Optimisation and application of the NAD/NADH Glo assay

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Talk Overview

NAD/NADH Glo Assay

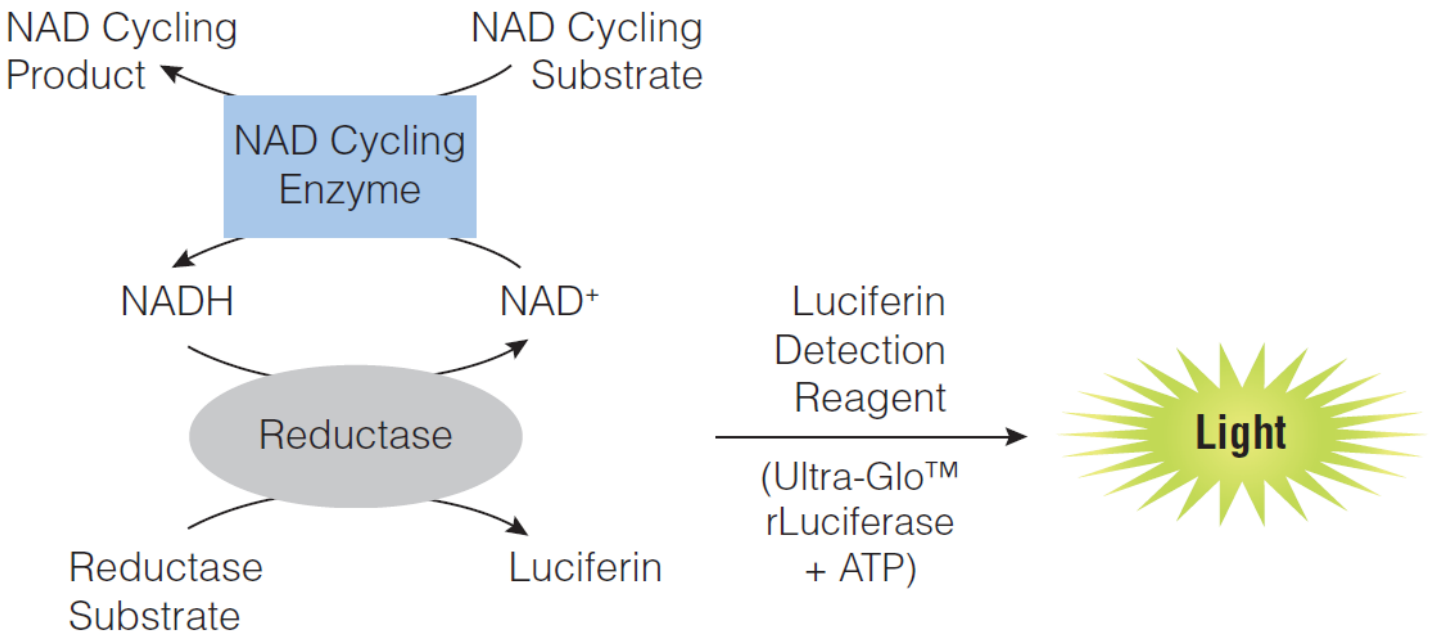
Assay optimisation

- Linearity and window
- Reduction of Assay variability

Introduction to NAD/NADH biology in cancer

Application of the assay to assess the effect of PARG and PARP modulators on NAD/NADH biology

NAD/NADH-Glo Assay



Detects total oxidised or reduced nicotinamide adenine dinucleotides (NAD^+ and NADH)

One step addition

Light signal increases over time and is proportional to the starting amount of NAD^+ and NADH

Assessment of assay linearity and window

Hela cells: seeded into a white walled 384 well plate with transparent bottom in a volume of 30ul.

