# An improved quantitative mass spectrometry analysis of tumor specific mutant proteins at high sensitivity

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New disease specific biomarkers, especially for cancer, are urgently needed to improve individual diagnosis, prognosis, and treatment selection, that is, for personalized medicine. Genetic mutations that affect protein function drive cancer. Therefore, the detection of such mutations represents a source of cancer specific biomarkers. Here we confirm the implementation of the mutant protein specific immuno-SRM (where SRM is selective reaction monitoring) mass spectrometry method of RAS proteins reported by Wang et al. [*Proc. Natl. Acad. Sci. USA* 2011, *108*, 2444–2449], which exploits an antibody to simultaneously capture the different forms of the target protein and the resolving power and sensitivity of LC-MS/MS and improve the technique by using a more sensitive mass spectrometer. The mutant form G12D was quantified by SRM on a QTRAP 5500 mass spectrometer and the MIDAS workflow was used to confirm the sequence of the targeted peptides. This assay has been applied to quantify wild type and mutant RAS proteins in patient tumors, xenografted human tissue, and benign human epidermal tumors at high sensitivity. The limit of detection for the target proteins was as low as 12 amol (0.25 pg). It requires low starting amounts of tissue (ca.15 mg) that could be obtained from a needle aspiration biopsy. The described strategy could find application in the clinical arena and be applied to the study of expression of protein variants in disease.

**Keywords:**
Biomarkers / Mutant proteins / Oncogene / Selected reaction monitoring / Technology

## 1 Introduction

Cancer is caused by gene mutations that result in proteins with altered activity [1,2]. Some of these changes are "drivers" and responsible for the generation of the tumors; the remainder are "passengers," providing no selective growth advantage to the tumor cells [3, 4]. In some cases the mutation leads only to a single amino acid change in a protein, which is responsible for the aberrant behavior. A method to detect and quantify such changes provides a direct way of typing tumors at the protein level. In contrast to the mutant proteins, protein biomarkers now in use to assess cancer [5] or the risk of developing cancer, such as carcinoembryonic antigen (CEA) [6] or prostate-specific antigen (PSA) [7], are neither causal

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**Abbreviations: AB**, antibody; **CE**, collision energy; **CRC**, colorectal cancer; **CXP**, cell exit potential; **DP**, declustering potential; **FA**, formic acid; **PAR**, peak area ratio; **WB**, Western blotting; **WT**, wild type

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nor specific to the tumor cells. This is a clear advantage of driver gene markers, since they are not simply associated with disease, but are a cause of tumorigenesis. It is not possible to use more widely applied techniques such as Western blotting (WB) or ELISA to detect these small changes in protein structure, since it is extremely difficult to raise antibodies of sufficient specificity [8]. Furthermore, each mutation will probably require a specific reagent, rendering this task even more formidable. The development of targeted mass spectrometry approaches to quantify proteins, especially SRM, is showing the broad applicability of this technique in proteomics and diagnostics [9–13]. MS is already applied in clinical analysis, for example, for the screening of newborns for congenital metabolic diseases, multianalyte therapeutic drug monitoring (TDM), and the determination of endogenous levels of steroid hormones [14–16].

Assays that use antibodies to enrich a tumor specific biomarker from tissue followed by LC-MS SRM-based quantification have been described [6] and a method for detection and quantification of the mutant proteins as tumor biomarkers has been reported [9]. Immunocapture improves the sensitivity and selectivity of MS-based protein assays as well as enabling the analysis of protein variants. [17–19]. In fact, the potential of KRAS mutation analysis by SRM as clini- cal marker has been already suggested [9]. The technique reported by Wang et al. uses an antibody that recognizes an epitope common to the wild type and mutant proteins to simultaneously capture different forms of KRAS, harnessing the resolving power of LC and SRM MS to distinguish between the different forms. In this way, wild type and mutant molecules can be processed and measured in a single assay avoiding the need to generate mutation specific antibodies. The incorporation of heavy isotope labeled peptides into the assay permits quantitation of the target proteins, which provides information about the mutational load and the relative levels of mutant versus wild type. *KRAS* is a key oncogene in several types of cancer, including pancreatic, colon, and lung cancers [20–23] and there is a known correlation between the presence of mutant *KRAS* and the effectiveness of some epidermal growth factor receptor-targeted therapies [24]. Therefore, measuring KRAS is a good illustration of this approach in a clinically relevant context [24].

Here we show the measurement of wild type (WT) and mutant KRAS (G12D) in a variety of fresh OCT embedded tissue samples obtained directly from patients or from human tumors xenografted into mice. An important consideration from the clinical standpoint is that the method requires low milligram amounts of tissue (ca. 15 mg) to capture the protein and only 500 μg tissue equivalent (corresponding to 50 μg total protein equivalent) is used for the MS analysis. This generally leaves sufficient material for more established techniques such as immunohistochemistry still to be performed. The method would also provide an excellent complement or alternative to the available PCR methods [25].

