Electronic Supplementary Material (ESI) for Analytical Methods.

Development of an automated direct blotting electrophoresis system for bioanalytical applications

Shinichi Goto[a,†], Nasa Savory[b,†], Koichi Abe[b], Hideki Kinoshita[a] and Kazunori Ikebukuro[b*]

[a]New Business Development Division, Sharp Corp., 1-9-2, Nakase Mihama, Chiba, Chiba 261-8520, Japan

[b]Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho, Koganei, Tokyo, 184-8588 Japan

[†]Shinich Goto and Nasa Savory contributed equally to this work.

*Corresponding author. Tel.: +81 42 388 7030; Fax: +81 42 388 7030

E-mail address: ikebu@cc.tuat.ac.jp (K. Ikebukuro)

Postal address: 2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan

Electronic Supplementary Information

MATERIALS AND METHODS

Reagents and materials

Precision Plus Protein Kaleidoscope Standards and Precision Plus Protein Dual Color Standards were purchased from Bio-Rad Laboratories (Hercules, CA). BLUE Star Prestained Protein-Ladder was purchased from Nippon Genetics (Tokyo, Japan). VEGF and human VEGF165 biotinylated antibody were purchased from R&D Systems (Minneapolis, MN). AFP was purchased from BBI Solutions (Cardiff, UK). The anti-AFP monoclonal antibody was purchased from Acris Antibodies (Herford, Germany). The Aβ1-40 peptide was purchased from Peptide Institute (Osaka, Japan). The Aβ 1-16 (6E10) monoclonal antibody was purchased from Covance (Princeton, NJ). The NeutrAvidin-HRP conjugate was purchased from Thermo Fisher Scientific (Waltham, MA). The anti-mouse IgG-HRP conjugate was purchased from Promega (Madison, WI). The anti-FITC antibody HRP conjugate was purchased from (Southern Biotechnology Associates, Birmingham, AL).

Automated direct blotting electrophoresis (AutoDBE)

The AutoDBE apparatus comprised a SDS-PAGE part and a blotting part with a power supply (PowerPac HC High-Current Power Supply, Bio-Rad Laboratories) (Fig.1A). In the SDS-PAGE part, a pair of glass plates (50 × 74 × 3 mm) were superposed and held with adhesive tape and the bottom edge was sealed with hydrophilic PVDF Durapore membrane (Merck Millipore, Billerica, MA). A polyacrylamide gel was prepared in the glass plates to a thickness of 1 mm and 0.5 mm at the edge of the gel, because the glass plates are tapered (Fig. 1B). A separation gel monomer solution [375 mM Bis-Tris buffer (pH 6.4) containing 10.0% (w/v) acrylamide, 0.3% (w/v) *N,N′*-

methylenebisacrylamide, 0.05% (w/v) ammonium persulfate and 0.1% (v/v) *N*,*N*,*N*′,*N*′-tetramethylethylenediamine] and a stacking gel monomer solution [375 mM Bis-Tris buffer (pH 6.4) containing 5% (w/v) acrylamide, 0.3% (w/v) *N,N*′-methylenebisacrylamide, 0.05% (w/v) ammonium persulfate and 0.1% (v/v) *N*,*N*,*N*′,*N*′-tetramethylethylenediamine] were used to prepare a 22 mm long separation gel and a 5 mm long stacking gel in the glass plate cavity.

The blotting part in AutoDBE is composed of a stepping motor (AZ46AKD-1, Oriental Motor, Tokyo, Japan), positioning arm and transfer membrane. The motor moves the positioning arm that carries the transfer membrane (Fig. 1C, D). The transfer membrane moves in a horizontal direction and the SDS-PAGE gel is placed vertically in contact the membrane to allow blotting of separated proteins that elute from the hydrophilic PVDF Durapore membrane at the end of the gel onto the transfer membrane (Fig. 1D).

To perform AutoDBE, Immobilon-FL PVDF (Merck Millipore) or 0.45-μm nitrocellulose membrane (Bio-Rad Laboratories) was positioned in an anode chamber containing 400 mL of electrophoresis buffer [100 mM MOPS, 50 mM Tris, 50 mM Bis-Tris and 20% (v/v) EtOH]. A 6.5, 10 or 15% polyacrylamide gel containing SDS was positioned in a cathode chamber containing 170 mL of electrophoresis buffer [100 mM MOPS, 50 mM Tris, 50 mM Bis-Tris and 0.5% (w/v) SDS]. Electrophoresis was performed at a constant current of 50 mA for 120–150 min. The transfer membrane was kept at the initial position for 20 min, after which the transfer membrane frame was moved to the front of the anode chamber at a speed of 0.1–3.3 mm/min.

Western analysis

Aβ oligomers were prepared according to previous reports[1, 2]. VEGF, AFP and Aβ oligomer samples prepared in an SDS sample buffer [62.5 mM Tris-Cl, 6% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 10% glycerol, pH 8.6] were applied to AutoDBE as described above. Protein-transferred PVDF and nitrocellulose membranes were incubated

with 4% (w/v) skim milk prepared in TBST (50 mM Tris-Cl, 150 mM NaCl, 5 mM KCl, pH 7.4) for 1 h at room temperature (r.t.). After washing in TBST, the membranes were incubated with the anti-VEGF biotinylated antibody, anti-AFP antibody and anti-Aβ antibody, respectively, for 1 h at r.t. The membranes were further incubated with the NeutrAvidin-HRP conjugate and anti-mouse IgG-HRP conjugate, respectively, for 1 h. After washing in TBST, HRP chemiluminescence was detected by adding Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) using ImageQuant LAS 4000 mini (GE Healthcare, Buckinghamshire, UK).

