

## InstantONE ELISA<sup>®</sup>



### Technology

*InstantOne ELISA assays use the traditional sandwich ELISA format, but with a major difference. InstantOne allows for greater flexibility, ease of use, and reduced assay time by allowing the target analyte to bind to the both of the two sandwich ELISA antibodies in solution as the capture antibody binds to the plate through a proprietary mechanism. This allows for both the sample and the assay reagents to be added to the InstantOne ELISA assay microplate at the same time. Unbound assay reagents and non-specific sample components are washed away just as in a traditional sandwich ELISA, while the specific analyte is detected through a colorimetric detection reagent. The whole process can take just over 60 minutes to complete. In addition to the ease that the 1 hour/ 1 wash InstantOne ELISA provides, it also adds a layer of flexibility not readily accessible with traditional sandwich ELISAs. As the antibodies are not pre-coated in the wells, several different targets can be analyzed simultaneously in the same plate in different wells. Simply add the sample lysate to the plate wells and add different antibody reagent cocktails to the different wells. It has never been easier to analyze both total and phosphorylated MAP Kinase family members or across pathways (e.g. ERK and AKT) in the same plate.*

**InstantOne ELISA Assay Flow Chart:**

**1. Prepare Sample Lysate**



**2. Add Sample to InstantOne ELISA Microplate Wells**

50  $\mu$ L of Sample Lysate  
or  
50  $\mu$ L of Lysis Mix (Negative Control)  
or  
50  $\mu$ L of Control Lysate (Positive Control)



**3. Add Prepared Antibody Cocktail**

Add 50  $\mu$ L of freshly prepared antibody cocktail to each of the test wells

**Remove** the Detection Reagent from 4°C and let warm

**Incubate** 1 hour at room temperature while shaking at 300 rpm



**4. Wash Plate**

Wash plate wells 3 times with 200  $\mu$ L/well



**5. Add Detection Reagent**

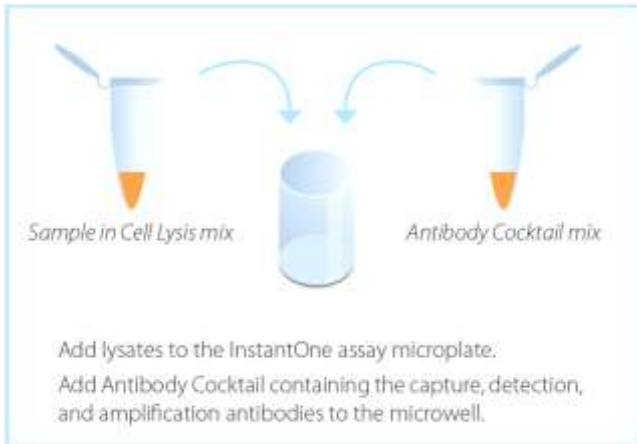
Add 100  $\mu$ L of Detection Reagent to each assay well run and incubate for 10-30 minutes



**6. Read Absorbance**

Add 100  $\mu$ L of Stop Solution and immediately read absorbance on colorimetric plate reader set to 450 nm

**InstantOne ELISA Assay Protocol Flow Chart:**



Sample in Cell Lysis mix      Antibody Cocktail mix

Add lysates to the InstantOne assay microplate.  
Add Antibody Cocktail containing the capture, detection, and amplification antibodies to the microwell.

1



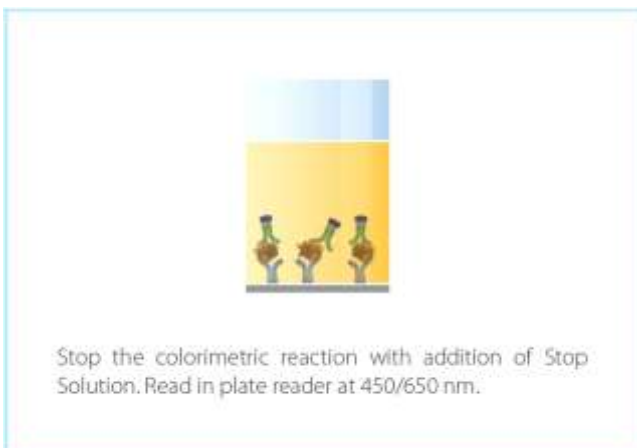
Incubate wells for 1 hour.  
Wash Microplate with included Wash Buffer.

