Generation of High Density Protein Microarrays by Cell-free *in Situ* Expression of Unpurified PCR Products*

Philipp Angenendt‡§, Jürgen Kreutzberger¶, Jörn Glökler‖, and Jörg D. Hoheisel‡

Due to the success of DNA microarrays and the growing numbers of available protein expression clones, protein microarrays have become more and more popular for the high throughput screening of protein interactions. However, the widespread applicability of protein microarrays is currently hampered by the large effort associated with their production. Apart from the requirement for a protein expression library, expression and purification of the proteins themselves and the lacking stability of many proteins remain the bottleneck. Here we present an approach that allows the generation of high density protein microarrays from unbound DNA template molecules on the chip. It is based on the multiple spotting technique and comprises the deposition of a DNA template in a first spotting step and the transfer of a cell-free transcription and translation mixture on top of the same spot in a second spotting step. Using wild-type green fluorescent protein as a model protein, we demonstrated the time and template dependence of this coupled transcription and translation and showed that enough protein was produced to yield signals that were comparable to 300 μg/ml spotted protein. Plasmids as well as unpurified PCR products can be used as templates, and as little as 35 fg of PCR product ($\sim$22,500 molecules) were sufficient for the detectable expression of full-length wild-type green fluorescent protein in subnanoliter volumes. We showed that both aminopropyltrimethoxysilane and nickel chelate surfaces can be used for capture of the newly synthesized proteins. Surprisingly we observed that nickel chelate-coated slides were binding the newly synthesized proteins in an unspecific manner. Finally we adapted the system to the high throughput expression of libraries by designing a single primer pair for the introduction of the required T7 promoter and demonstrated the *in situ* expression using 384 randomly chosen clones.   *Molecular & Cellular Proteomics 5:1658–1666, 2006.*

The understanding of complex cellular networks necessitates tools that are amenable to the analysis of different parameters in a highly parallel manner (1). Although in the last years DNA microarrays were the technology of choice to monitor the abundance of several thousands of mRNA transcripts at a time, such studies provide us with little information on the proteins that are encoded by these transcripts (2, 3). However, because proteins rather than DNA carry out cellular functions, there is large interest to analyze proteins and their entirety, the proteome, in a manner comparable to DNA microarrays. One technology that is envisaged to meet the demands of high throughput protein interaction and modification screening is protein microarray technology (4–8).

Protein microarrays have been applied in different areas of application, such as the analysis of protein-protein interactions (9–11), the identification of substrates for protein kinases (12–14), or the elucidation of potential diagnostic markers in bacterial or autoimmune diseases (15–18). All of them share the basic principle of production. It involves the generation of expression clones and the subsequent expression and purification of proteins off the chip followed by spotting them onto the microarray surface. Currently this process represents a major bottleneck in the production of protein microarrays because both the generation of the expression clones and the purification of the proteins are time- and cost-intensive even in low throughput applications (19, 20).

To circumvent this bottleneck, different approaches have been developed for the generation of protein microarrays. Madoz-Gurpide *et al.* prepared cell lysate from adenocarcinoma cell lines and fractionated the protein extract first by anion exchange and then by reverse phase liquid chromatography (21). The obtained fractions were then spotted and probed with antibodies against different proteins. Although this approach is truly advantageous with regard to proper post-translational modifications of the proteins, only mixtures of protein are displayed in which less abundant proteins may not be detectable. He and Taussig (22, 23) introduced the idea of creating protein arrays by cell-free transcription and translation of PCR products and the subsequent purification on nickel chelate-coated surfaces. However, their approach, termed "protein *in situ* array" (PISA), is limited by the large volume of 25 μl used for the expression and by the capture process that is not performed on microarrays but on nickel chelate-coated microtiter plates or magnetic agarose beads. A miniaturization of cell-free transcription and translation as an intermediate step toward the expression on the chip was

reported by Angenendt *et al.* (24), who performed an expression and subsequent functional assay in 100-nl volumes. Steffen *et al.* (25) finally performed transcription on a chip using a gene encoding enhanced green fluorescent protein. After overlaying the chip with a transcription mixture, they were able to detect the resulting mRNA in the mixture by RT-PCR.

Currently the only methodology for the production of protein microarray by expression of the proteins on the chip is the nucleic acid programmable protein array (NAPPA)[1] approach (26). It entails the immobilization of plasmids containing the cDNAs of interests as GST fusions on the surface in combination with an anti-GST capture antibody. The chip is then overlaid with a cell-free expression mixture, which causes expression of the protein and direct capture of the protein by the antibody via the GST domain. The NAPPA process is very cost-effective due to the small consumption of reagents and allows the production of proteins just prior to the microarray experiment, diminishing problems associated with the storage of protein microarrays. Nevertheless it is limited by several intrinsic drawbacks. For expression, NAPPA requires plasmids that contain the gene of interest as a GST fusion. This necessitates time-consuming cloning of cDNAs. Moreover it relies on the immobilization of the plasmid, which requires a biotinylation of the plasmid at defined stoichiometries to prevent dissociation from the array or a termination of transcription by biotin incorporation within the coding sequence. In addition, only low density protein microarrays with up to 512 spots per microscope slide can be produced because a higher density would cause proteins to diffuse to capture antibodies of adjacent spots.

To overcome these problems, an optimized technology should meet several criteria. (*a*) Expression should be directly possible from a wide variety of different templates, including PCR products. (*b*) Expression should be facile and inexpensive. (*c*) The generation of protein microarrays should be feasible within a short time period, just prior to their use, to avoid storage effects. (*d*) Mode of binding should permit the production of high density protein microarrays capable of displaying several thousand proteins on a single microscopic slide.

Here we present an approach that meets these demands. It is based on the multiple spotting technique (MIST) (27) and comprises the spotting of a DNA template in a first spotting and the transfer of a cell-free transcription and translation mixture on top of the very same spot in a second spotting run. Using this technique, high density protein microarrays with up to 13,000 spots per slide can be produced from a variety of different sources in an uncomplicated and inexpensive manner.

---

[1] The abbreviations used are: NAPPA, nucleic acid programmable protein array; APTES, aminopropyltrimethoxysilane; MIST, multiple spotting technique; wt-GFP, wild-type green fluorescent protein.

