Lumiex of homogenized mouse tissue

Day 1

Bead incubation

Materials	• Esky with wet ice, place all substances on ice
	• Assay Buffer (type 1 or two depending on the used beads)
	• Bead kit: only beads will be needed, antibody vial can stay in the fridge
	• Phosphatase inhibitors
	  ○ β-Glycerophosphate (from 50mM stock)
	  ○ Phosphatase inhibitor cocktail 2 (Sigma P5726)
	• Positive control cell lysates (supplied with beads)
	• Filter plate (such as☺

Plate preparation	• Add 25 µl of assay buffer to each of the target, control and background wells • Remove fluid with vacuum pump (only remove excess fluid, do not suck the wells completely dry. Stop once little white spot is visible) • Gently plot excess fluid from plate bottom on paper towel

Preparation of bead mixes	• Calculation based on 96 well plate:
	  ○ 2.5 ml assay buffer – bead volume (2.375 ml for single plex)
	  ▪ Add PIC2 at 0.5%
	  ▪ Add β-Gly at 0.1%
	  ○ 125 µl per bead set (1.3 µl/well), vortex for 10 sec, centrifuge briefly)
	  ○ !(total & phospho of same target cannot be multiplexed, neither can beads with the same bead number)
	• Include duplicates for each sample, background/negative control and positive control per plate
	  ○ Combine each mix (e.g. one for total and one for phosphorylated proteins), vortex and place on ice

Sample preparation	• Allow samples to slowly defrost on ice
	• Add assay buffer (0.5% PIC2, 0.1% β-Gly) to each sample for a final volume of 25 µl/well
	  ○ Product Manual says 1-25 µg protein per well, dilute accordingly if necessary
	  ○ Additionally, if the sample is likely to contain “larger” pieces which are likely to block the luminex suction probe, consider centrifuging and 1:1 dilution steps

Plate loading	• Add 25 µl of each bead mix to the relevant wells, including background/negative control, positive control and samples • Add 25 µl of each positive control to the relevant wells • Add 25 µl of each sample to the relevant wells (remember duplicates and possibly wells for total vs phospho) • Seal & cover plate, place on top of an empty 96-well plate, wrap silver foil around and place on shaker • Move shaker in to cold room (+4C) and set to 700-800 rpm for overnight incubation

Day 2

Luminex 200 startup routine	• Check the sheath fluid volume: 1L is the minimum for one run, refill if necessary (concentrate diluted in MilliQ H2O) • Turn on the sheath fluid pump, the two LX200 segments and the PC • Run the daily startup routine (prime, H2O and alcohol wash) • Run the start-up and verification routine
Additional maintenance runs	• If the machine hasn't been used for a while: return from storage routine • If the sheath fluid has been re-filled since last run: remove bubbles routine • If the machine hasn't been used for +1 month: recalibration routine

