UNIVERSIDADE DA BEIRA INTERIOR
Sciences

Biosynthesis, solubilization and purification of human membrane bound catechol-*O*-methyltransferase in *Brevibacillus choshinensis* cells

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Para a minha Mãe e o meu Avô,

Com todo o carinho e amor…

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Resumo

As proteínas membranares constituem cerca de 20 a 30 % de todas as proteínas codificadas pelo genoma de vários organismos. Elevadas quantidades de proteínas num estado de elevada pureza são necessárias quer para estudos farmacológicos, quer para estudos cristalográficos, daí a imperativa necessidade de desenvolver novos sistemas para a sobre-expressão heteróloga de proteínas membranares. Especificamente, nós testámos a aplicação de *Brevibacillus choshinensis* para a biosíntese da isoforma membranar da catecol-*O*-metiltransferase humana. No que diz respeito ao processo de produção, obteve-se uma moderada a elevada expressão num meio complexo com um valor de 45 nmol/h/mg para a actividade biológica da hMBCOMT, atingida às 20 horas de cultura a 37 ºC e 250 rpm. No que diz respeito à solubilização da proteína alvo, a eficiência de reconstituição para a hMBCOMT é nula na presença de detergentes iónicos tais como o SDS. No entanto, a aplicação de baixas concentrações de detergentes não-iónicos parece ser ideal para solubilizar a fracção membranar visto que a hMBCOMT recombinante retém elevados valores para a actividade biológica. Dos detergentes testados, a digitonina a 0.5 % (m/v) parece ser o mais adequado. De facto, o método descrito nesta tese é simples e poder-se-á tornar muito útil se aplicado num diagrama global para o isolamento da MBCOMT tendo em vista a sua caracterização bioquímica ou biofísica, onde se destaca a determinação da sua estrutura por cristalografia de raios-X ou estudos de interacção da hMCOMT com inibidores.

Palavras-chave

Proteínas membranares; MBCOMT humana; Produção heteróloga de proteínas; Detergentes; Doença de Parkinson

Abstract

Membrane proteins constitute 20-30% of all proteins encoded by the genome of various organisms. While large amounts of purified proteins are required for pharmaceutical and crystallization attempts, there is an unmet need for the development of novel heterologous membrane protein overexpression systems. Specifically, we tested the application of *Brevibacillus choshinensis* cells for the biosynthesis of human membrane bound catechol-*O*-methyltransferase (hMBCOMT). In terms of the upstream stage moderate to high expression was obtained for complex media formulation with a value near 45 nmol/h/mg for hMBCOMT specific activity achieved at 20 hours culture at 37 ºC and 250 rpm. Subsequently, the efficiency for reconstitution of hMBCOMT is markedly null in the presence of ionic detergents, such as SDS. However, the application of lower concentrations of non ionic detergents seems to be ideal to solubilize the membrane fraction since recombinant hMBCOMT retains more biological activity. From the detergents tested, digitonin at 0.5 % (w/v) appears to be the most suitable. Indeed, the straightforward method describe in this paper can be very useful and apply in a global flow sheet of MBCOMT isolation for biochemical or biophysical characterization including X-ray crystal structure determination or crosslinking interaction inhibitors studies.

Keywords

Membrane proteins; Human MBCOMT; Heterologous protein production; Detergents; Parkinson Disease

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Chapter I - Introduction

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List of Acronyms

AADC	Aromatic amino acid descarboxylase
B. chosinensis	*Brevibacillus choshinensis*
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CHAPS	3 – [(3-Cholamidopropyl) dimethylammonio] – 1 – propanesulfonate
CMC	Critic micellar concentration
COMT	Catechol-*O*-Methyltransferase
CTAB	Methylammonium bromide
DNA	Deoxyribonucleic acid
DTT	Dithiotreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
HIC	Hydrophobic Interaction Chromatography
hMBCOMT	Human Membrane bound Catechol-*O*-Methyltransferase
HPLC	High Performance Liquid Chromatography
IPTG	Isopropylthiogalactosidase
MBCOMT	Membrane bound Catechol-*O*-Methyltransferase
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
NLP	Nanolipoprotein
OD_{660}	Cell density at 660 nm
PCR	Polymerase chain reaction
PD	Parkinson disease
PVDF	Polyvinyl difluoride
rHDL	Reconstituted high density lipoproteins
SAH	S-adenosyl-*l*-homocysteine
SAM	S-adenosyl-*l*-methionine
SCOMT	Soluble Catechol-*O*-Methyltransferase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Chapter I

Introduction

Section I - Brevibacillus Expression System:

Subsection I - An Overview:

The *Brevibacillus* Expression System based on *Brevibacillus choshinensis* (*B. choshinensis*) cells is well-suited for secretory production of heterologous proteins with high efficiency [1]. This system presents several advantages such as the fact that the host bacterium secretes proteins very efficiently and produces low levels of extracellular proteases so that the products remained unscatched in the culture medium [1]. In addition, the proteins are produced as active forms; the host bacterium is a safe organism and is amenable to genetic engineering [1]. Many proteins from eukaryotic and prokaryotic organisms have been successfully produced in this system at high levels and with native biological activity [2 - 6]. In particular, for eukaryotic proteins with cystein residues that make intramolecular bonds and, consequently, its disulfide bonds must be formed at an exact location, it is very difficult to achieve an efficient production at traditional bacterial systems based on intracellular expression [1]. However, this can be overcome using extracellular expression strategies based on *B. choshinensis*.

Subsection II - *Brevibacillus choshinensis* cells:

B. choshinensis (figure 1) is a gram-positive bacterium that has excellent ability to produce many kinds of proteins in an extracellular model [1]. These cells exhibit high transformation efficiency by electroporation, which makes the construction of expression clones a straightforward process [1].

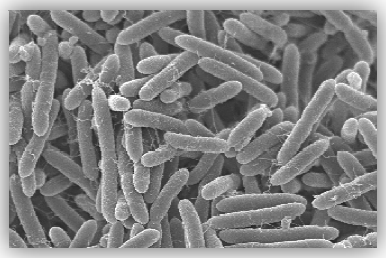


Figure 1 – Electron Microscopy Image of *B. choshinensis* cells [7].

In general, there are several expression vectors that can be used in the *Brevibacillus* expression system [1]. Some of them like the pNI or the pNI-His DNA allows preferentially an intracellular protein production and others such as the pNCMO2 or the pNY326 lead to an extracellular protein biosynthesis [1].

From the mentioned expression vectors, the one chosen for this work was the pNCMO2 (figure 2) which is a shuttle vector between *B. choshinensis* and *Escherichia coli* (*E. coli)* [1]. In order to construct an expression plasmid using this vector, first *E. coli* is used as the host and then the vector is transferred into *B. choshinensis* cells [1].

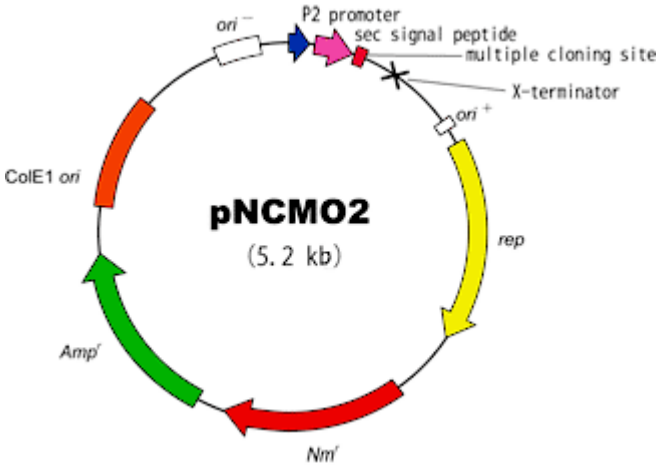


Figure 2 – pNCMO2 DNA vector map [1].

The pNCMO2 presents several unique features. Among others, this vector has a neomycin and ampicilin resistant genes as a selection marker in *B. choshinensis* and *E. coli*, respectively [1]. Also possesses a secretory signal segment that is responsible by the protein secretion directly into the medium culture [1]. Also, the pNCMO2 vector contains the P2 promoter (which is one of the five promoters driving the transcription of cell wall protein) that does not work in *E.*

coli. but has a strong promoter activity in *B. choshinensis*, enabling efficient protein production and making pNCMO2 suitable for cloning genes of which expressions are stressful for *E. coli* [1].

Section II - The COMT Enzyme:

Subsection I - COMT multiple functions:

Catechol-O-methyltransferase (COMT; EC 2.1.1.6) is a magnesium-dependent enzyme that catalyzes the methylation of catechol substrates using S-adenosyl-*l*-methionine (SAM) as a methyl donor and yielding, as reaction products, the O-methylated catechol and S-adenosyl-l-homocysteine (SAH) (see figure 3) [8].

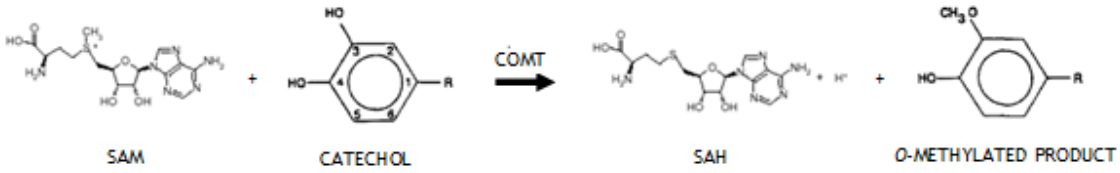


Figure 3 – The O-methylation of the catechol substrate catalysed by COMT (Adapted from [9, 10]).

Typically, the substrates of COMT in mammals include catecholamines with hormonal and neurotransmission activities such as dopamine, norepinephrine, epinephrine, catecholestrogens and their metabolites, ascorbic acid, some indolic intermediates of melanin metabolism and xenobiotic catechols, like carcinogenic catechol-containing flavonoids [9].

Subsection II - COMT Gene:

The protein COMT is present in prokaryotes and eukaryotes [8]. In mammals, it appears in two molecular forms, a soluble form (SCOMT) and in membrane form bound to the rough endoplasmatic reticulum membrane (MBCOMT) [8]. Both the isoforms are encoded by a single gene that, in humans, is located on chromosome 22 band q11.21 and is composed by six exons (see figure 4) [9]. The first two exons are non-coding and the translation initiation codons for

both the isoforms are located on the third exon [8]. In humans, two separate promoters direct the synthesis of two partially overlapped transcripts (see figure 4), one (P2) of 1.5 Kb that is constitutively expressed and another (P1) of 1.3 Kb is subject to tissue-specific regulation [9]. The short transcript translates SCOMT and the longer transcript translates MBCOMT but also the soluble form by the leaky scanning mechanism of translational initiation [8].

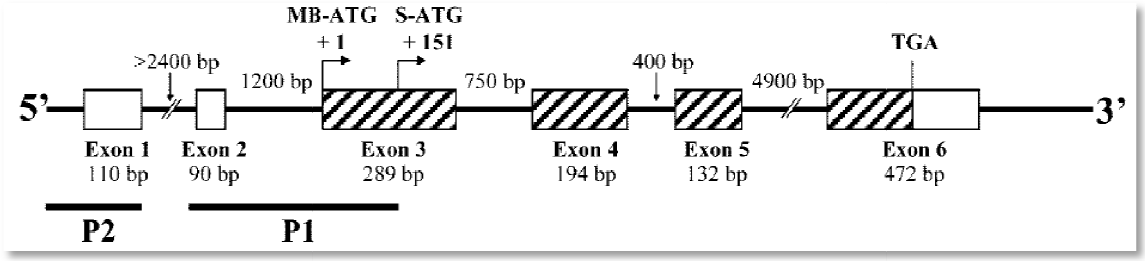


Figure 4 – Structure of human COMT gene [11].

Subsection III - COMT Genetic Polymorphisms:

COMT activity is ubiquitous in animal tissues but the COMT levels vary among different species, in individuals of the same specie as well as in tissues from the same individuals [8]. The highest enzymatic activity, within individuals, is present in liver, followed by kidneys and the gastrointestinal tract while the lowest enzymatic activity is reported to the cardiac tissue [8]. In humans, COMT activity can be distributed in three groups, one with high activity ($COMT^{H/H}$), one with intermediate activity ($COMT^{H/L}$) and other with low activity ($COMT^{L/L}$) [8]. The difference in activity is correlated with a functional COMT polymorphism at codon 108/158 (respectively for SCOMT and MBCOMT) involving a substitution of a methione to a valine in the polypeptide chain [9]. The Met 108/158 variant is associated with low enzymatic activity and decreased thermal stability, while the Val 108/158 is associated with high activity [8]. Several others polymorphisms have been reported to COMT but with irrelevant physiological significance [8].

Subsection IV

COMT and its implication in several human disorders:

During the last decades, the COMT enzyme has been implicated in several human disorders such as cardiovascular diseases [12], neurologic disorders [13 - 15] and estrogen-induced cancers [16, 17].

In particular, the metabolism of steroidal estrogens are depicted in figure 5 where the oxidation of estradiol (E2) occurs primarily at C-2 (and C-4) to form the 2-3 and 3-4 catechols, at C-17 to form estrone (E1) and C-16 to form the 16α-hydroxy (OH) E1 [17]. The E2 and the E1 catechols are intermediates in the generation of more reactive semiquinones and quinones which can serve as substrates for redox cycling and, consequently, generation of reactive oxygen species. Beyond the glucoronidation and sulfatation, the catechols are inactivated by O-methylation mediated by COMT [17].

The E2 catechols 2-OH and 4-OH are O-methylated by COMT with the metabolic clearance of 4-OH E2 being slower than that of 2-OH E2 due to the fact that probably the 2-OH E2 inhibits the O-methylation of 4-OH E2 that persists and participates in redox cycling to generate increased oxidative DNA damage [17]. Some studies with hamsters revealed that the inhibition of COMT potentiates tumorigenesis but it is still unknown whether the susceptibility to steroid-hormone cancers in humans is associated with the levels of COMT activity [17].

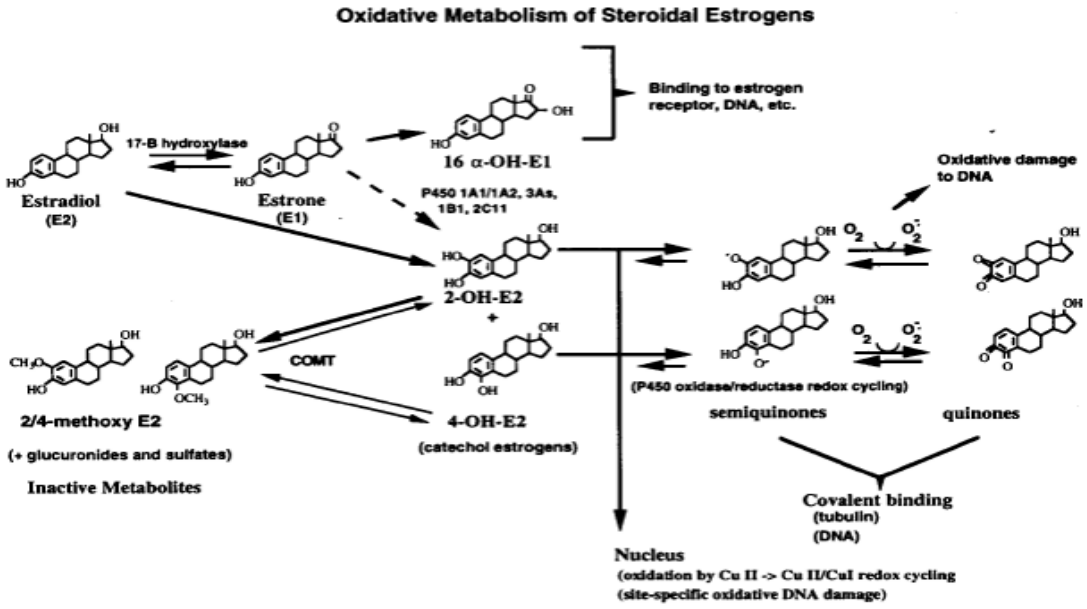


Figure 5 – Pathways for the oxidative metabolism, redox cycling and inactivation of estradiol and estrone in mammalian cells and tissues [17].

