Chapter 10

Measuring Gli2 Phosphorylation by Selected Reaction Monitoring Mass Spectrometry

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Abstract

Phosphorylation is an important mechanism by which Gli proteins are regulated. When the Hedgehog (Hh) pathway is activated, multiple serine and threonine residues of Gli2 are dephosphorylated, while at least one residue undergoes phosphorylation. These changes in phosphorylation have functional relevance for the transcriptional activity of Gli proteins, as shown by in vitro and in vivo assays on Gli mutants lacking the phosphorylated residues. Here, we describe a method of quantitatively monitoring the phosphorylation of Gli proteins by triple quadrupole mass spectrometry of Gli2 immunoprecipitated from cell lysates. This method is broadly applicable to the monitoring of phosphorylation changes of immunoprecipitated Gli proteins when the putative phosphosites are known.

Key words Gli proteins, Phosphorylation, Protein kinase A, Triple quadrupole, Mass spectrometry, Selected reaction monitoring

Abbreviations

AmBic	Ammonium bicarbonate
ACN	Acetonitrile
FA	Formic acid
Hh	Hedgehog
HPLC	High-performance liquid chromatography
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
nLC	Nanoliter-scale high-performance liquid chromatography
SRM	Selected reaction monitoring

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1 Introduction

Gli proteins, vertebrate homologs of the *Drosophila* protein Cubitus interruptus (Ci), are a family of transcription factors that mediate the effects of Hedgehog (Hh) signaling on gene expression [1]. The mammalian Gli2 and Gli3 proteins have long been known to be phosphorylated by protein kinase A (PKA) at six conserved serine/threonine residues, hereafter referred to as P1–P6, localized in their C-terminal part [2]. These phosphorylations were known to be required for the conversion of Gli2/Gli3 into truncated transcriptional repressors (GliR—Gli repressors) by the proteasome [3, 4]. More recently, we have shown that failure to phosphorylate these six residues results in the formation of constitutive Gli activators (GliA) capable of inducing the production of Hh target genes in the absence of any upstream signal [5]. Moreover, we showed that P1–P6 dephosphorylation is correlated with the phosphorylation of a serine at the N-terminus of Gli2, which we refer to as Pg [5]. Monitoring these phosphorylation events is important for the understanding of Gli protein regulation by PKA and other kinases.

Several methods can be used to measure protein phosphorylation in cells [6]. Labeling cells with radioactive [^{32}P]orthophosphate followed by electrophoresis (1-D or 2-D) and detection by film or phosphor imaging autoradiography is a well-established method in the field, but is generally limited to detection of phosphorylation at the level of a protein rather than quantification of phosphorylation at specific residues. Phosphospecific antibodies are an excellent tool for quantification of specific phosphosites [7]. The distinct advantage of this method is its sensitivity and ability to detect phosphoproteins by immunohistochemical methods in tissues and cells. However, a separate antibody must be produced for each phosphosite, which effectively limits the number of sites that can be monitored. In addition, phosphospecific antibodies of sufficient quality to provide quantitative information on phosphosite occupancy are often difficult to make, as has been the case with the Gli proteins.

Progress in mass spectrometric techniques has opened up new avenues for phosphosite analysis. In particular, large-scale "shotgun" LC-MS/MS-based screens have made it possible to discover and quantify new phosphosites in a global, high-throughput manner [8–10]. Chromatographic techniques based on metal ion affinity chromatography (IMAC) or titanium oxide are used to enrich phosphorylated species, thus reducing non-phosphorylated peptide abundance in the sample and increasing phosphopeptide detection sensitivity [10, 11]. However, these methods are unsuitable for the detection of less abundant phosphopeptides. In order to precisely quantify low-abundance

phosphorylated peptide species, a targeted MS-based approach known as selected reaction monitoring (SRM) is often employed using a triple quadrupole instrument [12–14].

In the SRM experiment, proteins are digested by trypsin into short (5–50 amino acids) peptides. These peptides are resolved using reverse-phase high-performance liquid chromatography (HPLC) and ionized by electrospray ionization (ESI). After the peptide ions enter the instrument, the first quadrupole is used to select the desired precursor peptide ion that contains the phosphosite of interest. The precursor ion is then fragmented by collision-induced dissociation (CID) in the second quadrupole. The third quadrupole is used to select fragment ions that had been found to give the most intense signal in preliminary experiments, and these fragment ions reach the detector (Fig. 1). The combination of a targeted peptide precursor ion with one of its specific fragments is known as a "transition." Usually 2–3 transitions are enough to quantify the amount of a peptide in the extract using an internal standard. Since no survey mass spectra are acquired in the quantification mode, many peptides in the extract can be quantified relatively quickly (approximately 200 peptides in 1 h). The two-level mass filtering in the first and third quadrupole and the high ion transmission used in SRM account for a much higher sensitivity compared to conventional proteomic techniques [15]. These SRM approaches have been shown to detect specific peptides and phosphopeptides in complex mixtures [13].

