PepCa10 510(k) Filing

"Mock 510(k)" for a Multiplex Diagnostic Test using Immunoaffinity Mass Spectrometry Protein Quantitation

Commented [A1]: FDA comment on regulatory path:

Depending on your final intended use (which would appear to be novel and currently not classified), de novo down-classification could be considered if there are special controls that can be identified to mitigate the risks. At this time, considering that the intended use has not been finalized, we do not have sufficient information on whether the risk associated with the use of this device can be mitigated with special controls, or whether a device would need to be a Class III and reviewed as a PMA.

A test used to determine whether a biopsy (for subsequent breast cancer diagnosis) would be conducted or not would most likely be a PMA.

CAVEATS:

This document contains only fictitious data. The protein targets and peptide analytes described as comprising the multiplex PepCa10 panel are NOT presented as real biomarkers of breast cancer or any other disease. Specific features attributed to the peptides shown here as examples should not be considered real.

The nanoflow liquid chromatography and triple-quadrupole mass spectrometer instruments are likewise presented here as generic instruments rather than specific real models.

The purpose of this document is to present a model approach to multiplex protein measurement using quantitative mass spectrometry based on real methods currently in use at a research stage.

The presentation approach is to give concrete (though fictional) explanations and data where a workable approach is clear, and to propose one or more alternative approaches where significant uncertainty remains as a means of soliciting regulatory guidance.

N. Leigh Anderson

Table of Contents

I. INTRODUCTION 4
A. Contact Information 4
B. Device Information 4
C. Regulatory Information for 510(k) submissions only (not for pre-IDE) 4
1. Regulation section: TBD 4
2. Predicate device. NA 4
3. Proposed package insert (see 21 CFR 809.10) 5

II. INTENDED USE AND INDICATIONS FOR USE 5
A. Intended Use 5
B. Indications for Use 5

III. DEVICE DESCRIPTION 5
A. Reagents and test components. 6
1. Assay components 7
2. Characterization of active reagents in the assay. 13
3. Other reagents, materials, equipment needed to run the assay but not provided in the kit. 14
4. Calibrators. 15
5. Controls 17
B. Instruments and Software 18
1. General laboratory instruments 18
2. TQMS Triple Quadrupole Mass Spectrometer Instrument 23
3. PepCa10 Program 27
C. Sample/specimen type and procedures for specimen collection 27
D. Principle of operation for the PepCa10 methodology. 28
1. Summary 28
2. Background 29
3. PepCa10 Workflow 43

IV. ANALYTICAL PERFORMANCE 49
A. Precision, using methods of CLSI, EP5-A2 49
B. Reproducibility, using methods of CLSI EP12-A 50
C. Analytical Specificity CLSI EP7-P, Interference) 51
1. Background for the approach used for interference detection 51
2. PepCa10 interference test method 54
3. Interference test results 55
D. Digestion efficiency 57
E. Recovery 60
F. Carryover 60
G. Normal Range/Cut-off , using methods of CLSI C28, CLSI EP-12 61
H. Analytical sensitivity (CLSI I/LA21-P). 63
I. Accuracy NA 63
J. Matrix comparison. NA 63
K. Other studies 63
1. Linearity over the reportable range (refer to CLSI EP-6P, H20-A and H26-A) 63

V. METHOD COMPARISON. NA 64
A. Predicate Device. Not applicable. 64
B. Reference method (Gold Standard comparisons). Not applicable. ..64
C. Literature Comparison. Not applicable.....64

VI. Study design.....64
A. Patient samples or specimens **.....69**
1. Inclusion Criteria.....69
2. Exclusion Criteria 69
3. Constraints on Eligibility 69
4. Specimen collection.....69
B. Analyses..... Error! Bookmark not defined.
1. Plan for data analysis (independent and dependent variables)..... **Error! Bookmark not defined.**
2. Cut-off and reference range determination and validation (NCCLS GP10-A; also see Analytical Performance, Section V, Part C).**Error! Bookmark not defined.**
3. Expected results and calculations. Determination of equivocal zones and resolution of discrepant results. .....**Error! Bookmark not defined.**
4. Expected rate of clinical false positives, false negatives if known. . **Error! Bookmark not defined.**
C. Interpretation..... Error! Bookmark not defined.
1. Interpretation criteria and/or cite literature for the meaning of positive, negative, or equivocal results. .....**Error! Bookmark not defined.**
2. Consequence of misdiagnoses.....**Error! Bookmark not defined.**
3. Recommendations for results in equivocal zone (if appropriate).... **Error! Bookmark not defined.**

I. INTRODUCTION

This document was prepared as a "mock 510(k)" filing for comment by FDA and NCI, technology developers, commercial instrument and reagent providers and biomarker researchers in order to:

- Provide FDA with a generic view of novel technology being advanced for future multiplex diagnostic tests
- Improve the biomarker research community's understanding of regulatory requirements for approval of mass spectrometry-based multiplex tests using the PepCa10 test as an example.

The principal distinctions between the methodology described here and that used in a conventional immunoassay test involve the use of a mass spectrometer (MS) as a detector. In particular the triple-quadrupole MS (TQMS) detector provides both wide dynamic range quantitation and detailed, sequence-based characterization of multiple analyte molecules (unlike optical or electrochemical signals generated as surrogates of the analyte molecule in conventional immunoassay). This capability for simultaneous measurement and characterization of analytes allows two important advances in protein assay. First, it permits facile multiplex measurement of many molecular species without interference. Second, it facilitates the use of analyte-identical internal standards (same structure, differing mass) to control all aspects of the assay workflow. These advantages constitute a major step forward in assay quality control, potentially shifting some of the performance and reliability burden from technical standardization of reagents and instruments to real-time observation and evaluation of the analyte molecules themselves.

Commented [A2]: This would need to be proven.

Commented [A3]: You should present qualitative and quantitative data based on sound clinical background in order to be able to state this. It would especially need to be evalauted considering known problems with MS standardization in the past.

A. Contact Information

Leigh Anderson
Plasma Proteome Institute
P.O.Box 53450
Washington DC 20009 USA
301-728-1451
leighanderson@plasmaproteome.org

B. Device Information

- PepCa10 test carried out on a TQMS instrument
- Common name: NA

C. Regulatory Information for 510(k) submissions only (not for pre-IDE)

1. Regulation section: TBD

2. Predicate device. NA

3. Proposed package insert (see 21 CFR 809.10)

Package Insert not included

II. INTENDED USE AND INDICATIONS FOR USE

A. Intended Use

The PepCa10 test is a quantitative in vitro diagnostic test using the PepCa10 kit and a TQMS instrument to measure 10 peptide analytes in a proteolytic digest of fresh EDTA plasma, yielding a single analytical result calculated from the 10 measurements. A negative result (PepCa10 < 10) indicates a lower risk of breast cancer than a positive result (PepCa10 > 10).

The test is intended for females 40 years or older following mammography of a breast lesion with a BI-RAD of 4 result to aid physicians in the decision to recommend a breast biopsy. The PepCa10 result is indicated for use by physicians as an aid in the differential diagnosis of a breast lesion only, along with other clinicopathological factors.

.

B. Indications for Use

Same as intended use.

III. DEVICE DESCRIPTION

The PepCa10 test is carried out using a PepCa10 reagent kit, a "TQMS" triple-quadrupole mass spectrometer with associated "NanoLC" nanoflow LC instrument, and a PepCa10 computer program for calculation of test results. The test measures the amounts of 10 tryptic peptides (p1…p10) in a digest of the patient EDTA plasma sample in comparison to 10 added stable isotope labeled internal standard peptides (s1…s10) having the same respective sequences. Measurements are carried out using commercially available liquid chromatography and triple quadrupole mass spectrometer instruments. The instrument measures 150 selected reaction monitoring (SRM) transitions corresponding to specific combinations of intact peptide and specific fragment masses: 5 transitions specific for each specimen-derived analyte peptide, and two parallel series' of 5 transitions for each of two corresponding stable isotope labeled internal standard peptides (one serving as internal quantitation reference

Commented [A4]: The word "analytical" should be deleted. The meaning of "analytical" is not clear for a result of a linear combination of 10 analytes.

Commented [A5]: This is very general and relative. Lower risk relative to what? How low or high is the risk for the positive result?

Commented [A6]: Note that the correct term is BI-RADS

Commented [A7]: Note that the correct term is BI-RADS (Breast Imaging-Reporting and Data System)

Commented [A8]: We do not see any data in the suggested clinical study about "differential diagnosis." How do you plan to demonstrate this?

Commented [A9]: Is this math correct – it's not completely clear how this calculates to 150. Please clarify.

Commented [A10]: Is this math correct – it's not completely clear how this calculates to 150 (for example, 5*10+5*10+2(5*2)= 120). Please clarify.

and the other as a reference for recovery measurement). The SRM measurements provide analyte-specific detection of specific peptide peaks in the LC chromatogram at specific retention times. Peak areas for each transition occurring at the expected chromatographic retention time are calculated in the primary analysis software provided with the MS instrument. Peak area measurements are transferred to a provided secondary analysis software program for QC analysis and computation of test result. The relative concentration of each peptide is derived from a weighted average of analyte:internal standard peak ratios obtained for each interference-free transition. The PepCa10 test result is computed using a linear combination of the resulting 10 peptide relative concentration measurements with coefficients obtained from a pilot clinical study. A positive test result in BI-RADS 4 women is defined as a PepCa10 result greater than or equal to 10.0, and indicates low probability of cancer and diminished need for a lesion biopsy at the time of sample acquisition.

Commented [A11]: If not occuring in the expected chromatographic retention time, are they interpreted as invalid? Not read by the software? Not caluclated in the result?

Commented [A12]: How do you assess that the transition is intereference free – do you mean in the absence of isobaric (same mass) peptides?

Commented [A13]: These results would need to be provided in the submission.

Commented [A14]: There will need to be a clarification of what one can expect to get in a sense of coefficients used for diagnostics from a combination of 10 proteins.

Commented [A15]: This is probably a typo – it should be "A negative result in BI-RADS 4 women is defined as a PepCa10 result less than 10.0.....

Commented [A16]: How low (percentage-wise)?

Commented [A17]: How long do you propose such a pilot study to last to provide a dependable set of data concerning probability of cancer based on variations in the expression of 10 proteins?

Commented [A18]: These would in most cases probably not be lesions, but various types of calcifications, calcification clusters, etc

Commented [A19]: One of the key points in this submission – use of capturing antibodies--has not been mentioned. Without their development and use the whole test may be meaningless.

A. Reagents and test components.

The kit is provided with sufficient reagents and materials to process 48 patient samples, as summarized in the following Table:

	Kit Component	Contents (each in a single tube except for 1, 11 and 12)	Storage Conditions
1	Digest plate (96-well)	96 well plate containing lyophilized urea (denaturant), TCEP (disulfide reductant), CHAPS (detergent) and C-SIS concatamer internal standard	-20C
2	Iodoacetamide	Lyophilized cysteine alkylation reagent	-20C
3	Digest diluent buffer	Buffer for reconstitution of lyophilized reagents 2, 4, 5	+4C
4	Trypsin	Lyophilized proteolytic enzyme	-20C
5	Trypsin inhibitor	Lyophilize aprotinin trypsin inhibitor	-20C
6	P-SIS peptide standard (labeled peptide mixture internal standard)	Lyophilized mixture of 10 stable isotope labeled synthetic peptides	-20C
7	Capture antibody mixture	Mixture of 10 capture antibodies in 50% glycerol	-20C
8	Magnetic beads	Paramagnetic beads coated with protein G for antibody capture	+4C
9	Magnetic bead wash solution	Buffer and detergent mixture for washing magnetic beads and reconstitution of reagent 7	+4C

Commented [A20]: Would need to be clearly specificed in the future PI that different components of the kit kept at different storage conditions: some at -20 and some at +4C.

10	Magnetic bead elution solution	Acidic buffer for elution of peptides from magnetic beads and reconstitution of reagent 6	+4C
11	Calibrators	6 lyophilized synthetic peptide mixtures providing response curves for all 10 peptide analytes	-20C
12	Controls	3 lyophilized human plasma samples providing High, Low and Near-cutoff analyte levels	-20C

Commented [A21]: Would these represent various mixtures of 10 peptides?

Commented [A22]: Please note that a near-cutoff value is related to the cutoff of the Index (linear combination of 10 variables). The different values of these 10 varaibles on the hyperplane are giving the same result of the Index. Therefore, more information should be provided about the samples with High Index, Low Index and Near-Cutoff Index.

The nomenclature used to refer to assay-related peptide and protein components is summarized in the following table and described in detail below.-+

Protein	Protein Common Name	Peptide Sequence Measured	Analyte Peptide	Internal Standard from C-SIS Concatamer	Internal Standard in P-SIS mix	Enrichment Antibody
P1	Osteopontin isoform A	YPDAVATWLNPDPSQK	p1	c1	s1	A1
P1	Osteopontin isoform A	AIPVAQDLNAPSDWDSR	p2	c2	s2	A2
P2	Mesothelin isoform 3	EIDESLIFYK	p3	c3	s3	A3
P2	Mesothelin isoform 3	LLGPHVEGLK	p4	c4	s4	A4
P3	Receptor tyrosine-protein kinase erbB2	VLGSGAFGTVYK	p5	c5	s5	A5
P3	Receptor tyrosine-protein kinase erbB2	ITDFGLAR	p6	c6	s6	A6
P4	LPS-binding protein	ITLPDFTGDLR	p7	c7	s7	A7
P4	LPS-binding protein	LAEGFPLPLLK	p8	c8	s8	A8
P5	Mucin-1 (Carcinoma-associated mucin) isoform 8	EGTINVHDVETQFNQYK	p9	c9	s9	A9
P5	Mucin-1 (Carcinoma-associated mucin) isoform 8	YVPPSSTDR	p10	c10	s10	A10

Commented [A23]: Capture antibodies

1. Assay components
   a) Sample digestion.

The kit includes a 96-well plate containing lyophilized reagents to which 20ul aliquots of patient EDTA plasma samples are added, as well as individual aliquots of reagents added subsequently.

Wells A1-E12 (i.e., the first 5 rows of 8) of the digest plate are to be used for sample preparation and contain, in each well, the lyophilizate of 20ul of a solution of

- 9M urea
- 54mM tris-carboxylethyl phosphine (TCEP)
- 0.2% w/v CHAPS detergent
- 0.1M TrisHCl pH 8.5
- 5 fmol/ul of C-SIS internal standard (labeled concatamer protein digest standard)

Direct addition of 20ul EDTA plasma to such a well dissolves the lyophilized reagents, which then results in denaturation of plasma proteins (due to the effects of urea and CHAPS), reduction of protein disulfide bonds (due to TCEP) and incorporation of stable isotope labeled internal standards (C-SIS concatamer protein).

Samples S1-S48 and Controls C1-C3 (respectively LOW, HIGH and NEAR_CUTOFF) are placed in the digest plate as follows prior to the initiation of sample preparation:

		Plate Column											
		1	2	3	4	5	6	7	8	9	10	11	12
Plate Row	A										Control1	Control2	Control3
	B	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
	C	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
	D	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36
	E	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46	S47	S48
	G												
	H												

Other reagents required for subsequent steps in sample digest preparation are provided in 3 separate vials:

- 15mg of lyophilized iodoacetamide used to alkylate protein cysteine residues (to make 2ml of 40mM solution when reconstituted in digest dilution buffer; 40ul added per well)
- 10ml digest dilution buffer (1mM CaCl2 in TrisHCl pH 8.5; 100ul added per well)
- 3.4mg of lyophilized porcine trypsin used to cleave sample proteins to peptides (to make 1ml solution when reconstituted in digest dilution buffer; 20ul added per well)
- 1mg of lyophilized bovine aprotinin (a trypsin inhibitor) used to stop trypsin activity at the end of digestion (to make 1ml solution when reconstituted in digest dilution buffer; 20ul added per well)

b) Stable isotope labeled internal standards.

The kit contains two sets of stable isotope labeled standards designed to provide accurate internal standardization of both sample digestion and of analyte recovery. The role of these standards is summarized in the following workflow schematic, and explained in detail in the discussion of the PepCa10 workflow. Briefly, the C-SIS concatamer standard is a labeled protein whose sequence

Commented [A24]: Is there some additional coding of the samples in wells (other than the plate row/column)?

Commented [A26]: How is this achieved? Were all reagents aliquoted into 96-well plate and then lyophilized? Are there any losses during and after lyophoilization that can influence denaturization? What will be QC for manufacturing of such plates? May be very complicated for users.

Commented [A25]: How is this achieved? Were all reagents aliquoted into 96-well plate and then lyophilized? Are there any losses during and after lyophoilization that can influence denaturization? What will QC for manufacturing of such plates be? May be very complicated for users.

Commented [A27]: What is the length of the denaturation step (seems like it would be mostly the effect of urea)?

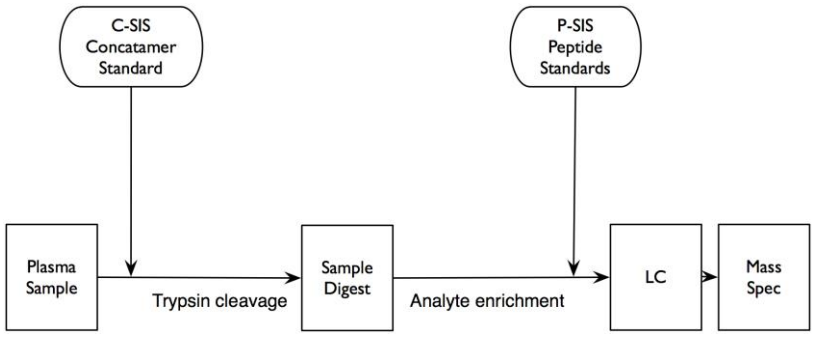
Commented [A28]: Would there be any difference if samples and controls are placed differently in the plate? For example, maybe it would be better to place controls between samples instead of all together at the side of the table. We would ask for the study showing no carryover or cross-reactivity within the plate.

Commented [A29]: Molarity?

Commented [A30]: What is the possible total plasma protein/trypsin ratio?

Commented [A31]: These standards would generally be termed “in-process controls” in diagnostic assays.

contains the analyte peptide sequences and is added to the patient sample prior to trypsin digestion (its cleavage parallels the cleavage of target proteins in the sample); the P-SIS standard is a mixture of synthetic labeled peptides added immediately prior to peptide quantitation in the MS (to provide a standard against which to measure analyte recovery). The stable isotope labeling methods used ensure that C-SIS and P-SIS versions of the target peptide sequences are distinct, on the basis of mass, both from one another and from the sample-derived unlabeled analytes.



C-SIS and P-SIS standards both contain stable isotope labeled peptides with the same sequences as the target analytes; however C-SIS is U13C labeled, while P-SIS is U13C, U15N labeled in the c-terminal amino acid (Lys or Arg) only. As a result of this differential labeling, all three versions of each target peptide (the unlabeled sample-derived analyte, the C-SIS derived, and the P-SIS peptide) are distinguished by the MS.

Internal standards for mass spectrometry function best when added to samples at levels near to or slightly above the levels of the respective sample components, such that measured amounts of standard and analyte are of similar magnitude. While mass spectrometric detectors have a dynamic range of ~1e4, optimal relative quantitation is achieved when analyte and standard are within 10-fold of one another, or ideally approximately equal. In the PepCa10 test, internal standard levels are adjusted to approximate the average analyte levels occurring in samples giving a test result near the cutoff, thus delivering optimal quantitative precision at this level. For this reason, the amounts of the different standards used are not in general equal.

Each of the 10 analyte measurements is generated as a ratio in which the amount (peak area) of the analyte peptide is divided by the peak area of the respective internal standard peptide derived from trypsin cleavage of C-SIS. Hence both analyte peptide and internal standard peptide are cleaved at identical sites on both ends and flow through the entire sample preparation process together, as desired for an effective internal standard.

P-SIS standards are added at known concentrations at the end of sample preparation, and used to assess analyte recovery for QC purposes.

Commented [A32]: Unless you have a calibration curve spanning the analyzed region.

Commented [A33]: This is confusing—do you mean ideally they are within 10-fold or ideally thay are approximately equal?

Commented [A34]: Interpretation

Commented [A35]: This looks problematic: the amount of standard peptide must come from P-SIS independent of the digest. Otherwise in each test results will depend not upon the amount of the internal standard but rather on the completion of the tryptic digest.

Commented [A36]: Effective internal standard also needs to be added at the last step to ensure minimal losses and maximal effectiveness of calculations.

Commented [A37]: Is analyte recovery included in any calculations?

(1) C-SIS internal standard (labeled concatamer protein digest standard)

The kit contains 200ul of a 5 fmol/ul solution of a recombinant stable-isotope labeled concatamer protein (C-SIS1; 513 amino acids, 56,013 dalton molecular weight) whose artificial sequence contains one to three copies of each analyte peptide sequence as defined in the table below, such that tryptic digestion of C-SIS1 protein releases each labeled peptide (c1 to c10) in a defined stoichiometric ratio. The number of copies of each of the peptide approximates the relative concentrations of its respective parent protein in human plasma. Each tryptic peptide analyte sequence (in **bold**) appears in the C-SIS1 protein with additional 5 amino acid n-terminal and 5 amino acid c-terminal sequence extensions present in the source protein sequence, thus providing the same sequence context for tryptic cleavage at both ends of the target peptide as occur in the target protein. C-SIS1 also contains a heme-binding domain sequence from cytochrome b5 (LTKFLEEHPGGEEVLREQAGGDATENFEDVGHSTDAREL SKTY), thus yielding a colored C-SIS1 protein product, and a His-6 tag to assist in purification of the recombinant protein. The C-SIS1 protein is produced in E coli and labeled by substitution of 12C by 13C during synthesis (i.e., U13C labeled).

Commented [A38]: We have some difficulty understanding how the digest of C-SIS can be used as an IS. What will happen if not all AA are replaced with stable isotope analogs? This could conceivably happen even with more than 90% inclusion of isotopes in the sequence. May be unreliable IS.

Peptide	Included Sequence (measured peptide in BOLD)	Concentration (fmol/ul)	Copies
c1	QLYNK**YPDAVATWLNPDPSQK**QNLLA	5	1
c2	NGAYK**AIPVAQDLNAPSDWDSR**GKDSY	5	1
c3	GKKAR**EIDESLIFYK**KWELE	15	3
c4	AEVQK**LLGPHVEGLK**AEERH	15	3
c5	LRKVK**VLGSGAFGTVYK**GIWIP	10	2
c6	PNHVK**ITDFGLAR**LLDID	10	2
c7	SELLR**ITLPDFTGDLR**IPHVG	5	1
c8	KFNDK**LAEGFPLPLLK**RVQLY	5	1
c9	TLAFR**EGTINVHDVETQFNQYK**TEAAS	10	2
c10	HTHGR**YVPPSSTDR**SPYEK	10	2

"Contextual" Peptide Standard Concatamer Sequence: C-SIS1

(measured peptides released by trypsin in BOLD, easily digested linker sequences gsgk in lower case)

MGSGKQLYNK**YPDAVATWLNPDPSQK**QNLLAgsgkNGAYK**AIPVAQDLNAPSDWDSR**GKDSYgsg
kGKKAR**EIDESLIFYK**KWELEgsgkGKKAR**EIDESLIFYK**KWELEgsgkGKKAR**EIDESLIFYK**
KWELEgsgkAEVQK**LLGPHVEGLK**AEERHgsgkAEVQK**LLGPHVEGLK**AEERHgsgkAEVQK**LLG**
PHVEGLKAEERHgsgkLRKVK**VLGSGAFGTVYK**GIWIPgsgkLRKVK**VLGSGAFGTVYK**GIWIP
gsgkPNHVK**ITDFGLAR**LLDIDgsgkPNHVK**ITDFGLAR**LLDIDgsgkSELLR**ITLPDFTGDLR**
IPHVGgsgkKFNDK**LAEGFPLPLLK**RVQLYgsgkTLAFR**EGTINVHDVETQFNQYK**TEAASgsgk
TLAFR**EGTINVHDVETQFNQYK**TEAASgsgkHTHGR**YVPPSSTDR**SPYEKgsgkHTHGR**YVPPSS**
TDRSPYEKgsgkLTKFLEEHPGGEEVLREQAGGDATENFEDVGHSTDARELSKTYHHHHHH

Commented [A39]: How did you determine how many copies of each peptide should be present?

(2) P-SIS internal standard (labeled peptide mixture standard)

The kit contains (in a single tube) a defined mixture of chemically synthesized peptide standards (s1-s10) labeled with U13C, U15N in the c-terminal amino acid (Lys or Arg as appropriate) giving respective mass increments of 8 or 10 amu. The relative concentrations of the 10 peptides in the mixture are in the same stoichiometric ratios as in the C-SIS concatamer protein.

