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Robust microarray production of freshly expressed proteins in a human milieu

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Abstract

Purpose—*In vitro* transcription/translation (IVTT) systems are widely used in proteomics. For clinical applications, mammalian systems are preferred for protein folding and activity; however, the level of protein obtained is low. A new system extracted from human cells (1-Step Human Coupled IVT) has the potential to overcome this problem and deliver high yields of protein expressed in a human milieu.

Experimental design—Western blots and self-assembled protein microarrays were used to test the efficiency of protein synthesis by 1-Step Human Coupled IVT (HCIVT) compared to rabbit reticulocyte lysate (RRL). The arrays were also used to measure the immune response obtained from serum of patients exposed to pathogens or vaccine.

Results—HCIVT performed better than RRL in all experiments. The yield of protein synthesized in HCIVT is more than 10 times higher than RRL, in both western blot and protein microarrays. Moreover, HCIVT showed a robust lot-to-lot reproducibility. In immune assays, the signals of many antigens were detected only in HCIVT-expressed arrays, mainly due to the reduction in the background signal and the increased levels of protein on the array.

Conclusion and clinical relevance—HCIVT is a robust IVTT system that yields high levels of protein produced in a human milieu. It can be used in applications where protein expression in a mammalian system and high yields are needed. The increased immunogenic response of HCIVT-expressed proteins will be critical for biomarker discovery in many diseases, including cancer.

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Conflict of interest statement

The authors KV, GK, PJ and BW are employees at Thermo Fisher Scientific. All the remaining authors declare that there are no conflicts of interest.

Keywords

NAPPA; protein microarray; *in vitro* transcription and translation; Human Coupled IVT

Protein microarrays are increasingly finding their way into clinical proteomics research. These tools display peptides and full-length proteins in high spatial density on a fixed matrix that can be probed with a variety of reagents to detect interactions, enzymatic modification and recognition by specific probes. They are particularly powerful in enabling researchers to screen through thousands of candidates to find a select few polypeptides of interest to a particular application or assay. Protein microarrays have been used in clinical research to look for protein levels [1], kinase activity [2], protein phosphorylation status [3], and proteomic signatures [4].

One particular clinical application for protein microarrays is in the study of immune responses where they effectively allow global mapping of humoral responses. This has been applied in infectious diseases, *Pseudomonas aeruginosa* [5], *Plasmodium falciparum* [6], *Mycobacterium tuberculosis* [7] to name a few; where such studies may lead to the development of diagnostics and perhaps provide clues for vaccine studies. In addition, protein microarrays have found use in studies on antibodies to self-proteins, termed autoantibodies. These responses occur in a number of autoimmune diseases: systemic lupus erythematosus [8], rheumatoid arthritis [9], multiple sclerosis [10], and diabetes [11]; as well as chronic diseases such as cancer [12, 13]. In a similar fashion, these specific immune responses can act as biomarkers to help inform prognosis, the stratification of patients into disease subtypes and possibly even act as early detection markers of disease [14–16].

A number of methods have been used to manufacture protein microarrays. In the most common approach, protein microarrays are produced by printing proteins purified from cells, including bacteria [17], yeast [18], plant [19] or mammalian [20, 21]. The growth and purification of thousands of proteins from cells can be tedious and costly, and often the yields of such proteins can vary over several orders of magnitude, which is then reflected on the arrays. Moreover, the purification of the proteins from cells may involve steps that affect protein folding and activity [22]. An alternative strategy is the expression of proteins *in vitro* using various cell-free extracts that provides the transcriptional and translational machinery necessary for protein synthesis [23]. This approach has been used successfully in protein microarrays, where the proteins were expressed with extracts from *E. coli* [24, 25], wheat germ [26] or rabbit reticulocytes (RRL) [27–29].

In circumstances when the use of mammalian ribosomes is preferred, such as in the production of functional human proteins, RRL has historically been the only mammalian system available for protein expression *in vitro*; however, the yield of recombinant protein is the lowest among these systems [30]. Moreover, the manufacture of RRL entails isolating reticulocytes from rabbit blood, which led to a significant problem with batch-to-batch (i.e., animal-to-animal) variation. Users had to test several lots of product to find one that gave similar results to a previous lot. This was not only true with respect to protein production, but other entities in the samples obtained from some animals (such as the rabbits' own antibodies) may contaminate the final product and interfere with some assays, such as the

screening for immune responses. For example, we have observed marked differences in general background in immune studies from one RRL batch to the next (data not shown).