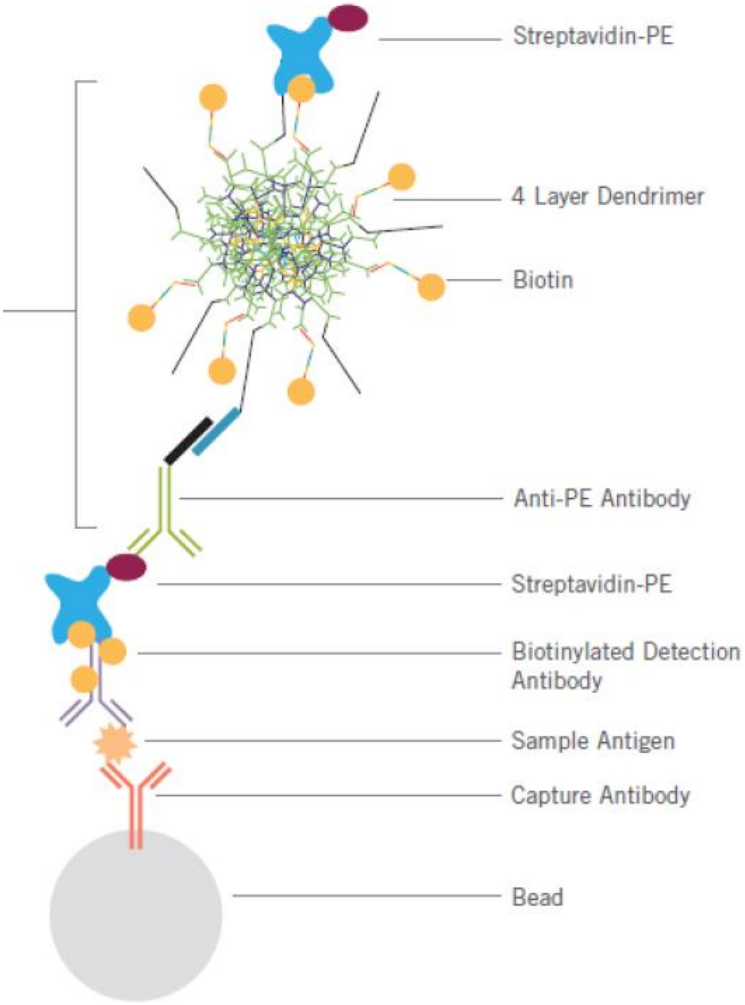
Antibody mix	• Calculation based on 96 well plate:
	  ○ 2.5 ml assay buffer – antibody volume (2.375 ml for single plex)
	  ○ 125 µl per antibody set (1.3 µl/well), vortex for 10 sec, centrifuge briefly)
	  ○ !(total & phospho of same target cannot be multiplexed, neither can targets with the same bead number)
	• Include duplicates for each sample, background/negative control and positive control in to calculation!
	• Combine each mix (e.g. one for total and one for phosphorylated proteins), vortex and place on ice

Wash & Incubation	• Remove lysate with vacuum pump and blot off excess fluid from beneath the plate with paper towel • TWO washing steps: add 100 µl assay buffer to each well, vacuum pump, blot, **REPEAT!** • Add 25 µl of the antibody mix to the appropriate well • Seal the plate, place it on empty 96 well plate, wrap in silver foil, place on shaker for 60 min @ RT @ 750 rpm
SAPE & Signal Amplification	• Prepare Streptavidin-Phycoerythrin (SAPE) mix ○ 0.1 ml SAPE + 2.4 ml Assay Buffer per 96 well plate • Remove antibody solution with vacuum pump and blot excess • Add 25 µl SAPE solution to each well • Seal the plate, place it on empty 96 well plate, wrap in silver foil, place on shaker for 15 min @ RT @ 750 rpm • **Add** 25 µl Amplification buffer to each well • Seal the plate, place it on empty 96 well plate, wrap in silver foil, place on shaker for 15 min @ RT @ 750 rpm

Prep for analysis	• Remove solutions with vacuum pump and blot excess
	• Add 150 µl assay buffer to each well
	  ○ If the plate will be analysed in batches, then only add 150 µl to the wells of the first batch and 25 µl to the wells of all the other batches. During the sampling phase, it is likely that the wells will lose some or all of their fluid and will have to be topped up anyway
	• Seal the plate, place it on empty 96 well plate, wrap in silver foil, place on shaker for 10 min @ RT @ 750 rpm
	• Remove seal from plate, place plate on luminex machine tray, fill reservoir with dH2O, start batch
	  ○ Once the first batch of one well is completed, take the plate out, top up the wells of the next batch and place on shaker for 5 min before returning it to the luminex machine

Slide covering	• Coverslip with DPX or entellan neu

Example visualisation mechanism:



- Bead attaches to sample antigen -> bead binds to all proteins of interest and can be captured by the LX200
- Biotinylated antibody only binds to the region of interest (e.g. a particular Phosphorylation site) and is amplified by Streptavidin-PE
    - Further dendrimers are added via the amplification buffer, containing PE antibodies and more biotin binding sites -> amplifying the original signal source
- The LX200 can then measure via it's lasers, which bead is currently analysed and via the streptaviding colour signal of it is of interest to the analysis
- Therefore, bead count does not represent actual target number, but the MFI (mean fluorescent intensity) value gives indication how much target material is present on 100 beads of interest

http://genisphere.com/sites/default/files/pdfs/UltraAmp_Signal_Amplifiers.pdf