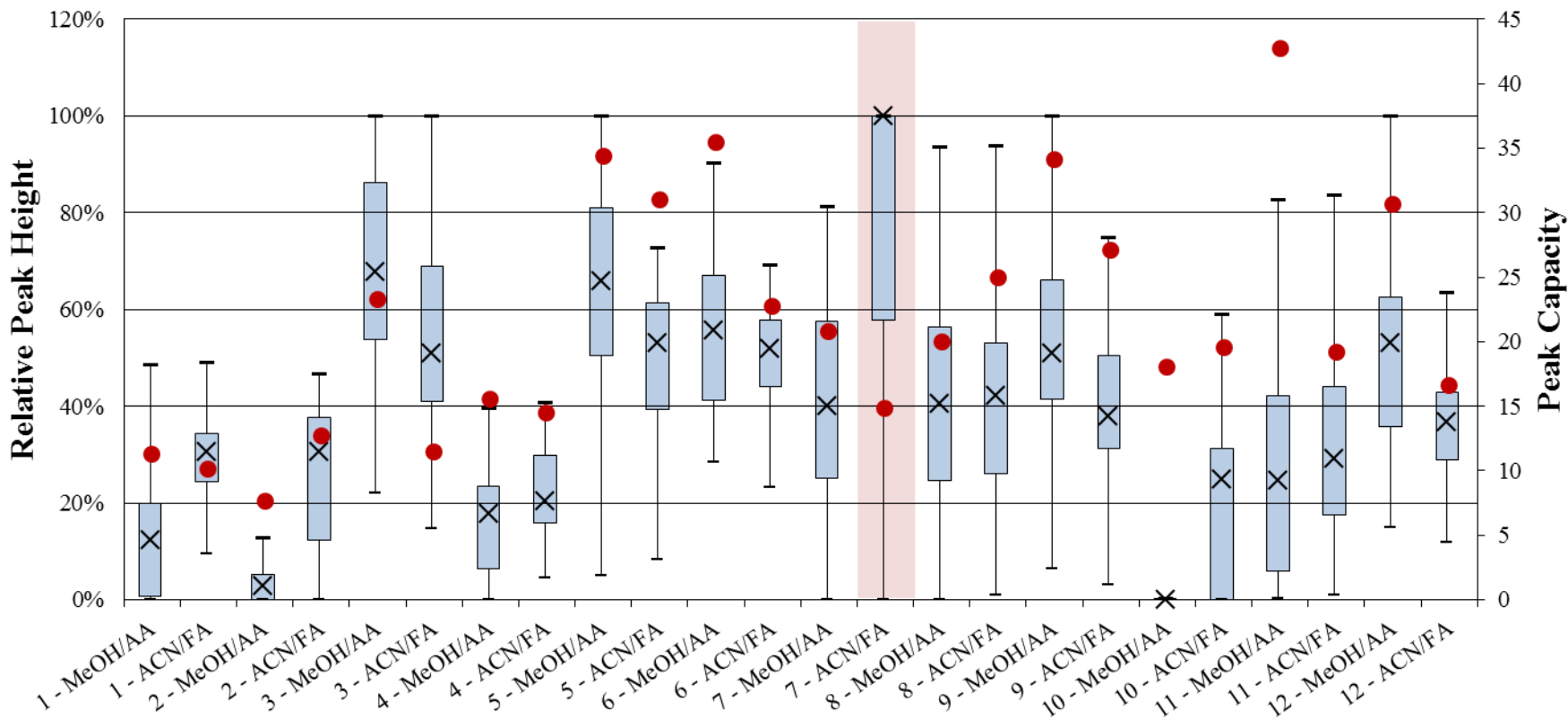
Tier 2 Column Screen

12 Columns (“Optimal” Mode)

2 Mobile Phase

(ACN-FA, MeOH-AA)

