CHAPTER 3

RESULTS

In an earlier study, rWbSXP-1 was identified as a suitable immuno-diagnostic candidate (Rao et al 2000). The rapid antibody flow through kit was developed to suit field conditions using finger prick blood samples collected on filter paper (Basker et al 2004). The ‘MF-Signal: rapid kit format’ was originally developed under the memorandum of understanding between Centre for Biotechnology Anna University, Chennai and SPAN Diagnostics Ltd., Surat, Gujarat, India.

Since the focus of the proposed work was on the application of protein for industrial level production of diagnostic kit, an attempt was made to optimize the conditions for large-scale protein expression and purification. In this work the recombinant Wuchereria bancrofti antigen SXP-1 was expressed in the salt inducible recombinant E.coli GJ1158 (referred as GJ1158) harboring pRSETB:WbSXP-1. The purified protein was used for production of a rapid antibody flow through test kit for diagnosis of Human Lymphatic Filariasis.

3.1 RECOMBINANT FILARIAL CLONE \( pRSETB:WbSXP-1 \) USED IN PRESENT STUDY

An orthologue of BmSXP-1 was identified from W. bancrofti L3 cDNA library using BmSXP-1 specific DNA primers as a probe (Rao et al 2000). The identified WbSXP-1 gene (Accession no. AF098861) was cloned
in EcoRI site of pRSETB vector and was expressed in E.coli BL21 to derive the diagnostic antigen rWbSXP-1 (Rao et al 2000). The identification of WbSXP-1 set a platform for the development of a specific diagnostic method to detect both Brugian and Bancroftian filariasis. In a previous study, development of rWbSXP-1 specific ELISA with predominantly IgG4 antibodies and a detection method for circulating filarial antigen in bancroftian and brugian filariasis using monospecific polyclonal antibodies were demonstrated (Lalitha et al 2002). Further, the rapid flow through antibody assay kit developed using the WbSXP-1 antigen underwent national (Basker et al 2004) and global evaluation (Lammie et al 2004).

The antibody test kit underwent field trials and showed 91 % sensitivity and 100 % specificity (Basker et al 2004, Lammie et al 2004). The technology transfer for production, quality control and application of recombinant antigen rWbSXP-1 was done as per the MoU between CBT, Anna University and Span Diagnostics Ltd. Considering an imminent requirement for large scale application of rWbSXP-1 antigen, there arises a need for economic production of the protein. In this scenario, the osmotically induced prokaryotic host E.coli GJ1158 was specifically preferred since the recombinant expression can be induced by using NaCl (Bhandari et al 1997). The plasmid construct pRSETB:WbSXP-1 was confirmed for the specific gene insert and transformed into E.coli GJ1158 host.

The objective of this research work is to study the suitability of E.coli GJ1158 host for development of methodology for large-scale production of rWbSXP-1, development of a suitable purification strategy and optimization of diagnostic kit for higher sensitivity. The purified antigen was also used for development of a monoclonal antibodies for the detection of circulating WbSXP-1 antigen in both bancroftian as well as brugian filariasis. The results of the research work are herewith presented.
3.1.1 Confirmation of recombinant clone rWbSXP-1

3.1.1.1 Restriction analysis of rWbSXP-1

As a prerequisite to confirm the recombinant clone, prior to expression studies in \textit{E.coli}, restriction analysis of the clone \textit{pRSETB: WbSXP-1} was carried out by digesting with EcoRI enzyme. This resulted in the release of the 726 bp pop out and the 2.9 kb linearized \textit{pRSETB} vector back bone, confirming the presence of the inserted gene (Figure 3.1).

![Restriction analysis of pRSETB:WbSXP](image)

**Figure 3.1** Restriction analysis of \textit{pRSETB:WbSXP} using \textit{EcoR I} and \textit{Pst I} enzyme

Approximately 2 µg of recombinant \textit{pRSETB: WbSXP-1} plasmid DNA was digested with \textit{EcoRI} and \textit{Pst I} and resolved on 1% agarose gel. Lane 1: 100bp ladder, Lane 2: \textit{pRSETB: WbSXP-1} undigested, Lane 3: \textit{Pst I} digest of \textit{pRSETB: WbSXP-1} showing linearization (3.6 kb), Lane 4: \textit{EcoRI} digest of \textit{pRSETB: WbSXP-1} showing the popout of 726bp and linearized 2.9 kb vector, Lane 5: \textit{λ Hind III} digest.
3.1.1.2 PCR analysis of *E. coli* GJ 1158 transformants

Transformation of *pRSETB*: *WbSXP*-1 plasmid into *E. coli* GJ1158 host was carried out using CaCl2 method. The transformation efficiency was estimated to be an average 45 colonies per μg of plasmid. Lysate-PCR of the transformants was carried out using *WbSXP*-1-specific forward and reverse primers. The amplification profile of the gene showed 573 bp, thereby confirming the SXP gene insert (Figure 3.2).

![Agarose gel pattern of PCR products amplified from E.coli GJ1158 transformants using SXP specific primers. Lane 1:100 bp ladder, Lane 2: positive control (previously tested pRSETB: WbSXP-1) Lanes 3-6: Transformants and Lane 7: negative control contains vector DNA.](image)

**Figure 3.2** Characterization of *pRSETB*: *WbSXP* by PCR

Agarose gel (1%) electrophoresis pattern of PCR products amplified from *E. coli* GJ1158 transformants using SXP specific primers. Lane 1:100 bp ladder, Lane 2: positive control (previously tested *pRSETB*: *WbSXP*-1) Lanes 3-6: Transformants and Lane 7: negative control contains vector DNA.

3.1.1.3 Comparison of Expression profile of Recombinant *E. coli* GJ1158 & BL21 *pRSETB*: *WbSXP*-1 Cultivated in LB media

The expression of the recombinant *pRSETB*: *WbSXP*-1 was compared in *E. coli* BL21 and *E. coli* GJ1158 hosts. The expression of the recombinant protein *WbSXP*-1 was studied under uninduced and induced conditions. The recombinant *WbSXP*-1 was expressed as a 26 KDa fusion
protein with 6(x) histidine tag. *E.coli* hosts containing only the *pRSETB* vector (without insert), induced under the same conditions were used as a control. In BL21, a low leaky expression of r*WbSXP*-1 was observed without induction, because the T7 vectors are prone for basal level expression of T7RNAP. However, an enhanced expression of r*WbSXP*-1 was clearly observed in BL21 after induction for 3 hours with IPTG at a final concentration of 1mM (Figure 3.3).

![Figure 3.3 SDS-PAGE analysis of pRSETB:WbSXP-1 protein expressed in E.coli BL21 with IPTG.](image)

**Figure 3.3** SDS-PAGE analysis of *pRSETB:WbSXP-1* protein expressed in *E.coli* BL21 with IPTG. Total protein extracts from recombinant *pRSETB:WbSXP-1* and control *pRSET B* were solubilized in 1 X SSB, separated on 12% SDS-PAGE gel and stained with CBB dye. 50 μg of protein was loaded in each of the respective lanes. Lane1: molecular weight marker (97, 66, 44, 30, 20, 14 kDa). Lane 2: BL21 Host induced. Lane 3: Uninduced BL21 with *pRSETB* vector without insert. Lane 4: Induced BL21 with *pRSETB* vector without insert Lane 5: BL21 with *pRSETB*: r*WbSXP*-1 uninduced >2.0 OD$_{600}$,Lane 6-8: BL21 with *pRSETB*: r*WbSXP*-1 induced. The recombinant protein expressed from the clone *pRSETB:WbSXP-1* is approx. 26kDa in size.
A similar expression study in *E.coli* GJ1158 was carried out and the protein profile of the batch course is shown in figure 3.4. Interestingly, in GJ1158 the T7 RNAP is under the control of ProUp promoter which is highly osmoreponsive. Consequently, there was low leaky expression of rWbSXP-1 observed without the addition of inducer (NaCl). The induction was performed using 250 mM NaCl for 3 hours and an enhanced expression of rWbSXP-1 was clearly observed.

**Figure: 3.4** SDS-PAGE analysis of recombinant protein expression profile of osmotically (NaCl) induced *E.coli* GJ1158. Total protein extracts from recombinant pRSETB:WbSXP-1 and control were solubilised in 1 X SSB, separated on 12% SDS-PAGE gel and stained with CBB dye. 50 µg of protein was loaded in each of the respective lanes. Lane 1: molecular weight marker. Lane 2: GJ1158 Host induced. Lane 3: Induced GJ1158 with pRSETB vector without insert. Lanes 4-7 and 9-11: GJ1158 with pRSETB: rWbSXP-1 induced at 0.8 OD<sub>600</sub>. Lane 8 – GJ1158 with pRSETB: rWbSXP-1 uninduced at 1.6 OD<sub>600</sub>.
3.1.1.3.1 Consideration of M9 media for Expression of Recombinant 

*E. coli* GJ1158 *pRSETB: WbSXP-1*

Since the purpose of this work is to design large cultivation, the experiments and media used were other than the LB media, which is primarily meant for lab level low-density cultivation. Growth in LB (without NaCl) and M9 basal medium were initially compared in terms of the maximal biomass attained and expression of recombinant protein levels in 3.0 L bioreactor. Though the expression patterns and maximal biomass were similar, in both the growth media, the duration of expression and quantity of protein expressed was higher in basal M9 medium due to slower growth rate in presence of glucose. *E. coli* has been reported to adjust to slower growth rates during cultivation (Natarajan et al 2002). In addition, the offline measurability of glucose made it easy for further study.