EXPERIMENTAL PROCEDURES

Materials—The Rapid Translation System (RTS) 100 *Escherichia coli* HY kit was obtained from Roche Diagnostics GmbH. The plasmid used for expression is an integral part of the kit and is based on the pIVEX2.3 plasmid containing a gene encoding wild-type green fluorescent protein (wt-GFP) fused to a C-terminal His_6 tag (pIVEX-GFP). PCR primers were synthesized by biomers.net GmbH, (Ulm, Germany). PCR Buffer E was purchased from Genaxxon Bioscience (Biberach, Germany), *Taq* polymerase was from Qiagen GmbH (Hilden, Germany), and dNTPs were from Fermentas GmbH (St. Leon-Roth, Germany).

Expression of GFP with Different Incubation Times—Dilutions of the pIVEX-GFP plasmid in water were prepared ranging from 1 $\mu g/\mu l$ to 1 ng/μl. The dilutions as well as a negative control containing only water were spotted on homemade aminopropyltrimethoxysilane (APTES) slides (prepared as described Ref. 28) using a NanoPlotter 2.0 non-contact spotting system equipped with a with a nanoliter pipette (Gesim mbH, Grosserkmannsdorf, Germany). The volume that was dispensed per spot was 350 pl. After spotting of the DNA template, the *in vitro* transcription and translation mixture (RTS 100 mixture) was prepared without any template DNA as recommended by the manufacturer and spotted onto the very same position as the template DNA. In addition, the cell-free transcription and translation mixture was spotted on negative control spots to which only water had been dispensed. After spotting, the slides were placed in humidified extradeep hybridization chambers (TeleChem International Inc., Sunnyvale, CA) and incubated at 32 °C in a water bath for different incubation times. After incubation, the slides were blocked in 3% (w/v) fat-free milk powder/Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T) for 15 min on a shaker at room temperature. The slides were rinsed with TBS-T and incubated for 30 min in 0.5 $\mu g/ml$ anti-GFP antibody (TP401, Acris Antibodies, Hiddenhausen, Germany) diluted in blocking buffer. After rinsing the slides with TBS-T, the slides were washed twice for 5 min in TBS-T and dried by pressurized air. Scanning was performed with a ScanArray 5000 (PerkinElmer Life Sciences). Analysis was done using GenePix Pro 5.0. All signal intensities used for analysis were signal intensities from which the local background had been subtracted.

Determination of the Absolute Yield of Expression—PCR templates containing the coding sequence of wt-GFP were generated by PCR of the pIVEX-GFP plasmid. PCR was performed in 50-μl volumes in 96-well polypropylene PCR plates (Steinbrenner Laborsysteme GmbH, Wiesenbach, Germany) with the following primers: forward primer, 5'-GTGGCGAGCCCGATCTTC-3'; reverse primer, 5'-GTC-AGGCACCGTGTATGAAATC-3'.

The composition of the reaction was 1× Buffer E (67 mM Tris-HCl, pH 8.8, 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.01% (v/v) Tween 20), 0.2 mM each dNTP, 0.2 μM each primer, and 2.5 units of *Taq* polymerase. Reactions were performed in a PTC-200 Thermocycler (Bio-Rad) at the following temperatures: denaturation at 95 °C for 2 min; 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s; and a final elongation step at 72 °C for 10 min.

PCR products were checked on agarose gels and purified using a Qiaquick PCR purification kit (Qiagen GmbH). DNA was quantified by absorption using a Nanodrop ND-1000 (Peqlab Biotechnologie GmbH, Erlangen, Germany). Dilutions of the PCR were prepared from 20 ng/μl to 100 pg/μl using negative control PCR mixture (templates were substituted by water) supplemented with 0.5 M betaine (final concentration) for dilution. wt-GFP calibration curves ranging from 1 mg/ml to 2 $\mu g/ml$ were prepared by diluting conventionally obtained wt-GFP with PBS and 0.5 M betaine (final concentration).

Expression and detection were done as described in the previous paragraph with the following modifications. Nickel chelate-coated
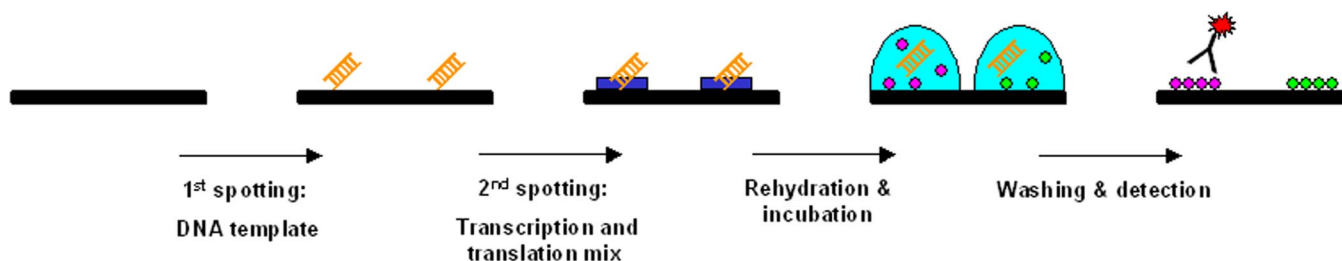
Fig. 1. **Schematic outline of MIST.**

slides (Xenopore, Hawthorne, NJ) were used as solid support. As a negative control, the diluent used for the dilution series was used. Expression was performed at 32 °C for 4 h, the slides were blocked in 3% (w/v) BSA in TBS-T, and detection was achieved by incubation of the slides in 0.1 μg/ml anti-penta-His antibody-Alexa Fluor 647 conjugate (Qiagen GmbH) diluted in blocking buffer for 1 h. Washing was performed for 10 min in TBS-T, and the slides were rinsed with PBS before scanning.

Effect of His Tagging on Nickel Chelate-coated Slides—A construct lacking the C-terminal His$_6$ tag was cloned using standard cloning methodology. The resulting plasmid was named pIVEX-GFP-notag, and the sequence was verified by sequencing. PCR of the pIVEX-GFP and pIVEX-GFP-notag plasmids was performed using the same primers and cycling conditions as described before. The PCR products were purified using a Qiaquick PCR purification kit (Qiagen GmbH) and quantified. PCR products were diluted down to 10 ng/μl in 10 mM Tris (pH 8.5) containing 0.5 M (final concentration) betaine and 0.5 ng/μl (final concentration) sonicated salmon sperm DNA (GE Healthcare).

