Development of a novel approach for immunohistochemical assay validation

Keith Page, Jessica Petty, Mozam Ali, Sam Ho and Amanda Woodroofe
Asterand Bioscience, 2a Orchard Road, Royston Hertfordshire, UK & TechOne Suite 501, 440 Burroughs, Detroit MI USA

INTRODUCTION

Immunohistochemical (IHC) assays are increasingly used to detect protein biomarkers. For an IHC biomarker assay to be used to its maximal utility, assay development and validation are of paramount importance. Assay development and validation typically follows the guidelines set out in Validation of Analytical Procedures: Text and Methodology Q2(R1) and Q2B Validation of Analytical Procedures: Methodology. Assay validation can include quantification and while quantitative immunohistochemistryis performed, relative measures (i.e. relative optical density) are frequently used. In the analysis of human protein biomarkers, an approach has been developed which accelerates the progression of an IHC assay through assay validation (ICH Guidelines) and allows quantitation of antibody performance with reference to the physical concentration of the target protein. In the evaluation of this approach, we assessed the performance of two rabbit monoclonal antibodies to human Met (high-affinity tyrosine kinase receptor for hepatocyte growth factor) in standard IHC assays. Met was selected as the target for the case study due to the availability of antibodies, cell lines and full length recombinant, human protein.

APPROACH & METHODS

TARGET: Met, high-affinity tyrosine kinase receptor for hepatocyte growth factor (HGF). Altered Met expreession and phosphorylation state is found in several human cancers, including gastric, renal, colon and breast.

CELL LINES:

Met-ir	Cell Line	Reference
High	SNU5 gastric	SNU-5 (ATCC® CRL-5973™)
Moderate	HT29 colorectal	HT-29 (ATCC® HTB-38™)
Low	MB231 breast	MDA-MB-231 (ATCC® HTB-26™)
Absent	SNU1 gastric (mets)	SNU-1 (ATCC® CRL-5971™)

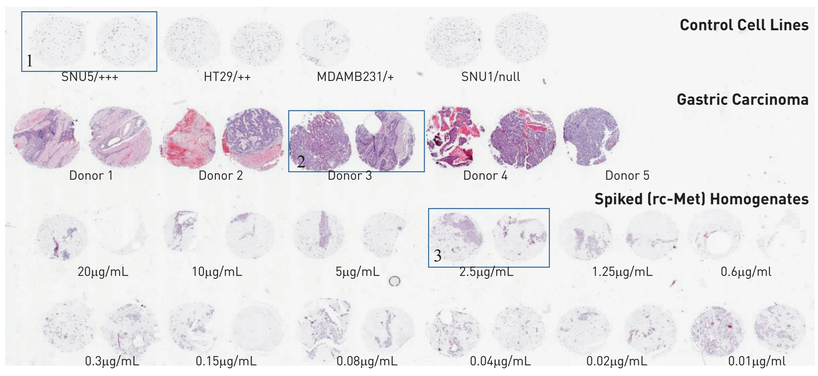
ANTIBODIES:

D1C2 Cell Signaling #8198 Met, Xp rabbit mAb-total Met, C-terminal
EP1454Y Abcam ab51067 rabbit mAb-total Met, N-terminal

PROTEINS:

ORIGENE TP317003

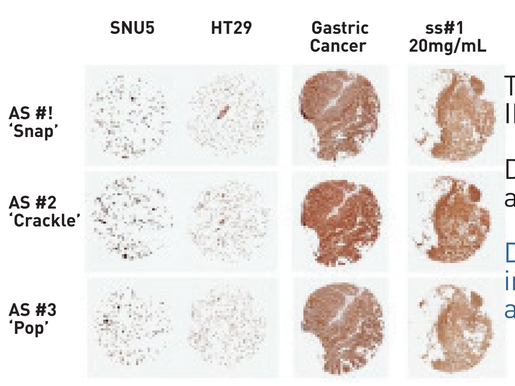
IHC VALIDATION ARRAY DESIGN:



ARRAY HAS THREE KEY FEATURES

1. Cores from four cell lines with known levels of MET expression
   SNU5>HT29>MDAMB231>SNU1
2. Tissue array cores from gastric cancer (n=5 donors)
   Cellular and sub-cellular distribution
3. Spiked homogenates, 12 concentrations of recombinant human met
   Linearity, range, detection limits, etc

