

ab323460 – SEAP Reporter Gene and Alkaline Phosphatase Activity Assay Kit (Luminometric)

For the measurement of alkaline phosphatase activity in various samples.

For overview, typical data and additional information please visit:

<http://www.abcam.com/ab323460> (use www.abcam.cn/ab323460 for China, or www.abcam.co.jp/ab323460 for Japan)

Storage and Stability: Store kit at -20°C in the dark immediately on receipt and check below for storage for individual components. Kit can be stored for 1 year from receipt if components have not been reconstituted.

Reconstituted components are stable for 2 months.

Aliquot components in working volumes before storing at the recommended temperature.

Avoid repeated freeze-thaws of reagents.

Materials Supplied:

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
ALP Assay Buffer II	50 mL	-20 °C	4 °C
ALP Luminescent Substrate	300 µL	-20 °C	-20 °C
ALP Lysis Buffer	50 mL	-20 °C	4 °C
Enzyme Mix II	1 vial	-20 °C	-20 °C

Materials Required, Not Supplied

Microplate reader capable of measuring luminescence

White 96 well plate with flat bottom

Dounce homogenizer (if using tissue)

Reagent Preparation: Briefly centrifuge small vials at low speed prior to opening.

- ALP Assay Buffer II and ALP Lysis Buffer:** Equilibrate to room temperature (RT) Allow to warm to RT prior to use. Store at +4°C.
- ALP Luminescent Substrate:** Divide into aliquots and store at -20°C, protected from light and moisture. Avoid repeated freeze-thaw cycles. Prior to use, warm solution to RT. After use, promptly retighten the cap to minimize adsorption of airborne moisture.
- Enzyme Mix II:** Reconstitute with 1000 µl of ALP Assay Buffer II. Store at -20°C and avoid repeated freeze-thaw cycles.

Sample Preparation

ΔNOTE: This assay was developed using human bone marrow-derived mesenchymal stem cells (BMSCs) as an example multipotent cell line and Jurkat cells as an example terminally-differentiated cell line. Cells were grown in T-25 sized culture dishes and seeded in a 96-well plate format at a density ranging from 1000 to 50,000 cells per well for ALP detection.

ΔNOTE: For investigating SEAP, use transfected cells with a promoter construct driving the expression of SEAP to seed the desired number of wells in a 96 well plate. Cells transfected with an empty vector should be used as a control.

- Culture cells to 80-90% confluence in a CO₂ incubator at 37 °C for the in appropriate growth, induction or differentiation medium according to the cell maintenance/differentiation protocol.

SEAP Samples:

- Transfer 10 µl of culture medium to a white 96-well plate and heat plate to 60 °C for 30 minutes.
- Bring the volume up to 50 µl per sample well with ALP Assay Buffer II.

Whole Cells (Pluripotent/Multipotent Stem Cells) and Cell Lysates Samples:

- For adherent cells, detach using a cell dissociation solution (e.g. 0.25% trypsin or non-enzymatic dissociation solution) or manually with a rubber cell scraper and transfer cells to a clean microfuge tube. Rinse cells with serum-free medium and pellet by centrifugation at 600 x g for 5 min.

ΔNOTE: We recommend a using non-enzymatic cell dissociation solution or manual scraping for whole embryonic, mesenchymal or induced pluripotent stem cells. Trypsin may cleave the membrane-associated placental-type ALP that is characteristically expressed by these cells.

- For suspension cells, pellet cells by centrifugation, rinse with serum-free medium and pellet again.
- For detection of extracellular surface ALP only (whole cells), aspirate serum-free medium and resuspend cells in ALP Assay Buffer II at a concentration of 1 x 10⁶ cells per ml of buffer.
- For detection of total ALP activity (including intracellular), aspirate serum-free medium, resuspend in ALP Lysis Buffer (1 x 10⁶ cells per ml of buffer), vortex thoroughly and incubate on ice for 15 min, vortexing every 5 min. Centrifuge lysate at 5000 x g for 10 min at 4 °C and transfer supernatant to a clean microfuge tube.
- Add sample volume that corresponds to the desired number of cells to sample wells (anywhere from 100-50000 whole cells or a corresponding volume of lysate) in an opaque white 96-well plate and bring the volume up to 50 µl per well with ALP Assay Buffer II.

Tissues:

- Soft tissues (~10 mg wet tissue) should be homogenized on ice with 100 µl cold ALP Lysis Buffer using a Dounce glass bead homogenizer or probe sonicator (adjust the amount of ALP Lysis Buffer based upon the tissue sample weight).
- Centrifuge homogenate at 10,000 x g for 15 min at 4°C and transfer the supernatant to a new micro-centrifuge tube.
- Add 2-50 µl of sample to desired wells in an opaque white 96-well plate and bring the volume up to 50 µl per well with ALP Assay Buffer II.

