Title: Utilising the Luminex magnetic bead based suspension arrays for rapid multiplexed phospho protein quantification.

Keywords: Luminex, suspension array, phospho protein, antibody, multiplex

Running Head: Magnetic bead based suspension arrays.

Summary

The study of protein phosphorylation is critical for the advancement of our understanding of cellular responses to external and internal stimuli. Phosphorylation, the addition of phosphate groups, most often occurs on serine, threonine or tyrosine residues due to the action of protein kinases. This structural change causes the protein to become activated (or deactivated) and enables it in turn to initiate the phosphorylation of other proteins in a cascade, eventually causing cell wide changes such as apoptosis, cell differentiation and growth (amongst others). Cellular phospho protein pathway dysregulation by mutation or chromosomal instability can often give the cell a selective advantage and lead to cancer. Obviously the understanding of these systems is of huge importance to the field of oncology.

This chapter aims to provide a ‘how to’ manual for one such technology, the 96 well plate based xMAP® platform from Luminex. The system utilises antibody bound free floating magnetic spheres which can easily be removed from suspension via magnetisation. There are 100 unique bead sets (moving up to 500 bead sets for the most recent system) identified by the ratio of 2 dyes coating the microsphere. Each bead set is conjugated to a specific antibody which allows targeted protein extraction from low concentration lysate solution. Biotinylated secondary antibodies /

streptavidin phycoerythrin (SAPE) complexes provide the quantification mechanism for the phospho protein of interest.

1.Introduction

Phosphorylation is one of the most important and best understood post translational modifications[1]. Proteins become phosphorylated by the action of protein kinases, of which there are thought to be approximately 480 [2]. It is believed that one third of all proteins are phosphorylated at some point[3]. The addition of a bulky negatively charged phosphate group activates or inhibits protein activity [4]. Coupled with the action of phosphatases, which dephosphorylate proteins, returning them to their original form, cells have a mechanism for regulatory control. Phosphorylation maybe activating or inhibitory, for example p-EGFR activates the EGFR signalling network[5] whilst hyper-phosphorylated Rb inhibits the transcription repression complex allowing the cell to enter the G_1/S transition of cell division[6,7]. Thus quantifying the dynamic changes in phosphorylation status gives researchers key insights into cancer biology. There are a number of technologies available to identify the presence of a protein in its phosphorylated state. These are commonly divided into two categories: those that require you to have a predefined protein target (generally antibody based) and those that don't. The former is limited by antibody availability, affinity and specificity[8,9]whilst the latter requires long preparation and post test data analysis times[8]. Examples of the latter include Mass spectrometry (MS) and 2D-Polyacrylamide Gel Electrophoresis (2D-PAGE). These procedures are very sensitive and allow discovery of new sites of phosphorylation and can quantify many thousand phosphoproteins at a time [9]. It can be time consuming in the set up and analysis, as well as potentially error prone due to deionization and batch variability[10]. The pros and cons of MS methodology have been discussed elsewhere in this book.

Antibody based technology covers a broad range of phosphorylation quantification methods. There are however a few limitations that affect some, if not all, of the most commonly used techniques. Most phospho-antibodies bind to only one or two phosphorylation sites on a given protein. Many key proteins have a significant number of sites, each involved in different aspects of cellular activity, sometimes even having opposite effects [9]. This makes a priori knowledge of the targeted pathway vital before designing the experiment and limits the scope for unexpected discovery. Being limited to the commercial availability of high affinity and specificity phospho antibodies (or antibody pairs used in sandwich based methods) means that many of the lesser studied or less popular phospho-sites are hard to test for [11]. Additionally many phospho proteins are expressed at low concentrations so that even though there is signal activity the sensitivity of the antibody might not be high enough to detect it [12] giving a false negative result. Utilising the epitope-antibody (or antibodies)-reporter molecule motif does allow for reliable and high through put phospho-protein quantification [8]. This introduction briefly discusses some pros and cons of the main antibody methods and how they compare to Luminex.

