CML648Mu, 96 Tests

Multiplex Assay Kit for KEAP1, etc.

by CBA (Cytometric Bead Array)

Organism Species: Mouse

Instruction manual

FOR RESEARCH USE ONLY

NOT FOR USE IN CLINICAL DIAGNOSTIC PROCEDURES

Version 5.5

Please read the instruction manual carefully before the experiment. Cloud-Clone also provides flow cytometry-based multi-factor kits with V-bottom plates and filter plates. For more information, please visit our official website.

[INTENDED USE]

The kit is a magnetic bead-based multiplex assay kit applies sandwich immunoassay for in vitro quantitative measurement of KEAP1 in serum, plasma, tissue homogenates, cell lysates, cell culture supernates and other biological fluids.

[REAGENTS AND MATERIALS PROVIDED]

Reagents	Quantity	Reagents	Quantity
96-well plate (8*12, detachable plate)	1	Instruction manual	1
Pre-Mixed Standard	2	Plate sealer for 96 wells	4
Pre-Mixed Magnetic bead	1×1mL	Standard Diluent	1×20mL
Setup Bead	0.4mL	Analysis buffer	1×20mL
Pre-Mixed Detection Reagent A	1×120μL	Assay Diluent A	1×12mL
Detection Reagent B	1×120μL	Assay Diluent B	1×12mL
Sheath Fluid	1×10mL	Wash Buffer (30 × concentrate)	1×20mL

[MATERIALS REQUIRED BUT NOT SUPPLIED]

1. Flow Cytometer: NovoCyte D2060R, Navios, FACSMelody, Gallios, DxFLEX, CytoFLEX S, etc. (It is recommended that the instrument be preheated, self-checked and calibrated before use).
2. Single or multi-channel pipettes with high precision and disposable tips.
3. Microcentrifuge Tubes.
4. Deionized or distilled water.
5. Magnetic frame(When the magnetic field strength is below 5000 Gauss, please extend the magnetic attraction time to 4 minutes).
6. Container for Wash Solution.
7. 0.01mol/L (or 1×) Phosphate Buffered Saline (PBS), pH 7.0~7.2.
8. Vortex oscillator.
9. Incubated Shaker.

[STORAGE OF THE KITS]

1. **For unused kit:** For experiment convenience, reagents should be stored separately, Standard,Standard Diluent,Assay Diluent A and Detection Reagent A should be stored at -20°C while the others could be at 4°C. Pre-Mixed Magnetic beads and Detection Reagent B should be protected from light.
1. **For used kit:** When the kit is used, the remaining reagents need to be stored according to the above storage conditions.

Note:

It is highly recommended to use the remaining reagents within 1 month provided this is prior to the expiration date of the kit. For the expiration date of the kit, please refer to the label on the kit box. All components are stable up to the expiration date.

[SAMPLE COLLECTION AND STORAGE]

Serum - Use a serum separator tube and allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 20mins at approximately 2,000 x g. Collect the supernates and then centrifuge them at approximately 16,000 x g at 2~8°C for 20-30mins. Assay freshly prepared serum immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15mins at 2,000 x g at 2~8°C within 30mins of collection. Collect the supernates and then centrifuge them at approximately 16,000 x g at 2~8°C for 20-30mins. Assay immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Tissue homogenates - The preparation of tissue homogenates will vary depending upon tissue type.

1. Tissues were rinsed in ice-cold PBS to remove excess blood thoroughly and weighed before homogenization.
2. Mince the tissues to small pieces and homogenized them in fresh lysis buffer (catalog: IS007, different lysis buffer needs to be chosen based on subcellular location of the target protein) (w:v = 1:20~1:50, e.g. 1mL lysis buffer is added in 20~50mg tissue sample) with a glass homogenizer on ice.
3. The resulting suspension was sonicated with an ultrasonic cell disrupter till the solution is clarified.
4. Then, the homogenates were centrifuged at 2~8°C for 20~30mins at 16,000 x g. Collect the supernates and assay immediately or aliquot and store at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Cell Lysates - Cells need to be lysed before assaying according to the following directions.