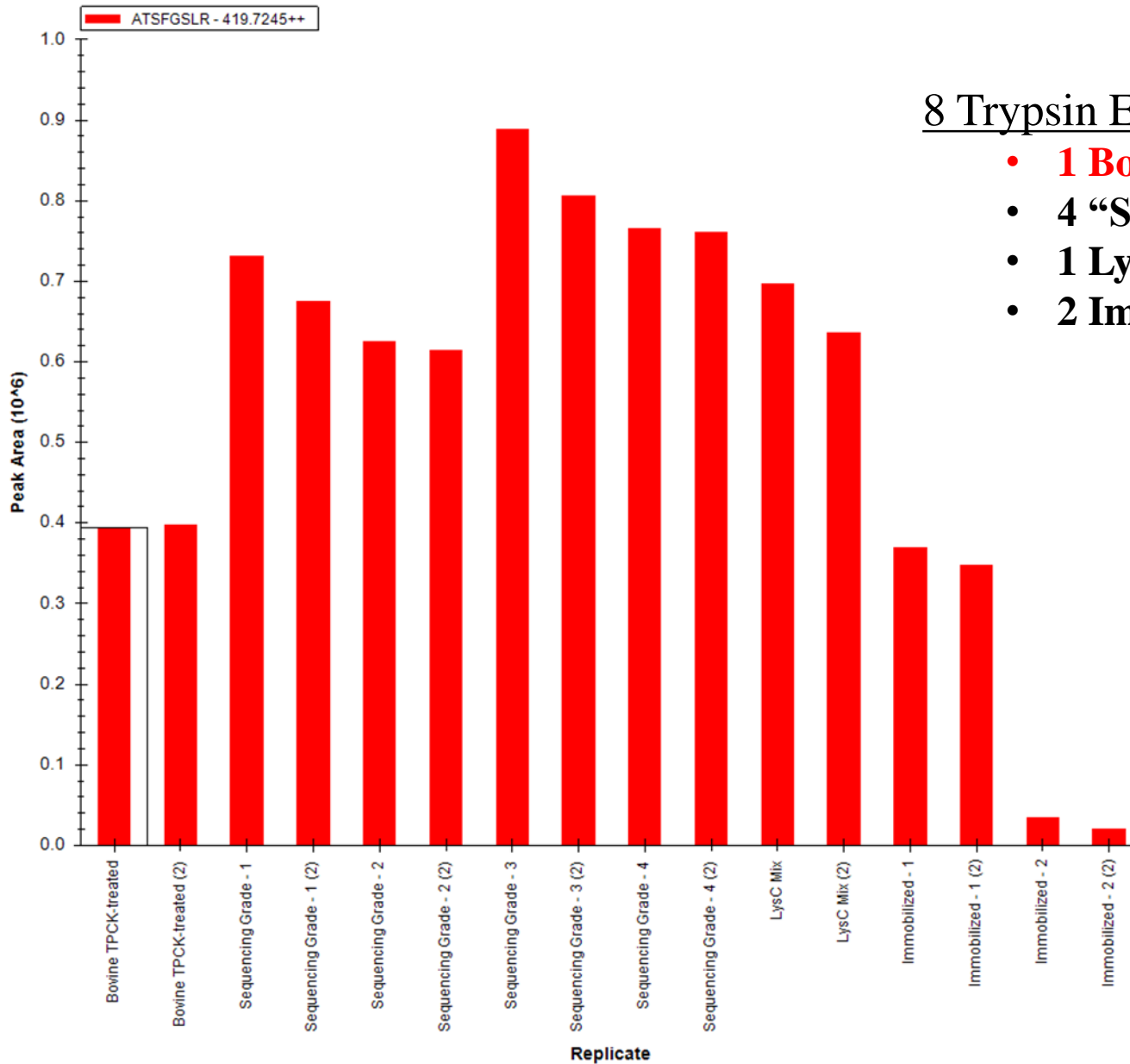
72 injections → 552 Chromatograms



Preliminary Digestion Screen

Peak Areas

⌵ ×



8 Trypsin Evaluation

- **1 Bovine TPCK-treated**
- **4 “Sequencing Grade”**
- **1 LysC/Trypsin Mixture**
- **2 Immobilized**

Preliminary Digestion Screen



Empirically Driven Peptide Attrition

28 Peptides
In-silico Filters



26 Peptides
Exclude
Poor Flyers



26 Peptides
Exclude
Poor Retainers



8 Peptides
Select
“Easy Digesters”

Targets

- R.WESQLPQPR.A [1270, 1278]
- R.LEDIPVASLPDLHDIER.A [1371, 1387]
- R.FTDLIQSGSFQLHLDISK.T [1398, 1414]
- K.TFPAETIR.F [1415, 1422]
- R.FLQGDHFVTSR.T [1423, 1434]
- K.VIFDANAPVAVR.S [1578, 1589]
- R.DALGNASK.A [1646, 1652]
- K.ATSFGLR.C [1653, 1660]
- R.SHGQDSPAVYK.K [1667, 1678]
- K.GQGSTTTLQK.R [1680, 1689]
- R.LGDQEFIK.S [1792, 1799]
- K.SLTPLEGTQDTFTNFQQVYLWK.D [1800, 1821]
- K.VILEDK.V [1954, 1959]
- R.HFDVAHVSTAATSNFSAVR.D [2108, 2126]
- R.EEATHIYR.K [2177, 2184]
- R.KPGISLLSYEASVSPVSTHGR.L [2185, 2207]
- R.SQAIQVGTSWK.Q [2212, 2222]
- R.VSLAADR.G [2379, 2385]
- R.GGADVASHLLTAR.A [2386, 2399]
- R.ATNSQLFR.R [2400, 2407]
- R.AQQQAIALAK.E [2428, 2437]
- K.TAFYQALQNSLGGEDSDAR.V [2536, 2554]
- R.SGNPNYPYEF.SR.K [2664, 2675]
- K.VPTFATPWPDFVPR.A [2677, 2690]
- R.AGGENYK.E [2691, 2697]
- K.EFSELLP.NR.Q [2698, 2706]
- K.TSADGAK.G [2726, 2732]
- R.EDLLSLQEPGSK.T [2752, 2763]

Targets

- R.LEDIPVASLPDLHDIER.A [1371, 1387]
- R.FTDLIQSGSFQLHLDISK.T [1398, 1414]
- K.TFPAETIR.F [1415, 1422]
- K.VIFDANAPVAVR.S [1578, 1589]
- R.DALGNASK.A [1646, 1652]
- K.ATSFGLR.C [1653, 1660]
- R.SHGQDSPAVYK.K [1667, 1678]
- K.GQGSTTTLQK.R [1680, 1689]
- R.LGDQEFIK.S [1792, 1799]
- K.SLTPLEGTQDTFTNFQQVYLWK.D [1800, 1821]
- K.VILEDK.V [1954, 1959]
- R.HFDVAHVSTAATSNFSAVR.D [2108, 2126]
- R.EEATHIYR.K [2177, 2184]
- R.KPGISLLSYEASVSPVSTHGR.L [2185, 2207]
- R.SQAIQVGTSWK.Q [2212, 2222]
- R.VSLAADR.G [2379, 2385]
- R.GGADVASHLLTAR.A [2386, 2399]
- R.ATNSQLFR.R [2400, 2407]
- R.AQQQAIALAK.E [2428, 2437]
- K.TAFYQALQNSLGGEDSDAR.V [2536, 2554]
- R.SGNPNYPYEF.SR.K [2664, 2675]
- K.VPTFATPWPDFVPR.A [2677, 2690]
- R.AGGENYK.E [2691, 2697]
- K.EFSELLP.NR.Q [2698, 2706]