Single cell mapping of predefined phosphorylation events are possible via two related procedures, immunofluorescence (IF) and flow cytometry [8,13]. Immunofluorescence requires a fluorophore tagged antibody to be incubated with the cells directly onto a glass slide or cover slip[14] and visualised under a microscope (confocal or fluorescent).

The main advantage of IF over flow cytometry and whole lysate based methods is the ability to map phosphorylation events across the cellular architecture. For example phosphorylation of CHK1, a protein associated with cell cycle arrest after DNA damage, can be seen to be localised to the nucleus [15]. Alternatively p-AKT and p-ERK upregulation in EGFR tyrosine kinase inhibitor resistant cell lines can clearly be detected in the cytoplasm [16]. It’s also has fairly low preparation time [8] and can be utilised with formalin fixed paraffin embedded

(FFPE) sections which retains extra-cellular structures allowing visualisation within a solid tumour or other tissues ex vivo [14].

IF does however suffer from a number of issues including insufficient antigen retrieval preventing antibody from accessing its target (in the case of FFPE) [17,12], fluorophore bleaching by light over time [14] and subjectivity of interpretation making objective quantification difficult.

Flow cytometry is solution based making it well suited to whole blood or ascites fluid [18]. After loading into the flow cytometer single cells pass a series of lasers measuring light scatter (to quantify cell size and granularity) and fluorescent emissions (via fluorophore labelled antibodies) [19]. Multiple filters can be used to measure different antibodies simultaneously however typical flow cytometers can only measure up to 3 colours reliably [8] due to fluorescence spectral overlap [20] (auto-fluorescence in certain cell types). Information regarding subpopulations with similar marker expression may also get lost in the large amount of data generated by this technology [19].

Single cell methods allow for a deeper look into single cell heterogeneity than protein lysate based methods reducing the requirement to purify out your population of interest. Multi population ex vivo tissue often doesn't lend itself to targeted cell removal [21] and hence these techniques can often be invaluable.

However single cell methods as a group can also suffer from a number of disadvantages. Being limited to running samples individually and low numbers of fluorescent filters makes multiplexing difficult and leads to low through put compared to other technologies. Fluorescence work can also be hampered by signal bleed through from one wavelength to another confusing the results [17]. Ex vivo sample quality also needs to be high as protein

structure degrades rapidly. Thought has to be given to how the sample was fixed [22] or how long outside of patient, although this is less of an issue with cell lines.

Unlike single cell phosphorylation analysis protein lysate based methods require initial cell population purification. Whilst not necessary for homogeneous cell populations e.g. cell lines; whole blood or ascites preparations need to have the target cell population isolated and removed from solution or risk the validity of the results. Using lysate also means that any information about cellular compartmentalisation or structure is lost. As an aside, thought needs to be given to the nature of target phosphoprotein(s) when selecting appropriate lysis buffer and technique as certain methods might damage the protein structure [23].

Much has been written on westerns blot analysis [24,25] and for many it seems to be the standard go to technique for phosphorylation qualification. It allows diverse sample lysates to be run side by side enabling rapid comparison of results. As it incorporates gel electrophoresis to separate out proteins by polypeptide length the misidentification (false positivity) of antibody cross hybridisation is removed, or at least limited.

It has a number of limitations such as challenges with reproducibility that can reduce its usefulness however, probably the most important of which is its low through put. Also relatively large amounts of protein are also required to get an adequate result. Limited dynamic range and difficulty of result quantification [24] contribute to making Western blot unsuitable for large scale phospho protein studies.

Enzyme linked immunosorbent assays (ELISAs) and forward-phase protein micro arrays (FPPA) are very similar techniques. The former uses phospho-protein antibodies (or total protein antibody for a non-discriminatory pull down) bound to a spot within a well (usually a 96 well plate format) whilst the latter is bound to an array chip/membrane (allowing for a greater number of analytes per lysate but fewer

samples measured) [8,26]. Both techniques have reasonably high through put but the former is limited to 1 to 10 phospho-targets per assay and the latter is limited to 1 sample per assay but can quantify 20+ phospho proteins. Both methods have good reproducibility, generally low set up times and can be relatively inexpensive to run (although the more sophisticated versions require expensive plate readers). Depending on the system used both techniques can provide data with a large dynamic range allowing minimally expressed phospho proteins to be detected alongside highly expressed phospho proteins.

