

A reversible fluorescent tag for mass spectrometry compatible detection and quantification of proteins and peptides.

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INTRODUCTION

- 2D gel electrophoresis still represents the principal method of analysis in proteomics.
- Contemporary proteomics relies on identification of proteins using peptide mass fingerprinting (PMF) LC-MS and sequencing so detection methods must be compatible with mass spectrometry techniques.
- The concentration of proteins in a sample can vary over several orders of magnitude and detection systems must be highly sensitive with a wide dynamic range.
- Proteomics is moving away from traditional stains such as Coomassie, which lacks the sensitivity to detect minor components, and silver which is known to interfere with identification by PMF.
- Fluorescent stains have become the detection method of choice due to their sensitivity and dynamic range but many new stains are difficult to remove or are covalently attached to the peptide core and may interfere with identification by mass spectrometry.
- Post-translational modifications such as glycosylation are also known to interfere with both staining and protein identification. However as many of the markers being investigated involve glycosylation variants it is essential to be able to identify these proteins.
- LavaPurple™ is based on a naturally occurring molecule that uses a novel mechanism of staining proteins. This fully reversible covalent attachment to proteins makes the stain easy to remove without the need for complicated and time consuming washing steps, and has been reported to result in superior matching results than many other stains.^{1,2}
- This study compares the compatibility of four commercially available fluorescent stains: LavaPurple (Fluorotechnics), Flamingo Pink™ (BioRad), Krypton™ (Pierce), and Sypro Ruby™ (Invitrogen).

LC-MS ANALYSIS

Methods

- GE low molecular weight SDS-PAGE calibration markers were loaded onto BioRad 4-20% Tris-glycine gels in a 2 x dilution series ranging from 4 pmol to 0.5 fmol of protein per band.
- 1 gel each was stained with LavaPurple, Flamingo Pink, Krypton or Sypro Ruby using the manufacturer's recommended high sensitivity protocol.
- The gels were imaged using a GE Typhoon™ scanner with the appropriate settings for each stain.
- The first 7 dilutions of the BSA Band were excised from each of the gels reduced and alkylated and digested with proteomics grade trypsin.
- The digest was extracted from the gel pieces and analysed with a ThermoFinnigan LCQ Deca Ion Trap mass spectrometer coupled to a nanoLC-MS system.

Results

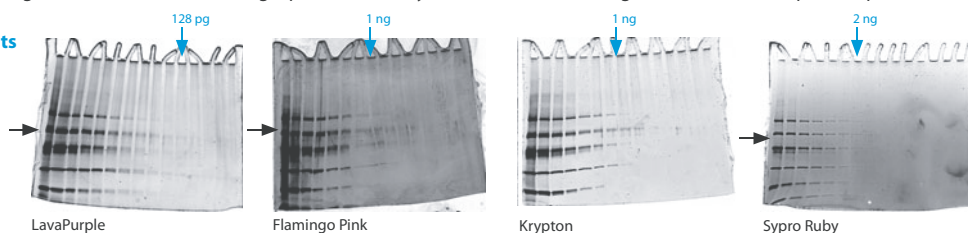


Figure 1. 1D SDS-PAGE gels stained with 4 Fluorescent stains. Arrows indicate the lane at which 5 of the 6 markers are visible. The BSA band (marked) was excised from the LavaPurple, Flamingo Pink, and Sypro Ruby from 256 ng (4 pmol) to 2 ng (0.5 fmol), digested and analysed by LC-MS. As Krypton does not fluoresce using the wave length of any commercially available transilluminator the bands could not be excised and it was as such eliminated from this study.

- It was not possible to accurately excise the bands from the Krypton gel.
- Using a minimum of 3 peptides for the study (99% certainty) LavaPurple identified BSA with certainty with 1 pmol of protein loaded onto the gel.
- Sypro Ruby identified BSA with certainty with 4 pmol of protein loaded onto the gel.
- Flamingo Pink did not identify BSA with certainty in any band.

MALDI-ToF PMF

Methods

- BioRad 2D page calibration standards were dissolved into reducing sample buffer so that 2.9 µL was loaded onto each strip.
- BioRad 11 cm pl 3-10 IEF strips were re-hydrated for 8 hours then focused on GE IEF apparatus for 100 000Vh.
- BioRad Criterion™ 8-16% Tris-glycine gels were used for the second dimension.
- 3 gels were selected at random and stained with LavaPurple, Flamingo Pink, Krypton or Sypro Ruby using the manufacturer's recommended high sensitivity protocol.
- The gels were imaged using a GE Typhoon scanner with the appropriate settings for each stain.
- 10 identical spots were cut from 2 gels of each stain using a cutting tool and submitted to the Australian Proteome Analysis Facility for MALDI-ToF PMF using an ABI 4700 ToF-ToF Mass Spectrometer.

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Results

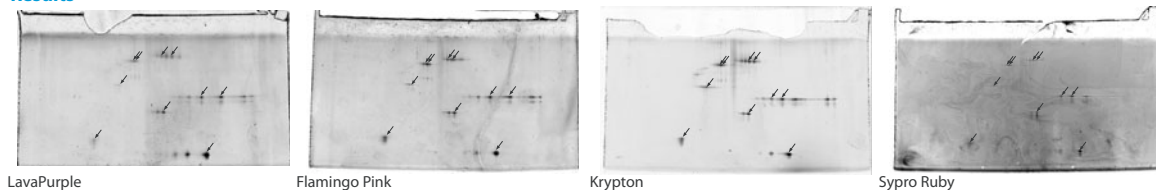


Figure 2. Representative gels of each of the stain types indicating which spots were cut. Note that even with a simple proteome such as that which is made of these 7 markers LavaPurple identifies more isoforms than the other three stains.

