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Certificate of Analysis

Beadlyte® 3-Plex Apoptosis Signaling Kit

(Active Caspase 3, GAPDH, and Cleaved PARP)
Catalog # 48-670
Lot #

Kit Components

Beadlyte® 3-plex Apoptosis, beads, Catalog # 42-670, Lot #. One vial containing **131µL** of anti-Caspase 3, GAPDH, and PARP Luminex® Beads at 2400/µL (20x):

Protein	Bead #
Active Caspase 3	6
GAPDH	15
Cleaved PARP	60

Beadlyte® 3-plex Apoptosis, biotin, Catalog # 44-670, Lot #. One vial containing **131µL** of (**20X**) biotin-conjugated anti-Active Caspase 3, GAPDH, and Cleaved PARP (Asp214) IgG in a proprietary formulation of Tris-buffered salts and animal protein containing 0.05% sodium azide as a preservative.

Beadlyte® Cell Signaling Universal Lysis Buffer, Catalog # 43-040, Lot #. One vial containing **50mL** of a proprietary formulation of Tris buffered salts, detergents, and phosphatase inhibitors including 1mM sodium orthovanadate.

Beadlyte® Cell Signaling Assay Buffer 1, Catalog # 43-010, Lot #. One vial containing **50mL** of a proprietary formulation of Tris buffered salts and animal protein containing 0.05% sodium azide as a preservative.

Beadlyte® Streptavidin-Phycoerythrin, Catalog # 45-001, Lot #. One vial containing **100µL** of a 1mg/mL solution of Streptavidin-Phycoerythrin in PBS, pH 7.5, containing 0.02% sodium azide. **Do not freeze. Store in the dark.**

Beadlyte® Jurkat Cell lysate: Anisomycin, Catalog # 47-207, Lot #102407. One vial containing lyophilized cell lysate made from anisomycin-stimulated Jurkat cells lysed in Beadlyte® Cell Signaling Universal Lysis Buffer and protease inhibitors. Make fresh for each assay. Reconstitute in 200µl of ultrapure water.

Storage and Stability: Stable for 1 year at 4°C from date of shipment. **Store in the dark.**

Multi-screen plate (Catalog # MSBVN1250)

Empty mixing vials

FOR RESEARCH USE ONLY
DO NOT USE FOR DIAGNOSIS OF DISEASE IN HUMANS OR ANIMALS
DO NOT USE IN HUMANS OR IN ANIMALS

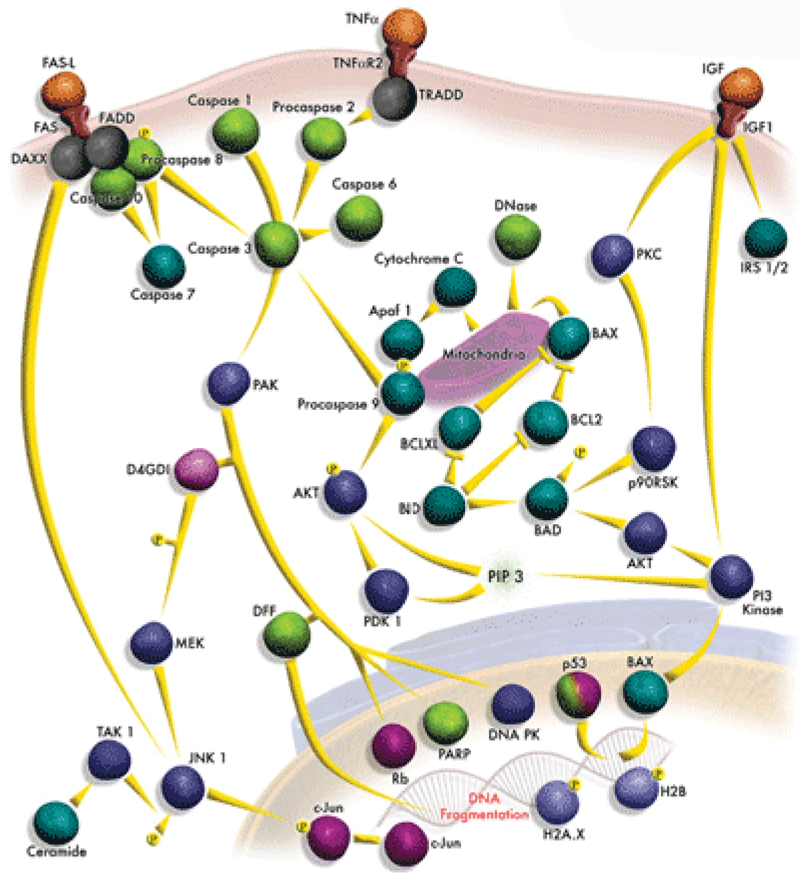
Other components required but not included as part of kit are:

- Cell lysates or cell extracts harboring protein(s) of interest
- Vortex mixer
- Plate shaker
- Timer
- Variable volume (5-200µL) pipette + tips
-
- Protease inhibitors (recommend *Complete protease inhibitor cocktail tablets*, Cat. 11 836 153 001, Roche Molecular Biochemicals, Inc., www.biochem.roche.com)
- Millipore multiscreen vacuum manifold (Catalog # MSVMHTS00)
- (Catalog # MSBVN1250)
- Luminex® System

3-plex Apoptosis Signaling Kit

The Beadlyte® 3-plex Apoptosis Signaling kit is used to detect changes in activated Caspase 3 and PARP in cell lysates using the Luminex® system. Total GAPDH is provided in the kit for total protein normalization. The detection assay is a rapid, convenient alternative to Western Blotting and immunoprecipitation procedures. Each kit has sufficient reagents for 100 individual assays.

Summary of Apoptosis Signaling Pathways



Apoptosis is an essential physiological process that plays a crucial role in normal development and homeostatic mechanisms of multicellular organisms. At least two major apoptotic pathways have been described in mammalian cells: the intrinsic pathway, in which death arises from mitochondrial dysfunction, and the extrinsic pathway, in which death is initiated from the activation of cell surface receptors. Both pathways lead to the activation of Caspase 3 and PARP.

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Materials and Methods

A. Preparation of cell lysates

Beadlyte® Cell Signaling Universal Lysis Buffer is supplied as **1X** stock solution. The lysis buffer contains phosphatase inhibitors *including* 1mM sodium orthovanadate (Na_3VO_4) but does ***NOT*** contain protease inhibitors. It is recommended that *Complete protease inhibitors (*Cat. 11 836 153 001) from Roche Molecular Biochemicals, Inc. be added immediately before use.

Suggested cell lysis protocol for cells

a. Pellet the cells by centrifugation (500 – 1000 x g) in a tabletop centrifuge for 5 minutes.
b. Wash the cells in ice cold TBS.
c. Add ice cold **1X** Beadlyte® Cell Signaling Universal Lysis Buffer containing freshly prepared protease inhibitors to cells (1mL per 1 x 10^7 cells).
d. Gently rock the lysate for 10-15 minutes at 4ºC.
e. Remove particulate matter by filtration.
f. Aliquot and store the lysate at –70°C. The lysate should be stable for several months.
g. It is recommended that the lysate be diluted at least 1:10 in PBS for determining the protein concentration with Coomassie-based assays.

Cell lysis protocol for cells in **96-well filter plates**

Adherent or non-adherent cells seeded or grown in sterile 96-well filter plates (See supplemental protocols) can be washed, treated, lysed and filtered in the same plate.

a. Wash the cells by placing the 96 well filter plate containing cells over a vacuum manifold to remove liquid.
b. Add 100µL of ice cold TBS then remove via vacuum.
c. To lyse the cells, add 200µL/well of ice cold **1X** Beadlyte® Cell Signaling Universal Lysis Buffer containing freshly prepared protease inhibitors.
d. Place the plate on an orbital shaker (600 – 800rpm) for 10-15 minutes at 4ºC.
e. Place a low protein binding, 96-well round bottom or V-bottom plate underneath the filter plate.
f. Centrifuge the plates in a microplate carrier for 5 minutes at 500 x g.
g. Store the filtered lysate at –70°C until ready for use.
h. It is recommended that the lysate be diluted at least 1:10 in PBS for determining the protein concentration with Coomassie-based assays.

