Chapter 2: Materials and Methods

2.1 In Situ Hybridisation

2.1.1 Probe Synthesis

2.1.1.1 RNA Extraction

RNA was extracted from a normal kidney cell line. The cells were cultured in a 25 cm^3 tissue culture plate with 10% Fetal Bovine Serum (FBS) DMEM until they reached confluency. The cells were grown in a monolayer that attached on the culture plate and washed with PBS (LSS). The cells were then homogenised by adding 2 ml TRIzol LS reagent per 25 cm^3 of culture plate with vigorous shaking for a period of 2 hours. For complete homogeneity the cells were passed through a pipette several times. The homogenate was transferred to a 2 ml eppendorf tube and 1 ml of chloroform was added to the homogenate, followed by 15 seconds of vigorous vortexing. The mixture was left at room temperature for 10 minutes, followed by a 1-hour centrifugation period at 4^OC to allow complete phase separation. The top aqueous phase layer was transferred to a sterilised 2 ml eppendorf tube, and 1 ml isopropyl alcohol was added and mixed by inverting the tube to precipitate the RNA. The sample was then incubated at room temperature and later incubated overnight at 4^oC to precipitate RNA. After 16 hours the sample was centrifuged for 30 minutes at 3000 rpm at 4^oC, and the supernatant was discarded. The RNA pellet was washed for 5 minutes at 3000 rpm at 4^oC, with 70 % ethanol, prepared with diethyl pyrocarbonate treated water, (DEPC H20), The pellet was resuspended in 100 µl 0.1 % DEPC treated H_2O and the RNA was stored at -70^oC.

2.1.1.2 Formaldehyde Agarose Gel Electrophoresis.

The RNA sample was then loaded onto a formaldehyde agarose gel to view whether RNA was intact or degraded. One gram of agarose was microwaved in 10 ml of 10X MOPS and 85 ml DEPC treated H_2O. This was cooled to 50^oC and then 5.4 ml of 37 % (v/v) formaldehyde was added, followed by 4 µl of 100 µg/ml ethidium bromide (EtBr). The mixture was set in a casting gel tray. 7 µl, 10 µl and 15 µl of the RNA sample was mixed with 7µl, 10µl and 15µl of freshly prepared formaldehyde gel loading buffer respectively (1:1). The samples were then heated at 65^{o}c for 5 minutes to denature RNA and cooled on ice for 10 minutes before loading on gel. The gel was electrophoresed with 1X MOPS at 50 V and viewed using a transilluminator.

2.1.1.3 Primer Design and Synthesis

Specific primers were designed for the amplification of DNA fragments for probe synthesis for PBR, the 5' and 3' ends of 1-ACBP and B-ACBP, and also for the open reading frames (ORFs) of PBR, 1-ACBP and B-ACBP. These primers were designed using the gene sequences obtained from the NCBI site (www.ncbi.nlm.nih.gov).

2.1.1.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR is a reverse transcription polymerase chain reaction technique that is carried out for the amplification of cDNA from mRNA. RT-PCR is a technique that utilises poly-T oligonucleotides to hybridise to the poly-A tails of mRNA in the reaction, and cDNA is synthesised by the Reverse Transcriptase enzyme.

Table 1: RT-PCR mixture

Reagents	**Volume/1Reaction**	**Final concentration**
5x reaction buffer	4.0 µl	1x
25mM $MgCl_2$	4.0 µl	5 mM
DNTPs mix	2.0 µl	1 mM
Oligo-p(dT)15	2.0 µl	0.04 A260 units
RNAse inhibitor	1.0 µl	50 units
RNA sample.	1.0 ul	1-2 ng
AMV Reverse Transcriptase	0.8 µl	+20 units
sdH_20	5.2 µl	-
Total Volume	20 µl	-

A negative control where sdH_2O is used instead of RNA was set up as well.

Table 2: RT-PCR Thermocycle Conditions

Temperature	**Time period**	**Rationale for each step**
25°C	10 mins	Primer hybridisation at poly-A sites.
42°C	60 mins	cDNA synthesis by Reverse Transcriptase.
99°C	5 mins	Inactivation of Reverse Transcriptase.
Hold @ 4°C	-	Samples cooled and ready to proceed to next step.

When proceeding to the next step, which is Polymerase Chain Reaction (PCR), make sure to keep samples on ice. Storing conditions are at –20°C.

2.1.1.5 Polymerase Chain Reaction (PCR)

PCR is a technique utilised for the successful amplification of a desired fragment of DNA, via repetitive cycles of DNA synthesis. PCR involves two oligonucleotide primers (usually 18-25 nucleotides long), which are complementary to and hybridises to the ends of the DNA sequence of interest, followed by DNA synthesis by the heat-stable Taq polymerase enzyme and free single oligonucleotides added to the reaction sample. These reactions occur at three different temperatures for 25-30 cycles in a thermocycler. The temperature, pH and salt content of the reaction mixture favour primer hybridisation only and not the re-hybridisation of the single-stranded DNA molecule generated.

The 1-ACBP, b-ACBP and PBR genes were amplified from cDNA (see RT-PCR section) using a specific set of primers for each gene during PCR. The reagents utilized in the reaction mixture (excluding the primers) were supplied by Promega™.

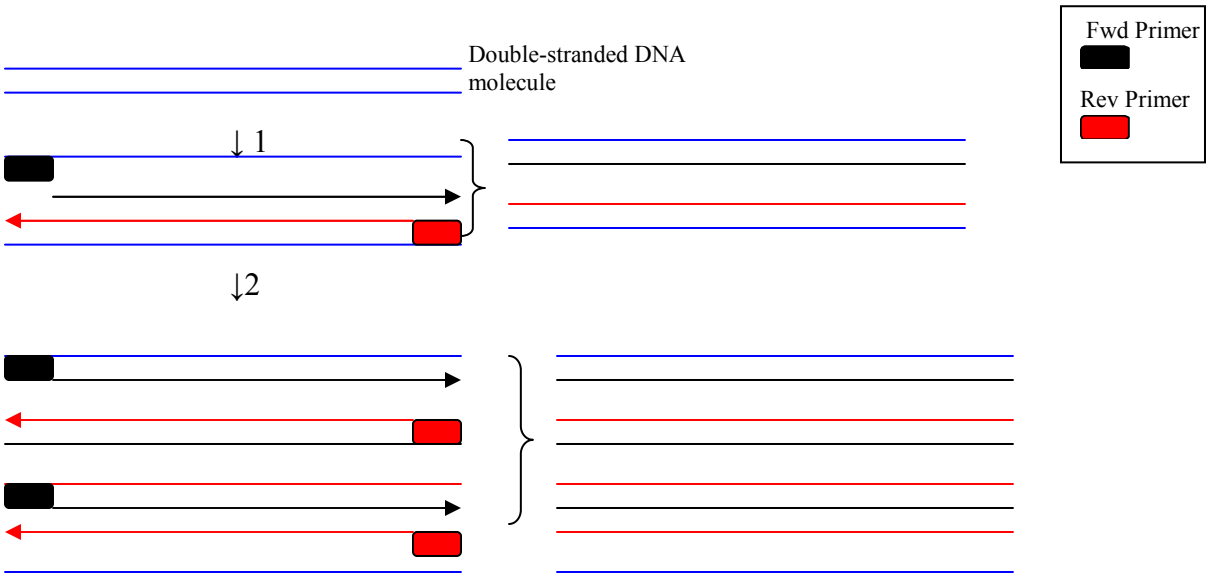

Figure 11: Illustration of 2 cycles of 30 cycles of PCR reaction

1-denaturation at 94°C → primers anneal at 56°C → extension/DNA synthesis at 72°C= results in 2 copies of the gene

2-denaturation at 94°C→ primers anneal at 56°C → extension/DNA synthesis at 72°C= results in 4 copies of the gene

Reagents	**Volume/1Reaction**
Master Mix (10x PCR buffer, $MgCl_2$, Taq)	12.5µl
Forward Primer	1.0µl
Reverse Primer	1.0µl
$MgCl_2$	2.0µl
Nuclease Free H_2O	7.5µl
Template DNA	1.0µl
Total Volume	25.0µl

A negative control was set up here as well, utilising the negative control from RT-PCR as template.