Stain	% Identified	Average Coverage	Average Peptides	Average Mowse Score	Average Missed Cleavage	Number of Incorrect Identifications
LavaPurple	89	58.5%	24.3	129.5	9.3	0
Flamingo Pink	63	58.0%	24.5	145.6	11.2	3
Krypton	80	53.1%	20.7	118.3	11.6	1
Sypro Ruby	58	40.0%	14.7	87.0	5.2	1

Table 1. Comparison of 4 fluorescent stains for ability to identify spots from 2D gels. At least one identification that was returned as real by the search software but was not present in the standard samples occurred in Flamingo Pink, Sypro Ruby and Krypton. Samples that contained keratin contamination were removed from the study so as to not bias results.

Stain	Number of Identities on Both Gels	Average Difference in Coverage	Average Difference in No. of Peptides
LavaPurple	6 (2)	5.10%	1.50
Flamingo Pink	4 (0)	7.25%	5.00
Krypton	7 (1)	9.86%	6.14
Sypro Ruby	4 (1)	7.50%	2.75

Table 2. Comparison of the four stains for consistency of results. The table demonstrates how many of the duplicate spots were identified in both of the gels that were used, and of those that returned a positive result in both how close each of the identifications were to each other. Numbers in parentheses indicate how many spots for each stain contained keratin contamination which may have affected results.

GLYCOPROTEIN ANALYSIS

Methods

- Invitrogen Candycane™ markers were loaded onto 4 x BioRad 4-20% Tris-glycine gels such that each band contained 62.5 µg of protein.
- 1 gel each was stained with LavaPurple, Flamingo Pink, Krypton or Sypro Ruby using the manufacturers recommended high sensitivity protocol.
- The gels were imaged using a GE Typhoon scanner with the appropriate settings for each stain.
- A cutting tool was used to remove plugs of equal size from the centre of each of the 4 glycoproteins in the markers.
- 4 plugs from each stain were submitted to the Australian Proteome Analysis Facility for MALDI-ToF PMF using an ABI 4700 ToF-ToF Mass Spectrometer.

Results

Sample	Protein	Sequence Coverage	Mowse Score	Number of Peptides	Missed Cleavages
SR1	α ₂ -macroglobulin [<i>Homo sapiens</i>]	24%	119	29	1
SR2	No significant hit				
SR3	No significant hit				
SR4	No significant hit				
LP1	α ₂ -macroglobulin precursor (Alpha-2-M)	32%	202	37	2
LP2	Glucose Oxidase	27%	71	12	1
LP3	α ₂ -acid glycoprotein [<i>Bos taurus</i>]	41%	70	9	4
LP4	Chain A, Crystal Structure of Avidin	78%	104	15	6
FL1	α ₂ -macroglobulin precursor (Alpha-2-M)	41%	169	37	2
FL2	Chain, Glucose Oxidase (E.C.1.1.3.4)	50%	101	22	1
FL3	α ₂ -acid glycoprotein [<i>Bos taurus</i>]	56%	74	12	7
FL4	No significant hit				
KR1	α ₂ -macroglobulin precursor (Alpha-2-M)	42%	209	49	4
KR2	No significant hit				
KR3	α ₂ -acid glycoprotein [<i>Bos taurus</i>]	56%	91	11 0.11 delta	7
KR4	Chain A, Recombinant Avidin	73%	85	12	4

Table 3. MALDI-ToF PMF of glycosylated proteins. Note that LavaPurple was the only stain to identify all of the proteins. Also Note that LavaPurple™ had the highest average coverage of these proteins and the lowest average missed cleavages. These results indicate that LavaPurple is least likely to interfere with MALDI-ToF MS analysis of proteins excised from polyacrylamide gels.

CONCLUSIONS

- All fluorescent stains tested demonstrated MS compatibility.
- The level of coverage and identification success rate of different stains varied considerably.
- In our hands Flamingo Pink was suitable for MALDI-ToF identification but had considerably reduced sensitivity than the other stains tested using LC-MS.
- Using traditional MALDI-ToF PMF Sypro Ruby exhibited the least successful matching and poorest recovery of peptides.
- LavaPurple exhibited consistently higher sensitivity, number of identifications, coverage and peptides recovered in both MALDI-ToF and LC-MS techniques.
- The differences seen in MS compatibility are likely explained by the level of interference to digestion and recovery of peptides. This is possibly the result of the different staining mechanisms of the stains tested.

REFERENCES

1. Coghlan DR, Mackintosh JA, Karuso P. (2005) Mechanism of reversible fluorescent staining of protein with epicocconone. *Org Lett.* Jun 9; 7(12): 2401-4
2. Tannu NS, Sanchez-Brambila G, Kirby P, Andacht TM. (2006) Effect of staining reagent on peptide mass fingerprinting from in-gel trypsin digestions: A comparison of Sypro Ruby and Deep Purple. *Electrophoresis*, 27: 3136-3143

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