Detection was achieved as described in the previous paragraph using a mixture of 0.1 μg/ml anti-penta-His antibody-Alexa Fluor 647 conjugate and 0.5 μg/ml anti-GFP antibody-Texas Red conjugate (Ab6660, Abcam Plc, Cambridge, UK) in blocking buffer.

High Throughput Expression of cDNAs—384 cDNAs were randomly chosen out of a human fetal brain library (hex-1) (29). Cells were inoculated in 2YT medium (16 g/liter tryptone, 10 g/liter yeast extract, 5 g/liter NaCl, pH 7.0) containing 100 μg/ml ampicillin, 15 μg/ml kanamycin, and 2% glucose and grown at 37 °C overnight. Individual wells of 50-μl volumes in 96-well polypropylene PCR plates were inoculated with 1 μl of bacterial culture. PCR was performed with the following primers: forward primer, 5'-GTAGCGGATCGAGT-TCTCGATCCCGCGACACTAATACGACTCACTATAGGGAGACCAC-AACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGA-TATACCATGAGAGGATCGCATCACCATCACCATCAC-3'; reverse primer, 5'-CGCGATCATGGCGACCACACCCGTCCTGTGGAGATCCAGATA-TAGTTCCTCCTTTCAGCATCTCCGATGCGGATGTACTAAAAACCCC-TCAAGACCCGTTTAGAGGCCCCAAGGGGTTCGACTCACTATAGG-GAGCGG-3'.

The composition of the reaction was 1× Buffer E (67 mM Tris-HCl, pH 8.8, 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.01% (v/v) Tween 20), 0.2 mM each dNTP, 0.2 μM each primer, 1 unit of *Taq* polymerase. Reactions were performed in a PTC-200 Thermocycler (Bio-Rad) at the following temperatures: denaturation at 95 °C for 10 min; 40 cycles of 95 °C for 60 s, 60 °C for 60 s, 72 °C for 120 s; and 72 °C for 10 min. Prior to spotting, 0.5 M (final concentration) betaine and 0.5 ng/μl (final concentration) sonicated salmon sperm DNA were added to the PCR products. Expression and detection were performed as described under "Determination of the Absolute Yield of Expression" above.

Protein expression in microtiter plates was performed with 48 clones in a reaction volume of 8 μl/reaction in 96-well polypropylene

PCR plates. The transcription and translation mixture was prepared as recommended by the manufacturer, and unpurified PCR products diluted 1:2 in water were used as templates. Incubation of the mixture was performed overnight at 30 °C in a PTC-200 Thermocycler followed by spotting of the mixture on nickel chelate-coated slides. The slides were stored overnight at 4 °C, and detection was performed as described under "Determination of the Absolute Yield of Expression" above.

RESULTS

Principle of Utilizing MIST for Expression—The *in situ* protein expression on the chip is based on the creation of separate reaction entities on each spot of the microarrays by MIST (Fig. 1). In a first step, DNA templates are spotted on an activated glass slide. On top of the DNA templates, a cell-free transcription and translation mixture is spotted in a second spotting run before the whole slide is incubated in a humid hybridization chamber, which causes the spots to rehydrate and the expression to start. After incubation, the slide is blocked, and the expressed proteins are detected by specific antibodies.

Dependence of the Expression on Incubation Time and Concentration of DNA Template—Transcription of DNA templates into mRNA and its subsequent translation into protein are a coupled enzymatic reaction mediated by T7 RNA polymerase and a complex cascade of enzymes within the ribosomal complex. As an enzymatic process, the amount of products that is produced is dependent on two factors: the amount of substrate, *i.e.* DNA template molecules, and time. To demonstrate this principle, a dilution series of plasmid encoding wt-GFP was applied to different APTES-coated microarrays. Then cell-free transcription and translation mixture was spotted on top, and the slides were incubated for different periods of time, starting directly after application of the expression mixture; the longest incubation was overnight (Fig. 2).

For analysis, the mean signal intensity of each dilution and time point was calculated, and the mean signal intensity of the negative control spots without template was subtracted (Fig. 3). The diagram shows an increase of signal intensity with increasing concentration of template molecules for the varies time points. In addition, an increase of signals with longer incubation times of up to 2 h was observed, whereas there was a slight loss of signal intensity after prolonged incubation times, such as overnight. The coefficient of variation was

computed for all signals >1000 and was determined to be in the range of 16–20%.

Determination of the Absolute Yield of Expression—To quantify the absolute amounts of wt-GFP that are produced and to demonstrate that unpurified PCR products can also be used as DNA templates, the gene coding for wt-GFP was

FIG. 2. **Dependence of on-chip expression on DNA template concentration and incubation time.** Different DNA concentrations ranging from 1 μg/μl (*top row*) to 1 ng/μl (*second row* from the *bottom*) were applied to the microarray as well as a negative control in which pure water was spotted (*bottom row*). In a second spotting step, cell-free transcription and translation mixture was applied on top of this template. Subsequently the slides were subjected to different periods of incubation ranging from 0 min to overnight. For detection, an anti-GFP antibody was used.

amplified by PCR, and a dilution series was prepared. In a first spotting run, the PCR products were spotted on nickel chelate-coated slides. Then the cell-free expression mixture was added. For absolute quantification, a dilution series of purified wt-GFP was spotted on the very same set of slides for calibration (Fig. 4). Detection was performed using an anti-penta-His antibody, which recognizes the C-terminal His$_6$ tag of both the on- and off-chip produced wt-GFP. Because of the terminal position of the His tag, only the quantification of full-length wt-GFP was possible. To be able to compare the signals from the expression of wt-GFP and the calibration series, it was assumed that the binding characteristics of both the newly synthesized wt-GFP and the purified wt-GFP were identical. A graph of spotted PCR template concentration *versus* expressed protein concentration was prepared by calculation of the mean of the signal intensities of the expression and normalizing them via the calibration data (Fig. 5). The graph displays the suitability of unpurified PCR products for expression on a chip with a saturation of the signals above 0.5 ng/μl DNA yielding full-length wt-GFP in the range of 300 μg/ml.