3.1.2 Optimization of M9 Media by Pulse Shift Experiment

The purpose of selecting GJ1158 is to take advantage of the NaCl induction as it works out to be economical as compared to IPTG. However, GJ1158 showed high basal level expression, which is an undesirable factor for high cell density cultivation. Thus, initial experiments were designed to modify M9 media in order to chart out the effect of media components on cultivation of GJ1158. The composition of standard M9 media (without NaCl) was used. Since the use of glucose for *E. coli* cultivation requires pH adjustment due to production of acids, all the optimization experiments were done in KLF 2000 with working volume of 2 litres.

Continuous culture experiments with GJ1158 were designed and carried out to optimize the M9 basal medium further. Continuous culture was established at dilution rates of 0.3 h\(^{-1}\) and 0.5 h\(^{-1}\). On reaching steady state at respective dilutions, different media components were pulsed into the
cultivation. The responses in terms of biomass (OD600) and dissolved oxygen (%pO2) were recorded every 10 min. and final response was derived (Table 3.1).

Table 3.1 Pulse shift experiment performed using different media components during continuous culture of recombinant E.coli GJ1158.

<table>
<thead>
<tr>
<th>Components Pulsed</th>
<th>Concentration</th>
<th>OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>pO2 %</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Extract</td>
<td>1 g</td>
<td>+++</td>
<td>--</td>
<td>Positive</td>
</tr>
<tr>
<td>Glucose</td>
<td>2 g</td>
<td>+++</td>
<td>---</td>
<td>Positive</td>
</tr>
<tr>
<td>Vitamin B Mix</td>
<td>10 mg</td>
<td>+</td>
<td>-</td>
<td>Positive</td>
</tr>
<tr>
<td>Trace metal mix [7]</td>
<td>1 ml</td>
<td>+</td>
<td>-</td>
<td>Positive</td>
</tr>
<tr>
<td>Thiamine (B1)</td>
<td>25 mg</td>
<td>+</td>
<td>-</td>
<td>Positive</td>
</tr>
<tr>
<td>Biotin</td>
<td>10 mg</td>
<td>+</td>
<td>-</td>
<td>Positive</td>
</tr>
<tr>
<td>Amino acids (based on pathway)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>10 mg</td>
<td>+</td>
<td>-</td>
<td>Positive</td>
</tr>
<tr>
<td>Serine group</td>
<td>10 mg</td>
<td></td>
<td></td>
<td>Neutral</td>
</tr>
<tr>
<td>Alanine group</td>
<td>10 mg</td>
<td>+</td>
<td>-</td>
<td>Positive</td>
</tr>
<tr>
<td>Glutamate group</td>
<td>10 mg</td>
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<td>N</td>
<td>N</td>
<td>Neutral</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>100 mg/ml</td>
<td>N</td>
<td>N</td>
<td>Neutral</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>200 mg/ml</td>
<td>-</td>
<td>+</td>
<td>Negative</td>
</tr>
<tr>
<td>NaOH (100 mM)</td>
<td>0.5 ml/L</td>
<td></td>
<td></td>
<td>Neutral</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt; (100 mM)</td>
<td>0.5 ml/L</td>
<td></td>
<td></td>
<td>Neutral</td>
</tr>
<tr>
<td>10% Ammonia</td>
<td>0.5 ml/L</td>
<td>+</td>
<td>-</td>
<td>Positive</td>
</tr>
<tr>
<td>10% Ortho-phosphoric acid</td>
<td>0.5 ml/L</td>
<td>+</td>
<td>-</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Table 3.1 response of GJ1158 to various media constituents during steady state: (D = of 0.3 h<sup>-1</sup> and 0.5 h<sup>-1</sup>); Filter sterilized 5 ml suspensions were pulsed. The (+/-) is a relative response showing the extent of increase or decrease respectively in OD<sub>600</sub> or pO2%. N is neutral response. At both dilution rates, the responses had a similar trend.

The media components that induced a positive response were added to growth medium and further optimised. The neutral response to 5 groups of
amino acid tested shows that the cells are not starved. Though histidine, alanine, B-vitamins and biotin were found to show a slight positive response independently, in the presence of complex Yeast extract in growth medium the responses to addition of amino acids and vitamins were neutral. The addition of trace metals showed positive trend towards enhancing growth. For pH adjustment during cultivation, ammonia and ortho-phosphoric acid were preferred for control instead of NaOH and $\text{H}_2\text{SO}_4$.

The biomass and substrate levels at various dilution rates during continuous culture is plotted as XsD diagram, Figure 3.5. The maximal growth rate for recombinant GJ1158 was estimated to be 0.7 h$^{-1}$ during which wash out occurred.

![Figure 3.5](image)

**Figure 3.5** The XsD diagram of continuous culture of *E.coli* GJ1158 in M9 media supplemented with glucose. The diagram shows wash out occurred at dilution 0.7 h$^{-1}$. Since at steady state $D=\mu$, The maximal growth rate is also 0.7 h$^{-1}$. M9 salts medium with 3 g L$^{-1}$ Glucose. Mean values of triplicate are represented.
3.1.3 **Optimization of *E.coli* Growth Media and Cultivation**

Key metabolic genes and their response to growth and protein over-expression are the cause of different glucose utilization pathways in recombinant *E.coli*. Therefore, based on the pulse shift experiment results, the cultivation of *E.coli* in M9 medium with varying glucose levels with and without the yeast extract was studied. The comparison of GJ1158 growth in different batches in terms of variation in biomass in response to different glucose concentration and yeast extract in M9 medium is shown in Figure 3.6.

![Optimization of Media](image)

**Figure 3.6** **Representation of Biomass (OD<sub>600</sub>) and (DW-g/L) in batch:**

with respect to variation in glucose. The biomass shows an increasing trend with increase in glucose (g L<sup>-1</sup>). Concentrations of glucose and yeast extract in basal M9 medium. **Batch 1:** M9/Gluc. 4 g L<sup>-1</sup>; **Batch 2:**M9/YE 4 g L<sup>-1</sup> Gluc. 4 g L<sup>-1</sup>; **Batch 3:**M9/YE 4g/L Gluc10g/L; **Batch 4:**M9/YE 4 g L<sup>-1</sup> Gluc 20 g L<sup>-1</sup>. Dry cell weight was calculated by drying 5 ml of culture at 60° C for 2 days. The data were analysed in triplicates at three parallel experiments. The mean values (+/-) deviations are represented. The data shows that batch 2 and 3 are optimal.
The yield coefficients of the batches were also compared, and the results are shown in Figure 3.7. As predicted the increase in substrate concentration led to the increase in biomass, however, the coefficient factor showed a different trend. The yield coefficient was maximal (0.5) in batch 2, in the medium containing 4 g L\(^{-1}\) glucose and 4 g L\(^{-1}\) yeast extract with

![Figure 3.7](image)

**Figure 3.7** Representation of yield coefficients (Yx/s) of batches shows the effect of glucose. Concentrations of yeast extract and glucose in basal M9 medium: Batch 1:M9/Gluc 4 g L\(^{-1}\), Batch 2:M9/YE 4 g L\(^{-1}\) Gluc 4 g L\(^{-1}\), Batch 3:M9/YE 4 g L\(^{-1}\) Gluc10 g L\(^{-1}\), Batch4:M9/YE 4 g L\(^{-1}\) Gluc 20 g L\(^{-1}\). The biomass and glucose data were analysed in triplicates from three parallel experiments. The mean values with deviations are represented. Dry cell weight was calculated by drying 5 ml of culture at 60\(^{\circ}\) C for 2 days. The Y x/s was significant and optimal in batches in batches 2 and 3.
maximal biomass reaching a mean 1.5 g L\(^{-1}\) dry cell weight. Batch3 with 10 g L\(^{-1}\) glucose and 4 g L\(^{-1}\) yeast extract was also found to be suitable with yield coefficient of 0.4 and a maximal biomass reaching a mean 2.9 g L\(^{-1}\) dry cell weight. The growth rate of these four different batches was also compared and the results are shown in Figure 3.8. The oscillation in growth rate was as such minimal in batch 2 and batch 3. Trend analysis on the growth

![Graph](image_url)

**Figure 3.8** Trend of specific growth rates in batches with respect to media. Batch-1 is without yeast extract. The addition of yeast extract in batches 2 and 3 had reduced the oscillations in growth rate and showed an optimal trend. Behaviour in Batch 4 is due to high glucose level. The data were analysed in triplicates at three parallel experiments. The mean values (+/-) deviations are represented. The R\(^2\) value using polynomial fit for batch 2 was 0.97 and batch 3 was 0.98 significant compared to R\(^2\) value for batch 1 and 4, which was less than 0.95.
rates in terms of $R^2$ value using a polynomial fit was determined and favours batch 2 and 3. It was found that the addition of yeast extract (4 g L$^{-1}$) had reduced the oscillations in growth and accelerated the growth rate during initial growth phase.

The complete batch kinetics showing the biomass and substrate profile in different modified M9 media is shown in figure 3.9. From the Figure 3.9, it is apparent that glucose confers to optimal growth and yield

![Graph showing batch growth kinetics of GJ1158 in varying glucose and yeast extract.](image)

**Figure 3.9a** Batch growth kinetics of GJ1158 in varying glucose and yeast extract. Concentrations of substrates added in basal M9 medium. Batch 1:M9/Gluc 4 g L$^{-1}$, Batch 2:M9/YE 4 g L$^{-1}$ Gluc 4 g L$^{-1}$, Batch 3:M9/YE 4 g L$^{-1}$ Gluc10 g L$^{-1}$, Batch4:M9/YE 4 g L$^{-1}$ Gluc 20 g L$^{-1}$. The data were analysed in triplicates from three parallel experiments. The mean values (+/-) deviations are represented.
concentrations at an optimal concentration of 4g to 10 g/L (Batches 2 and 3), but inhibits growth at 20g/L (Batch 4). This is possibly due to the feedback inhibition by bi-products of glucose utilization pathway produced during *E.coli* growth.

