A Robust Method for Quantitative High-throughput Analysis of Proteomes by $^{18}$O Labeling*[	extsuperscript{S}]

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MS-based quantitative proteomics plays an increasingly important role in biological and medical research and the development of these techniques remains one of the most important challenges in mass spectrometry. Numerous stable isotope labeling approaches have been proposed. However, and particularly in the case of $^{18}$O-labeling, a standard protocol of general applicability is still lacking, and statistical issues associated to these methods remain to be investigated. In this work we present an improved high-throughput quantitative proteomics method based on whole proteome concentration by SDS-PAGE, optimized in-gel digestion, peptide $^{18}$O-labeling, and separation by off-gel isoelectric focusing followed by liquid chromatography-LIT-MS. We demonstrate that the off-gel technique is fully compatible with $^{18}$O peptide labeling in any pH range. A recently developed statistical model indicated that partial digestions and methionine oxidation do not alter protein quantification and that variances at the scan, peptide, and protein levels are stable and reproducible in a variety of proteomes of different origin. We have also analyzed the dynamic range of quantification and demonstrated the practical utility of the method by detecting expression changes in a model of activation of Jurkat T-cells. Our protocol provides a general approach to perform quantitative proteomics by $^{18}$O-labeling in high-throughput studies, with the added value that it has a validated statistical model for the null hypothesis. To the best of our knowledge, this is the first report where a general protocol for stable isotope labeling is tested in practice using a collection of samples and analyzed at this degree of statistical detail. Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.003335, 1–14, 2011.

The analysis of differential protein expression is fundamental for the understanding of biological processes and plays an increasingly important role in biological and medical research (1). In recent years, numerous stable isotope labeling (SIL)$^{1}$ techniques have emerged as alternatives to the historically used two-dimensional-based approaches for semiquantitative proteomic studies. In these techniques the quantification is done in the same mass spectrometer where peptides are analyzed by tandem mass spectrometry (MS/MS), so relative quantification and peptide identification is performed at the same time. The differences among the several existing SIL approaches are mainly related to the way labels are introduced and the method used to perform the quantification by MS. Thus, in the SILAC method (2) labels are introduced metabolically at the protein level before peptides are generated from protein by enzymatic digestion, minimizing variability introduced by peptide preparation, whereas in the others labeling is performed postdigestion at the peptide level, either chemically in the iTRAQ method (3), or enzymatically in the $^{18}$O labeling method (4–6). In the iTRAQ method, quantification is made at the MS/MS level, allowing the possibility of performing multiplexed comparisons (7) whereas in SILAC and $^{18}$O methods peptides are quantified at the MS level and are mainly used for pairwise comparisons. In other SIL approaches, such as the ICAT method (8), labeled peptides are specifically recovered after an affinity purification approach; this allows reducing peptide complexity, which is particularly appropriate to selectively analyze peptide subpopulations, such as reduced or oxidized cys-containing peptides (9). The $^{18}$O labeling method has the advantage that labels are intro-

$^{1}$ The abbreviations used are: SIL, stable isotope labeling; MS/MS, tandem MS; RP-HPLC, reverse phase-high pressure liquid chromatography; IEF, isoelectric focusing; pl, isoelectric point; FDR, false discovery rate.
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Produced enzymatically using trypsin, so that eventually any kind of protein sample may be labeled, the same mass shift is introduced in all the peptides and secondary reactions inherent to chemical labeling are avoided. In addition, the reagent needed ($^{18}$O-labeled water) is extremely stable and, because of its relatively low price, labeling of peptides produced from large amounts of sample is possible. However, $^{18}$O labeling is considered a more delicate and less robust technique than the others, and if it is not carefully controlled, the complete $^{18}$O labeling of peptides is not always attained, producing quantitative artifacts. In addition, $^{18}$O labels are pH-sensitive (10, 11) and hence not all peptide manipulation steps are fully compatible with this labeling method. These problems have hindered the widespread use of this technique in comparison with the other SIL methods. Not surprising, a wide repertoire of sample preparation, proteome digestion, and $^{18}$O labeling protocols may be found in the literature. The quantitative analysis of proteomes from human cells at the depth of several thousand proteins using this method has recently been demonstrated by our laboratory (10), showing that a full $^{18}$O incorporation is possible in very complex samples. However, we also reported the existence of a number of potential artifacts related to the method used, including incomplete proteome digestion and differential methionine oxidation. Clearly, a universal, robust, and high-throughput $^{18}$O labeling protocol, capable of attaining a full $^{18}$O incorporation, which avoids $^{18}$O unlabeling and that minimizes digestion and oxidation artifacts is currently needed. Such a method would put the wide application of this promising technique at the same level as its other SIL counterparts.

Protein digestion is most commonly performed in solution after protein denaturation in the presence of high urea concentrations (12, 13, 10). The use of centrifuge spin filters to wash away contaminants that might hinder subsequent MS analysis has also been demonstrated (14, 15). In-solution digestion is thought to be particularly adequate in studies concerning post-translational modifications (16); (17), where as much as possible from the protein sequence should be recovered for analysis. In-solution digestion is commonly followed by a first step of peptide fractionation by cation-exchange chromatography, which is done prior to reverse phase-high pressure liquid chromatography (RP-HPLC)-MS analysis (18). We (10) and previously others (12) have shown that this approach is fully compatible with $^{18}$O labeling. An alternative to in-solution digestion of proteins followed by peptide fractionation is protein fractionation by one-dimensional SDS-PAGE followed by in-gel digestion and RP-HPLC-MS peptide analysis of each fraction separately. This method may not only be more effective for the analysis of hydrophobic proteins, such as membrane proteins, but by trapping the proteins within the gel matrix it also allows the effective removal of detergents and other contaminants that might hinder subsequent trypsin digestion or may be difficult to eliminate from the peptide pool to avoid interferences with MS analysis. In conjunction with SILAC labeling and previous subcellular fractionation, in-gel digestion of SDS-PAGE-separated proteins allowed the quantification of whole proteomes from cell cultures at a depth of several thousand proteins (19, 20). Very recently a method has been described that combines the advantages of SDS protein solubilization with in-solution digestion in presence of urea, using centrifuge spin filters (21).

Protein separation by SDS-PAGE prior to $^{18}$O labeling of peptides has also been used to study the differential complex formation around the NFkB transcription factor p65 upon TNF-$\alpha$ stimulation (22). Lane et al. 2007 (23) employed a similar approach to perform a comparative analysis of microsomal P450 proteins in liver from control and drug-treated mice. Although the combination of SDS-PAGE protein separation and $^{18}$O labeling was demonstrated to attain femtomolar sensitivity (22), the variability introduced by protein preparation in a postdigestion method like $^{18}$O when the peptides were quantified by MS has never been investigated.

A recent alternative for peptide fractionation is isoelectric focusing (IEF) (24, 13), which presents an excellent resolution and provides another criterion, the peptide isoelectric point (pI), to validate peptide identifications (25). The off-gel technique uses IPG strips conventionally used for two-dimensional electrophoresis protein separation and maintains IEF-separated peptides in solution (26). This technique has been demonstrated to be highly efficient and reproducible in resolving complex peptide samples (27–29). By subjecting whole cell extracts to in-solution digestion and off-gel separation, the same depth of analysis was obtained as with subcellular fractionation followed by the one-dimensional-SDS-PAGE approach (29), but with less time and effort. IEF separation of $^{18}$O labeled peptides in the pH range 3–11 was used to compare the relative abundances of nuclear proteins from a drug resistant MCF-7 cancer cell line with those from the drug susceptible parent cell line (24). However, extreme pH values have been reported to cause acid- or base-catalyzed oxygen back-exchange (4) and in that work the stability of $^{18}$O peptide labeling was not addressed. Therefore, it still remains unclear whether the off-gel is fully compatible with protein quantitation by $^{18}$O labeling.

In a recent work, a method to determine the extent of individual $^{18}$O labeling of each one of the peptides quantified in a paired comparison of proteomes was demonstrated in our laboratory (30). Using this procedure to control for labeling efficiency, we demonstrated the precise $^{18}$O quantification of proteomes at a depth of several thousand proteins (10). In that work, a statistical model was also developed for the analysis of results obtained by $^{18}$O labeling and linear ion trap mass spectrometry (10). The model decomposes the sources of variance at the scan, peptide and protein levels, allowing their separate analysis. Although variance at the scan level is mainly dependent on the MS setup and at the protein level on the preparation of protein samples, at the peptide level it
measures the dispersion of quantitative values obtained from different peptides belonging to the same protein. Because this dispersion depends critically on the procedure used for peptide preparation and labeling, analysis of variance at the peptide level would not only inform about the accuracy associated to the protocol but also indicate the existence of quantification artifacts. Using this statistical framework, in a previous work we were able to detect systematic errors associated to protein digestion and differential methionine oxidation, which are factors whose effect on quantification accuracy have not been analyzed previously (10). Taken together, the existence of computational tools for a systematic control of 18O labeling efficiency and for the analysis of variance at the peptide level opens the way to the development of peptide preparation procedures that optimize labeling efficiency, are fully compatible with 18O labels, and at the same time maintain protein quantification accuracy.

