# Transfection of bone marrow-derived mast cells for transcription factor luciferase reporter assays

**Kate Jeffrey**
The Garvan Institute of Medical Research
**Charles MacKay**
The Garvan Institute of Medical Research
**Tilman Brummer**
The Garvan Institute of Medical Research

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# Abstract

# Introduction

Mast cells are now being understood as important intitiators and regulators in both innate and adaptive immunity in addition to their traditional roles in allergic and IgE-mediated immune responses.Transfection of primary leukocytes has traditionally been a challenging but much desired protocol. It allows not only the analysis of cells in a more natural state to a cell line system, it enables the direct comparison of, for example, transcriptional activity using luciferase reporters, in immune cells taken from genetically-altered mice. In addition, importantly it allows for "rescue experiments" in knockout cells as well as the ability to over-express or reconstitute wild-type and/or mutated constructs into wild-type or knockout cells for analysis of functional responses such as cytokine production, apoptosis, migration etc.

# Reagents

Amaxa solution T, interleukin 3,

# Equipment

Amaxa Nucelofector, beta scintillator counter

# Procedure

1. Maturate mast cells from bone marrow using interleukin 3. Isolate bone from femurs and tibias of 8-12 Pac-1-/- and Pac-1+/+ week old mice. Grow cells at $4\times10^5$ cells/mL in RPMI 1640 medium \(Life Technologies, Rockville) supplemented with 10% fetal bovine serum \(HyClone, Logan), 100 µg each of penicillin and streptomycin per mL, 2mM L-glutamine \(Life Technologies), 50 µM β-mercaptoethanol, 1% HEPES and 5 ng/mL of recombinant murine IL-3 \(Peprotech) for 4-6 weeks at 37$^o$C in 5% CO2. By 4-6 weeks in culture, greater than 98% of cells should be c-kit and FcεRI positive as assessed by fluorescein isothiocyanate \(FITC)-labelled anti-c-kit \(BD, Pharmingen) and anti-DNP IgE \(Sigma) antibodies respectively using Flow Cytometry. Cell morphology should also be assessed by cytospin and staining with giemsa and toluidine blue. To stimulate, sensitise cells with anti-DNP IgE mAb \(100 ng/mL, Sigma) for 18 hours, wash twice to remove unbound IgE and stimulate with 20 ng/mL of DNP-HSA \(Sigma) for chosen times. 2. For the measurement of transcriptional Elk-1 activity, transfect 4 x $10^6$ BMMCs with 1.75 µg pG5E4D38–Luc, 1.75 µg pSG-Gal-Elk-1 \(both plasmids kindly provided by Dr. Peter Shaw, University of Nottingham, UK, Frost et al, 1997) and 1 µg of the lacZ-expression vector pCMVβ \(Clonetech, to allow standardization of the measured luciferase activity) using solution T and an Amaxa nucleofector device \(Köln, Germany; set to program T-016). In addition, “mock-transfect”cells with PBS in order to measure the background luciferase and β-galactosidase activities. This Elk-1 reporter system is based on a hybrid fusion protein consisting of the DNA binding domain of the yeast transcription factor Gal4 and the