Experiments were designed to determine the salt (NaCl) concentration for optimal induction of rWbSXP-1. Batch cultivation of recombinant *E.coli* using modified M9 medium was carried out. Different concentrations of NaCl were pulsed for induction. The level of NaCl at 200-250 mM (final concentration L$^{-1}$) was found to be optimal (Figure 3.9). The optimization of *E.coli* GJ1158 cultivation in the media was based on

![Graph showing the relationship between NaCl concentration and rWbSXP-1 expression levels.](image)

**Figure 3.9b Final expression levels of rWbSXP-1 (total) in GJ1158.**

Induction was done with NaCl. Growth conditions were identical with uniform induction time duration of 3hrs. The range of 200-250 mM NaCl was optimal for induction. The rWbSXP-1 was estimated using ELISA at 430nm. The experiment were performed in triplicates at three parallel runs. The mean values (+/-) deviations are represented.
biomass, yield coefficient, growth rate and suitability for over-expression upon induction. However, the rate and duration of recombinant protein expression varied with increase in inducer concentration (NaCl).

3.1.4 Batch Analysis of osmotically inducible *E. coli* GJ1158 *pRSETB* plasmid harboring Wb-SXP1 gene in modified M9 medium

3.1.4.1 Protein expression in batch

Recombinant *E. coli* GJ1158 (*pRSETB*:WbSXP1) was grown in modified M9 medium with 10 g L\(^{-1}\) glucose and 4 g L\(^{-1}\) yeast extract. The expression profile of rWbSXP-1 is shown in Figure 3.10. The rWbSXP-1

![SDS PAGE Profile of recombinant E.coli during batch cultivation in modified M9 medium.](image)

Figure 3.10 SDS PAGE Profile of recombinant *E.coli* during batch cultivation in modified M9 medium. Lane 1-Molecular weight marker; 1) Molecular weight marker Lanes 2 to 7-Uninduced culture Lane 2) 0.85 OD 3) 1.98 OD 4) 4.1 OD 5) 7.5 OD 6) 9.5 OD Lanes 7-10-Induced culture 7) 5.4 OD 8) 5.8 OD 9) and 10) 5.7 OD; after 1hr, 2hr, 3hr, 4hr respectively. Shows basal level expression. Equal volumes of samples were processed and 75 µL of each was loaded to compare time course.
expression was found to be optimal and maximum in this optimized M9 media. However, a high leaky expression of the recombinant protein was observed in uninduced cultures in batch mode at higher cell densities as demonstrated by SDS-PAGE profile (figure 3.10). Leaky expression has been reported in T7 expression system. However, in the original work on GJ1158 (Bhadari et al 1997) it has not been reported as the cultivation was done in LB/ON medium supporting low cell densities.

3.1.4.2 Western Blot Analysis of Protein expression in batch

The rWbSXP-1 expression in GJ1158 batch cultivation using modified M9 medium was analyzed. The protein expression profile as detected by western blot analysis of the batch is shown in Figure 3.11.

![Western blot Profile of rWbSXP-1 expressed in GJ1158 during batch cultivation in modified M9 medium](image)

**Figure 3.11** Western blot Profile of rWbSXP-1 expressed in GJ1158 during batch cultivation in modified M9 medium. Lane 1- Molecular mass (KDa) marker; Lanes 2,3,4-Uninduced Batch culture at 1.98 OD, 4.10 OD, 5.70 OD respectively. Lanes 5,6,7-Induced Batch culture (induced at 5 OD) after 1hr, 2hr, 3hr respectively. WbSXP-1 basal expression prior to induction is clearly seen in lane 2,3 and 4. This confirms the immunoreactivity of rWbSXP-1 expressed. Equal volumes of samples were processed and 75 µL of each was loaded.
From the profile it was clear that the recombinant protein expressed was immunoreactive. The Protein expression level increased with increase in the cell density. The protein was expressed even under uninduced conditions. This confirms the fact that T7 expression system in *E.coli* is prone to leaky expression. However, in GJ1158 a high level of basal expression occurred only at higher cell densities.

### 3.1.4.3 Growth Kinetics in Batch

The growth and substrate kinetics of both induced and uninduced culture during the batch cultivations are shown in the Figure 3.12. In the uninduced culture the growth occurred exponenentially till the carbon source was depleted, while in induced culture, the growth ceased within 30 minutes of induction since most of the cellular machinery was diverted towards recombinant protein production.

The induction of GJ1158 batch was optimized at $\frac{1}{2} \text{OD}_{600}^{\text{max}}$, which is half of the maximal biomass (OD$_{600}$) reached in a particular medium. For induction, 250 mM NaCl (final concentration L$^{-1}$) was found to be optimal.

Fed batch cultivation in general, is more efficient in recombinant protein production and gives higher productivity under optimized conditions of substrate feeding (Lee et al 1996). In this study substrate feed for fed batch with GJ1158 was designed based on the batch cultivation yield coefficient (0.4) and a condition to maintain the growth rate of 0.4 h$^{-1}$. However, there was no increase in cell density in fed batches using osmotically inducible *E.coli* GJ1158, with biomass not rising beyond densities marginally higher than batch cultivations. This was inspite of maintaining higher dissolved oxygen saturation above the critical 40% (Jeongseok et al 1999, Akesson et al 2001), well supported with optimized and controlled feeding strategy expected to maintain the growth rate. There was no significant growth observed after 15 OD$_{600}$. The biomass reached in fed batch was equivalent to
Figure 3.12 The glucose utilization and biomass profiles measured during batch course. Figure shows proportionate increase of biomass with glucose utilization. I (filled marker) and C (empty marker) represent induced batch and un-induced batch conditions respectively. Mean values plotted, were measured in triplicates from 3 batches (Mean ± deviations).

batch no 4 with 20 g glucose Figure 3.8. Beyond this cell density, cell lysis and high foaming were observed. On repeated attempts of fed batch cultivation and analysis it was found that the high basal level expression of the recombinant protein was occurring at higher cell densities even under uninduced conditions, which had eventually lead to cell lysis. The cell lysis may be due to over expression of recombinant protein and associated metabolic burden in the system.

3.1.5 Comparison of soluble protein synthesis rate and pure protein yields in *E.coli* BL21 and GJ1158

Further, the suitability of osmotically inducible *proUp* promoter system was evaluated by comparing the rWbSXP-1 protein synthesis rate in *E.coli* strains GJ1158 and BL21 with *pRSETB*:WbSXP-1.
Batch cultivation and induction was performed under identical conditions. The rate of total rWbSXP-1 expressed was determined in all hourly batch samples by densitometry. The rate of rWbSXP-1 protein expressed in soluble form was estimated using sandwich ELISA method (Figure 3.13).

Figure 3.13 Analysis of rWbSXP-1 synthesis rate in *E.coli* BL21 and GJ1158 in modified M9 medium with 10 g Glucose and 4 g yeast extract. Soluble protein synthesis rate was determined by Sandwich ELISA. The total rWbSXP-1 was estimated by densitometry. The (I) and (C) represent induced and uninduced batch. Values plotted are mean values measured from batch (n=3) in triplicates (Mean ± deviations).

The monoclonal antibody 1A6C5 (IgG1K) developed was found to be highly specific and could detect up to 4 ng of rWbSXP-1. The levels of soluble rWbSXP-1 in broth were calculated from the standard sandwich
ELISA with known concentrations of highly purified rWbSXP-1 protein. Though the rates of total rWbSXP-1 synthesis in both *E.coli* strains were comparable, remarkably high levels of protein was expressed in soluble fraction with GJ1158. However, the basal level expression in the total and soluble fractions was considerably high in uninduced GJ1158 compared to BL21.

The comparison of protein expression and formation of inclusion bodies was carried out in BL21 and GJ1158 cultivated in modified M9 media. The protein yields from soluble and insoluble fractions were compared and estimated by sandwich ELISA and Bradford methods. The high ratio of protein expressed in soluble fraction makes GJ1158 extremely advantageous (Table 3.2).

**Table 3.2** Comparison on *WbSXP-1* protein yields (mg L⁻¹ culture) between *E.coli* BL21 and GJ1158 grown under identical conditions in modified M9 medium. Induction was done at 5.0 OD₆₀₀. Mean values measured in triplicates (±deviation values) from 5 batches are represented.

<table>
<thead>
<tr>
<th>rWbSXP-1 Protein Expression</th>
<th><em>E.coli</em> BL21</th>
<th><em>E.coli</em> GJ1158</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total produced</td>
<td>16.0 ± 0.2</td>
<td>15.6 ± 0.2</td>
</tr>
<tr>
<td>Soluble form</td>
<td>4.2 ± 0.2</td>
<td>12.4 ± 0.2</td>
</tr>
<tr>
<td>Insoluble form</td>
<td>11.8 ± 0.2</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>Ratio (soluble : insoluble)</td>
<td>1: 2.8</td>
<td>3.87: 1</td>
</tr>
</tbody>
</table>

3.1.6 Study on *Wb*-SXP1 leaky expression in osmotically inducible *E.coli GJ1158*

The T7 based expression system is prone to basal level expression and is known to affect the recombinant *E.coli* growth adversely. This has been
reportedly prevented by the co-transformation with pLysS plasmid, which encodes T7 phage lysozyme, an antagonist of T7 RNAP and therefore serves to reduce the basal level of target gene expression (Studier et al 1990; Bhandari 1997). To control the basal level expression in *E.coli* GJ1158 (*pRSETB*: *Wb*-SXP1), it was co-transformed with pLysS and cultured in modified M9 medium. The SDS page profile of the batch cultivation is shown in Figure 3.14.

