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ICM-MS phenotyping in applied animal sciences: Two examples

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ICM-MS phenotyping in applied animal sciences: Two examples

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- Basic and applied animal sciences
- Reproduction and Behaviour
- Infectiology and Public Health
- Avian Research Unit



Intact Cells MALDI-TOF MS

- Identification of cell-specific peptide/protein MS fingerprints
- “Intact cell” means that whole cells are subjected directly to MS analysis without any preparatory steps
- Widely used in microbiology for biotyping
- Starting to be used in pathology and diagnostics

1

Phenotyping
chicken male
fertility



Why phenotyping male fertility?

Sperm quality tests evaluate **individual sperm cells functions**:
motility, integrity, acrosome reaction, etc.

Often **poorly correlated** with *real* fertility

A **new paradigm of sperm quality testing** is needed: global evaluation
of sperm functions

Background: Pilot study by Labas et al., 2014:

-Intact cell MALDI-TOF mass spectrometry (**ICM-MS**) = fast, reliable and relatively inexpensive tool to phenotype male fertility



molecular basis of fertility (top-down HRMS)

OBJECTIVES:



- 1- Acquisition of sperm cells ICM-MS profiles in a standardized and automated way.
- 2- Evaluation of the diagnostic performance of ICM-MS profiles comparison to predict fertility.
- 3- Identification of the endogenous peptides/proteins represented in the sperm cells ICM-MS spectra, particularly those linked with fertility.

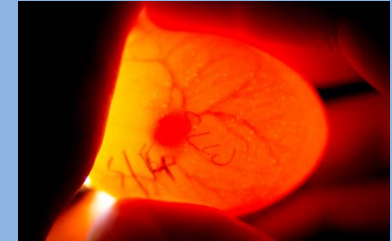
Animal model and reference fertility rates

Our model is.....the chicken!!!



72 roosters of 2 divergent pure genetic lines: meat line (n=36) and a egg laying line (n=36).

Fertility rates (% fertile/incubated eggs) : artificial insemination 10 females/male.



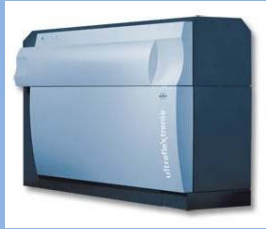
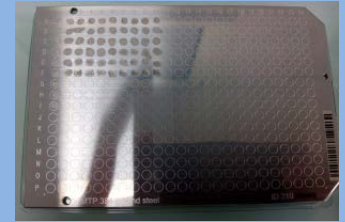
Fertility rates
cut-off values



Intact Cells MALDI-TOF MS

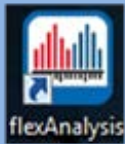
10⁶ of fresh, washed (Tris-Sucrose) sperm cells + Matrix (20mg/mL Sinapinic acid, 2% TFA, 50% ACN)

12 spots / sample, 3 consecutive runs ---- 36 technical replicates



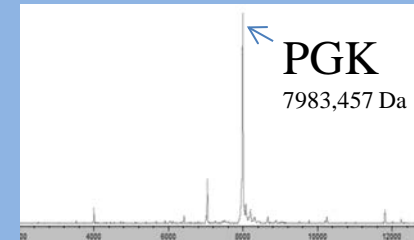
3 ejaculates per male (high day-to-day variability)

Spectra automatic acquisition in a Bruker UltrafleXtreme MALDI-TOF instrument (mass range 1-20 kDa).



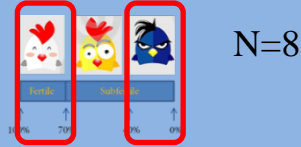
External calibration : Peptide/protein mix calibrant

Internal calibration: Lock-mass using peak 7983,457 Da



Pre-processing: ClinProTools 3.0 (Bruker)

Model construction



Quick Classifier
Supervised Neural Network
Genetic Analyser

Validation



Classification as Fertile
or Subfertile by >50%
spectra from each male

*Fertility-
predictive models
construction
using
ClinProTools 3.0*

Evaluation

Recognition capability: Ability
of each model to correctly
identify its component spectra

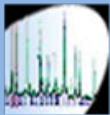
Cross-validation: Ability to
handle variability among spectra

Diagnostic Accuracy

Sensitivity / Specificity

Positive and negative
predictive values

Positive and negative
likelihood ratios



In-vitro sperm quality tests

- Viability
- Subjective mass motility evaluation
- Objective motility evaluation measured with computer-assisted sperm analysis (CASA) system with an HTM-IVOS

