

# **ClariCELL™ Kinase Assay Kit for BTK (cat. # CK-01-1001-384), or BTK [C481S] (cat. # CK-01-1002-384) in 384-well ELISA format**

## **User's Manual**

**V1.0 – 20131007**

**For Research Use Only. Not for use in diagnostic procedures, or in humans.**

Information in this document is subject to change without notice.

### **IMPORTANT SAFETY INFORMATION**

This assay kit is designed for use by trained scientific professionals following appropriate laboratory safety procedures. Appendix A outlines important general safety precautions for utilizing these materials.

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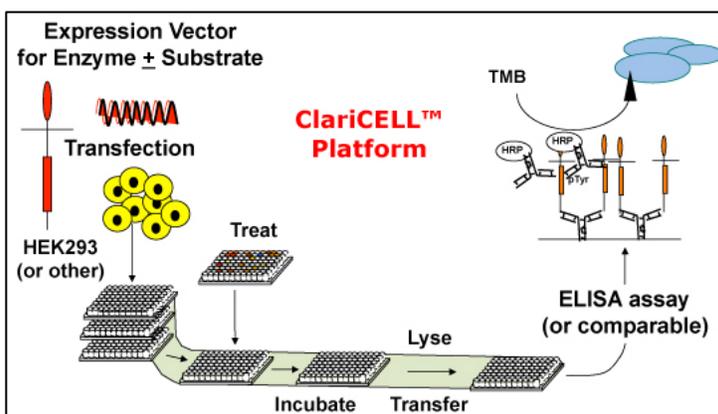
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## Introduction

BTK (Bruton's agammaglobulinemia Tyrosine Kinase) is a member of the Tec family of cytoplasmic tyrosine kinases. BTK is an important regulator of B-cell development and signaling (1), and as such, has been investigated as a target of inhibition for the treatment of B-cell malignancies, autoimmune disorders, and inflammation (2). The activity of BTK is regulated by a variety of mechanisms, including membrane translocation and phosphorylation. Key in the activation process is phosphorylation of two tyrosine residues, Y551 and Y223 (1). It is generally believed that Y551 of BTK is phosphorylated by a Src family kinase, likely Lyn, which subsequently leads to autophosphorylation of the Y223 site (3, 1). However, BTK has also been shown to autophosphorylate at the Y551 site, at least *in vitro* (4).

BTK [C481S] is a variation of wild type BTK where the cysteine at amino acid 481 has been mutated to serine. The clinical compound PCI-32765 (ibrutinib) irreversibly binds BTK by reacting with the cysteine at position 481 (5). As such, ibrutinib is predicted to exhibit reduced binding and potency toward BTK [C481S] compared to the wild type protein. Consistent with this prediction, the BTK [C481S] mutant has recently been reported in ibrutinib resistant patients (6).

ClariCELL™ kinase assays (7) represent an invaluable system for testing the inhibitory activities of small molecules against a specific kinase of interest in the context of human cells. Cell-based compound potency measurements are important components of the drug discovery process, since biochemical potency values often do not translate to cellular activity for a number of reasons, including compound membrane permeability, cellular ATP concentration, compound localization, etc. With the ClariCELL™ BTK and BTK [C481S] assay kits, autophosphorylation of human full-length wild type BTK or BTK [C481S] is quantified, and the cellular potencies of compounds that modulate these autophosphorylation events are measured. Figure 1 depicts an overview of the ClariCELL™ assay system.

