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Recombinant viral proteins for use in diagnostic ELISAs to detect virus infection

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Abstract

ELISAs provide a valuable tool in the detection and diagnosis of virus infection. The ability to produce recombinant viral proteins will ensure that future ELISAs are safe, specific and rapid. This latter point being the most crucial advantage in that even if a virus cannot be cultured, provided gene sequence is available, it is possible to rapidly respond to emerging viruses and new viral strains of existing pathogens. Indeed, ELISAs based on peptides (corresponding to epitopes) also hold great promise, as in this case no cloning or expression of a recombinant protein is required. Both recombinant protein and peptide based systems lend themselves to large scale production and purification. These approaches can also be used to distinguish recombinant vaccines from parental or wild type viruses.
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1. Introduction – detecting virus infection

Constant monitoring to determine virus exposure and to evaluate vaccine efficacy play an important role in controlling disease outbreaks. Several assays can be used to detect the presence of virus. Viral genomes and gene products can be detected using RT-PCR [1,2] and genetic micro-arrays [3,4]. Whereas virus neutralization, haemagglutination inhibition and enzyme-linked immunosorbent assay (ELISA) have been used for the detection of viral specific antibodies or viral proteins [5]. In several cases combinations of both genome and protein detection methods can be used as is the case with a combined RT-PCR-ELISA approach to identify and differentiate alphavirus infections [6].

However, as a rapid, simple and sensitive method, ELISA has been widely used in the serological profiling of viruses and many commercial kits are available. In general, inactivated virus was used as the coating antigen and whole virus applied in this way can detect antibodies against a number of viral structural proteins, which, depending on the virus, can include surface glycoproteins and/or viral nucleic acid binding proteins, which are generally termed capsid, nucleocapsid or nucleoproteins.

2. An overview of ELISA

ELISAs can be viewed as combining the specificity of antibodies and antigens with the sensitivity of simple enzyme assays coupled to an easily-assayed enzyme. Apart from detecting these specific molecules they may also provide a useful measurement of their concentration. The ELISA has high sensitivity in terms of ligand detection limits and can be viewed as a primary binding assay with an objective data output that can be automated for rapid, high throughput or alternatively run on a small scale. There are two main variations on this method. ELISA can be used to detect

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the presence of antigens that are recognised by an antibody [7] or it can be used to test for antibodies that recognise an antigen [8]. Setting up an ELISA involves a number of different steps including; coating the micro-titre plate wells with antigen/antibody; blocking all unbound sites to prevent false positive results; adding antibody/antigen to the wells (depending on the detection strategy); addition of a specific antibody conjugated to an enzyme; and reaction of a substrate with the enzyme to produce a soluble colour product, thus indicating a positive reaction. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction.

2.1. Obtaining viral proteins for ELISA

Detecting specific antibodies against an antigen has traditionally involved virus propagation and purification as a source of viral protein. However, whilst many viruses can be grown in cell culture or eggs, they may not grow to sufficient titre to produce enough protein for purification, coupled with the difficulties associated with purifying native un-tagged protein. Likewise some viruses cannot be cultured, yet gene sequence is available for their proteins. Therefore, given these potential limitations, recombinant protein expression technology has the potential to provide a reliable source of antigens but has not yet been widely used in ELISA kits for the diagnosis of viral disease.

2.2. Selecting recombinant proteins for ELISA

Several factors should be taken into consideration when selecting suitable antigens for protein-based expression, including specificity and post-translational modifications. The key factor being that the protein can detect antibody induced by natural or vaccine immunogens. When developing protein-based ELISA systems for the detection of viruses, as with preparing proteins from purified virus, several candidate proteins can be considered such as the surface glycoproteins and capsid proteins. Both have distinct advantages and disadvantages. Surface glycoproteins tend to be the major inducers of neutralizing antibodies and therefore the host is likely to produce antibodies to these proteins. However, because such proteins are modified post-translation with the addition of sugar moieties, the correctly folded protein can only be produced in a eukaryotic system. Additionally, given their role in virus attachment and the fact that they provoke a large antibody response these proteins are highly variable, and as such antibodies to them may only recognise certain strains of the same virus family. On the other hand the role of nucleocapsid proteins in binding to the virus genome is fundamental to the success of the virus life cycle. When considering RNA viruses, given the constraints on RNA sequence in terms of structure and function, such proteins tend to be more conserved between divergent strains than the proteins encoding surface glycoproteins. Therefore, a nucleocapsid protein from one strain when used in recombinant protein-based ELISA should be recognised not only by antibodies raised against itself but also by antibodies raised against nucleocapsid proteins from other strains of the same virus. Recombinant nucleocapsid proteins from different viruses have been used in a wide variety of ELISAs. For example, the Nipah virus nucleocapsid protein [9], the coronavirus nucleocapsid protein [8,10–13], the hepatitis C virus core protein [14] and even peptides [15,16] or sub-regions of these proteins can be used [17,18]. Therefore, careful selection of recombinant proteins can lead to the generation of both generic virus and strain-specific ELISAs. For example, a peptide ELISA based upon the ORF4 envelope glycoprotein of porcine reproductive and respiratory syndrome virus (PRRSV) can be used to differentiate between virus strains [19].