1. Adherent cells should be washed by cold PBS gently, and then detached with trypsin, and collected by centrifugation at 1,000 x g for 5mins (suspension cells can be collected by centrifugation directly).
2. Wash cells three times in cold PBS.
3. Resuspend cells in fresh lysis buffer with concentration of 10^7 cells/mL. If it is necessary, the cells could be subjected to ultrasonication (at 2-8°C) till the solution is clarified.
4. Centrifuge at 16,000 x g for 20~30mins at 2~8°C to remove cellular debris. Assay immediately or aliquot and store at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Cell culture supernates and other biological fluids - Centrifuge samples for 20~30mins at 16,000 x g at 2~8°C. Collect the supernates and assay immediately or store samples in aliquot at -20°C or -80°C for later use.

Avoid repeated freeze/thaw cycles.

Note:

1. **On the day of the assay, All fresh and previously frozen samples require centrifugation at 16,000 x g for 20-30 minutes immediately prior to use or dilution.**
2. Samples to be used within one week may be stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination.
3. Sample hemolysis will influence the result, so hemolytic specimen should not be used.
4. The lipids, colloids or precipitates in the sample will cause the aggregation and sedimentation of magnetic beads and influence the final detection result. It is recommended to centrifuge the sample first and test the sample supernatant.
5. Samples should be slowly equalized to room temperature before use, and should not be heated to melt.
6. Cell cultures with animal serum may contain high levels of latent factor in the preparation of cell culture media and will affect the results.

[REAGENT PREPARATION]

1. Bring all kit components and samples to room temperature (18~25°C) before use. If the kit will not be used up in one time, please only take out strips and reagents for present experiment, and leave the remaining strips and reagents in required condition.
2. **Standard -** Reconstitute the **Standard** with 0.5mL of **Standard Diluent**, kept for 10mins at room temperature,close the lid and invert repeatedly to mix,shake gently (not to foam). The highest concentration of the Standard is shown as tube "1" in the table below. Please prepare 6 tubes labeled STD2~7 containing 0.3mL Standard Diluent, produce a 4-fold dilution series according to the table shown below. Mix each tube thoroughly before the next transfer. Take 100μL to the next tube and mix, until the "STD6" tube with the lowest target concentration. The "STD7" tube only added Standard Diluent is the blank.

Analyte	**Region**	STD1	STD2	STD3	STD4	STD5	STD6	STD7	Concentration
KEAP1	***	10	2.5	0.625	0.156 3	0.039 1	0.009 8	0	ng/mL

3. Before using the **Pre-Mixed Magnetic Beads**, use a vortex oscillator to keep the Magnetic Beads suspended, Pre-Mixed Magnetic Beads should be protected from light.
4. **Detection Reagent A and Detection Reagent B -** Briefly spin or centrifuge the stock Detection A and Detection B before use. Dilute them to the working concentration 100-fold with **Assay Diluent A** and **B**, respectively. Detection Reagent B should be protected from light during the experiment.
5. **Wash Solution** - Dilute 20mL of Wash Solution concentrate (30×) with 580mL of deionized or distilled water to prepare 600mL of Wash Solution (1×).

Note:

1. Making serial dilution in the wells directly is not permitted.
2. Prepare standards within 15mins before assay. Please do not dissolve the reagents at 37°C directly.
3. Please carefully reconstitute Standards or working Detection Reagent A and B according to the instruction, and avoid foaming and mix gently until the crystals are completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to suck more than 10μL for one pipetting.

4. The reconstituted Standards, Detection Reagent A and Detection Reagent B can be **used only once**.
5. If crystals have formed in the Wash Solution concentrate (30×), warm to room temperature and mix gently until the crystals are completely dissolved.
6. Contaminated water or container for reagent preparation will influence the detection result.

[SAMPLE PREPARATION]

1. We are only responsible for the kit itself, but not for the samples consumed during the assay. The user should
2. calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
3. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments. Sample should be diluted by PBS.
4. If the volume of samples is limited, please dilute the samples with PBS to appropriate volume.
5. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
6. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected results due to the impacts from certain chemicals.
7. Due to the possibility of mismatching between antigen from other origin and antibody used in our kits (e.g. antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.
8. Influenced by the factors including cell viability, cell number or sampling time, samples from cell culture supernates may not be detected by the kit.
9. Fresh samples without long time storage is recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

[ASSAY PROCEDURE]

