Molecular BioSystems

METHOD

View Article Online
View Journal | View Issue

Rapid *in vitro* protein synthesis pipeline: a promising tool for cost-effective protein array design

Cite this: *Mol. BioSyst.*, 2014, **10**, 1236

Received 3rd January 2014,
Accepted 25th March 2014

DOI: 10.1039/c4mb00003j

www.rsc.org/molecularbiosystems

Mangesh Bhide,*[ab] Satheesh Natarajan,[a] Stanislav Hresko,[a] Carmen Aguilar[c] and Elena Bencurova[a]

Several protein expression systems for construction of protein arrays have been established in recent years. However, current protocols for protein synthesis are still time consuming, laborious and expensive. This study has established an alternative workflow that covers rapid construction of expression cassettes, in-tube and on-membrane synthesis of recombinant proteins, and straightforward screening of synthesized proteins. Eighteen membrane associated eukaryotic proteins and two secretory complement regulators (C1 inhibitor and vitronectin) were included in the study. To generate hybrid genes, double-overlap extension PCR was employed to fuse the 5′ fragment (consisting of a T7 promoter and a species independent translation sequence), ORFs of the target proteins, and the 3′ fragment (encompassing GFP fusion, Myc-tag and stop codon). OE-PCR generated fragments were directly mixed with the *Leishmania torentolae* lysate (translation mix) for protein synthesis. In order to establish a cheap and user-friendly alternative to existing cell-free protein array techniques, PCR products were spotted on the hydrophobic substrate (PVDF membrane), air-dried and covered with only 2 μL of translation mix. All synthesized proteins were spontaneously immobilized on the membrane due to the hydrophobic interaction between C-terminally fused GFP and PVDF. Synthesis and immobilization of proteins were confirmed simply by assessing the GFP chromophore under a laser scanner or a fluorescent microscope.

Introduction

Cell free protein expression is a high throughput methodology for the conversion of genetic information into protein.[1] It provides seamless intersection between nucleic acid and protein technologies and delivers a platform for exploration of fundamental biological principles.[2] A cell-free translation system enables rapid development of protein arrays in which required target concentrations are in micro or nanograms.

In spite of the fact that cell-free expression systems date back to the discovery of the genetic code, it has not become widespread as *in vivo* systems.[3] Moreover, many of the eukaryotic cell free systems such as the wheat germ extract (WGE), the rabbit reticulocyte lysate and the insect cell extract are costly and complex for preparation. The cost varies between 40–50 $ per reaction (30 μL reaction volume) for the WGE, 25–30 $ per reaction (30 μL reaction volume) for the rabbit reticulocyte and 40–70 $ per reaction (40 μL reaction volume) for the insect cell extract. Previous reports have shown the use of the WGE for synthesis of proteins, for structural and biochemical analysis and for construction of the expressed human proteome.[4–6] Despite these successes, preparation of a high quality wheat-based extract requires extensive optimizations[7] and the quality of commercially available lysates may vary. Complex plant genetics, a long life cycle of the host and laborious extract preparation also make the WGE system less popular. In a recent effort to develop a cell-free system based on an eukaryotic host amenable to rapid genetic modification, rapid and scalable cultivation, and straightforward lysate preparation, the unicellular flagellate *Leishmania tarentolae* is worthwhile of attention.[8–10] *Leishmania tarentolae*, a protozoan parasite of the gecko *Tarentolae annularis*, has been established as a new *in vivo* eukaryotic expression system for recombinant protein production.[11] As demonstrated for erythropoietin, the proteins produced in *Leishmania* possess mammal-like *N*-glycosylation,[11] which is one of the main advantages in effort to produce functional eukaryotic recombinant proteins. An *in vivo Leishmania* expression system has also been used for the production of various heterologous proteins such as proprotein convertase 4

[a] *Laboratory of Biomedical Microbiology and Immunology, Department of Microbiology and Immunology, University of Veterinary Medicine and Pharmacy, Komenskeho 73, 04181, Kosice, Slovakia. E-mail: mangeshbhide@me.com; Fax: +00421556323173; Tel: +00421915984604*
[b] *Institute of Neuroimmunology, Slovak Academy of Sciences, 84245 Bratislava, Slovakia*
[c] *Grupo de Genómica y Mejora Animal, Departamento de Genética, Universidad de Córdoba, Spain*

(a member of Ca^{2+} dependent mammalian subtilases), human laminin-332, a tissue type plasminogen activator, HIV-1 gag, GTPase Rab7 and Luciferase.[2,7,12–15] It was shown that extracts generated from *Leishmania* cells could be used for *in vitro* protein expression, and in ideal cases up to 200 μg mL^{-1} of recombinant protein could be produced within 2 hours.[14]

Rapid and efficient cloning is an important aspect of protein synthesis pipelines. Conventional cloning techniques are still routinely used in many laboratories. These techniques involve several time consuming steps like vector preparation, ligation, transformation and transformant selection (by PCR or sequencing). In this article, we provide a detailed description for double-overlap extension PCR (double OE-PCR) used to prepare expression cassettes (hybrid genes). Although, a similar type of OE-PCR was used elsewhere to construct expression cassettes,[8] which composed of two overlapping fragments and amplicons of the target genes, scanty details of the methodology were presented.

Various on-surface cell-free protein expression systems use DNA templates that have already been immobilized onto the protein capture surface. In an earlier nucleic acid programmable protein array (NAPPA), DNA was biotinylated and captured on the avidin pre-coated surface. The synthesized proteins in this array were C-terminal glutathione *S*-transferase (GST) tagged and simultaneously captured by the *anti*-GST antibody coated on the chips.[16] Some of the disadvantages of this array were laborious preparation steps in DNA biotinylation, impure protein co-localized with the capture antibody and the lack of some post-translation modification.[16] Presently used next-generation NAPPA is robust, less laborious and has enhanced DNA capture and translation efficiency. Next-generation NAPPA relies on dramatically improved binding of DNA to positively charged diamines in the presence of bovine serum albumin.[17] An *in situ* puromycin-capture array uses single stranded oligonucleotides (which serve to capture mRNAs) with two different tags – puromycin at one end and biotin at the other. Puromycin is tagged with a newly synthesized polypeptide, while biotin immobilizes surface proteins, which is coated with streptavidin.[18] Both NAPPA and puromycin-capture techniques are robust and give protein yield at a high density, however they are quite expensive due to the capture surfaces used in these assays. The protein *in situ* array (PISA), a more sophisticated technique, uses a highly automated system to ensure accurate and sequential supply of the reagents for transcription and translation reactions in a sub-nanoliter droplet.[19] However, the automated systems are highly expensive and limited mostly to the state-of-the-art laboratories. To that extent, here we developed a simple and cost effective method for protein translation and immobilization on the hydrophobic substrate with the help of a C-terminal hydrophobic green fluorescent protein (GFP) tag (Translation and Immobilization of Protein on Hydrophobic Substrate, TIPoHS).