Targets

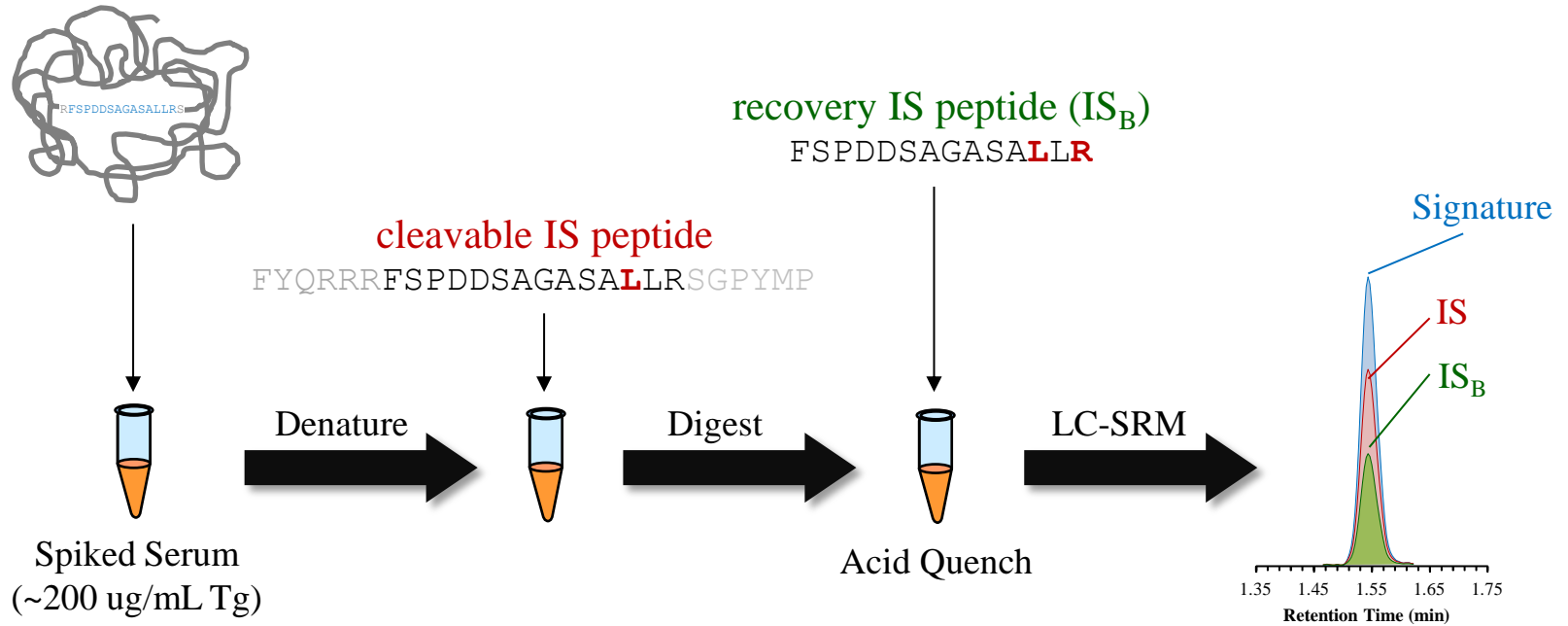
- R.LEDIPVASLPDLHDIER.A [1371, 1387]
- R.FTDLIQSGSFQLHLDISK.T [1398, 1414]
- K.TFPAETIR.F [1415, 1422]
- K.VIFDANAPVAVR.S [1578, 1589]
- R.DALGNASK.A [1646, 1652]
- K.ATSFGLR.C [1653, 1660]
- R.SHGQDSPAVYK.K [1667, 1678]
- K.GQGSTTTLQK.R [1680, 1689]
- R.LGDQEFIK.S [1792, 1799]
- K.SLTPLEGTQDTFTNFQQVYLWK.D [1800, 1821]
- K.VILEDK.V [1954, 1959]
- R.HFDVAHVSTAATSNFSAVR.D [2108, 2126]
- R.EEATHIYR.K [2177, 2184]
- R.KPGISLLSYEASVSPVSTHGR.L [2185, 2207]
- R.SQAIQVGTSWK.Q [2212, 2222]
- R.GGADVASHLLTAR.A [2386, 2399]
- R.ATNSQLFR.R [2400, 2407]
- R.AQQQAIALAK.E [2428, 2437]
- K.TAFYQALQNSLGGEDSDAR.V [2536, 2554]
- R.SGNPNYPYEF.SR.K [2664, 2675]
- K.VPTFATPWPDFVPR.A [2677, 2690]
- R.AGGENYK.E [2691, 2697]
- K.EFSELLP.NR.Q [2698, 2706]

Targets

Replicates: 4mg_mL_6_5_hrs_Rep 3

- R.FSPDDASAGASALLR.S [1009, 1022]
- R.LEDIPVASLPDLHDIER.A [1371, 1387]
- K.TFPAETIR.F [1415, 1422]
- K.VIFDANAPVAVR.S [1578, 1589]
- K.VILEDK.V [1954, 1959]
- R.SQAIQVGTSWK.Q [2212, 2222]
- R.GGADVASHLLTAR.A [2386, 2399]
- K.EFSELLP.NR.Q [2698, 2706]

Digestion Recovery



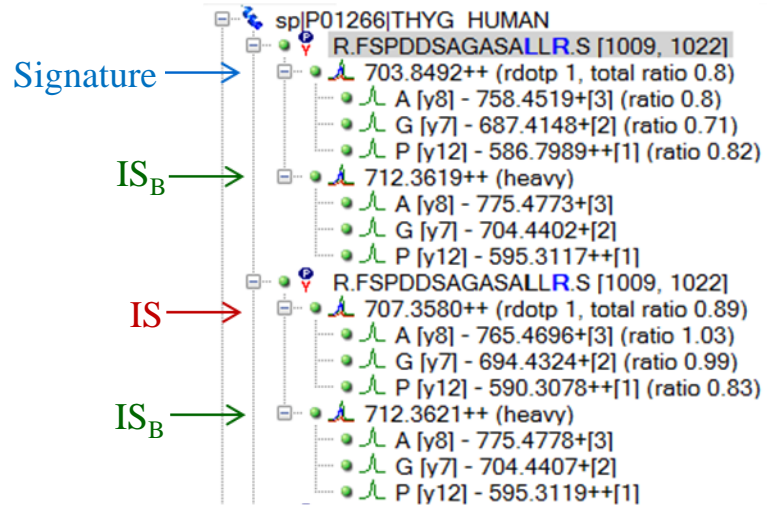
$$1 \quad \frac{A_{Sig}}{A_{IS_B}} \times [IS_B] = [Sig]$$

Recovery of **Signature** Peptide

$$2 \quad \frac{A_{IS}}{A_{IS_B}} \times [IS_B] = [IS]$$

Recovery of **Internal Standard** Peptide

Digestion Recovery



Edit Modifications

Structural:		Isotope heavy:
	F	
	S	
	P	
	D	
	D	
	S	
	A	
	G	
	A	
	S	
	A	Leu
	L	
	R	Arg

