# Chapter 34
# Evaluation of Recombinant Antibodies on Protein Microarrays Applying the Multiple Spotting Technique

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## 34.1 Introduction

In recent years, a number of semi-automated concepts have been introduced for the selection of recombinant binders from combinatorial phage display antibody libraries. These include the parallel selection of antibody-displaying phage molecules on targets immobilised in microtitre plates using ELISA washers (Krebs et al. 2001) or on targets attached to magnetic particles using magnetic particle processors (Walter et al. 2001; Konthur and Walter 2002). These methods are particularly of interest in large-scale antibody generation project, as initiated in many laboratories world-wide (Konthur et al. 2005; Taussig et al. 2007). Leaving aside the problem of antigen production and availability of multiple targets in time for selection, up to 96 parallel selections can be performed at a time, and hence, automating the panning process has largely increased the number of targets against which antibodies are selected. However, this also shifts the bottleneck of the overall selection pipeline further towards the isolation and evaluation of mono-specific binders.

Assuming that all 96 parallel selections resulted in polyclonal enrichment of phage particles and that for each of these selections, only 96 clones are analysed in soluble monoclonal antibody fragment ELISAs, 9,218 individual clones need to be processed. With picking robots able to grid ~ 3,000 colonies an hour into microtitre plates, the isolation of these numbers of individual colonies is obviously not an issue. However, screening on ELISA would already require 192 ELISAs to be performed, which can take longer by hand than the whole phage display selection process. In an automated set-up, the picture is completely different; Hallborn and Carlsson (2002) have demonstrated that setting up a fully automated robotic clone

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handling, cultivation and evaluation platform can handle up to 20,000 data points per day with minimal user intervention. However, fully-automated pipelines are very costly and are demanding in respect of assembly time and process development. More recently, Turunen et al. (2009) described an automated ELISA platform for scFv's (single-chain Fragment variable) derived from phage display libraries, and Buckler et al. (2008) reported on standardised assays and protocols developed with Dyax for tracking and identifying monoclonal binders in Fab format in their selection pipeline. Another recently emerging technique for screening and characterisation of binder-antigen complexes is the use of multiplexed bead-based assay formats using flow cytometry (Ayriss et al. 2007; Schwenk et al. 2007).

Alternatively, protein array based methods can be used. For instance, to evaluate monoclonal binders derived from phage display selection rounds, Ian Tomlinson and colleagues made 20 × 20 cm colony arrays consisting of around 20,000 *Escherichia coli* clones. All clones expressed a recombinant antibody molecule, which became accessible for analysis with directly-labelled selection targets after lysis of the cells (de Wildt et al. 2000). One disadvantage of this method, however, is the need for large amounts of directly-labelled target protein, the relatively low sensitivity and poor dynamic range of applicable non-radioactive readout systems and the low multiplexing potential.

Protein microarray applications, however, allow multiplexing, and a wide range of applications have been reviewed (Joos and Bachmann 2009; Hultschig et al. 2006). For the analysis of monoclonal antibody entities derived from phage display or animal immunisation and hybridoma technology, two protein array methods that allow multiplexing are applied. Sawyer and colleagues have set up a method to rapidly characterise primary cell-fusions for the expression of mouse monoclonal antibodies obtained after a multiplexed immunisation strategy (de Masi et al. 2005). For characterisation, cell culture supernatants of cell-fusions are spotted onto glass microarrays, which were earlier completely coated with 5 μg of the antigens used in the immunisation process. Using a set of different fluorescent-labelled secondary antibodies, isotype specific detection of monoclonal antibodies was achieved.

The other method allowing multiplexing is the use of the multiple spotting technique (MIST; Angenendt et al. 2003) developed in our laboratory. Here, we describe the application of MIST for the simultaneous evaluation of phage display derived soluble monoclonal antibody fragments on protein microarrays. The multiple spotting technique allows simultaneous evaluation of phage display derived soluble monoclonal antibody fragments on protein microarrays (Angenendt et al. 2004). The technique is based on the concept of printing multiple solutions in a sequel of spotting processes onto the same single positions on a microarray slide (Fig. 34.1, Table 34.1). The droplets on the microarray surface can be regarded as inverted wells in which specific interactions can occur. If multiple fields with different antigens are spotted on the slide and the remaining surface is blocked, a set of antibodies can be spotted onto the different antigens to not only find binders to each antigen but also to eliminate cross-specific binders at a very early stage of screening. Only when an interaction between the spotted antigen and antibody is established, the antibodies from the second spotting process are not washed away.

