# Alizarin Red S Staining of Cells Cultured in Mineralization Medium

(By Hongwei Cheng @ 06/11/01; updated by Yi Zhu @ 01/08/2024; Commented by TCH)

## A. Chemicals/Reagents for Mineralization Medium

1). L-Ascorbic acid (AA) ($C_6H_7O_6Na$)
2). Sodium β-glycerophosphate pentahydrate (bGP)(C3H19Na2O11P), MW 308.13 g/mol

## B. Stock Preparation

1). **L-Ascorbic acid (AA)** @ 0.25g/ml (= **500x stock**)
1.25g Ascorbic acid in 50ml 1x PBS; syringe-filtered and aliquoted/kept @ -20°C.
2). **Sodium β-glycerophosphate pentahydrate (bGP)** @ 1.0M (= **100x stock**)
3.081g β-glycerophosphate in 10ml 1x PBS; syringe-filtered and aliquoted/kept @ -20°C.
***NOTE****: It is more desirable to mix AA and bGP solutions before use.*

## C. Working/Cell Culture Medium Preparation

1). The total volume varies depending on experimental design.
2). For example, to prepare 50ml AA/bGP-containing working medium, add 100ul of **AA (500x stock)** and 500ul of **bGP (100x stock)** to 49.4ml **Complete DMEM** (in a 50-ml conical centrifuge tube) [*The final concentration for ascorbic acid is 50μg/mL, and β-glycerophosphate 10mM, respectively*].

## D. Cell Culture in Mineralization Medium

1). Seed cells in cell culture plates (24-, 12, or 6-well) for 4-6h to allow full attachment.
2). Replace the medium with the AA/bGP-containing mineralization medium prepared above.
3). You can add other treatment at this stage as well (e.g., adenoviral infection, etc.).
4). Depending on cell types and/or experimental design, keep the culture in a $CO_2$ incubator for 5-14 days, and proceed to Alizarin Red S staining (***see next section***).

(**NOTE**: *The culture medium may turn to yellowish color after 3-5 days. This is normal and very common. You* ***should NOT ever change the medium****. Instead, you can* ***add some fresh complete DMEM medium****, and let the culture continue till the endpoints of the assay).*

## E. Alizarin Red S Staining

1). Prepare **2% Alizarin Red S Solution** (usually stable for at least 2-3 weeks): Dissolve 2.0g of Alizarin Red S (Sigma-Aldrich) in 100ml dd$H_2O$. Mix well (*using magnetic stirring bar if necessary*)[***NOTE****: Use Whatman filter paper to remove debris o precipitates if necessary*]
2). Prepare **1x PBS pH 4.2 solution**: To 100ml 1x PBS, add 10% (v/v) HCl dropwise to adjust pH to 4.2 (*approx. 500μl of 10% HCl for 100ml 1x PBS*).
3). Fix cells in 2.5% glutaraldehyde (freshly prepared in 1x PBS buffer) for 10-15min at room temperature.
4). Remove fixation solution and wash the fixed cells with 1x PBS pH4.2 twice.

5). Add filtered 2% Alizarin Red Solution to the fixed cells and leave in 37°C incubator for 10-20min (Note: monitor staining under microscope every 2-5 min.).
6). Remove Alizarin Red Solution and rinse the cells with regular 1x PBS 1-2 times.
7). Record staining results with a bright field microscope (use non-phase contrast; positive stained nodules are in orange red).

## GENERAL COMMENTS

1). It is important to wash the fixed cells with PBS adjusted pH to 4.2 to get brilliant red color.
2). Alizarin Red can be quantified by dissolving the stain in a solution of 20% methanol and 10% acetic acid in water for 15min, followed by OD reading on a spectrophotometer at 450nm.
3). Staining should be stable for 1-2 weeks in either dry or wet condition, but the best images are obtained immediately after staining.

## SAMPLE STAINING RESULTS