Buttons: OK, Cancel, Create copy, Reset

Edit Modifications

Structural:		Isotope heavy:
	F	
	S	
	P	
	D	
	D	
	S	
	A	
	G	
	A	
	S	
	A	
Leu	L	
	L	
	R	Arg

Buttons: OK, Cancel, Create copy, Reset

Additive Screening

9 additives x 2 levels x 3 time points x 3 replicates = 162 injections

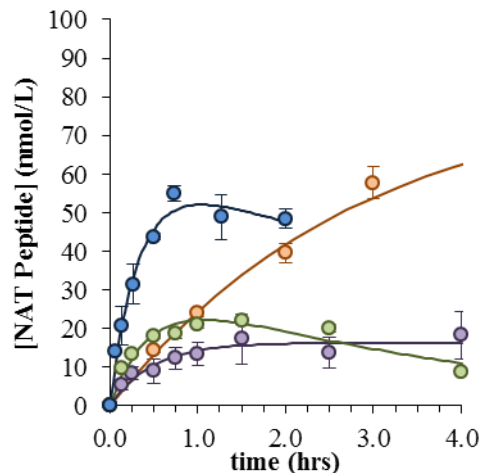
% of max yield	Additive	LED	TFP	VIF	VIL	SQA	GGA	EFS	FSP	# >85% of max
100%	0.25 M Urea	61.7	77.7	82.0	44.6	94.0	63.2	58.7	47.2	5
95%	1.0 M Urea	66.5	83.4	83.6	52.6	94.4	55.6	60.6	48.2	6
90%	0.25 M GnHCl	45.2	79.9	83.2	53.3	92.5	36.9	60.7	39.2	4
85%	1.0 M GnHCl	26.0	65.2	81.7	53.2	113.4	63.7	61.9	51.5	6
80%	0.0675 M Thiurea	52.2	74.8	81.9	42.8	90.5	57.5	57.1	49.0	4
70%	0.25 M Thiourea	66.4	82.0	80.7	55.5	94.7	54.7	64.2	48.5	6
60%	0.2% DOC	60.0	82.4	89.4	52.1	96.6	53.8	66.1	49.7	7
50%	0.8% DOC	16.7	76.4	91.9	49.4	83.1	42.0	60.6	58.2	5
40%	0.125% CHAPS	31.7	69.1	81.5	26.7	88.2	49.3	41.0	34.6	1
30%	0.5% CHAPS	69.8	76.2	76.5	50.0	89.0	47.3	56.1	46.5	3
20%	1.25% TFE	69.6	83.1	83.4	55.3	88.8	55.9	63.3	55.3	7
10%	5.0% TFE	57.3	76.3	81.3	57.8	83.4	54.6	60.5	57.8	6
0%	10% ACN	33.8	77.0	93.4	21.5	100.6	56.5	64.3	35.3	5
	37.5% ACN	3.0	13.0	65.9	3.9	45.3	14.4	5.3	9.1	0
	10% MeOH	1.9	7.7	53.4	1.0	35.6	5.3	3.1	6.5	0
	37.5% MeOH	16.3	64.1	80.5	40.7	58.2	30.2	44.5	26.9	1
	10% 1-PrpOH	21.1	21.1	77.7	44.7	79.9	43.0	41.6	38.3	0
	37.5% 1-PrpOH	1.0	1.0	56.8	2.3	27.3	6.1	2.1	9.3	0
	No Additive	1.3	3.1	56.2	0.6	29.6	3.1	1.8	7.7	0
	Maximum Recovered	69.8	83.4	93.4	57.8	113.4	63.7	66.1	58.2	
	% Recovery @ Max	44%	53%	59%	37%	72%	41%	42%	37%	

min time to 85% of max yield

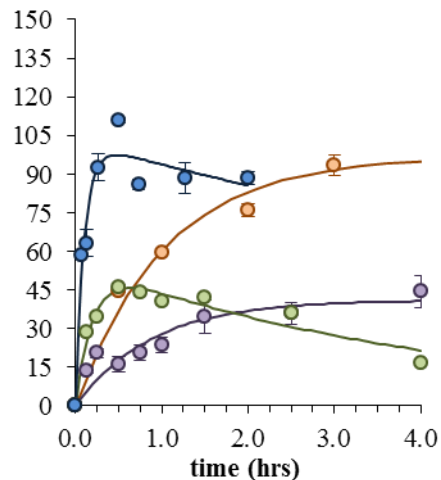
- 1 hr
- 4 hrs
- 20 hrs

“Enzyme Reactors”

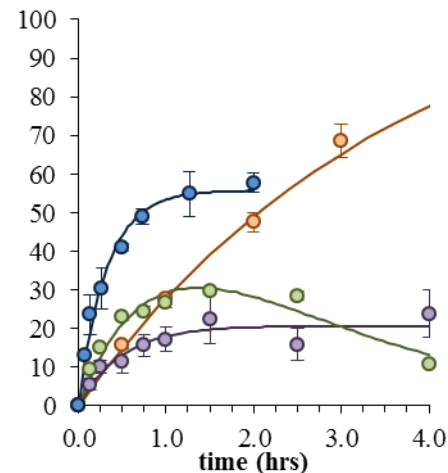
VIL



GGA



EFS



● Thermomixer® (Heat Block)
 ● Rapid Enzyme Digestion System (Microwave Reactor)
 ● MARS6 (Microwave Reactor)
 ● Barocyler® (Pressurized Reactor)

Peptide Recovery (nmole/L)

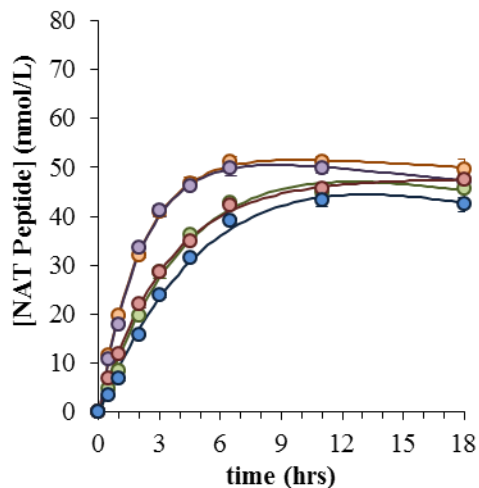
Peptide	Thermomixer	REDS	MARS6	Barocyler
LED	73.73	13.17	12.59	42.44
TFP	129.70	63.98	57.68	105.40
VIF	138.50	92.14	65.47	124.00
VIL	86.97	18.26	20.51	59.90
SQA	168.80	82.72	71.02	146.21
GGA	109.32	44.36	46.21	101.74
EFS	119.85	23.86	28.33	55.75
FSP	96.46	39.23	33.89	88.77

