

Appendix A – Materials and Methods

A.1 Chemicals, Reagents and Other Materials

All chemicals and reagents were supplied by Sigma-Aldrich (Poole, UK) unless stated otherwise and all equipment used is listed in *Appendix B*. The cell lines were either supplied by the European Collection of Cell Culture (ECACC, Salisbury UK) or the American Type Culture Collection (ATCC, Manassas VA USA).

A.2 Cell Culture

A.2.1 Media

A variety of cell lines were cultured and their names and cellular requirements are shown in *Table A.1*.

A.2.2 Cell Culturing

All cell lines were cultured in an incubator at 37°C with 5% (v/v) CO₂ and the medium was changed every other day. Once the cells reached 70-80% confluences they were rinsed in phosphate saline buffer (PBS) and passaged by trypsinisation with a 1x Trypsin solution (approximately 5ml of a 1x solution of trypsin were added/ T75 flask). The flasks were then incubated at 37°C for up to 5 minutes to allow the trypsin to dissociate the cells (cells were never trypsinised for longer than 5 minutes as cellular damage could occur). The cellular mixture was then transferred to a 15ml centrifuge tube (Fisher Scientific, Leicestershire UK) and an equal volume of medium containing serum was added to neutralise the trypsin. The cells were then centrifugated at 105 xg at 4°C for 5 minutes and the supernatant removed. The pellet was then re-suspended in an appropriate volume of medium for further culturing.

For cryo-preparation of cells, the pellet was re-suspended in foetal calf bovine serum (FCS) in a 1:1 ratio with 10% (v/v) DMSO in FCS, this was then stored at –80°C overnight and then transferred to liquid nitrogen. For slide preparation, slide flasks were seeded and once the cells reached a desired confluence the flasks were washed three times with cold phosphate saline buffer (PBS) (Invitrogen Corporation, Paisley UK)

(formulation in *Appendix B*) and fixed by immersion in cold methanol for 10 minutes. These were then left to dry at 4°C for 30 minutes and then wrapped in foil and placed at -20°C until required.

Table A.1. The cell lines used and their media requirements.

Cell Line name	Cell Type	Medium	Supplements
PNT1A (ECACC No. 95012614)	Normal Human Post Pubertal Prostate Epithelial Cells	RPMI-1640	2mM Glutamine, 10% (v/v) foetal calf bovine serum and 5units/ml of Penicillin-and 0.005mg/ml Streptomycin solution
PC-3 (ECACC No. 90112714)	Human Prostate Adenocarcinoma	Firstly Grown in: -1:1 ratio of Coon's modified F12 (Scientific laboratory Supplies Ltd, Leicester UK) and Kaign's modified F12 (ATCC, USA). Once Stocks obtained grown in :- Dulbecco's modified Eagle's medium/Nutrient F-12 Ham	2mM Glutamine, 7% (v/v) foetal calf bovine serum, 0.045mg/ml of Ascorbic acid, 0.018mg/ml solution of Myo-inositol and 5units/ml of Penicillin-and 0.005mg/ml Streptomycin
RT112/84 (ECACC No. 85061106)	Human Bladder slow growing tumour	Dulbecco's modified Eagle's medium/Nutrient F-12 Ham	1mM Glutamine, 10% (v/v) foetal calf bovine serum, 5units/ml of Penicillin and 0.005mg/ml Streptomycin
OE21 (ECACC No. 96062201)	Human Caucasian Oesophageal Squamous cell carcinoma.	RPMI-1640	10% (v/v) foetal calf bovine serum, 5units/ml of Penicillin and 0.005mg/ml Streptomycin
CaCO2 (ATCC No. HTB-37)	Colorectal Adenocarcinoma Colon	Dulbecco's modified Eagle's medium/Nutrient F-12 Ham	1mM Glutamine, 10% (v/v) foetal calf bovine serum, 5units/ml of Penicillin and 0.005mg/ml Streptomycin
PE/CA PJ41 (ECACC No. 98020207)	Oral Squamous epithelium carcinoma	Dulbecco's modified Eagle's medium/Nutrient F-12 Ham	1mM Glutamine, 10% (v/v) foetal calf bovine serum, 5units/ml of Penicillin and 0.005mg/ml Streptomycin

A.3 Cell Count

Once cells reached a desired confluence they were washed in PBS and then trypsinised as in Section A.2.2. The cellular pellet was then re-suspended in 2mls of PBS and 100 μ l transferred to a 0.5ml tube with 100 μ l of trypan blue (viability). These solutions were then mixed together making a final volume of 200 μ l and left to stand for 1 minute. While these solutions were resting the neubauer hemocytometer (Assistent, Germany) was prepared as shown in figure A.1.

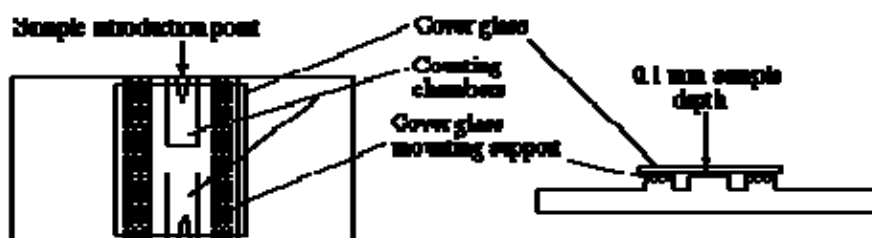


Figure A.1. <http://www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html>: A cover slip is moistened and placed on the hemocytometer covering the 2 central channels.

A drop of the cellular mixture was then placed underneath the cover slip at the sample introduction point and by capillary action the whole counting chamber filled.

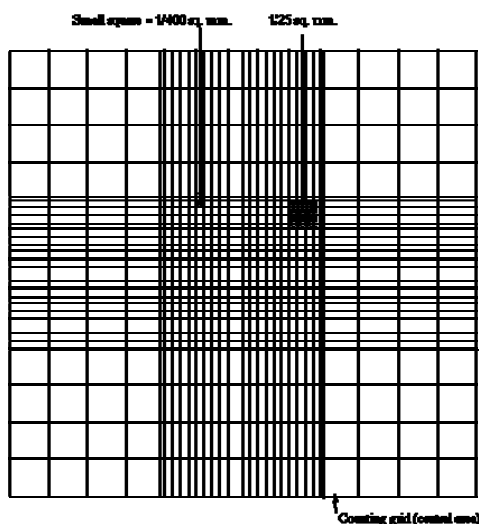


Figure A.2. Diagram of the grid on the hemocytometer each of the 9 square is 1mm and the depth between the coverslip and grid is 0.1mm.

<http://www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html>

A cell count was then performed by following these rules:- The hemocytometer contains 2 identical grids one on the upper counting chamber and one on the lower

counting chamber, shown in *Figure A.2*. Each grid consists of four corner squares containing 16 smaller squares and one central square containing 25 little squares. When viewed under a microscope if the cellular number exceeded 200 when counting the central square alone, then only the 2 central squares on both grids were counted and an average was calculated. However if the cellular number did not exceed 200, then all 4 corner squares were counted on each grid plus the central square. Once the cellular average was calculated the equation below was used.

Cellular viability was also assessed using the hemocytometer, by counting the number of white and blue cells, once calculated the equation below was used to calculate viability.

Once the average is calculated the equation below was used to calculate cellular number:

$$\text{Average} \times 10000^a \times 2^b = \text{Total number of cells in 2ml of Cellular suspension}$$

^a Conversion factor 1mm to 1 ml 0.1μl= 1mm)

^b Dilution Factor

To find the number of cells per ml:

Divide the total numbers of cells in 2ml by 2.

Once number of white to blue cell was calculated the equation below was used to calculate cellular viability:

$$\frac{\text{Number of white cells}}{\text{Total number of white and blue cells}} \times 100 = \% \text{ Viable Cells}$$

A.4 Immuno-cytochemistry (ICC) and Immuno-histochemistry (IHC)

All incubation periods for immunocytochemistry and immunohistochemistry were performed in a humidity chamber. Slides for immunocytochemistry were removed from storage at -20°C and incubated at room temperature (RT) for 10 minutes then an immedge pen (Vector labs Ltd, Burlingame UK) was used to draw an adequate grid.

Slides for immunohistochemistry were incubated at 60°C for 20 minutes and then placed in histoclear twice (2X) until completely de-waxed. These slides were then re-hydrated by placing them in various dilutions of ethanol (100%, 100%, 90%, and 70%) until finally they were placed in water. Once re-hydrated the slides were exhausted of any endogenous peroxidases by being placed in a 3% solution (v/v) of hydrogen peroxidase and methanol, for 15 minutes. After which antigen retrieval was performed by placing the slides in a 0.01M solution of Sodium Citrate Buffer pH6 (w/v) for 25 minutes, in a microwave at full power (optimisation of antigen retrieval for tissue refer to *Appendix D*). Once complete, the slides were then given a further 25 minutes to rest and washed in a 1M Tris Buffered Saline solution pH 7.5 (w/v) (TBS) (formulation *Appendix B*) for 5 minutes.

Once both preparation steps were complete the tissue sections and cell lines were treated the same way until mounting. Firstly the slides were blocked to ensure that no non-specific binding of the primary antibody could occur. This was done by applying a 20% secondary serum in 1M TBS pH 7.5 (v/v) (DakoCytomation Ltd, Cambridge UK) and leaving it to incubate at 37°C for 1 hour (hr). Once blocked the slides were then incubated with their optimal concentration of the primary antibodies (please ref. to specific chapter for appropriate concentration of antibody) for a desired time in a 1% secondary serum (v/v) in 1M TBS pH 7.5(w/v).

Following primary incubation the slides were washed in 1M TBS pH7.5 (w/v) for 5 minutes three times and then incubated with a secondary antibody which was biotinylated (DakoCytomation Ltd, Cambridge UK). This was applied to the slides for 1 hr at 37°C in a 1/200 dilution with a 1% secondary serum (v/v) in 1M TBS pH7.5 (w/v).

After the secondary incubation the slides were again washed as before and an ABcomplex (v/v) (DakoCytomation Ltd, Cambridge UK) was applied (formulation in *Appendix B*). This ABcomplex was left in the dark for 20 minutes before being applied to the slides and then incubated on the slide at 37°C for 30 minutes. The slides were then washed three times and placed in a 3,3 diaminobenzidine tetra hydrochloride (DAB) (w/v) solution (formulation in *Appendix B*) to allow the formation of a brown precipitate, which identified the proteins of interest. They were then counter-stained using haematoxylin, washed with de-ionised water for 5 minutes and mounted

For immunocytochemistry the slides were mounted using glycerol (DakoCytomation Ltd, Cambridge UK) while for immunohistochemistry the slides were first de-hydrated using ethanol (70%, 90%, 100%, and 100%) and then placed in histoclear 2X before being mounted with distyrene plasticizer xylene (DPX).

A.5 Protein extraction - CHAPS Lysis Buffer

Cells were cultured in T75 flasks until they reached a desired confluence, then the medium was removed and the cells washed in cold PBS. 10ml of protein inhibitor cocktail in PBS (v/v) (MP Biomedicals Inc, Iikirch France) (formulation *Appendix B*) was added, and the flask was scraped to dissociated the cells. The cellular suspensions were then transferred to a 15ml tube and centrifuged at 105 xg for 10 minutes at RT. The supernatant was then discarded and the pellets re-suspended in 1.5ml PBS, this was then transferred to a 2ml tube and centrifuged at 12,000 xg for 15 minutes at RT. The supernatant was discarded and 1.5ml of CHAPS Lysis Buffer (formulation *Appendix B*) was used to re-suspend the cellular pellets. This tube was then placed in liquid nitrogen for a few minutes to aid cell lysis via freeze-thaw, and once frozen the solution was quickly thawed and placed on ice for 1 hr. The sample was centrifuged at 12,000 xg at 4°C for 30 minutes, and the supernatant containing the cellular protein collected.

A.6 Protein Quantification

The concentration of cellular protein was calculated using the Bradford method and a standard curve of absorbency at 595nm was produced. A 1mg/ml protein standard solution of bovine γ globulin (Bio Rad lab, Hemel Hempstead UK) was used to generate

a dilution series (*table A.2*). To these solutions 1.5ml of Bradford reagent was added, vortexed and left to stand at RT for 30 minutes. The absorbance (595nm) of these samples was then measured using a spectrophotometer (WPA Lightwave UV/VIS Diode array Spectrophotometer version 1.5) and a standard curve was produced showing absorbance against concentration of protein.

The protein concentration of an unknown sample was then calculated. Firstly the sample was diluted in a 1:5 ratio with nuclease free water, then 1.5ml of bradford reagent was added and the sample was then treated the same way as the standard. The protein concentration for the unknown sample was then calculated, by reading the concentration from the standard graph produced for the bovine γ globulin. The concentration was then multiplied by the dilution factor to give the amount of protein present in the sample ($\mu\text{g/ml}$).

Table A.2. Preparation of standard for the Bradford method – to produce a graph of concentration against absorbency.

Standard Protein $\mu\text{g/ml}$	Amount of standard applied μl	Amount of water applied μl
0	0	50
100	5	45
200	10	40
400	20	30
800	40	10
1000 (1mg/ml)	50	0

A.7 Western Blotting

Western blotting was performed to assess if a desired protein was present in a sample and to semi-quantitatively compare differences in amounts between samples.

This technique was sub-divided into 3 stages.

A) SDS-PAGE Electrophoresis

B) Transfer of the protein to a solid phase

C) Immuno-detection and Identification of the protein

A) Electrophoresis SDS-PAGE

A polyacrylamide gel made up of a 10% resolving gel and 4% stacking gel of desired percentage was produced (formulation in *Appendix B*). The gel was then placed into the electrophoresis kit and running buffer added (formulation *Appendix B*). The protein samples were then thawed, and a loading dye added, at a ratio of 1:5 (formulation in *Appendix B*). These samples were boiled for 5 minutes, placed on ice for 1 minute and then loaded into the wells alongside, a protein molecular weight ladder (Autogen-Bioclear, Wiltshire UK).

Electrophoresis was performed using a Hoefer electrophoresis kit (Amersham Pharmacia biotech, Buckinghamshire UK) at 75 volts for 15 minutes and 115 volts for 2.15hrs. Once completed the gel was transferred to a solid support.

B) Transfer of the protein to a solid phase

For transfer from the gel to a membrane a Hoefer semiphor blotter was used (Amersham Pharmacia Biotech, Buckinghamshire UK). Eight pieces of Whatmann filter paper 3mm were cut and these along with the gel were soaked in transfer buffer for 1 minute (formulation *Appendix B*). Then an immobilon transfer membrane with 0.45µm pore (Millipore, Gloucestershire UK) was cut, soaked in methanol until opaque, water and then transfer buffer for 5 minutes. Once ready the blotter was stacked creating a sandwich of: anode (+), 4 filters, membrane, gel, four filters and cathode (-) and run at 70mA for 1 hr causing the proteins to transfer from the gel phase to the solid support (membrane).

C) Detection and identification of the protein

Once transfer was complete, the membrane was removed from the blotter and blocked in a 5% solution of low fat milk powder (w/v) (Marvel, Premier bands) in 1M TBS pH 7.5 at RT for 1 hr. This was performed on a rocker to help ensure all the binding sites were blocked. Once blocked, a rabbit polyclonal antibody to PSA RB-9065-P (Stratech, Cambridge UK) was applied in a 1/200 dilution in a 5% Marvel solution in 1M TBS pH7.5 (w/v) and incubated overnight at 4°C.

After incubation the membrane was washed in 1M TBS pH 7.5 with 0.2% Tween20 (v/v) for 5 minutes and this was repeated five times. Once washed, the membrane was incubated on a rocker for 1.5hr with donkey anti rabbit HRP (Autogen-Bioclear, Wiltshire UK) labelled secondary antibody, that was applied in a 1/2500 dilution in a 5% Marvel solution (w/v) in 1M TBS pH 7.5. Once complete, the membrane was washed as before and ECL detection was performed.

Chemiluminescent detection was performed using an ECL plus western blotting detection reagent (Amersham Bioscience, Buckinghamshire UK). Reagent A was mixed with reagent B in a 40:1 ratio, and applied to the membrane for 5 minutes, after which the excess liquid was blotted off using filter paper. The membrane was then sealed in a plastic bag and all the air removed. This was placed in a cassette with a film for an appropriate time depending on the strength of the signal. Once exposure was complete the film was placed in developer for a few seconds until the bands of interest appeared, then washed in water, fixed for 5 minutes and finally placed in running water for 5 minutes. Once complete the film was allowed to air-dry.

A.8 Dot Blot

A dot blot was performed to see which cell lines expressed a desired protein of interest. Firstly a Hybond-C nitrocellulose membrane (Amersham Biosciences, Buckinghamshire UK) was cut to the desired size and 2µl of protein was dotted onto the membrane and allowed to dry. Once dry the membrane was treated the same way as the membrane for western blotting *Section A.8C Detection and identification of the protein.*

A.9 PSA ELISA

96 well plate PSA ELISA kit (Calbiotech Inc, California USA), was used to test the six cell lines for secretion of PSA into the media. Firstly, the ELISA wells were allowed to warm up to RT, then 50µl of the PSA standards and the samples to be tested were pipetted into the individual wells, along with two negative controls (Negative controls = media DMEM F12 and RPMI media). To these wells 50µl of assay buffer was added and the plate was covered and incubated for 30 minutes at RT. Once incubated the well

content was removed and washed with 350µl of wash buffer three times and blotted on absorbent towel. Then 100µl enzyme conjugate was added to each well and incubated at RT for 30 minutes. The wells were washed as before and 100µl of TMS substrate was added and allowed to incubate for 15 minutes at RT. After incubation, 50µl of stop solution was added and mixed for 30 seconds. The absorbance was then read at 450nm within 30 minutes.

A.10 RNA Isolation

RNA isolation was performed using a number of techniques these were:- Micro-Fast Track™2.0 Kit (Invitrogen™ Living Science, Paisley UK), the RNeasy® Mini column (Qiagen, West Sussex UK) and Tri-Reagent (*Chapter 6 Section 6.2.3*).

A.10.1 Micro-Fast Track™2.0 Kit

When the cells reached a desired confluence they were washed in PBS then scraped from the flask in 10mls of PBS. This cellular mixture was then transferred to a 15ml centrifuge tube, and centrifuged at 105 xg for 10 minutes at 4°C. The supernatant was then discarded and 1.5mls of PBS was used to re-suspend the pellet. This was then transferred to a 2ml gamma-irradiated tube (Starlabs, Ahrensburg Germany) and centrifuged at 12,000 xg for 15 minutes at RT, again the supernatant was discarded.