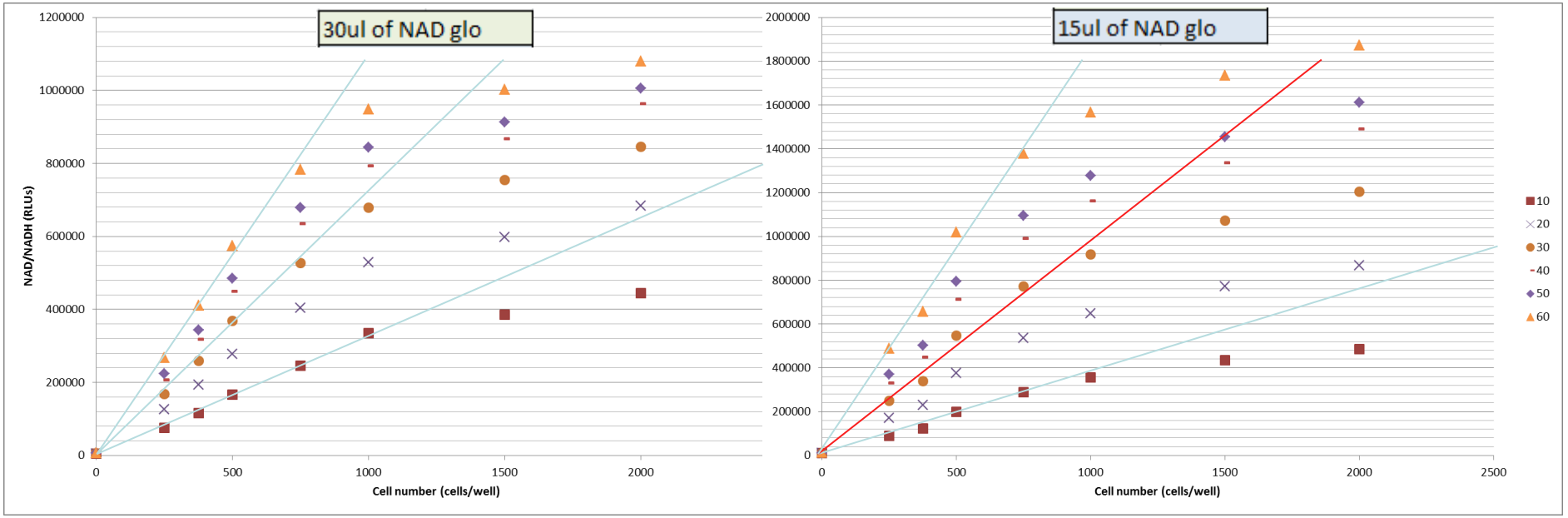
Important – centrifuge plate at 1000 rpm for 1 minute

	1	2	3	4	5	6	7	8	9	10
A										
B		2000	1500	1000	750	500	375	250	0	
C		2000	1500	1000	750	500	375	250	0	
D		2000	1500	1000	750	500	375	250	0	
E		2000	1500	1000	750	500	375	250	0	
F										

30ul of NAD glo

15ul of NAD glo

24hrs later: Kinetic experiment performed to establish the optimum time to read the luminescence. Every 5mins - 1hr. Important – equilibrate kit reagents to room temp



15ul of NAD/NADH glo reagent – 800 cells/well – 30mins incubation

Reduction of assay variability

Expanding the assay over a greater well number resulted in clear variability across the plate.

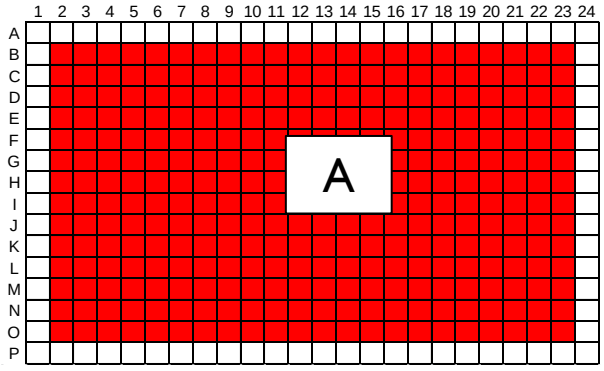
Solution:

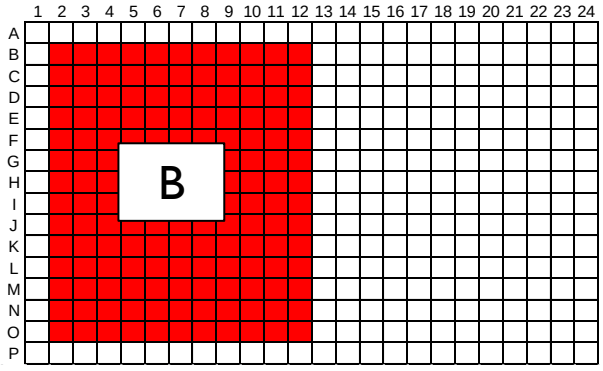
Stop reagent implemented to address this

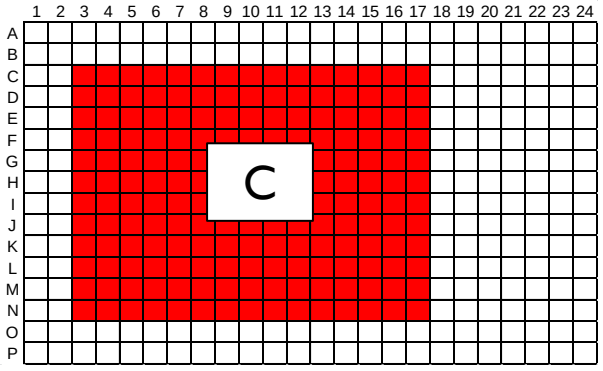
Establish layout of experimental wells which provide low CVs

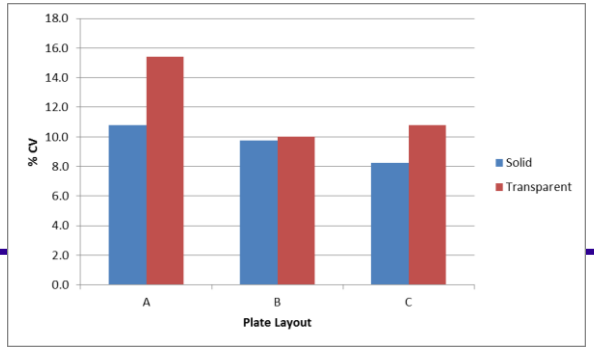
Plate type

- Hela cells seeded into white walled 384 well plates at 800cells/30ul/well
- Plates centrifuged at 1000rpm for 1minute
- 24hrs later 15ul of NAD/NADH Glo reagent applied, plates spun down.
- Following 30mins incubation 9ul of 1.375mM Menadione was added









Application of the Glo assay to longer time points

MDA MB 468 cells: seeded into a white walled 384 well plate with transparent bottom in a volume of 30ul.

Important – centrifuge plate at 1000 rpm for 1 minute

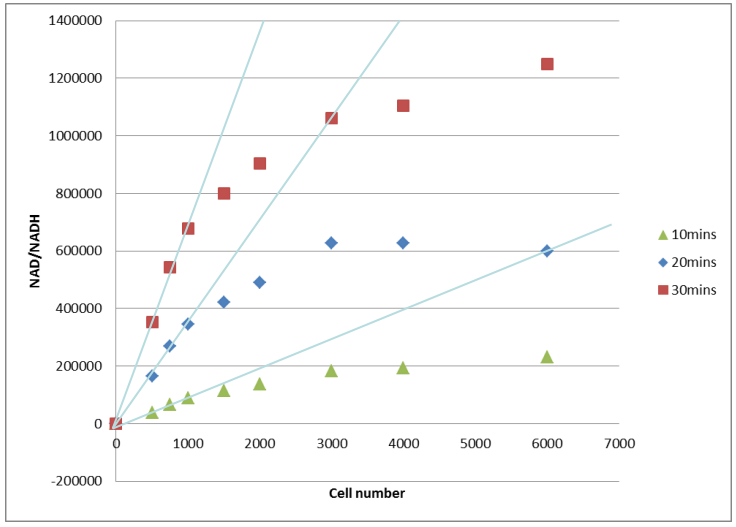
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C			6000	4000	3000	2000	1500	1000	750	500	0	
D			6000	4000	3000	2000	1500	1000	750	500	0	
E			6000	4000	3000	2000	1500	1000	750	500	0	
F												

NAD & NADH was assayed using the NAD/NADH Glo assay at 48, 72 and 96hrs.

