Published in final edited form as:

Methods Mol Biol. 2015 ; 1312: 165–173. doi:10.1007/978-1-4939-2694-7_20.

A miniaturized blotting system for simultaneous detecting of different autoantibodies

Ulrich Canzler, M.D.^{1,2}, Holger Bartsch, Ph.D.¹, Kai Großmann³, Werner Lehmann, Ph.D.³, Karsten Conrad, M.D.¹, Biji T. Kurien, Ph.D.⁴, Yaser Dorri, BS⁴, R. Hal Scofield, M.D.⁴, Michael P. Bachmann, Ph.D.^{1,*}

¹Institut für Immunologie, Carl Gustav Carus Technische Universität Dresden, Germany, Fetscherstrasse 74, 01307 Dresden, Germany;

²Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe, Universitätsklinikum Carl Gustav Carus, Germany, Fetscherstrasse 74, 01307 Dresden

³Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe, Universitätsklinikum Carl Gustav Carus, Germany, Fetscherstrasse 74, 01307 Dresden

⁴Arthritis and Clinical Immunology Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, OK 73104, USA

Summary

Sera of tumor patients frequently contain autoantibodies to tumor associated antigens. Here we describe a miniaturized immunoblot platform allowing us to screen sera of patients for the presence of autoantibodies to ten autoantigens in parallel.

Keywords

Multiparametric assay; Autoantibodies; Tumor-associated antigens

1. Introduction

Autoantibodies to cellular components are not only found in sera of patients with autoimmune diseases: using a variety of different techniques including for example cDNA library screening (SEREX technique), enzyme linked immunosorbant assay, immunoblotting, immunoprecipitation, and epifluorescence microscopy, autoantibodies were also detected in tumor patients (*e.g.* 1–6). The presence of such antibodies are of interest for several reasons: (i) these antibodies indicate that tumor cells are indeed recognized by both, the cellular and humoral arm of the immune system, and (ii) the antibodies against tumor associated antigens (TAAs) can perhaps be used as early indicators of a developing or already existing tumor or a relaps of a cancer. Many investigators have, therefore, been interested in the use of autoantibodies to TAAs as serological markers for cancer diagnosis (*reviewed in* 7). Enthusiasm for this approach has been tempered by the

*Corresponding author: tel. +49-351-458-6540; fax: +49-351-458-6308; michael.bachmann@mailbox.tu-dresden.de.

low sensitivity when individual antigen–antibody reactions were studied: According to these studies, antibodies to any individual antigen such as for example p53, c-myc, or p62 do not reach levels of sensitivity which could become routinely useful in diagnosis. However, it was proposed that this drawback can be overcome by using a panel of selected TAAs. Indeed, some published data support the idea that a multiparametric assay improves the sensitivity and specificity for a specific tumor entity when several selected TAAs are analyzed in parallel (*e.g.* 8). Here we describe a novel platform, a miniaturized immunoblot system (Fig. 1), allowing us to screen sera for the presence of different autoantibodies in parallel.

2. Materials

2.1. Bacterial Expression and Purification of Antigens

1. Luria-Bertani (LB) medium (10 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone, pH 7.0), store at 4°C.
2. Isopropyl β -D-1-thiogalactopyranoside (IPTG), 1M stock solution in water, store in aliquots at –20°C (*see* Note 1).
3. Bacterial expression clones (pET28 or equivalent) in BL21(DE3)pLysS bacteria (*see* Note 2).
4. Ni-NTA agarose (Qiagen, Hilden, Germany).
5. Lysis buffer (8 M urea, 100 mM NaH₂PO₄, 10 mM Tris/HCl, pH 8).
6. Wash buffer I (10 mM imidazole, 8 M urea, 100 mM NaH₂PO₄, 10 mM Tris/HCl, pH 8.0) and wash buffer II (20 mM imidazole, 8 M urea, 100 mM NaH₂PO₄, 10 mM Tris/HCl, pH 8).
7. Elution buffer (350 mM imidazole, 8 M urea, 100 mM NaH₂PO₄, 10 mM Tris/HCl, pH 8)
8. Suitable antibiotic stock, dependent on the used expression system.

2.2. Western Blotting for Preparing of the Microblot

1. Transfer buffer: Roti-Blot A and Roti-Blot K (Carl-Roth, Karlsruhe, Germany).
2. Nitrocellulose membrane: Porablot NCP, not enforced (Machery Nagel, Düren, Germany) (*see* Note 3).
3. 3MM Chr chromatography paper.
4. Tris-buffered saline with Tween-20 (TBST) (10x): 1.5 M NaCl, 0.5 M Tris-HCl, pH 8, 1% Tween-20. Dilute 100 mL with 900 mL water for use.
5. Blocking buffer: 5% (w/v) Blocking reagent (Roche, Mannheim, Germany) in TBST.
6. Ponceau S staining solution: 0.2% [w/v] Ponceau S in 0.3% [v/v] trichloroacetic acid.

