

Signal Transduction Inhibitors in Cellular Function

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Summary

Signal transduction pathways mediate cell–cell interactions and integrate signals from the extracellular environment through specific receptors at the cell membrane. They play a pivotal role in regulating cellular growth and differentiation and in mediating many physiological and pathological processes, such as apoptosis, inflammation, and tumor development. The mitogen-activated protein kinases (MAPKs) constitute a cascade of phosphorylation events that transmit extracellular growth signals through membrane-bound *Ras* to the nucleus of the cell. In this chapter, detailed protocols for analyzing the kinase activities of the key components of the MAPKs pathway—MEK1, ERK1, JNK, and p38 MAPK—are described. A brief introduction to the chemical inhibitors to the MAPKs pathway is provided in the method section of each kinase assay. Inhibitors of other signaling pathways are summarized in **Table 1**. The reporter assay of cyclin D1, a key downstream target gene of MAPKs pathway, is also described in detail.

Key Words: Signal transduction; MAPKs; chemical inhibitors; in vitro kinase assay.

1. Introduction

In multicellular organisms, gene expression is tightly controlled within the cell. Extracellular molecules, such as hormones, growth factors, and cytokines, communicate with the nuclear gene regulatory machinery through the interaction with receptors on the cell membrane and initiate intracellular signaling cascades. Signal transduction can occur between cells and within a single cell. In cancer cells, the integrity of signal transduction cascades is often disrupted by

Table 1. Signal Transduction Pathways and Inhibitors

Signal Transduction Pathways	Inhibitors	References
Tyrosine Kinase Growth Factor Receptors		
HER2/Neu inhibitor	Herceptin (anti-HER2 antibody)	(53,54)
EGF receptors	IMC-C225 (anti-EGFR antidody)	(55,56)
EGFR-Tyrosine kinase	ZD1839, pyridopyrimidines	(54,57,58)
Ras Signaling		
Inhibitors of <i>Ras</i> farnesyltransferase		
FPP analogues		
CAAX peptide analogues	BZA-5B, L-739, Cys-4-ABA-Met FT1-276, SCH44342, SCH66336	(57–59)
Bisubstrate inhibitors	BMS-182878, BMS-184467	(57–59)
Inhibitors of the Raf protein kinases	ISIS5132, BAY43-9006	(57–59)
Mitogen-Activated Protein Kinase Pathways		
Inhibitors of MEK	PD184352, PD098059, U0126, R009-22110	(17,20,21, 23–27)
Inhibitors of ERK1 and ERK2	PD098059, E64D, calpeptin	(5,28)
Inhibitors of JNKs	SB600125	(33,34)
Inhibitors of p38 kinases	SB203580, SB202190, SB242235, SB239063, SB220025, SB202474, SC68376, FR167653	(36,37, 39–44,46)
Inhibitors of PI3-Kinase Signaling Pathways	Wortmannin, LY294002	(60,61)
Protein Phosphatases Inhibitors	Microcystines, calyculins, cantharidin	(67)
Proteasome Inhibitors		
Peptide aldehydes	ALLN, MG132, PSI, MG115	(63)
Peptide boronates	MG262	(63)
Nonpeptide inhibitors	Lactacystin	(63)
DCI	3,4-DCI	(63,64)
Peptide vinyl sulfones	NLVS, YL3YS	(63)
Epoxyketones	Epoxomicin, eponemycin, Ac-hFLFL-epoxide	(63,65)
Bivalent inhibitors	Polyoxyethylene	(66,67)
Natural compound inhibitors	TMC-95A, gliotoxin, EGCG	(63,68)
Histone Deacetylase inhibitors		
Short-chain fatty acids	Butyrates	(70,75)
Hydroxamic acids	Trichostatin A, oxamflatin	(73)
Cyclic peptides	Trapoxin A, FR901228, apicidin	
Benzamides	MS-27275	(78,80)
SIR2 inhibitors	Nicotinamide, splitomicin	(78,79)

gene mutations or altered gene expression. Constitutive activation of signaling cascades contributes to uncontrolled cellular growth (1,2).

The elucidation of signal-transduction pathways in cancer cells, both at the proteomic and the genomic level, has provided the basis of rational screening for chemical inhibitors and targeted drug design. New therapeutics act at specific steps of the signal transduction cascade. The inhibitor may interfere with signaling processes by blocking binding of a ligand to a cell-surface receptor, by inhibiting the receptor tyrosine kinase (RTK) activity of a receptor or by inhibiting downstream components of a signaling pathway (3).

Protein kinases are enzymes that covalently attach phosphate to the side chain of serine, threonine, or tyrosine of specific proteins inside cells. Mitogen-activated protein kinases (MAPKs) are a family of protein kinases whose function and regulation have been conserved during evolution from unicellular organisms to complex organisms, including humans. Multicellular organisms have three subfamilies of MAPKs, namely ERK, JNK, and p38 protein Kinases, which control a vast array of physiological processes (4). The extracellular signal-regulated kinases (ERKs) are involved in the control of cell proliferation and division. The c-Jun amino-terminal kinases (JNKs) are critical regulators of apoptosis and gene transcription. The p38 MAPKs are activated by inflammatory cytokines and environmental stresses (5–7).