The pellet was then placed in liquid nitrogen for a few seconds (can stop here and place pellet in -80°C) and 1ml of Micro-Fast Track™ 2.0 Lysis Buffer (formulation in *Appendix A*) was added. This was then vortexed and the resulting solution passed through an 18-21-gauge needle (0.9mm diameter) till the viscosity reduced. This lysate was then placed in a water bath at 45°C for 20 minutes. The lysate was transferred to a fresh 2ml gamma-irradiated tube and 63µl of 5M NaCl solution was added, and passed through an 18-21-gauge needle.

The mixture was then transferred to an Oligo(dT) Cellulose powder tube and left for 2 minutes to swell, the mixture was placed on an orbital shaker for 20 minutes, and centrifuged at 4,000 xg for 5 minutes at RT. The supernatant was then discarded and

the sample was washed by re-suspending in a 1.3ml binding buffer solution that was centrifuged at 4,000 xg for 5 minutes at RT, this was repeated four times. Once completed the Oligo(dT) Cellulose was re-suspended in 0.3ml of binding buffer and transferred to a spin column, where the solution was centrifuged at 4,000 xg for 10 seconds at RT. The Oligo(dT) Cellulose was then washed by re-suspending in 0.5ml binding buffer and centrifuging at 4,000 xg for 10 seconds at RT, this was then repeated four times. Then the Oligo(dT) Cellulose was re-suspended in 0.2ml Low Salt Wash Buffer and centrifuged at 4,000 xg for 10 second at RT, this was repeated two times to complete the washes.

The sample was then ready for elution. The spin columns were placed in a fresh collection tube and the Oligo(dT) Cellulose re-suspended twice in 0.1ml of elution buffer, this was then centrifuged at 4,000 xg for 10 seconds at RT. The spin column was then removed and the flow through retained. To this 10µl of glycogen carrier, 30µl of sodium acetate and 600µl of 100% ethanol were added to precipitate out the mRNA. The solution was then either stored at -80°C until required, or placed in liquid nitrogen for a minute to freeze the sample. Once the sample was thawed it was centrifuged at 12,000 xg for 15 minutes at 4°C and the supernatant was discarded. The pellet was then slightly dried and 10µl of Elution buffer was applied to re-suspend the mRNA, this was then stored at -80°C until required.

The concentration of the mRNA was then measured, by testing an aliquot of the total RNA solution and measuring the absorbance at 260nm (A_{260}).

A.10.2 RNeasy® Mini column

When the cells reached, their desired confluence they were dissociated from the flask by trypsinisation (*Section A.2.2*) and pelleted. The supernatant was then discarded and the pellet re-suspended in 1.5mls of PBS, this was transferred to a fresh 2ml gamma-irradiated tube and centrifuged at 300 xg for 5 minutes at 4°C and the supernatant discarded. The cellular pellet was then placed in liquid nitrogen for a few seconds preparing it for the total RNA process.

The pellet was then thawed and 600µl of RTL buffer was added, this was passed through an 18-21 gauge needle (0.9mm diameter) 5X, after which 600µl of 70% ethanol was mixed with the lysate. Once the solutions were mixed 700µl of the mixture was transferred to the RNeasy mini column, which was spun at 8,000 xg for 15 seconds at RT. The flow was then discarded and this was repeated until the whole sample was transferred. Once complete 700µl of RWI buffer was added to the RNeasy mini column and again centrifuged at 8,000 xg for 15 seconds at RT, with the flow being discarded.

Once complete a new collection tube was then applied to the RNeasy mini column and 500µl of RPE buffer was added. This was then centrifuged at 8,000 xg for 2 minutes at RT. The collection tube was then discarded and a new 1.5ml collection tube was used to perform the elution step. For elution 50µl of RNase free water was added to the RNeasy mini column and again the column was spun at 8,000 xg for 1 minute at RT, this time however the flow was not discarded as this contained the total RNA.

The concentration of the total RNA was then analysed, by testing an aliquot of the total RNA solution and measuring the absorbance at 260nm (A_{260}).

A.10.3 DNase Treatment

Deoxyribonuclease-1 was used to DNase treat the samples. DNase treatment removes any DNA contamination from an RNA sample. For every 10µl of RNA, 2µl of Dnase-1 (RNase free) was added and 2µl of X10 reaction buffer (200mM Tris HCL with 20mM MgCl₂). This sample was then incubated at RT for 15 minutes, then 2µl of a stop buffer (50mM EDTA) was added and it was heated to 70°C for 10 minutes. Once completed any DNA contamination was removed

A.11 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR is divided into two stages:-

2.11.1 Reverse Transcription phase (RT) Utilising a Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen™, Paisley UK)

2.11.2 Polymerase chain reaction (PCR). Utilising a PCR kit (Qiagen distributor, West Sussex UK).

A.11.1 Reverse Transcription

RNA samples were obtained (*Section A.10 or Section 5.2.4*) and all components required for RT-PCR were placed on ice and thawed. Once thawed 1µl of (50µM) Oligo (dT)₂₀ primer was added to a 0.5ml PCR tube with 2µl of 10mM dNTP. To this a desired amount of RNA was added (either from the Micro-Fast Track™2.0 K or TRI-Reagent), and then the mixture was made up to 12µl by adding DEPC-treated water, this was then incubated for 5 minutes at 65°C.

After the incubation, a 5 X cDNA Synthesis Buffer was vortexed and 4µl added to the tube. As well as 1µl of 0.1M DTT, 1µl of (40U/µl) RNase OUT, 1µl of DEPC-treated water and 1µl of (15U/µl) Cloned AMV-RT was added. The tube was then vortexed and incubated for 1 hour at 50°C then 5 minutes at 85°C. At the end of this procedure cDNA was produced.

A.11.2 Polymerase Chain Reaction (PCR)

Following cDNA synthesis, PCR was performed using a master-mix the component and amounts are shown in *Table A.3*.

Table A.3. Shows the essential components of the master mix and the quantities.

Component added	Amount per tube	Amount for ten tubes
Nuclease Free Water	(varies depending on sample)	(varies depending on sample)
10 X PCR Buffer	5µl	50µl
dNTPs (10mM)	1µl	10µl
Taq (250units)	0.5µl	5µl
Forward Primer (20pmolar) (Appendix A)	0.5µl	5µl
Reverse Primer (20pmolar) (Appendix A)	0.5µl	5µl
Total	48µl	480µl
Final Volume	50µl	500µl

The master-mix was then placed into a fresh 0.5ml PCR tube and a desired amount of cDNA added, to make a final volume of 50µl. The mixture was then thoroughly mixed and placed in a PCR machine (Techne Flexigene, Cambridge UK) and the desired cycles run. RT-PCR was performed for MCM2, MCM5, MCM7 and β -Actin and primers and cycles are shown in *Appendix B*.

A.11.3 Gel Electrophoresis

Once the PCR process was completed, agarose gel electrophoresis was performed to identify whether the specific regions of interest had been sequenced and amplified by the PCR process.

A 1.5 % (w/v) agarose gel in TAE (formulation *Appendix B*) was made and the RT-PCR samples were prepared for loading by adding 1µl of 6X loading dye (Fisher Scientific, Leicestershire, UK) with 5µl of the PCR sample. In addition a 100bp/1Kda DNA ladder (Promega UK Ltd, Southampton, UK) was loaded, alongside the samples. Electrophoresis was then performed at 75 - 100Volts until the sample ran to the end. Once electrophoresis was complete the gel was placed in 40µg/ml of ethidium bromide

solution, for up to 45 minutes to allow the amplified PCR products to stain. The gel was then transferred to de-ionised water for 1 minute and then analysed after by being placed on an UV transilluminator and photographed digitally using gene snap syngene version 4.0. The amplified PCR region was then detected via fluorescence as the ethidium bromide intercalates with the DNA and then fluoresces, to indicate the amplified regions. This was then displayed on a computer by gene snap syngene Version 4.0 and the relative brightness of the bands was assessed.

The size of the products analysed are shown in *table A.4*

Table A.4. Primers used and size of PCR fragments obtained.

Target	Primers codes	Fragment Size
MCM2	2F & 2R	500bp
MCM5	5F & 5R	500bp
MCM7	7F & 7R	407bp
β -Actin	BAA & BAS	680bp

Appendix B

Formulations

Phosphate Buffered Saline (PBS)

One tablet of PBS (Invitogen™, Paisley)

Distilled Water 500mls

Tris Buffered Saline (TBS)

Firstly a 20x solution of Tris saline buffer is made

Tris 121.1grams

NaCl 175.3grams

Distilled Water 1 litre

pH 7.5

Then 50ml of the 20x is diluted to 1 litre with distilled water so a 1x TBS solution is obtained

AB Complex

1x TBS 500ml

Avidin 4µl

Biotin 4µl

DAB solution

1X TBS 100ml

Hydrogen Peroxide 200µl

3,3-Diamino-benzidine (DAB) 0.06grams

Protease inhibitor and Phosphate Saline Buffer

1X PSB 9ml

Complete protease inhibitor 1ml

Microfast track™2.0 Lysis Buffer

Stock Buffer 1ml

Protein/RNase degrader 20µl

Chaps Lysis buffer

1M Tris at pH8 1.000ml

4M NaCl 1.875ml

0.5M EDTA 100µl

Chaps 310mg

Complete protease inhibitor 1.000ml

Add these all together and make the final dilution up to 50ml with distilled water.

Polyacrylamide Gel

Resolving Gel 10%

1.5M Tris pH 8.8	2.50ml
30% 29:1 Bis/acrylamide	3.30ml
10% SDS	100µl
10% (w/v) Ammonium Persulphate	100µl
Distilled Water	4.00ml
TEMED	4µl

4% Stacking Gel

0.5M Tris pH7.5	1.25ml
30% (v/v) 29:1 Bis/acrylamide	0.650ml
10% (w/v) SDS	50µl
10% (w/v) Ammonium Persulphate	25µl
Distilled Water	3.05ml
TEMED	10µl

5x Loading Dye

0.5M Tris pH 7.5	5ml
Glycerol	5ml
10% (w/v) SDS	10ml
1% (v/v) Bromophenol Blue	5ml
Distilled Water	25ml

Running Buffer

Firstly a 10X solution is produced

Tris	30.3grams
Glycine	144grams
SDS	10grams
Distilled Water	1 litre

To make 1x running buffer 100mls of the 10x is diluted to make 1 litre

Transfer Buffer

Tris	2.905grams
Glycine	1.465grams
SDS	0.5 grams
Methanol	100ml
Distilled Water	500ml

50 x TAE

Tris base	605grams
EDTA	36.9 grams
Glacial Acetic acid	125mls
RO water to	2.5 litres

To make 1x TAE need 200ml of 50x TAE to 10 litres of RO water

Protease Inhibitor Cocktail

4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF)	40mg
Leupeptin	100MCG
Pepstatin A	100MCG
Ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA-Na ₂)	500mg

Equipment

Platform Shaker STR6 (Stuart Scientific, Surrey UK)
Universal Water bath (Grant, Cambridge UK)
WPA Lightwave UV/VIS Diode array Spectrophotometer version 1.5 (WPA, Cambridge UK)
CO₂ Incubator (Jencons-PLS, Leight-Buzzard UK)
Microflow bio Safe cabinet (Bioquell, Hany UK)
C-28 Cytospin (Boeco, Hamburg Germany)
PK 131 R multispeed refrigerated centrifuge (ALC, Porta Italy)
Techne genius PCR (Techne, Cambridge UK)
Hoefer miniVE vertical electrophoresis system (Amersham Pharmacia biotech, Buckinghamshire UK)
Hoefer TE77 Semi dry transfer units (Amersham Pharmacia biotech, Buckinghamshire UK)
BA Jouan Centrifuge (DJB Labcare, Buckingham UK)
Balance Oertling HC22 (Oertling, Yorkshire UK)
Vortex genie 2 (Fisher Scientific, Leicestershire UK)
UV Transluminator Gene genius Bio-imaging systems (Syngene, Cambridge UK)

Primer Design

MCM2, 5 and 7 primers were identified and produced by reading the cDNA sequence of the MCMs of interest, these were produced and supplied by Simone Degan while the β -Actin primers were supplied by Tracey Bailey.

Forward Primer MCM2 2F: 5'-TAT- GTC- CAG- CGG- CAC- CCT- GTC-A-3'
Reverse Primer MCM2 2R: 5'-GGT- GGA- CCC- TCT- CCT- TGG- CGT- A-3'

Forward Primer MCM5 5F: 5'-ATC-GCC-AAG-GCT-GGG-ATC-ACC-A-3'
Reverse Primer MCM5 5R: 5'-CCG-CAG-GGC-CTC-CTC-CAC-A-3'

Forward Primer MCM7 7F: 5'-TCC-CAG-CCC-CAA-GGG-TCT-AGG-A-3'
Reverse Primer MCM7 7R: 5'-GGT-GGA-CTG-TGG-CCG-GCC-AA-3'

Forward Primer β -Actin BAA: 5'- TCA-CGG-GGT-CAC-CCA-CAC-TGT-3'
Reverse Primer β -Actin BAS: 5'- CTA-GAA-GCA-TTT-GCG-GTG-GAC-3'

PCR cycle for MCM2, 5, 7.

Initiate denaturation 95°C for 2 minutes

NO HOT START

35 Cycles of: 94°C for 1 minute
61°C for 1 minute
72°C for 2 minutes

1 Cycle of: 94°C for 1 minute
61°C for 1 minute

FINAL EXTENSION 72°C for 5 minutes

PCR cycle for β -Actin.

Initiate denaturation 95°C for 4 minutes

NO HOT START

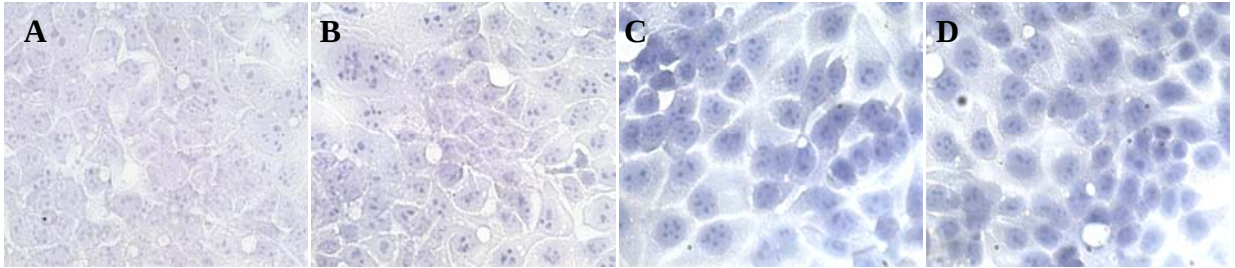
35 Cycles of: 94°C for 45 seconds
54°C for 30 seconds
72°C for 1.30 minutes

1 Cycle of: 94°C for 45 seconds
54°C for 30 seconds

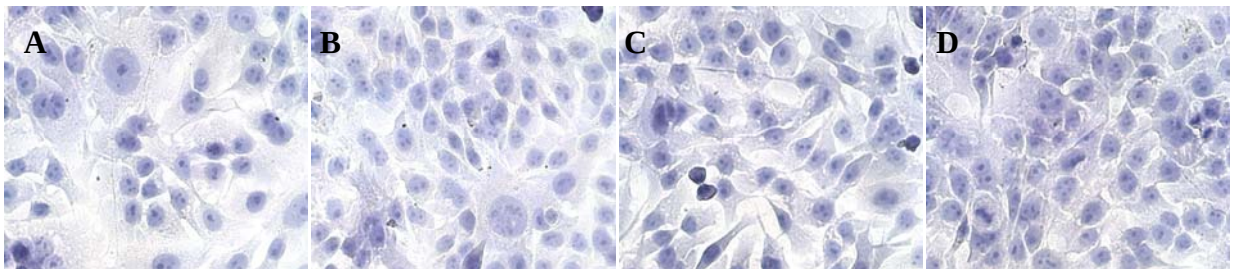
FINAL EXTENSION 72°C for 5 minutes

Appendix C

OE33



OE21



NCL H23

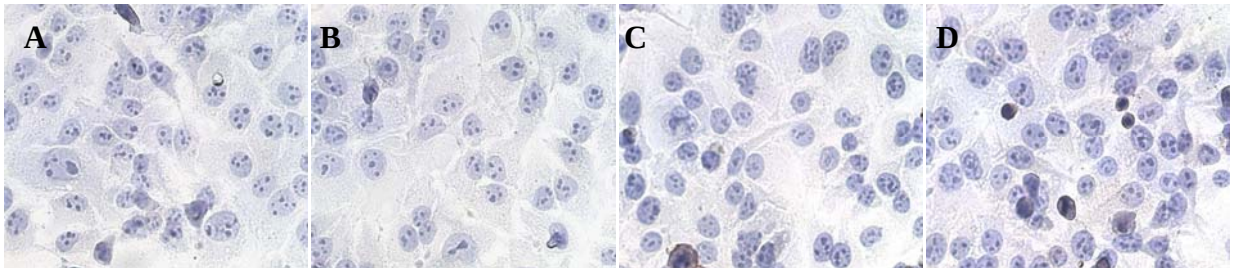


Figure c.1. ICC results for three cell lines tested using antibodies to PSA. A = Negative Control, No Monoclonal Antibodies to PSA applied, B = Positive Control, Monoclonal Antibodies to PSA applied, C = Negative Control, No Polyclonal Antibodies to PSA applied, D = Positive Control, Polyclonal Antibodies to PSA applied. No positive staining visualised as no brown precipitation. Magnification x400

Table c.1. Cellular proliferation rates for six different human cell lines cultured in 2x 12 well plates with normal medium over a maximum of 12 days. The starting seeding density was 2×10^4 and 2 wells from the 12 well plate were trypsinised and counted for each cell line every 2 days. All cell numbers recorded on the table are to $\times 10^4$.