Furthermore, we confirmed the value and efficacy of the method proposed by Wang et al. [9] and achieved an improvement of two orders of magnitude in sensitivity by implementing the method on a more sensitive mass spectrometer. The method of Wang et al. was streamlined by digesting the captured KRAS on column thereby avoiding the elution step. Although no improvement in the recovery ratio is observed, our method may be simpler. Also, the identity of the targeted peptides was confirmed in the same LC-MS SRM assay, since enhanced MS/MS spectra were acquired for the analytes in a targeted fashion, using the MIDAS workflow, which is a function supported by the QTRAP.

## 2 Materials and methods

### 2.1 Animal and human samples

Pancreatic and colorectal cancer (CRC) tumors were resected surgically from patients and directly implanted into the subcutaneous space of immune-deficient 4–6-week-old female athymic (nu/nu) mice to establish primary tumor xenografts as described [26]. Nontumoral colon and pancreas tissue samples were collected from healthy areas outside the tumor lesions in the resected pieces. Benign human skin tumors were collected as described elsewhere [27]. Xenograft tumors were harvested from mice and immediately stored at −80°C.

The studies using human material were approved by the Ethics Board of the John Hopkins Hospital, Grupo Hospital de Madrid, and Hospital del Mar (Barcelona). All subjects gave informed consent.

### 2.2 Genotyping

Genomic DNA from CRC and pancreatic tumor xenografts was isolated following the instructions of the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Exon 2 of human *KRAS* gene was then amplified by PCR using a previously published forward primer (GGCCTGCTGAAAATGACTGA) and reverse primer (GTCCTGCACCAGTAATATGC) [28]. The change of wild type codon 12, GGT (Gly, G), to mu- tant codon 12, GAT (Asp, D), creates a new restriction target for the BccI restriction enzyme in the *KRAS* gene sequence. This new BccI site can be used to discriminate between wild type and mutant *KRAS* alleles (Supporting Information Fig. S1). Human primary pancreatic and CRC tumor samples were directly genotyped using the CE-marked TheraScreen$^{QR}$ KRAS Mutation Kit (DxS, Manchester, UK). Skin sample *RAS* genotyping was carried out using Sequenom technology as previously described [27]. For further details on PCR analysis, please refer to Supporting Information.

### 2.3 Sample preparation for proteomic analysis

The workflow followed is outlined in Fig. 1. Aliquots of xenograft tumors (approximately 70 mg) were mixed with

