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## **Multiple Site-specific Phosphorylation of IDPs Monitored by NMR**

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**Running head:** IDP phosphorylation monitored by NMR.

## **Abstract**

In line with their high accessibility, disordered proteins are exquisite targets of kinases. Eukaryotic organisms use the so-called intrinsically disordered proteins (IDPs) or intrinsically disordered regions of proteins (IDRs) as molecular switches carrying intracellular information tuned by reversible phosphorylation schemes. Solvent exposed serines and threonines are abundant in IDPs, and, consistently, kinases often modify disordered regions of proteins at multiple sites. In this context, nuclear magnetic resonance (NMR) spectroscopy provides quantitative, residue-specific information that permits mapping of phosphosites and monitoring of their individual kinetics. Hence, NMR monitoring emerges as an *in vitro* approach, complementary to mass-spectrometry or immuno-blotting, to characterize IDP phosphorylation comprehensively. Here, we describe in detail generic protocols for carrying out NMR monitoring of IDP phosphorylation, and we provide a number of practical insights that improve handiness and reproducibility of this method.

## **Key words**

NMR spectroscopy, Intrinsically Disordered Proteins, IDP, post-translational modifications, phosphorylation, cell signaling, kinases, multiple phosphosites, quantitative NMR, time-resolved NMR.

## 1. Introduction

Living organisms use post-translational modifications (PTMs) as a convenient mechanism to transmit information in a fast and reversible fashion. PTMs can provoke changes in protein conformation or create/abolish binding sites, therefore regulating the activity of proteins. In eukaryotes, serine/threonine phosphorylation represents about 60% of the detected PTMs [1, 2]. Intrinsically disordered regions (IDRs) of proteins carry ~60% of the ~200,000 phosphosites found in the 13,000 human phosphoproteins [3, 4], in line with their importance in cell signaling [5]. Interestingly, phosphosites tend to cluster : about half of them are at a distance of four residues or less [6], while more than 50% of phosphoproteins have six phosphosites or more [7]. This generates potential crosstalks, collective effects and improved robustness of signaling [5, 8-10]. A large number of detected phosphorylation sites may also reveal loose specificities that result from the promiscuous access of kinases to IDP residues [11]: a majority of the detected phosphosites in cultured cells show a fractional occupancy of 25% or less, the functional role of such events being unclear [7, 12].

The most popular methods to detect phosphorylation are mass-spectrometry and Western-blotting, because of their ability to detect phosphosites in biological material with high sensitivity. However, multiple neighboring phosphorylation sites hamper immuno-recognition and prevent standard peptide isolation/enrichment/ionization/identification steps in the course of MS analysis, although technical advances have been achieved in this field [13]. Degenerate motifs are also problematic in western-blotting because they hinder site-specific immuno-recognition. Finally, improving phosphoproteome coverage is a perpetual battle: even though novel buffers, enrichment methods, chromatography, and digestion protocols progressively enhance sampling depth [14, 15], accessible data from immunoblots and MS can probably not provide a comprehensive view of the PTM schemes established on your protein of interest.

NMR spectroscopy is a complementary method, because of its intrinsic ability to provide quantitative, residue-specific information on IDPs in solution [16]. Using the “NMR isotope-filter”, <sup>15</sup>N-labeled proteins are the only observable species in the presence of natural abundance <sup>14</sup>N-containing material, which makes it possible to monitor phosphorylation reactions of a <sup>15</sup>N-labeled

IDP *in vitro* and in cell extracts [17, 18]. For example, it has been instrumental to solve complex phosphorylation cascades on PTEN [19], or to elucidate the respective kinetics and roles of degenerated Erk phosphosites on Elk1 [20].

NMR spectroscopy provides residue-specific information because nuclei resonance frequencies depend on their chemical environment. Upon phosphorylation, the chemical environment changes in the vicinity of the phosphorylated residues, provoking new chemical shifts for residues neighboring the phosphosites. We illustrate it here with the example of a BRCA2 fragment (aa48-218), phosphorylated at S193 and T207, which generates large chemical shift perturbations for S193 and T207, but also weak but discernable NMR signals displacements of their neighboring residues D189, S195, L198, A199, T203 and V211 (**Fig. 1a**) [16, 21]. Hence, by recording time series of NMR spectra during the phosphorylation reactions, we can obtain time-resolved quantification or reaction advancement at every phosphosite: new crosspeaks appear while some peaks progressively disappear, corresponding to phosphorylated and non-modified populations, respectively (**Fig. 1b**). More complex situations can emerge when close residues are concomitantly phosphorylated: two neighboring sites S1 and S2 (separated by ~4-6 amino acids or less) will generate a set of four peaks upon phosphorylation, corresponding to S1-S2, pS1-S2, S1-pS2 and pS1-pS2, where pS1-S2 and S1-pS2 peaks may increase and later decrease (see for example [19, 22, 23]).

In the past years, we have successfully monitored phosphorylation reactions using NMR on a large variety of IDPs and kinases. Here, we provide a generic protocol integrating a sum of technical solutions focusing on sample preparation, control of the reaction conditions and instrument parameters for generating reliable data. We also provide some explanations for the rationale and warnings to avoid misinterpretation (see **Note 1**). We illustrate the protocol with results obtained on a BRCA2 fragment (aa48-218) phosphorylated by Plk1.

[Fig 1 near here]

## 2. Materials

## 2.1 Stock Solutions Preparation and Storage

Prepare all solutions using milli-Q water (resistivity of 18.2 M $\Omega$ \*cm at 25 °C). Buffers, reagents stocks and proteins can be kept at -20°C for months, but fresh dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) should be added to the samples before performing experiments. DTT half-life in solution is indeed about one day at pH~7 and even decreases in presence of metal ions [24] so that buffers containing DTT should not be stored. Other reducing agents may be used with profit but also with careful attention (see **Note 2**).

We describe standard buffers using Hepes at 50 mM, pH 7.0. Any good buffering molecule in this range of pH would also be acceptable, e.g. phosphate, PIPES, MOPS, whose pKas also have low temperature coefficients (see **Note 3**).

