Molecular Serology Data Interpretation		
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Abbreviations

CPA - control's peak area
CV – coefficient of variation
CytoC – bovine cytochrome c standard peptide
Analysis)-Script FIS - fragment ion ratio score
RTCV - retention time coefficient of
variation SPA - sample's peak area
SPA-CPA – sample fold change
XIC - extracted ion chromatograms
MSDA (Molecular Serology Data

1 Result Calling Criteria

1.1 The Molecular Serology Body Fluid assay is a classification assay that determines whether a body fluid is present in or absent from a sample. It is not a quantitative assay and does not determine how much body fluid is present.

1.2 There are three parameters that are used to discriminate the quality of the data coming from the mass spectrometer in order to ascertain whether a sample (or item) is positive or negative for a specific body fluid: the intensities of ions of interest (peak areas), the ratio of the peak areas of fragment ions that come from the same parent ion, (fragment ion ratio scores), and the time at which targeted ions are detected after the beginning of a mass spectrometry run (retention time concordance).

1.2.1 **Peak Area** - Peak area is the area under the curve of extracted ion chromatograms (**XIC**) for fragment ions of a peptide of interest. Peak area is log2 transformed.

1.2.2 **Fragment Ion Score** - The intensity (peak areas) of marker peptide fragments ions is compared to generate a fragment ion ratio score. The ratio of a peptide's fragment ions to each other is consistent and stable. How well the fragment ion ratios match their expected ratio pattern is assessed by calculating the normalized contrast angle of scaled fragment ion peak areas to those fragments from synthetically made standards.

1.2.3 **Retention Time Concordance –** A marker peptide elutes from the HPLC column as a single molecule, which is fragmented in the mass spectrometer. Thus, the retention times recorded for each of the four fragment ions measured per peptide should be close. Larger than expected variation in fragment ion retention time may be evidence of an interference signal not originating from the target peptide. Retention time concordance is measured by calculating the coefficient of variation (**CV**) of recorded retention times of fragment ions for a peptide

1.3 The values for peptide peak area, FIS, and retention time CV are compared to those in the tables below.

1.3.1 To account for possible HPLC carryover from a previous sample, blank runs are performed prior to each sample run. A peptide fragment ion score (FIS) of 0.6 or greater in a blank run indicates the presence of carry-over. The low score threshold for carry-over detection in the blank runs allows for conservative detection of carry-over and more accurate identification of peaks attributable to carry-over verses true peptides present in the subsequent sample run.

- IF no carryover is detected, a sample peptide's Peak Area and Fragment Ion Score (FIS) values must be greater than the Peak Area and FIS values in table below, and Retention Time CV (RTCV) below the threshold values in the tables below for that peptide to pass (Detected).
- IF carryover is detected prior to a sample's run, the Sample's Peak Area (**SPA**) must be one or more times greater than the Control's Peak Area (**CPA**), in addition to the above criteria for Peak Area, FIS, and RTCV, for that peptide to pass (Detected). This fold change (**SPA-CPA**) is given in Column 6 in the tables below.

1.3.1.1 Cytochrome C Standard

1	2	3	4	5	6
Protein	Peptide	FIS	RTCV	Peak Area (log2)	SPA-CPA
Cyto C	EDLIAYLK	0.797	0.0042	12.80	1.00
	TGPNLHGLFGR	0.830	0.0091	11.62	1.00
	TGQAPGFSYTDANK	0.782	0.049	11.68	1.00

1.3.1.2 Blood

1	2	3	4	5	6
Protein	Peptide	FIS	RTCV	Peak Area (log2)	SPA-CPA
B3AT	ADFLEQPVLGFVR	0.713	0.0045	15.06	3.00
	ASTPGAAAQIQEVK	0.869	0.0167	13.80	2.83
	IPPDSEATLVLVGR	0.734	0.0242	15.23	3.30
HBB	LLVVYPWTQR	0.670	0.0044	23.80	2.75
	SAVTALWGK	0.670	0.0045	23.40	3.00
	VNVDEVGGEALGR	0.688	0.0045	22.83	3.03
HBA	FLASVSTVLTSK	0.737	0.0045	17.00	2.74
	TYFPHFDLSHGSAQVK	0.814	0.0045	19.55	3.31
	VGAHAGEYGAEALER	0.688	0.0045	23.55	3.00