In this work we apply these tools to demonstrate the existence of quantification problems associated to the combination of one-dimensional-SDS-PAGE protein fractionation and postdigestion 18O labeling. Also, we present a robust method that combines the advantages of both the SDS-PAGE and off-gel approaches, attains a full degree of 18O labeling, maintains 18O label stability, and keeps at a low and constant level peptide variance for proteomes obtained from a wide range of biological sources. According to our knowledge, this is the first systematic study of quantification error sources produced in a general sample preparation method for SIL.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Protein Extraction**—HepG2 and SK-N-MC human cell lines were obtained from American Type Culture Collection (ATCC no. HB-8065 and HTB-10TM). Cells were grown in MEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential aminoacids, and 0.05 mg/ml gentamicin, at 37 °C and 5% CO2. After trypsinization cells were plated out from 175-mm2 flasks to 100-mm dishes (Corning, Elmira, NY) at 3 × 106 cells/dish. After 24 h the cell pellet was washed twice with ice-cold phosphate-buffered saline, resuspended and incubated for 30 min in 350 l of 0.01 M ice-cold phosphate-buffered saline with 1% triton X-100 and EDTA-free Protease Inhibitor Mixture (Roche Applied Science) during 30 min at 4 °C. The suspension was homogenized in a Potter-Elvehjem homogenizer and centrifuged at 400 g for 5 min to remove cell debris. The supernatants were collected and protein concentration was determined by the Bradford method.

Cardiac mitochondria were isolated from rat hearts by differential centrifugation and Percoll-gradient ultracentrifugation as described previously (31). The purity of the mitochondrial preparations was assessed by Western blot analysis using antibodies for other cellular compartments. Protein concentration in mitochondrial extracts was measured using the Bradford protein assay.

J urkat T cells were grown in RPMI (GIBCO, Invitrogen) containing 10% fetal calf serum (Sigma) supplemented with L-glutamine plus antibiotics (100 units/ml penicillin and 100 l/g/ml streptomycin) until 300 × 10^6 cells were obtained, at 37 °C and 5% CO2. Cells were washed three times with serum-free RPMI and left to culture at 2 × 10^6 cells/ml in RPMI without serum. After 12 h, the conditioned medium was eliminated by three washes with phosphate-buffered saline and replaced by RPMI without serum. After 8 h conditioned media from two 150 ml flasks were combined, centrifuged at 200 × g for 5 min to remove cell debris and then at 100,000 × g for 1 h to remove intracellular vesicles. Supernatants were lyophilized, resuspended in 2.5 ml 25 mM ammonium bicarbonate, pH 8.8, desalted on PD-10 columns (GE Healthcare) equilibrated with the same buffer and lyophilized. Samples were taken up in 200 l water and protein concentration was assayed by the Bradford method.

The cytosolic fraction of Jurkat T cells stimulated with phorbol 12-myristate 13-acetate and calcium ionophore A23187 (lo) was obtained as described in (29). Briefly, after culturing cells for 12 h in RPMI without serum as described above, cells were washed thrice with phosphate-buffered saline and incubated for 8 h in RPMI without serum, supplemented with 20 ng/ml phorbol 12-myristate 13-acetate, 1 μM lo (Sigma). Control cells were cultured in serum-free media containing vehicle (dimethylsulfoxide). The cell pellet was incubated for 10 min in 800 l ice-cold lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.2% N-octylglucoside, and EDTA-free Protease Inhibitor Mixture). The suspension was homogenized in a Potter-Elvehjem homogenizer and centrifuged at 400 × g for 15 min to obtain a supernatant containing predominantly cytoplasmic proteins.

**Protein Quantification by Proteome Separation on SDS-PAGE and Postdigestion 18O Labeling**—A 300-μg aliquot of paired protein extracts were suspended in 100 μl sample buffer (5% (w/v) SDS, 10% (v/v) glycerol, 25 mM Tris-Cl, pH 6.8, 10 mM dithiotreitol, and 0.01% (w/v) bromphenol blue), separated on different lanes of a 1.5 mm thick, 10% SDS-PAGE gel, and visualized by Coomassie Brilliant Blue R-250 staining. Gel lanes were horizontally cut into 10 slices and each gel slice was cut into cubes (2 × 2 mm). The gel cubes were pooled in a tube and subjected to a standard overnight in-gel digestion at 37 °C (32) with 600 μl of 0.01 μg/μl sequencing grade trypsin (Pro- mega, Madison, WI, USA) in 50 mM ammonium bicarbonate, pH 8.8. The resulting tryptic peptides were extracted twice by 1-h incubation at room temperature using 400 μl of 12 mM ammonium bicarbonate, pH 8.8. Trifluoroacetic acid was added to a final concentration of 1% and samples were desalted on OMEI C18 tips (Varian) and dried-down.

Peptides from each gel slice were differentially labeled with either H216O or H218O (95%, Isotec, Miamisburg, OH) as previously described (10). After labeling, trypsin beads were removed using a physical filter (Wizard minicolumns; Promega, Madison, WI). The filtered samples were reduced with 10 mM dithiotreitol for 1 h at room temperature, and remaining trypsin activity was eliminated by alkylation by incubating with 50 mM iodoacetamide for 1 h at room temperature on the dark. The paired labeled samples from the corresponding gel fractions were then mixed, diluted to 2.5% acetonitrile (ACN), pH adjusted to 3 with 1 M ammonium formate, pH 3, and desalted onto C18 Oasis HLB Extraction cartridges (Waters, Milford, MA) using as elution solution 50% ACN in 5 mM ammonium formate, pH 3. The resulting peptides from each gel slice were dried down and analyzed separately by RP-HPLC-LIT.

**Protein Quantification by One-Step In-Gel Digestion, Peptide 18O Labeling, and IEF Fractionation**—The paired protein extracts, containing from 0.25 to 1 mg protein, were suspended in a volume up to 300 μl of sample buffer, and then applied onto 2.8-cm wide wells of a conventional SDS-PAGE gel (0.5 mm-thick, 4% stacking, and 10% resolving). The run was stopped as soon as the front entered 3 mm into the resolving gel, so that the whole proteome became concentrated in the stacking/resolving gel interface (Fig. 1). The unseparated protein bands were visualized by Coomassie staining, excised, cut into cubes (2 × 2 mm), and digested overnight at 37 °C with 60 ng/μl trypsin at 5:1 protein:trypsin (w/v) ratio in 50 mM ammonium bicar-
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Fig. 1. Scheme of the proposed $^{18}$O-based quantification protocol. For further details see the text. Numbers 1 and 2 indicate control points to check for labeling efficiency.

Dried peptides from the paired samples were subjected to differential $^{16}$O/$^{18}$O-labeling in 100 mM ammonium acetate, pH 6, 20% ACN, at 1:200 (v:w) immobilized trypsin/protein ratio (10). For the cytosolic fraction of Jurkat T cells, peptides from control cells were labeled with $^{16}$O, whereas peptides from activated cells were labeled with $^{18}$O. The extent of labeling reaction may be monitored at this point by taking up small aliquots, mixing them up and immediately analyzing them by HPLC-MS/MS. After labeling, trypsin activity was controlled by taking up small aliquots, mixing them up and immediately analyzing them by HPLC-MS/MS. After labeling, trypsin activity was controlled by taking up small aliquots, mixing them up and immediately analyzing them by HPLC-MS/MS.

For the experiments aimed to analyze the effect of activation on cytoplasmic proteins of Jurkat T cells, peptide samples differentially labeled with $^{16}$O/$^{18}$O were desalted separately on C18 Oasis cartridges, mixed at different ratios (1:1, 2:1, 4:1, 8:1, 1:2, 1:4, and 1:8) at a final content of 200 $\mu$g of peptides and dried-down. Each one of the peptide mixtures was taken up in 400 $\mu$l of 25% ACN in 5 mM ammonium formate pH 3, loaded onto MCX Oasis cartridges (Waters), eluted thrice with 100 $\mu$l 25% ACN in 1 mM ammonium formate pH 3, twice with 100 $\mu$l 25% ACN in 2 mM ammonium formate pH 3, and dried-down prior to RP-HPLC-LIT analysis.