1. Standard IDP Size-Exclusion Chromatography (SEC) buffer (SEC-B): 50 mM Hepes, 100 mM NaCl, pH 7.0.
2. Standard IDP frozen storage buffer (Stor-B): 50 mM Hepes, 100 mM NaCl, pH 7.0, 2 mM DTT, 1x ethylenediaminetetraacetic acid (EDTA)-free protease inhibitors.
3. Standard phosphorylation buffer (Phos-B): 50 mM Hepes, 100 mM NaCl, pH 7.0, 4 mM DTT, 1x EDTA-free protease inhibitors, 4 mM adenosine triphosphate (ATP), 10 mM MgCl<sub>2</sub>.
4. Urea buffer (UB): 50 mM Hepes, 100 mM NaCl, pH 6.5, 4 mM DTT, 10 M urea. High urea concentration buffers tend to progressively diverge towards higher pH when stored at room temperature for days.
5. ATP stock solution: 0.1 M ATP, pH 7.0 in water (10 mM Hepes can be added for convenient pH adjustment); store aliquots at -20 °C.
6. DTT stock solution: 1 M DTT in water; store aliquots at -20 °C.
7. MgCl<sub>2</sub> stock solution: 1 M MgCl<sub>2</sub>, pH 7.0 (10 mM Hepes can be added for convenient pH adjustment); store aliquots at -20 °C.
8. EDTA-free proteases inhibitors (PI): prepare a 25x stock solution (cOmplete EDTA-free tablets, or any other cocktail of inhibitors that does not contain EDTA). Store aliquots at -20 °C.

9.  $^{15}\text{N}$ -labeled IDP stock solution at a concentration greater than or equal to 200-250  $\mu\text{M}$  is stored at  $-80\text{ }^{\circ}\text{C}$ . Aliquoting prevents eventual aggregation that often appears upon multiple freeze-thaw cycles.
10. Kinase stock solution: active kinase is obtained either commercially or produced in-house. Prepare low volumes aliquots on ice (2 to 10  $\mu\text{L}$ , providing a phosphate transfer activity in the range of 50 to 500 pmol/min) and flash-freeze them before storage at  $-80\text{ }^{\circ}\text{C}$ . Aliquoting preserves kinase activity which may be lost upon freeze-thaw cycles, and will thus favor experimental reproducibility. Addition of 20 % glycerol promotes activity conservation.
11. EDTA or trans-1,2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid monohydrate (CyDTA) stock solution: 0.5 M EDTA or CyDTA solution, pH 7.0; store aliquots at  $-20\text{ }^{\circ}\text{C}$ .
12.  $\text{D}_2\text{O}$  at 99% deuterium enrichment.
13. 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) stock solution: 100 mM DSS in water; store at room temperature (RT).
14. Laemmli buffer 4x: 10 mL Tris (1 M, pH 6.8), 4 g sodium dodecyl sulfate (SDS), 20 mL glycerol, 10 mL  $\beta$ -mercaptoethanol, 0.1 g bromophenol blue, 6 mL  $\text{H}_2\text{O}$ .
15. Polyacrylamide gels for SDS-PAGE analysis.

## **2.2 Sample Preparation**

1. SEC column: preparative 16/600 Superdex column containing 75pg or 200pg resin depending on protein size.
2. Fast protein liquid chromatography (FPLC) system at  $4\text{ }^{\circ}\text{C}$  able to carry out SEC, i.e. applying constant flow rates of 0.3-1.5 mL/min at a pressure of 0.15 to 0.5 MPa.
3. 0.22  $\mu\text{m}$  pore-size membrane filter system.
4. Centrifugal concentrators with a pore size adapted to the IDP molecular weight.
5. Precise pH-meter combined with a pH microelectrode able to measure pH in  $\sim 100\text{ }\mu\text{L}$  samples.

## **2.3 NMR Spectra Acquisition and Analysis**

1. High-field NMR spectrometer (larger or equal to 600 MHz) equipped with a triple-resonance cryoprobe.
2. A  $^1\text{H}$ - $^{15}\text{N}$  SOFAST-HMQC pulse sequence program. This can be found in most of the standard pulse sequence libraries. Be careful with the default pulses used in libraries: the central band-selective  $180^\circ$  pulse should be a REBURP and not a r-SNOB to get maximal S/N, as shown by Schanda et al. [25]. Set interscan delays to  $\sim 100$  ms and use a high number of dummy scans ( $>64$ ). Set offset frequencies of shaped pulses to 8.5 ppm and use large pulse lengths ( $>2.4$  ms) to avoid saturation of water protons magnetization. Because of fast amide-water proton exchange in IDPs, water protons represent a magnetization reservoir enhancing the apparent amide interscan  $T_1$ -recovery [26]. For this reason, great S/N enhancement is obtained for IDPs using long shaped pulses at fields higher than 600-700 MHz, since amide and water proton frequencies become distant enough.
3. A  $^1\text{H}$ - $^{15}\text{N}$  HSQC pulse sequence program. Sensitivity enhanced versions of this pulse sequence provide better S/N for most IDPs. This can be found in most of the standard pulse sequence libraries.
4. NMR tubes. These can be 3 or 5 mm diameter regular tubes, which must be filled with minimal volumes of 150 or 475  $\mu\text{L}$ , respectively. In standard phosphorylation buffer conditions, 5 mm tubes offer about twice S/N than 3 mm tubes. Using 3 or 5 mm diameter Shigemi tubes optimizes sample efficiency by reducing sample volumes to 90 or 270  $\mu\text{L}$ , respectively. 4 mm Shigemi tubes (efficient volume: 180  $\mu\text{L}$ ) also exist.
5. NMR data processing and analysis softwares (e.g. Topspin, NMRPipe, CcpNmr Analysis, Sparky, ...).
6. Plotting and fitting software (e.g. Prism, Kaleidagraph, Gnuplot, ..).

### 3. Methods

The following protocol describes a generic strategy to monitor IDP phosphorylation using NMR spectroscopy. This requires the prior production and purification of the protein of interest



uniformly  $^{15}\text{N}$  or  $^{15}\text{N}/^{13}\text{C}$  labeled. Such labeling is usually obtained by recombinant protein expression in bacteria (see **Note 4**). Bacterial expression is preferred for this protocol because it generates recombinant, non-phosphorylated proteins in most cases (see **Note 5**). We will not detail the corresponding tasks, but we must stress a few points that should be considered with great care for later success in phosphorylation characterization. The IDP of interest can be purified using standard strategies adapted to the presence or absence of affinity tags. First, to avoid any artifactual influence of such tags, their cleavage and removal is generally preferred before executing PTMs kinetics experiments. Second, because of their high accessibility, IDP's cysteines are prone to establish non-native disulfide bonds *in vitro*. The complete reduction of cysteine thiols at every step of the purification is mandatory (see **Note 2**). Third, IDPs susceptibility to proteolysis must be addressed by the addition of protease inhibitors in lysis buffer and after every purification step.

The protocol starts with the last preparation steps of the IDP sample, then describes a method to optimize the phosphorylation conditions, and later a method to assign the NMR spectra in the phosphorylation conditions. The two final subsections detail the NMR data acquisition and analysis. We have introduced two possible methods to record NMR spectra, either by a continuous readout of a single sample during the phosphorylation reaction (3.4.1 NMR continuous readout), or by successive measurements of aliquots quenched at various time points (3.4.2 The “Quenched reaction” approach). The first approach is more convenient in many aspects, but it provides too weak S/N for a number of IDPs (see **Note 6**). All other paragraphs are common to all IDPs.

