

# Multiplexing of antibody and GFP-based translocation assays: A pathway to better profiling of cellular signalling

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## Pathway screening using Redistribution<sup>®</sup> and Cellomics<sup>®</sup> HCS Reagent Kits

Multiplexing of Cellomics<sup>®</sup> HCS Reagent Kits and Biomege Redistribution<sup>®</sup> assays enables easy acquisition of multiple readouts from the same cell in a single assay run. This approach is highly relevant for pathway analysis and hit selection/de-selection in High Content Analysis (HCA). Cellomics HCS Reagent Kits are based on immunofluorescence whereas Redistribution technology is based on GFP fusion proteins. Using the Cellomics ArrayScan<sup>®</sup> V<sup>®</sup> HCS Reader for automated image acquisition and analysis, we present several examples of multiplexing with these technologies.

### p38 multiplexing

The Biomege MK2 Redistribution assay monitors translocation of GFP-MK2 from the nucleus to the cytoplasm in response to stimulation of the p38 pathway by IL-1 $\beta$  or TNF $\alpha$ . By applying several different Cellomics HCS Reagent Kits to the MK2 Redistribution assay, we have created a multiplexed HCA system for deconvolution of p38 pathway activation, including assay readouts for MK2, p38, NFkB, and c-jun.

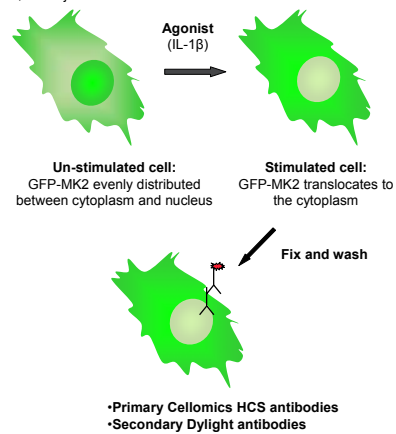


Figure 1. Illustration of the translocation event in the MK2 Redistribution assay and the subsequent application of Cellomics HCS Reagent kits.

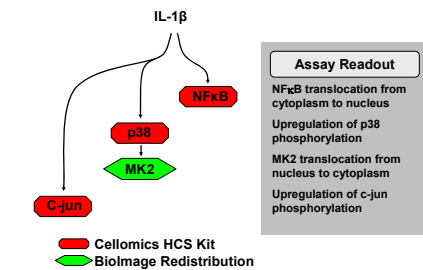


Figure 2. A schematic illustration of the target readouts from the p38 multiplex assay. Multiplexing of Cellomics HCS Reagent Kits and Biomege Redistribution assays enable easy acquisition of multiple readouts from the same cell in a single assay run. This approach is highly relevant for pathway analysis and hit selection/de-selection in High Content Analysis (HCA).

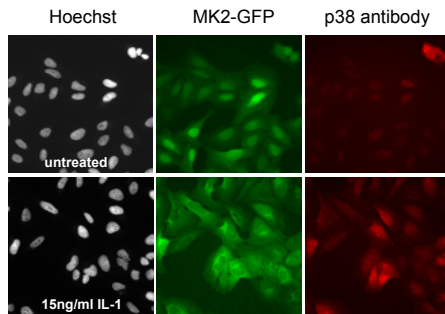


Figure 3. ArrayScan V<sup>®</sup> images of untreated (top row) and IL-1 stimulated (bottom row) MK2-GFP expressing cells stained with phospho-p38 primary antibody from the p38 activation Cellomics HCS reagent kit and a secondary GaR-Dylight-649 antibody. The images are obtained with a 40x objective with the following filter settings: Hoechst (XF100-Hoechst), GFP (XF100-FITC), Dylight-649 (XF110-Cy5, sensitive).