Peptide	Peptide Sequence Measured	Concentration (fmol/ul)
s1	YPDAVATWLNPDPSQK	5
s2	AIPVAQDLNAPSDWDSR	5
s3	EIDESLIFYK	15
s4	LLGPHVEGLK	15
s5	VLGSGAFGTVYK	10
s6	ITDFGLAR	10
s7	ITLPDFTGDLR	5
s8	LAEGFPLPLLK	5
s9	EGTINVHDVETQFNQYK	10
s10	YVPPSSTDR	10

c) Peptide capture antibodies.

The kit contains (in a single tube) a mixture of 10 rabbit monoclonal antibodies, each selected to bind one of the 10 analyte peptide sequences. Antibodies are produced by recombinant expression in a mammalian cell line and purified in a GMP environment. Binding capacity of the individual antibodies is assessed using Biacore SPR measurements and exceeds 75% of theoretical capacity. Different amounts of these antibodies are used to capture the amounts of different peptides required for optimal relative quantitation by mass spectrometry involving an internal standard. Since the ratio of analyte to internal standard yields the analytical result, and since the antibody binds the structurally identical analyte and internal standard peptides equally (not changing their ratio), incomplete capture by the antibody has no effect on the result as long as both peptides are captured in amounts sufficient to provide adequate measurement precision in the MS. Thus for a high abundance peptide, it is often sufficient to use an amount of antibody that captures a small percentage of the target peptide, while for a low abundance peptide near the detection limit of the MS, a larger amount of antibody capable of binding nearly all the analyte is used.

Antibody	Peptide Bound	Concentration (ug/ul)
A1	YPDAVATWLNPDPSQK	1
A2	AIPVAQDLNAPSDWDSR	1
A3	EIDESLIFYK	4
A4	LLGPHVEGLK	2
A5	VLGSGAFGTVYK	0.5
A6	ITDFGLAR	3
A7	ITLPDFTGDLR	2
A8	LAEGFPLPLLK	2
A9	EGTINVHDVETQFNQYK	1
A10	YVPPSSTDR	1

Commented [A40]: In a real submission, we would ask for more specifics about this for every antibody, to understand how antibodies were produced and how specific they are for their target.

Commented [A41]: Need more explanation: Since Ab does not discriminate between labeled and unlabeled peptides will different amounts of both kinds of peptides be captured? Such a scenario would affect the outcome. It would seem that all peptides would need to be captured for reliable results?

Commented [A42]: Is there a saturation point?

Commented [A43]: Capturing peptides with anti-peptide antibodies and using magnetic beads is a complex process that depends on a lot of variables. Therefore, thorough analytical evaluation studies would need to be provided to assure this is working properly.

d) Magnetic beads.

The kit contains 200ul of a 30mg/ml suspension of 1 micron diameter paramagnetic beads coated with staphyloccal protein G, a protein that binds the Fc region of rabbit antibodies with high affinity. These beads are used to capture anti-peptide antibodies (and the peptides they bind) from the sample digest, and thus transport the bound peptides out of the plasma digest matrix and through wash steps to an elution step that delivers the final peptide analyte sample for LC-TQMS analysis. Covalent binding of antibodies to the beads is unnecessary.

e) Magnetic bead wash solution

A solution of 0.1M Tris HCl pH 7.5 and 0.1% CHAPS in water.

f) Magnetic bead elution solution

A solution of 5% glacial acetic acid USP in water.

g) Calibrators (described below)

h) Controls (described below)

2. Characterization of active reagents in the assay.

a) Urea.

USP grade urea manufactured under cGMP is characterized by a certificate of analysis from the bulk manufacturer and lot tested for performance in the PepCa10 assay.

b) TCEP.

TCEP (tris(2-carboxyethyl)phosphine; CAS 51805-45-9) is ≥98.0 % pure by GC-MS, characterized by a certificate of analysis from the bulk manufacturer and lot tested for performance in the PepCa10 assay.

c) Tris-HCl.

Commented [A44]: Tris-HCl or TRIZMA base? You listed elsewhere using Tris buffer with pH 8.2.

USP Grade Tris-HCl (Tris(hydroxymethyl)aminomethane, CAS 77-86-1) is manufactured under cGMP, characterized by a certificate of analysis from the bulk manufacturer and lot tested for performance in the PepCa10 assay.

d) $CaCl_2$.

Calcium chloride USP is manufactured under cGMP, characterized by a certificate of analysis from the bulk manufacturer and lot tested for performance in the PepCa10 assay.

e) CHAPS detergent.

CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate, CAS No. 75621-03-3) is ≥98% pure by TLC, characterized by a certificate of analysis from the bulk manufacturer and lot tested for performance in the PepCa10 assay.

f) Iodoacetamide

Iodoacetamide (CAS 144-48-9) is ≥99% pure by TLC, characterized by a certificate of analysis from the bulk manufacturer and lot tested for performance in the PepCa10 assay.

g) Trypsin.

Bovine pancreatic trypsin USP (CAS 9002-07-7) is purchased from a commercial vendor providing a certificate of analysis and lot tested to pass three criteria: 1) >=2875 USP/NF trypsin activity units per mg protein; 2) <= 0.1 unit/mg chymotrypsin activity; 3) performance in the PepCa10 assay.

h) Aprotinin

Aprotinin USP (CAS 9087-70-1) is characterized by a certificate of analysis from the bulk manufacturer and lot tested for performance in the PepCa10 assay.

i) Stable isotope labeled recombinant protein C-SIS1.

The C-SIS1 protein is produced under cGMP by expression of a plasmid in E coli grown on a ^{13}C enriched (>98%) carbon source and purified by binding to an immobilized nickel column. Purified C-SIS protein is >95% pure by

electrophoresis, has the expected mass within 2 amu (by FT-ICR MS), and ^{13}C isotope substituted for ^{12}C at >98% of carbon positions. C-SIS is lot tested for performance in the PepCa10 assay. A tryptic digest of C-SIS produces c1-c10 labeled peptides whose relative abundances match those of P-SIS peptides within <= 5% by LC-TQMS.

j) Stable isotope labeled internal standard peptide mixture P-SIS1.

Each of the 10 P-SIS peptides s1-s10 is made by solid phase Fmoc synthesis incorporating either a labeled lysine or arginine c-terminal residue, and is provided by the manufacturer with a certificate of analysis. Each peptide meets two quality criteria: 1) purity greater than 95% as demonstrated by HPLC and MALDI mass spectrometry; 2) isotopic substitution at labeled residues of >98% for each isotope. Peptides are assayed in triplicate by acid hydrolysis and amino acid analysis to arrive at the known standard concentrations, and mixed in the appropriate ratios for incorporation into the PepCa10 kit. The P-SIS mixture is lot tested for performance in the PepCa10 assay, in particular to verify relative abundances when compared to C-SIS by LC-TQMS.

k) Anti-peptide antibodies.

Rabbit monoclonal antibodies are expressed in a mammalian expression system under GMP and purified by column affinity chromatography on protein A. Affinity constants (off-rate <1e-3 sec-1) and binding capacity (>75% of theoretical sites) with respect to the respective peptide ligands are assessed using Biacore SPR measurements. Each antibody is lot tested in the multiplex PepCa10 assay prior to mixing.

l) Magnetic beads

Protein G coated magnetic particles are manufactured under GMP, provided with a certificate of analysis, and lot tested for antibody binding capacity, non-specific peptide binding and performance in the PepCa10 assay.

m) Magnetic bead wash solution

Magnetic bead wash solution is lot tested in the PepCa10 assay.

n) Magnetic bead elution solution

Magnetic bead elution solution is lot tested in the PepCa10 assay.

3. Other reagents, materials, equipment needed to run the assay but not provided in the kit.

a) Multiwell plates

General-purpose sample/wash plates: Thermo KingFisher 96 KF plate (200 µl), Cat. No. 970 02 540

KingFisher 96 tip comb for PCR magnets,
(ThermoFisher Cat. No. 970 02 514)

Eluate collection plate: Axygen 96 well full skirt PCR microplate clear (Axygen Cat. No. PCR-96-FS-C)

Commented [A45]: This would be true in the case of 100% completeness of tryptic digest.

Commented [A46]: Page 9, paragraph 2. Sponsor states that P-SIS contains both 13C and 15N; not listed here.

Commented [A47]: If this is a synthesized peptide with labeled AA, thus no isotopic substitution – is this correct?

Commented [A48]: For ancillary reagents (reagents required but not provided) with specified catalog numbers, we would require some additional information to determine how the ancillary reagents will be controlled to provide consistent results.

b) LC buffer load solvent A

A solution of 0.1% (v/v) formic acid and 3% (v/v) acetonitrile is provided by the user.

c) LC buffer gradient solvent A

A solution of 0.1% (v/v) formic acid and 3% (v/v) acetonitrile is provided by the user.

d) LC buffer gradient solvent B

A solution of 0.1% (v/v) formic acid and 70% (v/v) acetonitrile is provided by the user.

e) C18 trap column

An LC Packings PepMap™ C18 trapping column 5 µm, 0.3 x 5 mm, 100 Å, or equivalent, is provided by the user.

f) C18 chromatography column

An LC Packings PepMap™ C18, 5 µm, 0.075 x 150 mm, or equivalent, is provided by the user.

4. Calibrators.

Five PepCa10 calibrators are provided containing a range of known amounts of each of the 10 analyte peptides spanning the measurement range (512-fold total span), together with a constant amount of the 10 internal standard peptides (see table below) to be used in a matrix of 5% acetic acid. A sixth calibrator containing internal standards but no unlabeled analyte peptide is also provided. Calibrators are provided as lyophilized material in evacuated vials. This material is dissolved before use in Magnetic bead elution solution (5% acetic acid). This matrix is the same as that in which analyte peptides and internal standards are delivered to the LC-TQMS for quantitative analysis

The calibrators are intended to establish quantitative calibration of the LC-TQMS analytical instrument, and thus approximate the composition of the enriched peptide samples resulting from antibody capture from patient sample digests, ready for injection into the LC-TQMS instrument.

The 20 peptide components of the calibrators are purified synthetic peptides (the 10 labeled version are identical to the P-SIS internal standards s1-s10). Each is made individually by solid phase synthesis and the resulting crude peptide material purified by HPLC to a >95% purity as assessed by integration of the UV peak collected from the HPLC. Each peptide sequence is confirmed using analytical LC-TQMS by comparison against the reference spectra of analyte peptides observed in human plasma digests, and against intact peptide (parent) and y-ion (fragment) masses predicted from the known sequence. Labeled and unlabeled versions of each peptide are quantitated separately by triplicate amino acid analysis. Relative amounts of labeled and unlabeled peptide in calibrator 3 (which should be equal) are measured by LC-TQMS: if these peak areas differ by more than 5% for any of the 10 peptides, the calibrator series is rejected.

Amounts of each peptide in each of the 6 total calibrators in fmol/ul of reconstituted 5% acetic acid solution is shown below.

Commented [A49]: Should be "purchased by the user" istead of "provided by the user"

Commented [A50]: Should be "purchased by the user" istead of "provided by the user." These will therefore be considered materials required but not provided.

Commented [A51]: Our underdstanding is that you plan to 1) construct a calibration curve using 6 calibrators; 2) the internal standards would always be your third type of "calibrators"; 3) to check whether there are problems with the calibration curves by checking only one point on each calibration curve.

Please note the calibration curves should be checked at the points where most of the values from the clinical patient samples fall.

Commented [A52]: Our underdstanding is that you plan to 1) construct a calibration curve using 6 calibrators; 2) the internal standards would always be your third type of "calibrators"; 3) to check whether there are problems with the calibration curves by checking only one point on each calibration curve.

Please note the calibration curves should be checked at the points where there are the most of the values from the clinical patient samples, and where the most of the values from the clinical patient samples fall.

		Calibrator (fmol/ul)					
		1	2	3	4	5	6
Analytes	YPDAVATWLNPDPSQK	0.156	1.25	10	80	640	0
	AIPVAQDLNAPSDWDSR	0.313	2.50	20	160	1280	0
	EIDESLIFYK	0.234	1.88	15	120	960	0
	LLGPHVEGLK	0.078	0.63	5	40	320	0
	VLGSGAFGTVYK	0.391	3.13	25	200	1600	0
	ITDFGLAR	0.469	3.75	30	240	1920	0
	ITLPDFTGDLR	0.313	2.50	20	160	1280	0
	LAEGFPLPLLK	0.234	1.88	15	120	960	0
	EGTINVHDVETQFNQYK	0.078	0.63	5	40	320	0
	YVPPSSTDR	0.156	1.25	10	80	640	0
Internal Standards	YPDAVATWLNPDPSQK	10	10	10	10	10	10
	AIPVAQDLNAPSDWDSR	20	20	20	20	20	20
	EIDESLIFYK	15	15	15	15	15	15
	LLGPHVEGLK	5	5	5	5	5	5
	VLGSGAFGTVYK	25	25	25	25	25	25
	ITDFGLAR	30	30	30	30	30	30
	ITLPDFTGDLR	20	20	20	20	20	20
	LAEGFPLPLLK	15	15	15	15	15	15
	EGTINVHDVETQFNQYK	5	5	5	5	5	5
	YVPPSSTDR	10	10	10	10	10	10

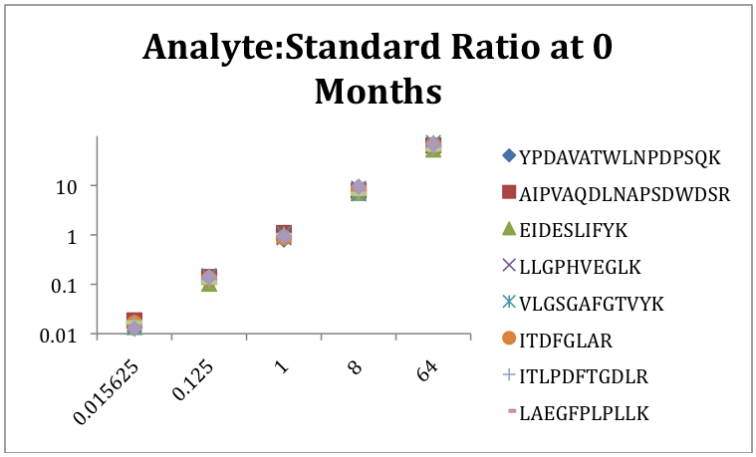
Aliquots of calibrators are dissolved in Magnetic bead elution solution and transferred to the elution plate in positions A1-A6 as shown in the following diagram, which also shows the positions after sample processing of Control and patient sample peptides:

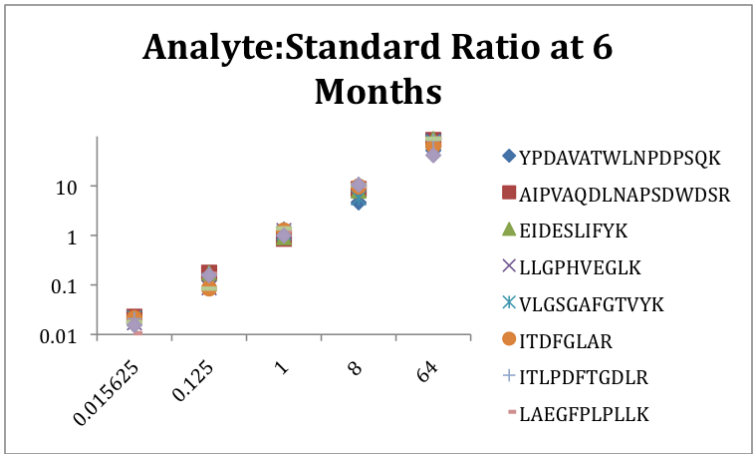
		Plate Column											
		1	2	3	4	5	6	7	8	9	10	11	12
Plate Row	A	Cal1	Cal2	Cal3	Cal4	Cal5	Cal6				Control1	Control2	Control3
	B	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
	C	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
	D	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36
	E	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46	S47	S48
	G												
	H												

Calibrator stability was tested by accelerated aging of packaged lyophilized material for 0, 1, 3 and 6 months at 37C. Ratios of SRM peak areas of analyte (unlabeled) and P-SIS (labeled) peptide were plotted for each peptide in each sample. Regression curves for each such curve were computed. R^2 was >0.98 for all peptides at all time points, indicating acceptable stability of the calibrators.

Commented [A53]: Real time stability should also be assessed. If submission is a PMA, stability protocols should be provided.

Commented [A54]: Please define what your parameters for "acceptable" are.





5. Controls

Three human plasma-based control materials are provided as part of the PepCa10 kit in addition to the set of 6 instrument response calibrants. Given that the PepCa10 result is calculated as a linear combination of 10 independent analytical results, there are many ways in which the relative concentrations of the target peptides in the control materials can yield the required 3 result levels (LOW, HIGH and NEAR-CUTOFF). The approach adopted is based on spiking recombinant versions of analyte peptide-containing proteins P1-P5 into a pool of human EDTA plasma characterized by a PepCa10 assay result near the 5th percentile (LOW) value observed among all the test subjects of a pilot clinical study.

Recombinant proteins P1-P5 are expressed, purified and spiked into the LOW control to generate analyte peptide levels upon digestion appropriate for NEAR-CUTOFF and HIGH controls.

a) LOW Control.

A pool of plasma from normal, healthy male donors verified as having, upon digestion, levels of the 10 analyte peptides that yield a PepCa10 test result at or below the 5th percentile of the subject population in the pilot clinical study.

b) HIGH Control.

Generated from the LOW control material by addition of amounts of recombinant, unlabeled proteins P1-P5 that yield, upon digestion, levels of the 10 analyte peptides producing a PepCa10 test result approximating 95th percentile observed in the subject population in the pilot clinical study.

c) NEAR CUTOFF (NC) Control.

Generated from the LOW control material by addition of amounts of recombinant, unlabeled proteins P1-P5 that yield, upon digestion, levels of the 10 analyte peptides producing a PepCa10 test result approximating the cutoff value (10) in the subject population in the pilot clinical study.

B. Instruments and Software

Four instruments are used in sample preparation and analysis for the PepCa10 test, as well as a final assay-specific computer program:

	Device	Function	Potential Risk
1	Clinical centrifuge	Remove cells from fresh blood	Low
2	Magnetic bead processor	Transport magnetic beads between solutions in 96 well plates	Low
3	Nanoflow liquid chromatograph	Resolve a simple peptide mixture and deliver to MS via electrospray interface	Low
4	Triple-quadrupole mass spectrometer	Select and measure specific peptides over duration of chromatography run; output peak area results	High
5	PepCa10 Program	Perform QC and compute test result from peak areas	High

1. General laboratory instruments

a) Blood Centrifuge.

A refrigerated clinical centrifuge is used to remove cells and cellular debris from 10ml patient blood samples prior to digestion. The centrifuge is not manufactured under cGMPs and is a conventional clinical centrifuge used for blood processing.

Operation is batched allowing 1 to 5 samples to be processed at once. The centrifuge is operated at 4º C, and samples are spun for 15 minutes (using the device's timer) and at speeds, set using the device's control setting, required to generate either 1500 or 2000 x g. The device is not computer-controlled, has no software, and is not connected to any computer network.

The rationale for using this device for blood clarification is that essentially all non-lipoprotein particulate material in blood has a density greater than plasma

Commented [A55]: These seem to be good ideas for controls, but labs might need to run real clinical specimens under CLIA, or other local or state requirements. Also, if you can provide or recommend controls that would appropximate clinical specimens from the test population (BI-RADS 4, etc), that would be helpful.

Commented [A56]: It is difficult to make a judgement about these controls in the absence of real data. When you have a combination of 5 proteins, in what ratio should you spike them to make a low cutoff 1:1:1:1:1, or some other? This would require further discussion.

Commented [A57]: All of the devices listed would be part of your test system. Therefore they would take on the risk of the entire system, regardless of their instrinsic, stand-alone risk. If your intended use were determined to be class II, then all these would be reviewed as class II.

Commented [A58]: All of the devices listed would be part of your test system. Therefore when they are a part of this device they would take on the risk of the entire system, regardless of their instrinsic, stand-alone risk. If your intended use were determined to be class II, then all these would be reviewed as class II. For details, please refer to specific software comments in the test of the review memorandum, including the level of concern.

Commented [A59]: Refer to specific software comments in the test of the review memorandum, including the level of concern.

and thus will sediment out of plasma upon centrifugation. In the context of the PepCa10 assay, the removal of cellular material is desirable in order to minimize the inclusion of cellular proteins in the plasma sample.

b) Magnetic Bead Processor

A Kingfisher 96 Magnetic Bead Processor (Cat. No. 540 05 00, with PCR magnet head Cat. No. 24073410) manufactured by Thermo Fisher Scientific Inc. (81 Wyman Street, Waltham, MA 02454) is used to carry out assay steps involving magnetic beads. For the purposes of this filing, the Kingfisher device is considered a Class I exempt device.

The rationale for using this device in the PepCa10 test is based on 1) the lower variability compared to manual procedures for processing a mass of magnetic beads through a series of solutions, primarily associated with the potential for progressive loss of beads during manual operation; and 2) the superiority of the Kingfisher approach of moving beads from plate to plate rather than moving liquid volumes into and out of the same sample well containing beads (resulting in lower carryover). All plates and tip combs are disposable polypropylene consumables.

The principle of operation of the Kingfisher device is summarized as follows: magnetic beads in the wells of a 96-well plate are attracted to one of an array of 96 permanent magnet probes lowered into the magnetic bead suspensions, and once the beads have coalesced into a compact mass they are raised from the plate and lowered into the next plate in the process, usually containing the solvent required for the next processing step. In the capture process, the magnet probes are sheathed in a 'tip comb' (each of the 96 probes extending into one of 96 narrow sheaths of thin polypropylene) so that neither beads nor solution contact the magnets directly. Upon delivery of beads into a plate, the magnet array is withdrawn from the tip comb, releasing the bead mass from the tip sheaths. The tip comb is typically agitated up and down by a built-in mechanical action to ensure that all beads are dislodged from the tip comb and effectively suspended and mixed with the receiving solution. This process of capture on a tip comb with magnets inserted, transport to a fresh plate, and release (and mixing) of beads in the fresh plate is carried out under control of a software protocol provided with the PepCa10 kit. The device has 8 onboard positions for 96-well plates, one of which (8) is typically occupied by the tip comb (loaded at the beginning of the protocol). The protocol is initiated by the operator using a front panel display and push button.

Sample identification in the Kingfisher protocol is based on position in input 96-well sample plate. Relative sample position (e.g., well B5) in the output 96-well plate (ready for LC-TQMS injection) is the same as in the input sample plate. No reagents are stored on the Kingfisher platform apart from their presence during execution of the test protocol.

The user selects a protocol for execution (PepCa10 is the only option on the device) and starts execution by front panel pushbutton once the device is loaded with required 96-well plates and reagents. The kingfisher control software used in the PepCa10 test (a 15 step program provided as Appendix 1) is stored

Commented [A60]: The device is the same class as the test system. Its standalone classification is not used.

Commented [A61]: The device would be the same class as the test system - its standalone classification would not be used when the performance of the whole system is reviewed.

Commented [A62]: Please provide a study assessing the carryover

Commented [A63]: Please provide a study assessing the carryover.

Commented [A64]: This software should be provided as a part of the submission for review (not just a 5-step program in Appendix 1)

on non-volatile memory within the Kingfisher device and is not accessible to operator modification. No data result is generated or communicated by the Kingfisher since it serves to enrich samples analyzed later in the process. The operator is prompted at the start of the protocol (through the front panel) to place the 8 required 96-well plates containing the appropriate assay components in their correct numbered positions on the instrument deck at the start of the protocol.

Failure of the Kingfisher instrument or software can result in failure to produce a test result (requiring a re-test) but it cannot produce an incorrect test result. The function of the Kingfisher process is to facilitate and standardize an enrichment process involving capturing test antibodies on protein G coated magnetic beads, washing these beads carrying antibody, and finally releasing the analyte and internal standard peptides bound by the antibodies as an enriched sample ready for quantitative LC-TQMS analysis. The test result is based on the ratios of the concentrations of 10 analyte peptides in a specimen digest relative to 10 respective added internal standard peptides. There is no known or suspected physical process forming part of the PepCa10 test procedure capable of altering these ratios: the analyte and internal standard peptides compared with one another are chemically indistinguishable (differing only by ^{13}C and/or ^{15}N stable isotopes) and thus cannot be significantly fractionated by simple laboratory processes. Since the ratios cannot be altered by the antibody enrichment process implemented on the Kingfisher, the Kingfisher process cannot alter the test result, except through loss of these peptides with consequent loss of signal. A decrease in signal through partial loss of a peptide will affect the analyte peptide and its corresponding internal standard peptide equally, leaving the ratio between them (and thus the test result) unchanged, provided both peptides are present at high enough levels to allow precise quantitation. Data quality tests applied to the results of the SRM quantitation (peak signal-to-noise etc) provide a rigorous means of detecting such decreases in peptide amount potentially affecting a test result. Lack of sufficient peptide to generate a sufficiently precise LC-TQMS measurement results in incomplete data and an automatic re-test within the PepCa10 procedure.

The following table lists identified risks associated with the Kingfisher device and the approach taken to mitigate each risk. The overall risk of between-sample contamination (i.e., from well to well) is low because all transfers from plate to plate are executed on all 96 wells simultaneously by a rigid magnet array.