2 Days				4 Days		
Cell No.	Well 1	Well 2	Mean	Well 1	Well 2	Mean
OE21	2.6	4.4	3.5	5.9	4.8	5.4
PNT1A	3.6	3.2	3.4	4.8	4.1	4.5
PC-3	3.8	5.6	4.7	6.2	3.9	5.1
RT112	6.6	3.8	5.2	5.1	5.9	5.5
CaCO2	2.6	2.8	2.7	6.5	4.4	5.5
PE/PJ41	2.6	3.4	3	7.4	3.6	5.5
6 Days				8 Days		
Cell No.	Well 1	Well 2	Mean	Well 1	Well 2	Mean
OE21	24.5	16.6	20.1	41.6	36.6	59.9
PNT1A	9.2	8.9	9.1	12.6	12	12.3
PC-3	11.7	9.3	10.5	25.6	26	25.8
RT112	37.5	42.1	39.8	45.6	57	51.3
CaCO2	5.4	8	6.7	41.1	49.9	45.5
PE/PJ41	7.4	8.4	7.9	29.3	29.5	29.4
10 Days				12 Days		
Cell No.	Well 1	Well 2	Mean	Well 1	Well 2	Mean
OE21	60.6	70.1	65.5			
PNT1A	29	29.8	29.4	31.1	41.6	36.4
PC-3	57.1	59.3	58.2	60	63.5	61.8
RT112	161	165	163			
CaCO2	41.2	47.2	44.2			
PE/PJ41	31.1	41.3	36.2	45	50.5	47.8

Table c.2. Mean proliferation rates for the six human cell lines cultured in 12 well plates in normal medium for a maximum of 12 days. All cell numbers recorded in the table are to $\times 10^4$.

Cell line	Days						
	0	2	4	6	8	10	12
OE21	2	3.5	5.4	20.1	59.9	65.5	
PNT1A	2	3.4	4.5	9.1	12.3	29.4	36.4
PC-3	2	4.7	5.1	10.5	25.8	58.2	61.8
RT112	2	5.2	5.5	39.8	51.3	163	
CaCO2	2	2.7	5.5	6.7	45.5	44.2	

Table c.3. PSA ELISA results obtained for the six human cell lines.

Absorbency reading obtained for:

Cell Lysate = protein extraction from cells (4mg/ml of protein lysate loaded for each sample).

Media = Absorbency reading for FCS free medium not grown with cells.

24 hours= Medium sample collected and concentrated after 24 hours growth with cells in FCS free media.

48 hours = Medium sample collected and concentrated after 48 hours growth with cells in FCS free media.

72 hours = Medium sample collected and concentrated after 72 hours growth with cells in FCS free media.

Standard Conc.= Standard PSA concentration ng/ml,

Standard Reading = Standards absorbency reading obtained for the different PSA standards

Absorbency Reading Obtained for PSA ELISA (nm)

Cell Line	Cell Lysate	Media (No FCS)	24 hours	48 hours	72 hours
PNT1A	0.0470	0.042	0.0500	0.0490	0.0500
PC-3	0.0520	0.060	0.0510	0.0480	0.0480
OE21	0.0540	0.042	0.0480	0.0510	0.0530
CaCO2	0.0540	0.060	0.0520	0.0480	0.0550
RT112	0.0540	0.060	0.0560	0.0450	0.0520
PE/PJ41	0.0500	0.060	0.0520	0.0580	0.0520

Standard Conc.	Standard Reading
0.0	0.052
1.0	0.119
2.5	0.176
5.0	0.400
10.0	0.492
25.0	0.933

Appendix D

Table d.1. Immunocytochemistry results obtained using antibodies to MCM2, 5, 7 at different confluence levels. The slides had the number of cells stained assessed by the scoring system given in *Table 3.1*. While approximate confluence is estimated using the Chark-ley point method (for > 100% - cells were grown after 100% confluence for a further 2 days *Section 3.2.2 and 3.2.4*).

Confluence PNT1A	MCM2	MCM5	MCM7	Confluence PC-3	MCM2	MCM5	MCM7
28	3	3	3	28	2	3	2
36	3	3	3	36	2	3	2
48	3	3	3	40	2	3	2
52	3	3	3	56	2	3	2
72	3	3	3	72	2	3	2
76	3	3	3	72	2	3	2
88	3	3	3	96	2	3	3
96	3	3	3	96	2	3	3
> 100	3	3	3	> 100	2	3	3
> 100	3	3	3	> 100	2	3	3

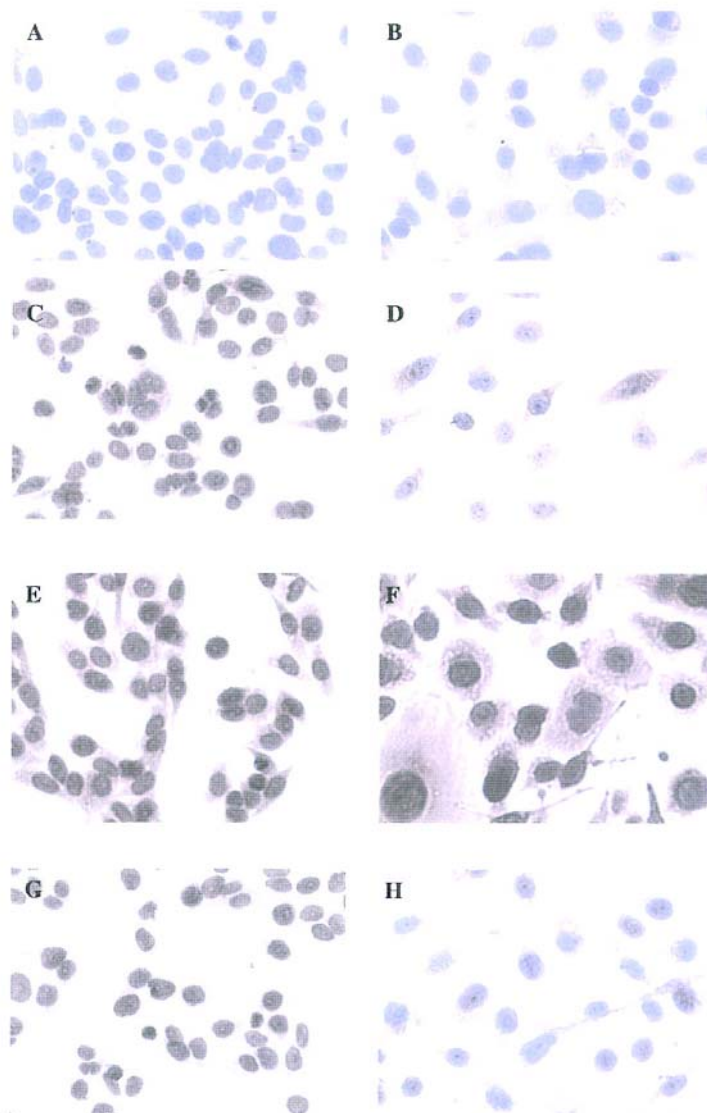


Figure d.1. Immunocytochemistry results for PNT1A and PC-3 at approximately 30% confluence (positive cells visualised by brown precipitate) . A = PNT1A No Antibody to MCM2, 5, 7 applied, Negative Control, B = PC-3 No Antibody to MCM2, MCM5, 7 applied, Negative Control, C = PNT1A MCM2 Antibody applied, D = PC-3 MCM2 Antibody applied, E = PNT1A MCM5 Antibody applied, F = PC-3 MCM5 Antibody applied, G = PNT1A MCM7 Antibody applied, H = PC-3 MCM7 Antibody applied. Magnification x400.

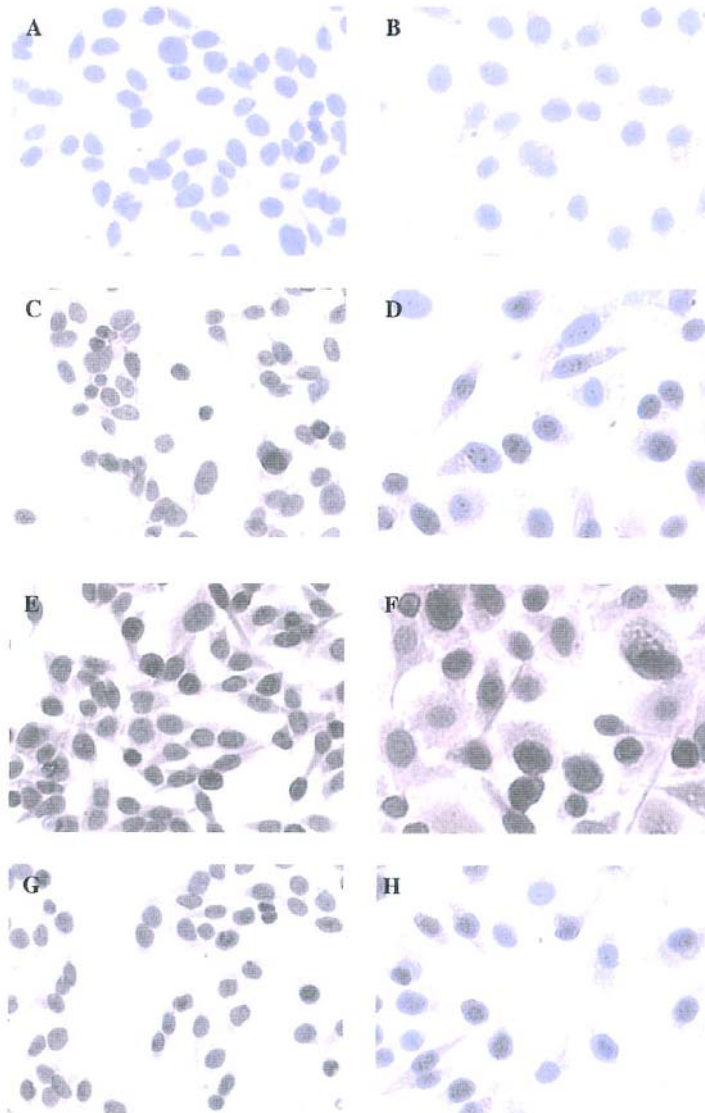


Figure d.2. Immunocytochemistry results for PNT1A and PC-3 at approximately 50% confluence (positive cells visualised by brown precipitate). A = PNT1A No Antibody to MCM2, 5, 7 applied, Negative Control, B = PC-3 No antibody to MCM2, 5, 7 applied, Negative Control, C = PNT1A MCM2 Antibody applied, D = PC-3 MCM2 Antibody applied, E = PNT1A MCM5 Antibody applied, F = PC-3 MCM5 Antibody applied, G = PNT1A MCM7 Antibody applied, H = PC-3 MCM7 Antibody applied. Magnification x400.

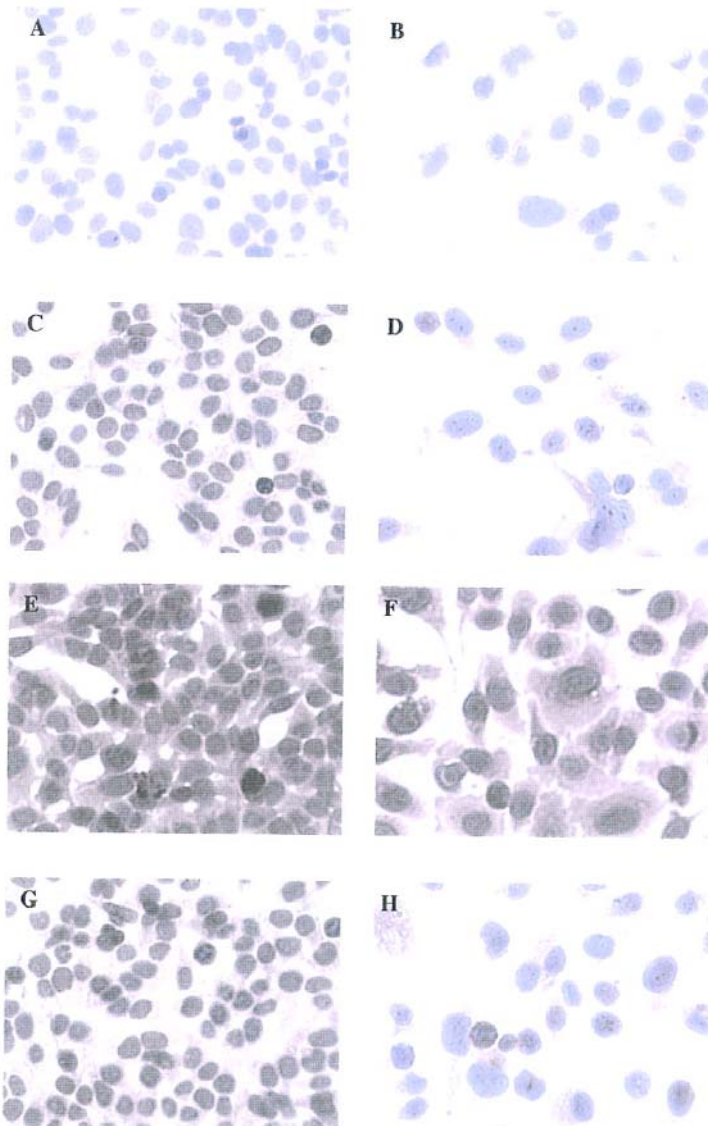


Figure d.3. Immunocytochemistry results for PNT1A and PC-3 at approximately 70% confluence (positive cells visualised by brown precipitate). A = PNT1A No Antibody to MCM2, 5, 7 applied, Negative Control, B = PC-3 No Antibody to MCM2, 5, 7 applied, Negative Control, C = PNT1A MCM2 Antibody applied, D = PC-3 MCM2 Antibody applied, E = PNT1A MCM5 Antibody applied, F = PC-3 MCM5 Antibody applied, G = PNT1A MCM7 Antibody applied, H = PC-3 MCM7 Antibody applied. Magnification x400.

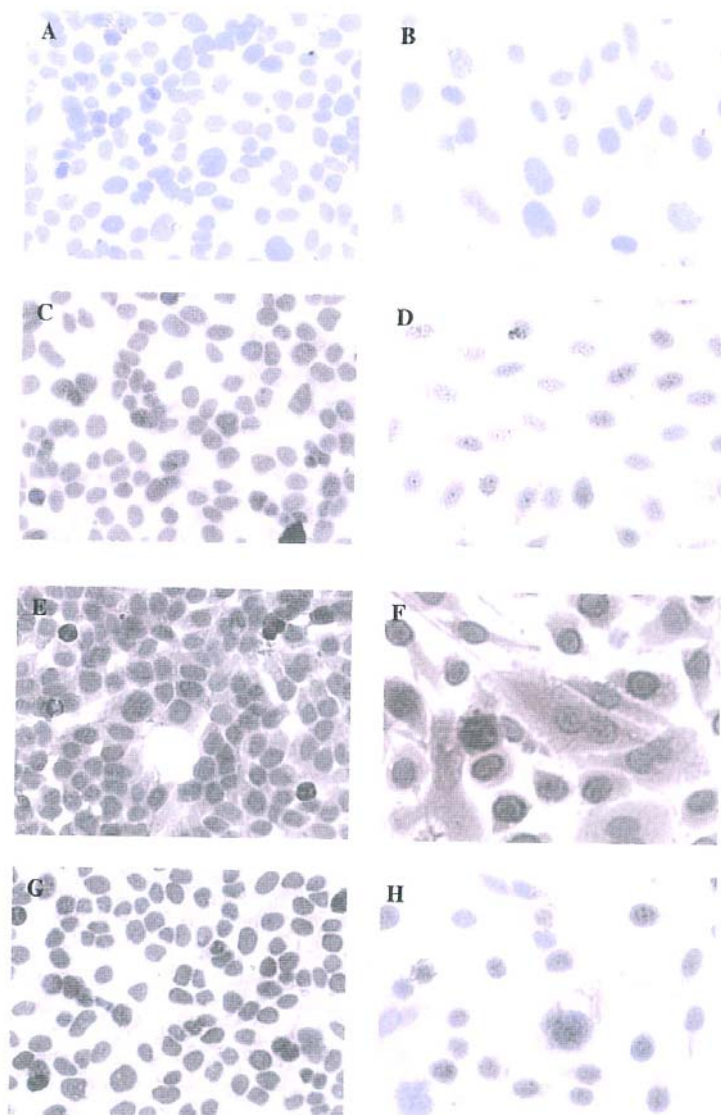


Figure d.4. Immunocytochemistry results for PNT1A and PC-3 at approximately 100% confluence (positive cells visualised by brown precipitate). A = PNT1A No Antibody to MCM2, 5, 7 applied, Negative Control, B = PC-3 No Antibody to MCM2, 5, 7 applied, Negative Control, C = PNT1A MCM2 Antibody applied, D = PC-3 MCM2 Antibody applied, E = PNT1A MCM5 Antibody applied, F = PC-3 MCM5 Antibody applied, G = PNT1A MCM7 Antibody applied, H = PC-3 MCM7 Antibody applied. Magnification x400.

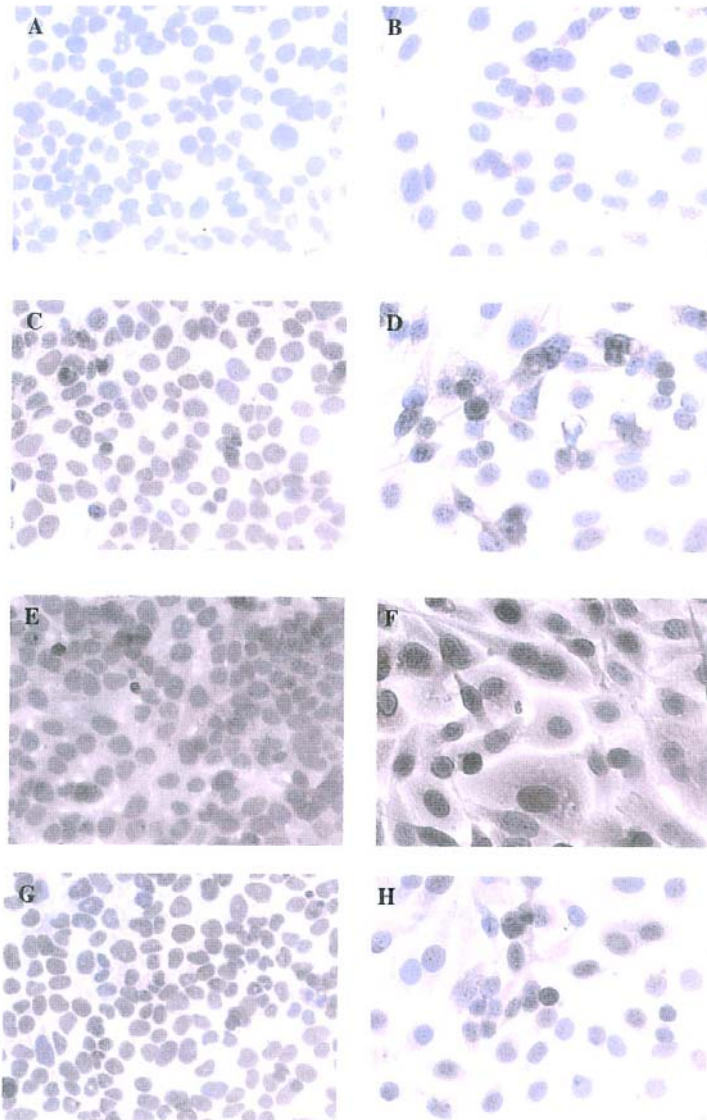


Figure d.5. Immunocytochemistry results for PNT1A and PC-3 at approximately >100% confluence (cells grown after 100% confluence for a further 2 days) (positive cells visualised by brown precipitate). A = PNT1A No Antibody to MCM2, 5, 7 applied, Negative Control, B = PC-3 No antibody to MCM2, 5, 7 applied, Negative Control, C = PNT1A MCM2 Antibody applied, D = PC-3 MCM2 Antibody applied, E = PNT1A MCM5 Antibody applied, F = PC-3 MCM5 Antibody applied, G = PNT1A MCM7 Antibody applied, H = PC-3 MCM7 Antibody applied. Magnification x400.