Signal transduction inhibitors have been developed to diverse signaling pathways. Limitations of using such inhibitors have been the temporal and spatial control of drug delivery. More recently approaches have been developed to target inhibitors to discrete subcellular compartments, or to activate compounds at a single-cell level using chemical “caging” (8). For example, it has been possible to screen for compounds that are selectively taken up by mitochondria and inhibit growth of tumor cell targets, in part owing to the altered mitochondrial membrane potential of malignant cells (9). Chemical “caging” of small molecules (e.g., ATP, NO, etc.), peptides and proteins, has been useful to define temporal relationships in biochemically mediated processes and to delineate the role of individual proteins in biological phenomena.

The recent application of caging ligands to regulate gene expression will provide important new insights into the mechanisms governing signal transduction in vivo (8). Using light to activate caged molecules at the single-cell level will allow the dissection of intracrine and paracrine signaling at an organismal level. Future development in signal transduction research will integrate microarray technology at a genome-wide level to identify novel signal-transduction inhibitors and, therefore, provide better chemotherapeutic approaches in the treatment of human diseases (8,10).

Here we briefly outline the MAPK signaling pathways and inhibitors that have proven useful for studying such pathways. Stepwise protocols for immunoprecipitating MEK1, ERK1, JNK, and p38 MAP Kinase are described,

along with assays for kinase activity. Because cyclin D1 is a key downstream target of the MAPK pathways, the utility of cyclin D1 promoter reporter assays to examine proliferative signaling pathways is also described.

2. Materials

2.1. Measuring MEK1 Kinase Activity (11,12)

1. Cell lysis buffer: 50 mM HEPES, pH 7.5, 150 mM NaCl, 0.5% deoxycholate, 1% Triton X-100, 1% NP-40, 50 mM sodium fluoride (NaF), 1 mM sodium orthovanadate (Na_3VO_4), 0.01% aprotinin, 4 $\mu\text{g}/\mu\text{L}$ pepstatin A, 10 $\mu\text{g}/\mu\text{L}$ leupeptin, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM dithiothreitol DTT. Add proteinase inhibitors immediately before use and keep solution on ice.
2. Phosphate-Buffered saline (PBS): For preparation of 10 L 1X PBS, dissolve 80 g NaCl, 2 g KCl, 14.2 g Na_2HPO_4 , and 2.4 g KH_2PO_4 in double-distilled H_2O . The pH should be between 7.28 and 7.60.
3. Anti-MEK1 antibody and Protein A-agarose (Santa Cruz Biotechnology, Santa Cruz, CA).
4. MAPK 2/Erk2, (inactive) (Upstate Biotechnology, Lake Placid, NY, cat. no. 14–198.)
5. Nonradioactive adenosine triphosphate (ATP) cocktail: 30 mM β -glycerol phosphate, 60 mM HEPES, pH 7.3, 4 mM EGTA, 1.5 mM DTT, 0.45 mM Na_3VO_4 , 30 mM MgCl_2 , 0.3 mM ATP, and 0.3 mg/mL BSA.
6. Radioactive ATP cocktail: 2 μCi (γ - ^{32}P)-ATP, 10 μg of myelin basic protein (MBP), 30 mM glycerophosphate, 60 mM HEPES, pH 7.3, 4 mM EGTA, 1.5 mM DTT, 0.45 mM Na_3VO_4 , 30 mM MgCl_2 , and 6 μg of BSA.
7. Myelin basic protein (MBP) (Research Diagnostics, Flanders, NJ, cat. no. RDI-TRK8M79).
8. Cell lines and cell-culture supplies.
9. Disposable cell lifter (Fisher Scientific, Pittsburg, PA).
10. PD-98059 stock (50 mM) in dimethyl sulfoxide (DMSO), store at -20°C (Calbiochem-Novabiochem, La Jolla, CA).
11. Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories).
12. Phosphorimager screen and phosphorimaging scanner (Strom, Amersham Biosciences, Piscataway, NJ).
13. Protein sample loading buffer: 50 mM Tris-HCl, pH 6.8, 10% glycerol, 1% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol.

2.2. In Vitro ERK1 Kinase Assay (12,13)

1. Cell lysis buffer: 50 mM HEPES, pH 7.5, 0.5% deoxycholate, 1% Triton X-100, 1% NP-40, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4 , 0.01% aprotinin, 4 $\mu\text{g}/\mu\text{L}$ pepstatin A, 10 $\mu\text{g}/\mu\text{L}$ leupeptin, 1 mM PMSF, 1 mM DTT. Add proteinase inhibitors immediately before use and keep solution on ice.

2. Anti-ERK1 antibody (Santa Cruz Biotechnology, cat. no. SC-94).
3. Protein A-agarose and Protein G-agarose (Santa Cruz Biotechnology).
4. Myelin basic protein.
5. Kinase reaction buffer: 10 μCi ($\gamma\text{-}^{32}\text{P}$)-ATP, 50 μM ATP, 20 mM HEPES, pH 8.0, 10 mM MgCl_2 , 1 mM DTT, 1 mM benzamidine.