Effect of His Tagging on Nickel Chelate-coated Slides— Surface activation of solid support plays a crucial role in the expression on the chip because it must provide an environment that prevents complete blocking by DNA templates and cell-free transcription and translation mixture on the one hand and on the other hand permits the newly synthesized protein to immobilize. APTES as well as nickel chelate coating were evaluated, and both performed well (Figs. 2 and 4) with nickel chelate displaying moderately brighter signals (data not shown). To elucidate whether this effect was due to the expected affinity capture of the His$_6$ tag or due to unspecific binding, a wt-GFP construct lacking the C-terminal His$_6$ tag was generated. Both genes were amplified by PCR and spotted on nickel chelate-coated slides. Detection was performed simultaneously by an anti-penta-His-Alexa 647 antibody conjugate and an anti-GFP-Texas Red conjugate (Fig. 6). Direct comparison of both constructs displayed comparable signal
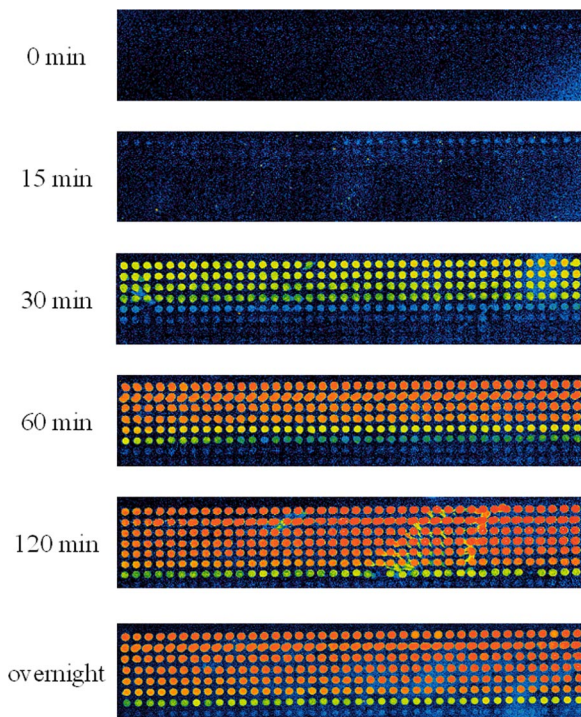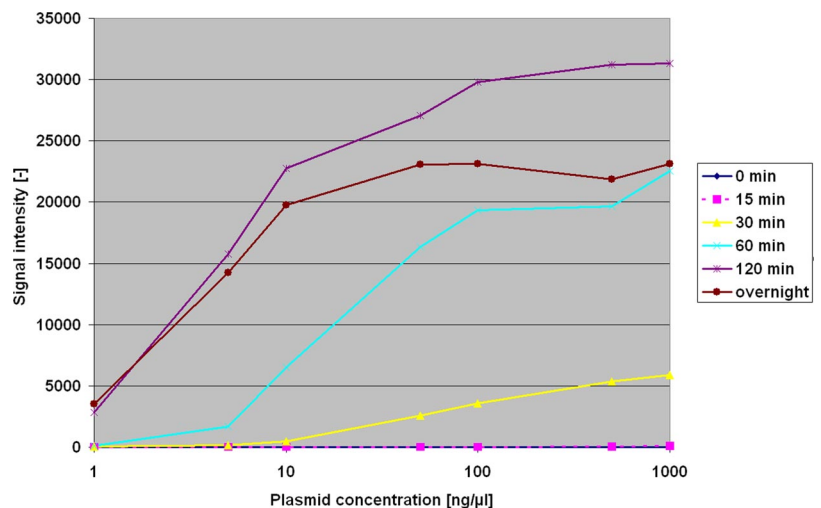
FIG. 3. **Diagram of plasmid concentration *versus* signal intensity.** For computation, the background-subtracted signals from Fig. 2 were quantified. The mean was calculated, and the signals from the negative control were subtracted.
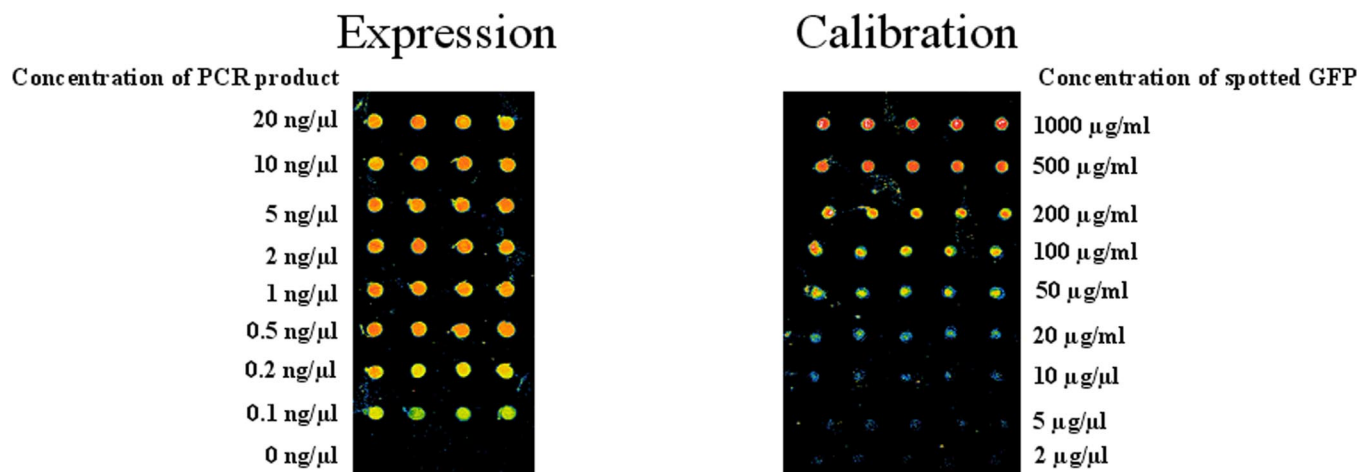
Expression

Concentration of PCR product

Calibration

Concentration of spotted GFP

FIG. 4. Absolute quantification of expression yield. For the determination of the absolute expression yield, different concentrations of unpurified PCR products ranging from 20–0.1 ng/μl as well as a negative control were used for expression. In parallel, a dilution series of purified wt-GFP was spotted for calibration. Detection was done with an anti-penta His antibody so that only full-length wt-GFP was identified.