3 ejaculates per male

Diagnostic accuracy

Sensitivity / Specificity

Positive and negative predictive values

Positive and negative likelihood ratios

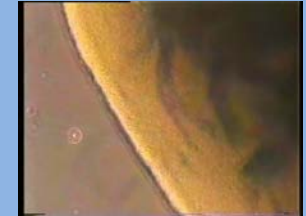


Viable

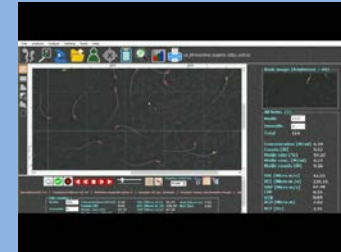
Not viable



Mass motility = 1/5



Mass motility = 5/5



CASA System

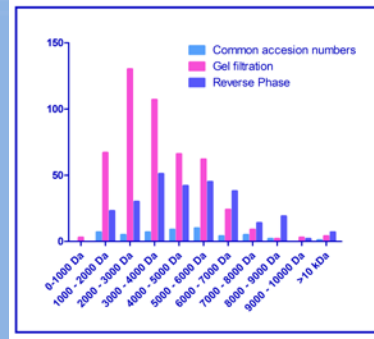
Top-down HRMS protein identification

Sperm cells
protein extracts
Tris-Urea 6M
Sonication

Fractionation



Reverse Phase Chromatography
XBridge BEH C18, Waters



Gel Filtration Chromatography
Superdex 75 10/300 GL, GE Healthcare

μ LC-MS/MS:



Monolithic PS-DVB
PepSwift, Dionex

LTQ Orbitrap Velos,
Thermo Scientific

Target resolution 100000

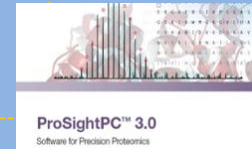
Range 400-2000 m/z

Isolation: top 10 most intense ions with charge states ≥ 2

Fragmentation/ HCD 38% Collision energy

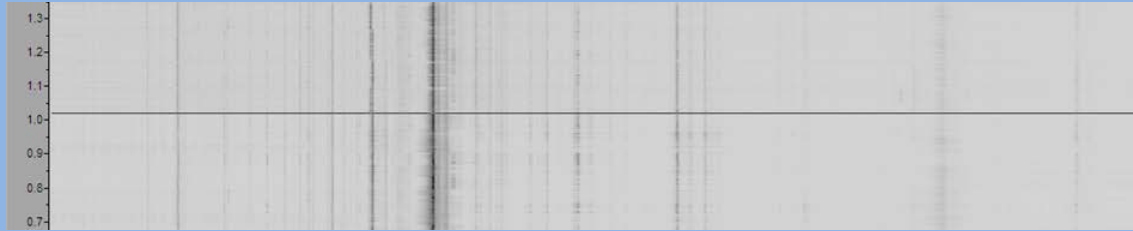
Ion selection thr: 500 counts; isol: m/z 3

Identification and structural characterization:
Search 1: Monoisotopic mass, 10ppm tolerance
Search 2: Average mass, 3 kDa tolerance
Search 3: Average mass, 25k Da tolerance
 p -score $\leq 1 \times 10^{-5}$



ICM-MS- based models vs. Traditional tests

85,5 % of shared m/z masses but differences in **TIC** values : no common model



Meat line (GA)

| Recognition Capability | Cross Validation |
|------------------------|------------------|
| 99.4 % | 93.48 % |

| Parameter | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | LR+ | LR- |
|---------------|-----------------|-----------------|---------|---------|-----|-----|
| ICM-MS | 84.6 | 55.0 | 55.0 | 84.6 | 1.9 | 0.3 |
| Mass motility | 69.2 | 55.0 | 50.0 | 73.3 | 1.5 | 0.6 |
| Viability | 76.9 | 35.0 | 43.5 | 70.0 | 1.2 | 0.7 |
| % Motiles | 61.5 | 40.0 | 40.0 | 61.5 | 1.0 | 1.0 |