In order to address some of these limitations, a new cell free expression system has been developed that is produced from a human cell line (HeLa) called 1-Step Human Coupled IVT (HCIVT – Thermo Scientific) that has theoretical advantages over previous systems. HCIVT enables the expression of human proteins by human ribosomes and in the presence of human chaperones, increasing the likelihood for successful folding. In addition, the use of a cell line as the biological source offers the possibility of a much more stable and consistent extract than relying on different animals. Finally, there is much lower likelihood of contamination of the extract with immunoglobulins and thus a potential for a reduced background in immune assays.

Here we test the use of this novel source of human ribosomes in the production of protein microarrays to determine if it can outperform existing systems. We have chosen to use Nucleic Acid Programmable Protein Array (NAPPA) for this test. In NAPPA, cDNAs coding for the genes of interest fused to a carboxyl-terminal GST tag plus an antibody against the GST-tag are printed on the array. Printed slides are expressed at the time of use with an *in vitro* transcription and translation system (IVTT) and the newly synthesized proteins are captured by the anti-GST antibody (Figure 1A). The final product is a functional protein array displaying thousands of proteins for use in functional assays [29]. NAPPA arrays have been successfully implemented in cancer biomarker discovery for breast cancer [15, 31].

As a first test, four genes selected from our NAPPA collection [32], myosin heavy chain 9 (MYH9), collagen alpha 1 chain type I (Col1A1), cyclin-dependent kinase 2 (CDK2), and Fas were expressed in solution. The samples were prepared with 1 ug of plasmid DNA in a 20 µl reaction using either RRL or HCIVT, according to the manufacturers' instructions. The expression was carried out for 1h 30min at 30°C and the product was analyzed in a western blot probed with mouse anti-GST antibody (Cell Signaling Technology) followed by anti-mouse IgG, HRP-linked antibody (Cell Signaling Technology). The signal was detected with Super Signal Femto Maximum Sensitivity Substrate (Thermo Scientific). As indicated in Figure 1B, all of the genes yielded proteins of the appropriate size, with no signal in the negative controls (no DNA in the expression mix). The level of protein expressed with HCIVT was consistently higher. This difference was particularly notable for the large protein MYH9 (250 kDa including the GST tag), which showed no detectable product in the RRL reaction and a pronounced protein band with HCIVT.

The high quantity of protein produced by HCIVT in solution suggested that this reagent would be valuable for protein microarrays. Thus, NAPPA arrays with 2000 unique cDNAs were printed and the levels of protein displayed were measured using anti-GST antibody followed by cy3-labelled secondary antibody (Jackson ImmunoLab), as described previously [33]. The capture and detection antibodies were produced in distinct hosts and recognize distinct epitopes in the GST tag, allowing them to be used simultaneously. The transcription/translation was performed with RRL or HCIVT for 1h 30mins at 30°C and the images were obtained in the PowerScanner (Tecan) with 20% gain and 25% laser intensity.

Both expression systems led to broad display of nearly all proteins on the slides; however, when scanned at settings that are ideal for displaying protein for the RRL system, the signal intensity obtained with HCIVT was saturated (Figure 1C-insert). When the same slides were re-scanned at a lower scanning setting that prevented saturation of HCIVT (10% gain and 10% laser intensity), it was difficult to appreciate protein display on the RRL slide (Figure 1C).

The signal intensity of each slide was quantified by the Array-ProAnalyzer 6.3 (Tecan), using the default settings. Our data showed that the median signal for all the spots in the slides expressed with HCIVT was 10 times higher than the median for the slides generated by RRL (Figure 1D). Normalizing the slide median with the median signal of the purified mouse IgG, which is present in a fixed concentration across the different experiments, it is possible to compare the performance of both systems. In the HCIVT this ratio was 0.98, whereas in RRL it was 0.069, showing that the signals obtained with HCIVT were comparable with the purified mouse IgG and approximately 14 fold higher than RRL. Taken together these results corroborate our previous data, suggesting that the levels of protein displayed with HCIVT is considerably higher than the one obtained with RRL. Moreover, for the first time we generated an array with proteins expressed in a human milieu *in vitro*.