Table 4: PCR Thermocycle conditions

Temp.	**Time period**	**Rationale for each step**	
95°C	2 mins	Double stranded DNA denatured to single strands	
94°C	30 secs	DNA denatured at start of each cycle to single strands	30 cycles
56°C	30 secs	Primers anneal to single strand template DNA	
72°C	1 min	DNA extension or synthesis by Taq polymerase	
72°C	10 mins	Final extension step	
4°C	-	Holding step, Sample ready to proceed to next step	

2.1.1.6 Agarose Gel Electrophoresis

Agarose gel electrophoresis is carried out to determine whether the PCR reaction was successful. Normally for each amplified product a band of a single molecular weight is expected in the gel. A 0.8% gel was prepared by boiling 0.8g agarose in 100ml 1x Tris Borate EDTA (TBE) buffer until agarose was dissolved. 3µl Ethidium bromide (EtBr) was added to the gel when it reached a temperature of ~50°C and poured into a gel-casting tray. The PCR products were mixed with 6x orange-blue loading dye (Promega™), and were loaded onto 0.8% agarose gels together with a 100bp molecular weight maker for size determination. Gels were run at 70-100 Volts in 1x TBE running buffer for one hour and were viewed with a transilluminator thereafter.

2.1.1.7 Cloning of PCR products into pGEM-T-Easy vectors

Cloning means isolating a gene of particular interest and making many copies of it for further analysis. The procedure commences with the desired gene being ligated into a suitable plasmid (vector). Ligafast pGEM-T-Easy cloning system kit (Promega™) was used to clone the PCR products, view figure 12.

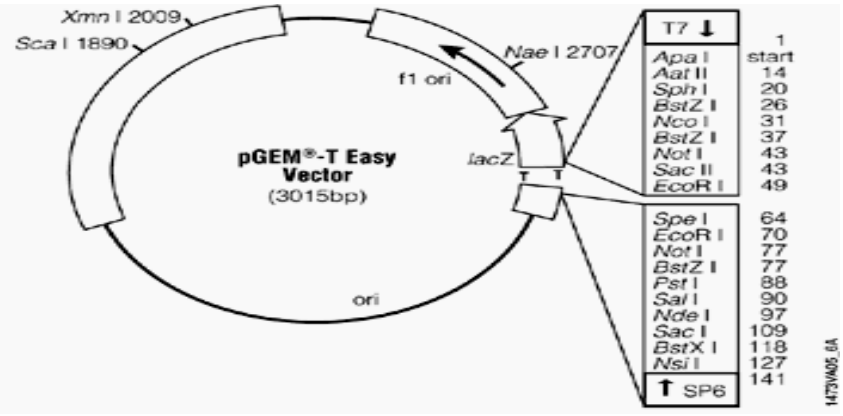

Figure 12: pGEM-T-Easy Vector

Once ligated transforming bacteria (usually *Eschericia coli*) to make millions of copies of the gene of interest. pGEM-T-Easy was the vector of choice as it is a simple cloning system with an ampicillin resistant gene for selection purposes, and the cleaning up of PCR products were not necessary for successful cloning.

Table 5: Ligation of PCR products into pGEM-T-Easy

Reagents	**Experimental Reaction**	**Positive Control Reaction**	**Negative Control Reaction**
PCR Product	1.0 µl	1.0 µl (+ve control)	-
10x Ligation buffer	5.0 µl	5.0 µl	5.0 µl
pGEM-T-Easy	1.0 µl	1.0 µl	1.0 µl
T_4 Ligase	1.0 µl	1.0 µl	1.0 µl
Nuclease Free H_20	2.0 µl	2.0 µl	2.0 µl
Total Volume	10.0 µl	10.0 µl	10.0 µl

The experimental reaction contained PCR product, positive control reaction contained an insert supplied with the kit and the negative control contained sdH_20 (background control). These reactions were subjected to a 2-hour incubation period at room temperature. Transformation was the next step. T4 ligase was the enzyme used for the ligation of the PCR product into the pGEM-T-Easy vector, in the appropriate ligation buffer supplied with the kit.

2.1.1.8 Transformation

Transformation is the step that introduces the recombinant vector into the appropriate bacterial cell to make millions of copies it. The strains used for these experiments were

competent MC1061 *E.coli* strains, which were thawed on ice and 100 µl of these cells were added to each ligation reaction. A negative control was set up here, which only contained competent MC1061 *E.coli* strains with no plasmid. Each reaction was incubated on ice for a period of 30 minutes followed by a 5-minute 37 °C incubation period to heat shock the cells, thereafter the reactions were incubated again on ice but for a 2-minute period. 900 µl of Luria broth (LB) lacking ampicillin (Amp) was added to the reactions and incubated at 37°C for 1 hour to allow the cells to grow. All reactions were plated on LB plates containing ampicillin and incubated at 37°C overnight. Ampicillin LB plates are used as a selective medium for *E.coli* cells containing a ligated pGEM vector. pGEM contains an ampicillin resistant gene that is expressed only if ligated and transformed into the appropriate bacterial cell, eliminating all those bacteria that were not successfully transformed. The following day colonies are selected and screened for positive clones via colony PCR.

2.1.1.9 Screening for positive colonies using colony PCR

Colony PCR is an effective technique that utilises normal PCR methods (see table 3 & 4), but the template used is 1.0 µl colony mix (1 colony resuspended in 10 µl sterile distilled water). This method is utilized to confirm that the insert has been cloned into the vector by amplification of the insert with degenerate primers specific for the insert. The colony is degraded at 95 °C in the thermocycler, exposing its plasmid DNA and if the insert was successfully cloned into pGEM the primers would bind to the specific sites of the insert and amplify up a fragment of desired size. The PCR product is then loaded onto a 0.8% agarose gel to determine whether the insert is present or not. Once it was confirmed that

the insert was present within the vector, the specific colony was miniprepped by adding the remainder of the colony mix (9 µl) to 5 ml Luria broth containing 5 µl ampicillin and left overnight at 37°C in a shaker at 120 rev/min. Miniprepping is a means of acquiring many copies of the specific clone in solution. This turbid solution (indicating growth) was then used the following morning for plasmid extraction.