FIG. 5. Diagram of spotted PCR template concentration *versus* expressed protein concentration. The background-corrected signals obtained in Fig. 4 were quantified and normalized using the calibration series of wt-GFP.
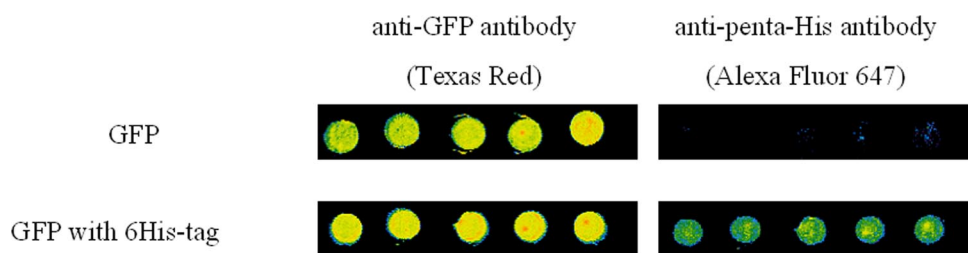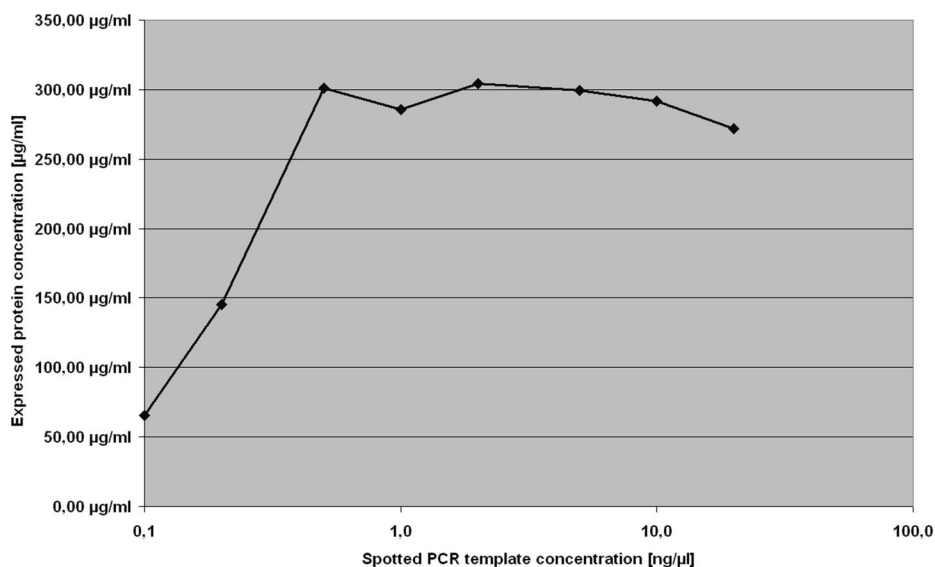

FIG. 6. Effect of His tagging on nickel-chelate coated slides. To elucidate whether the His$_6$ tag is promoting a specific and oriented attachment to nickel-chelate slides, two wt-GFP constructs, one with and one without the His$_6$ tag, were prepared and used for expression. Detection was performed simultaneously with an anti-penta-His antibody-Alexa 647 conjugate and an anti-GFP Texas Red conjugate.

intensities for the anti-GFP antibody, whereas only the construct with the His$_6$ tag generated signals with the anti-penta-His antibody.

High Throughput Expression of cDNAs—384 clones from a human fetal brain expression library were randomly chosen to elucidate whether the principle of the on-chip expression of proteins could be extended to the high throughput expression of proteins from an expression library. The clones were originally cloned in the pQE-30NST expression vector that carries a phage T5 promoter and two *lac* operators for isopropyl β-D-thiogalactoside-inducible recombinant protein expression. In addition, the cDNAs were flanked by a T7 and an SP6

FIG. 7. **High throughput expression of cDNAs.** PCR of 384 randomly chosen cDNAs was conducted with a single primer pair introducing the required promoter sequences. The PCR products were used as templates in the subsequent *in situ* expression, and proteins were visualized by their N-terminal His_6 tag and an anti-penta-His antibody-Alexa 647 conjugate. Negative control reactions were performed by using water instead of template in the PCR, and expression was performed in the two quadruplicates at the *bottom right*.
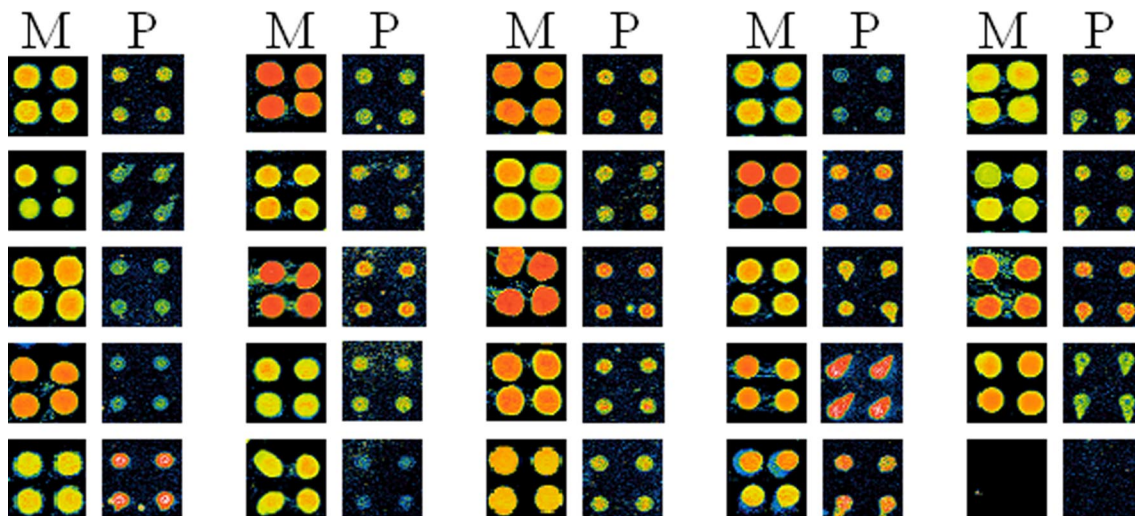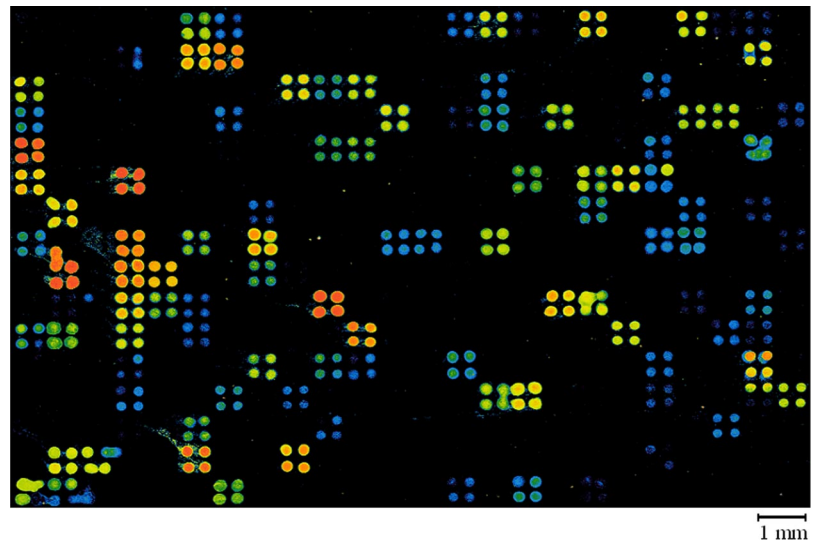

