Compatibility of the Criterion Stain Free™ Gel Imaging System with Mass Spectrometric Protein Analysis

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Introduction

Bio-Rad's Criterion Stain Free gels are Tris-HCl polyacrylamide gels containing unique trihalocompounds that allow rapid fluorescent detection of proteins separated by electrophoresis using the Criterion Stain Free gel imaging system (McDonald et al. 2008). The unique compounds react with tryptophan residues in a UV light–induced reaction to produce fluorescence, which can be easily detected by the Criterion Stain Free imager within gels and on blots. The modification of tryptophan residues raises the question of compatibility with downstream applications. We demonstrated in a previous study that the use of Criterion Stain Free gels is compatible with western blot applications (Elbaggari A et al. 2008). The present study was designed to determine whether the activation of Criterion Stain Free gels by UV light affects protein identification by liquid chromatography–tandem mass spectrometry (LC-MS/MS) or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Methods

Materials

All reagents and equipment were from Bio-Rad Laboratories, Inc., unless otherwise specified.

Cell Culture and Protein Sample Preparation

HeLa cells (ATCC #CCL-2) were cultured to confluence in 100 mm petri dishes. The cells were rinsed in phosphate buffered saline (PBS) three times before being lysed in a buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 40 mM Tris-HCl, 2 mM tributylphosphine TBP, 1 mM EDTA, 2.5 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and one Complete Mini protease inhibitor cocktail tablet (Roche Applied Science).

2-Dimensional (2-D) Gel Electrophoresis, Spot Excision, and Trypsin Digestion

HeLa cell lysate was treated with the ReadyPrep™ 2-D cleanup kit and the resulting protein pellets were resolubilized in IPG rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 3 mM TBP, 0.2% Bio-Lyte® 3/10 ampholyte, and 0.001% bromophenol blue). About 150 µg of protein was loaded onto each 11 cm ReadyStrip™ IPG strip pH 3–10 and isoelectric focusing was performed at 8,000 V for 35,000 V-hr. Proteins on the IPG strips were reduced with dithiothreitol, alkylated by iodoacetamide, and equilibrated in Tris/glycine/SDS buffer. The 2-D SDS-PAGE separation was performed using 8–16% Criterion Stain Free or control Criterion™ Tris-HCl precast gels at 200 V for 1 hr.

For LC-MS/MS analysis, the Criterion Stain Free gel was exposed to UV light for 5 min prior to Coomassie staining and spot picking. The control gel and the Criterion Stain Free gel were stained with Bio-Safe™ Coomassie gel stain. 2-D images were analyzed using PDQuest™ 2-D analysis software. Gel plugs 1.5 mm in diameter were excised from spots of interest using the EXQuest™ spot cutter. Eight pairs of spots were randomly selected from the control and Criterion Stain Free gels for MS analysis. The gel plugs were destained in 50% acetonitrile and 50 mM NaH_4CO_3 prior to overnight trypsin digestion at 37ºC. The peptides were recovered from the gel plug by collecting the trypsin solution followed by an extraction with 67% acetonitrile and 3% formic acid. The peptide samples were vacuum-dried to remove most of the acetonitrile prior to LC-MS/MS analysis.

For MALDI-TOF-MS analysis, the Criterion Stain Free gel was exposed to UV light for 5 min. The control gel and the Criterion Stain Free gel were stained with Bio-Safe Coomassie gel stain. Forty-eight pairs of spots were randomly selected from the control and Criterion Stain Free gels for MS analysis. Trypsin digestion was performed using a Tecan Freedom EVO automated liquid handling system (Tecan Trading AG) under the control of Gemini version 4.2 software (Tecan). The gel plugs were destained in a 96-well filter plate using a 50% acetonitrile, 25 mM NaH_4CO_3 solution prior to a 3-hr trypsin digestion at 37ºC. The peptides were recovered by centrifugation.

Protein Identification by LC-MS/MS

Peptides were analyzed by LC-MS using an Agilent 1100 Series Capillary LC system (Agilent Technologies) interfaced to a ThermoFinnigan LTQ linear ion trap mass spectrometer (Thermo Scientific) equipped with a Michrom ADVANCE nanospray ionization source (Michrom Bioresources, Inc.). Peptides were separated on a 0.2 x 50 mm reversed-phase column with 3.0 µm C18 particles using a 10 min linear gradient from 95:5 water:acetonitrile + 0.1% formic acid to 65:35 water:acetonitrile + 0.1% formic acid, at a flow rate of

Table 1. Comparison of LC-MS/MS identification of 8 proteins on Criterion Stain Free and control gels.