transactivation domain of Elk-1 that binds via its Gal4 moiety to the pG5E4D38–Luc plasmid and drives the transcription of the luciferase gene only if it is phosphorylated by ERK within its Elk-1 moiety \(Kortenjann et al, 1994). 3. For the measurement of transcriptional NFAT:AP-1 activity, transfect BMMCs with 1 µg pCMVβ and 3.5 µg of the NFAT reporter plasmid pNFAT-Luc \(kindly provided by Dr. Gerald Crabtree, Stanford University, Stanford, USA) as described above. This NFAT reporter carries three tandem copies of the distal NFAT:AP-1 composite element of the human IL-2 promotor \(Durand et al, 1988), which were cloned upstream of the minimal IL-2 promotor \(from position -89 to +51) and the luciferase reporter gene. Consequently, this reporter is only transactivated when both NFAT and AP-1 are present in the nucleus and assembled to a ternary complex \(Fiering et al, 1990; Macian et al., 2001). This widely used reporter plasmid responds to all known NFAT family members and can be activated in a NFAT-dependent manner in a variety of cell types. 4.Following transfection with reporter plasmids, plate BMMCs in 3 mL culture medium \(RPMI 1640 medium \(Life Technologies) supplemented with 10% fetal bovine serum \(HyClone), 100 µg each of penicillin and streptomycin per mL, 2mM L-glutamine \(Life Technologies), 50 µM β-mercaptoethanol, 1% HEPES and 5 ng/mL of recombinant murine IL-3 \(Peprotech)) and allow to recover for 24 h. Then sensitise with 100 ng/mL anti-DNP IgE antibodies for 18 hours. Subsequently, split the cells and either stimulate with 20 ng/mL DNP-HSA for 6 h \(Elk-1) or 8 h \(NFAT:AP-1) or leave untreated. Following stimulation, lyse the cells in luciferase lysis buffer \(Promega) and determine the raw luciferase activities using a luciferase activity kit \(Promega). As an internal control for transfection efficiency, measure the β-galactosidase \(β-gal) activities using Galactostar and Galacton reagents \(Tropix). The background β-gal activity of mock-transfected cells should be subtracted from the lysates of transfected BMMCs and the relative β-gal activity of each sample was calculated. In order to obtain the standardised luciferase activity, divide the luciferase activity of each sample by the corresponding relative β-gal activity. 5. For over-expression or rescue experiments, transfect mast cells with 2ug of WT Pac-1, C257S Pac-1 or pMIG vector control.

# Timing

4-6 weeks for mast cell maturation, 48 hours for transfection, 6-8 hours stimulation and 2 hours for luciferase assay

# References

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# Figures

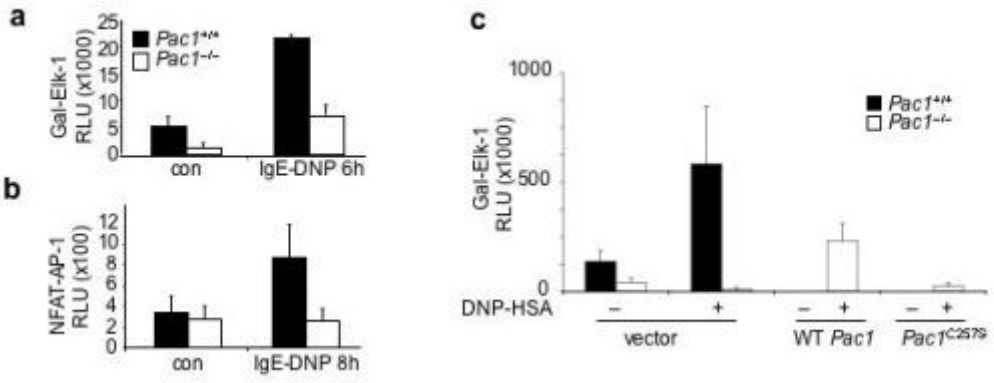


**Figure 1**

Reduced transcriptional Elk-1 and NFAT:AP-1 activity in Pac1 minus/minus BMMCs. Re-expression of Pac1 but not Pac1C257S could enhance Elk-1 activity in Pac1 minus/minus BMMCs. a, Reduced transcriptional Elk-1 and b, NFAT:AP-1 activity in Pac1 minus/minus BMMCs as measured by standardized luciferase activity of Gal-Elk-1 or NFAT:AP-1 reporter in Pac1+/+ and Pac1 minus/minus BMMCs transfected using Amaxa Nucleofector and activated for 6 h (Elk-1) or 8 h (NFAT:AP-1) with Fc_RI crosslinking. RLU is relative luciferase activity. c, (Re)introduction of Pac1 but not Pac1C257S into Pac1 minus/minus BMMCs increases Elk-1 activity. BMMCs were co-transfected with Gal-Elk-1 reporter constructs and empty vector alone, full length WT Pac1 or Pac1C257S. Error bars represent standard deviation of triplicate samples. Similar results were obtained in 5 independent experiments.