Table d.2. The raw data obtained for PNT1A and PC-3 when viability at different confluence was tested. Each confluence for each cell line had it viability tested twice and the method used is shown in *Appendix A Section A.3* (Confluence calculated by performing a cell count *Appendix A Section A.3*).

PNT1A Confluence	Viability	Trypan Blue (% death)	PC-3 Confluence	Viability	Trypan Blue (% death)
30	98	2	26	85	15
31	93	7	28	94	6
50	96	4	52	88	12
53	92	4	53	85	15
69	96	4	67	94	6
69	87	13	67	85	15
100	96	4	100	93	7
100	96	4	100	90	10
155	97	3	136	70	30
162	93	7	197	70	30

Table d.3. The raw data obtained when immunocytochemistry was performed on antibodies to Ki67. The Chark-ley point method (*Section 3.2.4*) was used to assess the data and to calculate percent confluence. Each confluence level was assessed twice for each cell line.

Confluence PNT1A	% of Ki67 Staining	Confluence PC-3	% of Ki67 Staining
28	84	28	72
36	84	36	56
48	88	40	52
48	88	56	36
72	72	72	16
76	48	72	16
88	36	96	16
96	36	100	8
> 100	72	> 100	44
> 100	72	> 100	36

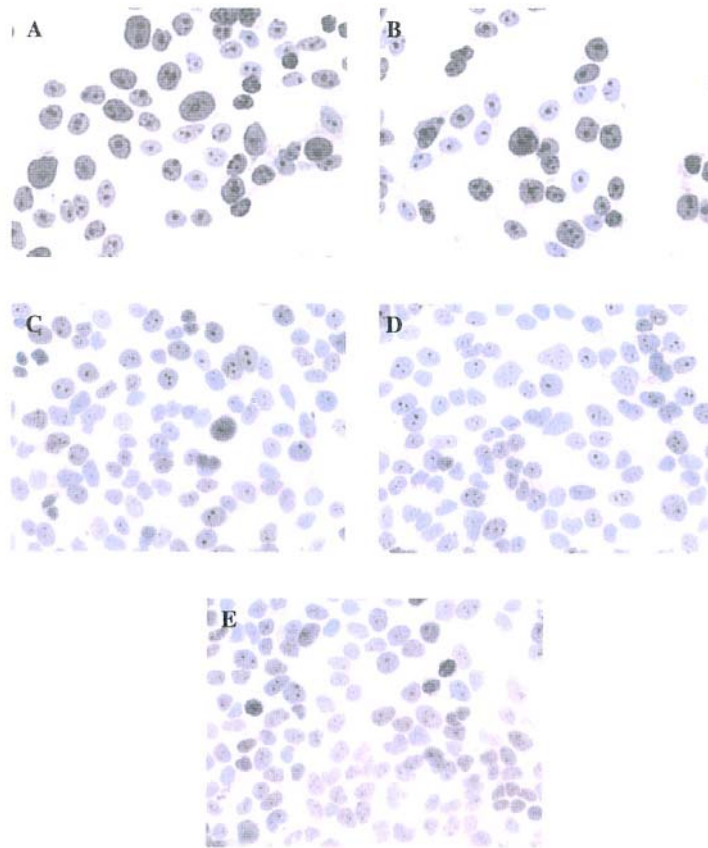


Figure d.6. Immunocytochemistry results for PNT1A at different confluence level (positive cells visualised by brown precipitate). A = 30% Confluence Positive Ki67, B = 50% Confluence Positive Ki67, C = 70% Confluence Positive Ki67, D = 100% Confluence Positive Ki67, E = More than 100% Confluence Ki67. All these had antibody to Ki67 applied. Negative Controls (No antibody to Ki67 applied) for 30% Confluence d.1A, for 50% Figure d.2A, for 70% Confluence Figure d.3A, for 100% Figure d.4A and for more than 100% Confluence Figure d.5A. Magnification x400.

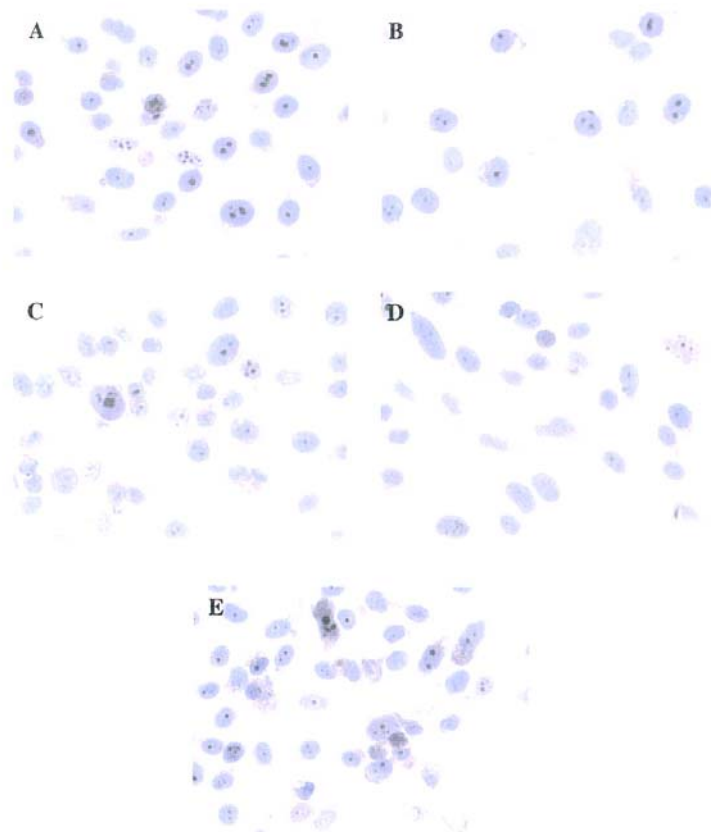


Figure d.7. Immunocytochemistry results for PC-3 at different confluence level (positive cells visualised by brown precipitate). A = 30% Confluence Positive Ki67, B = 50% Confluence Positive Ki67, C = 70% Confluence Positive Ki67, D = 100% Confluence Positive Ki67, E = More than 100% Confluence Ki67. All these had Antibody to Ki67 applied. Negative Controls (No Antibody to Ki67 applied) for 30% Confluence d.1B, for 50% Figure d.2B, for 70% Confluence Figure d.3B, for 100% Figure d.4B and for more than 100% Confluence Figure d.5B. Magnification x 400.

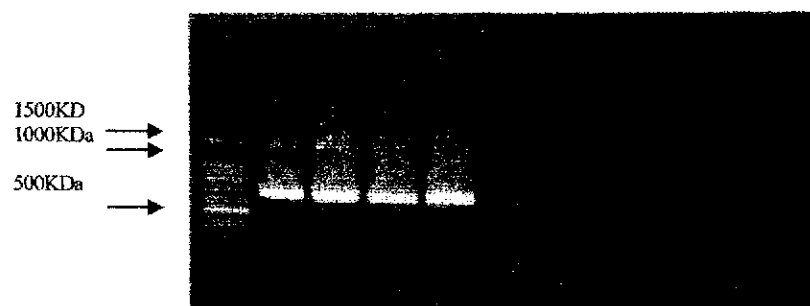


Figure d.8. Ethidium bromide gel showing the RT-PCR results for β -Actin. L= Ladder, 1= RT-PCR on 200 μ g/ μ l RNA from PNT1A cell line using Micro-Fast Track™2.0 Kit, 2 = RT-PCR on 200 μ g/ μ l RNA from PC-3 using Micro-Fast Track™2.0 Kit, 3= RT-PCR on 200 μ g/ μ l RNA from PNT1A using RNeasy® Mini columns, 4= RT-PCR on 200 μ g/ μ l RNA from PC-3 using RNeasy® Mini columns, 5= PCR on 200 μ g/ μ l RNA from PNT1A no RT using Micro-Fast Track™2.0 Kit , 6 = PCR on 200 μ g/ μ l RNA from PC-3 no RT using Micro-Fast Track™2.0 Kit, 7= PCR on 200 μ g/ μ l RNA from PNT1A no RT using RNeasy® Mini columns, 8 = PCR on 200 μ g/ μ l RNA from PC-3 no RT using RNeasy® Mini columns, 9 = Negative Control No RNA Loaded.

Table d.4. Raw data obtained when RT-PCR is performed at different cycle numbers for MCM2 on 200 μ g/ μ l of RNA from 30% confluence PNT1A cells. The experiment was repeated 3 times (Results1 – 3) and then the average relative brightness (rbu) was calculated.

30% PNT1A No. of cycles	MCM2 Raw Volume			
	Results 1	Results 2	Results 3	Average
15	0.00	0.00	0.00	0.00
20	778.67	0.00	422.69	660.01
22	716.86	972.83	1016.73	902.14
24	2963.48	-	2869.76	2916.62
26	4005.92	3804.25	4387.48	4065.88
28	5577.26	4827.01	5941.95	5448.74
30	6140.28	6006.60	7519.31	6555.40
32	5902.83	6459.83	7418.58	6553.75
35	7578.37	6145.93	5994.34	6572.88
40 (final)	5846.14	6200.83	6531.18	6152.72

Table d.5. Raw data obtained when RT-PCR is performed at different cycle numbers for MCM5 on 200µg/µl of RNA from 30% confluence PNT1A cells. The experiment was repeated 3 times (Results1 – 3) and then the average relative brightness (rbu) was calculated.

30% PNT1A No. of cycles	MCM5 Raw Volume			
	Results 1	Results 2	Results 3	Average
15	0.00	0.00	0.00	0.00
20	0.00	0.00	484.37	161.46
22		1215.77	1071.76	1751.65
24	2733.99	2204.94	2735.21	2558.05
26		3218.91	4572.95	5895.93
28		3721.41	6161.72	4541.54
30	5481.95	5271.71	6915.79	5869.82
32		6009.43	8445.54	7227.49
35	5810.41	6804.42	5933.51	6182.78
40 (final)	6060.35	5226.19	6340.01	5875.52

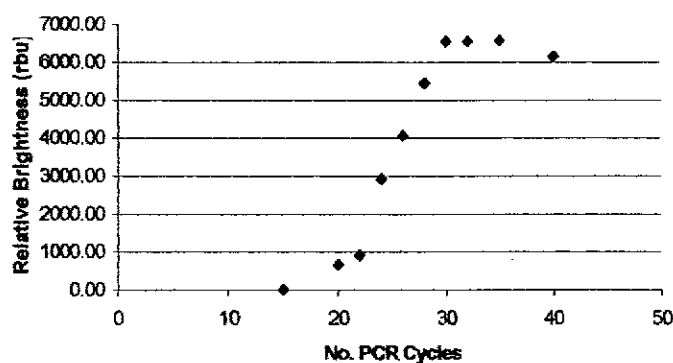
Table d.6. Raw data obtained when RT-PCR is performed at different cycle numbers for MCM7 on 200µg/µl of RNA from 30% confluence PNT1A cells. The experiment was repeated 3 times (Results1 – 3) and then the average relative brightness (rbu) was calculated.

30% PNT1A No. of cycles	MCM7 Raw Volume			
	Results 1	Results 2	Results 3	Average
15	0.00	0.00	0.00	0.00
20	0.00	0.00	0.00	0.00
22	0.00	0.00	0.00	0.00
24	0.00	0.00	0.00	0.00
26	0.00	0.00	0.00	0.00
28	0.00	0.00	0.00	0.00
30	0.00	0.00	0.00	0.00
32	0.00	0.00	1480.60	493.53
35	437.47	1918.53	1745.73	1367.24
37	841.46	8254.54	5353.70	4816.57
40	Band Disappears			

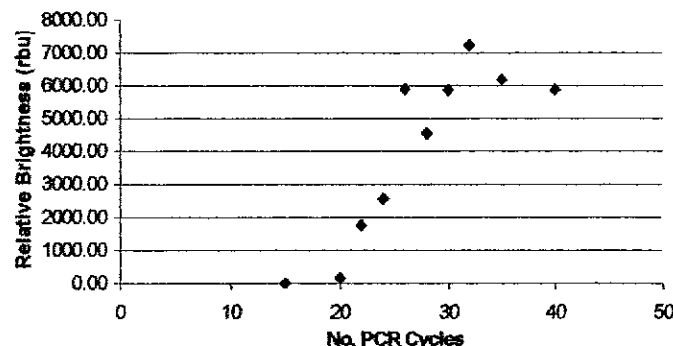
Table d.7. Mean RT-PCR relative brightness Reading (rbu) obtained for MCM2, 5 and 7 when different cycle numbers were tested on 200µg/µl of RNA from 30% confluence PNT1A cells.

No.Cycles	MCM2	MCM5	MCM7
15	0	0	0
20	660.01	161.46	0
22	902.14	1751.65	0
24	2916.62	2558.05	0
26	4065.88	5895.93	0
28	5448.74	4541.54	0
30	6555.4	5869.82	0
32	6553.75	7227.49	493.53
35	6572.88	6182.78	1367.24
37	-	-	4816.57
40 (Final)	6152.72	5875.52	Band disappears

A



B



C

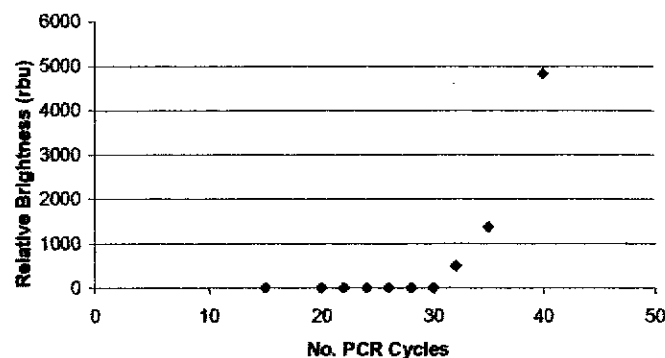


Figure d.9. A line graph of the PCR cycle number against mean relative brightness (rbu) readings for A - MCM2, B - MCM5, C - MCM7.

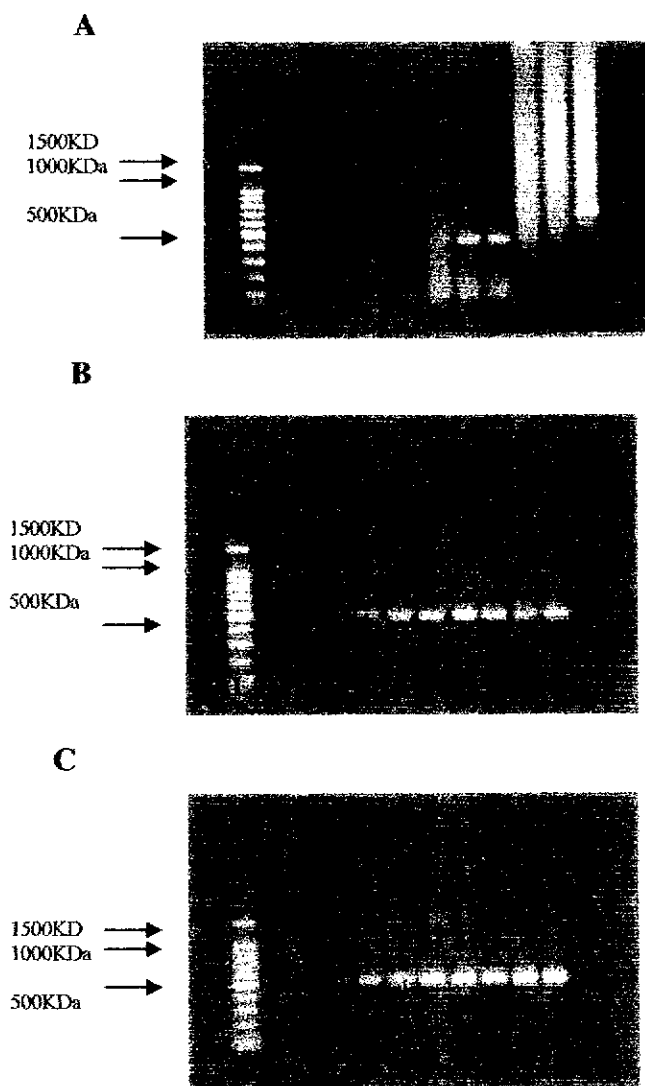


Figure d.10. A – Ethidium bromide gel showing the RT-PCR results obtained for MCM2 (500bp bands visible) using 200µg/µl of cDNA from PNT1A cells at different cycle numbers where L = Ladder, 1 = 15 Cycles, 2 = 20 Cycles, 3 = 22 Cycles, 4 = 24 Cycles, 5 = 26 Cycles, 6 = 28 Cycles, 7 = 30 Cycles, 8 = 32 Cycles, 9 = 35 Cycles, 10 = 40 Cycles, 11 = No cDNA .

B– Ethidium bromide gel showing the RT-PCR results obtained for MCM5 (500bp bands visible) using 200µg/µl of cDNA from PNT1A cells at different cycle numbers where L = Ladder, 1 = 15 Cycles, 2 = 20 Cycles, 3 = 22 Cycles, 4 = 24 Cycles, 5 = 26 Cycles, 6 = 28 Cycles, 7 = 30 Cycles, 8 = 32 Cycles, 9 = 35 Cycles, 10 = 40 Cycles, 11 = No cDNA .

C – Ethidium bromide gel showing the RT-PCR results obtained for MCM7 (407bp bands visible) using 200µg/µl of cDNA from PNT1A cells at different cycle numbers where L = Ladder, 1 = 15 Cycles, 2 = 20 Cycles, 3 = 25 Cycles, 4 = 27 Cycles, 5 = 30 Cycles, 6 = 32 Cycles, 7 = 35 Cycles, 8 = 37 Cycles, 9 = 40 Cycles, 10 = No cDNA, 11 = β-actin Positive for 200µg/µl of cDNA from PNT1A cells.