Here we present details of: (1) rapid construction of expression cassettes with double OE-PCR, (2) in-tube and on-membrane protein translations using an eukaryotic *Leishmania* translation system, (3) simultaneous immobilization of the translated proteins on membranes and (4) rapid detection of the translated protein with a GFP reporter. With the minimum labour, cost, and time, TIPoHS can be used effectively to construct in-house protein arrays for various biological experiments like the study of protein–protein or protein–nucleic acid interactions, enzyme activities, functional analysis of a large number of mutants and disease diagnosis.

Materials and methods

Construction of expression cassettes

Two universal fragments F1 and F2 were designed first by PCR. The pLEXSY_invitro_2 vector (Jena biosciences, Germany) was used as a template. Obtained amplicons were again modified with series of PCR in a such a way that the final F1 fragment contained a T7 promoter, a species independent translation sequence (SITS, which encompasses poly TTTTA region[9]), a start codon and an overlapping region for double OE-PCR. The F2 fragment was consisted of an overlapping region for double OE-PCR, a cleavage site for Factor Xa, eGFP, Myc-tag, stop codon and 3′ UTR.

Open reading frames of a C1 inhibitor, vitronectin, ICAM-1 and its five domains (Ig-like C2-type 1–5), CD40 and its four domains (D1-D4), annexin 1, 2, 3, 5 and 6, interferon-induced transmembrane protein 3 (IFITM3) and vimentin were amplified from rat or human cDNA. Forward primers used to amplify the ORFs contained 5′ overhang complementary to the overlapping region in the F1 fragment, while reverse primers contained 3′ overhang complementary to the overlapping region in the F2 fragment (Table 1 and Fig. 1).

All amplicons were synthesized in 25 μL of the reaction mixture consisting of 1X GC Buffer, 0.2 mM each of dNTPs, 10 ng of template DNA, 500 nM of each primers and 20 U mL^{-1} of Phusion polymerase (Thermo Scientific, Finland). Fragments F1 and F2 were amplified under following conditions: 2 min at 98 °C followed by 35 cycles of amplification (10 s at 98 °C and 30 s at 72 °C) and finally 10 min at 72 °C. Cycling conditions for ORFs were as follows: 2 min at 98 °C, 35 cycles of amplification (10 s at 98 °C, 30 s at 61 °C and 30 s at 72 °C) and finally 10 min at 72 °C. PCR products were resolved on 0.7% TAE agarose gel. Bands of the corresponding size were excised and amplicons were purified with a QIAquick Gel extraction kit (Qiagen, Germany).

Double OE-PCR was used to fuse F1, ORFs and F2 fragments. In order to have double OE-PCR work optimally, it is crucial that the fragments are combined at an equimolar ratio. The first round of double OE-PCR reaction was set up in 25 μL and contained 1X reaction buffer, 5 nM of gel purified ORF fragments, 0.2 mM of each of dNTPs and 20 U mL^{-1} of Taq polymerase (Jena Biosciences, Germany). Note that no primers were added in this reaction. Cycling conditions used in this round were: 2 min at 94 °C followed by 11 cycles of amplification (30 s at 94 °C, 30 s at 53 °C and 60 s at 72 °C). The product of this PCR reaction was directly purified using the QIAquick PCR purification kit (Qiagen, Germany). The reaction mixture for the second round of double OE-PCR contained 1X reaction buffer, 10 ng of the purified product from the first round, 0.2 mM of each of dNTPs, 20 U mL^{-1} of Taq polymerase (Jena Biosciences, Germany) and 200 nM of the primers. The forward

Table 1 Primers used in the study