2.3. Development of Microblot

1. The manufacturing principle of the microblot (9,10) is shown in Fig. 1. Briefly, protein antigen bearing lines excised from stained western blots as well as marker bands for software based data analysis are stacked and embedded in paraffin. Sections of 10 μm are cut by a microtome, paraffin is removed and nitrocellulose slices are mounted to a solvent resistant support membrane by organic solvents. The resulting microblots ($6 \times 2 \text{ mm}$) are fixed to a plastic holder and integrated into modules of a standard 96-well microtitre plate. Each microblot contains 10 autoantigen bands, seven marker bands and a conjugate reaction control line.
2. The solutions requested for development of the microblots are provided by Attomol GmbH (Lipten, Germany) upon delivery of your microblot test system. These ready-to-use solutions include a sample diluent and a precipitating 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution for horseradish peroxidase (HRP), concentrated wash buffer (10x), and sheep anti-human IgG-HRP-conjugate (27x). The wells containing the microblots are stored at room temperature (RT). The 10x washing buffer has to be stored at RT. The anti-IgG-HRP-conjugate as well as the TMB substrate has to be stored at 4°C. The sample diluent solution should be stored at -20°C.
3. For data collection, a scanner with a suitable depth of focus (e.g. Plustek Optic Pro ST48) and a PC for documentation and analysis is required. The results can be automatically evaluated with the Attomol® Microblot-Analyzer software.
4. For washing of the 96-well microtitre plates, an ELISA washer, adjusted to 400 μL wash volume set to overflow can be used. Adjust the washer needle maximal eccentricity in order to provide the microblot from being touched (see Note 4). An automatic plate washer from Tecan GmbH (Germany) can be employed.

3. Methods

As patient sera are often limited, it is desirable to evaluate as much parameters with as little sera probe as possible. Therefore, multiparameter assays have been developed. Other problems often confronted with when screening patient sera for specific antigen recognition are limitations concerning the used antigens. Often, these are difficult to purify or, if purchased, very expensive.

One technique, which is able to address both problems at one time (limited availability of serum sample and antigen) is the use of miniaturized multiparametric assays as for example the microblot technique used herein. Here, one can use very little amount of antigen, which does not need to be absolutely pure, because there is an additional SDS-PAGE step for separation of the protein of interest from undesired contaminants. In addition, in one well of a 96 well plate the simultaneous screening of up to ten different antigen reactivities can be accomplished. Also, the procedure is related to a normal ELISA assay, which makes it easy to perform. Hence, the microblot is very suitable for a multiparametric evaluation of precious patient sera.

3.1. Preparation of Samples; Bacterial Expression and affinity Purification of His₆ Tagged Proteins

1. If not already available, start with cloning of the desired antigens into a bacterial expression system. We have good experience using the pET system provided by Merck KGaA (Darmstadt, Germany), but other systems might work as well.
2. The day before the bacterial expression pick an isolated colony of the desired clone and incubate in 10 mL LB medium supplemented with the appropriate antibiotic in a rotating incubator at 37°C.
3. Transfer 8 mL of the overnight culture to 800 mL of freshly prepared LB medium and continue to incubate at 37°C in a rotating incubator (*see Note 5*).
4. Monitor the growth of the bacterial culture by measuring the optical density₆₀₀ (OD₆₀₀) and continue the culture until the OD₆₀₀ reaches 0.5.
5. Induce the culture by adding 1 mM IPTG.
6. Grow the culture for additional three hours and keep monitoring OD₆₀₀ at least every hour. If the OD₆₀₀ is not increasing any further, you can stop the culture.
7. Pellet the bacterial suspension at 5000 × g for 20 min at 4°C. Discard the supernatant.
8. Store the cell pellet at –20°C until use or proceed immediately with the cleaning procedure.
9. Resuspend the pellet in 10 mL lysis buffer, add 1 mM PMSF (*see Note 6*).
10. Disintegrate the bacterial suspension by incubating for 15 min at room temperature and periodical mixing. The suspension will become very viscous due to the released nucleic acids.
11. Degrade the nucleic acids by applying ultrasound in short pulses. Keep the protein suspension on ice during the whole procedure (*see Note 7*).
12. Centrifuge the lysate for 15 min at 10000 × g (4°C) and save the supernatant for purification.
13. Prepare a Ni-NTA column as follows: fill the bottom of a 5 mL syringe with some sterile glass wool and wet the glass wool with water to remove any air that is trapped. Add 500 µL Ni-NTA agarose beads (approximately 1 mL Ni-NTA slurry) on top of the glass wool and equilibrate the prepared column with 10 mL lysis buffer.
14. Apply the supernatant saved after the centrifugation step onto the column. Keep the flow through and reapply once more. If desired keep an aliquot for control purpose.
15. Wash the column once with 10 mL lysis buffer.