The implication of COMT in cardiovascular diseases is related to SAH (see figure 6), one of the products formed from the O-methylation of catechol substrates by COMT, commonly described as an endogenous inhibitor of COMT-mediated O-methylation of endogenous as well as exogenous catechols [12]. In the case of catecholamines, the inhibition of its methylation by SAH, results in accumulation and, subsequently, in the overstimulation of the adrenoreceptor-mediated functions of the cardiovascular system [12]. Also, the constant exposure of the vascular endothelial cells to high levels of circulating catecholamines would lead to their chronic cumulative damage due to the large amounts of the oxidative products (catechol quinones or semiquinones and oxyradicals) formed from the endogenous catecholamines. [12].

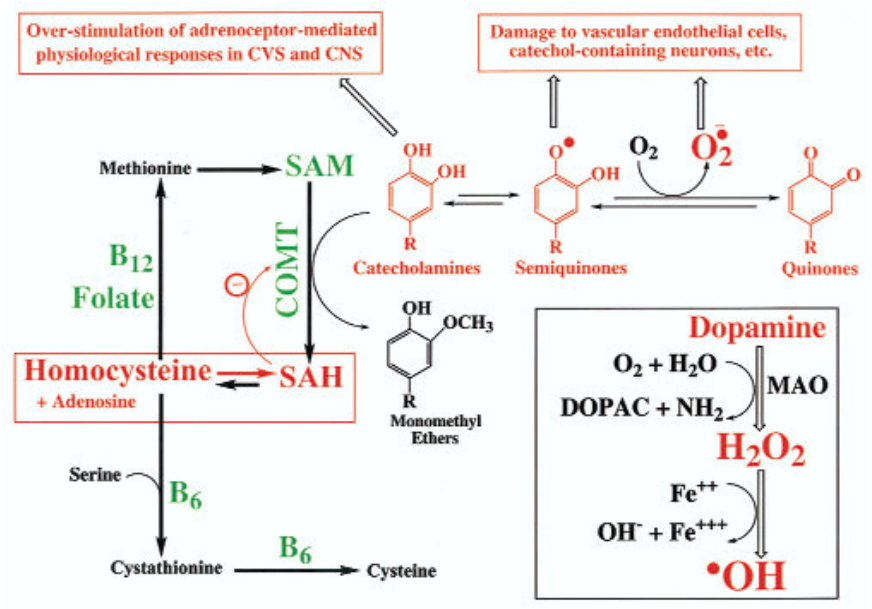


Figure 6 - Proposed mechanism of homocysteine pathophysiology and pathogenesis based on accumulation of intracellular SAH [12].

Parkinson's disease (PD) is a neurological disorder characterized by the degeneration of dopaminergic neurons, with consequent reduction in striatal dopamine levels leading to characteristic motor symptoms [8]. The most appropriated treatment for this disease is the dopamine replacement therapy with levodopa together with an inhibitor of aromatic amino acid descarboxylase (AADC) [8]. It has been stated that the use of COMT inhibitors as adjuvants to levodopa/AADC inhibitor therapy significantly improves the clinical benefits of this treatment [8]. The first-generation COMT inhibitors (figure 7) comprise several compounds such as Pyrogallol and tropolone that contain a catechol substrate or some related

bioisosteric moiety [8]. Tipically, these compounds are competitive substrates of COMT leading, in some cases to lowers efficacies *in vivo* and extremely toxic [8]. The second-generation COMT inhibitors (figure 7) (tolcapone, nebicapone, nitecapone, among others) constituted a new class of di-substituted catechols whose enhanced potency is assigned to the substitution with electron-withrawing groups at a position *ortho* to a hydroxyl group of the catechol moiety [8]. For these compounds, the necessary concentration to inhibit 50 % of COMT activity is in the nanomolar range (increased potency when compared with the first-generation inhibitors whose concentration is at a micromolar range) [8]. It has been discussed whether COMT inhibition should be restricted to the peripheral tissues (like the Nitecapone) or, alternatively, a COMT inhibitor should present broad tissue selectivity (like tolcapone) [8]. Since is attributed to SAM an antidepressive effect and it is well known that the COMT inhibition both central and peripherally leads to the depletion of SAM in the striatum, it is accepted that the COMT inhibitors should present a limited access to the brain in order to minimize the side effects of the PD adjuvant therapy [8].

Nowadays, there is a third class of COMT inhibitors – late atypical inhibitors (figure 7) – that comprises both the bisubstrate inhibitors and the bifunctional inhibitors. In spite of the first ones were designed to target simultaneously the catechol and the SAM binding sites, the bifunctional inhibitors were made by including two catecholic pharmacofores in the same inhibitor molecule [8]. In what concerns to this last class of compounds, it seems that they have more potency *in vitro* than the compounds previously described but more studies are necessary to address several issues like its efficacy *in vivo* or its safety [8].

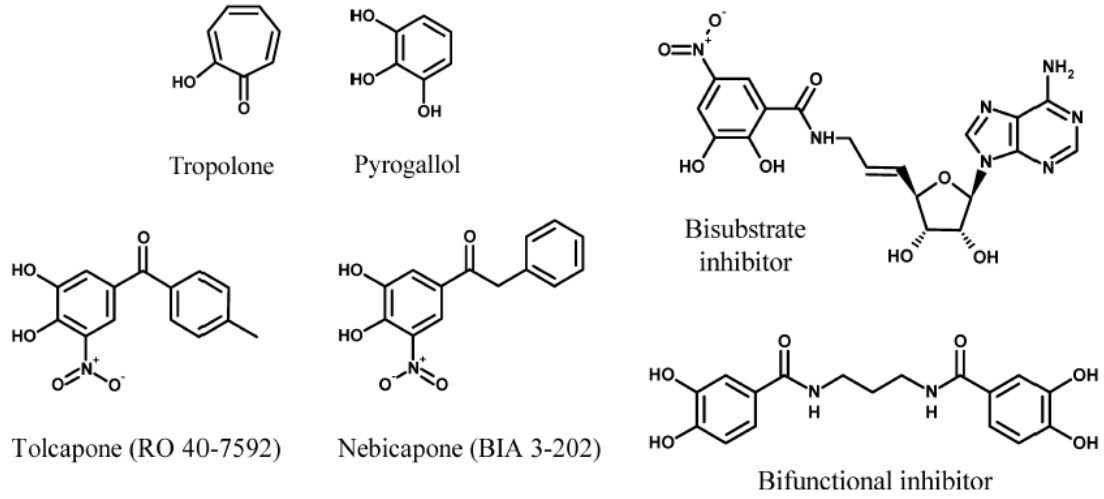


Figure 7 – Representative structures and examples of the three major classes of COMT inhibitors (Tropolone, Pyrogallol refers to the first-generation inhibitors, Tolcapone and Nebicapone to the second generation and the Bisubstrate and bifunctional inhibitors represents the late atypical inhibitors) [8].

Section III – The COMT isoforms:

In mammals, COMT is present in two molecular forms: a soluble form (SCOMT) that contains 221 amino acid residues and a molecular weight of 24.7 KDa (humans) and in another form associated with the membrane (MBCOMT) [8]. The last one, beyond the 221 amino acids from the soluble isoform, has an additional peptide in its amino terminal of 50 amino acid residues corresponding to a molecular weight of 30 KDa (humans) [8]. This extra peptide contains a stretch of 21 (humans) hydrophobic amino acid residues that constitute the membrane anchor region [8]. MBCOMT is an integral membrane protein with the carboxy-terminal portion of the polypeptide (the region catalytically active) exposed to the cytoplasmic side of the membrane [18].

Subsection I - Intracellular localization of COMT isoforms:

In general, the enzyme SCOMT is present in the cytoplasm but it also has been reported in the nucleus of rat transfected COS-7 cells [9] as well as in mammary epithelial cells under certain circumstances such as increased levels of catecholestrogens [19]. On the other hand, MBCOMT, previously assigned to the outer mitochondrial membrane [20] and plasma membrane [21], actually is attributed to the rough endoplasmic reticulum membrane [22].

Subsection II - Relative distributions of S and MBCOMT:

In most human and rat tissues, the levels of SCOMT greatly exceed the levels of MBCOMT (see tables 1 and 2), except for the human brain where it only represents 30 % of the total COMT [8]. This difference in the soluble to MBCOMT ratios (where mainly the long transcript is found) between the rat and human brains is probably due to the fact that in humans the MB-AUG initiation codon is located in a more favorable context than the S-AUG initiation codon [8]. Therefore, most of the translation is initiated from the MB-AUG site [18].

Table 1 – Relative quantification of SCOMT and MBCOMT proteins in rat tissues expressed as % of total COMT in the immunoblot [9].

Tissue	*SCOMT*	*MBCOMT*
Liver	93	7
Kidney	75	25
Heart	79	21
Cerebellum	86	14
Telencephalon	69	31

Table 2 – Relative quantification of SCOMT and MBCOMT proteins in human tissues and cells expressed as % of total COMT in the immunoblot [9].

Tissue	*SCOMT*	*MBCOMT*
Liver	85	15
Kidney	77	23
Adrenal	74	26
Duodenum	89	11
Brain	30	70
HeLa-Cells	35	65
MCF7-Cells	92	8

Subsection III - Analytical methods in COMT assays:

Tipically, a COMT activity trial usually consists on the handling of the sample and incubation followed by separation and detection of the reaction products [23]. The several analytical methods described for COMT activity analysis are depicted in figure 8 [23}. Some of these methods involve the direct quantification of the substrate or the product and don't include a separation step between the *O*-methylated products and the substrate or co-substrate [23]. During the last decades, more reliable and sensitive methods have been developed, involving liquid-liquid extraction or chromatographic techniques with different types of detection [23]. The first methods to separate an O-methylated metabolite from the parent compound included liquid-liquid extraction with organic solvents [23]. After extraction, the organic phase was usually evaporated and the products assess by fluorometric or radiochemical detection [23]. Paper and thin layer chromatography has been used to identify the *O*-methylated products, extracted from COMT incubation mixtures, by comparing the *Rf* values with standard reference compounds [23]. Also, gas chromatography have been applied to separate regioisomeric *O*-methylated products from each other [23] This kind of methods usually require derivatisation of the products and the use of an internal standard [23].

In spite of the methods described above, High Performance Liquid chromatography (HPLC) is the most common technique used for COMT activity measurements. Normally, HPLC with UV detection shows poor selectivity and moderate sensitivity [23]. On the other hand, HPLC with fluorescence detection can be very sensitive and allows the analysis of Omethylated compounds if they exhibit native fluorescence themselves or if they can be derivatised with fluorogenic reagents [23]. The sensitivity of HPLC with radiochemical detection is comparable with other types of detection but when compared with other systems, are restricted to special cases, in order to avoid working with radioactive material [23].

The catechols and phenolic hydroxils of the *O*-methylated products are easily oxidized, which allows electrochemical detection in COMT activity assays [23]. This technique can be used in a wide range of substrates (catecholamines, L-dopa, among others) and it has been applied to determination of the inhibitory effect of COMT inhibitors [23].

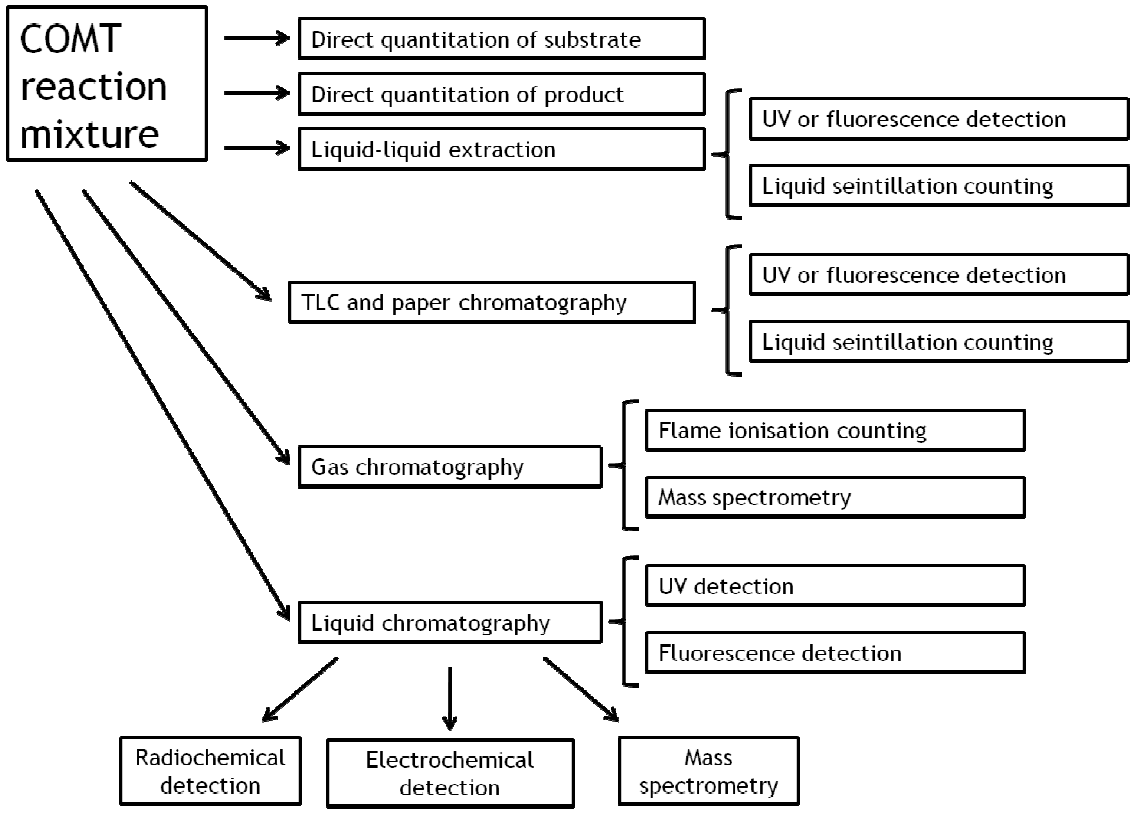


Figure 8 – Analytical methods applied to COMT activity analysis [23].

Section IV – MBCOMT:

Many kinetic and biochemical properties of MBCOMT are similar from those described to SCOMT isoform. Specifically, the mechanism reaction, Ca^{2+} inhibition, pH optimum, Mg^{2+} requirement and similar Km values for the methyl donor AdoMet [24]. However, there are several differences between the two enzymes. MBCOMT is an integral membrane protein [18, 24] with pI values higher from those of SCOMT [20]. Another important difference between the COMT isoforms is that with exception of estrogen catechols, which possess similar K_M values for both isoforms of COMT, all other catechol substrates including catecholamines have lower K_M values for MBCOMT [24]. Interestingly, the substrate concentration seems to influence the kinetic parameters of both isoforms since at low concentrations of catecholamines, *O*-methylation by the low K_M membrane-bound form of COMT would predominate, and only when this enzyme becomes saturated with substrate, does the contribution of the high K_M, soluble form of COMT becomes significant [24]. The catecholamine substrates have a higher affinity but lower reaction velocity with MBCOMT

than SCOMT, suggesting that MBCOMT is more important for the metabolism of catecholamines *in vivo* [24, 25].

Subsection I - Recombinant hMBCOMT Production:

Membrane proteins and COMT, in particular, are implicated in several disorders (previously described). However, studies of these disorders are hampered by a lack of information of the proteins involved which makes the knowledge of the structure of hMBCOMT an essential prerequisite for understanding its function and, further, how its function can be modified by small molecules, one of the major goals of pharmaceutical industry [26]. As the majority of medically and pharmaceutically relevant membrane proteins, hMBCOMT is present in tissues at low concentration, which makes heterologous expression in large-scale production-adapted cells a prerequisite for structural studies [26].