Positive Control:

- For Positive Control, dilute reconstituted Enzyme Mix II stock 100 -fold with ALP Assay Buffer II (mix 5 µl ALP Positive Control stock and 495 µl ALP Assay Buffer II). This stock may be used to build an optional standard curve.
- Dilute this 50-fold by mixing 10 µl with 490 µl of ALP Assay Buffer II.
- Add 50 µl of the diluted solution to a designated positive control.

Reagent Blank:

- In addition to sample wells, prepare a reagent blank well (background control) by adding 50 µl of ALP Assay Buffer II to a designated well.

ΔNOTE: If measuring secreted alkaline phosphatase in cell culture supernatant, reagent blank should be made with a proportionate amount of growth medium (20% growth medium, 80% ALP Assay Buffer II).

Standard Curve (Optional)

Always prepare a fresh set of standards for every use. Discard working standard dilutions after use as they do not store well. The following section describes the preparation of a standard curve for duplicate measurements (recommended).

1. Dilute reconstituted Enzyme Mix II stock 100-fold with ALP Assay Buffer II (mix 5 μL ALP Positive Control stock and 495 μL ALP Assay Buffer II). (Positive Control Step 1). This is the 200 μU/μL **Stock Standard** Solution.
2. Label eight tubes, Standards 1– 8.
3. Add 490 μL of ALP Assay Buffer II into tube number 1 and 150 μL of ALP Assay Buffer II into numbers 2-8. If samples have low predicted activity, the standard curve may be expanded to include 3 additional points (1.563, 0.781 and 0.391 μU).

ΔNOTE: If using the standard curve to measure secreted alkaline phosphatase in cell culture supernatant, custom diluent should be made with a proportionate amount of growth medium (20% growth medium, 80% ALP Assay Buffer II).

4. Use the **Diluted Stock Standard** to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:

Standard #	Dilution Sample	Volume to Dilute (μL)	Volume of Diluent (μL)	Final activity (μU /well)
1	Stock Standard	10	490	200
2	Standard#1	150	150	100
3	Standard#2	150	150	50
4	Standard#3	150	150	25
5	Standard#4	150	150	12.5
6	Standard#5	150	150	6.25
7	Standard#6	150	150	3.125
8	Blank Control	0	150	0

5. Add 50 μL of Standard to each respective standard well.

ΔNOTE: Since standard and positive control is built out of heat intolerant alkaline phosphatase, these should not be heated.

Assay Procedure

Equilibrate all materials and prepared reagents to RT just prior to use and gently agitate. Assay all standards, controls and samples in duplicate.

1. For each reaction well (including reagent blank, positive control wells, and standard curve wells if applicable), prepare 50 μL of Sample Reaction Mix according to the table below. Prepare sufficient volume of each type of mix to add 50 μL to all assay wells. by mixing 1 μL of ALP Luminescent Substrate and 49 μL ALP Assay Buffer II. Make a sufficient amount of the Reaction Mix for all of the assay wells (we recommend preparing a minimum of 5 reaction wells to ensure pipetting accuracy).

Component	Reaction Mix (μL)
ALP Luminescent Substrate	1
ALP Assay Buffer II	49

2. Add 50 μL to each reaction well using a multichannel pipette and mix reagents completely by gently tapping plate.
3. Start measuring luminescence intensity in kinetic mode, reading luminescence every 1-2 minutes for at least 30 minutes.

ΔNOTE: Peak luminescence is typically achieved within 5-10 min and signal intensity begins to decay after 15-30 minutes, depending upon the sample ALP activity.

Data analysis

1. Calculate the net luminescence signal (L) by subtracting the reagent background (BC) RLU reading from each of the corresponding Sample RLU readings:

$$L = RLU_{Sample} - RLU_{BC}$$

Optional: Using the standard curve to calculate ALP activity

2. Plot the activity of the alkaline phosphatase vs luminescence for the chosen time point.
3. Find the equation of the line in the form $y=mx + b$.
4. Use the luminescence signal for each sample and the equation of the line to determine the alkaline phosphatase activity in the well (x).
5. Determine the activity of the sample by accounting for the volume of sample added to the well and any sample dilutions.

$$Activity = \frac{x}{V} * D$$

Where: x is the activity in the well determined using the standard curve (μU)

V is the volume of sample added to the well (in μL)

D is the dilution factor for samples diluted prior to adding the sample to the well

Technical Support

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