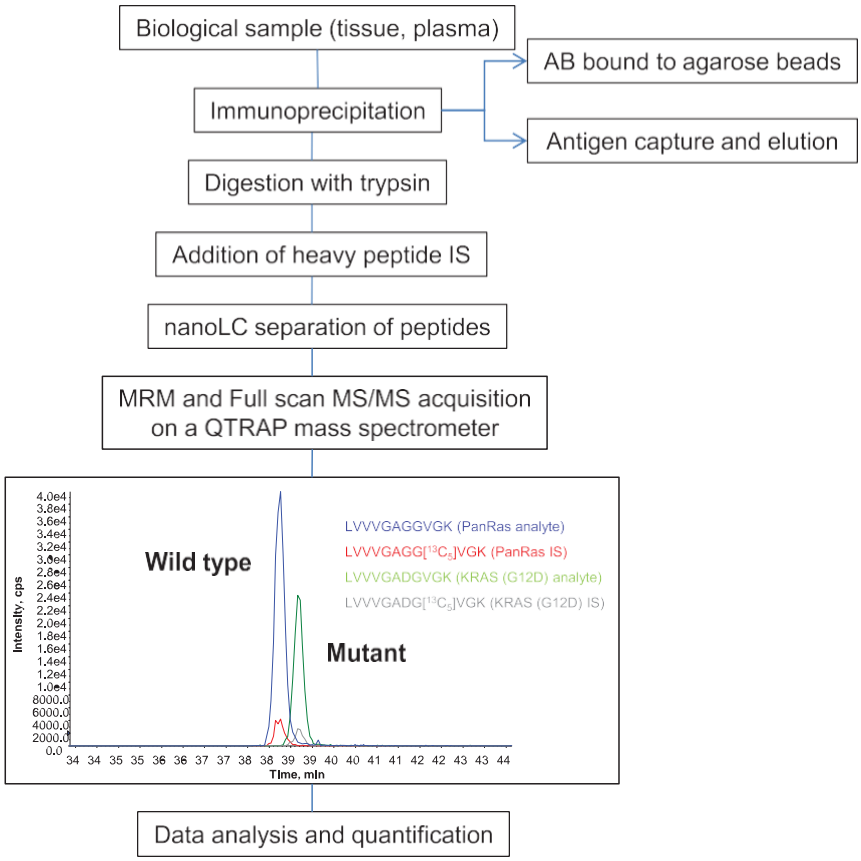


**Figure 1.** An overall flow diagram of the process used for the KRAS assay. Tissue was extracted with RIPA buffer plus protease inhibitors. Protein extract pools were incubated with the immo- bilized antibody resin to capture HRAS, NRAS, and KRAS. Samples were digested with trypsin, evaporated to dryness, re- constituted in loading buffer, and spiked with a standard mixture of heavy $^{13}C$- labeled peptides, prior to LC/SRM anal- ysis. The extracted ion chromatogram shows targeted peptides LVVVGAGGVGK (WT) and LVVVGADGVGK (G12D), and their heavy version, LVVVGAGG[$^{13}C_5$]VGK and LVVVGADG[$^{13}C_5$]VGK. All samples were an- alyzed by nano-LC/SRM on a 5500 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer. AB, antibodies; IS, in- ternal standard.

1 mL of RIPA buffer (20 mM TrisHCl pH 7.4, 37 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% NaDeoxycholate) plus protease inhibitors and homoge- nized for 1 min, followed by centrifugation at 4°C and 16 100 $\times$ *g* for 20 min. All further patient samples (pancreas and colon tumors ca. 15 mg, benign skin tumors 40 mg) were processed in the same way. Healthy human pancreas and colon tissues were processed as described above and pooled to obtain a control matrix. Protein concentration of clari- fied extracts was determined with a Lowry-based assay us- ing BSA as standard (DC-Protein Assay kit, Bio-Rad, Her- cules, CA). The average amount of total protein in tissue extracts was approximately 7 mg/mL. Protein extract pools from tissue in Dulbecco's PBS (DPBS) (Euroclone, Pavia, Italy) were incubated overnight at 4°C with gentle shaking with the antibody resin Anti-V-H-Ras (Ab-1) Rat mAb (Cal- biochem, NJ, USA) which binds HRAS, NRAS, and KRAS and derives from rat mAb Y13-259 (ATCC number CRL-1742). The epitope is located between residues 62 and 76 of v-H-Ras. Unbound proteins were removed by washing the resin five times alternating between 1 mL of DPBS and DPBS containing 0.5 M NaCl and finally washed with 1 mL of 50 mM ammonium bicarbonate. Bound proteins were digested on resin with trypsin (Promega, Madison, WI, USA) for 5 h at 37°C and the released peptides transferred to a clean tube and evaporated to dryness. Samples were reconstituted in 0.1% formic acid and 2% ACN for a final total protein concentration of 5 µg/µL and spiked with a standard mix- ture of heavy $^{13}C$-labeled peptides for a final concentration of 0.5 fmol/µL, prior to LC-SRM analysis. Stable isotope-labeled standard peptides were added immediately following tryptic digestion to avoid inconsistent results [29].

## 2.4 Targeted peptides

The targeted peptides were LVVVGAGGVGK (WT) (MW 955.16 amu) and LVVVGADGVGK (G12D) (MW 1013.19 amu). The tryptic peptide carrying the mutation is both unique and MS detectable [30]. Both peptides show high response in electrospray LC-MS/MS. The mutant G12D is a signature peptide, with a unique sequence when searched against a nonredundant human protein database (ftp://ftp.ncbi.nih.gov/genomes/H_sapiens/protein, 235519 sequences). WT is common for NRAS, HRAS, and KRAS. Synthetic unlabeled $^{12}C$ forms of each peptide (LVVVGAGGVGK and LVVVGADGVGK) were purchased from GenScript (Piscataway, NJ, USA). Two heavy peptides (LVVVGAGG[$^{13}C_5$]VGK and LVVVGADG[$^{13}C_5$]VGK) derived from the targeted proteins were synthesized with a single uniformly labeled valine (+5 Da) (JPT Peptide Technologies, Berlin, Germany). All synthetic peptides were quantified by amino acid analysis. Moreover, $^{13}C$ peptides were used to

determine percentage of isotopic impurity due to unincorporated $^{13}C$ in the heavy amino acid valine by monitoring the *m/z* channel for the all-$^{12}C$-analytes. Using peak areas for the $^{12}C$ and $^{13}C$ transitions we determined that there was ca. 1% residual for each $^{13}C$-peptide standard, which is similar to published values [31] (Supporting Information Fig. S2).