In the last years, recombinant hMBCOMT was produced in several eukaryotic systems such as Sf9 insect cells [21], in transfected human embryonic kidney fibroblast cell lines [27] and in human HeLa and hamster BHK-cells [28].

The system reported by Ulmanen *et al*. (1992) based on Sf9 insect cells for recombinant hMBCOMT production yielded great amounts of hMBCOMT proteins whose polypeptide wasn't processed in the lower-molecular mass SCOMT form [21]. Moreover, they also evaluated the effects of Triton X-100 treatment on substrate methylation of hMBCOMT containing fractions and found that the meta/para methylation ratio increases upon treatment with Triton X-100 [21].

In what concerns to human kidney cell lines, its transfection with the plasmid containing the MBCOMT cDNA, yielded a value of 206 U/mg of protein (using 2.5 mM pyrocatechol as substrate), comparatively to the 9,98 U/mg of protein in the untransfected cells [27]. Also in this work, the inhibitory effect of Ro 40-7592 and tropolone on MBCOMT activity was assessed. Indeed, it was found that these two compounds inhibited hMBCOMT in a dose-dependent manner with an IC_{50} (700 nM and 1mM for Ro 40-7592 and tropolone respectively) similar from those obtained for the native enzyme [27].

Lundstrom *et al*. *(*1997) described a system for hMBCOMT production in human HeLa and hamster BHK-cells from which they assessed the intracellular localizations of COMT isoforms [28]. Indeed, they found that hSCOMT is predominantly found in the cytoplasm but it also could be detected in nuclei [28]. On the other hand, hMBCOMT was attributed to rough endoplasmic reticulum membrane [28].

A common characteristic from almost all works published concerning recombinant hMBCOMT production were largely based on fractionations of cell or tissue homogenates since in that time there weren't available any hMBCOMT-specific antibodies [28].

Several prokaryotic systems have been described for recombinant hMBCOMT production with one major common characteristic, the employment of *E. coli* as the host. In one of these systems, it wasn't able to express the recombinant protein and the authors supposed that the hydrophobic sequence that anchors MBCOMT to the membrane was toxic for the bacteria [9]. In spite of these results, recombinant hMBCOMT in a catalytical active form was produced at relatively high levels in *E. coli* SG 13009 [29, 30] and BL21 strains [11].

The experiments for hMBCOMT production using *E. coli* SG 13009 under the IPTG inducible T7 promoter were carried out with two complementary Deoxyribonucleic acid (cDNA), one coding for the MBCOMT and the other lacking the 24 hydrophobic N-terminal amino acids of MBCOMT [29, 30]. These two cDNAs were introduced into the expression vector pDS56/RBSII and relatively high levels of catalytically active MBCOMT were obtained while the levels of the truncated COMT protein were much lower [29, 30].

A recently work from Zhu *et al*. (2007) presented a system for hMBCOMT production, from which the authors performed a wide set of kinetic analyses using several substrates, computational modeling studies in order to understand the molecular mechanisms underlying the catalytic behavior of hMBCOMT with respect to cofactor SAM, various substrates and the regioselectivity for the formation of mono-methyl ether products [11]. In fact, the enzyme kinetic analyses indicated that the produced MBCOMT was functionally active and the homology models developed for MBCOMT showed that the binding energy values calculated for most substrates agreed well with measured kinetic parameters [11]. Also, the model developed predicted precisely the regioselectivity for the *O*-methylation of several substrates at different hydroxyl groups since the predicted values matched the experimental data [11].

Subsection II - Recombinant MBCOMT Purification:

Actual scientific literature fails in terms of relevant information concerning purification trials for MBCOMT isolation. However, there is a report [31] where the authors described a partial purification by anion-exchange chromatography of a fraction containing rat MBCOMT previously isolated from rat liver homogenates and solubilized with Triton X-100 0,5 %. In this strategy, the authors used a Resource Q column, prepacked with Source 15Q, combining high speed, elevated capacity with high resolution [31]. The figure 9 represents a typical chromatogram obtained with a Resource Q column for the purification of MBCOMT [31].

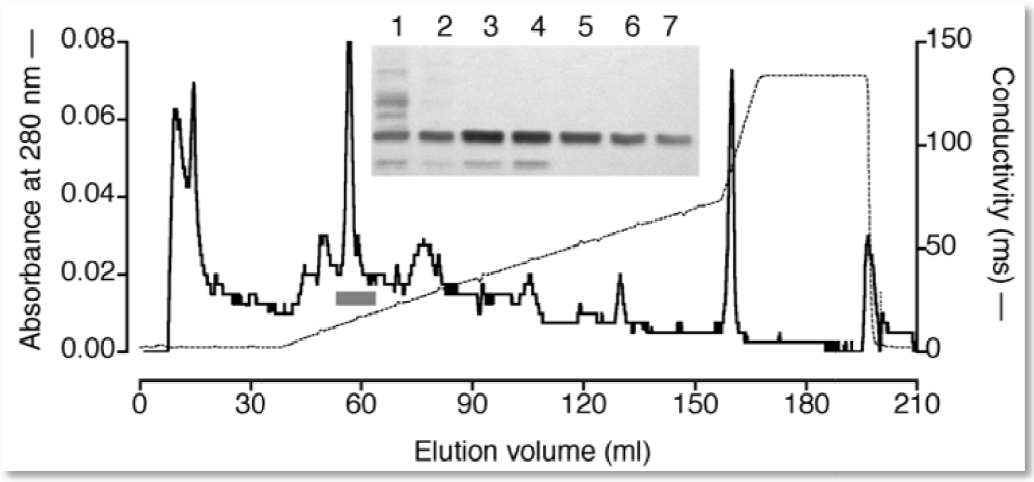


Figure 9 – Elution of MBCOMT from a Resource Q column at pH 7.8 by a NaCl gradient. The full line represents the absorbance at 280 nm and the dashed line represents the conductivity read on-line. The grey bar indicates the fractions positive for COMT activity. The inset represents an analysis by western-blot of the MBCOMT microssomal fraction (1), the solubilized MBCOMT fraction (2) and the Resource Q fractions positive for COMT activity (3 – 7) [31].

Section V – The mimics of the lipid bilayer: a mandatory tool for membrane protein production:

There are several difficulties concerning the study of membrane proteins [32]. The fact that membrane proteins are naturally embedded in a mosaic lipid bilayer, which in even the simplest organism is a complex, heterogenous and dynamic environment limits (but does not preclude) the use of many standard biophysical techniques to determine protein structure and function such as NMR, X-ray christallography, among others [32]. Such methods require previous protein extraction from its native membrane and studied in a detergent or a lipid environment *in vitro* [32].

In addition, membrane proteins are not generally soluble in aqueous solution, so they need to reside in surroundings that satisfy their high hydrophobicity. This can be accomplished using special synthetic systems for *in vitro* trials [32]. In figure 10 are depicted several solubilization techniques used for functional and structural characterization of membrane proteins [22].

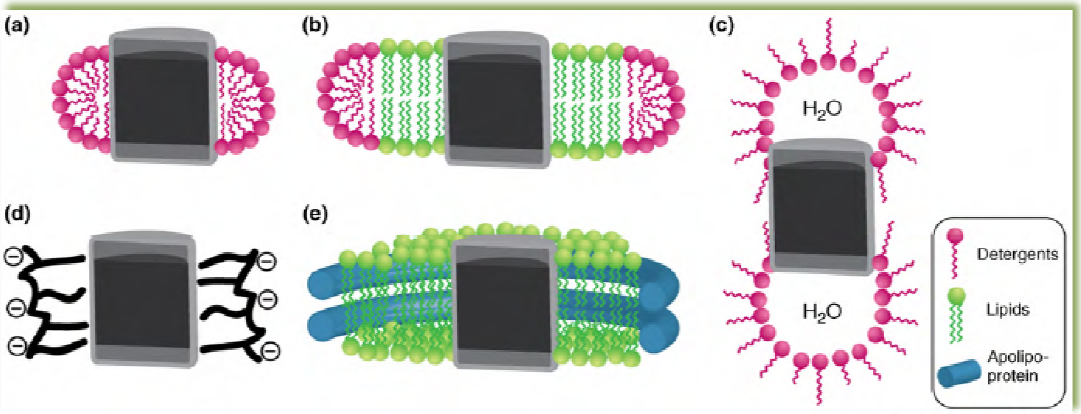


Figure 10 - Schematic representation of different solubilization methods for integral membrane proteins. Micelle (a), bicelle (b), reverse micelle (c), amphipol (d) and nanolipoprotein particle (e). The membrane protein is depicted in grey with its hydrophilic and hydrophobic regions in light and dark grey [22].

Subsection I - Detergents:

The importance of detergents as tools for the study of membrane proteins cannot be underestimated since they are usually vital in the isolation and purification of the protein and applied in the primary solubilization step of reconstitution [32]. Detergents are amphipatic molecules, consisting of a polar head group and a hydrophobic chain that exhibit unique properties in aqueous solution by forming spherical micellar structures in which membrane proteins are usually soluble (see figure 10 a) [32].

Detergents are classified in four major classes of detergents (whose structure is depicted in figure 11), the ionic, non-ionic, zwiterionic and bile acid salts detergents [32]. The ionic detergents contain a head group with a net charge that can be anionic or cationic and a hydrophobic hydrocarbon chain or steroidal backbone [32]. This type of detergents comprises sodium dodecyl sulphate (SDS) which is extremely effective in the solubilization of membrane proteins but it can promote irreversible denaturation [32]. The bile acid salts are ionic detergents which differ from SDS in their backbone, which consists on a rigid steroidal group [32]. As a result, they have a polar and apolar face instead of a well-defined head group, with the capacity to form small kidney-shaped aggregates unlike the spherical micelles formed by the traditional ionic detergents [32]. Non-ionic detergents contain uncharged hydrophilic head groups of either polyoxyethilene or glycosidic groups. Typically, are considered to be mild and non-denaturing as they break lipid-lipid and lipid-protein interactions rather than protein-protein contacts [32]. This allows membrane protein solubilization and isolation in a biological active form [32]. Finally, zwiterionic detergents combine the properties of ionic

and non-ionic detergents and, in general have more deactivating characteristics than non-ionic detergents [32].

The critic micellar concentration (CMC) can be defined as the minimum concentration of detergent for individual detergent molecules to cluster and form micelles promoting a sudden change in surface tension and other pertinent physical properties [32].

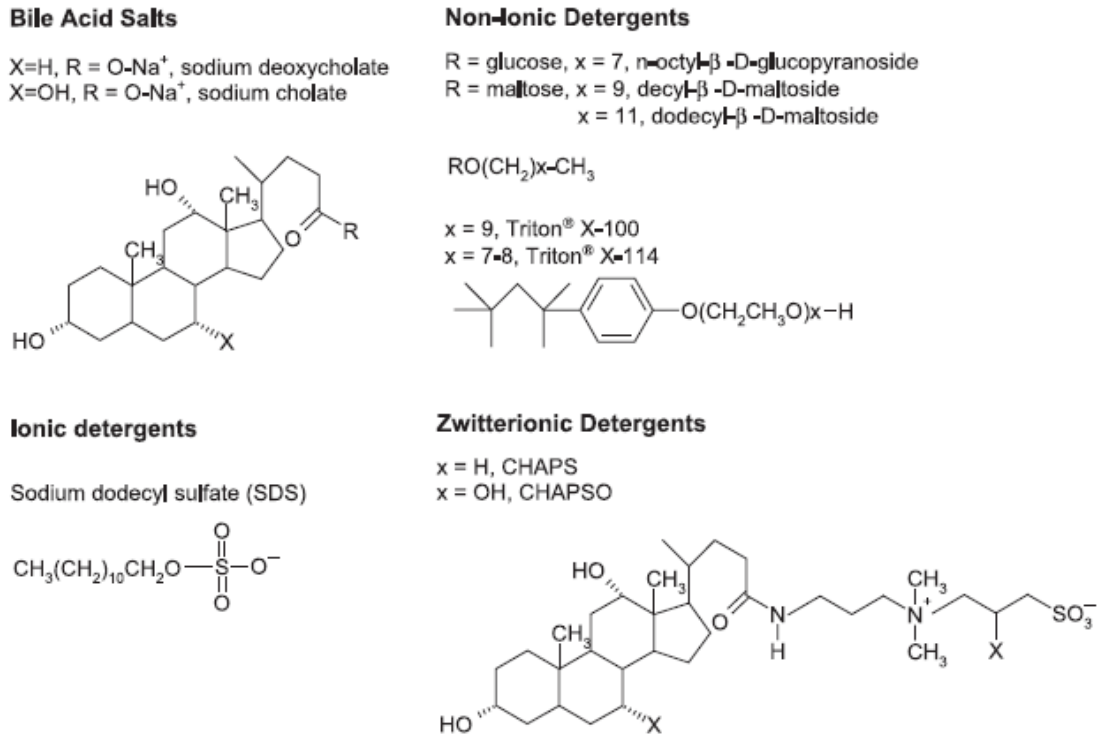


Figure 11 – Structure of the different types of detergents [32].

Subsection II - Non-micellar membrane-mimicking systems:

The appropriated function of membrane proteins is likely to depend strongly on the chemical and physical properties of the membrane [22]. A major limitation for their functional and structural characterization is thus the requirement for an artificial environment that mimics the native membrane to preserve the integrity and stability of the membrane protein [22]. The most commonly employed methods are detergent micelles, which can be detrimental to membrane protein activity and stability [22]. Therefore, there is an imperative need for the development of alternative nonmicellar solubilization techniques.

The amphipols (see figure 10 d) were designed to minimize the micellar phase in order to limit the membrane protein instability in detergents [33].The amphipols are composed of a hydrophilic polymeric chain onto which hydrophobic alkyl chains are randomly grafted [33]. Thus, these molecules possess a high affinity for the surfaces of the protein (multipoint attachment) and even very low concentrations of free surfactant allow the protein to remain soluble [33]. Furthermore, the diminished protein-protein interaction and the reduced dynamics of conformational transitions in the helix bundle may limit protein aggregation and unfolding phenomena [33].

The amphipols, as well as detergent micelles, are able to stabilize membrane proteins in aqueous solution, but they don't closely resemble the native lipid bilayer [22]. Therefore, a membrane mimic comprising a lipid bilayer is preferable, as it may better maintain the structural integrity of a membrane protein [22]. This requirement can be achieve applying four groups of membrane mimics, the lipossomes, reverse bicelles, bicelles and nanolipoproteins [22].

Specifically, coupling of proteins to the liposome surface offers the possibility of cell-specific targeting with important therapeutic implications [34]. Several methods have been described for the insertion of membrane proteins into artificial membranes and it usually involves the use of detergents [34]. Recently, a three steps method for transmembrane proteins has been described, focus the solubilization of liposomes by a detergent, addition of the protein and the detergent removal [34]. However, liposomes can limit solubility, improve the formation of multilamellar vesicles and the inaccessibility of the vesicle interior may interfere with functional investigations [22].

Reverse or inverted micelles (see figure 10 c) contain hundreds of surfactant molecules with geometric and charge properties that drive the spontaneous organization of a typically spherical particle with an inner water core [35]. In these systems, integral membrane proteins should be transferred from an aqueous detergent solubilized state to the reverse micelle system maintaining the structural integrity of the protein [35]. This can be accomplished by employing a special kind of surfactant (such as methylammonium bromide – CTAB), one acting as an aqueous detergent and as a reverse micelle surfactant [35]. These two characteristics showed by the surfactants are sometimes conferred by the use of co-surfactants such as hexanol [35].

The bicelles are binary, water-soluble assemblies of lipids and detergents [22]. In a bicelle (see figure 10, the lipids form the central part and the detergents form the edge of a disc-shaped assembly (see figure 10 b) [22]. For NMR experiments, micelles offer an advantage over bicelles since its large molecular weight leads to broad resonance lines [22]. However, the use of bicelles is preferable for the study of interactions that are not retained in micelles [22].