2.3. *In Vitro* JNK Kinase Assay (14)

1. Cell lysis buffer: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4 , 1 mM β -glycerol phosphate, 1 mM PMSF, and 1 $\mu\text{g/mL}$ leupeptin.
2. Anti-JNK antibody (Cell Signaling Technology).
3. Protein A-agarose and Protein G-agarose (Santa Cruz Biotechnology).
4. ATF2 fusion protein (Cell Signaling Technology).
5. Kinase buffer: 25 mM Tris-HCl, pH 7.5, 5 mM β -glycerol phosphate, 2 mM DTT, 0.1 mM Na_3VO_4 , 10 mM MgCl_2 and 100 μM ATP.
6. 6X SDS sample buffer: For 100 mL, add 35 mL 1 M Tris-HCl (pH 6.8), 10.28 g SDS, 36 mL Glycerin, 9.2 g DTT, 12 mg Bromophenol Blue, adjust volume with dd H_2O to 100 mL. Store in aliquots at -20°C .
7. Potter Elvehjem tissue grinder.

2.4. *In Vitro* p38 MAPK Assay (15)

1. Cell lysis buffer: 50 mM HEPES, pH 7.6, 150 mM NaCl, 10% glycerol (v/v), 1% Triton X-100 (v/v), 30 mM $\text{Na}_4\text{P}_2\text{O}_7$, 10 mM NaF, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 1 mM Na_3VO_4 , 1 mM DDT, and 100 nM okadaic acid.
2. Anti-p38 MAP kinase antibody (Santa Cruz Biotechnology).
3. Protein A- and Protein G-agarose.
4. ATF2 fusion protein (Cell Signaling Technology) or ATF-2 peptide (New England BioLabs, Beverly, MA).
5. Kinase buffer: 50 mM Tris-HCl, pH 7.5, 10 mM MgCl_2 , and 1 mM dithiothreitol and 100 μM ATP.
6. Whatman P81 phosphocellulose filter (Whatman, cat. no. 3698-023).
7. 175 mM phosphoric acid.
8. Potter Elvehjem tissue grinder.
9. Polyvinylidene flouride (PVDF) membrane.

2.5. *Cyclin D1* Reporter Assay

1. Cell line: Breast cancer cell line MCF-7. Cells are maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-streptomycin at 37°C in the presence of 5% CO_2 .

2. Plasmid DNA: Mammalian expression vector pSV2, pSV2Neu-T, and luciferase reporter constructs 1745D1-LUC, Cyclin A-LUC and *c-fos*-LUC (**16**).
3. Transfection reagents: SuperFect reagent (Qiagen, cat. no. 301305, 1.2 mL). Store at 4°C.
4. MEK inhibitor: PD098059 (2-amino-3-methoxyflavone) (Calbiochem, cat. no. 513000, 5 mg, M.W. 267.3) Stock Solution: 10 mM in DMSO. (Dissolve 5 mg PD098059 in 1.87 mL DMSO, aliquot into 100 µL/tube and store at -20°C.)
5. 0.5 M Glycylglycine (Glygly) buffer: dissolve 33.05 g in 500 mL distilled water, adjust pH to 7.8 with KOH, store at 4°C.
6. 100 mM Potassium phosphate (K-Phos): Mix 90.8 mL of 1 M K₂HPO₄ with 9.2 mL 1 M KH₂PO₄, adjust volume to 1 L with distilled water and determine if pH is 7.8.
7. 1 M DTT in distilled water, stored at -20°C in 1-mL aliquots.
8. 200 mM ATP in distilled water, store at -20°C in 400 µL aliquots.
9. 1 mM Luciferin substrate (Molecular Probes, cat. no. L-2911, 25 mg). Dissolve in 78.51 mL distilled water, store at -20°C in 1-mL aliquots. Protect from light using aluminum foil.
10. 1 M MgSO₄, store at room temperature.
11. GME buffer: 25 mM Glygly, 15 mM MgSO₄, 4 mM EGTA, 5 mL 0.5 M glygly, 1.5 mL 1 M Mg MgSO₄, 0.8 mL 0.5 M EGTA, adjust volume to 100 mL with distilled water, store at 4°C.
12. Extraction buffer: 1% (w/v) Triton X-100 and 1 mM DTT in GME buffer. To prepare, add 0.5 mL Triton X-100 to 50 mL GME buffer, mix well, and then add 50 µL of 1 M DTT. Prepare freshly before use.
13. ATP assay buffer: For each assay point, mix 300 µL GME buffer with 60 µL 100 mM K Phos buffer, 0.4 µL 1 M DTT, and 4 µL 200 mM ATP. Prepare freshly before use.
14. Luciferin solution: Prepare 100 µL per assay. A 5 mL preparation will be enough for 40 samples (1 mL of 1 mM Luciferin, 4 mL GME buffer, and 50 µL of 1 M DTT). Make fresh and protect from light by wrapping the tubes with aluminum foil. Leave on ice before use.
15. Luciferase assay tubes (Becton Dickinson Labwares, cat. no. 352008).
16. Luminometer (i.e., Autolumat, Model LB953, Berthold).