### 3.1. Preparation of the IDP Stock

We start the protocol with the last preparation step of the IDP, which consists of a SEC. This permits i) to polish the protein purification and ii) to transfer the IDP into the exact salt and buffer conditions used for the phosphorylation reaction.

1. Equilibrate the SEC column with two column-volumes of SEC-B, previously filtered on a 0.22  $\mu\text{m}$  pore-size membrane. Using an FPLC system at 4°C will lower the risks of IDP proteolysis.
2. Concentrate the purified IDP to a final volume of 2 mL in the presence of 2 mM DTT and PIs (final concentration 1x), using concentrators with centrifugation steps at 10 °C (4,000 x g for swinging buckets, 5,000 x g for fixed angle rotors).
3. Add DTT to a final concentration of 10 mM DTT to the concentrated sample and incubate it for 30 min at RT to ensure that all thiols are reduced.
4. Centrifuge the sample for 5 min at 16,000 x g and 4 °C to remove aggregates and inject the supernatant on the equilibrated SEC column.
5. Run the SEC with parameters recommended by manufacturers and check the purity of the eluted fractions by SDS-PAGE.
6. Pool the fractions of interest, supplement them with 2 mM DTT (final concentration) and PIs (final concentration 1x), and concentrate the protein to 250  $\mu\text{M}$  by centrifugation at 10 °C (4,000 x g for swinging buckets, 5,000 x g for fixed angle rotors).
7. Flash freeze the sample in liquid nitrogen and store it at -80 °C.

### 3.2. Optimization of Phosphorylation Conditions

For every IDP:kinase pair, phosphorylation kinetics can be first evaluated by SDS-PAGE in order to identify the appropriate concentration of kinase and, eventually, the best buffer conditions. This relies on the fact that phosphorylated amino acids tend to slow down protein migration during SDS-PAGE (**Fig. 2**). SDS-binding to phosphoproteins is thought to be weaker, which provokes a decreased electrophoretic mobility [27]. However, this effect can be negligible for proteins carrying large excess (~15 or more) of negative charges due to a biased composition between Glu/Asp and Arg/Lys; this situation calls for directly moving on to NMR monitoring. Here, we describe the procedure for the SDS-PAGE evaluation of phosphorylation efficiency.

1. Thaw the IDP sample, and prepare 3 samples of 25  $\mu\text{L}$  containing the IDP at 25  $\mu\text{M}$  in Phos-B.
2. Calculate the amount of kinase to add. As a first estimation, this quantity should correspond to a theoretical capacity to phosphorylate all predicted phosphosites in 1 hour, with no consideration for the  $K_m$ .

Commercial kinases activity has normally been evaluated, and this information is often presented in  $\text{nmol} \cdot \text{min}^{-1}$  of phosphate transfer per mg of kinase (together with the kinase concentration). The quantity of kinase to use is thus obtained using the equation:

$$M_{\text{kinase}} = N_{\text{sites-predicted}} * C_{\text{IDP}} * V_{\text{reaction}} / (Kin_{\text{activity}} * t_{\text{reaction}})$$

With  $N_{\text{sites-predicted}}$  the number of phosphorylation sites predicted from the known kinase consensus site (see **Note 7**),  $C_{\text{IDP}}$  is the IDP concentration in molar,  $V_{\text{reaction}}$  the reaction volume in liters,  $Kin_{\text{activity}}$  the activity of the kinase in  $\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ , and  $t_{\text{reaction}}$  the incubation time in minutes.  $M_{\text{kinase}}$  is thus in mg.

Hence, our rough evaluation gives  $M_{\text{kinase}} \sim N_{\text{sites-predicted}} * 10^{-11} / Kin_{\text{activity}}$ , which often translates into volumes lower than 1  $\mu\text{L}$  of kinase stock solution. Prepare a diluted kinase stock to make it convenient to pipet.

For in-house produced kinase, whose activity has not yet been characterized: i) if the kinase is activated (phosphorylated or in complex with activators), use  $M_{\text{kinase}} \sim 10$  ng (kinase:IDP molar ratio of 1:2500); ii) if the kinase is not activated, use  $M_{\text{kinase}} \sim 100$  ng (kinase:IDP molar ratio of 1:250).

For phosphorylation of IDP by cell extract, see **Note 8**.

3. Spike  $V_{\text{kinase}} = M_{\text{kinase}} * [\text{kinase}]$ ,  $3 * V_{\text{kinase}}$  and  $9 * V_{\text{kinase}}$  in the three pre-phospho mixes prepared in step 1. Incubate at 298 K.
4. Take 4  $\mu\text{L}$  of the samples at 0 min, 10 min, 30 min, 1 h and 2 h, mix them to 4  $\mu\text{L}$  of 2x Laemmli buffer and heat them immediately for 5 min at 95  $^{\circ}\text{C}$ .
5. Load the samples on a polyacrylamide gel.

6. Run the SDS-PAGE, stain with Coomassie Blue or any other better suited staining method. Pick the kinase concentration that will permit to distinguish phosphorylation events using the NMR time resolution, i.e. 15-30 min per time point over 3 to 5 hours (most kinases lose their activity after a few hours at 25 °C).
7. Buffer conditions can also be evaluated. To this purpose, prepare stock solutions of the various conditions to test. One may test a range of pH values (pH 7-7.5-8) and of salt concentrations (50-100-150 mM NaCl). Repeat steps 1-6. If the reaction is much faster at high pH, adopting the “quenched reaction approach” may be the method of choice to monitor phosphorylation by NMR. However, if the activity of your kinase is acceptable at pH 7.0, we recommend running the phosphorylation reaction at this pH. For most kinases, pH 7 corresponds to an acceptable compromise between kinase activity and decent quality of IDPs' NMR spectra.  
[Fig 2 near here]

### 3.3. Assignment of NMR Spectra in the Conditions used for Phosphorylation Reactions

To extract residue-specific information, assignment of the IDP's NMR spectra is a prerequisite. While phosphorylation reactions will typically be executed at 298 K and pH~7, assignment is usually performed at conditions favorable for NMR analysis of IDPs, i.e. low temperature (~283 K) and pH (~6.5), which decrease water/amide proton exchange and consequently maximize S/N. We will not detail this primary assignment strategy relying on the use of 3-dimensional spectra establishing connections between spins of backbone nuclei (the most useful ones in the case of IDPs being HNCO, HN(CA)CO, HNCACB and (H)N(CA)NH). We describe below the next step that aims at inferring the  $^1\text{H}$ - $^{15}\text{N}$  crosspeaks assignment in the conditions used for phosphorylation. It relies on a temperature and pH gradient from the assignment conditions to the phosphorylation conditions, using DSS as an internal reference. 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC or SOFAST-HMQC pulse sequences can be used. SOFAST-HMQC will be better suited at higher pH and temperature because of its higher S/N efficiency.