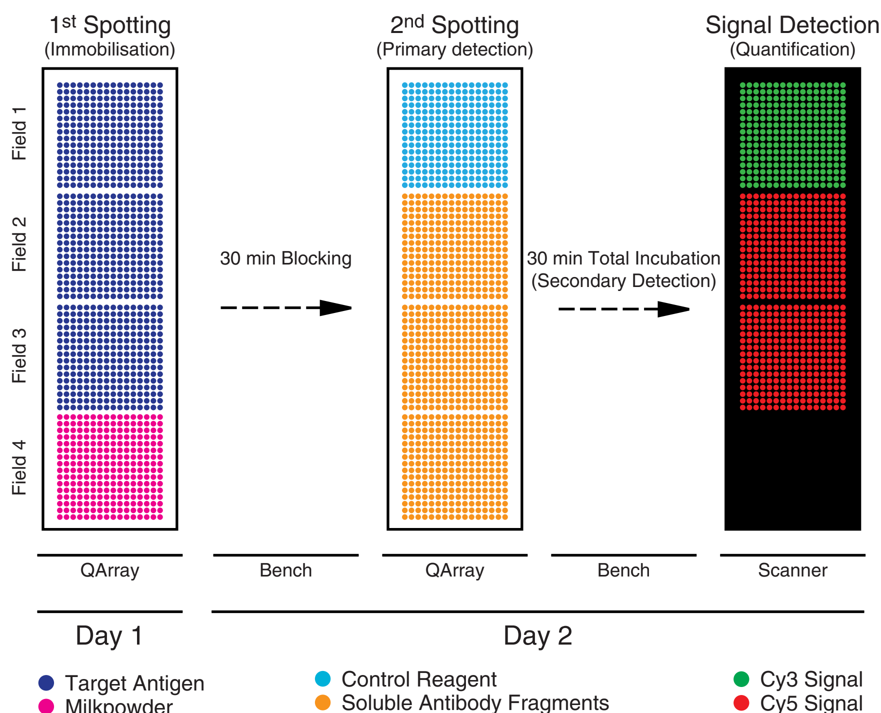


**Fig. 34.1** Flowchart of all work-steps involved in the evaluation of soluble recombinant antibody fragments on protein microarrays applying the multiple spotting technique. During first spotting, target antigen and background control antigen are immobilised. Next day, microarray slides are blocked and in the second spotting step the primary detection reagents (soluble recombinant antibody fragments and control reagent) are printed. After total incubation with secondary detection reagent, slides are washed and scanned. Fluorescence signals are detected and quantified. Work-steps take place either in the QArray instrument, on the laboratory bench or in a microarray fluorescence scanner

**Table 34.1** Slide printing scheme applying the multiple spotting technique

| Printing step | Field(s) | No. of stamps/spot | Day |
|---|---|---|---|
| Target spotting | 1, 2, 3 | 2 | 1 |
| Background spotting | 4 | 2 | 1 |
| Antibody fragment spotting | 2, 3, 4 | 2 | 2 |
| Control reagent spotting | 1 | 2 | 2 |

The workflow and technical parameters of the process are described in more detail in Sect.34.3.

In summary, by applying the multiple spotting technique, multiplexed analysis of hundreds of antibody fragments against a given set of target proteins on a single protein array can be performed.