3. Methods

3.1. Measuring MEK1 Kinase Activity

Ras interacts with and activates Raf1, which in turn phosphorylates and activates the dual-specificity kinase MEK1 (MAP kinase kinase) on two distinct serine residues. Activated MEK1 catalyzes the phosphorylation of p44MAPK (ERK1) and p42MAPK (ERK2) on a tyrosine and a threonine

residue (Y183 and T185). These MAP kinases can phosphorylate a variety of substrates, including transcription factors and cell-cycle control genes. The small-molecule inhibitor of MEK, PD184352, directly inhibits MEK1 with a 50% inhibitory concentration (IC_{50}) of 17 nM. PD184352 produces a dose-dependent block in the G1 phase of the cell cycle in colon cancer cells. The cell-culture and in vivo efficacy studies indicate colon tumors are especially sensitive to MEK inhibition (17). When human multiple myeloma or leukemia cell lines are exposed to the MEK/MAPK inhibitor PD184352 and the cell-cycle checkpoint inhibitor UCN-01 the cells show dramatic mitochondrial damage and apoptosis (18,19).

PD098059 is a synthetic inhibitor that selectively blocks the activation of MEK-1 and, to a lesser extent, the activation of MEK-2 (20). The inhibition of MEK-1 activation prevents activation of the MAPKs ERK-1/2 and subsequent phosphorylation of MAPK substrates both in vitro and in intact cells. PD098059 reversed the transformed phenotype of *Ras*-transformed mouse fibroblasts and rat kidney cells and blocked induction of cyclin D1 and cell-cycle progression (21–24). PD098059 does not inhibit JNK/SAPK and the p38 pathways at the concentrations that inhibit ERK activity, demonstrating its specificity for the ERK pathway (25).

U0126 is a newly discovered potent inhibitor of the dual-specificity kinases MEK1 and MEK2 (26). Like PD98059, U0126 is a noncompetitive inhibitor of MEK1/2. U0126 displays significantly higher affinity for all forms of MEK than PD098059. U0126 inhibits phosphorylation of MEK1/2 and ERK1/2, inhibits the invasion of human A375 melanoma cells, and decreases c-Jun expression, a major component of the transcription factor AP-1 (27). U0126 inhibits T-cell proliferation in response to antigenic stimulation and cross-linked anti-CD3 plus anti-CD28 antibodies. U0126 has an inhibitory concentration (IC_{50}) of 50–70 nmol/L, whereas PD098059 has an IC_{50} of 5 μ mol/L. Ro 09-2210, another inhibitor of MEK-1 and MEK-2, also inhibits other dual-specificity kinases such as MKK-4, MKK-6, and MKK-7, albeit at 4 to 10-fold higher IC_{50} concentrations compared with its effect on MEK-1 (25).

1. After treatment of the cells with proper kinase inhibitors, such as 5 μ M PD098059 for 24 h, aspirate the culture medium from the tissue-culture plates.
2. Wash the cells twice with 15 mL ice-cold PBS.
3. Put the culture plates on ice, add 300–500 μ L cell lysis buffer. Scrape the cells from the culture plates using a disposable cell lifter. Transfer the cell lysate to an ice-cold 1.5-mL Eppendorf tube.
4. Freeze-thaw twice using liquid nitrogen or a dry ice-ethanol mix.
5. Vortex for 30 s and centrifuge at 14,000 rpm in a microcentrifuge at 4°C for 10 min.

6. Transfer the supernatant to a 1.5-mL Eppendorf tube.
7. Measure the protein concentration by using the Bio-Rad Protein Assay Reagent.
8. Dilute 300–600 μg cell lysate in 400 μL cell lysis buffer; add 10 μL anti-MEK1 antibody. Incubate for 1 h at 4°C, rotating to thoroughly mix the sample.
9. Add 20 μL of Protein A-agarose bead slurry, washed according to the manufacture's instruction, and incubate for 2 h at 4°C with constant rotation to immunoprecipitate the kinase.
10. Pellet the agarose by centrifuging for 15 s at 14,000 rpm in a microcentrifuge.
11. Remove the supernatant fraction and wash the protein A agarose beads twice with 800 μL ice-cold lysis buffer and 800 μL once with ice-cold PBS.
12. Add 5 μL of inactive ERK2 (250 $\mu\text{g}/\text{mL}$) and 10 μL of nonradioactive ATP cocktail and incubate for 10 min at 30°C on a shaking incubator to mix the sample thoroughly.
13. Add 20 μL of the (γ - ^{32}P)-ATP mixture and incubate for an additional 10 min (see **Note 1**).
14. Stop the reaction by adding 40 μL sample buffer and boil at 95°C for 5 min in heat block, then cool on ice for 2 min (see **Note 2** and **3**).
15. Vortex vigorously for 30 s, and centrifuge at 14,000 rpm at room temperature for 5 min.
16. Electrophorese 15 μL of the supernatant fluid on an 15% SDS-polyacrylamide gel (PAGE) gel.
17. Transfer proteins from the SDS-PAGE onto a nitrocellulose membrane and determine the amount of radiolabeled ERK2 by phosphor imager analysis.

