Robust and Sensitive iTRAQ Quantification on an LTQ Orbitrap Mass Spectrometer*

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Isobaric stable isotope tagging reagents such as tandem mass tags or isobaric tags for relative and absolute quantification enable multiplexed quantification of peptides via reporter ion signals in the low mass range of tandem mass spectra. Until recently, the poor recovery of low mass fragments observed in tandem mass spectra acquired on ion trap mass spectrometers precluded the use of these reagents on this widely available instrument platform. The Pulsed Q Dissociation (PQD) technique allows negotiating this limitation but suffers from poor fragmentation efficiency, which has raised doubts in the community as to its practical utility. Here we show that by carefully optimizing instrument parameters such as collision energy, activation Q, delay time, ion isolation width, number of microscans, and number of trapped ions, low m/z fragment ion intensities can be generated that enable accurate peptide quantification at the 100 amol level. Side by side comparison of PQD on an LTQ Orbitrap with CID on a five-year old Q-Tof Ultima using complex protein digests shows that whereas precision of quantification of 10–15% can be achieved by both approaches, PQD quantifies twice as many proteins. PQD on an LTQ Orbitrap also outperforms “higher energy collision induced dissociation” on the same instrument using the recently introduced octapole collision cell in terms of lower limit of quantification. Finally, we demonstrate the significant analytical potential of iTRAQ quantification using PQD on an LTQ Orbitrap by quantitatively measuring the kinase interaction profile of the small molecule drug imatinib in K-562 cells. This article gives practical guidance for the implementation of PQD, discusses its merits, and for the first time, compares its performance to higher energy collision-induced dissociation. Molecular & Cellular Proteomics 7:1702–1713, 2008.

Quantitative measurements are becoming increasingly important in proteomics, and a multitude of mass spectrometry-based methods have been developed for this purpose over the past few years (recently reviewed by Bantscheff et al. (1) and Ong and Mann (2)). Among these, stable isotope labeling of proteins and peptides have enjoyed particular success because of the fact that for every peptide measured, there is an internal reference standard of the same peptide in the experiment. Today, there is a clear trend toward multiplexing quantitative measurements to enable the study of more complex systems such as time-resolved experiments or multiple experimental conditions. In addition, multiplexing can lead to a higher confidence in the obtained quantification values by e.g. increasing the number of biological replicates or the addition of label swap experiments to detect and correct systematic bias (3–5). Metabolic labeling as well as most chemical labeling procedures of proteins and peptides lead to an increased peptide mass via the incorporation 13C and/or 15N atoms, which is determined and quantified in ordinary mass spectra. For most practical applications, these methods are confined to the introduction of 2 or 3 different mass labels, which limits the extent to which multiplexing can be achieved and, in turn, increases the number of experiments required for the analysis of complex experiments (6–10). As an alternative, isobaric labeling techniques such as tandem mass tags, (11) and isobaric tags for relative and absolute quantification (iTRAQ)1 (12) have emerged, which allow the introduction of 4–8 different mass labels into peptides with a concomitant increase in the number of conditions that can be compared in one experiment. During peptide sequencing by tandem mass spectrometry, these mass labels give rise to distinct low-mass peptide fragment ions from which quantification values are determined. Mass spectrometers such as quadrupoles and TOF instruments have the inherent capability of detecting low m/z fragment ions whereas for ion traps, the recovery of fragment ions below ~30% of the precursor ion mass is very poor (13). This limitation can, in principle, be overcome by pulsed Q dissociation (PQD) (14) or high amplitude short time excitation (13) both of which improve the stabilization of low m/z fragment ions.

In two recent papers, the Griffin laboratory has shown that PQD on a commercial linear ion trap mass spectrometer offers similar quantification performance compared with a quadrupole TOF instrument (15, 16). However, the practical utility of PQD is still controversially debated in the field in part

1 The abbreviations used are: iTRAQ, isobaric tags for relative and absolute quantification; HCD, higher energy collision-induced dissociation; PQD, pulsed Q dissociation; BSA, bovine serum albumin; FibA, fibrinopeptide A; LC-MS/MS, liquid chromatography tandem mass spectrometry; CID, collision-induced dissociation; TOF, time-of-flight.
because of the very low observed fragmentation efficiency and the concomitant low apparent signal intensity. To this end, the recent addition of an octapole collision cell to the back-end of the LTQ Orbitrap, which enables so-called “higher energy collision-induced dissociation” (HCD) might offer improvements (17) as it provides straightforward control over the balance with which high and low mass fragment ions are generated by varying the collision energy.

In this study, we optimized and implemented PQD on an LTQ Orbitrap mass spectrometer and compared its performance to HCD on the same instrument and to CID on a quadrupole TOF mass analyzer. The data obtained from the analysis of iTRAQ-labeled analytical standards as well as complex peptide mixture show that after careful optimization of instrument parameters, PQD quantification significantly outperforms both HCD- and CID-based quantification. To demonstrate the merits and practical utility of the iTRAQ-PQD approach, we measured the dose-response characteristics and selectivity profile of the small molecule kinase drug imatinib against the protein complement of K-562 myelogenous leukemia cells.

