InstantOne ELISA™ 96 Well Test Manual

Doc. Part No. ELISA05673-1 **Pub. No.** MAN0017040 **Rev.** A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

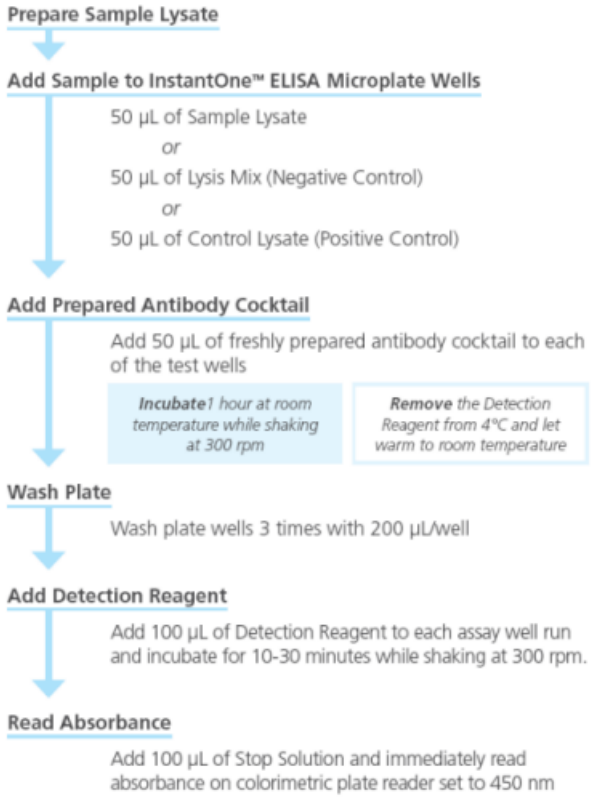
Assay principle

InstantOne ELISA™ assays use the traditional sandwich ELISA format, but with a major difference. InstantOne ELISA™ allows for greater flexibility, ease of use, and reduced assay time by allowing the target analyte to bind to 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 nonspecific sample components are washed away just as in a traditional sandwich ELISA, while the specific analyte is detected though a colorimetric detection reagent. The whole process can take just over 60 minutes to complete.

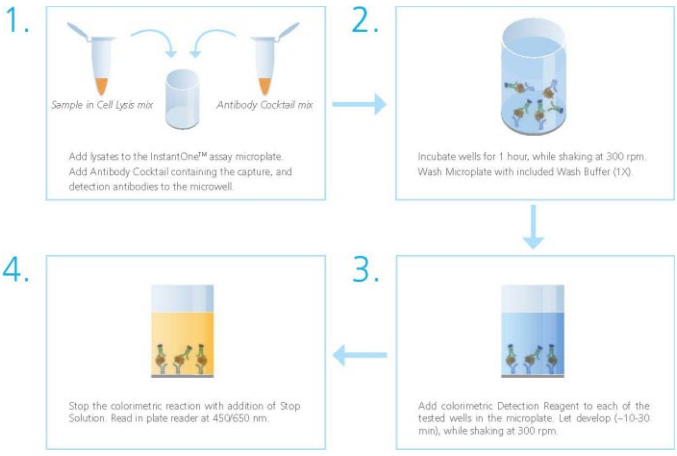
In addition to the ease that the 1-hour/1-wash InstantOne ELISA™ provides, it also adds a layer of flexibility not readily accessible with traditional sandwich ELISAs. As the antibodies are not precoated in the wells, several different targets can be analyzed simultaneously in the same plate in different wells. Simply add the sample lysate to the plate wells and add different antibody 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 overview