Cell lysis protocol for cells in sterile **96-well tissue culture plates**

Adherent or non-adherent cells seeded or grown in sterile 96-well tissue culture grade plates (See supplemental protocols) can be washed, treated, and lysed in the same plate, but need to be filtered in a separate 96-well filter plate.

a. Wash the cells by centrifugation in a microplate carrier 2 min at 500 x g.
b. Remove the supernatant via aspiration and add 100µl of ice cold TBS.
c. Centrifuge and remove supernatant via aspiration.
d. Add 200µL/well of ice cold **1X** Beadlyte® Cell Signaling Universal Lysis Buffer containing freshly prepared protease inhibitors.
e. Place the plate on an orbital shaker (600 – 800 rpm) for 10-15 minutes at 4ºC.
f. Transfer the lysate to a 96-well filter plate that has been pre-wetted with **1X** lysis buffer.
g. Place a low protein binding, 96-well round bottom or V-bottom plate underneath the filter plate.
h. Centrifuge the plates in a microplate carrier for 5 minutes at 500 x g.
i. Store the filtered lysate at –70°C until ready for use.
j. It is recommended that the lysate be diluted at least 1:10 in PBS for determining the protein concentration with Coomassie-based assays.

B. Preparation of antibody Apoptosis beads

Beadlyte® capture beads are provided as a **20X** stock solution and should be protected from light.

a. Gently resuspend the **20X** stock capture beads with a pipette, then sonicate for 15 seconds.
b. Dilute the beads to **1X** by adding 0.125mL of beads with 2.475mL of Beadlyte® Cell Signaling Assay Buffer 1. Use the empty mixing vial provided.
c. Gently mix the **1X** stock capture beads with a pipette and sonicate for 15 seconds.

C. Preparation of biotin-labeled reporters and Streptavidin-PE

Beadlyte® reporter antibodies are provided as a **20X** stock solution.

a. Gently vortex and centrifuge the **20X** reporter stock for 1 minute at 1000 x g.
b. Dilute the reporter antibody to **1X** by adding 0.125mL of reporter antibody with 2.475mL of Beadlyte® Cell Signaling Assay Buffer 1. Use the empty bead-mixing vial provided.
c. Dilute Beadlyte® Streptavidin-Phycoerythrin 1:25 by adding 0.1mL of Streptavidin-Phycoerythrin with 2.5mL of Beadlyte® Cell Signaling Assay Buffer 1. Use the empty mixing vial provided.

D. Multiplexing additional Beadmates™ with 3-plex Apoptosis Signaling Kit.

Additional Cell Signaling Phospho-Beadmates™ can be combined with this kit. *Please note that Total Beadmate™ pairs should not be multiplexed with the 3-plex Apoptosis Signaling Kit.*

a. Gently resuspend the **20X** stock capture beads for each additional Beadmate with a pipettor, then sonicate for 15 seconds.
b. Refer to the Bead/Reporter Dilution Table below for details on adding the **20X** stock beads to the 3-plex Apoptosis beads.
c. Gently vortex and centrifuge the **20X** reporter stock for each additional Beadmate for 1 minute at 1000 xg.
d. Refer to the Bead/Reporter Dilution Table below for details on adding **20X** reporter stock to the 3-plex Apoptosis biotin.

Bead/Reporter Dilution Table for Multiplexing Additional Beadmates™

Number of Additional Beadmate™ Pairs added		Amount of additional **20X** Beads or **20X** Reporter (mL)		Amount of 3-plex Apoptosis **20X** Beads or **20X** Reporter (mL)		Total Bead or Reporter (mL)		Cell Signaling Assay Buffer 1 (mL)		Total **1X** volume (mL)
0	X	0	+	0.125	=	0.125	+	2.475	=	2.6
1	X	0.125	+	0.125	=	0.250	+	2.350	=	2.6
2	X	0.125	+	0.125	=	0.375	+	2.225	=	2.6
3	X	0.125	+	0.125	=	0.500	+	2.100	=	2.6
4	X	0.125	+	0.125	=	0.625	+	1.975	=	2.6
5	X	0.125	+	0.125	=	0.750	+	1.875	=	2.6
6	X	0.125	+	0.125	=	0.875	+	1.725	=	2.6
7	X	0.125	+	0.125	=	1.000	+	1.600	=	2.6
8	X	0.125	+	0.125	=	1.125	+	1.475	=	2.6
9	X	0.125	+	0.125	=	1.250	+	1.350	=	2.6

Luminex® Instrument Settings

1. Make sure lines are free of air bubbles (an alcohol flush, followed by a wash will often help).
2. Check to make sure the lids of the sheath fluid containers are tightly fastened.
3. Choose the 100 region bead map by going through options from the main page of the Luminex® Data Collector.
4. Select **Bead #s** for the protein detection assay. (See page 1 of each Beadmate™ COA).
5. Read 50-100 events per bead.
6. Set sample size = 75µL. Set gate to 8,000-13,500.
7. Adjust needle height using 2 disks and the same plate type as the plate used in assay performed.
8. Bio-Plex Instruments (Bio-Rad) should be calibrated with "Low" assay values on the calibration 2 bottle.