1	**Risk**	**Incorrect reagent plates loaded by operator**
	Effect	Target peptides not captured; no test result
	Mitigation	Device platform labeled to identify correct plate for each of 8 locations
2	**Risk**	**Plate loaded in incorrect orientation**
	Effect	Plate rotated 180 : wells in rows A, B, C contain no peptides
	Mitigation	Device platform labeled to enforce well A1 as upper left corner
3	**Risk**	**Incorrect instrument protocol attempted**
	Effect	Target peptides not captured; no test result

Commented [A65]: Is this "cutoff" built into the assay software, or provided as interpretation criteria?

Commented [A66]: You need to decide upfront what output will be provided to a doctor – qualitative (positive/negative) or quantitative. These different outputs would require different studies to evaluate them.

Commented [A67]: As noted above, this will need to be shown experimentally.

Commented [A68]: The carry-over study should be conducted.

	Mitigation	Device loaded with a single firmware program initiated from front panel
4	**Risk**	**Magnetic beads not transferred effectively**
	Effect	Peptides not delivered to Bead Eluent plate; no test result
	Mitigation	Operator observes beads remaining in incorrect plate at end of process; MS fails to detect peptides (no result reported)
5	**Risk**	**Magnetic beads left in final eluate**
	Effect	Potential to clog LC column
	Mitigation	Visual inspection of eluates by operator to detect brown beads

Commented [A69]: Is this effective enough?

c) Nanoflow Liquid Chromatography Instrument

A NanoLC liquid chromatography instrument, controlled by NanoGradient software, is used to concentrate and separate the analyte peptides by nanoflow liquid chromatography prior to their introduction by electrospray ionization into the mass spectrometer.

Commented [A70]: Will need to be reviewed. See FDA's software guidance.

(1) NanoLC device.

A NanoLC nanoflow liquid chromatography instrument is used to deliver enriched peptide samples to the mass spectrometer. For the purposes of this filing, the NanoLC device is considered a Class I 510(k) exempt device (regulation 862.2260), manufactured under GMP. Operation is serial (one sample processed after another) but effectively batched by virtue of sample input in 96-well plates.

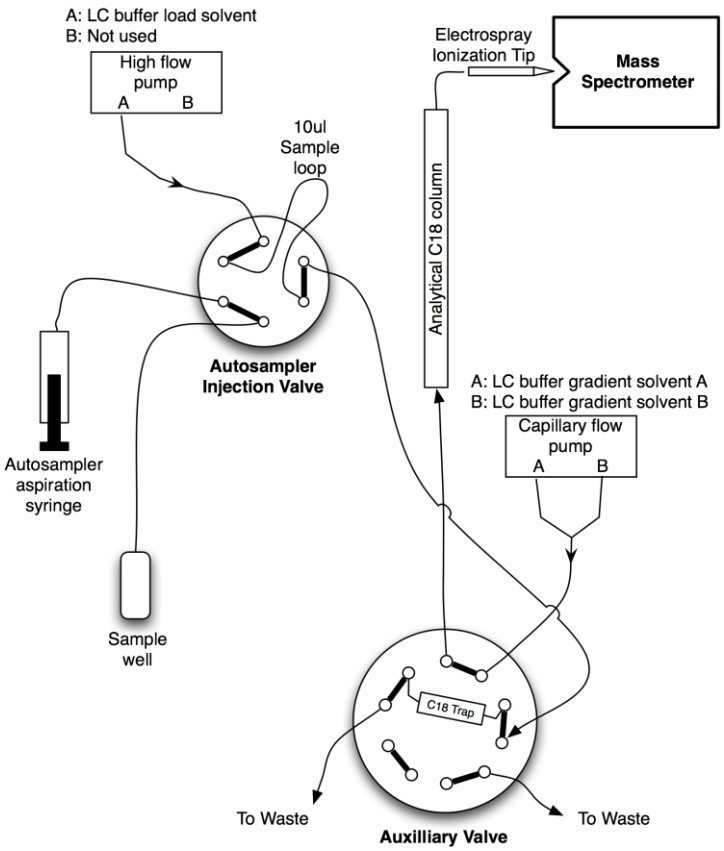
Commented [A71]: This device takes on the classification of the test system. Its standalone classification is not used.

Commented [A72]: Would there be carryover issues?

The rationale for using this device is based on 1) the need to concentrate the enriched peptides to achieve the desired detection efficiency in TQMS (TQMS sensitivity is concentration dependent); 2) the desirability of chromatographically separating the peptide analytes to provide added discrimination based on retention time; and 3) the superiority, compared to higher flowrate regimes, of nanoflow chromatography coupled with nanospray ionization in maximizing analyte TQMS signal.

The nanoflow liquid chromatography instrument is comprised of a commercial nanoflow pump instruments with associated computer control, and capillary plumbing system depicted in the following diagram:

Commented [A73]: Any? Are there required specs? Or Is NanoLC a specific instrument?



(2) NanoGradient Software

Software of Unknown Pedigree: The NanoGradient software used in conjunction with the NanoLC device (version 1.5.4 used with device firmware version 1.1) is Software of Unknown Pedigree (SOUP), provided by the vendor for general laboratory use. The PepCa10 NanoGradient process protocol was designed as part of the development of the PepCa10 test, and is executed through the NanoLC device software.

Failure of the NanoLC device or NanoGradient software can result in failure to produce a test result (requiring a re-test) but it cannot produce an incorrect test result. The function of the NanoLC device is to remove a selected sample from an input 96-well sample plate, subject the peptides in the sample to reversed-phase separation by nanoflow liquid chromatography and finally spray the resulting flow into the entrance of the MS instrument for quantitative LC-TQMS analysis. The PepCa10 test result is based on the ratios of the

concentrations of 10 analyte peptides in a specimen digest relative to 10 respective added internal standard peptides. There is no known or suspected physical process forming part of the PepCa10 test procedure capable of altering these ratios: the analyte and internal standard peptides compared with one another are chemically indistinguishable (differing only by 13C and/or 15N stable isotopes) and thus cannot be significantly fractionated by reversed phase chromatography. Since the ratios cannot be altered by reversed phase chromatography in the NanoLC device, the LC process cannot alter the test result, except through loss of these peptides with consequent loss of signal. A decrease in signal through partial loss of a peptide will affect the analyte peptide and its corresponding internal standard peptide equally, leaving the ratio between them (and thus the test result) unchanged, provided both peptides are present at high enough levels to allow precise quantitation. Data quality tests applied to the results of the SRM quantitation (peak signal-to-noise etc) provide a rigorous means of detecting such decreases in peptide amount potentially affecting a test result. Lack of sufficient peptide to generate a sufficiently precise LC-TQMS measurement results in incomplete data and an automatic re-test within the PepCa10 procedure.

The following table lists identified risks associated with the NanoLC device and the approach taken to mitigate each risk.

Commented [A74]: Please comment on HPLC column QC and how long HPLC columns will be used (how many runs).

1	**Risk**	**Sample injection fails**
	Effect	No peptides detected, no result
	Mitigation	Re-run sample
2	**Risk**	**LC column clogs, interrupting flow**
	Effect	Instrument overpressure, alert operator
	Mitigation	Suspends operation until problem resolved; re-run sample
3	**Risk**	**Chromatography retention time drifts**
	Effect	One or more peptides drifts outside quantitation time window; no result
	Mitigation	Re-run sample
4	**Risk**	**Nanospray source fails to spray**
	Effect	No peptides detected, no result
	Mitigation	Re-run sample
5	**Risk**	**Solvents exhausted during run**
	Effect	No peptides detected, no result
	Mitigation	Re-run sample

Commented [A75]: It appears that for several of these risks and effects in the tables, it is possible that a result could be generated, which might be wrong (e.g. wrong ratio possibly)?

2. TQMS Triple Quadrupole Mass Spectrometer Instrument

A triple-quadrupole mass spectrometer serves as quantitative specific analyte detector for peptides in the PepCa10 test. Whereas the instruments considered above are used in sample preparation, none participates directly in the generation of a quantitative result, and their performance is internally controlled by added labeled standards. The mass spectrometer, on the other

Commented [A76]: ?

hand, must be capable of distinguishing multiple analytes and standards, and delivering accurate relative abundance values. An undetected erroneous peak area measurement could lead to an incorrect analyte/standard ratio, and a consequent incorrect test result. An incorrect test result could cause a physician to make an incorrect treatment decision regarding the patient's breast lesion. For these reasons, the MS required for the PepCa10 test is likely to be considered a Class II device.

No suitable instruments are currently produced under GMP, with associated hardware design process documentation and software validation required to be approvable as a Class II device. Existing instruments and associated software programs, including those used in development of the PepCa10 technology, were designed for research applications. Many of these applications are governed by GLP- or GMP-like regulations, since they generate basic data required for FDA approval of drugs, and in some cases, in vitro diagnostic test results in reference laboratories. Given that there are an estimated 10,000 TQMS instruments currently deployed to generate accurate quantitation of molecular analytes, with a substantial fraction of these generating data used in FDA regulatory submissions, there is a strong argument that the technology currently functions at a level adequate to ensure reliable results.

Commented [A77]: For diagnostic use, they would need to be produced under GMP/QSR.

In all likelihood, efforts will be made by industry to design and market approvable MS instruments and software within the next 3 to 5 years. In the interim, this submission describes an approach using a generic fictitious TQMS device that, together with controls and modifications intended to improve robustness of the PepCa10 assay, provide a workable system for delivering reliable clinical test results. Retrospective validation of such a system, based on existing records and quality systems could provide a means to advance MS-based diagnostic test improvements into general use.

a) TQMS Device

A TQMS model XYZ device manufactured by TQMS Co. is used to provide quantitative measurement of analyte and internal standard peptides. The instrument is manufactured under an ISO 1345 quality system, but is not manufactured under cGMPs. Installation parameters are determined by TQMS Co. employees during instrument installation. Internal process controls are provided by analysis of peptide measurements produced by the instrument and analyzed in the PepCa10 software.

Operation is serial, since the TQMS functions as a mass-resolving quantitative detector monitoring the fluid stream exiting the NanoLC. The device is not connected to any computer network. No reagents are stored on the TQMS instrument. The instrument, associated computer and software installation are dedicated to running the PepCa10 assay.

The rationale for using this device for peptide quantitation is based on 1) extensive history of TQMS in precise and accurate quantitation of small molecules including drugs and metabolites in clinical samples; 2) the superiority of TQMS specificity in comparison with conventional immunoassay technologies;

3) the inherent robustness of TQMS with respect to multiplex measurement of multiple analytes without inter-assay interference; 4) the potential in TQMS to detect and identify potential assay interferences from other sources; and 5) the wide linear dynamic range (>1e4) of the detector.

The primary approach taken to address potential risks in the use of the TQMS instrument involves incorporation of multiple layers of internal controls within the PepCa10 test. Because of the molecular specificity of the TQMS as a detector, and its basis in well-understood physical and chemical principles, substantially more information is available through TQMS than can be obtained from immunoassay detectors at the point of use. This information provides detailed assessment of the function of both the TQMS instrument and the preceding steps of sample processing. Hence three series of QC runs are performed at the start of each daily batch of patient samples (see test protocol description below). These runs verify:

QC parameter	**Approach to verification**
Instrument detection linearity and dynamic range	6 calibrant samples with varying amounts of each target peptide with constant amounts of labeled internal standard
Peptide LC retention times	All 10 peptide analytes tested to confirm LC elution in expected time window
Mass accuracy	Transitions examine expected and offset mass windows to verify strongest response at expected mass settings
Quantitative reproducibility	CV's of peak areas from 3 replicate injections of 10 target peptides evaluated to ensure required performance
Test result on control samples	Controls processed through complete test protocol and quantitative PepCa10 index verified with limits

Primary functional specifications of the model XYZ TQMS device:

Mass range	400 – 1,600 m/z in Q1 and Q3
Mass accuracy	+/- 0.2 amu in both Q1 and Q3
Mass calibration stability	<0.1amu drift in 24hr in both Q1 and Q3
Sensitivity in peptide quantitation	Median LOQ for PepCa10 peptides p1-p10 of 100 amol delivered via nanoLC
Multiplex SRM capability	150 SRM's schedulable in 10 or more time windows
Quantitative reproducibility	Median CV <5% for repeat injections of 100 fmol PepCa10 peptides
Control of NanoLC	Ability to communicate sample identification information and run initiation commands via electronic interface

Commented [A78]: What are the amounts? Are the high and low end of the range included?

Commented [A79]: Provide more details: a) amounts of the 6 calibrant samples; b) how linearity is evaluated (see CLSI EP6-A); c) what deviation from linearity is considered unacceptable.

Commented [A80]: Mass accuracy is defined by the instrument manufacturer. How do you plan to deal with TQMS instruments produced by different companies? Test each time whether a particular instrument corresponds to a desired mass accuracy?

Commented [A81]: At what level is CV calculated? What level of CV is considered as an unacceptable level of variability?

Commented [A82]: This needs to be evaluated and provided as a part of the submission, for every peptide if each result is reported separately

Commented [A83]:
The basic parameters of the analytical performance such as limit of blank, limit of detection and limit of quantitation (if quantitative assay) should be evaluated for each analyte.

Detailed information might need to be provided on how the linear combination of 10 analytes is calculated under different situations as:
1) Level of analyte X is below the limit of blank for this analyte. How is analyte X calculated in the linear combination.
2) Level of analyte X is above the limit of blank but below limit of quantitation. How is analyte X calculated in the linear combination.

Commented [A84]: The basic parameters of the analytical performance such as limit of blank, limit of detection and limit of quantitation (if quantitative assay) should be evaluated for each analyte.

Detailed information might need to be provided on how the linear combination of 10 analytes is calculated under different situations as:
3) Level of analyte X is below the limit of blank for this analyte. How is analyte X calculated in the linear combination.
4) Level of analyte X is above the limit of blank but below limit of quantitation. How is analyte X calculated in the linear combination.

Commented [A85]: Needs to be shown for relevant concentration as a part of reproducibility study.

Commented [A86]: How connected to NanoLC? Software?

b) TQMS software

The current software used to control the TQMS device and to reduce the collected SRM data to peak areas was designed and is widely used for research applications. The software development processes for the TQMS software have not been previously submitted or reviewed by FDA. The design methods employed require it to be classified as Software of Unknown Pedigree. For use in the PepCa10 tests, the software is modified to incorporate additional constraints on user operations.

Commented [A87]: Please refer to our software specific review comments.

The TQMS software runs on a dedicated generic PC under the Windows operating system. The PepCa10 analytical method and TQMS software are stored locally (on the PC) which is not connected to a network. Quantitative test measurements produced by the TQMS software are output as CSV files for use by the PepCa10 program, also running on the PC computer attached to the TQMS instrument.

A workable approach to enable use of TQMS software for the PepCa10 test is to 'wrap' the existing research-use software application with fixed parameters in a software package that controls user access and ensures data integrity. Several facilities are available in the Windows operating system to implement such controls, including file and directory level ownership protection, access control lists and file checksums. Program, analytical protocol and analytical data files are stored in secure folders protected from unauthorized access and verified by checksum and other verification means. Secure mechanisms are used to construct a simplified and secure PepCa10 user interface that allows access to a very restricted set of options in the TQMS software. Through this interface, a specific authorized user can carry out instrument calibration and tuning, access sample lists, initiate batch runs and observe SRM data collection. Collection of SRM data and its processing to yield peak areas is carried out without user input inside the wrapper, and the results written out automatically as CSV data files into a secure checksum-verified directory.

The following table lists identified risks associated with the TQMS device and software and the approach taken to mitigate each risk.

1	**Risk**	**Mass calibration drifts**
	Effect	SRM transitions detect incorrect molecules
	Mitigation	Daily within-run check of mass accuracy in Q1 and Q3; built-in interference detection and rejection
2	**Risk**	**Incorrect SRM peak integration**
	Effect	Error in relative abundance of one or more peptides
	Mitigation	Operator review of automatic peak integration results
3	**Risk**	**MS sensitivity decreases**
	Effect	Peptides appear to be below LOQ
	Mitigation	Correct instrument problem and re-run

Commented [A88]: How? What would define the problem?

3. PepCa10 Program

The PepCa10 program reads CSV peak areas produced by the TQMS software, performs QC tests and test result calculations, and outputs a printed report. The program runs on the PC attached to the TQMS and is implemented as a Microsoft Excel spreadsheet. The structure and format of the spreadsheet is locked to prevent user alterations, with only a designated input area available for importation of CSV peak area data. MS Windows user authorization controls are employed to protect program code and results from alteration by unauthorized users, including operators.

C. Sample/specimen type and procedures for specimen collection

The PepCa10 test is intended for use on EDTA plasma samples obtained by venipuncture using the following protocol:

- Patient position: Patient should be seated at least 5 minutes before the draw and the arm should be positioned on a slanting armrest in a straight line from the shoulder to the wrist. The arm should not be bent at the elbow.
- Source of blood: Median, cubital, basilic, or cephalic veins (never from a port)
- Tourniquet technique
  - Apply a tourniquet 2 inches above the antecubical fossa or above area to be drawn with enough pressure to provide adequate vein visibility. Have the patient form a fist. Select the site for venipuncture.[1]
  - Clean the forearm of the patient with antiseptic wipe in a circular motion beginning at the insertion site. Allow the antiseptic to dry.[1]
  - Anchor the vein by placing the thumb 2 inches below the site and pulling the skin taut to prevent the vein from moving. The holding finger is placed below the site, not above, to prevent accidentally sticking the finger with the needle.[1]
  - Using the dominant hand, insert either the vacutainer needle or the butterfly needle (if using vacutainer needle, attach hub first). Push the evacuated tube onto the vacutainer hub or the Luer adapter if using a butterfly.[1]
  - Release the tourniquet once blood flow is established. [The elapsed time for the tourniquet should be less than 1 minute. In the case that additional time is required, the tourniquet must be removed in a fashion that restores both the circulation and normal skin color.]
  - Make sure that tube additives do not touch the stopper or the end of the needle during venipuncture.[2]
- Drawing blood into tubes
  - Pre-chill 10 mL lavender-top K2 EDTA BD Vacutainer® venous blood collection tubes (BD 366643, 10 mL plastic, whole blood EDTA tube with lavender top) on ice for at least 5 minutes.[2]
  - Aspirate and discard approximately 3 mL of blood prior to collecting the EDTA plasma for the study. [If EDTA tube for the study is in a later order of draw of multiple tubes, there is no need to collect this discard.]

- Completely fill the tubes. Carefully remove the tubes when full without dislodging the needle.
- Inversion of EDTA tubes
- Immediately after allowing the lavender-top Vacutainer® tube to completely fill, slowly and gently invert the tube 8-10 times
- Immediately insert the tube into wet ice
- Immediately place on ice
- Sample processing and freezing must be completed within 90 min of collection

- Plasma processing
  - Centrifugation I
    - Within 30 minutes of collection, centrifuge at 1500 g for 15 min in a refrigerated centrifuge (4 ºC).
  - Collection of supernatant I
    - Transfer plasma (using sterile disposable 10 cc pipette) to centrifugation tubes (BD 352196, 15 mL polypropylene Falcon tube), taking care to not disturb the buffy coat.
  - Centrifugation II
    - The secondary tubes are then centrifuged at 2000 g at 4º C for 15 minutes to remove all potentially remaining cells.
  - Collection of supernatant II
    - After second centrifugation, transfer the top 2.5 ml of the supernatant into a 3 ml cryovial (Simport Cryovial Sim-T309-3A; sterile cryovials with silicone washer seal and external threads; self-standing; certified DNase-free, RNase-free, DNA-free and Pyrogen free; available through LABSCO).
    - Additional aliquoting and storage to be determined by each site at their discretion.
- Storage and shipment
  - Biospecimens should be immediately placed on dry ice or in a -70 to -80ºC freezer.
  - Biospecimens should be stored at -70 to -80ºC before shipment to the biorepository.
  - Biospecimens should be shipped to the analysis laboratory on at least 5 lbs of dry ice.

D. Principle of operation for the PepCa10 methodology.

1. Summary

The PepCa10 test measures the concentrations of 10 tryptic peptides (2 peptides from each of five proteins) in a digest of patient plasma. The peptide analytes are captured from the digest by specific affinity reagents (anti-peptide antibodies) and measured by quantitative triple-quadrupole mass spectrometry in relation to 10 respective internal standards (stable isotope-labeled peptides of identical sequence) to obtain individual analyte measurements. The 10 individual

Commented [A89]: My understanding is that quantitative measurements are obtained through the calibration curves with 6 calibrators for each curve?

Commented [A90]: Our understanding is that quantitative measurements are obtained through the calibration curves with 6 calibrators for each curve – is that correct?

measurements are combined using the proprietary PepCa10 algorithm to yield a single diagnostic result.

The analytes are:

Protein	Common Name	SwissProt Accession ID	Peptide	Peptide Sequence Measured
P1	Osteopontin isoform A	P10451	p1	YPDAVATWLNPDPSQK
P1	Osteopontin isoform A	P10451	p2	AIPVAQDLNAPSDWDSR
P2	Mesothelin isoform 3	Q13421	p3	EIDESLIFYK
P2	Mesothelin isoform 3	Q13421	p4	LLGPHVEGLK
P3	Receptor tyrosine-protein kinase erbB2	P04626	p5	VLGSGAFGTVYK
P3	Receptor tyrosine-protein kinase erbB2	P04626	p6	ITDFGLAR
P4	LPS-binding protein	P18428	p7	ITLPDFTGDLR
P4	LPS-binding protein	P18428	p8	LAEGFPLPLLK
P5	Mucin-1 (Carcinoma-associated mucin) isoform 8	P15941	p9	EGTINVHDVETQFNQYK
P5	Mucin-1 (Carcinoma-associated mucin) isoform 8	P15941	p10	YVPPSSTDR

2. Background

The PepCa10 assay makes use of a novel workflow, including proteolytic digestion of plasma samples, and a structurally specific analytical detector (triple-quadrupole mass spectrometer) to measure the amounts of multiple specific biomarker peptides derived from proteins in patient samples. This approach provides improved assay specificity and facile multiplexing without interference between assays. The rationales for these departures from conventional immunoassay practice are outlined below.

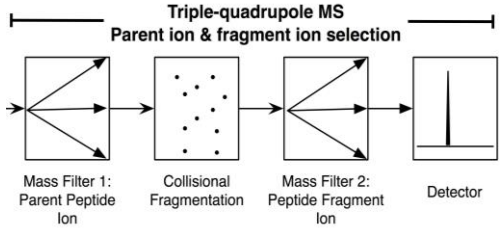
a) Structural variation in protein analytes

Proteins in plasma occur in a variety of different structural forms, due to covalent chemical modification (glycosylation, phosphorylation, proteolytic cleavage, etc.) and non-covalent shape changes (denaturation/misfolding, associations with other proteins, etc.) In many cases these forms have different clinical significance. An ideal protein assay would accurately report individual concentrations of one or more of these precise structural forms. Unfortunately the full complexity of these variations is not comprehensively measurable by present methods, and as a result it may be unclear which forms of a protein analyte are measured by a specific antibody pair (in the case of sandwich ELISA assays), or what the effect of protein unfolding or complexation will be on assay result. These uncertainties give rise to imperfect convertibility between assays for the same protein, and to assay interferences (recognized and unrecognized).

b) Structural specificity in an analytical detector: mass spectrometry

Specificity and accuracy of protein assays would be improved if methods were available for complete structural characterization of the analyte. While this goal is beyond the reach of current mass spectrometry (and other) technologies in the context of large proteins, it is achievable for short (8-20 amino acid) peptides through the use of a triple-quadrupole mass spectrometer (TQMS).

The TQMS is used in "selected reaction monitoring" (SRM) mode, in which the peptides emerging from the nanoflow LC are introduced into the TQMS using an ionizing nanospray interface. Within the TQMS, the peptide ions generated in the spray interface 'fly' through a first mass analyzer (MS1) set to pass the parent molecule (the intact peptide analyte), rejecting components of other mass-to-charge ratios (m/z). The analyte then passes into a collision chamber where it is fragmented by controlled collisions with gas atoms ("collisionally-induced dissociation", or CID). The resulting peptide fragments are passed to a second mass analyzer (MS2) set to pass a known specific fragment, which then enters a quantitative detector capable of counting the arriving fragment molecules. Each of these mass filters has a resolution allowing it to reject ions more than +/-0.5 atomic mass units (amu) different from the programmed setting. This two-stage selection of parent and fragment ions (selected reaction monitoring: SRM) affords great specificity, with the result that the detected signal traces a peak in the chromatogram at the expected LC retention time corresponding to the selected analyte. Integrating this peak gives a measure of the relative quantity of the analyte.