3. Protein expression strategies

There are several options for expressing recombinant proteins for ELISA and these depend on how much yield is required per unit volume of culture and perhaps more crucially whether post-translational modifications are required for antigen specificity. One of the most commonly used expression systems in use is the bacterium *E. coli*. Proteins can be produced in large quantities due to the robust nature of the culture conditions; however, these proteins are not post-transitionally modified. Alternatively, the protein can be produced in insect cells utilising baculovirus expression vectors which encode the recombinant protein of interest [20]. Here, the protein is post-translationally modified and typical yields of the recombinant protein can be similar to those from *E. coli* [21]. The only caveat with insect cells such as SF9 cells is that certain post-translational modifications will be different than if the protein were expressed in mammalian cells [22,23]. This is especially true of glycosylation since in insect cells the number of glycan residues added is typically less than mammalian cells.

Recombinant proteins are phosphorylated in insect cells and at least in the case of the coronavirus infectious bronchitis virus (IBV) nucleocapsid protein the sites of phosphorylation are the same as in mammalian or equivalent cells [24]. Obviously, protein expression in species-specific cells is optimal. However, typical protein yield can be substantially lower than either in *E. coli* or insect cells. This is probably reflected in the fact that both *E. coli* and Sf9 cells can be grown easily in suspension culture whereas this can be problematic for mammalian cells.

In general most recombinant proteins used in diagnostic ELISA are synthesized in either *E. coli* or insect cells. Although post-translational modification should always be considered in some cases protein produced in *E. coli*, and therefore not modified, is a more effective antigen than the protein produced in the eukaryotic cell systems, e.g. insect cells [8]. As discussed, the modifications added by insect cells to exogenous proteins may be different from those added in the native infected cells [25] and may therefore affect their

ability to efficiently distinguish between different viral strains [8]. For example, in the case of PRRSV this is particularly relevant when considering the N-linked glycosylaiton of the GP5 protein, in which the loss of glycan residues in the ectodomain of GP5 enhances both the sensitivity of these viruses to *in vitro* neutralization and the immunogenicity of the nearby neutralization epitope [26].

If a protein is toxic for the three expression systems described, or there are too many rare codons, or its gene cannot be cloned then *in vitro* (cell free) protein expression systems can be used [27–29]. One such system is Roche's ProteoMaster which is designed for optimum cell free protein expression utilising simultaneous transcription and translation. Proteins for expression can either be cloned into an appropriate expression vector or templates synthesized by PCR, in this latter instance a prokaryotic promoter (e.g. the T7 promoter), affinity tags and other features are incorporated in non-templated primers. The cell free expression system utilises translation competent extracts from either *E. coli* or wheat germ, and different amounts of protein can be produced. In large scale preparations, the reaction chamber is divided in two. One which contains is a reaction compartment for coupled transcription and translation from the translation extract, and the other a feeding compartment for substrates and energy components, thus simplifying purification of the recombinant protein. Up to 150 mg of protein can be produced in 24 h, although currently the cost per mg is greater than conventional expression in *E. coli*. The ProteoMaster itself provides optimal protein expression conditions by regulating the reaction temperature and by smooth shaking that supports the continuous exchange between reaction and feeding chamber.

An alternative to recombinant protein expression is the use of peptides as candidate diagnostic antigens (or even vaccine immunogens). These may be especially useful where all cloning and expression attempts have failed. Suitable epitopes (and hence candidate peptides) may have been identified experimentally [30–34]. Alternatively, several prediction methodologies are available that allow the analysis of a given protein sequence for potential epitopes [35–38]. The disadvantage of a peptide based approach over producing the whole recombinant protein is that the epitopes have to be mapped and evaluated in ELISA, which can require time and expense. Although as with recombinant proteins, several peptides from the same of different antigens could be used to improve sensitivity. In general peptide synthesis can achieve lengths of 30 amino acids, which is normally enough to cover an epitope. The advantage of using peptides is the ease of synthesis and purity of preparation.

4. Protein purification strategies

As previously described, extracting native protein from mature virions gives rise to a large number of practical problems, namely producing high enough titres of virus stocks from which to extract protein. Recombinant proteins can circumvent these problems but several factors should be taken into consideration.

