

Highly accurate detection of ovarian cancer using CA125 but limited improvement with serum matrix-assisted laser desorption/ionization time-of-flight mass spectrometry profiling

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1 **Highly accurate detection of ovarian cancer using CA125 but limited improvement**
2 **with serum MALDI-TOF MS profiling**

3 **Short title:** Ovarian cancer detection using MALDI-TOF MS and CA125

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16
17 **Keywords:** serum profiling, ovarian cancer, CA125, mass spectrometry, biomarkers, peptidome

18
19 **Non-standard abbreviations:** AUROC, area under the receiver operating characteristic curve; kNN,
20 k-nearest neighbours; MALDI, matrix-assisted laser desorption/ionisation; MS, mass spectrometry;
21 PPV, positive predictive value; ROC, receiver operating characteristic; TOF, time-of-flight; UKOPS,
22 United Kingdom Ovarian Cancer Population Study.

1 **Abstract**

2 **Objectives** Our objective was to test the performance of CA125 in classifying serum samples from a
3 cohort of malignant and benign ovarian cancers and age-matched healthy controls and to assess
4 whether combining information from MALDI-TOF profiling could improve diagnostic performance.

5 **Methods/Materials** Serum samples from women with ovarian neoplasms and healthy volunteers were
6 subjected to CA125 assay and MALDI-TOF MS profiling. Models were built from training datasets
7 using discriminatory MALDI MS peaks in combination with CA125 values and tested their ability to
8 classify blinded test samples. These were compared to models using CA125 threshold levels from 193
9 patients with ovarian cancer, 290 with benign neoplasm and 2236 post-menopausal healthy controls.

10 **Results** Using a CA125 cut-off of 30 U/mL, an overall sensitivity of 94.8% (96.6% specificity) was
11 obtained when comparing malignancies vs. healthy post-menopausal controls, while a cut-off of 65
12 U/mL provided a sensitivity of 83.9% (99.6% specificity). High classification accuracies were obtained
13 for early-stage cancers (93.5% sensitivity). Reasons for high accuracies include recruitment bias,
14 restriction to post-menopausal women and inclusion of only primary invasive epithelial ovarian cancer
15 cases. The combination of MS profiling information with CA125 did not significantly improve the
16 specificity/accuracy compared to classifications based on CA125 alone.

17 **Conclusions** We report unexpectedly good performance of serum CA125 using threshold classification
18 in discriminating healthy controls and women with benign masses from those with invasive ovarian
19 cancer. This highlights the dependence of diagnostic tests on the characteristics of the study population
20 and the crucial need for authors to provide sufficient relevant details to allow comparison. Our study
21 also shows that MS profiling information adds little to diagnostic accuracy. This finding is in contrast
22 with other reports and shows the limitations of serum MS profiling for biomarker discovery and as a
23 diagnostic tool.

24

1 **Introduction**

2 Ovarian cancer is the leading cause of death from gynaecologic malignancy in the western world which
3 is mainly attributable to its diagnosis at an advanced stage ^(1, 2). This suggests that detecting ovarian
4 cancer at an earlier stage may improve survival. Crucial to early detection is the identification of
5 accurate biomarkers. Serum CA125 is the most extensively assessed biomarker for ovarian cancer with
6 elevated levels of CA125 found in >90% of patients with advanced disease. However, CA125 has been
7 shown to lack sensitivity (50-60%) for early-stage disease detection ^(1, 3-8) and its expression is not
8 specific to malignant ovarian cancers ⁽⁹⁾. Indeed, CA125 can be elevated in women with benign
9 gynaecological conditions such as ovarian cysts, endometriosis, and uterine fibroids, as well as in other
10 cancers (breast, bladder, pancreatic, liver, lung) ⁽¹⁰⁾. Efforts have therefore been made to identify
11 additional biomarkers to complement CA125.

12 Over the last two decades, dozens of new biomarkers of ovarian carcinomas have been proposed,
13 with combinations of these biomarkers with or without CA125 reported to significantly increase the
14 accuracy in detecting ovarian cancer at both early and late stages ^(6, 8, 11-16). Some of the multiple marker
15 panels achieved the important benchmark value of >99.6% specificity that is required to achieve a
16 positive predictive value of 10% (for an incidence rate of 40 per 100,000 women). However, this was
17 accompanied by a fall in sensitivity values to <60% for early-stage and <77% for late-stage cancer ⁽¹⁶⁾.

18 In the context of ovarian cancer screening, CA125 interpreted using a Risk of Ovarian Cancer
19 algorithm has a high sensitivity and specificity for detecting primary invasive ovarian and tubal
20 malignancies. For multimodal screening using annual CA125 screening with transvaginal ultrasound
21 scan as a second-line test, the sensitivity for primary ovarian and tubal malignancies was 89.4% at a
22 specificity of 99.8% ⁽¹⁷⁾. Whilst the performance of screening strategies has greatly improved in recent
23 years, the need for additional screening modalities providing both high sensitivity and specificity
24 remains. Likewise, the differential diagnosis of symptomatic patients would also benefit from improved

1 and simpler tests. With this in mind, the objective of this study was to evaluate whether combinations
2 of serum CA125 and mass spectrometry (MS) profiling data could enhance the identification of ovarian
3 cancer patients from benign cases and healthy controls compared to the use of CA125 values alone.

4

1 **Material and Methods**

2 **Subjects, sample collection and handling**

3 The study was approved by the local ethics committee (MREC 05/Q0505/58) and written informed
4 consent was obtained from all donors. Women were recruited to the UK Ovarian Cancer Population
5 Study (UKOPS) from ten NHS Trusts across the UK. Patients were recruited at gynaecological
6 oncology departments and healthy volunteers were recruited from women attending annual screening in
7 the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) ^(17, 18). Supplemental Data 1
8 provides details on the initial set of subjects, sample collection, transport and storage. For the combined
9 MS profiling and CA125 assay analyses we used the same sample set as previously reported ⁽¹⁹⁾. After
10 excluding samples with missing CA125 values and those from borderline ovarian cancer cases, the data
11 from 321 women were used for identifying the best classification models when comparing malignant
12 vs. healthy and malignant vs. benign. Sixty seven samples were from individuals newly diagnosed with
13 invasive epithelial ovarian cancer, 84 were from women diagnosed with benign ovarian neoplasm and
14 170 were from age-matched healthy controls. For model generation and validation, samples were
15 divided into two training and test sets (see Table 1). Figure 1A shows CA125 assay values across the
16 groups. For the extended CA125 analysis, CA125 serum levels were evaluated from 2719 women.
17 Supplemental Data 2 shows the division of this set into the three classes (malignant, benign and
18 healthy), FIGO stage distribution and average and median age in each class and stage group.

19 **CA125 immunoassays**

20 Samples collected, processed and frozen at the regional centres were transported on dry ice to the UCL
21 laboratory and thawed. After thawing, samples were mixed by gentle inversion and CA125 analysis
22 was performed using an electro-chemiluminescence immunoassay (ECLIA) on a Roche Elecsys 2010
23 analyser (Roche Diagnostics, Burgess Hill, UK). The assay uses monoclonal antibodies OC125 as the

1 detection antibody and M11 as the capture antibody (Fujirebio Diagnostics; Oxford Biosystems,
2 Oxford, UK).

3 **MALDI-TOF MS-based profiling**

4 Samples were processed and analysed in two batches. Samples in each batch were randomized at the
5 UCL laboratory, thawed and aliquoted into 96-well plates, then transported on dry ice to the BioCentre
6 at the University of Reading and stored at -80°C. For MS serum polypeptide profiling, samples were
7 prepared according to previously published methods ⁽¹⁹⁾ ⁽²⁰⁾ and profiled using an Ultraflex II MALDI-
8 TOF/TOF mass spectrometer (Bruker Daltonics, Coventry, UK). Various spectral quality control
9 criteria were implemented with adequate quality assurance for the entire sample preparation process,
10 data collection and analysis ⁽¹⁹⁾ ⁽²⁰⁾. Supplemental Data 3 provides details on sample preparation, data
11 acquisition and pre-processing.

12 **Data processing and classification**

13 Raw spectral data was processed using algorithms developed in-house ⁽¹⁹⁾. Data from the two batch
14 analyses were combined with corresponding CA125 values and used to construct two training and
15 blinded test sets for classification (Table 1). Prediction models were constructed for two types of
16 discrimination independently, malignant vs. healthy and malignant vs. benign, and compared to simple
17 classification using a CA125 cut-off value of 30 U/mL. Multiple models using the weighted k-nearest
18 neighbours algorithm (kNN), logical combinations of cut-off rules, cut-off rules for linear combinations
19 and support vector machine (SVM) with various kernels were applied to subsets of peaks of certain
20 cardinality (usually a small number). Cross-validation was performed by randomising sample labels in
21 1,000 iterations and calculating *p*-values (Monte-Carlo method) for the randomly permuted and
22 correctly labeled samples. The models performing best on the training sets (all weighted kNN models)
23 were then validated on the blinded test sets. For calculation of significance of improvement through
24 addition of MS profiling data, a Monte-Carlo test was applied that measured the chance to get the

1 accuracy greater than or equal to the best accuracy achieved on the test set if peak intensities were
2 reshuffled across samples at the given CA125 accuracy. The *p*-value was calculated as the proportion
3 of iterations with accuracy greater than or equal to the accuracy achieved with the best model. For
4 analysis of the extended sample set using CA125, various cut-off values were tested and ROC analysis
5 was applied using GraphPad Prism v5.0 software (GraphPad Software Inc., La Jolla, CA, USA).

6 **Peak identification by MALDI-QTOF MS/MS**

7 Peak identifications in this study were obtained by analysing a pool of serum samples on a Premier Q-
8 TOF mass spectrometer (Waters, Manchester, UK) in the MALDI mode using a comparable MALDI
9 sample preparation method that was only changed to account for the different type of MALDI target
10 used.