3.2. *In Vitro* ERK1 Kinase Assay (12,13)

ERK1 and ERK2 are widely expressed and are involved in the regulation of meiosis, mitosis, and postmitotic functions in differentiated cells (5). ERKs 1 and 2 are both components of a three-kinase phosphorylation module that includes the MKKK c-Raf1, B-Raf, or A-Raf, which can be activated by the proto-oncogene *Ras*. Oncogenic *Ras* persistently activates the ERK1 and ERK2 pathways, which contributes to the increased proliferative rate of tumor cells (5). PD098059 specifically inhibits the ERK pathway (25). Interestingly, inhibition of cysteine proteinases by either E64D or calpeptin leads to a dramatic inhibition of ERK activity (28).

1. Aspirate the culture medium from the tissue culture plates.
2. Wash the cells twice with 15 mL ice-cold PBS.
3. Put the cell-culture plates on ice and add 0.5 mL ice-cold cell lysis buffer. Scrape the cells from the culture plates using a disposable cell lifter.

Transfer the cell lysate to a 1.5-mL Eppendorf tube and incubate on ice for 30 min (*see* **Notes 4 and 5**)

4. Freeze-thaw twice using liquid nitrogen or a dry ice ethanol mix.
5. Vortex 30 s and centrifuge at 14,000 rpm in a microcentrifuge at 4°C for 10 min.
6. Transfer the supernatant to a new 1.5-mL Eppendorf microcentrifuge tube.
7. Measure the protein concentration Bio-Rad Protein Assay Reagent.
8. Dilute 500 µg of cell lysate in 500 µL cell lysis buffer, and incubate with 2 µg ERK1 anti-antibody for 2 h at 4°C, rotating to thoroughly mix the sample.
9. Add 30 µL of washed Protein A Plus-agarose bead slurry and incubate for another 2 h at 4°C to immunoprecipitate the kinase, rotating thoroughly to mix the sample.
10. Pellet the agarose beads by centrifugation in a microcentrifuge at 14,000 rpm for 15 s.
11. Remove the supernatant and wash the pellet twice with 800 µL ice-cold lysis buffer and twice with 100 mM NaCl in 50 mM HEPES buffer, pH 8.0.
12. Incubate the immunoprecipitated complexes with 0.3 mg/mL MBP at 37°C for 15 min in kinase reaction buffer. Use a shaking incubator to thoroughly mix the sample.
13. Stop the reaction by adding 40 µL sample buffer, boil at 95°C for 5 min, then cool on ice for 2 min (*see* **Note 3**).
14. Vortex vigorously for 30 s, and centrifuge at 14,000 rpm at room temperature for 5 min to pellet the beads.
15. Electrophorese 15 µL of the supernatant fraction on to an 15% SDS-PAGE gel.
16. Transfer proteins from the SDS-PAGE onto a nitrocellulose membrane and determine the amount of radiolabeled MBP by phosphorimager analysis.

3.3. *In Vitro* JNK Kinase Assay (14)

The JNKs are stress-activated protein kinases (29,30). The JNKs bind and phosphorylate c-Jun, a component of the AP-1 transcription complex, and increase its transcriptional activity (7,31,32). AP-1 is involved in regulation of many cytokine genes and is activated in response to environmental stress, radiation, and growth factors, all stimuli that activate JNKs. The inhibition of JNKs enhances chemotherapy-induced inhibition of tumor cell growth, suggesting that JNKs may provide a molecular target for the treatment of cancer. JNK inhibitors have shown promise inhibiting tumor cell growth and in the treatment of rheumatoid arthritis (5).

SP600125 is a JNK inhibitor that completely blocks IL-1-induced expression of c-Jun and collagenase mRNAs. The inhibitor suppressed IL-1-induced accumulation of phosphorylated-c-Jun in synoviocytes (33,34).

Bioactive cell-permeable peptide inhibitors of JNK were engineered by linking the minimal 20-amino acid inhibitory domains of the IB proteins (the islet-brain [IB] 1 and 2 proteins, which inhibit JNK signaling) to the 10-amino acid HIV-TAT sequence that rapidly translocates peptides into cells. Addition of the peptides to the insulin-secreting betaTC-3 cell line resulted in a marked inhibition of interleukin-1 (IL-1)-induced c-jun and c-fos expression, indicating inhibition of JNK signaling (35).