4.1. Affinity tags

The use of affinity tags to facilitate purification, aid in solubility and allow for simple detection is well documented [39–41]. Affinity tags are normally (but not necessarily) short amino acid motifs which have a high affinity for an antibody or metal, which can be used to facilitate protein purification or identification in a cell – e.g. using immunofluoresence. They are added N- or C-terminally to a protein during the cloning step. For example, large soluble tags placed N-terminally to the protein of interest can greatly increase solubility and enhance expression as the ribosome will encounter the tag first, allowing it to begin elongation before reaching the gene to be translated [42]. Indeed some studies indicate that addition of a his-tag increases expression in *E. coli* [43]. N-terminal tags do however confer a major disadvantage when attempting to purify expressed protein. Expression of an exogenous protein often leads to the production of truncated peptides due to premature termination of translation caused by rare codons and inefficient translation due to the activation of host stress responses. When expressed with an N-terminal tag these truncated products can be affinity purified alongside full-length protein and can be to difficult to remove even by size-exclusion methods, as they are often very similar in size to full-length product. Whereas affinity purification via a C-terminal tag can more easily be used to determine whether any protein bound to the affinity matrix is full length (if no other antibodies are available) [8]. Double tag purification systems may overcome this difficulty [44].

Polyhistidine tags provide a convenient means of purifying recombinant protein under non-denaturing conditions, which leads to the production of correctly folded protein. Immobilised metal-affinity chromatography relies on the stable interactions between histidine and a metal such as nickel or zinc [45]. This interaction is extremely stable, owing to electron donor groups on the imidazole ring of histidine forming coordination bonds with the zinc. Due to their small size, often just six amino acids in length or sometimes placed in tandem [46], histidine tags are thought to have little effect on the overall structure of the purified protein (over half of the proteins on the protein data base contain a his-tag), alleviating the requirement for cleavage post-purification.

Due to the extensive use of histidine tags there are a many commercially available purification systems, each of which have slightly differing properties and may cause variations in binding affinity of recombinant protein for the charged matrix. Despite these systems being based on the same basic principle, these variations can preclude efficient purification of recombinant protein and thorough investigation into a number of products is required to achieve optimal purification. The major disadvantage of utilising a histidine tag in

purification is the existence of host cell proteins that may contain a number of adjacent histidines, these proteins also have high affinity for the transition metal matrix and are often affinity purified with the protein of interest [47]. This may lead to the requirement of a two-step purification strategy [42] in which the affinity purification is followed by a size-exclusion or ionic exchange chromatography step. This need for further purification following histidine affinity purification can be time consuming and may lead to reductions in the yield of purified protein produced. A number of larger tags which confer greater specificity, such as glutathione S transferase (GST) [48,49], alleviate this requirement for a second step to remove contaminating proteins, as the occurrence of host proteins with similar domains is uncommon. These larger tags do however necessitate a second step in which the large tag is cleaved and removed by a further round of affinity purification. Therefore, if the smaller and more convenient histidine tag is suitable then its use allows rapid and simple purification of recombinant protein to be achieved.

4.2. Rare codon usage

One difficulty often encountered when expressing an exogenous protein in bacterial systems is the existence of rare codons. These rare codons occur when the mRNA of the protein to be expressed contains codons for which the host only has a small percentage of tRNAs. For example, the tRNA AGA is responsible for over 15% of arginines in a mammalian cell compared to only 2.6% in *E. coli*. When rare codons occur within the gene of interest at a high frequency the resulting protein product is often truncated [50], due to translation stuttering or stopping at each codon for which the host doesn't have sufficient amino-acyl tRNAs [51]. Many systems are available for addressing this problem such as the use of specific *E. coli* strains, cell free systems and plasmids encoding rare tRNAs [52,53].

4.3. Avoiding aggregation of recombinant protein

Recombinant protein expression is used to obtain a high degree of accumulation of soluble product in *E. coli*. This over expression is not always well tolerated by the metabolic system of the host and in some situations a cellular stress response is encountered. One phenomenon to be avoided during over expression is the accumulation of over expressed proteins into insoluble aggregates known as inclusion bodies. These aggregated proteins are in general misfolded and thus biologically inactive. This can be avoided in part by slowing down expression rate using low temperatures, indeed, the expression and activity of a number of *E. coli* chaperones is increased at 30 °C [54,55] hence increasing protein folding efficiency and reducing precipitation into inclusion bodies. Expression can also be slowed by induction with low concentrations of isopropyl β-D-1-thiogalactopyranoside (IPTG).