EXPERIMENTAL PROCEDURES

Sample Preparation—Carboxymethylated BSA was purchased from Michrom Bioresources Inc. (Auburn, AL), and Fibrinopeptide A was purchased from Bachem AG (Bubendorf, CH).

Kinobead pulldowns were performed as described previously (18). Briefly, for the dilution experiments (data in Fig. 2) a 1:1 mixture of Jurkat and Ramos cell lysates was used at a final protein concentration of 5 mg/ml. 1-ml lysate aliquots were incubated with 35-µl kinobead slurry for 1 h, and bound kinases were eluted after washing using 50-µl × 2 SDS sample buffer. Aliquots of 20, 10, 5, and 2.5-µl eluates were run into a denaturing gel for 20 min to remove reagents incompatible with tryptic digestion. For comparison of iTRAQ quantification by PQD and HCD on the LTQ Orbitrap (data in Fig. 4), kinobead pulldowns from HeLa and Jurkat cell lysates were mixed at a ratio of 5:1 (iTRAQ labels 114–116) and quantified against the same amount of a 1:1 mixture of kinobead pulldowns from the same lysates (iTRAQ 117). Thus, expected protein ratios varied from 0.33–1.66 depending on relative expression levels of kinases in these cell lines. Protein fold changes measured by PQD and HCD, respectively, were averaged (114 versus 117, 115 versus 117, and 116 versus 117). The kinobead profiling experiment with imatinib (data in Fig. 5) was performed as described previously (18) with the modifications described in the “Results” section.

Tryptic in-gel digestion, iTRAQ labeling, and phosphopeptide enrichment was done as described (18). Briefly, reduced and carboxymethylated kinase eluates were concentrated on 4–12% NuPAGE gels (Invitrogen) by running sample ~1 cm into the gel to remove reagents incompatible with tryptic digestion and iTRAQ labeling. After staining with colloidal Coomassie, gel lanes were cut into three slices and subjected to in-gel digestion. Subsequently, peptide extracts were labeled with iTRAQ™ reagents (Applied Biosystems) by adding 10-µl reagent in ethanol and incubation for 1 h at 20 °C in 60% ethanol, 40 mV triethylammoniumbicarbonate, pH 8.53. After quenching of the reaction with glycine, all labeled extracts of one-gel lane were combined and mixed with differently labeled extracts. For the kinobead profiling experiment with imatinib, phosphopeptides were enriched using immobilized metal affinity chromatography (Phos-Select; Sigma).

Mass Spectrometry—Experiments were performed on a Thermo LTQ Orbitrap mass spectrometer that was coupled to a split-less Eksigent NanoLC or coupled to a Surveyor LC system operated in a vented column set-up (19) or operated in infusion mode and on a Waters Q-Tof Ultima coupled to a Waters CapLC. The Orbitrap mass spectrometer was operated using the XCalibur Developers kit 2.0. Intact peptides were detected in the Orbitrap at 60,000 resolution. Internal calibration was performed using the ion signal of \((\text{SiCH}_{3}O)_{6}H^{+}\) at \(m/z\) 445.120025 as a lock mass (20). Maximal ion accumulation time allowed on the LTQ Orbitrap was 1 s for all scan modes; automatic gain control was used to prevent over-filling of the ion trap. Critical instrument parameters are described in the “Results” section. Unless stated differently, 5 × 10⁶ ions were accumulated in the ion trap for generation of PQD spectra and a total of 3 × 10⁷ ions were accumulated in the C-trap in three steps for the generation of HCD spectra. HCD normalized collision energy was set to 45%, and fragment ions were detected in the Orbitrap at a resolution of 7500. For LC-MS/MS analysis, up to six PQD or three HCD spectra were acquired following each full scan. On the Q-Tof Ultima, MS scans were acquired for 1 s followed by 3 MS/MS spectra for 2 s each.

For LC-MS/MS analyses on the systems described above, acidified peptide extracts were dried in vacuo. Samples were then re-suspended in 0.1% formic acid in water, and aliquots of the sample were separated on custom-made 20 cm × 75 µm (identification) reversed phase columns (Reprosil, Maisch, Germany). Gradient elution was performed from 2 to 40% acetonitrile in 0.1% formic acid within 4 h for kinobead experiments and within 40 min for tryptic BSA digests.

