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Metabolic labeling of leucine rich repeat kinases 1 and 2 with radioactive phosphate

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Editor, ***JoVE***
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Dear Editor of *JoVE*, Dear Kira Henderson,

Please find enclosed our revised manuscript entitled: "**Metabolic labeling of leucine rich repeat kinases 1 and 2 with radioactive phosphate**" by Jean-Marc Taymans, Fangye Gao, Veerle Baekelandt. All reviewer comments have been taken into account and we have tracked all changes in the revised document we are now submitting. A detailed account of the comments and action taken is attached to the revision submission as a separate document.

We thank you for your kind consideration and hope you will find our revised manuscript suitable for publication in *JoVE*.

Sincerely,

Jean-Marc Taymans

Metabolic labeling of leucine rich repeat kinases 1 and 2 with radioactive phosphate

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Keywords: LRRK1, LRRK2, metabolic labeling, ^{32}P orthophosphate, immunoprecipitation, autoradiography.

Short abstract
Leucine rich repeat kinases 1 and 2 (LRRK1 and LRRK2) are multidomain proteins which encode both GTPase and kinase domains and which are phosphorylated in cells. Here, we present a protocol to label LRRK1 and LRRK2 in cells with ^{32}P orthophosphate, thereby providing a means to measure their overall cellular phophorylation levels.

Long abstract
Leucine rich repeat kinases 1 and 2 (LRRK1 and LRRK2) are paralogs which share a similar domain organization, including a serine-threonine kinase domain, a Ras of complex proteins domain (ROC), a C-terminal of ROC domain (COR), and leucine-rich and ankyrin-like repeats at the N-terminus. The precise cellular roles of LRRK1 and LRRK2 have yet to be elucidated, however LRRK1 has been implicated in tyrosine kinase receptor signaling[1,2], while LRRK2 is implicated in the pathogenesis of Parkinson's disease[3,4]. In this report, we present a protocol to label the LRRK1 and LRRK2 proteins in cells with ^{32}P orthophosphate, thereby providing a means to measure the overall phophorylation levels of these 2 proteins in cells. In brief, affinity tagged LRRK proteins are expressed in HEK293T cells which are exposed to medium containing

^{32}P-orthophosphate. The ^{32}P-orthophosphate is assimilated by the cells after only a few hours of incubation and all molecules in the cell containing phosphates are thereby radioactively labeled. Via the affinity tag (3xflag) the LRRK proteins are isolated from other cellular components by immunoprecipitation. Immunoprecipitates are then separated via SDS-PAGE, blotted to pvdf membranes and analysis of the incorporated phosphates is performed by autoradiography (^{32}P signal) and western detection (protein signal) of the proteins on the blots. The protocol can readily be adapted to monitor phosphorylation of any other protein that can be expressed in cells and isolated by immunoprecipitation.

Introduction/Abstract

Leucine rich repeat kinases 1 and 2 (LRRK1 and LRRK2) are multidomain paralogs which share a similar domain organization. Both proteins encode a GTPase sequence akin to the Ras family of GTPases (Ras of Complex Proteins, or ROC) as well as a C-terminal of ROC domain (COR), effectively classifying both proteins to the ROCO protein family[5,6]. N-terminal of the ROC-COR domain tandem, both proteins encode a leucine-rich repeat domain as well as an ankyrin-like domain, while only LRRK2 encodes an extra armadillo domein[6-8]. C-terminal of ROC-COR, both proteins share a serine-threonine kinase domain while only LRRK2 encodes a WD40 domain in the C-terminal region[8]. The precise cellular roles of LRRK1 and LRRK2 have yet to be elucidated, however LRRK1 has been implicated in tyrosine kinase receptor signaling[1,2], while genetic evidence points to a role for LRRK2 in the pathogenesis of Parkinson's disease[3,4].

The phosphorylation of proteins is a common regulatory mechanism in cells. For example, phosphorylation can be essential for the activation of enzymes or for the recruitment of proteins to a signaling complex. The cellular phosphorylation of LRRK2 has been extensively characterized and phosphosite mapping has shown a majority of cellular phosphorylation sites to occur in a cluster between the ankyrin repeat and leucine rich repeat domains[9-11]. Although LRRK1 cellular phosphorylation sites have yet to be mapped, evidence from studies using phosphoprotein staining of blots of immunoprecipitated LRRK1 protein from COS7 cells suggests that LRRK1 protein is phosphorylated in cells[12].