Spot	Gel	Probability	Cross-correlation Score (XC)	% Coverage	Peptide Sequences	Protein	Number of Trp-Containing Peptides: No Modification*	Number of Trp-Containing Peptides: Modification*
1	Criterion Stain Free gel	1.7 e-013	80.2	40.49	8	Heat shock 27 kD protein 1	1	
	Control	5.6 e-016	90.3	44.39	9	Heat shock 27 kD protein 1	1	
2	Criterion Stain Free gel	4.4 e-014	100.3	33.21	10	YW HAZ protein		
		1.6 e-012	100.3	51.22	11	Tyrosine 3-monooxygenase	1	
		9.2 e-011	48.3	28.14	5	14-3-3 protein eta chain		
		3.6 e-010	78.2	30.36	8	14-3-3 gamma protein		
	Control	6 e-012	124.3	40.36	13	YW HAZ protein		
		3.9 e-012	98.3	39.18	9	Tyrosine 3-monooxygenase		
		5 e-011	68.3	42.71	7	14-3-3 protein eta chain		
		1.1 e-008	68.2	26.72	7	14-3-3 gamma protein		
3	Criterion Stain Free gel	5.1 e-010	174.3	50.93	19	Mutant beta-actin	1	
	Control	.2 e-009	164.3	50.40	19	Mutant beta-actin	1	
4	Criterion Stain Free gel	5.6 e-013	38.3	13.13	4	Phosphopyruvate hydratase		
		6.1 e-013	118.4	47.02	12	Alpha enolase		
		2.3 e-006	20.2	4.14	2	6-phosphogluconate dehydrogenase		
	Control	3.9 e-012	50.3	19.59	6	Phosphopyruvate hydratase	1	
		7.8 e-015	130.3	52.68	14	Alpha enolase	3	
		1.1 e-011	70.3	20.08	7	6-phosphogluconate dehydrogenase	2	
5	Criterion Stain Free gel	1 e-030	364.3	52.14	36	Heat shock protein 70 kD 5		2
		2.4 e-009	16.2	4.93	2	Similar to 78 kD glucose-deregulated protein precursor		
		3 e-009	190.2	33.02	19	Protein disulfide isomerase related protein		
		2.3 e-008	58.2	7.87	6	Heat shock protein 90-beta		
	Control	1 e-030	348.3	51.83	36	Heat shock protein 70 kD 5	2	
		2 e-010	26.2	4.93	3	Similar to 78 kD glucose-deregulated protein precursor		
		4 e-007	100.2	16.12	11	Protein disulfide isomerase related protein	1	
		1.1 e-010	68.3	12.02	7	Heat shock protein 90-beta		
6	Criterion Stain Free gel	2.4 e-012	86.2	21.09	9	HSPCA protein		
		9.7 e-012	312.3	38.95	32	Heat shock protein 90-beta	1	1
		2.2 e-011	78.3	28.85	8	Unnamed protein product		1
	Control	3.9 e-013	138.2	33.41	15	HSPCA protein	1	
		2.4 e-010	308.3	37.15	32	Heat shock protein 90-beta	2	
		1.4 e-009	102.3	38.14	11	Unnamed protein product	2	
7	Criterion Stain Free gel	2.9 e-012	208.3	31.7	21	ACTN4 protein		1
		9.5 e-012	60.3	8.14	6	Alpha actinin 4		
		6.9 e-010	124.2	25.14	13	Unnamed protein product	2	3
		1.1 e-009	46.2	13.69	5	Hypothetical protein DKFZp434K0126.1		
	Control	1.6 e-012	240.3	42.27	24	ACTN4 protein	2	
		5.7 e-013	90.3	9.95	9	Alpha actinin 4	1	
		8.9 e-012	138.3	28.14	14	Unnamed protein product	5	
8	Criterion Stain Free gel	3.3 e-009	126.3	42.54	13	TRA1 protein		
		1.1 e-007	168.2	17.65	17	Heat shock protein gp96 precursor		
		8.8 e-007	20.1	2.74	2	Tumor rejection antigen		
	Control	1.4 e-009	150.3	45.71	16	TRA1 protein	1	
		1.8 e-009	268.3	30.95	27	Heat shock protein gp96 precursor	6	
		2.9 e-007	20.2	2.74	2	Tumor rejection antigen		

* Database searches were performed without and with dynamic modification of Trp.