The disadvantages of this method are that (1) only a limited number of fragment ions can be detected in each sample and (2) the precision of mass measurement is limited to around 0.3 Da around the precursor, sometimes leading to false positives with similar MS/MS fragmentation patterns being detected in place of the less abundant true peptide of interest. With those limitations in

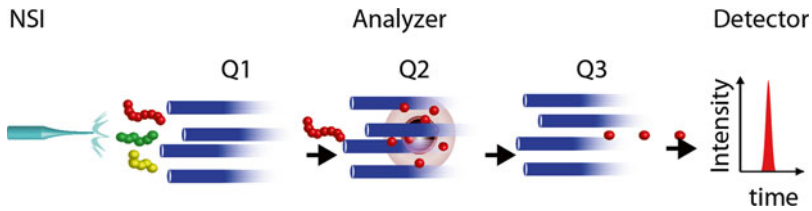

Fig. 1 Diagram illustrating the operation of the triple quadrupole instrument. The peptide solution is ionized in the nanospray ionization source (NSI). Precursor ions enter the first quadrupole (Q1). Precursor peptide ions within a narrow mass to charge (m/a) ratio (ideally representing a single precursor) pass through Q1 and enter Q2. In Q2, precursor ions are dissociated into fragment ions. Fragment ions enter Q3 and are filtered so that only fragment ions within a narrow m/a range pass onto the detector. A combination of a precursor ion and a specific fragment ion is known as a transition. The instrument cycles through multiple transitions and measures the intensity of each transition over time, which is known as the extracted ion current (XIC) for this transition (graph on the right). The total amount of the peptide in the solution is proportional to the area under the curve of the XIC

mind, the assay design must be undertaken with care to maximize sensitivity and specificity, and internal isotope-coded standard peptides have to be applied as an additional control.

Phosphopeptides of interest can be quantified and validated using isotope-labeled reference peptides in combination with SRM-MS. This simple method, based on the classical principle of isotope dilution, was introduced by the Gygi Lab in 2003 and uses synthesized isotope-labeled reference peptides as internal standards [13–16]. To develop a suitable reference peptide, a peptide out of the digested sample is selected based on the applied proteases, retention behavior, ionization efficiency, and fragmentation pattern. The selected peptide is then synthesized by solid-phase synthesis with light amino acids and one isotopically labeled "heavy" amino acid. This results in the synthesis of a chemically identical peptide homolog that differs from the endogenous peptide only in mass (8–10 Da). Since the endogenous (light) and the internal standard (heavy) peptides differ in mass, the heavy peptide transitions can be easily distinguished by triple quadrupole mass spectrometry from those of the endogenous peptide. Thus, the heavy peptide can be used as reference for the light peptide derived from the endogenous protein. Since the reverse-phase separation, ionization, and fragmentation are identical for the isotopically labeled and the corresponding endogenous peptides, the reference peptides elute at the same time as the endogenous peptides and are fragmented in an identical pattern. Consequently, if the concentration of the "heavy" peptide in the sample is known, the concentration of endogenous peptides can be directly measured as a ratio over the added isotopically labeled peptides (absolute quantification—AQUA). Alternatively, if the "heavy" peptide is added at an unknown but constant concentration to all samples, the relative increase or decrease in the abundance of the endogenous peptide can be quantified (relative quantification). The advantage of the latter method is that the isotopically labeled peptides do not need to be precisely titrated and can be purchased in an unpurified and therefore much more economical format.

Here, we describe relative quantification SRM assays that we designed for the measurement of the phosphorylation of Gli2 at five distinct sites, but the same methodology can be applied to any proteins that can be isolated at sufficient quantities and purity levels from cell lysates or tissues.

2 Materials

2.1 Cell Culture Materials

1. Cells: NIH/3T3 (ATCC).
2. High-serum culture media: DMEM containing 10 % fetal bovine serum (FBS), 1× GlutaMAX, 1× nonessential amino acids, 1× sodium pyruvate, 1× penicillin/streptomycin solution (Life Technologies).

3. Low-serum culture media: Same as high-serum media, except made with 0.5 % FBS instead of 10 %.
4. Cell culture dishes: Nunc Nunclon tissue culture-treated 10 and 15 cm diameter round dishes or equivalent.
5. Bortezomib stock solution: 10 mM bortezomib (LC Laboratories) in DMSO; store at –20 °C.