Peptide and Protein Identification—Mascot™ 2.2 (Matrix Science) was used for protein identification. For Orbitrap data, 5-ppm mass tolerance was allowed for intact peptide masses and 0.8 Da for PQD fragment ions detected in the linear ion trap and 10 mDa for HCD fragment ions detected in the Orbitrap. For Q-Tof Ultima data, 0.4 Da mass tolerance was allowed for intact peptide and fragment ions (for a plot of the distribution of measured precursor mass deviation see supplemental Fig. S1). For BSA in-solution digests the data was searched using carboxymethylation as a fixed modification. In all other experiments, carbamidomethylation of cysteine residues was set as a fixed modification. In addition, iTRAQ modification of lysine residues was set as fixed modifications and Ser, Thr, Tyr phospho-rylation, methionine oxidation, N-terminal acetylation of proteins, and iTRAQ modification of peptide N termini were set as variable modifications. We searched an in-house curated version of the human International Protein Index database combined with a decoy version thereof (21). Our database contains a total of 203,788 protein sequences (50% forward, 50% reverse) and represents a non-redundant composite of International Protein Index versions 1.0–3.30. It is further supplemented with protein sequences of bovine serum albumin, porcine trypsin, and mouse, rat, and sheep keratinous. Protein identification acceptance criteria for kinobead pulldown samples were solely based on spectrum to peptide sequence assignments that represented the best match (bold red definition of Mascot) and had a >10:1 higher Mascot probability than the second best match. For protein identifications with only one single peptide meeting these criteria, we required the Mascot score to be at least 37 for Orbitrap data and 42 for Q-Tof data. Experiments with a decoy data base indicate a <1% false discovery rate for protein identification. For identifications where multiple peptides met these criteria, the decoy search results indicate <0.1% false discovery rate.

Peptide and Protein Quantification—Centroied iTRAQ reporter ion signals were computed by the XCalibur software operating the mass spectrometer and extracted from MS data files using in-house developed software. Only peptides unique for identified proteins were used for relative protein quantification. iTRAQ reporter ion intensities were multiplied with the ion accumulation time yielding an area value
to yield a total concentration of 330 fmol/µl and iTRAQ 117-labeled FibA was mixed at a ratio of 1:0.8 was used to optimize instrument parameters for PQD. iTRAQ intensities of two conditions are plotted on the x and y axis. The slope of the trend line is a measure for the determined fold change, and the standard error can be used as a measure for precision (see “Results” for further details). For the BSA dilution series, we required iTRAQ reporter ions to be detected under all conditions applied and reporter ion areas to be above the electronic noise level (200 for PQD spectra and 2000 for HCD spectra).

### RESULTS

**PQD Optimization**—iTRAQ-labeled Fibrinopeptide A (FibA) was used to optimize instrument parameters for PQD. iTRAQ 114 and iTRAQ 117-labeled FibA was mixed at a ratio of 1:0.8 to yield a total concentration of 330 fmol/µl and continuously infused into the mass spectrometer at 250 n/minute using a syringe pump. Initially, activation Q was set to 0.7, and activation time was 0.1 ms as suggested by the manufacturer. To optimize collision energy settings, we monitored four different fragment ion signals for their intensities at different normalized collision energies (iTRAQ 114.1, iTRAQ 117.1, Y1, at m/z 1077.5, and Y2 at m/z 1350.5). As shown in Fig. 1A, fragment ion intensities maximize at similar normalized collision energies within a narrow window (around 29%). This substantially differs from CID where fragment ion intensities of FibA remain relatively stable over a wide range of collision energies (data not shown). At collision energy of 29%, by far the most abundant ion in the PQD spectrum is intact doubly charged FibA (m/z 741.3). iTRAQ reporter ions were detected at ~20% relative intensity, and similar intensities were observed for other fragments (Fig. 1D). In a next step, fragment ion intensities of FibA were monitored during a stepwise increase of the delay time at activation Q of 0.7. The resulting plot revealed an optimum for iTRAQ reporter ions at 0.1 ms delay time (Fig. 1B) whereas high m/z fragment ions continued to increase in intensity with increasing delay times. We then investigated the influence of activation Q on fragment ion intensities. Increasing activation Q above 0.7 typically gave spectra of lower intensity (data not shown). Conversely, high intensity fragment ions were observed at activation Q < 0.7 if the delay time was concurrently increased. Interestingly, at Q = 0.55, iTRAQ reporter ion intensities were a lot less sensitive to changes in delay times than observed at higher Q values (Fig. 1C) but again, high m/z fragment ion intensity continued to benefit significantly from higher delay times. Under the optimized conditions (Q = 0.55, delay time 0.4 ms), relative intensities of fragment ions were generally weak (<10%) compared with the intact precursor ion (Fig. 1D and E). However, this visual impression is somewhat misleading: when compared with the initial settings (Q = 0.7, delay time 0.1 ms), absolute intensities of iTRAQ reporter ions were slightly increased; total fragment ion intensity below the precursor m/z value was doubled and increased by a factor of four for ions above the precursor m/z value. Hence, the optimization of PQD parameters improved the data quality for peptide identification as well as quantification purposes. Still, it should be noted that the total fragment intensities achieved by PQD are typically 3–4 times lower than those for CID on the same instrument (data not shown).