2.1.1.10 Plasmid DNA extraction

Plasmid DNA extraction was performed utilising the WizardPlus SV Miniprep DNA purification System kit (Promega™). This kit provides a safe, convenient and reliable way to extract plasmid DNA from bacteria (*E. coli* in this case). Manufacturer's instructions were followed and each step was carried out at room temperature:

The overnight culture was centrifuged at maximum speed for five minutes to pellet the cells. 250 µl Cell Resuspension Solution provided with the kit was then added to the pellet and was resuspended into solution using a pipette. Once completely resuspended, 250 µl Cell Lysis Solution was added to the solution and inverted four times to lyse the cells and allow plasmid DNA to be released. Five minutes after the cell lysis solution addition, 10 µl of Alkaline Protease Solution was added to the tube and inverted four times to inactivate the lysis solution. After 5 minutes, 350 µl Neutralising Solution was added to the tube and inverted four times as well. Thick cell debris occurred in the tube at this point and was centrifuged at maximum speed for 10 minutes. After spinning, the cell debris precipitated to the bottom and ~ 850 µl of the supernatant present was transferred to a spin column inserted into a collection tube. This collection tube containing the spin

column and supernatant was centrifuged at maximum speed for 1 minute to allow all the other solutions except the DNA to pass through the spin column into the collection tube. The next step was washing the DNA contained in the spin column by adding 750 µl Wash Solution and centrifuging at maximum speed for 1 minute. The flow through was decanted from collection tube and the wash was repeated but with 250 µl Wash Solution and centrifugation carried out for 2 minutes at maximum speed. Thereafter the spin column was transferred to a sterile 1.5ml eppendorf tube to which 100 µl nuclease free water was added to elute the DNA during centrifugation for 1 minute at maximum speed. After centrifugation the spin column could be removed and the 1.5ml eppendorf containing the eluted DNA was ready for restriction digestion analysis. This purified DNA was stored at –20°C.

2.1.1.11 Restriction Digestion of purified clones

Restriction digestion analysis of the clone is a method utilized to determine whether the clone still contains the insert by cutting the insert out of the vector and also determining whether the vector containing the insert can be linearised. Linearization is necessary for probe synthesis to be carried out. The two enzymes utilized are EcoRI (releases the insert) and PstI (linearizes the vector). EcoRI has two sites within the multiple cloning site of the vector pGEM-T-easy, and it flanks both sides of the insert, so when EcoRI cuts it cuts only at these two sites of the vector releasing the insert. PstI has only one site on the vector, downstream the insert, so upon cutting the vector is linearized. Table 6 below contains the reagents used (Promega™) for restriction digestion.

Table 6: Restriction digestion by EcoRI and PstI

	EcoR1 digest	**PstI digest**
PGEM-T-easy clone purified	**10.0** µl	**10.0** µl
Buffer H 10X	**1.0** µl	**1.0** µl
Enzyme	**1.0** µl	**1.0** µl
SdH_2O	**18.0** µl	**18.0** µl
Total Volume	**30.0** µl	**30.0** µl

Both tubes were left in a 37°C waterbath for 1 hour. Thereafter both tubes were subjected to a temperature of 65°C for a period of five minutes to denature or inactivate the enzymes. The digests were run on 0.8 % agarose gels to determine if EcoRI successfully released the insert and if PstI linearized the vector. Once successful digestion has been obtained, the next step would be to sequence the insert cloned into the vector to determine whether the correct fragment as been amplified and cloned.

2.1.1.12 Sequencing

The samples were sent to Inqaba Biotech, located in Pretoria, for sequencing. Sequences obtained were analysed using the bioinformatic tool, Blast (www.ncbi.nlm.nih.gov). Once the sequence obtained was confirmed to be correct, the next step was to linearize the clone for DIG labelling.

2.1.1.13 Clone Linearization

Linearization of the clone is important for labelling. Once confirmed that the insert cloned is correct, the orientation of the insert in the vector has to be verified. Orientation has to be known as it determines which enzyme has to be used to linearize the vector. If the insert is cloned from 5' to 3' into the vector from the T7 site to the Sp6 site, PstI is utilized to linearize the vector. However if the insert has been cloned from 3' to 5', from the T7 site to the Sp6 site, the enzyme ApaI is used to linearize the vector. The reason for this is to make sure that the antisense strand of the insert is being synthesised for probe synthesis and not the sense strand, else hybridisation will not take place during *in situ* and northern blot analysis. The sense strand was only used as a control. Table 7 below represents the reagents used for the digests.

Table 7: Restriction digests with PstI and ApaI

	PstI digest	**ApaI digest**
pGEM-T-easy clone purified	10.0 µl	10.0 µl
Buffer	1.0 µl	1.0 µl
Enzyme	1.0 µl	1.0 µl
sdH_2O	18.0 µl	18.0 µl
Total Volume	30.0 µl	30.0 µl

The reactions were incubated in a 37°C waterbath for 1 hour. The enzymes were denatured at 65°C for 5 minutes and the samples were then run on a 0.8% agarose gel and viewed under a low UV light lamp. The gel had single bands indicating a successful cut

and subsequently the DNA was cut out of the agarose gel utilising sterile blades. Once cut out, the DNA was purified from the remainder of the gel slice using a special kit which is described in the next step.

2.1.1.14 Purification of linearized Clones from Agarose Gel

After the band was cut out of the gel, the gel slice containing the desired DNA fragment was transferred to a pre-weighed eppendorf tube, so the weight of the fragment could be calculated and the correct amount of solution could be added to purify the DNA from the gel slice. The fragment was purified utilising a specialised purification kit, Wizard SV gel and PCR clean-up system. For every 10 ng gel slice obtained, 10 µl membrane binding solution was added. This was incubated at 65°C for 10 minutes or until gel slice had been dissolved, with vortexing in between the melting period. Once melted the solution was transferred to a spin column in a collection tube and left to incubate at room temperature for 1 minute. The spin column in the collection tube was then centrifuged at 10 000 rpm for 1 minute. The flow through was then discarded from the collection tube and the mini column reinserted into the collection tube. To the mini column 700 µl of membrane Wash solution diluted with 95 % ethanol was added and then centrifuged at 10 000 rpm for 1 minute. The flow through was discarded again from the collection tube and the mini column was washed again using 500 µl of membrane wash solution but centrifugation carried out for 5 minutes. It is important that after this step the mini column does not touch the membrane wash solution and if so, it is recommended to spin again for 1 minute after decanting the flow through. Once effectively washed the mini column was transferred to a 1.5 ml eppendorf tube to which 50 µl of DEPC treated water was added

and left to incubate at room temperature for one minute. Subsequently centrifugation at 10 000 rpm for 1 minute at room temperature was followed and the DNA was quantified utilising a spectrophotometer. Storage of this DNA was at -20°C. Next step was synthesising and labelling cRNA using the T7 or Sp6 polymerase DIG labelling kit.