InstantOne ELISA™ assay workflow



InstantOne ELISA™ assay protocol workflow



Target overview

MAP kinase family

Target	Alternate name	Analyte description
ERK 1/2	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 is 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. When 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.
p38α	SAPK2A, Stress-activated protein kinase 2α, MAPK14, Mitogen-activated protein kinase 14	The p38 MAPKs, referred to as SAPK2/3/4 (Stress Activated Protein Kinases), are a subfamily of the JNK/SAP family of MAP kinases. There are four isoforms of p38 MAPK, denoted α (SAPK2A), ß (SAPK2B), γ (SAPK3b), and Δ (SAPK4). The p38 MAP kinases are activated via phosphorylation of the TxY motif, just as the ERK and JNK kinses are. In the case of p38α, it is the sites Thr180/Tyr182 that become phosphorylated that result in activation. The p38 MAPK 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. When 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.
JNK 1/2	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 proinflammatory 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
AKT	PKB, Protein Kinase B, RAC-PK-α	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, TSC 1, TCS 2, 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.
GSK3ß	Glycogen Synthase Kinase-3	GSK3 (Glycogen Synthase Kinase 3) is a Ser/Thr kinase that exists as two isoforms, GSK3α and GSK3ß. Unlike many other kinases, GSK3 is active in the absence of phosphorylation on residues Ser 9 and Ser21 for α and ß, respectively. GSK has been implicated in a number of diseases that include cancer and diabetes through its phosphorylation of Glycogen Synthase. GSK3 has also been implicated in neurodegenerative diseases such as Alzheimer's disease through its kinase activity on Tau.
p70 S6K	Ribosomal protein S6 kinase ß-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.
S6RP	40S ribosomal protein S6,RPS6, Phosphoprotein NP33	The S6 Ribosomal Protein (S6RP) is a subunit of the 40S ribosome and is involved in translation of particular classes of mRNA. A key kinase regulating phosphorylation of S6RP is p70 S6 Kinase. Phosphorylation can occur on multiple sites including Ser235, 236, 240, and 244. Phosphorylation is mitogen activated, and dephosphorylation occurs under growth arrest conditions, including rapamycin treatment. The cellular functions of S6RP appear to include roles in cellular growth and cell cycle progression.

NF-κB pathway

Target	Alternate name	Analyte description
NF-κB p65	Nuclear Factor κB, p65 subunit, RelA, NF-κB3	NF-κB (Nuclear Factor κB) is a family of transcription factors with five members that includes Rel (c-Rel), RelA (p65), RelB, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100). NF-κB members can exist as either homo- or heterodimers. NF-κB dimers containing p65 are activators of transcription. In a majority of unstimulated cells, NF-κB remains in its inactive form and is retained in the cytoplasm by the bound inhibitory IkB proteins. Upon stimulation by inducers such as TNFa, IL-1, or PMA, IkBa is phosphorylated and degraded. This results in the release of the NF-κB complex from the IKK 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-κBα, causing an auto-regulatory mechanism of NF-κB. NF-κB 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. NF-κB 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.
I-κB	I-κB	I-κB proteins are present in the cytosol where they are bound to NF-κB/Rel transcription factors to form an inactive complex. For NF-κB to become activated, it must first disassociate from the inhibitor I-κB. This occurs via the phosphorylation of I-κB on Ser32 and Ser36. I-κB phosphorylation is stimulated by many extracellular signals that include inflammatory cytokines, growth factors, and chemokines. This phosphorylation marks I-κB for ubiquination and destruction by the proteosome. This results in the release of NF-κB 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 NF-κB activation.

STAT family

Target	Alternate name	Analyte description
STAT	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 nonstimulated 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 tranlocated into the nucleus. Dephosphorylation of the STATs in the nucleus occurs rapidly and triggers its transport back into the cytoplasm.
STAT1	Signal transducer and activator of transcription 1-α/ß, 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-α and IFN-ß) binding that activates the JAK kinase or through homodimerization in response to IFN-γ 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.
STAT3	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 α, but not STAT3 ß, 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.
STAT5	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
SMAD1	Mothers against decapentaplegic homolog 1, MAD homolog 1, Mad-related protein 1, SMAD family member 1, transforming growth factor-ß signaling protein 1	SMAD1 falls into the class of receptor-regulated Smad proteins along with SMAD2, 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 Morphogenic 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.
SMAD3	Mothers against decapentaplegic homolog 2, MAD homolog 2, MADH2	SMAD2 and 3 are considered receptor-activated SMADs. SMAD2 and 3 are phosphorylated downstream of TGF-ß that is believed to help regulate its activity. In response to a TGF-ß signal, SMAD2 and 3 are phosphorylated by the TGF-ß receptors. The phosphorylation induces the dissociation of this protein with SARA and the direct association with the family member Smad4. Phosphorylated SMAD2 and 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

