Suggested Stimulation Conditions for Phosphoprotein Detection

Important Considerations when Stimulating Cells for Phosphoprotein Analysis

- Exogenous stimuli must be bioactive to induce a cellular response. Stimuli should be obtained from sources that have been tested for use in functional assays or reported in the literature.
- Many signaling responses vary considerably between cell types, with differences in the phosphorylation events induced, the response magnitude and kinetics, and the stimulus concentration required for a response. Additionally, responses in cell lines are not always representative of cognate primary cells.
- For some stimuli, the concentration required for optimal induction of a phosphorylation response varies between whole blood and PBMCs. Cell lines typically show phosphorylation responses at stimulus concentrations similar to those used for PBMCs.
- Cell signaling events occur with rapid kinetics. Most responses are maximal after 1 to 30 minutes at 37°C, with phosphorylation decreasing rapidly afterwards. The stimulation time required for an optimal phosphorylation response may vary with cell type, stimulus, and cell signaling molecule. Reverse time courses (staggering stimulation start times such that all samples are fixed simultaneously) are useful for studies of phosphorylation kinetics.
- Cellular stress can alter the signaling responses and/or the basal phosphorylation states of some signaling proteins. Prior to performing any cell signaling study, care should be taken to minimize cell manipulation. Rest periods may allow cells to recover from stressful harvest procedures (See the BD Phosflow™ Protocols for Human PBMCs and the BD Phosflow™ Protocols for Mouse Splenocytes or Thymocytes). However, the optimal recovery period varies by cell type and signaling pathway, and recovery periods can interfere with certain signaling responses. Polypropylene tubes are recommended to minimize cell adherence during recovery periods.
- When performing any cell signaling study in cell lines, cells should be healthy and subconfluent. Serum starvation may reduce the basal phosphorylation levels of many signaling proteins.
- Since Ca^{++} is required for many cell signaling responses, stimulations should be performed in complete media or PBS containing $CaCl_2$ and $MgCl_2$.
- Since stimulation kinetics are strongly influenced by temperature, use of a properly calibrated 37°C water bath, rather than a cell culture incubator, is recommended to allow cell suspensions to quickly equilibrate to 37°C for stimulation periods.

Recommendations provided below are for guidance only. Due to variation in methods and kinetics of activation for different cell types and phosphoproteins, stimulation conditions should be optimized for each application.

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Human Whole Blood and PBMCs

Stimulus	Recommended Concentration	Approximate Stimulation Time at 37°C	Vendor Information	Phosphorylation Events Induced*
IL-2	0.1 µg/mL	15 minutes	BD Cat. No. 554603	Stat5 pY694 (T, NK)
IL-4	0.1 µg/mL	15 minutes	BD Cat. No. 554605	Stat6 pY641
IL-6	0.1 µg/mL	15 minutes	BD Cat. No. 550071	Stat3 pY705 (T, mono), Stat1 pY701 (<; T, mono)
IFN-α	40,000 U/mL (whole blood) or 10,000 U/mL (PBMC)	15 minutes	PBL InterferonSource Cat. No. 11101-2	Stat1 pY701, Stat3 pY705 (<), Stat4 pY693 (<; T, NK), Stat5 pY694 (<), Stat6 pY641 (<)
PMA	400 nM (whole blood) or 40 nM (PBMC)	15 minutes	Sigma Cat. No. P8139	Stat1 pS727, Stat3 pS727, ERK1/2 pT202/pY204, p38 pT180/pY182, AKT pS473, NF-κB p65 pS529
LPS	10 µg/mL	15 minutes	Sigma Cat. No. L3137	p38 pT180/pY182 (mono)
CD3/CD28 Activation	See BD Phosflow™ Protocols for TCR Stimulation: Human	2–5 minutes	See BD Phosflow™ Protocols for TCR Stimulation: Human	CD3ζ pY142, LCK pY505, SLP-76 pY128

Mouse Splenocytes

Stimulus	Recommended Concentration	Approximate Stimulation Time	Vendor Information	Phosphorylation Events Induced*
IL-6	0.1 µg/mL	15 minutes	BD Cat. No. 554582	Stat3 pY705 (T, B), Stat1 pY701 (T)
IL-7	0.1 µg/mL	15 minutes	R&D Systems Cat. No. 407-ML-005	Stat5 pY694 (T)
IFN-α	1,000 U/mL	15 minutes	PBL InterferonSource Cat. No. 12100-1	Stat1 pY701
PMA	40 nM	15 minutes	Sigma Cat. No. P8139	Stat3 pS727, ERK1/2 pT202/pY204, p38 pT180/pY182, AKT pS473

* Weak or minor phosphorylation events are indicated by "<". For stimuli with considerable cell type selectivity, responsive cell types are listed: T cells (T), B cells (B), NK cells (NK), and monocytes (mono).

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