upstate | CHEMICON | Linco
THE EXPERTISE OF UPSTATE®, CHEMICON® AND LINCO® IS NOW A PART OF MILLIPORE

Quantitative Alkaline Phosphatase ES Characterization Kit

CATALOG NUMBER: SCR066

LOT NUMBER:

QUANTITY: 1 Kit

DESCRIPTION: Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass (ICM) of preimplantation embryos and are capable of unlimited, undifferentiated proliferation *in vitro* under appropriate cell culture conditions. Undifferentiated embryonic stem cells are characterized by high expression levels of stage-specific embryonic antigens (SSEA-1, 3 and 4), tumor rejection antigens (TRA-1-60 and TRA-1-81), Oct-4 and alkaline phosphatase. These undifferentiated markers are downregulated upon spontaneous or induced differentiation.

Alkaline phosphatase (AP) is a hydrolase enzyme responsible for dephosphorylating molecules such as nucleotides, proteins, and alkaloids under alkaline conditions. The enzyme is present within all tissues of the body but is elevated in cells of the liver, kidney, bone, placenta, embryo and under specific disease states. Under alkaline conditions (pH>10), AP can catalyze the hydrolysis of p-nitrophenylphosphate (p-NPP) into phosphate and p-nitrophenol, a yellow colored by-product of the catalytic reaction. The amount of p-nitrophenol produced is proportional to the amount of alkaline phosphatase present within the reaction. The amount of AP can thus be reliably quantified by reading the amount of p-nitrophenol amassed after the catalytic reaction at 405 nm on a spectrophotometer.

p-nitrophenylphosphate —**Alkaline Phosphatase (pH>10)**→ **phosphate + p-nitrophenol (yellow)**

Millipore's Quantitative Alkaline Phosphatase ES Characterization Kit allows researchers to easily quantify the amount of alkaline phosphatase present within their embryonic stem cell cultures using a convenient 96-well colorimetric enzymatic assay. This kit can be used in conjunction with Millipore's Alkaline Phosphatase Detection kit (Cat. No. SCR004) to fully characterize the levels of alkaline phosphatase present within an embryonic stem cell culture.

KIT COMPONENTS: Sufficient for 100 Reactions

p-NPP Substrate Concentrate (50X): (Part No. ES008-200UL)	200 µL
p-NPP Buffer: (Part No. ES011-100ML)	100 mL
Reaction Stop Solution: (Part No. CS200653)	5 mL
1X Wash Solution: (Part No. CS200652)	2 X 125 mL
Recombinant Alkaline Phosphatase Standard: (Part No. CS200654)	1 µg (10 µg/mL)

STORAGE/ HANDLING: All reagents are to be stored at 2° to 8°C until expiration date. p-NPP substrate concentrate and p-NPP buffer are light sensitive and should not be exposed to light. Reaction stop solution contains 1N sodium hydroxide and is corrosive. Avoid skin contact and inhalation.

USA & Canada • Phone: +1(800) 437-7500 • Fax: +1 (951) 676-9209
www.millipore.com

Important Note: During shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. For products with volumes of 200 µL or less, we recommend gently tapping the vial on a hard surface or briefly centrifuging the vial in a table-top centrifuge to dislodge any liquid in the container's cap.

ASSAY PROTOCOL: We recommend that an 8-point standard curve for alkaline phosphatase (including blank) be included in triplicate for every assay run. Total reaction volume = 100 µL. Recombinant alkaline phophatase is provided in the kit.