Target	Alternate name	Analyte description
ß-Catenin	CTNNB1, CTNNB, Catenin beta-1	ß-Catenin lies downstream of the WNT/Frizzled ligand/receptor activation pathway. ß-Catenin binds to the cytoplasmic domain of E-Cadherin, a protein that helps govern cell-cell adhesion. Excess ß-Catenin that is not acting in a structural role is associated with a number of proteins including the tumor suppressor Adenomatous Polypopsis Coli (APC). GSK3 phosphorylates both ß-Catenin and APC marking them for destruction. Upon Wnt binding to its receptor Frizzled, GSK3 becomes phosphorylated, thus reducing its kinase activity. Phosphorylation of ß-Catenin is reduced and is no longer destroyed and enters into the nucleus. It is here that ß-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.
CREB	Cyclic AMPresponsive elementbinding 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, and stress signals. CREB can homodimerize or form heterodimers with related family members such as CRaEB 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 it non-activated form, PKA resides in the cytoplasm as an inactive heterotetramer of paired regulatory and catalytic subunits. Stimulation of cAMP causes the releases of the catalytic subunits of PKA, thereby allowing phosphorylation of CREB on Ser133. Other kinases are believed to phosphorylate CREB as well, including MAPK.
p53	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.
Jun	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. When 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. The can result in the canonical Jun activation via JNK as it associates with and phosphorylates c-Jun on Ser63 and Ser73.

Assay kit components and storage

Components

Reagents and wells for 96 reactions

Quantity: Sufficient reagents and wells to perform 96 reactions per kit.

Alternatively: Both 384-well and nonstrip-well plate formats are available upon request for use in high throughput and automation settings. Additional Wash Buffer (10X) and Cell Lysis Buffer (5X) are also available. Contact Technical Support for further information.

- **InstantOne ELISA™ Assay Plate:** One (1) 96-well strip-well plate specifically designed and manufactured for this assay. Use only InstantOne ELISA™ Assay plates for InstantOne ELISA™. The plate is specifically designed to work with this assay and cannot be substituted with other 96-well microplates. Plates should be stored at 2–8°C. Allow plate to equilibrate to room temperature prior to opening the pouch, to minimize condensation from forming in the wells. Unused wells should be stored dry at 2–8°C and used within 1 month of opening the microplate foil bag.

  Note: Nonstrip-well format and 384-well versions of the plate are available for special purchase. Contact Technical Support for further information.

- **Cell Lysis Buffer:** The Cell Lysis Mix is a combination of the Cell Lysis Buffer and Enhancer Solution. The Cell Lysis Buffer (5X) contains a combination of detergents, phosphatase inhibitors, salts, and buffers. Cell Lysis Buffer (5X) is supplemented with Enhancer Solution to yield a versatile Cell Lysis Mix that can be applied to many cells and tissues. Note the difference in names. Cell Lysis Mix is referred to heavily in the assay protocol.
  - The Cell Lysis Mix (5X) is used to lyse cells in the presence of culture medium and is typically used to lyse nonadherent cells.
  - The Cell Lysis Mix (1X) is used to lyse cells after the removal of culture medium, and is typically used to lyse adherent cells or nonadherent cells that have been harvested by centrifugation. Cell Lysis Mix (1X) should be used as the diluent for any dilution of cellular lysates that are required.

  Note: Supplementing Cell Lysis Mix with extra components (e.g., protease inhibitors, chelating agents, detergents) should be tested on a case-by-case basis for compatibility with InstantOne ELISA™ assays.
- **Wash Buffer (10X):** The Wash Buffer, supplied as a 10X concentrate, is used for washing the InstantOne ELISA™ assay microplate. It is a simple mix of buffer, salts, and mild detergent. Alternatively, a PBS, 0.05% (v/v) Tween™ 20 solution may be substituted as a wash solution. If washing wells with a microplate washer, use 3X washes with a 10-second mixing cycle.
- **Detection Reagent:** The emission filter should be in the range of 450 nm, with bandwidths ≤30 nm. The signal in the wells should be developed for around 15 minutes. Best results will be obtained if the microplates are developed in the dark (e.g., by covering the microplate with foil). It is recommended to protect the plate from light while undergoing development.
- **Stop Solution:** The Stop Solution is used for stopping HRP-mediated colorimetric conversion. When added to the wells, the HRP enzyme activity stops and the detection reagent turns from blue to yellow with deeper yellow indicating a higher concentration of target over a lighter development. The plate should be read immediately after the addition of the stop solution.