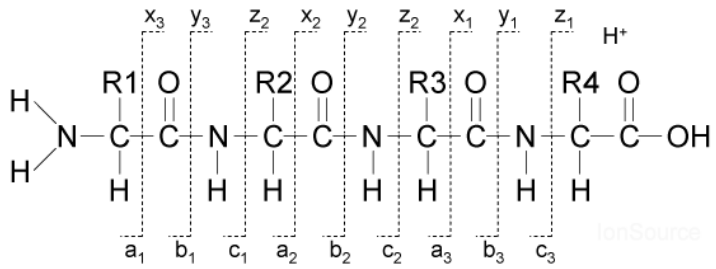
Virtually any alteration in the chemical nature of the peptide involves a change in mass of 1 amu or more, with the exception of structural rearrangements of the same set of atoms (e.g., changes in the order of constituent amino acids) or substitutions with identical mass (e.g., a change of isoleucine for leucine). By examining a series of large and small fragments of the peptide, sufficient additional sequence information is obtained to achieve reasonable certainty, in the context of the known human genome sequence, that the peptide has the structure expected of the desired analyte. This structural specificity in an analytical detector provides a major advance in clinical assay specificity.

Because tryptic peptides always contain a positively-charged amino acid (lysine or arginine) at their c-termini (reflecting the specificity of trypsin to cleave after these amino acids) in addition to the +1 positive charge of the n-terminal

amino group, tryptic peptides typically fly as ions of +2 charge (or +3 etc if they contain additional positive amino acids such as histamine). Because quadrupole mass filters actual select ions based on mass divided by charge (m/z), the setting of the MS1 filter for a tryptic peptide is typically the mass of the peptide plus 2 protons (giving +2 charge) divided by 2.

In practical use, particularly for multiplexed analyses such as the PepCa10 test, many SRM measurements can be carried out in a single analytical run. This is achieved by cycling through a series of SRM's repeatedly, i.e., by measuring first using a pair of SRM parameters (MS1, MS2; called a "transition") corresponding to the parent and fragment m/z values for a first peptide, followed by a switch to the SRM parameters for a second peptide, and so on through the complete list of peptides to be measured. As discussed below, it is desirable to measure multiple different SRM's for the same peptide (generally with the same parent MS1 setting, and different MS2 settings corresponding to different y-ion fragments) to confirm detection specificity. This is practical because the TQMS instrument can switch between SRM settings very rapidly (5 milliseconds) and can deliver adequate signal intensity measurements in 10 msec. Thus the set of 150 SRM's specified in the PepCa10 test are measured in ~2.3 sec, providing a minimum number of points across a ~15sec wide peptide peak to deliver reliable peak area measurements for all SRM's. Recent advances in TQMS control software allow SRM's to be scheduled, such that the transition is only measured during a time window in which the respective peptide peak is expected to be delivered by the LC. This approach radically decreases the number of SRM's observed at any one time in the PepCa10 test to a maximum of 45 (allowing measurement of each SRM every 0.7 sec, or 20 points across the peak), since the 10 peptides to be observed are spread over ~8 minutes of chromatographic elution and have peak widths of ~15 sec.

SRM measurement of peptides in a TQMS instrument is particularly informative with respect to analyte structure (and hence identity) because of the specific mechanisms by which peptide fragmentation occurs in CID. In general, most fragmentation occurs by breakage of a bond in the peptide backbone, generating two fragments: one containing the amino acids n-terminal to the break and one containing the c-terminal amino acids. In a peptide backbone, there are three possible bonds that can break and the resulting fragments are labeled according to a standard scheme (as shown below). In practice most breaks occur at the C-N peptide bond, yielding b-ion (amino acids n-terminal of the break) and y-ion (amino acids c-terminal to the break) fragments. The y-ions are most frequently used in SRM.



A synthetic stable-isotope labeled internal standard peptide that is labeled by inclusion of a single labeled c-terminal amino acid (i.e., a labeled lysine or arginine) produces y-ions that each contain the labeled amino acid and thus show the same mass increment with respect to the analyte as the parent peptide. This feature provides a useful simplification in production of the labeled standards and in calculating the mass of the labeled internal standard peptide and its fragments, while ensuring that both MS1 and MS2 settings differ between analyte and standard (allowing unambiguous separate detection). Fragments of an internal standard labeled uniformly (e.g., by U13C) will retain only part of the parent's mass increment relative to the unlabeled analyte, requiring calculation of mass increments from the elemental composition of each fragment.

The complete y-ion (or b-ion) series provides an almost completely unambiguous readout of the amino acid sequence of the peptide. The following figure shows the structures of the series of y-ions resulting from fragmentation of the peptide TATSEYQTFFNPR (from human prothrombin), labeled from y13 (the complete, unfragmented peptide) to y1 (the c-terminal arginine).

n-terminus	T	A	T	S	E	Y	Q	T	F	F	N	P	R	c-terminus		Fragment ion
	T	A	T	S	E	Y	Q	T	F	F	N	P	R			*y13*
		A	T	S	E	Y	Q	T	F	F	N	P	R			*y12*
			T	S	E	Y	Q	T	F	F	N	P	R			*y11*
				S	E	Y	Q	T	F	F	N	P	R			*y10*
					E	Y	Q	T	F	F	N	P	R			*y9*
						Y	Q	T	F	F	N	P	R			*y8*
							Q	T	F	F	N	P	R			*y7*
								T	F	F	N	P	R			*y6*
									F	F	N	P	R			*y5*
										F	N	P	R			*y4*
											N	P	R			*y3*
												P	R			*y2*
													R			*y1*
	T															*b1*
	T	A														*b2*
	T	A	T													*b3*
	T	A	T	S												*b4*
	T	A	T	S	E											*b5*
	T	A	T	S	E	Y										*b6*
	T	A	T	S	E	Y	Q									*b7*
	T	A	T	S	E	Y	Q	T								*b8*
	T	A	T	S	E	Y	Q	T	F							*b9*
	T	A	T	S	E	Y	Q	T	F	F						*b10*
	T	A	T	S	E	Y	Q	T	F	F	N					*b11*
	T	A	T	S	E	Y	Q	T	F	F	N	P				*b12*
	T	A	T	S	E	Y	Q	T	F	F	N	P	R			*b13*

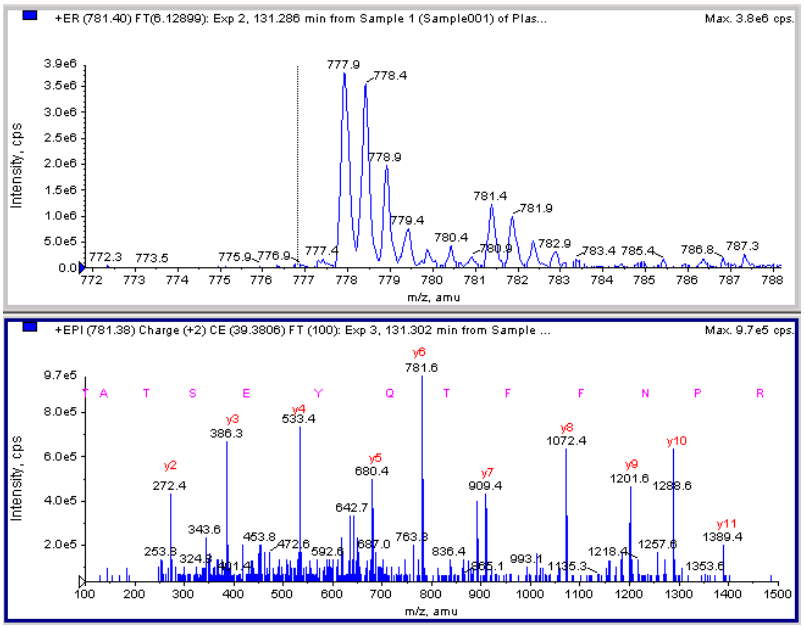
Given the accurately known masses of the amino acids, the masses of all these fragments can be computed exactly:

Sequence: **TATSEYQTFFNPR**, pI: **5.66367**

Fragment Ion Table, monoisotopic masses

Seq	#	B	Y	# (+1)
T	1	102.05555	1561.72874	13
A	2	173.09266	1460.68106	12
T	3	274.14034	1389.64395	11
S	4	361.17237	1288.59627	10
E	5	490.21496	1201.56424	9
Y	6	653.27829	1072.52165	8
Q	7	781.33687	909.45832	7
T	8	882.38454	781.39974	6
F	9	1029.45296	680.35206	5
F	10	1176.52137	533.28365	4
N	11	1290.56430	386.21524	3
P	12	1387.61706	272.17231	2
R	13	1543.71817	175.11955	1

Using these masses, the various fragments can be identified with high confidence. When all fragments are recorded, by performing full scans of the fragments using a TQMS mode different from SRM, most observed fragment masses can be accounted for as y- or b-ions derived from the expected peptide sequence. The restricted nature of the fragment pattern and the high resolution of two-stage SRM detection provide great specificity for peptide detection.



In the example, intense y-ion fragments at m/z 533.28, 781.40, 1072.52, 1201.56, and 1288.59 (i.e., y4, y6, y8, y9, and y10 ions) could be monitored as SRM fragments of a peptide with intact mass 1561.7 (m/z 781.4 with charge +2). Their joint occurrence strongly support presence of a peptide having a sequence of (TAT)SE(YQ)(TF)(FNPR), where the amino acids in parenthesis could in principle be in any order. However the expected arrangement can be tested against all protein sequences in the human proteome (translated expressed genome) and found to be the only alternative that actually occurs.

An additional advantage of mass spectrometry as a detector for peptide measurement is the precision and wide linear dynamic range (~1e4) available in quantitative applications. Triple-quadrupole mass spectrometers have been in routine use for many years providing accurate quantitation of small molecule analytes, and their quantitative accuracy is very well established. Recently a significant body of published research has demonstrated that the desirable features of TQMS quantitation can be extended from small molecules to short peptides, providing the basis of the methodology used in the PepCa10 test.

c) Unique signature peptides for each protein analyte

Key to the exploitation of this approach for protein measurement is the ability to identify peptide sequences unique to a desired analyte protein in the human proteome (so-called "proteotypic" peptides), coupled with the ability to release and recover these peptides from the analyte protein at high efficiency. As indicated below, proteotypic peptides exist for most human proteins (i.e., peptides not found in any other human protein), and these peptides can be efficiently released by digestion of the analyte protein with a proteolytic enzyme such as trypsin. Proteotypic peptides can be found by testing the predicted tryptic peptides against the human genome, using e.g., BLASTP, and finding only a single occurrence. As noted, essentially all human proteins contain proteotypic peptides.

Under conditions where trypsin releases the peptide completely (complete digestion), the molar amount of the peptide released is equal to the molar amount of the protein from which it was derived times the number of copies of the peptide in the protein sequence (typically 1 copy, with exceptions recognizable from the protein primary sequence). This equivalence, subject to quantitative release and recovery, provides a quantitative peptide surrogate for measurement of the protein. In practical use, it has proven to be more appropriate to focus on the directly observed peptide (the real analyte) rather than inferences with respect to the protein parent molecule.

d) Proteolytic digestion as a pre-analytical step

Digestion of the proteins in a patient sample to peptides results in a substantial stabilization of the sample. The activity of plasma enzymes (themselves proteins) is abolished, reducing the probability of post-collection chemical changes. Protein denaturation, unfolding, and complexation with other biomolecules (major sources of immunoassay interference) are features of intact proteins, and are eliminated once the proteins are digested to short tryptic peptides. In the context of the PepCa10 assay, proteolytic digestion is carried out under controlled conditions in the assay workflow, monitored by specific QC indices, and begins with a patient EDTA plasma sample. In this approach, endogenous post-collection proteolysis (such as occurs spontaneously in serum samples allowed to clot) is intentionally avoided, providing a critical distinction between the PepCa10 workflow and previous tests involving analysis of peptide fragments in serum resulting from autolysis.

e) Standardization of proteolytic sample digestion using a labeled recombinant concatamer protein

To obtain reproducible results from quantitative measurement of peptides liberated from plasma proteins by proteolytic digestion, the digestion process must be reproducible. In the ideal case digestion would be complete (quantitative stoichiometric yield of the relevant peptides from the proteins containing them). Since the result of the PepCa10 test depends on relative amounts of 10 peptide analytes in the digested sample, absolute peptide or

Commented [A91]: Correct, but first in vitro experiments would need to be carried out to identify which of the unique peptides is produced reliably and elicits the strongest MS peak. Not all unique peptides are created equally.

Commented [A92]: However, a number of AA in those released peptides may become more susceptible to oxidation and other chemical modifications.

Commented [A93]: QC or any control of tryptic digest completeness?

Commented [A94]: Should be evaluated as a part of reproducibility/precision analytical study.

protein quantitation is of secondary interest in comparison with accurate relative quantitation among the peptide analytes. Uncompensated differences in relative peptide yield in digestion could lead to error.

Commented [A95]: How do you determine that these do not happen?

It is known that trypsin cleaves with slightly different relative efficiencies depending on the specific amino acids present at positions n-terminal and c-terminal to the lysine or arginine residue after which the enzyme cleaves the polypeptide chain. Therefore the occurrence of a lysine or arginine residue in different sequence contexts can result in different cleavage rates, as shown in the following figures. For example, if a proline residue follows either Lys or arg, the cleavage efficiency is drastically reduced (which is why peptides containing a KP or RP dipeptide at either end are not selected for use in the PepCa10 assay).

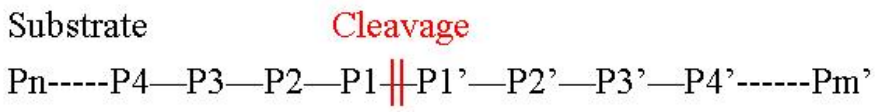


Fig.1 Schematic representation of enzyme-substrate complex with eight binding sites. Positions Pn to Pm' in the substrate are counted from the bond between P1 and P1', where the cleavage occurs.

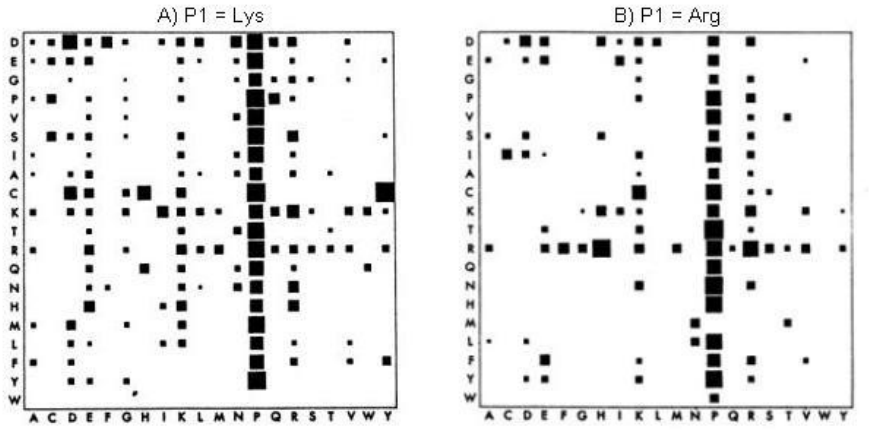


Fig. 3) Negative influence of residues in position P2 (vertical scale) and P1' (horizontal scale) on the cleavage for P1 = Lys (A) and P1 = Arg (B) by **trypsin**. The surfaces of black square on the chessboards represent the percentage of inhibition in the corresponding cross-section for each tripeptide sequence P2-Lys-P1' or P2-Arg-P1' (According to Keil, 1992)

Ideally an internal standard capable of mimicking the tryptic cleavage rates at the n-terminus and c-terminus of the target tryptic peptides in the sample proteins would provide the best standardization. The C-SIS1 labeled concatamer protein contained in the PepCa10 kit digest plate provides such a standard. The sequence of C-SIS1 includes each of the 10 target peptide sequences as well as

additional surrounding sequence from the target proteins. Specifically, each tryptic cleavage site required to generate the c1-c10 labeled peptide standards is surrounded with at least 5 amino acids of target protein sequence in both n-terminal and c-terminal directions. The following figure shows the relationships between the 3 forms of the peptide AIPVAQDLNAPSDWDSR (peptide p2; 1 of 2 peptides representing human osteopontin isoform 1) involved in the assay. The sequence environments of the n-terminal and c-terminal trypsin cleavage sites at which cuts are required to liberate the peptide from osteopontin (in the sample) or labeled C-SIS1 internal standard protein are identical, ensuring similar cleavage efficiencies for the analyte and the standard. The labeled peptide s2, used to assess recovery of the peptide, requires no cleavage. Different labeling schemes ensure that the peptide version from the 3 sources can be distinguished by mass.

Source	Mass (Bold Seq)	Trypsin ↓ Trypsin ↓
Osteopontin isoform 1	1854.9	***ESEELNGAYK**AIPVAQDLNAPSDWDSR*GKDSY*ETSQL***
C-SIS1 protein	1934.9	***AGSGKNGAYK**AIPVAQDLNAPSDWDSR*GKDSY*GSGKG***
P-SIS peptide s2	1864.9	**AIPVAQDLNAPSDWDSR**

The principal advantage gained by concatenating into one protein the peptide standards comprising the C-SIS1 molecule is the guarantee of defined stoichiometry between the component peptides. Once the sequence of the C-SIS1 protein is confirmed during production, the molar ratios between its component peptides is defined exactly. During tryptic digestion, the relative yields of the component standards closely approximate the yields of the respective analyte peptides, thus providing effective internal standardization of any variation in tryptic cleavage efficiency.

Commented [A96]: Sounds attractive but beeds to be demonstrated with actual data. If this piece is not supported by actual data it could cause a domino effect. It may still be problematic to use as an IS for quantitation.

f) Use of antibodies to enrich peptide analytes

Antibodies are used in classical immunoassays to specifically bind to analytes, and thereby determine which sample molecules give rise to an output signal. In the typical sandwich ELISA format, the use of one antibody to capture the analyte and a second antibody (binding to a different analyte epitope) to generate a detectable signal (e.g., through a fluorescent label) confers added specificity by requiring two intact epitopes. Nevertheless immunoassays of this type cannot provide structural information beyond the presence of epitopes, and indeed the definition of an epitope in precise structural terms can be ambiguous. In addition, antibodies are large protein structures with numerous potential interaction sites, including epitopes, of their own that can be involved in interactions causing interferences (HAMA for example).

Antibodies can be made against short peptide linear epitopes as well as intact proteins, and hence can serve to bind the tryptic proteotypic peptides used in the PepCa10 test and separate them from a large mass of unbound peptides. This specific affinity enrichment of peptide analytes from a complex digest serves to: 1) enrich and concentrate the analyte peptides from larger amounts of plasma digest than could otherwise be introduced into the mass spectrometer, and 2)

deplete other peptides in the digest, particularly those derived from high-abundance proteins, and thus decrease interfering signals. In practice this enrichment (>100,000-fold relative to high-abundance serum albumin peptides) deliver analyte in a matrix (Magnetic Bead Elution buffer; 5% acetic acid) much less complex than the plasma digest. For this reason it is appropriate to perform instrument calibration using peptide standards in 5% acetic acid.

It is important to note that only one antibody is required for each peptide, and that this antibody's only function is to bind, and thus enrich, a specific peptide sequence from a complex digest of the patient plasma. The antibody binds the sample-derived target peptide and the spiked internal standard (a stable isotope labeled version of the same peptide sequence) equally, there being no basis for preferential binding of one isotopic version compared to the other. Since the quantitative assay result is derived from the ratio of the two peptide forms (analyte vs. internal standard), the antibody has no direct role in analyte quantitation (which is carried out by the mass spectrometer). Hence the assay is largely insensitive to the fraction of the peptide recovered by the antibody, provided there is sufficient analyte and internal standard to generate accurate MS measurements. If the antibody fails to capture sufficient peptide to generate an adequate MS signal for either peptide form, no result is produced. This contrasts with immunoassays, in which antibody binding per se generates a signal, and variation in binding alters the test result.

Commented [A97]: This would need to be shown

Commented [A98]: Should be enriched equally.

An important consequence of the use of antibodies for analyte enrichment only (and not for detection) is the fact that binding of one antibody to another has no effect on the assay result. Antibody crosslinking through interaction with other proteins (as in HAMA interference in ELISA) is avoided in the first instance through digestion of the plasma sample to peptides (removing proteins capable of binding antibody reagents) and finally because antibody aggregation, if it did occur, has little effect on the ability of antibodies to bind peptide ligands and thus enrich them.

One can therefore look upon the PepCa10 workflow as one in which the anti-peptide antibody enriches an analyte peptide but does not otherwise participate directly in the quantitative measurement – quantitation is instead based on the ratio of analyte to internal standard as measured in the mass spectrometer. By analogy with the conventional sandwich ELISA, the PepCa10 workflow uses an anti-peptide antibody as the first (capture) antibody, and a mass spectrometer in the role of an improved second antibody (one that is universal for all peptide analytes and capable of providing near-absolute structural specificity).

g) QC Assessment of peptide analyte recovery using labeled synthetic peptide standards

A second set of labeled peptide standards (s1-s10, provided as a mixture) is added to the LC-TQMS sample prior to LC-TQMS analysis. The s1-s10 peptides are present in the mixture in the same relative molar quantities as the c1-c10 peptides are present in the sequence of the concatamer protein C-SIS1. The molar amount of each s1-s10 peptide added per sample is likewise the same as the molar amount of C-SIS1 protein added (adjusted for the multiplicity of

Commented [A99]: Would this mean that if the ratio of labeled tryptic peptides from concatamer does not coincide with the ratio of s1-s10 peptides, the digest is not complete or reliable and the analysis needs to be repeated?

some of the peptide sequences in C-SIS1). Thus if tryptic cleavage of C-SIS1 is stoichiometric, releasing the peptides at 100% efficiency, and analyte recovery through the antibody capture process is 100%, then equal amounts of c1 and s1, equal amounts of c2 and s2, and so on, would be detected by the MS. Deviations from these equalities indicate less than perfect recovery of the peptides due to incomplete tryptic cleavage of the parent proteins, incomplete recovery of peptide by antibody capture, or other losses. Analyte recoveries less than a specified minimum acceptable value alert the user to a technical problem in the assay. [Once sufficient experience is gained in the use of the PepCa10 workflow, it is expected that the use of recovery standards may be eliminated to decrease assay complexity.]

Commented [A100]: It appears plausible that deviations will happen in the majority of the assays. How will you define an acceptable value? (Particularly considering set of 10 peptides from 5 proteins.)

h) Combination of Multiple Analyte Measurements Into a Single Test Result

A combination of multiple analytes offers the potential for improved specificity and sensitivity compared to a single analyte test. Correlated analytes can be combined to increase statistical power for detecting change in a single biological pathway, for example, while uncorrelated analytes can be combined to probe alternative mechanisms contributing to a single disease. Major challenges in the multiplex approach are the selection of analytes that contribute most to the aggregate result, and design of a robust algorithm for combining analyte measurements.

i) Selection of PepCa10 Target Peptides

Ten tryptic peptides (p1-p10; two each from 5 cancer biomarker proteins P1-P5) were selected in a training study comprising half of the samples in the clinical study described below. Measurements were made by mass spectrometry using the same general protocol as described for the PepCa10 test.

A proprietary iterative statistical approach was used to evaluate the contributions of peptide measurements to incremental improvements in ROC curve performance. Initial work indicated that the balance between addition of beneficial signal versus addition of unwanted noise occurred at ~10 peptide analytes (more analytes thereafter adding more noise than signal to the assay). The best performing panel of peptides (best combination of sensitivity and specificity for the selected diagnostic application) was chosen as the basis of the PepCa10 test, and applied without modification in the second set of samples from the clinical study (the validation set).

Commented [A101]: What weights were used in the combination of sensitivity and specificity? Do you plan to use the cutoff that corresponds to these levels of sensitivity and specificity? Please note that for the women with BI-RADS 4, the level of sensitivity is very important because (depending on the intended use) one would probably need to have extremely high NPV.

Commented [A102]: What are the differences between the first and second set (i.e. is it the same set randomly split into "training" and validation sets)?

The selected tryptic peptides representing each protein were chosen to optimize a combination of features in addition to their contribution to assay sensitivity and specificity. These features included:

Uniqueness in the human proteome (i.e., absence from the sequences of other human proteins, thus ensuring the peptide can be derived only from the intended target protein)

Absence of known sequence polymorphisms and post-translational modifications (avoiding the possibility of varying amounts of multiple forms of the peptide analyte)

Low frequency of amino acids with potential for pre-analytic modification, e.g., Cys, Met, Gln, Asn, Trp

Mass between 800 and 2,500 Daltons (optimal range for quantitation by SRM)

Relative signal strength in SRM

Success in producing a corresponding anti-peptide antibody with a kinetic off-rate <1e-3/sec

Reproducible yield in the tryptic digestion protocol (especially avoidance of KP or RP sequences at either terminus)

Reproducible chromatographic performance (good peak shape and reproducible retention time)

Features 1-4 are computed from genome data and help ensure assay specificity. SRM signal strength (5) and antibody kinetics (6) contribute jointly to assay sensitivity. Digestion yield (7) and chromatographic performance (8) contribute to assay robustness.

In the case of three proteins (P1, 2, 5) the ratio between two peptides derived from different regions of the protein sequence proved to be better cancer/benign discriminators than the abundance of either alone, likely indicating that two forms of these proteins are present in different amounts and that these forms are differentially affected in breast cancer.