Fragments	Primer	Sequence 5′–3′	Length[a]
Fragment F1	F1 forward F1 reverse	GGGTTATTGTCTCATGAGC **TGTGTCTAGAGAGGCGCCAGC**CTCCATGGTTTCACTTACG	274 bp
Fragment F2	F2 forward F2 reverse	**AAGGGTACCATAGAAGGGAGA**AGCAAGGGCGAGGAGCTGTTCACC CATCTATAGAGAAGTACACGTAAAAG	905 bp
C1 inhibitor	C1 inhibitor forward C1 inhibitor reverse	**GCTGGCGCCTCTCTAGACACA**ATCCAAATGCTACCAGCTCC **GCTTCTCCCTTCTATGGTACCCTT**AAGCCTGGGGTCATATACTCGCC	1433 bp
Vitronectin	Vitronectin forward Vitronectin reverse	**GCTGGCGCCTCTCTAGACACA**GCCCTGCTGGCATGGGTTCTC **GCTTCTCCCTTCTATGGTACCCTTA**AGAGCAATGGAGCGTGGGTAGGG	1369 bp
ICAM-1$_{(\text{Ig-like C2-type 1 to 5})}$	ICAM-1 forward ICAM-1 reverse	**GCTGGCGCCTCTCTAGACACA**GGTGCTCAGGTATCCATC **GCTTCTCCCTTCTATGGTACCCTT**AGGGGGGAGGCGGGGCTTGTAC	1318 bp
ICAM-1$_{(\text{Ig-like C2-type 1})}$	IG1 forward IG1 reverse	**GCTGGCGCCTCTCTAGACACA**AGGTGCTCAGGTATCCATCCAT **GCTTCTCCCTTCTATGGTACCCTT**AGGTCCACTCGC TCTGGGAA	280 bp
ICAM-1$_{(\text{Ig-like C2-type 2})}$	IG2 forward IG2 reverse	**GCTGGCGCCTCTCTAGACACA**ACAGCAGGTGGGCAAGAAC **GCTTCTCCCTTCTATGGTACCCTT**AGGAGGTCAGGGGTGTC	307 bp
ICAM-1$_{(\text{Ig-like C2-type 3})}$	IG3 forward IG3 reverse	**GCTGGCGCCTCTCTAGACACA**ACCACAAGGGCTGTCACTGTTC **GCTTCTCCCTTCTATGGTACCCTTA**GGGTCTTCTCCATCTCCAGGGTCTG	331 bp
ICAM-1$_{(\text{Ig-like C2-type 4})}$	IG4 forward IG4 reverse	**GCTGGCGCCTCTCTAGACACA**AATCCTGACCCTGAGCCAG **GCTTCTCCCTTCTATGGTACCCTT**AGGGACTTCCCATCCACCTCCAA	237 bp
ICAM-1$_{(\text{Ig-like C2-type 5})}$	IG5 forward IG5 reverse	**GCTGGCGCCTCTCTAGACACA**TGGACAAGAAGGACTGC **GCTTCTCCCTTCTATGGTACCCTT**AGGTCCCTGGTGATACTCCC	217 bp
CD40$_{(\text{TNFR-Cys 1 to 4})}$	CD_{D1-D4} forward CD_{D1-D4} reverse	**GCTGGCGCCTCTCTAGACACA**GACAAACAGTACCTCCAAGGT **GCTTCTCCCTTCTATGGTACCCTT**AAGCATCCGGGGCTGGAAACC	494 bp
CD40$_{(\text{TNFR-Cys 1})}$	CD_{D1} forward CD_{D1} reverse	**GCTGGCGCCTCTCTAGACACA**GACAAACAGTACCTCCAAGGT **GCTTCTCCCTTCTATGGTACCCTT**AAGGTCGCACGGTTGGCATTGGGT	104 bp
CD40$_{(\text{TNFR-Cys 2})}$	CD_{D2} forward CD_{D2} reverse	**GCTGGCGCCTCTCTAGACACA**GAGAAGACCCAATGCCAACCG **GCTTCTCCCTTCTATGGTACCCTT**AAGGCAGTGCTGCCCTTCCTTGCA	170 bp
CD40$_{(\text{TNFR-Cys 3})}$	CD_{D3} forward CD_{D3} reverse	**GCTGGCGCCTCTCTAGACACA**ACCGCGGTTTCAGACACT **GCTTCTCCCTTCTATGGTACCCTT**AAGGAAGAATCCGACCGGGCA	167 bp
CD40$_{(\text{TNFR-Cys 4})}$	CD_{D4} forward CD_{D4} reverse	**GCTGGCGCCTCTCTAGACACA**GATACTGTCTGCCAACCCTGC **GCTTCTCCCTTCTATGGTACCCTT**AAGCATCCGGGGCTGGAAACC	158 bp
Annexin A1	Annexin A1 forward Annexin A1 reverse	**GCTGGCGCCTCTCTAGACACA**GGATCCTCAGCAGTGAGCCCCTACCCT **GCTTCTCCCTTCTATGGTACCCTT**AAGGTCGACTCCACACAGAGCCACCAGGAT	993 bp
Annexin A2	Annexin A2 forward Annexin A2 reverse	**GCTGGCGCCTCTCTAGACACA**GGATCCCATTCTACACCCCCAAGTGCC **GCTTCTCCCTTCTATGGTACCCTT**AAGGTCGACGTCGTCCCCACCACACAGGTA	1029 bp
Annexin A3	Annexin A3 forward Annexin A3 reverse	**GCTGGCGCCTCTCTAGACACA**GGATCCGCGTCTTTGTGGGTTGGACCT **GCTTCTCCCTTCTATGGTACCCTTA**AGGTCGACGCAGCCGTAGTGCTTCTTGAA	948 bp
Annexin A5	Annexin A5 forward Annexin A5 reverse	**GCTGGCGCCTCTCTAGACACA**GGATCCGGCAGGGCTGATGCCGAAGTT **GCTTCTCCCTTCTATGGTACCCTT**AAGGTCGACAGAGTACAGGGACGTGGCGAA	906 bp
Annexin A6	Annexin A6 forward Annexin A6 reverse	**GCTGGCGCCTCTCTAGACACA**GCGGCCGCTATCACCTCCCGCAGCAACAAG **GCTTCTCCCTTCTATGGTACCCTT**AAGGTCGACTTCTCCACCACAGAGCGCCAG	1941 bp
IFITM3	IFITM3 forward IFITM3 reverse	**GCTGGCGCCTCTCTAGACACA**GGATCCAACCACACTTCTCAAGCCTTC GCTTCTCCCTTCTATGGTACCCTTAAGGTCGACATGGTCAGGCACAGAGACCTC	228 bp
Vimentin	Vimentin forward Vimentin reverse	**GCTGGCGCCTCTCTAGACACA**GGATCCTCCACCCGCACCTACAGCCTA GCTTCTCCCTTCTATGGTACCCTTAAGGTCGACCCTGCTCTCCTCCCCTTCCAG	1191 bp