1. Record a 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of the IDP at ~100  $\mu\text{M}$  in the pH ( $\text{pH}_{\text{assign}}$ ) and temperature ( $T_{\text{assign}}$ ) conditions used for prior assignment.

2. Record 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC or SOFAST-HMQC spectra at the intermediate temperature  $(298\text{K}-T_{\text{assign}})/2$  and at  $298\text{ K}$ .
3. DSS-reference your spectra in both  $^1\text{H}$  and  $^{15}\text{N}$  dimensions (or apply a 10.5 ppb/K shift if you center the water signal at 4.7 ppm). Then, apply a temperature correction of -7.5 ppb/K in the proton dimension and -6.5 ppb/K in the nitrogen dimension –do not forget that  $\gamma(^{15}\text{N})$  is negative. This will account for the average temperature shifts of random coil amino acids [28], and make peaks tracking easier.
4. Assign crosspeaks at  $298\text{ K}$  by tracing lines through every set of 3 resonances across the temperature range (see **Fig. 3**).
5. Apply the same logic for shifting pH progressively from  $\text{pH}_{\text{assign}}$  to  $\text{pH}_{\text{phosphorylation}}$ . Record 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC or SOFAST-HMQC spectra at 3 pH values, DSS-reference your spectra (or apply a 20 ppb/pH unit correction if you center the water peak at 4.7 ppm). In the range of the pH values considered here, i.e. pH~6.5-7.5, crosspeak frequencies should not shift significantly except histidine residues and their close neighbors.

[Fig. 3 near here]

### 3.4. Phosphorylation Monitoring by NMR

A number of IDPs provide poor S/N at pH 7 or higher, due to their high content in hydrophilic amino acids that show fast water:amide proton exchange (Gly, His, Lys, Arg, Ser, Thr).

Continuous readout at  $298\text{ K}$  can thus be greatly hampered by the absence of reliable reporter crosspeaks. In such situations, it may be more adapted to perform the phosphorylation reaction on the bench, to remove aliquots and quench the reaction at regular intervals, and to record NMR spectra of these aliquots later. This “quenched reactions” strategy has an advantage: low pH and large NMR acquisition times can be used to enhance S/N and resolution. However, the limited number of time points can hinder the robust fitting and interpretation of phosphorylation kinetics. In the following, we describe protocols for the continuous readout NMR approach (3.4.1) and for the discontinuous “quench reaction” approach (3.4.2). To choose between these 2 strategies, you must evaluate the quality of the 2D  $^1\text{H}$ - $^{15}\text{N}$  SOFAST-HMQC spectra recorded at  $\text{pH}_{\text{phosphorylation}}$  and  $298\text{ K}$  (see 3.3). If a number of reporter crosspeaks provide S/N ratios above 10, the

continuous readout is probably feasible. S/N will depend of course on the IDP concentration and the tube diameter/volume.

### **3.4.1 Phosphorylation Monitoring using Continuous NMR Readout**

1. Define the final volume of your sample. This ranges from 90 to 475  $\mu\text{L}$  depending on the NMR tube you want to use (see Materials 2.3.4.). Pipetting losses are difficult to avoid, so start with initial volumes 10-20% higher than the measured ones.
2. Calculate the amount of kinase to add according to 3.2, i.e. the amount leading to measurable levels of phosphorylation within 2 to 5 hours. The reaction should not be too fast because the NMR timeframe is on the order of magnitude of 15-30 min in the described phosphorylation conditions. If you skipped 3.2 and did not test the kinase activity, you may try an IDP concentration of 50  $\mu\text{M}$ , and a kinase:IDP molar ratio of 1:2500 for an activated kinase, or 1:250 for a non-activated kinase.
3. Prepare the pre-phospho mix containing the IDP at 50  $\mu\text{M}$  in Phos-B supplemented with 3%  $\text{D}_2\text{O}$  and 50  $\mu\text{M}$  DSS. Check the pH at room temperature using a micro-pH meter and adjust it to 7.0 if necessary.
4. Transfer the pre-phospho mix into the NMR tube, insert the tube in an appropriate spinner and then into the NMR spectrometer equilibrated at 283 K.
5. Tune and match the  $^1\text{H}$  circuit of the probehead, lock the HOD signal and shim the sample tube. Determine the  $^1\text{H}$  90° pulse.
6. Record 283 K reference 1D  $^1\text{H}$  spectra and 2D  $^1\text{H}$ - $^{15}\text{N}$  2D SOFAST-HMQC spectra using a FID resolution of ~15 Hz in both  $^1\text{H}$  and  $^{15}\text{N}$  dimensions. This step is recommended to check the quality of your sample (pH control, ATP/ADP concentrations, IDP degradation) and serves to measure IDP reference peak intensities. The acquisition time of the 2D spectrum should be between 30 minutes and 2 hours. Ideally, the S/N of peaks of interest should be 20:1 or more for an accurate quantification of later phosphorylation advancement.
7. Equilibrate the spectrometer sample temperature at 298 K and re-perform wobbling, shimming and pulse calibration.

8. Record a 298 K reference 1D  $^1\text{H}$ -spectrum, record two 2D  $^1\text{H}$ - $^{15}\text{N}$  SOFAST-HMQC reference spectra using FID resolutions of 15 Hz in  $^1\text{H}$  dimension and i) 15 Hz and ii) 30 Hz in  $^{15}\text{N}$  dimension. The final acquisition times of the 2D spectra should be about 15 or 30 minutes. Evaluate the quality of these spectra, try different apodization functions, and pick the parameters that provide the best compromise between resolution and S/N. These will be used for time-resolved NMR monitoring of the phosphorylation reaction.
9. Extract the sample from the tube and add the kinase in quantities defined in 3.4.1, step 2. Mix well by pipetting and load the phosphorylation sample into the same NMR tube.
10. Insert the sample in the magnet, wait 1 minute for temperature equilibration, lock and shim. Tuning, matching and pulse calibration should not change after addition of the kinase, unless the kinase stock is very diluted in a very peculiar buffer.
11. Record a 1D  $^1\text{H}$ -spectrum at 298 K and, then, a series of  $^1\text{H}$ - $^{15}\text{N}$  2D SOFAST-HMQC spectra over 4-12 hours, using the parameters defined in 3.4.8.
12. At the end of the series, shim again and record a 1D  $^1\text{H}$  spectrum to evaluate the ADP/ATP ratio (see **Note 9**).
13. Take back the sample from the tube. At this stage, phosphorylation should not progress significantly because of the progressive loss of kinase activity. In the case of a heat resistant IDP, to definitely stop any enzymatic activity, boil the sample at 90 °C for 5 min, centrifuge 5 min at 16,000 xg and recover the supernatant. Avoid boiling IDPs containing Asn-Gly motifs that may suffer from deamidation. You can alternatively spike EDTA or CyDTA at a final concentration of 15 mM to chelate  $\text{Mg}^{2+}$  ions, which will not prevent potential interactions between the IDP and the kinase and their consequences on IDPs NMR peak intensities.
14. Measure pH and adjust it to the initial value if necessary. ATP, DTT and PI hydrolysis usually acidify the solution.
15. Fill the NMR tube with the pH-readjusted sample. Insert it in the magnet, set the sample temperature to 283 K, tune/match, lock and shim. Record 1D  $^1\text{H}$  spectrum and a 2D SOFAST-HMQC spectra at 283 K using the same parameters than in 3.4.6. These spectra constitute the endpoint references.