1. Add 200μL analysis buffer solution to each well of the plate for pre-wetting. Shake the plate at room temperature for 10mins on shaker. Then, remove the liquid from the well.
2. Determine wells for diluted standard, blank and sample.Add 100μL each of dilutions of Standard (follow Reagent Preparation step 2), blank and samples into the appropriate wells. Use vortex oscillator to suspend the magnetic beads, add 10μL evenly suspended magnetic beads in each well. Cover with the Plate sealer. Incubate for 120mins protect from light at 25ºC on shaker with speed of 800±50rpm, amplitude of 2~4mm, to avoid settling of magnetic beads. Note: During beads addition, shake mixed beads bottle intermittently to avoid bead settling.
3. Using a magnetic device designed to accommodate a microplate, allow 2 mins before removing the liquid,don’t wash,Uniform removal of liquid is essential for good performance. Note: Do NOT blot; this may cause a loss of microparticles.
4. Add 100μL of **Detection Reagent A** working solution to each well, cover the wells with the plate sealer and incubate for 60mins protect from light at 25ºC on shaker with speed of 800±50rpm, amplitude of 2~4mm.
5. Using a magnetic device designed to accommodate a microplate, allow 2 mins before removing the liquid,don’t wash,Uniform removal of liquid is essential for good performance. Note: Do NOT blot; this may cause a loss of microparticles.

6. Remove the magnetic frame, add 100μL of **Detection Reagent B** working solution to each well, cover the wells with the plate sealer and incubate for 30mins protect from light at 25ºC on shaker with speed of 800±50rpm, amplitude of 2~4mm.
7. Using a magnetic device designed to accommodate a microplate, wash by applying the magnet to the bottom of the microplate, allow 2 mins before removing the liquid, filling each well with Wash Buffer (100 μL) and allow 2 mins before removing the liquid. Uniform removal of liquid is essential for good performance. Note: Do NOT blot; this may cause a loss of microparticles.This process could be performed by magnetic plate autowasher.
8. Remove the magnetic frame, add 100μL of sheath fluid to each well, cover the wells with the plate sealer and protect from light, shake the plate at 25ºC for 10mins on shaker with speed of 800±50rpm, amplitude of 2~4mm. Make the Magnetic beads in suspension, then run the machine and conduct the reading.Preferably within the same day of the assay (Note: Prolonged sample storage can lead to reduced signal.)

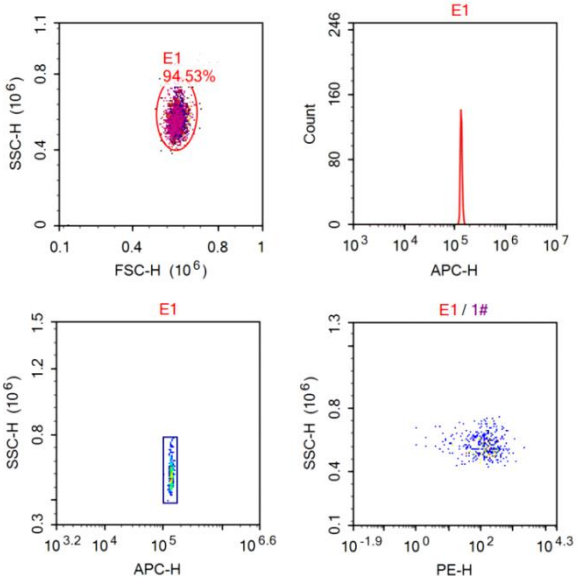
[INSTRUMENT SETTING]

NovoCyte D2060R	
Analytes volume	50μL
Fluorescent	☑APC ☐APC-Cy7 ☑PE
Total Event	100*N
Median Fluorescence Intensity (MFI)	Median

Note: N is the number of targets detected by this kit.

Instrument Setup with NovoCyte D2060R and Beads

1. Start up NovoCyte D2060R and go through Power On Self Test.
2. Add Setup Beads(evenly suspended magnetic beads) into a tube for sampling. Start the NovoCyte D2060R software.
3. Proceed to Instrument Setup with the Setup Beads and establish the template.
4. Analyzing samples by the template created by "step 3".
5. Create a statistical table, collect the PE median values, and export the data.



Note:

1. **Samples or reagents addition: Please use the freshly prepared Standard.** Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10mins. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and samples, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of standards, samples, and reagents. Also, use separated reservoirs for each reagent.
2. **Incubation:** To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents are added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be controlled. To avoid fluorescence quenching, the incubation process should be performed protect from light.
3. **Washing:** The wash procedure is critical. Complete removal of liquid at each step is essential for good performance. But in order to avoid the loss of Magnetic Beads, please perform the wash step on the magnetic frame. DO NOT blot the plate on absorbent paper. Insufficient washing will result in poor precision and false elevated reading.
4. If the humidity in the laboratory is below 60%, it is recommended to use a humidifier to increase the humidity.
5. Before reading, adjust the needle of the instrument to the appropriate height (two magnetic gaskets) from the bottom of the plate wells.
6. When the magnetic field strength is below 5000 Gauss, please extend the magnetic attraction time to 4 minutes.
7. When removing liquid from wells, keep pipette tips away from the magnetically concentrated bead area to prevent accidental aspiration. When adding liquid to wells, direct tips toward the bead accumulation zone to facilitate resuspension.