Two peptides with highly correlated behavior were included from each of two other proteins (P3 and P4) to add statistical power and also provide a further quality check on sample preparation, specifically on completeness of tryptic digestion. In each case, there was no evidence that the peptides are present in unequal amounts in the patient samples (indicating that the corresponding proteins are probably not subject to cleavage in vivo). However, for each protein one of the peptides is released early in the course of tryptic digestion and one is released more slowly. By comparing the relative amounts of these two peptides, and specifically requiring that they are present at near-equal levels, a measure of digestion completeness is obtained (see below).

The peptides selected are shown underlined and in bold in the proteins sequences below:

P1: Osteopontin isoform 1
>sp|P10451|OSTP_HUMAN Osteopontin OS=Homo sapiens GN=SPP1 PE=1 SV=1
MRIAVICFCLLGITCAIPVKQADSGSSEEKQLYNK**YPDAVATWLNPDPSQK**QNLLAPQN
AVSSEETNDFKQETLPSKSNESHDHMDDMDDEDDDDHVDSQDSIDSNDSDDVDDTDDSH
QSDESHHSDESDELVTDFPTDLPATEVFTPVVPTVDTYDGRGDSVVYGLRSKSKKFRRP
DIQYPDATDEDITSHMESEELNGAYK**AIPVAQDLNAPSDWDSR**GKDSYETSQLDDQSAE
THSHKQSRLYKRKANDESNEHSDVIDSQELSKVSREFHSHEFHSHEDMLVVDPKSKEED
KHLKFRISHELDSASSEVN

Commented [A103]: Data?

Commented [A104]: Does this mean that the ratio of two peptides from the same protein is a cancer discriminator? Will need to see the actual data.

Commented [A105]: Data?

Commented [A106]: Length of digestion?

Commented [A107]: This is confusing. If the digest is performed overnight how do you plan to determine kinetics of the tryptic peptides release? What are the conditions for tryptic digest?

P2: Mesothelin isoform 3
>sp|Q13421-2|MSLN_HUMAN Isoform SMRP of Mesothelin OS=Homo sapiens GN=MSLN
MALPTARPLLGSCGTPALGSLLFLLFSLGWVQPSRTLAGETGQEAAPLDGVLANPPNIS
SLSPRQLLGFPCAEVSGLSTERVRELAVALAQKNVKLSTEQLRCLAHRLSEPPEDLDAL
PLDLLLFLNPDAFSGPQACTRFFSRITKANVDLLPRGAPERQRLLPAALACWGVRGSLL
SEADVRALGGLACDLPGRFVAESAEVLLPRLVSCPGPLDQDQQEAARAALQGGGPPYGP
PSTWSVSTMDALRGLLPVLGQPIIRSIPQGIVAAWRQRSSRDPSWRQPERTILRPRFRR
EVEKTACPSGKKAR**EIDESLIFYK**KWELEACVDAALLATQMDRVNAIPFTYEQLDVLKH
KLDELYPQGYPESVIQHLGYLFLKMSPEDIRKWNVTSLETLKALLEVNKGHEMSPQVAT
LIDRFVKGRGQLDKDTLDTLTAFYPGYLCSLSPEELSSVPPSSIWAVRPQDLDTCDPRQ
LDVLYPKARLAFQNMNGSEYFVKIQSFLGGAPTEDLKALSQQNVSMDLATFMKLRTDAV
LPLTVAEVQK**LLGPHVEGLK**AEERHRPVRDWILRQRQDDLDTLGLGLQGGIPNGYLVLD
LSVQGGRGGQARAGGRAGGVEVGALSHPSLCRGPLGDALPPRTWTCSHRPGTAPSLHPG
LRAPLPC

P3: erbB2
>P04626|23-1255
TQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFLQDIQEVQ
GYVLIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNGDPLNNTTPVTGASPGGLREL
QLRSLTEILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLIDTNRSRACHPCSPMC
KGSRCWGESSEDCQSLTRTVCAGGCARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLHF
NHSGICELHCPALVTYNTDTFESMPNPEGRYTFGASCVTACPYNYLSTDVGSCTLVCPL
HNQEVTAEDGTQRCEKCSKPCARVCYGLGMEHLREVRAVTSANIQEFAGCKKIFGSLAF
LPESFDGDPASNTAPLQPEQLQVFETLEEITGYLYISAWPDSLPDLSVFQNLQVIRGRI
LHNGAYSLTLQGLGISWLGLRSLRELGSGLALIHHNTHLCFVHTVPWDQLFRNPHQALL
HTANRPEDECVGEGLACHQLCARGHCWGPGPTQCVNCSQFLRGQECVEECRVLQGLPRE
YVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMP
IWKFPDEEGACQPCPINCTHSCVDLDDKGCPAEQRASPLTSIISAVVGILLVVVLGVVF
GILIKRRQQKIRKYTMRRLLQETELVEPLTPSGAMPNQAQMRILKETELRKVK**VLGSGA**
FGTVYKGIWIPDGENVKIPVAIKVLRENTSPKANKEILDEAYVMAGVGSPYVSRLLGIC
LTSTVQLVTQLMPYGCLLDHVRENRGRLGSQDLLNWCMQIAKGMSYLEDVRLVHRDLAA
RNVLVKSPNHVK**ITDFGLAR**LLDIDETEYHADGGKVPIKWMALESILRRRFTHQSDVWS
YGVTVWELMTFGAKPYDGIPAREIPDLLEKGERLPQPPICTIDVYMIMVKCWMIDSECR
PRFRELVSEFSRMARDPQRFVVIQNEDLGPASPLDSTFYRSLLEDDDMGDLVDAEEYLV
PQQGFFCPDPAPGAGGMVHHRHRSSSTRSGGGDLTLGLEPSEEEAPRSPLAPSEGAGSD
VFDGDLGMGAAKGLQSLPTHDPSPLQRYSEDPTVPLPSETDGYVAPLTCSPQPEYVNQP
DVRPQPPSPREGPLPAARPAGATLERPKTLSPGKNGVVKDVFAFGGAVENPEYLTPQGG
AAPQPHPPPAFSPAFDNLYYWDQDPPERGAPPSTFKGTPTAENPEYLGLDVPV

P4: LPS-BP
>P18428|26-481
ANPGLVARITDKGLQYAAQEGLLALQSELLR**ITLPDFTGDLR**IPHVGRGRYEFHSLNIH
SCELLHSALRPVPGQGLSLSISDSSIRVQGRWKVRKSFFKLQGSFDVSVKGISISVNLL
LGSESSGRPTVTASSCSSDIADVEVDMSGDLGWLLNLFHNQIESKFQKVLESRICEMIQ
KSVSSDLQPYLQTLPVTTEIDSFADIDYSLVEAPRATAQMLEVMFKGEIFHRNHRSPVT

LLAAVMSLPEEHNKMVYFAISDYVFNTASLVYHEEGYLNFSITDDMIPPDSNIRLTTKS
FRPFVPRLARLYPNMNLELQGSVPSAPLLNFSPGNLSVDPYMEIDAFVLLPSSSKEPVF
RLSVATNVSATLTFNTSKITGFLKPGKVKVELKESKVGLFNAELLEALLNYYILNTFYP
KFNDK**LAEGFPLPLLK**RVQLYDLGLQIHKDFLFLGANVQYMRV

P5: Mucin-1 isoform 8
>P15941-8
MTPGTQSPFFLLLLLTVLTVVTGSGHASSTPGGEKETSATQRSSVPSSTEKNALSTGVS
FFFLSFHISNLQFNSSLEDPSTDYYQELQRDISEMFLQIYKQGGFLGLSNIKFRPGSVV
VQLTLAFR**EGTINVHDVETQFNQYK**TEAASRYNLTISDVSVSDVPFPFSAQSGAGVPGW
GIALLVLVCVLVALAIVYLIALAVCQCRRKNYGQLDIFPARDTYHPMSEYPTYHTHGR**Y
VPPSSTDR**SPYEKVSAGNGGSSLSYTNPAVAAASANL

j) Derivation of Coefficients in PepCa10 Algorithm

A linear combination approach for combining the 10 peptide measurements was selected to allow simple interpretation of the direction and relative significance of change in any of the peptides.

Coefficients were derived using logistic regression on the estimated concentrations (log scale) of the ten target analytes measured in the samples from the training set from the proposed clinical study, and involves all 10 analytes

These values were incorporated into the PepCa10 test as coefficients in the linear combination of the 10 peptide measurements.

When used with log-transformed relative concentration ratios (analyte peak area divided by internal standard peak area), these coefficients produce a test result that is consider positive when >10.

Commented [A108]: Needs to be upfront in the Interpretation of the results part.

k) Selection of SRM Transitions Measured by the Mass Spectrometer

Five SRM transitions are measured for each peptide. These transitions were selected as the best 5 y-ions in experiments where all high-mass y-ions were measured. In each case the principal adjustable parameter governing fragmentation (the collision energy) was optimize individually for each transition. Source ionization parameters were fixed at a constant value for all peptides and transitions.

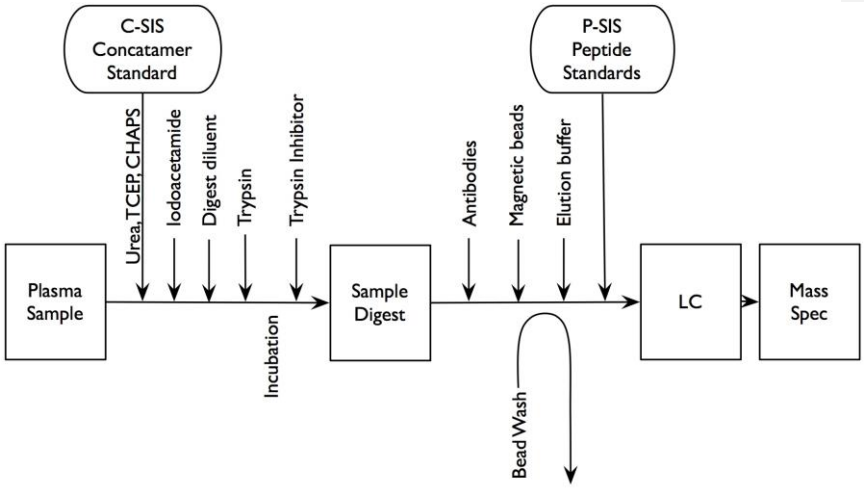
Commented [A109]: This energy along with other paramenters will require adjustment with different MS instruments. How do you plan to instruct the user in this regard?

l) Generation of Antibody Reagents

The antibodies used in the PepCa10 test are rabbit monoclonals generated by immunization with KLH-conjugated synthetic target peptides, and selected by a hybridoma screening process yielding affinity reagents with a kinetic off-rate lower than ~1e-3/sec (giving a half-time before dissociation of ~20min) and an affinity of ~1e-9 (as measured by Biacore using immobilized antibody and flowing peptide). This slow off-rate ensures that the target peptide, once captured, remains bound to the antibody during an extensive washing and separation process on magnetic beads prior to final elution and LC-TQMS analysis.

3. PepCa10 Workflow

The PepCa10 kit is used in a workflow to a) digest the proteins of an aliquot of the plasma sample to yield tryptic peptides; b) capture specific analyte peptides on anti-peptide antibodies, and finally c) elute the bound analyte peptides and present them in the mass spectrometer where they are measured using pre-specified selected reaction monitoring (SRM) transitions. The workflow for sample and kit reagents is shown in the following schematic. As described above, the C-SIS standard is cleaved by trypsin in parallel with the sample proteins to yield labeled internal standard peptides at the start of sample processing. The P-SIS labeled peptide standards (different label) are added at the end of sample processing to allow measurement and QC of analyte recovery.



a) Plasma sample preparation

Patient plasma samples are collected in conventional EDTA plasma tubes, and processed by centrifugation to remove cellular material. Sample stability studies have shown that samples frozen for up to 4 weeks at –20C or 1 year at –80C, with or without up to 3 freeze/thaw cycles, show no significant change in assay result.

Commented [A110]: Would need to provide protocols on how these were done.

b) Digestion

Digestion of the patient plasma specimen to yield a tryptic digest is accomplished with the reagent kit provided. The specimen is thawed at room temperature and a 20ul volume is added to a lyophilized aliquot of urea, TCEP, CHAPS detergent, Tris HCl pH 7.5 and C-SIS labeled concatamer standard in one well of the 96-well “Digest” plate provided as part of the kit. The plate is then shaken on a plate shaker for 30 sec to complete dissolution of the reagents.

After a subsequent 30 minute incubation at 50C, 10ul of iodoacetamide solution is added, mixed on the plate shaker and the mixture further incubated for 10 minutes in the dark at 37C. Digestion is accomplished by addition of 150ul of the provided trypsin solution and shaking, followed by incubation overnight at 37C. Finally 20ul of reconstituted aprotinin (trypsin inhibitor) is added to each well to stop trypsin activity.

Commented [A111]: How long? Overnight might be 8 hours, 10 h, or? In many cases, overnight digestion is performed at 4C (not at 37C). Please specify conditions for this critical step.

c) Addition of peptide capture antibodies

Peptide capture antibodies (a mixture of 10 rabbit monoclonals) in 50% glycerol are diluted 1:10 by addition of 500ul of Magnetic Bead Wash Solution, and 10ul of the resulting solution added to each digest-containing well. After shaking to mix the contents, the plate is incubated for 2hr at 37C to allow the antibodies to capture their target peptides from the digest.

Commented [A112]: May need some data to confirm incubation time.

d) Analyte enrichment

Affinity enrichment of analytes is completed by capturing the antibodies (with their bound peptide cargo) on protein G-coated magnetic beads, washing the beads and finally eluting the bound peptides to yield a sample of enriched peptides ready for introduction to the LC-TQMS. These steps are executed using a ThermoFisher Kingfisher-96 magnetic bead processor operating under a program protocol provided with the kit. The Kingfisher is loaded with the following 96-well plates in the 8 available positions:

Magnetic bead suspension (50ul/well)
Bead wash solution (150ul/well)
Patient sample digests (100ul/well)
Bead wash solution (150ul/well)
Bead wash solution (150ul/well)
Distilled water (150ul/well)
Bead Eluent (20ul/well, 5% acetic acid containing added QC peptides)
Empty plate with unused Kingfisher tip-comb

The protocol directs the Kingfisher to pick up the unused tip comb (8), transfer magnetic beads (1) to a wash plate (2), where the suspension is agitated for 1 minute, and then into the patient sample digests plus capture antibodies (3), in which the bead suspension is agitated for 1 hour. The beads, carrying bound antibody and analyte peptides, are then removed from the digest, and transported sequentially through 2 washes (4,5; each agitated for 1 minute) in bead wash solution and a final wash in distilled water (6; agitated for 1 minute), followed by elution (with agitation for 1 minute) in 20ul of Bead Eluent solution (7). Following elution the beads are removed from the Bead Eluent and placed, with the used tip-comb, in position 8 for disposal.

The enriched analyte + peptide standard samples are collected in plate 7 in Magnetic Bead Eluent buffer. MS calibrants 1-6 are loaded manually into wells A1-A6 of plate 7. Plate 7 is thereafter used to transport the samples to the LC instrument for analysis.

Commented [A113]: Both ones from concatamers and ones in stoichiometric amounts?

		Plate Column											
		1	2	3	4	5	6	7	8	9	10	11	12
Plate Row	A	Cal1	Cal2	Cal3	Cal4	Cal5	Cal6				Control1	Control2	Control3
	B	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
	C	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
	D	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36
	E	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46	S47	S48
	G												
	H												

e) LC-TQMS analysis: Daily QC standards

At the start of each day's LC-TQMS run, instrument calibration is verified by injecting in successive runs 10ul of Calibrants 1-6 from wells A1-A6. The resulting instrument response curves for each target peptide are evaluated for linearity.

Second, a series of 3 replicate injections (10ul each) is made from well A3. A total of 100 SRM's are measured in each run, comprising 5 transitions for each of 4 peptides (20 transitions), each at 5 different m/z offsets in MS1 and MS2 respectively: (0,0), (-0.5,0), (+0.5,0), (0,-0.5), (0,+0.5). The resulting data provides a QC check of 1) LC elution time of target peptides; 2) MS1 and MS2 mass accuracy (by comparing signal at the expected maximal m/z settings with data collected at +/- 0.5 amu offsets in MS1 and MS2 separately); and 3) CV's of the resulting peak areas across 3 replicate injections.

			Nominal m/z		Decrement MS1		Increment MS1		Decrement MS2		Increment MS2	
QC peptide	Sequence	LC Elution Time (min)	MS1	MS2	MS1 - 0.5	MS2	MS1 + 0.5	MS2	MS1	MS2 - 0.5	MS1	MS2 +0.5
1	YPDAVATWLNPDPSQK	32	905.438	467.3	904.94	467.30	905.94	467.30	905.44	466.80	905.44	467.80
			905.438	679.3	904.94	678.80	905.94	679.80	904.94	678.80	905.94	679.80
			905.438	793.4	904.94	792.90	905.94	793.90	904.94	792.90	905.94	793.90
			905.438	1193.6	904.94	1193.10	905.94	1194.10	904.94	1193.10	905.94	1194.10
			905.438	1264.6	904.94	1264.10	905.94	1265.10	904.94	1264.10	905.94	1265.10
2	AIPVAQDLNAPSDWDSR	25	932.949	839.9	932.45	839.40	933.45	840.40	932.45	839.40	933.45	840.40
			932.949	870.4	932.45	869.90	933.45	870.90	932.45	869.90	933.45	870.90
			932.949	1055.5	932.45	1055.00	933.45	1056.00	932.45	1055.00	933.45	1056.00
			932.949	1283.6	932.45	1283.10	933.45	1284.10	932.45	1283.10	933.45	1284.10
			932.949	1482.7	932.45	1482.20	933.45	1483.20	932.45	1482.20	933.45	1483.20
3	EIDESLIFYK	20	632.821	465.2	632.32	464.70	633.32	465.70	632.32	464.70	633.32	465.70
			632.821	578.3	632.32	577.80	633.32	578.80	632.32	577.80	633.32	578.80
			632.821	778.5	632.32	778.00	633.32	779.00	632.32	778.00	633.32	779.00
			632.821	907.5	632.32	907.00	633.32	908.00	632.32	907.00	633.32	908.00
			632.821	1022.5	632.32	1022.00	633.32	1023.00	632.32	1022.00	633.32	1023.00
4	LLGPHVEGLK	16	535.816	553.3	535.32	553.30	536.32	553.30	535.82	552.80	535.82	553.80
			535.816	690.4	535.32	690.40	536.32	690.40	535.82	689.90	535.82	690.90
			535.816	787.4	535.32	787.40	536.32	787.40	535.82	786.90	535.82	787.90
			535.816	844.5	535.32	844.50	536.32	844.50	535.82	844.00	535.82	845.00
			535.816	957.5	535.32	957.50	536.32	957.50	535.82	957.00	535.82	958.00

Finally a single injection is made of each processed Control sample (LOW, HIGH and NEAR-CUTOFF) in wells A10 – A12. PepCa10 results for these controls must fall within established limits, as determined in the PepCa10 program.

Commented [A114]: Are these the same for every lot of e.g. calibrators?

f) LC-TQMS analysis: Patient samples

A 10ul aliquot of the Bead Eluent from the Kingfisher process (i.e., the enriched peptide analyte sample (plate 7)) is injected into the LC-TQMS instrument for analysis. Operating under the control of the manufacturer's instrument software with run parameters specified in a provided method file, the instrument resolves sample peptides by C18 reversed phase chromatography and introduces the resolved peptides into the triple quadrupole mass spectrometer using the nano-electrospray interface.

Commented [A115]: ?

During the analytical run, a series of 150 specific transitions are observed. The observation periods for each peptide are scheduled to occur during a window of time during which it is expected to elute from the chromatography column, so that a maximum of 45 transitions are monitored at any one time. Using an SRM dwell time of 10msec dwell time, observations are made at least every 800msec. These transitions (table below) consist of 5 each for the 10 analyte peptides (p1 – p10), 5 each for the 10 corresponding C-SIS1-derived internal standard peptides (c1 – c10), and 5 each for the 10 corresponding P-SIS1 recovery standard peptides (s1 – s10). As explained above, p1, c1 and s1 peptides all have the same sequence and chemical structure but differ from one another in mass: p1 is sample-derived and unlabeled (i.e., natural preponderance of 12C and 14N stable isotopes), c1 is U12C labeled during expression of a concatamer protein, and s1 is U12C, U15N labeled in the c-terminal residue during chemical synthesis.

Analyte peaks observed in the resulting SRM traces at the expected elution times for each peptide are computed by the TQMS instrument manufacturer's peak integration software using parameters supplied with the PepCa10 kit. Peak area values for each transition are output as a CSV file for input to the PepCa10 program.