### 3.4.2 Phosphorylation Monitoring using Quenched Reactions

1. Quenching can be performed using heat inactivation or urea denaturation. Verify that the studied IDP is resistant to 5 minutes heating at 90 °C. Test it on an aliquot at the concentration and in the buffer conditions used for the phosphorylation reaction. After 5 minutes, centrifuge 5 minutes at 16,000 xg, separate the supernatant and the eventual aggregated material and analyze it using SDS-PAGE and NMR. Pay a particular attention to the NMR crosspeaks of Asn and Gln, whose decreased intensities may reveal deamidation occurring upon heating.
2. If your protein is not heat resistant, you can quench the phosphorylation reaction by adding phosphorylation reaction buffer supplemented with 10 M urea buffer to the aliquots. The final urea concentration should be over 4 M. This will require a novel NMR assignment at the final urea concentration, which can be performed using a urea gradient with the same logic than for temperature in 3.3. 2D  $^1\text{H}$ - $^{15}\text{N}$  SOFAST-HMQC spectra of IDPs in urea at pH 7 and 283 K are usually of high quality.
3. Define the experimental time points (i.e. the number of quenched aliquots) you want to have, and the final volume and concentration of the measured samples. The corresponding aliquots can be diluted after being quenched. Such dilution permits a better control of the later pH adjustment.
4. Calculate the amount of kinase to add according to 3.2 and the time frame you choose. If you skipped 3.2 and did not test the kinase activity, you may try an IDP concentration of 50  $\mu\text{M}$ , a total reaction time of 5 hours, and an kinase:IDP molar ratio of 1:2500 for an activated kinase, or 1:250 for a non-activated kinase.
5. Prepare the pre-phospho mix containing the IDP at 50  $\mu\text{M}$  in Phos-B supplemented with 3%  $\text{D}_2\text{O}$  and 50  $\mu\text{M}$  DSS. Check the pH at room temperature using a micro-pH meter and adjust it to  $\text{pH}_{\text{phosphorylation}}$  if necessary.
6. Remove an aliquot and heat it 5 min at 90 °C or dilute it in a urea buffer, according to 3.4.2 step 1. Then, flash-freeze the samples and keep them at -80 °C.
7. Add the kinase in quantities defined in 3.4.2 step 4. Mix well and place it at 298 K in a dry bath.



8. Remove aliquots at the defined time points, and heat them 5 min at 90 °C or dilute them in a urea buffer. Then, flash-freeze the samples and keep them at -80 °C.
9. After thawing the samples, centrifuge the samples 5 minutes at 16,000 xg if you heated them. Take the supernatant and complete with the volume of phosphorylation buffer PhosB to reach the final volume that will be measured by NMR.
10. Adjust the pH to 6.5. Flash freeze the aliquots that you will not measure in the next hours, keep the other ones on ice.
11. Transfer the sample into the NMR tube, insert the tube in an appropriate spinner and then into the NMR spectrometer equilibrated at 283 K.
12. Tune and match the  $^1\text{H}$  circuit of the probehead, lock the HOD signal and shim the sample tube. Determine the  $^1\text{H}$  90° pulse.
13. Record 283 K spectra of every aliquot: a 1D  $^1\text{H}$ -spectrum and a  $^1\text{H}$ - $^{15}\text{N}$  2D SOFAST-HMQC spectrum using a FID resolution of ~15 Hz in both  $^1\text{H}$  and  $^{15}\text{N}$  dimensions. 1D  $^1\text{H}$ -spectra are useful to compare the eventual differences of IDP concentration in the aliquots. They should thus be recorded with a high S/N. They also permit to check the pH and the ATP/ADP concentrations. The acquisition time of the 2D spectrum should be between 30 minutes and 2 hours. Ideally, the S/N of peaks of interest should be 20:1 or more for an accurate quantification of the phosphorylation advancement.

### 3.5. Analysis of NMR Data

Although a novel assignment of the phosphorylated IDP spectra is not always necessary (see **Note 10**), it can be very useful in the case where too many phosphosites generate large number of spectral changes. We will not detail the assignment method relying on the prior phosphorylation of a  $^{13}\text{C}$ - $^{15}\text{N}$  labeled IDP and the use of 3-dimensional pulse sequences to establish sequential connections (see 3.3). A single 3D HNCACB spectrum is often sufficient to assign the phosphorylated population. We will not elaborate extensively on curve fitting: because of the progressive kinase loss of activity, the potential IDP degradation, or the competition between phosphosites, fitting models may often become too complicate to provide consistent fitted values. In most cases, it is safer to only consider the order of phosphorylation events and

their initial rates. Crosspeaks whose intensities vary during the phosphorylation reaction reveal phosphorylation advancement, and will be called “reporters”. In the following, we describe how to monitor and quantify the reaction advancement in a residue specific fashion. We will name “non-phosphopeaks” all crosspeaks found in the reference spectra before phosphorylation, and “phosphopeaks” those appearing in spectra upon phosphorylation.