[TEST PRINCIPLE]

Anti-KEAP1 antibodies were coated on the surface of magnetic beads, to make solid phase carrier. Standard or sample were added to the magnetic beads, respectively, in which KEAP1 was bound to the antibody connected to the solid phase carrier, and then biotinylated anti-KEAP1 antibodies were added. After the unbound biotinylated antibody was washed, PE-labeled avidin was added. There is a positive correlation between the concentration of KEAP1 in the sample and corresponding value of MFI (Median Fluorescence Intensity). Flow Cytometer analyzer was used to determine and calculate the sample concentration.

[CALCULATION OF RESULTS]

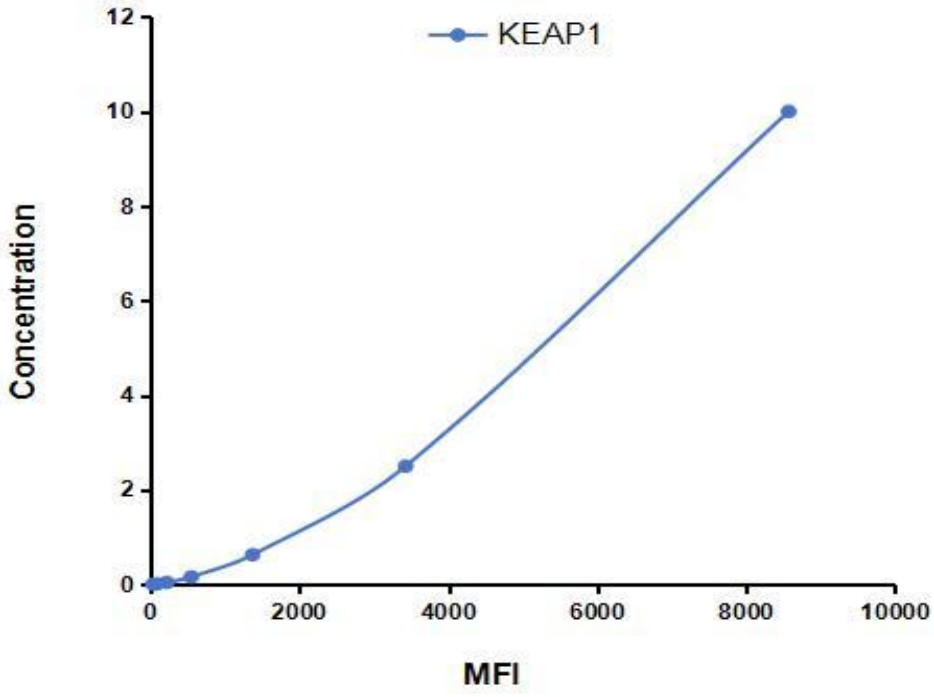
Median MFI value of each standard, control and samples subtract the zero standard MFI value. Create standard curves with KEAP1 concentration on the Y-axis and corresponding MFI on the X-axis, respectively. Draw a best fit curve through the points and it can be determined by regression analysis. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

[TYPICAL DATA]

In order to make the calculation easier, we plot the corrected MFI value of the standard as the horizontal axis (X-axis) against the concentration of the standard as the Y-axis, although the concentration is the independent

variable and the MFI value is the dependent variable. In order to make the experimental results intuitive, the original data rather than logarithm values are provided in the figure. Logarithmic values are recommended for standard graphs.

However, the MFI of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), plotting log of the data to establish standard curve for each test is recommended. Typical standard curve below is provided for reference only.



X-axis: MFI, Y-axis: Concentration

Typical Standard Curve for Multiplex Assay Kit.

Analyte	Region	Detection range	Sensitivity
KEAP1	***	0.0098-10ng/mL	0.0033ng/mL

The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined by adding two standard deviations to the mean MFI value of twenty zero standard replicates and calculating the corresponding concentration.

[SPECIFICITY]

This assay has high sensitivity and excellent specificity for detection of KEAP1. No significant cross-reactivity or interference between the targets and analogues was observed.