On the downside both techniques are generally limited to pre-defined 'off the shelf' phospho protein sets so have limited room for multiplexing together diverse antibodies to suit specialised projects. Quantification of phosphorylation using FPPA generally requires image analysis software. This, coupled with probe spotting inconsistencies can often introduce artefact to results[8]. Multiplex ELISAs, due to the close proximity of different antibody spots within the individual well, can suffer from signal bleed through.

Another common antibody based technology is the reverse phase protein array (RPPA). Small amounts of tissue or lysate are spotted to a membrane and probed with a single antibody with reporter molecule attached (as opposed to indirect or sandwich based methods). This low sample requirement makes it ideal for clinical research where material per patient is often limited[11]. Hundreds of spots can be applied to each slide allowing rapid detection of a large number of samples in one assay[8]. Due to the low antibody per membrane limitation this technology is limited in the number of phosphorylation events that can be measured per round. The direct antibody detection method (using a single primary antibody bound to a reporter molecule) can

cause high background noise [8] and because of this the antibody used needs rigorous validation to assess its specificity[27], more so than other systems.

Antibody barcoding is an emerging technology using the Nano String platform that has the potential to indirectly quantify 50+ phospho proteins in a single cell. Antibodies tagged to single stranded DNA or mRNA probes are hybridised to a fluorescently barcoded reporter molecule allowing DNA labelling. Barcoding technology allows for a large number of unique reporter molecules. Protein levels can then be extrapolated by normalising out various measurable parameters [28].

The final technology to be looked at, and the purpose for this chapter, is bead-based suspension arrays, and more specifically the Luminex system. This technique follows a standard ‘sandwich’ approach. A magnetic bead covalently bound to a capture molecule (in the interest of simplicity the focus will be on bound antibodies, however other options are possible such as Avidin or nucleic acid sequences) is incubated with the lysate sample in a 96 well plate. The magnetic nature of the bead allows the protein of interest to be pulled out of solution and the remaining unwanted protein to be washed off. A biotinylated secondary phospho antibody targeting the phospho protein site of interest added to solution binds and allows the subsequent addition of Streptavidin R-Phycoerythrin (SAPE) to act as a reporter molecule (Figure1.) via the formation of a biotin/avidin complex. As mentioned above the beads are coated with 2 dyes, via a swelling/soaking/shrinking process[29], the ratio of which allows each bead set to be given a unique emission figure print. It is this finger print that allows multiple bead sets to be multiplexed together to create customisable data rich phospho protein quantification panels.

The Luminex plate reader removes the bead/protein/reporter molecule structure in solution, moving each bead in single file through a detection chamber that simultaneously excites at 635nm and 525nm[29] measuring the emission profile. The former allows the identification of the unique dye ratio of each bead and thus classifies the protein bound. The latter quantifies the amount of phycoerythrin which is proportional to the amount of phospho antibody bound to the protein. Each data point is sent to the system software and reported as median fluorescent intensity (MFI) for each analyte.

Whilst in theory this system can multiplex as many beads together as there are unique bead sets available (500 at time of writing) in practise this is limited by antibody cross reactivity (causing high background noise) to around 15 per well [8]. Even with this limitation the technology has more scope for simultaneous multiplexing of phospho antibodies than either western blot or RPPA. Flexibility of assay composition is also enhanced as multiple off the shelf singleplex/multiplex bead kits can be mixed together ad hoc. Conjugating beads to antibodies in-house is also an option, provided suitable optimisation is carried out, allowing greater project specific customisation of phospho protein panels compared to ELISA. These two factors allow a large amount targeted quantifiable information to be generated at high through put with limited laboratory time. The quantifiable nature of the data generated removes the subjectivity that plagues other methods such as IF.