11

1 **Results**

2 **Classification performance using MS profiling data and CA125 values**

3 As shown in our previous study⁽¹⁹⁾, MALDI-TOF MS profiling was robust and reproducible with inter-
4 assay coefficients of variance <15%. Nonetheless, MALDI MS profiling data alone had only limited
5 diagnostic value for ovarian cancer, particularly when compared with recent reports using multi-
6 biomarker panels^(6, 8, 11-17, 21). Consequently, we have evaluated the performance of classification
7 models derived from the combination of MS profiling and CA125 immunoassay data.

8 Our first analysis employed randomly selected sets for training and testing as detailed in the upper
9 part of Table 1. Modelling for the separation of women with invasive epithelial ovarian cancer from
10 healthy controls revealed that weighted kNN algorithms performed best, with several models
11 outperforming simple CA125 cut-off classification (at a 30 U/mL threshold) in the training set. Three
12 models were chosen for validation in the blinded test set selecting each for highest sensitivity,
13 specificity and quality, respectively. One of these models performed better than CA125 alone in the test
14 set with an accuracy of 100% (Table 2; upper part), although this improvement was not statistically
15 significant ($p=0.24$). Comparison of invasive epithelial ovarian cancer (malignant) and benign cases
16 showed inferior performance based on overall accuracy and quality in the test set, although several
17 models outperformed the CA125 cut-off classification in the training set (Table 2; lower part).

18 This analysis demonstrates that a classification model utilising CA125 values alone using a cut-off
19 level of 30 U/mL performs extremely well in this sample set. In this specific case, all malignant
20 samples in the test set had CA125 values >30 U/mL, making it impossible to improve on sensitivity.
21 Similarly, only one healthy sample had a CA125 value >30 U/mL, giving little space for improvement
22 in specificity. As a consequence, we reshuffled the training and test set according to two conditions.
23 First, the ratio between the training and test set was set at ~2:1 for all classes. Second, in both sets each
24 class had the same ratio of samples above and below 30 U/mL CA125 (lower part of Table 1).

1 Modelling analysis using these new training and test sets showed more models were now able to
2 improve on specificity in comparison to CA125 alone. Nonetheless, the improvement in discriminating
3 malignant from healthy controls was still limited to a maximum of two additional correctly classified
4 healthy samples in the test set, whilst sensitivity could not be further improved. Likewise, for
5 classification of malignant vs. benign cases there was improvement in specificity for many models in
6 the test set, but none matched the sensitivity when using CA125 alone, limiting overall accuracy (Table
7 3). Improvement in overall accuracy with the best model, from 76.9% (CA125 cut-off model) to 78.9%
8 (5 out of 10 model), was not significant ($p=0.72$). Furthermore, neither of the discriminatory peaks
9 (m/z 2755 and 2094) in this model was found in any of the best models obtained from the initial sample
10 sets. These two peaks were identified as fragments of serum albumin (25-48; Swiss-Prot entry P02768)
11 and fibrinogen α -chain (605-624; Swiss-Prot entry P02671). Only one peak (m/z 4787) from the
12 models in Table 3 was also used in the best models from the first analysis (cf. Table 2).

13 **Classification performance using simple CA125 cut-off models**

14 As a consequence of the good performance of CA125 cut-off classification, we further investigated an
15 extended set of UKOPS samples looking at CA125 alone for classification. This extended set
16 comprised 2236 healthy controls (median age of 64.31), 290 benign (median age of 57.96) and 193
17 invasive ovarian cancers (median age of 63.88), of which 48.2% were FIGO stage I ($n=74$) or II ($n=19$)
18 (see Supplemental data 2). In the comparison of malignant and healthy samples, using a 65 U/mL cut-
19 off level, only 10 out of 2236 healthy women had elevated CA125 giving a specificity of 99.6% (95%-
20 CI of 99.1-99.8%) and a sensitivity of 83.9% (95%-CI of 78.0-88.8%). At a 30 U/mL cut-off level, a
21 sensitivity of 94.8% (95%-CI of 90.7-97.5%) and specificity of 96.6% (95%-CI of 95.5-97.3%) were
22 obtained. For malignant vs. benign, the 65 U/mL cut-off gave a specificity of 76.2% (95%-CI of 70.9-
23 81.0%) at a sensitivity of 83.9%, whilst a 30 U/mL cut-off gave 53.4% specificity (95%-CI of 47.5-
24 59.3%). The area under the receiver operating characteristic curve (AUROC) for this classification was

1 0.877 ($p<0.0001$; 95%-CI of 0.846-0.908) (Figure 2A). Analysis of early-stage cancer vs. benign
2 revealed that at the 65 U/mL CA125 threshold, the sensitivity was 77.4% (95%-CI of 67.6-85.5%) and
3 at 30 U/mL, was 92.5% (95%-CI of 85.1-96.9%). Due to the relatively low number of stage II samples
4 (n=19), these sensitivity values only changed marginally when only stage I samples (n=74) were used;
5 the AUROC for stage I vs. benign cancer was 0.842 ($p<0.0001$; 95%-CI of 0.794-0.891) (Figure 2B)
6 with sensitivity values of 54.1%, 33.8% and 27.0% for specificity values of 90%, 95% and 98%,
7 respectively.
8

1 **Discussion**

2 We have further evaluated our earlier reported MALDI MS profiling study by combining profiling data
3 with pre-operative CA125 serum levels. The rationale was to explore if this combination could improve
4 in discriminating healthy women or those with benign masses from women with invasive epithelial
5 ovarian cancer. Two different training and test sets were employed, one using representative sampling
6 with respect to CA125 value distribution above and below 30 U/mL. Although improvements in
7 classification performance for discriminating healthy or benign samples from malignant samples were
8 apparent in the training sets (compared to a standard 30 U/mL CA125 cut-off classification), only
9 marginal and statistically insignificant improvement on performance was achieved in the test sets. This
10 is in keeping with our earlier observation that MS profiling alone is limited in its ability to discriminate
11 malignant ovarian cancer samples from benign or healthy controls ⁽¹⁹⁾. However, the unexpectedly
12 good performance of the CA125 immunoassay on its own made it virtually impossible to improve on
13 performance.

14 We next used an extended set of over 2700 samples to investigate further this better-than-expected
15 CA125 performance. At a threshold of 65 U/mL CA125, only 10 out of 2236 healthy controls were
16 misclassified providing a specificity of 99.6%. At a 30 U/mL cut-off, the specificity was 96.6%, while
17 the sensitivity for correctly identifying malignant samples was 94.8%. For early-stage disease (stage I
18 & II) the sensitivity was still 92.5% at 30 U/mL and 90.3% at 35 U/mL, and above reported values. It is
19 also noteworthy that our CA125 classification of early-stage cancer vs. healthy performed as well as, or
20 better than, classification models based on multiple biomarkers ^(8, 11, 16). CA125 also showed improved
21 accuracy for discriminating malignant vs. benign cases compared to recent literature. For example, the
22 pooled sensitivity of CA125 in a meta-analysis on diagnostic strategies for distinguishing adnexal
23 masses was 78% at a threshold of 35 U/mL, with individual study sensitivities ranging from 45-100%
24 ⁽²²⁾. For stage I cases alone, comparison with a recent study ⁽¹²⁾, showed that our sensitivity values are

1 more than twice as high for 90%-specificity (54.1%) and 95%-specificity (33.8%) and more than thrice
2 as high for 98%-specificity (27%).

3 The high sensitivity of CA125 in this study may reflect the fact that samples were obtained from
4 women referred to specialist gynaecological cancer centres who may in part have been referred on the
5 basis of elevated CA125. This is in keeping with a recent report that over-representation of operative
6 cases, especially from academic facilities, exaggerates the performance of CA125 in regard to
7 sensitivity and PPV ⁽²²⁾. The good performance may also in part be explained by the exclusion of pre-
8 menopausal women from our cohort, since both sensitivity and specificity of CA125 are consistently
9 higher in post-menopausal women. This is the rationale underlying restricting participation in ovarian
10 cancer screening trials such as UKCTOCS ^(17, 18) to only post-menopausal women. The definition of
11 malignancy is another factor that can influence test accuracy. In this study, samples were restricted to
12 those from cases of primary invasive epithelial cancer, the most common ovarian cancers and the main
13 contributor to the high case fatality ratio associated with the disease. This further increased our
14 accuracy as we excluded both non-epithelial ovarian malignancies and borderline/low malignant
15 potential ovarian cancers, both of which are less likely to produce CA125. Staging of ovarian cancer
16 and the CA125 immunoassay are other sources for potential bias, but both procedures are relatively
17 standardised and, therefore, less likely to have contributed to the observed higher accuracies.

18 In conclusion, we report the unexpectedly good performance of simple serum CA125 threshold
19 classification in discriminating healthy and benign from malignant samples for the detection of ovarian
20 cancer. Compared to the data on CA125 assays published so far, a substantially increased accuracy was
21 obtained. Reasons for this increase include recruitment bias in the specialist gynaecological oncology
22 centres participating in sample collection, restriction of the study to post-menopausal women and
23 restricting the definition of ovarian malignancy to primary invasive epithelial cancer. The performance
24 characteristics of the CA125 immunoassay in our study highlight its dependence on the study

1 population and the crucial need for authors to provide sufficient detail on relevant characteristics of
2 study populations to allow comparisons.

3 The collection of serum samples and their subsequent handling followed a strict protocol designed
4 for optimal proteomic profiling with the aim of minimising post-sampling difference due to proteolysis.
5 However, the combination of CA125 with MS profiling data provided only marginal improvement.
6 Unfortunately, due to the good performance of CA125 as a discriminatory biomarker, the benefit of
7 MS profiling to provide additional classification power is difficult to judge. In this context, the
8 additional benefit of MS profiling should be evaluated in combination with other biomarkers and/or
9 using study groups where sensitivity values can be improved upon. Here, the MS identification of
10 proteins of low specificity (serum albumin and fibrinogen α -chain) as the source of potentially
11 discriminatory peaks further supports a more careful approach to MALDI MS profiling for clinical
12 diagnostics.

