Protocol for Invitro Kinase Assay:

- Wash Buffer:
- Controls required:
    - Kinase substrate combination that is known to work
    - GST-fusion purified form of the kinase (might or might not be active)

This particular experiment:
Aim: To check if LANA is phosphorylated by Pim1
Cells: 293T
Vectors:
Control, pA3M
Kinase, pA3M-Pim1, pA3M-CyclinA
Substrates: pA3M-LANA

CyclinA-myc IP brings down attached CDKs which efficiently phosphorylate HistoneH1.
Purified histoneH1 was bought commercially.

S#	Kinase (Transfection)	Substrate (Transfection)	Information
1	pA3M	HistoneH1	
2	pA3M-CyclinA	HistoneH1	
3	GST	pA3M-LANA	LANA is not phosphorylated by anything other than Pim1
4	GST-Pim1	pA3M	Pim1 is not phosphorylating a molecule other than LANA
5	GST-Pim1	pA3M-LANA	Pim1 phosphorylates LANA
6	pA3M	pA3M-LANA	
7	pA3M-Pim1	pA3M	
8	pA3M-Pim1	pA3M-LANA	

- Transfect 10-12x10^6 cells with 10ug of plasmid by electroporation.
- Seed in a 100mm dish.
- 24hrs later (ideally 36hrs) harvest cells
    - Wash with ice cold PBS
    - Shear off surface
    - Pellet
    - Resuspend in 500uL RIPA supplemented with protease and phosphatase inhibitors
    - Incubate on ice for 1hr with intermittent vortexing
    - Pellet debris and transfer supernatant to a new tube.
    - Measure protein concentration using Bradford assay
    - Save 200ug of each sample for western blot analysis
- Preclear the lysate with proteinA or proteinG sepharose beads
    - For a Mouse Ab, use a 1:1 mix of ProteinA and ProteinG beads (25ul per tube)
    - For a Rabbit Ab, use ProteinA beads (25ul per tube)
    - Add beads to each tube and rotate for 1hr at 4^{o}C
- Pellet beads and transfer lysate to a new tube.
- Add required amount of Ab (2uL of the sera for a Myc IP), and rotate overnight at 4^{o}C.
- Capture Ab by rotating with 35uL of corresponding beads
- Pellet and wash 2X with RIPA buffer
- Wash one final time with Wash buffer.
- Pellet and resuspend in 30uL Wash buffer + 10mM cold ATP (3.5uL of 100mM stock) + 0.2uCi/uL ^{32}P γATP (0.7uL of stock) + Beads with substrate (as the case maybe) + Beads with Kinase (as the case maybe). For the HistoneH1 sample add 4ug substrate per sample. For the GST samples add ?????
- Incubate for 30min at 37^{o}C.
- Stop reaction by adding 35uL SDS loading buffer and heating to 95^{o}C for 10min
- Resolve labeled substrate by SDS PAGE
- Dry gel?????
- Quantification?????