**Figure 3.14  SDS PAGE Profile of recombinant *E.coli* with pLysS during batch cultivation in modified M9 medium.** Lane 1 -Molecular weight marker; Lanes 2 to 9 - Uninduced cultures at 0.15 OD, 0.45 OD, 0.97 OD, 2.08 OD, 3.58 OD, 5.40 OD, 8.10 OD, 9.00 OD respectively; Lanes 10,11- Induced cultures after 1 hr , 2 hr respectively. Equal volumes of samples were processed and 75 µL of each was loaded to compare time course.

Interestingly, the comparison of time course for protein profile of osmotically inducible recombinant *E.coli* with and without co-transformation
of pLysS (Figures 3.10 and 3.14 respectively) in modified M9 medium, demonstrates that the basal level expression of recombinant protein Wb-SXP1 was higher in both instances. This makes it evident that the basal level expression of recombinant protein was only due to the osmotic induction of the cells by metabolites. In addition, the expression of protein in the pLysS co-transformed system was comparatively higher in uninduced cultures, which might be the effect of chloramphenicol to amplify the plasmid leading to higher gene dosage and higher product formation. The effect of chloramphenicol has been discussed in detail (Kyslik et al 1993, Teich et al 1998). Thus, the pLysS co-transformation in osmotically induced system led to responses that were more complex and was ineffective in controlling the basal level expression in salt inducible host. This outcome of metabolites inducing the system was further substantiated by the conductivity and osmolarity data obtained during the batch cultivation.

During the cultivation, medium conductivity and osmolarity are positively correlated (Suresh et al 2001). Consequently, in this study the medium conductivity and osmolarity were analysed for batch cultivation using an offline conductivity meter and osmometer, to comprehend if the secreted ionic metabolites increased the medium osmolarity. During the cultivation, 10% ammonia was used to control the pH. Ammonia is a weak ion and contributed negligibly to conductivity. The use of ortho-phosphoric acid was negligible as cultivation pH trend was acidic. The conductivity and osmolarity were directly proportional to biomass increase and glucose utilization during cultivation Figure 3.15. Conductivity was easier to measure online and was preferred for further work.
Figure 3.15 The osmolarity and conductivity profiles measured during batch course (modified M9 medium). Figure shows proportionate raise with increase in biomass and glucose utilization. I (filled marker) and C (empty marker) represent induced batch and un-induced batch conditions respectively. Mean values plotted were measured in triplicates from 3 batches (Mean ± deviations).

3.1.7 Identification of metabolites causing the osmotic induction

Metabolites such as acetate and pyruvate are secreted during E. coli cultivation. To ascertain the cause of induction by osmotically inducible E. coli, we tried to identify certain major metabolites i.e. acetate, which might be secreted by recombinant E. coli during cultivation in modified M9 medium. Samples from batch course were analyzed for metabolites by HPLC and molar concentrations were determined. The metabolites secreted by E. coli into the medium, particularly acetate and other metabolites such as malate and succinate increased the osmolarity by greater than 100 mOsmol / kg during
batch course. The glucose utilization and metabolite levels in the broth during the batch course are plotted in Figure 3.16.

**Figure 3.16** Metabolites profile during batch cultivation (modified M9 medium) of recombinant *E.coli* GJ 1158 and their additive levels. Acetate was observed to be the major secreted metabolite. C (filled marker) and I (empty marker), represent uninduced and induced batch conditions. Mean values derived from batches (n=3) with (Mean ± deviations) are plotted.

The pattern of osmolarity and conductivity in cultivation due to increase in metabolites level is already represented in Figure 3.15.

### 3.1.8 Continuous cultivation: Effect of Dilution rates on Plasmid and leaky expression

To observe the cell behaviour and basal level expression, continuous cultivation was carried out in the modified M9 medium under the uninduced condition with increasing dilution rates. The protein expression profile by
SDS-PAGE analysis in at various dilution rates is shown Figure 3.17. At the lower dilution rates, the basal level expression was higher and correspondingly the plasmid instability was higher in spite of continuous feeding of the medium with selection pressure. With the increasing dilution rates the basal level expression of the recombinant protein decreased resulting in higher plasmid stability. The plasmid stability data at various dilution rates is shown Figure 3.18.

Figure 3.17. SDS PAGE Profile of recombinant *E.coli* during continuous cultivation in modified M9 medium. Lane 1- Molecular weight marker; Lanes 2, 3- Steady state culture at 0.05 D; Lanes 4, 5- Steady state culture at 0.10 D; Lanes 6, 7- Steady state culture at 0.30 D; Lanes 8, 9- Steady state culture at 0.50 D

This behaviour of protein expression was due to the dilution of medium metabolites at higher dilution rates resulting in the lesser osmolarity of the growth medium. The dilution rate of 0.3 h⁻¹ and above was found to be optimum in alleviating the metabolite effect on osmotic induction. Washout of cells occurred in this medium at the dilution rate of 0.7 h⁻¹.
Figure 3.18  Steady state plasmid instability at different dilutions rates during continuous cultivation of recombinant *E.coli* GJ1158. With increase in dilution rates, the plasmid instability decreased. The data were analysed in triplicates at two different time points during the steady state at every dilution rate. The mean values (+/-) deviations are represented. The results were derived from spread plate studies from fermenter samples.

### 3.1.9 Total Cell Retention culture

The total cell retention culture was attempted with complete cell recycling and dilution of culture medium by removal of permeate along with exponential substrate feeding and buffer addition simultaneously. The permeate removal was designed so as to maintain 0.5 h⁻¹, at which dilution rate the recombinant protein basal level expression was found to be the lowest with higher plasmid stability during continuous cultivation. The specific
Figure 3.19  Biomass, substrate and conductivity profile during total cell retention cultivation of recombinant *E.coli* GJ1158. The biomass concentration increased with controlled specific growth rate, and the residual substrate concentration in permeate was maintained less than 0.05 g L\(^{-1}\). During TCRC, conductivity remained at a possible low as at the start of batch. Data shown are mean values (± deviation) of 5 experiments. Each estimation variable per batch were estimated in triplicates.

The growth rate of the organism was maintained at 0.4 h\(^{-1}\) with optimized substrate feeding based on monad kinetics, through a peristaltic pump controlled by a computer unit. The reactor volume was maintained by addition of buffer containing M9 salts, ampicillin and trace metal components using load cell. The cultivation pH was also controlled due to the addition of M9 salts buffer. However, the controller was set to maintain the pH at 7.0 with 10% ammonia. The TCRC experiment was started with modified M9
medium with low glucose concentration. When the batch attained log phase and prior to exhaustion of glucose, TCRC was started. The dissolved oxygen concentration was maintained above the critical 40% saturation throughout the cultivation. The medium conductivity was monitored online to understand the overall medium osmolarity.

The biomass, substrate and conductivity profile during the cultivation is shown in Figure 3.19 while the protein expression profile is shown in Figure 3.20. The biomass concentration increased with controlled specific growth rate as expected, and the residual substrate concentration analyzed from permeate was maintained at less than 0.05 g L\(^{-1}\) throughout the cultivation.

The TCRC graph in Figure 3.19, shows a slight increase in conductivity at the initial stage of batch mode of cultivation. As the permeate removal was started in TCRC mode the medium conductivity dropped back and maintained the lowest possible as it was at the start of the cultivation. The cell density increased up to 38 OD\(_{600}\) without any hindrances and the culture was induced at this concentration to check the recombinant protein productivity. The protein expression profile clearly shows that the basal level expression of the recombinant protein by metabolite induction was controlled till the induction of the system with NaCl, and expression of the recombinant protein transpired from that point onwards.

A comparison of maximal biomass and rWbSXP-1 concentration achieved in batch, fed batch and TCRC operations in modified M9 medium is shown in Table 3.3.
Figure 3.20  SDS PAGE Profile of recombinant *E.coli* during total cell retention cultivation in modified M9 medium. Lane 1- Molecular weight marker; Lanes 2 to 10 - Uninduced cultures at 1.1 OD, 1.6 OD, 2.4 OD, 4.5 OD, 7.0 OD, 10.0 OD, 24.0 OD, 34.0 OD, 38.0 OD respectively; Lanes 11,12,13 - Induced cultures after 1 hr, 2 hr, 3 hr respectively. Equal volume of 75 µL sample at each hour was used for processing. An equal volume of sample was loaded to justify the time course profile.
Table 3.3 Comparison of maximal biomass and rWbSXP-1 concentration achieved in batch, fed batch and TCRC modes of cultivation using GJ1158: The increase in biomass was relatively higher in TCRC mode than in fed batch mode. The protein yield was in TCRC was comparable to batch proving the effectiveness in the process strategy. Averages of 5 batch data represented with deviations.

