Chapter 2

Cloning of Rat nNOS in E. coli
2.1. Introduction

Molecular Cloning is an important research tool in genetic engineering which involves the insertion of desired fragment of DNA in the specific vector to produce multiple copies of the desired fragment (Brown 2001). Recently, Dr. Gurdon and Dr. Yamanaka were awarded the Nobel prize of 2012 in Physiology or Medicine for their discoveries concerning the manipulation of living cells, using the techniques for cloning animals and generating stem cells. Proteins that are normally produced in very small amounts include growth hormone, insulin in diabetes, Interferon in few immune disorders and blood clotting factor VIII in hemophilia, are known to be missing or defective in various disorders. Prior to the advent of gene cloning and protein production via recombinant DNA techniques, these molecules were purified from animal tissues or donated human blood. But both sources have drawbacks, including slight functional differences in the non-human proteins and possible viral contamination. (e.g. HIV, CJD). Production of recombinant protein from a cloned gene in a defined, non pathogenic organism has circumvent these problem (Brown and 1998). The affinity tags have aided tremendously in the production and purification of recombinant proteins as well as in the biochemical characterization and functional elucidation of proteins (Walls and Loughran 2011). The most commonly employed affinity tags range from short polypeptide sequences, such as polyhistidine tags which can bind to immobilized metal chelates, as well as the myc-tag, FLAG-tag, and streptavidin specific Strep-tag which can confer advantageous solubility effects. In addition, large whole proteins, such as, glutathione S-transferase and the maltose-binding protein fusion peptides, aided the solubility of many proteins (Waugh 2005; Young et al. 2012)

Mechanistic studies of structure and function of nNOS had initially been extremely difficult due to the minute amounts of nNOS protein that can be purified from cerebellar tissue. However, cloning of nNOS (Bredt et al 1991) sparked the interest of several researchers resulting in the cloning and expression of other NOS isoforms (Bredt et al. 1991; Forstermann et al. 1994; Nadaud and Soubrier 1995; Ogura et al. 1993; Sessa 1994). nNOS cloned by earlier workers improved the yield but the purified protein showed 100 fold less specific activity due to the expression of insoluble and
inactive recombinant nNOS. Roman et al (1995) successfully obtained highly expressed functional rat nNOS in the \textit{E. coli}. The method includes the 2’,5’-ADP-Sepharose affinity purification followed by size exclusion filtration with S-200 beads. (Berka et al. 2008; Gerber and Ortiz de Montellano 1995) simultaneously cloned rat nNOS with slight modification by using calmodulin sepharose column followed by 2’,5’-ADP-Sepharose. Both the methods were tedious and time consuming with the chances of protein degradation in the column. Later (Berka et al. 2008) cloned the oxygenase domain of nNOS with His-tag, which required removal of imidazole during purification which resulted in denaturation of the protein (Walls and Loughran 2011). Full length nNOS appended with His tag and biotin tag has been expressed first time in this work. A new cloning strategy has been developed to express full length rat nNOS in high yield with easy purification of nNOS protein for the first time in our laboratory.

\section*{2.2 Objectives}

1. The cloning of Recombinant nNOS in \textit{E. coli}.
   a. Modification of pAT 223 expression vector at the Multiple Cloning Site
   b. Incorporation of nNOS gene in the modified pAT 223 vector.
   c. Expression of recombinant rat nNOS in \textit{E. coli}.
   d. Functional activity of rat nNOS using Griess reagent.

\section*{2.3 Methods and Material}

\subsection*{2.3.1 Chemicals}

All chemicals and biochemicals were purchased from the following standard commercial sources such as: Sigma Chemical Co., St. Louis, USA; Bio-Rad, Richmond, USA; New England Biolabs Inc, Beverly, MA, USA; Himedia India Ltd, Bangalore Genei India Pvt. Ltd., Bangalore, India. Tris Base, Agarose, Dithiothreitol, 2-Mercaptoethanol, glycine, IPTG and EDTA were purchased from Sigma Chemicals. Potassium dihydrogen phosphate, disodium hydrogen phosphate, monosodium dihydrogen phosphate from Qualigens. Propanol, Sodium Chloride, Potassium Chloride, cobalt chloride, Glacial Acetic Acid, Methanol from Merck, Taq Polymerase from Gene Aid.
Coomassie Brilliant Blue, Bromophenol Blue, PMSF from Imperial Chemicals, India. Culture media including Luria Bertani Medium and Luria Bertani Agar, Neutravidin agarose from Pierce biotechnology Pvt Ltd.