2.1.1.15 DIG Labelling of Probes

The purified linearized clones were then used as the template to generate antisense and sense cRNA strands. Antisense strands were generated by the T7 polymerase, if the insert's orientation was from 5' to 3' in the vector, and SP6 polymerase was used if the inserts orientation was from 3' to 5'. The labelling reaction protocol for the antisense, sense and control cRNA strands was as follows:

Table 8 Cocktail for DIG Labelling

Reagents	**Antisense cRNA**	**Sense cRNA**	**Control cRNA**
Linearized plasmid-containing insert (2µg)	28.0 µl	28.0µl	-
Control DNA PSPT 18-Neo/PvuII (2µg)	-	-	8.0 µl
10X NTP labelling mix	4.0 µl	4.0 µl	4.0 µl
10X Transcription buffer	4.0 µl	4.0 µl	4.0 µl
T7 / Sp6 RNA polymerase (2U/µl)	4.0 µl	-	-
T3 RNA polymerase (2U/µl)	-	4.0µl	-
Sp6 RNA polymerase (2U/µl)	-	-	4.0 µl
DEPC-treated water	-	-	20.0 µl
Total volume	**40.0µl**	**40.0 µl**	**40.0 µl**

The DIG RNA labelling kit (Roche Diagnostics™) reagents were utilized. 2µg of the linearized and purified vectors containing the specific genes were incubated for two hours with a mixture containing DIG NTPs, T7 or SP6 polymerase (depends on insert's orientation and enzyme used to linearize, see section 2.1.1.11), to generate antisense strands. Sense strands of the clones were also generated utilising the T3 polymerase, and a control (DNA PSPT 18-Neo/Pvu II) provided with the kit was used to generate DIG labelled cRNA utilising the SP6 polymerase. Control cRNA was labelled to verify whether reagents were functional. Antisense, Sense and Control cRNA reactions were PCR-incubated for 2 hours at 37ºC. Thereafter the reactions were stopped by adding 4µl of 0.2 M EDTA (pH 8.0). The reactions were mixed and then pulse spinned to collect all reactants at the bottom of the tubes. 4.4µl of 4M Lithium Chloride was then added to the tubes followed by the addition of 150µl cold absolute ethanol to precipitate the labelled cRNA. A quick mix and a pulse spin to collect reactants at the bottom of tube was performed and the all tubes were then subjected to a 2 hour -20ºC incubation period. After precipitation the samples were pelleted by centrifugation at 13 000 rpm for 15 minutes at 4ºC. The supernatant was then decanted and the pellet was washed with 200µl of cold 70% ethanol and centrifuged again at 13 000 rpm for 15 minutes at 4ºC. After centrifugation the supernatant was removed with a pipette to avoid disturbing or losing the pellet. The pellet was dried under a laminar flow for two hours followed by resuspending the pellet in 50µl of sterile DEPC treated water. The tubes were then left at 4ºC for 1 hour to allow complete resuspension. The DIG labelled cRNA transcripts were then stored at -70ºC in aliquots of 10µl.

2.1.1.16 Probe concentration estimated

The concentrations of the probes were estimated using a guideline described in DIG user's guide (Roche Diagnostics™). The probes were diluted as described in the table below to determine the best concentration for localisation studies.

Table 9 dilutions to estimate concentration of probe

Dilution	**DIG-labelled control RNA**	**DIG-labelled cRNA to control DNA-Pspt-18-Neo**	**DIG-labelled probe (Antisense)**	**DIG-labelled probe (Sense)**
Initial conc.	10µg/100µl	250ng/50µl	~ 10µg/50µl	~ 10µg/50µl
Dilution 1	(1:5) 1µl + 4µlsdH_2O		(1:10) 1µl +9µl sdH_2O	(1:10) 1µl +9µl sdH_2O
Dilution 2	(1:20) 2µl+38µl sdH_2O	(1:5) 2µl+8µl sdH_2O	(1:20) 2µl+38µl sdH_2O	(1:20) 2µl+38µl sdH_2O
Dilution 3	5µl+45µl sdH_2O	5µl+45µl sdH_2O	5µl+45µl sdH_2O	5µl+45µl sdH_2O
Dilution 4	5µl+45µl sdH_2O	5µl+45µl sdH_2O	5µl+45µl sdH_2O	5µl+45µl sdH_2O
Dilution 5	5µl+45µl sdH_2O	5µl+45µl sdH_2O	5µl+45µl sdH_2O	5µl+45µl sdH_2O
Dilution 6	5µl+45µl sdH_2O	5µl+45µl sdH_2O	5µl+45µl sdH_2O	5µl+45µl sdH_2O
Dilution 7	5µl+45µl sdH_2O	5µl+45µl sdH_2O	5µl+45µl sdH_2O	5µl+45µl sdH_2O

A DIG-labelled control RNA sample was also provided with the kit and was diluted with the other DIG labelled samples according to table 9. The DIG-labelled control RNA supplied with the kit is the guideline to estimate the probes concentration. Once dilutions were carried out for each DIG labelled sample, DIG users guide was used (Roche Diagnostics™) to estimate the concentration of the probe that is described below:

Spot points were marked lightly using a pencil, in a straight line order on the membrane (Hybond) and then 1µl of each dilution was pipetted on each spot on the membrane. The membrane was then air-dried for 10 minutes followed by fixing the probe to the membrane by exposing the membrane to UV light for 5 minutes. The membrane was then subjected to different washes which all took place at room temperature on a gentle shaker. The first wash was with 1X washing buffer together for 5 minutes followed by blocking with 1X blocking buffer for 30 minutes. Thereafter the membrane was incubated in anti-DIG Alkaline phosphatase, diluted 1:10000 with 1X blocking buffer, for 30 minutes. The membrane was then washed twice with 1X washing buffer, 15 minutes each. The membrane was then immersed in 1X detection buffer for 2 minutes followed by incubation overnight with NBT/BCIP (diluted 1:50) in detection buffer. This step allowed the colour to develop in the dark overnight. The reaction was terminated by placing the membrane in 50 ml TE buffer for 5 minutes with shaking. The membrane was air-dried and the probe concentrations were estimated by comparing the spot intensities with that of the known DIG-labelled control RNA concentrations. The concentration was found to be between 10pg/µl and 100pg/µl. This probe was then used for In Situ Hybridisation.

2.1.2 LOCALIZATION OF 1-ACBP, B-ACBP and PBR mRNA

Colorimetric and flourescent detection methods were used for In Situ Hybridisation.

2.1.2.1 Preceding ISH

Initially 4% paraformaldehyde (PFA) was prepared by dissolving 4g PFA in 100ml 1M phosphate buffer at 60ºC with stirring (NB not to exceed 60ºC). Once the PFA was completely dissolved it was cooled to room temperature. TBS was chilled at 4ºC.

2.1.2.2. Pre-hybridisation treatment of sections

4 µm cut sections of oesophageal carcinoma tissue sections were obtained from Prof Stewart Goetsch at the University of the Witwatersrand in the Department of Anatomical pathology. Ethics clearance was obtained from the Human Ethics Committee, University of the Witwatersrand. The normal tissue sections were obtained from Prof M. Scholtz at the University of the Witwatersrand Medical School, Department of Forensic Pathology.

The 4 µm cut tissue sections were dewaxed in clean xylene (Saarchem) for 30 minutes (three changes of 10 minutes each) at room temperature. This was followed by rehydration of the tissues in fresh absolute ethanol for 6 minutes (2 changes of 3 minutes) at room temperature. Further rehydration in 90%, 70% and 50% ethanol and DEPC treated water for 3 minutes in each solution at room temperature was carried out. The sections were then fixed in freshly prepared 4% PFA for 20 minutes at room temperature followed by 3 rinsing periods in fresh TBS (3 changes of 1 minute each) at room temperature. Proteins were denatured by immersing the slides in 0.1MHCl for 10 minutes

at room temperature. The sections were again subjected to 3 rinses in fresh TBS (3 changes of 1 minute each) at room temperature. Non-specific labelling was limited by placing slides in freshly prepared 0.5% Acetic Anhydride, in 100mM Tris (pH 8), for 10 minutes at room temperature. These were again rinsed in TBS for 3 minutes (3 changes of 1 minute each) and cell membranes were permeabilised by adding 20µg/µl Proteinase K (prepared in TBS from 10µg/ml) to each section and incubating for 20 minutes at room temperature. This step is performed to ensure the cells are opened up and the mRNA is exposed for hybridisation purposes. The slides were then rinsed in TBS for 3 minutes again (3 changes of 1 minute each). Proteinase K activity was terminated by incubating slides in TBS for 5 minutes at 4ºC. The sections were then dehydrated in 50%, 70%, and 90% ethanol for 1 minute in each solution, followed by dehydration in absolute ethanol for 2 minutes, 2 changes of 1 minute each. They were then dried in chloroform for 10 minutes in the fume hood. Slides were stored in a closed slide box.