Protein Name	Protein	Peptide	Transition	SP #	Target peptide sequence	Parent ion charge	Fragment ion	Frgment ion charge	CE	Analyte MS1	Analyte MS2	C-SIS1 Internal Standard MS1	C-SIS1 Internal Standard MS2	P-SIS1 Internal Standard MS1	P-SIS1 Internal Standard MS2
Osteopontin	1	1	1	Q15683	YPDAVATWLNPDPSQK	2	y4	1	27.64	901.44	459.30	942.44	478.30	905.44	467.30
Osteopontin	1	1	2	Q15683	YPDAVATWLNPDPSQK	2	y6	1	27.64	901.44	671.30	942.44	699.30	905.44	679.30
Osteopontin	1	1	3	Q15683	YPDAVATWLNPDPSQK	2	y7	1	27.64	901.44	785.40	942.44	817.40	905.44	793.40
Osteopontin	1	1	4	Q15683	YPDAVATWLNPDPSQK	2	y10	1	27.64	901.44	1185.60	942.44	1238.60	905.44	1193.60
Osteopontin	1	1	5	Q15683	YPDAVATWLNPDPSQK	2	y11	1	27.64	901.44	1256.60	942.44	1312.60	905.44	1264.60
Osteopontin	1	2	1	Q15683	AIPVAQDLNAPSDWDSR	2	y15	2	28.43	927.95	835.90	967.95	871.40	932.95	839.90
Osteopontin	1	2	2	Q15683	AIPVAQDLNAPSDWDSR	2	y7	1	28.43	927.95	862.40	967.95	898.40	932.95	870.40
Osteopontin	1	2	3	Q15683	AIPVAQDLNAPSDWDSR	2	y9	1	28.43	927.95	1047.50	967.95	1090.50	932.95	1055.50
Osteopontin	1	2	4	Q15683	AIPVAQDLNAPSDWDSR	2	y11	1	28.43	927.95	1275.60	967.95	1328.60	932.95	1283.60
Osteopontin	1	2	5	Q15683	AIPVAQDLNAPSDWDSR	2	y13	1	28.43	927.95	1474.70	967.95	1535.70	932.95	1482.70
Mesothelin	2	3	1	P01033	EIDESLIFYK	2	y3	1	22.90	628.82	457.20	658.32	481.20	632.82	465.20
Mesothelin	2	3	2	P01033	EIDESLIFYK	2	y4	1	22.90	628.82	570.30	658.32	600.30	632.82	578.30
Mesothelin	2	3	3	P01033	EIDESLIFYK	2	y6	1	22.90	628.82	770.50	658.32	809.50	632.82	778.50
Mesothelin	2	3	4	P01033	EIDESLIFYK	2	y7	1	22.90	628.82	899.50	658.32	943.50	632.82	907.50
Mesothelin	2	3	5	P01033	EIDESLIFYK	2	y8	1	22.90	628.82	1014.50	658.32	1062.50	632.82	1022.50
Mesothelin	2	4	1	P01033	LLGPHVEGLK	2	y5	1	22.90	531.82	545.30	556.32	569.30	535.82	553.30
Mesothelin	2	4	2	P01033	LLGPHVEGLK	2	y6	1	22.90	531.82	682.40	556.32	712.40	535.82	690.40
Mesothelin	2	4	3	P01033	LLGPHVEGLK	2	y7	1	22.90	531.82	779.40	556.32	814.40	535.82	787.40
Mesothelin	2	4	4	P01033	LLGPHVEGLK	2	y8	1	22.90	531.82	836.50	556.32	873.50	535.82	844.50
Mesothelin	2	4	5	P01033	LLGPHVEGLK	2	y9	1	22.90	531.82	949.50	556.32	992.50	535.82	957.50
erbB2 - Receptor tyro	3	5	1	P04626	VLGSGAFGTVYK	2	y5	1	24.48	599.82	567.30	627.82	593.30	603.82	575.30
erbB2 - Receptor tyro	3	5	2	P04626	VLGSGAFGTVYK	2	y6	1	24.48	599.82	714.40	627.82	749.40	603.82	722.40
erbB2 - Receptor tyro	3	5	3	P04626	VLGSGAFGTVYK	2	y8	1	24.48	599.82	842.40	627.82	882.40	603.82	850.40
erbB2 - Receptor tyro	3	5	4	P04626	VLGSGAFGTVYK	2	y9	1	24.48	599.82	929.50	627.82	972.50	603.82	937.50
erbB2 - Receptor tyro	3	5	5	P04626	VLGSGAFGTVYK	2	y10	1	24.48	599.82	986.50	627.82	1031.50	603.82	994.50
erbB2 - Receptor tyro	3	6	1	P04626	ITDFGLAR	2	y3	1	21.32	446.75	359.20	466.75	374.20	451.75	367.20
erbB2 - Receptor tyro	3	6	2	P04626	ITDFGLAR	2	y4	1	21.32	446.75	416.20	466.75	433.20	451.75	424.20
erbB2 - Receptor tyro	3	6	3	P04626	ITDFGLAR	2	y6	1	21.32	446.75	678.40	466.75	708.40	451.75	686.40
erbB2 - Receptor tyro	3	6	4	P04626	ITDFGLAR	2	y6	2	21.32	446.75	339.70	466.75	354.70	451.75	343.70
erbB2 - Receptor tyro	3	6	5	P04626	ITDFGLAR	2	y7	1	21.32	446.75	779.40	466.75	813.40	451.75	787.40
LPS-binding protein	4	7	1	P18428	ITLPDFTGDLR	2	y5	1	23.69	624.33	561.30	652.33	583.30	629.33	569.30
LPS-binding protein	4	7	2	P18428	ITLPDFTGDLR	2	y6	1	23.69	624.33	708.40	652.33	739.40	629.33	716.40
LPS-binding protein	4	7	3	P18428	ITLPDFTGDLR	2	y7	1	23.69	624.33	823.40	652.33	858.40	629.33	831.40
LPS-binding protein	4	7	4	P18428	ITLPDFTGDLR	2	y8	1	23.69	624.33	920.40	652.33	960.40	629.33	928.40
LPS-binding protein	4	7	5	P18428	ITLPDFTGDLR	2	y10	1	23.69	624.33	1134.60	652.33	1184.60	629.33	1142.60
LPS-binding protein	4	8	1	P18428	LAEGFPLPLLK	2	**b5**	1	23.69	599.36	518.10	628.86	543.10	599.36	518.10
LPS-binding protein	4	8	2	P18428	LAEGFPLPLLK	2	y6	1	23.69	599.36	680.30	628.86	714.30	603.36	688.30
LPS-binding protein	4	8	3	P18428	LAEGFPLPLLK	2	y7	1	23.69	599.36	827.40	628.86	870.40	603.36	835.40
LPS-binding protein	4	8	4	P18428	LAEGFPLPLLK	2	y8	1	23.69	599.36	884.30	628.86	929.30	603.36	892.30
LPS-binding protein	4	8	5	P18428	LAEGFPLPLLK	2	y9	1	23.69	599.36	1013.40	628.86	1063.40	603.36	1021.40
Mucin-1 isoform 8 (Ca	5	9	1	P15941	EGTINVHDVETQFNQYK	2	y7	1	28.43	1011.48	928.45	1055.48	970.45	1015.48	936.45
Mucin-1 isoform 8 (Ca	5	9	2	P15941	EGTINVHDVETQFNQYK	2	y8	1	28.43	1011.48	1057.50	1055.48	1104.50	1015.48	1065.50
Mucin-1 isoform 8 (Ca	5	9	3	P15941	EGTINVHDVETQFNQYK	2	y9	1	28.43	1011.48	1156.60	1055.48	1208.60	1015.48	1164.60
Mucin-1 isoform 8 (Ca	5	9	4	P15941	EGTINVHDVETQFNQYK	2	y10	1	28.43	1011.48	1271.60	1055.48	1327.60	1015.48	1279.60
Mucin-1 isoform 8 (Ca	5	9	5	P15941	EGTINVHDVETQFNQYK	2	y11	1	28.43	1011.48	1408.70	1055.48	1470.70	1015.48	1416.70
Mucin-1 isoform 8 (Ca	5	10	1	P15941	YVPPSSTDR	2	y3	1	22.11	511.25	391.10	533.25	405.10	516.25	399.10
Mucin-1 isoform 8 (Ca	5	10	2	P15941	YVPPSSTDR	2	y4	1	22.11	511.25	478.10	533.25	495.10	516.25	486.10
Mucin-1 isoform 8 (Ca	5	10	3	P15941	YVPPSSTDR	2	y5	1	22.11	511.25	565.20	533.25	585.20	516.25	573.20
Mucin-1 isoform 8 (Ca	5	10	4	P15941	YVPPSSTDR	2	y6	1	22.11	511.25	662.20	533.25	687.20	516.25	670.20
Mucin-1 isoform 8 (Ca	5	10	5	P15941	YVPPSSTDR	2	y7	1	22.11	511.25	759.20	533.25	789.20	516.25	767.20

Commented [A116]: Would be helpful to know if data in this table is real or made up for this mock submission.

g) Assay computations carried out in the PepCa10 program

The computations proceed in the following stages:

- Input peak area values for the 150 transitions for each sample as CSV file.
- Compute two raw ratios for each of the 150 transitions.
    - A raw recovery ratio obtained by dividing the peak area of the C-SIS1-derived labeled peptide by P-SIS1 peak area (e.g., c1/s1, etc) for the given transition. This ratio compares the two different labeled versions of each peptide added at the same molar amount, one of which is added prior to sample digestion in the form of a recombinant protein, and the other added after digestion and sample workup in the form a purified synthetic peptide. The ratio expresses the recovery of a peptide through sample preparation, digestion and workup.

- A raw relative concentration ratio obtained by dividing analyte peak area by C-SIS1 peak area (e.g., p1/c1, etc). This ratio compares the amount of peptide analyte with the amount of a labeled version added, in the form of a recombinant protein, to the sample prior to sample digestion.

- Apply outlier interference detection to the raw ratios, considering in each case the 5 transitions measured for each peptide, eliminating the outlier transitions (see description below in section on interference detection). This method uses a robust linear model with high breakdown point (such as 40%) and provides a robust mean value over the 5 transitions, automatically down-weighting any outliers (transitions with interference).
  - Compute a final recovery ratio for each peptide as the robust mean of the raw recovery ratios.
  - Compute a final relative concentration ratio R1 – R10 for each peptide as the robust mean of the raw relative concentration ratios.
  - If any of the 10 recovery ratios is less than 40%, the PepCa10 test is rerun.
- Log10 transform the final relative concentration ratios for each peptide (R1 – R10). Since plasma protein concentrations span orders of magnitude, the natural logarithm is applied to the absolute concentrations to transform their distribution to one more closely resembling a Gaussian distribution and for which additivity is more applicable. Both of these properties underlie many standard statistical models including the models used to develop the formulas for the PepCa10 result.
- Compute the PepCa10 test result. Assay computations are carried out in the PepCa10 program provided with the kit. The PepCa10 test metric is obtained according to formula 1 below, which was derived using logistic regression on the estimated concentrations (log scale) of the ten target analytes measured in the samples from the training set from the proposed clinical study, and involves all 10 analytes.

$$PepCa10 = \begin{bmatrix} R1 & R2 & R3 & R4 & R5 & R6 & R7 & R8 & R9 & R10 \end{bmatrix} \bullet \begin{bmatrix} -3.3 \\ +2.7 \\ +1.2 \\ -0.8 \\ +1.1 \\ +1.1 \\ -0.9 \\ -0.9 \\ +2.1 \\ -1.6 \end{bmatrix}$$ Formula 1

A value of PepCa10 > 10 indicates a positive test result.

IV. ANALYTICAL PERFORMANCE

The analytical performance of each of the 10 component assays in the PepCa10 test is characterized individually, using data obtained when the assays are carried out together in multiplexed test format. The test analytes are thus the 10 peptides. Measured values for each analyte in each sample are log-transformed concentration ratios (R1 – R10). The PepCa10 test result is also reported.

Specific Performance Parameters

A. Precision, using methods of CLSI, EP5-A2

A precision study was performed at Study Site A with a design based on CLSI, EP5-A2. In this study, 3 samples (High, Low, Near-Cutoff control samples) were analyzed in duplicate analyses per run (total 6 samples per run), with 2 runs per day for 20 days. Duplicate sets of the Calibrators 1-6 samples were included in each run at the point samples were presented to the LC-TQMS instrument.

A total of 40 runs, comprising 80 analyses of each of the 3 samples were performed (240 total samples analyzed), with 10 analyte values reported for each (2,400 measurements). The PepCa10 result was also reported for each of the 240 analyses and its performance characterized as well. All runs were performed with single lots of test samples, calibrators and PepCa10 test reagents.

Commented [A117]: Are the results interepreted and reported as both individual analyte results and as composite result? What is the meaning of each?

Commented [A118]: Now called CLSI.

Commented [A119]: Should be real samples, assessed starting from proteolytic digest, to evaluate variability of the whole process. If individual analyte results are reported (and the assay is qualitative), each of the analytes would have to be represented in 3 different concentrations.

Commented [A120]: Cutoff determination?

Commented [A121]: How many isntruments, operators, reagent lots? In general, at least 3 of each should be evalauted.

We can discuss the number of days needed for the whole assay, depending on what other sources of variability need to be assessed. See comment below in reproducibility section.

Commented [A122]: Calculation of the number of measurements for all samples can be confusing. Precision is normally estimated for each sample (concentration) separately.

Commented [A123]: For each sample (Low, Near-cutoff and High), PepCa10 (composite score) has 80 measurements.

Commented [A124]: Were all runs performed on the same C18 column?

Commented [A125]: Precision at internal site and reproducibity studies can be discussed together.

Generally, real patient samples should be used in these studies and the values of calibrators and controls should be provided also.

			Analyte										PepCa10
			p1	p2	p3	p4	p5	p6	p7	p8	p9	p10	result
Repeatability	Std Dev	Low	0.0438	0.0330	0.0215	0.0167	0.0612	0.1249	0.0292	0.0228	0.0156	0.0591	0.3145
		Near-Cutoff	0.1081	0.1049	0.0480	0.0631	0.1115	0.2578	0.0907	0.0688	0.0660	0.0888	0.4583
		High	0.1285	0.1849	0.0684	0.0940	0.1946	0.2063	0.1464	0.1223	0.1341	0.2190	0.6777
	CV	Low	10.5%	16.7%	7.0%	7.1%	14.0%	26.8%	14.6%	11.7%	10.5%	18.4%	6.8%
		Near-Cutoff	13.0%	17.7%	6.8%	9.8%	13.4%	29.1%	14.6%	11.3%	11.5%	12.0%	4.1%
		High	10.2%	16.7%	5.7%	8.3%	14.0%	14.8%	13.5%	11.0%	12.9%	17.2%	3.6%
Within-device precision	Std Dev	Low	0.0466	0.0455	0.0270	0.0222	0.0822	0.1776	0.0435	0.0239	0.0212	0.0789	0.3608
		Near-Cutoff	0.1577	0.1399	0.0561	0.0783	0.1221	0.3291	0.1183	0.0757	0.0826	0.1174	0.4599
		High	0.1576	0.2411	0.0788	0.1145	0.2717	0.2796	0.2013	0.1533	0.1952	0.3031	0.9936
	CV	Low	12.3%	23.3%	8.9%	9.5%	15.1%	38.0%	19.5%	14.7%	13.2%	26.1%	9.4%
		Near-Cutoff	16.7%	23.4%	7.4%	14.5%	17.0%	37.9%	18.1%	13.3%	14.4%	16.6%	4.5%
		High	12.0%	18.0%	7.4%	9.9%	19.0%	18.4%	18.2%	15.5%	13.0%	24.7%	3.6%

B. Reproducibility, using methods of CLSI EP12-A

Reproducibility was measured using test results from three sites (A, B, C), each of which analyzed three samples (High, Low, Near-Cutoff control samples) in duplicate in one batch on each of 10 days (60 total samples analyzed; 600 analyte values generated). Duplicate sets of the Calibrator 1-6 samples were included in each run at the point samples were presented to the LC-TQMS instrument.

			Analyte										PepCa10
		Sample	p1	p2	p3	p4	p5	p6	p7	p8	p9	p10	result
Repeatability	Std Dev	Low	0.0438	0.0330	0.0215	0.0167	0.0612	0.1249	0.0292	0.0228	0.0156	0.0591	0.3145
		Near-Cutoff	0.1081	0.1049	0.0480	0.0631	0.1115	0.2578	0.0907	0.0688	0.0660	0.0888	0.4583
		High	0.1285	0.1849	0.0684	0.0940	0.1946	0.2063	0.1464	0.1223	0.1341	0.2190	0.6777
	CV	Low	10.5%	16.7%	7.0%	7.1%	14.0%	26.8%	14.6%	11.7%	10.5%	18.4%	6.8%
		Near-Cutoff	13.0%	17.7%	6.8%	9.8%	13.4%	29.1%	14.6%	11.3%	11.5%	12.0%	4.1%
		High	10.2%	16.7%	5.7%	8.3%	14.0%	14.8%	13.5%	11.0%	12.9%	17.2%	3.6%
Within-device precision	Std Dev	Low	0.0508	0.0400	0.0295	0.0246	0.0801	0.1633	0.0303	0.0279	0.0211	0.0841	0.4700
		Near-Cutoff	0.1146	0.1517	0.0535	0.0650	0.1651	0.3727	0.1315	0.0832	0.0864	0.1322	0.6515
		High	0.1619	0.2187	0.0843	0.1312	0.2243	0.2554	0.1486	0.1350	0.1538	0.2580	0.9641
	CV	Low	11.9%	19.0%	7.6%	7.2%	18.4%	37.5%	17.0%	15.6%	14.7%	24.1%	8.2%
		Near-Cutoff	16.1%	24.0%	8.9%	12.3%	14.5%	40.9%	17.2%	16.5%	13.2%	15.7%	5.2%
		High	12.5%	22.1%	7.1%	8.5%	19.5%	21.8%	14.2%	13.6%	16.0%	22.7%	3.8%
Reproducibility	Std Dev	Low	0.0755	0.0502	0.0371	0.0355	0.0922	0.2333	0.0387	0.0300	0.0285	0.1035	0.4750
		Near-Cutoff	0.1660	0.1898	0.0616	0.0752	0.2186	0.4420	0.1693	0.1083	0.0866	0.1705	0.8276
		High	0.1653	0.2906	0.1173	0.1941	0.2495	0.3699	0.1816	0.1516	0.1582	0.3748	1.1769
	CV	Low	14.3%	25.9%	8.7%	7.9%	21.7%	50.0%	18.3%	21.8%	17.8%	34.7%	8.3%
		Near-Cutoff	18.4%	31.9%	12.4%	15.2%	18.0%	54.0%	23.0%	17.7%	17.7%	21.5%	6.0%
		High	15.4%	32.7%	7.1%	8.5%	29.2%	31.4%	18.4%	17.7%	18.6%	31.4%	4.5%

Additional FDA comments (originally provided in the first round of the review):

- *Main issues that should be addressed in precision/reproducibility studies include identifying major sources of variability, which should be evaluated in a site-to-site study. This study should be performed at three different sites, at least two of which should represent intended users of the assay (e.g. clinical laboratories, CLIA high complexity), while one site can be internal (manufacturer site). If your assay will be a single site laboratory service, a demonstration at 3 sites may not be necessary; however, reproducibility amongst multiple users and multiple instruments may be required. Additionally, since there appears to be a protocol for sample collection and processing that is to occur at the local site of collection (prior to sending to service lab), the reproducibility of sample collection and processing may be necessary.*

Commented [A126]: We assume the PepCa result is the "composite" result?

How many isntruments, sites, operators, reagent lots? In general, at least 3 of each should be evalauted.

We can discuss the number of days needed for the whole assay, depending on what other sources of variability need to be assessed in the site-to-site vs in-house reproducibility/precision.

Please also prvide mean value/result obtained for each anayte and the PepCa result, plus SD and CV.

Please provide the way results were calculated for both precision and reproducibility. For example, what was all included in within-device precision as opposed to repeatability?

Commented [A127]: Just for reviewer orientation – are these results real or fictitious data for this mock submission?

Commented [A128]: This is the wrong document – CLSI EP12-A2 is about qualitative tests. EP5 would be more appropriate especially if this test is quantitative.

Commented [A129]: Please see comments on precision above.

- *Please clarify whether you considered different lots of reagents (plus different lots of calibrators) in your precision study. How many different instruments? (We recommend at least 3 of each.)*
- *At least three different reagent lots, different calibrator lots, and three different instruments should be evaluated. If there are multiple instruments used successively in the assay, best study design covering variability of these instruments should be discussed. It is possible that some of these precision studies can be performed in-house at the manufacturer site.*
- *Please provide more details about how frequently a calibration should be done (i.e., every day? every week? monthly?)*
- *Provide a description of the 3 study samples, including whether they were pooled samples or individual patient samples.*
- *Depending on the way test results are interpreted, we might recommend additional real patient samples be run.*
- *If duplicate sets of calibrators are included in each run in the studies, is this what would be recommended to the users in the package insert / instructions for use?*
- *Provide a demonstration of the biological intra-variability for the 10 analytes: collect [x number] samples at different time points within a day and between days [x number]. Additionally, a specific patient posture for blood collection is recommended in the submission. Sponsor should ideally demonstrate the variability that occurs when the position of the patient during blood collection is different.*
- *Provide the results from the within-run precision and between-run precision. Depending on the way results are interpreted, the study results may need to be supplied for each of the 10 peptides and the overall impact on the final result.*
- *Demonstrate the precision of the controls.*
- *Similar questions apply to both in-house precision and reproducibility study.*
- *Precision and reproducibility results may need to include the peak area measurement for each peptide or transition in addition to end results.*

C. Analytical Specificity (CLSI EP7-A, Interference)

1. Background for the approach used for interference detection

Analytical specificity is considered separately for each of the 10 component peptide assays in the PepCa10 test. Each analyte signal (measurand) obtained is the result of five specific sequence-dependent peptide analyte selection steps in the assay:

1) Cleavage at both ends by trypsin (cleaving c-terminal to lysine or arginine);
2) Affinity selection from a sample digest using a specific anti-peptide antibody;
3) Reversed-phase liquid chromatography delivering a peak at the expected retention time;

4) Mass of the intact analyte peptide (selected within a +/- 0.5 m/z window, equivalent to +/- 1 amu for a typical doubly-charged peptide ion); and
5) Mass of a specific fragment of the peptide generated by collisionally-induced dissociation (CID) inside the MS (selected within a +/- 0.5 m/z window, equivalent to +/- 0.5 amu for a typical singly-charged peptide fragment). The specificity of the combined fractionation/resolution by is extremely high.

Nevertheless, given the large number of peptides present in a plasma digest, interferences can occur that produce a detectable signal at the expected peptide mass, fragment mass and elution time of a measured transition. In order to produce a valid result from the ratio of the sample-derived analyte peptide and the stable-isotope labeled internal standard peptide, each of these two forms of the peptide must generate a valid measurement free of interference.

A stringent specificity test is incorporated into the data processing component of the PepCa10 test to detect and eliminate the effects of interferences detected in the TQMS. For each peptide, the assay method contains internal consistency checks designed to reveal the existence of an interference and where possible to eliminate it. Thus the component assays are intended to provide 'real-time' interference detection and elimination from both known and previously unknown sources.

The specificity test confirms the correct molecular structure of the analyte peptide by measuring multiple analyte transitions and assessing consistency between the transitions' intensities and the intensities observed for the same transitions in the stable isotope labeled internal standard peptide. Since a spiked stable isotope standard is known to have the correct peptide structure (its having been chemically synthesized and its structure verified by TQMS), the relative intensities of the 5 transitions observed represent the correct reference values for this sequence. If the analyte peptide detected also has the correct structure, the intensities of the same 5 transitions will have the same relative values as the spiked standard, and therefore the ratio of analyte-to-internal standard peaks areas for each transition will be the same within measurement error.

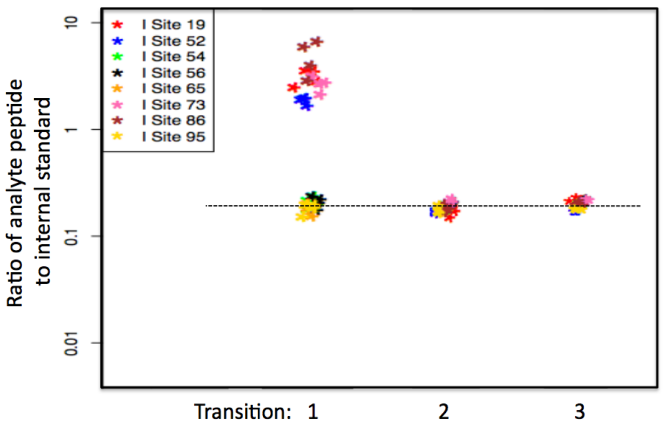
In the following example, 3 transitions were measured in quadruplicate at 8 performance sites for a sample containing 8.5fmol/ul of a peptide derived from myelin basic protein and 50fmol/ul of a stable isotope labeled internal standard peptide of the same sequence. The 3 transitions yield extremely similar values of the analyte:standard ratio, except for transition 1, for which higher than expected results were obtained at sites 19, 52, 73 and 86.

Commented [A130]: Is this interferences to getting appropriate result, or is this cross-reactivity (i.e. wrong analyte giving a "positive" result?

Commented [A131]: Can interference be a plasma protein tryptic peptide which co-elutes with the analyte peptide, that is not recognized by SRM but contributes to the LC peak area?

Commented [A132]: This seems to address "cross-reactivity," not "interferences".

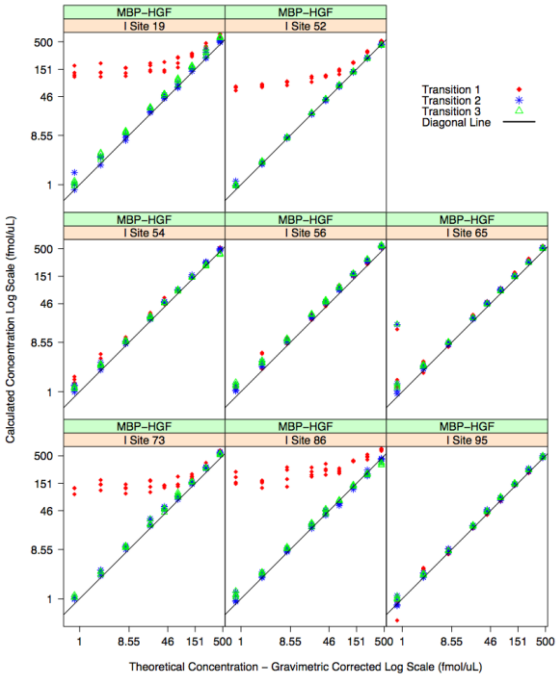
Commented [A133]: How?



CPTAC verification study: MBP-HGF peptide at 8.5fmol/ul

This consistency of ratios for most measured transitions allows reliable rejection or automatic down-weighting of outliers based on well-developed statistical techniques such as a robust linear model with high breakdown point. The results shown above for one concentration are consistent with the observations in full-dilution curve interference assessments carried out at the same 8 sites for these peptides:

Commented [A134]: What is the explanation for transition 1 being so different? What is the consequence – is it just rejected like it didn't exist?



In this example, based on results at a single analyte and standard concentration, Transition 1 would be down-weighted or excluded from the average of transitions in the results at sites 19, 52, 73 and 86, whereas at the other sites all 3 transitions would be used.

2. PepCa10 interference test method

The interference test is applied to the results of each patient PepCa10 test as part of data analysis occurring in the PepCa10 program. Each analyte peptide transition peak area is divided by the peak area of the respective transition for the stable isotope labeled internal standard to yield a ratio. The 5 ratios corresponding to the 5 transitions are log transformed.

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Each analyte peptide transition peak area is divided by the peak area of the respective transition for the stable isotope labeled internal standard to yield a ratio. The 5 ratios corresponding to the 5 transitions are log transformed.

Outlier detection is performed by estimating the mean and standard deviation with a robust linear model with high breakdown point such as 40%. The statistical model is robustness with respect to the deviations from the standard

Gaussian distribution assumption. Robust methods with high breakdown point (e.g. 40%) provide estimates of mean and standard deviation that are negligibly influenced by data with up to 40% of the points being outliers. When no outliers are present, their efficiency is close to that obtained assuming a Gaussian distribution. Multiple robust methods have high breakdown points. As examples, two methods that have shown to be useful in practice are Tukey's biweight which has a re-descending influence function, thus limiting the influence of large outliers to practically nil, and the use of the Student's *t*-distribution with low degrees of freedom commonly between 4 to 6, which also has a re-descending influence function. The use of Student's *t*-distribution has the additional advantage of allowing the implementation of Bayesian and likelihood approaches. With experience, the Bayesian approach enables prior information on the likely size of the standard deviation between transitions to be incorporated into the analysis, thus enhancing the ability of the method to down-weight outliers. The size of the standard deviation is usually dependent on the concentration of the target analyte, increasing with lower concentration, and with a sample of unknown concentration, care will have to be taken with form of the prior information. On the other hand, interference most often has a greater affect at the lower end of the concentration range and is therefore more readily detected in these concentration ranges.