LC-MS/MS Analysis and Peptide Identification—All samples were analyzed by LC-MS/MS using a Surveyor LC system coupled to a linear ion trap mass spectrometer model LTQ (Thermo-Finnigan, San Jose, CA) as previously described (34, 35). The LTQ was operated in a data-dependent ZoomScan- and MS/MS-switching mode (36). Zoom target parameters, number of microscans, normalized collision energy, and dynamic exclusion parameters were as previously described (34). Protein identification was carried out as previously described (34) using SEQUEST algorithm (Bioworks 3.2 package, Thermo Finnigan), allowing optional (methionine oxidation, lysine and arginine modification of +4 Da) and fixed modifications (cysteine carboxamidomethylation), two missed cleavages, 2 atomic mass units, or 1.2 atomic mass units mass tolerance for precursor or fragment ions, respectively. The MS/MS raw files from brain (SK-N-MC) and liver (HepG2) cell samples were searched against the Human Swissprot database (Uniprot release 14.0, 19929 sequence entries for human) supplemented with porcine trypsin, whereas those from rat samples were searched against the Mammal Swissprot database (Uniprot release 54.4, 56413 sequence entries for mammal). The same collections of MS/MS spectra were also searched against inverted databases constructed from the same target databases. SEQUEST results were analyzed using the probability ratio method (37) and FDR (false discovery rates) of peptide identifications were calculated from the search results against the inverted databases using the refined method (38).

When the off-gel technique was used to separate peptide pools, we used an improved version of the probability ratio method that took into account the isoelectric point (pI) of the peptides to improve peptide identification. The peptides were first identified by the conventional method using FDR<$\sim$0.01 as a criterion and the median pI of the corresponding peptides was then calculated in each off-gel fraction. An arbitrary window around the mean pI is then applied so that peptides whose pI are outside this window are considered as false identifications and a new peptide identification versus FDR curve is constructed. The width of the pI window is then iteratively varied and the FDR curves recalculated until an optimum FDR value is obtained. An example of the improved efficiency in peptide separation obtained using this algorithm can be found in Supplementary Fig. S1. This procedure is robust against potential problems arising during IEF peptide separation, because in no case the final performance is lower than the one obtained without the pI information. The procedure has been implemented into our probability ratio software, which is freely available upon request.
Peptide Quantification and Statistics—Peptide quantification from ZoomScan spectra and calculation of labelling efficiencies of all the identified peptides with a FDR lower than 5% were performed as described (39, 30) using QuiXoT, a program written in C# in our laboratory. Statistical analysis of the data was done on the basis of a novel random-effects model recently developed in our laboratory that includes four different sources of variance: at the spectrum-fitting, scan, peptide, and protein levels (10). The log2-ratio of peptide concentration in samples A (nonlabeled) and B (labeled) determined by scan s coming from peptide p derived from protein q is expressed as \( x_{qp} = \log_2(A/B) \). The statistical weight associated to the scan, \( w_{qps} \), is calculated from the spectrum fitting and the scan variance, \( \sigma^2_{qs} \), as described (10). The log2-ratio value associated to each peptide, \( x_{qp} \), is calculated as a weighted average of the scans used to quantify the peptide, and the value associated to each protein, \( x_q \), is similarly the weighted average of its peptides. Besides, a grand mean, \( \bar{x} \), is calculated as a weighted average of the protein values. In turn, the statistical weight associated to each peptide, \( w_{qp} \), is calculated from the corresponding scan weights and the peptide variance, \( \sigma^2_{qp} \), and that of each protein, \( w_q \), is calculated from the corresponding peptide weights and the protein variance, \( \sigma^2_p \). In all cases the statistical weights are the inverses of variances. Outliers at the scan and peptide levels are detected by calculating the probability that the measurements deviate from the expected average according to their respective variances, and controlling for the false discovery rate at each level, \( \text{FDR}_{qps} \) and \( \text{FDR}_{qp} \) respectively. Details about the statistical model and the algorithm used to calculate the variances at the scan, peptide, and protein levels can be found in our previous work (10). Raw quantification data may be found at: ftp://150.244.205.155/raw_quantif_data/raw_quantif_data.xls.

RESULTS

Quantitative Analysis of Proteomes by One-Dimensional SDS-PAGE Fractionation, In-Gel Digestion and 18O Labeling—We first analyzed whether one-dimensional-SDS-PAGE protein separation was suitable to perform a relative quantification of two protein preparations by 18O labeling. Two different proteome extracts from rat heart mitochondria, prepared under the same conditions, were separated by SDS-PAGE in two adjacent gel lanes and each protein lane was horizontally cut into 10 pieces at the same places. The 20 resulting gel pieces were subjected to in-gel trypsin digestion separately and the peptides were extracted, desalted, and subjected to trypsin-catalyzed labeling, as described under “Experimental Procedures,” the peptides from one sample in the presence of unlabeled water and those from the other in the presence of 18O-labeled water. To assure a complete elimination of residual trypsin activity, a step critical to avoid oxygen back-exchange of labeled peptides in the presence of nonlabeled water (11), we used a two-step procedure. First, immobilized trypsin beads were used for labeling, and the beads were separated by filtration; second, the potentially remaining trypsin activity in the filtrate was inhibited by reduction followed by alkylation. The two peptide samples corresponding to each gel fraction were then mixed and the resulting 16O/18O-labeled peptide pairs analyzed by RP-HPLC-MS/MS in a LTQ linear ion trap mass spectrometer, making a total of 10 HPLC runs. The LTQ was programmed to perform a Zoom scan spectrum and then an MS/MS spectrum over the six most intense ions detected in a survey MS scan, as described (39). The first scan was used for 16O/18O-labeled peptide pair quantification, and the second scan for peptide identification. 18O labeling efficiency of each one of the quantified peptide pairs was calculated automatically using an algorithm described previously (30).

As shown in Fig. 2A, using this procedure the majority of peptides were labeled with an efficiency of 0.9, and only a small amount had an efficiency lower than 0.8; this result was representative of several different experiments performed using this protocol. Because labeling efficiency is defined as the fraction of labeled oxygen atoms, in these conditions the remaining fraction of nonlabeled peptide belonging to the labeled sample was lower than 0.04; therefore the effect of labeling efficiency on the calculated ratio was in no case superior to 4% and could be efficiently corrected (30).

However, in the same figure it was observed that the cloud of quantifications had a greater dispersion in the log2-ratio scale than that usually observed in previous analysis using in-solution digestion (10). When quantifications from different gel fractions were analyzed separately, we observed that each fraction’s cloud of points was slightly displaced in relation to the others (Fig. 2A, black points), thus explaining the increased overall dispersion of quantifications in relation to the grand mean. Although this effect could be partially alleviated by subtracting to the quantifications in each fraction its own grand mean, this procedure introduced numerical errors and consistently produced higher variances than those observed when all the proteins were digested together in-solution (data not shown).

The sources of error associated to this protocol were studied in more detail by using a statistical random-effects model developed for the analysis of 18O labeling data by linear ion trap mass spectrometry (10). This model assigns to every individual quantitative measurement a different variance, calculated from the fitting of a theoretical isotope profile to the ZoomScan spectra (10); peptide means and variances are then calculated from the different scans by which each peptide is quantified and finally protein means and variances from the different peptides belonging to the same protein (10). One of the advantages of this method is that the variances at the scan level can be used to detect the presence of outliers, i.e. of scans that deviate from peptide mean more than expected from their estimated variance. With this protocol we routinely found a striking large proportion of scan outliers (15% of the total number of scans in the example presented in Fig. 2B, see black points). A detailed analysis of these outliers revealed that they were produced by peptides coming from proteins that were quantified in different fractions because the proteins were located in the frontier between two adjacent gel slices. Accordingly, when proteins identified in different fractions were statistically treated as if they were...
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**Fig. 2.** Analysis of quantification artifacts associated to $^{18}$O labeling by the SDS-PAGE protein fractionation technique. A. Distribution of labeling efficiencies as a function of log2-ratios in a representative experiment (gray points). Scans corresponding to a representative gel fraction are indicated by black points. B and C. Analysis of outliers at the scan level. The statistical weight associated to each quantification is plotted against the difference between the scan measurement and their corresponding peptide average in order to highlight the presence of outliers. Black points indicate outliers at the scan level (FDRqps < 5%). In (C) the peptides and proteins which were quantified in different gel fractions were considered as a different species. D. Effect of alkylation on quantifications at the peptide level. The statistical weight associated to each peptide is plotted against the difference between the peptide and their corresponding protein average. Peptides that were acetylated in Lys are indicated by black points, while these that were also found in non-acetylated form are indicated by white points. E. Analysis of proteins quantified in more than one gel fraction. The plot shows the protein averages corrected for the grand mean, assuming that the proteins which were quantified in different gel fractions are different entities; the lines join averages from the same protein when they were quantified in different fractions. For all the proteins, the averages having higher protein weights are plotted first. Note that the lines cross the horizontal axis when the protein log-ratios have different sign in the gel fractions.