Laying line (SNN)

| Recognition Capability | Cross Validation |
|------------------------|------------------|
| 92.28 % | 80.3 % |

| Parameter | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | LR+ | LR- |
|---------------|-----------------|-----------------|---------|---------|-----|-----|
| ICM-MS | 100.0 | 75.0 | 53.3 | 100.0 | 4.0 | 0.0 |
| Mass motility | 71.4 | 78.6 | 45.5 | 91.7 | 3.3 | 0.4 |
| Viability | 71.4 | 57.1 | 29.4 | 88.9 | 1.7 | 0.5 |
| % Motiles | 71.4 | 78.6 | 45.5 | 91.7 | 3.3 | 0.4 |

ICM-MS: *Global analysis* of sperm cells functions

119/256
m/z
masses
identified

GO - Biological Process

Respiratory Electron Transport Chain

Cellular Metabolic Process

Small Molecule Metabolic Process

Protein Polymerization

Microtubule Cytoskeleton Organization

Microtubule-based Process

GO - Molecular Function

Structural Constituent of Cytoskeleton

ATPase Activity

Proton-transporting ATP Synthase Activity, Rotational Mechanism

Transmembrane Transporter Activity

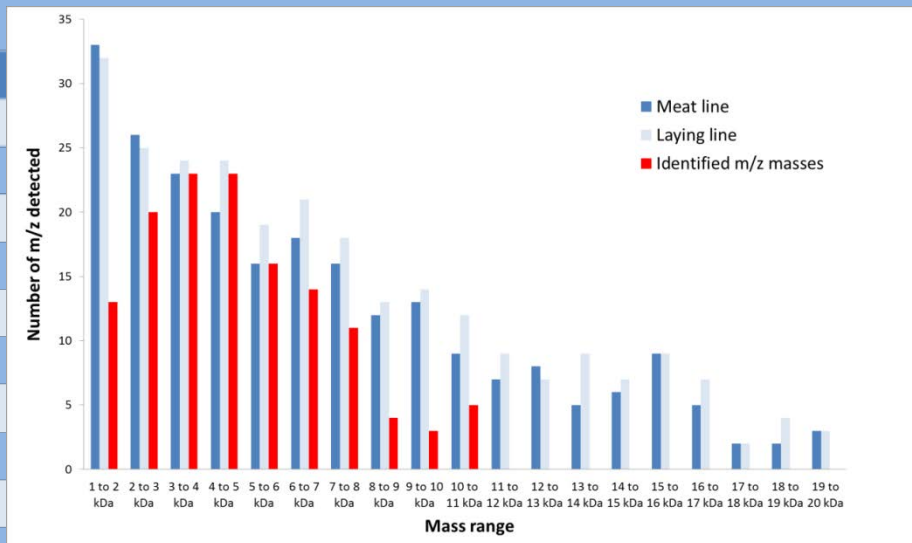
Creatine Kinase Activity

MHC Class II Protein Complex Binding

Protein Binding Involved in Protein Folding

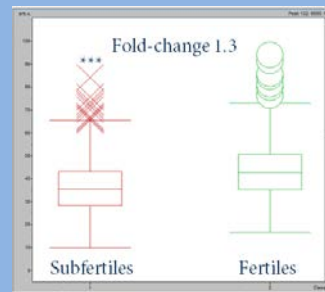
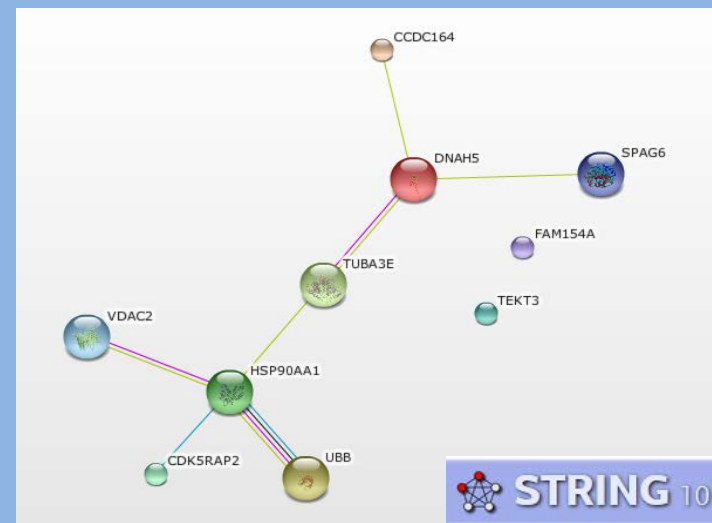
ATP Binding

