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

Mangesh Bhide,⁎a,b) Satheesh Natarajan,a) Stanislav Hresko,a) Carmen Aguilar,c) and Elena Bencuurova,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 tarentolae 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 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 optimizations7 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

⁎ Laboratory of Biomedical Microbiology and Immunology, Department of Microbiology and Immunology, University of Veterinary Medicine and Pharmacy, Komenského 73, 04181, Kosice, Slovakia. E-mail: mangeshbhide@me.com; Fax: +00421556323173; Tel: +00421915984604
a Institute of Neuroimmunology, Slovak Academy of Sciences, 84245 Bratislava, Slovakia
b Grupo de Genómica y Mejora Animal, Departamento de Genética, Universidad de Córdoba, Spain
system to ensure accurate and sequential supply of the reagents for 
(PISA), a more sophisticated technique, uses a highly automated 
charged diamines in the presence of bovine serum albumin.17

DNA capture and translation efficiency. Next-generation NAPPA

generation NAPPA is robust, less laborious and has enhanced 
protein expression, and in ideal cases up to 200

Leishmania

cassettes with double OE-PCR, ( 2) in-tube and on-membrane 

Hydrophobic Substrate, TIPoHS).

translation and immobilization on the hydrophobic substrate 

Here we developed a simple and cost effective method for protein 

diversity pipelines. Conventional cloning techniques are still 

Fast and efficient cloning is an important aspect of protein 
synthesis pipelines. Conventional cloning techniques are still 

in situ

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 

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 
relied 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 oligo-
nucleotides (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_in vitro_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 region1), 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 
mixing 1X GC Buffer, 0.2 nM each of dNTPs, 10 ng of 
template DNA, 500 nM of each primers and 20 U mL
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
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 nM of each of dNTPs, 20 U mL
of Taq polymerase (Jena 
Biosciences, Germany) and 200 nM of the primers. The forward
## Table 1  
Primers used in the study