1. Determine the total reaction volumes required to carry out the experiments in replicates and to include the 8-point standard curve with blank in replicates. For example if there are 2 experimental conditions, the calculations are as follows:

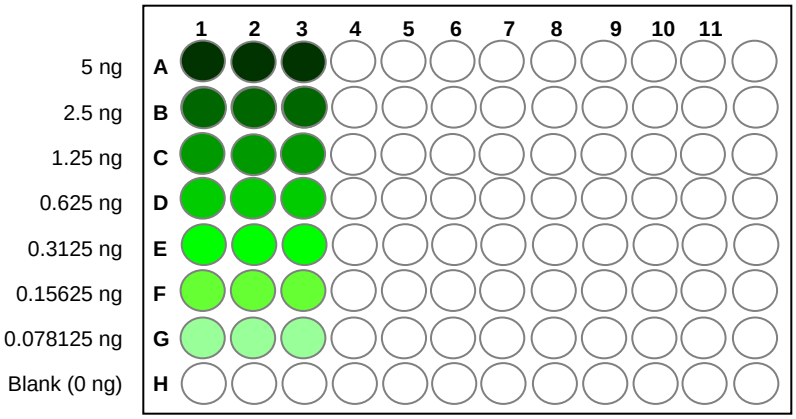
   2 experimental conditions X 3 replicates = 6 total reactions
   8 reactions (i.e. 8-point standard curve) x 3 replicates = 24 total reactions
   30 total reactions X 100 µL reaction volume = 3000 µL total volume

   Assuming ~ 20% volume overage = 3000 + 600 = 3600 µL or ~ **3.6 mL total reaction volume**
2. Prepare a 2X p-NPP Substrate solution such that the total volume is one half of the total reaction volume. Thus using the example provided above, make up 1.8 mL of a 2X p-NPP Substrate solution by diluting 72 µL of 50X p-NPP Substrate Concentrate with 1728 µL of p-NPP Buffer. Set aside.

   Note: 2X p-NPP Substrate Solution should always be prepared fresh before each assay run and should be protected from light.
3. Aspirate the culture medium from the ES cells or other cells to be analyzed.
4. Wash the cells with 10 mL 1X PBS to remove any residual serum.
5. Detach the ES cells to be analyzed with Accutase (Cat. No. SCR005).
6. Collect the cells in a 15 mL conical tube.
7. Centrifuge the tube at 2000 rpm for 5 minutes at room temperature to pellet the cells.
8. Remove the supernatant and resuspend the cell pellet in 2 mL 1X Wash Solution.
9. Count the number of cells using a hemacytometer.
10. Aliquot the desired number of cells (we recommend 20,000 cells per reaction) to three Eppendorf tubes (for replicates). Do this for each of the cell sample to be analyzed.
11. Spin at 2000 rpm for 5 minutes at room temperature to pellet the cells.
12. Remove the supernatant and resuspend each cell pellet in 50 µL p-NPP Buffer. Transfer each of the three cell suspensions to one well of the 96-well assay plate for a total of three wells. For example, using the example provided above regading 2 experimental samples, use A4, A5, A6 for 1 sample and B4, B5, B6 for the 2nd experimental sample. At this time, the enzymatic reaction has not been initiated. Enzymatic reaction is initiated when 50 µL 2X p-NPP Substrate Solution is added to the cell suspension (i.e. Step 14).

    ***Note**: It is important to include experimental replicates. Avoid plating cell suspension onto the first 3 columns (A1-3 through H1-3) of the 96-well plate which should be reserved to run the 8-point alkaline phosphatase standard curve.*

13. Prepare an 8-point standard curve in triplicates. This is done by serial dilutions of the AP standard in triplicates, corresponding to 5, 2.5, 1.25, 0.625, 0.3125, 0.15625 and 0.078125 ng AP and a blank control, as described below:



- Using a multichannel micropipette, aliquot 50 µL of p-NPP Buffer into the first three columns (A1-3 through H1-3) of the 96-well plate. Total number = 24 wells.
- Aliquot an additional 49 µL of p-NPP Buffer into the first three wells (A1, A2, A3). Total volume in these 3 wells after the addition is 100 µL.
- Using an appropriate micropipette and tips, transfer 1 µL of AP standard into each of the three wells, A1, A2, A3, containing the 100 µL p-NPP Buffer. Use a fresh tip for each transfer.
- Using a multichannel micropipette set to 50 µL, carefully mix the contents of wells A1, A2, A3 and then transfer 50 µL to the next row of wells (B1, B2, B3).
- Repeat the mixing and transfer 50 µL to the next row of wells (C1, C2, C3). Repeat the mixing and transfer of 50 µL, going consecutively down each row to obtain in total seven serial dilutions of the AP standard, in triplicates, corresponding to 5, 2.5, 1.25, 0.625, 0.3125, 0.15625 and 0.078125 ng AP per well.
- Discard the excess 50 µL solution from wells G1, G2, G3.
- Wells H1, H2, and H3 should contain only the p-NPP Buffer and serve as a blank or 0 ng AP control.