WARNING! Take caution because the Stop Solution is acid.

Part. No.	Description	Amount
IOAP96	InstantOne ELISA™ Assay Plate	1 × 96 wells
IOCLB1	Cell Lysis Buffer (5X)	1 × 10 mL
IOES1	Enhancer Solution	1 × 1 mL
IOWB1	Wash Buffer (10X)	1 × 15 mL
IODR1	Detection Reagent	1 × 12 mL
IOSS1	Stop Solution	1 × 12 mL
IOPS	Plate Seals	1

Assay target specific reagents

- **Capture Antibody Reagent (Part No. Kit Specific):** Contains the Capture Antibody Reagent that will be mixed in equal parts to the Detection Antibody Reagent to yield the Antibody Cocktail (ELISA antibody sandwich pair).
- **Detection Antibody Reagent (Part No. Kit Specific):** Contains the Detection Antibody Reagent that will be mixed in equal parts to the Capture Antibody Reagent to yield the Antibody Cocktail (ELISA antibody sandwich pair). The Antibody Cocktail can be prepared by adding an equal volume of Capture Antibody Reagent and Detection Antibody Reagent, and mixing by inversion prior to each experiment.
- **Positive Control Cell Lysate (Part No. Kit Specific):** Positive Control Cell Lysate is prepared from various cell types, which have been cultured and prepared to optimize the activation of the intracellular pathway of interest.
  - The Positive Control Cell Lysate is intended for use as an assay positive control only, and should not be used for the absolute quantification of a particular protein or phosphorylated target. In combination with negative control wells containing Cell Lysis Mix (1X) only, the Positive Control Cell Lysate can be used to give an indication of the expected signal range for a given assay.
  - The Positive Control Cell Lysate controls are supplied lyophilized, and should be reconstituted with 250 µL of reagent grade dd H_2O. If required, Positive Control Cell Lysate can be further diluted with Cell Lysis Mix (1X), and frozen at less than –20°C in aliquots for subsequent use.

	Single target kits	Total/phospho kits	Triple target kits
Capture Antibody Reagent 1	1 × 3 mL	1 × 1.5 mL	1 × 1.0 mL
Capture Antibody Reagent 2	—	1 × 1.5 mL	1 × 1.0 mL
Capture Antibody Reagent 3	—	—	1 × 1.0 mL
Detection Antibody Reagent 1	1 × 3 mL	1 × 1.5 mL	1 × 1.0 mL
Detection Antibody Reagent 2	—	1 × 1.5 mL	1 × 1.0 mL
Detection Antibody Reagent 3	—	—	1 × 1.0 mL
Positive Control Lysate	1 × 0.25 mL (lyophilized)	1 × 0.25 mL (lyophilized)	1 × 0.25 mL (lyophilized)

Materials required but not supplied

- Colorimetric plate reader capable of detecting 450 nm
- Multichannel pipet (optional)
- Reagent grade water

Storage conditions

Store kit components at the temperatures indicated on the labels. When handled as described below, the kit is stable for 6 months from date of receipt.

Store all reagents at 2–8°C. Do NOT freeze the kits.

Unopened kit	Item	Handling
Opened/reconstituted kit reagents	Capture Antibody Reagent	Store at +2–8°C after opening, until expiry date.
	Detection Antibody Reagent	
	Cell Lysis Buffer (5X)	
	Enhancer Solution	
	Wash Buffer (10X)	
	Detection Reagent	
	Stop Solution	
	InstantOne ELISA™ Assay Microplate	Return unused wells to foil pouch with dessicant pack, and seal with tape. May be stored at 2–8°C for up to 1 month after opening.
	Positive Control Cell Lysate	Aliquot and store at <–20°C for up to 1 month.