13

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5

1 **References**

- 2 [1] Jacobs IJ, Menon U. Progress and challenges in screening for early detection of ovarian cancer.
3 *Mol Cell Proteomics*. 2004;**3**: 355-66.
- 4 [2] Schwartz PE. Current diagnosis and treatment modalities for ovarian cancer. *Cancer Treat Res*.
5 2002;**107**: 99-118.
- 6 [3] Fritsche HA, Bast RC. CA 125 in ovarian cancer: advances and controversy. *Clin Chem*.
7 1998;**44**: 1379-80.
- 8 [4] Nossov V, Amneus M, Su F et al. The early detection of ovarian cancer: from traditional
9 methods to proteomics. Can we really do better than serum CA-125? *American journal of obstetrics*
10 *and gynecology*. 2008;**199**: 215-23.
- 11 [5] Jacobs I, Bast RC, Jr. The CA 125 tumour-associated antigen: a review of the literature. *Hum*
12 *Reprod*. 1989;**4**: 1-12.
- 13 [6] Visintin I, Feng Z, Longton G et al. Diagnostic markers for early detection of ovarian cancer.
14 *Clin Cancer Res*. 2008;**14**: 1065-72.
- 15 [7] Skates SJ, Horick N, Yu Y et al. Preoperative sensitivity and specificity for early-stage ovarian
16 cancer when combining cancer antigen CA-125II, CA 15-3, CA 72-4, and macrophage colony-
17 stimulating factor using mixtures of multivariate normal distributions. *J Clin Oncol*. 2004;**22**: 4059-66.
- 18 [8] Gorelik E, Landsittel DP, Marrangoni AM et al. Multiplexed immunobead-based cytokine
19 profiling for early detection of ovarian cancer. *Cancer Epidemiol Biomarkers Prev*. 2005;**14**: 981-7.
- 20 [9] Kabawat SE, Bast RC, Jr., Bhan AK et al. Tissue distribution of a coelomic-epithelium-related
21 antigen recognized by the monoclonal antibody OC125. *Int J Gynecol Pathol*. 1983;**2**: 275-85.
- 22 [10] Sjovall K, Nilsson B, Einhorn N. The significance of serum CA 125 elevation in malignant and
23 nonmalignant diseases. *Gynecol Oncol*. 2002;**85**: 175-8.
- 24 [11] Zhang Z, Yu Y, Xu F et al. Combining multiple serum tumor markers improves detection of
25 stage I epithelial ovarian cancer. *Gynecol Oncol*. 2007;**107**: 526-31.
- 26 [12] Moore RG, Brown AK, Miller MC et al. The use of multiple novel tumor biomarkers for the
27 detection of ovarian carcinoma in patients with a pelvic mass. *Gynecol Oncol*. 2008;**108**: 402-8.
- 28 [13] Woolas RP, Xu FJ, Jacobs IJ et al. Elevation of multiple serum markers in patients with stage I
29 ovarian cancer. *J Natl Cancer Inst*. 1993;**85**: 1748-51.
- 30 [14] Mor G, Visintin I, Lai Y et al. Serum protein markers for early detection of ovarian cancer.
31 *Proc Natl Acad Sci U S A*. 2005;**102**: 7677-82.

- 1 [15] Kozak KR, Su F, Whitelegge JP et al. Characterization of serum biomarkers for detection of
2 early stage ovarian cancer. *Proteomics*. 2005;**5**: 4589-96.
- 3 [16] Havrilesky LJ, Whitehead CM, Rubatt JM et al. Evaluation of biomarker panels for early stage
4 ovarian cancer detection and monitoring for disease recurrence. *Gynecol Oncol*. 2008;**110**: 374-82.
- 5 [17] Menon U, Gentry-Maharaj A, Hallett R et al. Sensitivity and specificity of multimodal and
6 ultrasound screening for ovarian cancer, and stage distribution of detected cancers: results of the
7 prevalence screen of the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS). *Lancet*
8 *Oncol*. 2009;**10**: 327-40.
- 9 [18] Menon U, Gentry-Maharaj A, Ryan A et al. Recruitment to multicentre trials--lessons from
10 UKCTOCS: descriptive study. *BMJ (Clinical research ed)*. 2008;**337**: a2079.
- 11 [19] Timms JF, Cramer R, Camuzeaux S et al. Peptides generated ex vivo from serum proteins by
12 tumor-specific exopeptidases are not useful biomarkers in ovarian cancer. *Clin Chem*. 2010;**56**: 262-71.
- 13 [20] Tiss A, Smith C, Camuzeaux S et al. Serum Peptide Profiling using MALDI Mass
14 Spectrometry: avoiding the Pitfalls of Coated Magnetic Beads using Well-established ZipTip
15 Technology. *Proteomics*. 2007;**7 Suppl 1**: 77-89.
- 16 [21] Menon U, Skates SJ, Lewis S et al. Prospective study using the risk of ovarian cancer algorithm
17 to screen for ovarian cancer. *J Clin Oncol*. 2005;**23**: 7919-26.
- 18 [22] Myers ER, Bastian LA, Havrilesky LJ et al. Management of adnexal mass. *Evid Rep Technol*
19 *Assess (Full Rep)*. 2006: 1-145.

20

21

Table 1 Sample sets used for combined MS profiling and CA125 analysis

Randomised sets – no representative sampling*

	Training Set	Test Set	Total
Healthy	104	66	170
Benign	62	22	84
Malignant	38	29	67
Total	204	117	321

Randomised sets – with representative sampling*

	Training Set	Test Set	Total
Healthy	113	57	170
Benign	55	29	84
Malignant	44	23	67
Total	213	108	321

* See text for further details.

Table 2 The performance of two peak weighted k-nearest neighbour models in comparison to the >30 U/mL CA125 threshold model for the classification of ovarian cancer samples using the randomized training and blinded test set without representative sampling. Cross-validation was performed by randomizing sample labels in 1,000 iterations and calculating *P*-values (Monte-Carlo method) for the randomly permuted and correctly labelled samples.

Malignant vs. Healthy

kNN threshold / kNN total	Peak 1 (m/z)	Peak 2 (m/z)	TRAINING SET					TEST SET			
			Sensitivity	Specificity	Accuracy	Quality*	p-value for 1000 iterations	Sensitivity	Specificity	Accuracy	Quality*
1 out of 2	2661	2770	97.4%	99.0%	98.6%	97.9%	0.001	100.0%	98.5%	99.0%	99.5%
2 out of 5	972	2367	94.7%	100.0%	98.6%	96.5%	0.001	100.0%	100.0%	100.0%	100.0%
1 out of 4	3952	1114	100.0%	92.3%	94.4%	97.4%	0.001	100.0%	86.4%	90.5%	95.5%
CA125 >30 U/mL cut-off			94.7%	96.2%	95.8%	95.2%		100.0%	98.5%	99.0%	99.5%

Malignant vs. Benign

kNN threshold / kNN total	Peak 1 (m/z)	Peak 2 (m/z)	TRAINING SET					TEST SET			
			Sensitivity	Specificity	Accuracy	Quality*	p-value for 1000 iterations	Sensitivity	Specificity	Accuracy	Quality*
1 out of 3	4787	2367	97.4%	75.8%	84.0%	90.2%	0.001	93.1%	59.1%	78.4%	81.8%
1 out of 4	741	3241	97.4%	75.8%	84.0%	90.2%	0.001	93.1%	54.6%	76.5%	80.3%
2 out of 9	1467	2430	97.4%	75.8%	84.0%	90.2%	0.001	96.6%	40.9%	72.6%	78.0%
2 out of 5	4054	3507	94.7%	85.5%	89.0%	91.7%	0.001	93.1%	63.6%	80.4%	83.3%
CA125 >30 U/mL cut-off			94.7%	64.5%	76.0%	84.7%		100.0%	59.1%	82.4%	86.4%

* Quality = (2xSensitivity + Specificity)/3

Table 3 The performance of two peak weighted k-nearest neighbour models in comparison to a >30 U/mL CA125 threshold model for classification of malignant vs. benign ovarian cancer samples using randomized training and blinded test sets with representative sampling. Cross-validation was performed by randomizing sample labels in 1,000 iterations and calculating *p*-values (Monte-Carlo method) for randomly permuted and correctly labelled samples.

kNN threshold / kNN total	Peak 1 (m/z)	Peak 2 (m/z)	TRAINING SET					TEST SET			
			Sensitivity	Specificity	Accuracy	Quality*	p-value for 1000 iterations	Sensitivity	Specificity	Accuracy	Quality*
1 out of 4	3263	4787	100%	76.4%	86.9%	92.1%	0.001	87.0%	69.0%	76.9%	81.0%
4 out of 8	1742	3971	97.7%	90.9%	93.9%	95.5%	0.001	69.6%	72.4%	71.2%	70.5%
5 out of 10	2755	2094	93.2%	92.7%	92.9%	93.0%	0.001	69.6%	86.2%	78.9%	75.1%
CA125 >30 U/mL cut-off			97.7%	63.6%	78.8%	86.4%		95.7%	62.1%	76.9%	84.5%

* Quality = (2xSensitivity + Specificity)/3

kNN threshold / kNN total	Peak 1 (m/z)	Peak 2 (m/z)	TRAINING SET					TEST SET			
			Sensitivity	Specificity	Accuracy	Quality*	p-value for 1000 iterations	Sensitivity	Specificity	Accuracy	Quality*
1 out of 9	1618	4787	100.0%	69.1%	82.8%	79.4%	0.001	87.0%	58.6%	71.2%	68.1%
4 out of 8	1742	3971	97.7%	90.9%	93.9%	93.2%	0.001	69.6%	72.4%	71.2%	71.5%
5 out of 10	2755	2094	93.2%	92.7%	92.9%	92.9%	0.001	69.6%	86.2%	78.9%	80.7%
2 out of 4	1520	3966	90.9%	94.6%	92.9%	93.3%	0.001	65.2%	75.9%	71.2%	72.3%
1 out of 1	2094	1114	86.4%	98.2%	92.9%	94.2%	0.001	69.6%	79.3%	75.0%	76.1%
CA125 >30 U/mL cut-off			97.7%	63.6%	78.8%	75.0%		95.7%	62.1%	76.9%	73.3%

* Quality = (Sensitivity + 2xSpecificity)/3

Figure Legends

Figure 1 Distribution of CA125 levels in the extended sample set.