There are two methods for proceeding. The first is to estimate the number of robust standard deviations each data point is from the robust mean, reject any outliers more than four standard deviations distant, and then re-estimate the mean as the value of the log-concentration with which to go forward. The second method is to use the robust mean itself as the estimate of concentration, without removal of the outliers, as the robust method automatically down-weights the influence of the outliers. Experience with the data from Addona et. al. has shown there is little difference between these two approaches. With more experience, the field will be able to make stronger recommendations as to which approach is preferable. While such experience is being accumulated, the second approach of using the robust mean has the advantage of not requiring any further computation.

The preferred approach is use of the robust mean and standard deviation, the robust mean being carried forward as the analytical result for the respective peptide to the calculation of the PepCa10 algorithm.

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3. Interference test results

In using the robust mean and standard deviation to asses the data generated in the multi-site reproducibility study, the frequency with which outliers (>= 4 SD from robust mean) occurred was tabulated for each of the 150 transitions.

Commented [A135]: If this is about the clinical study and you plan to remove some patient results as "outliers" based on statistical methods, this is not an appropriate approach. All data should be included in the clinical study data.

Are you referring to measurements for the linear combination? Do you plan to use prior information for the Bayesian approach? Please note that Pepca10 algorithm should be defined completely ("frozen") before the validation set of the clinical study.

Commented [A136]: While this is an interesting approach, how did you experimentally test that it works?

Commented [A137]: What would this mean for the test result for a particular specimen?

Commented [A138]: Intereference and/or cross-reactivity (i.e. analyticial specificity) testing might need to include e.g. some similar peptides that might cross-react and check whether their results would end up being called outliers, or similar studies. Also, whether any other substances found in blood would interefere with calling of the results.

Commented [A139]: This section does not describe an interference study. We recommend you follow CLSI EP7-A. This sentence should be moved to the section about precision. You can tabulate the frequency of statistical outliers but all data should be included in the evaluation of precision.

Target peptide sequence	Fragment ion	Analyte MS1	Analyte MS2	polySIS1 Internal Standard MS1	polySIS1 Internal Standard MS2	C-SIS1 Internal Standard MS1	C-SIS1 Internal Standard MS2	P-SIS1 Internal Standard MS1	P-SIS1 Internal Standard MS2	
YPDAVATWLNPDPSQK	y4	4	3	2	0	3	1	4	1	18
YPDAVATWLNPDPSQK	y6	1	1	0	0	3	1	0	0	6
YPDAVATWLNPDPSQK	y7	0	0	0	0	0	1	2	0	3
YPDAVATWLNPDPSQK	y10	0	0	0	0	0	0	0	0	0
YPDAVATWLNPDPSQK	y11	0	0	4	0	0	0	0	0	4
AIPVAQDLNAPSDWDSR	y15	0	1	0	3	3	0	0	0	7
AIPVAQDLNAPSDWDSR	y7	4	2	0	0	0	3	0	0	9
AIPVAQDLNAPSDWDSR	y9	0	0	0	2	1	0	0	3	6
AIPVAQDLNAPSDWDSR	y11	1	0	3	0	0	0	0	0	4
AIPVAQDLNAPSDWDSR	y13	0	0	1	0	0	0	2	0	3
EIDESLIFYK	y3	0	2	4	0	3	0	1	2	12
EIDESLIFYK	y4	0	0	0	0	4	0	0	0	4
EIDESLIFYK	y6	0	0	0	0	3	0	0	0	3
EIDESLIFYK	y7	2	0	0	0	1	0	0	0	3
EIDESLIFYK	y8	0	0	0	2	1	0	0	1	4
LLGPHVEGLK	y5	4	3	0	0	0	0	0	0	7
LLGPHVEGLK	y6	0	0	0	0	0	0	0	0	0
LLGPHVEGLK	y7	0	0	0	0	0	0	0	0	0
LLGPHVEGLK	y8	0	0	0	3	3	1	0	0	7
LLGPHVEGLK	y9	3	0	2	4	0	0	2	2	13
VLGSGAFGTVYK	y5	0	0	0	0	3	0	1	0	4
VLGSGAFGTVYK	y6	2	0	3	0	0	4	1	1	11
VLGSGAFGTVYK	y8	1	1	2	2	0	1	0	0	7
VLGSGAFGTVYK	y9	0	0	0	0	0	0	0	0	0
VLGSGAFGTVYK	y10	1	0	0	1	1	2	3	0	8
ITDFGLAR	y3	1	1	0	0	1	0	0	0	3
ITDFGLAR	y4	0	0	0	0	0	1	0	0	1
ITDFGLAR	y6	4	0	0	0	0	0	2	0	6
ITDFGLAR	y6	0	0	0	0	1	0	0	2	3
ITDFGLAR	y7	0	0	0	0	0	0	3	2	5
ITLPDFTGDLR	y5	3	1	0	0	2	0	0	1	7
ITLPDFTGDLR	y6	2	0	1	0	3	0	0	0	6
ITLPDFTGDLR	y7	1	3	0	4	0	3	3	0	14
ITLPDFTGDLR	y8	3	0	0	0	0	0	1	0	4
ITLPDFTGDLR	y10	4	0	0	0	1	0	3	0	8
LAEGFPLPLLK	b5	1	0	0	0	0	4	0	2	7
LAEGFPLPLLK	y6	0	4	0	0	1	0	0	0	5
LAEGFPLPLLK	y7	0	0	0	0	1	0	0	4	5
LAEGFPLPLLK	y8	0	0	1	1	1	1	2	0	6
LAEGFPLPLLK	y9	0	0	1	0	0	0	2	0	3
EGTINVHDVETQFNQYK	y7	0	4	0	0	1	0	0	3	8
EGTINVHDVETQFNQYK	y8	0	0	0	0	0	0	0	0	0
EGTINVHDVETQFNQYK	y9	0	0	0	0	0	1	3	2	6
EGTINVHDVETQFNQYK	y10	1	0	0	0	0	0	0	0	1
EGTINVHDVETQFNQYK	y11	0	4	4	4	0	0	0	0	12
YVPPSSTDR	y3	4	0	0	0	0	0	3	0	7
YVPPSSTDR	y4	0	0	1	2	2	0	0	0	5
YVPPSSTDR	y5	0	0	1	0	0	0	2	0	3
YVPPSSTDR	y6	0	1	3	4	0	1	4	0	13
YVPPSSTDR	y7	0	0	0	0	0	0	0	0	0
		47	31	33	32	43	25	44	26	

Additional FDA comments on analytical specificity:

Cross-Reactivity

- *Provide results from protein databases searched for known polymorphisms of peptides and demonstration that the peptide sequences are unique to the proteins of interest.*
- *Indicate whether there are any sequence differences in the population that might alter the peptide sequence or affect the trypsin digestion sites.*
- *Indicate whether the peptides are from proteins in families that share homologies, or if known isoforms of the proteins exist.*

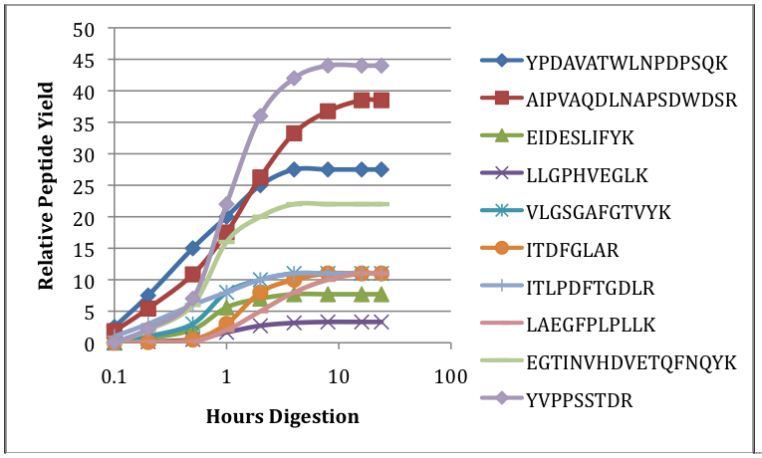
- *Indicate if the peptides have any post-translational modification sites, and impact on results.*

Interference

- *Demonstrate that common interferents, e.g., hemoglobin (as would result from grossly hemolyzed samples), bilirubin, and triglycerides, do not interfere with the assay.*
- *Demonstrate that the presence of heterophilic antibodies does not interfere with the assay (HAMA). It may be important to demonstrate that common medical substances do not interfere with the assay as well. You may need to demonstrate the effect on each peptide and on the overall effect on the final result before and after the addition of interferent.*
- *Demonstrate that cross-interference among the peptides does not occur.*
- *Since total protein can affect the trypsin digestion process, interference by excess total protein should be evaluated as well.*

D. Digestion efficiency

The reproducibility of tryptic digestion of sample proteins is an important pre-requisite for accurate PepCa10 test results. The digestion protocol and reagents provided in the PepCa10 kit have been demonstrated to yield recoveries of 80-110% for all 10 peptides specified in the test, as shown in the following timecourse study. Provided that trypsin cleavage is carried to asymptotic endpoint, reproducible amounts of each peptide, equivalent to stoichiometric yield, are produced. In the study shown, 16hr digestion yields the required stable amounts.



Commented [A140]: Data?

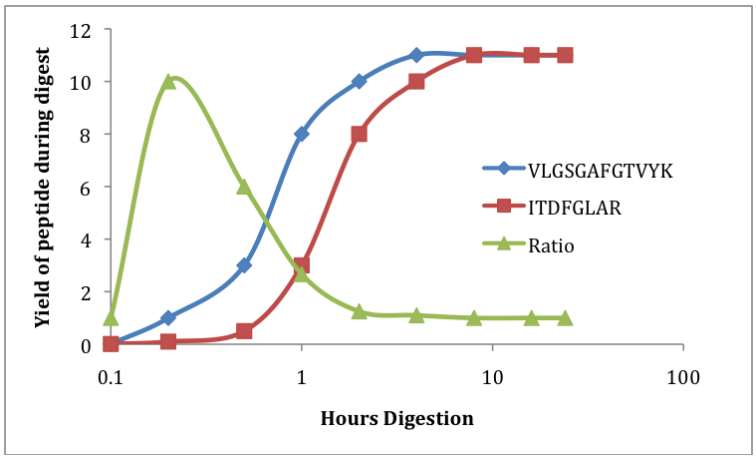
Commented [A141]: Is this what would be recoomended in the instructions for use of the test?

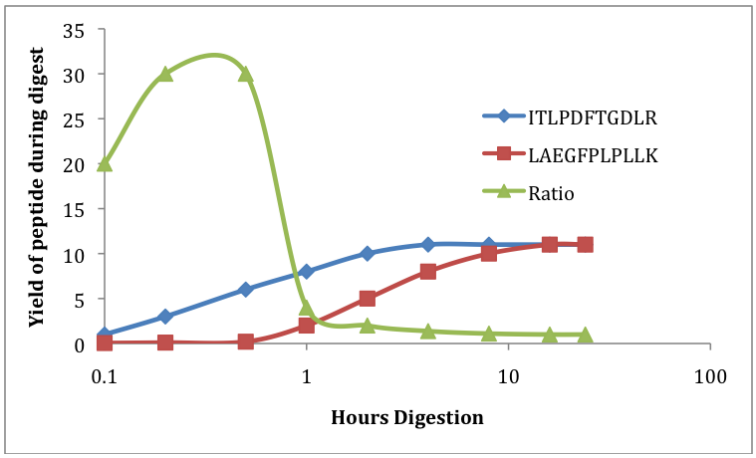
Commented [A142]: Are the ratios for analyte digestion vs standard digestion, or vs stoichiometric amount of standard added in the end?

These seem to all be relative amounts so not completely clear how the recovery can be called at 100%, even though this may appear to be an appropriate way of assessing relative digestion rates.

Commented [A143]: Relative yields seem to be far from 80-110% recovery claimed above by the sponsor.

In addition, two internal ratio tests are provided within the test to detect incomplete protein digestion. In these ratio tests, peptides p5 & p6 (contained in protein P3, which is known to be present in plasma intact, and thus containing equal amounts of peptides p5 & p6) should be produced in equal stoichiometry by tryptic digestion, and thus should appear in equal amounts relative to their respective internal standards (which are spiked at equal concentrations). Similarly peptides p7 and p8 (derived from protein P4) should be produced in equal molar amounts. However the rates of release of the two members of each pair are different: one is released earlier in the timecourse of digestion than the other, and hence the ratio between the amounts of the two peptides changes over the course of digestion, only reaching the asymptotic value of 1 late in the timecourse. Thus a value of this ratio >1 signifies incomplete digestion.





Daily average data from the precision study over 20 days was tabulated to characterize the ratios between these two pairs of 'early' and 'late' peptides. Here the values for each peptide are represented by the ratio of the analyte peak area (e.g., p1) to the recovery standard peak area (e.g., s1), thus standardizing each peptide against a constant level. The ratios of the appropriate pairs of peptides then give the relative release in digestion. Ratios for the two peptides from protein P3 ranged from 99.6% to 106.1%, while ratios for the two peptides from protein P4 ranged from 98.8% to 105.5%.

Commented [A144]: Should we assume that in all these graphs ratios are calculated this way?

	P3				P4		
	VLGSGAFGTVYK	ITDFGLAR	Ratio		ITLPDFTGDLR	LAEGFPLPLLK	Ratio
	p5/s5	p6/s6			p7/s7	p8/s8	
1	0.876595838	0.89380569	102.0%		-0.658892551	-0.6688972	101.5%
2	0.798480672	0.81681038	102.3%		-0.566256687	-0.5708946	100.8%
3	0.789118901	0.81627894	103.4%		-0.454427594	-0.4527997	99.6%
4	0.705784788	0.7144555	101.2%		-0.648627036	-0.6446963	99.4%
5	0.985096786	0.9812755	99.6%		-0.619972749	-0.6281248	101.3%
6	0.780026117	0.79854338	102.4%		-0.586421335	-0.6099159	104.0%
7	0.900371472	0.93775354	104.2%		-0.761570229	-0.7847062	103.0%
8	0.888894802	0.92961272	104.6%		-0.493730627	-0.4926888	99.8%
9	0.761812923	0.77540354	101.8%		-0.644491635	-0.6521684	101.2%
10	0.906793612	0.93020005	102.6%		-0.543886901	-0.5414754	99.6%
11	1.077472647	1.08245714	100.5%		-0.719172671	-0.7584979	105.5%
12	0.735339268	0.76795376	104.4%		-0.589400288	-0.604869	102.6%
13	0.813258498	0.84665039	104.1%		-0.703263562	-0.7226911	102.8%
14	0.783147782	0.77973775	99.6%		-0.72967658	-0.7357423	100.8%
15	1.015498003	1.01677779	100.1%		-0.500287002	-0.5030974	100.6%
16	0.614965001	0.65111934	105.9%		-0.646634616	-0.6588254	101.9%
17	0.714415249	0.72884822	102.0%		-0.680705373	-0.702876	103.3%
18	0.867908779	0.88723567	102.2%		-0.514883091	-0.5088573	98.8%
19	0.818412132	0.86804358	106.1%		-0.571273855	-0.5774281	101.1%
20	0.783866745	0.81090297	103.4%		-0.755791906	-0.7782645	103.0%
		Min	99.6%			Min	98.8%
		Average	102.5%			Average	101.4%
		Max	106.1%			Max	105.5%

E. Recovery

The PepCa10 test includes internal standards providing a measure of recovery for each of the 10 peptide analytes in every sample.

Results for the 20 day precision study, in which the daily average values of digest standard c1 are divided by the daily averages for recovery standard s1 (none of the results are log-transformed in this case) gives the percentage recovery of the peptide, since the two standard versions of each peptide are added in equal molar amounts and thereafter differ only in the effect of digestion and antibody capture on the release of the concatamer standards (c1-c10).

Recovery values ranged from 49.2% to 91.5% for the various peptides.

	YPDAVATWLNPDPSQK	ALPVAQDLNAPSDWDSR	EIDESLIFYK	LLGPHVEGLK	VLGSGAFGTVYK	ITDFGLAR	ITLPDFTGDLR	LAEGFPLPLLK	EGTINVHDVETQFNQYK	YVPPSSTDR	Average	CV
1	79.1%	78.1%	51.4%	91.1%	97.7%	52.3%	89.9%	73.0%	92.2%	83.8%	78.9%	20.4%
2	75.9%	68.2%	40.1%	82.3%	98.0%	59.5%	88.6%	62.4%	85.6%	71.3%	73.2%	22.9%
3	83.0%	78.1%	41.5%	80.1%	82.1%	51.0%	74.8%	77.1%	82.5%	75.7%	72.6%	19.7%
4	72.6%	65.9%	55.0%	84.7%	96.6%	52.7%	77.1%	69.1%	85.4%	89.0%	74.8%	19.4%
5	74.3%	77.5%	50.2%	93.8%	99.2%	60.8%	85.3%	70.5%	87.7%	76.8%	77.6%	19.1%
6	76.9%	73.2%	52.9%	89.6%	93.5%	61.8%	77.0%	66.8%	89.3%	73.5%	75.5%	17.1%
7	85.6%	76.8%	46.9%	98.6%	98.8%	55.8%	86.9%	63.7%	98.0%	84.4%	79.5%	23.2%
8	81.1%	77.9%	41.5%	91.6%	95.7%	52.4%	76.4%	62.1%	92.8%	83.5%	75.5%	23.9%
9	88.3%	75.6%	56.3%	86.8%	82.2%	69.1%	89.2%	73.8%	87.3%	84.0%	79.3%	13.4%
10	71.4%	72.4%	43.2%	86.5%	99.3%	56.7%	83.6%	70.9%	99.9%	84.1%	76.8%	23.2%
11	72.6%	63.8%	42.7%	89.6%	97.9%	66.9%	77.6%	77.4%	80.9%	72.8%	74.2%	20.2%
12	76.4%	77.7%	47.1%	82.5%	89.0%	57.3%	80.5%	76.3%	88.4%	83.7%	75.9%	17.8%
13	87.7%	79.8%	50.3%	80.0%	86.6%	54.8%	84.4%	80.0%	90.3%	87.9%	78.2%	18.0%
14	84.1%	72.2%	55.5%	93.7%	92.3%	67.6%	81.4%	60.9%	87.5%	78.2%	77.3%	16.8%
15	75.9%	65.2%	53.9%	91.5%	81.4%	50.9%	79.5%	64.0%	90.6%	76.7%	73.0%	19.3%
16	78.8%	74.5%	44.5%	88.6%	93.0%	50.5%	82.5%	74.6%	90.3%	89.1%	76.6%	21.8%
17	88.2%	79.4%	51.6%	80.2%	92.4%	69.8%	79.6%	72.9%	94.2%	80.2%	78.8%	15.7%
18	81.8%	65.0%	46.0%	86.9%	82.7%	68.0%	86.3%	75.8%	91.5%	84.7%	76.9%	17.9%
19	70.0%	62.4%	56.3%	84.2%	85.0%	66.9%	70.4%	71.7%	96.5%	71.9%	73.5%	16.1%
20	75.1%	64.1%	57.0%	92.0%	86.1%	64.1%	86.8%	71.3%	88.7%	72.3%	75.8%	16.0%
Average	78.9%	72.4%	49.2%	87.7%	91.5%	59.4%	81.9%	70.7%	90.0%	80.2%		
CV	7.3%	8.4%	11.5%	5.9%	7.1%	11.6%	6.5%	8.1%	5.3%	7.4%		

F. Carryover

A study was performed to evaluate carryover in which 10 aliquots of the HIGH control sample were processed through the entire sample workup interspersed with pairs of blank samples (20ul of phosphate buffered saline in place of plasma), for a total of 30 analyses (i.e., HIGH, blank, blank, HIGH…). The values examined are the peak areas for the peptide (not ratio'ed or log transformed). This approach tests for carryover in all sample processing steps up to and including the LC and TQMS. All 3 forms of each of the 10 peptides were evaluated for carryover (30 carryover measurements). Carryover to the first

Commented [A145]: In general we also ask for high positive specimens interspersed with high negatives.

Commented [A146]: While this is useful, the final outputs of the assay should also be examined.

Blank ranged from 2.1% to 5.2%, and averaged 3.73% over the 30 peptides. Carryover to the second Blank was generally below the peak area threshold indicating a detected peak (~1e3).

		Peptide	Average Control	First Blank	Second Blank		Average Carryover
1	YPDAVATWLNPDPSQK	p1	3.20E+04	1.49E+03	8.65E+01		4.67%
		c1	3.38E+04	1.62E+03	9.27E+01		4.80%
		s1	3.45E+04	1.60E+03	9.42E+01		4.64%
2	AIPVAQDLNAPSDWDSR	p2	5.49E+05	2.67E+04	1.62E+03		4.86%
		c2	5.52E+05	1.62E+04	1.43E+03		2.93%
		s2	6.92E+05	1.55E+04	1.94E+03		2.24%
3	EIDESLIFYK	p3	2.65E+05	1.11E+04	7.35E+02		4.21%
		c3	3.09E+05	1.51E+04	8.05E+02		4.91%
		s3	4.00E+05	1.95E+04	1.18E+03		4.86%
4	LLGPHVEGLK	p4	3.90E+06	1.45E+05	1.01E+04		3.73%
		c4	4.21E+06	1.57E+05	1.19E+04		3.72%
		s4	5.07E+06	2.02E+05	1.44E+04		3.99%
5	VLGSGAFGTVYK	p5	1.09E+06	3.99E+04	2.98E+03		3.66%
		c5	9.36E+05	4.38E+04	2.48E+03		4.68%
		s5	1.08E+06	2.56E+04	3.06E+03		2.37%
6	ITDFGLAR	p6	2.11E+06	7.11E+04	5.84E+03		3.36%
		c6	2.48E+06	1.29E+05	6.48E+03		5.18%
		s6	2.69E+06	8.12E+04	7.70E+03		3.01%
7	ITLPDFTGDLR	p7	8.49E+04	1.81E+03	2.21E+02		2.13%
		c7	7.56E+04	2.92E+03	1.89E+02		3.86%
		s7	1.11E+05	4.11E+03	3.25E+02		3.69%
8	LAEGFPLPLLK	p8	1.06E+05	2.23E+03	2.65E+02		2.11%
		c8	8.07E+04	2.03E+03	2.10E+02		2.51%
		s8	1.02E+05	3.72E+03	2.95E+02		3.66%
9	EGTINVHDVETQFNQYK	p9	4.91E+04	1.81E+03	1.43E+02		3.69%
		c9	3.67E+04	1.06E+03	9.76E+01		2.89%
		s9	4.77E+04	1.59E+03	1.27E+02		3.33%
10	YVPPSSTDR	p10	9.26E+04	4.45E+03	2.44E+02		4.81%
		c10	1.13E+05	4.93E+03	3.25E+02		4.35%
		s10	1.40E+05	4.21E+03	4.07E+02		3.00%
						Min	2.11%
						Average	3.73%
						Max	5.18%

G. Normal Range/Cut-off , using methods of CLSI C28, CLSI EP-12

Parameters used in the combination of the measured concentrations of the 10 analytes to yield the PepCa10 test result (the PepCa10 algorithm) were determined in a study comparing two groups of clinical specimens. The specimens were collected from female patients immediately prior to the performance of a breast biopsy to determine histologic malignant/benign status

Commented [A147]: How many specimens gave a wrong call because of carryover?

Commented [A148]: Carryover study details should be provided. The basic idea of the carry-over study is 1) Blank samples should be measured N times, then averages of each of 10 peptides are calculated. 2) Blank samples interspersed with High positive are measured N1 times, averages of each of 10 peptides are calculated for the blank samples. 3) Carry-over effect for each peptide is estimated as the difference between the average of the blank samples interspersed with High samples minus the average of the blank samples without presence of the high positive.

Commented [A149]: This section would benefit from revision. Please consult our previous review comments on this issue.

You should investigate whether the different reference intervals are needed for different subpopulations (for example, pre-menopausal women and post-menopausal women).

Reference intervals and the cutoff selection are separate issues to consider and should be provided in separate sections.

For reference intervals we recommend you consult CLSI C28-A3. Reference intervals should be provided for healthy women who are not part of the intended use population. Reference intervals should be provided for pre-menopausal and post-menopausal women separately. In general, at least 120 subjects should be used for evaluation of the reference intervals. 2.5th and 97.5 th percentiles should be calculated.