Assay preparation

Buffer preparation

Note: Avoid vortexing the Capture Antibody Reagent or Detection Antibody Reagent, as vigorous mixing can damage some antibodies.

Reagent	Instructions
Cell Lysis Mix (5X)	Prepare Cell Lysis Mix (5X) by diluting Enhancer Solution 10-fold in Cell Lysis Mix (5X) (e.g., mix 900 µL Cell Lysis Buffer (5X) and 100 µL Enhancer Solution). Cell Lysis Mix (5X) is used to directly lyse cells in cell culture medium. Prepare immediately prior to use. Discard unused Cell Lysis Mix (5X). Control Cell Lysate can be used to give an indication of the expected signal range for a given assay.
Enhancer Solution	Supplied as a concentrate. Precipitation will occur during storage at 4°C, which is normal. To re-dissolve, warm to 37°C and mix by inversion prior to use.
Cell Lysis Mix (1X)	Prepare immediately prior to use. Discard unused Cell Lysis Mix (1X). Cell Lysis Mix (1X) is used to directly lyse cells when the medium has been aspirated. Prepare Cell Lysis Mix (1X) by diluting Cell Lysis Mix (5X) prepared (as instructed above) 5-fold in ddH_2O (e.g., mix 200 µL 5X Lysis Buffer and 800 µL ddH_2O).
Antibody Cocktail[1] (Capture Antibody Reagent + Detection Antibody Reagent)	Prior to each experiment, prepare by adding an equal volume of Capture Antibody Reagent and Detection Antibody Reagent, and mixing by inversion. Prepare enough to use 50 µL/well (e.g., for a single 8-well strip, prepare 400 µL Antibody Mix).
Positive Control Cell Lysate	The control is supplied lyophilized, and should be reconstituted with 250 µL of ddH_2O. If required, control lysates can be further diluted with Cell Lysis Buffer Mix (1X). When reconstituted, aliquot and store at <–20°C for up to 1 month.
Wash Buffer (10X)	Dilute Wash Buffer (10X) to 1X with ddH_2O (e.g., mix 1 mL of Wash Buffer (10X) and 9 mL of water). Prepare at least 600 µL of Wash Buffer (1X) per well to allow for the 3 washes.
Detection Reagent	Allow Detection Reagent to warm to room temperature prior to use for optimal assay performance.

[1] Bring all reagents to room temperature before use.

Assay protocols

Method 1: Prior sample preparation protocol

Use this method when samples are grown, treated/stimulated, and lysed prior to the assay.

Sample preparation

1. For adherent cultured cells, remove any media from the cells and gently wash cells with PBS.
   a. For cells cultured in 96-well microplates, lyse the cells with 100 µL of freshly prepared Cell Lysis Buffer Mix (1X).

      Lysis volume should be adjusted depending on the desired lysate concentration. Lysates in the range of 0.1–0 .5 mg/mL protein are usually sufficient. However, preparing more concentrated lysates can help with the detection of low abundance analytes.

   b. Shake cells (~300 rpm) at room temp for 10 minutes.
2. For nonadherent cells, centrifuge the cells, gently remove the media while leaving the cells undisturbed. It is recommended, but not required, to wash the cells in PBS. Resuspend the cell pellet at an appropriate density in HBSS containing 5% FBS. A cell density that yields cellular lysate at a protein concentration of 0.1–0.5 mg/mL is suitable for many cell lines.

   Alternatively, resuspend cells in cell culture medium if necessary for the cells.
3. Return cells to a 37°C incubator for 1–2 hours.

   For certain pathways, this can allow handling-mediated pathway activation to subside. This step is optional, and depends on the activation status of your cells following resuspension.
4. At the completion of the treatment, lyse cells with 20% final volume of Cell Lysis Mix (5X), with shaking (~300 rpm) at room temp for 10 minutes (e.g., for 40 µL of cells, use 10 µL of Cell Lysis Mix (5X).
5. Alternatively cells can be harvested by centrifugation and lysed with Cell Lysis Mix (1X).

Assay protocol

Remove Detection Reagent from refrigerator and allow to equilibrate to room temperature.