16. Wash four times with 1 mL wash buffer I each, followed by four additional wash steps with 1 mL wash buffer II each. If desired keep an aliquot for control purpose.
17. Elute the protein with elution buffer. Apply six times 500 μ L each and save in separate sample tubes.
18. Continue with an SDS-PAGE (*see* Section 3.2) in order to analyze the efficacy of your protein purification. Load samples of each elution fraction and, if desired samples of your washing steps, flow through and crude extract.

3.2. SDS-PAGE

1. Carry out SDS-PAGE (1 mm thick mini gels) essentially according to Laemmli (11) (*see* **Chapters 11, 34**).
2. Load 30 μ L (approximately 3 μ g protein) of each sample in a well. Include one well for prestained molecular weight markers Page Ruler TM. Load either the samples for the control or for the western blot.
3. Stain the control gel in Coomassie Blue staining solution for 1 h and destain until protein bands are clearly visible with a nearly clear background, or continue with western blotting.

3.3. Western Blotting

1. After separation by SDS-PAGE, the samples are transferred to nitrocellulose membranes electrophoretically. These directions assume the use of a semi-dry system provided by Bio-Rad (*see* **Chapter 31**). Two trays with transfer buffer Roti-Blot A and transfer buffer Roti-Blot K respectively are prepared with a size slightly bigger than the dimension of the gel.
2. Cut the nitrocellulose membrane and eight pieces of Whatman 3MM paper to the size of the separating gel. Cut one edge of the membrane for later orientation.
3. Four sheets of 3 MM are moistened in transfer buffer Roti-Blot A and transferred to the anode plate of the blotting device. On top of this stack, the nitrocellulose membrane is added.
4. The gel unit is disconnected from the power supply and disassembled. The stacking gel is removed and discarded and one corner (corresponding to the membrane) is cut from the separating gel to allow its orientation to be tracked. The separating gel is then laid on top of the nitrocellulose membrane.
5. Another four sheets of 3 MM paper are wetted in transfer buffer K and carefully laid on top of the gel, ensuring that no bubbles are trapped in the resulting sandwich. You can remove air bubbles by carefully rolling a glass pipette on top of the stack.
6. The lid is put on top of the stack and the power supply activated. Transfers can be accomplished at 0.8 mA/cm² blot size for 1 h.

7. Once the transfer is complete the stack is carefully disassembled. The 3 MM paper and the gel can then be discarded. If you want, you can stain the gel in Coomassie blue staining reagent for one hour and destain thereafter in order to check for complete transfer of the separated proteins. On the nitrocellulose membrane the coloured molecular weight markers should be clearly visible on the membrane.
8. Stain the membrane with Ponceau S staining solution for 5 min at room temperature (RT). Destain the blot with water until the protein band is clearly visible.
9. This protein band together with similarly prepared nine additional protein bands can then be used for the assembly of a stack of protein blots as schematically summarized in Fig. 1. This sophisticated assembly of the microblot is made commercially available from the company Attomol GmbH (Lipten, Germany). At present, up to ten different proteins have been used for the assembly of one microblot.

3.4. Detection of Autoantibodies using Microblots

1. Equilibrate assay reagents and microtitre modules to room temperature (RT), vortex reagents and insert the desired modules into a microplate frame.
2. Prepare 1x washing buffer by adding 900 mL aqua dest. to 100 mL concentrated washing buffer. The ready-to-use buffer is stable for at least two weeks if stored at 4°C.
3. Warm sample buffer to 37°C for 15 min in order to solubilize all components. Mix thoroughly. After usage, store the remaining buffer at -20 °C.
4. Thaw the needed sera and warm to RT, mix well. Dilute the sera 1:100 with sample buffer and mix well.
5. Fill each well with 100 µL diluted sera sample and cover the microtitre modules. Incubate for 60 min at RT on a rotary shaker.
6. Carefully remove the diluted sera and wash each well six times with 300 µL diluted wash buffer for 200 sec at RT (*see* Note 8). Remove any remaining liquid by tapping the plate on a pile of filter paper.
7. During the washing steps prepare the HRP-conjugate always freshly by diluting one to 27 in ready-to-use wash buffer.
8. Fill each well with 100 µL diluted HRP-conjugate. Incubate the covered microtitre modules for 60 min at RT on the rotary shaker.
9. Carefully remove the HRP-conjugate and wash each well six times with 300 µL ready-to-use wash buffer for 200 s at RT. Remove any remaining liquid by tapping the plate on a pile of filter paper.
10. Fill each well with 100 µL substrate solution. Incubate the covered microtitre modules for 20 min at RT on the rotary shaker. Protect from light!