1. Fourier transform the spectra recorded at 283 K before and after the phosphorylation reaction (see 3.4.1 step 6 and 3.4.1 step 15 or 3.4.2 step13) using Topspin or any equivalent NMR-data processing software. Process these data with an adapted apodization function, and perform baseline corrections if necessary. The 2D SOFAST-HMQC pulse sequence often generates distorted baselines in the  $^{15}\text{N}$ -dimension, which alters absolute peak intensities.
2. Load these 2D 283 K-spectra in an NMR data analysis software (i.e. Ccpnmr or Sparky). Measure intensities of every isolated peak in the reference spectrum ( $I_{ref}$ ) and after the phosphorylation reaction ( $I_{final}$ ).
3. Identify the peaks that faded. Because the corresponding residues are either phosphorylated or close to phosphorylated residues, these peaks permit identification of the residues or the segments where phosphorylation reactions occurred. The intensity ratios  $I_{final}/I_{ref}$  of these peaks reveal the final degrees of advancement of these reactions.
4. If you used the “continuous readout approach”, Fourier transform the spectra recorded at 298 K during the phosphorylation reaction and apply baseline corrections if necessary (see 3.5.1). Load these 298 K-spectra in an NMR data analysis software.
5. Measure intensities of every isolated peak in every spectrum, including novel peaks appearing upon phosphorylation. Pay attention to eventual peak displacements due to pH shifts in the time series (see **Note 11**).
6. Plot the evolution curves of every peak using a plot/fit software (Kaleidagraph, Prism, Gnuplot, ...). If you used the “continuous readout approach”, set the time of the first spectrum to  $(t_{set-up} + t_{acq-spectrum}/2)$ , and the later time points to  $(t_{set-up} + t_{acq-spectrum} * (n-1/2))$ .  $t_{set-up}$  should be about 5 minutes. Decay of connected non-phosphopeaks, either of phosphosites or of neighboring residues, should show similar trends.

7. Pay attention to the fact that a given residue can sense more than one phosphorylation reaction, and the decay of its non-phosphopeak may report on more than one phosphorylation event in its vicinity. Such a situation can be recognized by the appearance of more phosphopeaks than there were non-phosphopeaks.
8. After identifying non-phosphopeaks that report the same phosphorylation reaction, add their individual peak intensities at every timepoint (**Fig. 1c**). This will increase the S/N of the phosphorylation build-up curves. For every phosphorylation site, evaluate the total intensity  $I_{t=0}$  at time=0 according to the initial slopes of the decay curves. Plot the phosphorylation build-up curve  $\%phospho(t) = 1 - I(t)/I_{t=0}$ .
9. You may try to assign the phosphopeaks. The peaks corresponding to phosphorylated residues should shift down-field according to the published reference chemical shifts; the neighboring residues should show weaker peak displacements and remain close to the non-phosphopeaks. In both cases, peak intensities of phospho- and non-phospho-populations evolve in a complementary fashion.
10. If no reliable non-phosphopeak reporter can be isolated in the 298 K-spectra, plot the intensities of the assigned phosphopeaks. Evaluate their intensity  $I_{endpoint}$  at the end of the time series, according to the last 298 K-spectra (**Fig. 1c**). Normalize their build-up curve according to the ratio  $I_{final}/I_{ref}$  of non-phosphopeaks measured at 283 K.
11. You can also add build-up curves from related phosphopeak and non-phosphopeaks to increase S/N. Perform it before intensity normalization and normalize the final build-up curves. Depending on the shape of the build-up curves there are four possible scenarios:
12. If the curve looks like a mono-exponential (**Fig. 4a**), fit it using  $\%phospho(t) = \%phospho_{t \rightarrow \infty} (1 - \exp(-R_{phos} * t))$ ;
13. If the initial IDP concentration is above the  $K_m$  of a phosphosite, the corresponding decay curve may look more linear than exponential (**Fig. 4b**); in this case, fit it using  $\%phospho(t) = \%phospho_{t \rightarrow \infty} (1 - (\ln(1 + (e^K - 1) * \exp(-R_{phos} * t))) / K)$ , with  $K = [IDP]/K_m$ ;
14. If the curve has an S shape, it may reveal a conditional phosphosite P2 that requires a prior phosphorylation of a neighboring site P1 (**Fig. 4c**), which may be fitted using  $\%phospho_{P2}(t) = 1 - [R_{phosP1} * \exp(-R_{phosP2} * t) - R_{phosP2} * \exp(-R_{phosP1} * t)] / [R_{phosP1} - R_{phosP2}]$ . In such situation, a set of two P1

phosphopeaks should appear, corresponding to P1-noP2 and P1-P2. You can fit  $\%phospho_{P1-noP2}(t) = R_{phosP1}/[R_{phosP1}-R_{phosP2}][\exp(-R_{phosP2} * t) - \exp(-R_{phosP1} * t)]$ , and  $\%phospho_{P1P2}(t) = \%phospho_{P2}(t)$ .

15. An S shape can also reveal the late phosphorylation of a poor  $K_m$ , slow phosphosite Pslow after the early phosphorylation of a faster site Pfast (**Fig. 4d**). This situation does not provoke necessarily the apparition of multiple peaks like in 14. Such a build-up curve is fitted

approximately by  $\%phospho_{Pslow}(t) = 1 - [1 - \%phospho_{Pfast}]^{\delta}$ , with

$\delta = (V_{maxPslow} * K_{MPfast}) / (V_{MaxPfast} * K_{MPslow}) < 1$ . Do not pay too much attention to the individual values obtained upon fitting, but consider only the initial rates calculated from fitted parameters (see **Note 12**).

[Figure 4 near here]

## 4. Notes

1. The lowest IDP concentration to obtain sufficient S/N is about 12.5  $\mu$ M in most cases.

Phosphorylation monitoring by NMR is however often performed at much higher concentrations in the literature, sometimes at several hundreds of micromolar. Importantly, at high IDP concentrations, kinases are likely to steadily phosphorylate non-specific sites with very high  $K_m$ . NMR concentrations may thus generate questionable results if this aspect is neglected.  $K_m$ s of kinases for their cognate targets span a wide range of values, from 1 to 100  $\mu$ M, while  $k_{cat}$  values can vary between 0.01 and 100  $s^{-1}$  (we did not find an article providing statistics, these are numbers based on long-term experience, you can try <https://www.brenda-enzymes.org/>). We highly recommend to measure kinetics at a given concentration and at either half or twice this concentration. The measured initial rates (in  $mol.s^{-1}$ ) will reveal whether the used concentrations are below or above the  $K_m$  of every detected phosphosite, and whether some sites emerge only because of high concentrations. There are three possibilities: i) if the molar phosphorylation rate is proportional to the substrate concentration, we are well below the phosphosite  $K_m$ , which may

be odd at concentrations above 100  $\mu\text{M}$  for example; ii) if it is the same at both concentrations, we are an order of magnitude above the  $K_m$ , which may call for running the phosphorylation reaction at lower concentration; iii) intermediate situations mean that we are at a concentration in the order of magnitude of the  $K_m$ . Of course, it is ideally preferable to determine  $k_{cat}$  and  $K_m$  more precisely by measuring initial rates at multiple concentrations following the Michaelis-Menten approach.