### 2.5 LC-MS/MS and optimization of the SRM method using synthetic peptides

Transitions for the assay were chosen based upon relative abundance in the full-scan MS/MS spectrum recorded on the 5500 QTRAP mass spectrometer. Supporting Informa- tion Fig. S3 shows the fragment ions obtained after per- forming full scan MS/MS of the [$^{12}C$] synthetic peptides (see Supporting Information for full scan MS/MS acquisition details). Declustering potential (DP), collision energy (CE), and cell exit potential (CXP) were optimized using the Compound Optimization function provided in Analyst 1.5. Unlabeled $^{12}C$ forms of each peptide standard (500 fmol/μL) were dissolved in ACN/$H_2O$ (50:50) with 0.1% formic acid and directly infused at 5 μL/min. Q1 scans with a DP voltage ramp were used to determine the optimal DP voltage for doubly charged parent ion of WT and G12D (Supporting Information Fig. S4A). Signal intensities from all SRM Q1/Q3 ion pairs for each peptide were ranked to ensure selection of the most intense precursor and fragment ion pair for SRM-based quantification. This approach resulted in selection of CE voltages that maximize the generation of each fragment ion species (Supporting Information Fig. S4B). Identical DP, CE, and CXP values were used for each $^{12}C/^{13}C$ pair. Finally, five SRM transitions per peptide were monitored and acquired at low resolution in Q1 and unit resolution in Q3 quadrupoles with 80-ms dwell time. A complete list of parent and product ions that were used for SRM together with their optimal DP, CE, and CXP is provided in Supporting Information Table S1. Low resolution is run with an offset drop from unit of about 0.05. This results in a transmission window of about 1.1 Da. Therefore, Q1 allows not only the monoisotopic peak to enter the Q2, but also additional parts of the isotopic envelope, with a consequent increase in sensitivity. A full scan in Q3 using the trap capabilities confirms that the correct transitions are being monitored. Cycle time was 3.3676 s and a minimum of 10 data points were collected per peak, sufficient for accurate quantitation. Following construction of the final SRM method, LC retention, MS detectability, and linearity of response of the WT and G12D peptides were evaluated (see Supporting Information for details).

All samples were analyzed by nano-LC-SRM on a 5500 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex, Toronto, Canada) equipped with a NanoSpray III source with a PicoTip emitter (360 μm od, 20 μm id, 10 ± 1 μm id at the tip, noncoated, New Objective, Woburn, MA). Chromatography was performed using an Eksigent nano-LC ultra system (Eksigent, Dublin, CA, USA). Solvent A was 0.1% formic acid (FA) and Solvent B ACN in 0.1% FA. Samples (10 μL injections) were loaded onto a reversed-phase IntegraFrit, 100 μm × 25 mm, ProteoPep2 C18, 300 A°, 5 μm (New Objective) trapping column, and washed for 10 min at 5 μL/min with Solvent A. The peptides were separated on a RP Chromolith CapRod, 150 × 0.05 mm, monolithic capillary column (kindly provided by MERCK, Darmstadt, Germany) The following gradient was used: 0–5 min 5% B, 5–35 min 5–40% B, 35–36 min 40–95% B, 36–46 min 95% B, 47–55 min 5% B and the flow rate was 300 nL/min. Data acquisition was performed with an ion spray voltage of 2800 V, curtain gas of 15 psi, ion source gas 35 psi, and an interface heater temperature of 150°C.

### 2.6 Evaluation of the SRM method and data analysis

The limit of detection (LOD), limit of quantitation (LOQ), assay accuracy, precision, and endogenous levels were determined for PanRas and KRAS (G12D) peptides by the method of standard addition. A control matrix was used to define these values and to mimic matrix effects during sample preparation. Protein extraction, immunoprecipitation, and digestion of control matrixes were carried out as described above. Each $^{12}C$-peptide, WT, and G12D was spiked into tryptic digests ranging from 0.01 to 5 fmol/μL. This concentration range is equivalent to 2.16–21,656 pg/mL for WT KRAS and 2.17–21,713 pg/mL for G12D KRAS. Labeled internal standard peptides were spiked in at a constant concentration of 0.5 fmol/μL prior to the analysis of 10 μL of sample by LC-SRM/MS. Quantification was performed using MultiQuant™ software (AB Sciex, Foster City, CA, USA). Typical integration settings were a Gaussian smooth width of 1 point and a peak splitting factor of 4. Peak integrations were reviewed manually and result tables were exported as Excel files. The peak area ratio (PAR) of light peptide (analyte) to heavy peptide (internal standard) was determined from the XIC of the most abundant transition for each peptide as follows: the pair 478.3/743.4 and 480.9/748.4 (heavy) for the peptide WT and the pair 507.3/312.2 and 509.7/312.2 (heavy) for the peptide G12D. Calibration curves for each biological matrix were obtained by plotting the PAR of light peptides versus heavy peptides against the nominal amount of the light peptides spiked into control pancreatic and colon tissue immunoprecipitates. The regression equation for the calibration curve was used to back-calculate the measured concentration at each standard level, and the result was compared with the theoretical concentration to obtain the accuracy, expressed as a percentage of the theoretical value, for each standard level measured. To determine the reproducibility (intra-assay precision) of the analysis, each point of the calibration curve was repeated three times. Signal to noise ratio (*S*/*N*) was calculated by dividing peak intensity at the apex by the background intensity (cps). These noise levels are conservative estimates based upon visual inspection of preceding regions and postregions around the analyte chromatographic peak. Here we de-