1.3.1.3 Saliva

1	2	3	4	5	6
Protein	Peptide	FIS	RTCV	Peak Area (log2)	SPA-CPA
AMY1	ALVFVDNHDNQR	0.708	0.0045	18.31	3.03
	IYVSDDGK	0.726	0.0131	15.90	2.77
	LSGLLDLALGK	0.736	0.0045	14.04	1.62
CYTT	ALHFVISEYNK	0.819	0.0073	13.60	2.72
	ATEDEYYR	0.708	0.0048	13.15	3.06
	SQPNLDTCAFHEQPELQK	0.694	0.0094	15.71	2.78
HIS1	EFPFYGDYGSNYLYDN	0.734	0.0121	11.24	1.32
LEG1H	ESPGQLSDYR	0.754	0.0149	11.15	1.83

1.3.1.4 Semen

1	2	3	4	5	6
Protein	Peptide	FIS	RTCV	Peak Area (log2)	SPA-CPA
SEMG1	EQDLLSHEQK	0.649	0.0139	11.16	1.55
	HLGGSQQLLHNK	0.832	0.0075	11.19	1.91
	SQIQAPNPK	0.714	0.0049	14.22	3.00
KLK3	IVGGWECEK	0.731	0.0097	15.85	1.85
	LSEPAELTDAVK	0.702	0.0045	16.95	2.65
	SVILLGR	0.700	0.0045	16.32	2.78
SEMG2	GQLPSGSSQFPHGQK	0.621	0.0188	12.37	1.38
	GSISIQTEEK	0.736	0.0045	14.78	2.72
	LWVHGLSK	0.702	0.0046	15.44	1.00

1.3.2 A negative result (**NOT DETECTED** for all three body fluids, see 1.3.4 below) is only valid if at least one CytoC standard peptide is detected (pass).

1.3.2.1 If no CytoC standard peptides are detected in a negative sample, sample failed to inject or a system error occurred, and sample must be re-injected.

1.3.3 Detection (passing) of ALL 9 blood target peptides from ALL 3 blood protein markers are required for a sample to be **DETECTED** for blood. Detection of at least 7 out of 9 semen peptide markers, including ALL 3 target peptides for KLK3 and 2 of 3 peptides markers for each SEMG1 and SEMG2 are required for a sample to be **DETECTED** for semen. Detection of at least 7 of 8 saliva peptides markers, including ALL 6 AMY1 and CYTT peptides and EITHER 1 LEG1 OR 1 HIS1 peptide, are required for a sample to be **DETECTED** for saliva.

1.3.3.1 If one peptide is missing from the required number of peptides for the detection of a body fluid, the missing peptide may be the result of a polymorphism in that peptide and may not indicate that the body fluid is not detected. Under these circumstances, an analyst may consult with the Technical Lead to make a final decision.

1.3.4 If three or less peptides of the target peptides as described above are detected (i.e., criteria stated above are not met for the specific peptide), that body fluid is **NOT DETECTED**.

1.3.4.1 If four or more peptides but LESS THAN a full marker peptide profile (as defined in 1.3.3) are detected, that sample or item is **INCONCLUSIVE** for that body fluid. In addition, an **INCONCLUSIVE** result for saliva requires the presence of at least one AMY1 peptide. If no AMY1 peptides are present, the sample is **NOT DETECTED** for saliva.

1.3.4.2 An inconclusive sample may be re-injected.

1.4 Negative controls will pass if they display three or fewer peptides. All samples within a batch will be re-injected if the Negative Control shows presence of four or more peptides. If on reinjection the Negative Control still shows four or more peptides, the batch fails, and new extractions must be made.

1.5 All positive controls must be detected, see 1.3.3 for detection criteria.

2 Viewing Marker Peptide Spectrum on Skyline

2.1 Peptide data that meet the requirements for peptide detection described in (1.3.1.1 – 1.3.1.4) cannot be reinterpreted by the analyst.

2.2 If peptide data does not meet the requirements for peptide detection described in (1.3.1.1 – 1.3.1.4) the peak selection in Skyline may be edited by the analyst as described below (2.6 and 2.7) and the new selection analyzed according to the peptide detection criteria described in (1.3.1.1 – 1.3.1.4).

2.3 Open the Skyline document for your batch. Each Skyline document has several files of the same name associated with it, to open the document double click on the file with type “Skyline Document”.