3 µl/min. Eluted peptides were analyzed by MS-MS using data-dependent acquisition in which the top five peptide precursor ions in each survey scan were selected for MS-MS. Proteins were identified from MS-MS data by search of the human FASTA database using TurboSEQUEST with BioWorks 3.2 software (Thermo Scientific). To account for trihalocompound modification of Trp, data files were also searched with a 58 Da dynamic modification.

Protein Analysis by MALDI-TOF-MS

Each sample (1 µl) together with 2 µl of matrix solution (α-cyano-4-hydroxycinnamic acid) was spotted onto a 600 µm anchor of a Bruker AnchorChip var/384 target (Bruker Corporation). After the peptide samples crystallized at room temperature, each spot was rinsed with 5 µl of 10 mM $(NH_4)_3PO_4$ and 0.1% trifluoric acid (TFA) for 5 sec to remove extra salt. The rinse solution was removed, and each peptide sample was recrystallized by applying 0.5 µl EtOH:acetone:0.1% TFA (6:3:1) and letting the sample dry again at room temperature. Each sample on the AnchorChip target was analyzed by the Bruker autoFlex II MALDI-TOF system (Bruker Daltonics) to obtain its peptide mass fingerprint (PMF). Proteins were identified by searching the human FASTA database using the MASCOT search engine for PMF searches. We considered a MASCOT score of 65 (statistical significance threshold) or higher to denote confidence in protein identification.

Results

Visualization of Proteins After 2-D Gel Electrophoresis

Proteins separated on Criterion Stain Free gels can be visualized within 5 min after gel electrophoresis. Figure 1 shows an image of a HeLa cell lysate fractionated by 2-D electrophoresis on a Criterion Stain Free gel.

Fig. 1. 2-D gel image produced with the Criterion Stain Free gel imaging system. 100 µg of HeLa cell lysate protein was separated on an 11 cm ReadyStrip IPG strip, pH 5–8, followed by separation on an 8–16% Criterion Stain Free gel. Image was captured using the Criterion Stain Free gel imaging system after exposure to UV light for 5 min.

Criterion Stain Free System Compatibility with LC-MS/MS

To determine whether activation of proteins in the Criterion Stain Free gel interferes with LC-MS/MS protein identification, eight protein spots with medium to high Coomassie stain intensity were selected randomly from a 2-D Criterion Stain Free gel (Figure 2) for mass spectrometric analysis. The same set of eight spots was also picked from a control 2-D Criterion gel for protein identification.

Results of the LC-MS/MS analysis are summarized in Table 1. Each pair of spots picked from the two types of gels were identified as the same protein (MASCOT scores ≥ 65). The peptide sequence coverage in the database identification search was high for both sets of peptides. An average of 13.5 peptides and 11.2 peptides for proteins were extracted from the control gels and the Criterion Stain Free gels, respectively. The probabilities and cross-correlation (XC) scores indicated high confidence in the identity of the protein spots (MacCoss et al. 2002). These results indicate that the use of Criterion Stain Free gels is compatible with protein identification using LC-MS/MS.

In some cases, we saw significantly lower peptide sequence coverage for protein spots extracted from the Criterion Stain Free gel. For example, ACTN4, an annotation of protein spot 7, had 21 peptides and 24 peptides covered for the Criterion Stain Free gel spot and control gel spot, respectively. Two of the control gel spot peptides missing in

Table 2. Summary of differences in MALDI-TOF-MS protein identification on Criterion Stain Free and control gels.

Spot #	Control Gel	Criterion Stain Free Gel
1	Keratin 9, type I, cytoskeletal, human	—
3	Thioredoxin (validated), human	—
4	—	Translation initiation factor eIF-5A (validated), human
6	Keratin, hair, basic, 3, *Homo sapiens* (human).	Calpain (EC 3.4.22.17) small chain, human
16	HUMHSP60A NID, *Homo sapiens*	—
25	Keratin 10, *Homo sapiens* (human).	—
26	Acidic ribosomal protein P0, cytosolic (validated), human	—
27	Capping protein alpha subunit isoform 1, human	—
31	—	Cofilin, human
35	Keratin 9, type I, cytoskeletal, human	DJ-1 protein, human
37	—	Cofilin, human
43	Transformation-sensitive protein IEF SSP 3521, human	—

— Not identified.