30ul of detection reagent was used – higher seeding density needed due to grow issues

Kinetic Luminescence data collected every 5 minutes

Example
48hr data



Important – cells need to be growing in linear phase throughout course of experiment

NAD/NADH in Cancer

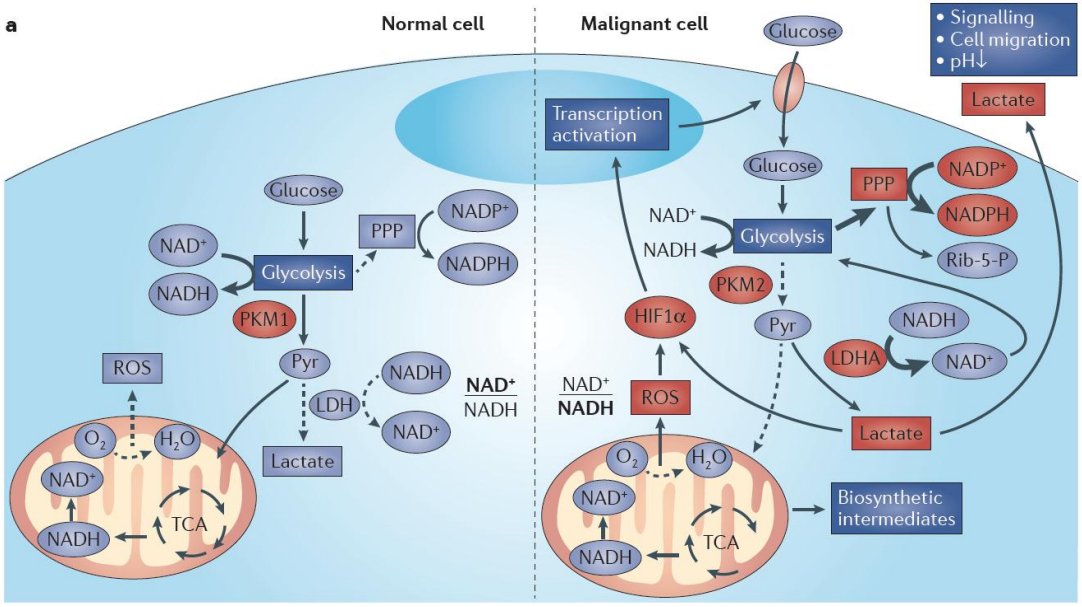
Nicotinamide adenine dinucleuotide (NAD) is a vital molecule in all organisms