Figure 2 A. ROC analysis of the CA125 cut-off model the classification of malignant vs. benign samples using the extended sample set. **B.** ROC analysis of the CA125 cut-off model the classification of stage I vs. benign samples using the extended sample set.

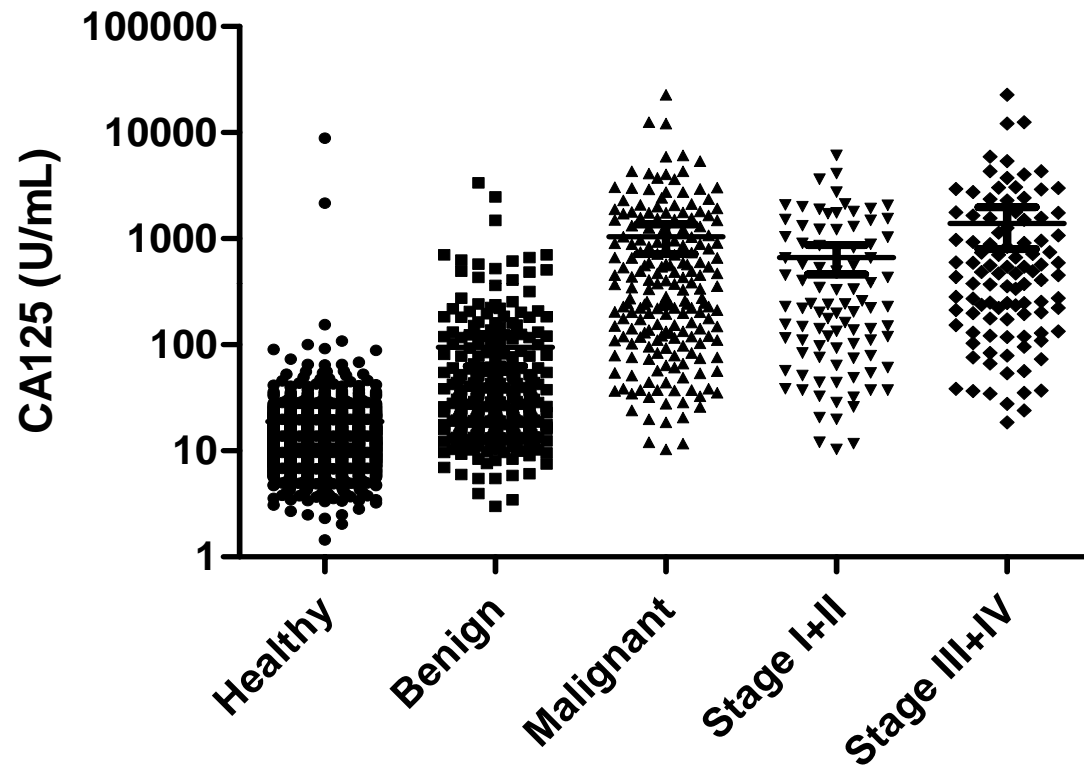
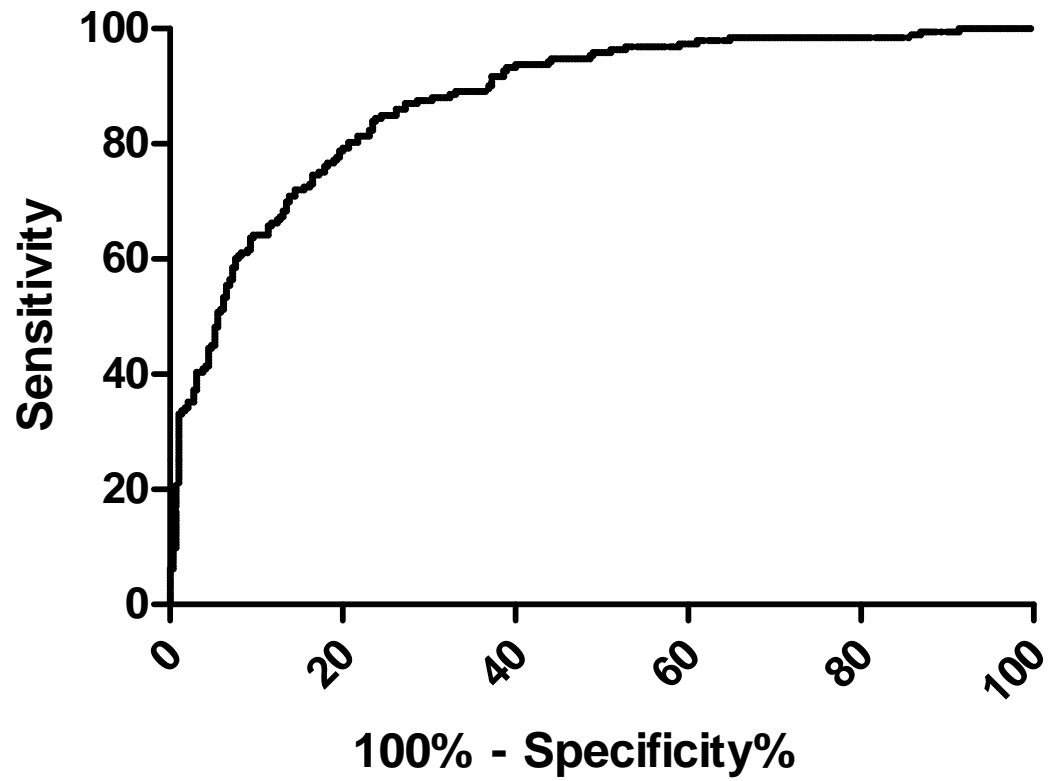
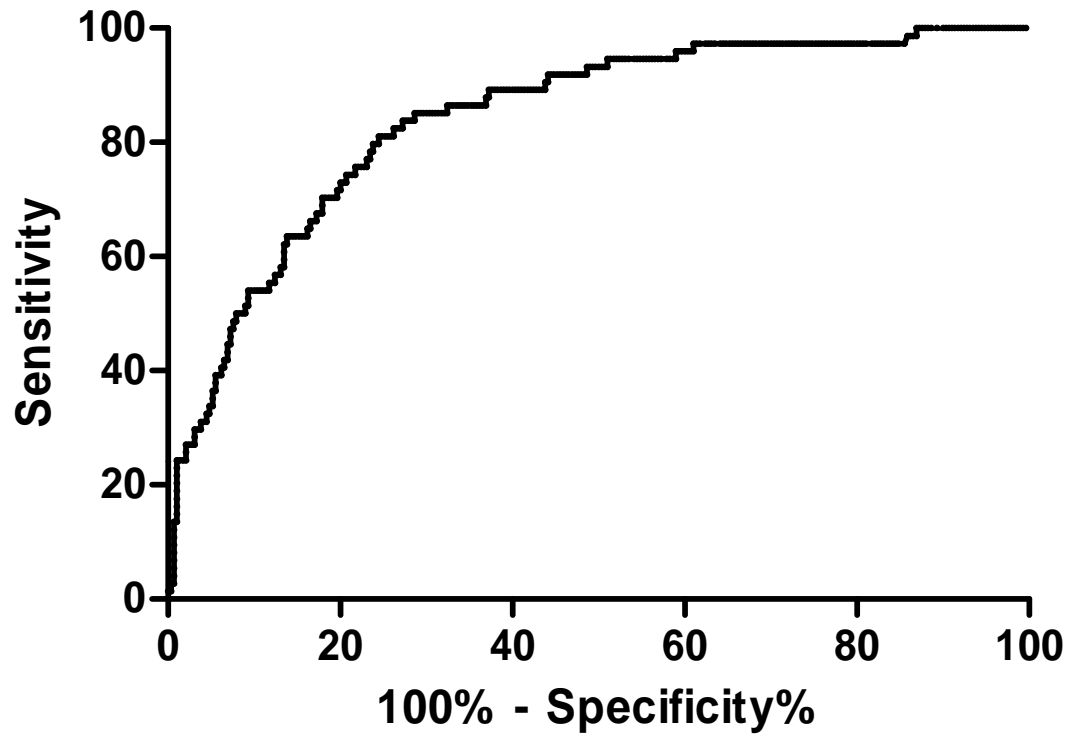


Figure 1



Area	0.8769
Std. Error	0.01578
95% confidence interval	0.8459 to 0.9078
P value	< 0.0001

Figure 2A



Area	0.8424
Std. Error	0.02493
95% confidence interval	0.7936 to 0.8913
P value	< 0.0001

Figure 2B

Supplemental Data 1

Details on the initial set of subjects, sample collection, transport, and storage

Serum samples from patients were collected prior to surgery for an ovarian neoplasm and the diagnosis of malignant or benign ovarian neoplasm was confirmed by independent review of notes and histopathology reports. The healthy volunteers had no significant family history of ovarian cancer and no diagnosis of a cancer during follow up after sample collection. All samples were collected and processed according to a strict protocol as previously described (Villanueva J, Martorella AJ, Lawlor K, Philip J, Fleisher M, Robbins RJ, Tempst P. Serum peptidome patterns that distinguish metastatic thyroid carcinoma from cancer-free controls are unbiased by gender and age. *Mol Cell Proteomics* 2006;5:1840-52). Briefly, venous blood was collected in BD Vacutainer ('red top') 10mL-tubes (Becton Dickinson, Oxford, UK; cat. no. 367985). Tubes were inverted 5 times and left at room temperature in a vertical position to clot for 1 hr. Samples were then placed on ice for no more than 2 hrs, centrifuged at 1,500 g for 10 min at room temperature, aliquoted into 10mL-Sarstedt tubes (Sarstedt, Leicester, UK; cat. no. 60.551.001) and frozen at -80°C. All samples were then transported to the Institute for Women's Health Laboratory at UCL on dry ice where they were thawed for 30 minutes at room temperature, mixed by gentle inversion, assayed for CA125 and aliquoted into bar-coded straws, re-frozen at -80°C for 24 hours and then transferred to liquid N₂ for long-term storage. The CA125 assays were performed centrally at the Institute for Women's Health at UCL while the MS analysis was performed in the BioCentre at the University of Reading as described in earlier publications (first paper; Tiss A, Smith C, Camuzeaux S, Kabir M, Gayther S, Menon U, et al. Serum peptide profiling using MALDI mass spectrometry: avoiding the pitfalls of coated magnetic beads using well-established ZipTip technology. *Proteomics* 2007;7 Suppl 1:77-89).