2.1.2.3 Hybridisation

The probes stored at -70ºC were thawed on ice. 10µl of melted Herring Sperm DNA (HSD) (10µg/ml) was added to 990µl of hybridisation buffer and vortexed thoroughly to enhance hybridisation. 3µl of the probe was added to 900µl of hybridisation buffer and HSD and was subjected to rapidly boiling water for 5 minutes, followed by cooling on ice. The other 100µl of hybridisation buffer + HSD was used as a negative control since no probe was added. 70µl of each probe preparation was spread on an appropriate section limited by an adhesion square/gene frame (Southern Cross Biotechnology.). To limit evaporation of the hybridisation mix a solution of 5X SSC (3M NaCl, 0.3 M Na-citrate,

pH 7.0) buffer and 50% formamide was prepared and poured into the hybridisation chamber. Hybridisation was carried out in a Hybaid Omnislide Flat Block Humidity Chamber (Hybaid USA) at 55ºC overnight.

2.1.2.4 Post-hybridisation washes and detection of bound probes

After hybridisation, sections were washed in 2X SSC for 30 minutes at 37ºC in a hybridisation oven, to remove or wash off excess unbound probe. The sections were then incubated with RNAase for 30 minutes at 37ºC, followed by 2X SSC, 1X SSC and 0.1X SSC washing respectively, at 55ºC for 20 minutes. The slides were then rinsed in TBS for 3 minutes, 3 changes of 1 minute each at room temperature. Non-specific binding was blocked by adding 100µl of 1X blocking buffer to each slide and incubating in a humid chamber for 2 hours.

2.1.2.5 Colorimetric method protocol for in situ hybridisation

The sections were incubated with anti-DIG IgG conjugated with alkaline phosphatase diluted 1:500 in 1X blocking solution, 100µl per slide was used. This was incubated in a humid chamber for one hour at room temperature. After this slides were rinsed in TBS for 3 minutes (3 changes of 1 minute each) at room temperature. The slides were then incubated with the chromogen NBT/BCIP (Roche Diagnostics) diluted 1:50 in 1X detection buffer. 100µl of this preparation was added to each slide. This was left to develop in a dark humid chamber overnight at room temperature. The reaction was stopped by placing slides in 1X TE for 5 minutes. The slides were washed in running tap

water for 5 minutes at room temperature. Counterstaining with Mayer's Hematoxylin was then done at room temperature for 5 minutes. The slides were then placed in running tap water for 10 minutes at room temperature. After this the slides were mounted with permanent aqueous glycerol medium. These were allowed to dry and then viewed under the light microscope. Images were captured using the Zeiss Microscope Camera using the Axio software package.

In the colometric protocol the 2° antibody is anti-DIG conjugated to the alkaline phosphatase enzyme, which in the presence of NBT/BCIP substrate forms an insoluble blue precipitate. The preparation is relatively permanent and results can be viewed and visualised over a prolonged period of time. Care must be taken to block endogenous enzymes to the NBT/BCIP substrate.

2.1.2.6 Fluorescent method Protocol for in situ hybridisation

In the fluorescence method no substrate is added, as the 2º antibody is anti-DIG conjugated to fluorescein isothiocynate (FITC) which when excited at a wavelength of 490 nm gives off a bright apple green fluorescence. It is instantly visible but the preparation is not permanent and tends to fade under exposure.The method followed is the same as those in 2.2.1.3, 2.2.1.4 and 2.2.1.5, followed by the washing of slides in TBS-TWEEN. The slides are then incubated in TNB for 30 minutes at 37ºC. 75µl of AntiDig-fluor, diluted in TNT (1:500), is added to each section of tissue and left for 30 minutes at room temperature. The slides are then washed in TBS-TWEEN, 3 washes of 5 minutes each, before being mounted using the SlowFade Light Antifade kit (Molecular Probes, USA). Manufacturer's instructions were followed.

2.2 Protein Expression

Protein expressions of the three genes were carried out to raise antibodies for localisation studies. The amplified coding regions of the three genes from RT-PCR, utilising specific primers, were cloned into pGEM vectors for sequencing purposes. Once the correct sequences were attained the inserts were cut from the clones utilising specific restriction enzymes, and cloned into pGEX vectors that were subsequently transformed into *E.coli* cells for recombinant protein expression.

2.2.1 pGEX expression vectors

pGEX is an expression vector that forms the basis of the system GST expression. There are three major types of pGEX vectors, T, P and X, which differ in their cleavage sites, Thrombin, PreScission or Factor Xa respectively, see figure 13 below.

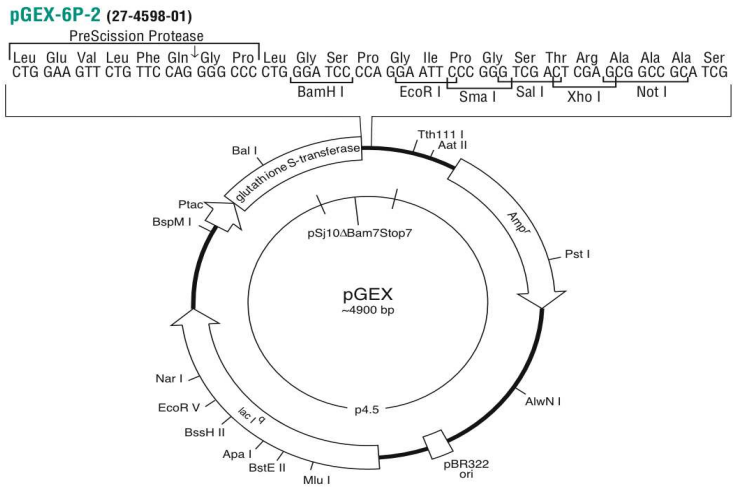

Figure 13: pGEX-6P-2 cloning vector

The plasmid consists of an upstream poly-linker region and the genes cloned are expressed as fusions with 26 kDa GST (glutathione-S-transferase) protein from *Schistosoma japonicum*. High level expression is controlled by a tac promoter in the pGEX vector.

2.2.2 Preparation of *E. coli* competent cells for transformation

The bacterial strain used for protein expression was *E. coli* BL 21 Gold with the genetic markers *F^- ompT hsdSR2 (rB^- mB^-) dcm^+ Tetr gal endA Hte.*

A single colony was taken from a freshly streaked nutrient agar plate and transferred to 20 ml of TYM and grown with vigorous shaking at 37 ˚C overnight. 1ml of the cells were added to 100ml TYM and grown to OD_{550}=0.2. The culture was transferred to 400 ml TYM and grown under the same conditions to OD_{550}=0.5. The cells were then rapidly chilled at 0˚C and pelleted by centrifugation at 3000 rpm for 10 minutes at 4˚C. The bacterial pellet was resuspended in 250 ml of cold Tfb1 and incubated at 4˚C for 30 minutes. The cells were pelleted again by centrifugation at 3000 rpm for 10 minutes at 4˚C. The pellet was gently resuspended in 50 ml Tfb2, then divided into 500 µl aliquots into 500 µl eppendorf tubes, which was frozen in liquid nitrogen before long term storage at -70˚C. This method yielded transformation efficiencies of 1-5 x 10^7 transformants per µg plasmid DNA.