The nanolipoprotein (NLPs) or reconstituted High Density Lipoproteins (rHDLs) particles provided a novel tool for studying membrane proteins in a native-like membrane environment

[22]. NLPs consist of a noncovalent assembly of phospholipids arranged as a discoidal bilayer, surrounded by amphipatic apolipoproteins (see figure 10 e). In their native context, apolipoproteins assemble into roughly spherical HDL particles [22]. The *in vitro* reconstitution of HDLs has served as a base for the development of disc-shaped rHDLs for membrane protein solubilization [22]. In general, the native-like bilayer architecture provided by NLPs is likely to support both protein stability and functionality of an incorporated membrane protein [22]. The *in vitro* reconstitution of NLPs from purified lipids or membrane extracts offers the unique possibility to precisely mimic the native composition of a particular membrane and to probe the effect of selected lipids on membrane-protein function [22]. The major advantage of these systems is render membrane proteins water-soluble in the complete absence of detergent molecules [22].

Chapter II

Materials and Methods

Section I - Materials:

High fidelity PCR enzyme mix, Nco I and EcoR I restriction enzymes were purchased from Fermentas International Inc (Thermo Scientific). Taq DNA polymerase, Wizard Plus SV Minipreps DNA Purification System and pGEM-T easy vector system were obtained from Promega (Madison, USA). PCR clean-up Gel extraction was obtained from Macherey-Nagel (Duren, Germany). *Escherichia coli* competent cells XL1B and Top 10 were obtained from Invitrogen (USA). The pNCMO2 expression vector, pNCMO2-BLA control vector and *B. choshinensis* Electro-Cells were purchased from Takara Bio Inc. Ultrapure reagent-grade water was obtained with a Mili-Q system (Milipore/Waters). Ampicilin (sodium salt), neomycin (trisulfate salt hydrate), isopropylthiogalactosidase (IPTG), glucose, calcium chloride dihydrate, yeast extract, ferrous sulfate heptahydrate, manganese sulfate monohydrate, zinc sulfate heptahydrate, magnesium chloride anhydrous, lysozyme, dithiotreitol (DTT), S-(5'-Adenosyl)-L-methionine chloride, CAPS, DNase, RNase, epinephrine (bitartrate salt), disodium Ethylenediamine tetraacetic acid (EDTA), sodium octil sulfate, dibutylamine, Bovine serum albumin (BSA), LB-Agar and Agar were obtained from Sigma Chemical Co (St Louis, MO, USA). Potassium acetate (anhydrous), potassium chloride and sodium chloride were supplied by Fluka (Buchs, Switzerland). Bacto Soytone and Polypeptone were purchased from Becton Dickinson (New Jersey, USA). Bis-Acrylamide 30 % was obtained from Bio-RAD, Hercules, CA. The High-Range Rainbow molecular weight markers used for estimation of subunit molecular weight and the anti-rabbit IgG alkaline phosphatase secondary antibody were purchased from GE Healthcare Biosciences (Uppsalla, Sweden). Polyclonal rabbit anti-COMT antibody was produced in Bial using purified recombinant rat COMT [36]. All other chemicals were of analytical grade and used without further purification.

Section II – Methods:

Subsection I - Strains, plasmids and media:

The *B. choshinensis* cells were used in this study for protein recombinant production. In addition, *E. coli* TOP 10 and XL1B cells were applied for deoxyribonucleic acid (DNA) manipulations. The vector pGEM-T easy was applied for DNA manipulations and the plasmid pNCMO2, the *E. coli - B. choshinensis* shuttle vector, was used as the expression vector. Usually, a LB formulation medium was essential to allow the growth of *E. coli* TOP 10 cells. Alternatively, for B. choshinensis cells, we used the 2SYNm medium (20.0 g/L glucose, 40.0 g/L Bacto Soytone, 5.0 g/L Bacto Yeast Extract, 0.15 g/L $CaCl_2.2H_2O$ and 50.0 µg/ml Neomycin), the MTNm liquid medium (10.0 g/L glucose, 10.0 g/L Polypeptone, 5.0 g/L Bacto Yeast extract, 10.0 mg/L $FeSO_4.7H_2O$, 10.0 mg/L $MnSO_4.4H_2O$, 1.0 mg/L $ZnSO_4.7H_2O$, 4.1 g/L $MgCl_2$ and 50.0 µg/ml Neomycin) and MTNm plates (MT Liquid medium, 3.75 g/L Agar and 10.0 µg/ml Neomycin).

Subsection II - Construction of pNCMO2-hMBCOMT expression vector:

Brevibacillus Expression system (Takara Bio Inc) was used for the expression of human MBCOMT in its native form and all the process was carried out according to manufacturer's instructions. Briefly, a 813 bp DNA fragment coding for MBCOMT was obtained using normal human liver cDNA as template. Polymerase chain reactions (PCR) were carried out using specific primers (forward primer, 5' TACCATGGCTTTCGCTATGCCGGAGGC 3'; reverse primer, 3' TAGAATTCTCAGGGCCCTGCTTC 5') with Nco I and EcoR I restriction sites for directional cloning. PCR was conducted as follows: denaturation at 95 ºC for 5 minutes, followed by 30 cycles at 95 ºC for 30 seconds, 64 ºC for 30 seconds and 72 ºC for one minute, and a final elongation step at 72ºC for 5 minutes. The amplified cDNA was purified by low melting agarose gel electrophoresis and was cloned into pGEM-T easy vector. The construct was transformed into *E. coli* Top 10 cells and grown overnight at 37 ºC in plates with LB-Agar medium containing ampicilin (50.0 µg/ml), IPTG (0.1 mM) and X-GAL (40.0 µg/ml). Next, some white positive colonies were inoculated in 2.0 ml of LB medium and grown at 37 ºC and 250 rpm overnight. From these cultures, highly purified plasmids were prepared using Wizard SV Plus Minipreps (Promega) and were sequenced (Stabvida, Oeiras, Portugal) to confirm the identity of the amplicon [37].

The MBCOMT DNA cloned into pGEM-T easy was digested with Nco I and EcoR I, purified by low melting agarose gel electrophoresis and was then cloned into the expression vector

pNCMO2 (previously digested with the same restriction enzymes) by T4 DNA ligase. This construct was transformed into *E. coli* Top 10 cells, grown overnight at 37 ºC in plates with LB-Agar medium containing ampicilin (50.0 µg/ml) and colonies were screened for the presence of the construct pNCMO2-hMBCOMT. Therefore, some colonies were inoculated in 2.0 ml of LB medium and grown at 37 ºC and 250 rpm overnight. From these cultures, highly purified plasmids were prepared using Wizard SV Plus SV Minipreps and digested with Nco I and EcoR I restriction enzymes to confirm the insert. Next, pNCMO2-hMBCOMT vector was sequenced to confirm the identity of the amplicon, orientation and frame. Since the sequence was confirmed to correspond to human MBCOMT [37], the target plasmid was introduced into B. choshinensis by electroporation, according to the manufacturer's instructions and grown overnight at 37 ºC in MTNm plates containing neomycin (50.0 µg/ml). After, some colonies were inoculated in 2.0 ml of MT medium and grown overnight at 30 ºC and 120 rpm for screening the presence of the construct pNCMO2-hMBCOMT.

Subsection III – Agarose gel electrophoresis:

The DNA electrophoresis was performed on a gel containing 1% agarose (Hoefer, San Francisco, CA, USA). The electrophoresis was carried out in Tris-acetic acid (TAE) buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0) and runed at 120 V for 30 min. The bands corresponding to DNA was visualized under ultra violet light after staining the gels with 0.01% ethidium bromide. The gels were visualized under UV light in a Vilber Lourmat system at 320 nm (ILC Lda, Lisbon, Portugal).

Subsection IV - Recombinant hMBCOMT Production:

Unless otherwise stated, recombinant hMBCOMT was carried out according to the following protocol. Cells containing the expression construct were grown overnight at 37 ºC in MTNm plates. A single colony was inoculated in 62.5 ml of 2SYNm medium in 250 ml shake flasks. Cells were grown at 30 ºC and 120 rpm until the cell density at 660 nm (OD_{660}) reached 2.6. Subsequently, an aliquot was added in 62.5 ml of 2SYNm medium on 250.0 ml shake flasks, since the inoculation volume was fixed to achieve an initial OD_{660} of 0.2 units. After a 48 h growth at 30 ºC and 120 rpm, cells were harvested by centrifugation (5000 x g, 25 min, 4 ºC) and stored frozen at – 20.0 ºC until use.

Subsection V - Cell Lysis:

The bacterial cell pellet (62,5 ml or 125,0 ml) was ressuspended in 10,0 ml of an appropriate Buffer (150 mM NaCl, 10 mM DTT, 50 mM Tris, $MgCl_2$ 1 mM, pH 8,0) with protease inhibitors (5,0 µg/ml leupeptin and 0,7 µg/ml pepstatin), disrupted by lysozyme treatment (10,0 mg/ml) for 15 minutes at room temperature and followed by six freeze (- 196 ºC in liquid nitrogen)/thaw (42 ºC) cycles. Then, deoxyrribonuclease (1,0 mg/ml) was added to the lysate and the soluble material removed by centrifugation (16000 x g, 20 min, 4 ºC).

Subsection VI - MBCOMT Solubilization:

Unless otherwise stated, solubilization was carried out by incubating the pellet (containing the total membrane) obtained after freeze thaw/lysis with 1.0 % (v/v) Triton X-100 in an appropriate buffer (150 mM NaCl, 10 mM dithiotreitol, 5 µg/ml leupeptin, 50 mM Tris pH 8.0) at4 ºC until full solubilization of the pellet.

Subsection VII - Total protein quantification:

Protein content in samples was measured by the Pierce BCA Protein Assay Kit (Thermo Scientific, USA), using BSA as the standards (0.025 – 2.0 mg/ml), according to manufacturer's instructions.

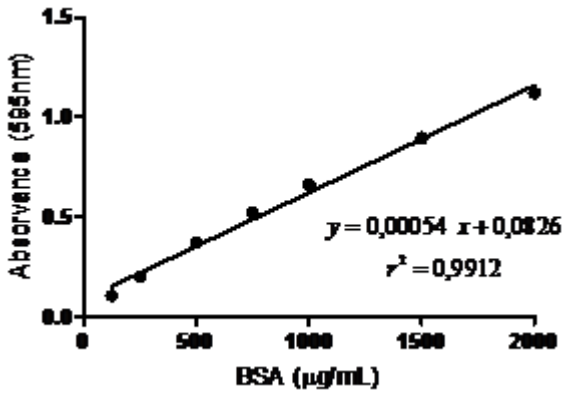


Figure 1 - BSA (bial buffer with Triton X-100 1% (v/v)) calibration curve.

Subsection VIII - MBCOMT Enzymatic assay:

The methylating efficiency of recombinant MBCOMT was evaluated by measuring the amount of metanephrine formed from epinephrine as substrate as previously described [38]. To determine the recombinant MBCOMT kinetics parameters K_M and V_{max}, aliquots of the solubilised membrane (previously optimized for 2.0 mg of total protein per ml) were added to increasing concentrations of epinephrine (20 – 400 μM) (maintaining cofactor SAM concentration on 250 μM) during 15 min (previously optimized) at 37 ºC. The reaction was stopped with 2 M of percloric acid and the samples were processed as described elsewhere [38].Finally, the incubation samples were injected and analyzed in a HPLC with an electrochemical amperometric detection system.

Subsection IX - SDS-PAGE and Western-blot:

Reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [39] and as previously described [40]. Samples were boiled in a loading buffer containing 500 mM Tris-CL (pH 6,8), 10 % (w/v) SDS, 0,02 % bromophenol blue (w/v), 0,2 % glycerol (v/v), 0,02 % Β-mercaptoethanol (v/v) for 5 min and then run on 4 % stacking and 12,5 resolving gels containing 0,1 % SDS, with a running buffer containing Tris (25 mM), glycine (192 mM), SDS (0,1 % w/v) at 150 V for 95 min. Then, one gel was stained by Coomassie brilliant blue and the other gel was transferred to a polyvinylidene difluoride (PVDF) membrane, in to perform the western blots experiments. The proteins were transferred over a 44 min period at 750 mM at 4 ºC in a buffer containing 10 mM CAPS and 10 % (v/v) of methanol. After the blotting, the membranes were blocked with TBS-T (pH 7,4) containing 5 % (w/v) non-fat milk for 65 min at room temperature and exposed overnight at 4 ºC to a rabbit anti-rat MBCOMT polyclonal antibody, that cross reacts with the human protein, at 1:2500 dilution in TBS-T 1 %. The filters were washed three times (15 min each) with TBS-T and adherent antibody was detected by incubation for 1 h with an anti-rabbit IgG alkaline phosphatase secondary antibody at a 1:10000 dilution in TBS-T 1 %. The PVDF membranes were air dried, incubated with 200 ml of ECL substrate for 5 min and enhanced by exposure to chemiluminescence's detection.

Subsection X - Determination of Cell density and Dry cell weight:

Cell density (OD_{660}) was measured spectrophotometrically and the assessment of dry cell weight was performed as previously described [41]. Specifically, for this recombinant strain, one unit of OD_{660} was found to correspond to a dry *Brevibacillus choshinensis* weight of 0.4415 g/L.

Subsection XI - Hydrophobic interaction Chromatography:

All chromatographic separations were performed at room temperature on an AKTA purifier system (GE Healthcare Biosciences, Uppsalla, Sweden). All buffers pumped in AKTA system were filtered through a 0.20 μM pore size membrane (Schleicher Schuell, Dassel, Germany) and degassed ultrasonically. The Hydrophobic Interaxtion Chromatography (HIC) stationary phase on analysis was butyl-Sepharose. The hydrophobic medium was packed according to company guidelines (11 ml of gel volume) into a C_{16} glass column purchased from GE Healthcare Biosciences. The columns were initially equilibrated with 0.4 M ammonium sulfate in Tris-Cl 10 mM, pH 7.8. Aliquots of recombinant MBCOMT-containing pellet were loaded onto the columns and an isocratic elution at 1.0 ml/min was performed with the same concentration of ammonium sulfate. After elution of unretained species, the concentration of ammonium sulfate was decreased to zero in a step mode, by eluting the column with Tris-Cl 10 mM, pH 7.8. This condition was maintained during the appropriate time in order to elute the bound and weakly retained species. In all separations, the optical density was monitored at 260 and 280 nm throughout the entire chromatographic run, while 10 ml and 7 ml fractions (for peak 1 and 2 respectively) were collected and evaluated for MBCOMT specific activity using a specific enzymatic assay. The “active fractions” were further analysed for purity and immunoreactivity respectively by SDS-PAGE and Western-blotting.

Chapter III

Results and Discussion

Section I - Construction of the pNCMO2-hMBCOMT expression vector:

Using a human liver cDNA as a PCR template, the gene of interest is typically amplified, cloned, sequenced and then sub-cloned into one or more expression vectors [42]. This initial cloning into a non-expression vector is often a big time saver for several reasons [42]. In first place, unintended expression of the gene of interest can cause toxicity and make it difficult to clone some genes directly into an expression vector [42]. Secondly, many expression vectors are large (complicating cloning) or have low copy number (complicating both cloning and sequencing). Thirdly, many genes, even those with good profile, contain sequence errors or single-nucleotide polymorphisms [42]. Thus, preliminary cloning into a smaller, higher copy vector facilitates both the cloning itself and also sequence confirmation of the gene and motifs that have been added via PCR primers to improve protein expression and purification [42]. The ideal promoter for the expression of recombinant proteins directs efficient transcription to allow high level protein production and is tightly regulated to minimize metabolic burdens and toxic effects [43]. Inexpensive and automatic (without operator intervention) induction is often desirable for large-scale production [44].

The first step on the construction of the expression vector pNCMO2-hMBCOMT is the gene amplification by PCR. In general, the magnesium ion concentration and the annealing temperature are the two major factors affecting the optimization of a PCR [45]. In order to assess the effect of the annealing temperature in the gene amplification, PCR reactions using specific primers were performed for hMBCOMT gene (see table 3), at three different annealing temperatures (figure 1 a).

Table 1 – Nucleotide sequence of Primers.