Table d.8. RT-PCR results for MCM2 on 200µg/µl of RNA extracted from PNT1A and PC-3 cells. RNA was extracted from two T75 flasks for each confluence level (Table A and Table B) and then RT-PCR was performed three times and the relative brightness (rbu) was assessed (Result 1 to Result 3).

Table A

PNT1A	Results1	Results2	Results3	Average	PC-3	Results1	Results2	Results3	Average
25	3156.38	3194.63	2680.91	3010.64	20	1606.34	1742.39	1080.89	1476.54
53	5715.78	3590.16	3181.21	4162.383	51	1837.63	2580.44	1182.4	1866.823
69	1886.99	2101.43	840.8	1609.74	77	2669.8	1863.58	934.28	1822.553
100	4510.99	4450.72	3557.84	4173.183	100	0	986.56	0	328.8533

Table B

PNT1A	Results1	Results2	Results3	Average	PC-3	Results1	Results2	Results3	Average
35	4194.07	5138.72	4360.44	4564.41	34	3118.00	3438.85	1352.03	2636.293
54	5633.14	5266.84	5287.11	5395.697	58	3210.67	3393.31	1994.37	2866.117
69	2417.41	2689.55	1860.81	2322.59	75	3068.36	2677.59	1172.9	2306.283
100	4196.2	4430.88	3277.19	3968.09	100	1574.77	1999.5	711.67	1428.647
139	4154.62	3342.12	1431.19	2975.977	136	881.69	1579.59	705.17	1055.483
162	4124.13	5236.07	3710.3	4356.833	197	744.61	1469.75	557.21	923.8567

Table d.9. RT-PCR results for MCM5 on 200µg/µl of RNA extracted from PNT1A and PC-3 cells. RNA was extracted from two T75 flasks for each confluence level (Table A and Table B) and then RT-PCR was performed three times and the relative brightness (rbu) was assessed (Result 1 to Result 3).

Table A

PNT1A	Results1	Results2	Results3	Average	PC-3	Results1	Results2	Results3	Average
25	3293.61	3963.1	3542.38	3599.697	28	1436.6	3693.7	1377.31	2169.203
53	4329.7	5644.7	4321.39	4765.263	51	1783.21	3915.05	2288.2	2662.153
69	1957.06	3041.44	1218.03	2072.177	77	2300.71	3013.99	1918.92	2411.207
100	3869.81	4435.75	3572.67	3959.41	100	0.00	1047.85	223.11	423.6533

Table B

PNT1A	Results1	Results2	Results 3	Average	PC-3	Results1	Results2	Results3	Average
35	4765.14	4746.35	2749.36	4086.95	34	3015.72	5024.73	1304.7	3115.05
45	5101.19	6775.19	3269.92	5048.767	58	3149.47	5343.93	1517.99	3337.13
69	1871.58	3455.34	832.85	2053.257	75	2195.21	4501.59	1270.04	2655.613
100	4288.55	5314.49	1831.11	3811.383	100	1717.95	3454.75	593.88	1922.193
139	2144.04	4209.63	1148.8	2500.823	136	1019.32	3254.63	574.25	1616.067
162	4287.67	5565.71	2548.61	4133.997	197	0.00	2972.74	0.00	990.9133

Table d.10. RT-PCR results for MCM7 on 200µg/µl of RNA extracted from PNT1A and PC-3 cells. RNA was extracted from two T75 flasks for each confluence level (Table A and Table B) and then RT-PCR was performed three times and the relative brightness (rbu) was assessed (Result 1 to Result 3).

Table A

PNT1A	Results 1	Results 2	Average	PC-3	Results 1	Results 2	Average
25	8139.01	13279.92	10709.47	28	2159	8402.65	5280.83
53	8379.14	13307.56	10843.35	51	5258	13643.92	9450.96
69	2982.22	4308.84	3645.53	77	1626.24	2743.2	2184.72
100	0.00	0.00	0.00	100	1519.99	1746.9	1633.45

Table B

PNT1A	Results 1	Results 2	Average	PC-3	Results 1	Results 2	Average
35	1361	2042.35	1701.68	34	3572.16	4202.44	3887.3
45	819.94	1184.35	1002.15	58	6785.4	7547.37	7166.39
69	1140.45	1574.61	1357.53	75	5269.91	6050.69	5660.3
100	0.00	0.00	0.00	100	3884.77	4474.9	4179.84
139	642.93	1090.38	866.66	136	0.00	0.00	0.00
162	221.8	3060.9	1641.35	197	0.00	0.00	0.00

Table d.11. Average relative brightness (rbu) obtained when RT-PCR is performed for MCM2 on PNT1A and PC-3 cells at different confluence. Each confluence level was tested twice by growing 2 flasks per confluence (1,2) and each confluence level had RT-PCR repeated 3 times to produce an average (A,B). The total mean average and total mean confluence was then obtained by adding together (1,2) and (A,B) and then dividing by 2 (Raw data table d.8).

1) PNT1A Confluence	A) Average	2) PNT1A Confluence	B) Average	PNT1A total mean Confluence	Total mean Average
25	3010.64	35	4564.41	30	3787.53
53	4162.38	54	5395.70	54	4779.04
69	1609.74	69	2322.59	69	1966.17
100	4173.18	100	3968.09	100	4070.64
		139	2975.98	151	1487.99
		162	4356.83		
1) PC-3 Confluence	A) Average	2) PC-3 Confluence	B) Average	PC-3 total mean Confluence	Total mean Average
20	1476.54	34	2636.29	27	2056.42
53	1866.82	58	2866.12	56	2366.42
69	1822.55	75	2306.28	72	2064.42
100	328.85	100	1428.65	100	878.75
		134	1055.48	166	527.74
		197	923.86		

Table d.12. Average relative brightness (rbu) obtained when RT-PCR is performed for MCM5 on PNT1A and PC-3 cells at different confluence. Each confluence level was tested twice by growing 2 flasks per confluence (1,2) and each confluence level had RT-PCR repeated 3 times to produce an average (A,B). The total mean average and total mean confluence was then obtained by adding together (1,2) and (A,B) and then dividing by 2 (Raw data *table d.9*).

1) PNT1A Confluence	A) Average	2) PNT1A Confluence	B) Average	PNT1A total mean Confluence	Total mean Average
25	3599.70	35	4086.95	30	3843.32
53	4765.26	54	5048.77	54	4907.02
69	2072.18	69	2053.26	69	2062.72
100	3959.41	100	3811.383	100	3885.40
		139	2500.823	151	3317.41
		162	4134.00		
1) PC-3 Confluence	A) Average	2) PC-3 Confluence	B) Average	PC-3 total mean Confluence	Total mean Average
20	2169.20	34	3115.05	27	2642.13
53	2662.153	58	3337.13	56	2999.64
69	2411.207	75	2655.61	72	2533.41
100	423.65	100	1922.19	100	1172.92
		134	1616.07	166	1303.49
		197	990.91		

Table d.13. Average relative brightness (rbu) obtained when RT-PCR is performed for MCM7 on PNT1A and PC-3 cells at different confluence. Each confluence level was tested twice by growing 2 flasks per confluence (1,2) and each confluence level had RT-PCR repeated 2 times to produce an average (A,B). The total mean average and total mean confluence was then obtained by adding together (1,2) and (A,B) and then dividing by 2 (*Raw data table d.10*).

1) PNT1A Confluence	A) Average	2) PNT1A Confluence	B) Average	PNT1A total mean Confluence	Total mean Average
25	10709.47	35	1701.68	30	6205.58
53	10843.35	54	1002.15	54	5922.75
69	3645.53	69	1357.53	69	2501.53
100	0	100	0	100	0
		139	866.66	151	1254.01
		162	1641.35		
1) PC-3 Confluence	A) Average	2) PC-3 Confluence	B) Average	PC-3 total mean Confluence	Total mean Average
20	5280.83	34	3887.30	27	4584.07
53	9450.96	58	7166.39	56	8308.68
69	2184.72	75	5660.30	72	3922.51
100	1633.45	100	4179.84	100	2906.65
		134	0	166	0
		197	0		

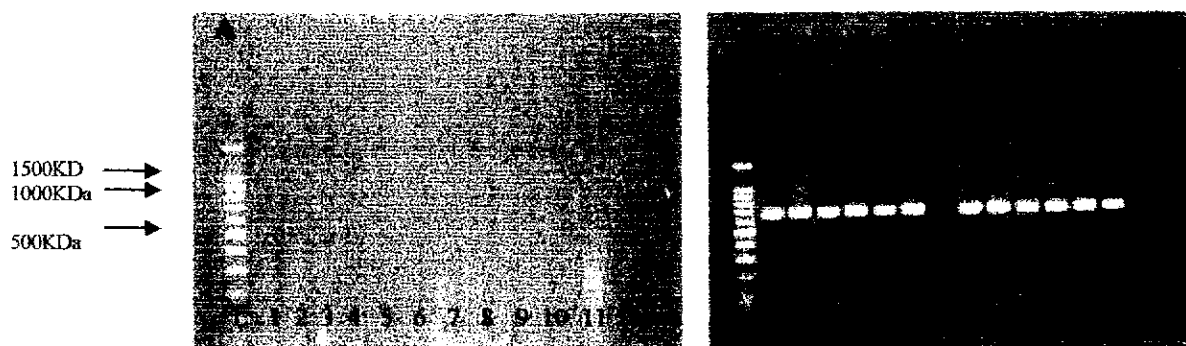


Figure d.11. Ethidium bromide stained gels showing the RT-PCR results when 200 μ g/ μ l of RNA was loaded from different confluence levels for PNT1A and PC-3. d.11A show negative No reverse transcription for PNT1A and PC-3, while d.11B shows the RT-PCR results for β -Actin (680bp band visible) when 200 μ g/ μ l of each sample is loaded. Ladder, 1 = PNT1A cells at 35% confluence, 2 = PNT1A cells at 54% confluence, 3 = PNT1A cells at 69% confluence, 4 = PNT1A cells at 100% confluence, 5 = PNT1A cells at 139% confluence, 6 = PNT1A cells at 162% confluence, 7 = Negative Control 8 = PC-3 cells at 34% confluence, 9 = PC-3 cells at 58% confluence, 10 = PC-3 cells at 77% confluence, 11 PC-3 cells at 100% confluence, 12 = PC-3 cells at 136% confluence, 13 = PC-3 cells at 197% confluence.

Table d.14. Immunocytochemistry results obtained for antibodies to MCM2, 5, 7 and Ki67 at different synchronisation times. Two slides were stained and assessed for each synchronisation time (Table A and Table B) and for each cell line the number of cells stained were given a score using Table 3.1.

Table A

Cell Line	Incubation Time	Ki67	MCM2	MCM5	MCM7
PNT1A	48 Hrs	0	1	3	3
PNT1A	48 Hrs 8 Hrs	1	2	3	2
PNT1A	48 Hrs 24 Hrs	2	0	3	0
PNT1A	48 Hrs 30 Hrs	2	3	3	3
Cell Line	Incubation Time	Ki67	MCM2	MCM5	MCM7
PC-3	48 Hrs	2	2	3	2
PC-3	48 Hrs 8 Hrs	1	0	3	1
PC-3	48 Hrs 24 Hrs	2	2	3	2
PC-3	48 Hrs 30 Hrs	2	3	3	3

Table B

Cell Line	Incubation Time	Ki67	MCM2	MCM5	MCM7
PNT1A	48 Hrs	0	1	3	3
PNT1A	48 Hrs 8 Hrs	1	1	3	2
PNT1A	48 Hrs 24 Hrs	2	0	3	0
PNT1A	48 Hrs 30 Hrs	2	3	3	3
Cell Line	Incubation Time	Ki67	MCM2	MCM5	MCM7
PC-3	48 Hrs	2	2	3	2
PC-3	48 Hrs 8 Hrs	1	0	3	1
PC-3	48 Hrs 24 Hrs	2	2	3	2
PC-3	48 Hrs 30 Hrs	2	3	3	2

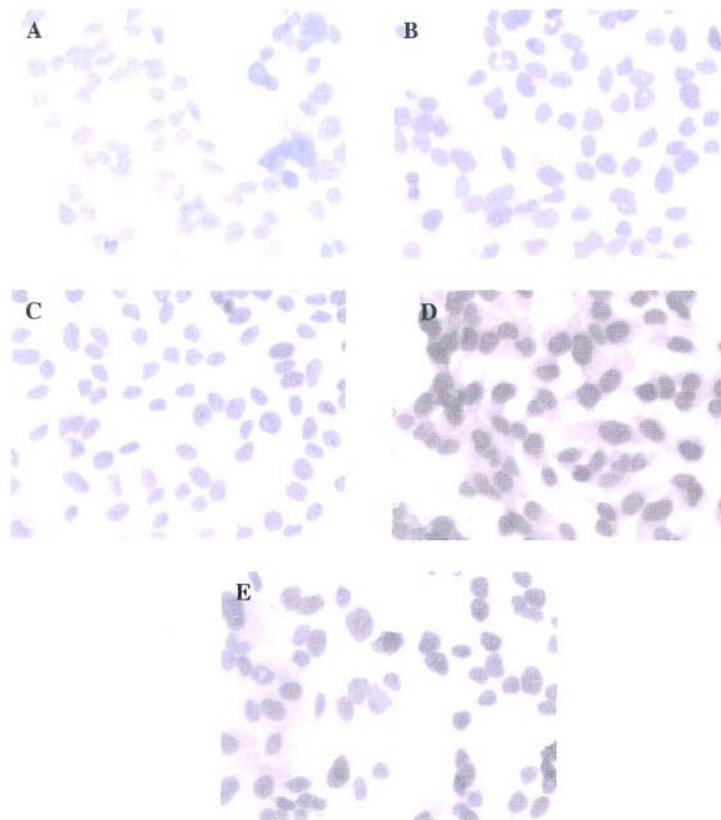


Figure d.12. Immunocytochemistry results for PNT1A at the synchronisation time of 48 hours No FCS (brown precipitate visualised indicates positive result). A = No Antibody to MCM2, 5, 7, Ki67 applied, Negative Control, B = Antibody to Ki67 applied, C = Antibody to MCM2 applied, D = Antibody to MCM5 applied, E = Antibody to MCM7 applied. Magnification x400.

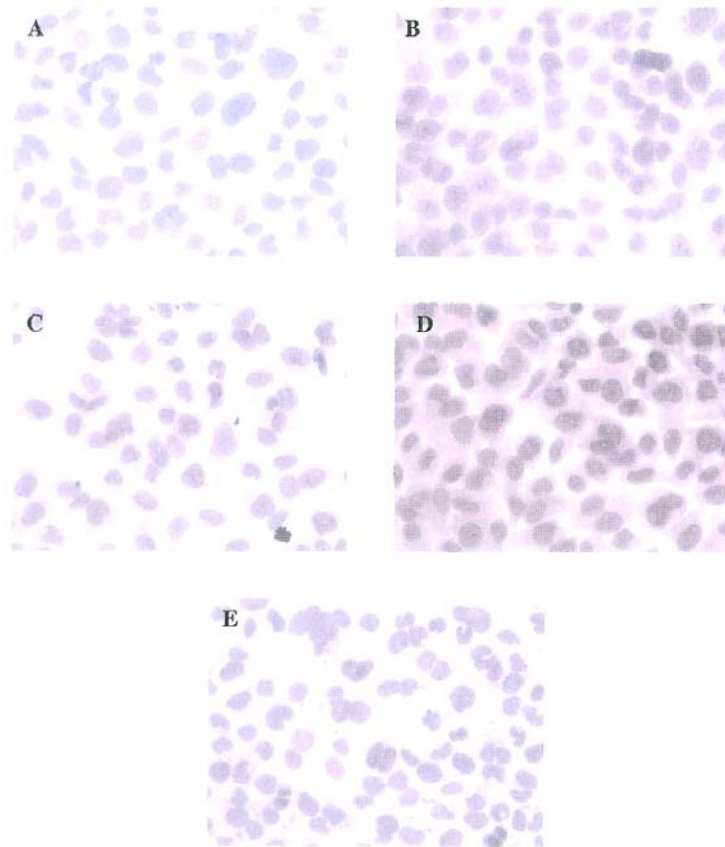


Figure d.13. Immunocytochemistry results for PNT1A cells at the synchronisation time of 48 hours No FCS then 8 hours normal media (brown precipitate visualised indicates positive result). A = No Antibody to MCM2, 5, 7 or Ki67 applied, Negative Control, B = Antibody to Ki67 applied, C = Antibody to MCM2 applied, D = Antibody to MCM5 applied, E = Antibody to MCM7 applied. Magnification x400.

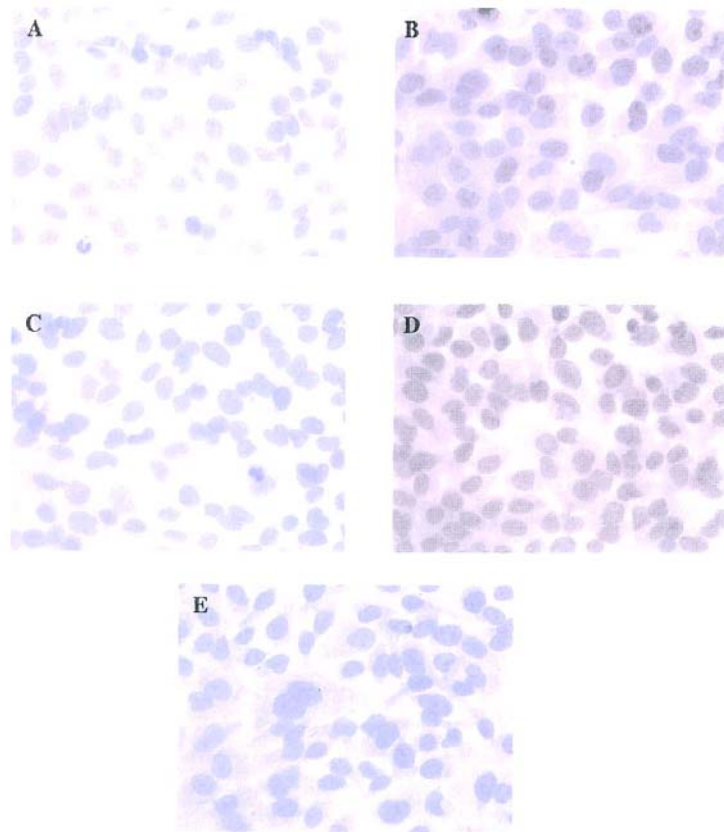


Figure d.14. Immunocytochemistry results for PNT1A cells at the synchronisation time of 48 hours No FCS then 24 hours normal media (brown precipitate visualised indicates positive result). A = No Antibody to MCM2, 5, 7 or Ki67 applied, Negative Control, B = Antibody to Ki67 applied, C = Antibody to MCM2 applied, D = Antibody to MCM5 applied, E = Antibody to MCM7 applied. Magnification x400.

