Western Blotting via Proximity Ligation for High Performance Protein Analysis*

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Western blotting is a powerful and widely used method, but limitations in detection sensitivity and specificity, and dependence upon high quality antibodies to detect targeted proteins, are hurdles to overcome. The in situ proximity ligation assay, based on dual antibody recognition and powerful localized signal amplification, offers increased detection sensitivity and specificity, along with an ability to identify complex targets such as phosphorylated or interacting proteins. Here we have applied the in situ proximity ligation assay mechanism in Western blotting. This combination allowed the use of isothermal rolling circle amplification of DNA molecules formed in target-specific ligation reaction, for 16-fold or greater increase in detection sensitivity. The increased specificity because of dual antibody recognition ensured highly selective assays, detecting the specific band when combinations of two cross-reactive antitubulin antibodies were used (i.e. both producing distinct nonspecific bands in traditional Western blotting). We also demonstrated detection of phosphorylated platelet-derived growth factor receptor β by proximity ligation with one antibody directed against the receptor and another directed against the phosphorylated tyrosine residue. This avoided the need for stripping and re-probing the membrane or aligning two separate traditional blots. We demonstrate that high-performance in situ proximity ligation-based Western blotting described herein is compatible with detection via enhanced chemiluminescence and fluorescence detection systems, and can thus be readily employed in any laboratory. *Molecular & Cellular Proteomics 10: 10.1074/mcp.O111.011031, 1–9, 2011.

Western blotting (WB),1 one of the gold standard protein analytical techniques, has been routinely used in most molec-...
by no more than a few tens of nanometers allows hybridization of two oligonucleotides, which can then be ligated into a circle, in reactions templated by the oligonucleotides attached to the antibodies. Finally, the circularized DNA strand is locally amplified by RCA for enhanced detection, in a reaction primed from one of the oligonucleotides attached to the antibodies.

The opportunity to employ the requirement for dual recognition in order to discriminate between closely similar proteins and to directly detect post-translational modifications (14) while generating a locally amplified signal prompted us to investigate the in situ PLA WB as a means to overcome obstacles encountered in traditional WB.

In this article, we establish PLA WB as a strategy for high performance protein analyses beyond the scope of current WB capabilities.

**EXPERIMENTAL PROCEDURES**

*PLA Probes—Duolink® II PLA probes (OLINK Bioscience), composed of secondary antirabbit, antimouse and antichicken IgG antibodies conjugated with the oligonucleotides 5′-AAA AAA AAA ATA TGA CAG AAC TA GAC ACT CTT and 5′-AAA AAA AAA AGA CGC TAA TAG TTA AGA CGC TTU UU, respectively, were used for PLA WB detection.*

*Antibodies and Their Specificities—The following antibodies were used for both traditional WB and PLA WB: Polyclonal rabbit antihuman transferrin antibody (Dako) specific for human transferrin, mouse...*
anti-β-tubulin (Sigma, T4026) specific for all five isoforms of β-tubulin, (β1–β5), and rabbit anti-PDGFRβ (28E1) mAb (Cell Signaling, Danvers, MA; #3169) specific for endogenous PDGFRβ were applied to detect human transferrin, β-tubulin, and PDGFRβ, respectively. Two pairs of antibodies were used to detect phosphorylated PDGFRβ: Mouse antiphospho-PDGFRβ (Tyr751) (88H8) (Cell Signaling, #3166), specific for PDGFRβ phosphorylated at tyrosine 751, was paired with rabbit anti-PDGFRβ (28E1), and mouse anti-PDGFRβ (2B3) mAb (Cell Signaling, #3175) specific for endogenous PDGFRβ, was paired with rabbit antiphospho-PDGFR α/β (C43E9) mAb (Cell Signaling, #3170) specific for PDGFRα phosphorylated on Tyr849 and PDGFRβ phosphorylated on Tyr857 to detect PDGFRβ phosphorylation. Finally, polyclonal rabbit antitubulin whole antiseraum (Sigma, T3252) targeting tubulin, was paired with mouse anti-β-tubulin, or polyclonal chicken anti-TUBB2A (Sigma, GW21263) specific for tubulin on beta polypeptide, to specifically detect human β-tubulin.