Bold letters represent overlap sequences. [a] Amplicon size of ORF (except for F1 and F2 fragments).

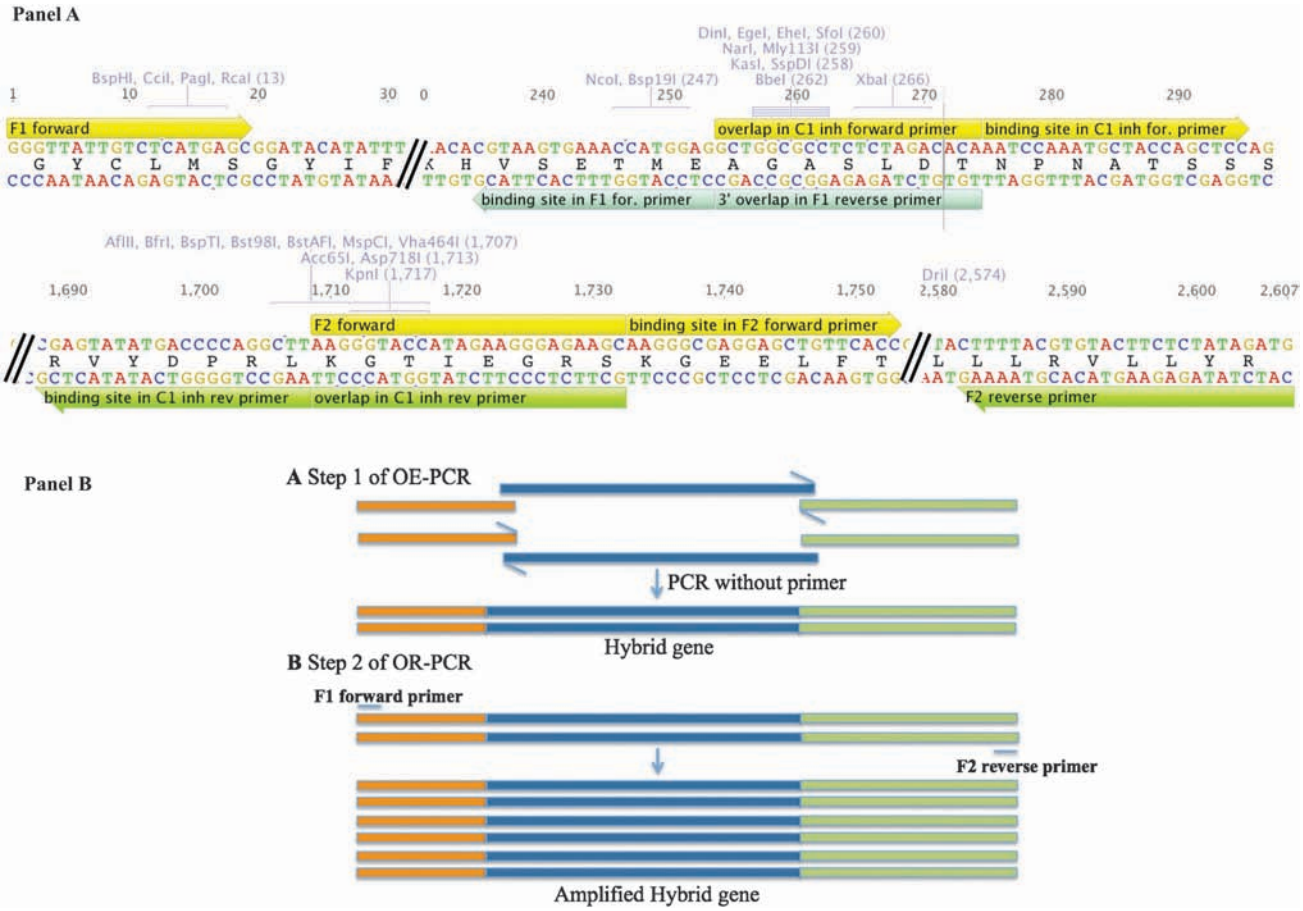

Fig. 1 Primer design strategy and principle of double OE-PCR. Panel A: primer design strategy used to construct F1, insert genes and F2 fragment are presented. Sense oligos are on the top of the sequence, while antisense oligos are at the bottom. Restriction sites built in overlaps are also presented. Researchers may use these sites to fuse the fragments with the help of classical cloning methods. Panel B: (A) in the first step of the double OE-PCR, which occurs in the absence of primers, overlapping segments (slanting bars) act as priming sites. Consequently, three fragments get fused during the extension step and form a single hybrid gene. (B) In the second step of double OE-PCR the hybrid gene is specifically amplified with end primers (F1 forward and F2 reverse primers).

primer used to construct the F1 fragment and the reverse primer used to amplify the F2 fragment were used in this case (Fig. 1). The reaction was performed as follows: 2 min at 94 °C followed by 30 cycles of amplification (30 s at 94 °C, 30 s at 53 °C and 1.20 min at 72 °C) and final extension for 2 min at 72 °C. Amplified expression cassettes were checked on 0.7% agarose gel.

Cell free protein translation

The translation reaction setup requires mixing of the PCR product from second OE-PCR with the *Leishmania* cell lysate (Jena Biosciences, Germany) and adjustment of the final volume with water. The in-tube translation was performed in a reaction mixture of 4 μL, which contained 111 ng of the purified expression cassette in 1.2 μL of water and 2.8 μL of supplemented cell lysate. Tubes were incubated for 2 h at 20 °C for translation and then kept at 4 °C overnight for protein maturation. In-tube translation can be scaled up easily.

In the case of TIPoHS, translation was performed on the hydrophobic substrate (Immobilon-FL membrane, pore size 0.4 μM, Millipore, USA). In short, expression cassettes were spotted (111 ng of DNA in 0.6 μL of water) on the membrane and allowed to air dry. Each dried DNA spot was covered with a drop (2 μL) of *Leishmania* cell lysate (1.4 μL of cell lysate and 0.6 μL of water). The Immobilon membrane was incubated for 2 h at 20 °C in a humid chamber and then at 4 °C overnight.

Negative controls contained *Leishmania* cell lysate without exogenous DNA. As a positive control, purified pLEXSY_invitro_2 plasmid was added in translation mix, which contains a sequence for expression of GFP only.

Analysis of in-tube synthesized proteins

In-tube translated proteins were purified with 4 μL of EZview Red *anti*-c-Myc affinity beads (Sigma, USA). Beads were washed two times with RIPA buffer (Sigma) and added to the translated proteins. Total volume was set to 10 μL with RIPA buffer and beads were incubated for 1 h on ice. Affinity beads were washed three times for 5 minutes with RIPA buffer and checked under a fluorescence microscope to confirm protein capture with the help of the GFP reporter. To elute the captured proteins, beads were either incubated with 0.1 M glycine HCl (pH 2.8) or with

20 μL of 1X SDS sample buffer for 5 min. Proteins eluted in SDS sample buffer were resolved on SDS-PAGE (10% polyacrylamide) and transferred onto the nitrocellulose membrane (30 V for 1 h in X-cell miniblotter, Invitrogen). pH of the proteins eluted with 0.1 M glycine HCl was adjusted to 7.0 with Tris HCl pH 9.0 and 2 μL of each protein was spotted on a nitrocellulose membrane. Proteins were air-dried.

Western blotting

Nitrocellulose membranes (from electrotransfer or dot blot) were blocked for 45 min in blocking buffer (TBS containing 0.05% Tween 20 and 5% skimmed milk), and then incubated with the *anti*-Myc tag antibody (HRP conjugated, 1 : 50 000 dilution, Abcam, UK) for 1 h. Membranes were washed five times for 5 min in washing buffer (TBS containing 0.05% Tween 20). Membranes were then incubated in the enhanced chemiluminescence substrate (SuperSignal West Pico chemiluminescence substrate, Pierce) for 5 min and signals were captured on X-ray film.

Detection of on-membrane translated (TIPoHS) protein

The Immobilon membrane was washed 5 times for 2 min in ultra-pure water, dried and the presence of GFP tagged proteins was detected by scanning at 488 nM (Molecular imager, Pharos FX system, Bio-rad) or by fluorescent microscopy. The presence of proteins on the membrane was also confirmed with the *anti*-Myc tag antibody as described above.

Results

Speeding-up the workflow of preparation of translation cassettes

Laborious molecular cloning steps are needed to generate a template for translation. This labor limits the throughput of the cell-free protein production especially when the parallel translation of many sequence variants is carried out. Double OE-PCR was used to overcome this obstacle, in which ORFs were fused with F1 and F2 to obtain expression cassettes (Fig. 1). In the first step all fragments were hybridized due to the complementary overlaps present between F1, gene inserts and F2. These overlaps ensured directional fusion of all three fragments as well as served as priming sites for elongation in PCR. In the second step, fused fragments were amplified with the end primers (Fig. 1), which generated full-length expression cassettes (Fig. 2).

Protein translation with the *L. tarentolae* cell extract

Cell free translation reactions were primed with purified double OE-PCR products and were incubated at 20 °C for 2 h for translation and at 4 °C overnight for protein maturation. One of the main advantages of cell-free protein production is its speed and amenability to miniaturization. Many downstream applications require proteins in pure form or at least captured on the substrate like affinity beads. Myc-tag at the C-terminus of the translated proteins facilitated easy and rapid capture of the proteins on affinity beads, while the GFP tag enabled direct screening of the presence of proteins on the beads by simple

