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Sample complexity reduction for two-dimensional electrophoresis using solution isoelectric focusing prefractionation

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Abstract

Despite its excellent resolving power, 2-DE is of limited use when analyzing cellular proteomes, especially in differential expression studies. Frequently, fewer than 2000 protein spots are detected on a single 2-D gel (a fraction of the total proteome) regardless of the gel platform, sample, or detection method used. This is due to the vast number of proteins expressed and their equally vast dynamic range. To exploit 2-DE unique ability as both an analytical and a preparative tool, the significant sample prefractionation is necessary. We have used solution isoelectric focusing (sIEF) via the ZOOM[®] IEF Fractionator (Invitrogen) to generate sample fractions from complex bacterial lysates, followed by parallel 2-DE, using narrow-range IPG strips that bracket the sIEF fractions. The net result of this process is a significant enrichment of the bacterial proteome resolved on multiple 2-D gels. After prefractionation, we detected 5525 spots, an approximate 3.5-fold increase over the 1577 spots detected in an unfractionated gel. We concluded that sIEF is an effective means of prefractionation to increase depth of field and improve the analysis of low-abundance proteins.

Keywords

2-DE; *Escherichia coli*; Narrow pH range; Prefractionation; Solution isoelectric focusing

1 Introduction

Despite significant advances in proteomic technologies, especially in MS, 2-DE remains the workhorse of the field because of its preparative and analytical nature, reliable and reproducible quantitation, and ability to resolve differentially modified proteins. Unfortunately, researchers using 2-DE cannot detect simultaneously all expressed proteins within a complex mixture. The vast number of proteins expressed and the similarly vast dynamic range of protein expression in most biological samples exceed 2-DE's capabilities that are limited by physical space for protein separation (gel format) and by protein detection (stain/dye sensitivity) [1]. It is

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estimated that proteins encoded by at least 15 000 genes are expressed at any given time, many with multiple modifications, and protein concentration differences spanning six orders of magnitude or more in a typical eukaryotic cell [2]. Even the most sensitive fluorescent stains, with the LOD of hundreds of picograms and linear ranges of four orders of magnitude [3], render even the highest quality 2-D gels capable of detecting only a fraction of the proteome. Thus, it is clear that prefractionation is a necessary step in reducing sample complexity and increasing depth of field to detect low-abundance proteins.

Several commercial tools are now available to accomplish this. Invitrogen's ZOOM[®] IEF Fractionator is based on Zuo and Speicher's original concept for solution IEF (sIEF) fractionation [4] that expounded upon the pioneering work of Bier [5] and Righetti [6] and avoids significant protein loss that accompanies the use of narrow-range IPG strips alone. The apparatus produces well-resolved fractions based on the pI of each protein in the mixture. These fractions can then be focused in the first-dimension using narrow-range IPG strips and separated by mass in the second dimension, using standard SDS-PAGE. Because each fraction necessitates a new 2-D gel, it provides the aforementioned necessary physical space for protein separation and overcomes a major limitation of traditional 2-DE. sIEF is accomplished in a conveniently low-volume platform [4,7], using a series of chambers connected in tandem and separated by thin membranes that contain Immobilines, covalently attached buffers of defined pH. The protein sample is loaded into the chambers separated by these disks and spacers and is subjected to sIEF. The result is a significant reduction in original sample complexity in the form of a set of five highly resolved protein fractions suitable for further analysis by 2-DE [1].

Even the prokaryotic proteome, with fewer genes and less post-translational modification (PTM) than eukaryotes, requires sample complexity reduction for global 2-DE analysis. In this regard, *Escherichia coli* is an important model organism and an ideal specimen to evaluate prefractionation strategies because of its relatively simple proteome and fully sequenced genome. It was our goal in this study to evaluate sIEF as a means of prefractionation to enhance the detection and analysis of the *E. coli* proteome.

2 Materials and methods

2.1 Chemicals and reagents

Ultrapure protein solubilization and electrophoretic reagents were obtained from Bio-Rad (Richmond, CA), Sigma Chemical (St. Louis, MO), BDH (Poole, UK), and National Diagnostics (Atlanta, GA). Broad pH range IPG strips (pH 3–10, 24 cm) were also purchased from Bio-Rad. Narrow pH range IPG strips (pH 3.5–4.5, 4.5–5.5, 5.3–6.5, 6.2–7.5, and 6–9, 24 cm) were obtained from Amersham Biosciences (Piscataway, NJ). ZOOM[®] IEF Fractionator and associated reagents were purchased from Invitrogen (Carlsbad, CA).