## 34.2 Materials

### *34.2.1 Production of Soluble Monoclonal Antibody Fragments in Microtiter Plates*

- 96-well U-bottom polypropylene (PP) microtitre plates (Nunc, Wiesbaden, Germany).
- AeraSeal breathable sealing film (Sigma-Aldrich, Taufkirchen, Germany).
- 2YT medium: 1.6% (w/v) tryptone, 1% (w/v) yeast extract, and 0.5% NaCl, pH 7.0.
- 2YT-AG-2: 2YT medium containing 100 μg/mL ampicilin, 2% (w/v) glucose.
- 2YT-AG-0.1: 2YT medium containing 100 μg/mL ampicilin, 0.1 % (w/v) glucose.
- 20 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

### *34.2.2 Release of Soluble Antibody Fragments from* E. coli *Periplasma into Supernatant*

- 3X PE buffer: 60% Sucrose, 150 mM Tris, 3 mM EDTA, pH 8.0.

### *34.2.3 Production of Protein Microarrays*

- Double-distilled water (dd$H_2O$).
- 80% (v/v) technical Ethanol.
- 384-well V-bottom polypropylene (PP) Microtiter plates X5005 (Genetix Ltd, New Milton, Hampshire, UK).
- SuperEpoxy2 Slides (ArrayIt Corporation, Sunnyvale, CA, USA).
- 2 × Protein Printing Buffer (PPB; ArrayIt Corporation, Sunnyvale, CA, USA).
- Phosphate-buffered saline (PBS): 8 g/L NaCl, 0.2 g/L KCL, 1.44 g/L $Na_2HPO_4 \cdot 2\ H_2O$ and 0.24 g/L $KH_2PO4$, pH 7.4.

### *34.2.4 Evaluation of Antibodies by the Multiple Spotting Technique*

- Phosphate-buffered saline Tween (PBST): PBS + 0.1% (v/v) Tween-20.
- Phosphate-buffered saline Tween-20 & Triton X-100 (PBST+T): PBST + 0.1% (v/v) Triton X-100.
- Blocking buffer: 3% (w/v) non-fat dry milk powder in PBST; prepare fresh.
- Bovine Serum Albumin (BSA): 10 mg/mL stock solution in PBS.
- Recombinant Protein L, Cy5 conjugated (see Sect. 34.5, Note 1).
- Streptavidin, Cy3 conjugated (see Sect. 34.5, Note 1).

### *34.2.5 Equipment and Software for Microarray Work*

- QArray Spotting Robot (Genetix Ltd, New Milton, Hampshire, UK).
- ArrayIt® Stealth Pin, Solid pin type, tip diameter 150 µm (ArrayIt Corporation, Sunnyvale, CA, USA).
- QSoft MicroArraying Software (Genetix Ltd, New Milton, Hampshire, UK).
- 428™ Array Scanner (Affymetrix, Santa Clara, CA, USA).
- GenePix Pro 4.1, Microarrays Image Analysis Software (Molecular Devices, Sunnyvale, CA, USA).
- Plastic slide rack and incubation chamber with lid (Carl Roth GmbH, Karlsruhe, Germany).

## 34.3 Methods

The multiple spotting technique is based on the simple but effective concept of addressing single positions on a chip multiple times. After spotting several fields of antigens on the slide and blocking the remaining surface, a set of antibodies can be spotted onto the different antigens to find binders to each antigen and to eliminate cross-specific binders at the same stage of screening. The workflow of the method is outlined in Fig. 34.1 and Table 34.1. The procedure takes only two days including soluble antibody fragment expression, 1st and 2nd spotting routines as well as for signal detection. In addition to standard laboratory equipment, only a contact or non-contact microarray instrument without any further modifications and a conventional microarray scanner is required. In our laboratory, we are applying a Genetix QArray Microarray instrument with stealth solid 150 micron pins. The printing gadget can hold up to 20 pins. For the evaluation of recombinant antibodies on protein arrays applying the multiple spotting technology we are, however, only using a 16-pin 4 × 4 printing gadget in combination with a 16 × 16 spotting pattern on 4 fields (Fig. 34.2). Spotting order, instrument settings and spotting conditions of samples are summarised in Tables 34.1–34.3. As substrates, microarray slides with different surface chemistry can be used. MIST works with self-coated Poly-L-lysine slides as well as with SuperEpoxy2 slides. Currently, SuperEpoxy2 slides are our choice of substrate for all MIST Experiments. Between the spotting of different samples, a wash routine was applied, which washed the head twice with dd$H_2O$ and once with 80% (v/v) technical ethanol.