Energy (TCA cycle) and signalling transduction.... DNA repair

Most cancer cells have an altered metabolism

Increased gylcolysis

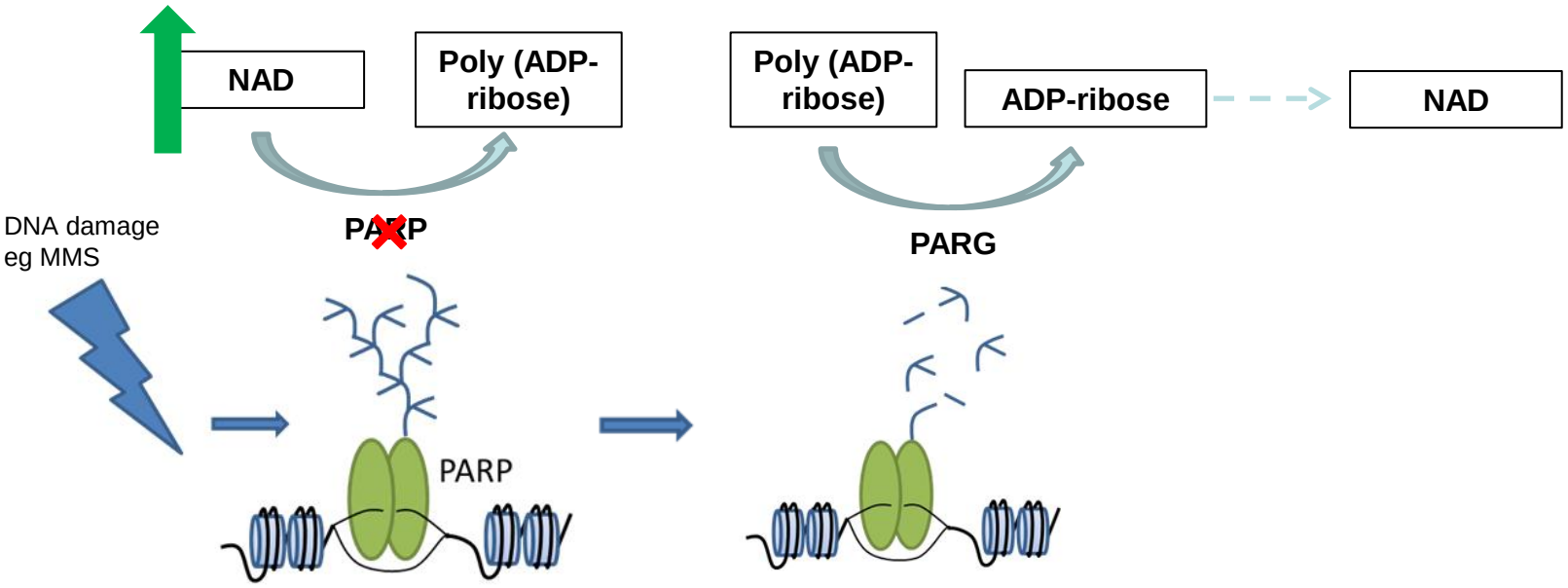
Have a low NAD to NADH ratio



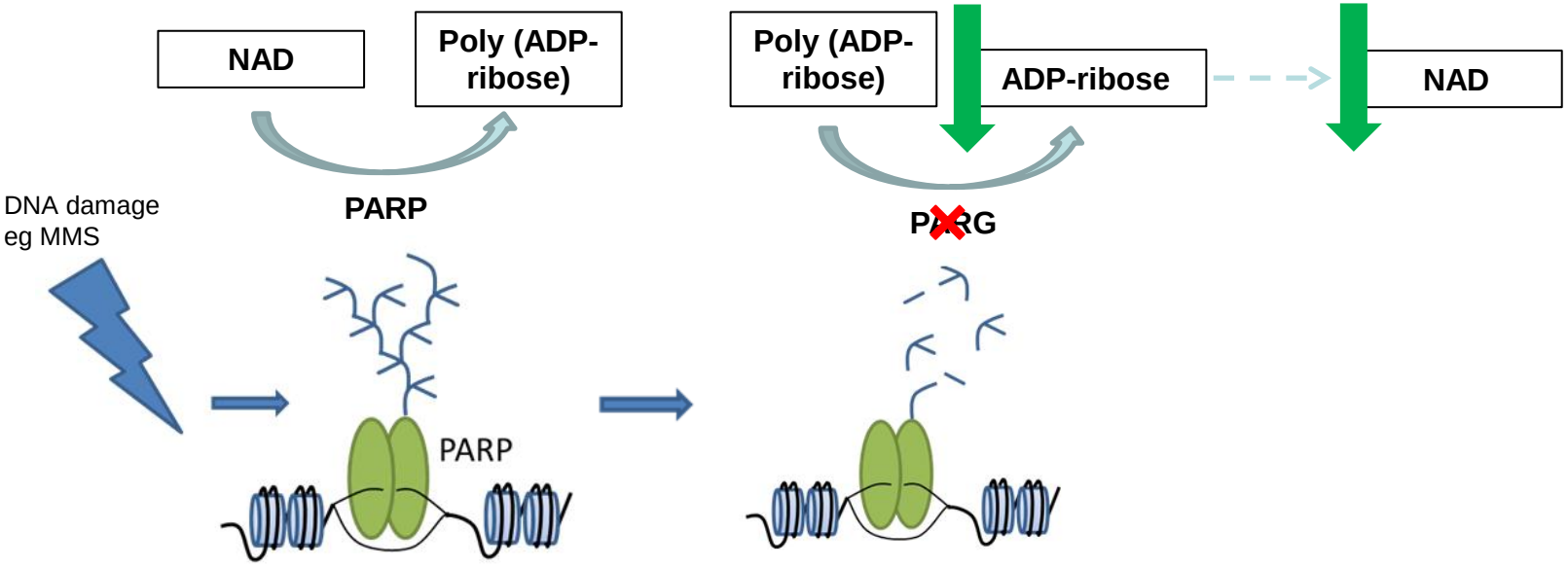
Low NAD levels → DNA repair?