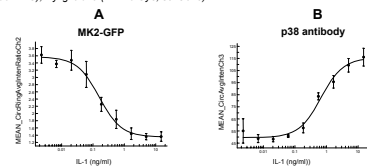


Figure 4. Dose-response curves for IL-1 stimulation. A) Translocation of MK2-GFP has an EC50=0.13ng/ml based on the MEAN\_CircRingAvgIntenRatioCh2 output parameter. B) Stimulation of p38 phosphorylation has an EC50=0.58 ng/ml based on the MEAN\_CircAvgIntenCh3 output parameter.

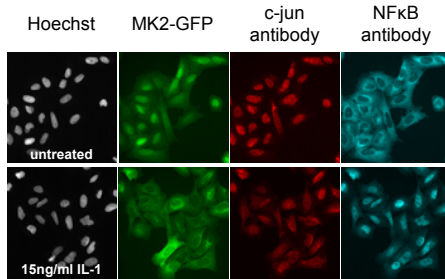


Figure 5. ArrayScan V<sup>®</sup> images of untreated (top row) and IL-1 stimulated (bottom row) MK2-GFP expressing cells stained with phospho-c-jun or NFkB primary antibody from the respective Cellomics HCS Reagent kits and a secondary GaR-Dylight-549 antibody or GaR-Dylight-649 antibodies respectively. The images were obtained with a 40x objective with the following filter settings: Hoechst (XF100-Hoechst), GFP (XF100-FITC), Dylight-549 (XF93-TRITC) and Dylight-649 (XF110-Cy5, sensitive).

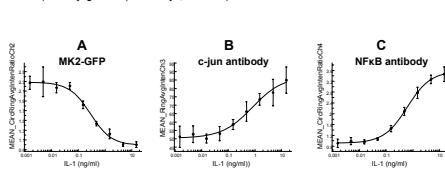


Figure 6. Dose-response curves for IL-1 stimulation. A) Translocation of MK2-GFP has an EC50=0.28ng/ml based on the MEAN\_CircRingAvgIntenRatioCh2 output parameter. B) Stimulation of c-jun phosphorylation has an EC50=0.8 ng/ml based on the MEAN\_RingAvgIntenCh3 output parameter. C) Stimulation of NFkB phosphorylation has an EC50=0.61 ng/ml based on the MEAN\_CircRingAvgIntenRatioCh4 output parameter.

## ERK activation: Multiplex of ERF1 Redistribution assay and Cellomics ERK HCS Reagent Kit

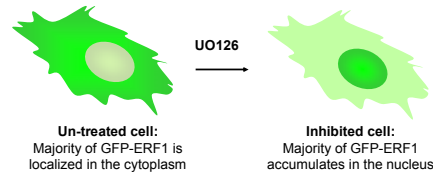


Figure 7. Illustration of the translocation event in the ERF1 Redistribution assay.

The Biomege ERF1 Redistribution assay reports on activity through the RAS-RAF-MEK-ERK pathway. We have multiplexed the ERF1 Redistribution assay with the Cellomics HCS Reagent kit for ERK activation to simultaneously monitor ERK activation by MEK and ERK kinase activity. By using this multiplexed assay approach inhibitors of ERK may be characterized for their ability to inhibit ERK activation (phosphorylation) and ERK kinase activity in a single assay run. The MEK inhibitor UO126 may be used as reference compound for inhibition of both ERK activation and ERK kinase activity.

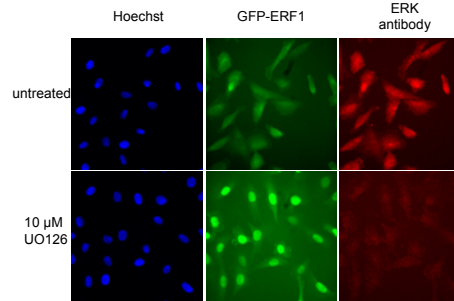


Figure 8. ArrayScan V<sup>®</sup> images of the ERF1 Redistribution cell line (T24 cells) untreated (top row) and treated with the MEK inhibitor UO126 (10  $\mu$ M) (bottom row). ERK activation/phosphorylation is monitored by the ERK Cellomics HCS Reagent kit and a secondary Dylight-649 antibody while ERK kinase activity is measured by ERF1 translocation. T24 cells express a constitutively active variant of Ras. The images were obtained with a 40x objective with the following filter settings: Hoechst (XF100-Hoechst), GFP (XF100-FITC), Dylight-549 (XF93-TRITC) and Dylight-649 (XF110-Cy5, sensitive).