This paper provides a basic protocol for assaying general phosphorylation level of LRRK1 and LRRK2 in cell lines using metabolic labeling with ^{32}P-orthophosphate. The overall strategy is straightforward. Affinity tagged LRRK proteins are expressed in HEK293T cells which are exposed to medium containing ^{32}P-orthophosphate. The ^{32}P-orthophosphate is assimilated by the cells after only a few hours of incubation and all molecules in the cell containing phosphates are thereby radioactively labeled. The affinity tag (3xflag) is then used to isolate the LRRK proteins from other cellular components by immunoprecipitation. Immunoprecipitates are then separated via SDS-PAGE, blotted to pvdf membranes and analysis of the incorporated phosphates is performed by autoradiography (^{32}P signal) and western detection (protein signal) of the proteins on the blots.

Protocol Text:

The present protocol uses radioactive ^{32}P-labeled orthophosphate to follow cellular phosphorylation of LRRK2. It is important to bear in mind that all operations with radioactive reagents should be performed using appropriate protective measures to minimize exposure of radioactive radiation to the operator and the environment. Compounds containing isotopes that emit ionizing radiation can be harmful to human health and strict licensing and regulations at an institutional and national level control their use. The experiments in this protocol were carried

out following training in open source radiation use at Katholieke Universiteit Leuven (KU Leuven) and following the good laboratory practice guidelines provided by the health, safety and environment department at the university. Several steps in our protocol are widely deployed such as cell culture, SDS-PAGE, western blotting and given here are details of the protocol as applied in our laboratory. It should be noted that precise experimental conditions vary from laboratory to laboratory; therefore specific measures to ensure proper handling of radioactive material should be adapted to each new laboratory setting.

Use of open source radiation is subject to prior regulatory approval and the regulatory body responsible for open source radiation in laboratory research varies from country to country. Users should consult with their institutional radiation safety officer in order to ensure that procedure conform to local rules and regulations. Information on regulatory bodies can be found: in Belgium, the Federal Agency for Nuclear Control (http://www.fanc.fgov.be, website in French or Dutch), in the United Kingdom, the Health and Safety Executive (http://www.hse.gov.uk/radiation/ionising/index.htm), in the United States the Nuclear Regulatory Commission (http://www.nrc.gov/materials/miau/regs-guides-comm.html), in Canada the Canadian Nuclear Safety Commission (http://nuclearsafety.gc.ca/eng/), and in Germany Das Bundesamt für Strahlenschutz (http://www.bfs.de/de/bfs).
Safety precautions relevant to this protocol have been noted in the text, highlighted with the radioactive trefoil symbol (☢).

1. Metabolic labeling of cells

1.1. Prepare cells for labeling.

1.1.1. Culture HEK293T cell lines according to standard culture conditions (37°C, 5% CO_2) in DMEM with 8% fetal calf serum and gentamycin.

1.1.2. Expand cells sufficiently to obtain at least 1.10^6 cells per sample to test.

1.1.3. Trypsinize cells and plate out into 6 well plates (35 mm diameter) at 10^6 cells/well.

1.1.4. 24 hours after plating out cells, express 3xflag-LRRK2 protein via transfection or lentiviral vector mediated transduction.

1.1.4.1. For transfection, mix per sample 4 µg DNA (pCHMWS-3xflag-LRRK2 plasmid[13-15] or pCHMWS-3xflag-LRRK1 plasmid[15]) and 8 µl of linear polyethyleneimine (linear PEI, 1 mg/ml) into 80 µl DMEM (without additions). Allow to complex for 15-30 minutes then add complex to cells by mixing well into medium present.

1.1.4.2. For lentiviral vector mediated transduction, dilute lentiviral vector encoding 3xflag-LRRK1 or -2 (LV-3xflag-LRRK1, LV-3xflag-LRRK2, as a rule of thumb, transduce with twice as many transducing units, ie number of functional vector particles, of lentivector as there are cells) into the culture medium. A description of the production of LV-3xflag-LRRK1/2 has previously been described[15].

1.1.5. When cells are 80-100% confluent (about 48 hours after transfection or transduction), rinse cells with prewarmed (37°C) DMEM without phosphates.

1.2. Label cells with ^{32}P-ortho-phosphate.

1.2.1. Keep in mind general principles of safety when working with radiation.

1.2.1.1. ☢☢☢Perform all operations with ^{32}P in a designated radiation area.

1.2.1.2. ☢Suitable personal protective equipment should be worn - under standard operating procedure in our laboratory these include lab coat, double gloves and protective goggles.

1.2.1.3. ☢All work with ^{32}P should be shielded from users by 6 mm Perspex screens to minimize exposure.

1.2.1.4. ☢Personal monitoring devices should always be used - within KUL all certified open source radiation user wears a film badge attached to the breast pocket of the lab coat to monitor radiation exposure during experiments.