<table>
<thead>
<tr>
<th>Fragments</th>
<th>Primer</th>
<th>Sequence 5'-3'</th>
<th>Length(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fragment F1</strong></td>
<td>F1 forward</td>
<td>GGGTTATGGTTGCTCATGAGG</td>
<td>274 bp</td>
</tr>
<tr>
<td></td>
<td>F1 reverse</td>
<td>TGTTCTGTAGAGGAGGGCCAGCCCTTCATTAGGTTTCCAAGCTTACCTGAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C1 inhibitor forward</td>
<td>GCTGCGGCGGCTCCTCTAGACACAAATCCAAATGCTACCAAGCTCC</td>
<td>1433 bp</td>
</tr>
<tr>
<td></td>
<td>C1 inhibitor reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>Vitronectin</strong></td>
<td>forward</td>
<td>GCTGCGGCGGCTCCTAGAAGACAGCAGGTTGACTTACCTG</td>
<td>1369 bp</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>ICAM-1 (Ig-like C2-type 1 to 5)</strong></td>
<td>ICAM-1 forward</td>
<td>GCTGCGGCGGCTCCTAAGACAGCAGGTTGACTTACCTG</td>
<td>1318 bp</td>
</tr>
<tr>
<td></td>
<td>ICAM-1 reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>ICAM-1 (Ig-like C2-type 1)</strong></td>
<td>IG1 forward</td>
<td>GCTGCGGCGGCTCCTCTAAGACAGCAGGTTGACTTACCTG</td>
<td>307 bp</td>
</tr>
<tr>
<td></td>
<td>IG1 reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>ICAM-1 (Ig-like C2-type 2)</strong></td>
<td>IG2 forward</td>
<td>GCTGCGGCGGCTCCTCTAAGACAGCAGGTTGACTTACCTG</td>
<td>237 bp</td>
</tr>
<tr>
<td></td>
<td>IG2 reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>ICAM-1 (Ig-like C2-type 3)</strong></td>
<td>IG3 forward</td>
<td>GCTGCGGCGGCTCCTCTAAGACAGCAGGTTGACTTACCTG</td>
<td>217 bp</td>
</tr>
<tr>
<td></td>
<td>IG3 reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>ICAM-1 (Ig-like C2-type 4)</strong></td>
<td>IG4 forward</td>
<td>GCTGCGGCGGCTCCTCTAAGACAGCAGGTTGACTTACCTG</td>
<td>167 bp</td>
</tr>
<tr>
<td></td>
<td>IG4 reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>ICAM-1 (Ig-like C2-type 5)</strong></td>
<td>IG5 forward</td>
<td>GCTGCGGCGGCTCCTCTAAGACAGCAGGTTGACTTACCTG</td>
<td>158 bp</td>
</tr>
<tr>
<td></td>
<td>IG5 reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>CD40 (TNFR-Cys 1 to 4)</strong></td>
<td>CD40 forward</td>
<td>GCTGCGGCGGCTCCTCTAAGACAGCAGGTTGACTTACCTG</td>
<td>494 bp</td>
</tr>
<tr>
<td></td>
<td>CD40 reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>CD40 (TNFR-Cys 1)</strong></td>
<td>CD1 forward</td>
<td>GCTGCGGCGGCTCCTCTAAGACAGCAGGTTGACTTACCTG</td>
<td>104 bp</td>
</tr>
<tr>
<td></td>
<td>CD1 reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>CD40 (TNFR-Cys 2)</strong></td>
<td>CD2 forward</td>
<td>GCTGCGGCGGCTCCTCTAAGACAGCAGGTTGACTTACCTG</td>
<td>170 bp</td>
</tr>
<tr>
<td></td>
<td>CD2 reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>CD40 (TNFR-Cys 3)</strong></td>
<td>CD3 forward</td>
<td>GCTGCGGCGGCTCCTCTAAGACAGCAGGTTGACTTACCTG</td>
<td>167 bp</td>
</tr>
<tr>
<td></td>
<td>CD3 reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>CD40 (TNFR-Cys 4)</strong></td>
<td>CD4 forward</td>
<td>GCTGCGGCGGCTCCTCTAAGACAGCAGGTTGACTTACCTG</td>
<td>158 bp</td>
</tr>
<tr>
<td></td>
<td>CD4 reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>Annexin A1</strong></td>
<td>Annexin A1 forward</td>
<td>GCTGCGGCGGCTCCTCTAAGACAGCAGGTTGACTTACCTG</td>
<td>993 bp</td>
</tr>
<tr>
<td></td>
<td>Annexin A1 reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>Annexin A2</strong></td>
<td>Annexin A2 forward</td>
<td>GCTGCGGCGGCTCCTCTAAGACAGCAGGTTGACTTACCTG</td>
<td>1029 bp</td>
</tr>
<tr>
<td></td>
<td>Annexin A2 reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>Annexin A3</strong></td>
<td>Annexin A3 forward</td>
<td>GCTGCGGCGGCTCCTCTAAGACAGCAGGTTGACTTACCTG</td>
<td>948 bp</td>
</tr>
<tr>
<td></td>
<td>Annexin A3 reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>Annexin A5</strong></td>
<td>Annexin A5 forward</td>
<td>GCTGCGGCGGCTCCTCTAAGACAGCAGGTTGACTTACCTG</td>
<td>906 bp</td>
</tr>
<tr>
<td></td>
<td>Annexin A5 reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>Annexin A6</strong></td>
<td>Annexin A6 forward</td>
<td>GCTGCGGCGGCTCCTCTAAGACAGCAGGTTGACTTACCTG</td>
<td>1941 bp</td>
</tr>
<tr>
<td></td>
<td>Annexin A6 reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>IFITM3</strong></td>
<td>IFITM3 forward</td>
<td>GCTGCGGCGGCTCCTCTAAGACAGCAGGTTGACTTACCTG</td>
<td>228 bp</td>
</tr>
<tr>
<td></td>
<td>IFITM3 reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
<tr>
<td><strong>Vimentin</strong></td>
<td>Vimentin forward</td>
<td>GCTGCGGCGGCTCCTCTAAGACAGCAGGTTGACTTACCTG</td>
<td>1191 bp</td>
</tr>
<tr>
<td></td>
<td>Vimentin reverse</td>
<td>GCTTCTCGCTCTATGTTACCTAAGGGGAGGGCGGTTGTACG</td>
<td></td>
</tr>
</tbody>
</table>

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

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-lg-linc2-type1iso5 and Annexin A6 (correct amplicon size for these genes is depicted with arrows).
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. 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. In contrast to other assays, like...
NAPPA or puromycin-capture, no immobilizing antibody or linker 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, CD40 and TNFR-Cys domains were used to assess their binding with OspA of Borrelia, 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. For GFP to form its chromophore, it needs first to be folded correctly. 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. This approach was successfully used by Wurth and colleagues to study misfolding and aggregation of beta amyloid. 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. 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. Although, the GFP reporter helps to judge the proper folding of the target protein, in some cases results should be interpreted with caution.
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. Experimental results showed that a DNA fragment that contains SITS, which by means of promoting the assembly of the active ribosome, replaces the untranslated 5' region (5' UTR) in mRNAs that are recognized by the translation initiation machinery, apparently initiates translation without the requirements for species dependent translation initiation factors. Earlier study successfully used SITS to translate mRNAs with five eukaryotic cell lysates and even *E. coli*. 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.\textsuperscript{25,30} Eukaryotic cell-free systems can overcome this problem, however the cost and complexity of the preparation of the lysates still remain a major hurdle. \textit{Leishmania} based cell-free eukaryotic expression system is available at low cost (approximately 5 $ per 20 $\mu$L of reaction volume and approximately 0.5 $ per spot on membrane) and creates less ethical problems. The cell-free \textit{Leishmania} expression system used in this study can be the plausible alternative to \textit{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 \textit{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).

**References**