2

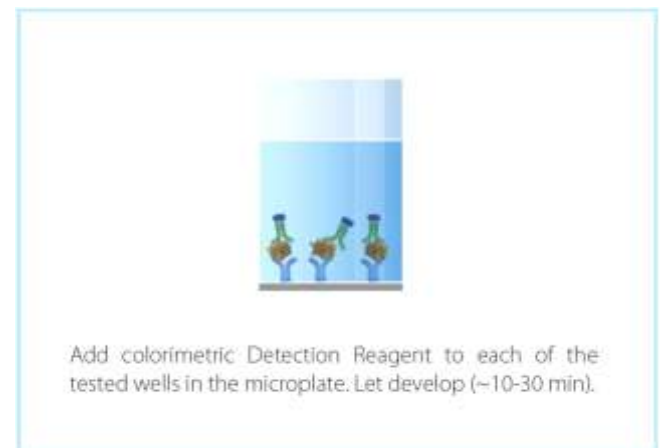
4



3



Stop the colorimetric reaction with addition of Stop Solution. Read in plate reader at 450/650 nm.



Add colorimetric Detection Reagent to each of the tested wells in the microplate. Let develop (~10-30 min).

## TARGET OVERVIEW:

### MAP Kinase Family

Target	Alternate Name	Analyte Description
<b>ERK 1/2</b>	p42/p44, MAP Kinase, MAPK, ERK1, ERK2	ERK, or MAP Kinases (Mitogenic Activated Protein Kinase), are the key kinases of the classical MAPK pathway. There are two ERK kinases, ERK1 (p44, MAP kinase 3) and ERK2 (p42, MAP kinase 2). Both are activated via the ERK/MAPK pathway, which are downstream of various Receptor Tyrosine Kinases (RTKs) or GPCRs. In the classical MAPK pathway, ligand binding results in the activation of an RTK. This initiates a signaling cascade that results in the activation of the GTPase Ras and the Ser/Thr kinase Raf (MAP Kinase Kinase Kinase). Raf then binds to and activates MEK (MAP Kinase Kinase) by phosphorylating it. MEK, a dual kinase (Ser/Thr and Tyr kinase), phosphorylates and activates ERK on the TXY motif of Thr202/Tyr204 and Thr185/Tyr187 for ERK1 and ERK2, respectively. Both phosphorylation sites are required for activation of ERK. There are no known mutations that exist for the constitutive activation of ERK 1/2. As such, detection of TXY dual phosphorylation is efficient to detect its activation as well as its upstream kinase, MEK, that has become a very popular drug target over the past number of years.  Once activated, ERK phosphorylates the PxS/TP motifs in many different proteins that regulate a large number of cellular processes that include cell division, proliferation, survival, differentiation, apoptosis, motility and metabolism. This is one reason why the MAPK signaling pathway is a highly sought after oncology drug target.
<b>p38<math>\alpha</math></b>	SAPK2A, Stress-activated protein kinase 2 $\alpha$ , MAPK14, Mitogen-activated protein kinase 14	p38 MAPKs, referred to as SAPK2/3/4 (Stress Activated Protein Kinases), are a sub-family of the JNK/SAP family of MAP Kinases. There are four isoforms of p38 MAPK, denoted $\alpha$ (SAPK2A), $\beta$ (SAPK2B), $\gamma$ (SAPK3b) and $\delta$ (SAPK4). p38 MAP Kinases are activated via phosphorylation of the TXY motif, just as the ERK and JNK kinases are. In the case of p38 $\alpha$ , it is the sites Thr180/Tyr182 that become phosphorylated that result in activation. p38 is activated as a result of cellular stresses, most notably inflammatory cytokines, irradiation, UV light, osmotic shock, lipopolysaccharides, and certain toxins such as anisomycin. The activating kinases of p38 MAPKs are MEK3 and MKK6. Once activated, p38 phosphorylates numerous targets that include the transcription factors such as ATF2 and ELK1, and kinases such as MAPKAPK2. As such, it plays a critical role in the production of many cytokines, including IL-6. SAPKs have been implicated in cellular responses that include inflammation, cancer, and neurodegenerative diseases.
<b>JNK 1/2/3</b>	c-Jun N-Terminal Kinase 1/2, MAPK8/9, Stress-activated protein kinase JNK1/2, SAPK 1/2	The JNK (c-Jun N-terminal Kinase) kinases are a family of MAP Kinases and part of the Stress-Activated Protein Kinase 1 (SAPK1) family. JNK is phosphorylated on the TXY motif on residues Thr183/Tyr185 and activated downstream of environmental stress and pro-inflammatory cytokines. This activation results in the phosphorylation of many downstream transcription factors that include the AP-1 family, such as Jun, as well as ATF2, and is required for the polarized differentiation of T cells into Th1 cells.