1.2.1.5. ☢All experimental surfaces should be assessed for radioactivity before and after use with a Geiger counter.

1.2.1.6. ☢All potentially contaminated consumables should be disposed of in strict adherence to institutional guidelines for radioactive waste disposal.

1.2.2. Under a laminar flow, prepare a falcon tube with 2,1 ml of DMEM without phosphates (pre-warmed to 37°C) per 6-well plate of cells to label.

1.2.2.1. For instance, to label cells in all wells of a 6-well plate, prepare 12,6 ml medium (=6x2,1). This is to provide for 2 ml medium to be used per 6-well plate well of cells with a 5% excess in volume.

1.2.3. ☢☢☢Prepare the bench at which the experiments with ionizing radiation will be performed. The working space is covered by a spill mat upon which a protective liner of absorbent material is placed. In case you are using a liner with one waterproof surface, place it with the absorbent side up.

1.2.4. ☢☢☢Also provide for a Perspex jar on the work space and place the tube of phosphate free medium in it.

1.2.5. ☢☢☢Take the lead lined container with the vial of ^{32}P labeled orthophosphate out of the fridge and bring it to the radioactivity bench. Monitor the container for external radioactive contamination using a Geiger counter.

1.2.6. ☢Dilute ^{32}P labeled orthophosphateinto the tube of DMEM without phosphates at a concentration of 24 µCi per ml.

1.2.6.1. Note: at 2 ml per 6-well plate well of cells, this corresponds to 5 µCi ^{32}P labeled orthophosphate per cm^2 of cultured cells.

1.2.6.2. ☢Keep the tube in the Perspex jar.

1.2.7. ☢Close the container with the remainder of the ^{32}P labeled orthophosphate and replace in the fridge.

1.2.8. ☢Remove the 6-well plates with cells to be labeled from the incubator and place on the radioactivity bench.

1.2.9. ☢Remove medium supernatant and discard. Add 2 ml of the phosphate-free medium containing ^{32}P labeled orthophosphate per well.

1.2.10. ☢Place the culture plates into a Perspex box then monitor the container for external radioactive contamination using a Geiger counter.

1.2.11. ☢Transfer the Perspex box with cells to a eukaryotic cell incubator dedicated to isotopic metabolic labeling.

1.2.12. ☢Incubate for 1-20 hours at 37°C in 5% CO_2.

1.2.12.1. In general, an incorporation time of 3 hours or more is advised. The optimal incubation time may be assessed through time course experiments for each specific protein as desired.

1.2.13. ☢Optional: treat cells with compound.

1.2.13.1. In experiments with compound treatment (such as a kinase inhibitor), a compound treatment step is included after an initial incubation time without compound to allow for the labeling. After the desired incubation time, the Perspex box containing the culture plates are removed from the incubator and brought to the radioactivity bench.

1.2.13.2. ☢Labeling medium is removed and replaced by prewarmed phosphate free medium into which the compound is diluted at the desired concentration. Discard medium in a 50 ml tube for waste collection which is placed in the Perspex jar.

1.2.13.3. ☢Cells are replaced in the Perspex box and placed in the cell incubator for the desired contact time.

1.3. ☢Collect lysates of labeled cells.

☢

1.3.1. ☢Remove medium from cells and discard into the waste collection tube which is placed in the Perspex jar.

1.3.2. ☢☢☢Rinse cells 2 times with ice cold Tris buffered saline (Tris 50 mM, NaCl 150 mM, pH 7,4, 2 ml per rinse), discarding rinse solution into a waste collection tube placed in the Perspex jar.

1.3.3. ☢☢☢Add 0,5 ml of ice cold immunoprecipitation (IP) lysis buffer to each well and collect lysate by pipetting lysate up and down in order to loosen all lysed cells.

1.3.3.1. Prepare the required volume of IP lysis buffer (0,5 ml per sample plus 5% excess) ahead of time, adding the Protease inhibitor cocktail and phosphatase inhibitor cocktail fresh just before use.

1.3.3.2. The composition of the lysis buffer is Tris 20 mM pH 7.5, NaCl 150 mM, EDTA 1 mM, Triton 1%, Glycerol 10 %, protease inhibitor cocktail and phosphatase inhibitor cocktail.

1.3.4. ☢☢☢Transfer the lysate to a microcentrifuge tube and incubate on ice at least 10 minutes.

1.3.5. ☢☢☢Centrifuge the lysates in a microcentrifuge at >5000g for 10 minutes.

1.3.6. ☢☢☢Dispose of radioactive waste in dedicated waste bins which are stored behind Perspex shields.