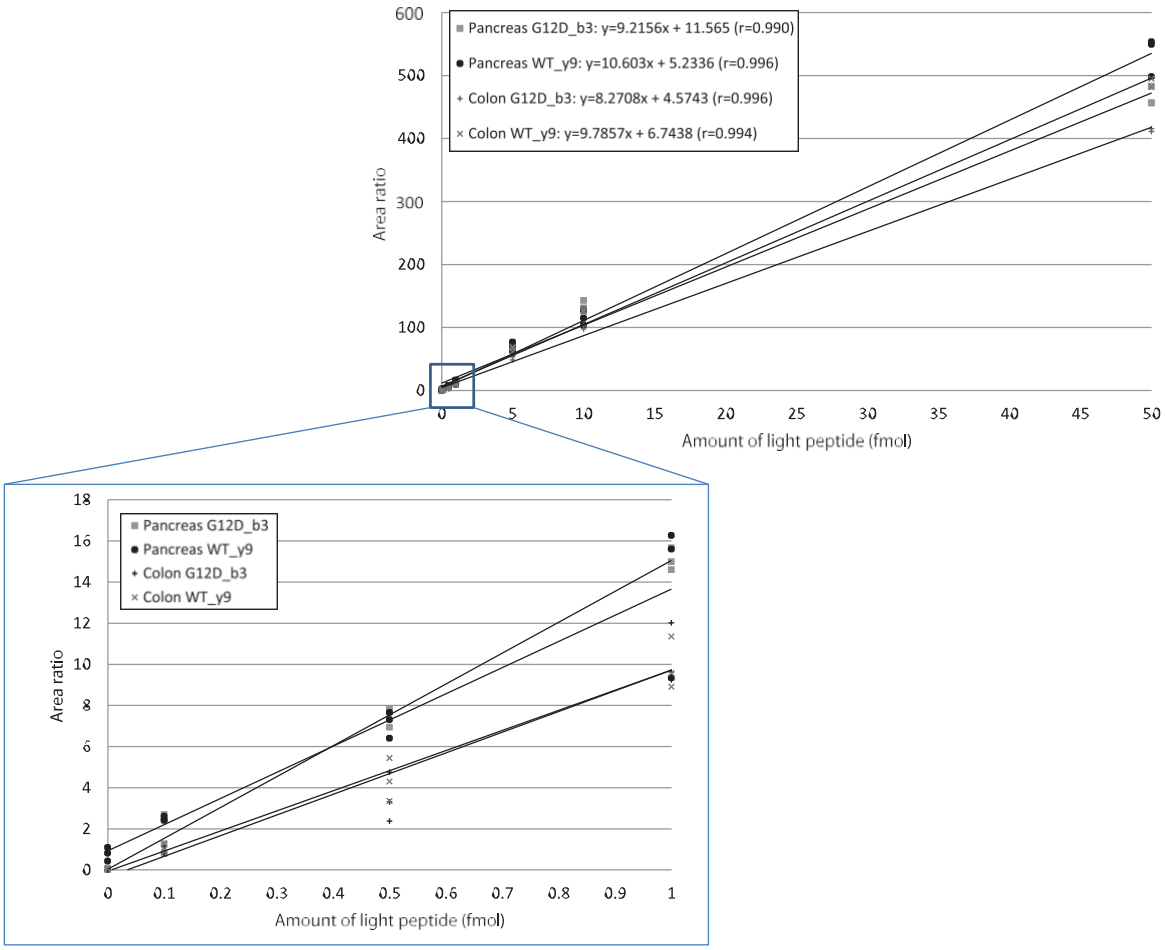


**Figure 2.** Calibration curves for signature peptides spiked in biological matrix. Representation of PAR versus concentration ratio between light and heavy synthetic standard peptides over the range of 0–50 fmol of light peptide in pancreas and colon matrix showing monitored SRM transitions used for quantitation. Triplicate injections monitoring [$^{12}C/^{13}C$] transitions were performed.

fine LOQ and LOD as the concentration at which the *S*/*N* of the analyte is equal to 10 and 3, respectively [32]. Also, the endogenous concentrations in pooled normal tissue were calculated from the slopes and intercepts, since endogenous KRAS in the matrix causes the nonzero intercept. Finally, regression equations obtained from each calibration curve were used to calculate concentrations of target proteins in primary and xenograft tumor samples.

