Stem Cell Research 23 (2017) 158–162

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

# A multiplexed screening method for pluripotency

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ARTICLE INFO

*Article history:*
Received 3 April 2017
Received in revised form 15 June 2017
Accepted 11 July 2017
Available online 12 July 2017

*Keywords:*
Alkaline
Phosphatase
Differentiation
Screening
ATP
Drug

ABSTRACT

Measurement of Alkaline Phosphatase (ALP) level is a widely used procedure in clinical and basic research. We present a simple and inexpensive luminescence-based method that allows multiplexed measurement and normalization of intracellular ALP levels in one sample well. The method comprises two commercially available reagents enabling quantification of ALP levels and cell number by two sequential luminescence readouts. Using this method we were able to detect and analyze somatic reprogramming into pluripotent stem cells. The method is highly applicable for High Throughput Screening (HTS) campaigns and analysis.



## 1. Introduction

Alkaline phosphatase (ALP) is a ubiquitous membrane-bound glycoprotein that catalyzes the hydrolysis of phosphate monoesters at basic pH values. Examination of expression of ALP at intracellular and extracellular level is a widely-used procedure in both clinical practice and basic research. In the clinic, changes of normal ALP level in plasma is a routine diagnostic marker for various pathological processes (Sharma et al., 2014). At the basic research level, ALP quantification is frequently used in the study of cancer physiology and for evaluation of pluripotency in stem cells. Intracellular expression of ALP is very high in induced pluripotent (iPS) and embryonic stem cells (ES) and therefore can be used as unique and unambiguous biomarker of stem cells (Martins et al., 2014; Stefkova et al., 2015). In cancer, ALP expression inversely correlates with disease severity in advanced colon cancer and positively correlates with the ability of cells to differentiate (Shin et al., 2015).

Currently, there are a number of methods for ALP detection. However, all of them have certain disadvantages such as: cost, multiplicity of reagents and washing steps, low-precision methods of detection, and the necessity of dedicated instruments. While these limitations may not be major liabilities in low-throughput analysis, drug discovery modalities typically require an optimized and affordable platform to perform automated High Throughput Screening (HTS) where the use of 384 and 1536-well microtiter plate formats is the standard.

Interpretation of HTS data typically relies on a cascade of assays whereby small organic compounds are sequentially tested and filtered by a series of single dimension assays and counter-assays. Multiplexing several readouts from the same sample can streamline this process and provide a significant advantage in cost-effectiveness and improvement of data quality by a more informed interpretation. The current state of the art methods for quantifying ALP levels improve greatly when normalizing to cell number. Although cytotoxicity monitoring is the most intuitive use for such a multiplexed assay, the transition from pluripotent/proliferative to differentiated states will also impact cell number, and therefore a dual read-out of ALP and cell number will increase assay sensitivity and reduce false negatives in an HTS campaign.

Multiplexing is common in dual luciferase reporter assays, where transfection of *Renilla* and Firefly luciferase reporter plasmids are performed in batch and then read sequentially (Liu et al., 2009). A combination of two detection methods, such as luminescence and fluorescence, is also possible although frequently there is a loss of sensitivity as optimal conditions for one platform may not be compatible with the other and requires either expression of fluorescent biomarkers like Green Fluorescent Protein (GFP) or live staining of cells with a fluorescent dye, such as Calcein AM.

We describe here a new protocol using commonly available reagents and assays which can be multiplexed to increase sensitivity and robustness of cell-based HTS, where ALP is the primary biomarker being evaluated.

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http://dx.doi.org/10.1016/j.scr.2017.07.014

## 2. Materials and methods

### 2.1. Mouse stem cells and fibroblasts tissue culture and handling

Mouse embryonic stem cell line (KH2 ES cells) (Hochedlinger et al., 2005) were expended on feeders (irradiated DR4 Mouse Embryonic Fibroblasts) and 0.2% gelatin coated plates. KH2 cells grew in Serum/LIF conditions (also known as – mouse metastable naïve conditions): 425 ml High-Glucose DMEM (Invitrogen - 41,965), 15% USDA certified and heat inactivated Fetal Bovine Serum (FBS) (Biological Industries), 1 mM L-glutamine (Biological Industries), 1% nonessential amino acids (Biological Industries), 0.1 mM β-mercaptoethanol (Invitrogen), penicillin/streptomycin (Biological Industries) and 20 ng/ml recombinant human Leukemia Inhibitor Factor (LIF; produced in-house). Cells were expanded in 20% $O_2$; 5% $CO_2$ at 37 °C. Cells were passage following single cells trypsinization (0.25% Trypsin – Biological Industries) every 4–5 days. Exclusion of Mycoplasma contamination was monitored and conducted by monthly routine tests with Mycoalert kit (LONZA). Mouse Embryonic Fibroblasts (MEFs) grew in ES media conditions (without addition of human LIF) and expended in 5% $O_2$; 5% $CO_2$ at 37 °C.