Supplemental Data 2

Extended sample set used for CA125 threshold model analysis

	Median age	Mean age	SD	No.
Healthy	64.31	64.69	6.16	2236
Benign	57.96	57.32	13.25	290
Malignant	63.88	63.27	11.32	193
Stage I+II	64.41	63.52	11.87	93
Stage III+IV	63.85	63.04	10.84	100

Supplemental Data 3

Details on the MALDI MS sample preparation, data acquisition and pre-processing

Polypeptides were enriched from 5 μ L of serum sample using a semi-automated protocol based on reversed phase pre-packed tips (C18 ZipTips). A CyBi™-Disk robot (CyBio AG, Jena, Germany) equipped with a 96-piston head for 25 μ L-tips was adapted and used for this purpose. After C18 ZipTip purification, enriched polypeptides were eluted from the ZipTips, mixed with CHCA matrix and spotted in four replicates of 0.8 μ L onto a 600 μ m-AnchorChip™ target plate (Bruker Daltonics) and allowed to dry at room temperature.

Mass spectrometric serum polypeptide profiles were acquired on an Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Coventry, UK) in the linear mode. The overall performance of the mass spectrometer was thoroughly checked every 2-3 weeks using peptide standards and a tryptic digest of bovine serum albumin. In addition, a commercial human serum standard (Sigma, # S7023) (previously prepared using the ZipTip protocol, aliquotted and frozen) was used prior to each run for a general systems check. Using this standard serum sample, the resolution and intensity of five major peaks across the mass range of 1,800 to 8,200 Da were checked against previously obtained data and, if necessary, the laser power was adjusted to keep the intensity and resolution of these peaks within their predetermined range of values of ± 2 SD. All samples were analysed at least in triplicate on separate days. The same commercial, pooled serum standard as above was freshly prepared 3 times within each batch of serum samples as an internal quality control to assess intra- and inter-run variations. ClinProTools software V2.1 (Bruker Daltonics) was used for this purpose and allowed to calculate the coefficient of variation (CV) of each of the individual peak areas (more than 100 peaks per spectrum) as well as the mean CV and standard deviation for all of the peaks within the same run or between runs. Furthermore, the following criteria were checked for each sample: (1) for each run at least 3 out of the 4 spot replicates contained 1,000 laser shots and were not rejected on grounds of low analyte signal or excessively high matrix signal and (2) at least 2 different runs satisfied condition (1). However, all samples yielded sufficiently reproducible spectra and none of the samples was excluded.

More information can be found in the SOP below:

Standard Operating Protocol for Blood Serum Polypeptide Profiling Using MALDI-TOF Mass Spectrometry

(January 2009, University of Reading)

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1. Materials

1.1. Equipment

- Scissors
- Pipettors (Fisher Scientific, UK, cat. nos. PMP-124-020E, PMP-124-040V, PMP-124-080J, PMP-124-120A, PMP-133-010G)
- Barcode reader and software (Barcode Solutions, UK, cat. no. Z3071WA)
- Centrifuge
- Freezer at -80°C
- CyBi™-Disk liquid handling robot (CyBio, Northern Europe, cat. no. 0501115-00) housed in a cabinet
- Humidifier unit (Fakir, Germany, Ultronic LB10)
- Mini-centrifuge (Fisher Scientific, UK, cat. no. TEC-100-010Y)
- Vortex Mixer (Fisher Scientific, UK, cat. no. FB15024)
- Ultraflex MALDI-TOF mass spectrometer and FlexControl/FlexAnalysis software (Bruker Daltonics, Germany)
- AnchorChip-600 384-spot MALDI target plates (Bruker Daltonics, Germany, cat. no.209513)
- Laminar Flow Safety Cabinet
- Desk- or lap-top computer

1.2. Consumables

1.2.1. Blood collection and preparation (in clinical lab)

- Vacutainer SST tube (BD, USA, cat. no. 367988)
- Cryovials (straws)
- Pipettor tips: 10 µl, 100 µl, 1000 µl (Fisher Scientific, UK, cat. nos. PMP-885-501W, PMP-885-507K, PMP-885-511T)
- Cryoboxes for sample storage (Fisher Scientific, UK, cat. no. FB71035)
- Natural Multi PCR tubes: 0.2ml and 1.5ml (Bioquote, UK, cat. nos. 16950F and 16130)
- Ice
- Laboratory timer (Fisher Scientific, UK, cat. no. FB51458)

1.2.2. Sub-sampling (in clinical or MS lab)

- Human serum, pooled (Sigma-Aldrich, UK, cat. no. S7023)
- Pipettor tips: 10 µl, 100 µl, 1000 µl (Fisher Scientific, UK, cat. nos. PMP-885-501W, PMP-885-507K, PMP-885-511T)
- CyBi™ tips (CyBio, Northern Europe, cat. no. OL3800-25-533-N)
- Cryoboxes for sample storage (Fisher Scientific, UK, cat. no. FB71035)
- Natural Multi PCR tubes: 0.2ml and 1.5ml (Bioquote, UK, cat. nos. 16950F and 16130)
- Thermostrips + lids (Abgene Ltd, UK, cat. no. AB-0773)
- 96-well microtitre plates (Fisher Scientific, UK, cat. no. TUL-962-060A)
- Universal microtitre plate lids (Camlab Ltd, UK, cat. no. WN\77041001)

- Ice
- Virkon[®] disinfection solution (Fisher Scientific, UK, cat. no. HYG-205-030J)

1.2.3. Polypeptide extraction, purification and analysis (in MS lab)

- Acetonitrile Chromasolv Plus for HPLC (Sigma-Aldrich, UK, cat. no. 34998-1L)
- Acetone Chromasolv (Sigma-Aldrich, UK, cat. no. 650501-1L)
- Ethanol 99.8% (Fisher Scientific, UK, cat. no. E/0665/DF/15)
- α -cyano-4-hydroxycinnamic acid (CHCA) (Bruker Daltonics, Germany, cat. no. 201344)
- Human serum, pooled (Sigma-Aldrich, UK, cat. no. S7023)
- Protein and peptide calibration standards (Bruker Daltonics, Germany, cat. nos. 206355 and 222570)
- Trifluoroacetic acid (TFA) spectrophotometric grade (Sigma-Aldrich, UK, cat. no. 302031-100ml)
- Water HPLC grade (Rathburn Chemicals, UK, cat. no. RH1020)
- Pipettor tips: 10 μ l, 100 μ l, 1000 μ l (Fisher Scientific, UK, cat. nos. PMP-885-501W, PMP-885-507K, PMP-885-511T)
- CyBi[™] tips (Cybio, Northern Europe, cat. no. OL3800-25-533-N)
- ZipTips-C18 (Millipore, supplied by Fisher Scientific, UK, cat. no. FDR-597-040G)
- Ice
- Modular reservoir, quarter module (Beckman Coulter, UK, cat. no. 372788)
- Isofreeze PCR rack (Alpha-Laboratories, UK, cat. no. LW5990Y)
- 384-well plates (Greiner Bio-One, UK, cat. no. 781280X)
- Teflon reagent reservoirs (CyBio, Northern Europe, cat. no. OL3391-146-24)
- Small plastic bags and rubber bands for plate storage
- Natural Multi PCR tubes of 0.2ml and 1.5ml (Bioquote, UK, cat. nos. 16950F and 16130)
- 96-well microtitre plates (Fisher Scientific, UK, cat. no. TUL-962-060A)
- Universal microtitre plate lids (Camlab Ltd, UK, cat. no. WN\77041001)
- pH 0-6 indicator sticks (Fisher Scientific, UK, cat. no. FB33005)

2. Methods

Disclaimer: *This protocol has been developed and tested for use only in a research laboratory, not in a clinical environment. No claims are made for its usefulness, accuracy or safety, and no liability can be accepted for any damages, losses or other expenses of any nature whatsoever arising from its use or supply.*

Users of this protocol should have read and understood all relevant Risk and COSHH Assessments and SOPs, in particular health and safety rules and regulations and SOPs for the handling of blood and blood-derived samples such as SOP039 “Safety Considerations in the Handling of Serum Samples from the MRC Project” as used in the MRC-funded project G0301107 at the University of Reading.

2.1. Collecting blood samples and preparing serum

WARNING: *For all blood and blood-derived samples, always observe precautions: Handle all biological samples as a potential source of pathogens/hazards, use the appropriate protective attire (lab coats, safety glasses, nitrile gloves etc.) and dispose of all (bio)hazardous materials in an appropriate manner.*

1. Collect venous blood in a Vacutainer tube and label it with the corresponding barcode.
2. Gently invert the tube five times to mix the clot activator with the blood. Allow blood to clot for 1 h at room temperature keeping the tube in the upright position.
NOTE: *Blood should be allowed to clot for exactly 1 h. If not, artificial differences in the mass spectra of peptides will result from different clotting times of the samples.*
3. Keep the Vacutainer tubes on wet ice in the upright position until the serum separates (up to 2h).
4. Label cryovials (straws) with the corresponding barcode.
5. Spin Vacutainer tubes at 1,400 × g for 10 min at room temperature.
6. Transfer the serum (upper phase) to the appropriately labelled straws. The volume of serum per straw should be approximately 500 µl.
7. Immediately store all samples at –80 °C.

8. Transport samples to the MS laboratory on dry ice. On arrival they must immediately be stored at -80°C again.

NOTE: *Avoid freeze-thaw cycles at all steps because they induce proteolysis (in the absence of anti-proteases) and the precipitation of peptides/proteins.*

2.2. Sub-sampling of serum samples

NOTE: Before aliquotting serum samples, they must be randomised to avoid any systematic error due to the sample order or history.