**2.** Cysteine reduction can usually be obtained by supplementing the lysis buffer with 10 mM DTT (final concentration), and by spiking 5 mM DTT (final concentration) in protein samples after every purification step. Avoid  $\beta$ -mercaptoethanol: it is a poor reducing agent and it easily forms mixed disulfides with cysteines. TCEP is advantageous in many regards, but its notorious stability is affected by the presence of phosphate and EGTA [24, 31], it requires pH adjustment after solubilization, and it may cleave progressively the protein backbone near cysteines [32].

**3.** IDPs are generally very well expressed in *E. coli* strains optimized for protein production. Bacteria are grown in minimum media containing  $^{15}\text{NH}_4\text{Cl}$  and  $^{13}\text{C}$ -glucose as sole sources of nitrogen and carbon, respectively. Recipes can be found at [https://www.embl.de/pepcore/pepcore\\_services/protein\\_expression/ecoli/n15/](https://www.embl.de/pepcore/pepcore_services/protein_expression/ecoli/n15/) or in [29]. IDPs can sometimes be obtained in inclusion bodies: in such situation, they can be first purified in urea using a Histidine tag, before being transferred in a non-denaturant buffer using SEC.

**4.** High buffering capacity is important because ATP and DTT hydrolysis release protons and can provoke deleterious pH variations. A careful control of the pH is indeed essential to generate NMR data of high quality: crosspeak intensities of IDPs amide functions can vary up to ~10% for a pH shift of 0.1 at pH 7 and 298 K (This is due to the pH-dependency of water/amide proton exchange [37], hence to the residence time of the detected amide protons). Because we calculate phospho and non-phospho populations based on NMR crosspeak intensities, pH variations can thus bias this quantification.

**5.** Recombinant expression in insect or mammalian cells may produce prephosphorylated protein samples. This may be detected using the Pro-Q Diamond Phosphoprotein Gel Stain kit, Phos-Tag SDS-PAGE [30], Mass Spectrometry or Western blot with an antibody directed against phosphoSer/Thr. It can also be detected using 2D  $^1\text{H}$ - $^{15}\text{N}$  NMR spectroscopy, given the recognizable chemical shifts adopted by phosphoSer/Thr residues [16, 21]. Tyrosine phosphorylation can be nailed using  $^1\text{H}$ - $^{13}\text{C}$  NMR spectroscopy because of the  $\text{C}_\epsilon$  chemical shifts perturbation [21]. If prephosphorylation is ascertained, a phosphatase treatment may be carried out during the purification.

**6.** Adding 10% glycerol to the reaction mix often improves S/N and decreases the pH-dependency of NMR crosspeak intensities. It provokes chemical shift perturbations, which may be large enough to force a supplementary assignment step. This can be carried out similarly to what has been described for temperature-dependent assignment in 3.3.

**7.** Kinases achieve substrate specificity in many ways [33]. Several servers exist predicting phosphorylation sites and their associated kinases. They rely mostly on kinase consensus site analysis, reported detection and existing annotation or interaction networks in databases [34, 35]. An extensive list of methods is available in [36]. A simple consensus site analysis can be obtained using <http://www.cbs.dtu.dk/services/NetPhos/>, lists of detected phosphorylation sites is available on <https://www.phosphosite.org/>.

**8.** Monitoring IDP phosphorylation in cell extracts is a challenging task. Which cell line? Which lysis conditions? In exponential growth phase, or after cell-cycle arrest and at which stage? Whole cell extract or separation of specific cell compartment? These parameters will generate very different extracts with highly variable phosphorylation activities. For this reason, we thought it was difficult to provide a generic protocol, and we prefer to refer to guidelines published previously [17]. Producing active extracts requires fast manipulation of the cellular material. Lysis buffers should of course be non-denaturing and contain large concentrations of protease and phosphatase inhibitors. ATP hydrolysis is usually intense because of the combined activity of

several cellular kinases. Extract concentration should be high (1-10 mg.mL<sup>-1</sup>). The experimental timeframe is usually about one hour, although it can be difficult to establish: early phosphorylation can be rapidly compensated by dephosphorylation activity of cellular phosphatases, which often take over anyway after ATP exhaustion. We finally warn the reader that interpretation of phosphorylation kinetics in cell extracts should be cautious: native cellular organization and colocalization is essential in cell signaling, and is lost in cell extracts; the IDP of interest may interact with enzymes that it would not meet in a real cell, and kinase or phosphatase activity regulation may quickly degenerate.

**9.** Kinases affinity for ATP is in the range of 10 to 100  $\mu$ M, and up to the millimolar range in a minority of cases [38]. Affinity for ADP is in the same range and very well correlated to that of ATP [38]. Hence, a large excess of ATP is recommended for monitoring phosphorylation reactions *in vitro* to avoid strong inhibition by growing concentrations of ADP. Moreover, we observed in most cases that kinases hydrolyze more ATP molecules than they transfer phosphate moieties to their substrate. We recommend to measure 1D <sup>1</sup>H spectra before and after the reaction to evaluate the ratio ATP/ADP (**Fig. 5**), which may help in interpreting the progressive loss of kinase activity.

[Figure 5 near here]

**10.** The most prominent chemical shift changes occur for phosphorylated residues. Their crosspeaks show strong downfield-shifts in <sup>1</sup>H-<sup>15</sup>N 2D spectra upon phosphorylation: due to a hydrogen bond between the new side-chain phosphate and the backbone amide function, their <sup>1</sup>H resonances move to 8.5-9.5 ppm, which is out of the regular spectral window (7.5-8.5 ppm) for disordered peptides [16, 21]. However, the corresponding peaks move upfield when pH acidifies, and their absolute intensities are also pH dependent. In surprising cases, crosspeaks in this “phospho-region” of 8.5-9.5 ppm can reveal a H-bond between the side-chain phosphate of a phosphorylated residue and the amide proton of a non-phosphorylated residue [39]. <sup>13</sup>C $\alpha$  and <sup>13</sup>C $\beta$  chemical shifts provide unambiguous information on the nature of these <sup>1</sup>H-<sup>15</sup>N crosspeaks

(phosphoSer/Thr, or neighboring non-modified residue establishing a H-bond with a phosphate). Recording a 3D HNCACB spectrum is thus often of good help in the analysis of the NMR data, which requires a  $^{13}\text{C}/^{15}\text{N}$ -labeled IDP.

**11.** Because phosphoSer and phosphoThr have pKa values of about 6 and 6.5, respectively [21, 40], their protonation state vary with the potential pH evolution during the phosphorylation reaction, as evoked in note 3. This provokes chemical shift perturbations of the phosphorylated residues and of their neighbors. Hence, it is important to verify that intensities are measured at the peak centers in every spectrum.