### 2.2. Reprogramming of mouse somatic cells

Induced pluripotent stem cells (iPSCs) were generated from two secondary transgenic reprogrammable mouse fibroblasts cell lines which harbor: constitutive expression of the rtTA and tetO OKSM cassette by viral integration or targeted into the *Rosa26* locus and the *Col1a1* locus respectively in addition to Oct4-GFP reporter (Yoshimizu et al., 1999). The first cell line is Mbd3/Nurd depleted as previously described (Rais et al., 2013); the second cell line carries no further manipulation (WT). For the screening platform, cells were seeded in 10 or 5 cells/well density and analyzed by day 8 following the addition of doxycycline. Plates were coated with 0.2% gelatin prior to the addition of the feeder cells (which were added together with the reprogrammable fibroblasts in density of 5000 cells/well). Reprogramming enhancer Forskolin (F6886, Sigma Aldrich) was added twice to growth medium of WT cells - at 1 and 4 days after cell plating in a final concentration of 5 μM.

### 2.3. Multiplex luminescence assay

For detection of ALP level in cells we apply CDP-star Chemiluminescent Substrate (CDP) from Sigma-Aldrich (C0712). CDP is commonly used as a substrate for ALP-conjugated reactions in Northern, Southern, and Western blotting applications. We found that it can be used for a precise detection of ALP in cell lysates as well.

For detection of viable cells in same samples we apply either CellTiter-Glo® Luminescent kit (CTG) from (Promega) or ATPlite® kit (Perkin Elmer). Both are homogeneous methods to determine the number of viable cells in culture based on quantitation of ATP, which signals the presence of metabolically active cells. Other viability assay kits from Sigma (FLAA), Abcam (AB-ab65314), Biotium (BTM-30020-T) and Lonza (BELT07-221) contained 6 different buffers were purchased and tested as well.

### 2.4. Multiplex assay protocol (all procedures are performed at room temperature)

- Wash cells 4 times with Phosphate Buffered Saline (Biological Industries, 02-023-1A) (PBS), leaving 7–10 μl/well in 384-well plate.
- Add 25 μl of Lysis buffer (LB) 0.2% Triton X-100 (Sigma) in PBS (Biological Industries, 02-023-1A) supplemented with a protease inhibitor cocktail (Sigmafast Protease Inhibitor Cocktail Tablets, EDTA free, Sigma, #S8830) for 25 min.
- Add 20 μl of CDP; incubate 25 min in the dark.
- Measure luminescence in plate reading luminometer: ALP amount per sample/well.
- Add 20 μl of CTG (or ATPlite) reagent (prepared according to manufacturer's details); Incubate 10 min.
- Measure luminescence: viable cell amount per sample/well.
- Normalize level of ALP per sample by dividing of CDP-star/CTG luminescence.

### 2.5. In-vitro ALP assays

Calf Intestinal ALP and 10× CutSmart Reaction buffer were purchased from New England BioLabs (#M0290). ALP enzyme was dissolved in LB (as above) with 1× CutSmart Reaction buffer for desired concentrations. Addition of CDP, CTG or ATPlite was performed as in the protocol above.

### 2.6. HTS equipment

The assays were performed using the HTS equipment: MultiDrop 384 (Thermo Scientific), Washer Dispenser II (GNF, San Diego, CA, USA), EL406 Microplate Washer Dispenser (BioTek, Winooski, VT, USA), Bravo Automated Liquid Handling (Agilent, Santa Clara, CA, USA). Luminescence signals were detected by luminescence module of PheraStar FS plate reader (BMG Labtech, Ortenberg, Germany). ImagExpress Micro XL high content microscope (Molecular Devices, Sunnyvale, CA, USA) was used to acquire images and metaExpress software used to analyze colonies of pluripotent cells.

### 2.7. Statistical analysis

Z′ is a widely used HTS statistical parameter for assessment of assay quality control and predict the ability to identify screening hits with high confidence. Z′ range from 0.5 to 1 is regarded as describing an excellent HTS assay (Zhang et al., 1999). The Student's *t*-test was used for analysis of significance between non-treated, Forskolin-treated and Mbd3/NuRD-depleted mouse embryonic fibroblasts.

## 3. Results

In order to examine whether CDP could be effectively used as a reagent for detection of ALP in a multiplexed assay with metabolic proliferation reagents *in-vitro*, we prepared calf intestinal ALP into 1xCutSmart Reaction Buffer and then diluted in LB with serial dilutions to simulate conditions of cell lysis. Following CDP addition, we saw a linear luminescence signal in all range of concentrations: from 0.017 nM to 20 nM (data not shown). To further evaluate the compatibility of the ALP reaction with metabolic detection reagents, we added LB, CTG buffer, or ATPlite buffer. While addition of LB had no effect on the CDP signal, both CTG and PE buffers markedly quenched the CDP signal (Fig. 1, A). Notably, 6 other buffers used for cell viability detection were purchased from other companies (see Materials and methods) and had no effect on CDP signal (data not shown). Quenching of the CDP signal by subsequent addition of a viability reagent is useful as the secondary luminescent, reading from the viability reagent will not be convoluted by luminescence from two different enzymes in the same homogenous reaction.

CTG is a reagent used to determine the number of viable cells in culture based on quantification of the total ATP correlating with cell number. Since ATP and ALP are both presented in cell lysates, it was important to exclude the possibility that one of the agents impacts the luminescent signal of the other. We measured ALP activity between 0 and 1000 nM by CDP reagent in the presence (10, 100, or 1000 nM) or absence of ATP, and observed no effect of ATP on ALP signal (Fig. 1, B). Conversely, there was no effect of ALP