INTERMEDIATE PRECISION



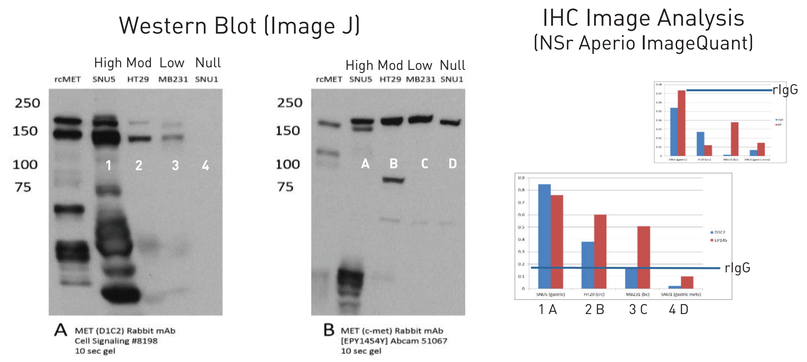
Three assays on three IHC machines

Data set (from three assays) shown

Data illustrates Met-ir in cell lines, tissues and homogenates

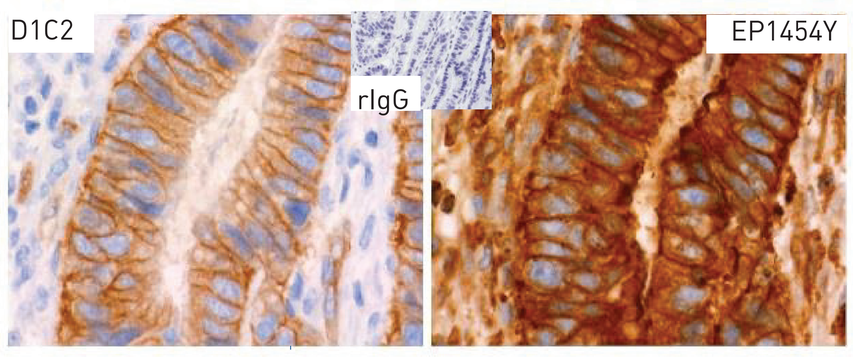
SPECFICITY

IHC & WESTERN ANALYSIS: CELL LINES AND LYSATES



Western and IHC analysis using D1C2 (panel A) more closely reflects the known levels of Met expression in the four cell lines. NSr data expresses the proportion of measured high-intensity pixels relative to all measured pixels.

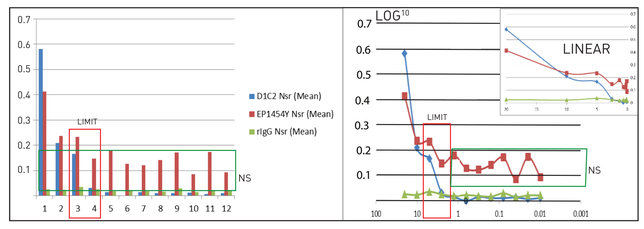
QUALITATIVE ANALYSIS IN GASTRIC CANCER CORES



Digital scans (20X0 showing Met-ir in matched regions of one representative gastric carcinoma core. Two antigen retrievals (pH 6-9 and pH 9, shown) and five antibody concentrations (o.5mg/5mL shown) were tested. Non-immune rabbit IgGs 90.5mg/mL shown) and no primary negative controls (not shown) were run.

D1C2 Membrane staining in cancer cells
EP1454Y Cytoplasmic plus membrane staining in cancer and mesenchymal cells

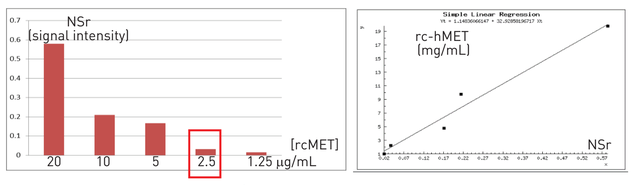
QUANTITATIVE ANALYSIS OF SPIKED HOMOGENATES



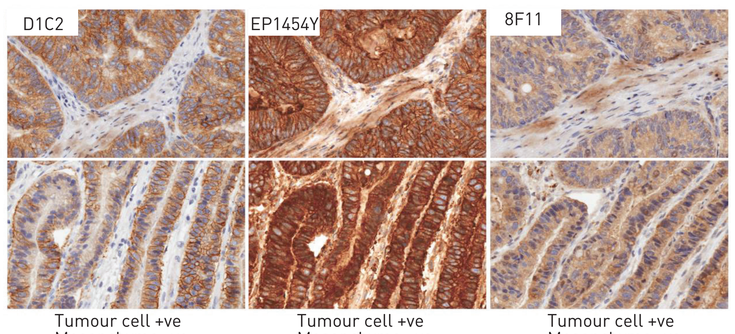
Both D1C2 and EP1454Y generate staining intensities which correlate with concentration of spiked recombinant-MET.