2. Analyze labeling of proteins of interest

2.1. ☢Isolate protein of interest by immunopurification (IP).

2.1.1. ☢☢☢Transfer the microcentrifuge tubes with centrifuged lysates back to ice and pipette the supernatant into a microcentrifuge tube containing 10 µl bed volume of equilibrated flag-M2 agarose beads.

2.1.1.1. Prepare the tubes with equilibrated flag-M2 agarose beads ahead of time.

2.1.1.2. For this, pipette a volume of flag-M2 agarose slurry corresponding to 10 µl bed volume per sample plus a 5% excess.

2.1.1.2.1. Generally, a 10 µl bed volume of beads corresponds to 20 µl slurry. Refer to the product data sheet for more details.

2.1.1.3. Equilibrate the beads by rinsing 3 times in 10 volumes (relative to bed volume) of IP lysis buffer.

2.1.1.4. Distribute equilibrated beads evenly at 10 µl bed volume per tube into as many tubes as there are samples. Label the tubes with an identifier for each sample.

2.1.2. Transfer the microcentrifuge tubes to 50 ml tubes (about 6 microcentrifuge tubes per 50 ml tube) labeled with a radioactive trefoil symbol and keep on ice.

2.1.3. Transfer samples to a rotating device behind a perspex shield in the designated area of a cold room for end over end mixing at 4°C for 1 to 20 hours.

2.1.4. Transfer the samples to a designated work space on ice.

2.1.5. Spin down the protein bound flag-M2 agarose beads in a microcentrifuge (1000 g, 1 minute) and discard the supernatant into a waste collection tube.

2.1.6. Wash the protein bound flag-M2 agarose beads by resuspending in 1 ml IP wash buffer.

2.1.6.1. Composition of IP wash buffer: Tris 25 mM pH 7.5, NaCl 400 mM, Triton 1%. It is recommended to also include protease and phosphatase inhibitors in the wash buffer for proteins sensitive to degradation by co-purifying proteases or to dephosphorylation by co-purifying phosphatases.

2.1.6.2. Spin down the protein bound flag-M2 agarose beads in a microcentrifuge (1000 g, 1 minute) and discard the supernatant into a waste collection tube.

2.1.6.3. Repeat the wash step 3 times.

2.1.7. After the washes, resuspend the beads into 1 ml IP rinse buffer (Tris 25 mM pH 7.5, $MgCl_2$ 10 mM, dithiothreitol (DTT) 2 mM, Triton 0,02%, beta-glycerophosphate 5 mM, Na_3VO_4 0.1 mM).

2.1.8. Spin down the protein bound flag-M2 agarose beads in a microcentrifuge (1000 g, 1 minute) and discard the supernatant into a waste collection tube. Remove all excess buffer.

2.1.9. Resuspend beads into 40 µl of IP sample SDS loading buffer (Tris-HCl 160 mM pH 6.8, SDS 2%, DTT 0.2 M, glycerol 40%, bromophenol blue 2 mg/ml).

2.1.9.1. Samples can be analyzed immediately or stored in a -20°C freezer for ulterior analysis.

2.1.9.1.1. For storage of samples at -20°C, place samples in tube holders or boxes in a Perspex box in a radioactive trefoil symbol labeled freezer dedicated for storage of radioactive samples.

2.1.10. Dispose of radioactive waste in dedicated waste bins which are stored behind Perspex shields.

2.2. Resolve IP samples via SDS-PAGE and blot to pvdf membrane.

2.2.1. Heat samples in loading buffer to 95°C for 2 minutes and centrifuge for 1 minute at >1000 g to pellet the beads.

2.2.2. Prepare the protein gel electrophoresis module on the radioactivity bench behind a Perspex screen.

2.2.3. Load samples onto a 3-8% tris-acetate SDS-PAGE gels.

2.2.3.1. This type of gel is suited for resolving high molecular weight (HMW) proteins. Other gel types may also be suited, such as a 4-20% Bis-Tricine gel orTris-glycine 4-20% gels.

2.2.3.2. Include a molecular weight marker which is suitable to discern sizes of HMW proteins.

2.2.4. Perform electrophoresis at 150V for 1 hour.

2.2.5. After electrophoresis, remove the gel from its plastic casing and transfer the gel to a container with western blotting transfer buffer.

2.2.5.1. Composition of western blotting transfer buffer: Tris 50 mM, Glycine 40 mM, SDS 0,04%, Methanol 20%.

2.2.5.2. Cut off the parts of the gel which stick out such as the well separators and bottom portion of the gel which sticks out.