The standard operating procedures provided in this section include the expression of soluble antibody fragments, the spotting protocols and data evaluation schemes. Applying these procedures and standardised pipetting templates, the migration from the semi-automated selection process described in Chap. 18 to the application of MIST for primary evaluation of monoclonal entities becomes straightforward. All steps and templates are adapted for use of 8-channel multipipettes for maximum convenience and minimum handling. Applying the multiple spotting technique

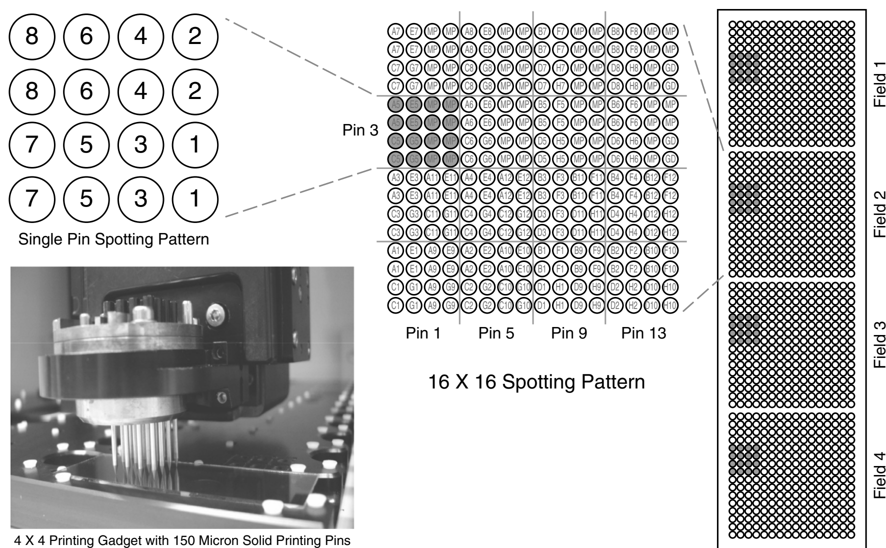


**Fig. 34.2** The applied microarray slide layout and spotting pattern. The slide is spotted with a 16-pin 4 × 4 printing gadget. A 16 × 16 spotting pattern is generated accommodating 96 soluble recombinant antibody fragments (A1–H12) and 32 controls (*MP* Milk powder, *GD* Guide dot) in duplicate per field. One slide contains four fields with 256 spots each

allows to reduce time, material and waste, and extends automation beyond the selection process applying conventional microarray machinery.

### *34.3.1 Production of Soluble Monoclonal Antibody Fragments in Microtiter Plates*

Soluble monoclonal antibody fragments are expressed from individual clones of a selection round. In our case, the clones are derived from the human single fold scFv antibody phage display library I (see Sect.34.5, Note 2) selected according to the semi-automated magnetic bead-based selection protocol described in Chap. 18. Prior picking, the selection rounds were tested in a polyclonal ELISA and the original *E. coli* host strain TG1 was switched to *E. coli* strain HB2151 (see Sect. 34.5, Note 3).

1. Prepare a fresh overnight culture from *mother plate* glycerol stock by inoculating fresh 96-well U-bottom polypropylene (PP) microtitre plate containing 190 μL 2YT-AG-2 with 10 μL of glycerol stock and incubate overnight at 37°C and 1,400 rpm (see Sect. 34.5, Note 4).

2. Next day, inoculate fresh 96-well U-bottom PP microtitre plate containing 180 μL 2YT-AG-0.1 with 20 μL of the overnight culture and incubate *daughter plate* for 2 h at 37°C and 1,400 rpm.
3. Induce soluble antibody fragment production in *daughter plate* by adding 10 μL 20 mM IPTG (final conc. 1 mM) to each well and continue incubating overnight at 30°C and 1,400 rpm.
4. Pellet bacteria by centrifugation of microtitre plates for 10 min at 3,000 rpm (see Sect. 34.5, Note 5).
5. Transfer soluble monoclonal antibody fragment containing culture supernatant into fresh 96-well U-bottom PP microtitre plate, seal with tape to stop evaporation and store until further use at 4°C. Discard pellet-containing plate.