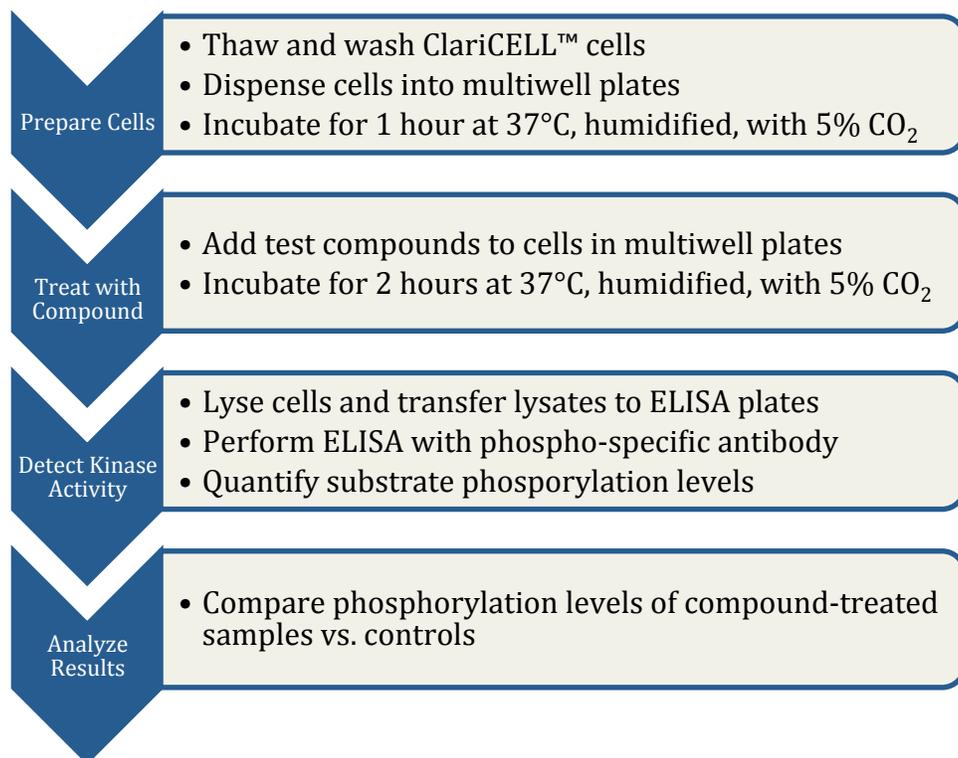


**Figure 1 – Basis of ClariCELL™ kinase cell-based assay technology.** HEK293 cells are first transiently transfected with a vector encoding a kinase of interest with or without a separate protein substrate. Following compound treatment and cell lysis, phosphorylation of the substrate is quantified by ELISA using an antibody to the phosphorylation site.

The ClariCELL™ BTK and BTK [C481S] kinase assay kits provide HEK293 cells that have already been transfected with a vector encoding sequence verified full length human BTK or BTK [C481S], plus reagents suitable for ELISA detection of BTK

or BTK [C481S] autophosphorylation levels. The reagents have been characterized extensively and titrated appropriately, such that the kit can easily be utilized without further optimization for testing the ability of small molecules to modulate the kinase activity of BTK or BTK [C481S] in human cells, in a high-throughput manner.

## Overview of Experimental Procedure



## Materials Supplied and Storage Conditions

- (1) vial (cryotube) of ClariCELL™ BTK cells (1.5 x 10<sup>6</sup> cells in 500 µL volume) or BTK [C481S] cells (1.3 x 10<sup>6</sup> cells in 500 µL volume) per assay plate, to be stored at -80°C for short term (3 months or less). For longer term, store in the vapor phase of liquid nitrogen. Be sure to follow relevant safety precautions, including the use of appropriate gloves and a face shield, when removing vials from liquid nitrogen.
- (1) 384-well high binding ELISA plate per assay, sealed with a clear plate seal, to be stored at room temperature. (Provided for orders of 5 assays or less. For orders of 6 or more assays, these can be purchased from Corning Costar - cat. #3700)
- (1) tube of coating antibody (red-capped tube. 125 µL per assay, at 1 mg/mL) to be stored at -20°C.
- (1) tube of primary antibody (yellow-capped tube. 1 µL per assay, at 0.2 mg/mL) to be stored at +4°C.

- (1) tube of secondary detection antibody (purple-capped tube. 1.25  $\mu$ L per assay) to be stored at  $-20^{\circ}\text{C}$ .
- (1) tube of 5x lysis buffer base per assay (15 mL tube. 3.75 mL per assay), to be stored at  $-20^{\circ}\text{C}$ .