Having optimized the basic PQD parameters, we investigated the relative variation of iTRAQ reporter ion signal observed in subsequent scans with the FibA standard peptide. Ion statistics is expected to be an important factor with respect to the precision of iTRAQ quantification, and two instrument parameters influencing ion statistics are the total number of ions accumulated in the linear ion trap (target value of automatic gain control) and the number of microscans that are averaged per scan. With a target value of 10,000 ions in the linear ion trap and a single microscan, we measured a relative standard deviation of 27% in a series of 50 scans (Fig. 1F). Increasing the number of microscans to 2, 5, or 10 reduced the relative standard deviation to 24, 12, and 10%, respectively. Increasing the target value to 50,000 reduced the relative standard deviation to 13.5% at 2 microscans, which is similar to the result obtained for 5 microscans at the lower target value setting. Although the precision of relative quantification of these acquisition regimes was rather similar, and no degradation of mass accuracy was observed at the 50,000-target value because of space charging, there was a significant difference in the time required per tandem mass spectrum. In particular, at a scan time of 200 ms and a requirement of 30 ms to accumulate 10,000 ions, 5 microscans add up to a total scan time of 1.15 s. In contrast, for 2 microscans and a target value of 50,000 ions, the total scan time is just 0.7 s. As a consequence, more tandem MS spectra can be acquired per unit of time enabling a higher depth sampling.

Quantification in MS/MS spectra should, in theory, provide a high dynamic range because tandem MS spectra typically exhibit less signal complexity than their single MS counterparts. However, matrix contributions such as co-eluting and nearly isobaric peptides and non-peptide contaminants can in practice significantly limit dynamic range and, consequently, accuracy of -fold change determinations. We have addressed the effect of matrix contribution on iTRAQ quantification by spiking 10 fmol of an iTRAQ-labeled BSA digest into a trypsin digest of a kinobead pulldown experiment (containing ~300–500 proteins (18)). The BSA digest was applied at a ratio of 3.5:1 iTRAQ 114/iTRAQ 117 and mixed with equal amounts of the kinobead digest labeled with all four iTRAQ reagents. In this spiking experiment, iTRAQ reporter ion signals at m/z 115 and 116 in spectra matching the BSA peptides can be used to determine background contributions. Four LC-MS/MS runs were performed from this sample using different precursor ion isolation widths for triggering tandem mass spectra. In all cases, the measured -fold change for BSA was significantly lower than the spiked 3.5:1 ratio (Fig. 1G). At narrow isolation widths (1 or 2 Th) ratios of 2.7:1 (iTRAQ 114/iTRAQ 117) were measured. Increasing the isolation width to 5 Th further re-
duced the observed -fold change to 2.2:1. This shows that the presence of increasing amounts of co-eluting and nearly iso-
baric peptides significantly contribute to the total measured iTRAQ reporter ion intensities. Under the assumption that matrix contributions are identical for all iTRAQ reporter ions, the average measured background reporter ion contribution can be subtracted from the measured BSA peptide ions, and -fold changes can be corrected (supplemental Fig. S2). Un-

Fig. 1. Optimization of PQD parameters using a mixture of iTRAQ 114- and iTRAQ 117-labeled FibA. A, B, and C, relative fragment ion intensity as a function of normalized collision energy (A), activation time at activation Q = 0.7 (B), and activation time at activation Q = 0.55 (C). iTRAQ reporter ions are shown as blue (114) and red lines (117); fragment ion intensities of the signals at m/z 1077.5 and 1350.5 are shown in green and brown, respectively. D, E, representative PQD spectra of iTRAQ-labeled FibA at different parameter settings. Color arrows indicate the fragment ions plotted in panels A, B, and C. Selected peaks are labeled with absolute signal intensities. Numbers above the red line refer to the summed signal intensities of all fragment ions at m/z values below and above the precursor. F, relative standard deviations measured for the ratio of reporter ion signal intensities 114 versus 117 at different microscan and target value settings and the required data acquisition time for respective tandem spectra. G, ratios of iTRAQ signal intensities for a BSA digest mixture (3.5:1, iTRAQ 114, 117) spiked into a complex peptide background (equal amounts of iTRAQ 114, 115, 116, 117) as a function of precursor ion isolation width.
Fortunately, this correction procedure cannot be generally applied because recalculation of \( \text{-fold} \) changes requires knowledge about background contributions to each protein. Therefore, it appears advisable to apply narrow isolation widths to generate trustworthy \( \text{-fold} \) changes. However, this comes at the cost of sensitivity. Supplemental Fig. S2 shows that increasing isolation width from 1 to 2–3 Th leads to an increase of iTRAQ reporter ion intensities of BSA peptides by factor 2.4 and 5.8, respectively. No further increase in signal is observed at 5 Th. Unfortunately, the signal contribution of co-fragmenting peptides and other background ions increases at about twice the rate than that of the analyte. As a result, an isolation width of \( \sim 2 \text{ Th} \) appears to be a reasonable compromise.

We tested the optimized PQD parameter set on a dilution series of iTRAQ-labeled BSA digest. For mixtures containing iTRAQ 114 and iTRAQ 117 BSA peptides at a ratio of 70:30 at a total of 100 amol on column, unambiguous protein identification was achieved (supplemental Fig. S3). Furthermore, by comparing the signal intensities of iTRAQ 114 and iTRAQ 117, a \( \text{-fold} \) change of 2.3 was determined, which is in very good agreement with the expected 70:30 ratio.