Proton-transporting ATPase Activity, Rotational Mechanism



Masses employed to generate models 12/27

| Mass (kDa) | Gene Name | Ratio SubF/F | Protein name | Role associated with sperm cells functionality |
|------------|-----------|--------------|---|--|
| 7035,24 | LRD | 1,8 | Left-right dynein protein | Flagellar motility |
| 6539,45 | TUBA3E | 1,7 | Tubulin, Alpha 3e | Cell integrity and Flagellar motility |
| 5226,48 | CDK5RAP2 | 1,6 | CDK5 regulatory subunit associated protein 2 | Centrosome organization during fertilization |
| 6695,03 | HSP90AA | 1,3 | Heat shock protein 90 alpha | Chaperone, stabilisation of key proteins |
| 2574,18 | TEKT3 | 1,2 | Tektin 3 | Flagellar motility |
| 8569,95 | UBB | 1,2 | Ubiquitin | Protein degradation |
| 4538,03 | SPAG6 | 1,1 | Sperm associated antigen 6 | Cell integrity and Flagellar motility |
| 4010,91 | FAM154A | 1,05 | Family with sequence similarity 154, member A | Flagellar motility |
| 6615,77 | VDAC2 | 0,9 | Voltage-dependent anion channel | Motility/capacitation/fertilization |
| 4777,2 | DRC1 | 0,9 | Dynein Regulatory Complex Subunit 1 Homolog | Flagellar motility |
| 2400,65 | FER1L4 | 0,8 | Fer-1-like protein 4 / Otoferlin | Membrane traffic/Sperm activation |
| 2187,08 | LV1L2 | 0,8 | Ig lambda chain V-1 region-like | Motility |



Immunoblotting



Subfertiles Fertiles

Fold-change 1.6

Conclusions

Fertility-predictive models can be generated from sperm cells ICM-MS spectra with high diagnostic performance

Allows for global testing of different sperm cell functions

Interpretation of molecular basis of fertility





2

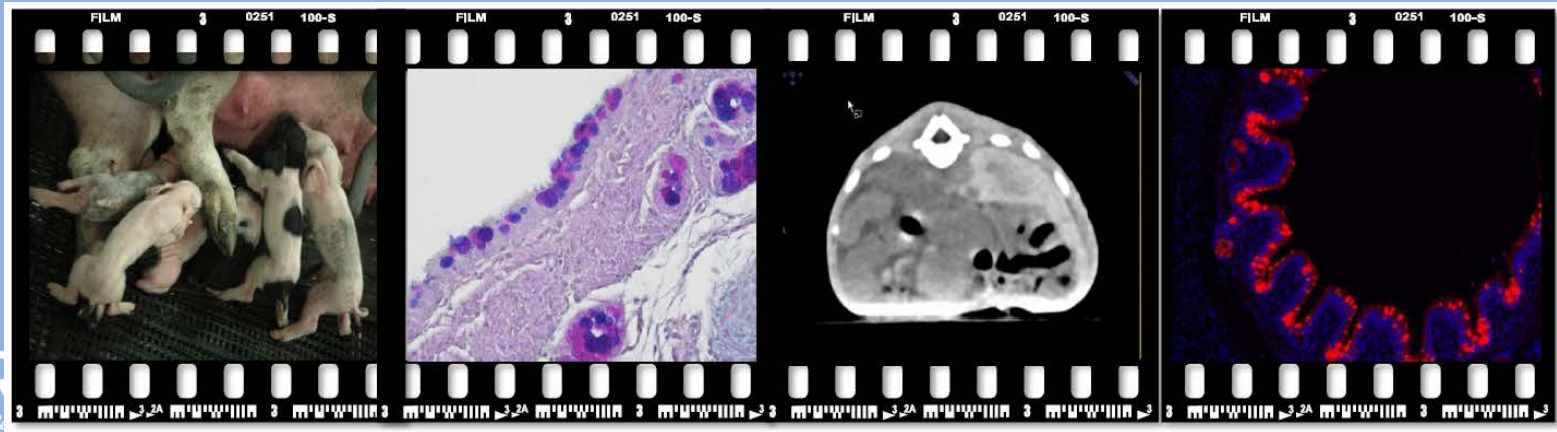
Phenotyping of
Swine $CFTR^{+/+}$
vs. $CFTR^{-/-}$
neutrophils

Example 2: Swine Cystic Fibrosis-mutant neutrophils phenotyping (*Ignacio Caballero-Posadas*)

Caused by the presence of mutations in both copies of the gene for the cystic fibrosis transmembrane conductance regulator (CFTR) protein.