2.3.2 Procurement of Strains and Vectors

All the strains and vectors were procured from various laboratories as gift.

1. **Bacterial strains:** DH5α, BL21(DE3)-pLyS S, XL-BLUE, C-43, BL21(DE3)-CodonPlus-pRIL strain of E. Coli were procured from Prof. Daman Saluja, of Dr. B R Ambedkar Center for Biomedical Research, University of Delhi, India.

2. **Expression vectors**
   - Rat and Human nNOS bacterial plasmid (pCWori nNOS) - Prof. Bettie Sue Siler Masters and Dr Linda Roman of University of Texas Health Science Center, San Antonio, Texas, USA.
   - pGroESL (Bacterial chaperones) - Prof. P. J. Thomas laboratory by Dr. Linda Millen of UT Southwestern Medical Center at Dallas.
   - pBirA-histag (biotin ligase) - Dr Steven Polyak of School of Molecular and Biomedical Science, University of Adelaide, USA.
   - pAT223 (bacterial expression vector) - Prof. Andreas Plückthun’s laboratory by Dr. Birgit Lindner of University of Zurich, Switzerland.
   - pJET1.2 vector (TA cloning vector carrying lethal gene mutation) - Thermo Fisher Scientific Pvt Ltd,
   - Recombinant pAT223 vector (our laboratory product)

Vector map of all above plasmid sequences (Appendix)

2.3.3 Antibodies

**Primary antibody:** Antibody: sc-7271, NOS3 (C-6) were procured from Santa Cruz Biotechnology, Inc.

**Secondary antibody:** Goat anti-mouse IgG(2005) were procured from Santa Cruz Biotechnology, Inc.
2.3.4 Antibiotics
Ampicillin and chloroamphenicol were purchased from Sigma chemicals, USA and Himedia India Ltd respectively.

2.3.5 Kits Used
TA cloning kit, gel extraction kit, T4 DNA ligation kit and Quick ligation kit were purchased from New England Biolabs Inc, Beverly, MA, USA.

2.3.6 Restriction Enzymes Used
BamH1, HindIII, Cla1, Not1, Sal1, were procured from Fermentas Pvt Ltd and Taq polymerase was bought from Gene Aid.

2.3.7 Primers
All the primers used in the study were custom synthesized from either Sigma Chemical Co. St. Louis, USA or Biolinkk Pvt Ltd. The primers were designed using Gene Runner software, NEB cutter V 2.0 and BLAST software of NCBI.

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<thead>
<tr>
<th>S.No.</th>
<th>Name of primer</th>
<th>Primer sequence</th>
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<tr>
<td>1</td>
<td>pJET forward</td>
<td>5’-CGACTCACTATAGGGAGAGCGGC-3’</td>
</tr>
<tr>
<td>2</td>
<td>pJET reverse</td>
<td>5’-AAGAACATCGATTTTCCATGGCAG-3’</td>
</tr>
<tr>
<td>3</td>
<td>Forward primer (recomb pAT)</td>
<td>5’-AATCGATGCGGCCGCGTC-3’</td>
</tr>
<tr>
<td>4</td>
<td>Reverse primer</td>
<td>5’-CTTGCTGCAACTCTCTCTCA-3’</td>
</tr>
<tr>
<td>5</td>
<td>Forward recRNOS</td>
<td>5’-GAGAGCTGTACACTGCTA-3’</td>
</tr>
<tr>
<td>6</td>
<td>Reverse NOS</td>
<td>5’-GTCAGATCTGCTATG-3’</td>
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2.3.8 Methodology
Cloning of nNOS was done according to the planned strategy (figure 1). The detail of the basic technical protocols used in the present work is given in appendix.
2.4 Results

2.4.1 Cloning of nNOS in *E. coli*

To initiate the cloning of mammalian nNOS, the original clone of rat and human nNOS (in pCWori vector) acquired as a gift (McMillan et al. 1992; McMillan and Masters 1995; Roman et al. 1995) did not possess any affinity tag, hence it was tedious to purify the protein after expression. Later, the work published from Andreas Plückthun’s laboratory (Parmeggiani et al. 2008) described pAT223 expression vector possessing His-tag and Avitag (biotin tag). However, cloning of rat nNOS required the modification in the multiple cloning sites (MCS) of the expression vector pAT223 because cla1 and not 1 restriction sites were not available for the insertion of nNOS gene present in the mammalian pCWori nNOS flanking with restriction sites cla1 and
not 1. Therefore, re-cloning of mammalian nNOS gene present in the pCWori nNOS was carried with the strategy given in Figure 1.