### AKT Pathway

Target	Alternate Name	Analyte Description
<b>AKT</b>	PKB, Protein Kinase B, RAC-PK-alpha	AKT is one of the principle kinases downstream of PI3 Kinase. The activation of PI3 Kinase and the resulting generation of PIP3 result in the subsequent phospholipid binding and activation AKT with its phosphorylation on residues Thr308 and Ser473 by PDK1 and the mTOR TORC2 complex, respectively. These two phosphorylations additively activate the AKT Ser/Thr kinase activity. There are over 50 known substrates for AKT that include GSK3, AS160, PRAS40, TSC1, TSC2, Raf-1, Bad, and PFK2, and the FoxO family of transcription factors. As such it has become one of the most highly sought candidates for oncology drugs.
<b>GSK3B</b>	Glycogen Synthase Kinase-3	GSK3 (Glycogen Synthase Kinase 3) is a Ser/Thr kinase that exists as two isoforms, GSK3 $\alpha$ and GSK3 $\beta$ . Unlike many other kinases, GSK3 is active in the absence of phosphorylation on residues Ser 9 and Ser21 for $\alpha$ and $\beta$ , respectively. GSK has been implicated in a number of diseases that include cancer and diabetes through its phosphorylation of Glycogen Synthase. GSK3 has also implicated in neurodegenerative diseases such as Alzheimer's disease through its kinase activity on Tau.
<b>p70 S6K</b>	Ribosomal protein S6 kinase beta-1, RPS6KB1, STK14A	p70 S6K is a member of the ribosomal S6 kinase family of Ser/Thr kinases. p70 S6K activity is controlled by multiple phosphorylation events, including phosphorylation of Thr389 in the linker domain, which is required for full activation. p70 S6K is regulated through multiple pathways, including the MAPK pathway, the phosphoinositide-3 kinase (PI3K) pathway, and the mTOR pathway. Activated p70 S6K phosphorylates several residues on the S6 ribosomal protein, which leads to an increase in protein synthesis.

### NFkB Pathway

Target	Alternate Name	Analyte Description
<b>NFkB p65</b>	Nuclear Factor Kappa B, p65 subunit, RelA, NFkB3	NFkB (Nuclear Factor kappa B) is a family of transcription factors with five members that includes Rel (c-Rel), RelA (p65), RelB, NFkB1 (p50 and its precursor p105), and NFkB2 (p52 and its precursor p100). NFkB members can exist as either homo- or heterodimers. NFkB dimers containing p65 are activators of transcription. In a majority of unstimulated cells, NFkB remains in its inactive form and is retained in the cytoplasm by the bound inhibitory I $\kappa$ B proteins. Upon stimulation by inducers such as TNF $\alpha$ , IL-1, or PMA, I $\kappa$ B $\alpha$ is phosphorylated and degraded. This results in the release of the NFkB complex from the I $\kappa$ B complex and the p105 subunit is cleaved into its active p50 form. Subsequently the p50/p65 translocates to the nucleus where it activates transcription of many genes, including its own inhibitor I $\kappa$ B $\alpha$ , causing an auto-regulatory mechanism of NFkB.  NFkB is known to regulate numerous genes that include cytokines, chemokines, adhesion targets, and acute phase proteins. These are involved in both cellular and physiological processes such as growth, development, apoptosis, immune and inflammatory response, and activation of various viral promoters.
<b>I<math>\kappa</math>B</b>	kappa B	I $\kappa$ B proteins are present in the cytosol where they are bound to NFkB/Rel transcription factors to form an inactive complex. For NFkB to become activated, it must first disassociate from the inhibitor I $\kappa$ B. This occurs via the phosphorylation of I $\kappa$ B on Ser32 and Ser36. I $\kappa$ B phosphorylation is stimulated by many extracellular signals that include inflammatory cytokines, growth factors and chemokines. This phosphorylation marks I $\kappa$ B for ubiquitination and destruction by the proteasome. This results in the release of NFkB from the complex and its subsequent translocation into the nucleus. As such the use of these phosphorylation sites has been widely used as a good marker of NFkB activation.
<b>IKK<math>\alpha</math></b>	I-kappa-B kinase alpha, I $\kappa$ BKA, CHUK	IKKs (I $\kappa$ B kinases), IKK $\alpha$ (IKK1) and IKK $\beta$ (IKK2), phosphorylate and trigger the degradation of the cytoplasmic NFkB inhibitors I $\kappa$ B (I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ and I $\kappa$ B $\gamma$ ). This phosphorylation and subsequent protein degradation results in the release of NFkB proteins from its inhibitory complex and thus allows for the translocation of NFkB into the nucleus.