11. Carefully remove the substrate solution and wash each well by applying 300 μ L ready-to-use wash buffer. Incubate for 5 min at RT on the rotary shaker. Wash finally with 300 μ L aqua dest. per well for 5 min on the rotary shaker. Remove any remaining liquid by tapping the plate on a pile of filter paper.
12. Dry microtitre modules for 2 hours at RT. Clean the lower surface of all wells free of lint. Scan the microblots from the bottom of modules (within the microplate frame) by using the scanner (resolution 1200 dpi). Analyse the immunoreactions on the enlarged scan image. An immunostained microblot is shown in Fig. 1. The control band displays a blue staining if the detection reagent does function and if the test was correctly carried out. Differentially stained antigen bands reflect the autoantibody content of the serum sample. The use of the automatic image processing algorithms of the Microblot-Analyzer software facilitates the evaluation as it detects marker bands and measures signals in all antigen areas densitometrically. The data are displayed as negative, borderline or positive results in relation to negative control sera defined cut-off values.

4. Notes

1. Unless stated otherwise, all solutions should be prepared in water that has a conductivity of 0.056 μ S/cm and total organic content of less than five parts per billion. This standard is referred to as “water” in this text.
2. There are plenty different expression systems commercially available, some of them for special purpose (e.g. for expression of sequences including rare used codons in bacteria). You should evaluate the system which works best for your purpose.
3. The usage of the nitrocellulose membrane porablot NCP, not enforced from Machery Nagel (Düren, Germany) is mandatory, as the preparation of the microblots is adjusted to that membrane. Other membranes might not work with this system. Especially the use of reinforced membrane must be avoided!
4. If there is no automated washer available, use an eight channel pipette for washing instead.
5. Use a flask which has 10 times the size of the used expression volume or e.g. Fernbach-Flasks as alternative.
6. There is no need for native purification, as the proteins are run on denaturing SDS-PAGE later on. Normally, the denaturing cleanup procedure is more effective. If purification under native condition is superior, one can use this procedure as well.
7. The settings one will need for this ultrasound step are extremely dependent on your specific device and have to be determined individually.
8. If possible use a commercial automated washer for all washing steps.

Acknowledgement

This work was supported in part by grants from the Medical Faculty of the TU-Dresden and the BMBF (Germany). We thank Martina Franke for her excellent technical support.

References

1. Tan EM (1989) Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. *Adv Immunol.* 44, 93–151. [PubMed: 2646863]
2. Cheever MA, Disis ML, Bernhard H, Gralow JR, Hand SL, Huseby ES et al. (1995) Immunity to oncogenic proteins. *Immunol Rev.* 145, 33–59. [PubMed: 7590829]
3. Tan EM (2001) Autoantibodies as reporters identifying aberrant cellular mechanisms in tumorigenesis. *J Clin Invest.* 108, 1411–1415. [PubMed: 11714730]
4. Stockert E, Jager E, Chen YT, Scanlan MJ, Gout I, Karbach J et al. (1998). A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. *J Exp Med.* 187, 1349–1354. [PubMed: 9547346]
5. Soussi T (2000) p53 Antibodies in the sera of patients with various types of cancer: a review. *Cancer Res.* 60, 1777–1788. [PubMed: 10766157]
6. Forti S, Scanlan MJ, Invernizzi A, Castiglioni F, Pupa S, Agresti R, et al. (2002) Identification of breast cancer-restricted antigens by antibody screening of SKBR3 cDNA library using a preselected patient's serum. *Breast Cancer Res Treat.* 73, 245–256. [PubMed: 12160330]
7. Conrad K, and Bachmann M Tumor-Associated Autoantibodies. In: *Autoantibodies*, 2nd Ed (ed. by Shoenfeld Y, Gershwin ME, Meroni PL), Elsevier 2006, pp 423–436
8. Zhang JY, Casiano CA, Peng XX, Koziol JA, Chan EK, and Tan EM (2003) Enhancement of antibody detection in cancer using panel of recombinant tumor-associated antigens. *Cancer Epidemiol Biomarkers Prev.* 12, 136–143. [PubMed: 12582023]
9. Lehmann W (2001) Procedure for production of an array for detection of components of a biological sample (Patent DE 101 10 511).
10. Canzler U, Bartsch H, Grossmann K, et al. (2009). A miniaturized blotting system for simultaneous detecting of different autoantibodies. *Methods Mol Biol* 536,129–37. [PubMed: 19378052]
11. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685. [PubMed: 5432063]

Figure 1:
Schematic view of the microblot manufacturing steps.