## 2.7 Evaluation of AB capture, digestion efficiency, and assay recovery

In order to test the efficiency of antibody capture of the KRAS protein, 500 fmol of recombinant KRAS (Prospec, East Brunswick, NJ, USA) was spiked into 2 mL of control matrix extract containing no endogenous KRAS, as it had been previously depleted, and processed as previously described. An aliquot of 10% of the immunoprecipitated samples was used for analysis of the bound KRAS protein by WB. The remaining sample was digested with trypsin and analyzed by WB to assess protease efficiency. The supernatant of the immunoprecipitation (IP) was subjected to a second IP and also analyzed by WB to determine if all the KRAS was captured by the AB. Antibodies were accepted as displaying a single predominant band at the expected molecular weight. For further details on WB, please refer to Supporting Information.

To further assess the efficiency of the combined steps involved in our approach, we added a known amount of recombinant KRAS protein (1 fmol, equivalent to 2.32 pg of protein) to 1 mL of control matrix extract containing no endogenous KRAS. In parallel, 1 mL of control matrix extract containing no endogenous KRAS was processed as described above, spiking 1 fmol of WT synthetic peptide after digestion. Both samples were processed as described above and 10% of each sample was analyzed by LC-MS/MS. The efficiency of the assay was calculated by comparing the PAR of light peptide to internal standard (IS) as previously explained between both samples.

**Table 1.** Evaluation the assay using synthetic peptides in pancreas and colon tissue

| Matrix | Peptide | LOD ($S/N = 3$)[a] | LOQ ($S/N=10$)[a] | Precision (%)[b] | | | Accuracy (%)[c] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 0.1 | 1 | 10 | 0.1 | 1 | 10 |
| Human pancreas | WT | 13 | 14 | 4.39 | 2.94 | 10.44 | 124.2 | 95.97 | 82.82 |
| | G12D | 17 | 18 | 12.11 | 3.55 | 6.21 | 84.46 | 102.46 | 92.52 |
| Human colon | WT | 12 | 14 | 19.53 | 12.73 | 2.12 | 112.71 | 102.26 | 98.98 |
| | G12D | 12 | 16 | 19.33 | 14.61 | 3.01 | 128.57 | 105.74 | 115.74 |

a) Results for LOD, LOQ are amol of protein on-column.
b) Expressed as relative standard deviation (RSD): (standard deviation/mean) $\times$ 100 of PARs for different amount of peptides (0.1, 1, and 10 fmol) injected on-column.
c) Calculated using MultiQuant™ 2.0.2 software (AB/MDS Sciex).

## 3 Results

### 3.1 Evaluation of SRM method

In all experiments, SRM transitions from analyte peptides were confirmed by chromatographic co-elution with heavy isotope-labeled peptides, as shown in the extracted ion chromatogram (Fig. 1). Although quantification of proteins in tissue is the primary objective of this work, peptide response curves were also used to define the assay performance. Many potential sources of sample loss and interference resulting from tryptic digestion of the protein in tissue extracts are not factors when response curves are generated using pep- tides instead of proteins [33]. The plot showing amounts of light WT and G12D peptides versus the PAR of the light to heavy peptide in each biological matrix (Fig. 2 and Supporting Information Fig. S7) illustrates the assay is linear between 0.1 and 50 fmol of peptide with $R^2 > 0.99$, with similar response in pancreas and colon, proving the versatility of the assay. The lower range is amplified, also showing linearity in the region where quantitation of real samples is performed.

The LOD and the LOQ of KRAS (G12D) in pancreatic and colorectal tissues was shown to be as low as 14 and 12 amol, respectively (Table 1). Also, Table 1 shows that precision at 0.1, 1, and 10 fmol of injected peptides is better than 20% in all cases. Furthermore, calculated accuracy reflects how close the computed concentration at those amounts of peptide is to the expected concentration. Moreover, the concentration of endogenous PanRas (H, N, and KRAS) in pancreatic tumors was 3.17 pg (150 amol)/mg of tissue and 2.16 pg (102 amol)/mg of tissue in colorectal tumors. The full MultiQuant Results Table datasets for standard analyses can be found in Supporting Information Tables S2 and S3.

### 3.2 Evaluation of AB capture, digestion efficiency, and assay recovery

Analysis of the IP results by WB with an antibody that reacts with KRAS is shown in Supporting Information Fig. S6. The KRAS-specific band in lane one shows that the spiked recombinant protein was captured successfully from the control matrix extracts, while the lack of a KRAS band in lane two indicates good capture efficiency. Furthermore, the tryptic digest analyzed by WB in lane three shows that no detectable KRAS or antibodies were present after proteolysis, indicating very high protease efficiency.

Regarding the efficiency of the assay, from the quantitative experiment where recombinant KRAS was spiked into KRAS depleted matrix and analyzed by MS, we found that 22.8 $\pm$ 0.4% of the input KRAS protein was recovered in the MS analysis, comparable to the result obtained by Wang et al. (22.4 $\pm$1.4%). We used this correction factor to calculate the amount of protein present in tissues.

