Cell Assay Innovations

ClariCELL™ Kinase Assay Kit for BTK (cat. # CK-01-1001), or BTK [C481S] (cat. # CK-01-1002)

User's Manual

V1.4 – 20130918

For Research Use Only. Not for use in diagnostic procedures, or in humans.
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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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Cell Assay Innovations

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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.

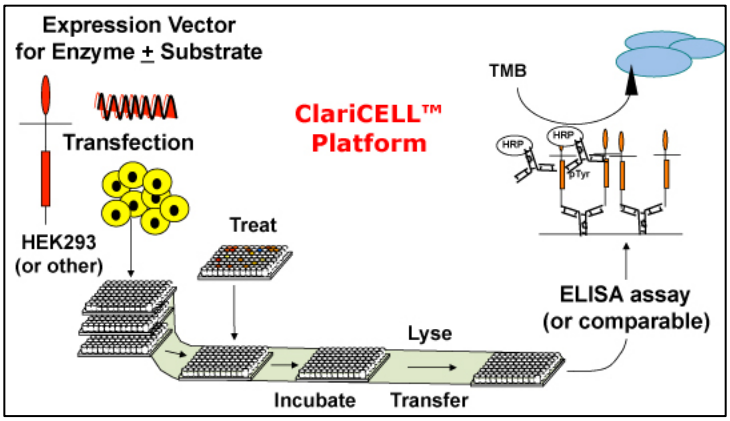
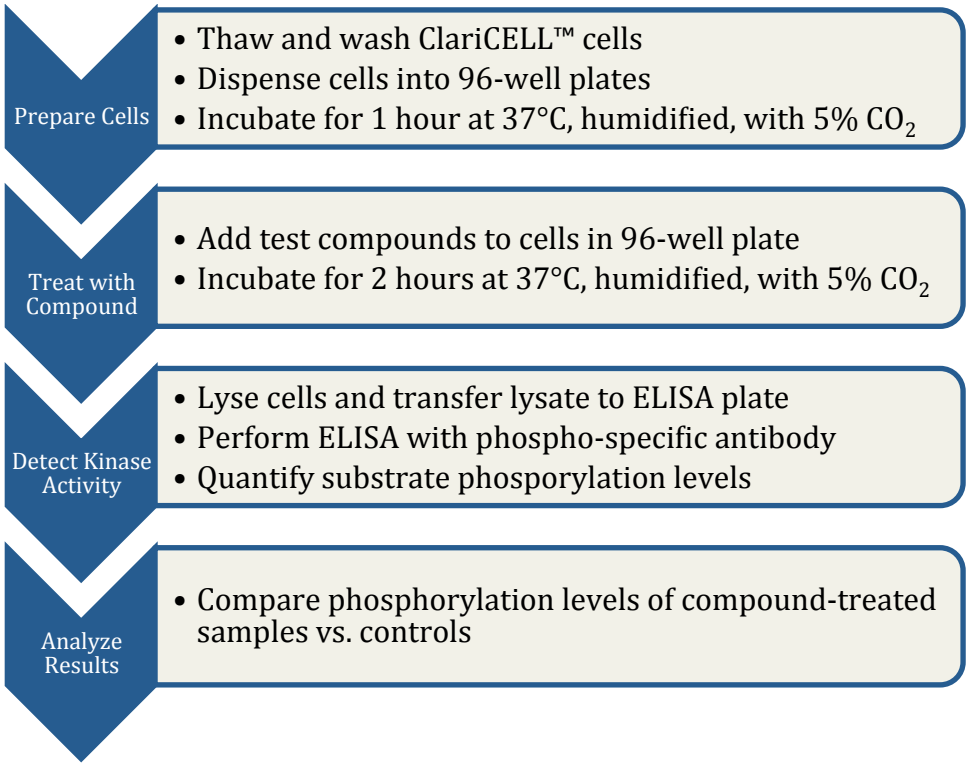


Figure 1 – Basis of ClariCELL™ kinase cell-based assay technology. HEK293 cells are first transiently transfected with a vector encoding a kinase of interest with or without a separate protein substrate. Following compound treatment and cell lysis, phosphorylation of the substrate is quantified by ELISA 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 10^6 cells in 500 µL volume) or BTK [C481S] cells (1.3 x 10^6 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) 96-well half area 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. #3690)
- (1) tube of coating antibody (red-capped tube. 50 µL per assay, at 1 mg/mL) to be stored at -20°C.
- (1) tube of detection antibody (yellow-capped tube. 1 µL per assay, at 0.2 mg/mL) to be stored at +4°C.