The number of scan outliers became negligible, just as observed in a previous work (10) (Fig. 2C), indicating that these outliers were not consequence of measurement errors but were produced by the “gel-cutting effect.” Further evidence of this effect was provided by plotting the log$_2$-ratios of proteins that were quantified separately in adjacent fractions (Fig. 2E). As shown, in most cases the protein ratios in one fraction were clearly different to those in the other. We have observed this effect to occur systematically in all experiments performed following this protocol, even when the two samples were checked in advance to have the same electrophoretic behavior and the cuts were carefully performed in gel zones where no proteins were detected by Coomassie staining (data not shown).

A further technical problem associated to this protocol is that it requires two reduction/alkylation steps, being the first one necessary to achieve a good yield during in-gel trypsin digestion, and the second to inactivate residual trypsin activity after the labeling step. To analyze whether the double alkylation treatment affected amine groups, known to react to a certain extent with iodoacetyl moieties, and whether these secondary reactions could affect protein quantification at the peptide level, the database search was repeated by setting as variable modification carbamidomethylation at Lys residues and N-terminal peptide ends. We observed that a surprisingly large proportion (20%) of these amine groups was alkylated. Furthermore, peptides containing alkylated amino groups were often detected as peptide outliers, because they tended to produce a ratio significantly different from other peptides belonging to the same protein (Fig. 2D). Clearly, an alternative, artifact-free method was also required to attain trypsin inactivation.

**Quantitative Analysis of Proteomes by One-Step In-Gel Digestion, $^{18}$O-Labeling and Off-Gel Peptide fractionation: Digestion and Labeling Efficiencies**—To circumvent the above mentioned limitations, we devised a new strategy, which kept the SDS-PAGE protein treatment step but avoided the gel-cutting effect. In the new protocol, schematized in Fig. 1, voltage was stopped when the front of the run entered 3 mm into the resolving part of the gel, so that all proteins got inside the stacking gel but remained unseparated forming a single band around the stacking and resolving gel interface. The bands from each sample were then cut into pieces and subjected to in-gel trypsin digestion in one single tube per sample. The resulting peptides were then extracted, $^{16}$O/$^{18}$O-labeled and mixed together.

In-gel digestion conditions were performed using always the same gel geometry, protein concentration, and volume, and protein:trypsin ratios, as described under “Experimental Procedures.” To optimize the efficiency of in-gel digestion as much as possible, the detergent 5-cyclohexyl-1-pentyl-$\beta$-o-maltoside was also added to the digestion buffer (33). In test experiments, we observed that the presence of 5-cyclohexyl-1-pentyl-$\beta$-o-maltoside not only increased the number of peptides that could be identified in the extract, but also the relative proportion of larger peptides (data not shown). Digestion efficiency was assessed by calculating the proportion of identified peptides containing one or more missed cleavages, i.e. non-C-terminal Arg or Lys residues not flanked by Pro residues. The new in-gel digestion approach produced a proportion of missed cleavages lower than 10% (Table I), which...
was lower than that obtained using the in-solution digestion protocol in the presence of urea (10) (Table I). This digestion efficiency was consistently maintained when proteomes from a variety of different sources, including those rich in membrane proteins, were analyzed, demonstrating the good reproducibility of the protocol (Table I).

After digestion, peptide labeling was performed at pH 6 using ammonium acetate as a buffer (Fig. 1). After the labeling step, and to avoid secondary reactions of residues other than Cys during the alkylation step, in the new protocol trypsin inactivation was achieved by incubating the labeled peptides with the irreversible trypsin inhibitor TLCK (Fig. 1). This procedure was found to be effective to prevent label back-exchange reactions because of residual trypsin activity (see also below).

In the new protocol the pool of labeled peptides were desalted and IEF-separated into 24 fractions (Fig. 1) using the off-gel technique. Because this technique allows an efficient separation of peptides according to their pi, this parameter was used as an additional criterion to improve peptide identification (see Experimental Procedures and Supplemental Fig. S1). A representative distribution of identified peptides along a 4–7 pi range is presented in Fig. 3A; we observed that this pi distribution of peptides was essentially similar when proteomes from other sources were analyzed using this protocol (data not shown) and agreed well with previously published studies (26, 27, 40). We also noticed that the peptide desalting step before off-gel fractionation (Fig. 1) was highly advisable to optimize the IEF peptide separation. In these conditions, more than 65% of the identified peptides were found in only one fraction and more than 75% in one or two fractions (Fig. 3B). The peptide redundancy, defined as the ratio of peptides that are identified adding up all the fractions by the final number of unique peptides, was usually lower than two.

### Table I

| Proteomes            | % Partial Digestion | Scan Variance (S, 95% C.I.) | Peptide Variance (P, 95% C.I.) | Protein Variance (Q, 95% C.I.) |
|----------------------|---------------------|----------------------------|--------------------------------|--------------------------------|
| H1                   | 5.3                 | 0.011 (0.010–0.012)        | 0.019 (0.018–0.020)            | 0.005 (0–0.011)                |
| H2                   | 4.2                 | 0.019 (0.018–0.020)        | 0.017 (0.016–0.018)            | 0.005 (0–0.009)                |
| S1                   | 2.8                 | 0.017 (0.016–0.018)        | 0.018 (0.018–0.020)            | 0.005 (0–0.009)                |
| Mit                  | 6.7                 | 0.012 (0.011–0.013)        | 0.019 (0.018–0.020)            | 0.006 (0–0.010)                |
| HUVEC                | 8.0                 | 0.019 (0.017–0.021)        | 0.014 (0.014–0.019)            | 0.0007–0.004                  |
| Mit                  | 12.9–39.8           | 0.018–0.031                | 0.018–0.040                    |                                |

a Partially digested peptides were assumed to be those containing at least one missed cleavage site that did not contain a proline residue after the Arg or Lysine residue.

**FIG. 3.** Analysis of peptide identifications by the IEF off-gel technique. A, Peptide fractionation profile, B, distribution of peptides according to the number of different fractions where they were identified in a representative experiment. These results were obtained from the analysis of a protein extract from HepG2 cells, whose labeled peptides were fractionated into a 4 to 7 pH range. Figures indicate the number of unique peptides (NP) and proteins (NQ) identified.
The compatibility of this protocol to separate $^{18}$O-labeled peptides was then investigated by analyzing the labeling efficiency of all the quantified peptides. The procedure included two desalting steps that were performed at a controlled pH 3.0 using ammonium formate buffer (Fig. 1); this pH was low enough to allow quantitative recovery of peptides from C18 solid-phase extraction cartridges and at the same time was high enough to avoid acid-catalyzed oxygen back-exchange reactions. As shown in Fig. 4, the peptides remained effectively labeled during the two desalting steps and all the time needed to run the off-gel fractionations, even when pI ranges of 3 to 10 were used. In addition no tendency to acid or base-catalyzed unlabeling was observed even at the more extreme pH fractions (Fig. 4). The distribution of labeling efficiencies obtained using the new protocol was remarkably similar to the one obtained using the previously proposed protocol (Fig. 4B and C). These results demonstrate for the first time that the off-gel peptide separation method is fully compatible with $^{18}$O labeling.

Quantitative Analysis of Proteomes by One-Step In-Gel Digestion, $^{18}$O-Labeling, and Off-Gel Peptide Fractionation: Analysis of Variances—To assess the sources of variability of the new method we applied the statistical model previously developed for $^{18}$O quantification by linear ion trap MS (10). We observed that the proportion of scan outliers was negligible (typically no more than three scan outliers among 1300 scans), in agreement with the results obtained previously (10). Besides, and as shown in Table I (second row), the scan variances obtained from a number of different proteomes were all similar to those obtained in a previous work (10). Also, as shown in Table I (fourth row), in none of the proteomes analyzed in this study was the protein variance significantly different from zero. This result just indicated that the different cell cultures and subproteome fractionations used in this study were performed in a way that did not introduce appreciable error sources for relative comparison of protein levels.

Analysis of the variance at the peptide level, a parameter more critical to judge the suitability of the proposed method based on postdigestion SIL, was then performed in detail for each one of the proteomes analyzed in Table I. As shown in Fig. 5, left panels, the peptide quantifications containing missed cleavage sites (black points) or completely digested subpeptides derived from the former (white points) did not show an appreciable deviation from the rest of the peptides, indicating that in all cases the digestion was reproducible in the two samples that were compared. A similar result was obtained when the quantifications of peptides containing ei-
ther nonoxidized (white points) or oxidized Met residues (black points) were analyzed (Fig. 5, right panels), indicating that no quantification artifacts were introduced by the protocol because of differential Met oxidation in the two samples. Similar results to those presented in Fig. 5 were obtained with the other proteomes listed in Table I (data not shown). In consistency with these results, the number of peptide outliers yielding a log2-ratio significantly displaced from the protein average, as judged by the analysis of the FDRp parameter (see Experimental Procedures) was found to be negligible (less than four per every 1800 quantified peptides) in all the proteomes analyzed. Moreover, and as shown in Table I (third row), the calculated variances at the peptide level were remarkably similar in all proteomes studied and essentially identical to those obtained by in-solution digestion and labeling (10).