### **Materials Required but not Supplied**

- 15 mL centrifuge tubes
- (1) 384-well high binding clear ELISA plate per assay (For orders of 6 or more assays - Corning Costar - cat. #3700. For orders of 5 or less assays, plates are included in the kit.)
- PBS (e.g. Fisher Scientific cat. # BP2438-20)
- 1% BSA solution in PBS (e.g. 1% w/v solution of Sigma cat. #A7888 dissolved in PBS)
- HEK293 culture medium (DMEM + NEAA + 10% FBS)
- (1) 384-well tissue culture plate per assay, sterile, with lid (eg. Corning Costar cat. #3701)
- PMSF (Phenylmethanesulfonyl Fluoride, e.g. Sigma cat. # 78830), dissolved in isopropyl alcohol, ethanol, or methanol to 200 mM.
- Control BTK inhibitor – ibrutinib (e.g. Selleck Chemical cat. # S2680)
- DMSO
- TBST (e.g. Fisher Scientific cat. # PI-28360 for 20x concentrate)
- 1-Step Slow TMB-ELISA (e.g. Pierce cat. # 34024)
- 50 mL amber (dark) tubes or aluminum foil
- 2 M  $\text{H}_2\text{SO}_4$
- Optional: Trypan Blue

### **Instrumentation and Equipment Required**

- Centrifuge suitable to spin 15 mL tubes
- Absorbance microplate reader (450 nm)
- $37^{\circ}\text{C}$  water bath
- Cell Culture Incubator, humidified, at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$
- Optional: Hemocytometer

### **Assay Protocol**

1. Coat the wells of a 384-well high binding ELISA plate with the provided coating antibody (red-capped tube). Dilute the coating antibody to 0.01 mg/mL in PBS (For each 384-well plate, add 125  $\mu$ L of coating antibody to 12.5 mL of PBS), and add 25  $\mu$ L solution per well. Incubate for 2 hours at room temperature (RT) or overnight at  $+4^{\circ}\text{C}$ .

2. Remove the antibody solution from the 384-well ELISA plate. Add 70  $\mu$ L 1% BSA solution per well for blocking. Incubate for 30 minutes at RT, or overnight at +4°C.
3. Prepare the cell suspension from frozen cells as follows:
  - Thaw one cryotube of BTK or BTK [C481S] ClariCELL™ frozen cells (per assay plate) by placing it in a 37°C water bath (~2 min). Be careful to not incubate the cells longer than is necessary to thaw the cells, as the viability may be impacted.
  - Dispense 5 mL of HEK293 culture medium into a 15 mL centrifuge tube and add the thawed cells to the tube.
  - Rinse the cryotube with an additional 1 mL of medium, then combine the rinse with the solution in the 15 mL tube.
  - Spin the cells for 6 minutes at 140 g (approximately 900 rpm).
  - Carefully remove and discard the supernatant without disturbing the cell pellet.
  - Resuspend the cells in 500  $\mu$ L of HEK293 culture medium. Pipet up and down several times with a 1 mL pipettor and tip to break up cell clumps.
  - Optional: Count the cell number and determine the cell viability using standard techniques such as trypan blue staining. An optimal dilution for counting using a hemocytometer is 1:4 dilution of cells, followed by 1:2 with trypan blue. For example, use 10  $\mu$ L of cells plus 30  $\mu$ L of medium plus 40  $\mu$ L of trypan blue for counting.

*Note: The cell viability should be  $\geq 60\%$ , and the total cell number should be  $\sim 1.5 \times 10^6$  cells per vial for BTK and  $\sim 1.3 \times 10^6$  cells per vial for BTK [C481S].*

- Dilute the cells with HEK293 culture medium. Add 12 mL of culture medium for a total volume of 12.5 mL. Pipet up and down to ensure that the cells are evenly distributed.
- Dispense 25  $\mu$ L of the cells per well to a 384-well tissue culture plate. Ensure that the cells are evenly distributed by mixing during transfer to the plate.

*Note: Variations in cell number from well to well will adversely affect the results in terms of data variability. The final cell number in the assay should be 1,500 – 3,000 viable cells per well, with a consistent number of cells from well to well.*

*Note:* 8-16 wells per plate should be left empty (no cells) for background controls.

- Cover the plate with a lid and incubate in a humidified 5% CO<sub>2</sub> incubator at 37°C for 1 hour before compound addition.
4. Prepare inhibitors at 6x final assay concentration in 3% (v/v) DMSO. When preparing dilution curves, always dilute compounds in 100% DMSO before adding water or medium in the final step.
  5. Add 5 µL of diluted compound to the cells for a final assay concentration of 1x compound and 0.5% DMSO. For positive assay controls (full activity), add 5 µL of 3% DMSO, and for negative controls (no activity), add 5 µL of 60 µM ibrutinib in 3% DMSO (final concentration of ibrutinib will be 10 µM). Cover the plate with a lid and incubate in a humidified 5% CO<sub>2</sub> incubator at 37°C for 2 hours.
  6. Prepare complete 5x lysis buffer by adding 200 mM PMSF to the supplied 5x lysis buffer base to a concentration of 5 mM (94 µL of 200 mM PMSF to the supplied 3.75 mL 5x lysis buffer base).