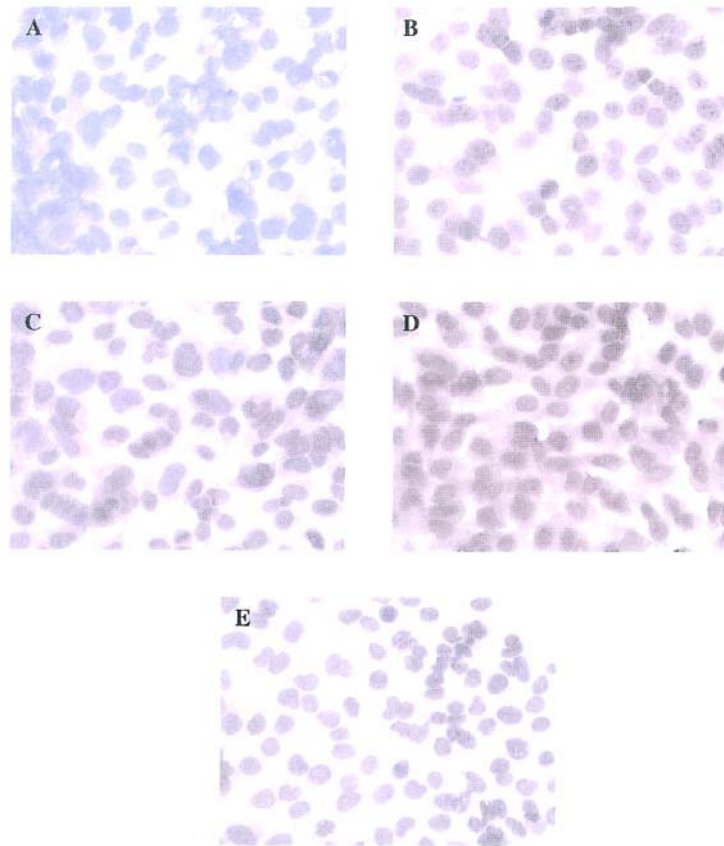


Figure d.15. Immunocytochemistry results for PNT1A cells at the synchronisation time of 48 hours No FCS then 30 hours normal media (brown precipitate visualised indicates positive result). A = No Antibody to MCM2, 5, 7 or Ki67 applied, Negative Control, B = Antibody to Ki67 applied, C = Antibody to MCM2 applied, D = Antibody to MCM5 applied, E = Antibody to MCM7 applied. Magnification x400.

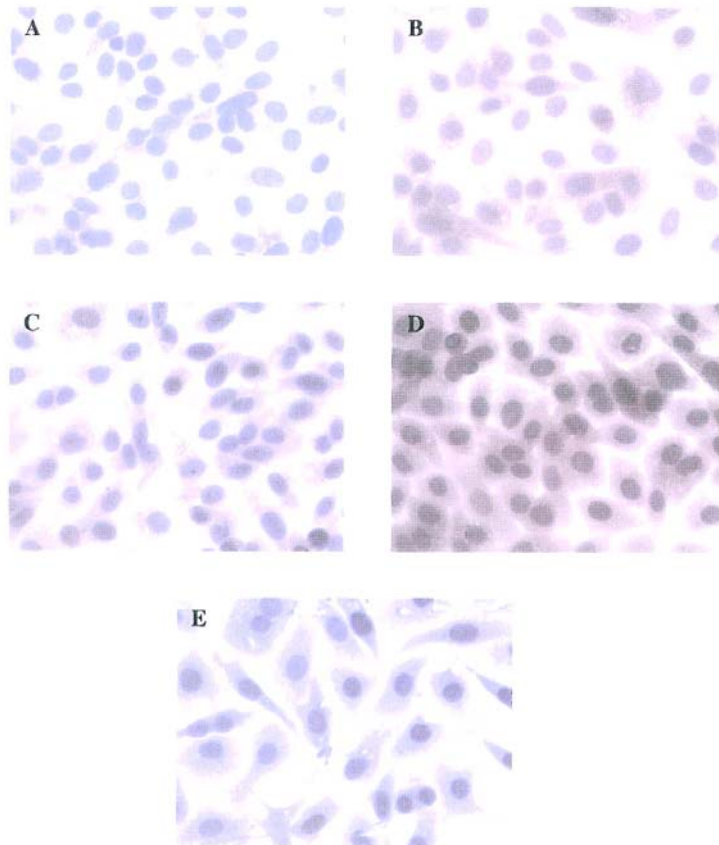


Figure d.16. Immunocytochemistry results for PC-3 cells at the synchronisation time of 48 hours No FCS (brown precipitate visualised indicates positive result). A = No Antibody to MCM2, 5, 7 or Ki67 applied, Negative Control, B = Antibody to Ki67 applied, C = Antibody to MCM2 applied, D = Antibody to MCM5 applied, E = Antibody to MCM7 applied. Magnification x400.

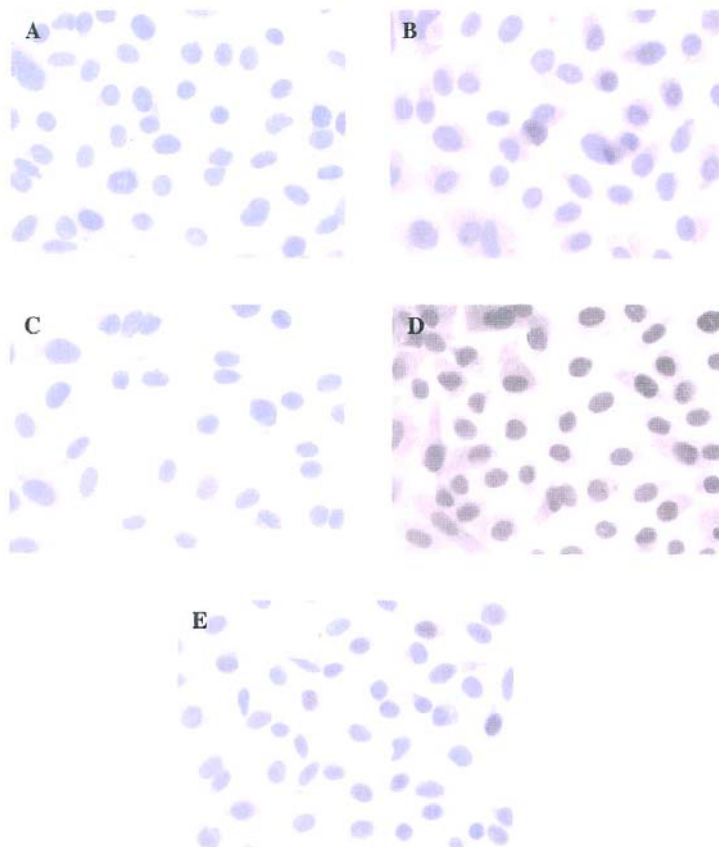


Figure d.17. Immunocytochemistry results for PC-3 cells at the synchronisation time of 48 hours No FCS then 8 hours with normal media (brown precipitate visualised indicates positive result). A = No Antibody to MCM2, 5, 7 or Ki67 applied, Negative Control, B = Antibody to Ki67 applied, C = Antibody to MCM2 applied, D = Antibody to MCM5 applied, E = Antibody to MCM7 applied. Magnification x400.

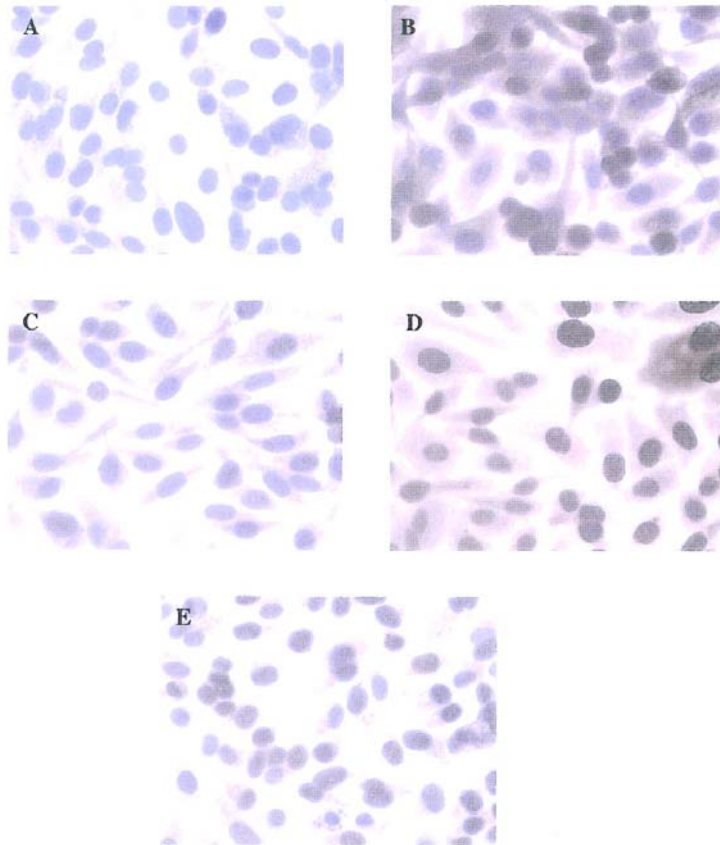


Figure d.18. Immunocytochemistry results for PC-3 cells at the synchronisation time of 48 hours No FCS then 24 hours with normal media (brown precipitate visualised indicates positive result). A = No Antibody to MCM2, 5, 7 or Ki67 applied, Negative Control, B = Antibody to Ki67 applied, C = Antibody to MCM2 applied, D = Antibody to MCM5 applied, E = Antibody to MCM7 applied. Magnification x400.

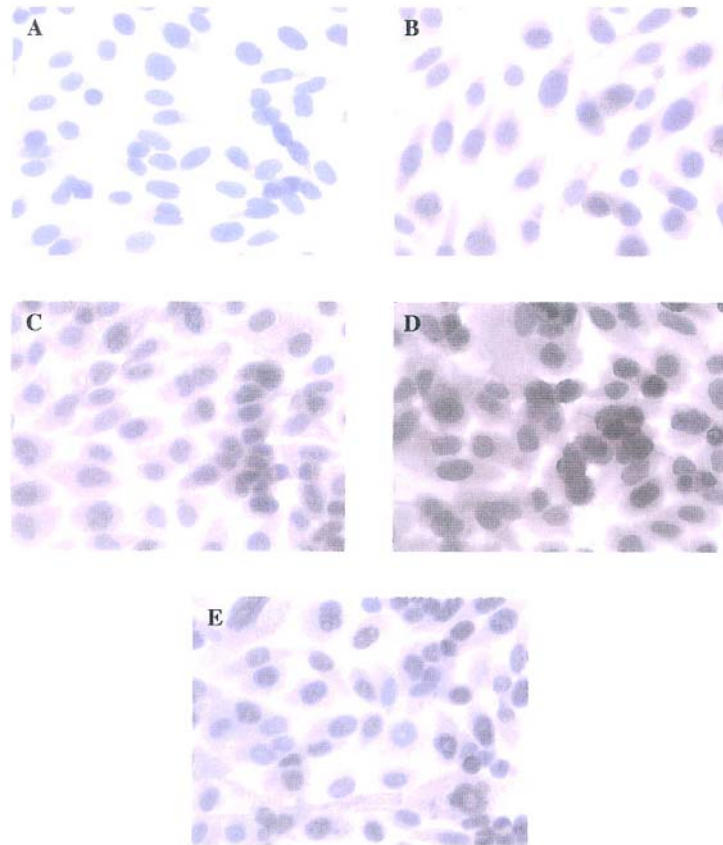


Figure d.19. Immunocytochemistry results for PC-3 cells at the synchronisation time of 48 hours No FCS then 30 hours with normal media (brown precipitate visualised indicates positive result). A = No Antibody to MCM2, 5, 7 or Ki67 applied, Negative Control, B = Antibody to Ki67 applied, C = Antibody to MCM2 applied, D = Antibody to MCM5 applied, E = Antibody to MCM7 applied. Magnification x400.

Table d.15. RT-PCR results for MCM2 for 200µg/µl of RNA extracted from PNT1A cells (table A) and PC-3 cells (table B). RNA was extracted from two T75 flasks for each cell line and each synchronisation time. Then RT-PCR was performed three times on flask 1, for each synchronisation time (Table A PNT1A 1, Table B PC-3 1) and twice on flask 2, for each synchronisation time (Table A PNT1A 2, Table B PC-3 2). An average was then calculated for each flask at each synchronisation time for each cell line.

Table A
PNT1A 1

Syn Time	Result 1	Results 2	Results 3	Average
48	2886.20	2320.54	1407.76	2204.83
48/8	724.47	755.33	531.40	670.40
48/24	5258.15	5105.00	4448.52	4937.22
48/30	3574.60	4514.41	4027.52	4038.84

PNT1A 2

Syn Time	Result 1	Result 2	Average
48	2241.91	1394.49	1818.20
48/8	1537.42	1179.90	1358.66
48/24	5055.42	4156.26	4605.84
48/30	3713.79	2515.36	3114.58

Table B
PC-3 1

Syn Time	Result 1	Result 2	Result 3	Average
48	2075.01	2859.44	3052.42	2662.29
48/8	1281.56	1460.98	1129.80	1290.78
48/24	2762.40	2991.63	2610.02	2788.02
48/30	892.95	774.19	1131.58	932.91

PC-3 2

Syn Time	Result 1	Result 2	Average
48	2279.13	4522.42	3400.78
48/8	592.44	1178.42	885.43
48/24	2746.47	4554.16	3650.32
48/30	770.00	3008.35	1889.18

Table d.16. RT-PCR results for MCM5 for 200µg/µl of RNA extracted from PNT1A cells (table A) and PC-3 cells (table B). RNA was extracted from two T75 flasks for each cell line and each synchronisation time. Then RT-PCR was performed three times on flask 1, for each synchronisation time (Table A PNT1A 1, Table B PC-3 1) and twice on flask 2, for each synchronisation time (Table A PNT1A 2, Table B PC-3 2). An average was then calculated for each flask at each synchronisation time for each cell line.

Table A
PNT1A 1

Syn Time	Result 1	Result 2	Average
48	2759.62	4619.90	3689.76
48/8	1637.83	2398.85	2018.34
48/24	7630.63	7229.33	7429.98
48/30	7042.87	5739.19	6391.03

PNT1A 2

Syn Time	Result 1	Result 2	Average
48	4451.62	3072.89	3762.26
48/8	4209.46	2628.09	3418.78
48/24	6689.50	7820.97	7255.24
48/30	5261.49	3933.92	4597.71

Table B
PC-3 1

Syn Time	Result 1	Result 2	Average
48	4138.75	5136.32	4637.54
48/8	3083.77	3011.50	3047.64
48/24	4678.37	4858.61	4768.49
48/30	1964.72	2655.29	2310.01

PC-3 2

Syn Time	Result 1	Result 2	Average
48	3280.99	4157.70	3719.35
48/8	3546.03	4490.35	4018.19
48/24	4085.14	5275.31	4680.23
48/30	1699.64	2315.67	2007.66

Table d.17. RT-PCR results for MCM7 for 200µg/µl of RNA extracted from PNT1A cells (Table A) and PC-3 cells (Table B). RNA was extracted from two T75 flasks for each cell line and each synchronisation time. RT-PCR was performed twice on both flasks for each synchronisation time and each cell line. An average was then calculated for each flask at each synchronisation time for each cell line.

Table A

PNT1A 1

PNT1A 2

Syn Time	Result 1	Result 2	Average	Syn Time	Result 1	Result 2	Average
48	841.42	918.22	879.82	48	861.49	1982.29	1421.89
48/8	1477.67	2647.87	2062.77	48/8	2330.68	3310.21	2820.445
48/24	3135.02	5814.17	4474.595	48/24	2756.09	5285.09	4020.59
48/30	2151.81	2951.31	2551.56	48/30	2187.69	2208.75	2198.22

Table B

PC-3 1

PC-3 2

Syn Time	Result 1	Result 2	Average	Syn Time	Result 1	Result 2	PC-3
48	2580.83	3254.28	2917.555	48	3360.85	3336.34	3348.595
48/8	961.06	1324.21	1142.635	48/8	1655.06	1407.64	1531.35
48/24	2508.87	2705.15	2607.01	48/24	2423.06	2993.91	2708.485
48/30	0	0	0	48/30	0	0	0

Table d.18. Average relative brightness (rbu) obtained for RT-PCR for MCM2 on PNT1A and PC-3 at different synchronisation times. Each cell line was grown twice per synchronisation time (1,2) and each synchronisation level had RT-PCR repeated at least twice to produce an average. The average total and total synchronisation time was then obtained by calculating the mean of the two set levels (*Raw data appendix d.15*).

1) PNT1A Syn Time (hours)	1) Average	2) PNT1A Syn Time (hours)	2) Average	Total PNT1A Syn Time (hours)	Total Average
48	2204.83	48	1818.20	48	2011.52
48/8	670.40	48/8	1358.66	48/8	1014.53
48/24	4937.22	48/24	4605.84	48/24	4771.53
48/30	4038.84	48/30	3114.58	48/30	3576.71
1) PC-3 Syn Time (hours)	1) Average	2) PC-3 Syn Time (hours)	2) Average	Total PC-3 Syn Time (hours)	Total Average
48	2662.29	48	3400.78	48	3031.53
48/8	1290.78	48/8	885.43	48/8	1088.11
48/24	2788.02	48/24	3650.32	48/24	3219.17
48/30	932.91	48/30	1889.18	48/30	1411.04

Table d.19. Average relative brightness (rbu) readings obtained for MCM5 on PNT1A and PC-3 at different synchronisation times. Each cell line was grown twice per synchronisation time (1,2) and each synchronisation level had RT-PCR repeated at least twice to produce an average. The average total and total synchronisation time was then obtained by calculating the mean of the two set levels (*Raw data appendix d.16*).

