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Simple Western Size™, qPCR

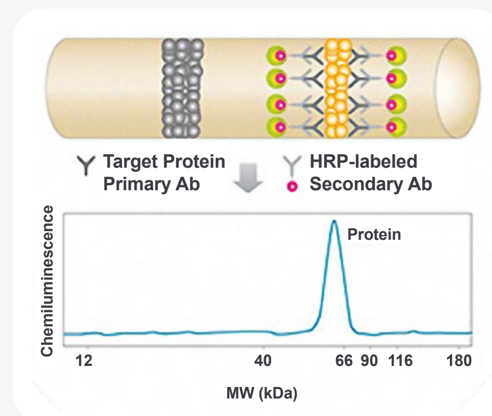
Detection of AXL RTK in minute amounts of human samples – development of a Simple Western Size™ assay

In order to optimize drug development and therapy, the efficacy of targeted therapy of patients has to be monitored by taking and analyzing biopsies on a regular basis. Therefore, laboratory methods are needed to handle minute amounts of clinical biospecimens, e.g. biopsies or microdissected material. Here, we demonstrate the development and validation of a Simple Western SizeTM assay for the detection of the AXL receptor tyrosine kinase, using only ng amounts of tissue lysates. The data was compared to gene expression data, measured by qPCR.

The AXL receptor tyrosine kinase is expressed in a variety of cancers and has been revealed as the most highly expressed gene in preclinical models with acquired resistance to targeted therapy. AXL upregulation was shown to be the second most common alteration in EGFR (epidermal growth factor receptor) inhibitor-resistant tumors, after the T790M mutation. Upregulation and/or activation of AXL are also shown to be predictive of lack of response to ErbB family receptor-targeted inhibitors, such as Her2-targeted agents.

Keywords:

- Simple Western Size™
- qPCR
- AXL Expression



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Material and Methods

Two assays were developed for the detection of AXL in tissue samples: a Simple Western Size™ assay (SWS) and a quantitative real-time PCR. For the SWS assay, the anti-AXL antibody from Cell Signaling Technology performed best in antibody selection procedures and was therefore chosen for development. High and low AXL expressing breast cancer cell lines were used to establish "fit for purpose" assays. Subsequently, Her2-positive and Her2-negative samples from breast cancer patients were analyzed by screening for AXL gene and protein expression.

Samples: Fresh-frozen tumor tissue samples of three Her2- positive (W831-Tp1, W887-Tp1, Z381-Tc6) and three Her2- negative (W628-Tp1, W642-Tc5, W836-Tp1) breast cancer patients were analyzed. Tissues were quality controlled – acceptable tumor content was determined to TC ≥50%. Additionally, three breast cancer cell lines MDAMB-231, BT474, and BT549, were used in this study.

Simple Western Size™: The Simple Western Size™ assay used in this study is based on a nano volume size-based protein separation and was run on the Peggy Sue™ instrument (Protein-Simple™). Proteins are separated by size in capillaries and immobilized to the capillary wall via a proprietary UV capture chemistry. Target proteins are then detected using a primary antibody and subsequently using a horseradish peroxidase (HRP)-conjugated secondary antibody. The peaks were assigned according to their expected molecular size and analyzed using the area under the curve (AUC). Results are shown in two different viewing formats: graph view, which shows an electropherogram image for each target protein and lane view, which shows a virtual Western Blot image.
The Simple Western Size™ 12 kDa to 230 kDa Masterkit (ProteinSimple™) was used and assays were performed in accordance with the ProteinSimple™ user manual. Validation of the assay included the determination of appropriate assay conditions, such as antibody dilution and protein loading. Assay validation with respect to linearity and precision was conducted in three independent runs [1]. Protein expression data was displayed using GraphPad Prism 5.0 (GraphPad Software, Inc.; USA).

Quantitative real-time PCR (qPCR): RNA isolation was performed with the RNeasy® Plus Mini Kit (Qiagen) and RNA quality and integrity was analyzed by using the Agilent 2100 bioanalyzer (Agilent Technologies). 1 µg RNA was reverse transcribed with the QuantiTect® Reverse Transcription Kit (Qiagen). qPCR was performed using the 2x SsoAdvanced Universal SYBR® Green Supermix as well as the AXL, UBC, and ACTB PrimePCR™ SYBR® Green Assays (both Bio-Rad) according to the supplier's instructions.