Preparation of BJ hTert Cell Lysate—An aliquot of 10⁶ human foreskin fibroblast BJ hTert cells were seeded on a 10-cm dish and grown in Dulbecco’s Modified Eagle Medium/F-12 Ham’s nutrient (Sigma) 1:1 mix containing 10% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin and 1 mM L-glutamine, for 2 days until the cells reached about 80% confluence. After starvation overnight in the Dulbecco’s Modified Eagle Medium/F-12 medium containing 0.5% fetal calf serum, the cells were kept on ice for about 10 min and subsequently incubated with or without human platelet-derived growth factor (PDGF) BB (PeproTech, Rocky Hill, NJ; 100 ng/ml working concentration) on ice for 1 h, gentle shaking. Cells were washed once with 2 ml ice cold PBS buffer, then lysed for 5 min on ice by adding 700 μl cold cell lysis buffer (150 mM NaCl, 10 mM Tris, 0.5% Nonidet P-40, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, pH 7.4) per 10-cm dish followed by detaching the cells with a rubber policeman. To protect cellular proteins against activities of protease and phosphatase, one tablet of Complete Mini and PhosSTOP (Roche) was freshly diluted 1:2000 before incubation with the membrane. Rabbit antitubulin (1:200 dilution) and chicken anti-TUBB2A antibodies (1:3000 dilution) were used for detection specificity study of β-tubulin. After primary antibody probing, the membranes were rinsed once and washed with TBST (TBST with 0.1% Tween 20) for 3 × 9 min, gently rocking at RT. Horse radish peroxidase (HRP) (Jackson ImmunoResearch, diluted 1:50 000) or ECL Plex Cy™ 5 (GE Healthcare, diluted 1:2500) conjugated antiamouse, antirabbit, or antichicken IgG secondary antibodies were incubated with the membrane for 1 h with gentle rotation at RT. After rinsing once and washing 3 × 9 min with TBST, the probed membrane was immersed in TBS buffer for 2 min before detection. ECL detection using ECL Plus reagent (GE Healthcare) was performed according to the manufacturer’s instruction. Before fluorescence detection the membrane was dried overnight at RT or for 1 h at 37 °C protected from light.

Traditional WB Detection of Phosphorylated PDGFRβ—Two sets of primary antibodies were used for detection of phosphorylated PDGFRβ. Cell lysates from control and stimulated BJ hTert cells (5 × 10⁵ cells/well) and PDGF-induced lysate of CCD-1064sk (1.9 μg/ml) were applied to Western blot and assayed either by traditional WB or dual recognition PLA WB assay (Fig. 1C) with ECL readout. Rabbit anti-PDGFRβ (28E1) (1:2000 dilution) and mouse antiphospho-PDGFRβ (Tyr751) (88H8) (1:1300 dilution) antibodies were paired to identify phosphorylated PDGFRβ in BJ hTert cell lysates. Mouse anti-PDGFRβ (2B3) mAb (1:850 dilution) and rabbit antiphospho-PDGFR α/β (C43E9) mAb (1:1000 dilution) antibodies were used to detect phosphorylation of specific receptor (PDGFRβ) in lysates from CCD-1064sk cells. For traditional WB detection, membranes were probed separately either with rabbit or mouse anti-PDGFRβ antibodies overnight at 4 °C and the corresponding antispecies IgG secondary antibodies (1 h at RT) to detect the presence of PDGFRβ. Alternatively they were treated with mouse antiphospho-PDGFRβ and rabbit antiphospho-PDGFR α/β (C43E9) antibodies overnight at 4 °C and the secondary antispecies IgG antibodies for 1 h at RT to detect the corresponding phosphorylated tyrosine residues.

PLA WB Assay—The general procedure for PLA WB is described in Fig. 1. The blotted membrane was blocked with PLA membrane blocking buffer (3% BSA (w/v), 0.1% Tween 20, 100 μg/ml salmon sperm DNA in TBS buffer) for 1 h with gentle orbital rotation at RT. The membrane was then incubated with the same primary antibody dilution as in the traditional WB protocol described above. All the following reactions were performed in a 5-ml plastic tube with gentle orbital rotation and TBST buffer was used for washes. After rinsing once and washing 3 × 9 min, a pair of PLA probes were diluted in PLA buffer (0.5 mg/ml BSA, 5 μg/ml salmon sperm DNA, 5 mM EDTA, 0.05% Tween 20 in TBS buffer) to a final concentration of 0.2 μg/ml and incubated with the membrane for 1 h, with gentle rotation at RT. The
PLA probes are a pair of antispecies antibodies with attached DNA strands, either specific for primary antibodies from the same species (for recognition of individual bound antibodies) or for those from two different species (for recognition of pairs of primary antibodies).