2.2.1. Preparations for sub-sampling

WARNING: All sub-sampling must be carried out in an adequate safety cabinet. Equipment and consumables used for the purpose of sub-sampling should be housed inside the cabinet and should be labelled with biohazard labels (PCR tubes, thermostrips, solutions, scissors etc.). Before starting work, ensure that there are two 1-litre Virkon[®] solutions available that are less than one week old and still pink. One of the solutions should be placed in a wide-mouthed screw cap jar for disposal of plastic consumables, and the other should be in a screw cap bottle for dealing with accidental spills.

The amount of time that individual samples are outside of the -80°C freezer must be kept to a minimum. The barcode reader should be plugged in and ready to be used before samples are removed from the freezer. Lab books and any other stationery should be kept outside the safety cabinet with any pens, markers etc. that might be needed. The sample label file should be opened on a computer.

Consumables needed for each sample include 1 × 1.5ml-PCR tube, 1 × thermostrip and lid, up to 20 × 200µl-PCR tubes. A 96-well microtitre plate (parent plate) is used for up to 90 sample aliquots to be run on a liquid handling robot. The remaining 6 wells are used for control samples as detailed later. All sample vials, tubes and plates must be kept on wet ice during the entire sub-sampling procedure.

2.2.2. Sub-sampling from straws

9. Remove the first straw from the freezer, and scan the barcode on the straw directly into the sample label file, and check that the numbers appearing in this file are the same as those written beside the straw bar code.
10. Using the scissors, cut the straw just below the closure at the top (green plug end down). Dispose of the cut-off end of the straw in the Virkon[®] solution for disposables.
11. Invert the tube so that the green plug is now at the top and the other end in a 1.5mL-PCR tube. Cut the straw again, just below the white section and dispose of the cut-off piece in the Virkon[®] solution. Wash the scissors by spraying the blades with ethanol from a wash bottle.

12. Lift the straw slightly to allow the thawed serum to drain into the PCR tube. Use the pipettor with a tip set at 50 µl to blow air through the tube from the top down, in order to expel the last few drops of serum into the tube. Place the empty straw into the Virkon[®] solution. Use the pipettor with the same tip still attached to mix the contents of the PCR tube, to ensure that the whole sample is thoroughly thawed and homogeneous. Dispose of the pipette tip in the Virkon[®] solution.
13. Transfer 50 µl of serum to the corresponding well in the parent microtitre plate. From the remaining serum volume sub-sample as many aliquots as are required into 200µl-PCR tubes or into thermostrips (these can be later used for manual or single point analysis). A volume of 50 µl of sample will later provide sufficient aliquots for 9 child plates (5 µl per plate).

NOTE: *The overall available sample volume determines the number of child plates that can be generated. Each sample preparation requires a volume of 5 µl of sample, so a total sample volume of 50 µl will realistically provide not more than 9 child plates since $50 \mu\text{l} / 5 \mu\text{l} = 10$ if the entire volume can be utilised - which is, however, practically impossible.*

14. Ensure that all of the tube lids are firmly pushed down, then change gloves.
15. Record the details of the sample code, date, and number and size of aliquots taken.
16. Label all non-plate aliquots with the correct barcode and store them at -80 °C.
17. Repeat steps 9-16 for a total of up to 90 straws (i.e. one microtitre plate-load).
18. Add 50 µl aliquots of the standard pooled serum sample (thawed and homogenised) to 3 of the 6 empty wells on the microtitre plate. This standard serum will be used as initial control for the whole run. The 3 remaining wells are left empty so that there are free spots on the MALDI target plate. A post-preparation control sample is later spotted onto these empty sample spots (**see SST solution, step 31**).

NOTE: *Serum must be kept on ice during all aliquotting steps. Prior to use, the standard serum sample should be aliquotted at 200 and 500 µl into PCR tubes and stored at -80 °C until required.*

2.2.3. Sub-sampling from parent microtitre plate

NOTE: *The following example of sub-sampling utilises a CyBi™-Disk robot (CyBio, Northern Europe) equipped with a 96-piston head for 25µl-tips and 10 microtitre plate positions and specifically set-up for this task (**see Annexe 1**). Thus some steps will most likely differ when using another liquid handling robot.*

19. Open the customised method “Sub-sampling” from the CyBi™ control software. This method allows replicating the parent plate into 9 child plates transferring 5 µl each time (see **Annexe 2A**).
20. Load a new CyBi™ tips rack.
21. Place 9 empty 96-well microtitre plates on the CyBi™-disk platform at positions 2 to 10.
22. Place the serum parent plate on the platform at position 1 (see **Annexe 1**).
23. Check the simulation of the “Sub-sampling” method for errors, and then run it.
24. Cover all the plates with labelled lids and secure the lids with a rubber band. Put each plate inside a small clear plastic bag, and place the 9 bags together with a copy of the plate plan in a labelled sample box. Store at -80°C until required.

2.3. Preparing solutions

25. **2% TFA:** Mix 98 ml of HPLC grade water with 2 ml of TFA. Check that the pH is ca. 0.5.
26. **0.1% TFA:** Mix 95 ml of HPLC grade water with 5 ml of 2% TFA. Check that the pH is ca. 2.
27. **Elution solution (50% ACN/0.1% TFA):** Mix 50 ml ACN with 5 ml TFA 2% and 45 ml HPLC grade water.
28. **Matrix solution (0.5 mg/ml CHCA in ethanol:acetone; 2:1):** Add 1 ml of ethanol to an 1.5ml-PCR tube containing 3 mg of CHCA and mix until the CHCA is dissolved. Transfer this solution to a 20ml-Teflon bottle, and add 4 ml ethanol and 1 ml acetone. Vortex the solution and keep at 4 °C in the dark until needed.

***NOTE:** The solutions of 2% TFA, 0.1% TFA and 50% ACN/0.1% TFA should be replaced weekly. The matrix solution should be freshly prepared on the day of the experiment.*
29. **Virkon® solution:** Add 10 g of Virkon® powder to 1 l of water. Mix thoroughly until completely dissolved to form a pink coloured solution. Replace the solution when the pink colour has gone.
30. **Polypeptide Calibrant Standard (PCS) solution:** Add 125 µl of the 0.1% TFA solution to a vial of peptide calibration standard II and vortex thoroughly to solubilise the peptides.

Do the same with a vial of protein calibration standard I. Transfer 25 µl from the first vial to a PCR tube and add all the contents of the second vial plus 600 µl of 0.1% TFA. Mix and aliquot at 4 µl. Store the aliquots at -20°C (**see Annexe 3 for a list of PCS components**).

31. **System Suitability Test (SST) solutions:** A 96-well plate containing 5 µl of the standard serum sample in each well is prepared using the protocol detailed in section 2.4, i.e. steps 32-63, for 96 samples. The 96 eluates are pooled, mixed and then aliquotted at 4 µl. Aliquots are stored at -80 °C until required.

NOTE: SST solutions are used as controls to check both the reproducibility of performance of the mass spectrometer (see Section 2.5.1, step 77, and Annexe 6) and the sample preparation (see Section 2.5.2.1).

2.4. Extracting polypeptides from serum

NOTE: This protocol utilises a CyBi™-Disk robot (CyBio, Northern Europe) equipped with a 96-piston head for 25µl-tips and 10 microtitre plate positions and set-up for this task (**see Annexe 1**). Some steps in the protocol will therefore differ when using another liquid handling robot. Duplicate MALDI target plates are spotted with the prepared samples to provide a back-up in case of problems during the MS acquisition or one-off irregular MALDI sample crystallisations.

2.4.1. Preliminary preparations

32. Turn on the CyBi™-Disk robot.
33. If the ambient relative humidity is less than 50%, increase relative humidity to around 50%, e.g. turn on a humidifier, set it to 50%, turn on a circulating fan in the CyBi™ cabinet and close the cabinet door.
34. Ensure the solvents and other solutions are less than 1 week old and check that the MALDI targets are clean (follow the manufacturer's protocols for cleaning the MALDI targets). Place the targets on the platform at positions 8 and 10, covered with the lid.
35. Check that the isofreeze racks are frozen and keep them in the freezer until required.
36. Prepare the matrix solution and keep it in the fridge until needed.
37. Transfer the ZipTips to their special CyBi™ holder.

38. Print the 96-well sample plate plan and mark the positions on the CyBi™ magazine that are to be kept clear for SST spotting. Replace the corresponding tips with sawn-off tips so that nothing is aspirated and dispensed by them.

NOTE: Ensure that all details of the sample preparation are recorded in the lab book, especially noting any deviation from this protocol and important observations made during the procedure.

2.4.2. Aliquotting ACN

39. Open the method “setup ACN” in the CyBi™ Control software (see **Annexe 2B**).

40. Load CyBi™ tips for pipetting ACN.

41. Place a Greiner 384-well plate on the platform at position 3 (see **Annexe 1**).

42. Place the empty bulk reservoir on the platform at position 9 and carefully fill with ca. 20 ml ACN.

43. Check the method “setup ACN” for errors and run it.

44. Cover the 384-well plate with a lid until needed.

45. Carefully remove the bulk reservoir and dispose of the excess ACN to the waste solvent bottle.

NOTE: Tips and plates used for ACN aliquotting can be reused for the same purpose.

2.4.3. Aliquotting 0.1% TFA

46. Open the method “setup TFA” in the CyBi™ Control software (see **Annexe 2C**).

47. Load CyBi™ tips for pipetting TFA.

48. Place a Greiner 384-well plate on the platform at positions 4 and 5 (see **Annexe 1**).

49. Place the empty bulk reservoir on the platform at position 9 and carefully fill with ca. 20 ml of 0.1 % TFA.

50. Check the method “setup TFA” for errors and run it.

51. Cover both 384-well plates with lids until needed.

52. Carefully remove off the bulk reservoir and dispose of the excess 0.1% TFA in the sink.
53. Keep the CyBi™ tips for the next step.

2.4.4. Preparation of plates, prior to extraction

54. Pour ca. 5 ml of the 2% TFA solution into the front section of a modular reservoir. Using a multi-channel pipettor, pipette 10 µl of the 2% TFA solution into each well of a 96-well microtitre plate. Place the plate on the platform at position 1 and cover it with a lid until needed.