Amplification was carried out in a reaction volume of 20 µl on Bio-Rads C1000 Touch™ Thermal Cycler by using a standard PrimePCR™ protocol including a melt curve analysis. Samples as well as controls were measured in triplicates. Based on similar efficiencies of target (AXL) and reference (UBC, ACTB) genes the ΔΔCq method was applied for quantification of respective mRNA expression of AXL. Gene expression data were displayed using GraphPad Prism 5.0 (GraphPad Software, Inc.; USA).

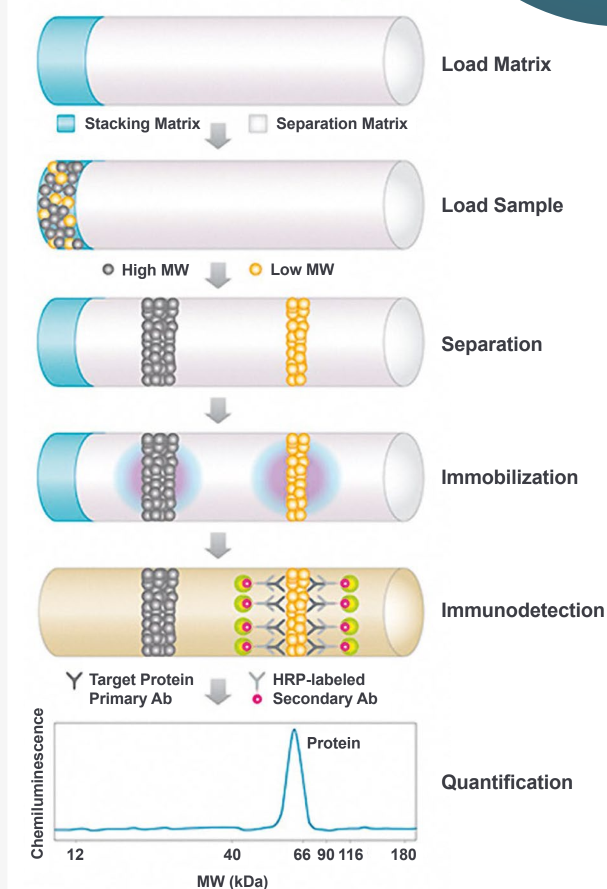


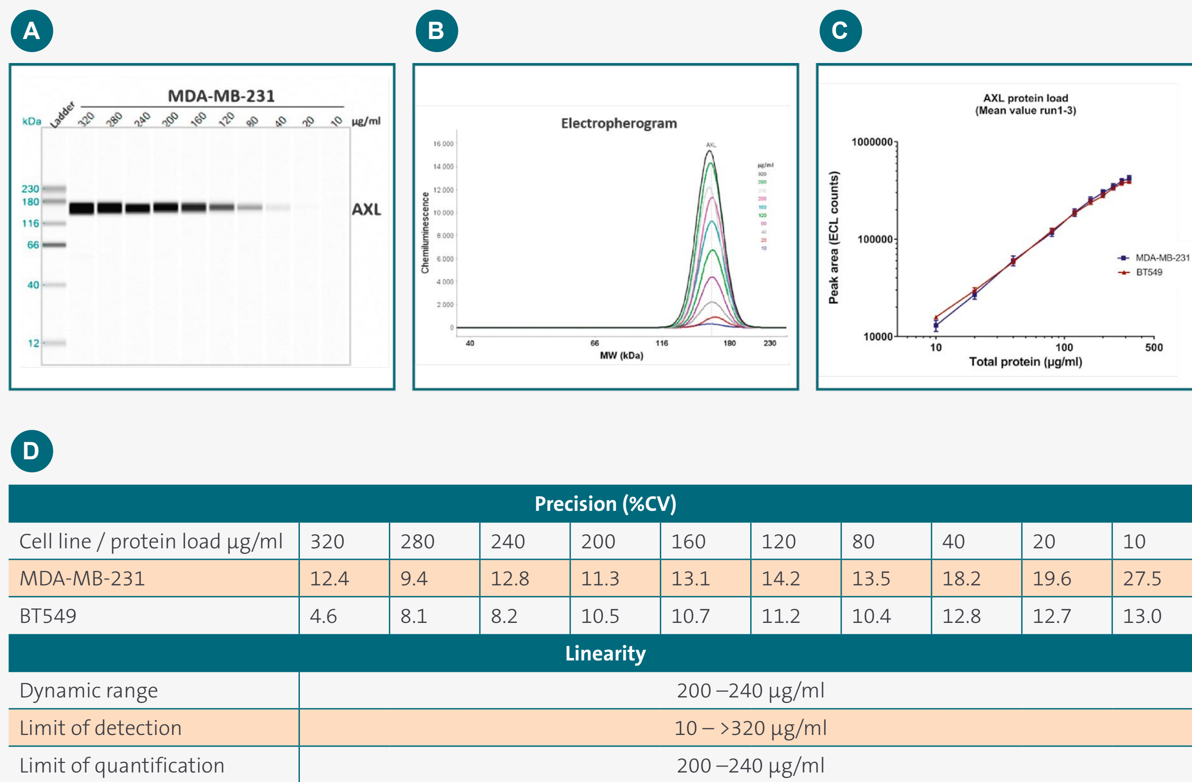
Figure 1: Schematic illustration of the technical workflow of the Simple Western Size™ technology
(adapted from http://www.proteinsimple.com)