D1C2 limit of detection is 21.5mg/mL 'LIMIT'
EP1454Y reatins non-specific staining throughout 'NS'

LINEARITY OF ASSESSED NSr: SPIKED HOMOGENATES



Using D1C2 at 0.5mg/mL, recombinant human Met is detectable to 2.5mg/mL. Staining intensity is linear across the measured range (2.5-20mg/mL, ANOVA F-99).



Tumour cell +ve
Mesenchyme -ve
Membrane +ve

Tumour cell +ve
Mesenchyme +ve
Membrane +ve

Tumour cell +ve
Mesenchyme -ve
Membrane -ve

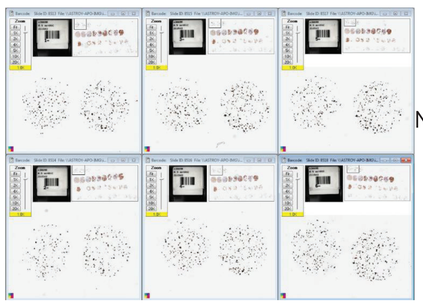
D1C2 demonstrates known cellular and sub-cellular expression

LINEARITY ASSESSED USING NSr AND Met-ir IN SPIKED HOMOGENATES

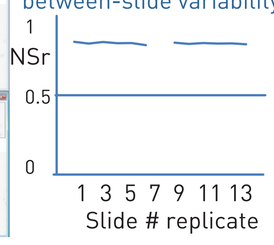
Quantitation Limit	IHC measurement determinable with quantitation	2.5mg/ml, when [D1C2] used at 0.5mg/mL*
Linearity	Over measureable Met concentration range*	Linear Regression: F-Test 99.2 (<5% signficance)
Range	Interval between lower and upper limits	>2.5mg/mL to 20 mg/mL, when [D1C2] used at 0.5mg/mL*

REPEATABILITY

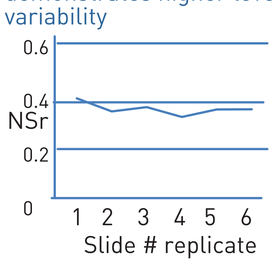
Six replicated assays



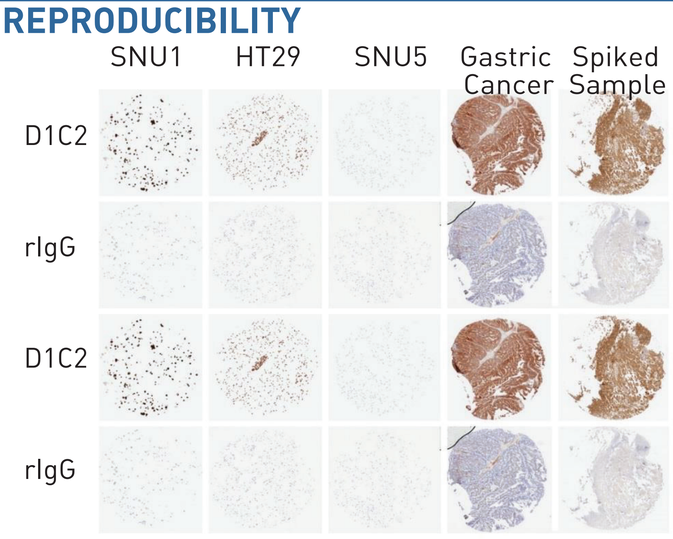
Measurement of NSr in cell lines demonstrates minimal between-slide variability



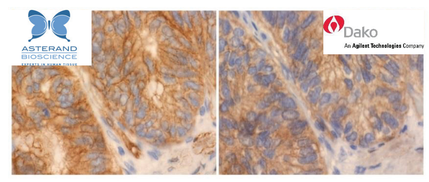
Meaurement of NSr in gastric cancer cores demonstrates higher level of between-slide variability



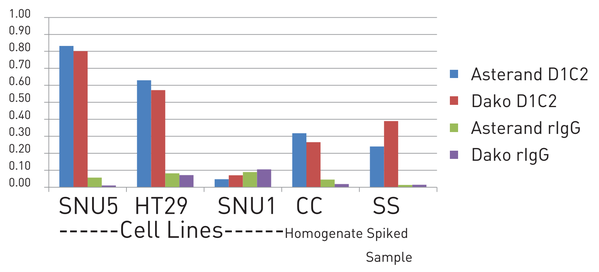
REPRODUCIBILITY



Example data demonstrating that analysis of human Met in the validation arrays is reproduced by two independent laboratories.



Images confirming assay precision between laboratories



Conclusions

- Custom validation arrays offer efficiences that can accelerate progression through IHC assay validation
- Combinations of cell lines, human tissues and spike homogenates provide complementary data
- Spiked homogenates analyzed using NSr generate informative and credible quantitative IHC data