**12.** Complex mechanisms emerging from multiple phosphorylation sites can take place. Assigning all peaks when multiple neighbor phosphosites evolve concomitantly can be tedious. Fitting evolution curves in such situations, or in the case of large  $K_m$  differences between phosphosites is always possible. Equations that can be used to fit complex phosphorylation schemes have been described extensively earlier [17]. However, the fitted parameters should be used cautiously: little differences in the range of the S/N (and thus inside the error bars) for a couple of early points can provoke large differences in the fitted values; moreover, the progressive loss of kinase activity during the hours-long reaction is very difficult to evaluate. Hence, we argue that one should limit the interpretation of the recorded kinetics to i) the ranking between phosphorylation sites and ii) the initial rates of phosphorylation.

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## Figure Captions

**Fig. 1:** NMR analysis of BRCA2(aa48-218) phosphorylation by Plk1 ([BRCA2]=50  $\mu\text{M}$ , [Plk1]=200 nM, spectra recorded at 700 MHz). **a)** Overlay of  $^1\text{H}$ - $^{15}\text{N}$  2D HSQC spectra recorded at 283 K before (reference, black) and after the phosphorylation reaction ( $t_{\text{final}}$ , red). Arrows highlight NMR chemical shift perturbations upon phosphorylation. The two phosphosites S193 and T207 crosspeaks show large perturbation; their neighbor residues D189, S193, S195 and L198, T203 and V211 also shift and can be used as phosphorylation reporters. Measuring the crosspeak intensities of the non-modified species (called later “non-phosphopeaks”) in the two 283 K spectra provides  $I_{\text{final}}/I_{\text{ref}}$  ratios, which later permit normalization of the phosphorylation build-up curves. **b)** Depiction of three time-points in the time series of 2D  $^1\text{H}$ - $^{15}\text{N}$  SOFAST-HMQC spectra recorded at 298 K during the reaction (grey spectra). We superimposed them on the reference spectrum at  $t=0$  (black spectrum). Close-up views show the evolution of 3 couples of “non-phosphopeaks” and “phosphopeaks”: A199 crosspeaks (orange) report on S193 phosphorylation,

while T207 (magenta) and V211 (blue) crosspeaks report on T207 phosphorylation. The non-phosphoT207 peak partially overlaps with other resonances, and is thus a reporter of poor quality. **c)** Treatment of peak intensities from raw data, to normalized intensities and interpretation in %phosphorylation. The value  $I_{t=0}$  of a non-phosphopeak is obtained from the tangent at  $t=0$  of a non-phosphopeak evolution curve, and can then be used for normalizing this evolution. Build-up curves of phosphopeaks are normalized by intensity ratios measured at 283 K  $I_{\text{final}}/I_{\text{ref}}$  (see 3.5). To make the graphs clearer, we show only A199 crosspeaks evolution as a reporter of S193 phosphorylation; S193, S195 and L198 crosspeaks can also be used, see a). Similarly, T203 crosspeaks can be used as reporters of T207 phosphorylation. Phosphorylation build-up curves are preferably obtained from non-phosphopeaks decay analysis. They can be normalized easily using the reference intensities at 0% phosphorylation; at the opposite, because a phosphorylation reaction rarely goes to completion, the reference intensities of phosphopeaks at 100% phosphorylation can be difficult to determine.

**Fig. 2.** SDS-PAGE analysis of the phosphorylation kinetics of a BRCA2 fragment presenting 5 phosphosites. Phosphorylation of the wild-type fragment and a mutant fragment were evaluated at  $t_0$ ,  $t_{10\text{min}}$ ,  $t_{30\text{min}}$  and  $t_{2\text{hrs}}$ . This gel exemplifies the typical phosphorylation shifts provoked by successive phosphorylation events. Although it is not fully characterized, it appears that SDS binds less to phosphoproteins, which slows down electrophoretic migration [27]. Peptides carrying a high net negative charge (~15 or more) do not shift as well upon phosphorylation. (15% polyacrylamide gel stained with Coomassie blue, lane X shows a default in gel loading).

**Fig. 3.** Temperature gradient from assignment conditions (283 K) to phosphorylation conditions (298 K) allows fast  $^1\text{H}_\text{N}$  and  $^{15}\text{N}$  assignment at 298 K. 2D  $^1\text{H}$ - $^{15}\text{N}$  SOFAST-HMQC spectra of BRCA2(aa48-218) were recorded at 283 K, 290 K and 298 K, and spectral referencing were temperature-corrected according to the rule presented in 3.3.3. All spectra are shown at the same contour level. A number of crosspeaks fade away at higher temperature. This is typical of IDPs NMR spectra. The spectra were acquired at pH 7.0 and 700 MHz.

**Fig. 4:** Theoretical phosphorylation build-up curves. **a)** Build-up curves in the situation where the phosphorylation target is at a concentration well below its  $K_m$ . **b)** Build-up curves in the situation where the phosphorylation target is at concentrations above its  $K_m$  (black:  $[\text{substrate}] \ll K_m$ ; brown:  $[\text{substrate}] = 2 \cdot K_m$ ; orange:  $[\text{substrate}] = 5 \cdot K_m$ ; red:  $[\text{substrate}] = 10 \cdot K_m$ ). **c)** Build-up curves in the situation where the substrate carries 2 phosphorylation sites and the 2<sup>nd</sup> phosphosites (in blue) is phosphorylated only once the 1<sup>st</sup> phosphosite (in red) has been modified. **d)** Build-up curves in the situation where the substrate carries 2 phosphorylation sites with  $K_m(P1) = 10 \cdot K_m(P2)$  (P1 is in red, P2 in blue).

**Fig. 5:** NMR analysis of the ATP/ADP concentrations. *Upper panel:* Overlay of 1D  $^1\text{H}$  spectra of pure ATP (blue) and ADP (red) showing the purine  $^1\text{H}_{35}$  resonances. *Lower panel:* 1D  $^1\text{H}$  spectrum of BRCA2(aa48-218) at 50  $\mu\text{M}$  at the end of the phosphorylation reaction by Plk1. The close-up view shows the ATP and ADP  $^1\text{H}_{35}$  signals allowing their quantification. All spectra were recorded in the phosphorylation buffer (50 mM Hepes, 100 mM NaCl, pH 7.0, 4 mM DTT, 1x EDTA-free protease inhibitors, 4 mM ATP, 10 mM  $\text{MgCl}_2$ ) at 283 K and at 700 MHz. ATP and ADP  $^1\text{H}_{35}$  chemical shifts depend on pH and on the presence of  $\text{Mg}^{2+}$  ( $\sim 0.03$  ppm).