2.2.6. Prepare one polyvinylidene fluoride (PVDF) membrane per gel by dipping in methanol for 1 minute, then place in transfer buffer.

2.2.6.1. Membranes are cut to the same size as the gel plus a margin of 3 mm.

2.2.7. Place a semi-dry blotting module on the radioactivity bench and remove the cover and upper electrode plate.

2.2.8. Prepare the blotting sandwich on the surface of the semi-dry blotting module.

2.2.8.1. Wet an extra thick (2,5 mm thick, 7,5x10 cm large) blotting filter in transfer buffer and place on bottom plate of the blotting module.

2.2.8.2. Place the pre-wet PVDF membrane on the blotting filter.

2.2.8.3. Carefully place the gel on the PVDF membrane and remove any air bubbles.

2.2.8.4. Complete the blot sandwich by wetting an extra thick blotting filter in transfer buffer and place on the bottom plate of the blotting module. Remove all air bubbles eventually present in the blotting sandwich.

Please note that the description given here is compatible with the BioRad trans-blot SD system where electrodes are such that proteins migrate downwards onto the membrane. Other blotting systems are also compatible with these steps with minor adaptations such as those eventually needed to take into account another blotting direction or, in the case of tank blotting, extra liquid waste to be disposed of in the same way as the electrophoresis buffer above.

2.2.9. Remove all excess buffer with an absorbent tissue and place the top plate and the cover of the semi-dry blotting module.

2.2.10. Transfer proteins at 15 Volts for 1-2 hours.

2.2.11. During this time, clean up the electrophoresis module.

2.2.11.1. Dispose of radioactive waste in dedicated waste bins which are stored behind Perspex shields.

2.2.11.2. Rinse the electrophoresis module with distilled water (AD) and discard the rinse water in the radioactive liquid waste container.

2.2.12. After transfer, remove the PVDF membrane with blotted proteins from the blotting module.

2.2.13. Optional: perform a Ponceau S staining of blotted proteins to visualize proteins.

2.2.13.1. Transfer the blot to a shallow blot incubation vessel containing Ponceau S solution and incubate for 5 minutes.

2.2.13.2. Rinse twice quickly in AD.

2.2.14. Dry the membrane.

2.3. Perform autoradiography.

2.3.1. Expose the membrane to a phosphorescence plate for 1-5 days.

2.3.2. Read the ^{32}P off of the exposed phosphorescence plate using a Storm 840 phosphorescence scanner or equivalentand save the image as a high resolution tiff.

2.4. Detect protein levels via immunodetection.

2.4.1. Rehydrate the membranes by dipping them briefly into methanol, then transfer to a shallow blot incubation vessel with PBS.

2.4.2. Block the membranes in PBS-T (PBS with 0,1% Triton) containing 5% milk.

2.4.3. Incubate the blots with anti-LRRK2 antibody[13,16] or anti flag antibody and process further with appropriate wash steps and secondary antibody incubation.

2.4.4. Perform chemiluminescence detection to confirm the relative protein levels of LRRK2.

2.5. Quantify incorporation of ^{32}P in LRRK2.

2.5.1. Perform densitometric analysis of the bands on the blot autoradiograms and immunoreactivity using appropriate software such as ImageJ software, a freeware program available on the National Institutes of Health website (http://rsbweb.nih.gov/ij/).

2.5.2. Calculate levels of phosphate incorporation as the ratio of the autoradiographic signal over the immunoreactivity level.

Results

In order to compare overall phosphorylation levels of LRRK1 and LRRK2 in cells, 3xflag tagged LRRK1 and LRRK2 were expressed in HEK293T cells[15]. Cells were cultured in 6-well plates and labeled with ^{32}P and analyzed as described above in the protocol text. Figure 1 shows representative results for metabolic labeling of LRRK1 and LRRK2 in HEK293T cells. Radioactive phosphate incorporation is observed for both LRRK1 and LRRK2. Upon quantification of the ^{32}P levels normalized to the protein levels as measured by densitometric analysis of the immunodetection with anti-flag antibody, it was found that LRRK1 had an average phosphorylation level which is lower than LRRK2 under the conditions tested, although statistical significance is not reached (P>0,05).