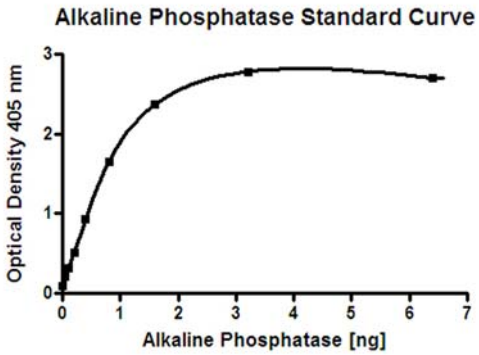
14. Using a multichannel pipette, aliquot 50 µL of the 2X p-NPP Substrate Solution into each of the wells containing the 8-point standard curve (A1-3 to H1-3) and also to each of the unknown samples (i.e. A4, A5, A6 and B4, B5, B6) so that the final reaction volume is 100 µL. Addition of 2X p-NPP Substrate Solution initaties the enzymatic reaction.
15. Incubate for 20 minutes at room temperature in the dark.

    Note: The reaction is time dependent. Do not leave the reaction for longer than 20 minutes (i.e. one hour),as all the samples (regardless of the amount of input AP) will then be read at the maximum absorbance.

16. Using a multichannel pipette, add 50 µL of Reaction Stop Solution to each reaction. Total volume per well = 150 µL.
17. Read the absorbance at 405 nm.

DATA:

A.



B.

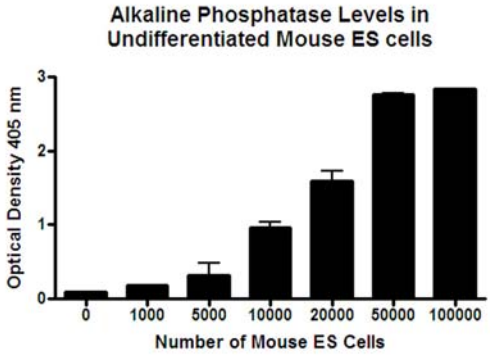


Figure 1. Standard curve of recombinant alkaline phosphatase **(A)**. Optimal number of cells to use per reaction was determined by assaying discrete numbers of undifferentiated murine embryonic stem cells (Cat. No. SCR012) cultured in media containing 15% serum and 1000 units/mL LIF/ESGRO **(B)**.

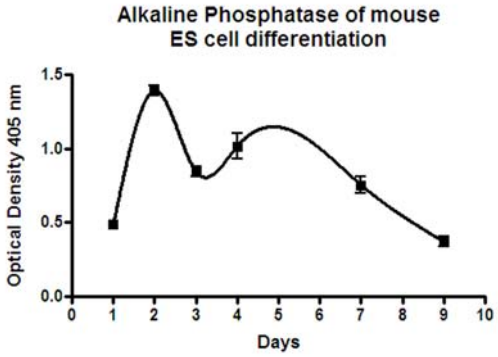


Figure 2. Mouse ES cells (Cat No. SCR012) were cultured in media containing 15% serum and 1000 units/mL LIF/ESGRO for 2 days. After the second day, differentiation was induced by the removal of LIF and β-mercaptoethanol (BME) from the culture media for 9 days. Removal of LIF and BME correlates with a decrease in alkaline phosphatase levels using SCR066. For each time point, approximately, 20,000 cells were assayed in triplicate as per protocol instructions.

FOR RESEARCH USE ONLY; NOT FOR USE IN DIAGNOSTIC PROCEDURES. NOT FOR HUMAN OR ANIMAL CONSUMPTION