Pepca10 values for the women with other cancers can be also provided.

occasioned by a positive mammogram. Collection of these specimens from a single cohort of women before malignant/benign status was known serves to eliminate potential biases that could affect the test results. After collection and storage of samples, two sets were selected based on malignant/benign status that were also matched with respect to age, hormonal status, race, smoking and BMI.

i. Group A consisted of EDTA plasma samples obtained from 100 patients whose breast lesions were determined to be malignant
ii. Group B consisted of EDTA plasma samples obtained from 100 patients whose breast lesions were determined to be benign

Additional FDA comments on Reference Interval/Cutoff

- *Please note that the reference interval is related to the healthy subjects who do not belong to the intended use population. Therefore, it is not obvious why the reference interval should be used for establishing the cutoff. Please note that the cutoff of PepCa10 is probably trying to distinguish benign vs malignant (not healthy vs malignant).*
- *It appears that you plan to use an ROC curve for determining the cutoff. Please note that an approach of using the empirical ROC curve of the pivotal study for determining an optimal cutoff can produce biased estimation of performance. One can consider two approaches which can give unbiased estimation of performance: 1) the cutoff was established using a pilot study; 2) the clinically acceptable level of, for example, sensitivity is pre-specified; then the cutoff is selected in the pivotal study using only the diseased subjects as a corresponding percentile. For details see, Kondratovich, Marina V. and Yousef, Waleed A. (2005) Evaluation of Accuracy and Optimal Cutoff of Diagnostic Devices in the Same Study. Proceedings of the 2005 Joint Statistical Meeting, Biopharmaceutical Section, p.2547-2551.*

Reference Interval

- *We recommend that you follow CLSI document C28-A3 for the study design and calculation of the reference interval.*
- *You should investigate whether different reference intervals are needed for different subpopulations (for example, pre-menopausal women and post-menopausal women).*
- *For establishing a reference interval, at least 120 subjects are recommended (see CLSI C28-A3).*
- *Please use non-parametric procedures for the estimation of 2.5th and 97.5th percentiles.*

Approach remains under development; not complete at time of mock submission

Commented [A150]: This is a crucial part of the test. It would be very useful if at least the approach contemplated was described.

H. Analytical sensitivity (CLSI I/LA21-P).

- Limit of blank: usually the mean of the blank determinations ± 2-3 standard deviations
- Limit of detection: lowest level of analyte detected ≥ 95% of the time
- Analytical sensitivity: intercept of calibration curve when the concentration = 0
- Functional sensitivity: lowest analyte concentration where the %coefficient of variation (CV) is acceptable, typically %CV < 20%.

The advantage of method #4 is that it does not rely on the distribution of the test results in blank samples. The issue with blank samples when the target analyte is in a complex mixture is the definition of an appropriate blank matrix. Ideally, a blank should be the same matrix as used for measuring concentration of the target analytes, in this case plasma, except for removal of all targets. However, this is very difficult if not impossible to achieve. If instead a simple matrix such as PBS (phosphate buffered saline) is used for the blank, the variation in the measurements of the target analytes is likely to be much less than what would be observed in an ideal blank sample. A possible compromise is the use of a complex matrix similar to human plasma that does not have the target analytes nor their proteotypic peptides. Mammalian plasma such as bovine, equine, or other, may satisfy this requirement, and might be a suitable surrogate blank.

However, since method #4 does not require results in "blank" samples, it may be the preferred approach to assessing analytical sensitivity for analytic targets in a complex matrix.

Approach remains under development; not complete at time of mock submission

I. Accuracy NA

J. Matrix comparison. NA

K. Other studies

1. Linearity over the reportable range (refer to CLSI EP-6P, H20-A and H26-A)

Approach remains under development; not complete at time of mock submission

Commented [A151]: For terminology, we recommend you consult CLSI EP17-A.

Commented [A152]: CLSI EP17

Commented [A153]: LoB, LoD and LoQ should be estimated for each peptide.

Commented [A154]: This is correct only if the distribution is normal. In a general case, the 95th percentile should be evaluated – we recommend that you consult CLSI EP17 for details on how to do this.

Commented [A155]: We recommend that you use the CLSI definition.

Commented [A156]: How is this acceptability determined? It should be related to some type of relevant clinical measure.

Commented [A157]: Neither does method 2.

Commented [A158]: In general, we recommend the approach from CLSI EP17 – which is #2.

Either way, there is no analytical sensitivity/LoD study provided in this submission.

Commented [A159]: All questions about performance at low analyte levels are important for the calculation of the linear combination of 10 analytes. How are the low values used in the calculation of the linear combination?

Commented [A160]: No linearity study submitted. We recommend that you follow CLSI EP6-A for design and statistical analysis of a linearity study.

V. METHOD COMPARISON. NA

The PepCa10 test is a multiplex test employing some novel analytes and a novel methodology. Hence there is no predicate device or gold standard.

Commented [A161]: While this is correct, there might be a need for some type of analytical comparator for proteins in the assay.

A. Predicate Device. Not applicable.

B. Reference method (Gold Standard comparisons). Not applicable.

C. Literature Comparison. Not applicable

VI. Study design

The criteria for an FDA OIVD is that it has to be safe and effective. We assume that a plasma biomarker test requiring a venipuncture is regarded as safe.

Then there is the crucial issue of interpretation of "clinical effectiveness". Two interpretations of "clinical effectiveness" are possible and guidance from the FDA as to which is most appropriate interpretation for this intended use would be very helpful:

1. The test is effective if it achieves an objective measure such as a pre-specified level of sensitivity at a given level of specificity.
2. The test is effective if it significantly improves on the medical judgment made without the use of the test. (As indicated by the FDA in discussions related to another 510k application for a differential diagnostic test)

We propose a study to determine the effectiveness of a test for differential diagnosis of breast lesions with BI-RADS 4 results using the first criterion, and then discuss the requirements for the second criterion.

First Criterion

The study will be conducted by enrolling patients following mammography for breast lesions with a BI-RADS 4 result at multiple sites, including the four 'CPTAC' sites:

1. Fred Hutchinson Cancer Research Center – via UW SSCA
2. Vanderbilt University through the Breast Clinic
3. UC San Francisco
4. Memorial Sloan Kettering Cancer Center

The accrual goal at each site is 500 patients with a BI-RADS 4 breast lesion, for a total of 2,000 patients satisfying the inclusion/exclusion criteria listed below. This sample size will provide the following expected number of patients with conditions as determined by subsequent breast biopsy diagnosis:

Commented [A162]: You should address the potential problem of verification bias.
Your inclusion criterion is "women undergoing biopsy procedure". Note that the intended use population for the PepCa10 test is women with BI-RADS 4 and the PepCa10 test will be applied to all women with BI-RADS 4, not only to the women with biopsy results. Women with BI-RADS 4 and biopsy results may be a non-representative sample of all women with BI-RADS 4; the women who were referred to a biopsy procedure are likely to have a higher risk of malignancy.

Please provide a list of covariates that will be collected in the clinical study. In order to address the potential problem of verification bias, all important covariates should be collected.

1. 25% with breast cancer (500) (Cases)
2. 75% with benign disease (1500) (Controls)

Of the patients with breast cancer, half (250) are expected to have ductal carcinoma in situ (DCIS) and half (250) are expected to have invasive breast cancer. Within each of these three groups, patients will be randomly split between a training set and a validation set. PepCa10 will be developed and optimized on the training set. It is important to note that the cut-off for PepCa10 is developed by applying the test to women in the intended use population (BI-RAD 4) and **not** in a population of *healthy women.* When the final algorithm and cut-points have been "written in stone" the test will be applied in a blinded manner to the validation set. The proportion of patients in the training set will be set at 50%.

Commented [A163]: We recommend you use the terminology of the STARD initiative (target condition present /target condition absent).

Commented [A164]: This is one way of doing the study. However, any bias in a particular center would be randomized accross both sets, and results might be different when introducing the test in clinical use at another center. Perhaps less bias would be introduced if data from 1-2 centers were used for training, and another 2 or 3 centers for validation.

Commented [A165]: Again, training set should be data from one or more sites and the validation set should be data from other independant sites.
If the clinical studies at the training data sites and at the validation data sites are going in parallel, we should discuss how to assure that the data from the validation set is not available during the development of the linear combination.

Sample Size

To determine a realistic combination of sensitivity and specificity with a plasma biomarker test, the separation between cases and controls observed in another plasma biomarker test is used for guidance. The measure of separation is the number of standard deviations the tests results are apart between cases and controls, and is termed the *effect size.* This measure is *only* being used to determine a clinically realistic difference that might be expected for plasma PepCa10 results between the breast cancer patients and patients with benign breast disease. From this measure of separation between the two distributions, the sensitivity can be set, and the expected performance of specificity can be estimated. The effect size is used for no other purpose.

We expect differentiating between a benign and malignant breast lesion with a plasma marker to be more difficult than using CA125 to differentiate between a benign and malignant pelvic mass. However, with 10 biomarkers together forming a combined marker, the effect size to differentiate breast lesions may achieve similar separation as CA125 does between benign and malignant pelvic masses. This analogy is purely for illustration of a realistic effect size for oncology diagnostics based on a test in current clinical use. In an actual breast cancer diagnostic study, there would be pilot data to estimate effect size. Here we proceed assuming the effect size is the same as for CA125 in differentiating benign from malignant disease. In patients with ovarian cancer, CA125 has a median value of 120 U/mL with a between patient CV of 50%, while in patients with a benign pelvic mass, CA125 has a median value of 20 with a between patient CV of 50%. On the natural logarithm scale, the effect size or the number of standard deviations by which the two means differ, is log (120/20)/$\sqrt{(0.50^2 + 0.50^2)}$ = 2.53. Since pilot data are absent in this hypothetical example, we proceed assuming the effect size of PepCa10 is the same size as for CA125 in differentiating malignant from benign pelvic masses Then PepCa10 could achieve the following operating characteristics.

Commented [A166]: Please note that what you called "effect size" is related to an area under the ROC curve. Area under the ROC curve is sensitivity averaged over all specificities; therefore, when your test is supposed to be used with a particular cutoff, area under the ROC curve is not an appropriate measure of the test performance.

Commented [A167]: It is our undrestanding there is no basis to expect this test would have the same size effect and this in only used for illustration of a realistic effect size before pilot data become available (at which time pilot data will be used to estimate effect size or other parameters, in place of this assumption).

The intended use of the PepCa10 test is to differentiate patients with BI-RAD 4 results into two groups, namely patients with a low probability of having cancer for whom the physician may recommend waiting a few months for subsequent testing, and thus avoid the morbidity associated with a biopsy, and all other patients for whom a biopsy would be recommended as currently occurs under standard of care. The definition of low probability for having cancer is for this group of patients to have the same probability as patients with a BI-RAD 3 result, namely 2% or lower, since BI-RAD 3 patients are usually recommended not to have a biopsy and wait a few months for further tests.

Commented [A168]: This is one way to look at it.

A probability for malignancy of 2% for women with a negative test means the negative predictive value is 98% (in other words, among 100 women with negative test results, two women have malignancies). With a prevalence of 25% of patients with a BI-RAD 4 result with malignancy (π=25%),, 75% of patients will have benign breast disease (1-π=75%). If PepCa10 has a specificity of 50%, half of the patients with benign breast disease will avoid having an unnecessary biopsy. This goal would seem to be clinically significant and therefore the sensitivity required to achieve it is now calculated. The sensitivity required to achieve an NPV of 98% with a specificity of 50% and prevalence of 25% is 96.9% (for Se=96.9%, Sp=50%, π=25%; the NPV is 98.0% and PPV is 39.2%; percent of subjects with negative test results is 38.3%).

With an effect size of 2.53 as hypothesized above, a plot of the NPV versus specificity is given in the Figure below, showing that an NPV exceeding 98% is achieved for all specificities of 80% or less. The clinical benefit will mainly be on the patients with benign disease for whom a biopsy is not recommended based on the test results, so that they will not have to undergo the morbidity of an unnecessary biopsy.

As a specific example, if the specificity is 75% (the proportion of benign patients for whom a unnecessary biopsy will be avoided is 75%), the sensitivity is 97% (the proportion of malignant patients for whom necessary biopsy will be missed is 3%), and the prevalence is 25% (pre-test probability of malignancy is 25%); then NPV is 98.7%, PPV is 56.4%, and percent of patients with negative test results is 57.0%. It means that

i) 57% of women with BIRAD of 4 can avoid a biopsy because of the negative test results; among them, probability of malignancy is 1.3% (1-NPV);
ii) 43% of women with BIRAD of 4 will be recommended for biopsy because of the positive test results; among them, probability of malignancy is 56.4% (pre-test probability of malignancy was 25%).

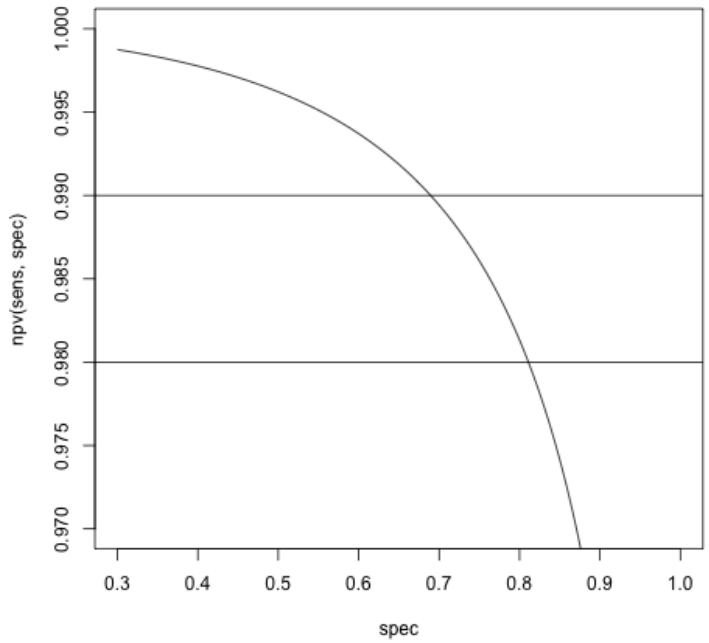


Figure: Negative predictive value of PepCa10 if the mean plasma test in patients with the target condition is 2.53 standard deviations above mean plasma test in patients without the target condition (on logarithmic scale) and the prevalence of the target condition is 25%.

To achieve an estimate of the sensitivity and specificity with small uncertainty, narrow confidence intervals are required. With a sample size of n=144 cancer cases and n=288 patients with benign disease in the validation set, the 95% confidence interval width is expected to be ±5% assuming a specificity of 75% and for sensitivities exceeding 90%. The 95% confidence intervals for NPV of 98% (96.4%, 99.4%), for an NPV of 99% it is (97.8%, 99.9%), and for an NPV of 99.5% it is (98.4%, 99.95%).

Commented [A169]: What level of sensitivity and specifcity did you consider in these calculations? Did you use exact confidence intervals for the negative likelihood ratios when you calculated the 95% CI for the NPV?

To determine whether PepCa10 provides a contribution beyond available clinical information, we will include the PepCa10 test results (continuous) in a logistic regression and assess whether the PepCa10 coefficient is significantly different from zero (which would imply no additional contribution). The clinical information would include age, menopausal status, family history, and other known risk factors for breast cancer.

Commented [A170]: Provide a list of these risks factors and how they will be be included in the logistic regression analysis.

Second Criterion for Effectiveness

To address the second criterion, we will collect the judgment of the physician (usually radiologist) following mammography and identification of the lesion as to whether the lesion is benign or malignant. The medical action will be altered if the test indicates a difference from the physician's judgment, and will be clinically relevant if 5% or more of patients have a better judgment with the test, where biopsy results will be considered the "gold standard". The validation sample will have an equal number of cancer cases and benign lesions. The test will be effective if the 95% confidence interval of the improved proportion rules out 1% or less. The confidence interval for a proportion is derived from the standard error for a binomial proportion that is $\sqrt{(p(1-p)/n)}$. With a total sample size of n=232, or 116 cases and 116 controls, and (net?) 5% of the patients have an improved judgment by the test, then we have 80% power to rule out 1% improvement or less at 95% two-sided confidence.

Commented [A171]: The "clinical effectiveness" should be demonstrated. This section seem to be a continuation of "clinical effectiveness", dealing with the available clinical information for a patient with BI-RADS 4 and available PepCa10 result.
See FDA comments (below) for a continuation of this comment.

Commented [A172]: By a pathologist reading biopsy results?

Commented [A173]: Please clarify how you propose to use PepCa10 test in a real life setting, considering a radiologist would normally read a mammogram and may provide results according to BI-RADS assesment categories.

See FDA comments (below) for a continuation of this comment.

Commented [A174]: The design of this study is not clear; therefore, it is difficult to comment on its value.

Continuation of comments regarding "Second Criterion for Effectiveness"

- *The "clinical effectiveness" should be demonstrated. This section seem to be a continuation of "clinical effectiveness", dealing with the available clinical information for a patient with BI-RADS 4 and available PepCa10 result.*

  For example, consider a hypothetical case where "additional clinical information" such as whether a woman is pre-menopausal, no family history, comparison to previous mammogram, no risk factors, etc (we would need to consult expert physicians/ radiologists on what information they use and how they actually account for it). Then all patients with BI-RADS of 4 may be divided into 4 groups: clinical information positive, PepCa10 positive; clinical information positive, PepCa10 negative; clinical information negative, PepCa10 positive and clinical information negative, PepCa10 negative. You would need to investigate the percent of referral to biopsy in each group separately.

- *Please clarify how you propose to use PepCa10 test in a real life setting, considering a radiologist would normally read a mammogram and may provide results according to BI-RADS assessment categories*

  In the hypothetical case that this type of test were available, and if the BI-RADS results are 4, when making a decision about referral to biopsy the physician would be able to decide whether to take this test into account. Depending on how the clinical study and intended use population is defined, the decision may not necessarily be about "malignant/benign" but about "refer to biopsy/not to refer to biopsy."

A. Patient samples or specimens

1. Inclusion Criteria

Women with no prior cancer history who are undergoing image-guided breast biopsy for lesions of unknown diagnosis in the breast imaging clinics at UW and SCCA, the outpatient clinical site for the FHCRC/UW Cancer Consortium, and the other CPTAC sites, where all patients have a BI-RAD 4 result.

Approximately 800 image-guided core needle biopsy procedures are performed annually in the breast imaging clinics at the University of Washington (UW) and Seattle Cancer Care Alliance (SCCA) (ca. 450 US-guided procedures, 250 stereotactic (mammography)-guided procedures, 100 MR-guided procedures). It is anticipated that at least 50% of these patients will meet criteria for study inclusion. Thus, we expect to have the opportunity to approach approximately 1000 patients and enroll approximately 500 of those individuals during the 2–2.5 years of clinical sample collection. The expected distribution of diagnoses in those 500 study participants is approximately 375 with benign breast disease and 125 with cancer. Of the 125 with cancer, it expected that 50% (approximately 62) would have invasive disease.

2. Exclusion Criteria

- Known (biopsy-proven) current breast cancer
- Any other *in situ* or invasive cancer
- Prior chemo, radiation or hormonal (e.g., tamoxifen, AIs) therapy; HRT OK
- Current pregnancy
- Blood transfusion within the last 6 mos.
- Those not competent to provide informed consent

3. Constraints on Eligibility

- Patients must have an undiagnosed breast lesion prior to blood draw
- Blood must be drawn prior to breast biopsy diagnosis
- Patients must sit quietly for five minutes prior to draw
- Blood must be processed, aliquoted, labeled and stored within <u>75 min.</u> of collection

NOTE: A prior benign breast biopsy is <u>not</u> an exclusion criterion.

4. Specimen collection

Specimen collection will be done as a collaborative effort between the UW/SCCA Breast Imaging group and the FHCRC/UW Breast Specimen Repository and Registry (BSRR), and will be performed similarly in the other CPTAC sites. Patients will be identified, approached and consented to the BSRR by a Clinical Research Coordinator or Nurse (RC). At the time of blood draw, the RC will page the BSRR Tissue Collection Specialist who will retrieve, process, aliquot and store the blood samples and enter specimen-related

information into the BSRR database. Prospective specimen and data collection protocols are well established at both sites.

Additional FDA comments on specimen:
You have provided specific instructions for blood collection tube handling and specimen processing. You will need to demonstrate the stability of the specimens across the extremes of these parameters (e.g., temperature, time to freezing, freeze-thaw, and shipping).
Sample amount: You have not provided information on how the blood sample volume was determined. What happens if the user obtains a short-draw sample?

Shipment study
Samples are processed at the collection site but shipped to the test site. You will need to perform a shipment study to validate the recommended shipping conditions and also test extreme shipping conditions. The number of days between collection and testing was not evaluated.

Stability data: Stability data should include different peptide analyte concentrations.

Approach remains under development; not complete at time of mock submission

To be developed:

Additional FDA comments:

The description of the proposed studies lacks sufficient detail to determine how the studies will support the proposed intended use. The following are some general comments:

- *Sponsor should be prepared to provide information related to the patient (in addition to age, menopausal status, smoking habits and BMI) co-existing or previous medical conditions, mammography method (e.g., digital, x-ray, w/ or w/o CAD), size of the lump (if there is one). How will other variables be accounted for in the enrollment to avoid bias?*
- *Please provide more information about how patient samples will be chosen for either the training set or validation set.*
- *Indicate whether sub-analyses based on test performance by stage is intended.*

Test reporting and test score

Minimal to no information on the test score and interpretation is provided in the current draft. Also, a draft patient test report would need to be supplied in the real submission.

- *Depending on how the validation studies were performed, sponsor may need to provide a description of the algorithm, and indicate how the selection of transitions was optimized to account for potential background signal, interference, biological effects, beyond the signal intensity of each transition of the peptide.*
- *Currently, the the submission is very focused on the individual components of the score/classifier; if test score is ultimately used we may need some information on rationale for the choice of 10 protein analytes, as well as what is the classifier.*
- *Information provided about the training and proposed studies for evaluation of the classifier is minimal. More information on cutoff determination and evaluation studies would be required.*

Expected Values in benign and malignant conditions

The target population may have a wide variety of conditions unrelated to cancer but present at the time a breast mass has been identified. These other conditions could dramatically affect the analytes in question (especially considering that you plan to test plasma specimens) and confound interpretation of results. Please demonstrate the results of your assay results in patients with other benign and malignant conditions that may be occurring concurrently. Include ovarian cancer, cervical cancer, GI cancers and disorders, lung, leukemia/lymphoma, liver, renal, endometriosis, diabetes, cardiac disorders, autoimmune disorders such as SLE, rheumatoid arthritis, infection, and anemia of chronic disease.

Software and Risk Hazard Analysis: *Software information and a risk hazard analysis for the assay should be submitted.*

FDA comments related to software/instrument:

- *Your submission implies the use of specified components (instruments) in the system, although it has not been specified whether all components or just some of the components be provided to the end user. Even if you do not market all components it appears likely that you will recommend them as validated for use with your assay, therefore evaluation of all will be required as a part of the review. Alternatively, you could make generic recommendations if there are similar components out there for use, but it appears the only such component might be the centrifuge.*
- *Once when the issues above are at a more defined stage for your system/assay, we can provide more specific regulatory requirements needed to support the test system's claims. Overall, you would need to ensure that all components of the test system (other than perhaps the centrifuge) are controlled under FDA's Quality System, which includes the need for design and purchasing controls for the components of the system. Regarding the submitted material, the recommended software*

documentation is summarized below and should be documented at a moderate level of concern. Software validation requirements can be found in the "Guidance for the Content of Premarket Submissions for Software Contained in Medical Devices" (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm089543.htm) and the "Guidance for Industry, FDA Reviewers and Compliance on Off-The-Shelf Software Use in Medical Devices" (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm073778.htm).

- *The centrifuge, magnetic bead processor, liquid chromatograph may not require software documentation to be submitted because the failure of these systems should cause a failure to obtain a result, and may or may not be considered off-the-shelf software (consult guidances listed above for requirements), however this will need further clarification and follow-up discussion. The NanoGradient software may be considered high risk and be subject to audit under OTSS. You also should ensure this software is registered and listed, and understand that it subject to inspection and FDA's Quality System requirements. Regarding the centrifuge, listing any generic centrifuge may be appropriate, but the required centrifugal force, timing and temperature should be validated.*
- *The triple-quadrupole mass spectrometer and the PepCa10 Program are key components of the test system in which a failure could produce incorrect results. These components should have complete software documentation submitted based on the level of concern for these devices (as described in premarket software guidance above).*
- *You also state the following in the submission for the mass spec: "No suitable instruments are currently produced under GMP… Many of these applications are governed by GLP- or GMP-like regulations, since they generate basic data required for FDA approval of drugs, and in some cases, in vitro diagnostic test results in reference laboratories. Given that there are an estimated 10,000 TQMS instruments currently deployed to generate accurate quantitation of molecular analytes, with a substantial fraction of these generating data used in FDA regulatory submissions, there is a strong argument that the technology currently functions at a level adequate to ensure reliable results." If this device is to be used for a regulated assay, then the instrument would need to be produced under FDA's Quality System requirements, even though some GMP-like components appear to have been met. Although there is some indication that this device may currently function at a level adequate to ensure reliable results, this claim needs to be tested and validated in terms of the intended use of the assay. The fact that there are numerous instruments in use for research does not negate the need for adequate validation.*

TQMS Device and all associated software

- *You state a TQMS model XYZ device manufactured by TQMS Co. is used to provide quantitative measurement of analyte and internal standard*

peptides. The instrument is manufactured under an ISO 13485 quality system, which is similar but not identical to FDA's Quality System regulation. The device used should be produced in compliance with the Quality System regulation.