Discussion:

This paper provides a basic protocol for assaying general phosphorylation level of LRRK1 and LRRK2 in cell lines using metabolic labeling with ^{32}P-orthophosphate. The overall strategy is straightforward. Affinity tagged LRRK proteins are expressed in HEK293T cells which are exposed to medium containing ^{32}P-orthophosphate. The ^{32}P-orthophosphate is assimilated by the cells after only a few hours of incubation and all molecules in the cell containing phosphates are thereby radioactively labeled. The affinity tag (3xflag) is then used to isolate the LRRK proteins from other cellular components by immunoprecipitation. Immunoprecipitates are then separated via SDS-PAGE, blotted to pvdf membranes and analysis of the incorporated phosphates is performed by autoradiography (^{32}P signal) and western detection (protein signal) of the proteins on the blots. This protocol is to be distinguished from the protocol to measure LRRK2 autophosphorylation[17] in that the labeling of LRRK1 or LRRK2 is performed in cell culture rather than in an in vitro phosphorylation reaction with purified proteins.

It should be noted that the detailed protocol presented here can be adjusted to accommodate for multiple variations depending on experimental needs. For instance, as labeling is efficient in most common laboratory cell lines, this protocol is not restricted to the use of the HEK293T cell line. Also, other affinity tags may be used as an alternative to 3xflag, such as HA, myc, V5, GFP or other tags[18] as multiple tags can be used to efficiently immunoprecipitate LRRK1 or LRRK2.

In case a protein-specific antibody is available for the protein that is suited for immunoprecipitation, as is the case for LRRK2[11], this can be implemented as well. With an immunoprecipitation grade protein-specific antibody, it is also feasible to perform metabolic labeling of LRRK proteins endogenously expressed in cell lines. In the case of LRRK2, several monoclonal antibodies have been described which can immunoprecipitation of endogenous LRRK2[19]. Finally, the metabolic labeling protocol, described here for LRRK1 and LRRK2 can also be adapted to any other protein which can be immunoprecipitated from cell lines using the general strategy described above.

A key consideration before performing metabolic labeling of proteins in cell culture is how this technique compares to other methods available to determine cellular protein phosphorylation. For instance, phosphorylation at specific sites can be monitored by immunoblotting using a phospho-specific antibody. This method follows similar steps to those described here, excluding the isotopic labeling steps, and for this reason, this technique is often favored over metabolic labeling with ^{32}P-orthophosphate when it is available. Metabolic labeling with ^{32}P-orthophosphate provides a signal which is representative of the overall phosphorylation state of the protein, therefore it cannot provide information on the phosphorylation of specific sites. For proteins with multiple phosphorylation sites, as it is the case for LRRK2[10,11], the metabolic labeling technique provides a one-step assessment of the overall phosphorylation level which can ascertained with phospho-specific antibodies only pending multiple immunodetection steps. For instance, LRRK2 is highly phosphorylated in its ANK-LRR interdomain region, i.e. the S910/S935/S955/S973[11,20] sites as well as in other regions[10] including the recently characterized S1292 site[21]. In order to dissect out the roles of individual phosphosites, it is recommended to prefer experiments with phosphosite specific antibodies. For example phosphosite specific antibodies have allowed to discern that the S910/S935/S955/S973 phosphosites are dephosphorylated in several pathogenic mutants such as R1441C/G, Y1699C, I2020T, but not in the G2019S [11,22], while LRRK2 disease mutant forms generally show higher phospho-S1292 levels[21]. However, metabolic labeling are useful for a number of other studies of cellular phosphorylation. Metabolic labeling is always an applicable technique for instance in cases of unknown phosphorylation sites, or when phospho-antibodies are not available or of low sensitivity. Finally, metabolic labeling allows comparing overall phosphorylation levels of different proteins (as shown here comparing cellular phosphorylation levels of LRRK1 and LRRK2, figure 1), a comparison which is challenging to do with phosphosite-specific antibodies given differences in sensitivity from one antibody to another.

In conclusion, the present protocol allows efficient assessment of the overall phosphorylation levels of LRRK proteins in cells. The protocol can readily be adapted to monitor phosphorylation of any other protein that can be expressed in cells and isolated by immunoprecipitation. Use of this protocol is recommended when phosphosite-specific antibodies are not available for the protein in study or as a step in their validation. This protocol is especially useful when the experimental goal is to compare overall phosphorylation of 2 or more different proteins as such comparisons via metabolic labeling are not biased by differences in sensitivity of detection of phosphorylation from one protein to another. Specifically for LRRK1 and LRRK2, this technique can be used to comparatively monitor activity dependent changes in phosphorylation of LRRK1 and LRRK2, given that such changes have begun to be described for LRRK2[11,13,23], while LRRK1 phosphorylation regulation is poorly understood.

Disclosures:
Authors have nothing to disclose.

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Figure legend:

Figure 1. Metabolic labeling of LRRK1 and LRRK2. A. LRRK1 and LRRK2 expressed in HEK293T cells were metabolically labeled with ^{32}P as described in the protocol and results sections. Depicted here are representative autoradiograms (upper panel) of the 32P incorporation as well as representative western blots (lower panel) of LRRK1 and LRRK2 detection via their 3xflag tags. B. Quantification of the comparative metabolic labeling of LRRK1 and LRRK2 (N=4).