1. Determine and remove the desired number of InstantOne ELISA™ microplate strips needed for the experiment including the Positive Control Cell Lysate and negative control. Return unused microplate well strips to storage pouch with desiccant and seal.
2. Add negative control, positive control, and sample lysate to assay wells
   a. Add 50 µL/well of prepared sample lysate (as described above) to be tested to each of the InstantOne ELISA™ assay wells.
   b. Add 50 µL/well of Cell Lysis Mix (1X) (negative control) and 50 µL/well of Positive Control Cell Lysate to separate wells for assay controls. The negative control can also act as the blank when the plate is read.
3. Add 50 µL/well of prepared Antibody Cocktail to each of the testing wells. Cover the microplate with adhesive seal and incubate for 1 hour at room temperature on a microplate shaker (~300 rpm).
4. Wash wells with 200 µL/well of Wash Buffer (1X) (repeat 3 times). After final wash, completely remove any remaining wash solution from wells by inverting on a paper towel.
5. Add 100 µL of the Detection Reagent to each of the wells. Incubate for 10–30 minutes with shaking at 300 rpm. Watch color development as high abundance targets/samples will take significantly less time than lower abundant targets.
6. Stop the reaction by adding 100 µL of Stop Solution to each well.
7. Read the plate by measuring the absorbance of the samples using a colorimetric (spectrophotometric) plate reader set at 450 nm. Plate should be read within 1 hour of adding the Stop Solution.

Method 2: All-in-one-well protocol

This protocol allows for the preparation of cellular lysates and their subsequent analysis in the InstantOne ELISA™ assay microplate. This protocol avoids lysate transfer steps.

Sample preparation

1. Harvest cells by centrifugation, and resuspend at an appropriate density in HBSS containing 5% FBS. A cell density that yields 10,000–25,000 cells/well (in 20 µL volume) is suitable for the analysis of many cell lines.

   Alternatively, resuspend cells in cell culture medium if necessary for the cells, using Method 1.
2. Determine the desired number of InstantOne ELISA™ assay plate strips needed. Remove unused strips from frame and return to storage pouch and with desiccant seal.

3. Rinse wells for use in the assay 2 times with sterile H_2O to remove preservatives.
4. Add 20 µL cells/well to the InstantOne ELISA™ assay microplate.
5. Incubate cells at 37°C for 1–2 hours. For certain pathways, this can allow handling-mediated pathway activation to subside. This step is optional, and depends on the activation status of your cells following resuspension.
6. Add 20 µL treatment/well to cells (e.g., 2X agonists and/or antagonists) for the desired time period. The final volume in wells prior to lysis should be 40 µL.
7. Directly add 10 µL Cell Lysis Mix (5X) (not prediluted) to each of the wells after desired treatment time with shaking (~300 rpm) at room temperature for 10 minutes. This results in cell lysis.

Assay protocol

Remove Detection Reagent from +4°C and allow to equilibrate to room temperature.

1. Add 50 µL/well of Cell Lysis Mix (1X) (negative control) and 50 µL/ well Positive Control Cell Lysate (positive control) to separate assay wells for controls.
2. Add 50 µL/well of prepared Antibody Cocktail to each of the testing wells. Cover the microplate with adhesive seal and incubate for 1 hour at room temperature on a microplate shaker (~300 rpm).
3. Wash wells with 200 µL/well of Wash Buffer (1X) (repeat 3 times). After final wash, completely remove any remaining wash solution from wells.
4. Add 100 µL of the Detection Reagent to each of the assay wells. Incubate for 10–30 minutes with shaking at 300 rpm. Watch color development as high abundance targets/samples will take significantly less time than lower abundant targets.
5. Stop the reaction by adding 100 µL of Stop Solution to each well.
6. Read the plate by measuring the absorbance of the samples using a colorimetric (spectrophotometric) plate reader set at 450 nm. Plate should be read within 1 hour of adding the stop solution.