Picture taken from Chiarugi et al. The NAD metabolome – a key determinant of cancer biology. Nature Reviews Cancer (12) p741

NAD and DNA damage repair



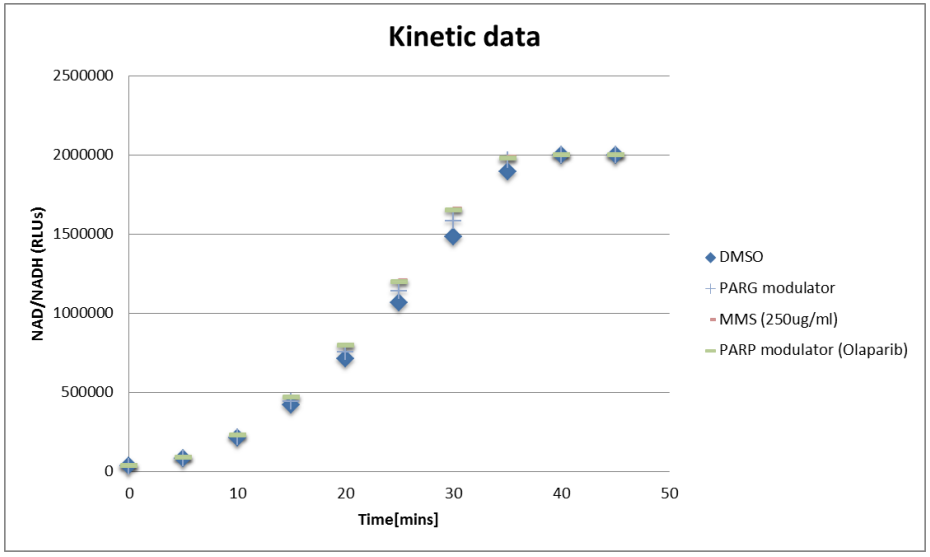
NAD and DNA damage repair

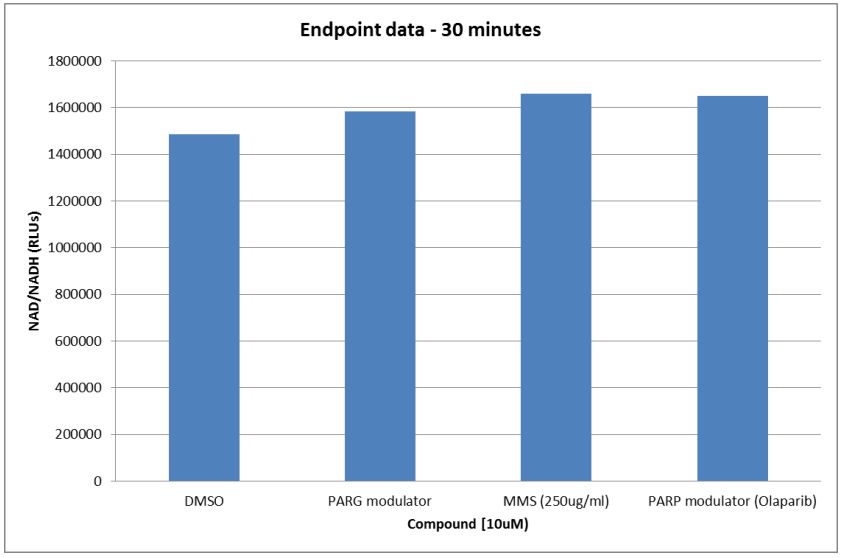


Aim – To investigate how modulating PARP and PARG activity effects NAD^+/NADH depletion following MMS treatment in cancer cell lines