2.2 Cell Lysis/ Immunoprecipitation Materials

1. 1× phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 m M KH_2PO_4; chill to 4 °C and adjust pH to 7.4.
2. Urea lysis buffer: 100 mM NaH_2PO_4*H_2O, 10 mM Tris base, 8 M urea, 300 mM NaCl, pH 8.0. Prepare fresh before each experiment and chill to 4 °C (*see* **Note 1**).
3. Cell lifters: Corning cell lifters or equivalent. Lifters can be rinsed in water, blotted dry, and reused.
4. High-salt RIPA buffer: 50 mM Tris, pH 7.4, 370 mM NaCl, 50 mM NH_4Cl, 25 mM glycinamide, 2 % Nonidet P-40 (or IGEPAL CA-630), 0.25 % sodium deoxycholate, 1 μM bortezomib, 1× SigmaFAST protease inhibitor cocktail, 1 mM β-glycero-phosphate, 1 μM microcystin LR, 1 mM NaF, 1 mM activated Na_3VO_4. Prepare fresh before each experiment and chill to 4 °C.
5. Ultracentrifuge and ultracentrifuge tubes that will hold at least 20 mL of liquid and withhold 100,000 × *g* centrifugation.
6. Anti-Gli2 antibody: Any anti-Gli2 antibody suitable for immunoprecipitation can be used (*see* **Note 2**).
7. Anti-Gli2 beads: Couple anti-Gli2 antibody protein A-conjugated Dynabeads (Life Technologies). For each 100 μL of Dynabeads slurry, use 20–40 μg of the affinity-purified antibody. Cross-link antibody to beads using dimethylpimelidate. Wash thoroughly and store at 4 °C in 0.2 M ethanolamine, pH 8.5, 0.2 M NaCl, 0.1 % Nonidet P-40, 10 % glycerol, 0.01 % NaN_3.
8. Acid wash solution: 100 mM glycine, pH 2.5.
9. Magnetic holders for 1.5 mL microcentrifuge tubes and 15 mL or 50 mL conical tubes (DynaMag-2, DynaMag-15, DynaMag-50 from Life Technologies or equivalent).
10. Bead wash buffer: Mix 1 part urea lysis buffer with three parts high-salt RIPA buffer (final urea concentration 2 M).
11. SDS elution buffer: 50 mM Tris, pH 6.8, 2.5 % sodium dodecyl sulfate, 5 % glycerol. Add dithiothreitol to 65 mM before each experiment.

2.3 Gel Electrophoresis/ Staining Materials

1. Polyacrylamide gels: Novex Tris-Glycine 8 % mini-protein gels 1.5 mm, 10 wells (Life Technologies) or equivalent with an appropriate electrophoresis box (*see* **Note 3**).

2. Gel running buffer: 25 mM Tris base, 192 mM glycine, 0.1 % sodium dodecyl sulfate. Make a 10× concentrate and sterile-filter. Do not adjust pH. Dilute with ultrapure water before use.
3. Coomassie blue solution: GelCode Blue (Pierce) or equivalent Coomassie G-250 solution.

2.4 Gel Cleaning/ Digest/Extraction Materials

All reagents used at stages following gel fragment excision *must be HPLC or MS grade* to ensure the absence of trace contaminants that can interfere with nLC separation and MS detection.

1. Ammonium bicarbonate (AmBic) solution: 50 mM AmBic in HPLC-grade water. Store for a few weeks at 4 °C.
2. Acetonitrile (ACN): HPLC-grade acetonitrile (100 %).
3. AmBic/ACN solution: Mix 1 part AmBic solution with 1 part ACN. Store for a few weeks at 4 °C.
4. Vacuum centrifuge resistant to small quantities of organic solvents and a vacuum pump.
5. Trypsin solution: Sequencing-grade modified trypsin (Promega) resuspended in the supplied buffer to 200 ng/μL. Aliquot and store frozen at −80 °C.
6. Eppendorf Protein LoBind microcentrifuge 1.5 mL tubes.
7. ACN/formic acid (FA) solution: 5 % formic acid, 65 % ACN, 30 % HPLC-grade water.
8. FA solution: 5 % formic acid in HPLC-grade water.
9. 2 % ACN/0.1 % FA solution in HPLC-grade water.
10. Sonicating water bath.
11. Tabletop centrifuge with adapters for 96-well plates. We used the Beckman Allegra 6R with a GH-3.8A rotor.
12. Peptide cleanup plate: Waters Oasis HLB μElution 96-well plate (30 μm particle size).
13. Concentrated formic acid solution: 98 % formic acid.
14. 100 % methanol.
15. 0.04 % trifluoroacetic acid.
16. 80 % ACN solution in HPLC-grade water.

2.5 HPLC/MS Materials

1. Standard peptide mix: Heavy arginine ($[^{13}C]_6[^{15}N]_4$—total mass increase 10 Da)-labeled unphosphorylated and phosphorylated peptides can be purchased from multiple vendors. We have purchased peptides from JPT Peptide Technologies (SpikeTides-L) in the unpurified format. Solubilize peptides to approximately 1 mM in 80 % AmBic solution/20 % ACN and sonicate for 5 min in an ice-cold sonicating water bath. Add all the heavy peptides needed for the experiment to AmBic solution to a final concentration of 1 μM and store the peptide mix, as

well as the remainder of the concentrated individual peptide solutions, aliquoted at −80 °C (*see* **Note 4**).