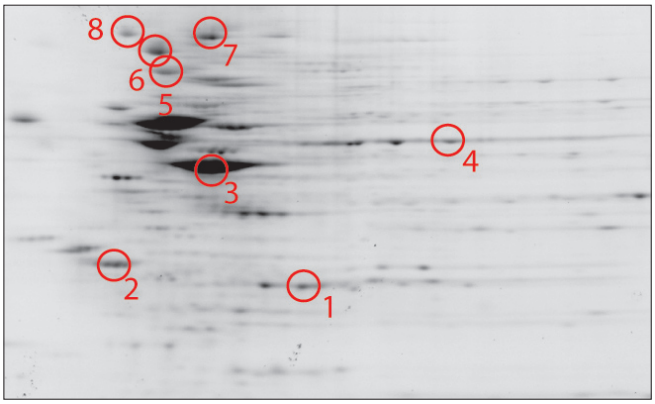

Fig. 2. Eight pairs of spots (indicated by red circles) were selected from control and Criterion Stain Free gels for LC-MS/MS analysis. 150 µg of HeLa cell lysate protein was separated on 11 cm ReadyStrip IPG strips (pH 3–10) followed by 2–D electrophoresis on either an 8–16% Criterion Stain Free gel or a control gel and extracted from each gel system for LC-MS/MS analysis.

the Criterion Stain Free gel spot peptide sequence coverage contained tryptophan residues (Table 1, penultimate column). Similar results were observed for 6-phosphogluconate dehydrogenase in spot 4 and heat shock protein gp96 precursor in spot 8. For all these protein spots, tryptophan-containing peptides were missing in the sequence coverage of Criterion Stain Free gel spots, because the database was searched without modification settings. To recover the missing peptides, we implemented the dynamic modification of Trp for database searches. However, only one of the two tryptophan-containing peptides of ACTN4 was recovered in a database search using dynamic modification of Trp (Table 1, last column).

We also observed that without the use of dynamic modification of Trp settings for the database search, a few peptides containing tryptophan were found among the sequence coverage peptides for Criterion Stain Free gel protein spots 1, 2, 3, 6, and 7 (Table 1). This suggests that not all Trp residues in the analyzed proteins were modified in the Criterion Stain Free gel.

Criterion Stain Free Gel Compatibility with MALDI-TOF-MS

To determine whether Criterion Stain Free gel activation interferes with protein identification by MALDI-TOF-MS, 48 protein spots with medium to high intensity (Figure 3) were selected randomly from a Criterion Stain Free 2-D gel for MS analysis. The same set of 48 spots was also picked from a control gel for protein identification.

The differences in protein identification for these 48 spots on the control and Criterion Stain Free gels are summarized in Table 2. MALDI-TOF-MS returned reliable protein identification for 44 spots (91%) from the control gel and 40 spots (83%) from the Criterion Stain Free gel. Identical protein identification was seen for 34 pairs of spots (73%) extracted from both gel systems. One spot was not identified in either of the two gel systems. Among the 13 pairs that returned different identities, three proteins were identified with high confidence in the Criterion Stain Free gel but not on the control gel; conversely, seven proteins were identified with high confidence on the control gel but not on the Criterion Stain Free gel; one pair could not be identified in either gel system; and finally, two pairs of spots had high XC scores but did not agree with each other. Because tandem MS usually gives more reliable protein identification data than does MALDI-TOF-MS, the 13 unmatching samples were then reanalyzed by LC-MS/MS. Three of the 13 pairs of protein spots analyzed returned the same identities on the two gel systems. Thus, in 48 pairs of protein spots, a combination of MALDI-TOF-MS and LC-MS/MS identified 38 pairs (79%) as matching on the two gel systems.

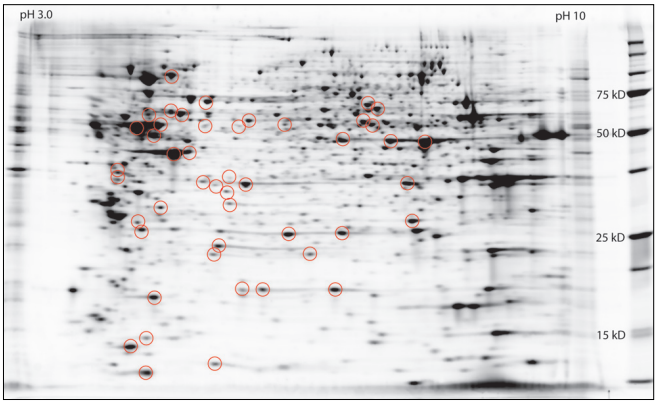

Fig. 3. 48 pairs of spots were selected from the control and Criterion Stain Free gels for MALDI-TOF-MS analysis. 100 µg of HeLa cell lysate protein was separated on each 11 cm ReadyStrip IPG strip (pH 3–10 NL) followed by 2-D separation on an 8–16% Criterion Stain Free gel or on a control Criterion gel. The same 48 spots, indicated by red circles, were selected and extracted from the two gel systems for LC-MS/MS analysis.