#### STAT Family

Target	Alternate Name	Analyte Description
<b>STAT</b>	Signal transducer and activator of transcription	STATs (Signal Transducers and Activators of Transcription) are transcription factors that are primarily activated by the JAK kinase in response to various stimuli such as cytokines. STATs are able to increase the transcriptional activity of various genes in a matter of minutes. In non-stimulated cells, STAT exists in an inactive state in the cytoplasm. Molecules such as cytokines, growth factors, and some peptides, bind to cell surface receptors and activate tyrosine kinases causing the phosphorylation of the STATs. Upon STAT activation, most STATs, with the exception of 2 and 6, form homodimers. STAT1 and STAT2, as well as STAT1 and STAT3, are known to form heterodimers. After dimerization, the STATs translocate into the nucleus. Dephosphorylation of the STATs in the nucleus occurs rapidly and triggers its transport back into the cytoplasm.
<b>STAT 1</b>	Signal transducer and activator of transcription 1-alpha/beta, Transcription factor ISGF-3 components p91/p84	STAT1 is known to mediate IFN (interferon) signaling. STAT1 accomplishes this by either forming a heterodimer with STAT2 as a result of Type I IFN (IFN $\alpha$ and IFN $\beta$ ) binding that activates the JAK kinase or through homodimerization in response to IFN $\gamma$ activation. In the canonical STAT activation, JAK kinases are activated downstream of cytokine signaling and phosphorylate various STAT family proteins. This phosphorylation allows for the dimerization of the STATs through their SH2 domains. This dimer then binds other proteins and translocates into the nucleus where it binds to DNA, most notably, to the interferon stimulated response element (ISRE) that drives the cell into an antiviral state.
<b>STAT 3</b>	Signal transducer and activator of transcription 3, Acute-phase response factor, APRF	STAT3 phosphorylation has been found to be induced by many stimuli. STAT3 is phosphorylated on Tyr705 by activated JAK kinases or by various receptor tyrosine kinases. This phosphorylation induces STAT dimerization and translocation into the nucleus. STAT3 $\alpha$ , but not STAT3 $\beta$ , is also phosphorylated on Ser727, which enhances its transcriptional activity. STAT3 is believed to be one of the main mediators of IL-6 signaling. STAT3 has also been linked to many cellular activities that are linked to tumor progression. STAT3 suppression results in impaired apoptosis, impaired cell migration, as well as the up-regulation of transcription of angiogenic proteins and immune suppressive proteins.
<b>STAT 5</b>	Signal transducer and activator of transcription 5A/B	STAT5 (STAT5A and STAT5B) are activated by tyrosine phosphorylation, usually by JAK kinases, on Tyr694 and Tyr699 for STAT5A and STAT5B, respectively. STAT5A and STAT5B show differential, cell-specific regulation. STAT5A expression is predominantly in mammary tissue, while STAT5B expression is more abundant in muscle and liver tissues. STAT5 plays an integral role in immune cell development and regulation, and is an important mediator of IL-2 and IL-15 signaling in regulatory T cells.