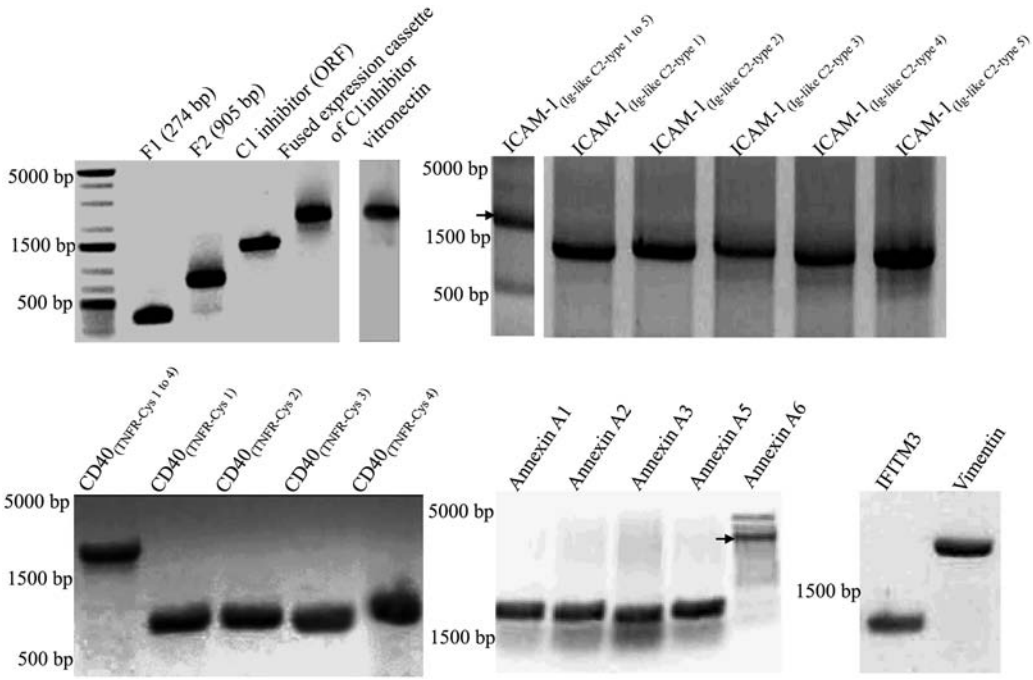

Fig. 2 Double OE-PCR and translated protein. Agarose gel showing amplicons of the F1, F2 fragments, the ORF fragment of a C1 inhibitor (representative candidate) and fused expression cassettes. For all other genes only fused expression cassettes are presented. No non-specific amplification was observed in OE-PCR except for ICAM-1$_{(Ig\text{-}like\ C2\text{-}type\ 1\ to\ 5)}$ and Annexin A6 (correct amplicon size for these genes is depicted with arrows).

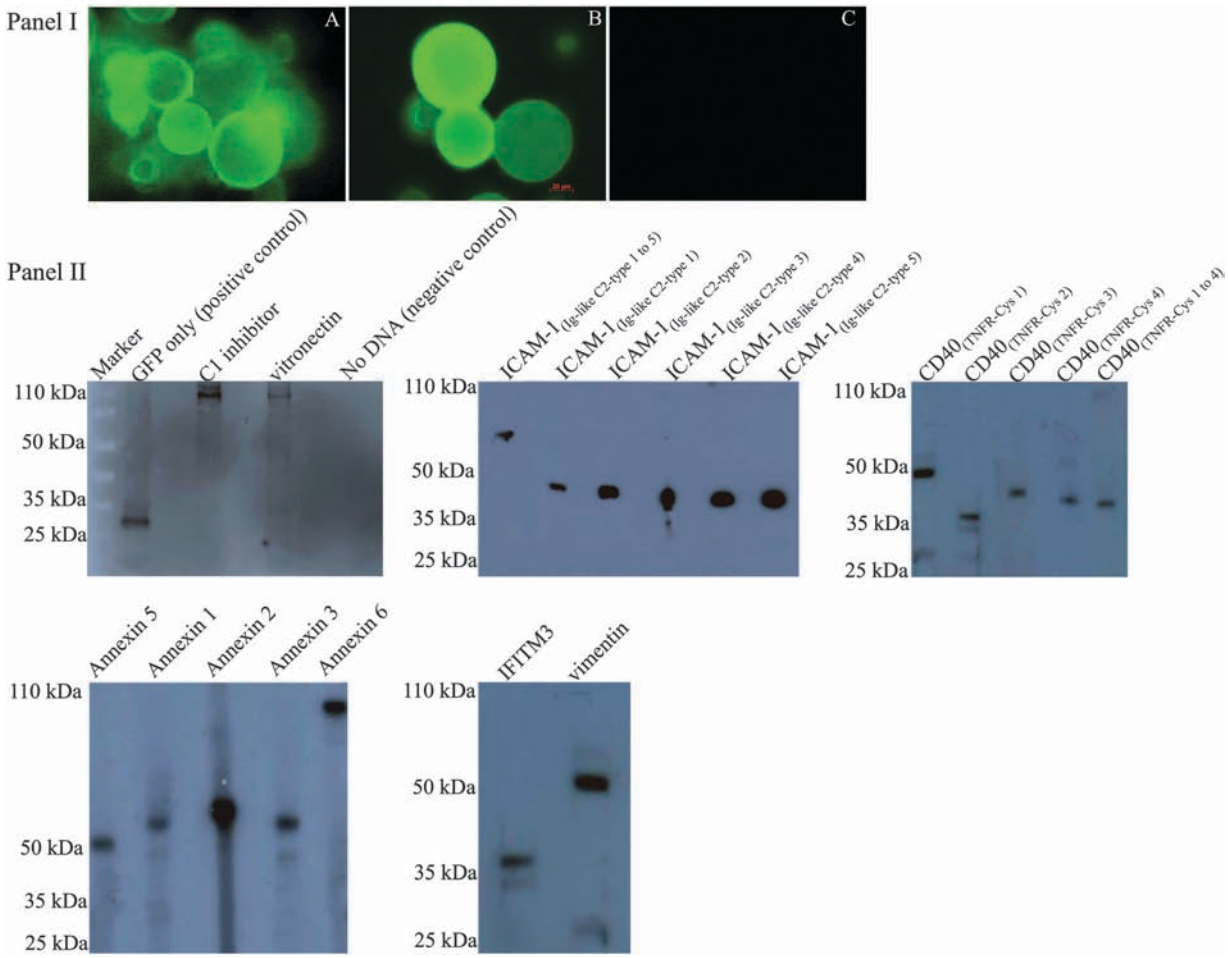

Fig. 3 In-tube translated proteins. Panel I – presents proteins captured on Myc-affinity beads and detected with GFP epifluorescence. Only the C1 inhibitor on affinity beads is presented as an example. (A) Positive control – only GFP is expressed, (B) the C1 inhibitor with C-terminal GFP fusion, (C) negative control – no exogenous DNA. Panel II – presents western blotting of the translated proteins, positive control (only GFP was translated) and negative control (no template DNA was added in translation mix).

fluorescent microscopy (Fig. 3, panel I). Beads incubated with translation reaction without exogenous DNA (negative control) showed no fluorescence.

Proteins captured on Myc-affinity beads were fractionated on SDS-PAGE, transferred onto nitrocellulose membranes and detected with the *anti*-Myc antibody (Fig. 3, panel II). Slight protein degradation was observed in some cases, while all protein bands were corresponded with their predicted molecular weight. Proteins were also detected simultaneously with dot blotting (Fig. 4).

To demonstrate the use of a PVDF membrane as a substrate for cell-free translation and direct protein immobilization, without any additional chemical coating, the reaction volume was reduced to 2 μL and translation was successfully performed on air-dried DNA. GFP fusion served as a tether between target protein and the hydrophobic PVDF substrate (Fig. 5) as well as an excellent reporter to check proper translation of target proteins simply by observing the spots under a fluorescent microscope (Fig. 6, panel I) or scanning the membrane at 488 nM on a Pharos FX system (Fig. 6, panel II).