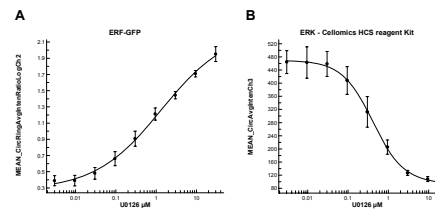


Figure 9. Dose-response curves of the MEK inhibitor UO126 in the ERF1 Redistribution assay cell line. The cell line is based on T24 bladder cancer cells that express a constitutively active variant of Ras. A) Translocation of GFP-ERF1 from the cytoplasm to the nucleus in response to inhibition of MEK and thereby of ERK by UO126. The EC50 of UO126 in this assays is ~1  $\mu$ M based on the MeanCircAvgIntenRatioLog output parameter. B) Phosphorylation of ERK as detected by the Cellomics ERK HCS Reagent kit and a secondary Dylight-649 antibody. The EC50 of UO126 is ~0.5  $\mu$ M based on the MeanCircAvgInten output parameter.

## FOXO1A Redistribution assay with FOXA3A HitKit

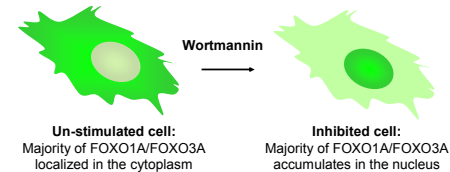


Figure 10. Illustration of the translocation event in the FOXO1 (FKHR) Redistribution assay and the FOXO3A HitKit assay.

The Biomege FOXO1A (FKHR) Redistribution assay monitors the translocation of the forkhead transcription factor from the cytoplasm to the nucleus. The Redistribution assay has been combined with the Cellomics FOXO3A Transcription Factor Activation Kit for measurement of both FOXO3A and FOXO1A translocation to the nucleus in the same cell.

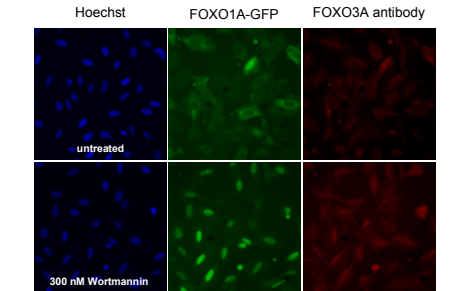


Figure 11. ArrayScan V<sup>®</sup> images of untreated (top row) and wortmannin (300 nM) stimulated (bottom row) FOXO1A/FKHR Redistribution cell line expressing FOXO1A-GFP and stained with FOXO3A primary antibody from the Cellomics HCS Reagent assay and a secondary Dylight-649 antibody.

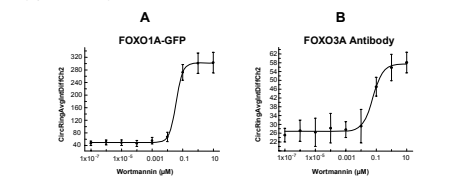


Figure 12. Dose-response curves of Wortmannin inhibition. A) Translocation of FOXO1A (FKHR) to the nucleus has an EC50=37nM based on the CircRingAvgIntenRatioCh2 output parameter. B) Translocation of FOXO3A to the nucleus has an EC50=61nM based on the CircRingAvgIntenRatio output parameter.

## Summary

- Multiplexing with Cellomics HCS Reagent kits and Biomege Redistribution assays provides a unique opportunity to enhance HCA assay quality
- Saves valuable time in drug discovery
- From a single assay run it is possible to obtain several assay readouts
- Helps the user to identify the mode of action of test compounds