The 96 well format usually utilised allows for a higher though put of samples compared to FPPA. Low protein requirement (0.2ug/ul or 10ug in 50ul total volume is a good pre optimisation starting point) places this technology at the more efficient end of the spectrum [30], beating Western blot and MS.

As discussed above the downside of protein lysate/antibody techniques, that they lose cell structural and location data, holds as true for bead based suspension arrays as it does any other similar system. It is also similarly limited to answering only predefined questions, and only if adequate antibody pair quality is available. This is however traded off for high through put and ease of data interpretation compared to technology unhindered by these requirements such as MS.

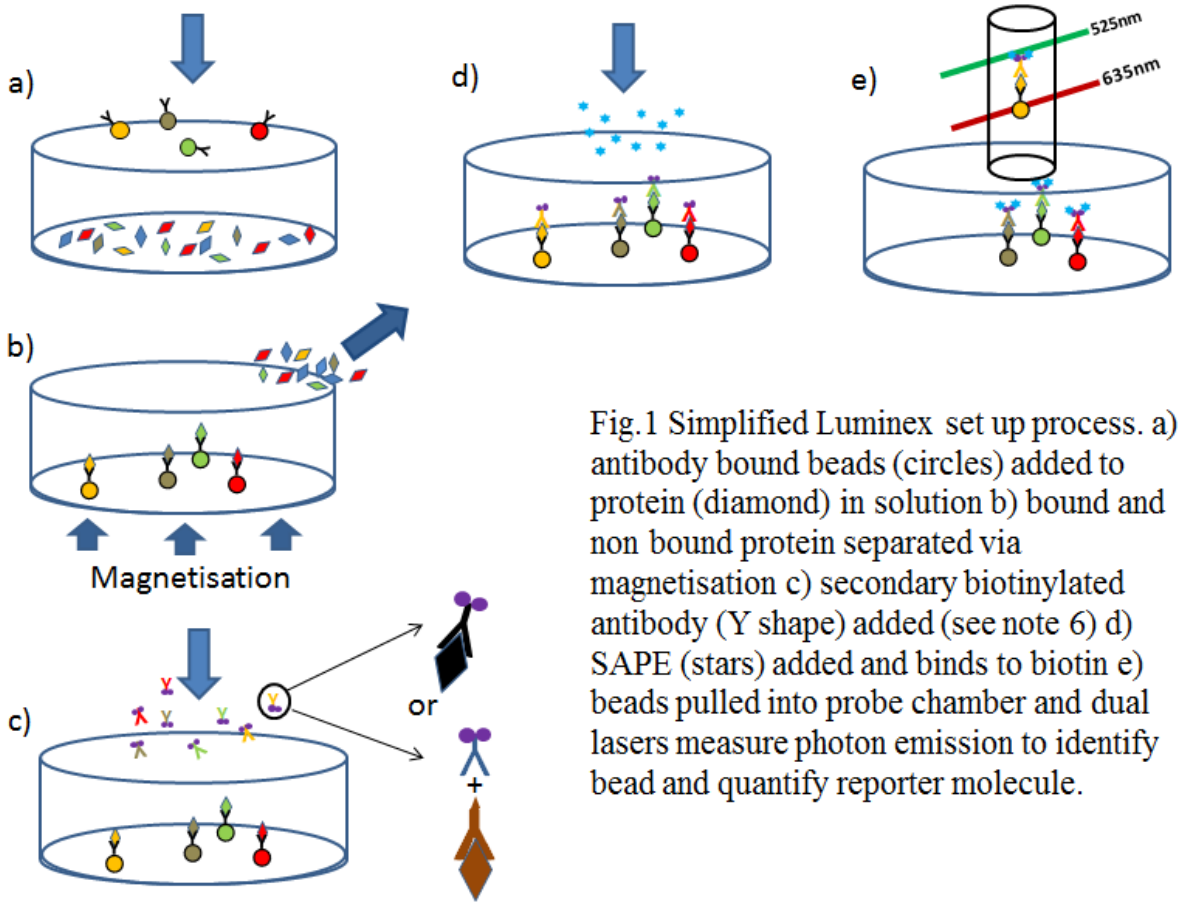

Fig.1 Simplified Luminex set up process. a) antibody bound beads (circles) added to protein (diamond) in solution b) bound and non bound protein separated via magnetisation c) secondary biotinylated antibody (Y shape) added (see note 6) d) SAPE (stars) added and binds to biotin e) beads pulled into probe chamber and dual lasers measure photon emission to identify bead and quantify reporter molecule.

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binds to biotin e) beads pulled into probe chamber and dual lasers measure photon emission to identify bead and quantify reporter molecule.

2.1 Materials:

1. Lysis buffer: Cell Signalling Lysis Buffer (Millipore, 43-040), supplemented with 1x PhosSTOP (Roche, 04906837001), made by the addition of one PhosSTOP tablet to 1ml of de-ionised water (giving a x50 working solution) as well as 1x Protease Inhibitor Cocktail (Sigma-Aldrich, P8340, supplied at 100x concentrate).

2. Wash buffers: Cell Signalling Assay Buffer 1 (Millipore, 43-010) and Assay Buffer 2 (Millipore, 43-041). Different Millipore MagPlex kits require and supply one of these two buffer types. For example Milliplex map AKT/mTOR phosphorylation magnetic bead kit (Millipore, 48-611MAG) requires Buffer 2 to perform optimally. Bio-Rad, another provider of bead sets, requires their own set of buffers to run their products (Bio-Plex pro Cell signalling Reagent kit, 171304006M). Alternatively 0.85% Sodium Chloride + 1% Foetal Bovine Serum (FBS) can be used (see Note 8).