2.2 Sample preparation

E. coli from the BL21 strain were grown in standard media and conditions; 1.5 mL culture media were transferred to microcentrifuge tubes, centrifuged at $15\,000 \times g$ for 1 min, and the supernatant was discarded. The pellet was washed twice with wash buffer (25 mM glucose, 50 mM Tris, 20 mM EDTA), and bacteria resuspended by briefly vortexing. The suspension was centrifuged as before, and the supernatant discarded. Pellets were weighed (~38 mg each) and homogenized in 8 volumes of solubilization buffer (9 M urea, 4% CHAPS, 1% DTT 65 mM, and 2% ampholytes pH 3–10) using a ground-glass tube/pestle. The protein solution remained at room temperature for 1 h with intermittent manual agitation. The sample was sonicated with a Fisher[®] Sonic Dismembrator using 3×2 s bursts at instrument setting no. 3. Sonication was performed every 15 min for 1 h at room temperature, after which the lysate was clarified

by ultracentrifugation at $100\,000 \times g$ for 20 min at 20°C. Following sample solubilization, a protein assay was performed using the RC DC Protein Assay kit (Bio-Rad) according to the manufacturer's protocol. Samples were stored at -45°C until prefractionation.

2.3 Prefractionation

Aliquots of the *E. coli* lysate were prefractionated by sIEF using the ZOOM® IEF Fractionator (Invitrogen) according to the manufacturer's protocol. Alkylation and reduction was not performed prior to sIEF, according to the manufacturer's established protocol, and the protocols published in the scientific literature upon which the commercialized technique is based. A total of 10 mg of lysate was loaded into the ZOOM® IEF Fractionator, 2 mg/chamber, and fractionated at room temperature under standard focusing conditions (100 V for 20 min, 200 V for 80 min, and 600 V for 80 min), resulting in five sIEF fractions, enriched in proteins with *pI* values of 3–4.6, 4.6–5.4, 5.4–6.2, 6.2–7, and 7–10.

2.4 2-DE

First-dimensional IEF was performed by loading 1 mg of protein from the unfractionated lysate on broad-range Bio-Rad IPG strips (pH 3–10, 24 cm, Fig. 2) and by loading 90% of the chamber volumes of the five ZOOM® IEF fractions on the five corresponding 24-cm narrow-range Amersham IPG strips (3.5–4.5, 4.5–5.5, 5.3–6.5, 6.2–7.5, and 6–9) (Figs. 3–7), as well as 10% of chamber volumes on broad-range IPG strips (Fig. 1). The cell lysate was diluted with rehydration buffer (8-M urea, 2% CHAPS, 15-mM DTT, 0.2% ampholytes pH 3–10) and passive rehydration of IPG strips was carried out overnight at room temperature. The proteins were focused using a Protean IEF Cell (Bio-Rad) at $\leq 50 \mu\text{A}/\text{strip}$ at 20°C, using a program of progressively increasing voltage (150 V for 2 h, 300 V for 4 h, 1500 V for 1 h, 5000 V for 5 h, 7000 V for 6 h, and 10 000 V for 3 h) for a total of 100 000 Vh. Second-dimensional separation was accomplished on linear 11–19% acrylamide gradient slab gels (20 cm $\times$ 25 cm $\times$ 1.5 mm), poured and cast reproducibly using a computer-controlled gradient maker. First-dimensional IPG strips were loaded directly onto the slab gels following equilibration for 10 min in Equilibration Buffer I and 10 min in Equilibration Buffer II (Equilibration Buffer I: 6 M urea, 2% SDS, 0.375 M Tris-HCl pH 8.8, 20% glycerol, 130 mM DTT; Equilibration Buffer II: 6 M urea, 2% SDS, 0.375 M Tris-HCl pH 8.8, 20% glycerol, 135 mM iodoacetamide). Gels were run simultaneously in a single tank for 18 h at 160 V and 8°C.