Digestion Time (hours)

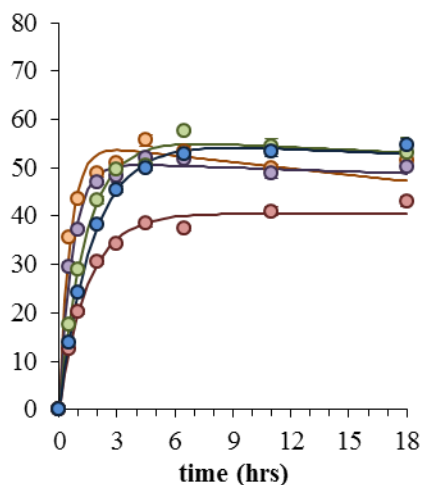
Peptide	Thermomixer	REDS	MARS6	Barocyler
LED	18.00	2.86	0.84	0.51
TFP	3.85	4.00	0.63	0.33
VIF	3.42	4.00	0.46	0.43
VIL	13.18	2.19	2.13	1.46
SQA	3.86	4.00	0.61	0.35
GGA	4.74	4.03	0.89	0.47
EFS	16.90	2.28	5.48	1.47
FSP	5.19	4.14	1.32	0.52

Trypsin Type Comparison

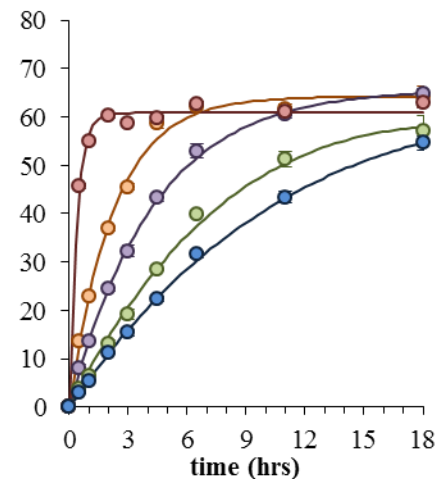
VIL



GGA



EFS



● Bovine
 ● Modified Porcine A
 ● Modified Porcine B
 ● Modified Porcine C
 ● LysC/Trypsin Mix (TPCK-treated)

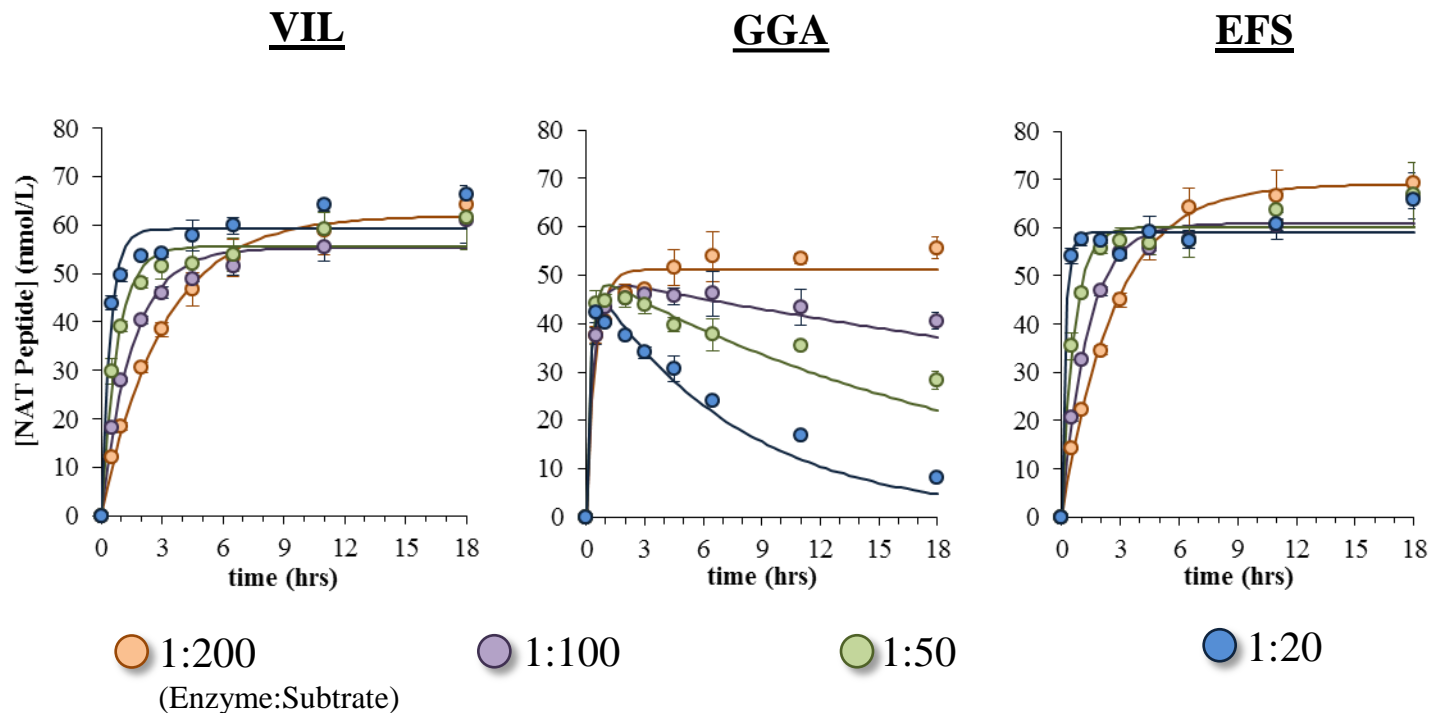
Peptide Recovery (nmole/L)