2. HPLC buffers: A, 0.1 % FA in HPLC-grade water; B, 0.1%FA in ACN.
3. Nano-HPLC instrument: EASY-nLC (Proxeon) equipped with 35 mm × 0.1 mm C18 trapping column (Michrom C18, 5 μm, 120 Å) and a 200 mm × 0.075 mm diameter reverse-phase C18 capillary column (Maisch C18, 3 μm, 120 Å).
4. MS instrument: TSQ Vantage (Thermo Fisher Scientific) triple quadrupole instrument with a Proxeon nanospray ionization source.

2.6 Data Analysis Materials

1. Xcalibur 2.2.44, data analysis and instrument control.
2. Skyline 2.5 software suite—available for free from the MacCoss Lab website: https://brendanx-uw1.gs.washington.edu/labkey/project/home/software/Skyline/begin.view
3. MS Excel or any other statistical software.

3 Methods

3.1 Cell Culture

1. Culture NIH/3T3 cells in high-serum culture media (*see* **Note 5**). For the experiment, prepare 4–6 15 cm round dishes of fully confluent cells for each experimental condition (*see* **Note 6**).
2. After the cells have reached full confluence (look "over-crowded"), change the media to low-serum culture media for 36 h (*see* **Note 7**).
3. 4–6 h before harvesting, add bortezomib to 1 μM to block proteasomal degradation of Gli2.
4. Add hedgehog-modulating drugs as needed for 1–4 h before harvesting (*see* **Notes 8** and **9**).

3.2 Cell Lysis

All procedures in this section should be carried out at 4 °C (in the cold room). Dishes can be processed in groups of 4–6.

1. Decant media from cells and wash briefly twice with ice-cold PBS.
2. Carefully remove as much of PBS as possible by aspirating or blotting the sides of each plate with a paper towel.
3. To each plate, add 0.8 mL of urea lysis buffer and scrape the cells off with a cell lifter. The cells will lyse as they are lifted, and the solution will become viscous. Pipet the solution into a 15 mL conical tube using a 1 mL tip with the end cut off.
4. Allow the cells to lyse for 15 min at 4 °C (do not put on ice or the urea will precipitate).

5. Sonicate the lysate to reduce viscosity (*see* **Note 10**).
6. Dilute the lysate with high-salt RIPA buffer. Calculate the volume of the buffer to adjust the final urea concentration to 2 M.
7. Ultracentrifuge the samples at 100,000 × *g* for 30 min to remove debris and protein aggregates. Keep a small aliquot of the supernatant as "total cell lysate."
8. Decant the supernatant into a conical tube.

3.3 Immunoprecipitation of Gli2

1. Prewash anti-Gli2 beads in acid wash solution for 5 min at room temperature. Wash twice with high-salt RIPA buffer. For each 15 cm dish of cells, use 16 µL of the bead slurry, corresponding to 3.2–6.4 µg of coupled antibody (*see* **Note 11**).
2. Add beads to lysate. Rotate at 4 °C for 24–40 h.
3. Magnetize beads on a magnetic stand. Keep a small aliquot of the supernatant as "flowthrough."
4. Remove supernatant from beads. Resuspend beads in 1 mL bead wash buffer and transfer to 1.5 mL microcentrifuge tubes.
5. Spin beads down briefly and magnetize. Aspirate the wash buffer and remove from the magnetic stand.
6. Resuspend beads in 50 µL SDS elution buffer.
7. Elute protein from beads with vigorous agitation at 37 °C for 30 min (*see* **Note 12**).

3.4 Electrophoresis and Gel Staining

From this point on, great care must be taken to avoid keratin contamination of samples. Always use fresh gloves, and if possible, perform all steps under laminar airflow or in a tissue culture hood. All containers and tools used for gel manipulation should be pre-cleaned by soaking overnight in 1 % SDS solution and rinsing several times with ultrapure water and kept clean by careful handling.

1. Load samples onto the gel and run the gels at 100 V constant voltage until the gel front has reached approximately 1/2 of the gel height. In order to avoid band "spreading" or "smiling," load SDS elution buffer into empty wells of the gel.
2. Remove the gel from its casing and wash 3 × 15 min in ultrapure water. Avoid touching the gel even with a gloved hand. Use a clean spatula instead.
3. Stain the gel with Coomassie blue according to the manufacturer's instructions. Destain the gel until clear bands at around 200 kDa are visible (*see* Fig. 2).

3.5 Gel Destaining and Trypsin Digestion

1. Transfer the gels onto a clean glass plate and excise the bands with a freshly opened scalpel blade. Cut the gel bands into pieces approximately 1 mm^3 in size. Transfer gel pieces into sterile 1.5 mL microcentrifuge tubes (*see* **Note 13**).