1 mm

FIG. 8. **Comparison of microarray-based and microtiter-based expression.** 24 clones showing high expression yields in the *in situ* expression and 24 clones without expression were selected and subjected to an expression in a microtiter format. After expression, transcription and translation mixture containing the expressed proteins was spotted on nickel-chelate coated slides, and the resulting recombinant proteins were detected by an anti-penta-His antibody-Alexa 647 conjugate. Scans from the 24 clones showing expression are displayed for the *in situ* expression in a microarray setup (*M*) and for the microtiter-based expression (*P*). Negative control reactions lacking any DNA template were performed in both formats and are shown at the *bottom right*. The 24 clones without detectable signals in the *in situ* expression on the microarray did not show any expression in the microtiter setup.

promoter for transcription in sense and antisense directions.

To make the cDNAs available for cell-free expression, they were amplified directly from the colony by PCR using a single primer pair harboring a T7 promoter along with the ribosomal binding site and part of the T7 terminator sequence. This led to a replacement of the original T5 promoter sequence as well as the antisense T7 promoter and provided the cDNAs in a format ready for spotting without any purification of the PCR products. The DNA fragments were spotted in quadruplicates, and detection was performed via their N-terminal His_6 tag and an anti-penta-His antibody (Fig. 7).

Expression of 24 clones showing high yields and 24 clones without expression was also carried out in microtiter plates to verify whether the unsuccessful expression of clones was due to the microarray setup. After expression, the mixture was spotted on nickel chelate slides, and an anti-penta-His antibody was applied for detection. All of the 24 clones showing high expression on a chip were also expressed in the microtiter format (Fig. 8), and none of the 24 clones without expression on the chip displayed a detectable expression in the microtiter plate.

DISCUSSION

The generation of high density protein microarrays is a cost- and time-intensive process. Reasons for this are not so much the spotting process itself but the production of the

proteins, which requires the generation of expression clones and the subsequent expression and purification of the proteins. Currently the only methodology to avoid this laborious task is the NAPPA technique, which was introduced by Ramachandran *et al.* (26). Although this approach provides a more feasible methodology than the separate expression and purification of the proteins off the chip, it is still limited by several intrinsic drawbacks. NAPPA requires the generation of an expression library, which harbors the coding sequences as GST fusions downstream of a T7 promoter. For expression, these constructs have to be purified and biotinylated at defined stoichiometries. In addition, the density of the resulting protein microarrays is limited to 512 spots per microscopic slide because at higher densities proteins might diffuse to adjacent spots.

To overcome these limitations and to enable the generation of high density protein microarrays with minimal manual interference, we developed an alternate strategy for an *in situ* expression of proteins. As a proof of principle, the expression of a plasmid harboring wt-GFP on the chip was performed. To demonstrate the dependence on time and amount of substrate different concentrations of the plasmid were applied to the chip, and the microarrays were incubated for different periods of time. As a solid support, slides coated with APTES were used because it has proven its ability to retain proteins in microarray applications (28, 30, 31). As expected, an increase of signal intensity was observed with increasing amount of plasmid as well as increasing incubation times of up to 2 h. Upon overnight incubation, the signals decreased again. This may be caused by proteolytic degradation by residual proteases of the cell-free transcription and translation mixture. The control, in which water instead of DNA template was spotted, resulted in very low signals, yielding signal to control ratios of up to 270.

To evaluate the possibility of using unpurified PCR products and to compute the absolute yield of expression, the gene encoding wt-GFP was amplified by PCR, and different concentrations of PCR products were applied to nickel chelate-coated glass slides. In parallel, a dilution series of purified wt-GFP was spotted as a calibration sample (Fig. 4). For detection, an antibody was used that recognizes the C-terminal His_6 tag of the wt-GFP, hence only recognizing full-length protein. To be able to determine the absolute amount of protein that is expressed and to compare the signals of the expression with the ones obtained from the calibration set, we inferred that the binding kinetics of the newly synthesized wt-GFP and the purified GFP are identical. From both dilution series, the signal intensities were determined, and a diagram of expressed protein concentration *versus* spotted PCR template concentration was drawn (Fig. 5). The diagram shows that as little as 0.1 ng/μl PCR product can be used for expression and that up to 300 μg/ml protein or 3.7 fmol can be produced on the spots. This amount seems to be remarkable considering that the amino acids present in each droplet

would already limit synthesis to 30.67 fmol. If the purified wt-GFP and the on-chip synthesized wt-GFP do not have equal binding kinetics, it can be expected that the purified wt-GFP will bind much more efficiently to the surface than the newly synthesized wt-GFP, since the latter has to compete with an excess of proteins from the cell-free transcription and translation mixture. This would mean that even more wt-GFP would have been produced so that equal amounts of protein can be detected.