### *34.3.2 Release of Soluble Antibody Fragments from* E. coli *Periplasma into Supernatant*

1. Culture *E. coli* cells as in Sect.34.3.1 until step 3.
2. Next day, release soluble antibody fragments from the *E. coli* periplasm into supernatant by adding 50 μL pre-chilled 3X PE-buffer to each well, mix gently and incubate on ice for 20 min (see Sect.34.5, Note 6).
3. Pellet bacteria by centrifugation of microtitre plates for 10 min at 3,000 rpm (see Sect.34.5, Note 5).
4. Transfer soluble monoclonal antibody fragment containing culture supernatant into fresh 96-well U-bottom PP microtitre plate and store until further use at 4°C. Discard pellet-containing plate.
5. Prepare *antibody plate* for spotting. Prefill positions A1–A6 to P1–P6 of a 384-well V-bottom PP microtitre plate with 15 μL 2 × blocking buffer.
6. Add 15 μL soluble monoclonal antibody fragment containing culture supernatants of columns 1 and 2 with an 8-channel multipipette to A1–O1 and B1–P1, respectively. Continue re-arraying with remaining 10 columns of soluble monoclonal antibody fragment containing culture supernatants, accordingly. Add 30 μL blocking buffer to positions A7–A8 to P7–P8 as negative controls (see Sect. 34.5, Note 7). Incubate plate for 30 min at RT.
7. Seal *antibody plate* with tape to stop evaporation and store until spotting at 4°C.

### *34.3.3 Production of Protein Microarrays*

An overview of the individual spotting routines is given in Table 34.1 and Fig. 34.1.

1. Dissolve 200 μg biotinylated target antigen (see Sect. 34.5, Note 8) in 500 μL PBS. Add same volume 2 × PPB to antigen solution and mix gently pipetting up and down (see. Sect. 34.5, Note 9).

2. Prepare *target plate*. Pipette 30 μL antigen solution into the position A1–A4, B1–B4, C1–C4 and D1–D4 of a 384-well PP microtitre plate (see Sect. 34.5, Note 10).
3. Prepare *background plate*. Pipette 30 μL background antigen solution (in our case 3% milk powder in PBST) into the position A1–A4, B1–B4, C1–C4 and D1–D4 of a 384-well PP microtitre plate (see Sect. 34.5, Note 11).
4. Place *target plate* in source plate holder in QArray instrument.
5. Place microarray slides (in our case SuperEpoxy2) on slide holder tray (see Sect. 34.5, Note 12).
6. Start spotting *target plate* using the microarray settings displayed in Tables 34.2 and 34.3. Target proteins should be spotted only onto fields 1–3 according to slide printing scheme in Table 34.1 (see Sect. 34.5, Note 13).
7. Next, spot *background plate* only onto field 4 according to slide printing scheme in Table 34.1 (see Sect. 34.5, Note 13).
8. After spotting is completed, leave microarray slides in QArray for 30 min before overnight storage at 4°C. Next day, start processing slides according Sect. 34.3.4.

### *34.3.4 Evaluation of Antibodies by the Multiple Spotting Technique*

This step is equivalent to day 2 of the spotting routines given in Table 34.1 and Fig. 34.1.

**Table 34.2** Overview of essential QArray micorarrayer settings used for protein microarray production and multiple spotting technique

| Field | Setting |
|---|---|
| Source plate holder | Type: Genetix plate ×5005 |
| Field settings | 3x 1″, 16 pins/4 fields |
| Humidity | 60% |
| Maximum stamps per ink | 1 |
| Number of stamps per spot | 2 |
| Stamp time | 50 ms |
| Inking time | 50 ms |
| Print adjustment | 200μ |