2.5 Staining and scanning

Slab gels were stained using a colloidal CBB G-250 procedure [8]. Gels were fixed overnight at room temperature in 1.5 L of 50% ethanol/2% phosphoric acid, followed by three 30-min washes in 2 L of deionized water. Gels were transferred to 1.5 L of 30% methanol/17% ammonium sulfate/3% phosphoric acid for 1 h followed by an addition of 1 g of powdered CBB G-250 stain. After 96 h, gels were washed several times with water and scanned at 95.3 $\mu\text{m}/\text{pixel}$ using a GS-800 Calibrated Imaging Densitometer (Bio-Rad). The resulting 12-bit images were analyzed using PDQuest™ software (Bio-Rad, v.7.1). Background was subtracted and peaks for the protein spots located and counted.

2.6 Simulated analysis

Virtual 2-D gels were created using JVirGelv2.2.3b [9]. A tool was used to create boundaries around each narrow *pI* range listed in Table 1 with the MW range limited to 6–200 kDa, and the total number of spots in each box was given automatically and recorded. This process was repeated for four different *E. coli* strains – EDL933, K12, Sakai, and UT189 (Table 1).

2.7 PMF

Protein spots were cut manually from the gels with a 1.5-mm gel-cutting tool placed in a 96-well plate, along with a recombinant human grp78 standard (StressGen), and processed automatically using the MultiProbe II Station robot (PerkinElmer). Gel plugs were destained with 50 mM ammonium bicarbonate/50% ACN followed by 100% ACN, reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, and digested overnight with modified trypsin (Promega) at 6 ng/ μ L. The peptides were eluted by the addition of 25 μ L 0.2% formic acid and 7 μ L ACN, cleaned-up/desalted and preconcentrated by micro solid-phase extraction using disposable ZipTip[®] technology and manually spotted on the MALDI-MS sample target along with α -cyano-4-hydroxycinnamic acid matrix. The target was analyzed using the M@LDI[™] (Waters) system. Monoisotopic peptide masses were submitted to online interrogation of the ProFound[™] Peptide Mass Database.

3 Results and discussion

To verify that the *E. coli* whole-cell lysate was successfully fractionated, we focused the resulting fractions on pH 3–10 IPG strips. As expected, each fraction contained only proteins corresponding to the approximate *pI* values of the chamber in which they were resolved (Fig. 1). Consistent with previous observations in *E. coli* 2-DE literature, broad-range 2-DE (pH 3–10) resulted in the detection of 1577 protein spots (Fig. 2). In contrast, the sum of five sIEF fraction gels (Figs. 3–7) yielded 5525 protein spots, a significant improvement in depth of field. This is also a vast improvement over previously published work using sIEF to fractionate the *E. coli* proteome, such as that of Smejkal and Lazarev [10]. It is important to note that there may be some redundancy as certain proteins may appear in two gels near the ends of the IPG strips where *pI*'s are shared, *e.g.* proteins with a *pI* of 5.4 may appear in both the 4.6–5.4 and 5.4–6.2 fractions. Table 2 lists those *E. coli* proteins identified in each *pI* fraction (Figs. 3–7), indicated by white dots (simulating a cutout), using MALDI-TOF-MS peptide mass fingerprinting. As expected, protein *pI*'s correspond to the fraction in which they were resolved by 2-DE.

To compare our fractional protein resolution to what one might expect to achieve, we used virtual 2-D gel software (JVirGel v2.2.3b [9]; <http://www.jvirgel.de>) to determine the theoretical number of protein spots expected in each narrow-range *pI* fraction, based on theoretical protein MW and *pI* of four different strains of *E. coli* (Table 1). The average total number of spots expected from all five fractions (*pI* range 3.5–9.0) was 3502. In the 5.4–7.0 fractions, we observed 1979 more spots than expected in that range. Interestingly, JVirGel predicted the various *E. coli* proteomes to contain an average of 1203 spots with *pI* at or above 9.0, which suggests that, in our sample, proteins with *pI* >7.0 might be modified, consequently acquiring a lower *pI* and resolving in the 5.4–7.0 range. Alternatively, if the pH of the basic chambers increased during focusing, proteins with a higher *pI* could accumulate in those chambers. Including proteins with a *pI* >9.0, the average expected to be detected by sIEF, is 4705 spots. Tonella *et al.* [11] determined that *E. coli* produced about 1.28 protein spots/ORF. Assuming the number of ORF is similar to the number of annotated proteins determined by JVirGel, we could expect to detect 6022 spots (4705×1.28), on average, in five 2-D gels using sIEF. According to this calculation, sIEF in this study enabled detection of about 92% of the *E. coli* proteome detectable by 2-DE.