Overall, time required to produce recombinant proteins was reduced to 2 days (Fig. 7).

Discussion

Protein arrays are a powerful tool for the study of biological functions of a large set of proteins, however, it is not widely used because of the cost and sophistication needed. Many of the available protein array synthesis platforms prefer to produce proteins separately and spot them on a chip with different cross-linking chemicals.[10,20,21] For a technique to be widely accepted the protein produced should be of good yield, stable, and must be conducive for further functional studies, besides with modest cost, labour and time. Here we have streamlined an alternative approach (TIPoHS) to chip based protein translation, in which GFP fusion servers as a reporter as well as a tether molecule. Translated proteins were captured on the Immobilon membrane thanks to the inherent affinity of GFP for the hydrophobic substrate.[22] In contrast to other assays, like

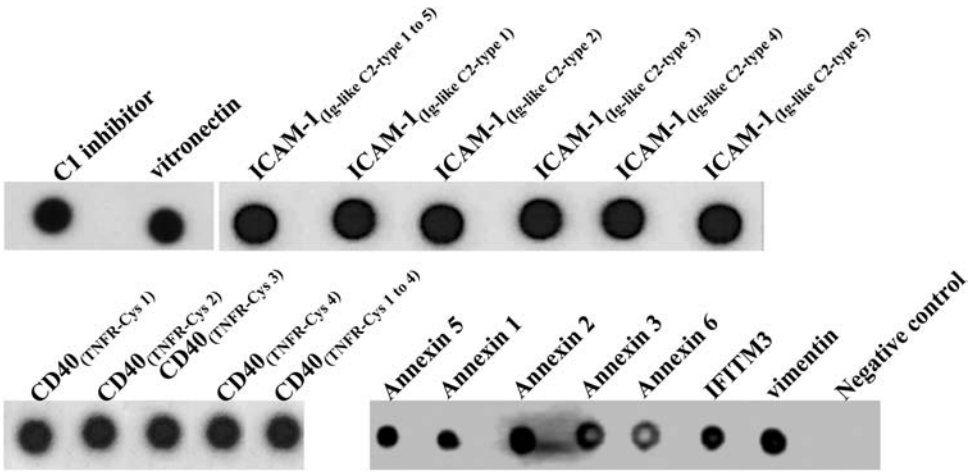

Fig. 4 Dot blotting of in-tube translated proteins. Negative control – no exogenous DNA was added in the translation reaction.

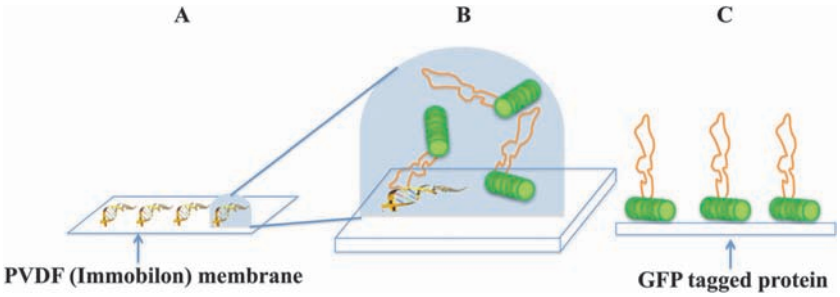

Fig. 5 Self-assembling protein microarray on the Immobilon membrane. (A) Expression cassettes were spotted on the Immobilon membrane and allowed to air dry. (B) *Leishmania* cell lysate was spotted on the dried expression cassettes. Diameter of each spot was approximately 1 mM and the distance between two spots was 3 to 4 mM. Due to the hydrophobic nature of the Immobilon membrane, the lysate was not diffused, but remained as a droplet. (C) Translated fusion proteins get attached to the immobilon through hydrophobic interaction between GFP and the membrane, most probably keeping the protein free for analysis, like protein–protein interactions or functional studies.

NAPPA or puromycin-capture, no immobilizing antibody or linker[16,18] was necessary in TIPoHS, this might be helpful to minimize chances of any non-specific reactivity observed in downstream experiments because of the presence of an immobilizing antibody on the chip.

In-tube and on-membrane translated ICAM-1 and its domains were tested for their affinity for PilE4 of *Francisella*,[23] CD40 and TNFR-Cys domains were used to assess their binding with OspA of *Borrelia*,[24] while annexins, C1inh, vitronectin and vimentin were used in pull down and blotting experiments to judge their interactions with *Trypanosoma* surface proteins (data not shown). Binding affinities of the proteins translated in-tube or on-membrane were consistent to their ligands. Most of the proteins translated here are membrane proteins (except C1inh and vitronectin), usually challenging to produce in heterologous expression systems. Thus, TIPoHS can be a primary tool to produce a repertoire of the proteins in a short time for screening of various biological functions.

Protein folding is another key requirement for investigating protein–protein interactions when performing functional assays. The reporter of protein folding has to achieve several criteria, such as thermal and chemical stability, protein-based and that requires no exogenous cofactors. GFP is one of the candidates for a reporter with these characteristics.[25] For GFP to form its chromophore, it needs first to be folded correctly.[26] All proteins translated in our study showed strong intensity of green fluorescence (Fig. 3 and 6). Previous work has shown that GFP as a reporter at C-terminus of the protein of interest gives a signal directly proportional to the amount of correctly folded protein without need for functional assay.[26] This approach was successfully used by Wurth and colleagues to study misfolding and aggregation of beta amyloid.[27] Rapid misfolding of the beta amyloid moiety blocked maturation of the GFP fluorophore and thus reduced the fluorescence. Nevertheless, in the recent study Wright and co-workers found that fluorescent aggregates form in nearly all cells expressing a misfolded product of exon I of Huntington gene with a N-terminal GFP reporter, while total fluorescence output per cell was equivalent to that of GFP alone.[28] This phenomenon might be explained by the differences in the folding rates of the reporter and target. If the folding rate of the target protein is slower than GFP folding time, the reporter maintains its chromophore even though the target is misfolded.[28] Although, the GFP reporter helps to judge the proper folding of the target protein, in some cases results should be interpreted with caution.