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*Figure
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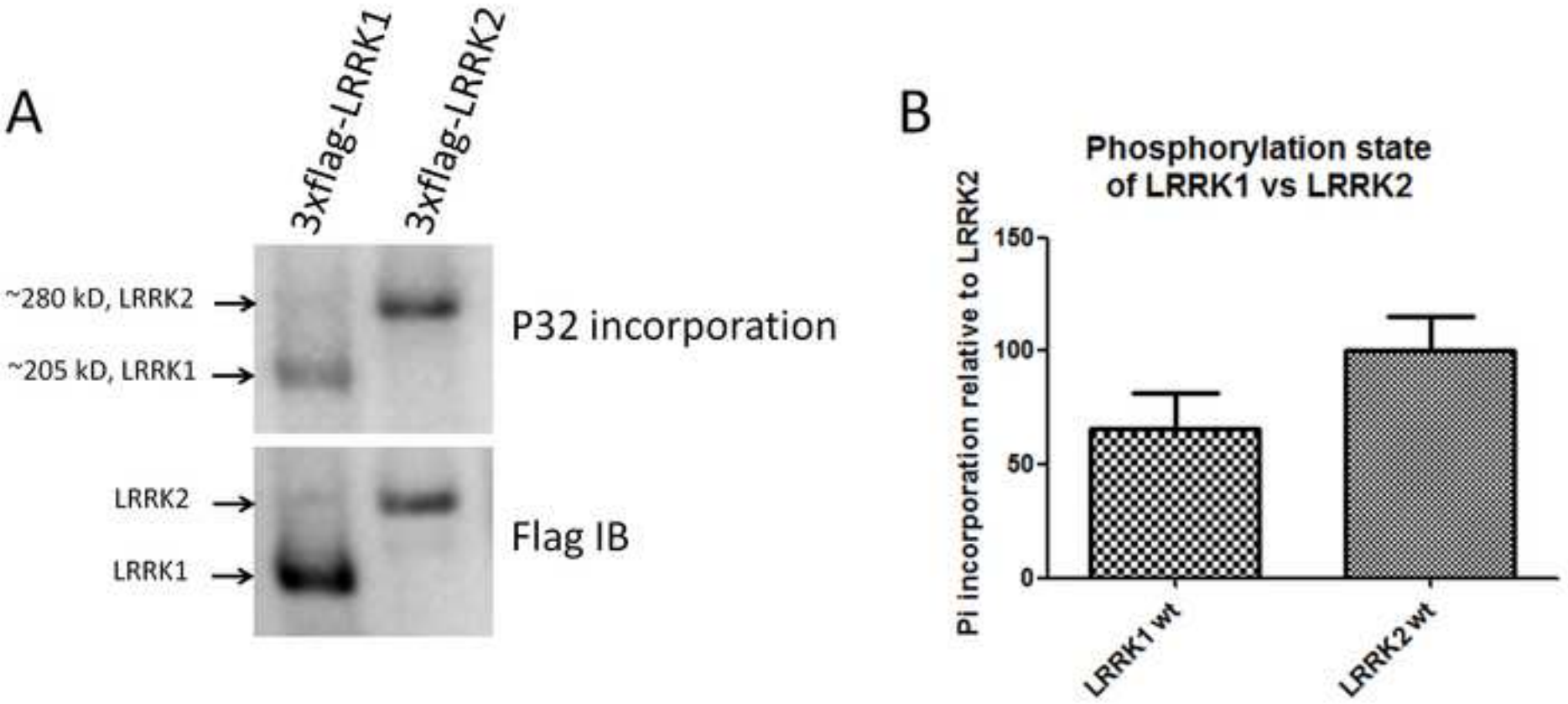

*Table of Reagents/ Materials Used

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Name of the reagent	**Company**
Phosphorus-32 Radionuclide, 1 mCi, buffer disodiumphosphate in 1 ml water	Perkin Elmer
Dulbecco's Modified Eagle Medium (D-MEM) (1X), liquid (high glucose)	Invitrogen
Anti Flag M2 affiinty gel	Sigma
Extra thick blotting filter	Bio-Rad
Ponceau S solution	Sigma

Catalogue number	Comments (optional)
NEX011001MC	
11971-025	This medium contains no phosphates
A2220	For an equivalent product with red colored gel (useful to more easily visualize the beads), use cat. No. F2426.
1703965	
P7170	

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Author(s): Jean-Marc TAYMANS, Fangye GAO, Veerle BAEKELANDT

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Article Title: Metabolic labeling of leucine Rich repeat kinase 1 and 2 with radioactive phosphate

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Response to reviewer comments on JoVE50523R3: "**Metabolic labeling of leucine rich repeat kinases 1 and 2 with radioactive phosphate**" by Jean-Marc Taymans, Fangye Gao, Veerle Baekelandt. A detailed account of the comments and action taken is given below.