**Table 34.3** Wash settings used during protein microarray production and multiple spotting technique

| Wash Station | Wash Solution | Wash Time (ms) | Dry Time (ms) |
|---|---|---|---|
| Position 1 | Double-distilled water | 8.000 | 2.000 |
| Position 2 | Double-distilled water | 8.000 | 2.000 |
| Position 3 | 80% Ethanol | 10.000 | 8.000 |

1. Prepare *control reagent plate*. Pipette 30 μL control reagent (in our case Streptavidin-Cy3; 1 μg/mL in blocking buffer + 5% glycerol) into the position A1–A4, B1–B4, C1–C4 and D1–D4 of a 384-well PP microtitre plate.
2. Take protein microarrays prepared according to Sect. 34.3.3, rinse with PBS and block for 30 min at room temperature with blocking buffer using a plastic slide rack and incubation chamber with lid.
3. Rinse slides with PBS and spin dry in centrifuge for 1 min RT at 1,000 rpm using slide holder adaptors (see Sect. 34.5, Note 5).
4. Place microarray slides back into QArray instrument. Use same positions on tray and keep slide order (see Sect. 34.5, Note 12).
5. Place *antibody plate* in source plate holder in QArray instrument.
6. Start spotting *antibody plate* using the microarray settings displayed in Tables 34.2 and 34.3. Soluble monoclonal antibody fragment should be spotted only onto fields 2–4 (see Table 34.1).
7. Next, spot *control reagent plate* only onto field 1 (see Sect. 34.5, Notes 13 and 14).
8. Directly after spotting has finished, rinse slides with PBS.
9. Incubate with 800 μL Protein L-Cy5 (1 μg/mL Protein L-Cy5 in blocking buffer) for 30 min at RT in the dark (see Sect. 34.5, Notes 15 and 16).
10. Wash slides 1 × 10 min with PBST and 2 × 10 min in PBST-T using a plastic slide rack and incubation chamber with lid (dark).
11. Rinse slides with PBS and spin dry in centrifuge for 1 min RT at 1,000 rpm using slide holder adaptors.
12. Scan slides using a microarray scanner adjusting the gain for optimal dynamic range of the signals. (see Sect. 34.5, Note 17 and 18).
13. Slides were analysed using GenePix Pro 4.1 software. Quantification of fluorescent signals was performed using "local feature" background subtracted median signal intensities of each spot.

## 34.4 Results and Conclusion

The multiple spotting technique allows the simultaneous analysis of hundreds of antibody fragments on a defined set of target and control antigens in a single experiment. The complete workflow is depicted in Fig. 34.1. Spotting of samples is carried out with a 16-pin (4 × 4) printing gadget in a Genetix QArray instrument. The microarray slide is custom designed to contain 4 fields of 256 spots each, representing 128 samples in duplicate (16 × 16 spotting patter, see Fig. 34.2). According to our standard operating procedure, field 1–3 are spotted with target antigen, field 4 is spotted with an appropriate background control antigen, which we chose according to the applied blocking reagent during phage display antibody selection (Chap. 18). In most cases, this is milk powder. In the second spotting step, 92 antibody fragments are spotted onto fields 2–4. Additional 32 positions are spotted with negative control antigens, directly-labelled guide dot reagents or

secondary detection reagents. For instance, Streptavidin–Cy3 or Streptavidin-Cy5 can be added in defined positions. Streptavidin will bind to biotinylated antigens and will serve as a positive control for the first spotting routine, or will simply serve as guide dot defining slide orientation. In the example in Fig. 34.3, BSA-Cy5 was spotted as guide dots (marked with *yellow circles*). As an additional control, an antigen-specific secondary detection reagent – for instance directed against a tag-sequence of the recombinant antigen – can be applied to field 1 to evaluate the transfer efficiency of the individual pins in the print gadget. Detection of the bound soluble antibody fragments is achieved by total incubation with Protein L-Cy5. Fluorescence signal intensities are visualised by scanning slides at respective excitation and emission wavelength with a laser intensity set to ~55 db. From the scanned images, signal intensities are calculated using GenePix Pro 4.1 microarray image analysis software. The software calculates the signal intensity of each spot by determining the arithmetic mean of the raw pixel intensities from each spot and hence, is independent of the spot diameter. Next, we use the mean values for each spot pair to monitor correlation between duplicate spots within a field and between the fields (Fig. 34.3, *top panel*). At this stage, the negative controls spotted in fields