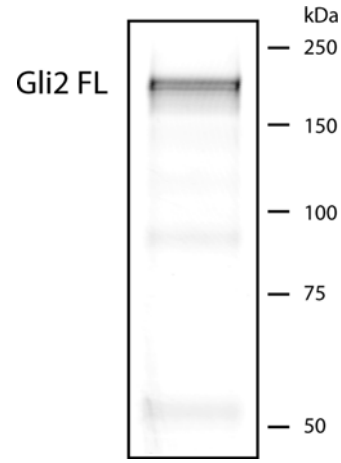

Fig. 2 Coomassie blue-stained gel of Gli2 immunoprecipitated from NIH/3T3 lysates

2. To each tube, add 250 μL AmBic solution. Mix vigorously at 37 °C for 30 min (a heating tube mixer, such as the Eppendorf ThermoMixer, can be used for this and subsequent steps). Spin down briefly.
3. Discard the supernatant and add 150 μL AmBic/ACN solution. Mix vigorously at 37 °C for 15 min. Spin down briefly.
4. Discard the supernatant, add 200 μL AmBic solution, and mix vigorously at 37 °C for 10 min. Spin down briefly.
5. Repeat **steps 3** and **4**. At this point, the gel pieces should be completely transparent without any traces of Coomassie blue staining. If there is still some blue staining remaining, **steps 3** and **4** can be repeated again.
6. Discard the supernatant. Add 200 μL ACN. Mix vigorously for 5 min. The gel slices should become completely opaque and dehydrated. Spin down briefly.
7. Discard the supernatant. Dry the gel pieces completely for 10 min in a vacuum centrifuge (*see* **Note 14**).
8. Put tubes with the dried gel pieces on ice. Take out one aliquot of trypsin solution and dilute 40× in ice-cold AmBic solution (final concentration, 5 ng/μL).
9. Add 30 μL of diluted trypsin to each tube with gel pieces. Leave on ice for 30 min until gel pieces swell and become transparent again.
10. Remove excess trypsin from gel pieces. Cover the pieces with 30 μL of AmBic solution.
11. Leave at 37 °C overnight (for 16–24 h).

3.6 Tryptic Digest Extraction

1. Add 10 µL of HPLC-grade water to each tube. Mix vigorously at 37 °C for 30 min. Spin down briefly.
2. Transfer supernatant to an Eppendorf Protein LoBind tube. Add 5 µL of the ACN/FA solution and 3 µL standard peptide mix to the tube with supernatant (*see* Table 1).
3. To the gel pieces, add 50 µL of FA solution. Mix vigorously at room temperature for 30 min. Spin down briefly.
4. Add the supernatant to the extract from **step 2**.
5. Repeat **steps 3** and **4**.
6. Repeat **step 3** and then sonicate gel pieces for 5 min in ice-cold water in a sonicating water bath. Spin down briefly.
7. Add supernatant to extracts from previous steps.
8. Repeat **steps 6** and **7**, except use 100 µL of FA solution for the final extraction.

3.7 Peptide Cleanup (*See* Note 15)

1. Check the pH of the sample by placing 1–2 µL of the sample on a pH indicator paper. If the pH is above 3, acidify with 1–2 µL of concentrated formic acid.
2. Condition the columns of the peptide cleanup plate with 250 µl 100 % methanol.
4. Centrifuge at 200 × *g* for 2 min. If liquid is still visible, repeat the centrifugation step.
5. Equilibrate the column with 250 µl of 0.04 % trifluoroacetic acid.
6. Centrifuge at 150 × *g* for 2 min.
7. Load sample (up to 250 µl).
8. Centrifuge at 200 × *g* for 3 min.
9. Wash sample with 250 µl of 0.04 % trifluoroacetic acid, pH 2–3.
10. Centrifuge at 150 × *g* for 2 min.
11. Add 100 µl 80 % acetonitrile in H_2O to elute peptides.
12. Centrifuge at 150 × *g* for 5 min.
13. Transfer each eluted peptide sample to a fresh Eppendorf Protein LoBind tube. Evaporate the peptide solution to complete dryness in a vacuum centrifuge (*see* **Note 16**). Resuspend the peptide pellet (which may not be visible at this point) in 15 µL of 2% ACN/0.1% FA solution. Pipet up and down 30 times and sonicate in an ice-cold sonicating water bath for 5 min.
14. Transfer the peptides into a nLC vial and store at 4 °C. If possible, analyze the sample on the same day.