Reviewers' comments:

Reviewer #1:

Minor Concerns:

The authors should list the equipment used, for example it isn't clear what transfer apparatus they are using and steps such as the transfer top do will vary considerably between different semi dry transfer modules. The authors should also take into account that people carrying out the protocol may not have access to exactly the same equipment and make the protocol as generalisable as possible.

> **Response**: The authors agree that the protocol should be as generalisable as possible and several brand names of products have for this reason already been removed from the manuscript text. The authors have also now removed the mention of the NuPAGE brand of SDS PAGE gels as gels with the given compositions can be obtained from several different suppliers. The name of the used semi-dry apparatus has now been mentioned, as well as further information on compatibility with other semi-dry blotters as well as tank blotters.

Reviewer #2:

Minor Concerns:

I think the authors need to add the necessary caveats of the meaningfulness of total phosphorylation vs site specific phosphorylation. Total phosphorylation may tell little of LRRK2 function activity whereas specific sites may be more important. In addition, the authors have selected Flag tagged LRRk2 and M2 resin as it could be applicable to other tagged proteins, however, if they were to also describe a LRRK2-specific antibody pulldown, then at least the method would apply to endogenous LRRK2. Without that, this method is only useful for a crude measure of the total phosphorylation of over-expression LRRK2 (or other protein), limiting physiological relevance. Perhaps total phosphorylation only reports proper folding of the protein when compared to various mutants, for example.

> **Response**: The authors agree with these points. There are now several reports on phosphosites of LRRK2 and clues are beginning to emerge indicating different functional roles for different phosphosites. Mention has been made of this in the text. The mention of a LRRK2-specific antibody pulldown is indeed a good idea and has already been mentioned in the discussion text. In this revision, we now also include a recent publication comparing 10 different monoclonal anti-LRRK2 antibodies including for immunoprecipitation of endogenous LRRK2. We would find no mention of whether total phosphorylation is a marker for protein folding, however as mentioned in the text, this method has the advantage over phospho-antibody based methods to detect phosphorylation in that different proteins can be quantitatively compared for their total phosphorylation levels given that incorporated

radioactive phosphates show the same activity from one protein to another. The discussion text has been modified to take these points into account.

Reviewer #3:

Minor Concerns:

1) Section 1.1.4.2: It is not clear how much lentivirus was used for transduction experiments.

Response: Twice as many transducing units, ie functional vector particles, should be added relative to the number of cells in the culture vessel. This information has been added to point 1.1.4.2.

2) Section 1.2.13: It could be nice if authors used the kinase inhibitor (for example IN1 for LRRK2) and demonstrated decrease in overall phosphorylation as an example/control.

Response: The authors agree that this would be useful to show, however this result has currently been included in another manuscript in submission and we are therefore not at liberty to include this result here.

3) Section 2.1.6. It is important to use phosphatase inhibitors not only in lysis buffer but also in washing buffers during IP procedure, since absence of phosphatase inhibitors may result in protein dephosporylation by phosphatases. It is possible that this might not be critical for LRRK, but might be critical for other proteins assayed using this technique.

Response: The authors agree that this is a useful extra measure to increase sensitivity of the detection. The same holds true for protease inhibitors. This information has been added to section 2.1.6.

4) Lines 416, 432: 3xflag is not labeled consistently throughout the manuscript (lowercase vs. uppercase letters)

Response: Where needed, the text was modified for the 3xflag to be consistently labelled throughout the manuscript.

5) Punctuation is not consistently used throughout the article. Please consider whether full stop should be used for the following lines: 116, 121, 123, 126, 131, 140, 186, 188, 203, 211, 225, 244, 246, 249, 253, 259,268, 282, 284, 286, 289, 291, 298, 311, 313, 316, 339, 344, 349, 354, 356, 367, 373, 378, 381, 383, 385, 387, 394, 398, 405. Please check 324 for a possibly missing comma.

Response: The punctuation has been reviewed for consistency. In particular, full stops have been included in protocol steps, as well as a comma at line 324.

6) Typo in figure 1, line 497, "comparative". Consider changing for "comparative" or another equally powerful word.

Response: This typo has been corrected.