E. Preparation of lyophilized Beadlyte® Jurkat Cell Lysate: Anisomycin (Catalog # 47-207).

Beadlyte® Jurkat Cell Lysate: Anisomycin is provided as a lyophilized stock of cell lysates prepared from Jurkat cells stimulated with 25μM of anisomycin (4 hours). The cells were lysed in Beadlyte® Cell Signaling Universal Lysis Buffer containing protease inhibitors, mixed together, and lyophilized for stability. The lysate can be used as a positive control sample or alternatively, to create standard curves for relative quantification of different phosphorylated or actived protein analytes.

Beadlyte® Jurkat Cell Lysate: Anisomycin as a positive control

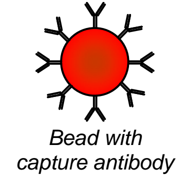
a. Reconstitute the lyophilized cell lysate in 200μl of ultrapure water.
b. Gently vortex and incubate the reconstituted lysate for 5 min at RT (store on ice).
c. Dilute 1μL of the reconstituted Beadlyte® Jurkat Cell Lysate: Anisomycin into 24μL of Beadlyte® Cell Signaling Assay Buffer 1 and mix with 25μl of 3-plex Apoptosis Signaling beads (steps 3 and 4 of main Assay protocol). This will provide MFI values comparable to a freshly made Jurkat cell lysates at 40 μg/mL.

F. Considerations for Cell Stimulation.

a. Treating cells with anisomycin or other apoptotic compounds induce apoptosis pathways. The duration of stimulation in addition to the concentration of the respective factor/compound should be considered since they influence the degree of phosphorylation/activation of any given analyte.
b. Cellular responses to apoptotic compounds are typically improved when cells have been serum starved prior to treatment.
c. Cell lines will differ in the robustness of their signaling response for any given stimulation.

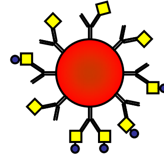
Detection Protocol Summary

Pre-wet filter plate and add 25µL of diluted cell lysate to each well containing 25µL of 1X beads



Bead with capture antibody

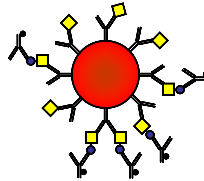
Overnight; dark (4ºC shaking)



Capture antibody binds target proteins

Wash twice with 100 µL of Beadlyte® Cell Signaling Universal Assay Buffer. Add 25µL of 1X reporter

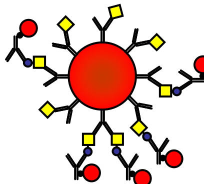
1 hour; dark (RT shaking)



Biotinylated reporter binds target proteins

Remove reporter and add 25µL of diluted Streptavidin-PE

30 min; dark (RT shaking)



Streptavidin-PE binds Biotinylated reporter

Remove Streptavidin-PE and resuspend beads in 100µL of Beadlyte® Cell Signaling Assay Buffer 1. Read results using Luminex®.

The Beadlyte® Cell Signaling assay is a simple fluorescence bead-based antibody sandwich immunoassay that is sensitive and easy to perform. A cell lysate or other test sample is incubated with beads coupled to a capture antibody that binds the target protein. After an overnight incubation, the beads are washed and mixed with a biotinylated reporter antibody which binds to an epitope distinct from the capture antibody. The beads are then incubated with streptavidin-phycoerythrin, washed, and the target protein is quantified using the Luminex® system.