Effect of test compounds on assay enzymes

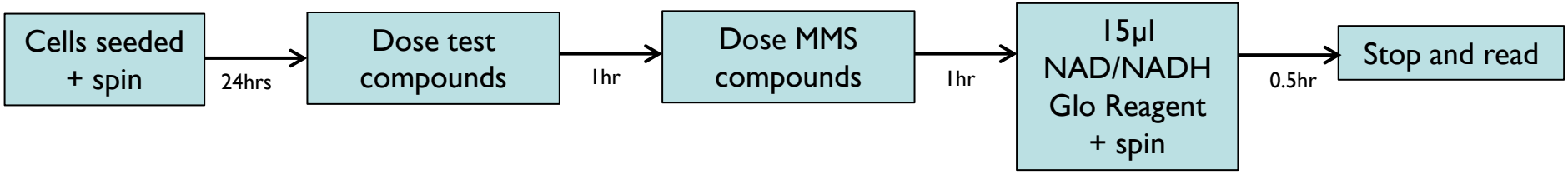
Assessing whether the test compounds effect the NAD/NADH Glo kit enzymes
25µl of 10nM NAD was plated into a white 384 well plate
Test compounds dosed at 10µM (MMS at 250µg/ml)
Kinetic experiment performed



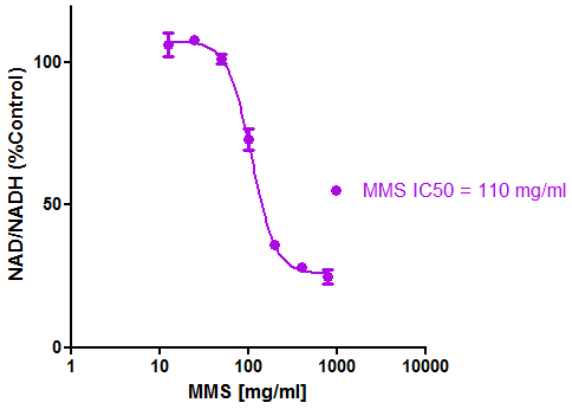


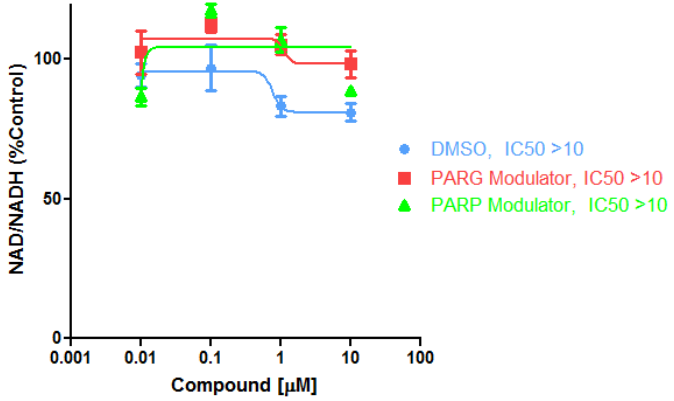
The effect of PARG and PARP modulation on NAD/NADH following MMS treatment

Experimental method in brief

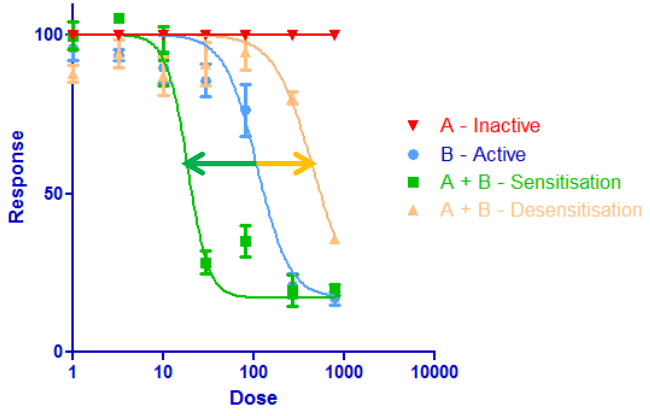


To effectively investigate combinations an IC50 for the test compounds must first be established