Making certain assumptions, we have estimated that with the current 2-DE technology and the prefractionation strategy we have outlined here, it should be possible to detect a single copy of a protein expressed in *E. coli* [12]. We estimate that a single bacterium contains approximately 155 fg of protein [13]. A single copy of a 30-kDa protein in this complex mixture would therefore contribute approximately 5×10^{-5} fg to this total. We have already established that 5×2 mg of protein (10 mg total) is possible for fractionation by sIEF (ZOOM[®] IEF) and

loading onto five narrow-range IPG strips. With 1-ng staining sensitivity using CBB now possible [14], and, if we assume that the *E. coli* cells harvested are in the final stage, with 10 mg protein loading, the 30-kDa protein should be present in one of the five narrow-range gels at 3.3 ng [e.g. $10 \text{ mg} \times (5 \times 10^{-5} \text{ fg})/155 \text{ fg}$], falling well within the LOD of colloidal CBB. Thus, by using ZOOM® IEF as a prefractionation strategy, it may be possible to detect and analyze the protein product(s) of every expressed gene in the *E. coli* proteome.

4 Concluding remarks

sIEF prefractionation of *E. coli* lysates followed by 2-DE using 24-cm, narrow-range IPG strips that approximate the sIEF ranges, results in a significant improvement in the number of proteins resolved and thus analytical “depth of field.” 2-DE, especially large-format 2-DE, is known for being labor intensive and requiring a high degree of technical proficiency. Adding a prefractionation step enhances these drawbacks. However, the power of the outcome is undeniable and justifies the required time and effort. Application of this approach to various experimental designs necessitates parallel 2-DE capabilities for reproducible separations. Using ZOOM® IEF in combination with other prefractionation strategies could enable the total detection of the most complex proteomes such as human plasma samples.

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Abbreviation

sIEF solution isoelectric focusing

Figure 1.

Narrow-range ZOOM[®] IEF fractions separated on broad range IPG strips (pH 3–10, 24 cm) and large-format 2-D gels (20 × 25 × 0.15 cm) stained with colloidal CBB.

Figure 2.
Large-format 2-D gel of whole-*Escherichia coli* lysate (500 µg protein loaded, 24-cm IPG strip pH 3–10, nonlinear gradient). PDQuest software detected 1577 spots.

Figure 3. *Escherichia coli* sIEF fraction 3–4.6 focused on pH 3.5–4.5 narrow-range IPG strip. PDQuest software detected 369 spots. White dots indicate spots that were cut out for identification along with the corresponding 96-well plate well number.

Figure 4.
Escherichia coli sIEF fraction 4.6–5.4 focused on pH 4.5–5.5 narrow-range IPG strip. PDQuest software detected 932 spots. White dots indicate spots that were cut out for identification along with the corresponding 96-well plate well number.

Figure 5.
Escherichia coli sIEF fraction 5.4–6.2 focused on pH 5.3–6.5 narrow-range IPG strip. PDQuest software detected 1772 spots. White dots indicate spots that were cut out for identification along with the corresponding 96-well plate well number.

Figure 6.

Escherichia coli sIEF fraction 6.2–7 focused on pH 6.2–7.5 narrow-range IPG strip. PDQuest software detected 1,601 spots. White dots indicate spots that were cut out for identification along with the corresponding 96-well plate well number.

Figure 7.
Escherichia coli sIEF fraction 7–10 focused on pH 6–9 narrow-range IPG strip. PDQuest software detected 851 spots. White dots indicate spots that were cut out for identification along with the corresponding 96-well plate well number.