Results

Determination of linear dynamic range and reproducibility of Simple Western Size™: Assay development and validation was conducted using AXL-expressing cell lines and comprised the determination of optimal antibody concentration, linear (dynamic) range, and reproducibility of the assay. To determine the linear range and to demonstrate reproducibility, cell line lysates were serially diluted from 320 µg/ml to 10 µg/ml and analyzed in three independent experiments.

Figure 2 shows an exemplary Simple Western Size™ electropherogram image and accompanying lane view of the protein loading series for the MDA-MB-231 cell line. The measured target peak area values showed a linear relationship to the total protein concentrations. The linear (dynamic) range detected within the protein loading series of lysates showed a lower limit of detection of 10 µg/ml for both MDA-MB-231 and BT549 cell lines, which corresponds to a total amount of 0.4 ng of protein lysate. The protein loading series was performed three times, in three independent runs, in order to analyze interrun reproducibility of the assay.

The SWS assay for the detection of AXL consistently showed linearity from 20 to 240 µg/ml total protein load for both cell lines. The dynamic range also defines the limits of quantification; the limits of detection were 10 µg/ml (LLoD) and >320 µg/ml (ULoD). Results are highly reproducible with a %CV of <15% within the dynamic range.



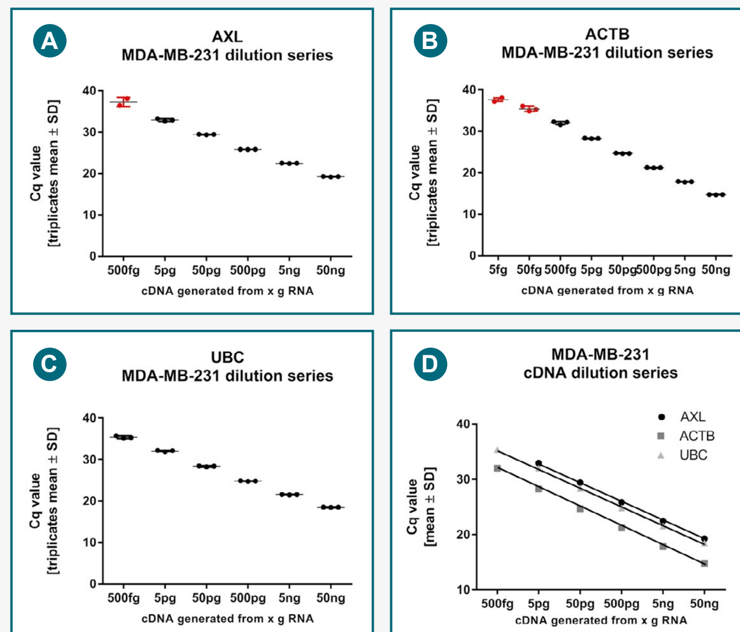
Precision (%CV)										
Cell line / protein load µg/ml	320	280	240	200	160	120	80	40	20	10
MDA-MB-231	12.4	9.4	12.8	11.3	13.1	14.2	13.5	18.2	19.6	27.5
BT549	4.6	8.1	8.2	10.5	10.7	11.2	10.4	12.8	12.7	13.0
Linearity										
Dynamic range	200 –240 µg/ml									
Limit of detection	10 – >320 µg/ml									
Limit of quantification	200 –240 µg/ml									

Figure 2: Determination of linear (dynamic) range and reproducibility

MDA-MB-231 and BT549 lysates were serially diluted from 320 µg/ml to 10 µg/ml total protein load.
A: Representative image of Simple Western Size™ lane view.
B: Representative image of Simple Western Size™ electropherogram view.
C: Linear analysis of software generated peak area of three independent runs (mean value).
D: Precision is expressed as percent coefficient of variation (%CV) of three independent runs. Limits of linearity are derived from the protein loading curve.