Peptide	Unmod.	Mod. A	Mod. B	Mod. C	LysC Mix
LED	39.11	41.81	28.35	23.23	28.52
TFP	71.10	70.81	71.53	71.62	68.21
VIF	76.64	76.22	75.72	78.28	71.84
VIL	50.61	48.92	45.32	42.42	47.71
SQA	83.99	83.85	81.65	80.89	72.65
GGA	51.67	51.12	56.37	55.80	40.53
EFS	64.21	66.19	57.12	54.59	60.81
FSP	50.41	48.25	46.00	44.27	35.00

Digestion Time (hours)

Peptide	Unmod.	Mod. A	Mod. B	Mod. C	LysC Mix
LED	15.64	23.07	19.54	16.75	22.08
TFP	1.58	0.94	1.83	2.40	1.14
VIF	0.73	0.14	0.89	0.66	0.46
VIL	9.84	10.05	20.97	29.30	15.14
SQA	1.40	0.86	1.18	1.57	1.11
GGA	2.57	3.12	6.35	8.10	6.77
EFS	10.30	19.69	61.53	67.59	1.73
FSP	1.86	2.08	2.25	2.54	2.51

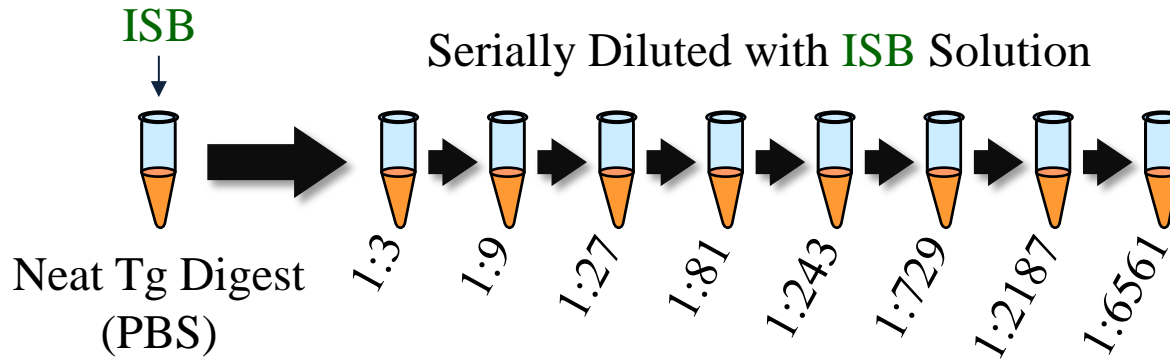
Trypsin Titration (Bovine TPCK-treated)



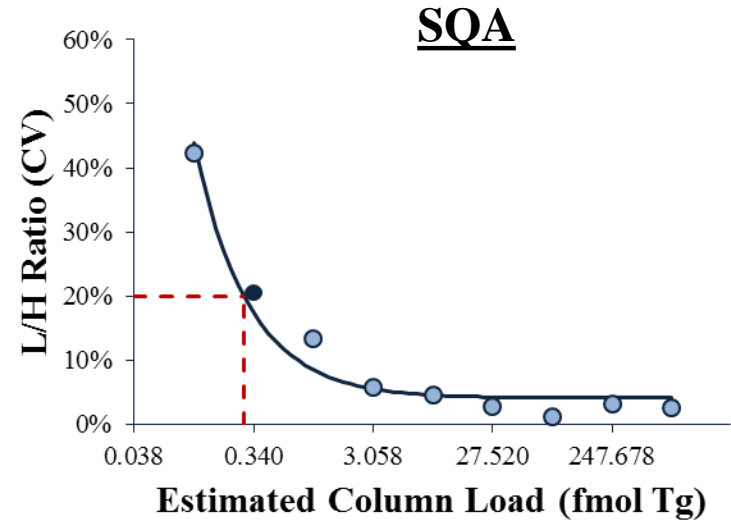
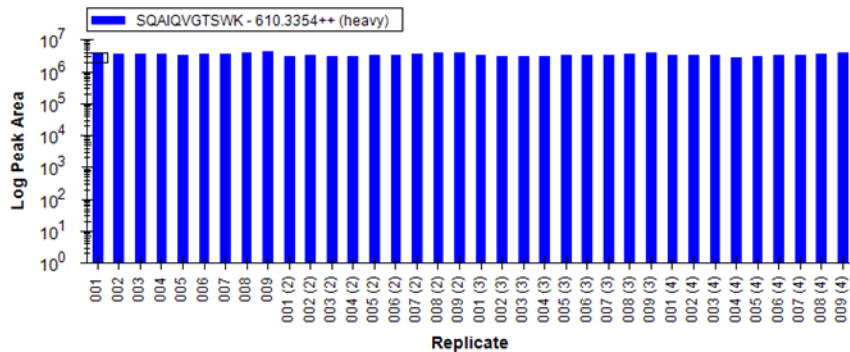
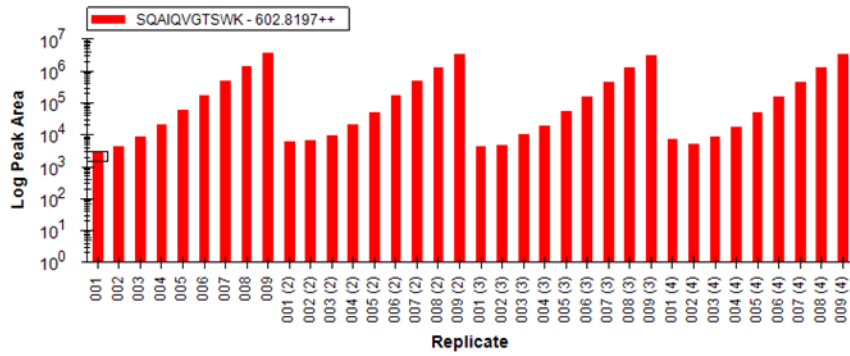
Peptide	Peptide Recovery (nmole/L)			
	1:200	1:100	1:50	1:20
LED	46.02	52.94	60.09	58.22
TFP	69.72	67.49	69.69	69.91
VIF	74.27	69.91	71.13	70.93
VIL	61.91	55.21	55.55	59.14
SQA	81.01	77.92	78.07	74.30
GGA	51.20	44.37	43.55	40.08
EFS	69.02	60.87	60.07	59.00
FSP	48.08	50.27	52.00	50.46