Table 1
List of transitions for monitoring Gli2 phosphorylation

Phosphosite	Peptide sequence	Precursor neutral mass	Heavy precursor neutral mass	Precursor charge	Fragment ion	Fragment neutral mass	Heavy fragment neutral mass	Fragment charge
Control	AHTGGTLDDGIR	1211.6	1221.6	2	y9	902.4	912.5	1
					y8	845.4	855.4	1
					y5	574.3	584.3	1
Control	YAAATGGPPPTPLPGLDR	1749.9	1759.9	2	y11	1158.6	1168.6	1
					y10	1061.6	1071.6	1
					y7	766.4	776.4	1
P1	RDSS[+80]TSTM[+16]SSAYTVSR	1830.7	1840.8	3	y7	782.4	792.4	1
					y6	695.4	705.4	1
					y5	624.3	634.3	1
P2	RSS[+80]GISPYFSSR	1422.6	1432.6	2	y9	1012.5	1022.5	1
					y7	842.4	852.4	1
					y6	755.4	765.4	1
P5	RGS[+80] DGPTYSHGHGHGYAGAAPAFPHEGPNSSTR	3411.5	3421.5	5	y8	846.4	856.4	1
					y7	717.3	727.3	1
					b4–98	397.2	397.2	1
P6	RAS[+80]DPVRRPDPLILPR	1937.0	1947.0	3	y8	919.5	929.6	1
					y6	707.5	717.5	1
					b4–98	411.2	411.2	1
					b6–98	607.3	607.3	1

(continued)

Table 1
(continued)

Phosphosite	Peptide sequence	Precursor neutral mass	Heavy precursor neutral mass	Precursor charge	Fragment ion	Fragment neutral mass	Heavy fragment neutral mass	Fragment charge
dephospho-P6	ASDPVR	643,3	653,3	2	y5	572.3	582.3	1
					y4	485.3	495.3	1
					y3	370.2	380.2	1
Pg	TS[+80]PNSLVAYINNSR	1614.7	1624.7	2	y8	935.5	945.5	1
					y7	836.4	846.4	1
					y6	765.4	775.4	1
dephospho-Pg	TSPNSLVAYINNSR	1534.8	1544.8	2	y8	935.5	945.5	1
					y7	836.4	846.4	1
					y6	765.4	775.4	1

3.8 Selected Reaction Monitoring (SRM) Mass Spectrometry

1. Set up the following program for the separation/quantitation of the peptides:
   (a) Separation: linear gradient from 5 % to 45 % ACN over 70 min with a 300 nl/min flow rate for separation and a 4 μl/min flow rate for sample loading.
   (b) Positive polarity (tune file).
   (c) Resolution for Q1 and Q3: 0.7u FWHM (method file).
   (d) Emitter voltage: 1200–1,500 V (tune file).
   (e) Temperature of the transfer capillary: 270 °C (tune file).
   (f) Scheduled SRM: maximum window 5 min, cycle time 1 s, average dwell time 26 ms (*see* **Note 17**). Set the transitions as indicated in Table 1 (*see* **Note 18**).
2. Inject 3–5 μL of the sample and start the run. Repeat the run twice for each sample to account for run-to-run variability. Example extracted ion currents for one of our runs are shown in Fig. 3.
3. To verify proper operating performance and to determine if the LC/MS system needs cleaning or calibration, perform SRM on a standard peptide mixture (6 Bovine protein digest equal molar mix, Michrom, USA) at regular intervals between batches of samples.

3.9 Data Analysis

1. Import raw data into Skyline.
2. Export peak data from Skyline to a tab-separated text file (*see* **Note 19**). Export the following parameters: PeptideSequence, ProteinName, ReplicateName, PeptideRetentionTime, and RatioToStandard. Only export the transition with the highest peak for each sample and peptide.
3. Take the RatioToStandard as the "uncorrected abundance" of a specific peptide in the sample.
4. To correct for unequal loading and extraction of the sample from the gel, divide the uncorrected abundance of the non-phosphorylatable "control" peptides (first two entries in Table 1) by the average uncorrected abundance of the same peptide in the technical replicates of the untreated sample. Average of these values over the two control peptides will be denoted as the "loading ratio" of the sample. The loading ratio of the untreated sample is, by definition, 1.
5. Divide each uncorrected abundance value of the phosphorylated peptide of interest by the loading ratio for the respective sample to obtain the "corrected abundance value."
6. Divide the corrected abundance values by the average corrected abundance value of the untreated sample technical replicates to obtain fold increase of the specific phosphorylated or