2.2.3 Transformation of *E. coli* cells

Competent E. coli strains were transformed with plasmid DNA as follows: Frozen competent cells were thawed on ice and 100 µl of these cells were added to 1- 10ng of DNA and incubated on ice for 30 minutes. The mixture was heat shocked at 37˚C for 5 minutes and then 1ml LB was added to each tube and allowed to incubate for a further 30 minutes at 37˚C, to allow antibiotic expression of the ampicillin antibiotic resistance markers. The transformed cells were then plated onto ampicillin containing plates and incubated overnight at 37˚C.

2.2.4 Large –scale preparation of plasmid DNA

A 500-1000 ml culture of a transformed *E. coli* colony was prepared in the presence of 100 µl.ml^{-1} ampicillin, and grown overnight with shaking at 37˚C. The bacteria culture was then pelleted by centrifugation at 5000 rpm for 10 minutes at 4˚C. The pellet was resuspended in 4 ml GTE and incubated on ice for 5 minutes. The cells were lysed by the addition of 8 ml NaOH/SDS with gentle swirling and incubated on ice for 5 minutes. 6 ml of pre-chilled 3:5 M KOAc was added and mixed gently to neutralise the alkali, and the mixture was incubated on ice for 5 minutes. The precipitate of cell debris, chromosomal DNA and SDS was removed by centrifugation at 10 000 rpm for 15 minutes at 4˚C. The supernatant was filtered through glass wool to remove particulate material and the nucleic acids precipitated by the addition of 1 volume propan-2-ol, followed by incubation at -20˚C for 30 minutes. The precipitate was pelleted by centrifugation at 10 000 rpm for 15 minutes at 4 ˚C. Plasmid DNA was separated from

RNA by either polyethylene glycol (PEG) precipitation or double CsCl/ethidium bromide fractionation.

2.2.5 PEG precipitation

The pellet was resuspended in 5 ml TE. 100 µg of RNase was added and incubated at 37 ˚C for 60 minutes. The RNase was removed by a single extraction with 1:1 phenol:chloroform. The supernatant was transferred to clean a tube, 0.3 M NaOAc was added and the nucleic acids precipitated by the addition of 2.5 volumes of ethanol and incubated at – 20 ˚C for 30 minutes. The pellet was then collected by centrifugation at 10 000 rpm for 10 minutes at 4 ˚C and resuspended in 2 ml 2.5 M NaCl by vortexing. 2 ml 13 % polyethylene glycol with molecular weight 8, 000 was added to precipitate the DNA. The mixture was vortexed thoroughly and incubated on ice for 30 minutes. The precipitate was then pelleted at 10 000 rpm for 10 minutes at 4 ˚C. The pellet was washed with 70 % ethanol and resuspended in 100 µl TE.

2.2.6 Small scale preparation of plasmid DNA

For the analysis of transformants, plasmid DNA was isolated from overnight cultures of E. coli picked from single colonies. This method yielded DNA of sufficient quality and quantity to perform restriction analyses.

A 1.5 ml saturated overnight culture of plasmid-containing *E. coli* were grown in LB containing 100 $\mu g.ml^{-1}$ ampicillin. The bacteria were then pelleted in a microfuge at 6000 rpm for 10 minutes and resuspended in 200 µl GTE after which the mixture was incubated at room temperature for 5 minutes. 400 µl NaOH/SDS was added to lyse the

cells and incubated at room temperature for 5minutes. The mixture was then neutralised by the addition 300 µl 3:5 KOAc, mixed gently and incubated at room temperature for 5 minutes. The precipitate was then pelleted at 13 000 rpm for 15 minutes. 800 µl of the supernatant was added to 600 µl propan-2-ol in a fresh tube and incubated at -20 ˚C. The precipitate of nucleic acid was pelleted at 13 000 rpm for 10 minutes, then washed with 70 % ethanol and resuspended in 500 µl TE. 100 µg/ml RNase was added and incubated at 37 ˚C for 1 hour and RNase was removed by single extraction with 1:1 phenol chloroform. The DNA was recovered by ethanol precipitation in which 1/10 Volume 3M NaOAc and 2.5 volumes of ethanol were added to precipitate the DNA and the pellet was once again washed with ethanol and resuspended in 50 µl TE.

2.2.7 Restriction digests of pGEX-6P-2 including alkaline phosphatase treatment.

Restriction enzymes used were *Xho* I and *Bam* HI, both supplied by Roche™. Both enzymes were used in a single digestion reaction as one buffer was found to be compatible with both enzyme requirements. Shrimp alkaline phosphatase (SAP) was included in the digestion of the vector in order to remove phosphate groups at the end of single digested vector fragments to prevent recircularization of vector. This leads to high background in plates. The digestion mixture as shown in table below was incubated at 37 ˚C for 3 hours to allow sufficient cleavage, followed by a 80 ˚C for 20 minutes incubation period to inactivate the enzymes. The digestion sample was then loaded through QIAprep spin column from QIAGEN™ to purify the digested vector DNA.

Table 10: Restriction digestion

Restriction digestion Reagents	Volumes
pGEX-6P-2 (200 µg/µl)	5.0 µl
10X Restriction buffer	5.0 µl
BSA	0.5 µl
dH_2O	36.5 µl
Bam HI (10 U/µl)	1.0 µl
Xho I (10 U/µl)	1.0 µl
SAP (1/10X dilution)	1.0 µl
Total Volume	**50.0 µl**

2.2.8 PCR Amplification of the coding regions for 1-ACBP, b-ACBP and PBR

Two sets of specific forward and reverse primers were designed and synthesised for each open reading frame (ORF) region of the three genes, to increase specificity. The first set of primers were designed to be specific to the gene and once the desired fragment was obtained, the second set of primers designed with restriction enzyme sites, was used to amplify a fragment containing these restriction enzyme sites. A *Bam* HI restriction enzyme site was introduced into each forward primer for cloning purposes. Six bases were incorporated before the *Bam* HI site to allow efficient cleavage of the insert from the vector. The reverse primer of each gene was designed to complement and cover the stop codon at the end of the open reading frame followed by an *Xho* I restriction enzyme site and six bases for efficient cleavage. The PCR products were successfully amplified using PCR master mix and the PCR thermocycle conditions shown in the tables 11, 12 and 13.

Table 11:PCR reagents

Reagents	**Volume/1Reaction**
Master Mix (10x PCR buffer, $MgCl_2$, Taq)	12.5 µl
Forward Primer	1.0 µl
Reverse Primer	1.0 µl
$MgCl_2$	2.0 µl
Nuclease Free H_2O	7.5 µl
Template DNA	1.0 µl
Total Volume	25.0 µl

Table 12: Thermocycle conditions: for each set of primer for each gene

Temp.	**Time period**	**Rationale for each step**
95°C	2 mins	Double stranded DNA denatured to single strands
94°C	30 secs	DNA denatured at start of each cycle to single strands
56°C	**30 secs**	**Primers anneal to single strand template DNA**
72°C	1 min	DNA extension or synthesis by Taq polymerase
72°C	10 mins	Final extension step
4°C	-	Holding step, Sample ready to proceed to next step

30 cycles

Table 13: Annealing temperatures

Genes	**AT for primer set:1**	**AT for primer set:2**
b-ACBP	56 °C	58 °C
1-ACBP	56 °C	56 °C
PBR	58 °C	64 °C

2.2.9 Cloning into pGEM for sequencing

Once the desired sized fragments were obtained they cloned into pGEM vectors and sent for sequencing at Inqaba Biotech. Once correct sequence was attained the insert was cut out of the pGEM vector using *Bam* HI and *Xho* I for cloning into pGEX vector.