- (1) tube of 5x lysis buffer base per assay (green-capped tubes. 1.46 mL each), to be stored at -20°C.

Materials Required but not Supplied

- 15 mL centrifuge tubes
- (1) half area 96-well high binding clear ELISA plate per assay (For orders of 6 or more assays - Corning Costar - cat. #3690. 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) 96-well tissue culture plate per assay, sterile, with lid
- PMSF (Phenylmethanesulfonyl Fluoride, e.g. Sigma cat. # 78830), dissolved in isopropyl alcohol 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)
- 15 mL amber (dark) tubes or aluminum foil
- 2 M H_2SO_4
- Optional: Trypan Blue

Instrumentation and Equipment Required

- Centrifuge suitable to spin 15 mL tubes
- Absorbance microplate reader (450 nm)
- Microplate Shaker
- 37°C water bath
- Cell Culture Incubator, humidified, at 37°C and 5% CO_2
- Optional: Cell Counter

Assay Protocol

1. Coat the wells of a 96-well half area ELISA plate with the provided coating antibody (red-capped tube). Dilute the coating antibody to 0.01 mg/mL in PBS (For each 96-well plate, add 50 µL of coating antibody to 5 mL of PBS), and add 50 µL solution per well. Incubate for 2 hours at room temperature (RT) or overnight at +4°C.

2. Shake off the antibody solution from the 96-well ELISA plate. Add 85 µL 1% BSA solution per well for blocking. Incubate for 30 minutes at RT with shaking, or overnight at +4°C.

Note: *After shaking off the solution from plates, tap the plate on absorbent paper to blot off the residual liquid. The same technique can be used for plate washing in subsequent steps.*

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.

   - Transfer the cells to a 15 mL centrifuge tube and add 5 mL of HEK293 culture medium.

   - 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 µL 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 µL of cells plus 30 µL of medium plus 40 µL of trypan blue for counting.

     Note: The cell viability should be $\geq$70%, and the total cell number should be ~1.5 x 10^6 cells per vial for BTK and ~1.3 x 10^6 cells per vial for BTK [C481S].

   - Dilute the cells with HEK293 culture medium. For wild type BTK, add 5.5 mL of culture medium for a total volume of 6 mL, and for BTK [C481S] cells, add 5 mL of culture medium for a total volume of 5.5 mL. Pipet up and down to ensure that the cells are evenly distributed.

   - Dispense 45 µL of the cells per well to a 96-well tissue culture plate. Ensure that the cells are evenly distributed during transfer to the plate by pipetting up and down after addition to each row or column.

     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 7,000 – 10,000 viable cells per well, with a consistent number of cells from well to well.

- Cover the plate with a lid and incubate in a humidified 5% CO_2 incubator at 37°C for 1 hour before compound addition.

4. Prepare inhibitors at 10x final assay concentration in 5% (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 5% DMSO, and for negative controls (no activity), add 5 µL of 100 µM ibrutinib in 5% DMSO (final concentration of ibrutinib will be 10 µM). Cover the plate with a lid and incubate in a humidified 5% CO_2 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 (green-capped tube) to a concentration of 5 mM (38 µL of 200 mM PMSF to the supplied 1.46 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 12.5 µL of the complete 5x lysis buffer directly into each well of the tissue culture plate to lyse the cells. No medium removal or washing of the cells is necessary. Shake 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 ELISA plate by shaking off the 1% BSA blocking solution. Transfer 50 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 with shaking.

   Note: *The majority of the sample is transferred to the ELISA plate (only ~12.5 µL of 'dead volume'). To ensure that the full amount can be aspirated from the wells, it is useful to tilt the plate and pipet carefully from the bottom edges of the wells.*

9. Shake off the cell lysate solution from the ELISA plate and wash 3x with TBST.

   Note: *Utilize 150 µL per well of wash solution to ensure thorough washing.*

10. Dilute the provided detection antibody (yellow-capped tube) 1:5000 in TBST and add 50 µl/well. (For each 96-well plate, add 1 µL of antibody to 5 mL of TBST). Incubate for 1 hour at RT with shaking.

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

11. Take 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 13) 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.

12. Shake off the detection antibody solution from the ELISA plate and wash 3x with TBST.

13. Add 50 µL per well of TMB-ELISA solution and shake for 10 to 15 minutes to allow the blue color to develop. Stop the reaction by adding 50 µL of 2M H_2SO_4. The blue color should change to yellow.

