# Kinase Assay

| Lysis buffer (final conc.): | for 20 ml: |
|---|---|
| • 20 mM Tris/HCl pH7.5 | 400 µl 1 M |
| • 150 mM NaCl | 600 µl 5 M |
| • 25 mM β-glycerophosphate | 500 µl 1 M |
| • 2 mM EDTA | 80 µl 0.5 M |
| • 2 mM pyrophosphate | 400 µl 0.1 M |
| • 1 mM orthovanadate | 200 µl 0.1 M |
| • 1% Triton X-100 | 2 ml 10% |
| • 1 mM DTT | 20 µl 1 M |
| • 1 mM NaF | 20 µl 1 M |
| • A. dest. | 15.8 ml |

Protease Inhibitors: added before use (Leupeptin, Pepstatin, Pefa-Block) according to stock

| Kinase buffer (final conc.): | for 20 ml: |
|---|---|
| • 20 mM Tris/HCl pH7.5 | 400 µl 1 M |
| • 20 mM β-glycerophosphate | 400 µl 1 M |
| • 100 µM orthovanadate | 20 µl 0.1 M |
| • 10 mM $MgCl_2$ | 200 µl 1 M |
| • 50 mM NaCl | 200 µl 5 M |
| • 1 mM DTT | 20 µl 1 M |
| • 50 µM ATP | 50 µl 20 mM |
| • 1 mM NaF | 20 µl 1 M |
| • A. dest. | 18.7 ml |

1. Lyse cells (in 6 wells) with 500 µl per well of Lysis buffer (+ protease inhibitors): 20 min at 4°C.
2. Clear by centrifugation (14000 rpm, 4°C 15 min Eppendorf centrifuge). Save an aliquot (30 µl) for Western blotting.
3. Immunoprecipitate the kinase (e.g. with 10 µl anti-flag affinity matrix beads, Sigma, for flag-tagged transfected kinase; or with appropriate antibody for endogenous kinase + Protein A-Sepharose or directly coupled to agarose): 2h at 4°C (rotating).
4. Wash the beads: 3x with 1 ml PBS (4°C), 1x with 1 ml Kinase buffer (4°C): pellet the beads by centrifugation (14000 rpm, 4°C, 45sec) and remove the supernatant.
5. Prepare Kinase buffer: add $MnCl_2$ to 10 mM (stock: 1 M) and $^{32}$P-γ-ATP (5 µCi per sample, usually 1/10 volume, i.e. 1 µl of stock solution for one 10 µl assay) and preincubate at 30°C for 10 min.
6. Add 1 µg substrate: GST-IκB (1 µl) or mutant substrate (as control) to the beads; add 10 µl kinase buffer, mix gently and incubate at 30°C for 30 min (or longer).
7. Stop the reaction by addition of 4x SDS-sample buffer (4 µl) and perform SDS-PAGE with the samples, followed by fixation of the gel (10% methanol, 10% HAc), drying and autoradiography.

For detection with PhastGel: use only 5 µl beads, 5 µl kinase buffer, 0.5 µl substrate and 2 µl 4x SDS-sample buffer: Run a 12.5% PhastGel with 4 µl per sample