2.4.1.1 A. Modification of pAT 223 Expression Vector at the Multiple Cloning Site

The modification in the MCS region of the pAT 223 was carried by the insertion of an artificial construct (AC) having the Cla I and Not I site (Figure 2).

(i) Cloning of AC in the TA Vector

The artificial construct (AC) procured from Biolinkk Pvt Ltd. was a 56 base pair (bp) double standard structure without overhangs.

Therefore it was cloned in to a TA vector pJET 1.2 (procured from Fermentas Pvt Ltd.). This vector was chosen in particular due the presence of lethal gene, DNA insert if integrated into this vector will not show any false positive colony after transformation. The positive colony with DNA insert would appear on luria agar (luria agar) plate containing ampicillin antibiotic. The ligation of the AC can do by sticky end or blunt end. For cloning, both blunt end and sticky end ligation of AC into TA vector pJET1.2 was carried. The ligated product was transformed into DH5α strain of **E. coli** which was identified by plating on LA ampicillin containing plate. 25 clones were selected out of which 8 were of blunt end ligation and 13 were of sticky end ligation. All the 25 clones were screened by colony Polymerase Chain Reaction (PCR) by amplification of clones in cycler (ACER make) followed by analysis on agarose gel (1% with 0.5ug/ml EtBr). The PCR parameters for 35 cycles were: 95°C for 5 min for initial denaturation, cycling of 95°C for 30 sec, 60°C for 30sec, 72°C for 1 min, final extension at 72°C for 10 min with primer 1 and 2 (Table 1). The amplicons analyzed by agarose gel electrophoresis showed that all clones were positive and showed the expected band of 175bp (Figure 3 and 4). Among the 25 screened colonies, 5 colonies (colony no. 5, 13, 15, 19 and 21) were randomly selected and inoculated in 5 ml of luria broth to isolate plasmid for PCR amplification of the AC.

The amplicons of AC was eluted from the 1 % agarose gel and further viewed and verified by restriction digestion of AC with BamH1, Cla1 & Not1 enzymes (Figure 5) on 1.7% agarose gel.
(ii) Identification and Insertion of AC in pAT 223 Vector

After the verification of AC insert in TA vector, 5 colonies of TA vector carrying AC was amplified and eluted from the agarose gel. The amplicons of AC and plasmid of pAT223 (2 sample) was double digested with the restriction enzymes BamHI and HindIII exposing the GGATC and AAGCTT sites respectively. The results of the restrictions digestion shows the linearised band of 56bp in all the 5 colonies (5, 13, 15, 19, and 21) subjected to restriction digestion along with the presence of 4.3kb band in the 2 sample of pAT223 vector. (Figure 6). The restricted digested product obtained from above was ligated and transformed in to DH5α strain of E. coli. on LA amp plate yielding numerous colonies carrying recombinant of pAT223 vector.

(iii) Verification of Recombinant pAT223 Vector by Colony PCR

Randomly picked 15 colonies were subjected to colony PCR with annealing temperature 54 °C using primer 3 and 4 (Table 1). The results of Figure 7 indicates that colony number-2,3,4,6,7,8,9,10,12, and 13 were positive by the presence of 586 base pair band on the agarose gel. Randomly picked colony number 8 of recombinant vector was used for downstream cloning of nNOS.

2.4.1.2 Recloning of Rat nNOS from pcWori into Recombinant pAT223 Vector

(i) Ligation of nNOS Gene in to Recombinant pAT223 Vector

The rat nNOS gene from the original vector pcWori and recombinant pAT223 vector was subjected to restriction digestion with ClaI and NotI. The linearized vector and
nNOS gene insert obtained was ligated using quick ligation kit (procured from Fermentas Pvt. Ltd.). The ligation product obtained was finalized on agarose gel. The result in figure 8 shows the increase in the molecular size of recombinant vector pAT223 from 4.3 kb (uncut) to approx. 8.7kb due to the insertion of nNOS gene. The ligated product was transformed in to DH5α and plated on LA amp plate to yield numerous colonies carried recombinant Rat nNOS.