After a brief rinse and washing 2×9 min, 10 ml each of two oligonucleotides (5′ phosphate- CTA TGA GCC TAA GAA TGG CAC GAA GCA GCC GTC AAG AGT GTC TA and 5′ phosphate- GTT CTG TAT TAT TAA ACG GTC TTA A; Integrated DNA Technologies, Coralville, IA) were added and allowed to hybridize to the bound PLA probes. The added oligonucleotides pairs were ligated to form circular DNA strands, using T4 DNA ligase (Fermentas, 0.05 U/pairs) for 40 min at 37 °C in oligonucleotide hybridization-ligation buffer (200 mM NaCl, 10 mM Tris-Ac, 10 mM MgAc, 50 mM KAc, 0.25 mg/ml BSA, 0.05% Tween 20, 0.5 mM ATP). The membranes were rinsed briefly and washed 2×6 min before the RCA step where 0.05 U/μl Phi29 polymerase (Fermentas) in RCA buffer (50 mM Tris-HCl, 10 mM MgCl2, 10 mM (NH4)2SO4, 0.16 mM each of four dNTPs, 0.25 mg/ml BSA, 0.05% Tween 20, pH 7.5) was added for 1 h at 37 °C. After two brief rinses, HRP (5 nM, Biomers) or Cy5 (15 nM, Integrated DNA Technologies) conjugated detection probe (5′ AAA AAA CAG TGA ATG CGA GTC CGT CT) in detection buffer (2×SSC, 0.5 mg/ml BSA, 2 μg/ml salmon sperm DNA, 5% formaldehyde, 0.05% Tween 20) was applied and hybridized to the RCA products for 30 min at 37 °C. The membranes were rinsed twice and washed 3×5 min, followed by a final rinse in TBS buffer to remove Tween 20 before detection.

PLA WB Detection of Transferrin, β-Tubulin and PDGFRβ—The PLA WB assay for detection of individual bound primary antibodies was applied to probe the precut membranes blotted with the dilution series of purified human transferrin and cell lysates using single primary antibodies. Binding of these antibodies were revealed by adding a pair of the corresponding PLA probes directed against immunoglobulin of the species of the primary antibodies. The DNA strands attached to the secondary antibodies guided the formation of an amplifiable DNA circle, and this was followed by RCA as described in the PLA WB assay.

PLA WB Detection of Phosphorylated PDGFRβ—The dual recognition PLA WB assay was performed as described above with the use of the same sets of antibodies applied in traditional WB. The primary antibody pair was incubated with the probing membrane, overnight at 4 °C. After washing, bound primary antibodies were detected by adding the corresponding antimouse and antirabbit PLA probes, followed by oligonucleotide ligation and RCA as described in the PLA WB assay.

PLA WB Specific Detection of β-Tubulin—Two pairs of antitubulin antibodies, i.e. rabbit antitubulin (1:200 dilution) and mouse anti-β-tubulin or rabbit antitubulin and chicken anti-TUBB2A antibodies (1:3000 dilution), were used for specific detection of β-tubulin. The same dual-recognition PLA WB detection (Fig. 1B) procedures as described above were followed.

Detection and Image Analysis—ECL signals were captured by exposing the membrane to Hyperfilm™ (GE Healthcare) and digitalized by using a CanoScan LIDE 200 scanner, or by using a CCD camera (ImageQuant LAS 4000 mini, GE Healthcare). The integrated signal intensities (pixel counts) of the target protein bands were measured using the software Adobe Photoshop CS4 or ImageQuant™ TL image analysis software (GE Healthcare).

Fluorescence signals were captured by employing a Typhoon™ FLA 9000 imager (GE Healthcare) to scan the probed membranes with the PMT setting 450 V. ImageQuant TL image analysis software was used to quantify the integrated signal intensities of the target bands.