The accuracy of the null hypothesis associated to the statistical model was tested in the proteomes analyzed. This was done, as in our previous work (10), by plotting the distribution of protein log2-ratios standardized according to their estimated variances ($z_q$ values) and fitting the curves to Gaussian functions. As shown in Fig. 6, the standard deviations of the curves were in no case significantly different from one, as expected. In addition, no deviations from normality were detected in any of the corresponding normal probability plots (Fig. 6, inset). Therefore, the errors produced by the proposed protocol were reproducible, well-controlled, and in good agreement with the null hypothesis of the statistical model used and the variance parameters in Table I.

Application of the Technique to Study Cytoplasmic Activation of Jurkat T cells and Analysis of Dynamic Range—To determine the dynamic range of quantification and at the same time demonstrate the utility of the technique for the analysis of protein expression changes within a physiological context, we used as a model cytosolic extracts of either control or phorbol 12-myristate 13-acetate/H11001-13-stimulated Jurkat T cells. These extracts were digested using the one-step in-gel protocol and the resulting peptides from the control and activated cells were labeled with either $^{16}$O or $^{18}$O, respectively. The two samples were then mixed at different ratios (1:1, 2:1, 4:1, 8:1, 1:2, 1:4, and 1:8), fractionated, and analyzed, so that the quantitative results from these seven independent experiments could be compared in terms of variances and of statistical significance of expression changes.

In each experiment the grand mean ($\bar{x}$) was calculated as the weighted mean of the log2-ratios of all quantified proteins (10). When the grand mean estimations were compared with the expected values, a good agreement was obtained in the entire range except for a slight underestimation of the expected value at the 8:1 ratio (Supplementary Fig. S2A). This effect is expected, since at these ratios the isotopic cluster peaks of $^{16}$O-labeled peptides significantly interfere with their $^{18}$O-labeled peptide counterparts, diminishing the accuracy of the estimated ratios. Accordingly, variances at the scan, peptide, and protein levels remained reasonably stable, and were similar to those obtained with the other proteomes analyzed at 1:1 ratios, except at the peptide level in the case of the 4:1 and 8:1 ratios, where, for the same reason, they showed a tendency to increase (Supplementary Table S1). The distributions of standardized log2-ratios, however, were in all cases in excellent agreement with the expected curves for a normal distribution with a variance of one, even at the two most extreme sample ratios (Supplementary Fig. S2B–D), demonstrating the validity of the null-hypothesis model in the entire dynamic range tested.
Further information about the performance of the method along the dynamic range was obtained by comparing the expression changes induced by activation of Jurkat T cells at the seven sample ratios. As shown in Table II, an excellent agreement in the magnitude and sign of the most significant expression changes was obtained in all the experiments. In addition, although the statistical significance of expression changes (expressed in terms of the standardized variable $Z_q$) showed a tendency to diminish at the more extreme sample ratios, as expected, the significance results were highly consistent in all the experiments.

The statistical model that bases our method allows accurate estimations of the dispersion of each one of the measurements at the scan, peptide, or protein levels by just calculating the variance as the inverse of the statistical weight (10). For illustrative purposes the dispersions of the log2-ratios obtained by using the proposed method are presented in Fig. 7. Note that these are typical results, because, as shown in Table I (and also Supplemental Table S1), the general variances are obtained in a reproducible manner in different kinds of samples. As shown, the total dispersion of log2-ratios tend to diminish at higher weights, but even the less accurate measurements rarely deviates more than twofold from the expected value (Fig. 7A). The dispersions of individual scans from their peptide mean (Fig. 7B) and of peptides from their protein mean (Fig. 7C) are very narrow and because of the effect of averaging produce an even narrower distribution of proteins around the grand mean (Fig. 7D). In these conditions,

### Table II

Comparative analysis of differential expression events upon Jurkat T cell activation when control and treated samples are mixed at different ratios

| Accession Number | Protein Description | Corrected log2-ratio | Fold change | $Z_q$ | FDRq |
|------------------|---------------------|---------------------|-------------|-------|------|
| P11021 | GP78 78 kDa glucose-regulated protein precursor | $-0.54$ | 1.47 | $-5.33$ | $<0.01$ |
| P00670 | VIME Vimentin | $-0.55$ | 1.45 | $-5.99$ | $<0.01$ |
| P16614 | DREM Dream | $-0.60$ | 1.50 | $-3.36$ | $<0.01$ |
| P43400 | NAMPT Nicotinamide phosphoribosyltransferase | $-0.75$ | 1.52 | $-3.77$ | $<0.01$ |
| Q6H91 | IMM JIMM-34 immunoglobulin | $-0.39$ | 1.31 | $-2.74$ | $<0.01$ |
| P35200 | PPARalpha 2-alpha fatty acid-binding protein | $-0.78$ | 1.61 | $-4.16$ | $<0.01$ |
| Q3UZ3 | STM2C Stem-to-leaf protein 2 | $-0.53$ | 1.28 | $-3.01$ | $<0.01$ |
| P27196 | VDAC Voltage-dependent anion-selective channel protein 1 | $-0.53$ | 1.40 | $-2.48$ | $<0.01$ |
| Q02680 | PAQR Preproglutamate-associated protein X | $-0.98$ | 1.34 | $-5.42$ | $<0.01$ |
| P14632 | HSP74 Heat shock 70 kDa protein 4 | $-0.63$ | 1.38 | $-3.37$ | $<0.01$ |
| Q9035 | MPPC Phosphoribosyl diphosphate | $-0.50$ | 1.27 | $-2.84$ | $<0.01$ |
| P41221 | PER Pernipherin | $-0.19$ | 1.60 | $-1.29$ | $<0.01$ |
| Q9VU1 | HOU1 Hypothetical protein 1 | $-0.15$ | 1.16 | $-1.31$ | $<0.01$ |
| P28880 | VDAC Voltage-dependent anion-selective channel protein 2 | $-0.50$ | 1.30 | $-2.75$ | $<0.01$ |
| P41025 | ACT5 C-T beta-casein | $-0.03$ | 0.99 | $-1.38$ | $<0.01$ |
| P68909 | RL31 O150 protein | $-0.23$ | 1.20 | $-1.39$ | $<0.01$ |
| P27004 | ATP synthase subunit alpha | $-0.23$ | 1.13 | $-2.21$ | $<0.01$ |
| P26889 | UBR Ubiquitin | $0.24$ | 1.15 | $1.36$ | $<0.01$ |
| P60174 | TPIS Trypsin-like proteinase | $0.37$ | 1.12 | $1.36$ | $<0.01$ |
| Q9S74 | OMGa Gm ratiation factor gamma | $0.48$ | 1.27 | $1.36$ | $<0.01$ |
| Q9S7M | FACP Falcocircum lineae group 1 protein | $0.48$ | 1.29 | $1.36$ | $<0.01$ |
| Q9V25 | SYLC Leucyl-tRNA synthetase | $0.20$ | 1.20 | $1.36$ | $<0.01$ |
| Q6643 | DREB DREBP | $0.47$ | 1.21 | $1.36$ | $<0.01$ |
| Q8TS5 | NPT1 Potassium channels H+ pump | $0.05$ | 1.33 | $1.36$ | $<0.01$ |
| P72502 | KCPA Protein kinase C alpha type | $0.50$ | 1.40 | $1.36$ | $<0.01$ |
| Q0076 | ACOD Acid hydrolase domain | $0.49$ | 1.37 | $1.36$ | $<0.01$ |
| Q4519 | KAD2 Adenylate kinase isoform 2 | $0.13$ | 1.33 | $1.36$ | $<0.01$ |
| P26217 | RPL6 ribosomal protein L8 | $0.37$ | 1.34 | $1.36$ | $<0.01$ |
| Q9N28 | DDBH Dibehexose | $0.13$ | 1.54 | $2.23$ | $<0.01$ |
| Q03N2 | WR56 WDR 18-containing protein 3 | $0.44$ | 1.30 | $2.35$ | $<0.01$ |
| Q147 | KIAA1430 ubiquitin conjugation enzyme family A1 protein | $0.62$ | 1.53 | $2.35$ | $<0.01$ |
| P07777 | INHBI Inhibin beta-1 | $0.47$ | 1.34 | $2.35$ | $<0.01$ |
| P01694 | STT1 Stathmin | $0.47$ | 1.34 | $2.35$ | $<0.01$ |
| P60440 | RB Retinoblastoma-associated protein | $0.37$ | 1.31 | $2.35$ | $<0.01$ |
| Q462 | MIPS Myosin | $0.54$ | 1.48 | $2.35$ | $<0.01$ |
| P99080 | DDBH Dibehexose | $0.35$ | 1.54 | $2.35$ | $<0.01$ |
| Q07L44 | MOLA Mps one binder kinase activo-xk1 | $0.40$ | 1.33 | $2.35$ | $<0.01$ |
| P33520 | RCH2 Ribosomal protein L2H3-phosphate dehydrogenase subunit M2 | $0.36$ | 1.30 | $2.35$ | $<0.01$ |
| P26820 | RH Rhinoceros | $0.49$ | 1.34 | $2.35$ | $<0.01$ |
| P07373 | TVM T-cell receptor beta chain V region 1T3 precursor | $0.13$ | 1.75 | $2.35$ | $<0.01$ |
| P60640 | CHI1104a heat shock protein | $0.41$ | 1.37 | $2.35$ | $<0.01$ |
| P37698 | P2M1 Mantle cell lymphoma | $0.37$ | 2.02 | $2.35$ | $<0.01$ |

a Proteins quantified in two or more experiments.