Among the 34 pairs of protein spots identity-matched by MALDI-TOF-MS, peptides containing tryptophan were often missing in the sequence coverage of spots from the Criterion Stain Free gel. Table 3 displays a sequence coverage list for a protein spot that was identified with high confidence as annexin V in both the control and Criterion Stain Free gels. Annexin V was covered by 24 peptides in each experiment.

However, two peptides containing tryptophan (185-191 and 185-199, Table 3) present in the coverage of the protein on the control gel were missing on the protein from the Criterion Stain Free gel. Interestingly, we found two peptides that did not contain tryptophan (78-87 and 200-206) in the Criterion Stain Free gel peptide list that were missing in the control peptide list. This indicates that there are other factors besides tryptophan modification that can affect peptide coverage as identified in MALDI-TOF-MS.

Table 3. Comparison of peptide coverage in MALDI-TOF-MS identification of annexin V protein spots on the control and Criterion Stain Free gels.

Control Gel				Criterion Stain Free Gel			
Start	End	Expected MW	Sequence	Start	End	Expected MW	Sequence
5	16	1339.5722	R. GTVTDFPGFDER. A	5	16	1339.6104	R. GTVTDFPGFDER. A
5	23	2095.9631	R.GTVTDFPGFDERADAETLR.K	5	23	2095.9070	R.GTVTDFPGFDERADAETLR.K
17	23	774.4354	R. ADAETLR. K	17	23	774.4220	R. ADAETLR. K
17	24	902.5143	R. ADAETLRK. A	17	24	902.5022	R. ADAETLRK. A
28	43	1703.7792	K.GLGTDEESILTLLTSR.S	28	43	1703.8259	K.GLGTDEESILTLLTSR.S
49	56	892.4865	R.QEISAAFK.T	49	56	892.4833	R.QEISAAFK.T
62	74	1445.7248	R.DLLDDLKSELTGK.F	62	74	1445.6900	R.DLLDDLKSELTGK.F
88	95	1013.5137	R.LYDAYELK.H	78**	87	1142.6514	K.LIVALMKPSR.L oxidation (M)
107	115	1000.5988	K.VLTEIIASR.T	88	95	1013.5293	R.LYDAYELK.H
125	149	2887.2774	K.QVYEEEYGSSLEDDVVGDTSGYYQR.M	107	115	1000.6029	K.VLTEIIASR.T
150	184	3810.8226	R.MLVVLLQANRDPDAGIDEAQVEQD AQALFQAGELK.W	125	149	2887.2332	K.QVYEEEYGSSLEDDVVGDTSGYYQR.M
				150	184	3811.1322	R.MLVVLLQANRDPDAGIDEAQVEQD AQALFQAGELK.W
185*	191	863.4194	K.WGTDEEK.F				
185*	199	1798.7709	K.WGTDEEKFITIFGTR.S	192	199	953.5685	K.FITIFGTR.S
192	199	953.5505	K.FITIFGTR.S	200**	206	825.5187	R. SVSHLRK. V
211	225	1817.8153	K.YMTISGFQIEETIDR.E oxidation (M)	211	225	1817.7600	K.YMTISGFQIEETIDR.E oxidation (M)
226	240	1612.7856	R.ETSGNLEQLLLAVVK.S	226	240	1612.8238	R.ETSGNLEQLLLAVVK.S
244	258	1732.8064	R.SIPAYLAETLYYAMK.G	244	258	1732.8142	R.SIPAYLAETLYYAMK.G
244	258	1748.7424	R.SIPAYLAETLYYAMK.G oxidation (M)	244	258	1748.8323	R.SIPAYLAETLYYAMK.G oxidation (M)
259	269	1154.5600	K. GAGTDDHTLIR. V	259	269	1154.5884	K. GAGTDDHTLIR. V
275	283	1105.5685	R.SEIDLFNIR.K	275	283	1105.5844	R.SEIDLFNIR.K
275	284	1233.6398	R.SEIDLFNIRK.E	275	284	1233.6667	R.SEIDLFNIRK.E
289	299	1273.5992	K.NFATSLYSMIK.G	289	299	1273.6371	K.NFATSLYSMIK.G
289	299	1289.6048	K.NFATSLYSMIK.G oxidation (M)	289	299	1289.6378	K.NFATSLYSMIK.G oxidation (M)
309	318	1117.5252	K.ALLLLCGEDD.-	309	318	1117.5389	K.ALLLLCGEDD.-

* Peptides containing Trp identified in control gel only. ** Peptides identified in Criterion Stain Free gel only.