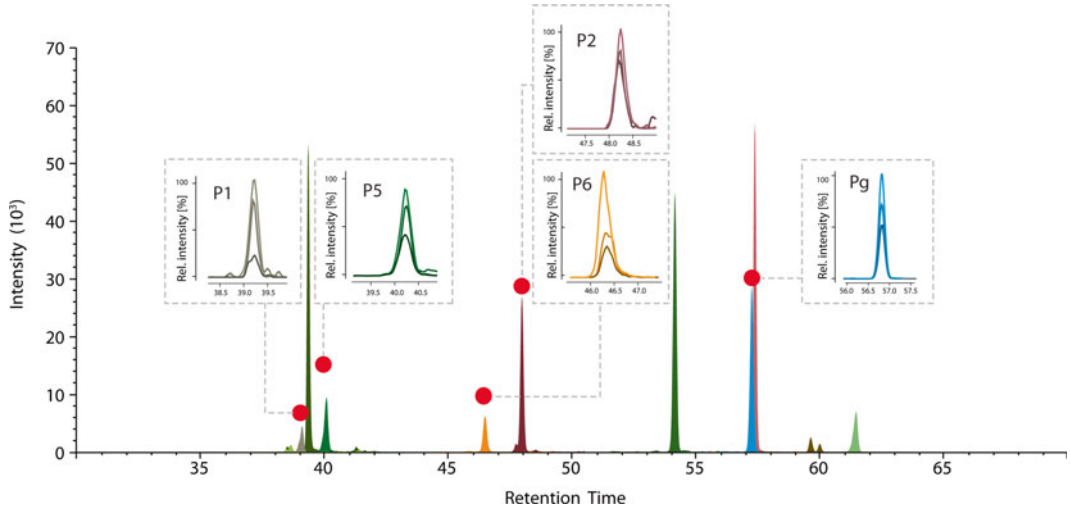

Fig. 3 Selected reaction monitoring (SRM) extracted ion currents (XICs) of Gli peptides. Chromatogram shows all monitored Gli phosphopeptides and Gli control peptides of approximately 250 ng Gli2 digest separated by reverse-phase nano-HPLC and analyzed by a triple quadrupole mass spectrometer. Each peptide XIC is displayed in a different color. The insets show XICs for individual transitions of the phosphopeptides used in this study

unphosphorylated peptide abundance over the untreated sample. The fold increase values should be reproducible between technical replicates and from experiment to experiment and can be reported. Example calculations are shown in Table 2.

4 Notes

1. The urea in the lysis buffer may precipitate in the cold. If it does, take the solution out to room temperature for a few minutes and resuspend the precipitated urea by vigorous mixing.
2. The anti-Gli2 antibody we used for immunoprecipitation of Gli2 was custom-made in rabbits from an antigen based on Cho et al. [17]. The antigen was a His-tagged fragment containing amino acids 1053–1264 of mouse Gli2 (VQYIKAH…AKPSHLG), and the sera were affinity purified on amino-link beads coupled to the same antigen.
3. We do not recommend pouring gels by hand, since it is very difficult to protect the gel from contamination with the ubiquitous keratins. There is no reason to use one brand over another, except the wells of the gel must be spacious enough to hold 50 µL of the sample.
4. If the peptides are designed for a new protein or a new phosphosite, they should only be ordered after an initial MS optimization phase to ensure identical sequences between the tryptic peptides from the sample and the synthetic standard peptides (*see* also **Note 17**).

Table 2
Example calculations of fold increase in the abundance of phosphopeptide P6 (RAS[+80]DPVRRPDPLILPR)

Site	Sample	Technical replicate	RatioToStandard = uncorrected abundance	Divided by control	Loading ratio	Corrected abundance	Fold increase
AHTGGTLDDGIR	Untreated	1	0.9035	1.006	1.000		
AHTGGTLDDGIR	SAG	1	0.8941	0.996	0.981		
AHTGGTLDDGIR	FSK	1	1.1101	1.236	1.277		
AHTGGTLDDGIR	Untreated	2	0.8923	0.994			
AHTGGTLDDGIR	SAG	2	0.8335	0.928			
AHTGGTLDDGIR	FSK	2	1.1501	1.281			
YAAATGGPPPTPLPGLDR	Untreated	1	0.4964	0.995			
YAAATGGPPPTPLPGLDR	SAG	1	0.4965	0.995			
YAAATGGPPPTPLPGLDR	FSK	1	0.6482	1.299			
YAAATGGPPPTPLPGLDR	Untreated	2	0.5015	1.005			
YAAATGGPPPTPLPGLDR	SAG	2	0.5016	1.005			
YAAATGGPPPTPLPGLDR	FSK	2	0.6436	1.290			
RAS[+80]DPVRRPDPLILPR	untreated	1	1.298			1.298	1.003
RAS[+80]DPVRRPDPLILPR	SAG	1	0.6155			0.627	0.485
RAS[+80]DPVRRPDPLILPR	FSK	1	3.5887			2.811	2.173
RAS[+80]DPVRRPDPLILPR	Untreated	2	1.2892			1.289	0.997
RAS[+80]DPVRRPDPLILPR	SAG	2	0.6886			0.702	0.543
RAS[+80]DPVRRPDPLILPR	FSK	2	3.8788			3.038	2.349

Non-phosphorylatable peptides AHTGGTLDDGIR and YAAATGGPPPTPLPGLDR are used for loading ratio calculations