Table 1
Predicted number of proteins from four different *Escherichia coli* strains in experimental pH ranges using Virtual Gel software

<i>Escherichia coli</i> strains						
pH range	EDL933	K12	Sakai	UT189	AVE	Zoom® IEF
3.5 - 4.5	331	226	320	250	282	369
4.6 - 5.4	1115	856	990	878	960	932
5.4 - 6.2	978	840	973	941	933	1772
6.2 - 7.0	471	399	494	481	461	1601
7.0 - 9.0	910	722	903	930	866	851
9.0+	1195	976	1281	1360	1203	-
Total	5000	4019	4961	4840	4705	5525

Averages for each fraction are calculated and compared with numbers observed in this study (Zoom IEF).

Table 2

List of protein identifications using MALDI-TOF peptide mass fingerprinting

Fig.	Spot No.	Protein ID	pI	MW	Prob	Z	%C
3	a1	orf, hypothetical protein; conserved hypothetical protein	4.0	18.7	0.002	-	57
3	a1	Colicin E7 immunity protein (ImmE7) (Microcin E7 immunity protein)	4.5	9.9	0.000001	2.43	76
3	b1	Glycine cleavage system H protein	4.0	13.9	0.0001	1.67	8
3	b2	Putative head-tail joining protein of prophage	4.6	13.6	0.0001	1.87	59
3	c1	Unidentified reading frame	4.6	12.4	0.000001	2.22	66
3	e1	Menaquinone biosynthesis, unknown	4.0	17.5	0.0001	1.70	41
3	f1	Putative minor tail protein	4.4	14.3	0.000001	2.43	41
3	gi	orf, hypothetical protein	4.0	16.3	0.000001	2.43	28
3	hi	mutT	4.6	12.8	0.000001	2.43	68
4	a4	Cell division; forms circumferential ring; tubulin-like GTP-binding protein and GTPase	4.6	40.3	0.000001	2.43	80
4	a5	Hsp 24 nucleotide exchange factor	4.7	21.8	0.000001	2.43	32
4	a6	DnaK suppressor protein	5.3	18.5	0.000001	2.43	57
4	b3	Trigger factor; a molecular chaperone involved in cell division	4.8	48.2	0.000001	2.36	62
4	b4	Matrix porin (Ompf) mutant with Gly 119 replaced by Asp (G119d)	4.6	37.1	0.000001	2.43	71
4	b5	Repressor for mtl	4.7	22.1	0.000001	2.43	31
4	b6	DNA-binding protein H-NS	5.2	15.0	0.000001	2.08	35
4	c3	Membrane-bound ATP synthase, F1 sector, beta-subunit	4.9	50.4	0.00001	1.96	73
4	c4	Trigger factor	4.7	48.0	0.000001	2.43	44
4	c5	1DKX, Chain A	5.1	23.8	0.000001	2.43	31
4	c6	Putative peptidase	5.3	39.8	0.0001	1.69	40
4	d2	Methyl-directed mismatch repair; methyl-directed mismatch repair	5.4	95.6	0.001	1.18	45
4	d3	Putative GTP-binding protein	4.9	40.0	0.000001	2.43	73
4	d5	Bacterioferritin, an iron storage homoprotein	4.7	18.5	0.000001	2.24	56
4	d6	Glutaryl-tRNA reductase (GluTR)	5.4	46.5	0.0001	1.43	18
4	e4	Polymerase III subunit alpha	4.8	37.2	0.000001	2.43	34
4	e5	Cytoplasmic ferritin (an iron storage protein)	4.8	19.5	0.0001	1.85	36
4	f2	Chaperone Hsp70; DNA biosynthesis; autoregulated heat shock proteins	4.7	69.5	0.000001	2.43	31
4	f4	Hypothetical protein ECs0712 [imported]	4.6	34.5	0.000001	2.43	54
4	f5	Structure of inorganic pyrophosphatase mutant D97n	5.1	19.7	0.000001	2.43	57