1) PNT1A Syn Time (hours)	1) Average	2) PNT1A Syn Time (hours)	2) Average	Total PNT1A Syn Time (hours)	Total Average
48	3689.76	48	3762.26	48	3726.01
48/8	2018.34	48/8	3418.78	48/8	2718.56
48/24	7429.98	48/24	7255.24	48/24	7342.61
48/30	6391.03	48/30	4597.71	48/30	5494.37
1) PC-3 Syn Time (hours)	1) Average	2) PC-3 Syn Time (hours)	2) Average	Total PC-3 Syn Time (hours)	Total Average
48	4637.54	48	3719.35	48	4178.44
48/8	3047.64	48/8	4018.19	48/8	3532.91
48/24	4768.49	48/24	4680.23	48/24	4724.36
48/30	2310.01	48/30	2007.66	48/30	2158.83

Table d.20. Average relative brightness (rbu) obtained for MCM7 on PNT1A and PC-3 at different synchronisation times. Each cell line was grown twice per synchronisation time (1,2) and each synchronisation level had RT-PCR repeated at least twice to produce an average. The average total and total synchronisation time was then obtained by calculating the mean of the two set levels (*Raw data appendix d.17*).

1) PNT1A Syn Time (hours)	1) Average	2) PNT1A Syn Time (hours)	2) Average	Total PNT1A Syn Time (hours)	Total Average
48	879.82	48	1421.89	48	1150.86
48/8	2062.77	48/8	2820.45	48/8	2441.61
48/24	4474.60	48/24	4020.59	48/24	4247.59
48/30	2551.56	48/30	2198.22	48/30	2374.89
1) PC-3 Syn Time (hours)	1) Average	2) PC-3 Syn Time (hours)	2) Average	Total PC-3 Syn Time (hours)	Total Average
48	2917.56	48	3348.60	48	3133.01
48/8	1142.64	48/8	1531.35	48/8	1336.99
48/24	2607.01	48/24	2708.49	48/24	2657.75
48/30	0	48/30	0	48/30	0

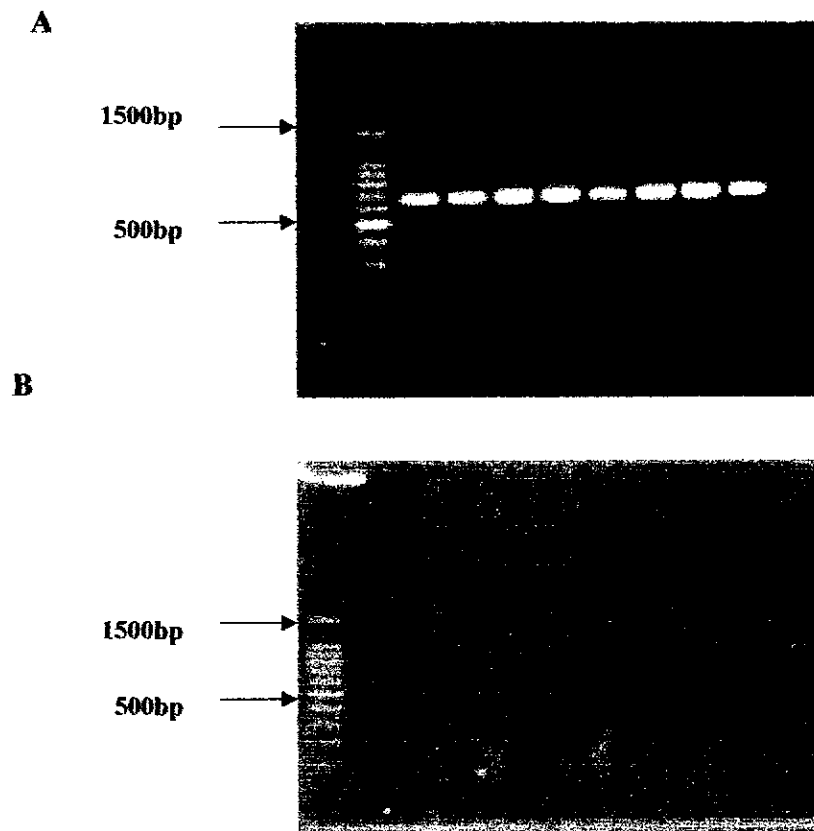


Figure d.20A. Ethidium bromide stained gel for RT-PCR for β -Actin (Positive control 680bp band visible) on 200 μ g/ μ l of cDNA from PNT1A cell lines which were synchronised as followed:- L = Ladder, 1 = PNT1A 48 hours serum free media, 2 = PNT1A 48 serum free 8 hours complete media, 3 = PNT1A 48 serum free 24 hours complete media, 4 = PNT1A 48 hours serum free 30 hours complete media, 5 = PNT1A 48 hours serum free media, 6 = PNT1A 48 serum free 8 hours complete media, 7 = PNT1A 48 serum free 24 hours complete media, 8 = PNT1A 48 hours serum free 30 hours complete media, 9 = No cDNA negative control .

Figure d.20B. Ethidium bromide stained gel when No Reverse Transcription is performed on 200 μ g/ μ l RNA and then PCR performed for β -Actin for PNT1A cell lines which were synchronised as followed:- L = Ladder, 1 = PNT1A 48 hours serum free media, 2 = PNT1A 48 serum free 8 hours complete media, 3 = PNT1A 48 serum free 24 hours complete media, 4 = PNT1A 48 hours serum free 30 hours complete media, 5 = PNT1A 48 hours serum free media, 6 = PNT1A 48 serum free 8 hours complete media, 7 = PNT1A 48 serum free 24 hours complete media, 8 = PNT1A 48 hours serum free 30 hours complete media, 9 = No cDNA negative control .

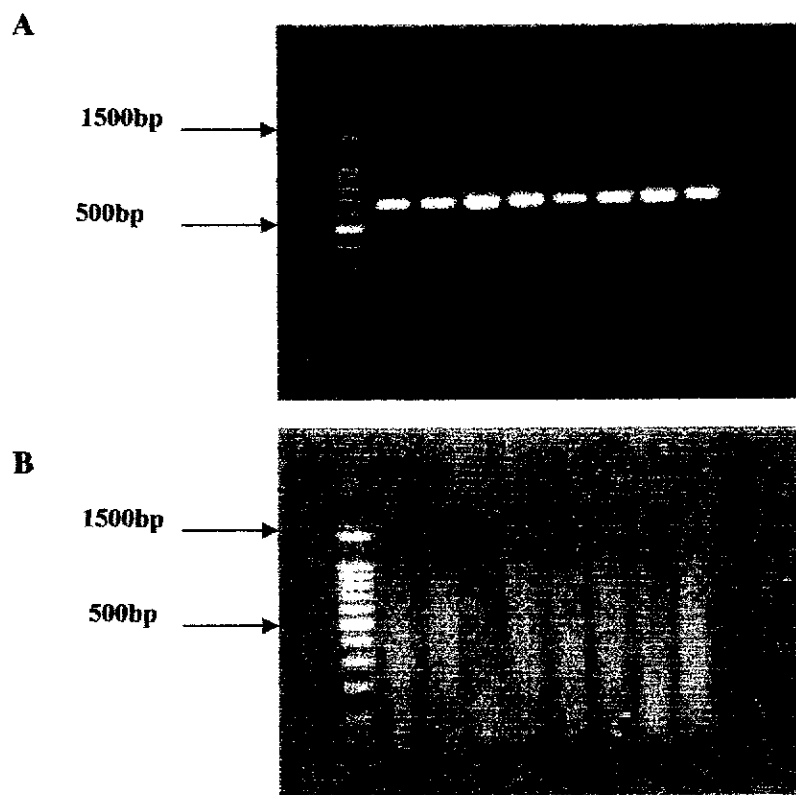


Figure d.21A. Ethidium bromide stained gel for RT-PCR for β -Actin (positive control) for 200 μ g/ μ l of cDNA from PC-3 cell lines which were synchronised as followed:- L = Ladder, 1 = PC-3 48 hours serum free media, 2 = PC-3 48 hours serum free 8 hours complete media, 3 = PC-3 48 hours serum free 24 hours complete media, 4 = PC-3 48 hours serum free 30 hours complete media, 5 = PC-3 48 hours serum free media, 6 = PC-3 48 hours serum free 8 hours complete media, 7 = PC-3 48 hours serum free 24 hours complete media, 8 = PC-3 48 hours serum free 30 hours complete media, 9 = No cDNA negative control.

Figure d.21B. Ethidium bromide stained gel when No Reverse Transcription is performed on 200 μ g/ μ l of RNA and then PCR is performed for β -Actin for PC-3 cell lines which were synchronised as followed:- L = Ladder, 1 = PC-3 48 hours serum free media, 2 = PC-3 48 hours serum free 8 hours complete media, 3 = PC-3 48 hours serum free 24 hours complete media, 4 = PC-3 48 hours serum free 30 hours complete media, 5 = PC-3 48 hours serum free media, 6 = PC-3 48 hours serum free 8 hours complete media, 7 = PC-3 48 hours serum free 24 hours complete media, 8 = PC-3 48 hours serum free 30 hours complete media, 9 = No cDNA negative control.

Appendix E

Ethical Approval and Trust Approval

Northamptonshire Local Research/Ethics Committee

Chairman: Dr Robin Sheppard
Administrative Assistant: Mrs Michelle Koornhof ☎(01604) 615363

Highfield
Cliftonville Road
Northampton
NN1 5DN

Our Ref:03/66

Tel: 01604 615000
Fax: 01604 615010

24 July 2003

Miss J Watkins
IBST
Cranfield University
Silsoe
Bedfordshire
MK45 4DT

Dear Jane

Re: 03/66 Novel Markers for the detection and management of diseases of the prostate

The Northamptonshire Local Research/Ethics Committee reviewed your application in relation to the above study at their meeting on 22 July 2003, and would like to thank you for attending.

Provided in the first instance only the first four markers under investigation are used, the members of the Committee present agreed there is no objection on ethical grounds to the proposed study. I am therefore pleased to inform you that Formal Ethical Approval has been granted.

If in the future you intend to carry out the other markers for investigation, this must first be sent to the Committee to review and approve as a protocol amendment.

Before the study can proceed, it is your responsibility to seek Trust approval through the Research and Development office. Please contact Julie Wilson, R & D Manager, on Knightley Ward (Telephone: 01604 545941).

I confirm that the Northampton Local Research/Ethics Committee operates according to Good Clinical Research Practice (GCP) principles, and enclose a copy of the Committee's Constitutions and Standing Orders.

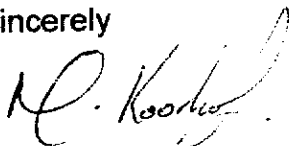
You will find details enclosed regarding a Regional funded project to record and analyse projects that have been submitted to this Ethics Committee. The letter enclosed explains the project in more detail. Please take time to read it, before completing the survey. Your participation is useful and necessary to the completion of a mapping exercise of research (any research) that is proposed, planned or taking part in Northamptonshire. Your record

of using resources would be helpful in shaping future funding of research and development in the county.

To complete our records regarding the project, please complete and return the form accompanying this letter.

Please let me know if the study has to be terminated or any ethical considerations arise which need to be discussed further by the Committee.

Yours sincerely

A handwritten signature in black ink, appearing to read 'M. Koornhof', with a stylized flourish at the end.

Michelle Koornhof
Administrative Assistant, Northamptonshire Local Research/Ethics Committee

cc. Dr John O'Donnell

Northampton General Hospital **NHS**

NHS Trust

**Research & Development
Knightley Ward**

Manager: Julie Wilson
e-mail : julie.wilson@ngh.nhs.uk

Tel : 01604 545941

Fax: 01604 603143

15 September 2003

Miss J Watkins
IBST
Cranfield University
Silsoe
Bedfordshire
Mk45 4DT

Dear Jane

Re: LREC 03/66 Novel Markers for the detection and management of diseases of the prostate

I am writing to advise you that the R&D office is in receipt of the following paperwork for the above study: -

- Trial protocol version 1, June 2003
- Your C.V and the research team
- A copy of the LREC approval letter ref. 03/66
- A copy of the approved patient information sheets and consent forms , version 1 dated July 2003
- A statement from Prof. O'Donnell outlining the finances of the study

There are no other outstanding issues and I can therefore confirm that you have Trust approval for this study.

Finally can I please request that you advise the R&D Office if you are named in any papers that are published as a consequence of this research

Best Wishes

Julie Wilson

Julie Wilson
R&D Manager

Cc Prof. J O'Donnell
Dr H Clarke



Study Protocol : Novel Markers for the Detection and Management of Diseases
of the Prostate

Recruitment of patients to the study:

- A provisional diagnosis of prostate disease is made during consultation in the out patient clinic.
- The normal procedure is performed to identify the need for a biopsy. This is done by either the identification of elevated PSA levels above the standard level or by DRE with the identification of an abnormal prostate.
- The patient is introduced to the study and asked if they would like further information given to them by the department. If in agreement, the patient will spend a few minutes with Mr M Miller having a verbal explanation of the study and its implications explained.
- The patient is given the information sheet to take away.
- They are then given at least 24 hours to decide if they wish to participate in the investigation.
- If not in agreement, the patient's care pathway continues in the normal fashion.
- When attending the department for the biopsy to be carried out the patient indicates their willingness or otherwise to take part in the study.
- Any further queries are addressed at this time.
- If willing to be a part of the study Mr M Miller will then ask them to sign the study consent form in addition to the standard hospital consent form.
- If consent is given then patients will be asked to provide a urine and blood sample

- If unwilling to be a part of the study the patients care pathway continues as normal.
- Mr M Miller will be responsible for the allocation of a reference number to each patient and he will also be responsible for a reference book, which will contain all the information about the patients and samples. The book will be kept in his office locked in a filing cabinet.
- The biopsy is then performed to normal procedure.
- Biopsy tissue is embedded in paraffin and sectioned. These sections then go through the normal procedure for histological diagnosis, if any tissue remains will be allocated for this investigation.
- The patient's involvement in the study ends when the biopsy specimen is taken. There after their care is identical to any other patient with similar complaint.

Recruitment of healthy volunteers to the study:

- Healthy volunteers will be recruited at Cranfield University, Silsoe via e-mail where they will be told of the study and asked if they wish to take part.
- Volunteers who wish to participate will be given a verbal explanation of the project by Miss J Watkins and will then be given an informaiton sheet to read.
- The volunteers are then allowed at least 24 hours to decide if they wish to take part.
- If the volunteers are willing to take part consent will be taken by Miss J Watkins
- The volunteer is asked to give a blood and urine sample, which will be taken by the nurse at Cranfield medical centre.
- The samples will be allocated a number and the nurse will identify what age group the volunteer falls between (Age groups = 18-29(A), 30-39(B), 40-49(C), 50-59(D), 60 and above (E)). No information derived from this study can be related back to the volunteer.

Analysis of Samples:

- Research samples will be analysed in the Biochemistry Department at Northampton General Hospital and at Cranfield University, Silsoe.
- The research analysis performed will be DNA, RNA and protein based techniques however no genetic testing will be performed.
- The results of the study will form the bases of a PhD theses and may generate publications

Study Protocol: Novel Markers for the Detection and Management of diseases of the Prostate – Molecular Markers to be examined.

Below is a list of some of the molecular markers which are currently known to be under investigation in relation to cancer and prostate cancer. They have been selected as they may have the potential to aid in diagnostic / prognostic processes. This study will look at some of the markers mentioned with the intention to identify molecules, which can show whether prostate disease is present and at what stage, with more specificity and sensitivity than the current methods used for diagnosis. The intention is to focus upon the MCM proteins, DNA methylation, PSA and PSMA, but due to the fluid nature of biomedical research it may later become necessary to study related molecules as listed. DNA, RNA and protein based techniques will be performed, for example PCR and RT-PCR to assess methylation status and gene expression. Western blots and immunohistochemistry will be performed to assess protein expression. No genetic testing will be involved in this study.

Markers under investigation:

MCMs 2, 3,4,5,6, 7,8	MiniChromosomal Maintenance Proteins: These are involved in cellular replication during the S phase of the cell cycle. They have been shown to vary in their level of expression within cancer cells.
DNA methylation	The study of DNA methylation is the analysis of genetic change within cellular DNA. For example GSTP1 (glutathione-S-transferase P1 gene) has been analysed and it has been shown that there is genetic alteration of this in prostate cancer.
PSA	Prostate Specific Antigen: This is secreted by prostatic epithelial cells and is already used as a molecular marker for the detection of prostate cancer. However the efficiency of PSA in prostate cancer diagnosis is under debate so we intend to use this marker in conjunction with others to try and improve on current diagnostic strategies.
PSMA	Prostate Specific Membrane Antigens: This is a 750-residue integral membrane glycoprotein, secreted by normal and cancerous prostate tissue. It is thought that in cancerous tissue its level of expression is higher than normal therefore this protein could be a good monitor for cancer progression.
CD44	This is a cell adhesion molecule whose expression has been shown to vary in number of cancers.
P53	This acts as a transcription factor and has been shown to control proliferation. In cancer cells its function has been shown to change and in some cases even been lost.
HA	Hyaluronic Acid is a glycosaminoglycan and regulates cell adhesion and migration. This has been shown to change in abundance within cancer cells.

Cyclin B	This is needed during cellular replication during the G2 phase and the early M phase. This has been shown to vary in its level of expression within cancer cells.
Cyclin D	This is needed during cellular replication during the mid and late G1 phase. This has been shown to vary in its levels of expression within cancer cells.
Phosphohistone-H3	This is needed during cellular replication during mitosis. This has been shown to vary in its levels of expression within cancer cells.
Ras protein	This is needed to control the signalling pathways during cell growth. Mutations can occur within the protein affecting cellular pathways. Therefore this protein can be looked at in relation to cancer development.
Cox-2	Cyclooxygenase 2: This is an enzyme and is required for the oxidation of arachidonic acid to produce prostaglandin. This has been shown to change in its level of expression within cancer cells.
Interleukins (IL)	These are cytokines and they have many physiological roles as well as being implicated in a number of pathophysiologic processes. It is known that IL can stimulate a number of tumour types and therefore contribute to progression of cancer. For example IL-6 activates a variety of signal transduction cascades some of which stimulate the development of prostate cancer.
5αreductase	This is a steroidal enzyme, which has been shown to vary in its level of expression within cancer cells.
Heparin-binding ECF	This is a polypeptide and has a molecular weight of 22KDa. These proteins bind and activate ECF receptors and have been shown to have different levels of expression within cancer cells.
Telomerase	These are ribonucleoproteins, which synthesise and repair telomeres. These have been shown to vary in their level of expression within cancer cell.
E-Cadherin	This is a cell adhesion molecule, which has been shown to have various levels of expression within cancer cells.
MIB-1	This is a proliferation marker, which has been expressed at various levels within cancer cells.
P27	This is a cell cycle protein, which has been shown to have various levels of expression within cancer cells.
Ki67	This is a human nuclear protein strictly associated with cellular proliferation. These have been shown to be expressed a various levels within cancer cells.
TGF	Tumour growth factors: These are active substrates produced by endothelium cells which have been shown to have a positive impact on cancer cells development and therefore could be studied as a potential marker.
Nitric oxide synthases	These are a family of enzymes responsible for generation of nitric oxide from the amino acid L-Arginine. These enzymes have been shown to have an association with cancer cells and it has been shown that different isoforms of these genes maybe present within different cancer types.

