Cell Labeling and Long Term Stability of CL-30Q02-6: Molday Ion(-)

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Authors: John Haserick and Ernest Groman

Purpose: This experiment was performed to determine if CL-30Q02-6 effectively labels NIH 3T3-fibroblasts after 70 days of storage at 4°C when used in conjunction with Poly-L-Lysine(PLL) per standard protocol.

Materials:

Catalog No.	Compound	Source
CL-30Q02-6	Molday ION(-) 10mg Fe/ml lot: 090323	BioPAL
CL-00-01	Poly-L-Lysine solution(10mg/ml)	BioPAL
CL-01-50	Prussian Blue solutions	BioPAL
PBS++	PBS with Mg and Ca (PBS++)	BioPAL
25% Glutaraldehyde	25% Glutaraldehyde	BioPAL
40% Formalin	40% Formalin	BioPAL
12mmx75mm borosilicate glass test tubes		Generic
NIH 3T3-fibroblasts grown to ~70% confluency in 24-well plate		ATCC
DMEM with 10% BCS and 1X AAS		Hyclone

Methods:

The cell labeling effectiveness of **CL-30Q02-6** [Molday ION(-)] is greatly increased by preparing the compound with PLL prior to diluting to the desired final concentration. Therefore, 200ul of **CL-30Q02-6** was mixed with 800ul of distilled water in a glass test tube, yielding a concentration of 2mg/ml Molday ION(-). To this solution, 60ul of PLL(10mg/ml) was added. Next, the test tube was gently vortexed to thoroughly mix the contents. The 2mg/ml **CL-30Q02-6**/PLL solution was allowed to "set" for 20 minutes at room temperature. After the 20 minute period had elapsed, the **CL-30Q02-6**/PLL complex was diluted to twice the target concentration with previously warmed and equilibrated complete DMEM medium (DMEM w/10% BCS and 1X AAS was placed in incubator for 30 minutes at 37°C and 5% CO_2 to equilibrate).

Four 2X target **CL-30Q02-6**/PLL concentrations used in this study were prepared as shown below:

A. 50.0 ul Molday(-)PLL conjugate + 450.0 ul DMEM = [200.0 ug Fe/ml]
B. 25.0 ul Molday(-)PLL conjugate + 475.0 ul DMEM = [100.0 ug Fe/ml]
C. 12.5 ul Molday(-)PLL conjugate + 487.5 ul DMEM = [50.0 ug Fe/ml]
D. 6.25ul Molday(-)PLL conjugate + 493.5 ul DMEM = [25.0 ug Fe/ml]

Each of the above solutions were thoroughly mixed by gently pipetting the solutions up and down. Next, the wells containing the NIH 3T3 cells were overlaid with 500ul of complete DMEM. Subsequently, the freshly mixed 500ul of the 2X **CL-30Q02-6**/PLL complex DMEM solutions (A, B, C, D) were added to the 500ul volume already on top of the cells. Thus achieving the final concentrations listed below.

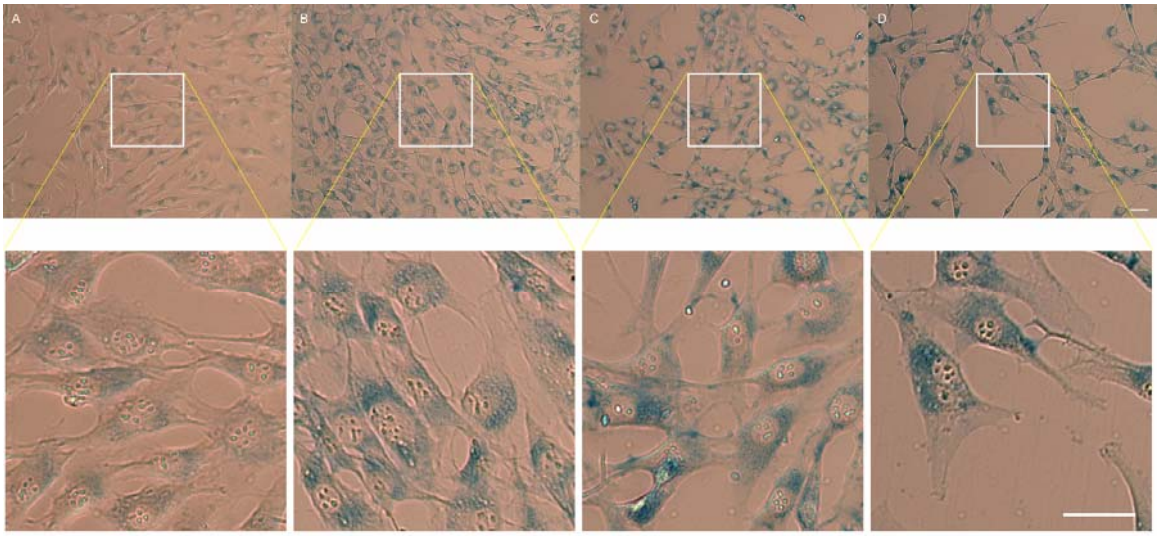
Final **CL-30Q02-6**/PLL concentrations on the cells:

A. 100.0 ug Fe/ml
B. 50.0 ug Fe/ml

C. 25.0 ug Fe/ml
D. 12.5 ug Fe/ml

When the addition was complete, the cells were placed into an incubator for 20.5 hours with 5% CO_2 with the temperature set at 37°C. The cells were removed from the incubator, the solution completely aspirated off and the cells washed with **PBS++** warmed to 37°C. The **PBS++** was aspirated off, then the cells were fixed using the **Cell fixation solution** (1) by adding (1ml/well). The fixative was allowed to remain on the cells for 10 minutes, then removed and washed twice with **PBS++**. Next, the Prussian blue solution (**CL-01-50**) was added to the cells and incubated for 10 minutes. The Prussian Blue solution was replaced with **PBS++** prior to imaging.

The cells were imaged using bright field optics and a 20X Plan Fluor objective (200x effective magnification) on a Nikon TE-2000S microscope. The exposure was 100ms with 1x software gain. Images were captured on a Diagnostic Instruments 18.2 Color Mosaic CCD camera. The captured images were arranged using the software program ImageJ version 1.21o.



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This montage shows NIH 3T3 cells after 20.5 hours of incubation with **CL-30Q02-6**/PLL. In this experiment, the level of iron staining is greatest at the lowest concentration of iron loaded onto the cells. Also, visual inspection suggests that the cells are healthy and the reagent is well tolerated even at the highest concentration. The lower half of the montage shows 400x400 pixel sections of selected areas from the upper montage to show detail. The concentration of the iron in A-D was: A. 100.0 ug Fe/ml, B. 50.0 ug Fe/ml, C. 25.0 ug Fe/ml, and D. 12.5 ug Fe/ml.

Supplemental:

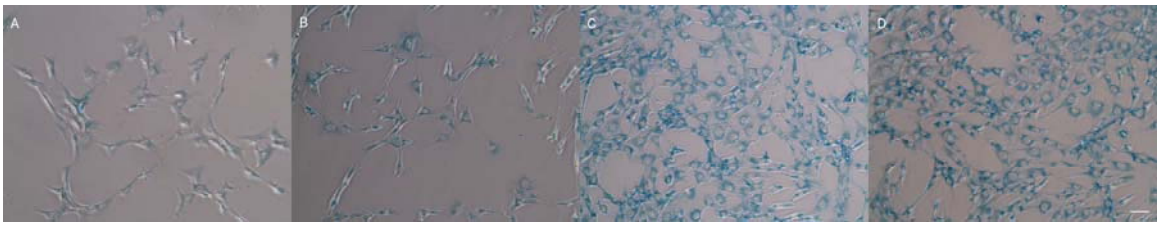
(1) **Cell fixation solution.** Prepare sufficient cell fixation solution consisting of: 2% formalin and 2.5% glutaraldehyde in PBS++. The recipe for 10ml of cell fixation solution is :

PBS++ 8.5ml
40% formalin 0.5ml
25% glutaraldehyde 1.0ml

Addendum:

CL-30Q02-6 lot: 090215 was tested for robustness by shipping to Johns Hopkins University stored for three months at various temperatures (4-25°C) and returned to BioPAL where it was left at room temperature for 10 days before testing using the aforementioned procedure. The results appeared to be identical to lot: 090323, which was manufactured 1.5 months later than lot 090215.

The material was prepared and added to NIH-3T3 cells in the exact same manner as the previously outlined protocol. The cells were incubated with the material for 21 hours before washout, fixation, and treatment with the Prussian Blue reagent.



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A. 100.0 ug Fe/ml
B. 50.0 ug Fe/ml
C. 25.0 ug Fe/ml
D. 12.5 ug Fe/ml