**Comparison of PQD on an LTQ Orbitrap with CID on a Q-Tof Ultima**—Precision and dynamic range of iTRAQ quantification by PQD was further assessed using a 1:2:4:8 dilution series of a complex protein digest. Four aliquots of a kinodepletion pulldown were applied on a gel at ratios of 1/0.5/0.25/0.125, digested with trypsin, and labeled with iTRAQ reagents 114–117, and digests were mixed prior to analysis. A total of four LC-MS/MS runs were acquired on the LTQ Orbitrap using PQD from two replicate experiments, and results were compared with those of the same digest analyzed in triplicate on a Q-Tof Ultima but using three times more material. PQD on the Orbitrap on average lead to the identification and quantification of 159 proteins (88 kinases) from 2062 tandem mass spectra. The respective figures for the Q-Tof Ultima were 79 proteins (54 kinases) and 528 spectra. Protein \( \text{-fold} \) changes were calculated as described above using the sample with the highest concentration as base. For both instruments, values obtained for individual proteins were in good agreement with the expected ratios (Fig. 2). Not surprisingly, variation was higher for proteins quantified from very few spectra. For these, precision was slightly better for the Q-Tof Ultima data (Fig. 2, B and D). Proteins quantified with three or more spectra gave relative standard deviations of 20\% or less for all ratios and both instruments. The Orbitrap quantified twice the number of proteins from three times the number of spectra despite the fact that only a third of the digest material was used compared with the Q-Tof Ultima. Therefore, the higher number of quantifiable spectra compensates for the slightly lower precision observed for proteins quantified with one or two spectra. In fact, there is no protein in the data for which the Q-Tof gave more quantifiable spectra than the Orbitrap, which further indicates the superior sensitivity of the Orbitrap for identification and quantification.

**Comparison of PQD and HCD on an LTQ Orbitrap**—Recently, HCD has been reported as an alternative approach for the detection of low-mass fragment ions in an LTQ Orbitrap (17). In this approach, classical CID is performed in an octopole collision cell at the back-end of the LTQ Orbitrap, and in contrast to PQD, fragment ions are analyzed in the Orbitrap rather than by the linear ion trap of the instrument. Because of the different fragmentation regime, the resulting HCD spectra differ significantly from PQD spectra. Notably, fragmentation efficiency is much increased and therefore, iTRAQ reporter ions may constitute the base peak in HCD spectra (supplemental Fig. S4). We investigated if this higher yield of iTRAQ reporter ion intensity translates into more precise and/or sensitive quantification by infusing a 1:10 mixture of iTRAQ 114- and iTRAQ 116-labeled FibA into the Orbitrap mass spectrometer. We found that the iTRAQ reporter ion signal was more stable between consecutive HCD spectra than between consecutive PQD spectra (supplemental Fig. S5). The relative standard deviation of determined peak ratios between 50 MS/MS scans was 20\% for PQD and 8.6\% for HCD indicating a significantly higher precision of quantitation for the latter technique. However, by the nature of the detection system, measuring HCD fragments in the Orbitrap requires significantly more ions than detection of PQD fragments in the linear ion trap. In typical PQD experiments, 1–5 \( \times 10^4 \) ions are accumulated in the linear ion trap whereas 1–5 \( \times 10^5 \) ions are accumulated for detection of HCD fragments in the Orbitrap, which requires a \( \sim 10 \times \) longer ion accumulation time.

To compare the lower limits of protein identification and quantification of PQD and HCD, we mixed iTRAQ-labeled BSA digests at ratios of 1:1:2:5:2.5 and subjected a dilution series of this mixture to LC-MS/MS analysis. Instrument parameters for PQD were set as described above, and up to 6 PQD spectra were acquired for each full scan. For HCD, 3 sequential isolations of \( 1 \times 10^5 \) ions were performed, and ions were accumulated in the C-trap before fragmentation. This allowed efficient isolation of precursor ions at an isolation width of \( \sim 2.5 \text{ Th} \). Because of the higher ion accumulation and ion transfer times required for HCD, total scan times were significantly longer than for PQD. To keep the cycle time below the chromatographic peak width, only 3 HCD spectra were acquired from each precursor scan. With both methods, BSA was identified at the level of 100 amol digest on column (Fig. 3). However, with PQD four out of the eight available spectra were suitable for quantification whereas none of the three HCD spectra contained all 4-reporter ion signals. For HCD, the lower limit of protein quantification in this experiment was 1 fmol BSA digest on column with 2 quantifiable spectra out of 26 available tandem MS spectra. At this level, PQD generated 58 BSA spectra, out of which 32 were used for quantification. At 10-fmol BSA digest on column, similar numbers of spectra were matched with PQD and HCD, but
again the fraction of quantifiable spectra was much higher for PQD (87 out of 99 versus 30 out of 91). As a result, PQD clearly outperforms HCD in terms of limit of quantification. The lower precision on a spectrum by spectrum basis is more than compensated for (indicated by smaller standard errors) by the much higher number of quantified spectra (Fig. 3B).