#### SMADs

Target	Alternate Name	Analyte Description
<b>SMAD 1</b>	Mothers against decapentaplegic homolog 1, MAD homolog 1, Mad-related protein 1, SMAD family member 1, Transforming growth factor-beta-signaling protein 1	Smad1 falls into the class of receptor-regulated Smad proteins along with Smads 2, 3, 5, and 9. These Smads couple to specific receptors and are phosphorylated by those receptors. Smad1, 5, and 8 are activated via signals from the BMP/GDF (bone morphogenetic proteins/growth differentiation factor) family. Under the canonical Bone Morphogenetic Protein (BMP) signaling, mediated via the activated BMP receptor kinase/ALK type I receptors, Smad1, 5 and 8 are phosphorylated in their SxS motif. The phosphorylated form of Smad1 forms a complex with Smad4, which is important for its function in the transcription regulation. These Smads are involved in a range of biological activities including cell growth, differentiation, apoptosis, morphogenesis, development, and immune responses.
<b>SMAD 2/3</b>	Mothers against decapentaplegic homolog 2, MAD homolog 2, MADH2	Smad 2 and 3 are considered receptor-activated Smads. Smad 2 and 3 (Smad2/3) are phosphorylated downstream of TGF $\beta$ that is believed to help regulate its activity. In response to a TGF $\beta$ signal, Smad2/3 are phosphorylated by the TGF $\beta$ receptors. The phosphorylation induces the dissociation of this protein with SARA and the direct association with the family member Smad4. Phosphorylated Smad2/3, along with Smad4, translocates to the nucleus to elicit the transcription of other genes. In the nucleus, it is believed to be involved in the regulation of multiple cellular processes that include apoptosis, cell proliferation, and differentiation.

#### Additional Targets $\beta$ -Catenin, Jun, CREB, p53

Target	Alternate Name	Analyte Description
<b><math>\beta</math>-Catenin</b>	CTNNB1, CTNNB, Catenin beta-1	$\beta$ -Catenin lies downstream of the WNT/Frizzled ligand/receptor activation pathway. $\beta$ -Catenin binds to the cytoplasmic domain of E-Cadherin, a protein that helps govern cell-cell adhesion. Excess $\beta$ -Catenin that is not acting in a structural role is associated with a number of proteins including the tumor suppressor Adenomatous Polyposis Coli (APC). GSK3 phosphorylates both $\beta$ -Catenin and APC marking them for destruction. Upon Wnt binding to its receptor Frizzled, GSK3 becomes phosphorylated, thus reducing its kinase activity. Phosphorylation of $\beta$ -Catenin is reduced and is no longer destroyed and enters into the nucleus. It is here that $\beta$ -Catenin functions as a co-activator of the TCF/LEF (T-Cell Factors/Lymphocyte-enhancer Factor) transcription factor family. This results in the activation of TCF responsive genes which are thought to play key roles in development and cancer progression.
<b>CREB</b>	Cyclic AMP-responsive element-binding protein 1	CREB (cAMP response element-binding protein) is a transcription factor that stimulates the expression of numerous genes in response to growth factors, hormones, neurotransmitters, ion fluxes, stress signals. CREB can homodimerize or form heterodimers with related family members such as CREB and ATF. CREB is activated downstream of extracellular ligand binding to cell surface receptors that relay their messages through various intracellular second messengers. As a result CREB is phosphorylated on Ser133; most notably by PKA (cAMP-dependent protein kinase). In its non-activated form, PKA resides in the cytoplasm as an inactive heterotetramer of paired regulatory and catalytic subunits. Stimulation of cAMP causes the release of the catalytic subunits of PKA thereby allowing phosphorylation of CREB on Ser133. Other kinases are believed to phosphorylate CREB as well, including MAPK.
<b>p53</b>	TP53, Cellular tumor antigen p53	p53, often referred to as the "guardian of the genome", is a tumor suppressor that can be induced by a range of stresses through transcriptional, post-transcriptional, and post-translational control mechanisms. It is believed to be fundamental at preventing tumor development, and it has been stated to be involved in 50% of all cancers (although this is somewhat debated). One of its functions is that of a transcription factor. In this capacity, p53 has many cellular effects that include apoptosis, cell cycle regulation, senescence, metabolism, angiogenesis, immune response, differentiation, and, migration. p53 is phosphorylated on multiple sites, including Ser15, which is a result of DNA damage, and is involved with many of the activities mentioned above.
<b>Jun</b>	Transcription factor AP-1, c-JUN	Jun is a member of the Activator Protein-1 (AP-1) family of transcription factors. The AP-1 family members can form homodimers with other Jun family members or itself or heterodimers with Jun and Fos family members. AP-1 transcription factors are highly regulated in normal cells. Phosphorylation of the AP-1 family members is required for their transcriptional activity. Once activated by stimuli, phosphorylated Jun/Jun or Jun/Fos dimers bind to DNA.  Jun is activated as either a homo- or hetero-dimers in response to growth factors, cytokines, and various other intra- and extracellular signals. This can result in the canonical Jun activation via JNK as it associates with and phosphorylates c-Jun on Ser63 and Ser73.