Primer	*Nucleotide Sequence*
hMBCOMT_Forward	5' TACCATGGCTTTCGCTATGCCGGAGGC 3'
hMBCOMT_Reverse	3' TAGAATTCTCAGGGCCCTGCTTC 5'
SP6 (pGEM-T easy)	5' – TATTTAGGTGACACTATAG – 3'
T7 (pGEM-T easy)	5' – TAATACGACTCACTATAGGG -3'
pNCMO2_Forward	5' – CGCTTGCAGGATTCGG – 3'
pNCMO2_Reverse	5' – CAATGTAATTGTTCCCTACCTGC – 3'

As we can see in the figure 1 a, all reactions amplified a DNA fragment with approximately 800 bp. Lanes II, III and IV correspond to PCR reactions with annealing temperatures at 55, 60.4 and 64 ºC, respectively. Since the band in the lane IV showed a higher density, the annealing temperature of 64 ºC was fixed in all the PCR performed in this work. In the lane IV was applied the negative control from these reactions and the band observed with low molecular weight would probably corresponds to primer dimerization.

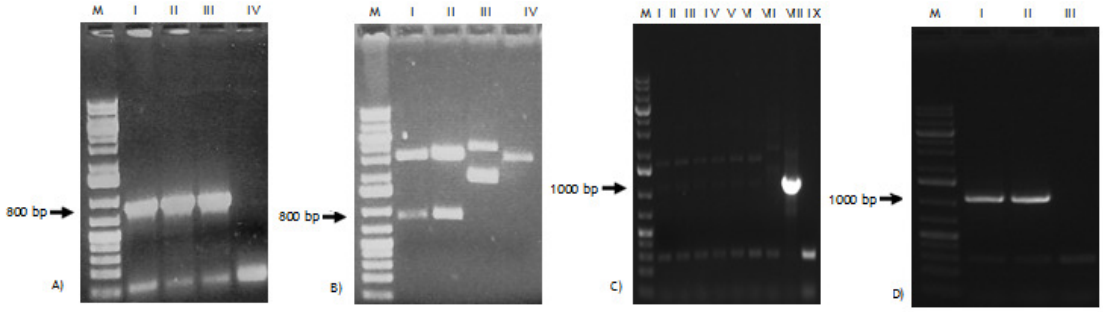


Figure 1 - Agarose gel electrophoresis pictures of the several stages during the construction of the expression vector pNCMO2-hMBCOMT. A) PCR products of selective amplification with hMBCOMT primers of human liver cDNA at three different annealing temperatures (55, 60.4 and 64 ºC); B) Purified pGEM-T easy vector cloned with hMBCOMT DNA insert from *E. coli*; C) Purified pNCMO2 vector cloned with hMBCOMT DNA insert from *E. coli* D) Purified pNCMO2 vector cloned with hMBCOMT DNA insert from *B. choshinensis*.

The PCR product with was cloned into pGEM-T easy vector using the T4 DNA ligase. Then, *E. coli* XL1B competent cells were transformed with this construct by heat shock (one hour in ice followed by one minute at 42 ºC) and inoculated at 37 ºC in LB plates (with ampicilin, X-GAL and IPTG) during 20 hours. Subsequently, up to 4 white colonies were picked and inoculated in 2.0 ml LB-broth at 37 ºC and 250 rpm overnight. From these colonies, highly purified plasmids were prepared using the Kit Wizard plus SV minipreps, a sequential digestion of cloned pGEM-T easy were done and the results were analyzed in an agarose gel electrophoresis (figure 1 b).

By the figure 1 b, it is possible to conclude that in the lane IV didn't occur the digestion and in the lane V, the plasmid was digested but didn't contain the insert. In what concerns to the lanes II and III (samples A. P. 1 and A. P. 2, respectively), the plasmids contained the DNA insert as we can see by the two bands that appear in the image, one with a molecular weight between 3000 and 4000 bp that corresponds to the linearized vector, and the other one with a molecular weight between 800 and 1000 bp, corresponding to the target DNA insert.

In order to assess if the nucleotide sequence cloned on the pGEM-T easy vector corresponded to the hMBCOMT gene nucleotide sequence, the vector was subjected to sequencing with specific primers (see table 3). The results achieving (see appendices 1) show a 100 % of homology between the sequence cloned on the vector and the one that corresponds to the hMBCOMT. Therefore, the sample A. P. 2 was digested with EcoR I and the DNA insert was separated from the vector in an agarose gel electrophoresis, from which the target DNA was purified. After this step, the DNA insert and the pNCMO2 vector were digested with the restriction enzymes Nco I and EcoR I. Then, the DNA fragment was cloned into pNCMO2 vector (at several ratios vector/insert DNA) using the T4 DNA ligase. Next, *E. coli* Top 10 cells were

transformed by heat shock treatment with this construct and spread onto LB plates with ampicilin and incubated overnight at 37 ºC. Several colonies were picked up and inoculated in 2.0 ml of LB-broth at 37 ºC and 250 rpm. Next, plasmid DNA was extracted from cells and a PCR screening was performed to identify plasmids with the insert. Then, each sample was applied on an agarose gel electrophoresis whose results are depicted in figure 1 c. In this image, we can conclude that plasmid at lane IX contains the DNA insert. The plasmid (from lane IX) was sequenced in order to confirm the identity of insert, orientation and frame.
At the end, *B. choshinensis* cells were transformed with the pNCMO2-hMBCOMT by electroporation. The transformation was made according the manufacturer's instructions and the cells incubated at 37 ºC on MTNm plates during 36 hours. Several colonies were inoculated in 2.0 ml of MTNm medium and incubated at 30 ºC and 120 rpm. Plasmid DNA was extracted and digested with restriction enzymes to confirm the insert.

Section II – Recombinant hMBCOMT production:

Up until now, almost all known production and recuperation processes for hMBCOMT have focused their attention in providing evidences that hMBCOMT is a distinct entity in comparison to SCOMT isoform. Specifically, in identifying the hMBCOMT cDNAs [27], finding precisely its intracellular localization [28] and assess the intrinsic kinetic parameters in several rat and human matrices [21, 11, 31, 46]. Indeed, there isn't any work publish regarding the optimization of hMBCOMT culture conditions and focusing a screening of the ideal detergent type and concentration for hMBCOMT solubilization obtained from recombinant systems. Since hMBCOMT exists in cells at low concentrations, the development of heterologous systems capable of delivery high concentrations of the recombinant enzyme for structural and pharmacological studies remains an essential goal to achieve.

Subsection I – Dry *Brevibacillus choshinensis* weight determination:

The dry *B. choshinensis* weight was determined (Figure 2) and one unit of OD_{660} was found to correspond to a dry *B. choshinensis* weight of 0.4415 g/L. The sizes of this cell vary from 0.4 to 0.6 x 1.5 to 4 μm [47], higher than those reported to *E. coli* 0.8 x 2 μm [48]. Previous results reported from Silva and co-workers (2009) specified that *E. coli*. DH5α retrieved a value of 0.25 g/l to one unit of OD (660 nm) [41]. Since the size of *B. choshinensis* is higher than reported to *E. coli* DH5α, it is highly expectable a relationship between dry *B.*

choshinensis weight and OD_{660} higher than *E. coli*. DH5α. Therefore, the value 0,4415 g/L for Dry *B. choshinensis* weight is in agreement with the values attributed for the size of the target cells.

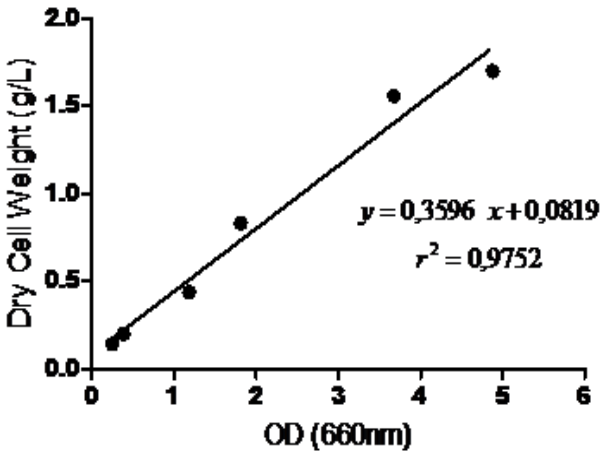


Figure 2 – Relationship between the OD (660 nm) and the dry *B. choshinensis* weight.

Subsection II – Analysis of the different fractions obtained in hMBCOMT production

Teorically, analysis by western-blot and the assessment of biological activity of the three different fractions obtained during the biosynthesis of hMBCOMT can provide pertinent information about its preferential location in cells. Because it is unpredictable whether membrane-bound COMT will end up in a membrane system or in inclusion bodies, the first step in monitoring membrane protein expression is usually fractionation the samples into soluble, insoluble and membrane fractions. Indeed, our results (Figure 3 a) indicate that the major percentage of hMBCOMT is present in the membrane fraction with a band close to the 31 KDa molecular weight marker, as expected, and only a basal level expression is detected in the cell lysis supernatant. In addition, the determination of biological activity in the culture medium, cell lysis supernatant and the solubilized membrane confirmed the hMBCOMT compartmentalization in the target cells. The following values 0; 1.21 and 26.62 nmol/h/mg of protein were obtained for the culture medium, cell lysis supernatant and the solubilized membrane, respectively. Thereby, the data obtained supports the fact that almost all the hMBCOMT produced in a biological active form is present in the membrane fraction and only a minor fraction can be found in the cell lysis supernatant.

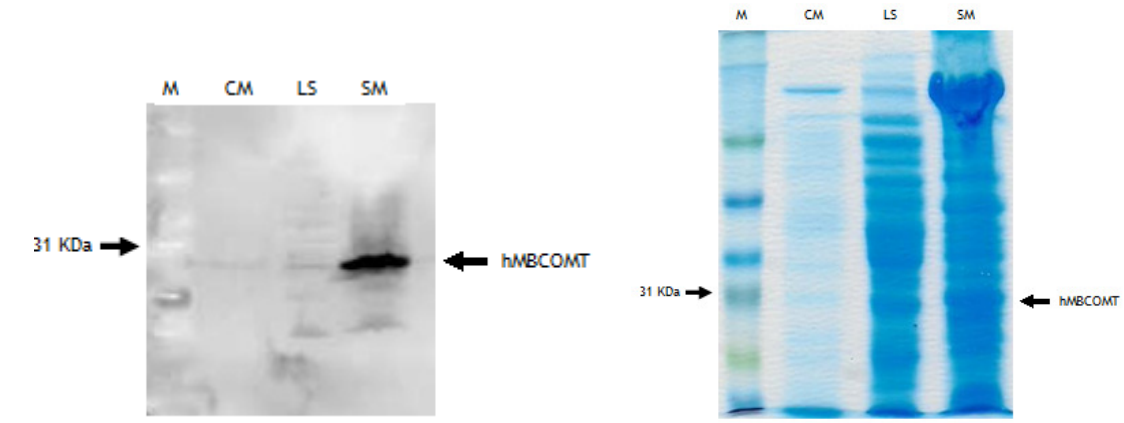


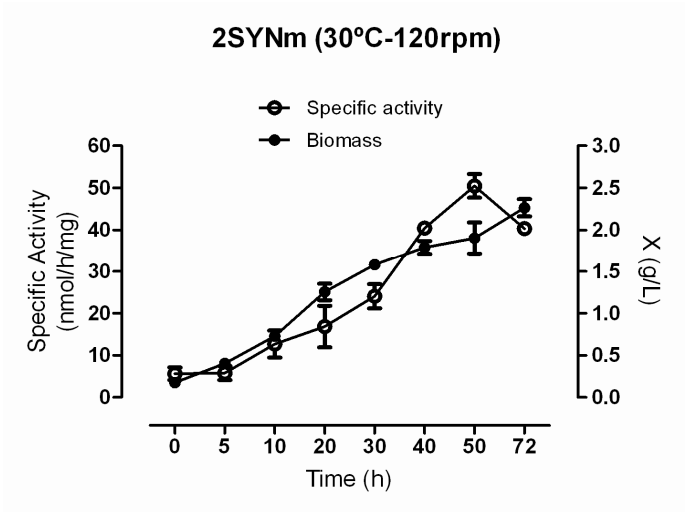
Figure 3 - Western-blot (a) and SDS-PAGE (b) analysis of recombinant human MBCOMT in the three different fractions obtained during the process of recombinant human MBCOMT production. M – molecular weight standards; Lane CM – Culture medium; LS – Cell lysis supernatant; SM – Solubilized membrane.

Subsection III – Time course profile of hMBCOMT production

In general, the media formulation for culturing *B. choshinensis* cells seems to require specific components for the development of a sustainable and successfully upstream stage. In particular, when we tried to produce recombinant hMBCOMT in a biological active form using several media (a semi-defined formulation with glycerol as the main carbon source, SOB, TB or LB) lacking specific compounds such as Bacto Soytone and Polypeptone, we couldn't detect any values for hMBCOMT biological activity. However, previous reports from our research group using the media formulation above for culturing *E. coli* yielded recombinant SCOMT protein with higher biological activity levels [49].

In this work, the recombinant human MBCOMT was produced in a genetically modified organism, *B. choshinensis* SP3, harboring the plasmid pNCMO2-hMBCOMT. In order to get an overview of the optimum conditions for overexpression of the target protein, several experiments varying the culture conditions were carried out. Therefore, the time course profiles of the biological activity of recombinant hMBCOMT as well as the biomass in the 2SYNm medium were analyzed. As we can see in the figure 4 a at 30 ºC and 120 rpm, the produced biomass increases during the 72 hours of fermentation while the biological activity of recombinant human MBCOMT reaches a peak at 50 hours, followed by a slight decrease. The analysis of the biological activity profile of hMBCOMT and the biomass levels in a MT medium formulation at identical operation parameters (figure 4 C) show the same behavior as the 2SYNm medium. Nevertheless, when we increased the fermentation velocity and temperature of the culture, maintaining the 2SYNm medium, we observe a significant

increment in biomass levels and a distinct profile in what concerns to the biological activity of hMBCOMT (figure 4 B). At these conditions, the highest value for the biological activity of hMBCOMT was achieved at 20 hours of culture with an identical value from the one achieved at 50 hours of culture at 120 rpm and 30 ºC.



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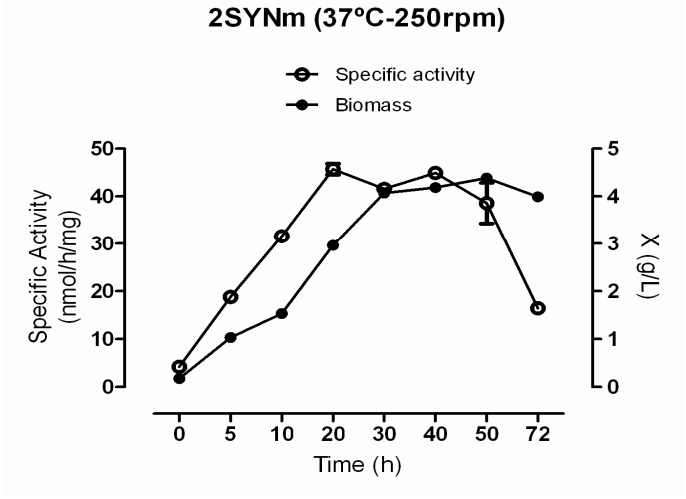

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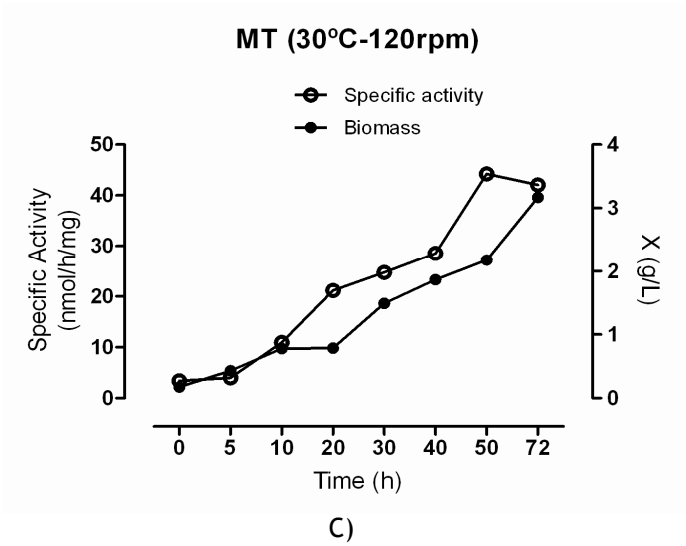


C)

Figure 4 - Growth profile of *B. choshinensis* and recombinant hMBCOMT specific activity profile (nmol/h/mg of protein) at different incubation periods (ranging from 0 to 72 hours) and different culture conditions. A) 30 ºC and 120 rpm in the 2SYNm medium; B) 37 ºC and 250 rpm in the 2SYNm medium; C) 30 ºC and 120 rpm in the MT medium.