3. Magnetic bead conjugated to a primary antibody with its counterpart secondary antibody bound to a biotin head. These bead sets are supplied either in a multiplex kit or as individual singleplex pairs. Alternatively they can be created ‘in house’. Unbound beads can be brought direct from Luminex Corp (MagPlex Microspheres) and conjugated to a commercially brought antibody via an xMAP Antibody conjugation kit (Luminex Corp, 40-50016). A complimentary commercial secondary antibody can then be biotinylated via an off the shelf biotin conjugation kit (for example the one supplied by Abcam, ab102865). This ‘in house’ method requires some optimisation (see Notes 2-9).

4. 1x SAPE, supplied as concentrate with various Milliplex kits. Alternatively 4ug/ml SAPE diluted in Sodium Chloride + 1% FBS can be used (SAPE can be purchased from Life Technologies, S866, at 1mg/ml).

5. xMAP Sheath Fluid, 20L (ThermoFisher Scientific, 4050000).

6. Di-ionised water.

7. 70% ethanol diluted in di-ionised water.

8. 10% bleach diluted in di-ionised water.

9. Calibration kit (Luminex, LX200-CAL-K25).

10. Verification kit (Luminex, LX200-CON-K25).

2.2 Equipment:

1. Luminex 200 with xPonent software or more recent combinations Luminex 200 system includes: Sheath fluid delivery system, XYP instrument, Luminex 200 analyser probe unit and attached computer.

2. Plate shaker capable of 600-800 rpm.

3. A method to maintain plate shaking at 4^{o}C in the dark overnight (a walk in cold room is ideal).

4. A magnetic particle separator capable of holding 96 well plates (or a vacuum pump if non-magnetic beads are to be used).

5. An opaque flat bottom 96 well plate. If using non-magnetic beads, a special 96 well plate that allows suction filtration to occur is required. These are supplied by Millipore in their Milliplex kits (See Note 10).

6. Luminex automated maintenance plate.

3. Methods:

The protocol below is an outline of the general principal used. Commercially available kits might not follow exactly the same outline so check manufacturers manual.

The lysate protein concentration of each sample needs to be quantified beforehand with a standard quantification method e.g. BCA assay.