1. Wash the treated cells twice with 10 mL of ice-cold PBS. Aspirate PBS completely after the second wash (*see Note 4*).
2. Add 0.5 mL of lysis buffer to the cells and incubate on ice for 20 min with occasional swirling.
3. Scrape cell lysate gently off the plate with a cell lifter and transfer the lysate to a sterile ice-cold 1.5-mL microcentrifuge tube. Disrupt cell lysate in a 2-mL Potter Elvehjem tissue grinder submerged in ice by using twenty up and down strokes (*see Note 5*).
4. Vortex 30 s and centrifuge in a microcentrifuge at 14,000 rpm at 4°C for 10 min.
5. Transfer the supernatant to a new ice-cold 1.5-mL Eppendorf tube and determine the protein concentration.
6. Adjust 300–600 µg of cell lysate in 500 µL of total lysis buffer.
7. Add 2 µg anti-JNK antibody and incubate at 4°C for 1 h on a rotating navigator.
8. Add 25 µL Protein-A agarose slurry and continue incubation at 4°C for 2 h or overnight on a navigator.
9. Pellet agarose beads for 10 min at 4°C by centrifugation in a microfuge at 1500g.
10. Remove supernatant and wash twice with 0.8 mL of ice-cold lysis buffer and twice with 0.5 mL cold kinase buffer.
11. Pellet agarose beads by centrifugation at 4°C for 10 min at 1500g in a microcentrifuge.
12. Remove the supernatant and suspend the pellets in 30 µL kinase buffer containing 200 µM ATP and 2 µg of ATF2 fusion protein.
13. Incubate for 30 min at 30°C on a shaking incubator.
14. Repeat **step 10**.
15. Wash the pellet three times with 0.8 mL of ice-cold cell lysis buffer.
16. Add 30 µL cell lysis buffer, 6 µL 6X protein loading buffer, boil at 95°C for 5 min, then cool on ice for 2 min (*see Note 3*).

17. Centrifuge at 14,000 rpm for 5 min in a microcentrifuge to collect the beads.
18. Electrophorese the supernatant fraction on to a 7% SDS-PAGE gel.
19. Transfer proteins from the gel onto nitrocellulose membrane. Detect the phospho-ATF2 signal by Western blotting using the phospho-ATF2 antibodies (*see* **Notes 6–10**).

3.4. *In Vitro* p38 MAPK Assay (15)

The p38 MAPKs are activated by inflammatory cytokines as well as by many other stimuli, including hormones, ligands for G protein-coupled receptors, stresses, and during activation of the immune response. Because the p38 MAPKs are key regulators of inflammatory cytokine expression, they appear to be involved in human diseases such as asthma and autoimmunity (**5**). Many inhibitors targeting p38 kinase have been developed, including SB203580, SB202190, SB242235, SB239063, SB220025, SB202474, SC68376, and FR167653 (**36–44**).

SB203580, a pyridinylimidazole compound, is a selective inhibitor of p38 MAP kinase that acts by competitive binding in the ATP-binding pocket. The p38 MAP kinase inhibitors are efficacious in several disease models, including inflammation, arthritis, septic shock, and myocardial injury (**45**). p38 MAPK is activated significantly in nitric oxide (NO)- or peroxynitrite-induced cell death in a time-dependent manner. Cell death and caspase-3 activation are markedly inhibited by SB203580 (**46**).

1. Same as in **Subheading 3.3**.
2. Add 0.3 mL of ice-cold cell lysis buffer to the 10 cm cell-culture dish (add 0.6 mL for 15 cm dishes) and incubate for 20 min on ice with occasional swirling.
3. Same as in **Subheading 3.3**.
4. Same as in **Subheading 3.3**.
5. Same as in **Subheading 3.3**.
6. Same as in **Subheading 3.3**.
7. Add anti-p38 MAP kinase antibody (2 μ g/reaction) precoupled to a 20 μ L mixture of Protein A- and Protein G-agarose beads and incubate at 4°C for 2–3 h with constant rotation. Antibody-coupled beads are washed twice with ice-cold PBS and once with ice-cold lysis buffer before use.
8. Pellet agarose beads for 10 min at 4°C at 1500 g in a microcentrifuge.
9. Remove supernatant and wash beads four times with 1 mL of wash buffer and twice with 1 mL of kinase buffer.
10. Pellet agarose beads for 10 min at 4°C at 1500 g in a microcentrifuge.
11. Remove the supernatant and suspend the pellets in 30 μ L of reaction mixture (kinase buffer containing 5 μ M ATP, 2 μ Ci of (δ -³²P)-ATP).

12. Incubate the reaction with 2 μg of ATF-2 fusion protein for 30 min at 30°C with constant agitation.
13. Pellet agarose beads for 10 min at 4°C at 1500 g in a microcentrifuge.
14. Transfer 30 μL of the supernatant onto a 2.1-cm diameter Whatman P81 cellulose phosphate filter circles.
15. Wash circles four times for 10 min with 3 mL of 175 mM phosphoric acid and once with 3 mL distilled water for 5 min.
16. Air-dry filters and then measure radioactivity in a liquid scintillation counter (*see Note 11–15*).

3.5. Cyclin D1 Reporter Assay (16)

The cyclin D1 gene encodes a labile growth factor and oncogene-inducible regulatory subunit of the holoenzyme that phosphorylates and inactivates the pRb protein. The abundance of cyclin D1 is rate-limiting in the induction of DNA synthesis by diverse mitogenic stimulus (47). The cyclin D1 gene is transcriptionally induced by mitogenic stimuli, including *Ras*, *Src*, *ErbB2*, and activated ERK, suggesting that the cyclin D1 promoter is a useful reporter of mitogenic intracellular signaling activity (16–50).