Capture of the newly synthesized wt-GFP onto the surface is a crucial step in the *in situ* expression. Two surfaces, APTES and nickel chelate, were used, and both demonstrated their applicability. Although in the case of the APTES surface the mode of binding is very likely to be unspecific ionic interaction, we wanted to test whether the binding of wt-GFP to the nickel chelate surface is due to an affinity binding between the His_6 tag and the nickel chelate microarray as widely applied in batch protein purification procedures (32, 33). The nickel chelate surface has been reported to bind His-tagged proteins in a specific and oriented manner in a microarray setup (10, 34, 35). However, to our knowledge, it has not yet been demonstrated that the binding is truly dependent on the His tag. To test this, a wt-GFP construct was cloned that was lacking the C-terminal His_6 tag. An expression on the chip was performed, and the resulting expression products were detected simultaneously by an anti-GFP antibody and anti-penta-His antibody (Fig. 6). Although the signals from the anti-GFP antibody were comparable, the signal from the anti-penta-His antibody was only detectable for the tagged wt-GFP. This result points toward the fact that unspecific binding of the protein is the major mode of binding rather than a specific and oriented attachment via the His_6 tag. This may be due to electrostatic interactions because high salt buffer conditions are often recommended for nickel-nitrilotriacetic acid chromatography to reduce background binding of untagged proteins.

To test whether the expression on a chip can be applied to proteins other than wt-GFP, 384 clones were randomly picked from a human fetal brain protein expression library. The coding sequences were amplified by PCR using a single primer pair carrying a T7 promoter, a ribosomal binding site, and a T7 terminator. Expression was performed on the chip using the unpurified PCR products as templates (Fig. 7). A multitude of the coding sequences revealed successful expression after detection by an anti-penta-His antibody, whereas negative controls (two quadruplicates at the *bottom right*), in which no template was added to the PCR, yielded signals in the background range. The comparison of *in situ* expression with conventional expression revealed an identical distribution of expressible and non-expressible clones, demonstrating that the success rate of expression is not affected by the miniaturization of the expression to subnanoliter volumes. This may be attributed to the nature of the proteins, which may contain hard-to-express secondary structures or structures that lead

to misfolding and subsequent degradation (36). Moreover the microarray-based expression seems to result in a more homogenous distribution of signal intensities in comparison with the microtiter-based expression, which is beneficial in high throughput analysis systems. Altogether the results show that that the principle of *in situ* expression on a microarray can be extended to a larger set of proteins and that the bottleneck of protein array generation could be overcome by this methodology.

In summary, we present an *in situ* expression of proteins on a chip as a new application of the multiple spotting technique that complements its application in the detection of proteins and antibodies (27), in the selection of single chain Fv fragments from phage display selections (37), and the characterization of enzymatic activities and inhibitors thereof (38). The *in situ* expression involves the spotting of DNA templates in a first spotting step followed by the spotting of a cell-free transcription and translation mixture in a second spotting step on top of the DNA templates. Rehydration of the spots creates separated reaction entities in which the expressions can proceed. This allows the generation of high density protein microarrays with densities of up to 13,000 spots per slide. The generation of templates by PCR permits the expression of coding sequences without the laborious cloning in expression vectors and facilitates the introduction of novel tags. In addition the PCR products can be used directly without purification, which minimizes manual interaction steps.