14. Measure the absorbance of the wells at 450 nm.

15. Calculate % inhibition values from the absorbance readings based on positive and negative control values, and according to the following formula: (%INH = ((positive control – sample)/(positive control – negative control))*100

16. If desired, calculate Z' values based on the following formula: 1-[(3*standard deviation of positives + 3*standard deviation of negatives)/(average positive – average negative)] (8). A Z' value of greater than or equal to 0.4 generally indicates an acceptable value.

17. For dose-response curves, plot % inhibition values vs. the log values of compound concentrations utilizing appropriate curve fitting software (e.g. GraphPad Prism software).

18. Fit the IC50 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 assay kit. 8 doses of ibrutinib were tested, starting at 1 µM 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 (uM)		
<>	1	2	3	4
A	POS	1.0	1.0	NEG
B	POS	0.33	0.33	NEG
C	POS	0.11	0.11	NEG
D	POS	0.037	0.037	NEG
E	NEG	0.012	0.012	POS
F	NEG	0.0041	0.0041	POS
G	NEG	0.0014	0.0014	POS
H	NEG	0.00046	0.00046	POS

Absorbance Reading at 450 nm:

<>	1	2	3	4
A	0.301	0.072	0.072	0.071
B	0.306	0.089	0.087	0.083
C	0.315	0.080	0.080	0.076
D	0.306	0.129	0.115	0.093
E	0.073	0.168	0.197	0.327
F	0.084	0.265	0.281	0.306
G	0.075	0.277	0.262	0.285
H	0.097	0.334	0.291	0.310

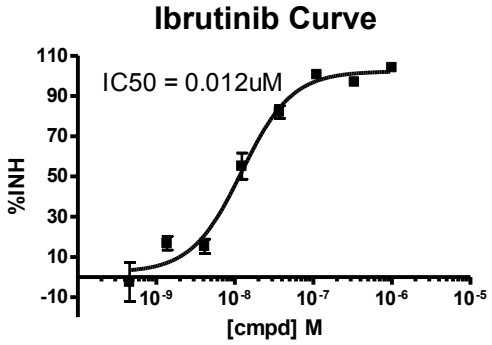
Plate Statistics:

average positive control – 0.307
average negative control – 0.081
signal to background – 0.307/0.081 = 3.8
Z' value – 0.71

Calculated Percent Inhibition Values:

<>	1	2	3	4
A	2.5	104.1	104.2	104.6
B	0.3	96.5	97.4	99.3
C	-3.6	100.7	100.6	102.6
D	0.4	78.9	85.1	95.0
E	103.9	61.6	48.6	-8.9
F	98.9	18.8	11.7	0.7
G	102.7	13.4	20.2	9.7
H	93.0	-12.1	7.2	-1.1

IC50 Curve Generated Using GraphPad Prism Software:



Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	2.219
TOP	102.3
LOGEC50	-7.913
HILLSLOPE	1.357
EC50	1.223e-008

References

1. Mohamed, AJ *et al*., "Bruton's tyrosine kinase (Btk): function, regulation, and transformation with special emphasis on the PH domain" (2009) *Immunol. Rev.*, 228, 58-73.
2. Vargas, L, Hamasy, A, Nore, BF, and Smith, CIE, "Inhibitors of BTK and ITK: State of the New Drugs for Cancer, Autoimmunity and Inflammatory Diseases," (2013) *Scand. J. Immunol.*, 78(2):130-9.
3. Rawlings, DJ, Scharenberg, AM, Park, H, Wahl, MI, Lin, S, Kato, RM, Fluckiger, AC, Witte, ON, and Kinet, JP, "Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases," (1996) *Science* 271, 822–825.
4. Dinh, M., Grunberger, D., Ho, H., Tsing, SY, Shaw, D, Lee, S, Barnett, J, Hill, RJ, Swinney, DC, and Bradshaw, JM, "Activation Mechanism and Steady State Kinetics of Bruton's Tyrosine Kinase," (2007) *J. Biol. Chem.* 282, 8768-8776.
5. Pan, Z *et al.,* "Discovery of Selective Irreversible Inhibitors for Bruton's Tyrosine Kinase" (2007) *ChemMedChem* 2, 58-61.
6. Chang, B *et al.* "Tumor genomic profiling reveals mechanisms of resistance to BTK inhibitor ibrutinib in chronic lymphocytic leukemia (CLL)" Poster Display and Discussion Session at ASCO, Chicago, IL, May 31- June 4, 2013.
7. www.cellassayinnov.com
8. Zhang, JH, Chung, TDY, Oldenburg, KR, "A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays," (1999) *J. Biomol. Screen*, 4, 67-73.

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.

Appendix B – Limited Use License Agreement

READ CAREFULLY

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