The performance of PQD and HCD was further compared using identical aliquots of the aforementioned kinobead pulldown sample. In two subsequent LC-MS/MS runs, PQD quantified 200 proteins (100 kinases) from 1740 tandem MS spectra, and HCD lead to the quantification of 131 proteins (74 kinases) from 1053 tandem MS spectra (Fig. 4). The quantification results on the protein level were very similar for both methods with a standard deviation of ~10% for proteins quantified with more than three spectra in HCD mode (Fig. 4B).

**Selectivity Profiling of the Kinase Drug Imatinib**—To demonstrate the practical utility of iTRAQ quantification by PQD, we applied the method to a kinobead selectivity profiling experiment of the marketed drug imatinib in K-562 cells, which express the imatinib target protein BCR-ABL. The
compound competition experiment was performed essentially as described before (18). Briefly, increasing concentrations of imatinib were incubated with Lys-562 cell lysates and subsequently subjected to an affinity pulldown using kinobeads. The dose-dependent reduction of binding of kinases to kinobeads was used to infer the binding potency of imatinib to these kinases. In our previous report, 6 point dose response curves via two separate iTRAQ experiments with overlapping compound concentrations and duplicate experiments were created, which identified known and novel interaction partners of imatinib and determined their binding strengths (IC₅₀). Here, we attempted to simplify the approach by reducing the effort to a single experiment with three compound concentrations (100 nM, 1 μM, and 10 μM and a Me₃SO vehicle control). In this experiment, dose response binding profiles of a total of 112 kinases (281 proteins) were computed (Fig. 5). Potencies for the three kinase targets BCR-ABL, ARG, DDR1, and their adaptor proteins as well as the novel non-kinase target NQO2 were found in good agreement to our previous study (Table I). These data indicate that the throughput of kinobead profiling experiments may be significantly increased without
strongly compromising the reliability of the information obtained.

**DISCUSSION**

**PQD Optimization**—The PQD approach for the detection of small m/z fragment ions in ion trap instruments has so far not been widely adopted in the mass spectrometry and proteomics community. The LTQ Orbitrap data presented here clearly show that PQD is a viable analytical option as it combines the excellent sensitivity and mass accuracy of the LTQ Orbitrap with the multiplexing capability of iTRAQ quantification. However, to achieve good performance, the optimization of some parameters requires particular attention. Notably, the window in which the collision energy can be varied is much narrower than that for conventional CID, and several microscans are needed to obtain acceptable quantification precision (Fig. 1). These two observations are in good agreement with results reported recently by the Griffin laboratory for the optimization of PQD on a LTQ linear ion trap mass spectrometer (16). Furthermore, we found that relatively low values for the activation Q parameter with a concomitant increase in delay times as well as increased automatic gain control target values help to improve the performance of PQD in terms of sensitivity and sampling speed. A distinct advantage of the Orbitrap over the linear trap is that the high mass resolution of precursor ion determination leads to an improved signal-to-noise ratio of full scan mass spectra. This enables more productive triggering of MS/MS events on multiply charged precursor ions compared with the low-resolution ion trap, which, in turn, results in higher sensitivity and reliability for peptide identification and quantification. Consequently, although 100-amol BSA digest on column provided robust data on the LTQ Orbitrap (Fig. 3), no information was obtained from the same sample analyzed in the linear ion trap part of the instrument. Similar observations can be made for complex samples for which the LTQ Orbitrap identifies ~30% more proteins than the LTQ (data not shown).

**Sensitivity of Quantification**—Unlike conventional CID spectra, typical PQD spectra were dominated by the unfragmented precursor ion (supplemental Fig. S4) indicating poor fragmentation efficiency, which in turn limits the sensitivity with which peptides can be quantified by the iTRAQ or tandem mass tags approaches. Despite the somewhat odd spectral appearance, we find that under optimized PQD conditions, signal intensity

![Fig. 4. Performance characteristics of quantification by PQD and HCD on a iTRAQ-labeled kinobead pulldown digest. A, proteins (left, y axis) and spectra (right, y axis) quantified with PQD and HCD, respectively. Quantified proteins are shown in blue, kinases in orange; quantified spectra are shown in yellow (all proteins) and turquoise (kinases). B, two-dimensional plot comparing average protein -fold changes determined by PQD and HCD. Each blue dot represents a protein, and only proteins are included for which least three spectra were identified. The slope of the linear regression line is 1.08.](image-url)
of iTRAQ reporter ions is still good at the level of 100 amol BSA on column, whereas iTRAQ signals are weak or absent in corresponding HCD spectra (Fig. 3); even though very strong iTRAQ reporter ion signals can in principle be generated by HCD (supplemental Fig. S4). It should be noted that this is not because of the difference in the number of acquired tandem mass spectra between LC-MS experiments using PQD (top 6 signals) and HCD (top 3 signals). In fact, in our experiments, about ten times more ions are subjected to fragmentation by HCD compared with PQD. This apparent discrepancy may be the result of a number of factors that are not compensated for by the higher iTRAQ fragment ion yield of HCD. For HCD, precursor ions are first isolated in the linear ion trap, then accumulated in the C-trap before transmission into the collision octopole, and fragments are then transferred to the Orbitrap for detection. The inevitable loss of some ions in this process does not apply for the linear trap. Second, the Orbitrap requires a minimum of 20 ions to detect a signal, whereas the electron multipliers employed on the linear trap are capable of detecting a single ion (22). The net effect of the above and possibly other factors is that iTRAQ quantification by PQD is ~10× more sensitive than that of HCD (Fig. 3). Despite