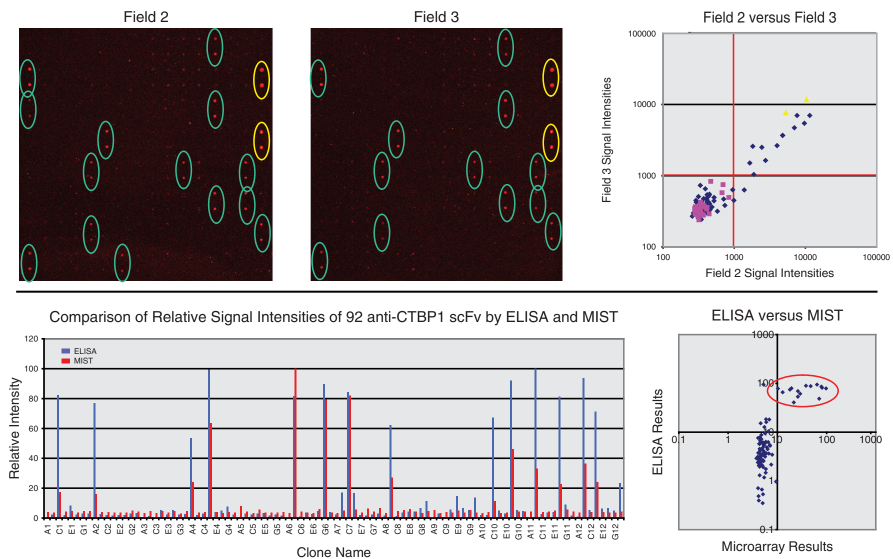


**Fig. 34.3** Comparison between ELISA and microarray results and reproducibility analysis of multiple spotting results on separate fields. (*Top panel*) Visual appearance of fields 2 and 3 of the same microarray slide. *Yellow circles* mark guide dots, green circles highlight the positives in respective fields. On the right, comparison of Field 2 vs. Field 3 signal intensities. Target antigen (CTBP1) values are blue, background control (Milk powder) values are pink and guide dots are yellow. For slide layout, see Fig. 34.2. (*Bottom panel*) Comparison of relative signal intensities between ELISA and Microarrays using the multiple spotting technology. On the right, ELISA versus Microarray results are shown in logarithmic scale. In both assay, values above 10 are positive, corresponding to 5-fold and 3-fold signal to background ratios for ELISA and micro-arrays, respectively

2 and 3 play an important role in slide evaluation. They are used to define background binding of primary or secondary detection reagents. All positive signals have to be a minimum of 3- to 5-fold higher than the median of all negative control signal intensities (Fig. 34.3, *top panel*, *pink values*). To evaluate the method and to correlate the outcome of microarray experiment based results using MIST, regular comparative studies are performed. The results of such a comparative study are shown in Fig. 34.3, *bottom panel*. Relative signal intensities of ELISA and MIST experiment of the same periplasmic antibody preparations are compared. A very good correlation between the results can be seen. In the right panel, all clones with a relative intensity above 10 are positive. A clone is scored positive, when in both assays the obtained values are minimum 3- to 5-fold higher than the median of all negative values. In general, good correlations between ELISA and MIST results are seen, but a lower dynamic range of the signal intensities are observed in the microarray experiments compared to ELISA. On the other hand, this can also be regarded as a beneficial effect of applying the multiple spotting technique for the evaluation of soluble monoclonal antibody fragments. Signals are predominantly obtained only for clones that show not only good binding but also good expression – a desired characteristics for recombinant antibodies. To further improve the dynamic range of signal intensities, primarily the protocols for soluble antibody fragment generation can be optimised. Only recently, Hust et al. (2009) have monitored the protein expression levels of soluble antibody fragments in microtitre plates under varying growth conditions. Applying these finding to our application could further increase the robustness and comparability of our method.

