AQT0912 – MAP3K14 (NIK) Assay Validation

PhosphoSens®-Red Assay Format

Outline for this Study

PhosphoSens-Red Assay Validation

Enzyme Source, Construct, and Lot Information:

Carna MAP3K14 (07-102/15CBS-0387E) amino acids 319-947(end); N-terminal GST tag

Experiments to be run:

PhosphoSens-Kinetic Assay

PhosphoSens-Red TRF End Point S:B Determination

Has this Sensor Peptide been fully validated in the PhosphoSens-Kinetic Assay Format?

Yes

Red-Shift TRF S:B Determination

Reaction Conditions and Set Up

Reaction Conditions:

54 mM HEPES, pH 7.5

1mM ATP

1.2 mM DTT

0.012% Brij-35

1% glycerol

0.2 mg/ml BSA

0.55 mM EGTA

10 mM $MgCl_2$

15 µM AQT0912

30nM MAP3K14

Notes:

Enzyme Dilution Buffer (EDB):20 mM HEPES, pH 7.5,0.01% Brij-35, 5% Glycerol, 0.5 mM EGTA, 1 mM DTT, 1 mg/ml Bovine Serum Albumin.

Reaction Set Up:

2 or 2.5 µL	10x Sensor Peptide
14 or 17.5 µL	Reaction Mix with ATP & DTT
4 or 5 µL	1x EDB or Kinase dilutions (5x in EDB)
20 or 25 µL	Final reaction volume

Reactions were run at 30˚C for 240 minutes in either Corning, low volume 384-well, white flat round bottom polystyrene NBS microplates (Cat. #3824) at 25 µL final well volume or in in PerkinElmer, ProxiPlate-384 Plus, white shallow well microplates (Cat. #6008280) at 20 µL final well volumeafter sealing using optically-clear adhesive film (TopSealA-Plus plate seal, PerkinElmer [Cat. #6050185]) in a Biotek Synergy Neo 2 microplate reader with excitation (360 nm) and emission (485 nm) wavelengths.

Red-Shift Reaction Set Up:

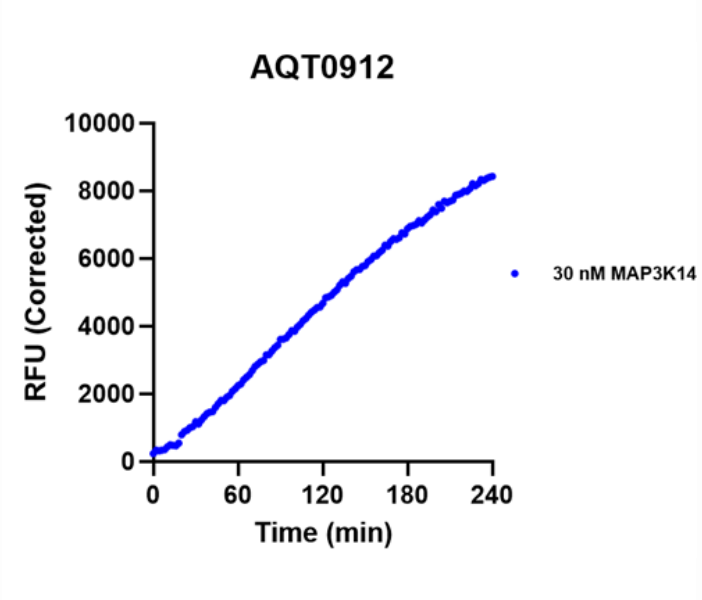
Following completion of the kinase reaction, remove plate from the reader and remove plate seal and add NaOH to a final concentration of 0.5 M, incubate for 5 minutes to adjust the pH, and then add Europium to a final concentration of 5 mM.

Incubate for 5 minutes at room temperature (RT), return to plate reader without seal and read in TRF mode with excitation (360 nm) and emission (620 nm) wavelengths.

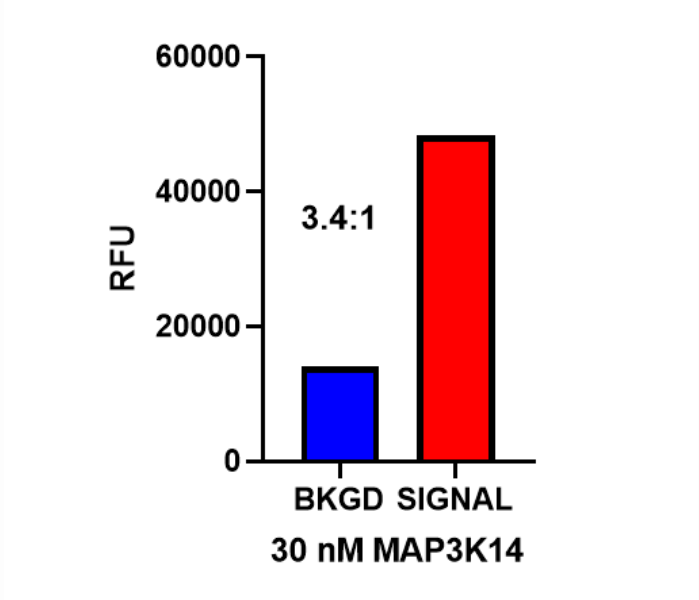
Red-Shift TRF S:B Determination

Progress and S:B Curves

PhosphoSens-Kinetic Progress Curves



PhosphoSens-Red S:B Chart



Using the reactions conditions described, TRF S:B for this assay is 3.4