Note:

Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between the targets and all the analogues, therefore, cross reaction may still exist.

[STABILITY]

The stability of Multiplex Assay Kit is determined by the loss rate of activity. The loss rate of this kit is less than 5% prior to the expiration date under appropriate storage condition.

To minimize extra influence on the performance, operation procedures and lab conditions, especially room temperature, air humidity, incubator temperature should be strictly monitored. It is also strongly suggested that

the assay is performed by the same operator from the beginning to the end.

[IMPORTANT NOTE]

1. Limited by the current conditions and scientific technology, we can't completely conduct the comprehensive identification and analysis on the raw material provided by suppliers. So there might be some qualitative and technical risks to use the kit.
2. The final experimental results will be closely related to validity of the products, so the kit should be used prior to the expiration date. And please store the kits exactly according to the instruction.
3. Kits from different batches may be a little different in detection range, sensitivity. Please perform the experiment exactly according to the instruction attached in kit while electronic ones from our website is only for reference.
4. Do not mix or substitute reagents from one kit lot to another. Use only the reagents supplied by manufacturer.
5. Protect all reagents from strong light during storage and incubation. All the bottle caps of reagents should be covered tightly to prevent the evaporation and contamination of microorganism.
6. Variation in sample preparation and each step of experimental operation may cause different results. In order to get better reproducible results, the operation of each step in the assay should be controlled.
7. Each kit has been strictly passed QC test. However, results from end users might be inconsistent with our in-house data due to some unexpected transportation conditions or different lab equipments. Intra-assay variance among kits from different batches might arise from above factors, too.
8. Kits from different manufacturers with the same item might produce different results, since we haven't compared our products with other manufacturers.
9. The standard of the kit and immunogen used for antibody preparation are commonly recombinant proteins, as different fragments, expression systems, purification methods might be used in recombinant protein preparation, we cannot guarantee the kit could detect recombinant protein from other companies. So, it is not recommended to use the kit for the detection of recombinant protein.
10. Please predict the concentration of target molecules in samples, or arrange a preliminary experiment, it is a good way to solve specific problem, e.g. the concentration of samples are beyond the detection range of the kit.
11. The kit might not be suitable for detection of samples from some special experiment, for instance, knock-out experiments, due to their uncertainty of effectiveness.

The kit is designed for research use only, we will not be responsible for any issue if the kit was used in clinical diagnostic or any other procedures

[TROUBLE SHOOTING]

Problem	Possible Source	Correction Action
Poor Standard Curve	Improper standard curve preparation	Ensure accurate operation of the dilution
	Incomplete washing and aspiration	Adequate washing and adequate aspiration
	Inaccurate Pipetting	Check and Calibrate pipettes
Poor Precision	Incomplete washing of wells	Ensure sufficient washing
	Inadequate mixing and aspiration reagents	Adequate aspiration and mixing reagents

	Reused pipette tips, containers and sealers	Change and use new pipette tips, containers and sealers
	Inaccurate Pipetting	Check and Calibrate pipettes
Low MFI	Inadequate reagent volumes added to wells	Calibrate pipettes and Add adequate reagents
	Incorrect incubation times	Ensure sufficient incubation times
	Incorrect incubation temperature	Reagents balanced to room temperature
	Loss of Magnetic Beads during the assay	Ensure the washing process performed on the magnetic frame, do not blot the plate on absorbent paper
	Conjugate reagent failure	Replace the conjugate reagent
	Incorrect Conjugate reagent dilution	Follow the assay protocol in the kit manual
	Read beyond suggested reading time	Read within the time recommended in the manual
Sample Values	Improper Sample Storage	Store the sample properly and use the fresh sample
	Improper sample collection and preparation	Take proper sample collection and preparation method
	Low quantity of analyte in samples	Use new sample and repeat assay
Insufficient Bead Count	Lipids, colloids or precipitates in sample leads to beads aggregation and sedimentation	Re-centrifuge sample before using and take the supernatant for detecting
	Beads are not fully suspended before adding into the plate well	Fully suspend the beads before adding into the plate well.
	Beads lost during washing process	Remove the wash buffer using single or multi-channel pipettes, the pitette tips should keep far away from each magnet on the magnetic frame, or decant the wash buffer by turn over the plate together with magnetic frame
	Beads are not fully suspended before reading	Fully suspend beads by vortex shaking or pipette blowing
	Probe may be clogged	Probe of the machine need to be cleaned, if needed, probe could be removed and sonicated