2.4 Use the Replicate drop down menu in the top left to select the specific sample run you wish to view.

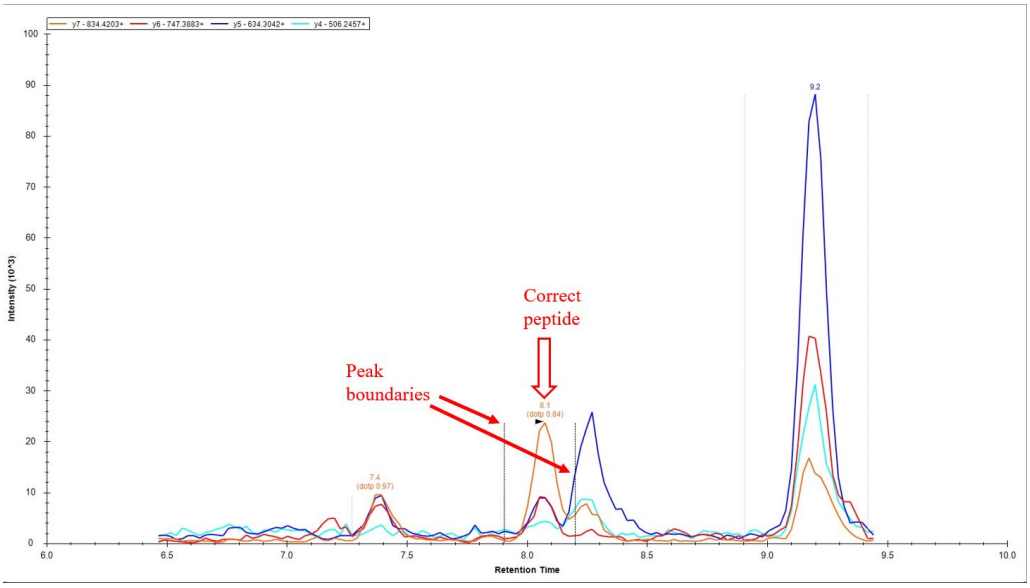
2.5 Use the targets pane on the left to select the peptide that you want to view. The peaks for all fragment ions for that peptide will be displayed.

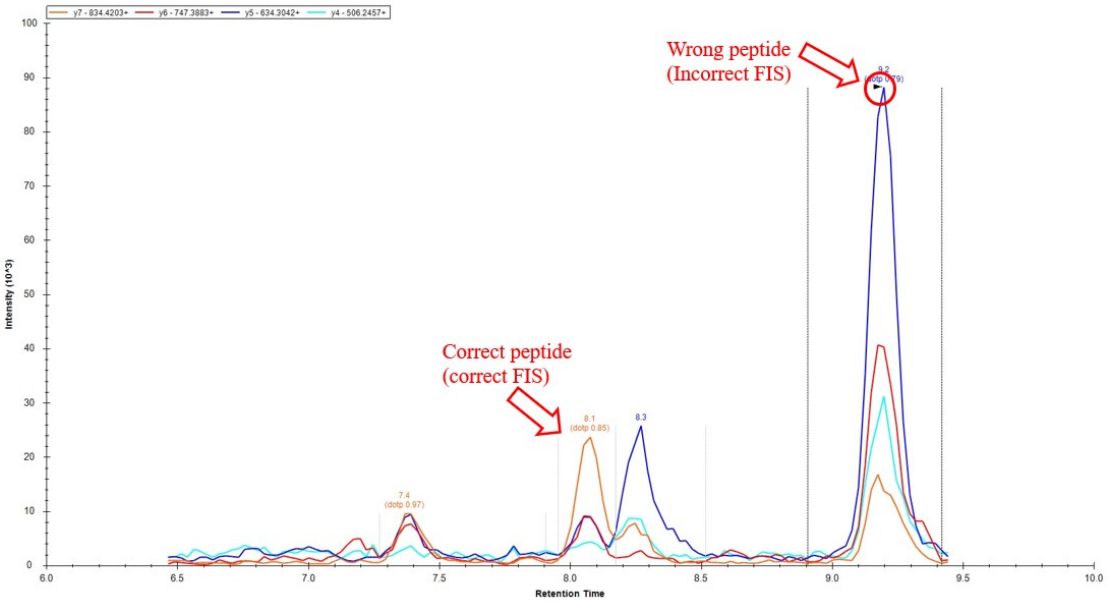
2.5.1 Note: The shape and apparent positions of transitions, as observed in the chromatograph, are not an accurate guide for evaluating a peptide. Peptides are statistically evaluated from the raw mass spectrometer data.

2.6 To select a different peak.

2.6.1.1 Open the Skyline document following 2.3and navigate to the chromatogram for the specific sample and peptide.

2.6.1.2 The selected peak is indicated in Skyline by a black triangle pointing at the apex of the peak. To change the selection, click on the retention time measurement displayed at the apex of the peak you wish to select (see figures below). The black triangle will move to the newly selected peak.