<table>
<thead>
<tr>
<th>Cultivation mode</th>
<th>Maximal Biomass (OD&lt;sub&gt;600&lt;/sub&gt;)</th>
<th>Maximal DCW (g/L) achieved</th>
<th>Post induction DCW (g) at harvest</th>
<th>Maximal Concentration mg/L</th>
<th>Yield mg/g DCW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td>9.8 ± 0.3</td>
<td>3.0 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>15.4 ± 0.2</td>
<td>8.57 ± 0.1</td>
</tr>
<tr>
<td>Fed Batch</td>
<td>15 ± 0.5</td>
<td>4.6 ± 0.2</td>
<td>4.5 ± 0.2</td>
<td>9 ± 0.2</td>
<td>2 ± 0.1</td>
</tr>
<tr>
<td>TCRC</td>
<td>38 ± 0.5</td>
<td>11.5 ± 0.3</td>
<td>10.2 ± 0.2</td>
<td>78.4 ± 0.3</td>
<td>7.68 ± 0.2</td>
</tr>
</tbody>
</table>

3.1.10 Batch cultivation of E.coli GJ1158 pRSETB-WbSXP in 30 L Airlift reactor

Recombinant E.coli GJ1158 was cultivated in modified M9 medium. The medium and cultivation conditions were optimized in the 3.0 L ALB (13) and were extended to 30 L ALB considering industrial applications. Growth kinetics of induced and uninduced batches and rWbSXP-1 protein expression in 30 L ALB was consistent in trial batches, Figure 3.21. The estimated mass transfer coefficient (kLa) in 3.0 L and 30 L ALB was comparable at varied aeration and agitation (data not shown). The maximal biomass achieved and specific rWbSXP-1 yields in 3.0 L and 30 L ALB’s shown in table3.4 was reasonable to comprehend the efficiency.
Figure 3.21  Batch cultivation of *E.coli* GJ1158 in 30 L airlift bioreactor with modified M9 medium. Experiments were done under induced condition and uninduced condition. The Glucose utilization, Biomass (OD$_{600}$) profiles and rWbSXP-1 concentrations after induction are shown. C and I denote control and induced conditions. rWbSXP-1 was estimated using sandwich ELISA with polyclonal rabbit and polyclonal mice Anti-WbSXP-1 specific antibodies.

Table 3.4  Comparison of maximal biomass (dry cell weight) and specific protein yield in airlift bioreactor. Averages and deviations were arrived from batches (n= 3) and estimated in triplicates. The scale up of volume by a factor of 10 shows comparable values in terms of biomass and Protein yield.

<table>
<thead>
<tr>
<th>Capacity</th>
<th>Maximal DCW (g/L)</th>
<th>Specific protein yield (mg/ g DCW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALB 3 L</td>
<td>3.72 ± 0.2</td>
<td>8.39 ±0.2</td>
</tr>
<tr>
<td>ALB 30 L</td>
<td>36.8 ± 0.3</td>
<td>8.18 ± 0.2</td>
</tr>
</tbody>
</table>
3.2 APPLICATION OF THE RECOMBINANT FILARIAL ANTIGEN \textit{WbSXP-1} FOR PRODUCTION AND EVALUATION OF RAPID IMMUNODIAGNOSTIC ASSAY OF HUMAN LYMPHATIC FILARIASIS

3.2.1 Down stream-processing and purification of \textit{rWbSXP-1} from \textit{E.coli} GJ1158

The recombinant protein \textit{pRSETB: WbSXP-1}, expressed as histidine tagged fusion protein in \textit{E. coli} GJ1158 under the T7 promoter system, forms less inclusion bodies. Earlier studies have shown that when foreign genes that are highly expressed under the T7 promoter system, they tend to form inclusion bodies when expressed in \textit{E.coli} BL21 (Bentley 1992, Belew 1994, Panda 2002). High expression rates allow insufficient time for the nascent polypeptide chain to fold into the native conformation leading to non-specific precipitation in cytoplasm and thus results in the formation of inclusion bodies. Despite various techniques like mechanical shearing or enzymatic lysis for solubilizing inclusion bodies, the use of chaotropic agents or detergents was preferred. The common chaotropic agents are Guanidine hydrochloride (GuHCl) and 8M-urea solution in phosphate buffer (0.1M pH8.0) to solubilize the proteins. In our initial study, the recombinant protein \textit{WbSXP-1} was expressed in BL21. After denaturation step with urea, protein solution was passed through the small 2.0 mL PD10 columns of IMAC chelating sepharose fast flow column to purify the proteins. The column was washed with wash buffer and eluted with competitive ligand, imidazole. Elution occurred at ~175-200 mM imidazole concentration as shown in SDS page Figure 3.21-a.
Figure 3.21A  **The purified recombinant rWbSXP-1 by IMAC using PD10 column.** Protein fractions was separated on 12% SDS-PAGE and stained with coomassie brilliant blue. Lane 1 Marker. Lane 2 & 3: Culture lysate and Flow through. Lane 4 wash 10 mM. Lane 5-6: wash 50mM imidazole. Lane 7-8 rWbSXP-1 wash with 175 mM Imidazole, Lane 9-10: Final wash with 500mM imidazole.

Further, the similar expression and purification of rWbSXP-1 was carried out with GJ1158 and the protein purified was purified from the soluble fraction using 7.5 ml bed volume IMAC column. The purification of the recombinant WbSXP-1 protein in soluble form (6xHis–tag fusion protein) was successfully done in IMAC column, result is shown in Figure 3.21-B. The binding and elution buffer conditions were initially optimized for native isolation of proteins and the combination of 50 mM TRIS-Sodium phosphate, 10 mM imidazole and 0.4 M NaCl was found to be successful. The pH of the binding buffer was optimized to be 8.0 and that of the elution buffer at 6.0. Elution gradient was with 0.5 M stock of Imidazole and rWbSXP-1 eluted at 250-275 mM Imidazole.
Figure 3.21b The purified recombinant rWbSXP-1 by IMAC using XK16 (7.5 ml Bed volume) column. The proteins fractions was separated on 12% SDS-PAGE and stained with coomassie brilliant blue. Lane 1 final wash with 500 mM Imidazole, lane 2-3: wash with 250 mM imidazole, lane 4-5 wash with 100 mM Imidazole, lane 6:10 mM lane 7 - 8: wash 50mM imidazole., lane 9 & lane 10 wash (W), Lane 11: Flow through (FT) and lane 12Culture lysate (B) and Lane 13 Marker (M). Note: The result was similar in 7.5 and 15 cm bed volume.
Figure 3.21c The run profile of IMAC with 7.5 ml bed volume using Akta chromatography is shown in Figure 3.21c, arrow points the rWbSXP-1 elution.

Silver staining of the purified rWbSXP-1 protein is shown in Figure 3.22.

Figure 3.22 SDS-PAGE analysis (silver staining) of purified rWbSXP-1 protein from PD10 column. The purified protein showed a 26 kDa single band, though some kinds break away or lower protein band could be seen. The purified protein was dialyzed against low salt PBS to remove excess salts and to facilitate the optimal application of the purified protein.
3.2.2 Western blotting

In order to study the immunoreactivity of the purified recombinant protein \( pRSETB:WbSXP-1 \), western blotting was carried out using various clinical groups of filarial patient sera. The purified protein \( pRSETB:WbSXP-1 \) was run on a SDS-PAGE with suitable molecular weight markers and then electro transferred onto an NCP membrane. The transferred protein in membrane was probed with different clinical group of filarial sera. The 26 kDa protein distinctively reacted only with the MF sera of \( W. bancrofti \) or \( B. malayi \) infection (Figure 3.23). No reactivity was observed with CP, EN and NEN sera.

![Western blot analysis of pRSETB:WbSXP-1](image)

**Figure 3.23** Western blot analysis of \( pRSETB:WbSXP-1 \): Induced \( pRSETB:WbSXP-1 \) protein was separated on SDS-PAGE, transferred to nitrocellulose membrane and probed with 1:200 diluted pooled (pool of 10 sera) \( W. bancrofti \) MF (\( Wb \) MF), \( B. malayi \) MF (\( Bm \) MF), CP, EN and NEN and Mouse sera. The 26 kDa recombinant protein is distinctly detected by bancroftian and brugian MF sera. Lane M=Molecular weight marker is shown on the left side. The recombinant protein is shown by arrow mark.
3.2.3 **Optimization of rWbSXP-1 purification**

*E. coli* lysis was carried out to recover rWbSXP-1 protein. Experiments on protein purification from GJ1158 were initially tried by IMAC in 2.5 mL bed volume using both urea solubilization (8M) and soluble fractions (native) or clarified lysates obtained after french pressure. Though the recovery of rWbSXP-1 is more in the urea solubilized purification, the contaminating proteins in the purified product was more, as evident from the lower specific activity and lesser fold purification. The purification from the soluble fraction had slightly lesser recovery but highly purified product was obtained (Table 3.5). Further, urea solubilized rWbSXP-1 has other problems such as much diluted product, removal of urea, refolding the protein and scale up etc., for larger application. On the other hand, purification from soluble fractions at higher scales with FPLC XK16 columns upto 15 mL bed volumes showed identical levels of purity and recovery of rWbSXP-1.

**Table 3.5** **Comparison of rWbSXP-1 recovered after purification using different chromatographic steps.** The final rWbSXP-1 concentration in broth (harvest) and in soluble fractions after lysis was estimated to be 15.6 mg/L (desitometry) and 12.4 mg/L (sandwich ELISA) respectively. Five purification runs were carried out and estimated in triplicates.

<table>
<thead>
<tr>
<th>Purification Steps</th>
<th>Total Protein mg</th>
<th>rWbSXP mg</th>
<th>Specific activity</th>
<th>Fold purification</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial broth sample</td>
<td>940</td>
<td>15.6</td>
<td>0.16</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>IMAC- Soluble fraction (Native)</td>
<td>16.3</td>
<td>12.02</td>
<td>0.741</td>
<td>44.7</td>
<td>77</td>
</tr>
<tr>
<td>IMAC (urea solubilization)</td>
<td>23.2</td>
<td>12.8</td>
<td>0.551</td>
<td>33.2</td>
<td>82</td>
</tr>
<tr>
<td>IMAC (Native) and Gel filtration</td>
<td>12.2</td>
<td>11.78</td>
<td>0.965</td>
<td>58.1</td>
<td>75.5</td>
</tr>
<tr>
<td>IMAC (Native) and Anion exchange</td>
<td>12.1</td>
<td>10.8</td>
<td>0.892</td>
<td>53.7</td>
<td>69.2</td>
</tr>
</tbody>
</table>
The rWbSXP-1 protein eluted at 250 mM imidazole. The concentrated IMAC purified samples had contaminating host proteins as evident in Figure 3.24, needed to be removed in the subsequent steps.