*Note:* PMSF is unstable in aqueous solution and should be added to the lysis buffer just prior to use. Note that a different stock concentration of PMSF can be utilized, with appropriate adjustment of the amount added, such that the final concentration is 5 mM.

7. Add 7.5 µL of the complete 5x lysis buffer directly into each well of the tissue culture plate and mix to lyse the cells. No medium removal or washing of the cells is necessary. Incubate the plate for 10 minutes at RT.

*Note:* Take care in pipetting the lysis buffer, as the solution is viscous and also may form bubbles if air is introduced by pipetting or shaking. Addition of the lysis buffer will change the color of the medium from pink to yellow/orange.

8. Prepare the coated and blocked ELISA plate by removing the 1% BSA blocking solution. Transfer 25 µL of the cell lysate from the tissue culture plate to each corresponding assay well of the ELISA plate, and incubate for 1hr at RT.
9. Remove the cell lysate solution from the ELISA plate and wash the wells 3x with TBST.

*Note:* Utilize 90 µL per well of wash solution to ensure thorough washing.

10. Dilute the provided primary antibody (yellow-capped tube) 1:20,000 in TBST and add 25 µL/well. (For one 384-well plate, add 1 µL of antibody to 20 mL of TBST.

For larger numbers of plates, add 0.625  $\mu\text{L}$  of antibody to 12.5 mL TBST per plate). Incubate for 1 hour at RT.

*Note: The plates can alternatively be incubated with antibody overnight at +4°C.*

11. Remove the primary antibody solution from the ELISA plate and wash the wells 3x with TBST.
12. Dilute the provided secondary detection antibody (purple-capped tube) 1:10,000 in TBST and add 25  $\mu\text{L}$ /well. (For one 384-well plate, add 1.25  $\mu\text{L}$  of antibody to 12.5 mL of TBST). Incubate for 1 hour at RT.

*Note: The plates can alternatively be incubated with antibody overnight at +4°C.*

13. Take 12.5 mL (per assay plate) of the 1-Step Slow TMB-ELISA solution out of the +4°C approximately 1 hour before the detection stage (step 15) to allow it to equilibrate to RT. To protect the 1-Step Slow TMB-ELISA from excess light, utilize amber (dark) tubes, or wrap the tubes in aluminum foil.
14. Remove the secondary detection antibody solution from the ELISA plate and wash the wells 3x with TBST.
15. Add 25  $\mu\text{L}$  per well of TMB-ELISA solution and incubate for 20-30 minutes at RT to allow the blue color to develop. Stop the reaction by adding 25  $\mu\text{L}$  of 2M  $\text{H}_2\text{SO}_4$  and mixing. The blue color should change to yellow.
16. Measure the absorbance of the wells at 450 nm.
17. Subtract the average background value (absorbance at 450 nm from wells that contained no cell lysate) from each absorbance value to obtain the 'corrected' absorbance values for each well.
18. Calculate % inhibition values from the corrected absorbance readings, based on positive and negative control values, and according to the following formula:  
$$\%INH = ((\text{positive control} - \text{sample}) / (\text{positive control} - \text{negative control})) * 100$$
19. If desired, calculate  $Z'$  values based on the following formula:  $1 - [(3 * \text{standard deviation of positives} + 3 * \text{standard deviation of negatives}) / (\text{average positive} - \text{average negative})]$  (8). A  $Z'$  value of greater than or equal to ~0.4 generally indicates an acceptable value.
20. For dose-response curves, plot % inhibition values vs. the log values of compound concentrations utilizing appropriate curve fitting software (e.g. GraphPad Prism software).
21. Fit the IC<sub>50</sub> curves utilizing standard techniques (e.g. sigmoidal dose response

curve fitting) to determine IC50 values.

Note that total phospho-tyrosine is the readout measured in these assays, and therefore there is a possibility that some tyrosine kinase activity in addition to that of BTK or BTK [C481S] can be detected. Also note that compounds exhibiting extreme cytotoxicity will appear to be BTK or BTK [C481S] inhibitors in the assays. However, since the compound incubation time is relatively short (2 hours), this risk is considered to be low. If suspected, cytotoxicity should be assessed in a separate assay.