Next, we analyzed the reproducibility of distinct lots of HCIVT. As noted above, there is often poor reproducibility between independent lots of RRL as shown in Figure 1E, with R^2 as low as 0.5. By contrast, slides expressed with HCIVT from distinct lots showed a very high reproducibility with typical R^2 higher than 0.9. Technical replicates, which use the same batch of slides and reagents, have a typical R^2 of 0.92 – 0.98, independent of the expression system (data not shown). Due to the variability among lots of RRL, we usually test more than 5 distinct lots of RRL to find one with expression equivalent to our old lot, i.e. average array intensity higher than 10^6 . It is not uncommon to find lots with poor performance, with an average array intensity lower than 10^5 , whereas every lot of HCIVT tested has had comparable signals to all other lots. These results suggest that HCIVT is very robust with minimal lot-to-lot variation, probably due to the use of a cell line as the biological source.

In addition to testing general protein display using the common GST tag on all expressed proteins, we wanted to confirm that the same trend held up when we tested individual proteins using protein-specific antibodies. Arrays containing 100 human genes printed in quadruplicate were expressed with RRL or HCIVT and probed with 10 protein-specific antibodies (aldose reductase (AKR1B1), annexin A1 (ANXA1), chromogranin A (CHGA), crystallin alpha B (CRYAB), fatty acid binding protein 5 (FABP5), fascin 1, glucose-6-phosphate isomerase (GPI), Serpin B3, peroxiredoxin 4 (PRDX4), and stratifin, all from the Office of Cancer Clinical Proteomics Research Antibody Portal [34]), for which the antigens were present on the slide (Figure 2A). All the antibodies showed a stronger signal for their target protein on arrays expressed with HCIVT. In contrast, arrays expressed with RRL showed no reactivity for half of the tested antibodies (ANXA1, CHGA, FABP5, Fascin 1 and Stratifin), even when the slides were scanned using higher laser intensity (data not shown).

The signal intensity of the antibody response was measured and the Z-score of each array was calculated against the average and the standard deviation of the raw signal intensity of the gene population (positive and negative controls were excluded for the calculation of the Z-score). The values obtained for the specific antigen of a given antibody were plotted as a function of the expression system used in the array (Figure 2B). Using a Z-score of 5 as cutoff, only 5 out of 10 antibodies tested in arrays expressed with RRL showed signals above the cutoff, whereas all antibodies in HCIVT-expressed slides passed the threshold value. For all the samples tested, the Z-score in HCIVT slides were higher or comparable to the values obtained in RRL slides. These results suggest that the sensitivity of the arrays expressed with HCIVT is greatly enhanced.

Finally, we tested the response obtained in serum immune assays performed in NAPPA arrays expressed with RRL or HCIVT. Arrays with more than 2000 unique features from *Vibrio cholerae* or *Bacillus anthracis* were screened with serum from a patient exposed to the pathogen or the vaccine (Anthrax Vaccine Adsorbed- AVA), respectively. In the vaccine experiment, serum was available from the same patient both pre- and post-AVA vaccination. The serum was diluted 1:500 and incubated with the slides O/N at 4°C, followed by incubation with HRP-linked secondary anti-human IgG antibody (Jackson labs). The signal was detected with Tyramide signal amplification reagent (TSA, Perkin Elmer). In slides expressed with HCIVT and probed with serum from Cholera or post-AVA patients, the immune response was very strong and could easily be visualized above the background (Figure 3). Pre-AVA patients showed a monotonic response, with the only easily detectable antigen being Epstein-bar virus nuclear antigen 1 (EBNA), to which most adults produce antibodies due to latent EBV infection (Figure 3B). Thus, we routinely use EBNA as a positive control in our assays. In contrast with our findings in the HCIVT-slides, arrays expressed with RRL, regardless of the serum tested, had higher background signal, which made it difficult to detect specific responses visually. This pattern was observed for two independent sets of arrays and serums, suggesting that the increase in the S/N in arrays expressed with HCIVT does not depend upon the biological model. Taken together, these data show that the level of protein displayed in HCIVT-expressed arrays is not only higher, but also that specific immunogenic responses are easier to detect, making it the preferred system for immune assays.