3.1 Day 1

1. Defrost protein lysate on ice.
2. Place 96 well plate on ice.
3. Pre wet well with 50ul of wash buffer, agitate manually and dispose buffer.
4. Add either 10ug of sample protein or control to each well and top the total volume per well to 25ul with lysate buffer (with protease/phosphate inhibitors).
5. Vortex beads for 20 seconds to break up clumps (sonication might help as well).
6. Add approximately 2500 beads per analyte in solution to each well and top up total volume to 50ul with wash buffer. Most commercial kits will dictate bead:buffer ratios and some will have separate bead suspension and wash buffers.
7. Cover plate with adhesive seal and aluminium foil cover (to avoid accidental exposure to light).
8. Place on plate shaker at 600-800rpm and leave overnight in the dark at $4^{o}C$.

3.2 Day 2: Plate set up

9. Place 96 well plate on magnetic separator for 90-120 seconds to pull the bead bound

protein out of solution.

10. Gently dump out the solution (with magnetic separator still attached to plate) into a waste reciprocal. Blot plate dry on tissue.
11. Add 100ul of wash buffer to each well, release plate from separator and gently tap (See Note 11).
12. Repeat stages 9-11 once more.
13. Add 25ul of diluted (in wash buffer) secondary antibody, cover with adhesive plate seal and foil cover. Commercial kits will dictate antibody:buffer ratios. For ‘in house’ secondary antibodies the dilution factor should be pre-optimised.
14. Incubate on plate shaker for 1 hour in the dark at room temperature.
15. Turn on Luminex 200 system: Sheath fluid delivery system, XYP instrument, Luminex 200 analyser probe unit and attached computer in that order (reverse when shutting down).
16. Repeat stages 9-11 twice.
17. Add 25ul of diluted (in wash buffer) SAPE, cover with adhesive plate seal and foil.
18. Incubate on plate shaker for 15-30 minutes in the dark at room temperature.
19. Run Luminex 200 system initialisation protocol (maintenance tab) using the automated maintenance plate (see Note 12).
20. Repeat stages 9-11 twice.
21. Add 150ul of buffer to each well to re-suspend the beads, cover with adhesive plate seal/foil cover.
22. Incubate on plate shaker for 5 minutes in the dark at room temperature.
23. If you intend to run the same bead set repeatedly its worth setting up a ‘protocol’ under the protocol tab. Run through the programme’s set up procedure (see Notes 13-14).

24. Remove foil cover and adhesive plate seal, eject motorised tray and place plate in.
25. Check that there is 2-3ml deionised water in the wash compartment if required. Retract tray and start the protocol.
26. The system will provide all raw data in a .csv format, usually found in a folder on the computer desktop.
27. Run the Luminex shut down protocol (maintenance tab), exit the software and turn the system off (see Note 12).

4. Notes:

1. If no commercially optimised buffer is available Sodium chloride +1%FBS is adequate as a wash buffer. Phospho buffered saline should be avoided when dealing with phospho protein antibodies.
2. Note that beads are photo sensitive and hence care should be taken to limit time sitting uncovered on the bench. For 'in house' bead sets wrap bead vials in foil and store in an opaque container at 2-8^{o}C.
3. When optimising a bead set developed in-house Luminex produce a free guide 'Luminex-xMAP_cookbook' (at time of writing) that is a good source of information on methodology and technique.
4. When producing large scale phospho antibody panels involving bead sets from multiple sources it's a good idea to keep track of all the bead regions used as doubling up within a well will make data interpretation impossible.
5. The affinity of an antibody to its target is often difficult to assess based on commercially provided information. Its suitability for any given experimental technique is also in question. It is therefore important to cross test a number of different bead bound primary antibodies to different secondary phospho

antibodies to discover which combination provides the best signal to noise ratio.