Fig.	Spot No.	Protein ID	pI	MW	Prob	Z	%C
4	g2	S1 ribosomal protein	4.9	61.1	0.000001	2.37	44
4	g4	Spermidine putrescine-binding protein complexed with spermidine	4.9	36.5	0.000001	2.43	49
4	g5	Alkyl hydroperoxide reductase, C22 subunit; detoxification of hydroperoxides	5.0	20.9	0.0001	1.84	71
4	h3	Putative uroporphyrin-III C-methyltransferase	4.6	43.9	0.001	1.08	55
4	h5	Chain A, crystal structure of <i>Escherichia coli</i> thiol peroxidase in the oxidized state	4.7	17.9	0.000001	2.43	49
5	a1	Damage-inducible protein J	5.2	9.4	0.000001	2.43	67
5	a2	Flavoprotein wrbA (Trp repressor binding protein)	5.9	20.8	0.000001	2.24	52
5	a7	Polynucleotide phosphorylase	5.4	79.9	0.0001	1.75	45
5	a8	Threonine-tRNA ligase	5.8	73.1	0.000001	2.43	35
5	a9	Putative glycoprotein	5.9	63.2	0.000001	2.43	37
5	a10	Glycerol kinase	6.0	60.7	0.00001	2.06	63
5	a11	ADP-glucose synthase	6.0	49.2	0.000001	2.43	35
5	b1	cysB	5.9	8.8	0.0001	1.38	51
5	b2	Hypothetical protein yidQ precursor	6.1	14.9	0.000001	2.43	77
5	b7	Initiation factor IF2-beta	5.6	79.8	0.000001	2.43	14
5	b8	NP 290772.1	5.5	54.7	0.0001	1.86	59
5	b9	2-C-Methyl-D-erythritol 4-phosphate synthase	5.7	43.8	0.000001	2.43	32
5	b10	ThiC	5.6	71.3	0.00001	1.96	27
5	b11	DNA helicase	5.7	47.1	0.000001	2.43	27
5	b12	Arginine-binding periplasmic protein 1 precursor	5.8	27.1	0.000001	2.43	42
5	c1	Shiga toxin 2 subunit B	5.2	10.0	0.000001	2.43	56
5	c2	Chromosomal replication initiator protein DNA A	6.1	22.9	0.0001	1.72	34
5	c7	TraI*	5.5	87.9	0.00001	2.08	39
5	c8	Fumarate reductase flavoprotein subunit	5.9	66.5	0.000001	2.43	47
5	c10	Replication protein P	5.5	54.3	0.000001	2.43	42
5	c11	Dihydrofolate synthetase; folypolyglutamate synthase	5.5	45.6	0.000001	2.43	32
5	d1	Protein yggX	5.9	11.0	0.000001	2.43	55
5	d2	Phenylacetic acid degradation protein	5.9	11.0	0.009	1.41	48
5	d7	Formate acetyltransferase 1; pyruvate formate lyase I, induced anaerobically	5.7	85.6	0.0001	1.49	31
5	d10	Enolase; phosphopyruvate hydratase	5.7	46.5	0.000001	2.02	42
5	e1	Chain A, purine nucleoside phosphorylase	5.5	25.9	0.000001	2.43	50
5	e2	KleA protein (KcrA1 protein)	6.1	8.7	0.0001	1.65	58