In conclusion, the multiple spotting technique allows to monitor the binding characteristics of hundreds of soluble monoclonal antibody fragments in parallel on a single protein microarray. In future, increasing the number of antigens and spot density on the slide can further enhance the multiplexing capability of the method. Thus far, the obtained microarray results show a high degree of conformity with ELISA experiments and clearly demonstrate the potential of the technique to be integrated in high-throughput recombinant antibody selection and screening pipelines (Konthur 2007).

## 34.5 Notes and Troubleshooting

1. Protein L-Cy5 and Streptavidin-Cy3 were generated using the Amersham Cy5 and Cy3 Mono-Reactive Dye Packs (GE Healthcare, Freiburg, Germany), respectively. Conjugation of Cy-dyes were performed as recommended by the manufacturer. Protein L and Streptavidin are available from Pierce (Thermo Scientific, Bonn, Germany).
2. The Human Single Fold scFv Libraries I + J (Tomlinson I + J) were created in Greg Winter's lab at the MRC Laboratory of Molecular Biology and the MRC Centre for Protein Engineering (Cambridge, UK). Further information on the libraries can be found at the distributor's website: http://www.geneservice.co.

uk/products/proteomic/scFv_tomlinsonIJ.jsp (Cited 7 May 2009). Using other than these combinatorial antibody phage display libraries might need some library specific adaptation to the individual protocols.

3. Host strain switching from *E. coli* strain TG1 to HB2151 is advantageous for high level expression of antibody fragments from the Tomlinson libraries I and J, since an amber stop codon is inserted between the antibody fragment and the gIII. In HB2151, this amber stop is not suppressed and therefore only antibody fragments without pIII fusion are produced. Furthermore, expression of the phage coat protein pIII can be toxic for the host at higher concentrations.
4. Dedicated microplate incubator shakers, such as iEMS (Thermo Scientific) or PST-60HL-4, Lab4You, Berlin, Germany) are able to shake >1,200 rpm ensuring best possible aeration of the cultures in combination with breathable sealing tapes. This is beneficial during soluble antibody fragment production.
5. Microtitre plates can be centrifuged in Eppendorf 5810 R with swing out rotor A-4-62 and microplate holders. Use Eppendorf CombiSlide adapters for centrifuging microarray slides.
6. Alternatively, incubation can be carried out overnight at 4°C.
7. These negative control values serve for internal normalisation of the obtained signals in individual spotting fields, as shown in Fig. 34.3.
8. In case the target antigen is not already biotinylated, it can be in vitro biotinylated with commercially available biotinylation reagent kits, such as the NHS-SS-Biotin (sulfosuccinimidyl-2-(biotinamido)ethly-1,3-dithiopropionate) from Pierce.
9. Alternatively, proteins can be directly spotted in PBS.
10. If denser protein arrays are prepared using printing gadgets of other type and of more than 16 pins, adjust the positions and the volume of the antigen solutions accordingly.
11. Alternatively other background control antigens, which were applied for blocking the selection matrix during the selection process, can be used.
12. Place slide accurately into holder. Consider substrate orientation, since only one side of SuperEpoxy2 Slides is suitable for printing.
13. For printing onto all positions of a field only from positions A1–A4 to D1–D4 of the source plate, use the "Print-test" routine (normally used for testing pins) defined by the software.
14. Any other adequate control antibody or reagent can be spotted onto field 1, which can serve as a control for the printing process of target antigen(s).
15. Recombinant Protein L binds only to human V-Kappa light chains. In case other than the Tomlinson I or J antibody phage display libraries are used, the recombinant ProteinL-Cy5 might need to be substituted with an appropriate, tag-dependent detection antibody, e.g. mouse anti-myc-tag monoclonal antibody (9E10, SIGMA-ALDIRCH).
16. When using <800 μL volumes for incubation, microarray slides should be covered with cover slip of appropriate size.
17. We use an Affymetrix 428 microarray scanner with gain setting of ~ 55 db for Cy5.

18. For the dyes Cy3 and Cy5 the following Excitation sources were used: Green HeNe (543.5 nm) and Red HeNe (632.8 nm) lasers, respectively. Emission maxima are at 570 and 670 nm, respectively.

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