b Log2-ratios were corrected by subtracting the grand mean value obtained in each experiment.

c Standardized normal values were obtained by dividing protein log2-ratios by their variance, as described (10).

d The fold change and the FDRq (false discovery rate at the protein level) were calculated by analyzing as a whole all the results from the seven experiments. Proteins are sorted by $Z_q$. The magnitudes of expression change and of the standardized variables are shown according to the color scales on the top.
expression changes as small as those of the protein vimentin (1.4-fold increase, Table II) are clearly rendered as statistically significant (Fig. 7, black points).

To determine what proteins showed significant expression changes after stimulation of Jurkat T cells, we analyzed as a whole the results obtained in all the experiments. Using a 10% FDRq threshold, corresponding to less than two false expression changes, 17 proteins were found up-regulated and 25 down-regulated (Table II). Among these, several proteins are known to be involved in T-cell signaling. Thus, NAMPT, STML-2, PA2G4, and PURA2, proteins playing a role in T-cell activation (41–45), were increased. The actin-binding protein Drebrin also decreased in the cytosolic fraction of activated Jurkat T cells, in line with recent findings demonstrating relocalization of this protein toward the immunological synapse at the plasma membrane in activated human T cells (46). In addition, a long-time treatment with phorbol 12-myristate 13-acetate plus lo has also been reported to induce apoptosis in Jurkat T cells (47); accordingly, a number of proteins known for participating in the cellular response to stress conditions were found increased in this analysis, including the ER-stress sensors GRP78 and HYOU1 (48, 49); a subunit of the ATP synthase complex, ATPA (50), and the mitochondria-mediated apoptosis proteins VDAC1 (51), VDAC2 (52), and IMMT (53). Some of the proteins whose levels decreased in the cytoplasm of our cells (H4 and KPCA) have recently been reported to increase in the lipid raft fraction of apoptotic Jurkat T cells (54). In these cells, KPCA disappears from the cytoplasm and is redistributed to lipid rafts of plasma membrane (54). Such relocalization of KPCA under apoptotic conditions is consistent with the reduction of KPCA in our cytosolic extracts. The list of down-regulated proteins also includes cytoskeletal proteins (DEST, LMNB1, STMN1, and MTPN), proteins playing a role in energy (TPIS) and lipid metabolism (ACOD, FDFT), and in protein synthesis and degradation (UBIQ, SYLC, and RL860S). All these results indicate that, although some accuracy is lost in the detection of changes taking place at high ratios, the proposed protocol allows full control over the sources of variance and accurate estimation of statistical significances for the detection of protein expression changes of physiological relevance in a dynamic range spanning about two orders of magnitude.

**DISCUSSION**

In this work we present a general protocol for 18O-based labeling tested on a set of samples of different nature. During optimization of experimental conditions in order to minimize quantification artifacts because of digestion, methionine oxidation, and incomplete labeling, this protocol was subjected to a profound statistical analysis on the basis of a previously published statistical model (10). As a first step toward this general 18O-labeling protocol, we performed a detailed statistical analysis of the widely used in-gel digestion method of SDS-PAGE-separated proteins (19, 20). SDS-solubilized proteins are known to be readily digested in-gel after SDS-PAGE protein separation and SDS elimination from the gel matrix. We reasoned that this kind of approach based on protein solubilization using SDS would be compatible with most kinds of proteomes, including those containing hydrophobic and membrane proteins. However, our data clearly demonstrates that although this protocol is satisfactory from the point of view of labeling efficiency, it suffers from an increased quantification variance, because each fraction has to be digested and labeled separately. This manipulation introduces systematic effects that are impossible to control at exactly the same extent in all the fractions. Moreover, even when the gel is cut into fractions at gel zones where proteins are not detected by staining, it is very difficult, if not impossible, to cut all proteins into fractions having exactly the same proportion in the two samples, especially in the case of proteins located at the cutting point between two adjacent gel slices. This makes unreliable the quantification of a protein located in adjacent fractions. Our data uncovers for the first time the "gel-cutting" effect associated to the one-dimensional-SDS-PAGE protein separation, demonstrating the problems encountered when this protocol is used with postdigestion SIL techniques. Also, we demonstrate that the reduction/alkylation step traditionally used for trypsin inactivation after 18O-labeling, modified a significant proportion of peptide amino groups, introducing an
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additional source of quantification artifacts at the peptide level. This effect was produced because, being amine alkylation a minor reaction, it is highly unlikely that it takes place at exactly the same extent in the two protein samples.

Taking into account these results we designed an improved high-throughput semiquantitative proteomics protocol, based on protein concentration on SDS-PAGE, followed by one-step in-gel digestion and peptide $^{18}$O-labeling. Because reproducibility and efficiency of the digestion step is critical to achieve a robust and artifact-free peptide-centric quantification, the in-gel digestion step was optimized, obtaining higher and more reproducible digestion efficiencies with proteomes of different nature, including membrane proteomes, compared with in-solution digestion. To minimize eventual losses of long peptides, which have been associated to in-gel digestion procedures (see for instance 55), the detergent 5-cyclohexyl-1-pentyl-β-D-maltoside was added to the digestion mixture. This detergent has been described to improve recovery of large hydrophobic peptides, reducing the risk of entrapment of proteolytic peptides within the gel matrix (33). Moreover, the good digestion efficiency of the new protocol was checked as not to be because of a bias against longer peptides partially retained by the gel matrix, because with this protocol long peptides containing missed cleavage sites were sometimes detected in higher amounts during the optimization of experimental conditions (not shown). After the labeling, the reduction/alkylation step was substituted by treatment with the irreversible trypsin inhibitor TLCK, a modification that was found to be effective in preventing label back-exchange throughout all the time of analysis. This step avoided the possibility of uncontrolled chemical modifications introducing quantification artifacts. Fractionation of labeled peptides was performed by off-gel IEF, a method that gave very satisfactory results in terms of reproducibility and peptide quantification performance, in agreement with other recent works (27, 28, 29). This method also allowed using the pI as an additional criterion to validate peptide identifications (25). As we show in the supplemental information, the number of peptides identified at the same error rate increased typically by 5%-7%, depending on the pH range used, being this effect more pronounced at narrow pH ranges. By analyzing the labeling efficiency of each one of the quantified peptides, we could demonstrate that the off-gel separation is fully compatible with $^{18}$O peptide labeling, even at rather extreme pH values of 3 or 10. Although $^{18}$O labels are long known to be pH-sensitive (10, 11) and of-gel IEF have been used before by others to fractionate $^{18}$O-labeled peptides (24), to the best of our knowledge the label stability as a source of potential artifacts has not been analyzed before and was not taken into account in previous studies devoted to protein quantification by SIL.

The suitability of a stable isotope labeling method for quantitative proteomics can only be established by analyzing the accuracy with which proteins are quantified, using an appropriate statistical framework. After analyzing a set of proteomes of different nature, together with a proteome exhibiting differential expression of proteins in which the control and treated samples are mixed at different relative proportions, we demonstrate that the variance at the scan level was essentially identical for all of them. Although this similarity was foreseen, because quantifications were made using the same MS method described before (39), we should note that this result reinforces the robustness of the statistical algorithm used to analyze this kind of data. On the other hand, the two main sources of variability at the peptide level are the partial digestions and methionine oxidation, which may occur at different extents in the two samples, making protein quantification unreliable. We demonstrate that in the novel protocol these two potential sources of quantification artifacts do not increase the variability for individual peptide quantification, rendering practically identical variances at the peptide level for all the proteomes analyzed. The variability because of the residual degree of partial digestion was minimized thanks to the efficient and reproducible in-gel digestion achieved in the new protocol. Also the new approach involved less sample manipulation and only about a third of the mass spectrometric analysis time compared with previous conditions (10), which probably might be the cause for the absence of Met oxidation artifacts. Hence, the new protocol allowed the inclusion in the analysis of all scans corresponding to partially digested and Met-containing peptides, which might amount up to 25% of those yielding positive peptide identification, thus increasing the number of quantified peptides without compromising protein quantification accuracy. We also analyze the dynamic range of quantification and demonstrate the utility of the method in the practice by showing the consistent detection of expression changes in a model of activated T cells. At the protein level, the source of variance is related to the method used to prepare the biological samples and to extract the proteins, and has little, if any, relation with the MS method used for protein quantification. The variance at the protein level was also similar among all the analyzed proteomes and close to zero, indicating that no biases were introduced during sample preparation from cell cultures, tissues, or subcellular fractionations. Finally, the distribution of quantified proteins, normalized according to its estimated variance, could be very accurately explained by a Gaussian function with a standard deviation of one in all the cases. This final and definitive result indicates that the variance integration at the three quantification levels was properly performed to calculate the final protein variances, which behaved as expected according to the statistical model.