2.4.5. Sample clean-up and MALDI sample preparation

55. Record the batch name of the sample (plate) to be run (including the date of preparation) on the plate plan and make a copy of the plan.
56. Remove the sample plate to be run from the freezer and place it on the platform at position 2.

***NOTE:** If for any reason the run is delayed, keep the sample plate on an isofreeze pack, covered with a lid until ready to use.*

57. Open the method “Sample Run” in the CyBi™ Control software and check it for errors.
58. Remove the lids from the plates on the CyBi™-Disk platform.
59. Run the method “Sample Run” **(see Annexe 2D for details)**.
60. When prompted, remove the CyBi™ tips, discard them, and load the ZipTips in their special holder.
61. After loading the samples, the ZipTips are washed with 0.1% TFA. During this washing step prepare the eluent plate as follows: Pour ca. 5 ml elution solution into the front section of a modular reservoir. From this reservoir use a multichannel pipettor to pipette 7 µl into each well of the 96-well plate and when prompted place the plate on the platform at position 6.
62. During the subsequent elution step prepare the matrix plate as follows: Pour ca. 5 ml matrix solution into the front section of a modular reservoir. From this reservoir use a

multichannel pipettor to transfer 18 μl into each well of the 96-well plate. Cover the plate with a lid and place it on an isofreeze pack until needed.

63. When prompted to change tips, remove and discard the ZipTips. Replace with a new set of CyBiTM tips, where the positions to be used for SST samples have been replaced by sawn-off tips. Place the matrix plate on the platform at position 7. Remove the lids from the matrix plate and MALDI targets.
64. Once the CyBiTM-Disk robot has sampled the eluates, remove the eluate plate from the platform, cover it with a pre-labelled lid and place it on an isofreeze pack. Mark the lid and plastic bag with "ELUATE". Secure the lid with a rubber band, and place the eluate plate back into the bag, together with a copy of the plate plan. Store the bag in the -80 freezer for future use.

NOTE: Ensure that the robot cabinet is only opened for the minimum time necessary, to keep the humidity unchanged. Record the humidity at the time of eluate spotting.

2.4.6. MALDI calibrant and control sample preparation

65. Keep the robot cabinet closed until the automatically spotted samples are completely dry.
66. Take a 4 μl aliquot of each of the SST and PCS solutions from the freezer and allow to thaw briefly. Spin the solution tubes to ensure that all of the tubes' contents are at the base of the tubes.
67. Transfer 27 μl of the matrix solution from the bottle to two 200 μl -PCR tubes, and close the lids tightly.
68. Add a 3 μl aliquot of the PCS solution to one of the tubes containing matrix solution, and mix thoroughly.
69. On both MALDI target plates spot 0.8 μl of the mixture onto each of 10 random calibrant positions. These spots are used for external calibration of the MS. The amount of each polypeptide spotted is ca. 10 fmol for peptides and ca. 50 fmol for proteins.
70. Add a 3 μl aliquot of the SST solution to the other tube containing matrix solution, and mix thoroughly.

71. On both MALDI target plates spot 0.8 µl of the mixture onto each of the positions (12 per MALDI target plate) marked for SST on the plate plan. These spots will be used to assess the performance of the MS analysis (see details below in section 2.5.2).
72. Close the robot cabinet immediately to maintain the humidity level and keep it closed until the spots are completely dry.
73. Turn off the robot, the humidifier and the re-circulating fan inside the robot cabinet (if used).

NOTE: For thorough calibration across the entire MALDI target plate, the method can be modified to allow automated spotting of PCS solution from a 96-well microtitre plate placed at position 9 on the CyBi™-Disk.

2.5. MALDI MS data acquisition and analysis

2.5.1. Data acquisition

NOTE: This protocol uses an Ultraflex II MALDI-TOF/TOF instrument (Bruker Daltonics) controlled by FlexControl software v.3.0 (Bruker Daltonics) with samples spotted on AnchorChip™ target plates having 384 anchors of 600µm diameter.

74. Open the FlexControl software and load optimised FlexControl and AutoXecute methods (see Annexes 4 and 5 for examples as used in the BioCentre at the University of Reading). These MALDI-TOF settings must be optimised for each individual instrument.
75. Take the first target plate from the robot and insert it into the MALDI-TOF mass spectrometer. The plate should be analysed by the mass spectrometer within 2 h of MALDI sample spotting. Keep the second target plate covered for possible later use.
76. Calibrate the mass spectrometer using a single calibrant spot containing the PCS mixture (see Annexe 3). Data should be collected automatically by clicking on the tab “Run method on current spot”.
77. Collect spectra from an SST spot to check the performance of the mass spectrometer. The signal-to-noise ratio and resolution of five well-selected peaks should be within their predetermined range of values of ± 2 SD (see Annexe 6). If this is not the case, re-adjust the laser power accordingly and collect spectra from another SST spot to fulfil

these specifications. Subsequently, data should be collected automatically by clicking on the Tab “Run method on current spot”.

78. Load the correct autoXsequence file (containing the names and positions of each sample on the target as well as the file name and path where the spectra will be saved on the hard disk). This file can be generated by using an Excel spreadsheet from the sample plate plan initially prepared. Save the file in text format and copy it into the AutoXecute sequence folder on the data drive of the Ultraflex desktop computer.
79. Start the automatic run by clicking on the tab “Start automatic run”. Data collection for a full 384-spot plate takes about 6 h.

2.5.2. Data evaluation, processing and preliminary analysis

NOTE: This protocol uses FlexAnalysis 3.0 and ClinProTools 2.1 software (Bruker Daltonics).

2.5.2.1 Quality control and data filtering/processing

80. Open the FlexAnalysis program once the data acquisition is complete. From the “Process” tab select “Batch check”. Load the folder containing all acquired spectra and set “the expected number of shots” to 1,000 and click OK. This will generate a list of any spectra having less than 1,000 shots. Any spectra with less than 1000 shots should be excluded from further processing.

NOTE: Poor quality spectra can often result from deterioration of the MALDI target anchors and/or poor crystallisation. Utilising the second MALDI target plate by acquiring spectra from the spots that are the duplicates of the spots that gave poor quality spectra at step 80 can sometimes give acceptable data for these samples.

81. Within the directory containing the MALDI files, create a sub-directory and name it “1000shots”. Transfer any spectra that gave less than 1000 shots to this directory. These spectra are excluded from further data processing and analysis.
82. Open the ClinProTools program to give a display with four quadrants. From the File tab, select “Open Model Generation Class” and browse to find the sub-directory containing all spectra files of the SST samples. Each of the SST spectra is then loaded into the software. Individual or average spectra are depicted in the top left quadrant, and all of the loaded spectra are depicted in a pseudo-gel view in the bottom left quadrant.

NOTE: The bottom left quadrant of the ClinProTools display depicts the result of clustering while the top right quadrant gives information about different models (see section 2.5.2.2).

83. Select “Open Model Generation Class” again and this time browse to find the sub-directory containing all the spectra given by the standard serum samples prepared with the batch of real samples. These spectra will be depicted in the pseudo-gel view alongside the spectra of the SST samples, thus allowing a visual comparison of their spectra. The 2 classes should give virtually identical spectra, in which case the overall preparation of the batch of samples is deemed acceptable. If the spectra are markedly different then further investigation will be needed to determine the cause.
84. Each sample preparation should yield a maximum of 4 spectra, depending on the number of spectra excluded at step 81 (if any). The spectra for each individual sample are loaded as a class into ClinProTools, and are visually inspected to determine any obvious differences within that class. If differences are found it may be necessary to exclude further individual spectra on the basis of poor quality.

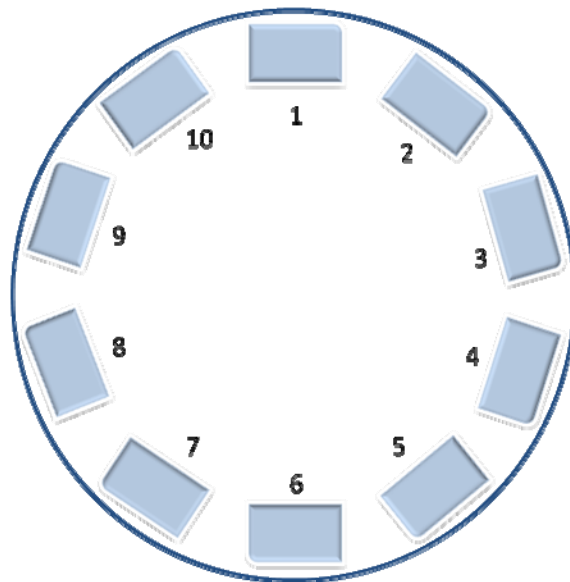
NOTE: If the number of replicate spectra per sample falls below 3 as a result of spectra exclusion, it may be necessary to repeat the sample preparation.

2.5.2.2 Preliminary Data Analysis

85. ClinProTools can also be used to identify peaks that are significantly different (in intensity) between 2 or more classes of spectra, by using the clustering feature. It is also possible to use the program to generate other classification models based on lists of discriminatory peaks. However, this higher level of data analysis and data mining is beyond the scope of this protocol.
86. ClinProTools is just one example of the large number of different bioinformatics programs that can be used for the more detailed analysis of the data. For compatibility with these programs spectra can be exported as tab-separated text files that contain the mass list with the corresponding intensities. This export facility is available within FlexAnalysis.

3. Annexes

Annexe 1. CyBi™-Disk plates plan



Position on CyBi™-Disk	Sub-sampling	ACN aliquotting	0.1% TFA aliquotting	Sample Cleaning and spotting
1	Parent 96-well MTP			2% TFA, 96-well MTP
2	Child 96-well MTP			Sample, 96-well MTP
3	Child 96-well MTP	384-well MTP		ACN, 384-well MTP
4	Child 96-well MTP		384-well MTP	0.1% TFA, 384-well MTP
5	Child 96-well MTP		384-well MTP	0.1% TFA, 384-well MTP
6	Child 96-well MTP			Eluate, 96-well MTP
7	Child 96-well MTP			Matrix, 96-well MTP
8	Child 96-well MTP			MALDI target 1
9	Child 96-well MTP	Bulk reservoir	Bulk reservoir	Empty*
10	Child 96-well MTP			MALDI target 2

** This position can be used for additional solutions such as calibrant solutions for automatic large-scale spotting of calibration samples.*

Annexe 2. CyBi-Disk™ Methods

(A): “Sub-sampling” method used to sub-sample serum from a parent microtitre plate:

1. Aspirate 5 µl aliquots of the serum samples from the parent plate at position 1 and dispense them into the equivalent wells of the child plate at position 2.
2. Repeat step 1 eight times each time dispensing the sample aliquots into the next sequential child plate (positions 3 to 10).