Subsequently, in order to compare the growth profile of *B. choshinensis* cells harboring the plasmid pNCMO2-hMBCOMT, *B. choshinensis* were transformed with the control vector pNCMO2-BLA, a plasmid that codes for *B. licheniformes* α-amylase, a protein with a molecular weight near 55 KDa. Maintaining the same operations parameters from the *B. choshinensis* cells transformed with pNCMO2-hMBCOMT, a batch run was performed at 120 rpm and 30 ºC. The results are depicted in figure 5. The comparison of growth profiles between these cells harboring different plasmids, we observed that the biomass level for the construct pNCMO2-hMBCOMT is higher than that from pNCMO2-BLA vector. This can be due to the fact that the DNA insert from pNCMO2-BLA is more toxic to the cells than the DNA that codes for hMBCOMT.

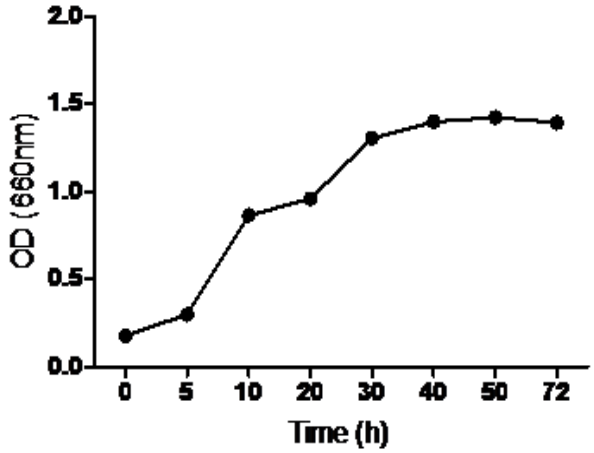
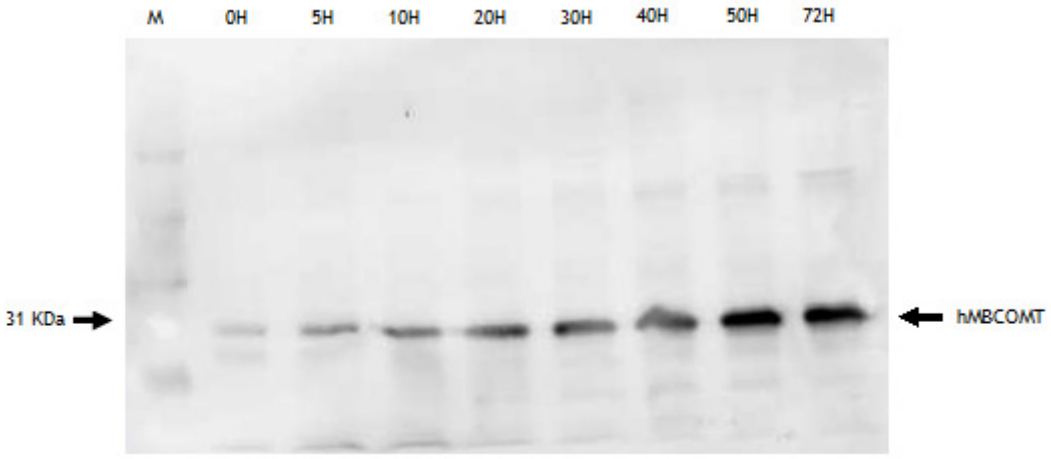
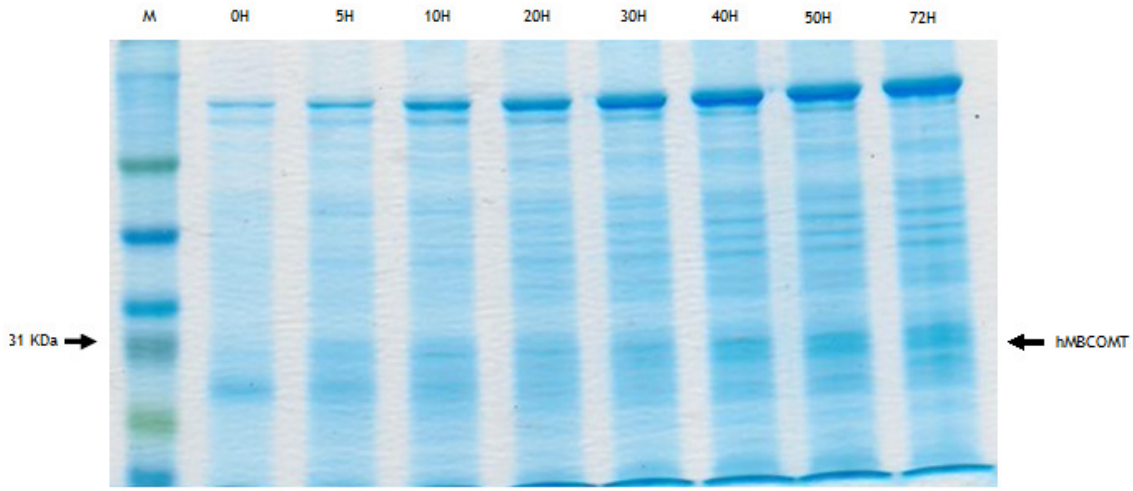


Figure 5 - Growth profile of *B. choshinensis* harboring the plasmid pNCMO2-BLA at different incubation periods (ranging from 0 to 72 hours) at 120 rpm and 30 °C.

Western-blot experiments were performed to assess the levels of hMBCOMT production in an immunologically active form at different incubation periods. However, in practice, owing to the hydrophobic nature of membrane proteins, their transfer from a gel to a blotting membrane can be cumbersome, making western blotting less suitable for quantitative purposes [17]. In spite of this observation and based on figure 6, relative hMBCOMT quantification was estimated by densitometric quantification (see figure 7) of the different bands obtained during a typical fermentation batch run. Specifically, when we compared the target bands with the control (0 hours), it was observe a progressive increase in the band density ranging from 5 to 72 hours, achieving a major peak near the 72 hours of culture (see figure 7).



A)



B)

Figure 6 - Western-blot and SDS-PAGE analysis of recombinant hMBCOMT at different times of incubation period (ranging from 0 to 72 hours at 120 rpm and 30 ºC) on 2SYNm medium. Lane I – molecular weight standards; Lane I to IX – Incubation period ranging from 0 to 72 hours.

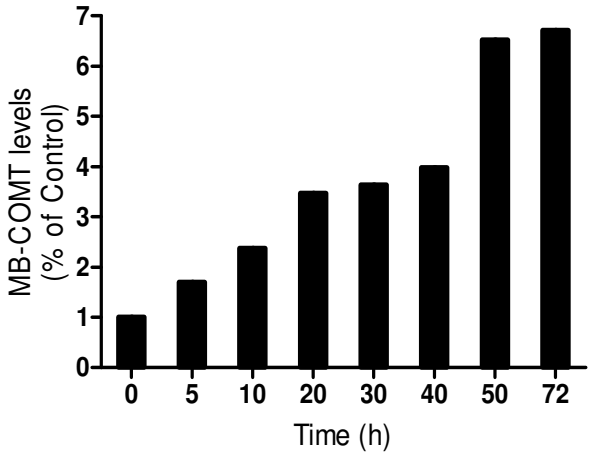


Figure 7 – Densitometric quantification based on figure 17 of the bands obtained at different times of incubation period (ranging from 5 to 72 hours) on 2SYNm comparatively to the control (0 hours).

Subsection IV – Detergent screening

Typically, in the hands-on with a membrane protein, in order to resemble the native environment of the lipid bilayer, it is highly desirable to transfer the protein for a more

hydrophobic environment. This can be achieved by treating the protein with detergents. The relevance of detergents as agents for the study of membrane proteins cannot be underestimated since they are, usually, vital for the isolation and purification of the target protein [32]. Detergents are amphipathic molecules, consisting of a polar head group and a hydrophobic chain that solubilises membrane proteins by creating a mimic of the natural lipid bilayer environment normally inhabited by the protein [32]. Preliminarily and in order to understand the influence of detergent selection, we assessed the effect of the type as well as concentration in the biological activity of recombinant human MBCOMT (figure 8).

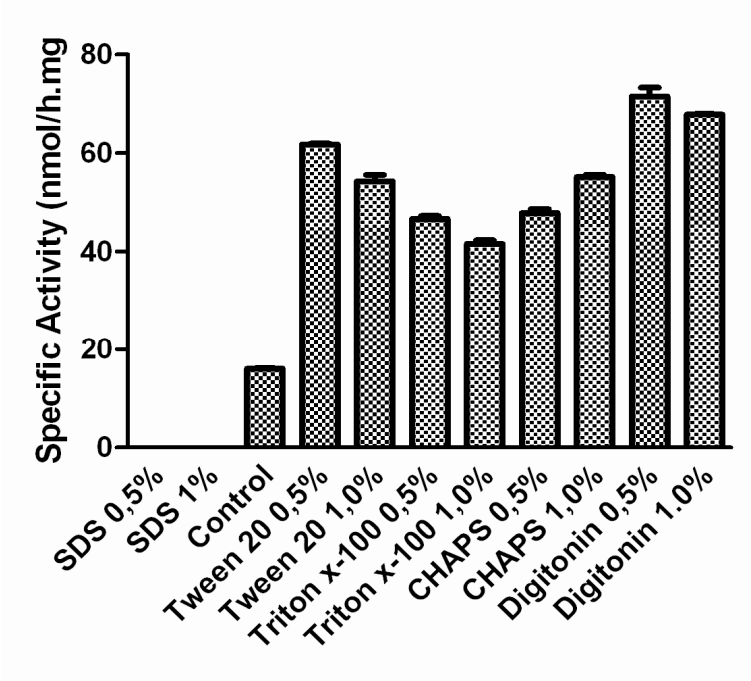


Figure 8 - Effect of concentration and type of detergent used to solubilize the membrane fraction on the human recombinant MBCOMT biological activity (nmol/h/mg of protein).

At this stage, we used three non-ionic, one ionic and one zwiterionic detergent. In what concerns to non-ionic detergents, the screening was made with Tween-20, Triton X-100 and Digitonin whose CMC are, respectively, 0.06 mM (20-25 ºC), 0.2-0.9 mM (20-25 ºC) and <0.5 mM (20-25 ºC). Also, we used 3 – [(3-Cholamidopropyl) dimethylammonium] – 1 – propanesulphate (CHAPS), a zwiterionic detergent that has a CMC of 6 mM (20-25 ºC) and the ionic detergent SDS with a CMC range from 7 to 10 mM (25 ºC). SDS is extremely effective in the solubilization of membrane proteins but it also promotes a great denaturation. Indeed, SDS incorporation as the principal solubilization agent of hMBCOMT protein leads to a null biological activity. With exception of CHAPS, for the three non-ionic detergents tested, we solubilized the target protein and maintained the native structure while higher values for

hMBCOMT biological activity were obtained applying a lower detergent concentration. However, the best results were achieved for the non-ionic detergent digitonin with an increase of hMBCOMT biological activity near 450 % (see figure 9) comparatively to the control (solubilization with a specific buffer on the absence of detergents). In general, until a detergent CMC of 1 mM, hMBCOMT shows more biological activity when solubilized at low detergent concentrations and for detergent CMC higher than 1 mM, hMBCOMT has more biological activity at higher detergent concentrations.

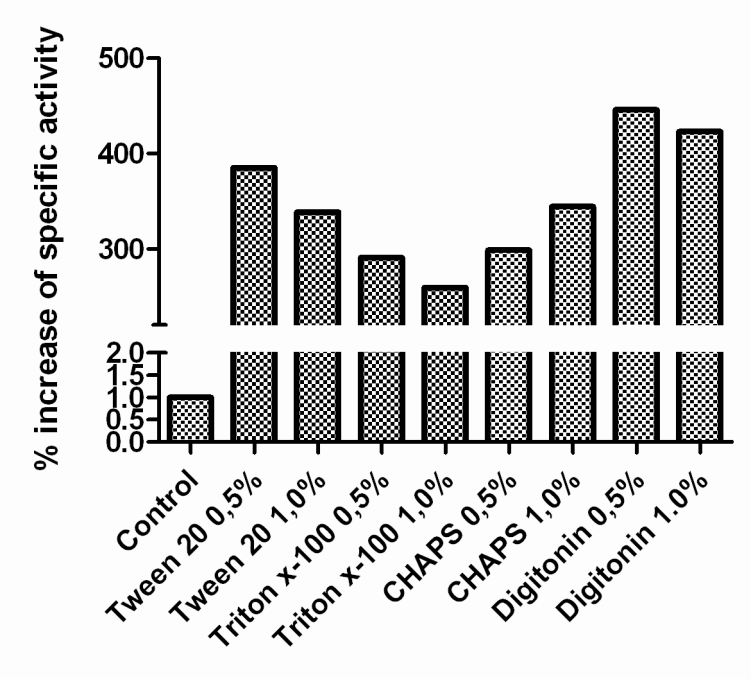


Figure 9 – Percentage of increase on hMBCOMT biological activity relatively to the control of the membrane fractions solubilized with different types and concentrations of detergents.

Subsection V – Kinetic characterization of hMBCOMT

The acquired stability during the protein solubilization stage allows an intrinsic kinetic characterization of hMBCOMT and infer if the presence of detergent lead to a conformational state that lower hMBCOMT activity. There are several factors that influence the MBCOMT enzymatic assay and therefore, preliminary optimization studies must be develop. Specifically, the results obtained for total protein concentration and incubation time for enzymatic activity trials are depicted in figures 10 and 11, respectively. The hMBCOMT specific activity is measured as the rate of formation of metanephrine using as substrate

epinephrine. According to the results obtained, the total protein concentration was fixed at 2.0 mg/ml with an incubation time of 15 minutes.

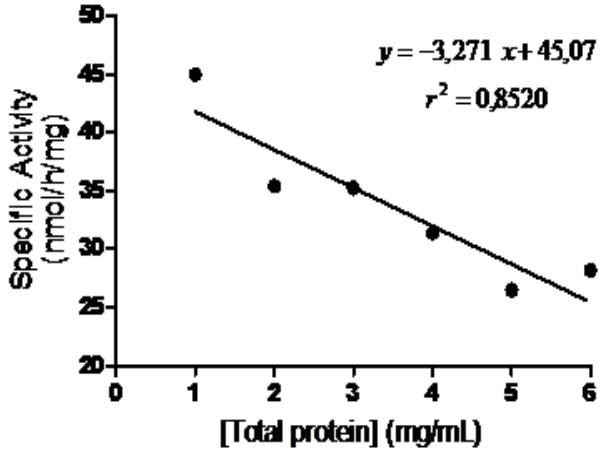


Figure 10 – Relationship between the total protein concentration and the hMBCOMT specific activity (nmol/h/mg protein).