Affects mostly the lungs but also the pancreas, liver, kidneys, and intestine.

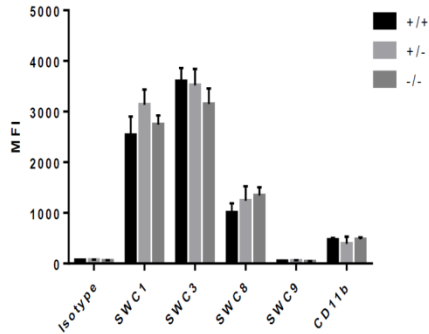
Pig transgenic model $CFTR^{-/-}$



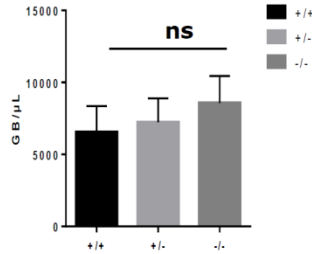
Altered neutrophil function?

- Presence of CFTR → Phagolysosome (Painter et al. 2006, 2008, 2010)
- Defective killing (chlorination phagolysosome)
- Altered chemotaxis
- Increased oxidative burst
- Increased protease release

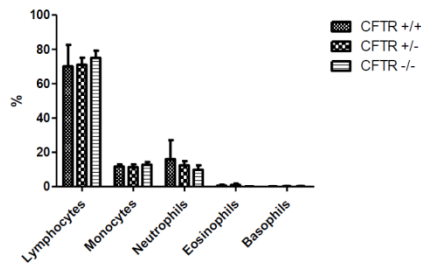
No differences in surface markers



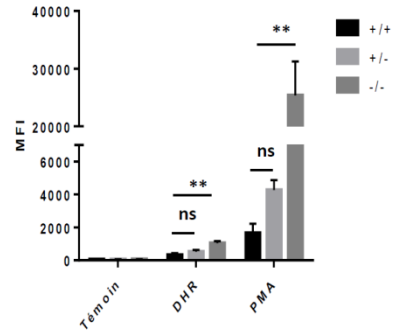
No difference in total and differential WBC count