In this present work we showed, for the first time, the production of a protein microarray with proteins expressed in human milieu. We demonstrated that HCIVT system is highly efficient for protein expression and the levels obtained were more than 10 times higher than RRL. Moreover, HCIVT was the only *in vitro* mammalian expression system able to express MYH9, a 250kDa protein. NAPPA arrays with cDNA from human, *Vibrio cholerae* or *Bacillus anthracis* were successfully expressed with HCIVT and the increase in protein levels combined with the protein expression in human milieu resulted in an enhanced sensitivity on the arrays. Several antibody responses not detected in RRL expressed slides were clearly detected in arrays expressed with HCIVT. We believe NAPPA coupled with HCIVT will provide a unique platform for the study of biomarkers for human diseases and the increased sensitivity will allow the identification of immune response even for autoantibodies present in low concentration in the serum.

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Abbreviations

AKR1B1	aldose reductase
ANXA1	annexin A1
AVA	Anthrax Vaccine Adsorbed
CDK2	cyclin-dependent kinase 2
CHGA	chromogranin A
Col1A1	collagen alpha 1 chain type I
CRYAB	crystallin alpha B
EBNA	Epstein-bar virus nuclear antigen 1
FABP5	fatty acid binding protein 5
GPI	glucose-6-phosphate isomerase
HCIVT	1-Step Human Coupled IVT
IVTT	<i>in vitro</i> transcription and translation system
MYH9	myosin heavy chain 9
NAPPA	Nucleic Acids Programmable Protein Array
PRDX4	peroxiredoxin 4
RRL	Rabbit Reticulocyte Lysate
TSA	Tyramide signal amplification

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Figure 1. Analysis of protein expression using RRL or HCIVT in Western Blot and NAPPA arrays

(A) Schematic representation of Nucleic Acids Programmable Protein Array (NAPPA). (B) *In vitro* transcription and translation of plasmids coding for the genes MYH9, Col1A1, Cdk2 or Fas by RRL or HCIVT. The product was analyzed by western blot with anti-GST antibody. (C) NAPPA arrays with more than 2000 cDNAs were expressed using RRL or HCIVT. The level of protein displayed on the array was measured using anti-GST antibody followed by a cy3-labeled secondary antibody. The inserts represent images obtained using higher sensitivity scanning settings (D) Box-and-whisker plot of the background-corrected intensities for each array.(E) Scatter plot of identical slides expressed with distinct lots of RRL or HCIVT.

Figure 2. Effect of the expression system on the antibody response in NAPPAs arrays
(A) NAPPAs arrays containing 100 unique human genes printed in quadruplicate were expressed with RRL or HCIVT, hybridized with primary antibodies (anti-AKR1B1, ANXA1, CHGA, CRYAB, FABP5, Fascin 1, GPI, Serpin B3, PRDX4, Stratifin or GST) followed by cy3-labeled secondary antibody. Images obtained for 2 of the protein-specific antibodies are shown. All images were acquired using the same settings, with the exception of the anti-GST signal for the array expressed with HCIVT, where the laser power was decreased to prevent saturation. Spots inside the yellow boxes are purified mouse IgG (positive control) and the arrows indicate the antigen spot specific for the antibody tested.
(B) The signal intensity of NAPPAs arrays was acquired using PowerScanner (Tecan) and Array-ProAnalyzer 6.3 (Tecan). The Z-score of each gene was measured against the average and standard deviation of the raw signal intensity for all gene spots. The dotted line represents the cutoff of 5.

Figure 3. Serum immune response in NAPPA arrays expressed with HCIVT or RRL

Two independent sets of arrays, each comprising 2000 unique genes from *Vibrio cholerae* (A) or *Bacillus anthracis* (B–C), were expressed with HCIVT or RRL. Arrays were probed with serum from patients exposed to *Vibrio cholerae* (A), pre-AVA vaccination (B) or post-AVA vaccination (C). The immune profile was revealed with HRP-labeled secondary antibody followed by TSA for detection. Red arrows show potential new antigens and yellow arrows point to EBNA (printed in triplicate).