Peptide	Digestion Time (hours)			
	1:200	1:100	1:50	1:20
LED	15.64	23.49	16.50	10.43
TFP	1.39	1.54	0.77	0.63
VIF	0.56	0.70	0.12	0.11
VIL	14.06	7.03	3.64	1.97
SQA	1.31	1.34	0.83	0.11
GGA	2.15	1.72	1.14	1.10
EFS	12.42	6.11	2.88	0.94
FSP	2.17	2.00	1.72	1.39

Functional Sensitivity

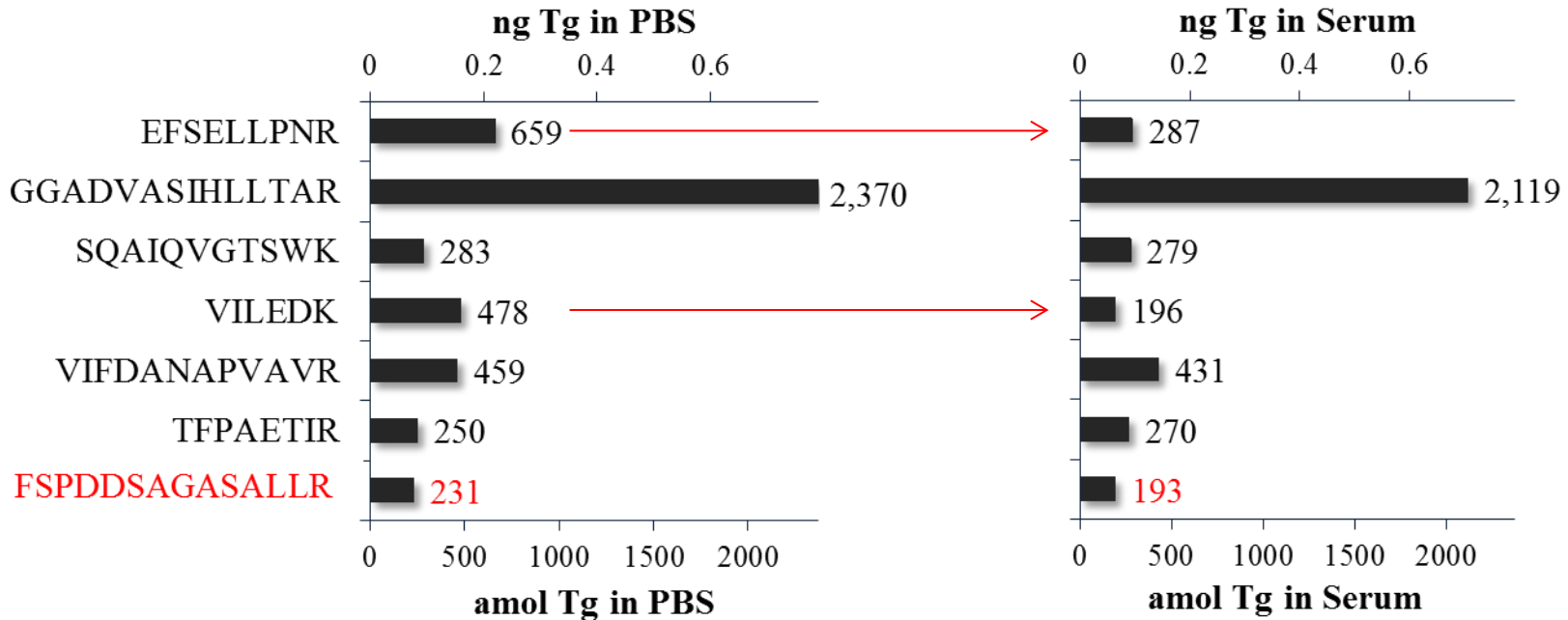
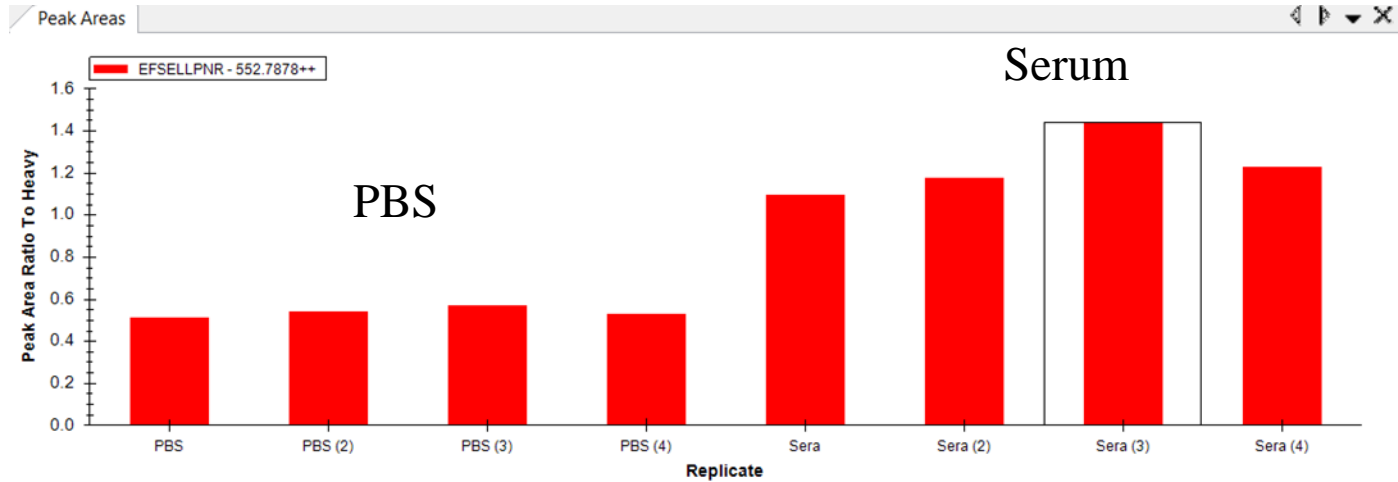