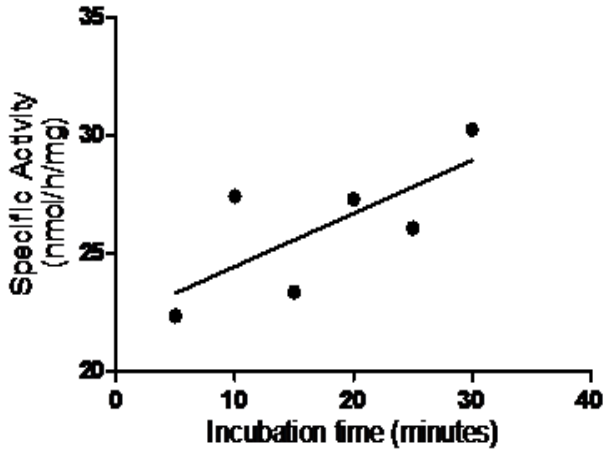
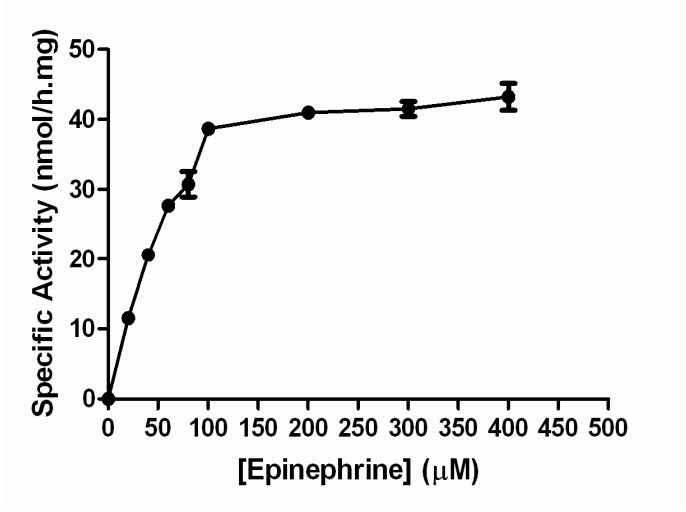


Figure 11 – Relationship between the incubation time and the hMBCOMT specific activity (nmol/h/mg protein).

The kinetic parameters Km and Vmax of the *O*-methylation reaction of epinephrine were determined to evaluate the functional properties of the recombinant protein produced in this work. As expected, the incubation of the enzyme preparations with increasing concentrations of epinephrine resulted in a concentration-dependent formation of metanephrine. The kinetic parameters Km and Vmax for epinephrine at a SAM concentration of 250.0 μM determined

from the fitted curve (Figures 12) were, respectively, 84.32 ± 18.46 μM and 61.25 ± 7.95nmol/h/mg. On the other hand, the kinetic parameters Km and Vmax at a SAM concentration of 100.0 μM for epinephrine determined from the fitted curve (Figures 13) were, respectively, 102.79 ± 18.06 μM and 90.22 ± 7.20 nmol/h/mg.

Specifically, these values are higher from those previously reported for hMBCOMT protein [11]. However, they are three times lower than those reported for recombinant hSCOMT [38].



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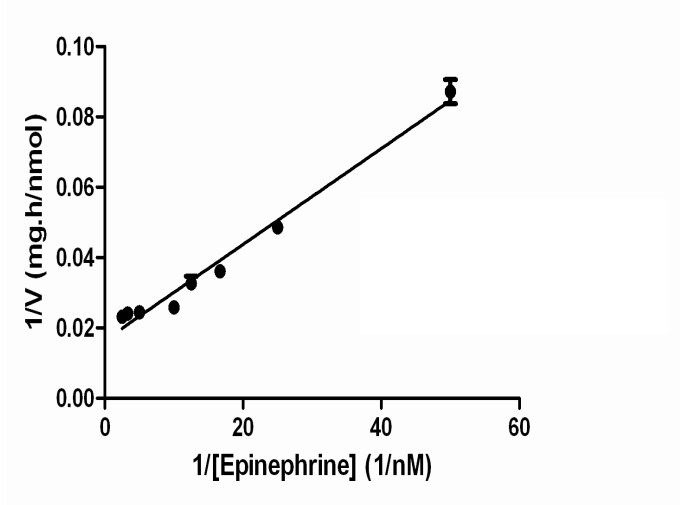
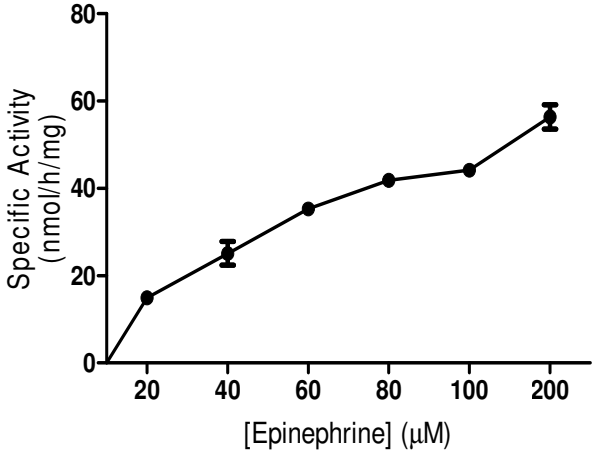


B)

Figure 12 - (A) Saturation curve of recombinant hMBCOMT ([SAM] = 250.0 μM) from a protein extract obtained after the solubilization of the membrane fraction with Triton X-100 1 % (v/v). COMT activity is shown as the rate of formation of metanephrine (nmol/h/mg of protein) vs. inverse concentration of

epinephrine. Vertical lines shown SD **(B)** Lineweaver-burk plot of the saturation curve of recombination hMBCOMT.



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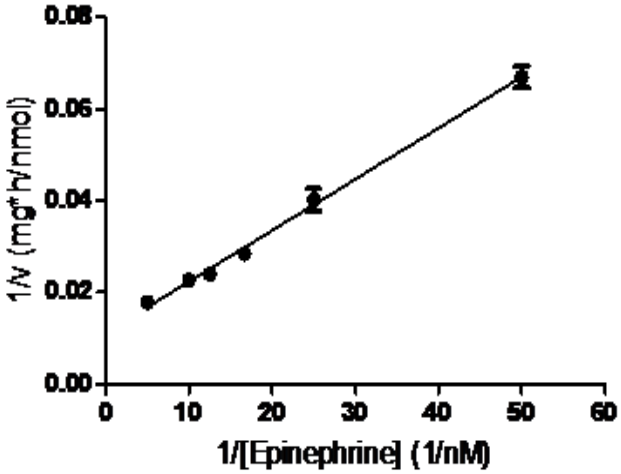


B)

Figure 13 - (A) Saturation curve of recombinant hMBCOMT ([SAM] = 100.0 µM) from a protein extract obtained after the solubilization of the membrane fraction with Triton X-100 1 % (v/v). COMT activity is shown as the rate of formation of metanephrine (nmol/h/mg of protein) vs. inverse concentration of epinephrine. Vertical lines shown SD **(B)** Lineweaver-burk plot of the saturation curve of recombination hMBCOMT.

It is well established that cofactor SAM concentration influences the values of the kinetic parameters Km and Vmax. At the concentrations used in our assays, there are only values described in literature for the assessment of these parameters at a SAM concentration of 100 µM. Indeed, concerning recombinant hMBCOMT solubilized without detergent, there is a report [11] in which the authors used SAM at 100.0 µM and obtained the following values: 28.8 µM and 46.3 nmol/mg/min, respectively, for Km and Vmax. The affinity values obtained for substrate in this work are slightly higher than those results. However, as stated before, the affinity of hMBCOMT decreases and, consequently, the Km increases, upon solubilization with detergent and they assign this change on the kinetics properties of hMBCOMT with the removal of the enzyme from its native environment [31]. Another hypothesis raised by Roth (1992) considers that a possible increase in the Km value for hMBCOMT upon solubilization is caused by apparent competitive inhibition of the enzyme by the detergent [24].

Section III - hMBCOMT purification Initial trials - HIC

Typically, in HIC, the proteins are adsorbed to hydrophobic matrices at salt concentrations that are higher but below the levels required for protein precipitation [50]. Desorption usually is achieved by reducing the salt concentration and the strength of the hydrophobic interaction also decreases when decreasing temperature and in general with increasing pH [50]. In laboratory routines, there aren't many examples with successful application of HIC to membrane proteins isolation due to the fact that detergents bind very strong to the matrix [50].

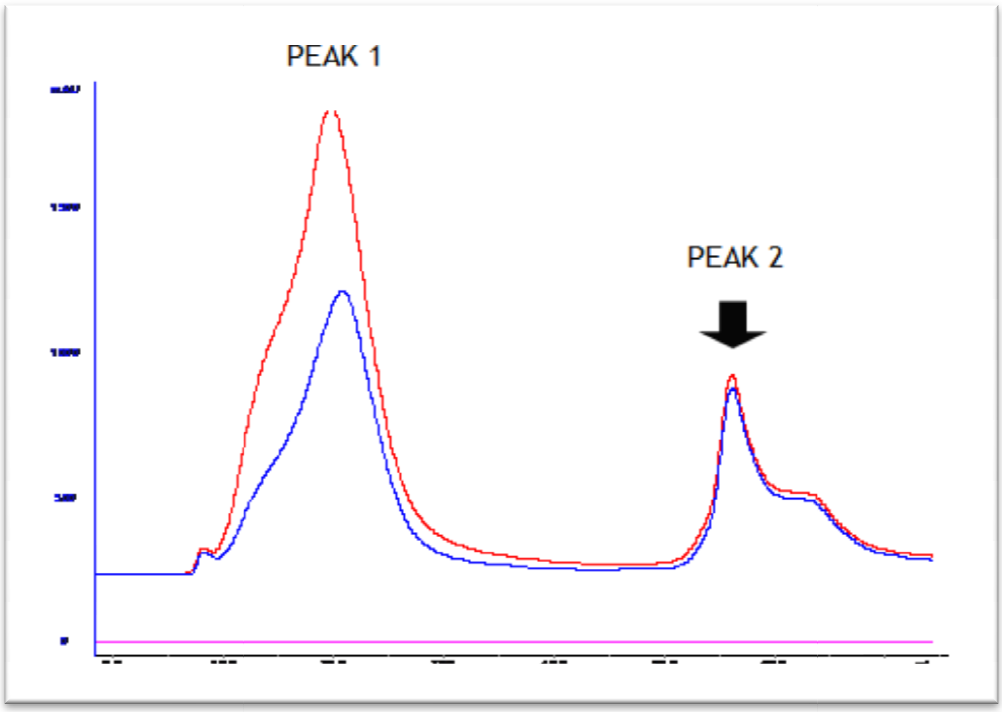
In this work, initials trials to hMBCOMT purification were carried out using a butyl-sepharose resin in which the adsorption of the protein was achieved with several concentrations of ammonium sulphate and desorption by changing the buffer to Tris-Cl (pH 7.8). The obtained results are depicted in figures 26 and 27.

In general, the elution profile is very similar for all the ammonium sulphate concentrations tested (figure 14).

In addition, the analysis of the fractions by SDS-PAGE showed that for 0.2 and 0.4 M ammonium sulphate concentrations, many contaminants are eluted in the peak I and, consequently, separated from our target protein. However, a distinct profile is observed when we performed a change in the ionic strength of the binding buffer. Indeed, the application of 0.6 M ammonium sulphate lead to a distinct elution profile, where apparently the peak II seems to have more proteins than the peak I.

In order to assess if the fractions recovered from the peaks I and II were immunologically and/or biological active, all these fractions were analyzed by western-blot and the hMBCOMT. Also, a enzymatic assay was performed. None of the fractions recover after this purification step showed any value for the biological activity. However, as we can see in the figure 15,

the fractions that corresponded to the peak 2 were immunologically active with an increasing of the COMT band density from 0.2 to 0.6 M ammonium sulphate.



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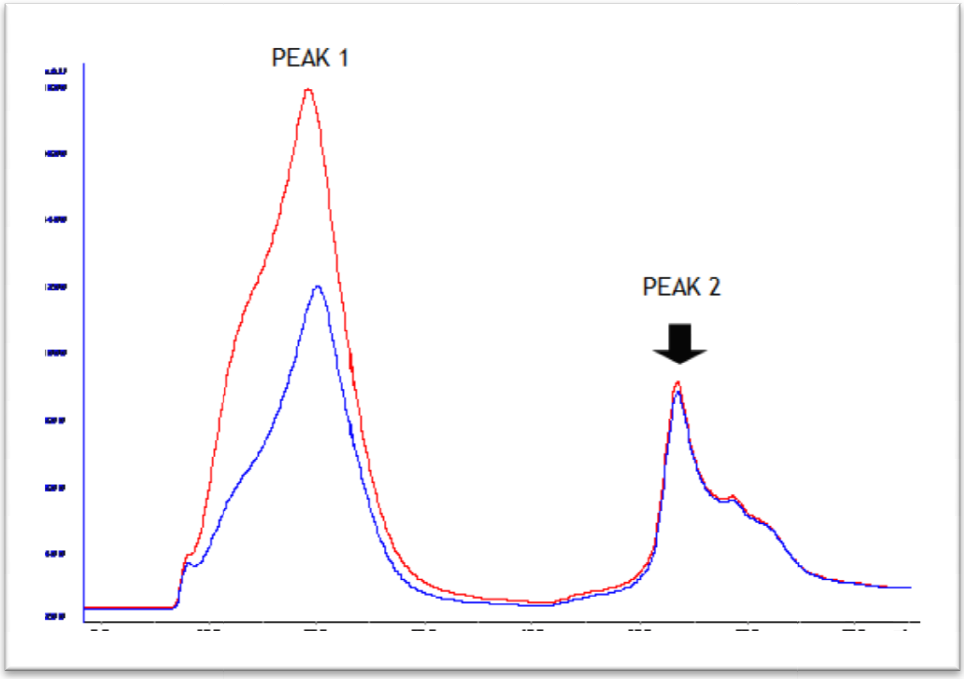