### Example Plate Layout and Expected Results

A dose response curve for ibrutinib was generated utilizing the ClariCELL™ BTK 384 well assay kit. 8 doses of ibrutinib were tested, starting at 1  $\mu$ M testing concentration and making 1 to 3 dilutions. The assay protocol was followed as outlined above, and the plate layout was as shown below:

		ibrutinib ( $\mu$ M)				
<>		1	2	3	4	5
A		POS	1.0	1.0	NEG	BKGRND
B		POS	0.33	0.33	NEG	BKGRND
C		POS	0.11	0.11	NEG	BKGRND
D		POS	0.037	0.037	NEG	BKGRND
E		NEG	0.012	0.012	POS	BKGRND
F		NEG	0.0041	0.0041	POS	BKGRND
G		NEG	0.0014	0.0014	POS	BKGRND
H		NEG	0.00046	0.00046	POS	BKGRND

Absorbance Reading at 450 nm:

<>		1	2	3	4	5
A		0.562	0.357	0.376	0.372	0.296
B		0.561	0.388	0.401	0.396	0.301
C		0.558	0.371	0.391	0.379	0.302
D		0.572	0.391	0.403	0.397	0.301
E		0.394	0.388	0.408	0.515	0.311
F		0.387	0.526	0.538	0.559	0.321
G		0.383	0.566	0.556	0.600	0.329
H		0.392	0.580	0.616	0.607	0.344

Background Corrected Reading at 450 nm:

<>		1	2	3	4
A		0.249	0.044	0.063	0.059
B		0.248	0.075	0.088	0.083
C		0.245	0.058	0.078	0.066
D		0.259	0.078	0.090	0.084
E		0.081	0.075	0.095	0.202
F		0.074	0.213	0.224	0.245
G		0.070	0.253	0.243	0.287
H		0.079	0.266	0.303	0.294

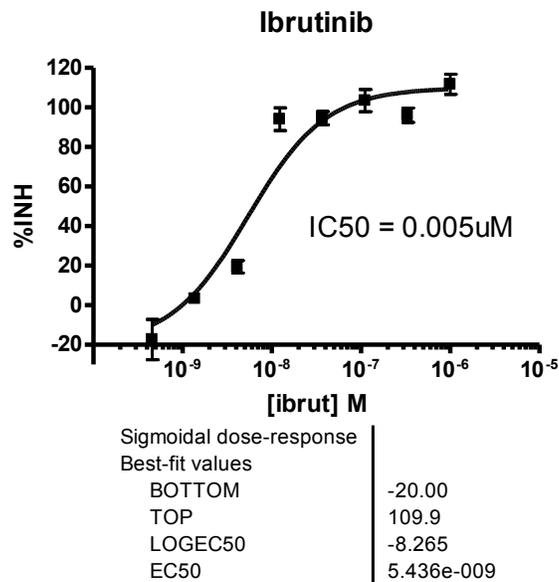
Plate Statistics:

avg pos	0.254
avg neg	0.074
st dev pos	0.028
st dev neg	0.009
s/b	3.41
z'	0.38

Calculated Percent Inhibition Values:

<>	1	2	3	4
A	2.6	116.8	106.5	108.5
B	3.1	99.6	92.4	95.3
C	4.7	109.1	97.8	104.9
D	-3.0	97.9	91.2	94.6
E	96.2	99.8	88.3	28.7
F	100.4	22.5	16.3	4.6
G	102.7	0.6	6.1	-18.4
H	97.5	-7.1	-27.5	-22.3

IC50 Curve Generated Using GraphPad Prism Software:



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7. [www.cellassayinnov.com](http://www.cellassayinnov.com)
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## Appendix A – Important Safety Information

The modified HEK293 cells included in this assay kit are classified as biosafety level 2 (BSL-2). Containment, waste disposal, and laboratory procedures appropriate for BSL-2 should be followed. For specific hazards associated with the kit components, refer to the MSDSs (Material Safety Data Sheets). For assistance in general guidelines and US regulations regarding BSL-2, please refer to the CDC (Center for Disease Control) Publication entitled, “Biosafety in Microbiological and Biomedical Laboratories.” As with other laboratory procedures, researchers utilizing this kit should wear appropriate PPE (Personal Protective Equipment) such as gloves, safety glasses, and a lab coat, to minimize the risk of exposure to the components.

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