2.2.10 Restriction Digestion of pGEM vectors plus insert

Once the correct sequence was confirmed, pGEM vectors containing the insert was cut with Bam HI and Xho I to release the insert. The same protocol was carried out as that in the pGEX-6p-2 restriction digestion, only deviation being the vector which is pGEM in this case. The samples were then loaded onto a 0.8 % agarose gel and the insert cut out and purified using the gel purification kit from Promega™.

2.2.11 Cloning into pGEX-6P-2vector

The *Bam* HI and *Xho* I cut insert was then cloned into previously *Bam* HI and *Xho I* cut pGEX-4T-3 vector, using T4 DNA ligase. The reagents used are shown in table below:

Table 14: Cloning into pGEX-6P-2 vector

Ligation reagents	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**
Digested Insert (<10 ng/ul)	4.0 µl	4.0 µl	4.0 µl	4.0 µl
Digested vector (<10 ng/ul)	-	2.0 µl	4.0 µl	8.0 µl
10X Ligation buffer	2.0 µl	2.0 µl	2.0 µl	2.0 µl
dH_2O	14.0 µl	12.0 µl	10.0 µl	6.0 µl
T4 DNA Ligase (3U/ul)	0.2 µl	0.2 µl	0.2 µl	0.2 µl
Total Volume	**20.2 µl**	**20.2 µl**	**20.2 µl**	**20.2 µl**

The ligation mixtures were left to incubate at room temperature for 16 hours. Sample 1 is the background control.

2.2.12 Transformation of *E. coli* with recombinant DNA

Frozen *E.coli* BL21 gold competent cells were thawed on ice and 50-100 µl of these cells were added to 1- 10ng of each 4 DNA samples and incubated on ice for 30 minutes. The samples were heat shocked at 37˚C for 5 minutes and put on ice for 2 minutes, then 900ml LB was added to each tube and allowed to incubate for 1 hour at 37˚C to allow antibiotic expression of the ampicillin antibiotic resistance markers. 100 µl transformed cells were then plated onto ampicillin containing plates and incubated overnight at 37˚C.

2.2.13 Colony PCR

Positive colonies containing the insert were verified by colony PCR utilising the specific primers for each gene. The same protocol was used as that in 2.1.1.7.

2.2.14 Sequencing of pGEX vector

Clones were sent to Inqaba Biotech (Pretoria, South Africa) for sequencing.

2.2.15 Protein Expression

Once confirmation was obtained, the positive *E. coli* transformed colonies were inoculated into 0.5ml LB with 100 µg/ml ampicillin and 0.3 % glucose to substitute aeration as this experiment was set up in 1.5ml centrifuge tubes. The tubes were incubated shaking at 37^{o}C for 4 hours. 3 µl of 0.1M IPTG was added to the tubes and

were incubated for further 2 hours. The cells were centrifuged and the supernatant was discarded from each tube. 0.5 ml PBS was used to resuspend the cells. 15 µl of each cell suspension and 5 µl gel dye were added and heated at 80°C for 5 minutes and were analysed on SDS/PAGE.

Alternative method:

Positive colonies were inoculated into 20 ml LB containing AMP (100 µg/ml) and incubated overnight at 37°C with vigorous shaking. The 20 ml culture was then transferred to 180 ml LB containing AMP and incubated at the same conditions but until an OD_{550}=0.4 was reached. IPTG (stock= 0.1M) was then added to each sample with varying concentrations to determine the optimum concentration for induction of the protein. The table below indicates the various IPTG concentrations used:

Table 15: Different IPTG concentrations for protein induction

Sample	IPTG Concentration	IPTG volume
Clone 1	0.1 mM	200.0 µl
Clone 2	0.3 mM	600.0 µl
Clone 3	1.0 mM	2.0 ml
Clone 4	3.0 mM	6.0 ml

The samples were shaken @ 110 rpm @ 25°C for overnight. The overnight culture was centrifuged at 10 000 rpm for 5 minutes at 4°C. The pellet was resuspended in 2 ml of PBS (phosphate buffered saline) and sonicated for 30 seconds. The sample was then

centrifuged at 10 000 rpm for 30 minutes at 4°C and the supernatant transferred to a new tube to be loaded onto an SDS gel.

2.2.16 SDS polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

The proteins were separated by denaturing discontinuous SDS polyacrylamide gel electrophoresis. The 12 % SDS gel reagents are shown in tables 16 and 17:

Table 16: Separating gel reagents

Reagents	**Volume**
dH2O	3.35 ml
1.5M Tris-Cl, pH 8.8	2.5 ml
10% SDS	0.1 ml
Acrylamide/bis (30%)	4.0 ml
10% APS	50.0 µl
TEMED	5.0 µl

Table 17: Stacking gel reagents

Reagents	**Volume**
dH2O	6.1 ml
0.5 M Tris, pH 6.8	2.5 ml
Acrylamide stock solution (30% T)	1.3 ml
10% SDS	0.1 ml
10% APS	50.0 µl
TEMED	5.0 µl

30 µl of the sample was added to 10 µl of the sample loading dye and boiled for 4 minutes. The sample was then loaded onto the protein gel and electrophoresed at 200 V for 45 minutes. Gels were stained in the staining solution, followed by multiple washes

with destaining solution. The gels were then viewed and images captured using a light box.

2.3 Comparison of mRNA expression levels of 1-ACBP, B-ACBP and PBR in an oesophageal cancer cell line determined by Quantitative PCR.

MRNA expression levels of 1-ACBP, B-ACBP and PBR were determined using the LightCycler FastStart DNA Master SYBR Green 1 (Roche®) system, to determine whether the expression of these genes were correlated in anyway.

The LightCycler FastStart DNA Master SYBR Green 1 is a "Hot Start" reaction mix for PCR in glass capillaries. It contains a modified Taq DNA polymerase, made from the usual thermostable recombinant Taq DNA polymerase, to remain inactive at room temperature and thus preventing elongation periods when primers can bind non-specifically and generate undesired products. This modification is the addition of heat-labile blocking groups on some of the amino acid residues of the enzyme, only being removed once exposed to a high temperature of 95 degrees Celsius for a maximum of 10 minutes.

Syber Green 1 is the dye used for detection of the amplified PCR products in this system. Syber Green 1 binds specifically to double-stranded DNA, and its fluorescence is greatly enhanced by this kind of binding. So during each phase of DNA synthesis, the SYBR Green 1 dye binds to the amplified DNA products and the amplicon is detected by its fluorescence.

The short cDNA transcripts of 1-ACBP, B-ACBP and PBR used for probe preparation, and the open reading frames cDNA transcripts of 1-ACBP, B-ACBP and PBR were used to determine their expression levels in an oesophageal cell line and compared to

determine whether any correlation occurred between the genes. See table below for Master Mix reaction mixture of 1 reaction.