### 3.3 Tissue analysis

The results of the assay of 11 primary tumor and xenograft tissue samples are shown in Table 2. There was complete agreement between genotype and proteotype, and quantitative data on the level of protein expression was obtained. In pancreatic tumors the overall level of PanRas proteins is quite variable, ranging from 18.9 to 150 pg of protein per mg of tissue, and the amount of KRAS (G12D) protein ranges from 9.3 to 75 pg of protein per mg of tissue. In the pancreatic xenograft samples, the amounts of mutant protein are similar to those of the wild type protein, whereas in the primary tumor samples the amount of mutant KRAS is much lower than in the wild type. This suggests that the xenograft tissue contains a higher proportion of tumor cells or perhaps that the proliferation of tumor cells carrying the mutant allele is favored in the xenograft microenvironment. By way of contrast, the levels of PanRas proteins in colon cancer tumors tends to be more consistent between samples, as is the ratio between mutant and wild type protein. The full MultiQuant Results Table datasets for sample analyses can be found in Supporting Information Tables S4 and S5. Moreover, the KRAS assay was successfully applied for the analysis of benign skin tumor samples (Fig. 3) and results were in complete agreement with the genotyping results [27].

**Table 2.** Genetic status of *KRAS* and absolute concentration of PanRas WT and G12D KRAS (G12D) proteins in different xenograft and primary tumors, for (A) pancreatic tissue and (B) colorectal tissue.

| | Genetic status of KRAS | | Absolute concentration (pg protein/mg tissue) | |
|---|---|---|---|---|
| | Wild type allele | *KRAS (G12D)* allele | PanRas proteins | KRAS (G12D) protein |
| **(A) Pancreatic tumours** | | | | |
| Xenograft tumors | | | | |
| PANC198 G12D/wt | + | + | 78.5 ± 14.3 | 75.0 ± 2.3 |
| PANC215 G12D/wt | + | + | 18.9 ± 1.6 | 14.8 ± 1.9 |
| PANC354 wt/wt | + | ND | 150 ± 12 | ND |
| Primary tumors | | | | |
| PT 3092 G12D/wt | + | + | 37.2 ± 3.6 | 13.4 ± 8.2 |
| PT 5778 G12D/wt | + | + | 62.1 ± 0.5 | 9.3 ± 2.3 |
| **(B) Colorectal tumours** | | | | |
| Xenograft tumors | | | | |
| CRC 010 G12D/wt | + | + | 26.3 ± 2.2 | 11.3 ± 0.2 |
| CRC 020 wt/wt | + | ND | 27.2 ± 0.5 | ND |
| CRC 026 wt/wt | + | ND | 23.5 ± 1.0 | ND |
| Primary tumors | | | | |
| CT 5669 G12D/wt | + | + | 29.3 ± 1.5 | 15.7 ± 0.7 |
| CT 5381 wt/wt | + | ND | 22.0 ± 12.6 | ND |
| CT 3056 wt/wt | + | ND | 40.2 ± 8.6 | ND |

Results for PanRas were estimated using the average Mw derived from KRAS, HRAS, and NRAS. Concentration results are presented showing average ± SD ($n$ = 3). ND indicates the G12D was not detected and therefore no quantitation could be carried out.

## 4 Discussion

The LC-MS SRM methods that use antibodies to simultaneously capture different forms tumor specific biomarker from tissues have several advantages over other methods: (i) they require a single antibody for enrichment, whereas a sandwich ELISA needs a matched pair of antibodies [6]; (ii) they could be multiplexed by using several capture antibodies in a single assay thereby allowing measurement of multiple analytes in a single assay; (iii) antibodies of lower specificity can be used, since the mass spectrometer provides the detection specificity [6]; (iv) from receipt of tissue sample to result requires 24 h; (v) the use of antibody to enrich the target analyte not only increases the concentration but also reduces the level of potentially interfering compounds; (vi) further assays will be relatively inexpensive to develop since the isotopically labeled peptides required as internal standards are readily available, whereas generating antibodies for ELISA is a costly and difficult undertaking [29].

Despite the high specificity and selectivity of the SRM assay, peptides and other small molecules in the biological matrix can produce interference in the $m/z$ channels monitored,