Determination of linear (dynamic) range quantitative real-time PCR:
To evaluate efficiency and dynamic range of the target (AXL) and reference (UBC, ACTB) primer assays, a standard curve was generated. The standard curve comprised an eight step 10- fold dilution series of cDNA from the AXL high expression cell line MDA-MB-231.

Figure 3A–C shows the linear relationship between Cq-value and cDNA dilution for the AXL, ACTB, and UBC primer assay. The mean standard curves with linear regression lines of evaluable data points are shown in Figure 3D. Information about the equation of the linear regression line, efficiency, and dynamic range of the individual primer assays are summarized in Figure 3E. Slope, coefficient of correlation, and efficiency of the equation of the linear regression lines are within the published acceptance criteria of PCR characteristics [2], reflecting optimized qPCR assays within the dynamic range. The AXL primer assay showed linearity between Cq-values ranging from 19 to 33. AXL expression between Cq-values of >33 and ≤37 is detectable but not quantifiable. Therefore, a Cq-value of 33 defines the lower limit of quantification (LLoQ) and Cq-values above 37 reflect the lower limit of detection (LLoD).



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Primer	Slope	Correlation coeefficent R2	PCR efficiency [8%]	Dynamic Range [Cq]	LLoQ [Cq]	LLoQ [Cq]
AXL	-3.4334	0.9995	95.5	19-33	>33	>37
ACTB	-3.4615	0.9991	94.5	14.7-32	>32	>37
UBC	-3.4172	0.9992	96.2	18.5-35.3	>35.3	>37
Acceptance criteria	-3.6 to -3.1	≥0.99	90-110%			

Figure 3: Determination of linear dynamic range

A–C: Primer efficiency of AXL, UBC and ACTB are determined by performing a serial dilution of MDA-MB-231 cDNA. An eight step 10-fold dilution series ranging from cDNA generated from 50 ng to 5 fg RNA are analyzed. Cq-values are displayed as scatter plots including mean value with SD of triplicates. Red dots do not fulfill qPCR criteria (SD of triplicates ≥ 0.5); black dots fulfill qPCR criteria and were used for calculation of regression line.

D: Mean standard curves with linear regression line of AXL, ACTB, and UBC primer assays.

E: Summary of amplification efficiency including acceptance criteria of PCR reactions as well as the dynamic range generated with AXL, UBC, and ACTB primer assays.
SD – Standard deviation, R2 – Correlation coefficient, Cq – value-quantification cycle, LLoQ – Lower limit of quantification, LLoD – Lower limit of detection, AXL – AXL receptor tyrosine kinase, UBC – Ubiquitin C, ACTB – Actin beta.

Analysis of AXL gene and protein expression in clinical biospecimen: Simple Western Size™ assays can be used to measure tumor biomarker protein levels in patient tissue samples. We used our validated Simple Western Size™ AXLassay to detect AXL protein expression in breast cancer patient tissue samples. Differential AXL protein expression levels were detectable in all tissue samples with a total protein concentration of 400 µg/ml (Figure 4). AXL protein expression results were highly comparable to AXL gene expression results generated by qPCR. These results show that the Simple Western Size™ assay provides a highly sensitive measurement of target proteins in small sample volumes.

Within this small cohort of patients no difference in AXL expression was detectable between Her2-positive and Her2-negative cases.

Results

In this study, we developed and validated an assay for the detection of AXL protein expression using the Simple Western Size™ technology. This highly sensitive technology enables high-throughput screening of extremely small sample amounts such as biopsies or laser capture microdissected material. Our results showed a broad linear (dynamic) range of the AXL SWS assay in both high and low AXL expressing cell lines, as well as a high reproducibility between multiple runs. The protein loading range suitable to detect AXL within whole cell lysates showed detection levels of AXL protein down to 2 ng of total protein.