3.5.1. Preparation of Cells for Transient Transfection

1. Plan the transfection experiment. For example, in this experiment, we will examine the effect of Her2/Neu signaling on the cyclin D1 transcription in MCF-7 breast cancer cells. We also want to know if Her2/Neu regulates cyclin D1 through a MAPK pathway and will examine this possibility by using the MEK inhibitor, PD098059 (*see Note 16*). In this protocol, we will use a reporter assay to address these questions. Cells will be transiently transfected with the mammalian expression vector of NeuT with cyclin D1 reporter construct, -1745 cyclin D₁-LUC. *c-fos*-LUC will be used as a positive reporter control because it is known that Her2/Neu signaling upregulates *c-fos* expression (51). Cyclin A-LUC will be used as a negative control. All transfections will be done in triplicate. The transfection plan is shown **Fig. 1** (*see Notes 17–19*).
2. Subculture MCF-7 cells for transfection. A day before transfection, seed 0.4×10^5 MCF-7 cells per well in 400 μL DMEM supplemented with 10% FBS into 24-well plates. By the time of transfection, the cells should reach 50–70% confluence (*see Note 20*).

3.5.2. Transient Transfection

1. For each well, dilute 1.2 μg reporter plasmid DNA along with either pSV2 control vector (75 ng) or expression vector pSV2-NeuT (100 ng) in 50 μL

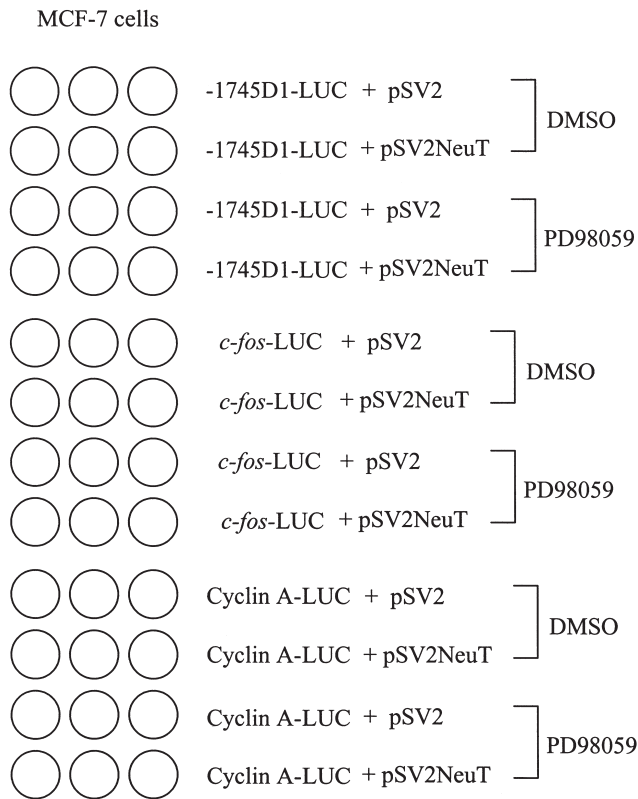


Fig. 1. Example of the transfection plan.

- DMEM (serum and antibiotic free medium). Mix well and sit at room temperature for 2 min.
2. Dilute 3–5 μL SuperFect reagent for each well in 50 μL DMEM (serum- and antibiotic-free medium). Mix well and sit at room temperature for 2 min.
 3. Combine the diluted DNA with diluted SuperFect, mix by pipeting up and down three to five times, and incubate at room temperature for 10 min to allow formation of the DNA-SuperFect complex.
 4. Add 100 μL of the transfection complexes directly into each well containing cell-growth medium. For SuperFect reagent, it is not necessary to change the cell-growth medium to serum- and antibiotic-free medium at this point. However, consult the manufacturer’s manual for transfection conditions with different reagents. Mix well by shaking the cell culture plate gently. Incubate the cells in a CO_2 incubator for 24 h.

3.5.3. Treatment of the Cells With PD98059

Twenty-four hours after transfection, replace the medium with 500 μ L fresh culture medium containing either DMSO (negative control) or 10 μ M PD98059. Incubate the cells for another 24 h (see **Note 21–23**).

3.5.4. Luciferase Assay

1. Lyse cells by aspirating the medium from the culture plate and adding 100 μ L of cell-extraction buffer. Rotate or shake the cells on a shaking platform at room temperature for 5–10 min.
2. For each sample, add 300 μ L of ATP assay buffer into luciferase assay tubes. Prepare six extra tubes as blank controls.
3. Transfer 100 μ L of cell lysate into the tube containing the ATP assay buffer and mix.
4. Load the samples onto the luminometer and put the substrate injector into the luciferin container (protected from light with aluminum foil). Make sure that the injector is submerged into the luciferin solution.
5. Measure the integrated light output for 10–60 s. At the end, wash the tubing of the luminometer with distilled water. (If renilla luciferin is used as an internal control, wash the tubing with 70% ethanol six times with six wash tubes and then repeat wash again with distilled water [52]).
6. Analyze the data statistically and graph as shown in **Fig. 2**.