5. The nidovirus (coronavirus and arterivirus) nucleocapsid protein as a specific example for use in ELISA

Coronaviruses cause a wide variety of disease, but can be viewed as principally causing either respiratory and/or gastro-intestinal infection. In terms of economic and health importance, three of the most important pathogens are IBV of chickens, transmissible gastroenteritis virus (TGEV) of pigs and severe acute respiratory syndrome coronavirus (SARS-CoV) of humans [56,57]. Serological monitoring for IBV is commonplace. During the 2003 SARS-CoV outbreak the ability to rapidly and accurately identify individuals infected with the virus was paramount (as early clinical symptoms were similar to influenza virus, yet the outcomes were very different – approximately a 10% mortality with SARS-CoV).

A number of wide ranging studies have involved the purification of coronavirus nucleocapsid proteins. Many of them utilising recombinant techniques [24,58]. Others used an immuno-precipitation approach in which native protein was isolated from infected cells using protein specific antibodies [59]. This technique has the major advantage of allowing purification of native protein from infected cells; however yields are extremely low compared to recombinant production, and bound antibody difficult to remove without denaturing the protein. In-gel purification, followed by mass spectroscopic identification also allows the isolation of native protein from infected cell lysate [60] but again leads to extremely low yields, in the nanomolar range, proving unsuitable for ELISA.

In order to generate recombinant nucleocapsid protein a wide variety of commercially available expression vectors, many of which have specialised functions such as antibiotic selection, rare codon expression, temperature sensitivity and host specificity can be used. For example, the gene sequence for IBV nucleocapsid protein was cloned into pTriEx1.1 (Novagen) a vector that allows expression of protein in *E. coli*, mammalian and insect (SF9) cells, upstream of a hexa-his-tag to generate vector pTriEx_IBV_N [61]. This vector can be utilised to express unmodified protein in *E. coli*, and post-translationally modified protein in mammalian or Sf9 cells to allow for in-depth functional analysis [24] as well as producing recombinant proteins for use in ELISA [8].

During purification and upon subsequent storage and handling of purified IBV N protein degradation products were formed, these lower weight proteins are recognised by anti-IBV antibodies but not by anti-his antibodies. This phenomenon is common with many proteins can be slowed in part by short term storage and reduced handling purified proteins. A number of research groups have used a stable fragment obtained through cleavage by *E. coli* proteases comprising of N-terminal residues from both IBV and SARS-CoV nucleocapsid proteins for structural studies [62,63].

Nucleocapsid protein has proved to be an excellent diagnostic reagent in combination with ELISA. Comparison using *E. coli* expressed SARS-CoV spike glycoprotein (a virion surface glycoprotein) versus the nucleocapsid protein indicated that the latter protein was a better candidate antigen for use in diagnostic ELISA [64]. However, subsequent research suggested that nucleoprotein-based ELISA lead to the false positive detection of two human coronaviruses (HCoV), HCoV-OC43 and HCoV-229E and that the additional use of a recombinant SARS-CoV spike protein western blot analysis differentiated between the different viruses [65]. Indeed false positives may be caused by the use of bacterial expressed antigens [66] with incorrect protein folding (and hence epitope presentation) through lack of suitable post-translational modification.

The combination of detecting two existing serotypes in only one ELISA based on the joint use of recombinant nucleoproteins from both type 1 and type 2 PRRSV strains permitted the development of a single, universal serologic tests for PRRSV. However, one potential problem with using recombinant nucleocapsid protein in this respect is that the catabolic rate of the nucleocapsid specific antibodies tends to be shorter than that of the antibody to the envelope glycoproteins during the long persistent infection of PRRSV. This determines that many animals in a herd become negative first by a nucleocapsid ELISA than when using a glycoprotein-based ELISA.

6. Recombinant protein-based ELISA – future perspectives

ELISAs provide a valuable tool in the detection and diagnosis of virus infection. The ability to produced recombinant proteins will ensure that future ELISAs are safe (as there is no live virus), low background (only virus antigens are present) and specific (single viral proteins can be used). The ability to synthesize recombinant proteins (or peptides) in the laboratory allows easy scale up for the commercial manufacture of ELISA kits [8]. The clear advantage of recombinant protein/peptide is that high quality assurance is clear, especially with peptides as these can be manufactured synthetically, in the absence of any other proteins. This latter point being the most crucial advantage of recombinant based proteins ELISAs in that even if a virus cannot be cultured, provided gene sequence is available, it is possible to rapidly respond to emerging viruses and new viral strains of existing pathogens. Combinations of peptides or chimeric recombinant proteins should allow the development of strain-specific and generic detection reagents. This is especially true in the case of differentiating vaccines based on recombinant (genetically manipulated) viruses. In this case virus proteins are modified to contain an exogenous epitope/tag, or a foreign protein is expressed in entirety. Thus because antibodies are raised against the tags, recombinant vaccine strains which can be readily distinguished from wild type and parental strains using recombinant protein/peptide based ELISA [43,67,68].

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