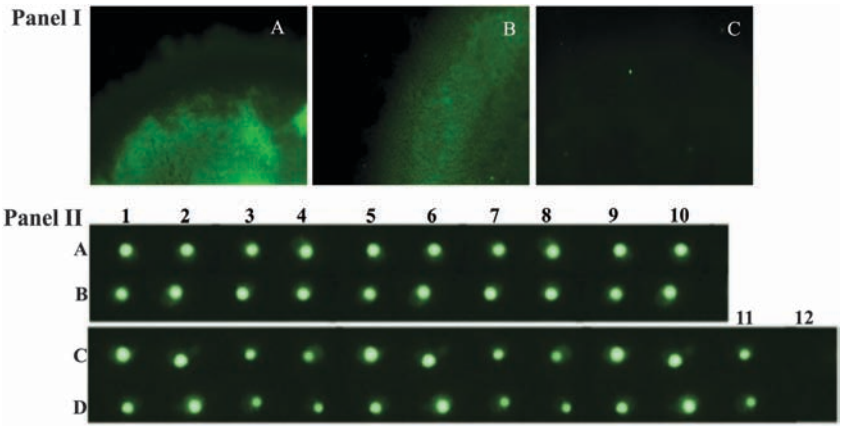

Fig. 6 Detection of on-membrane translated proteins. Panel I – assessment of the presence of protein on the membrane with fluorescent microscopy (10X). Proteins were translated on the PVDF membrane and the intensity of fluorescence in the protein spot was observed by placing the membrane directly under a microscope. (A) positive control, only GFP was expressed on-membrane, (B) C1 inhibitor fused with GFP (representative candidate), (C) negative control, no exogenous DNA in translation reaction. Panel II – assessment of the translation and the presence of protein with Pharose FX plus based laser scanning – A1 and B1 – C1 inhibitor; A2 and B2 – vitronectin; A3 and B3 – ICAM-1$_{(\text{Ig-like C2-type 1 to 5})}$; A4 and B4 – ICAM-1$_{(\text{Ig-like C2-type 1})}$; A5 and B5 – ICAM-1$_{(\text{Ig-like C2-type 2})}$; A6 and B6 – ICAM-1$_{(\text{Ig-like C2-type 3})}$; A7 and B7 – ICAM-1$_{(\text{Ig-like C2-type 4})}$; A8 and B8 – ICAM-1$_{(\text{Ig-like C2-type 5})}$; A9 and B9 – CD40$_{(\text{TNFR-Cys 1 to 4})}$; A10 and B10 CD40$_{(\text{TNFR-Cys 1})}$; C1 and D1 – CD40$_{(\text{TNFR-Cys 2})}$; C2 and D2 – CD40$_{(\text{TNFR-Cys 3})}$; C3 and D3 – CD40$_{(\text{TNFR-Cys 4})}$; C4 and D4 – Annexin A1; C5 and D5 – Annexin A2; C6 and D6 – Annexin A3; C7 and D7 – Annexin A5; C8 and D8 – Annexin A6; C9 and D9 -IFITM3; C10 and D10 – vimentin; C11 and D11 – positive control, only GFP; C12 and D12 negative control, no exogenous DNA in translation reaction.

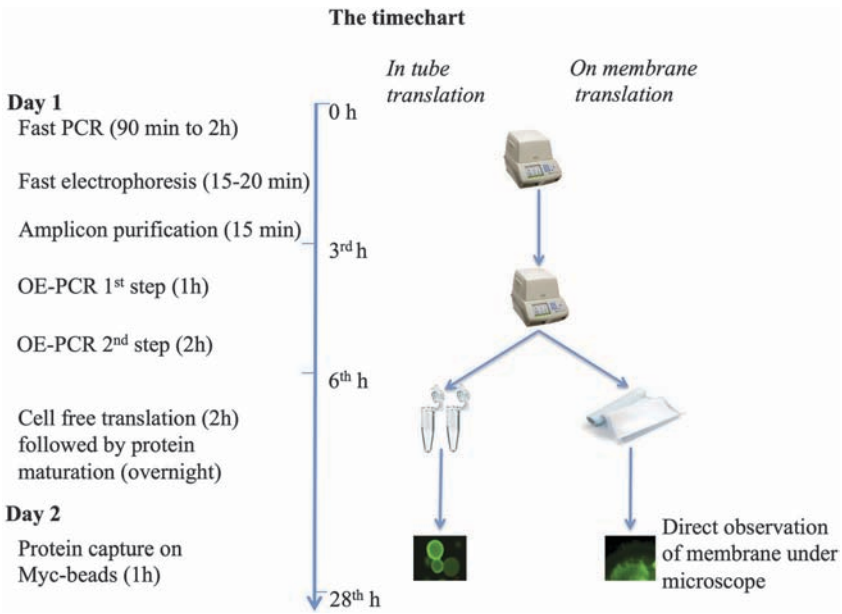

Fig. 7 Schematic outline of the established protocol. The time-chart highlighting overall pipeline for *in vitro* protein expression, detection and analysis of the protein protocol in terms of the expenditure of time presented.

One of the major advantages of the expression cassette used in this study is the presence of a Species Independent Translation Sequence (SITS), a translation initiation sequence.[9] Experimental results showed that a DNA fragment that contains SITS, which by means of promoting the assembly of the active ribosome, replaces the untranslated 5region (5′ UTR) in mRNAs that are recognized by the translation initiation machinery, apparently initiates translation without the requirements for species dependent translation initiation factors.[9] Earlier study successfully used SITS to translate mRNAs with five eukaryotic cell lysates and even *E. coli.*[9] Thus the SITS sequence in the expression cassette expands the organism range that can be used for cell-free protein expression.

E. coli is the most widely used expression system for production of recombinant proteins, however due to the lack of post-translation modifications its use for synthesis of eukaryotic proteins is limited. In addition, in the *E. coli* based cell-free expression system the translation may not be efficient because

the mRNA and DNA degrading enzymes originating from the cells often decrease the stability of the templates generated by the PCR.[29,30] Eukaryotic cell-free systems can overcome this problem, however the cost and complexity of the preparation of the lysates still remain a major hurdle. *Leishmania* based cell-free eukaryotic expression system is available at low cost (approximately 5 $ per 20 μL of reaction volume and approximately 0.5 $ per spot on membrane) and creates less ethical problems. The cell-free *Leishmania* expression system used in this study can be the plausible alternative to *E. coli* or other eukaryotic cell-free systems.

Conclusions

The protein expression pipeline described here offers a promising choice for construction of cost-saving protein arrays, without time consuming and laborious cloning steps. Within two days, twenty expression cassettes were prepared by double OE-PCR, translated into the proteins directly on membranes and used in downstream application to study protein–protein interactions. With several simplicities and advantages of the *in vitro* translation protocol described here, we hope that laboratories with limited assets can design their own small-scale protein arrays.

Acknowledgements

We thank Ing. Martina Cepkova for her excellent assistance in PCR and sequencing. The research activities were supported by the research grants APVV-0036/10, VEGA-1/0054/12 and structural funds for centers of excellence-26220120002 (INFEKTZOON). S.N. received funding for post-doctoral studies from SAIA. E.B is funded by ITMS 26220220185.