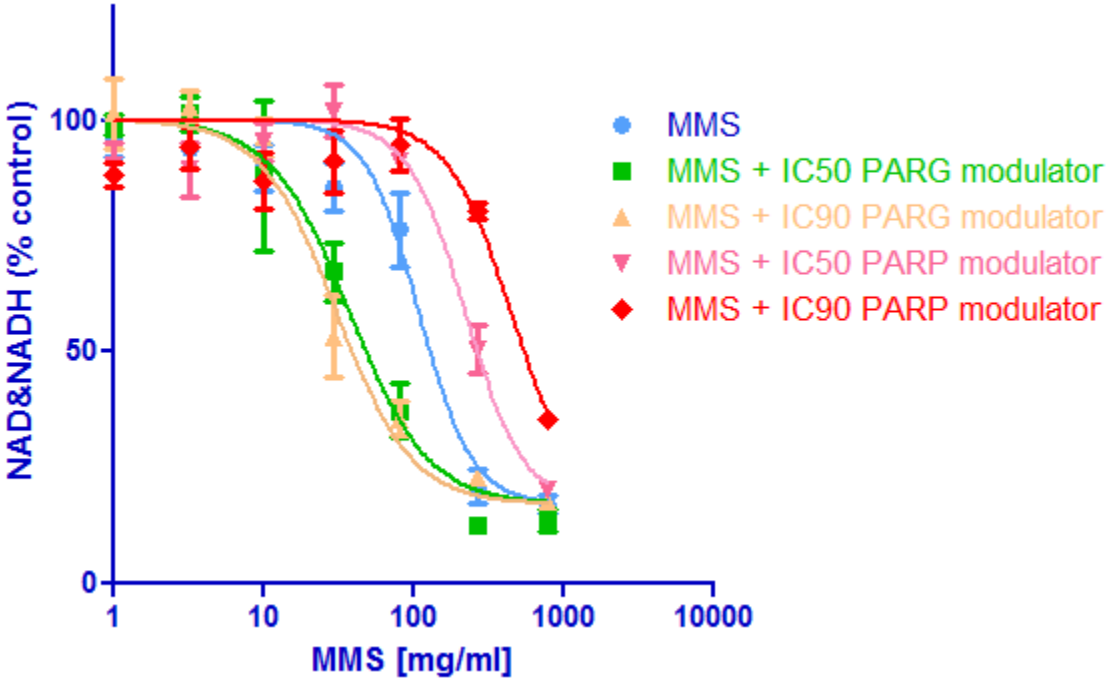
When one agent is active and the other inactive a curve shift analysis is performed.



The effect of PARG and PARP modulation on NAD/NADH following MMS treatment

Reduction in PARG activity sensitized all the tested cells (Hela, MDAMB468 and HCC1937) to NAD/NADH depletion by MMS

Reduction in PARP activity desensitised all cells to the depletion of NAD/NADH caused by MMS



Summary

The NAD/NADH Glo assay can be effectively optimised for small scale combination screening in cells

Key optimisations required

- Assay linearity
- Effect of test compounds on kit enzymes
- Variability of signal across test wells
- Stop reagent
- Plate type
- Integration time

This assay was used to effectively assess the combination of PARG and PARP modulators with MMS on NAD/NADH levels in Hela, MDA MB 468 and HCC1937 cancer cells

- Reduction in PARG sensitising cells to MMS induced NAD/NADH depletion
- Reduction in PARP desensitising cells to MMS induced NAD/NADH depletion

More broadly the NAD/NADH Glo assay provides a good tool for the study of NAD/NADH in metabolism and cancer