AQT0653 – JAK1 (JH1JH2) Assay Validation

PhosphoSens®-Red Assay Format

Outline for this Study

PhosphoSens-Red Assay Validation

Enzyme Source, Construct, and Lot Information:

SignalChem JAK1 (JH1JH2) (Cat/Lot #,J01-11G/E4140-3) (438 - end) was expressed as an N-terminal GST tag in SF9 insect cells.

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?

No

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 $MgCl_2$

20 µM AQT0653

15 nM JAK1(JH1 JH2)

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 Europium to a final concentration of 5 mM.

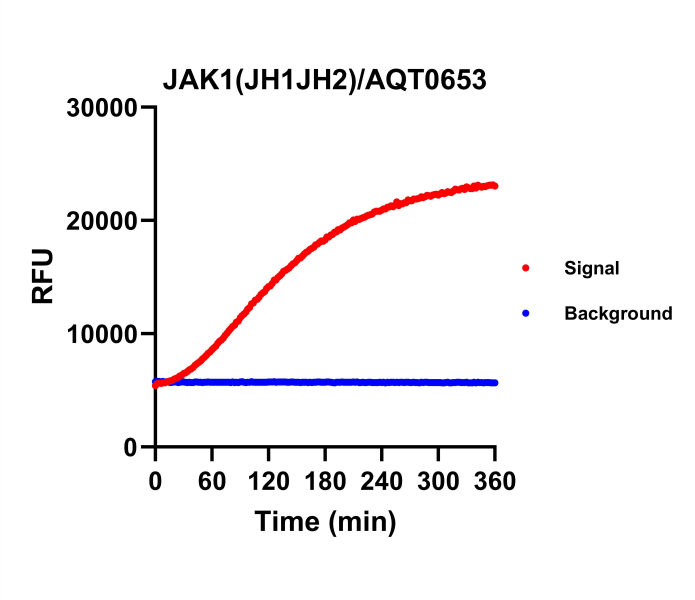
4 or 5 µL	30 mM Europium (6x)

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.

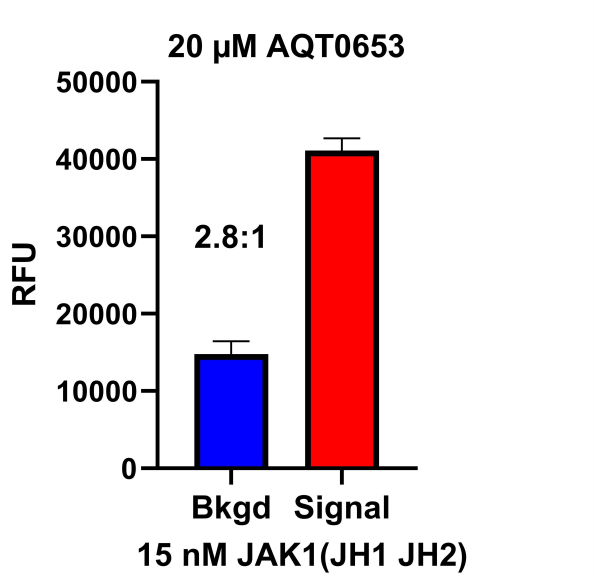
Red-Shift TRF S:B Determination

Progress and S:B Curves

PhosphoSens-Kinetic Progress Curves



PhosphoSens-Red S:B Chart



Using the reactions conditions previously described, the TRF S:B for this assay is 2.8