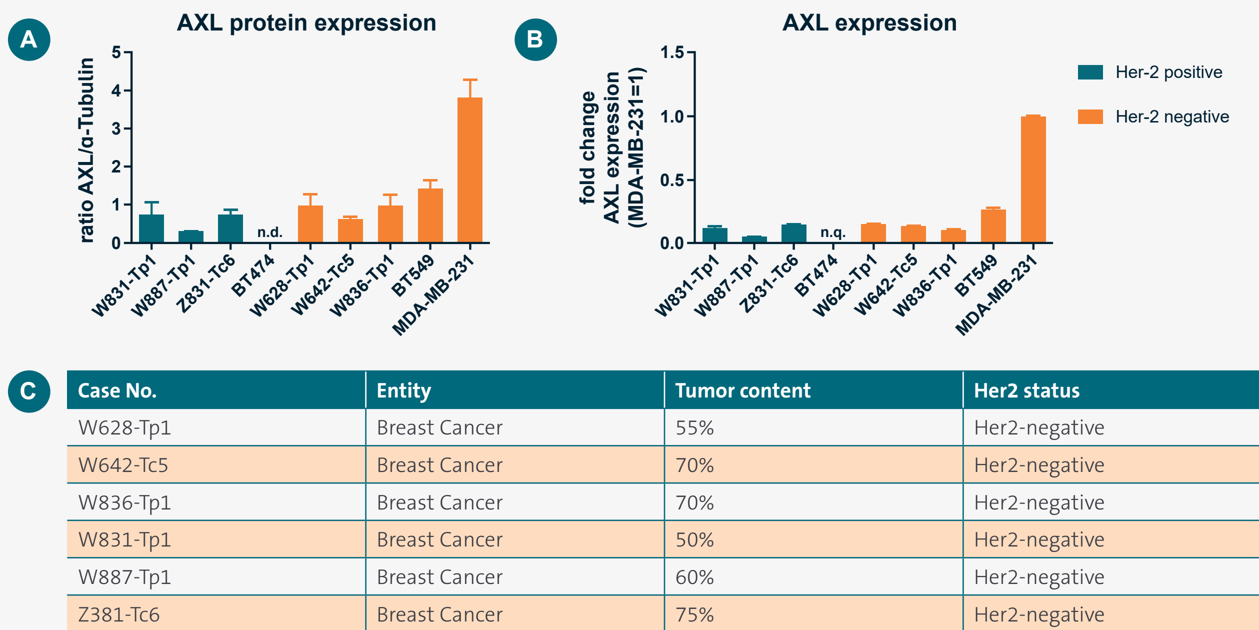
AXL protein expression results were confirmed by the AXL gene expression results in breast cancer cell lines and breast cancer patient samples. This newly developed Simple Western Size™ assay allows for the analysis and quantification of AXL protein expression profiles in breast cancer tissue (and subsequent correlation to Her2 expression status, for example).

Due to the high sensitivity and reproducibility, this quantitative SWS assay for the detection of AXL protein is highly qualified for implementation in the clinical setting (patient stratification).

Literature

1. Lee et al., Method validation and measurement of biomarkers in nonclinical and clinical samples in drug development: a conference report. Pharm Res. 2005; 22(4):499–511.

2. Raymaekers et al., Checklist for optimization and validation of real-time PCR assays. J Clin Lab Anal. 2009; 23(3):145–51.



Case No.	Entity	Tumor content	Her2 status
W628-Tp1	Breast Cancer	55%	Her2-negative
W642-Tc5	Breast Cancer	70%	Her2-negative
W836-Tp1	Breast Cancer	70%	Her2-negative
W831-Tp1	Breast Cancer	50%	Her2-negative
W887-Tp1	Breast Cancer	60%	Her2-negative
Z381-Tc6	Breast Cancer	75%	Her2-negative

Figure 4: Protein expression and qPCR gene expression analysis of AXL in breast cancer tissue samples and breast cancer cell lines

A: Breast cancer patient samples were analyzed in regard to overall tissue quality and tumor content. Tumor content was determined to ≥50%.

B: Protein expression of AXL from six breast cancer tissue samples and three breast cancer cell lines was measured by Simple Western Size™ and normalized to α-Tubulin. Each column represents the mean with SD of a measurement in duplicate.

C: Gene expression of AXL from six breast cancer tissue samples and three breast cancer cell lines was measured and normalized to reference gene expression of UBC and ACTB. Relative AXL gene expression is displayed as fold changes normalized to breast cancer cell line MDA-MB-231. Each column represents the mean with SD of a measurement in triplicate.

SD – Standard deviation, n.d.: not detectable, n.q.: not quantifiable

About Indivumed

Indivumed GmbH is a physician-led, integrated global oncology company for personalized medicine with the world's premier high-content tumor database and highest quality biobank. Indivumed's standard operating procedures are trusted as the global benchmark for biospecimen and clinical data collection.

Our long-standing biobanking expertise is ISO 9001-certified and includes strict protocols for tissue sampling and processing with regard to crucial parameters such as tumor region, ischemia time, and information management.

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