RESULTS

Signal Amplification and Detection Sensitivity—To determine if in situ PLA can provide enhanced sensitivity in WB, we compared PLA WB and traditional WB in studies of transferrin, β-tubulin, PDGFRβ, and phosphorylated PDGFRβ, using lysates from PDGF-BB stimulated human fibroblast BJ hTert cells and readout via ECL. A 2-fold dilution series of transferrin from 2.5 ng to 0.08 pg was analyzed using the CCD camera and the integrated signal intensities were quantified using the ImageQuant TL image analysis software (Fig. 2). After the sequential exposures from 10 s to 2 min, detection limits of 10 pg and 5 pg transferrin, respectively, were reached for traditional WB (Fig. 2A). To avoid signal saturation, only a 10 s exposure was used for PLA WB to detect the full dilution series. With this shorter exposure time, a detection limit of as little as 0.08 pg transferrin was reached, corresponding to around 60-fold increase in sensitivity over traditional WB (Fig. 2B). The dynamic range was similar for both PLA WB and traditional WB and was close to 2.4 orders of magnitude (Fig. 2C).

A fourfold dilution series of cell lysate was analyzed for β-tubulin using PLA WB with a single primary antibody, and ECL signals were captured by film exposure. Compared with traditional WB detection an ~8–21-fold increase in signal intensity was achieved that enabled PLA WB to detect two further serially diluted target bands, corresponding to a 16-fold increase in sensitivity (Fig. 3A). The signal intensity for PLA WB resulted in saturation for the highest protein concentration with 1 min film exposure. This suggested that the true signal amplification can be much larger. Similar results were obtained for PDGFRβ (Fig. 3B). The PLA WB signal was 25-fold stronger when an amount of lysate corresponding to 105 cells was applied and three further serial dilutions were detectable, representing a 64-fold increase in sensitivity compared with traditional WB. For detection of PDGFRβ phosphorylated on tyrosine751, the PLA WB provided an ~70-fold signal increase (Fig. 3C). Only a weak signal was visible for samples containing 105 cells by traditional WB, PLA WB enabled detection of the phosphorylated receptor from as little as 6000 cells using PLA WB with a pair of primary antibodies.

We investigated fluorescence PLA WB using a CyDye labeled detection probe for visualizing RCA products, followed by detection with a fluorescence Imager as an alternative to ECL readout. A 2-fold dilution series from 3.8 μg to 0.12 μg total cellular protein was prepared from a lysate of PDGF-BB induced sc-2264 cells. We compared detection of β-tubulin using either traditional fluorescence WB with a Cy5-labeled antimouse secondary antibody and single recognition PLA WB using a Cy5-labeled oligonucleotide for detection of RCA products. Fig. 4 shows the dramatic increases of signals using PLA WB, ranging from 25- to 50-fold, for all sample concentrations compared with stan-
standard WB detection. The relative sensitivity was greater for the lower sample concentrations.

Detection Specificity—In order to determine if also the specificity of detection can be improved by using PLA WB assays where two primary antibodies rather than one must bind the target molecules, human fibroblast BJ hTert cell lysates were subjected to SDS-PAGE and WB. β-tubulin was targeted using three different probing approaches, all visualized via ECL detection (Fig. 5). Traditional WB using rabbit antitubulin primary antibodies and HRP-conjugated secondary antibodies resulted in detection of several unspecific protein bands, in addition to the expected band at a molecular weight close to 55 kDa (Fig. 5A, top). Another, less unspecific mouse anti-β-tubulin primary antibody resulted in a single weak unspecific extra band corresponding to a molecular weight lower than 55 kDa, in addition to the specific β-tubulin band (Fig. 5A, middle). In this approach two secondary antirabbit IgG PLA probes were used to reveal the binding by mouse anti-β-tubulin antibodies via PLA WB for single primary antibodies. By contrast, in a dual recognition PLA WB experiment, simultaneous binding by both primary antibodies was required for detection via secondary antirabbit IgG and antimouse IgG antibodies with attached DNA strands as PLA probes. Under these conditions only the specific β-tubulin band was detected and no non-overlapping cross reactive bands appeared (Fig. 5A, bottom). The experiment in Fig. 5B produced similar results using unspecific chicken and rabbit antitubulin in traditional WB (Fig. 5B, top and middle), compared with the dual recognition PLA WB where both primary antibodies must recognize the target (Fig. 5B, bottom). The dual recognition PLA WB produced one specific β-tubulin signal, and thus avoided detection of the unspecific bands.