REFERENCES

1. Hoheisel, J. D. (2006) Microarray technology: beyond transcript profiling and genotype analysis. *Nat. Rev. Genet.* **7,** 200–210
2. Griffin, T. J., Gygi, S. P., Ideker, T., Rist, B., Eng, J., Hood, L., and Aebersold, R. (2002) Complementary profiling of gene expression at the transcriptome and proteome levels in Saccharomyces cerevisiae. *Mol. Cell. Proteomics* **1,** 323–333
3. Hack, C. J. (2004) Integrated transcriptome and proteome data: the challenges ahead. *Brief. Funct. Genomics Proteomics* **3,** 212–219
4. Bertone, P., and Snyder, M. (2005) Advances in functional protein microarray technology. *FEBS. J.* **272,** 5400–5411
5. Merkel, J. S., Michaud, G. A., Salcius, M., Schweitzer, B., and Predki, P. F. (2005) Functional protein microarrays: just how functional are they? *Curr. Opin. Biotechnol.* **16,** 447–452
6. Stoll, D., Templin, M. F., Bachmann, J., and Joos, T. O. (2005) Protein microarrays: applications and future challenges. *Curr. Opin. Drug Discov. Dev.* **8,** 239–252
7. Lueking, A., Cahill, D. J., and Mullner, S. (2005) Protein biochips: a new and versatile platform technology for molecular medicine. *Drug Discov. Today* **10,** 789–794
8. Angenendt, P. (2005) Progress in protein and antibody microarray technology. *Drug Discov. Today* **10,** 503–511
9. Boutell, J. M., Hart, D. J., Godber, B. L., Kozlowski, R. Z., and Blackburn, J. M. (2004) Functional protein microarrays for parallel characterisation of p53 mutants. *Proteomics* **4,** 1950–1958
10. Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., Lan, N., Jansen, R., Bidlingmaier, S., Houfek, T., Mitchell, T., Miller, P., Dean, R. A., Gerstein, M., and Snyder, M. (2001) Global analysis of protein activities using proteome chips. *Science* **293,** 2101–2105
11. Schweitzer, B., Predki, P., and Snyder, M. (2003) Microarrays to characterize protein interactions on a whole-proteome scale. *Proteomics* **3,** 2190–2199
12. Feilner, T., Hultschig, C., Lee, J., Meyer, S., Immink, R. G., Koenig, A., Possling, A., Seitz, H., Beveridge, A., Scheel, D., Cahill, D. J., Lehrach, H., Kreutzberger, J., and Kersten, B. (2005) High throughput identification of potential *Arabidopsis* mitogen-activated protein kinases substrates. *Mol. Cell. Proteomics* **4,** 1558–1568
13. Zhu, H., Klemic, J. F., Chang, S., Bertone, P., Casamayor, A., Klemic, K. G., Smith, D., Gerstein, M., Reed, M. A., and Snyder, M. (2000) Analysis of yeast protein kinases using protein chips. *Nat. Genet.* **26,** 283–289
14. Ptacek, J., Devgan, G., Michaud, G., Zhu, H., Zhu, X., Fasolo, J., Guo, H., Jona, G., Breitkreutz, A., Sopko, R., McCartney, R. R., Schmidt, M. C., Rachidi, N., Lee, S. J., Mah, A. S., Meng, L., Stark, M. J., Stern, D. F., De Virgilio, C., Tyers, M., Andrews, B., Gerstein, M., Schweitzer, B., Predki, P. F., and Snyder, M. (2005) Global analysis of protein phosphorylation in yeast. *Nature* **438,** 679–684
15. Horn, S., Lueking, A., Murphy, D., Staudt, A., Gutjahr, C., Schulte, K., Konig, A., Landsberger, M., Lehrach, H., Felix, S. B., and Cahill, D. J. (2006) Profiling humoral autoimmune repertoire of dilated cardiomyopathy (DCM) patients and development of a disease-associated protein chip. *Proteomics* **6,** 605–613
16. Steller, S., Angenendt, P., Cahill, D. J., Heuberger, S., Lehrach, H., and Kreutzberger, J. (2005) Bacterial protein microarrays for identification of new potential diagnostic markers for Neisseria meningitidis infections. *Proteomics* **5,** 2048–2055
17. Feng, Y., Ke, X., Ma, R., Chen, Y., Hu, G., and Liu, F. (2004) Parallel detection of autoantibodies with microarrays in rheumatoid diseases. *Clin. Chem.* **50,** 416–422
18. Robinson, W. H., DiGennaro, C., Hueber, W., Haab, B. B., Kamachi, M., Dean, E. J., Fournel, S., Fong, D., Genovese, M. C., de Vegvar, H. E., Skriner, K., Hirschberg, D. L., Morris, R. I., Muller, S., Pruijn, G. J., van Venrooij, W. J., Smolen, J. S., Brown, P. O., Steinman, L., and Utz, P. J. (2002) Autoantigen microarrays for multiplex characterization of autoantibody responses. *Nat. Med.* **8,** 295–301
19. Glokler, J., and Angenendt, P. (2003) Protein and antibody microarray technology. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **797,** 229–240
20. Labaer, J., and Ramachandran, N. (2005) Protein microarrays as tools for functional proteomics. *Curr. Opin. Chem. Biol.* **9,** 14–19
21. Madoz-Gurpide, J., Wang, H., Misek, D. E., Brichory, F., and Hanash, S. M. (2001) Protein based microarrays: a tool for probing the proteome of cancer cells and tissues. *Proteomics* **1,** 1279–1287
22. He, M., and Taussig, M. J. (2001) Single step generation of protein arrays from DNA by cell-free expression and in situ immobilisation (PISA method). *Nucleic Acids Res.* **29,** 73–73
23. He, M., and Taussig, M. J. (2003) DiscernArray technology: a cell-free method for the generation of protein arrays from PCR DNA. *J. Immunol. Methods* **274,** 265–270
24. Angenendt, P., Nyarsik, L., Szaflarski, W., Glökler, J., Nierhaus, K. H., Lehrach, H., Cahill, D. J., and Lueking, A. (2004) Cell-free protein expression and functional assay in nanowell chip format. *Anal. Chem.* **76,** 1844–1849
25. Steffen, J., von Nickisch-Rosenegk, M., and Bier, F. F. (2005) In vitro transcription of a whole gene on a surface-coupled template. *Lab Chip* **5,** 665–668
26. Ramachandran, N., Hainsworth, E., Bhullar, B., Eisenstein, S., Rosen, B., Lau, A. Y., Walter, J. C., and LaBaer, J. (2004) Self-assembling protein microarrays. *Science* **305,** 86–90
27. Angenendt, P., Glökler, J., Konthur, Z., Lehrach, H., and Cahill, D. J. (2003) 3D protein microarrays: performing multiplex immunoassays on a single chip. *Anal. Chem.* **75,** 4368–4372
28. Kusnezow, W., Jacob, A., Walijew, A., Diehl, F., and Hoheisel, J. D. (2003)

Antibody microarrays: an evaluation of production parameters. *Proteomics* **3,** 254–264

29. Büssow, K., Nordhoff, E., Lubbert, C., Lehrach, H., and Walter, G. (2000) A human cDNA library for high-throughput protein expression screening. *Genomics* **65,** 1–8

30. Angenendt, P., Glökler, J., Murphy, D., Lehrach, H., and Cahill, D. J. (2002) Toward optimized antibody microarrays: a comparison of current microarray support materials. *Anal. Biochem.* **309,** 253–260

31. Angenendt, P., Glökler, J., Sobek, J., Lehrach, H., and Cahill, D. J. (2003) Next generation of protein microarray support materials: evaluation for protein and antibody microarray applications. *J. Chromatogr. A* **1009,** 97–104

32. Hochuli, E., Dobeli, H., and Schacher, A. (1987) New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *J. Chromatogr.* **411,** 177–184

33. Paborsky, L. R., Dunn, K. E., Gibbs, C. S., and Dougherty, J. P. (1996) A nickel chelate microtiter plate assay for six histidine-containing proteins.

Anal. Biochem. **234,** 60–65

34. Ingvarsson, J., Lindstedt, M., Borrebaeck, C. A., and Wingren, C. (2006) One-step fractionation of complex proteomes enables detection of low abundant analytes using antibody-based microarrays. *J. Proteome Res.* **5,** 170–176

35. Wingren, C., Steinhauer, C., Ingvarsson, J., Persson, E., Larsson, K., and Borrebaeck, C. A. (2005) Microarrays based on affinity-tagged single-chain Fv antibodies: sensitive detection of analyte in complex proteomes. *Proteomics* **24,** 24

36. Spirin, A. S. (2004) High-throughput cell-free systems for synthesis of functionally active proteins. *Trends Biotechnol.* **22,** 538–545

37. Angenendt, P., Wilde, J., Kijanka, G., Baars, S., Cahill, D. J., Kreutzberger, J., Lehrach, H., Konthur, Z., and Glökler, J. (2004) Seeing better through a MIST: evaluation of monoclonal recombinant antibody fragments on microarrays. *Anal. Chem.* **76,** 2916–2921

38. Angenendt, P., Lehrach, H., Kreutzberger, J., and Glökler, J. (2005) Sub-nanoliter enzymatic assays on microarrays. *Proteomics* **5,** 420–425