Data analysis

- To analyze the data, calculate the averaged counts for untreated and treated cells. It is recommended to run the assay at least in duplicate wells (n = 2) to calculate a response, but triplicate is strongly advised.
- Dose response and dose inhibition curves can be fitted to 4 parameter nonlinear regression equations. These types of regression analyses output key parameters such as EC50 (or IC50), Min and Max signals, and Hillslope factors.
- Ensure that samples readings are within the linear range of the assay. This can vary based on reader performance, and analyte concentration. If a lysate sample generates a signal outside the linear range, the lysate samples should be diluted with Cell Lysis Mix (1X) and re-assayed.

Procedure limitations

- InstantOne ELISA™ kits are for Research Use Only. Not for use in diagnostic procedures.
- Do not use the kit reagents beyond the expiry stated on the label.
- Variations in general operator-related procedures, such as pipetting, washing, and incubation times, can cause variation in the final signal.
- The assay is designed to work for the detection of endogenous cellular proteins across a wide variety of cell lines. However, until each cell line in particular is tested, the possibility of the presence of interfering factors cannot be excluded.
- Users should ensure that their cell line has measurable levels of the pathway of interest. Expression levels of signaling proteins in different cell types vary widely.

Technical hints and troubleshooting

Issue	Solution
General cell handling	Cells should be harvested from flasks for seeding into microplates when approximately 70–90% confluent. The cells should be detached from the flasks using mild conditions (such as trypsin-free cell dissociation solutions where possible), accurately counted, and diluted to the appropriate density in fresh media. If using adherent cells, allow time for cells to regain full signaling capacity after harvesting.
Compatible cell types	The assay can be used for many adherent and nonadherent cell types, including transfected cell lines and primary cells. However, because kinase expression and phosphorylation conditions can vary from one cell line to another, some cells may be more amenable to particular assays than others. Parameters such as choice of stimulant, stimulation time, and cell number should be optimized for each cell line used. Cells over-expressing a receptor of interest have been shown to elicit good phosphorylation responses. When using overexpressed intracellular targets, ensure that the expressed target is full-length to ensure correct binding of assay antibodies. The concentration of cell lysate should be optimized to ensure the signal is within the working range of the assay.
Lysate viscosity and handling	Lysing cells with Cell Lysis Mix can yield lysates that are viscous and difficult to handle, particularly when concentrated lysates are required. Care should be taken when transferring lysates to minimize pipetting-related variability. To avoid this problem when using nonadherent cells, the cells can be transferred to the InstantOne ELISA™ assay microplate immediately prior to lysis, and subsequently lysed directly in the assay microplate (e.g., transfer 40 µL cells/well to an IInstantOne ELISA™ assay microplate, and lyse with 10 µL Cell Lysis Mix (5X)—see "Buffer preparation" on page 6). Alternatively, use the all-in-one-well assay procedure (see "Method 2: All-in-one-well protocol" on page 6).
Assaying for multiple targets from a single lysate	One of the features ofInstantOne ELISA™ protocols is the use of a common assay microplate for all assays. Therefore, a cellular lysate can be transferred to several replicate wells, and analyzed in parallel. This can enable a particular lysate to be sampled for both total and phosphorylated target on the same assay microplate, or for several assay targets on the same microplate.
Assay scalability	The InstantOne ELISA™ assay is optimized for equal 50 µL volumes of both the analyte and the Antibody Cocktail. However, in cases where lysate is limiting, as little as 25 µL of lysate can be used, in combination with 50 µL of Antibody Cocktail.
Assay incubation times	The general assay incubation times that are recommended are 1 hour after the addition of the Antibody Cocktail, and 15 minutes for the development of the Detection Reagent. Longer incubations (up to overnight) may be more convenient for certain assays, and can enhance sensitivity in some cases.
Buffer compatibility	Supplementing Cell Lysis Buffer with extra components (e.g., protease inhibitors, chelating agents, detergents) is typically okay, but should be tested on a case-by-case basis for compatibility with InstantOne ELISA™ assays. For example, strong detergents such as SDS can denature proteins, and is not recommended for use with InstantOne ELISA™ assays.
Lysis buffer formulation	1X Cell Lysis Buffer contains the following phosphatase inhibitors: • Sodium pyrophosphate • Sodium fluoride • Sodium orthovanadate The addition of other components to the Cell Lysis Buffer should be tested on a case-by-case basis.

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  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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