4. Notes

1. Safety warnings and precautions: Because the experiments described here involve the use of radioactive (γ - 32 P)-ATP, be sure to follow your institutional regulations relating to the handling, usage, storage and disposal of such materials. Always use protective barriers.
2. Alternatively, stop the reaction by adding 20 μ L of 100 mM EDTA, pH 7.5, centrifuge briefly, and spot 40 μ L of each supernatant onto phosphocellulose paper. The papers are then washed six times (5–10 min each) in 10% phosphoric acid, soaked briefly in 100% ethanol, and air-dried before analysis in a liquid scintillation counter (II).
3. When heating samples on the heating block, make sure that the microcentrifuge tubes are closed tightly. Place another heating block on top of the tubes will prevent the tops from popping open.
4. When harvesting the cell lysate, be sure to aspirate the PBS buffer completely from the plates. Residual PBS will dilute the concentration of the protein inhibitors in the cell lysis buffer.
5. Keep reconstituted lysis buffer on ice at all times.

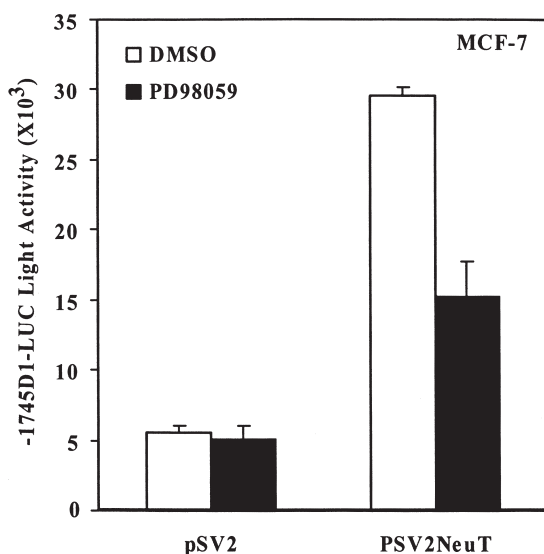


Fig. 2. Regulation of Cyclin D1 by NeuT. 0.4×10^5 MCF-7 cells are seeded into 24-well cell-culture plates and the cells are allowed to grow for 24 h to reach 70% confluence. 1.2 μ g of -1745D1-LUC and 100 ng of expression vector of NeuT and 75 ng of control vector are transfected as indicated using the proper transfection reagent. The cells are treated with 5 μ M PD98059 for 24 h and the luciferase activity is then measured.

6. Alternatively, incubate 300–600 μ g cell lysates with immobilized c-Jun (Cell Signaling Technology, cat. no. 9811) overnight at 4°C.
7. Pellet the agarose beads. Wash the immunoprecipitated products twice with the cell lysis buffer and twice with kinase buffer (**Subheading 2.3**).
8. Resuspend the pellets in the kinase buffer containing 100 μ M ATP.
9. Incubate the reaction for 30 min at 30°C in a shaking incubator.
10. Perform Western blot to detect the phospho-c-Jun signal.
11. Alternatively, stop the reaction by adding 30 μ L of 2X Laemmli sample buffer and heat for 5 min at 95°C (*see Note 3*).
12. Centrifuge at 12,000 rpm for 5 min.
13. Take 40 μ L of the supernatant and resolve on 13% SDS-PAGE gel.
14. Transferred onto polyvinylidene fluoride (PVDF) membranes.
15. Expose the PVDF membrane to a phosphorimager cassette and quantify the amount of radiolabeled phosphate substrate by using a molecular dynamics phosphorimager system.

16. **Table 1** summarizes the various inhibitors for different signaling pathways. Consult the literature for a more extensive list of inhibitors available for a particular pathway (5,28,33,34,36,37,39–44,46,53–79).
17. Different cell types may have a different genetic background. This is particularly true for the cancer cell lines. Careful selection of the cell type for studying a particular signal pathway is very important. For example, to study the effect of a signal pathway on the regulation of p53 expression, consider whether the p53 gene is expressed in the cell type you have chosen and whether or not the p53 gene is mutated. Also, consider whether the status of the particular pathway you are studying is altered (defective or constitutively active) in your chosen cells.
18. Transfection efficiency varies with cell types and different transfection reagents. Several internal control plasmids have been described, but these controls may independently affect activity of the promoter being assessed (52). We suggest using a green fluorescent protein (GFP)-expressing vector as a monitor of transfection efficiency.
19. If different cell types are used, a reporter control such as renillar luciferase (or β -galactosidase) should be included in order to adjust the data and make comparisons between different cell types.
20. Because the size of different cell types varies, the number of the cells to be seeded for transfection also varies. In general, the cells should reach 50–80% confluence by the time of transfection. Check the manufacture's manual for special requirements.
21. PD98059 is dissolved in DMSO or methanol. We use DMSO to make a 10 mM stock solution and store in small aliquot at -20°C . PD98059 should be protected from light. Always include the vehicle used to dissolve the inhibitor as a control.
22. For experiments where the ligand of hormone receptors, such as dihydrotestosterone (DHT) or estradiol are used, phenol-free medium and charcoal-stripped serum should be used when the cells are treated with hormones.
23. Treatment usually occurs after 24 h of transfection. The duration of the treatment varies depending on the reagents used and the targeted proteins or signaling pathways. Time-course and dose curves might be necessary.

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