**A**

![Graphs](image1.png)

**B**

![Graphs](image2.png)

**FIG. 5.** Kinase selectivity profile of imatinib in Lys-562 cell lysates. A, examples of competition binding curves calculated from iTRAQ reporter signals. Blue lines indicate the inflection point (50% of maximal competition) as well as 50% absolute competition. B, kinase-binding profile of imatinib for all 112 protein kinases simultaneously identified from Lys-562 cells in this experiment. Bars indicate IC_{50} values, defined as the concentration of imatinib at which half-maximal competition of kinobead binding is observed.

**TABLE I**

| Protein | Quantified spectra | Relative iTRAQ signal intensity | Potency determined in μM |
|---------|--------------------|--------------------------------|--------------------------|
|         |                    | Vehicle control | 33 nM imatinib | 330 nM imatinib | 10 μM imatinib | 3-point dose response (unicate) | 6-point dose response (duplicate) |
| BCR-ABL | 191                | 1               | 0.98           | 0.62           | 0.35           | 0.193                       | 0.25                        |
| ARG     | 78                 | 1               | 0.96           | 0.56           | 0.26           | 0.212                       | 0.272                      |
| DDR1    | 5                  | 1               | 0.65           | 0.42           | 0.28           | 0.041                       | 0.09                        |
| GRB2    | 17                 | 1               | 0.77           | 0.38           | 0.19           | 0.051                       | 0.143                      |
| INPPL1  | 34                 | 1               | 0.98           | 0.63           | 0.39           | 0.173                       | 0.242                      |
| STS-1   | 28                 | 1               | 0.92           | 0.72           | 0.47           | 0.271                       | 0.313                      |
| NQO2    | 221                | 1               | 0.70           | 0.38           | 0.25           | 0.036                       | 0.043                      |

Competition binding data for the kinase drug imatinib and proteins purified by kinobead pulldowns. IC_{50} values are based on inflection points of fitted dose-response curves (Fig. 5). Determined IC_{50} is below the lowest compound concentration used in assay.
the lower absolute sensitivity, it should also be noted that HCD produces tandem mass spectra with much better resolution and mass accuracy than PQD (<10 ppm versus ~200 ppm; supplemental Fig. S4), which can be of significant value for resolving ambiguities in fragment ion assignment (e.g., the b13/y14 ambiguity in supplemental Fig. S4) and thus improve peptide identification quality and the assignment of post-translational modifications.

Today, iTRAQ quantification data is most commonly generated on TOF instruments as these mass analyzers span the entire m/z range and do not suffer from the poor recovery of low m/z fragment ions observed for ion traps. A direct comparison of PQD on an LTQ Orbitrap with CID on a Q-Tof Ultima shows that the former identifies and quantifies a much higher number of peptides (and proteins) while using only a third of the material indicating better sensitivity and thus analytical depth for the PQD approach (Fig. 2). Given that the Q-Tof Ultima employed in this study is five years old, the latest generation quadrupole TOF instrumentation may provide more comparable performance. Still, in our hands, PQD currently offers the most sensitive way of iTRAQ quantification, and more improvements may be possible in the future by increasing the somewhat unsatisfactory fragmentation efficiency.

Reliability of Quantification — The precision with which peptides (and proteins) can be quantified by MS/MS-based methods such as iTRAQ depends on the quality of individual tandem mass spectra and the number of spectra from which quantification values can be calculated. An important thing to note is that many but not all tandem mass spectra contain detectable levels of iTRAQ reporter ions (Fig. 3A). Furthermore, the absolute signal intensity of iTRAQ reporter ions may vary by two orders of magnitude between tandem MS spectra of different peptides (supplemental Fig. S3). Therefore, the accuracy of quantification values derived from spectra with low reporter ion intensity often suffers from poor ion statistics. We believe that the frequently used method of simple averaging iTRAQ signal ratios between samples is not a reliable way to quantify relative differences because it does not distinguish between strong and weak spectra. Typically, this leads to large standard deviations of quantification because many of the underlying data points (spectra) contain an insignificant number of ions. In this study we instead followed an approach that is sensitive to differences in spectral quality. The details of the method are published elsewhere (18) but briefly, measured iTRAQ reporter ion intensities of individual spectra are multiplied with the ion trapping time resulting in a value that is directly proportional to the number of reporter ions detected. These values are then compared for each spectrum of the two (or four) iTRAQ-labeled samples in a two-dimensional plot, and the -fold change between the two (or four) states (i.e., the pairs or groups of reporter ions) is calculated using a two-sided linear regression analysis (supplemental Fig. S3). The slope of the trend line is a measure for the determined -fold change, and the standard error can be used as a measure of precision and to calculate confidence intervals. The quantification data obtained in this way and shown in Figs. 2, 3, and 4 indicate that overall precision is very similar between PQD, CID, and HCD. As one might expect, precision increases with the number of available spectra (Fig. 2, B and D). More specifically, at least three quantifiable spectra are required to distinguish confidently proteins changing by a factor of two between conditions in our experiments. We noticed that the spectrum to spectrum variation is much higher in PQD spectra than in HCD spectra (20% versus 9% relative standard deviation, respectively; supplemental Fig. S5). A similar observation is made for PQD versus CID (Fig. 2 and data not shown). Our interpretation of this observation is the better ion statistics of CID and HCD spectra compared with those of PQD spectra. However, given that many more quantifiable PQD spectra are available at any given protein amount compared with HCD or CID (both in terms of absolute number as well as fraction of all tandem mass spectra; Fig. 3), the larger spectrum to spectrum variation is compensated in the calculation of peptide and protein -fold changes.