Table 18: LightCycler Master Mix reaction mixture:

Components	**Volume**	**Final Concentration**
H_20, PCR grade	12.0µl	
$MgCl_2$	2.0µl	5mM
Fwd Primer Rev Primer	1.0µl 1.0µl	10pM 10pM
SYBER Green 1	2.0µl	1x
Total Volume	18.0 µl	

Experimental: add 2µl cDNA template (oesophageal cancer cDNA)

-ve control : add 2µl H_20

+ control : add 2µl cDNA template (ß-globin cDNA)

Once the Master Mix reaction mixture had been prepared, it was mixed gently and pipetted into the precooled LightCycler capillary. 2µl cDNA template was added for the experimental samples to each tube, water for the negative control, 2µl ß-globin cDNA for the positive control. Each capillary was then sealed with a stopper, and the adapters containing the capillary was placed into a standard benchtop microcentrifuge and centrifuged at 3000 rpms for 5 seconds. The capillaries were placed into the rotor of the LightCycler Instrument and the samples cycled according to the experimental protocol.

The experimental protocol consists of four programs:

Program 1: Pre-incubation and denaturation of the cDNA, where the target temperature is 95 degrees Celsius and the incubation temperature runs for a period of 10 minutes for 1 cycle. Program 2: Amplification of the target sequence which runs for 45 cycles at different target temperatures of 95, 58 and 72 degrees Celsius, at incubation times of 10, 5 and 10 minutes respectively. Program 3: Melting curve analysis for product identification that occurs for 1 cycle at three different temperatures of 95, 65 and 95 degrees Celsius, at incubation times of 0, 15 and 0 minutes respectively. Program 4: Cooling the rotor and thermal chamber at the end of the protocol contains 1 cycle at 40 degrees Celsius. When setting the fluorescence parameters, the display mode was set to channel 530 (530), using software version 4.x.

2.4 Solutions

Table 19: Composition of solutions used

Solution	**Composition**
10X TBE	0.9 M Tris (pH 8.3) 0.89 M Boric acid 25 mM EDTA
DNA loading buffer	30% Glycerol (v/w), 15mM EDTA (pH 8), 0.05% Bromophenol Blue (w/v)
Luria broth	1% Bacto-tryptone 0.5% Yeast Extract 1% NaCl
Ampicillin	0.1g Ampicillin in 1 ml sdH_2O
Formaldehyde gel loading buffer	720 µl formamide, 160 µl 10X MOPS buffer, 260 µl 37% formaldehyde, 100µl sdH_2O, 100 µl EtBr (10 mg/ml, 80µl BPB in DEPC H_2O.
GTE	1% glucose, 50mM Tris HCl, 10 mM EDTA
0.1 M PBS, pH 7.4 (w/v)	5 PBS tablets in 1000ml H_2O
20X SSC	175.3 g Trisodium citrate in 800 ml DEPC H_20, pH 7 with HCL and add up to a litre.
0.1 % v/v DEPC treated H_20	1 ml DEPC to 1 L dH_2O,shake overnight at 37^oC and autoclave.
TBS	1 M Tris (pH 7.5),5 M NaCl in DEPC H_2O
0.1 M Sodium Citrate, pH 6	29.4 g Tri-sodium citrate in 800 ml dH_2O, pH 6.0 add up to 1litre
10X TE	100 mM Tris-HCl (pH 7.5); 10 mM EDTA
4 m Lithium chloride	8.478 g LiCl in 50 ml DEPC H_20,store at room temperature
0.5 M EDTA, pH 8	18.612 g EDTA in 50 ml DEPC H_2O with continuous stirring and constant addition of NaOH drops until pH 8.0, add up to 100 ml with DEPC H20.
10 mg/ml Proteinase K	10 mg Proteinase K in 1000 µl DEPC H_20
10% SDS	10 g SDS in 100 ml DEPC H_2O
10X MOPS	200mM MOPS, 10mM EDTA, 50mM Sodium Acetate
0.1 M HCL	10 ml of 9M(32%) HCl in 990 ml DEPC H_20

Solution	Composition
0.5% Acetic Anhydride	0.25 ml Acetic Anhydride 49.75 ml Tris (pH 8)
Hybridisation Buffer	40 g Dextran sulphate sodium salt in 70 ml DEPC H_20 and heat at 68^oC for 3-4 hours and add up to 100ml, store at 4^oC
0.2 M Phosphate buffer	17.02 g Na_2HPO_4 and 12.48 g NaH_2PO_4 in DEPC H_20
0.85% Nacl	8.5 g NaCl in 1000 ml dH_2O,autoclave store at room temperature
4% PFA	4g PFA in 100 ml 0.1 M Phosphate buffer by slow heating to 60^oC, until solution becomes clear.
1 M Tris pH 8.0	Dissolve 121 g Trizma Base in 800 ml DEPC H_20, pH 8.0 add up to 1litre.
1 M Tris pH 7.5	121 g Trizma Base in 800 ml DEPC H_2O, pH 7.5 add up to 1 litre.
5 m NaCl	292.2 g NaCl in 1000 ml DEPC H_20
Biotinylated Nucleotide Mix	250 mM Biotinylated Nucleotide Mix 10 mM Tris-HCL (pH 7.6) 1 mM EDTA
Cell Resuspension Solution	500 mM Tris-HCL (pH 7.5) 10 mM EDTA 100 µg/ml RNase A
Cell Lysis Solution	0.2 M NaOH and 1% SDS
Neutralization Solution	4.09 M Guanidine Hydrochloride 0.75 M Potassium Acetate 2.12 M Glacial Acetic Acid (pH 4.2)
Column Wash Solution	60% Ethanol 60 mM Potassium Acetate 8.3 mM Tris-HCL (pH 7.5) EDTA
Membrane Wash Solution	10 mM Potassium Acetate (pH 5) 80% Ethanol 16.7 mM EDTA (pH 8)
Memebrane Binding Solution	4.5 M Guanidine Isothiocyanate 0.5 M Potassium Acetate (pH 5)
10X Detection Buffer	1 M Tris-HCL (pH9.5) 1 M NaCl

Equilibration Buffer	200 mM Patassium Cacodylate 25 mM Tris-HCL (pH 6.6) 0.2 mM DTT 2.5 mM Cobalt Chloride 0.25 mg/ml BSA

2.2.12 Abbreviations

ACBP- Acyl-CoenzymeA Binding Protein

PBR – Peripheral Benzodiazepine Receptor

BPB - Bromophenolblue

DEPC - Diethylpyrocarbonate

DIG dUTP – Deoxyuridine triphosphate

dNTP – 2'- deoxynucleotode 5'- triphosphate

DTT - Dithiothreitol

EDTA - Ethylenediaminetetraaceticacid

ETOH – Ethanol

FITC – Fluorescein isothiocynate

HCL – Hydrochloric Acid

HSD – Herring Sperm DNA

ISH – In Situ Hybridisation

kDA – Kilobase pairs

LB – Luria Broth

$MgCL_2$ - Magnesium Chloride

ml - millimeter

MOPS – [N-mophonolino] propane-sulphuric acid

NBT/BCIP - Nitro-blue-tetrazolium – 5-bromo-4-chloro-3-indolylphosphate

ºC – Degrees Celsius

PAP – Peroxidase Anti-Peroxidase

PBS-BSA – Phosphate Buffered Saline-bovine serum albumin

PCR – Polymerase Chain Reaction

PFA – Paraformaldehyde

RT-PCR – Reverse Transcripion -PCR

sdH_2O – Sterile Water

SDS – sodium dodecyl sulphate

SSC – Sodium Chloride Sodium Citrate

TBE – Tris Borate EDTA

TBS – Tris-buffered

TNB – Tris-sodium-blocking solution

µl - micro liter