Two-dimensional electrophoresis

Mouse liver tissue extract was prepared for electrophoresis according to a previous report[3], and proteins in the extract were fluorescently labelled with *N*-ethyl-*N*′-[5-(*N*″-succinimidyloxycarbonyl)pentyl]-3,3,3′,3′-tetramethyl-2,2′-indodicarbocyanine iodide (IC5) using an IC5-OSu special packaging (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. The mouse liver tissue extract was then subjected to isoelectric focusing (IEF) and equilibration on an Automated 2D Electrophoresis System (Auto2D) (Sharp Manufacturing Systems Co., Osaka, Japan) following a previous report[3]. The IEF part of the instrument (IEF chip for pH range 4.0–7.0, BM-114070, Sharp Manufacturing Systems Co.) was transferred into the groove containing 10 μL of the sample solution [2.5 μg of protein sample, 8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM DTT and 0.5% (v/v) carrier ampholyte] for 35 min. The voltage was linearly increased from 0 to 6,000 V over 25 min and maintained at 6,000 V for 5 min. The IEF part was transferred into the groove containing 700 mL of equilibrating buffer [500 mM Tris-Cl (pH 6.6), 4% (w/v) SDS, 12.5% (v/v) glycerol, 0.005% (w/v) bromophenol blue and 50 mM DTT] for 5 min and placed on the tapered surface of the polyacrylamide gel on the AutoDBE.

Image analysis

Protein bands were detected using the gel analysis software ImageQuant TL (GE healthcare) and ImageMaster 2D Platinum (GE Healthcare). To assign protein band positions, the mobility of each protein band was evaluated as a pixel count from the beginning of the separation gel region. The position of the protein spot was set at the position of its peak fluorescence signal. The signals used in these analyses were employed without background corrections.

Aptamer blotting assay

Five hundred micrograms of VEGF prepared in the SDS sample buffer were used for AutoDBE as described above. A protein-transferred nitrocellulose membrane was incubated with 4% (w/v) skim milk prepared in TBST for 1 h at r.t. After washing in TBST, the membrane was incubated with 500 nM VEGF-binding aptamer 3R02 bivalent for 1 h at r.t. The membrane was further incubated with an anti-FITC antibody HRP conjugate for 1 h at r.t. After washing in TBST, HRP chemiluminescence was detected by adding Immobilon Western Chemiluminescent HRP Substrate using ImageQuant LAS 4000 mini.

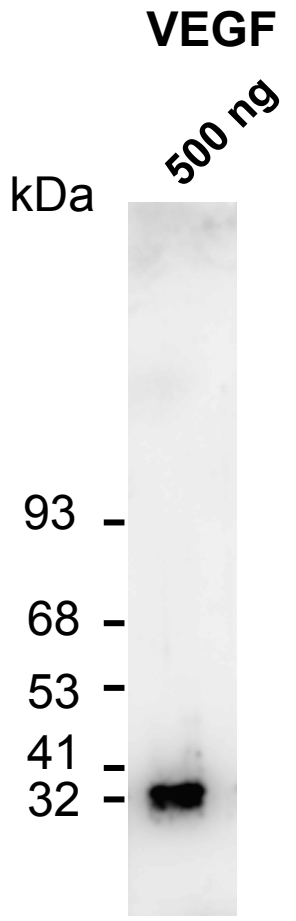

Fig. S1. Aptamer blotting analysis for VEGF proteins directly blotted on a nitrocellulose membrane using the AutoDBE apparatus. Five hundred micrograms of VEGF were blotted on a nitrocellulose membrane and incubated with the 5′-FITC modified VEGF-binding aptamer 3R02 bivalent. The binding of aptamer was detected using an anti-FITC antibody HRP conjugate.

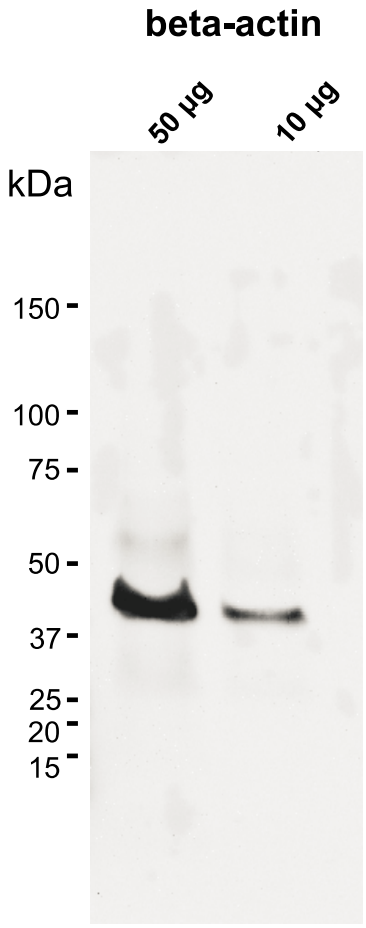

Fig. S2. Western analysis of beta actin protein in a mouse liver extract sample directly blotted on PVDF membranes using the AutoDBE apparatus. In Western analysis, 50 and 10 µg of mouse liver extract protein samples were subjected to AutoDBE. A 10% polyacrylamide gel was used for AutoDBE and separated proteins were directly transferred onto PVDF membranes for Western analysis with a mouse anti-beta actin antibody (Sigma-Aldrich, 1:2000 dilution).

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