Matrix Contributions to Quantification in Tandem Mass Spectra — Tandem mass spectra are typically very clean with respect to electronic or chemical noise, and signal intensities spanning 3–4 orders of magnitude can in principle be detected by a number of mass analyzers. However, in practical terms, the dynamic range of quantification of complex proteomic mixtures is often limited by ion statistics (Fig. 3) as well as matrix contributions, which constitute one of the main sources of uncertainty in quantitative measurements based on tandem mass spectrometry (supplemental Fig. S2). For quantification in precursor ion spectra (e.g., stable isotope labeling by amino acids in cell culture or other peptide labeling methods), interfering signals because of the presence of nearly isobaric peptide or non-peptide species can often be identified if high resolution data is available (e.g., Q-Tof, Orbitrap, or FT-ICR spectra). This enables correction of quantification data by considering signals that precisely match a particular peptide species only. For quantification in tandem MS spectra, high resolution MS data cannot be used for this purpose as it is impossible to predict to what extent an interfering species in the m/z window selected for fragmentation will contribute to the iTRAQ reporter ion signal. Therefore, the isolation width for precursor ion selection determines the level of interfering species that are co-fragmented along with the desired peptide and thus contribute to the overall iTRAQ signal. Consequently, the higher the isolation width and sample complexity, the larger the matrix contribution (supplemental Fig. S2). At the same time, the narrower the isolation width, the lower the signal intensity and the poorer the limit of quantification. Hence, any chosen set of parameters will have to represent a compromise between optimizing signal intensity and minimizing matrix contributions.
BSA spiking experiments show that determined -fold changes between two samples are systematically underestimated, and it is reasonable to expect that this effect will be more pronounced the more complex the peptide mixture is. The same experiment also shows that simple iTRAQ experiments (2 conditions), it is possible to determine the matrix contribution and correct the data accordingly. For more complex experiments (say 4 conditions) this is no longer straightforward as the extent of matrix contributions may be different for every measured protein. This effect is illustrated in the dose-response measurements shown in Fig. 5. Potent inhibition of binding to kinobeads is not always synonymous with strong reduction of signal intensity. In fact, residual signal intensity rarely falls below 10–20% and often level out at 40% even at high competitor concentrations indicating a very confined dynamic range of quantification for some proteins. In our experience, iTRAQ quantification of very complex mixtures offers a dynamic range of around 1:10 (1:20 for medium complex and 1:50 for simple mixtures). Interestingly, high residual signal intensities are found for proteins represented by relatively few spectra, and low residual signal is mostly associated with proteins for which many spectra are available. This probably reflects the abundance of a particular protein in the sample. Thus, high residual iTRAQ signal indicates a strong matrix contribution, and consequently, low residual iTRAQ signal indicates low matrix contribution. For the examples shown in Fig. 5 this means that inhibition data derived at 50% signal can represent a significant underestimate of the true value (as was the case in the BSA spiking experiment). Moreover, if only one single compound concentration had been used, inhibition values for different proteins might have been only marginal different. The solution to this problem lies in the use of the inflection point of the fitted dose response curve, which is independent of the absolute signal change and thus a much more reliable measure for inhibition of binding. This conclusion may be obvious in this particular case but the observation raises a more general and possibly very significant point: binary comparisons (e.g. two time points, two treatment conditions, mutant versus wild type etc.), which are most frequently found in the proteomic literature to date may suffer strongly from background contributions and thus may be highly prone to misinterpretations given our general inability to define the maximum possible signal in the system under study. This suggests that better accuracy may generally be achieved by systematically varying experimental parameters and measuring their effects rather than contrasting binary comparisons. The 3-point dose response data shown in Fig. 5 suggests that the extra effort may be manageable as the determined inhibition values for the drug imatinib and its cellular targets are very close to those determined for 6-point dose response experiments (Table I).

In conclusion, this report shows that sensitive and reliable iTRAQ quantification is possible using PQD on an LTQ Orbitrap and that it significantly outperforms other fragmentation methods in terms of sensitivity without compromising precision. We have furthermore demonstrated the practical utility of the method by determining the quantitative kinase selectivity profile of the drug imatinib. With the commercial availability of 6-plex tandem mass tags and 8-plex iTRAQ, we believe that the method holds significant potential for diverse future applications in proteomic research and drug discovery.

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