(B): “Setup ACN” method used to aliquot ACN into a 384-well microtitre plate:

NOTE: Each well of a 96-well microtitre plate is equivalent to a set of 4 wells (A, B, C, D) on the 384-well microtitre plate. The aliquot of ACN is delivered to well A and the other 3 wells are left empty to receive the waste during the ZipTip washing.

1. Aspirate 96 aliquots of 25 µl of ACN from the bulk reservoir at position 9 and dispense them into the 384-well microtitre plate (position 3 on the CyBi platform) at well A.
2. Repeat step 1 so that the total volume in each well A is 50 µl.

(C): “Setup TFA” method used to aliquot ACN into a 384-well microtitre plate:

NOTE: Each well of a 96-well microtitre plate is equivalent to a set of 4 wells (A, B, C, D) on the 384-well microtitre plate. The aliquot of 0.1% TFA is delivered to well A and the other 3 wells are left empty to receive the waste during the ZipTip washing.

1. Aspirate 96 aliquots of 25 µl of TFA 0.1% from the bulk reservoir at position 9 and dispense them into the first 384-well microtitre plate (position 4 on the CyBi platform) at well A.
2. Repeat step 1 so that the total volume in each well A is 50 µl.
3. Aspirate 96 aliquots of 25 µl of TFA 0.1% from the bulk reservoir at position 9 and dispense them into the second 384-well microtitre plate (position 5 on the CyBi platform) at well A.
4. Repeat step 3 so that the total volume in each well A is 50 µl.

(D): “Sample run” method used to extract polypeptides from serum samples:

1. Add 5 μ l 2% TFA to the samples in the 96-well MTP and mix 5 times by pipetting 8 μ l up and down.
2. Replace manually the CyBi[™] tips with a rack of 96 ZipTips.
3. Condition the ZipTips 3 times with ACN by aspirating 10 μ l ACN and dispensing it to the ACN waste wells.
4. Equilibrate the ZipTips 3 times with 0.1% TFA by aspirating 10 μ l of 0.1% TFA and dispensing it to the TFA waste wells.
5. Load the samples onto ZipTips by aspirating and dispensing 10 \times 10 μ l of the sample mixture. (Aspirating and dispensing of samples needs to be slow because of the viscosity of serum and the back pressure due to the packed phase in the ZipTips). An overstroke of 1 μ l is used at the last dispensing step to expel any remaining drops of serum from the ZipTips.
6. Wash the ZipTips by aspirating and dispensing 3 \times 10 μ l of 0.1% TFA. Each time the solution is dispensed into a new waste well to avoid cross-contamination. An overstroke of 1 μ l is also used at the last dispensing step to expel any remaining drops of wash solution from the ZipTips.
7. Elute the analytes from the ZipTips by aspirating and dispensing the eluent (7 μ l of 50% ACN / 01.% TFA) 5 times into the same well of the eluate plate.
8. Replace manually the ZipTips with a rack of 96-CyBi[™] Tips.
9. Transfer 2 μ l of the eluate to the matrix plate wells. Mix the eluate with the matrix solution by aspirating and dispensing 5 \times 15 μ l.
10. Aspirate 10 μ l of the eluate/matrix mixture solution and spot 0.8 μ l onto each of 4 subsequent positions of the MALDI target plate, so that there are 4 replicates for each prepared sample.

Annexe 3. Polypeptide Calibrant List

Polypeptide	Ion type	Average mass m/z
Peptide calibration standard II mixture		
<i>(Bruker Daltonics, #222570)</i>		
Bradykinin Fragment 1-7	[M+H] ⁺	757.858
Angiotensin II	[M+H] ⁺	1047.189
Angiotensin I	[M+H] ⁺	1297.486
Substance P	[M+H] ⁺	1348.642
Bombesin	[M+H] ⁺	1620.860
Renin Substrate Tetradecapeptide Porcine	[M+H] ⁺	1760.026
ACTH clip 1-17	[M+H] ⁺	2094.427
ACTH clip 18-39	[M+H] ⁺	2466.681
Somatostatin 28	[M+H] ⁺	3149.574
Protein calibration standard I mixture		
<i>(Bruker Daltonics, #206355)</i>		
Insulin	[M+H] ⁺	5734.56
Ubiquitin I	[M+2H] ²⁺	4283.45
Cytochrome C	[M+2H] ²⁺	6181.05
Ubiquitin I	[M+H] ⁺	8565.89

Annexe 4. FlexControl settings optimised for the analysis of polypeptides prepared from serum samples

The screenshot shows the 'Spectrometer' tab in the FlexControl software. The 'High Voltage' section is active, showing a 'Switched On' status and a 'READY' indicator. The settings are as follows:

- Ion Source 1: 25.00 (set) / 25.15 (actual) kV, set relative
- Ion Source 2: 23.70 (set) / 23.85 (actual) kV, 94.8 % IS1
- Lens: 6.00 (set) / 6.03 (actual) kV, 24.0 % IS1

The 'Pulsed Ion Extraction' section is set to 100 ns. The 'Polarity' is set to Positive. The 'Matrix Suppression' section has the following settings:

- Mode: Off, Deflection, Gating
- Gating strength: low, medium, high, maximum
- Suppress up to: 400 Da

A 'Calibration >>>' button is located at the bottom of the Matrix Suppression section.

The screenshot shows the 'Detection' tab in the FlexControl software. The settings are as follows:

- Mass Range:** A horizontal bar shows a range from 700 to 10000 Da. The 'Mode' is set to Low Range, Medium Range, and High Range.
- Detector Gain:** A linear scale from 1 x to 100 x is shown, with the current setting at 7.5 x.
- Sample Rate:** A scale from 0.05 to 2.00 GS/s is shown, with the current setting at 0.50 GS/s. A graph below shows the relationship between Mass Range and Resolution.
- Electronic Gain:** Buttons for Regular, Enhanced, and Highest are shown, along with a 100 mV setting.
- Realtime Smooth:** Off, High, Medium, Low.
- Spectrum:** Size: 32185 pts, Delay: 11783 pts.

AutoExecute Sample Carrier Spectrometer Detection Processing **Setup** Calibration Status

Mass Range Selector

Low Range
 Medium Range
 High Range

Define...

Laser

Frequency: 25.0 Hz

Automation

Autoteaching

Instrument specific settings

Digitizer

Trigger Level: 2000 mV
Digital Off Linear: 127 cnt
Digital Off Reflector: 127 cnt

Detector Gain Voltage Offset

Linear: 1300.00 V
Reflector: 1400.00 V

Laser Attenuator

Offset: 69 %
Range: 15 %

Laser Focus

Offset: 1 %
Range: 98 %
Value: 100.0 %

Electronic Gain Button Definitions

Gain	Offset Lin	Offset Ref	
regular:	100 mV	100 mV	200 mV/full scale
enh.:	50 mV	50 mV	100 mV/full scale
highest:	25 mV	25 mV	50 mV/full scale

current_settings_080723dak.isset

Save As... Load...

Annexe 5.

FlexControl autoXecute method optimised for the automatic analysis of polypeptides prepared from serum samples

The image displays three overlapping screenshots of the FlexControl software interface, showing different configuration tabs for an autoXecute method.

Top Screenshot (Evaluation Tab):

- Peak Selection:** Use masses from 1000 Da to 9000 Da for evaluation and processing. Use mass control list: none for evaluation and processing.
- Peak Exclusion:** Ignore the 2 largest peaks in the defined mass range.
- Peak Evaluation:** Processing Method: SN_equals_2. Smoothing: Off. Baseline Subtraction: Off. Peak Resolution must be higher than 300.
- Fuzzy Control:** Digest/Peptides (selected), Signal Intensity: Low, Maximal Resolution: 10 times above threshold.

Middle Screenshot (Laser Power Tab):

- Laser Power:** Fuzzy Control: Parent Mode: Off, Weight: 1.00. Fragment Mode: Off. Use: Initial laser power on new raster spot. Initial Laser Power: 32% or from Laser Attenuator. Maximal Laser Power: 65%.
- Matrix Blaster:** Fire initially 10% shots with a laser power of 37%.

Bottom Screenshot (Accumulation Tab):

- Fuzzy Control:** Parent Mode: On. Sum up 1000 satisfactory shots in 100 shot steps. Allow only 100 satisfactory shots per raster spot. Fragment Mode: Off. Sum up 300 satisfactory shots in 100 shot steps. Allow only 100 satisfactory shots per raster spot.
- Dynamic Termination:** Dynamic Termination: Off.
- MS:** Early Termination if reaching Signal/Noise value of 100 for this number of peaks 1.
- MS/MS:** Early Termination if reaching Signal/Noise value of 100 for this number of peaks 1.

Right Screenshot (Movement Tab):

- Random walk:** Shots at raster spot: 10.
- Measuring raster:** hexagon. Cyclic: Off.
- Maximal allowed shot number at one raster position:** Parent Mode: 100, Fragment Mode: 300.
- Ignore maximal shot number if signal is still good:** Parent Mode: On, Fragment Mode: Off.
- Quit sample after:** 20 subsequently failed judgments.

Annexe 6.

Peak list and parameters used for assessing the performance of the MS analysis (SST) based on a reference spectrum of a standard serum sample

m/z	Signal/Noise			Resolution		
	Mean (M)	M-2σ (min)	M+2σ (max)	Mean (M)	M-2σ (min)	M+2σ (max)
1808.82	1919	1214	2624	184	177	190
2981.54	2841	2399	3283	234	226	241
5003.29	4159	3330	4988	293	286	300
6447.50	959	749	1169	261	224	298
8126.35	1344	1073	1616	146	114	177