Taken together, all these results demonstrate that the proposed protocol for protein digestion and $^{18}$O labeling is suitable for performing quantitative studies on a wide variety of protein preparations. To the best of our knowledge, this is the first time that a general protocol for stable isotope labeling is tested in practice using a collection of samples of different origin and analyzed at this degree of statistical detail. The
development and testing of such protocol was possible thanks to the algorithm that calculates the individual $^{18}$O-labeling efficiency of all the peptides (30) and also to the existence of a statistical model for the null hypothesis (10) against which the different levels of variance associated to the experimental procedure can be tested. The similarity of the variances also suggest that following carefully the experimental conditions described here the null-hypothesis model defined by these variances can be considered as a good approach to detect the presence of significant expression changes in true quantitative experiments obviating the need for a careful adjustment of variances in each experiment. This would speed up the time required to interpret quantitative data generated in large-scale experiments. Similarly, the set of variances presented in this work can be used as reference for the detection of additional sources of error and artifacts. In our experience we have found some instances where an increase in variance at the scan level was indicative of malfunctions in the mass spectrometer, whereas at the peptide level they warned us against problems in the digestion step. We have also been able to detected significant increases in the variance at the protein level when experiments were performed trying to compare samples that required many steps of protein preparation (data not shown).

In summary, in this work we present a new protocol based on SDS-PAGE protein concentration, one-step in-gel digestion, peptide $^{18}$O-labeling, and fractionation by off-gel IEF. This protocol, together with our previously developed computational algorithm that allows semi-automated data analysis (30), could become a general approach to perform large-scale semiquantitative proteomic studies by $^{18}$O labeling and linear ion trap mass spectrometry. We believe that with these developments the $^{18}$O-labeling technique has reached its maturity, attaining the same level of reproducibility and stability than other SIL methods, with the added value that it has a computational algorithm that allows semi-automated data analysis of protein preparation (data not shown).

REFERENCES

1. Li, X.J., H. Zhang, J.A. Ranish, and R. Aebersold, (2003) Automated statistical analysis of protein abundance ratios from data generated by stable-isotope dilution and tandem mass spectrometry. Anal Chem, 75, 6648–57

2. Ong, S.E., B. Bioglov, I. Kratchmarova, D.B. Kristensen, H. Steen, A. Pandey, and M. Mann, (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics, 1, 376–86

3. Griffin, T.J., H. Xie, B. Bodnariu, K. Popko, A. Mohan, J.V. Carls, and L. Higgins, (2007) iTRAQ reagent-based quantitative proteomic analysis on a linear ion trap mass spectrometer. J Proteome Res, 6, 4200–9

4. Schnolzer, M., P. Jedrzelewski, and W.D. Lehmann, (1996) Protease-catalyzed incorporation of 18O into peptide fragments and its application for protein sequencing by electrospray and matrix-assisted laser desorption/ionization mass spectrometry. Electrophoresis, 17, 945–53

5. Yao, X., A. Freas, J. Ramirez, P.A. Demirev, and C. Fenselau, (2001) Proteolytic $^{18}$O labeling for comparative proteomics: model studies with two serotypes of adenovirus. Anal Chem, 73, 2836–42

6. Mirgorodskaya, O.A., Y.P. Kozmin, M.J. Titov, R. Korner, C.P. Sonksen, and P. Roepstorff, (2000) Quantitation of peptides and proteins by matrix-assisted laser desorption/ionization mass spectrometry using ($^{18}$O)-labeled internal standards. Rapid Commun Mass Spectrom, 14, 1228–32

7. Ross, A., R.D. Unwin, C.A. Evans, S. Griffiths, L. Carney, L. Zhang, E. Jaworska, C.F. Lee, D. Blinco, M.J. Okoniewski, C.J. Miller, D.A. Bitton, E. Spooncer, and A.D. Whetton, (2008) Eight-channel iTRAQ enables comparison of the activity of six leukemogenic tyrosine kinases. Mol Cell Proteomics, 7, 853–63

8. Gygi, S.P., B. Rist, S.A. Gerber, F. Turecek, M.H. Gelb, and R. Aebersold, (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol, 17, 994–9

9. Leichert, L., F. Gehrke, H.V. Gudiseva, T. Blackwell, M. Ilbert, A.K. Walker, J.R. Stahlr, P.C. Andrews, and U. Jakob, (2008) Quantifying changes in the thiol redox proteome upon oxidative stress in vivo. Proc. Natl. Acad. Sci. U.S.A., 105, 8197–202

10. Jorge, I., P. Navarro, P. Martinez-Acedo, E. Nunez, H. Serrano, A. Alfranca, J.M. Redondo, and J. Vazquez, (2009) Statistical model to analyze quantitative proteomics data obtained by $^{18}$O/$^{16}$O labeling and linear ion trap mass spectrometry: Application to the study of VEGF-induced angiogenesis in endothelial cells. Mol Cell Proteomics, 8, 1130–49

11. Staes, A., H. Demol, J. Van Damme, L. Martens, J. Vandekerckhove, and K. Gevaert, (2004) Global differential non-gel proteomics by quantitative and stable labeling of tryptic peptides with oxygen-18. J Proteome Res, 3, 786–91

12. Blonder, J., M.L. Hale, K.C. Chan, L.R. Yu, D.A. Lucas, T.P. Conrads, M. Zhou, M.R. Popoff, H.J. Issaq, B.G. Stiles, and T.D. Veenstra, (2005) Quantitative profiling of the detergent-resistant membrane proteome of iota-b toxin induced vero cells. J Proteome Res, 4, 523–31

13. Wang, N., L. Mackenzie, A.G. De Souza, H. Zhong, G. Goss, and L. Li, (2007) Proteome profile of cytosolic component of zebrafish liver generated by LC-ESI MS/MS combined with trypsin digestion and microwave-assisted acid hydrolysis. J Proteome Res, 6, 283–72

14. Manza, L.L., S.L. Stamper, A.J. Ham, S.G. Codreanu, and D.C. Liebler, (2005) Sample preparation and digestion for proteomic analyses using spin filters. Proteomics, 5, 1742–5

15. Liebler, D.C., and A.J. Ham, Spin filter-based sample preparation for shotgun proteomics. Nat Methods, 2009; 6(11): p. 785; author reply 785–6

16. Kaji, H., Y. Yamauchi, N. Takahashi, and T. Isobe, (2006) Mass spectrometric identification of N-linked glycopeptides using lectin-mediated affinity capture and glycosylation site-specific stable isotope tagging. J Proteome Res, 5, 2057–65

17. Smith, J.R., M. Olivier, and A.S. Greene, (2007) Relative quantification of peptide phosphorylation in a complex mixture using 18O labeling. J Proteome Res, 6, 357–63

18. Washburn, M.P., D. Wolters, and J.R. Yates, 3rd, Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat Biotechnol, 19(3): p. 242–7, 2001

19. de Godoy, L.M., J.V. Olsen, J. Cox, M.L. Nielsen, N.C. Hubner, F. Frohlich, T.C. Walther, and M. Mann, (2008) Comprehensive mass-spectrometry-based proteome quantification of haploid versus diploid yeast. Nature, 455, 1251–4
20. de Godoy, L.M., J.V. Olsen, G.A. de Souza, G. Li, P. Mortensen, and M. Mann, (2009) Status of complete proteome analysis by mass spectrometry: SILAC labeled yeast as a model system. Genome Biol, 2006; 7(6): p. R50

21. Wisniewski, J.R., A. Zougman, N. Nagaraj, and M. Mann, (2009) Universal sample preparation method for proteome analysis. Nat Methods, 6, 53–59.