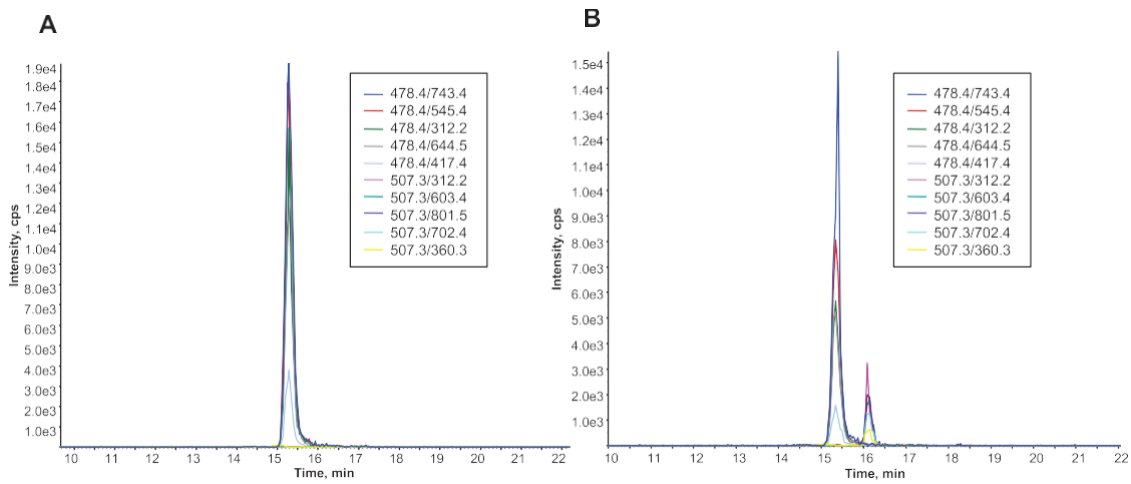


**Figure 3.** Analysis of benign skin tumors by SRM. Extracted ion chromatograms derived from the injection of 1 mg tissue-equivalent protein extract in buffer A, showing the ten transitions monitored for the peptides WT and G12D. These two samples correspond to benign skin tumors embedded in OCT and previously genotyped as (A) G12D/wt and (B) wt/wt using Sequenom technology as previously described [26] and confirmed by Sanger sequencing. cps, counts per second.

resulting in inaccurate quantitative measurements [34, 35]. The main limitation to targeted-based approaches is the extremely large concentration range and complexity of the proteins in the matrixes analyzed. Nevertheless, in this study the concentration and enrichment of the target using an antibody resulted in low background, high sensitivity, and specificity.

This work uses a concept similar to that of the SISCAPA (stable isotope standards and capture by anti-peptide antibody) method [36, 37], but SISCAPA requires numerous antipeptide antibodies to capture proteotypic peptides after proteolytic cleavage of the target proteins. It will however be difficult to generate the mutation specific antibodies needed to capture mutant peptides efficiently [8]. The potential of KRAS mutation analysis by SRM as clinical marker has been suggested [9]. However, in this work it was pointed that one potential limitation of the assay was its sensitivity. Based on the results presented in that work, the authors estimated that SRM could be used to detect mutant proteins reliably when they are present at levels above 25 fmol/mg of total protein.

Here, through the use of a different mass spectrometer (5500 QTRAP), we have reported LOD as low as 12 amol on-column, equivalent to 0.24 fmol/mg of total protein, showing an improvement of two orders of magnitude in sensitivity. Furthermore, by applying the MIDAS workflow [38] on the 5500 QTRAP, we were also able to confirm the sequence of the targeted peptides without a separate sequencing experiment. In addition, the on-bead proteolysis avoided the need to elute the bound proteins. Wang et al. calculated that there are between 1.5 and 8.6 million copies of WT and G12D KRAS, respectively, per cell in SW480 cell line. This is equivalent to 2.5 amol of WT and 14.3 amol of G12D KRAS per cell. While such concentrations may be present in a cell line, our results indicate far lower levels in tissue samples. The analysis of cancer cell lines, normal tissues, CRC tissue, and pancreatic cyst fluid was described [9]. We have extended this to xenografted tissue, pancreatic tumors, and benign skin tumors. We have shown that by using a peptide common to PanRas and another peptide specific for KRAS (G12D), these species can be specifically detected and quantified in the low pg/mg range in fresh patient tissues and xenografts that can also be used for immunohistochemistry. Here we show an improved implementation of the mutant protein specific Immuno-SRM mass spectrometry method of RAS proteins reported by Wang et al. [9], as well as demonstrating its first use to detect PanRAS and KRAS mutant proteins in embedded tissue at high sensitivity.

Moreover, the amount of tissue required for the assay is low milligrams, which can be obtained by needle aspiration biopsy. Therefore, the need for more invasive surgical techniques to obtain tissue can be avoided.

The method complements PCR-based assays [39] for typing tumor tissue sourced from patients, offering a means of rapidly and accurately detecting unequivocal structural features at the protein level, that is, driver point mutations. It would provide a rapid means of giving the clinician critical information about the oncogenic status of patients that can be used for therapy management. The development of similar assays and their multiplexing will be of value in personalized medicine. The approach is not restricted to point mutations in cancer driver genes but can be applied to other biologi- cal questions where point mutations are critical, for example, Huntington's disease, factor VII deficiency [40,41]. To explore the true clinical potential it would now be highly desirable to apply the KRAS assay to a large cohort of clinical samples, across multiple laboratories.

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