Fig. 2. Signal amplification, detection sensitivity and dynamic range in detection of transferrin. A twofold dilution series of purified transferrin protein was targeted in parallel by (A) traditional WB and (B) PLA WB using ECL readout, recorded by the CCD camera (ImageQuant LAS 4000 mini) and quantified by ImageQuant TL image analysis software. The exposure time was 10 s. C, The integrated signal intensities (pixel counts) for traditional WB (dark circle) and for PLA WB (gray square) were plotted as a function of the amounts of protein. The inset shows signals in the lower concentration range. Standard deviation from duplicate assays is shown.
that appeared when each of the primary antibodies was used individually. These results illustrate the improved detection specificity resulting from dual recognition PLA using two relatively unspecific primary antibodies, as the PLA WB requires that both primary antibodies bind to the same or closely proximal target molecules in order to produce a signal.

**Detection of Post-translational Modifications**—The requirement for dual recognition by PLA can also be of value to investigate more complex targets, e.g. interacting proteins or post-translational modifications. In order to investigate this point, lysates from human fibroblast control cells or cells stimulated with PDGF-BB were subjected to WB. A pair of antibodies directed against PDGFR\(\beta\)/H9252 and against the specific phosphorylation of tyrosine751 of the receptor were applied separately in traditional WB (Fig. 6A, top). In order to determine if the two antibodies bind to targets of the same approximate molecular weight, two parallel blots of the same sample need to be probed for ECL readout. Alternatively, the same blot can be investigated sequentially. Phosphorylation on tyrosine751 of PDGFR\(\beta\) in the lysate from stimulated cells was detected as a weak signal by traditional WB after 1 min film exposure. Using dual recognition PLA WB, simultaneous probing with both primary antibodies from different species produced one single band, indicating the presence of the tyrosine751-phosphorylated receptor (Fig. 6A, bottom). With only 10 s film exposure, the resulting PLA signals were already considerably stronger than the corresponding signals produced by traditional WB. In the absence of stimulation no PDGFR\(\beta\) phosphorylation was observed and accordingly no clear signal was detected for control cells (Fig. 6A, bottom).

PLA WB thus allows confident detection of a post-translationally modified protein in a single assay, whereas two separate or sequential probings are commonly undertaken in traditional WB. Also, PLA WB reveals molecular proximity between the binding sites for the two primary antibodies, rather than the more poorly resolved comigration of proteins on gels.

**Fig. 3.** Signal amplification and detection sensitivity for \(\beta\)-tubulin, PDGFR\(\beta\), and phosphorylated PDGFR\(\beta\). Fourfold dilution series of human fibroblast cell lysate were analyzed for \(\beta\)-tubulin, PDGFR\(\beta\), and phosphorylated PDGFR\(\beta\) by ECL via WB (dark circle) or PLA WB (gray square). Signal amplification and detection sensitivity were compared. The signal intensities (pixel count) were recorded for \(\beta\)-tubulin (A) and PDGFR\(\beta\) (B) after 1 min film exposure, for phosphorylated PDGFR\(\beta\) (C) after 2 min film exposure. Standard deviation from triplicate assays is shown.
Another example of detection of PDGF receptor modification is shown in Fig. 6B using lysates from PDGF-induced CCD-1064sk cells. This time another pair of primary antibodies was used for dual recognition PLA WB to specifically detect tyrosine phosphorylation of PDGFRα. A mouse anti-PDGFRα (2B3) targeted PDGFRα and a rabbit antiphospho-PDGFR α/β (C43E9) was specific for Tyr849 phosphorylation on PDGFRα and for Tyr857 phosphorylation on PDGFRβ. Using traditional WB with 10 min exposure to film, separate proings with these antibodies gave rise to several unspecific bands in addition to the expected bands (Fig. 6B, top and middle). In dual recognition PLA WB assay, only a specific band with the approximate molecular weight of 190 kDa was detected after 1 min film exposure indicating the phosphorylation on PDGFRβ (Fig. 6B, bottom).