Assay Protocol

1. Dilute filtered lysates at least 1:1 in Beadlyte® Cell Signaling Assay Buffer 1. The suggested working range of protein concentration for the assay is 1 to 10 μg of total protein/well (25μL/well at 40 to 400μg/mL).
2. Pre-wet filter plate with 25μL/well of Beadlyte® Cell Signaling Assay Buffer 1. Remove by vacuum filtration by placing the filter plate over a vacuum manifold and gently applying vacuum. Gently blot the bottom of the filter plate on a paper towel to remove excess liquid.
3. Gently mix the **1X** bead suspension with a pipette and sonicate for 10 seconds. Add 25μL of 1X bead suspension to each well.
4. Add 25μL of diluted cell lysate (or reconstituted Jurkat Cell Lysate: Anisomycin standard) to each well and incubate overnight[1] at 4°C (or 2 hours RT) on a plate shaker (600-800rpm) protected from light.
5. Remove the lysate by vacuum filtration.
6. Add 100μL/well of Beadlyte® Cell Signaling Assay Buffer 1. Remove buffer by vacuum filtration and gently blot the bottom of the filter plate on a paper towel.
7. Wash the beads a second time by repeating step 6.
8. Add 25μL/well of **1X** Beadlyte® Biotinylated Reporter.
9. Incubate on a plate shaker for 1 hour at room temperature, protected from light.
10. Remove reporter by vacuum filtration and gently blot the bottom of the filter plate on a paper towel.
11. Add 25μL of diluted (1:25) Beadlyte® Streptavidin-Phycoerythrin (Strep-PE).
12. Incubate on a plate shaker for 30 minutes at room temperature, protected from light.
13. Remove Beadlyte® Strep-PE by vacuum filtration and gently blot the bottom of the filter plate on a paper towel.
14. Resuspend beads in 100μl of Beadlyte® Cell Signaling Assay Buffer 1.
15. Analyze using the Luminex® system.

Step	Description
1. Prepare cell lysate samples	Dilute cell lysates in Beadlyte® Cell Signaling Assay Buffer 1
2. Pre-wet filter plate	Add 25μL/well of Beadlyte® Cell Signaling Assay Buffer 1, vacuum
3. Capture beads	Add 25μLl/well of **1X** Beads
4. Cell lysate/incubation	Add 25μl/well of cell lysate and incubate overnight[1] at 4°C (or 2 hrs RT) shaking, protected from light
5. Remove lysate	Remove lysate via vacuum
6. Wash	Wash wells with 100μl Beadlyte® Cell Signaling Assay Buffer 1
7. Second wash	Repeat step 6 a second time
8. Biotin reporter antibody	Add 25μL/well of **1X** Biotin-labeled Reporter
9. One hour incubation	Incubate 1 hr RT
10. Remove Reporter	Remove reporter via vacuum
11. Streptavidin-PE	Add 25μL/well of Streptavidin-PE (diluted 1:25)
12. 30 minute incubation	Incubate 30 minutes at room temperature, shaking and protected from light
13. Remove streptavidin-PE	Remove Streptavidin-PE from wells via vacuum
14. Resuspension in Beadlyte® Cell Signaling Assay Buffer 1	Add 100μL/well of Beadlyte® Cell Signaling Assay Buffer 1
15. Analysis	Analyze using the Luminex® system

[1] Beadmates™ can be incubated with cell lysate for 2 hrs at room temperature. However, overnight incubations provide better sensitivity.

Supplemental protocols

A. Analysis of viscous cell lysates

Some cell lysates may not flow through the filter plate efficiently due to high viscosity or the formation of particulate matter from long-term storage. For these samples, the initial capture and wash steps can be done in microcentrifuge tubes. The beads are then transferred into 96-well filter plates for the rest of the assay.

a. Add 25µL/assay point of 1X beads to a 500µL centrifuge tube.
b. Next, add lysate diluted in Beadlyte® Cell Signaling Assay Buffer 1 to a final volume of 100µL or higher.
c. Vortex the mixture at high speed for 15 seconds then sonicate for an additional 15 seconds.
d. Rotate the mixture overnight at 4°C, protected from light.
e. Centrifuge the beads for 1 min at 2,000 x g and carefully remove the supernatant to minimize bead loss.
f. Resuspend the pelleted beads in 25µl/assay point of Beadlyte® Cell Signaling Assay Buffer 1.
g. Transfer 25µL of the bead mixture to pre-wet filter plate wells and proceed to step 5 of the Main Assay protocol.

B. Growing or seeding cells in 96-well filter plates

Adherent or Non-adherent cells can be grown in sterile 96 well filter plates. Placing the cells over a gentle vacuum can simplify washing of cells, adding and removing cell treatments, and filtering lysed cells.