(ii) Identification and Verification of Recombinant nNOS

Randomly picked 3 colonies were subjected to colony PCR with annealing temperature at 52 °C with primer 5 and 6(Table 1). Figure 9 shows that all colonies were positive as observed by the presence of 1 kbp band in all lanes. Further validation of the recombinant rat nNOS was done by DNA sequencing (from TCGA). Re alignment of sequence obtained by the DNA sequencing with NCBI nucleotide database through BLAST. Figure 1 show 89% and 99% identity with recombinant pAT223 vector and rat nNOS gene respectively. Thus confirming the recloning of nNOS gene in to the recombinant pAT223 vector to yield recombinant rat nNOS (RRN).

Figure 3: TA cloning of Artificial construct (AC): PCR analysis of 15 colonies out of 25 selected colonies. All colonies were found to be positive with the expected band of 175bp. Key: M-Marker.
Figure 4: TA cloning of Artificial construct (AC): PCR analysis of remaining 10 colonies out of 25 selected colonies. All colonies were found to be positive with the expected band of 175bp. Key: M- Marker.

Figure 5: Restriction digestion analysis of the randomly selected five TA colonies no. 5, 13, 15, 19, and 21. All the selected colonies were subjected to restriction digestion with BamH1, Cla1, and Not 1. All the colonies showed the expected bands with BamH1 a broad band between ~120 to 60 bp (expected fragments: 112 & 62 bp), with Cla1 broad band between 100 to 60 bp (expected fragments: 106, 59 & 10bp) and with Not1 broad band between 80-60bp (expected fragment: 77, 78, & 20bp). Lane Key: a- BamH1, b- Cla1, c- Not 1, p- uncut pcr product, M-marker and SC-synthetic construct (AC).

Figure 6: Restriction digestion of the PCR product of TA colonies and pAT 223 plasmid vector. The positive TA colonies and pAT 223 vector were subjected to restriction digestion with BamH1 and HindIII to elute the AC as popout of 56bp for ligation into the linearised pAT 223 vector (2 plasmid sample). Key: UP- uncut plasmid, CP- digested plasmid, M-marker.
**Figure 7:** Colony PCR analysis of recombinant pAT 223 vector: 15 colonies were screened for the positive insert of AC in the pAT 223 vector to form recombinant pAT 223 vector. Out of 15, 10 colonies were positive by the presence of expected band of 586bp. Key: M-marker.

**Figure 8:** Ligation of nNOS gene insert into the recombinant pAT223 vector. Both rat and human linearised nNOS gene insert were ligated into the linearised recombinant pAT 223 vector and ligated product were analysed on 1% agarose gel to reveal the presence of expected ligated product of about ~10 kb in both human and rat. Key: LHN-ligated human nNOS, UHN- human nNOS insert, LRN- ligated rat nNOS, URN-rat nNOS insert.

**Figure 9:** PCR analysis of RRN showed the expected band of ~1000bp. M – Marker, 1,2,3,- colony picked after transformation of RRN.
Figure 10: Blast result of the TCGA sequence of recombinant rat nNOS: The sequencing result obtained from the TCGA was aligned with nucleotide database of NCBI using BLAST. The results show the similarity with rat nNOS and plasmid vector pAT223.

Figure 11: Western blot of C43, xl-blue, DE3 & PRIL strain of E.coli detecting the rat nNOS protein: the western blot shows the clear band of 174 KDa along with the broken fragments (produced by the denaturation of the intact protein) in the induced sample except C43 were the expression is also detected even in the uninduced sample in both with and without chaperone. key: C43- C43 strain, xl-xl-1 blue strain, G-with chaperone, D-BL-21DE3 strain, P-BL-21DE3-PRIL, UI-uninduced, I-induced, and M-marker.
2.4.1.3 Expression of Recombinant Rat nNOS in *E. coli*

Generally protein folding is accelerated in the presence of small proteins called chaperones (Hinault et al. 2011). Proper folding of RRN protein (a large size of 174 kDa) in *E. coli* requires over expression of bacterial molecular chaperones (normally present at basal level in *E. coli*) through the plasmid pGroESL encoding the bacterial molecular chaperones GroEL and GroES. RRN was core transformed with pGroESL plasmid in different strains of *E. coli* like BL21-DE3, C43, XL-BLUE and BL-21-PRIL and for comparison RRN was transformed in these four strains of *E. coli* without pGroESL plasmid. The positive transformed clone of RRN7 (with and without chaperone) were inoculated with overnight grown culture in 5 ml luria broth till the Optical Density reaches 0.4-0.6 nm, and the culture was induced for 24 hrs at 37°C with 0.5mM of IPTG (isopropyl-beta-D-thiogalactopyranoside). The western blot analysis of RRN revealed the band at 174 kDa in the induced lanes of all expressions strain (BL21-DE3, C43, XL-BLUE) and absence of band in uninduced lanes of all strains except for C43 showing 114 kDa in uninduced lane thus remaining unaffected by the induction by IPTG suggesting the leaky expression of rat nNOS in C43 strain. The expression of nNOSs in XL-BLUE and BL-21 PRIL without chaperone was observed better than the rest of strains as indicated by Figure 11.