2.6.1.3 Save the change and rerun the MSDA Script. The script will export and analyze data for the newly selected peak.

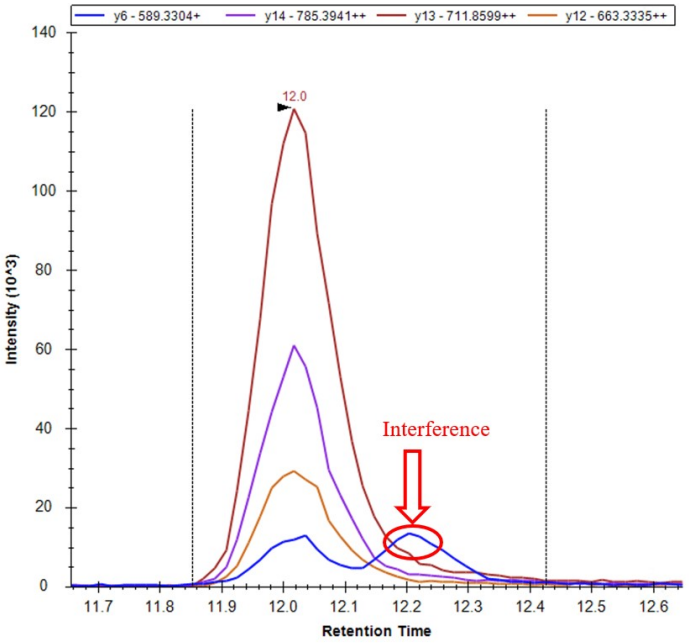
2.6.1.4 If the newly selected peak passes (meets criteria as described in (1.3.1.1 – 1.3.1.4)), the peptide is detected and cannot be reinterpreted by the analyst. If it does not pass, the selection process following 2.6.1.2-2.6.1.3may be repeated.

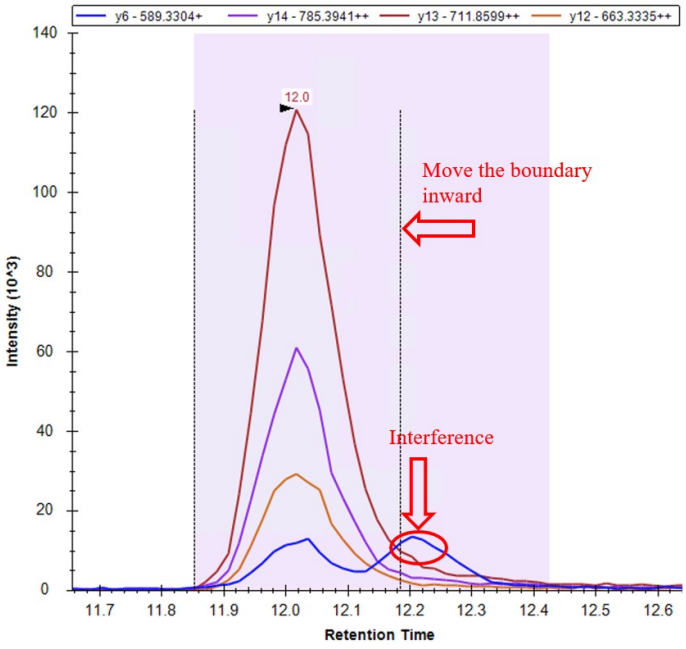
2.7 To change a peak's boundaries to exclude interference.

2.7.1 In some cases, interference from a complex sample matrix may have a mass close enough to a fragment ion to cause an interference signal that partially overlaps with a peptide peak that includes all four fragment ion signals. If the peak boundaries (dotted lines on either side of a peak) selected by Skyline include interference, the analyst may ONLY move the boundary inward (toward the peptide peak) in order to exclude interference. Interference may be excluded by the analyst ONLY if the interference has an apex distinct from the peptide peak AND that apex is higher than the apex of the peptide peak for the fragment ion displaying interference. See figures below.

2.7.1.1 Open Skyline document following 2.3 and navigate to the chromatogram for the specific sample and peptide.

2.7.1.2 Click on the boundary (dotted line) and move it inward until the interference is excluded, but with as much of the peptide peak as possible remaining within the boundaries. The apex of the peptide peak MUST remain within the peak boundaries.





2.7.1.3 Save the change and rerun the MSDA Script. The script will export and analyze data between the new peak boundaries.

2.7.1.4 If the newly selected peak passes (meets criteria as described in (1.3.1.1– 1.3.1.4)) the peptide is detected and cannot be reinterpreted by the analyst. If it does not pass, the boundary selection process following 2.7.1.2-2.7.1.3may be repeated.

3 Repeat Analyses

3.1 If additional LCMS runs of the same digest are completed and yield different results for detection of a body fluid, the best result is reported, and the Not Suitable for Interpretation Molecular Serology form filled out and attached to the case for the run that is not reported.

3.1.1 If additional LCMS runs of the same digest are completed and the best result for each body fluid occurs in different runs, both runs are reported.