Growing cells overnight in 96 well filter plates

a. Wash cells with sterile TBS or culture medium.
b. Gently resuspend the cell pellet in culture medium to give 1 x 10^5 cells per mL for adherent cells (such as HeLa or A431) or 2 x 10^5 cells per mL for non-adherent cells (e.g. Jurkat T cells).
c. Pre-wet the filter plate by adding 50µl of sterile TBS or culture medium per well of 96 well filter plate.
d. Remove liquid by placing the plate on a vacuum manifold and gently applying vacuum. Gently blot the bottom of the filter plate on a paper towel to remove excess liquid.
e. Add 100µL of cell suspension to each well.
f. Grow/treat cells under desired conditions.
g. Proceed to "Cell lysis protocol for cells in **96-well filter plates"** on pg. 3, Materials and Method section.

Seeding cells (short durations) in 96 well filter plates

a. Wash cells with sterile TBS or culture medium.
b. Gently resuspend the cell pellet in TBS or culture medium to give 1 x 10^6 cells per mL for non-adherent cells or adherent cells.
c. Pre-wet the filter plate by adding 50µL of sterile TBS or culture medium per well of 96 well filter plate.
d. Remove buffer by placing the plate on a vacuum manifold and gently applying vacuum. Gently blot the bottom of the filter plate on a paper towel to remove excess liquid.
e. Add 100µL of cell suspension to each well.
f. Treat cells under desired conditions.
g. Proceed to "Cell lysis protocol for cells in **96-well filter plates"** on pg. 3, Materials and Method section.

Technical Considerations

1. The suggested working range of protein concentration for the assay is 1 to 10 µg of total protein/well (25µL/well at 40 to 400 µg/mL).
2. Please note that multiplexing phospho-specific and total Beadmate™ pairs is not recommended due to reporter cross reactivity.
3. To view the dot plot, contact Luminex® Corporation to inquire how to enable the software to view all selected bead events in the dot plot (white oval target). Note that some beads may miss the white oval target; this is a result of variations in the calibration process. This will not affect results as only beads hitting the white target will be counted and read.

End-User License Agreement

By purchasing this product, which contains fluorescently labeled microsphere beads authorized by Luminex® Corporation, you, the customer, acquire the right under Luminex® Corporation's patent rights, if any, to use this product or any portion of this product, including without limitation the microsphere beads contained herein, only with Luminex's laser-based fluorescent analytical test instrumentation marketed under the name Luminex®. One or more of the following US patents covers this product and the use thereof: #6,046,807, #5,981,180.

Representative Data

Cleaved PARP

MFI
3000
2500
2000
1500
1000
500
0

NT
Anisomycin

0.0
0.1
1.0

Lysate (ug protein/well)

Active Caspase 3

MFI
2500
2000
1500
1000
500
0

NT
Anisomycin

0.0
0.1
1.0

Lysate (ug protein/well)

GAPDH

MFI
12000
10000
8000
6000
4000
2000
0

NT
Anisomycin

0.0
0.1
1.0

Lysate (ug protein/well)

Figure 1. Multiplex analysis of Jurkat cells treated with anisomycin. Jurkat cells were stimulated with 25μM of anisomycin for 4 hours (cat# 47-207). The cells were lysed in Beadlyte® Cell Signaling Universal Lysis Buffer containing protease inhibitors. 1μg of lysate diluted in Beadlyte® Cell Signaling Assay Buffer 1 was analyzed according the Assay protocol (lysate incubation at 4ºC). The median Fluorescence Intensity (MFI) was measured with the Luminex® system. The figures represent the average and standard deviation of three replicate wells.

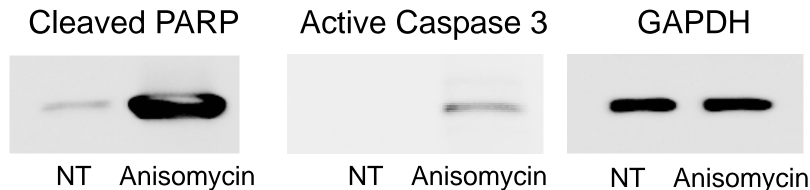


Figure 2. Immunoprecipitation/Western Blot analysis of multiplexed analytes in Jurkat cells. 20μg of lysates (described in Figure 1) were mixed with capture antibody beads to immunoprecipitate each respective protein. The immunoprecipitated proteins were separated on SDS-PAGE, transferred to nitrocellulose, and probed with biotin labeled active- or total reporter antibodies. The proteins were imaged using Streptavidin-HRP and chemiluminescence.