References

1 X. Ge and J. Xu, Cell-free protein synthesis as a promising expression system for recombinant proteins, *Methods Mol. Biol.*, 2012, **824**, 565–578.
2 M. Soleimani, F. Mahboudi, N. Davoudi, A. Amanzadeh, M. Azizi, A. Adeli, H. Rastegar, F. Barkhordari and B. Mohajer-Maghari, Expression of human tissue plasminogen activator in the trypanosomatid protozoan *Leishmania tarentolae*, *Biotechnol. Appl. Biochem.*, 2007, **48**, 55–61.
3 J. H. Matthaei and M. W. Nirenberg, Characteristics and stabilization of DNAase-sensitive protein synthesis in *E. coli* extracts, *Proc. Natl. Acad. Sci. U. S. A.*, 1961, **47**, 1580–1588.
4 K. Takai, T. Sawasaki and Y. Endo, The wheat-germ cell-free expression system, *Curr. Pharm. Biotechnol.*, 2010, **11**, 272–278.
5 K. Takai, T. Sawasaki and Y. Endo, Practical cell-free protein synthesis system using purified wheat embryos, *Nat. Protoc.*, 2010, **5**, 227–238.
6 M. Watanabe, K. Miyazono, M. Tanokura, T. Sawasaki, Y. Endo and I. Kobayashi, Cell-free protein synthesis for structure determination by X-ray crystallography, *Methods Mol. Biol.*, 2010, **607**, 149–160.
7 A. Basak, N. J. Shervani, M. Mbikay and M. Kolajova, Recombinant proprotein convertase 4 (PC4) from *Leishmania tarentolae* expression system: purification, biochemical study and inhibitor design, *Protein Expression Purif.*, 2008, **60**, 117–126.
8 O. Kovtun, S. Mureev, W. Johnston and K. Alexandrov, Towards the construction of expressed proteomes using a *Leishmania tarentolae* based cell-free expression system, *PLoS One*, 2010, **5**, e14388.
9 S. Mureev, O. Kovtun, U. T. Nguyen and K. Alexandrov, Species-independent translational leaders facilitate cell-free expression, *Nat. Biotechnol.*, 2009, **27**, 747–752.
10 G. MacBeath and S. L. Schreiber, Printing proteins as microarrays for high-throughput function determination, *Science*, 2000, **289**, 1760–1763.
11 R. Breitling, S. Klingner, N. Callewaert, R. Pietrucha, A. Geyer, G. Ehrlich, R. Hartung, A. Muller, R. Contreras, S. M. Beverley and K. Alexandrov, Non-pathogenic trypanosomatid protozoa as a platform for protein research and production, *Protein Expression Purif.*, 2002, **25**, 209–218.
12 M. Breton, C. Zhao, M. Ouellette, M. J. Tremblay and B. Papadopoulou, A recombinant non-pathogenic *Leishmania* vaccine expressing human immunodeficiency virus 1 (HIV-1) Gag elicits cell-mediated immunity in mice and decreases HIV-1 replication in human tonsillar tissue following exposure to HIV-1 infection, *J. Gen. Virol.*, 2007, **88**, 217–225.
13 T. Lang, S. Goyard, M. Lebastard and G. Milon, Bioluminescent *Leishmania* expressing luciferase for rapid and high throughput screening of drugs acting on amastigote-harbouring macrophages and for quantitative real-time monitoring of parasitism features in living mice, *Cell. Microbiol.*, 2005, **7**, 383–392.
14 H. P. Phan, M. Sugino and T. Niimi, The production of recombinant human laminin-332 in a *Leishmania tarentolae* expression system, *Protein Expression Purif.*, 2009, **68**, 79–84.
15 N. Hirano, T. Sawasaki, Y. Tozawa, Y. Endo and K. Takai, Tolerance for random recombination of domains in prokaryotic and eukaryotic translation systems: limited interdomain misfolding in a eukaryotic translation system, *Proteins*, 2006, **64**, 343–354.
16 N. Ramachandran, E. Hainsworth, B. Bhullar, S. Eisenstein, B. Rosen, A. Y. Lau, J. C. Walter and J. LaBaer, Self-assembling protein microarrays, *Science*, 2004, **305**, 86–90.
17 N. Ramachandran, J. V. Raphael, E. Hainsworth, G. Demirkan, M. G. Fuentes, A. Rolfs, Y. Hu and J. LaBaer, Next-generation high-density self-assembling functional protein arrays, *Nat. Methods*, 2008, **5**, 535–538.
18 S. C. Tao and H. Zhu, Protein chip fabrication by capture of nascent polypeptides, *Nat. Biotechnol.*, 2006, **24**, 1253–1254.
19 M. He and M. J. Taussig, Single step generation of protein arrays from DNA by cell-free expression and in situ immobilisation (PISA method), *Nucleic Acids Res.*, 2001, **29**, E73.
20 A. Lueking, M. Horn, H. Eickhoff, K. Bussow, H. Lehrach and G. Walter, Protein microarrays for gene expression and antibody screening, *Anal. Biochem.*, 1999, **270**, 103–111.

21 J. R. Newman and A. E. Keating, Comprehensive identification of human bZIP interactions with coiled-coil arrays, *Science*, 2003, **300**, 2097–2101.
22 W. W. Ward, *Methods of Biochemical Analysis, Green Fluorescent Protein: Properties, Applications and Protocols*, John Wiley & Sons, Inc., Hoboken, New Jersey, 2nd edn, vol. 47, 2005.
23 E. Bencurova, P. Mlynarcik, L. Pulzova, A. Kovac and M. R. Bhide, PilE4 may contributes in the adhesion of Francisella to brain microvascular endothelial cells, in *Farm animal proteomics 2013*, Wageningen Academic Publishers, 2013, pp. 99–102.
24 P. Mlynarcik, L. Pulzova, S. Hresko, E. Bencurova, S. Dolinska, A. Kovac, M. A. Dominguez, J. J. Garrido and M. R. Bhide, Identification of amino acid residues of OspA of *Borrelia* involved in binding to CD40 receptor, in *Farm animal proteomics 2013*, Wageningen Academic Publishers, 2013, pp. 107–111.
25 W. W. Ward, in *Bioluminescence and chemiluminescence*, Academic, New York, 1981.
26 G. S. Waldo, B. M. Standish, J. Berendzen and T. C. Terwilliger, Rapid protein-folding assay using green fluorescent protein, *Nat. Biotechnol.*, 1999, **17**, 691–695.
27 C. Wurth, N. K. Guimard and M. H. Hecht, Mutations that reduce aggregation of the Alzheimer's Abeta42 peptide: an unbiased search for the sequence determinants of Abeta amyloidogenesis, *J. Mol. Biol.*, 2002, **319**, 1279–1290.
28 O. Wright, L. Zhang, Y. Liu, T. Yoshimi, Y. Zheng and A. Tunnacliffe, Critique of the use of fluorescence-based reporters in *Escherichia coli* as a screening tool for the identification of peptide inhibitors of Abeta42 aggregation, *J. Pept. Sci.*, 2013, **19**, 74–83.
29 S. A. Lesley, M. A. Brow and R. R. Burgess, Use of in vitro protein synthesis from polymerase chain reaction-generated templates to study interaction of *Escherichia coli* transcription factors with core RNA polymerase and for epitope mapping of monoclonal antibodies, *J. Biol. Chem.*, 1991, **266**, 2632–2638.
30 K. A. Martemyanov, A. S. Spirin and A. T. Gudkov, Direct expression of PCR products in a cell-free transcription/translation system: synthesis of antibacterial peptide cecropin, *FEBS Lett.*, 1997, **414**, 268–270.