Peak Areas



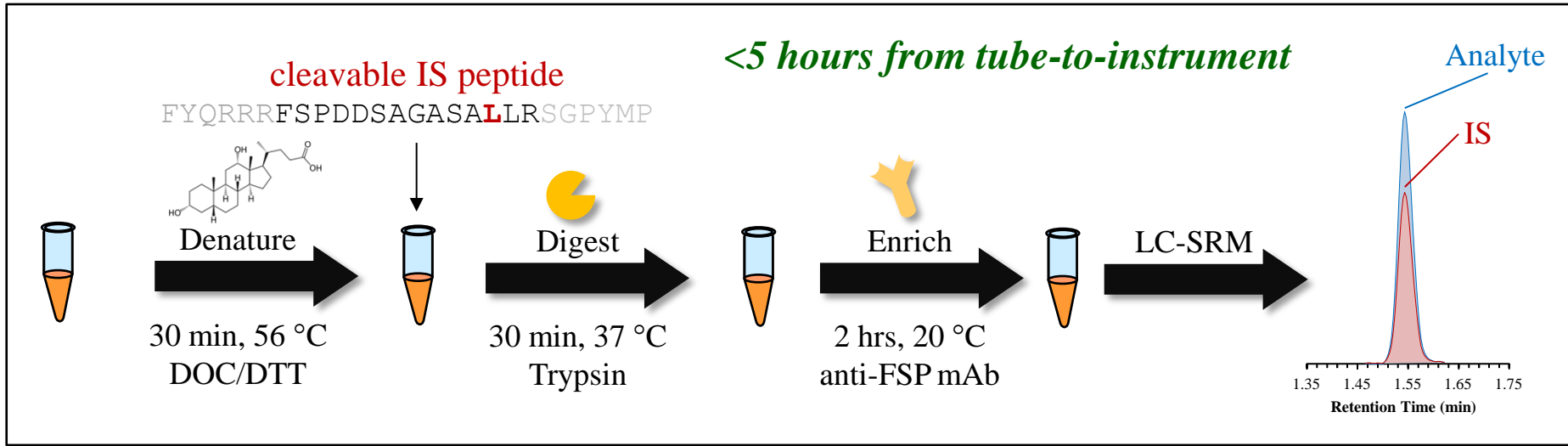
Evaluation with “top 3” transitions

Functional Sensitivity



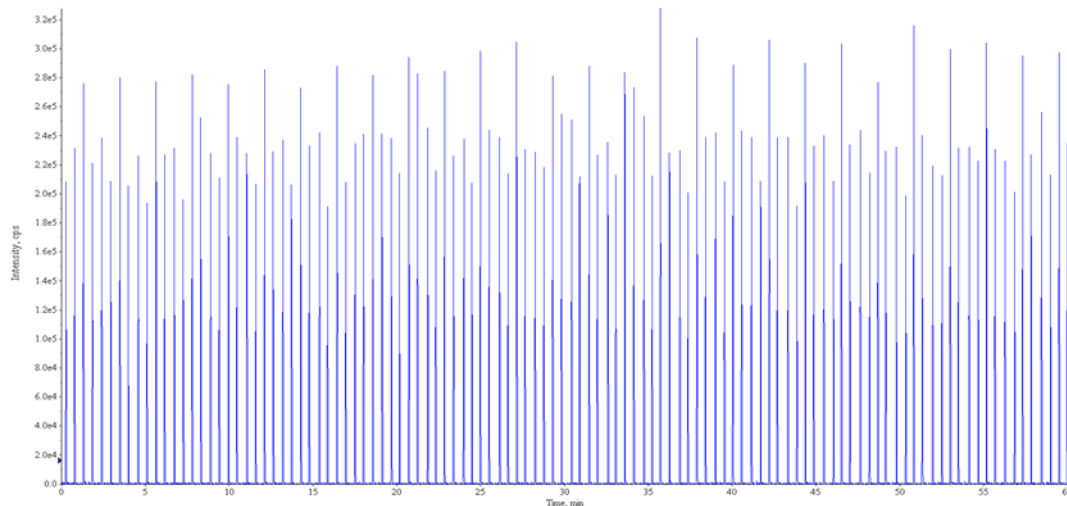
Estimate impacted by ionization/fragmentation efficiency AND digestion efficiency

After Re-optimization

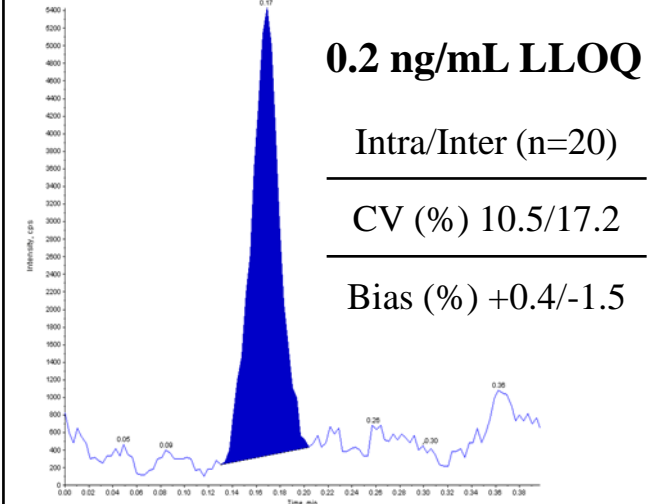


Capable >2000 patient samples per day on ONE LC-MS System

112 samples per hours (~30 seconds inject-to-inject)



200 amol on-column LLOQ



Summary

- Development of a front-line clinical proteomics assay is data intensive (data per unit time)
 - Considerations for 1) Throughput, 2) Cost, and 3) *Sensitivity*
 - Data volume is effeciently managed by [Skyline](#)
- CE Tuning via flow injection – facile alternative to LC with scheduled SRM
- Chemistry screening provides valuable repository of information
 - Critical for assay refinement/re-optimization
- Efficient digestion can be fast & cheap!
 - Optimize all conditions *as a function of time*

Acknowledgments

- Martin Green
- Pat Holland
- Matt Crawford
- Stacy Dee
- Yvonne Wright
- Russell Grant
- Eppendorf AG
Thermomixer®
- Hudson Surface Technology, Inc.
Rapid Enzyme Digestion System
- CEM Corporation
MARS6
- Pressure Biosciences, Inc.
Barocycler®