Data analysis of the unmatched pairs of protein spots indicated that missing peptides containing tryptophan contributed to a lack of confidence shown in the results for protein identification in some cases. Table 4 shows a sequence coverage list for the same spot from control and Criterion Stain Free gels. This protein was identified with high confidence in the control gel as the capping protein alpha subunit isoform 1. Although the same identity had the top score for this spot in the Criterion Stain Free gel, it did not pass the threshold criterion of high confidence. There were seven peptides in the sequence coverage for this spot on the control gel, including two peptides containing tryptophan. In contrast, there were only four peptides in the sequence coverage for this spot on the Criterion Stain Free gel. Three of the four peptides were also found in the control gel spot; the other peptide was detected only in the Criterion Stain Free gel. Two peptides containing tryptophan and two peptides without tryptophan that were detected in the control gel spot were missing on the Criterion Stain Free gel spot. This suggests that tryptophan modification in the Criterion Stain Free gel and other unknown factors contributed to the failure to detect these four peptides by MS, and this may be critical in identifying this specific protein.

Discussion

The Bio-Rad Criterion Stain Free gel imaging system allows quick visualization of proteins after electrophoresis through rapid in-gel, UV-induced modification of tryptophan residues in proteins. In this study, we demonstrated that tryptophan modification in Criterion Stain Free gels does not interfere with downstream applications such as MS identification of protein.

We conducted a large-scale study of a total of 56 protein spots selected from two sets of 2-D gels to characterize the compatibility of Criterion Stain Free gels with MS protein identification applications. We reported that most of the protein spots (48 of 56) on the Criterion Stain Free gels were identified by LC-MS/MS analysis, MALDI-TOF-MS analysis, or both. More importantly, 46 spots (8 from the LC-MS/MS and 38 from the MALDI-TOF-MS studies) had the same identities as those on the control gels. The results demonstrate that the Criterion Stain Free gel imaging system is compatible with MS protein identification by either LC-MS/MS or MALDI-TOF-MS.

We saw strong evidence that Criterion Stain Free gels are compatible with MS applications, but it should be noted that in our results, some peptides containing tryptophan

Table 4. Comparison of peptide coverage in MALDI-TOF-MS identification of capping protein alpha subunit isoform 1 protein spots on the control and Criterion Stain Free gels.

Control Gel				Criterion Stain Free Gel			
Start	**End**	**Expected MW**	**Sequence**	**Start**	**End**	**Expected MW**	**Sequence**
20	37	2087.9248	K.FITHAPPGEFNEVFNDVR.L	20	37	2087.9659	K.FITHAPPGEFNEVFNDVR.L
38	47	1196.6516	R.LLLNNDNLLR.E	38	47	1196.7170	R.LLLNNDNLLR.E
98	103	814.4558	K.FDHLRK.E	147	166	2313.0547	K.TIDGQQTIIACIESHQFQPK.N
134	146	1560.5954	K.DHYSNGFCTVYAK.T	179	193	1569.9037	K.FTITPPTAQVVGVLK.I
167*	172	792.3722	K.NFWNGR.W				
179	193	1569.7965	K.FTITPPTAQVVGVLK.I				
267*	273	903.4997	R.TKIDWNK.I				

* Peptides containing Trp identified in control gel only.

residue were missing in the peptide coverage in the Criterion Stain Free gel samples. A database search with a dynamic modification of the tryptophan residue was seen to recover a few but not all missing peptides containing trp. This database search modification may be helpful in the identification of low-abundance proteins on Criterion Stain Free gels. It should also be noted that tryptophan modification in Criterion Stain Free gels was not the only cause of the different peptide coverage seen after pairs of gel spots were analyzed for protein identification. Some peptides not containing trp were seen only on the Criterion Stain Free gel and not on the control gel. In fact, some protein spots were identified on the Criterion Stain Free gel but not on the control gel.

In summary, our study demonstrates that the quick and convenient Bio-Rad Criterion Stain Free gel imaging system can be compatible with such downstream applications as LC-MS/MS and MALDI-TOF-MS analysis.

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