5. Do not allow for the cells to reach full confluence until right before the experiment. It is best to split the cells every 2 days to keep them between 10 and 70 % confluent.
6. Full confluence is important for cell responsiveness to the signal and will also determine the quantity of Gli2 that can be isolated, which is decisive for the sensitivity of the method. For preliminary experiments, it may be worthwhile to increase the number of plates to 8–12 per condition.
7. Longer serum starvation seems to increase responsiveness, but will also make the cells more fragile. Starving for more than 40 h dramatically decreases cell viability.
8. For maximal dephosphorylation of sites P1–P6 and maximal phosphorylation of site Pg, 4 h treatment with 100 nM Smoothened agonist (SAG) is sufficient.
9. It is difficult to lyse more than 6 dishes at the same time, so treatment times must be spaced appropriately to allow time between batches of lysis.
10. Settings for the sonication must be adjusted for each sonicator individually. Avoid overheating the solution—use pulsed sonication in the cold room. The solution after the sonication should be easy to pipet with a standard 1 mL pipette tip.
11. Bead/antibody quantities should be adjusted based on the affinity of the antibody used. A pilot experiment with different bead to lysate ratios can be used for this purpose.
12. The elution from the antibody we used seems to be optimal at 37 °C, but with different antibodies, there may be some variability in the optimal elution conditions.
13. Gel pieces can be frozen at this point—add 50 μL of AmBic solution and place at −80 °C.
14. Dry gel pieces tend to be statically charged and may fly off the tubes through attraction to other statically charged objects, such as gloves.
15. We have found that peptide cleanup prior to running the reaction dramatically improved sensitivity. However, the cleanup may result in some losses of the material, so it is recommended to try different C18 materials for the cleanup in the optimization phase (Oasis HLB μElution 96-well plates worked best in our hands). One plate can be used for multiple experiments, but individual columns (wells) can be used only once. Mark columns that had been used to avoid contaminating your samples. Condition and equilibrate only the columns that will be used for the experiment in progress.
16. Drying the peptides may take a long time (up to 6 h), depending on how deep the vacuum is. If possible, use a high-performance solvent-resistant vacuum centrifuge connected to a vacuum pump of sufficient power.

17. Scheduled SRM must first be optimized in an unscheduled SRM run using a standard peptide mixture. Retention times for specific transitions may vary slightly from run to run depending on the composition of the sample and the performance of the instrument. Using scheduled rather than unscheduled SRM for the actual sample runs can dramatically improve sensitivity, since fewer transitions are monitored at the same time.
18. Selection of the right transitions for the phosphosites of interest takes significant up-front effort. For a new protein or a new phosphorylation site, it is recommended to initially run the sample on a tandem MS instrument to obtain an unbiased view of the peptide coverage of the protein, the tryptic digest pattern, and fragmentation spectra of specific peptides. Trypsin cleavage can be affected by phosphorylation, so the most abundant precursor peptides may have different lengths for the unphosphorylated and the phosphorylated peptide (see the P6 peptide of Table 1). For sites where phosphorylation stoichiometry is predicted to be significant (>20 %), it is sometimes advisable to monitor both the phosphorylated and the unphosphorylated peptide, so that absolute phosphorylation stoichiometry can be calculated. Even though fragmentation patterns may differ slightly between the tandem MS instrument and the triple quadrupole instrument, data obtained from a shotgun tandem MS experiment are a fairly good starting point for the setup of SRM transitions. Initially, at the optimization stage using a triple quadrupole instrument, it is recommended to try 5–6 transitions for each peptide, including transitions resulting from neutral loss of the phosphate moiety (−98 Da). Later, 2–3 most intensive transitions per peptide are selected for the actual experiments in order to maximize the dwell time and increase sensitivity. Since the standard heavy-labeled peptides are fairly expensive, they should only be ordered once the best precursor peptides and transitions have been selected based on the sample of interest. In the second stage of optimization, the heavy standard peptide extracted ion currents (peak height vs. retention time) are compared to those of the putative light peptide in the sample. If the retention time or fragmentation pattern differs between the heavy and the light peptide, chances are that the peptide detected in the sample is a false positive. In such case, the design of the peptide transitions should be started from scratch.

    It is also important to consider additional phosphorylation sites within the peptide of interest. It is possible that the changes in phosphorylation that one observes can be attributed to one of those additional sites, rather than the one we are interested in. If the transition peak retention time and relative intensities are identical between the standard heavy and the

light sample peptide, we can be fairly confident that the peptide we are monitoring is the same as the known standard peptide.

Oxidation status of methionine is another confounding factor for some peptides (see peptide P1 in Table 1). We were able to detect both the unoxidized and the oxidized form of P1, but the oxidized form was more abundant under standard handling conditions. We found that in vitro reduction of oxidated methionine resulted in some sensitivity loss, and therefore we used the oxidated peptide as our SRM precursor.

Finally, it must be mentioned that not all phosphorylation sites are amenable to SRM quantification. Some tryptic peptides are either too short or too long or have physicochemical properties that make them either elute poorly from the LC column or ionize/fragment poorly in the MS instrument. For those reasons, we were unable to quantify phosphorylation at a few sites that we were interested in (P3, P4).

19. Automatically assigned peak widths must sometimes be adjusted manually prior to export. Inspect automatic peaks for inaccuracies and artifacts. Peak boundaries must be the same for the sample peptide and the corresponding heavy standard peptide, but may differ slightly between runs.

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