![Silver stained SDS-PAGE showing quality of purified rWbSXP-1 after each step. Lane 1: protein molecular weight maker, Lane 2: IMAC, Lane 3: IMAC followed by Anion exchange, Lane 4: IMAC followed by Gel filtration.](image)

**Figure 3.24 Silver stained SDS-PAGE showing quality of purified rWbSXP-1 after each step.** Lane 1: protein molecular weight maker, Lane 2: IMAC, Lane 3: IMAC followed by Anion exchange, Lane 4: IMAC followed by Gel filtration.

Thus, a purification strategy involving anion exchange chromatography (7.5 ml bed volume) or gel filtration (50 cm bed height) as a second step to effectively remove the contaminating proteins was investigated. The IMAC purified rWbSXP-1 was dialyzed, freeze dried (10 mg/mL) and purified by either anion exchange chromatography or gel filtration. WbSXP-1 protein eluted at 400 mM NaCl in anion exchange. The purity of WbSXP-1 protein obtained after IMAC step followed by gel filtration and anion exchange chromatography was analyzed by SDS-PAGE, Figure 3.4, lanes 3 and 4. A higher level of purity was apparent in IMAC followed by gel filtration procedure. The protein was assessed for immuno-reactivity by western blot, figure 3.25.
Figure 3.25 Western blot analysis showing the Immuno-reactivity of purified rWbSXP-1 with different serum groups. Lane 1: molecular weight marker, lane 2: human microfilareamic positive serum, Lane 3: MAb-Anti-His antibody, Lane 4: murine Anti-WbSXP-1 polyclonal antibodies, Lanes 5 & 6: Human endemic normal serum and normal mouse serum used controls, which show no reactivity.

The rWbSXP-1 protein recovery was estimated after each purification step and compared to the total rWbSXP-1 protein concentration that was initially present in culture during the time of harvest. Fold purification was derived from the total protein and specific rWbSXP-1 concentration at each purification step. The results showed that the fold purification was maximal in IMAC followed by gel filtration, table 3.5. The recovery of rWbSXP-1 was 75% considering the initial concentration at harvest, whereas, it was 95% from the soluble fraction alone. The total protein were estimated by Bradford method and correlated by densitometry, whereas, the rWbSXP-1 protein was quantified by sandwich ELISA.
3.3 PRODUCTION OF RAPID FLOW THROUGH IMMUNO FILTRATION TEST KIT FOR THE DETECTION OF ANTIBODIES IN BANCROFTIAN AND BRUGIAN FILARIASIS USING THE RECOMBINANT FILARIAL ANTIGEN WbSXP-1

A rapid-format, simple and qualitative flow through immuno filtration test has been developed for the identification of total IgG antibodies to recombinant filarial antigen pRSETB:WbSXP-1. This test system employs colloidal gold-Protein-A as antibody capture reagent. The appearances of typical positive and negative test results are shown in (Figure 3.26).

![Figure 3.26](image)

**Figure 3.26** Interpretation of rapid flow through immuno filtration test in MF Signal: Photograph shows, A) positive test showing two red magenta spots indicating both control (C) and test sample (T) positive for filarial antibodies; B) Negative test showing only one red magenta spot on control (C) Negative for filarial antibodies.

The control spot containing goat anti-human IgG antibody serves as a control to ensure the stability of the test device, and it must give a positive reaction (a red magenta colour spot at the control area) after the completion of test. Failure of appearance of the control spot indicates a defective test kit. The test
spot contains the recombinant antigen \( pRSETB: WbSXP-1 \), which develops only when the serum contains anti filarial IgG antibodies specific to the antigen. Thus the final result in a positive test is the appearance of two magenta coloured spots in both control and test areas. When a negative serum is used, binding with the recombinant antigen does not occur, thus only the control spot will develop.

### 3.3.1 Enhancement of Sensitivity of \( rWbSXP-1 \) antigen based diagnostic assay

The sensitivity of \( rWbSXP-1 \) based kit was important for detecting even low levels of filarial antibody in individuals having the slightest exposure to the disease. The experimental plan was to obtain a balance between the concentration of \( rWbSXP-1 \) spotted and dilution of serum samples and to fine-tune the signal reagent concentration.

### 3.3.2 Categorizing the sera

On the basis of field and clinical requirement to enhance sensitivity and spot size for better performance of the already existing rapid flow through assay format (Basker et al 2004), we obtained and analysed different serum groups comprising of MF positives, CP, NEN and EN. The MF positive sera were analyzed for the antibody titre levels by ELISA, as shown in Figure 3.27, and were further classified into weak to very strong reactive patient groups exposed to lymphatic filariasis.
Figure 3.27 ELISA (coated 0.5 µg rWbSXP-1) showing titre values obtained on different serum groups. Serum titre of Microfilareamic positives MF1 (n=4); very strong reactivity, MF2 (n=3); strong reactivity MF3 (n=4); moderate reactivity MF4 (n=4); weak reactivity. The control groups include: chronic pathology (CP; n=15), endemic normal (EN; n=15) and non-endemic normal (NEN; n=15). The mean values and deviation are represented. Each sample was tested in triplicates. Undiluted serum (0) in control groups did not show reactivity (p<0.05).

The other cases of CP, EN and NEN sera were used as control. Of the various serum dilutions experimented for ELISA, 1:20 dilution was found to be optimal for any sera group. The enhancement of sensitivity in rapid format was optimized with this panel of known cases of human lymphatic filariasis with weak, moderate strong and very strong exposure to the disease.
For protein or antigen, the maximum concentration at which the undiluted control serum might just show a slight background is taken as the optimal cutoff (ELISA: mean \( \pm 3 \) SD). With the maximum protein concentration thus coated, the dilution of control group serum at which it does not produce a slight background is the optimal cut off for serum. However, background did not appear even in undiluted control serum. The range shows, weak serum with a value of 1 and very strong serum with a value of 4 in the relative spot intensity. The \( rWbSXP-1 \) protein requirement or cutoff value on enhanced rapid format was optimized to 0.5 \( \mu \)g in 0.7 \( \mu \)L per spot, which is 4 times higher than the concentration in ELISA format. The serum dilution for rapid format was optimized and 1:5 dilutions were effective for any MF serum group. The detection pattern of ELISA and rapid formats were compared, the ELISA and rapid formats showed similar trend in antibody reactivity levels and was clearly distinguished into weak to very strong, Figure 3.28.

### 3.3.3 Antibody Titre based Assay

The antibody titre levels for MF positive test groups based on degree of antibody reactivity were analyzed in both ELISA and rapid formats and the curves were compared, Figure 3.29. Thus, sensitivity to capture the circulating antibodies in serum using rapid format is similar to the response in ELISA format and shows that spot intensity was proportional to titre. The correlation of ELISA and rapid kit formats is shown in Figures 3.28 and 3.29. Results indicate that, during the optimization 100% concordance was achieved in both the formats on the MF and control groups tested.
Figure 3.28  ELISA (rWbSXP 125 ng, sera dilution of 1:20) and rapid kit (rWbSXP-1 500 ng and 1:5 sera dilution) based analysis of the MF groups classified based on degree of exposure. The ELISA values of CP (chronic pathology), NEN (non-endemic normal) and EN (endemic normal) control groups is reasonable and the cutoff value for a weak positive reaction was fixed at >0.9 OD after 10 min incubation. The CP, NEN and EN sera applied at 1:5 dilutions on kit did not show any colour spot. In ELISA mean values (triplicates) are with error bars are shown. The spot intensity is a relative value indicated as per industrial standards done in triplicates. (p<0.05).
Figure 3.29  Performance analysis of the rWbSXP-1 antigen (0.5 μg) in ELISA and Rapid formats. The assay is based on serum antibody titre in different microfilaremic positive (MF) groups showing weak, moderate, strong and very strong reactivity. The trend in detection of antibodies by ELISA and rapid kit is similar and gives a titre-based prediction to the exposure or antibody levels.

3.3.4  Evaluation of enhanced diagnostic kit format

The performance and efficacy of improvised rapid kit was studied by screening with human serum collected from healthy individuals (blood banks). The enhanced kit could detect cases positively exposed to the disease. We also tested the efficacy of the enhanced rapid diagnostic kit by screening with known MF positive serum samples obtained from institutes at different geographical locations, Table 3.6. Comparison was made using other commercial kits is shown in Figure 3.30.
Table 3.6  **Performance of rapid diagnostic kit.** The confirmed MF clinical cases were identified by night blood smears, whereas the serum of individuals tested during evaluation are donors in blood banks and do not represent night blood samples. The groups were categorized by titre based reactivity in the rWbSXP-1 kit and the commercial kits were used only to distinguish the specific causative infections.