2.4.1.4 Functional Activity of Rat nNOS using Griess Reagent

The functional activity for nitric oxide produced by nNOS in *E. coli* was estimated by Griess reagent (appendix). The quantitative estimation of NO concentration was done by using standard curve obtained from reaction of azo dye with various concentration of Sodium Nitrite to form purple colour complex. The Table 2 showed that IPTG induced bacterial culture of RRN with chaperone generate 6 mM nitric oxide. However negligible nitric oxide production.

<table>
<thead>
<tr>
<th>Table 2: Functional activity of rat nNOS was evaluated by Griess reagent</th>
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<tr>
<td><strong>Optical density</strong></td>
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<tr>
<td>Uninduced chaperone</td>
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<td>Induced chaperone</td>
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2.5 Discussion

High level of recombinant protein expression requires enhanced solubility and stability of the expressed protein in *E. coli* otherwise the expressed protein forms exclusion bodies and requires additional purification. Affinity tags make the expressed recombinant protein soluble and easy to purify (Walls and Loughran 2011). nNOS is a large protein with molecular weight of 160 kDa, and its cloning is a challenge due to increase chances of inclusion body formation, dysfunctional and unstable protein expression. Although, previous workers successfully cloned mammalian nNOS in bacterial expression system, but the expressed nNOS protein lacks affinity tag in the full length protein making its purification a tedious task (McMillan et al. 1992; McMillan and Masters 1995; Roman et al. 1995). In the present work, we attempted to rectify the purification method of rat nNOS. Therefore recloning of nNOS in a new expression vector was carried. The choice of adequate vector was the most important part in the recloning, the vector with biotin affinity tag should be able to express in a fusion protein. In this regard pAT223 vector possessed two affinity tags (Avi-tag or biotin tag and His-tag) and gene for Phage lambda (Parmeggiani et al. 2008). The expression from this vector would yield a fusion protein with phage λ protein D (pD). This is a major monomeric capsid protein with small size (110 aa; 11.6 kDa; pI=5.68), possessing high expression and resistance towards thermally induced irreversible aggregation. The N-terminal Avi tag employs a highly targeted enzymatic conjugation of a single biotin on a unique 15 amino acid peptide tag (GLNDIFEAQKIEWHE) using the biotin ligase (BirA) from *E. coli*. AviTag binds to streptavidin and modified avidin surfaces, to allows the elution of biotinylated recombinant proteins in native condition. The C-terminal His-tag can be used for immobilized metal ion affinity chromatography (IMAC) purification. (Forrer and Jaussi 1998). The vectors has been used for the purification of biotinylated proteins and peptides in phage display (Amstutz et al. 2005; Huang and Jacobson 2010; Kay et al. 2009; Milovnik et al. 2009; Steiner et al. 2008; Varadamsetty et al. 2012). However, the multiple cloning sites (MCS) of pAT223 vector lacked the restriction sites for ClaI and NotI, hence, was not non compatible with the ends of nNOS gene. Therefore MCS of pAT223 was swapped with the artifical construct (AC) carrying both restriction sites ClaI and NotI via cloning of AC into pJET
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TA vector. The re-cloning of rat nNOS gene present in pCWori vector was carried into the modified recombinant pAT223 expression vector. Expression of recombinant rat nNOS in various strains of *E. coli* like: BL21-DE3, C43, XL-BLUE and BL-21-PRIL was carried with/without chaperons (usually co-expressed for the proper folding of the overexpressed proteins), the results showed the higher expression of rat nNOS in all the strains used with maximum in XL-BLUE and BL-21-PRIL strains, other strains required chaperones, therefore BL-21-PRIL co-expressed with molecular chaperone was used for large scale isolation of recombinant rat nNOS. The functional activity by Griess Reagent also showed the reasonable amount nitrites produced. Hence, Expression and isolation of recombinant rat nNOS in recombinant pAT223 vector showed the highly stable expression, and easy purification of rat nNOS which can be used in pull down assays to find the novel interacting protein partners of nNOS.
References