6. Two possible methods for tagging ‘in house’ secondary antibody to your reporter molecule constructs should be assessed. The first requires you to chemically biotinylate your secondary antibody so that it can directly bind to the SAPE. Kits are available commercially that allow you to do this however they have a few draw backs depending on the method used. These can include overly diluting the antibody or not removing unbound biotin molecules. This free biotin can coat protein in solution and bind to SAPE, by passing the secondary antibody and give a false positive reading. One method of reducing this effect is to skip steps 8-11 above so that the free, non-bead bound, protein can act as a blocking agent getting removed by subsequent wash steps. The second method utilises a biotinylated tertiary anti-species antibody to act as a bridge between the secondary antibody and the SAPE in solution (and requires an additional incubation stage between steps 15 and 16 above). This technique requires the conjugated bead bound primary antibody be raised in a different species (e.g. mouse) to the secondary antibody (e.g. rabbit) so that the biotinylated tertiary (e.g. anti-rabbit) doesn’t cross hybridise to the primary. Drawbacks include the requirement that all multiplexed secondary antibodies are of the same species and every additional component to the bead/reporter molecule complex adds to its fragility and the complexity of optimisation.
7. Prior to first time use conjugated beads should be tested to make sure the antibody has bound successfully. This can be done by simply incubating your bead/antibody construct with an anti-species biotinylated secondary antibody (for example if you bind a mouse monoclonal antibody to your bead then use

an anti-mouse biotinylated antibody), washed, incubated with SAPE (at ~4ul per ml of buffer), washed, re-suspended in 150ml of buffer and then read on the Luminex system. Higher degree of binding leads to higher MFI readings. Note that this MFI reading doesn't tell you anything about the affinity of the chosen antibody for the protein target. You might get a high reading here but very poor back ground to noise ratio for your antibody pair and vice versa.

8. Things that need optimisation/titration: antibody pair choice, amount of primary antibody bound to bead (which in turn requires knowledge of your antibody's pre-bound concentration), amount of protein that gives the best signal:noise ratio, amount of secondary antibody to add (which again requires knowledge of its concentration in solution), reporter molecule binding method (see note 7), SAPE concentration and multiplexability (i.e. presence of cross hybridisation).
9. When making up your own bead set high quality positive controls are very important. It's worth testing the specificity of your phospho protein antibody to your positive control first (e.g. by western blot). Phosphorylation events can be hard to capture due to feedback loops rapidly de-phosphorylating the target protein.
10. Previous versions of the technology utilized polystyrene instead of magnetic beads so separation can be replaced by vacuum filtration if pumps/specialist plates are available and required. If using both types of beads, don't combine them in one assay.
11. During the wash steps it is important to lift the beads off the base of the plate to facilitated thorough washing. After the addition of the wash buffer, remove the plate from the magnetic holder and gently tap a few times to insure the beads mix properly.

12. The automated maintenance plate is supplied with the Luminex system. The initialisation protocol requires 3ml of di-ionised water and 3ml of 70% ethanol to be aliquoted into the designated wells on the plate. The shutdown protocol requires 3ml of di-ionised water and 3 ml of 10% bleach to be aliquoted into the designated wells. Once a week the system requires you to run a calibration/verification protocol. Use the appropriate kits (see Materials 9-10) vortex each bottle for 10-20 seconds and add five drops into the correct well as laid out by the software.
13. Create a protocol via the protocol tab. Bead option should be set to 'MagPlex' for any magnetic bead set. Total volume set to 100 ul and time out option ticked (200 seconds is fine). DD gating, the algorithm used to filter out any bead clumps, should be set to approximately 7500-15500 but refer to any product manual for exact figures. The analytes subsection requires information on the bead regions used in the assay. These can be found in your product manual. It's a good idea to click the total count option and allow for 100 beads per analyte as this should speed up the run. The plate layout sub section tells the software which wells on the 96 well plate requires testing. If you run different assays on a single plate it's a good idea to put a wash step in after the last well before the next assay starts.
14. Removal of beads from the probe between wells is often inefficient with a small number of beads showing up where they shouldn't. These can be removed automatically from the results via Admin tab, Batch options sub tab and putting the 'minimum bead count' to 3-5 (or more if you count a lot of beads per analyte).

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