<table>
<thead>
<tr>
<th>Degree of Antibody reactivity in Rapid format</th>
<th>Known MF cases (rWbSXP-1 kit)</th>
<th>Serum of Healthy Individuals screened</th>
<th>Bm-rapid*</th>
<th>Wb-ICT*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Tested</td>
<td>25</td>
<td>320</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Weak reaction</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Moderate reaction</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Strong reaction</td>
<td>12</td>
<td>5</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Very strong reaction</td>
<td>8</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Total Exposed or positive</td>
<td><strong>25</strong></td>
<td><strong>16</strong></td>
<td><strong>16</strong></td>
<td><strong>7</strong></td>
</tr>
<tr>
<td>Percent (%) exposed</td>
<td>100</td>
<td>5</td>
<td>100</td>
<td>43.75</td>
</tr>
<tr>
<td>Specificity%</td>
<td>100</td>
<td><strong>100</strong></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Sensitivity%</td>
<td>100</td>
<td><strong>100</strong></td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Bm-ICT* (antigen detection) and *Bm*-rapid do not indicate titre

** Rapid WbSXP-1 kit shows 100% concordance with rWbSXP-1-ELISA, the dilution pattern correlated with spot intensity values. Also, it shows 75% (43.75+31.25) concordance with *Bm*-rapid and Wb-ICT kits.
Figure 3.30 Kits used in comparative Study A) *Bm*-Rapid (antibody detection) B) *Wb*-ICT (Antigen detection)

Performance of rapid diagnostic kit was further evaluated using the positive cases identified from normal or healthy test group showing weak, moderate, strong, very strong antibody reaction. The *rWbSXP*-1 ELISA showed 100% concordance; *Bm*-rapid and *Wb*-ICT kits had 75% concordance (43.75+31.25) with *rWbSXP*-1 rapid format, whereas 25% cases in weak reaction group could not be detected by either of the commercial kits, Table 3.6. In strong reaction group 12.5 % (2/16) were positive in both *Bm*-dipstick and *Wb*-ICT kits showing some cross reactivity, while, it was 12 % (3/25) in the known MF cases tested. Thus, slightest antibody levels were enough to be positive in *rWbSXP*-1 kit. However, antibody detection cannot be taken into account to ascertain active filarial infections or it rather indicates presence of antibodies due to an exposure to disease.

3.3.5 Stability kinetics of Enhanced Diagnostic kit

Performance of the improvised diagnostic kit was subjected to accelerated stability test as per the guidelines of European pharmacopoeia, at 4° C, 37° C and 45° C. The kits were tested against dilutions of high titre MF serum and compared. The maximum dilution at which, there occurred a
positive spot and the final dilution at which no positive spot occurred were identified using the rapid kit format. The maximal dilution (1:320 in this study) there occurred a positive spot was tested for stable performance and consistency of the results, (Figures 3.31, 3.32, 3.33 and 3.34). Endemic normal serum at 1:5 dilutions was used as control and did not develop any positive spot. The kits were stored at 4°C and their stability was regularly monitored over a period of 1, 3, 6 and 12 months and assessed to be satisfactory as per the industrial norms.

![Stability Kinetics of kit at 4°C with high tire microfilareamic positive (MF) serum at different dilutions. Endemic normal (EN) serum (1:5) was used as control showed no spot.](image-url)
Figure 3.32 Stability Kinetics of kit at 37°C with high tire microfilareamic positive (MF) serum at different dilutions. Endemic normal (EN) serum (1:5) was used as control showed no spot.

Figure 3.33 Stability Kinetics of kit at 45°C with high tire microfilareamic positive (MF) serum at different dilutions. Endemic normal (EN) serum (1:5) was used as control showed no spot.
Figure 3.34 MF signal Kit: Stability Kinetics at 4°C with high tire microfilareamic positive (MF) serum at different dilutions. Endemic normal (EN) serum (1:5) was used as control showed no spot.

3.4 DEVELOPMENT OF MONOLCONAL ANTIBODIES TO RECOMBINANT FILARIAL ANTIGEN (pRSETB: WbSXP-1)

3.4.1 Hybridoma and monoclonal antibodies.

The recombinant filarial antigen WbSXP-1 was expressed in GJ1158. The purified protein was used for immunization and hybridoma development. A highly purified rWbSXP-1 established by SDS-PAGE and western blot analysis was used to develop monoclonal antibodies. Purified WbSXP-1 recombinant protein at a stock concentration of 1mg/ml was used for injection into homozygous balb/c Female Mice (3 nos). Hybridoma for the development of monoclonal antibodies resulted in several antibody secreting clones. Initially clones were screened through ELISA and those binding to rWbSXP-1 were selected.
3.4.2 Immunization and antibody titre

For immunization, purified recombinant WbSXP antigen 50 μg (per mouse) in Freunds complete adjuvant was injected subcutaneously as a primary dose and followed by two booster doses in Freunds incomplete adjuvant at a regular interval of 21 days. ELISA was performed to understand the progress of antibody titre every 10th day after booster. The animals were rested for 2 months to ensure that the antibody titer levels, particularly the IgM drop, thereby making the spleenocytes produce more of IgG if dosed with immunogen. A final booster of 250 μg in 0.4 ml PBS was injected intraperitoneally 3-4 days prior to fusion. A final serum titer of 1/30,000 was achieved after the immunization.

3.4.3 Myeloma cells - harvest and yield

A seeding cell density of $5 \times 10^4$ cells/ml worked well with Sp2/0 cells. Sp2/0 cells grew to a maximum density of $9 \times 10^5$ cells/ml, with a doubling time of approx. 20 hrs. A total of $1 \times 10^7$ Sp2/0 cells (ie 1:5 ratio to immune spleen cells) was used for fusion.

3.4.4 Mouse feeder cells - collection and yield

To maximize the yield of hybrids from the fusion and cloning procedures, feeder cells were required to be co-cultured with the hybrids. Mouse peritoneal cells, most of which were macrophages, have been found to be effective feeder cells, providing soluble growth factors for hybridoma cells. Approximately $5-7 \times 10^6$ peritoneal feeder cells were harvested from one mouse and the above concentration was enough to seed 100 wells (96 well plate) (ie. 3000-5000 cells/well) for conditioning and for removal of dead cells.
3.4.5 Cell fusion and hybrid yield

A yield of approximately 200 hybrids was obtained per spleen (per mice). The plates were observed and screened for clones secreting immunoglobulins. Around 100 clones (50%) of the hybrids were growing confluent and secreted immunoglobulins (either IgM or IgG), which was apparent by the colour change in culture medium. The supernatant from such clones were taken out to perform ELISA on recombinant WbSXP coated (2 μg/well) plate. The results showed that 15 clones (8% of the hybrids) secreted specific antibodies to recombinant filarial antigen, Figure 3.35.

Figure 3.35 Primary screening of hybrids from 96 well plates to select the clones for further analysis and scale up. 1A6 was a highly reacting to rWbSXP-1 in ELISA. ELISA OD values above 1.0 were considered for further study. A total of 5 fusions were screened.
3.4.6 Scale-Up of the Clones

The clones showing positively reacting (ELISA) antibodies to rWbSXP were scaled-up to 1ml culture in 24 well plate and retested by ELISA after 3-5 days of growth (approximately 5 – 10 × 10 cells/mL)

3.4.7 Sandwich Elisa Using Antibodies Raised To Recombinant Filarial Antigen (pRSETB:WbSXP-1)

Sandwich ELISA was done on all the 5 clones and 6 of the 15 clones (30%) of the clones 1A6, 1G5, 2G9, 2D7, 2A12 reacted positive for the sandwich ELISA done with both the purified recombinant and the crude Wb MF antigen separately.

3.4.8 Sub-cloning: Cloning by limiting dilution and derivation of stable clones

Cloning by limiting dilution was a standard method based on the poisson distribution. Dilution of cells to an appropriate number per well had maximized the proportion of wells that could contain a single clone. Hybridomas to be cloned were diluted to 1 cell/well. This kind of dilution provides approximately 35% of wells with 1 cell/well as per the poisson statistics. As a standard procedure, Hybridomas that yielded >90% antibody positive cultures upon recloning were considered to be stable. Those cultures that yielded <90% positive cultures were subjected to further cloning. At the end of this cloning process, IgG clones were selected and cryopreserved.

3.4.9 Kinetics of antibody production (hybridoma’s) with respect to viable cells

Productivity of the clones was calculated (viable cell density Vs amount of antibody secretion after 2\textsuperscript{nd} and 7\textsuperscript{th} day of inoculation) for the IgG
clones and IgM clones. At the end of this process three clones 2A6, 1G9 (Identified as IgG) and 2A12 (identified as IgM) were selected and cryopreserved. However, the clone 1G9 secreted highly reactive antibodies towards the recombinant antigen compared to the other two clones. The culture supernatants of IgG clones yielded approximately 10 µg/mL and 25 µg/mL of IgG antibodies respectively.

3.4.10 Selection of Monoclones

Further, we screened for stable and high secreting clones which showed affinity to *W. bancrofti* and *B. malayi* MF (isolated from serum) and rWbSXP-1. Finally, three probable clones 1A6C2, 2A12F8 that bind to rWbSXP and CMF giving a stable and highest values in sandwich ELISA were selected, whereas, the other clone 2G9B9 had affinity only to rWbSXP-1. On further ELISA analysis with MF serum and Crude MF, both the antibodies 1A6C2 and 2A12F8 showed higher values for circulating Microfilariae antigen isolated from infected patients by filtration method. While a slightly lower affinity was observed when MF serum samples were directly coated or applied in ELISA, Figure 3.36.

3.4.11 Affinity, sensitivity and immuno-characterization of Monoclones

The approximate affinity and sensitivity of the MAb antibodies to rWbSXP was determined by the dilution curve using rWbSXP-1 antigen ELISA, Figure 3.37. The result showed that only 1A6C2 had higher affinity to the rWbSXP-1 and the minimal detection of rWbSXP protein was determined to be 4 ng. Based on MAb affinity towards CMF, MF and rWbSXP, 1A6C2 and 2A12F8 were considered for further expansion and characterization. The clone 1A6C2 was identified to be *IgG* monoclonal antibody. The reactivity was characterized by western blot analysis to detect
rWbSXP and SXP in WbCMF isolated from serum, Figure 3.38. The A12F8 (IgM) antibody had relatively good affinity and would be useful in antigen capture in sandwich ELISA.