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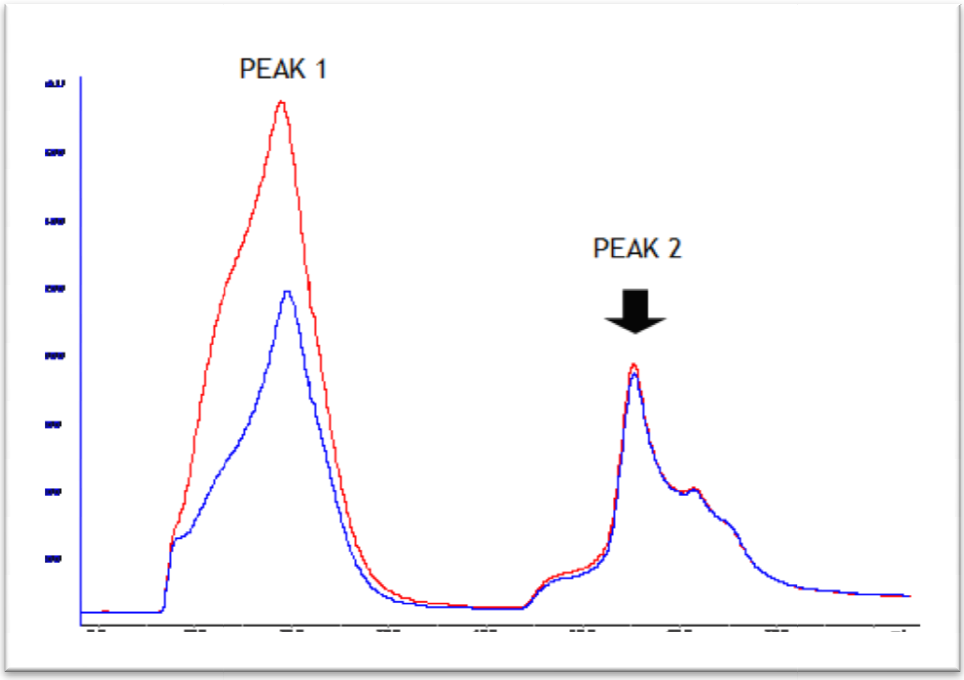
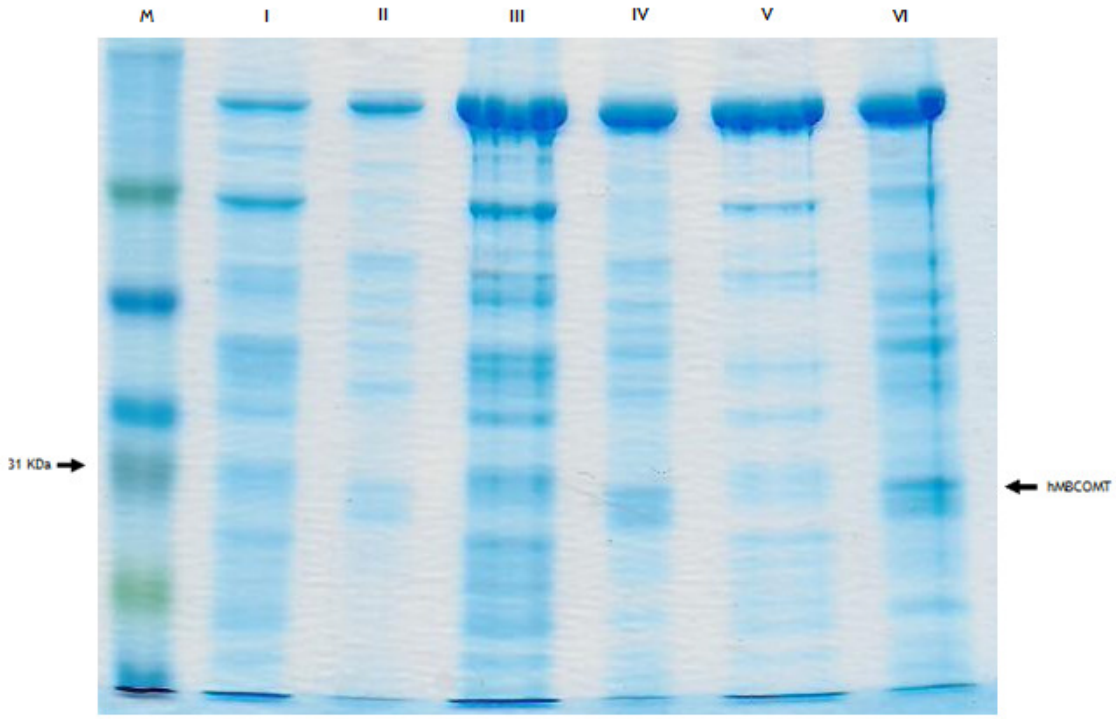
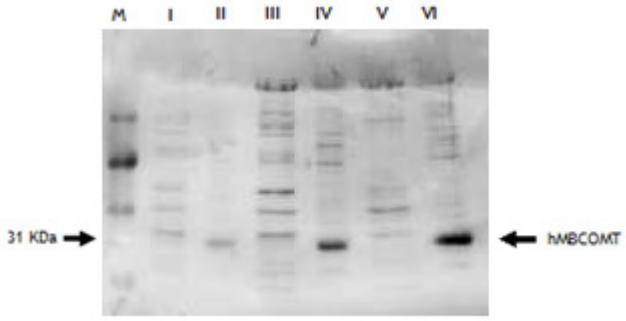


C)

Figure 14 - HIC on butyl-sepharose 4FF with 0.6 M, 0.4 M and 0.2 M ammonium sulphate, respectively for A, B and C in 10 mM Tris-Cl (pH 7.8).The position of recombinant hMBCOMT active fractions is indicated by the arrow. A blue was recorded the absorbance at 280 nm and a red the absorbance at 260 nm.



A)



B)

Figure 15 - SDS-PAGE (A) and Western-blot analysis (B) of recombinant hMBCOMT eluted at different concentrations of ammonium sulphate on the butyl-sepharose resin. Lane I – molecular weight standards; Lanes II, IV and VI – Peak I obtained at 0.2, 0.4 and 0.6 M ammonium sulphate, respectively; Lanes III, V and VII – Peak II obtained at 0.2, 0.4 and 0.6 M ammonium sulphate, respectively. The position of recombinant hMBCOMT active fractions is indicated by the arrow.

Chapter IV

Conclusions

Nowadays the rapidly growing knowledge of the biogenesis of membrane proteins, coupled with efforts to identify bottlenecks hampering membrane protein overexpression, has created new possibilities to design strategies for improving yields to host performance and protein levels.

The recombinant expression system developed in this work allows the production and recuperation, in a single step, of hMBCOMT protein, in an immunological and biological active form. Essentially, the recombinant enzyme is present in the membrane fraction and while is not aggregate as inclusion bodies, its recuperation is done easily by solubilization with suitable concentration of a non-ionic detergent. Instead of the three nonionic and zwiterionic detergent promotes an excellent recuperation of MBCOMT, the concentration requirements were distinctly.

Also, although the kinetic characterization of recombinant hMBCOMT produced in this work provided Km and Vmax values a little higher than expected, this fact it is probably due to the Triton X-100 solubilization step. Taking in account these results and the data from the western-blot analysis, we can say that the COMT isoform produced in this work is the membrane-bound and not the soluble one.

The apparent higher hydrophobicity of hMBCOMT when compared to hSCOMT led us to think that HIC could be a good technique for hMBCOMT purification and a suitable separation could be achieved with lower salt concentrations than those reported for hSCOMT protein. Indeed, the hMBCOMT purification by HIC on a butyl-sepharose resin from the membrane fraction solubilized with Triton X-100 1 % (v/v) didn't retrieve good results since the target protein was recovered at the different ammonium sulphate concentrations immunologically active but without biological activity.

Chapter V

Future perspectives

The system developed in this work allows the production and recuperation in a single step of hMBCOMT from the membrane fraction. It will be interesting to develop systems that combine *B. choshinensis* cells and plasmids that allow the extracellular production of hMBCOMT with a His-tag. The application of procedures based on membrane ultra-filtration should be useful for recovering the protein from the culture medium. Furthermore, the secretion of hMBCOMT coupled with a His-tag could be advantageous since it would allow hMBCOMT purification by affinity chromatography approaches.

In addition, the application of an ultracentrifugation step after the membrane solubilization with detergent should be useful since it would allow the removal of many contaminants.

In general, the use of detergents or other system that creates a hydrophobic environment that surrounds the target membrane protein is crucial in membrane protein studies. Indeed, besides the detergents used in this work, there are several systems that resemble the lipid bilayer in a better way than the detergents. It would be useful to test hMBCOMT behavior in these alternatively solubilization techniques such as bicelles, reverse-micelles, nanolipoproteins, among others.

Finally, factorial design studies should be applied to optimize culture conditions in order to obtain the most appropriated medium formulation, medium pH, temperature and velocity for culturing *B. choshinensis* cells for hMBCOMT production.

Chapter VI

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Chapter VII

Appendices

Appendicces 1 – pGEM-T easy DNA sequencing

AP-1 Clone with primer SP6

A01_AP-1+SP6_10.ab1 807 0 807 ABI trimmed

CGCGTTGCGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGATTCCGAATTCT
CAGGGCCCTGCTTCGCTGCCTGGGCC
CTTGTAGATGGCCTTCTCCAGGCCGTCCACCACCTCCCTGTATTCCAGGAACGATTGGTAGTGTGTGCA
CTCAAA
GCAGCTGCTCCCGCGCACGTGTGCTAGGAAGTCTGGCGCACCTGGGCAGATCACGTTGTCGGCCAGTA
GCACTGT
CCCCTTCCGCAGCAGGCCACATTCCTCCAAGAGAAGCGTGTCCGGCAGGTACCGGTCCTTCCAGTGGTC
GAGGAA
GACCATGTCCAGTGTGTCCACATCATACTTCTTCTTCAGCTGGGGGATGATGTCCTGGGACGCTCCAAC
CACAAG
GGTGACCTTGTCCTTCACGCCAGCGAAATCCACCATCCGCTGGGTGATGGCGGCACAGTCGGGGTTGA
TCTCGAT
GGTGATCAGCCTCGCCCCTGGTGACAGCAGGCGGGCCATGCGCACAGCTGAGTAGCCACAGTAGGCCC
CCAGCTC
CAGCAGCACGGAGGGCTGGTGCTCCTGAATCACGGCGTCCACGATCTTGCCTTTCTTGTCGCCCACGTT
CATGGC
CCACTCCTTCTGCTCGCAGTAGGTGTCAATGGCCTCCAGCACGCTCTGTGCGTTCCCGGGCTCCGCATG
CTGCAG
CACGTGGTTCAGGATGCGCTGCTCCTTGGTGTCACCCATGAGCAGGTTGTGGATGGGCTGCAGGATGA
ACTCCTT
CCAGCCGATAAGGAACAGGCCCCAGCCCCCAGTGCCT

AP-1 clone with primer T7

C01_AP-1+T7FwdpGEM_10.ab1 842 0 842 ABI trimmed

GCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTTCCCATGGCTTTCGCTATGCCGGAGGCCCCGC
CTCTGCTGTTGGCAGCTGTGT
TGCTGGGCCTGGTGCTGCTGGTGGTGCTGCTGCTGCTTCTGAGGCACTGGGGCTGGGGCCTGTGCCT
TATCGGCT
GGAACGAGTTCATCCTGCAGCCCATCCACAACCTGCTCATGGGTGACACCAAGGAGCAGCGCATCCTGA
ACCACG
TGCTGCAGCATGCGGAGCCCGGGAACGCACAGAGCGTGCTGGAGGCCATTGACACCTACTGCGAGCAG
AAGGAGT
GGGCCATGAACGTGGGCGACAAGAAAGGCAAGATCGTGGACGCCGTGATTCAGGAGCACCAGCCCTCC
GTGCTGC
TGGAGCTGGGGGCCTACTGTGGCTACTCAGCTGTGCGCATGGCCCGCCTGCTGTCACCAGGGGCGAGG
CTGATCA
CCATCGAGATCAACCCCGACTGTGCCGCCATCACCCAGCGGATGGTGGATTTCGCTGGCGTGAAGGACA
AGGTCA
CCCTTGTGGTTGGAGCGTCCCAGGACATCATCCCCCAGCTGAAGAAGAAGTATGATGTGGACACACTGG
ACATGG
TCTTCCTCGACCACTGGAAGGACCGGTACCTGCCGGACACGCTTCTCTTGGAGGAATGTGGCCTGCTGC
GGAAGG
GGACAGTGCTACTGGCCGACAACGTGATCTGCCCAGGTGCGCCAGACTTCCTAGCACACGTGCGCGGG
AGCAGCT
GCTTTGAGTGCACACACTACCAATCGTTCCTGGAATACAGGGAGGTGGTGGACGGCCTGGAGAAAGGC
ATCTACA
AGG

AP-2 clone with primer SP6

B01_AP-2+SP6_10.ab1 804 0 804 ABI trimmed

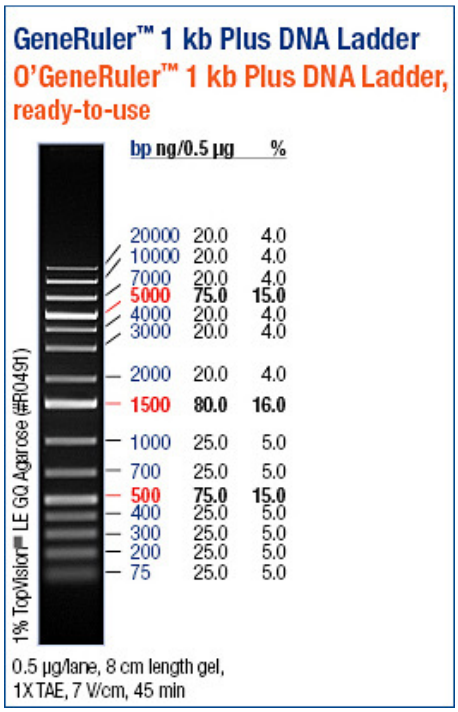
GCGTTGGGAGCTCTCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGATTCCGAATTCTCAG
GGCCCTGCTTCGCTGCCTGGGCCCTT
GTAGATGGCCTTCTCCAGGCCGTCCACCACCTCCCTGTATTCCAGGAACGATTGGTAGTGTGTGCACTC
AAAGCA
GCTGCTCCCGCGCACGTGTGCTAGGAAGTCTGGCGCACCTGGGCAGATCACGTTGTCAGCCAGTAGCA
CTGTCCC
CTTCCGCAGCAGGCCACATTCCTCCAAGAGAAGCGTGTCCGGCAGGTACCGGTCCTTCCAGTGGTCGAG
GAAGAC
CATGTCCAGTGTGTCCACATCATACTTCTTCTTCAGCTGGGGGATGATGTCCTGGGACGCTCCAACCAC
AAGGGT
GACCTTGTCCTTCACGCCAGCGAAATCCACCATCCGCTGGGTGATGGCGGCACAGTCGGGGTTGATCTC
GATGGT
GATCAGCCTCGCCCCTGGTGACAGCAGGCGGGCCATGCGCACAGCTGAGTAGCCACAGTAGGCCCCCA
GCTCCAG
CAGCACGGAGGGCTGGTGCTCCTGAATCACGGCGTCCACGATCTTGCCTTTCTTGTCGCCCACGTTCAT
GGCCCA
CTCCTTCTGCTCGCAGTAGGTGTCAATGGCCTCCAGCACGCTCTGTGCGTTCCCGGGCTCCGCATGCTG
CAGCAC
GTGGTTCAGGATGCGCTGCTCCTTGGTGTCACCCATGAGCAGGTTGTGGATGGGCTGCAGGATGAACT
CGTTCCA
GCCGATAAGGCACAGGCCCCAGCCCCAGTGCCTC

AP-2 clone with primer T7FwdpGEM

D01_AP-2+T7FwdpGEM_10.ab1 899 0 899 ABI trimmed

GCTCCGGCCGCCAT
GGCGGCCGCGGGAATTCGATTTCCCATGGCTTTCGCTATGCCGGAGGCCCCGCCTCTGCTGTTGGCAG
CTGTGTTGC
TGGGCCTGGTGCTGCTGGTGGTGCTGCTGCTGCTTCTGAGGCACTGGGGCTGGGGCCTGTGCCTTATC
GGCTGGA
ACGAGTTCATCCTGCAGCCCATCCACAACCTGCTCATGGGTGACACCAAGGAGCAGCGCATCCTGAACC
ACGTGC
TGCAGCATGCGGAGCCCGGGAACGCACAGAGCGTGCTGGAGGCCATTGACACCTACTGCGAGCAGAAG
GAGTGGG
CCATGAACGTGGGCGACAAGAAAGGCAAGATCGTGGACGCCGTGATTCAGGAGCACCAGCCCTCCGTG
CTGCTGG
AGCTGGGGGCCTACTGTGGCTACTCAGCTGTGCGCATGGCCCGCCTGCTGTCACCAGGGGCGAGGCTG
ATCACCA
TCGAGATCAACCCCGACTGTGCCGCCATCACCCAGCGGATGGTGGATTTCGCTGGCGTGAAGGACAAG
GTCACCC
TTGTGGTTGGAGCGTCCCAGGACATCATCCCCCAGCTGAAGAAGAAGTATGATGTGGACACACTGGACA
TGGTCT
TCCTCGACCACTGGAAGGACCGGTACCTGCCGGACACGCTTCTCTTGGAGGAATGTGGCCTGCTGCGG
AAGGGGA
CAGTGCTACTGGCTGACAACGTGATCTGCCCAGGTGCGCCAGACTTCCTAGCACACGTGCGCGGGAGC
AGCTGCT
TTGAGTGCACACACTACCAATCGTTCCTGGAATACAGGGAGGTGGTGGACGGCCTGGAGAAGGCCATC
TACAAGG
GCCCAGGCAGCGAAGCAGGGCCCTGAAAATTCGGAATCACTAGTGAATTCCCGGCCGCCT

Appendices II – Gene Ruler 1 Kb Plus DNA ladder bands map



Appendices III – High Range rainbow molecular weight marker