**DISCUSSION**

The requirement for dual antibody recognition of proximal epitopes on the same or interacting proteins in order to elicit a locally amplified detection signal are characteristics of in situ PLA that provide enhanced detection specificity and sensitivity. Here we demonstrate that this assay can be applied to WB in order to visualize proteins that have been separated by gel electrophoresis and transferred to a membrane. Com-
pared with reaction conditions for in situ PLA detection for microscopy, reagent concentrations in general must be lowered, and robust reaction mixing must be applied for detection of proteins on the highly adsorptive membranes in order to achieve optimal signal-to-noise ratios.

We show herein that PLA WB provides improved detection sensitivity compared with traditional WB, because of the specific recognition and the potent signal amplification via RCA. This offers a valuable opportunity to investigate molecules present in very few copies per cell, or in only a minority of cells within a heterogeneous population, and the improved sensitivity can also permit analysis of small amounts of sample, reducing consumption of precious samples.

It is well known that so-called sandwich immunoreactions, where antigens are detected via binding by two antibodies, generally provide greater specificity of detection compared with single binder assays. This dependence on dual binding serves to discriminate against cross reactive recognition that is not shared between two antibodies. In traditional WB, detection depends on recognition by single antibodies, whereas the molecular weight of the targeted protein provides an additional criterion for identification. Frequently, nonspecific bands are observed in WB. In such cases PLA WB can facilitate identification of the correct protein. For the pairs of antibodies tested herein PLA WB eliminated the nonspecific bands and the assay may thus also enable the use of antibodies that would give rise to cross reactivity in traditional WB. This feature of PLA WB can help reduce dependence on high-quality antibodies and molecular weight confirmation in WB analysis. However, any irrelevant protein detected by both antibodies will still appear in PLA WB.

Fig. 6. Detection of phosphorylated PDGFRβ using traditional WB and PLA WB. Phosphorylated PDGFRβ in the control and PDGF-BB-stimulated lysates from 5 × 10⁶ human fibroblast BJ hTert cells was targeted by traditional WB or dual recognition PLA WB with ECL readout. A. For traditional WB detection the receptor and the phosphorylated tyrosine751 were separately targeted using primary rabbit anti-PDGFRβ or mouse antiphospho-PDGFRβ (Tyr751) antibodies, respectively, and the corresponding antispecies IgG secondary HRP-conjugated antibodies (top image). PLA WB was performed using a combination of the same two primary antibodies, followed by secondary antirabbit and antimouse PLA probes to detect the phosphorylated receptor (bottom image). The traditional and PLA WB signals were captured by film exposure of 1 min and 10 s, respectively. B, PDGFRβ phosphorylation was also investigated using another pair of primary antibodies in lysate (1.9 μg) from PDGF-BB stimulated CCD 1064sk cells using traditional WB, probing with two antibodies in separate reactions (top and middle image) and by dual recognition PLA WB using the two antibodies in a single reaction (bottom image). Readout was via ECL. Rabbit antiphosphor-PDGFRα (Tyr849)/PDGFRβ (Tyr857) primary antibody was followed by a secondary antirabbit HRP (top image). Mouse anti-PDGFRβ primary antibody was revealed by a secondary antimouse HRP (middle image). A combination of these two primary antibodies were applied with secondary antirabbit and antimouse PLA probes (bottom image). The traditional and PLA WB signals were captured by the film exposure for 10 and 1 min, respectively.

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So far, in situ PLA has mainly been used to interrogate more complex targets such as protein interactions and post-translational modification of proteins, but we illustrate here that the technique brings a valuable increase of detection specificity to localized protein detection via the requirement for dual recognition. The assay could also be used to distinguish closely similar proteins, such as splice variants that share some but not all epitopes. We demonstrate herein that PLA WB facilitates detection of post-translational modified proteins, exemplified by the detection of phosphorylated PDGFRβ. The assay could also be used to demonstrate protein interactions such as receptor dimerization and aggregation products using native gel electrophoresis.

Compared with traditional WB, PLA WB requires more reaction steps and about 2 more hours. Work is in progress to simplify the process.

In conclusion, the PLA WB method presented herein provides several distinct advantages compared with traditional WB in terms of sensitivity, specificity, and the ability to distinguish closely similar protein variants. By visualizing detected proteins in blots as locally amplified DNA sequences it enhances their susceptibility to selected chemotherapeutic drugs in vitro and in vivo. "Int. J. Cancer 124, 1227–1234

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