**Figure 3.36** Mean ELISA values of each test with the number of serum samples tested. (n) = 5 (mean of triplicates +/- 3SD). Clone 1A6C2 showed higher affinity towards Circulating MF antigen in serum compared to other Monoclonal and polyclonal antibodies. SP2/0 cell supernatant was used as negative control and did show any immuno-reactivity. rPAb and mPAb:: are rabbit polyclonal and murine polyclonal respectively.
Figure 3.37 Reactivity of monoclonal antibodies against rWbSXP (2 fold serial dilution) using ELISA. 1A6C2 (IgG) The assay data plotted are Mean value of triplicates +/- deviations. 1A6C5 was omitted because it originated from same parent clone 1A6 and conferred a lower OD. The MAb 1A6C2 showed a maximal sensitivity in detecting up to 4 ng.
Figure 3.38  A) Western blot analysis showing the immuno-reactivity of anti-WbSXP 1A6C2 MAb on WbMF. Lane 1: Protein Molecular weight marker (KDa); Lane 2: pure rWbSXP-1, Lane 3: WbMF-SXP antigen (detected from Wb crude antigen); Lane 4: anti-WbSXP polyclonal (Mouse positive control) on rWbSXP Lane 5: anti-WbSXP polyclonal (Mouse positive control) on crude Wb-MF SXP; Lane 6: SP 2/0 (Negative control) on rWbSXP; Lane 7: SP 2/0 (Negative control) on crude Wb-MF antigen.

B) Western analysis of 1A6C2-MAb reactivity on BmMF where the specificity in immuno-recognition of Bm-SXP antigen from crude MF protein is shown. Lane 1: BmSXP (detected from Bm crude antigen) and Lane 2: rWbSXP-1 pure protein.

Immunoreactivity of 1A6C2 (IgG) monoclonal antibody was further characterized by the standard method using rabbit Ant-Ig capture (MAb-Isotyping kit). We also studied differential affinity of MAb towards rWbSXP, circulating microfilaria isolated from human blood (microfilaremic positive sera) and compared along with standard, Figure 3.39. It was observed that the
MAb 1A6C2 was highly specific to both recombinant WbSXP and circulating filarial SXP antigen. The 1A6C2 MAb was identified to be IgG1- kappa with high specificity to Wb-MF SXP antigen. The specificity of the 1A6C2 MAb was determined by ELISA with many different antigens of filarial parasite and other proteins, Figure 3.40.

Figure 3.39 Characterization of monoclonal antibody 1A6C2: ELISA was performed using the standard Rabbit Anti-Ig antibody based capture of MAb-1A6C2. Comparisons were made on antigen-based recognition using circulating microfilaria from MF positive sera and rWbSXP-1. This data shows 1A6C2-MAb is highly specific. Normal Rabbit sera was used as negative control and did not show reactivity. MAb did not show any reactivity to endemic normal sera. SP2/0 cell supernatant was used as negative control and did not show any immuno-reactivity. Mean value of duplicates are plotted.
Figure 3.40  Specific reactivity of monoclonal antibody 1A6C2 (IgG1k) using Sandwich ELISA with polyclonal Rabbit anti-WbSXP-1 IgG as capture. Reactivity to different antigens are shown; Bar: 1 Bovine serum Albumin control. Bars 2-5: other Wb-filarial recombinant antigens; rThioredoxin (TRX), rThioredoxin peroxidase (TRP), rTransglutaminase (TGA), rAbundant larval transcript ALT-2. Bar 6: E.coli host cell protein, Bar 7: Lysozyme, Bar 8: Pichia host cell protein, Bar 9: Wb-Microfilarea crude antigen. SP2/0 was negative control which did not react with any antigen. Mouse polyclonal antibody was used as positive control, which reacted with MF crude antigens and none to other antigen. The data represents mean ELISA values measured in triplicates and two replicates (+/-) deviations.
The monoclonal antibody 1A6C2 was evaluated for its immune-recognition to SXP-1 antigens in serum. We studied the response in sandwich ELISA with MF positive cases and used the EN and CP groups as controls. Though the level of antigen in serum samples and its bound state as immunocomplexes play a major role in detection, it was the MF isolated from whole blood of patients which helped to determine that WbSXP-1 is a prospective candidate for the development of antigen based diagnostic assays for lymphatic filariasis, Figure 3.41.

Figure 3.41 The Scatter plot of Sandwich-ELISA values of 1A6C2 monoclonal antibody on various serum groups; EN: Endemic normals, CP: Chronic pathology, Microfilareamics (MF: *Bm* and *Wb*). Sandwich was performed with enriched rabbit polyclonal anti-rWbSXP-1 antibodies for capture of MF antigen. mAb 1A6C2 reactivity to MF serum group is considerably higher than in control groups; EN & CP. (p<0.05).
The major aim of the present work is large scale production and purification of rWbSXP-1 protein. The Table 3.7 describes the key factors or conditions for development, the variable measured and the results.

The protein expression in osmotically inducible E.coli GJ1158 was successful and high cell densities were achieved in TCRC mode of operation at 0.5 D. The batch bioreactor scale up was successful from 3 L to 30 L in modified M9 glucose -yeast extract media.

The purification of rWbSXP-1 (6 His-tag) fusion protein was carried out in AKTA FPLC. The previously used urea solubilization was not scalable operation. In the present work, a methodology was optimized for purifying the protein from soluble fraction. The strategy was successful using immobilized metal affinity chromatography upto a bed volume of 15 cm in the capture step and followed gel filtration using superdex-100 (bed height 50 cm) in the polishing step. This is a significant improvement compared to the previous work with PD10 columns in manual mode of operation.

The purification strategy resulted in highly pure protein as demonstrated by silver staining and western analysis. The pure protein was successfully used for diagnostic application. The application of rWbSXP-1 on rapid ‘MF Signal’ format in terms of volume and concentration was optimized at as per customer requirement for better performance. The evaluated results in table shows 100% specificity and 100% sensitivity in the data obtained from experimental test groups used in this study. The mAb 1A6C2 developed against rWbSXP-1 was highly specific to circulating Wb and Bm antigens in MF positive serum. The affinity was moderate and considerably differentiated the MF antigen in microfilaremics compared to control groups.
Table 3.7 A summary of parameters involved in production of rWbSXP-1 and performance in different scales and optimization studies

<table>
<thead>
<tr>
<th>Condition</th>
<th>Measure</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein production:</strong></td>
<td><strong>Requirements: Speed, Effectiveness and Economical</strong></td>
<td></td>
</tr>
<tr>
<td>Expression in <em>E.coli</em> BL21 and GJ1158</td>
<td>rWbSXP-1 Protein Yield</td>
<td>BL21 and GJ1158 had comparable levels of expression and yields in a given medium. However, in GJ1158, the soluble protein content was 3 times higher than BL21. TCRC mode was suitable for GJ1158 due to osmo-induction.</td>
</tr>
<tr>
<td>Scale of operation</td>
<td>Bioreactor volume</td>
<td>BL21 and GJ1158 could be used in large scale. However, GJ1158 has the advantage of using NaCl as inducer, which is economical in large scale (30 L). Repeatability and reproducibility are consistent.</td>
</tr>
<tr>
<td>Purification</td>
<td>Protein recovery</td>
<td>BL21: the protein forms insoluble inclusion bodies, urea solubilization limits large-scale operation, 82% recovery. GJ1158: the protein is largely soluble, easy and effective downstream processing, 77% recovery.</td>
</tr>
<tr>
<td><strong>Kit production:</strong></td>
<td><strong>Customer requirement was bigger spot &amp; high sensitivity</strong></td>
<td></td>
</tr>
<tr>
<td>Spot size</td>
<td>Application volume</td>
<td>Previously the spot size was smaller with application volume of 0.3 µL /spot (Basker et al 2004). In this work, the optimization was done and spot volume was increased to 0.7 µL / spot, thereby increasing the spot area (Janardhan et al 2007)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>Concentration</td>
<td>The concentration was 125 ng/spot (Basker et al 2004). Increased to 500 ng/ spot. This detected even low levels of antibody in patient serum (Janardhan et al 2007).</td>
</tr>
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</table>

**Evaluation**

<table>
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<tr>
<th>Type of assay</th>
<th>(rWbSXP-1 kit)</th>
<th>rWbSXP-1 kit</th>
<th>WbSXP-1 ELISA</th>
<th>Tested in Bm-Rapid</th>
<th>Tested in Wb-ICT</th>
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<tbody>
<tr>
<td>Total Tested</td>
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<td>320</td>
<td>16 (IP)</td>
<td>16</td>
<td>25</td>
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<tr>
<td>Total Exposed /positive</td>
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<td>16 (IP)</td>
<td>16</td>
<td>7</td>
<td>11</td>
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<tr>
<td>Percent (%) exposed</td>
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<td>5%</td>
<td>100%</td>
<td>43.7%</td>
<td>44%</td>
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<tr>
<td>Known NEN (NIH)</td>
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<td>0</td>
<td>-</td>
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<tr>
<td>Known EN (DPH)</td>
<td>-</td>
<td>0/16</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Known CP (DPH)</td>
<td>-</td>
<td>0/12</td>
<td>0</td>
<td>-</td>
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**Monoclonal Antibody**

<table>
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<tr>
<th>WbSXP-1 (highly pure)</th>
<th>Moderate affinity</th>
<th>Detects both <em>Bm</em> and <em>Wb</em> serum (Basker 2003)</th>
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<tr>
<td><em>MAb 1A6C2 clone Moderate affinity</em></td>
<td>Highly specific to SXP-1 protein and detects <em>Bm</em> and <em>Wb</em> antigen in serum. (Present work)</td>
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