Fig.	Spot No.	Protein ID	pI	MW	Prob	Z	%C
5	e10	Chain A, beta-ketoacyl [acyl carrier protein] synthase I	5.8	44.3	0.000001	2.43	36
5	f1	Putative reductase	5.9	38.8	0.000001	2.13	44
5	f2	Cold shock protein 7.4	5.6	7.4	0.0001	1.53	60
5	f6	Aconitate hydratase 2	5.8	100.1	0.000001	2.34	51
5	f7	Putative DNA packaging protein of prophage; terminase large subunit	6.1	73.5	0.001	1.14	32
5	f8	N-(5-Phosphoribosyl)anthranilate isomerase and indole-3-glycerolphosphate synthetase	5.5	49.9	0.000001	2.43	24
5	f9	Lysine-, arginine-, ornithine-binding periplasmic protein	5.6	28.1	0.000001	2.43	77
5	f10	Glucosamine-1-phosphate N-acetyltransferase	6.1	49.4	0.001	1.06	23
5	f11	Hypothetical protein ECs2957	5.6	36.9	0.0001	1.51	26
5	g1	CysQ protein	5.6	27.3	0.000001	2.43	63
5	g2	Hypothetical protein ECs3591	6.1	9.4	0.000001	1.97	87
5	g6	Putative outer membrane protein	6.1	92.3	0.000001	2.43	17
5	g10	6-Phosphofructokinase isozyme I	6.0	39.1	0.0001	1.76	55
5	g11	Oxidoreductase upcA	5.7	34.3	0.0001	1.77	52
5	h1	A Chain A, triosephosphate isomerase tim	5.8	27.1	0.000001	2.43	67
5	h6	Chain A, structure of <i>Escherichia coli</i> malate synthase G; pyruvate: acetyl-coenzyme A	5.9	81.7	0.000001	2.43	22
5	h10	Putative regulator: TDP-4-oxo-6-deoxy-D-glucose transaminase	5.8	42.3	0.000001	2.43	37
5	h11	D-alucitol-6-phosphate dehydrogenase	6.0	29.1	0.0001	1.48	38
6	a3	rhsA protein in rhs element	6.5	156.9	0.000001	2.43	23
6	a4	orf, hypothetical protein: tyrosine-protein kinase ETK, autophosphorylates	6.2	81.2	0.000001	2.43	22
6	a5	DNA topoisomerase IV subunit A	6.2	83.9	0.000001	2.43	18
6	a6	Periplasmic dipeptide transport protein precursor	6.4	60.5	0.007	1.35	42
6	a7	BfpD	6.4	60.8	0.000001	2.43	36
6	a8	Putative multidrug resistance efflux pump protein, membrane protein	6.8	36.9	0.000001	2.43	25
6	a9	2-Dehydro-3-deoxyphosphooctonate aldolase	6.3	31.1	0.000001	2.43	42
6	a10	orf, hypothetical protein: conserved protein	6.4	19.0	0.000001	2.43	59
6	b3	RNA polymerase, beta prime subunit	6.7	156.0	0.000001	2.43	15
6	b5	Putative DNA helicase	6.4	79.7	0.000001	2.43	33
6	b6	Putative 2-component regulator: putative transcriptional regulator	6.3	57.6	0.000001	2.43	14
6	b7	E91021 probable enzyme (imported)	6.4	43.1	0.000001	2.43	32
6	c3	1008145A polymerase beta'y, RNA y	6.8	155.9	0.000001	2.43	15
6	c4	Thiolase I, 3-ketoacyl-CoA thiolase: acetyl-CoA transferase	6.2	41.2	0.000001	2.43	20

Fig.	Spot No.	Protein ID	pI	MW	Prob	Z	%C
6	c5	Putative DNA helicase	6.4	79.7	0.000001	2.43	17
6	c6	Exodeoxyribonuclease V alpha chain	6.4	67.3	0.0001	1.82	39
6	c9	Hypothetical protein vcfP	6.5	23.6	0.000001	2.43	83
6	d3	Excinuclease ABC subunit A	6.3	104.7	0.0001	1.66	22
6	d5	REP ECOLI ATP-dependent DNA helicase rep	6.6	77.2	0.000001	2.43	23
6	e3	DNA polymerase II	6.4	90.5	0.000001	2.03	37
6	e4	Putative regulator protein	6.3	35.6	0.000001	2.43	33
6	e9	Hypothetical protein ECs2496	6.5	18.0	0.0001	1.78	55
6	f3	YohA	6.2	86.2	0.000001	2.35	54
6	f8	Protein-glutamine gamma-glutamyltransferase	6.7	38.1	0.000001	1.97	30
6	h2	Respiratory nitrate reductase 2 a chain	6.2	141.1	0.000001	2.43	26
6	h3	RNase E	6.4	91.6	0.0001	1.61	54
6	h4	Putative membrane protein	6.4	38.7	0.000001	2.43	61
6	h9	orf18	6.8	8.9	0.000001	2.34	73
7	a11	Phosphogluconate dehydrogenase	9.5	8.3	0.000001	2.43	81
7	a12	Putative integrase	9.9	42.9	0.000001	1.95	70
7	c10	Hypothetical protein YeeR	7.3	92.7	0.000001	2.43	31
7	d10	SfaF protein	7.7	96.6	0.000001	2.43	48
7	c12	Periplasmic binding protein component of Pn transporter	8.5	37.4	0.000001	2.43	46
7	g10	HrpA, ATP-dependent helicase	7.5	144.8	0.000001	2.43	33
7	h10	Side tail fiber protein homolog from lambdoid prophage Rac	7.4	114.1	0.0001	1.87	27
7	h11	Hypothetical protein ECs1674	11.3	7.8	0.000001	2.43	51