Differential White Blood Cell Count



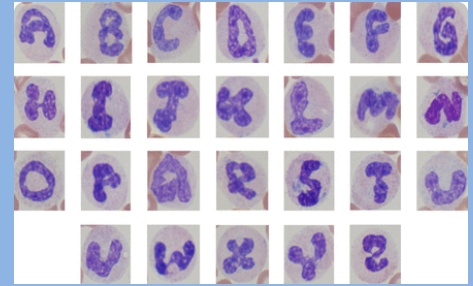
Increase in ROS secretion



5×10^5 cells/spot

7 CFTR^{+/+} and 7 CFTR^{-/-}

8 technical replicates

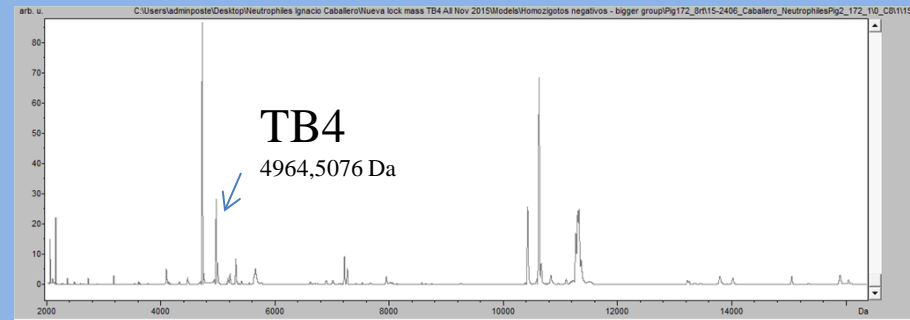


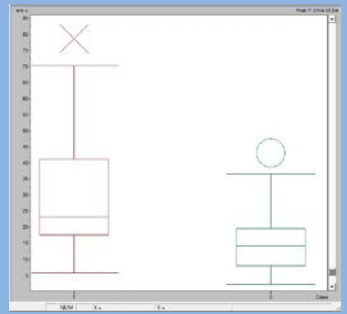
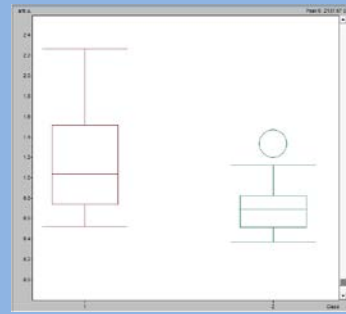
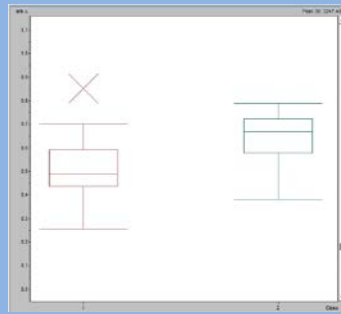
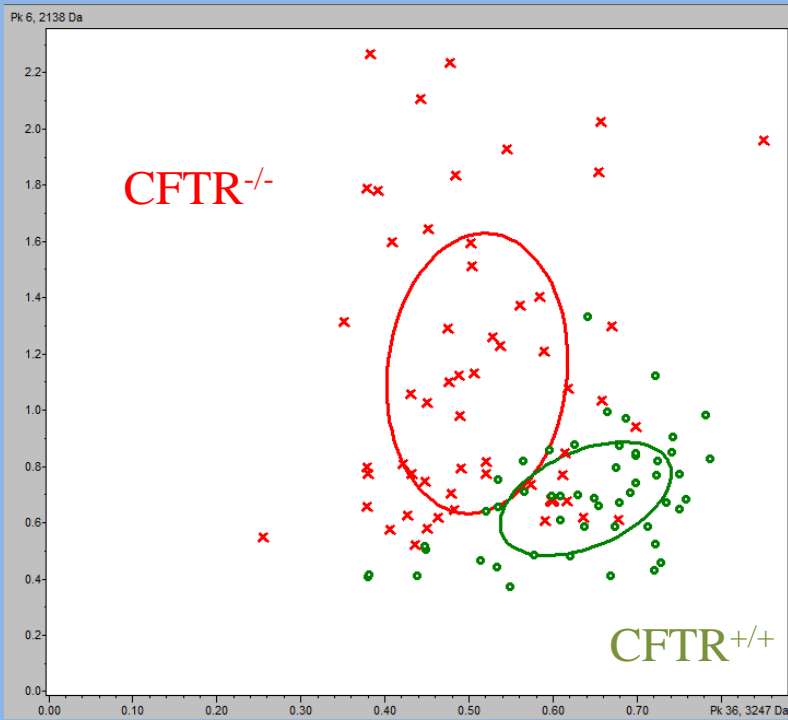
Spectra manual acquisition in a Bruker UltrafleXtreme MALDI-TOF instrument (mass range 1-20 kDa).

External calibration : Peptide/protein mix calibrant

Internal calibration: Lock-mass using peak 4964,5076 Da (**TB4**)

Pre-processing: ClinProTools 3.0 (Bruker)

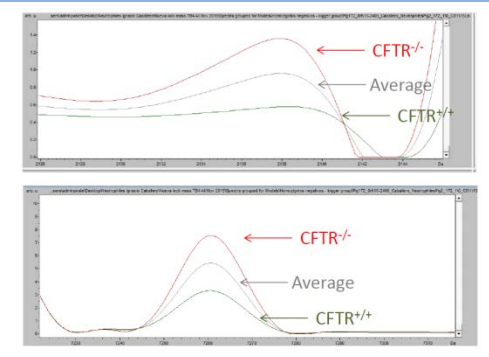


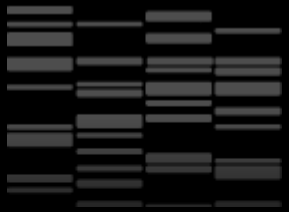


| Index | Mass | DAve | PTTA |
|-------|---------|-------|------------|
| 36 | 3247.48 | 0.14 | < 0.000001 |
| 6 | 2137.67 | 0.5 | 0.0000036 |
| 7 | 2154.58 | 17.17 | 0.00000367 |
| 114 | 7260.62 | 4.2 | 0.00000367 |
| 28 | 2980.76 | 0.13 | 0.00000367 |

Genetic Analyser

| Recognition Capability | Cross Validation |
|------------------------|------------------|
| 100 % | 92.73 % |





Acknowledgements_

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