22. Bantscheff, M., B. Dumpiefeld, and B. Kuster, (2004) Femtomol sensitivity post-digest (18)O labeling for relative quantification of differential protein complex composition. Rapid Commun Mass Spectrom, 18, 869–76

23. Lane, C.S., Y. Wang, R. Betts, W.J. Griffiths, and L.H. Patterson, (2007) Comparative cytochrome P450 proteomes in the livers of immunodeficient mice using 18O stable isotope labeling. Mol Cell Proteomics, 6, 953–62

24. An, Y., Z. Fu, P. Gutierrez, and C. Fenselau, (2005) Solution isoelectric focusing for peptide analysis: comparative investigation of an insoluble nuclear protein fraction. J Proteome Res, 4, 2126–32

25. Chenau, J., S. Michelland, J. Sidibe, and M. Seve, Peptides OFFGEL electrophoresis: a suitable pre-analytical step for complex eukaryotic samples fractionation compatible with quantitative iTRAQ labeling. Proteome Sci, 2008; 6: p. 9

26. Horth, P., C.A. Miller, T. Preckel, and C. Wenz, (2006) Efficient fractionation and improved protein identification by peptide OFFGEL electrophoresis. Mol Cell Proteomics, 5, 1968–74

27. Fraterman, S., U. Zeiger, T.S. Khurana, N.A. Rubinstein, and M. Wilim, (2007) Combination of peptide OFFGEL fractionation and label-free quantitation facilitated proteomics profiling of extraacellular muscle. Proc Natl Acad Sci USA, 104, 15404–16

28. Heller, M., M. Ye, P.E. Michel, P. Morier, D. Stalder, M.A. Junger, J.R. Aebersold, F. Reymond, and J.S. Rossier, (2005) Added value for tandem mass spectrometry shotgun proteomics data validation through isoelectric focusing of peptides. J Proteome Res, 4, 2273–82

29. Graumann, J., N.C. Hubner, J.B. Kim, K. Ko, M. Moser, C. Kumar, J. Cox, H. Scholer, and M. Mann, (2008) Stable isotope labeling by amino acids in cell culture (SILAC) and proteome quantitation of mouse embryonic stem cells to a depth of 5,111 proteins. Mol Cell Proteomics, 7, 672–83

30. Ramos-Fernandez, A., D. Lopez-Ferrer, and J. Vazquez, (2007) Improved method for differential expression proteomics using trypsin-catalyzed 18O labeling with a correction for labeling efficiency. Mol Cell Proteomics, 6, 1274–86

31. Boeniger, K., G. Dodoni, A. Rodriguez-Sinovas, A. Cabestrero, M. Ruiz-Meana, P. Gres, I. Konietzka, C. Lopez-Iglesias, D. Garcia-Dorado, D. Li, G. Heusch, and R. Schulz, (2005) Connexin 43 in cardiomyocyte mitochondria and its increase by ischemic preconditioning. Cardiovasc Res, 67, 234–44

32. Shevchenko, A., H. Tomas, J. Havlis, J.V. Olsen, and M. Mann, (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc, 1, 2866–60

33. Katayama, H., T. Tabata, Y. Ishihama, T. Sato, Y. Oda, and T. Nagasu, (2004) Efficient in-gel digestion procedure using 5-cyclohexyl-1-pentyl-beta-D-maltoside as an additive for gel-based membrane proteomics. Rapid Commun Mass Spectrom, 18, 2388–94

34. Lopez-Ferrer, D., S. Martinez-Bartolome, M. Villar, M. Campillos, F. Martin-Maroto, and J. Vazquez, (2004) Statistical model for large-scale peptide identification in databases from tandem mass spectra using SEQUEST. Anal Chem, 76, 6853–60

35. Ortega-Perez, I., E. Cano, F. Were, M. Villar, J.P. Garcia-Ruiz, and J. Vazquez, (2008) Properties of average score distributions of SEQUEST: the probability ratio method. Mol Cell Proteomics, 7, 1135–45

36. Navarro, P., and V.Z. JS, A refined method to calculate False Discovery Rates for peptide identification using decoy databases. J Proteome Res, 8(4): p. 1792–6, 2009

37. Lopez-Ferrer, D., A. Ramos-Fernandez, S. Martinez-Bartolome, P. Garcia-Ruiz, and J. Vazquez, Quantitative proteomics using 180/18o labeling and linear ion trap mass spectrometry. Proteomics, 2006. 6 Suppl 1: p. S94–11

38. Cargile, B.J., D.L. Talley, and J.L. Stephenson, Jr., Immobilized pH gradients as a first dimension in shotgun proteomics and analysis of the accuracy of pI predictability of peptides. Electrophoresis, 25(6): p. 936–45, 2004

39. Feske, S., J. Giltinan, R. Dolmetsch, L.M. Staudt, and A. Rao, (2001) Gene regulation mediated by calcium signals in T lymphocytes. Nat Immunol, 2, 316–24

40. Romo-Sosa, A., R.J. Shea, M.H. Mulks, D. Gigot, J. Urbain, O. Leo, and F. Andris, (2002) Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis. Eur J Immunol, 32, 3225–34

41. Kirchoff, M.G., L.A. Chau, C.D. Lemke, S. Vardhana, P.J. Darlington, M.E. Marquez, R. Taylor, K. Rizkalla, I. Blanca, M.L. Dustin, and J. Madrenas, (2008) Modulation of T cell activation by stomatin-like protein 2. J Immunol, 181, 1927–36

42. Sun, H., N. Li, X. Wang, T. Chen, L. Shi, L. Zhang, J. Wang, T. Wan, and X. Cao, Molecular cloning and characterization of a novel muscle adenylsuccinate synthetase, AdSS1. from human bone marrow stromal cells. Mol Cell Biochem, 2005, 269(1–2): p. 85–94

43. Clark, L., J.R. Matthews, and R.T. Hay, (1990) Interaction of enhancer-binding protein EBPI (NF-kappa B) with the human immunodeficiency virus type 1 enhancer. J Virol, 64, 1335–44

44. Perez-Martinez, M., M. Gordon-Afonso, J.R. Cabrero, M. Barrero-Villar, M. Rey, M. Mittelbrunn, A. Lamana, G. Morlino, C. Calabia, H. Yamazaki, T. Shirao, J. Vazquez, R. Gonzalez-Amaro, E. Veiga, and F. Sanchez-Madrid, F-actin-binding protein drehbin regulates CXCR4 recruitment to the immune synapse. J Cell Sci, 2010. 123(Pt 7): p. 1160–70

45. Rodriguez-Tarduchy, G., A.G. Sahuquillo, B. Alarcon, and R. Bragado, (1996) Apoptosis but not other activation events is inhibited by a mutation in the transmembrane domain of T cell receptor beta that impairs CD3zeta association. J Biol Chem, 271, 30417–25

46. Lopez-Anton, N., A. Ruddy, N. Barth, M.L. Schmitz, G.R. Pettit, K. Schulze-Osthoff, V.M. Dirsch, and A.M. Vollmar, (2006) The marine product stomatin-like protein 2. Modulation of T cell activation by stomatin-like protein 2. J. Biol. Chem, 281, 33073–86

47. Sanson, M., N. Auge, C. Vindis, C. Muller, Y. Bando, J.C. Thiers, M.A. Marachet, K. Zarkovic, Y. Sawa, R. Salvayre, and A. Negre-Salvayre, (2009) Oxidized low-density lipoproteins trigger endoplasmic reticulum stress in vascular cells: prevention by oxygen-regulated protein 150 expression. Circ. Res., 104, 328–36

48. Singh, S., and A. Kilar, (2008) Differential gene expression during apoptosis induced by a factor: role of mitochondrial F0-F1 ATP synthase complex. Apoptosis, 10, 1489–92

49. Godbole, A., J. Varghese, A. Sarin, and M.K. Mathew, VDAC is a conserved element of death pathways in plant and animal systems. Biochim Biophys Acta, 2003. 1642(1–2): p. 87–96

50. Roy, S.S., A.M. Ehrlich, W.J. Craigien, and G. Hajnoczky, (2009) VDAC2 is required for truncated BID-induced mitochondrial apoptosis by recruiting BAK to the mitochondria. EMBO Rep, 10, 1341–7

51. John, G.B., Y. Shang, L. Li, C. Renker, C.A. Mannella, J.M. Selker, L. Rangell, M.J. Bennett, and J. Zha, (2005) The mitochondrial inner membrane protein mitoflin controls cristae morphology. Mol. Biol. Cell, 16, 1543–54

52. Solstad, T., E. Bjorgo, C.J. Koehler, M. Strozymski, K.M. Torgersen, K. Tasken, and B. Thiede, Quantitative proteome analysis of detergent-resistant membranes identifies the differential regulation of protein kinase C isoforms in apoptotic T cells. Proteomics, 10(15): p. 2758–68, 2010

53. Villar, M., I. Ortega-Perez, F. Were, E. Cano, J.M. Redondo, and J. Vazquez, Systematic characterization of phosphorylation sites in NFATc2 by linear ion trap mass spectrometry, Proteomics, 2006. 6 Suppl 1: p. S16–27
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