Maspin	This is a member of the serpin family of serine proteases and functions as tumour suppressor. This has been shown to vary in its levels of expression within cancer cells
Endothelins	These are the most potent vasoconstrictor known and these are very widely distributed in tissues. They are essential for normal development and function as a survival factor for endothelial cells. Therefore within cancer cells normal development is lost so these molecules are expressed differently.
FGF	Fibroblastic growth factors: These are present in normal growing cells and are believed however to be a potential stimulator in cancer cells.
CSF	Colony-stimulating factors: These influence the differentiation, survival, proliferation, migration and metabolism of macrophages, granulocytes and dendritic cells. These factors have been shown to be secreted by a number of cancer cells types and therefore effect the proliferation and development of cancer.
HGF	Hepatocyte growth factors or 'scatter factors': These are growth factors and stimulate anchorage independent growth. These have therefore been shown to vary in their level of expression within some cancer types.
TF	These are tissue factors and have been shown to vary in their level of expression within cancer cells.
Myc genes	These are oncogenes and are needed in control of signalling pathways into cellular proliferation. These genes have been shown to differ in cancer cells therefore they could be studied as a potential marker.
Bcl-2	Bcl-2 is an apoptosis suppressor and its expression may be correlated to progression of cancer.
Bax	Bax proteins are apoptosis inducers and have been implicated in the progression of cancer.
CD40	These are antigens, which are normally expressed by many kinds of epithelium. In cancer however CD40 expression is reduced as the cancer becomes more aggressive.
E2F	These are transcription factors and are required for cell cycle completion. This has varying levels of expression within cancer cells.
Cyp1b1	This is a member of the cytochrome P450 family and has shown to vary in levels of expression within cancerous tissue.
DD3	This is a prostate specific gene and has been shown to vary in its expression within cancer cells.

Patient Information Sheet

1. Study Title

Novel Markers for the Detection and Management of Diseases of the Prostate.

2. Invitation paragraph

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and you'. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, LONDON N16 0BW.

Thank you for reading this.

3. What is the purpose of the study?

Current tests for prostate cancer offers a great deal of information for management of the disease, but these tests do have their disadvantages. This study will look at new molecules that show disease is present, with the hope that they will provide more detailed information. This information may help make diagnosis clearer and provide an idea of how the cancer may progress (prognosis). The study will start with investigating the presence of these molecules in prostate tissue, before moving to blood and other bodily fluids.

4. Why have I been chosen?

As you are aware your Clinician has informed you that a biopsy is going to be taken from your prostate as you have been shown to have raised PSA and/or an enlarged prostate. If you consent to taking part, a blood sample and urine sample will be collected before your biopsy. Once your biopsy has been examined any remaining tissue will be allocated to the study.

5. Do I have to take part?

It is up to you to decide whether or not you wish to take part. If you do decide to take part you will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without any reason. This will not affect the standard of care received.

6. What will happen to me if I take part?

If you decide to take part you will be asked to sign a consent form for this study. Before your biopsy is taken you will be asked to give a blood sample and a urine sample. When the biopsy has been looked at for your diagnosis any tissue left is allocated for this study.

7. What do I have to do?

We ask you read this information and take your time in deciding if you want to be part of this study. If you agree to take part you will be asked to sign a consent form then an extra blood sample and a urine sample will be taken. When the routine biopsy is taken the normal procedure is performed and any remaining tissue is allocated for this study.

8. What is the drug or procedure that is being tested?

No drug or procedure is being tested.

9. What are the side effects of taking part?

Taking a blood sample may cause slight discomfort and sometimes you can get localised bruising around the area from which the sample was obtained. The urine sample has no risks associated and the biopsy have no additional side effects associated, apart from those normally associated with the operation.

10. What are the possible disadvantages and risks of taking part?

There are no possible disadvantages or risks associated with taking part in this study.

11. What are the possible benefits of taking part?

There is no direct benefit to you but this study may help improve diagnosis and follow up of future patients with this disease.

12. What happens when the research study stops?

All tissue allocated for this study will be destroyed and the results will form the bases of a scientific thesis and may generate scientific publications.

13. What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspects of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms may be available to you.

14. Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised. All the samples will be analysed anonymously.

15. Who is organising and funding the research?

The research is being organised and funded by charities associated with Northampton General Hospital and Cranfield University, Silsoe.

16. Who has reviewed the study?

The study has been reviewed by: The Northamptonshire Medical Research Ethics Committee

17. Contact for further information

Mr M Miller

Department of Integrated Surgery

Northampton General Hospital NHS Trust

Cliftonville

Northampton NN1 5BD

Tel : 01604 544887

Thank you very much for taking the time to read the information sheet.

Consent Form

Patient identification number for this trial:.....

Title of Project: **Novel Markers for the Detection and Management of diseases of the prostate.**

Name of Researcher : Dr J. O'Donnell, Dr H Clarke, Mr M Miller, Miss J Watkins

1. I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions.

☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

☐

3. I understand that sections of my medical notes may be looked at by responsible individuals from Northampton General hospital or from the regulatory authorities where it is relevant to taking part in research. I give permission for these individuals to access to my records.

☐

4. I agree to take part in the above study.

5. I agree to samples being sent to Cranfield University, Silsoe.

6. I agree to anonymous data being taken out of the hospital.

Name of Patient:.....

Signature.....Date.....

Name of person taking consent.....

Healthy Volunteers information Sheet

1. Study Title

Novel Markers for the Detection and Management of Diseases of the Prostate.

2. Invitation paragraph

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and you'. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, LONDON N16 0BW.

Thank you for reading this.

3. What is the purpose of the study?

Current tests for prostate cancer offers a great deal of information for management of the disease, but these tests do have their disadvantages. This study will look at new molecules that show disease is present, with the hope that they will provide more detailed information. This information may help make diagnosis clearer and provide an idea of how the cancer may progress (prognosis). The study will start with investigating the presence of these molecules in prostate tissue, before moving to blood and other bodily fluids.

4. Do I have to take part?

It is up to you to decide whether or not you wish to take part. If you do decide to take part you will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without any reason.

5. What will happen to me if I take part?

If you decide to take part in this study you will be asked to sign a consent form. You will then be asked to give a blood sample and a urine sample for the investigation. These samples will be allocated a number and analysed anonymously. We will not be able to relate your sample back to you at any point in the study.

6. What do I have to do?

We ask you read this information and take your time in deciding if you want to be part of this study. If you agree to take part you will be asked to sign a consent form. Then a blood sample and urine sample will be taken.

7. What is the drug or procedure that is being tested?

No drug or procedure is being tested.

8. What are the side effects of taking part?

Taking a blood sample may cause slight discomfort and sometimes you can get localised bruising to the area from which the sample is obtained, the urine sample has no risks associated.

9. What are the possible disadvantages and risks of taking part?

There are no possible disadvantages or risks associated with taking part in this study.

10. What are the possible benefits of taking part?

There is no direct benefit to you but this study may help improve diagnosis of this disease.

11. What happens when the research study stops?

All samples allocated for this study will be destroyed and the results will form the bases of a scientific thesis and may generate scientific publications.

12. What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspects of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms may be available to you.

13. Will my taking part in this study be kept confidential?

No information will be collected about you during the course of the research. All samples will be allocated an ID number and analysed anonymously. It will not be possible to relate this number or your samples back to you.

14. Who is organising and funding the research?

The research is being organised and funded by charities associated with Northampton General Hospital and Cranfield University, Silsoe.

15. Who has reviewed the study?

The study has been reviewed by: The Northamptonshire Medical Research Ethics Committee

17. Contact for further information

**Dr A Woodman
IBST
Cranfield University
Barton Road
Silsoe
Bedfordshire
MK45 4DT**

Tel: 01525 863000

Thank you very much for taking the time to read the information sheet.

Healthy Volunteer Consent Form

Title of Project: **Novel Markers for the Detection and Management of diseases of the prostate.**

Name of Researcher : Dr J. O'Donnell, Dr H Clarke, Mr M Miller, Miss J Watkins

1. I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I agree to take part in the above study.

Name of Patient:.....

Signature.....Date.....

Name of person taking consent.....

Northamptonshire Local Research Ethics Committee**Chairman:** Dr Robin Sheppard**Administrator:** Mrs Michelle Spinks (01604) 615363

Our Ref: RS/MS/03/66

28 April 2004

Miss Jane Watkins
Cranfield University
Silsoe
Bedfordshire
MK45 4DT

Dear Miss Watkins

03/66 Novel markers for the detection and management of diseases of the prostate

Further to my letter of 16 April 2004, I am writing to confirm that the following amendments have been reviewed by the Chairman of the Northamptonshire Local Research Ethics Committee:-

Patient Information Sheet – Version 2 dated April 2004
Consent Form – Version 2 dated April 2004
Study Protocol – Version 2 dated April 2004

Ethical opinion

The Chairman, acting under delegated authority, is satisfied that these amendments accord with the decision made by the Committee at their meeting on 23 March 2004, and has given a favourable ethical opinion of the amendment.

Management approval

Before implementing the amendment, you should check with the host organisation whether it affects their approval of the research.

REC Reference: 03/66. Please quote this number on all correspondence.

Yours sincerely



Michelle Spinks
Administrator, Northamptonshire Local Research Ethics Committee

Northamptonshire Local Research Ethics Committee



Room 205
Highfield
Cliftonville Road
Northampton
NN1 5DN

Telephone: 01604 615363
Facsimile: 01604 615146

08 July 2005

Jane Watkins
Cranfield University Silsoe
IBST
Barton Road
Silsoe
Bedfordshire MK45 4DT

Dear Jane

REC Reference: 03/66
Study Title: Novel markers for the detection and management of diseases of the prostate
Protocol No: Version 3 dated April 2005
Amendment No: 2 dated April 2005

The above amendment was reviewed at the meeting of the Sub-Committee of the Research Ethics Committee held on Tuesday 28 June 2005. Thank you for attending the meeting to answer questions from the Committee.

Ethical Opinion

The Committee agreed that it is important to ensure that a sufficient amount of archive tissue will be left in the blocks as the tissue may be needed for further testing.

In response to these concerns, I have since received a copy of your letter dated 5 July 2005, which confirms that you will not collect archive tissue from blocks which are sparse in tissue. This answers the Committee's concerns satisfactorily. The members of the Committee present agreed to give a favourable ethical opinion of the amendment on receipt of this undertaking, and I am therefore pleased to confirm the Committee's approval of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

- Notice of Substantial Amendment Form dated 31 May 2005
- Amended REC Application Form
- Study Protocol, Version 3 dated April 2005
- *Letter from Jane Watkins dated 5 July 2005 subsequently reviewed by the Administrator on 06/07/2005*

Membership of the Committee

The members of the Ethics Committee who were present at the meeting were:

Dr Sue Price – Vice Chairman
Mr Michael Tawn – Lay Member
Mr Mike Newman – Expert Member
Mr Ron Wellings – Lay Member

Management approval

All investigators and research collaborators in the NHS should notify the R&D Department for the relevant NHS care organisation of this amendment and check whether it affects local management approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

REC Reference Number: 03/66 Please quote this number on all correspondence

Yours sincerely



Michelle Spinks
Committee Administrator

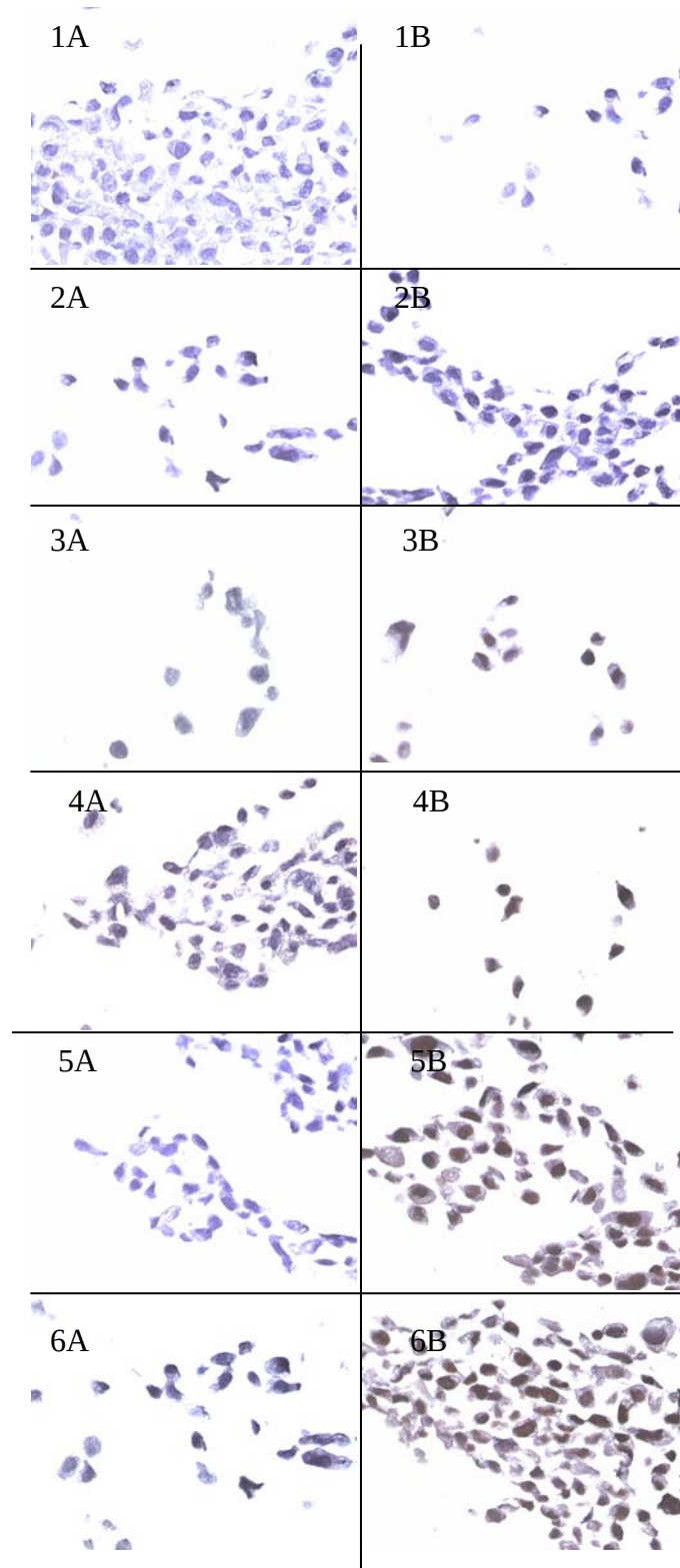


Figure e.1 IHC optimisation using paraffin embedded PC-3 cell lines and different antigen retrieval. A= Negative control, No antibodies applied B= Positive control, antibodies applied 1= 5min boil/ 5min rest, 2= 10min boil/ 10min rest, 3= 15min boil/15min rest, 4= 20min boil/ 20min rest, 5= 25minboil/ 25min rest, 6= 30min boil/ 35min rest. Antibodies to MCM5 applied. Brown precipitate indicates positive cells. Magnification X400.

Table e.1 Scores given to different neoplastic patients for IHC, using antibodies to MCM2, 5, 7 and PSA. The staining criterion used is shown in Table 4.1, ID No. = indicates individual patients and Score = indicates the Gleason score given by the consultant histopathologist.

ID No.	Score	MCM2	MCM5	MCM7	PSA
A12	4	0+	1+	1+	3+
A6	5	0+	1+	1+	3+
A14	5	0+	1+	1+	3+
A15	5	0+	2+	0+	3+
A17	5	0+	1+	1+	3+
A4	6	0+	2+	1+	3+
A5	6	1+	2+	1+	3+
A10	6	0+	2+	1+	3+
A22	6	1+	2+	2+	3+
12	6	0-	0+	0-	3+
45	6	0-	1+	2+	3+
13	6	0+	1+	1+	3+
15	6	0+	1+	1+	3+
49	6	0-	2+	1+	3+
23	7	1+	2+	1+	3+
3	7	1+	1+	1+	3+
53	7	0+	1+	1+	3+
33	7	1+	2+	1+	3+
A2	7	0+	2+	1+	3+
51	8	0-	2+	1+	3+
105	8	0+	1+	1+	3+
A9	8	0+	1+	1+	3+
A19	8	1+	2+	1+	3+
A20	8	0+	2+	1+	2+
A21	8	0+	1+	1+	1+
A23	8	1+	2+	2+	3+
5w	9	1+	2+	1+	3+
4	9	1+	2+	2+	3+
25	9	1+	2+	2+	3+
2w	9	1+	2+	3+	3+
93	9	1+	2+	2+	3+
A1	9	1+	2+	1+	3+
A3	9	0+	2+	2+	3+
A11	9	1+	2+	2+	3+
A16	9	1+	3+	2+	3+
A18	9	1+	1+	1+	3+
50	10	2+	2+	3+	2+
18	10	1+	1+	2+	3+
74	10	2+	3+	3+	1+
A7	10	1+	2+	2+	2+
A8	10	1+	2+	2+	2+
A13	10	0+	2+	2+	2+

Table e.2 Score given to the different patients with BPH using IHC for antibodies to MCM2, 5, 7 and PSA. The staining criterion used is shown in Table 4.1 and the ID No. = indicates individual patients.

3w	0-	0-	0-	3+
1	0-	0-	0-	2+
2	0-	0-	0-	3+
7	0-	0-	0-	3+
8	0-	0-	0-	3+
14	0-	0-	0-	3+
19	0-	0-	0-	3+
21	0-	0-	0-	3+
22	0-	0-	0-	3+
24	0-	0+	0-	3+
26	0-	0+	0-	3+
28	0-	0+	0-	3+
32	0-	0+	0-	3+
35	0-	0-	0-	3+
31	0-	0-	0-	3+
41	0-	0-	0-	3+
43	0-	0-	0-	3+
42	0-	0-	0-	3+
37	0-	0-	0-	3+
39	0-	0-	0-	3+
48	0-	0-	0-	3+
52	0-	0-	0-	3+
73	0+	0+	0-	3+
103	0-	0-	0-	3+
61	0-	0+	0+	3+
34	0-	0+	0+	3+
47	0-	0+	0+	3+
101	0-	0+	0+	3+
114	0-	0+	0+	3+
112	0-	0+	0+	3+
123	0+	0+	0+	3+
115	0-	0-	0+	3+

Appendix F

Table f.1. PSA readings for each consented patient, their Gleason score and age range.

No.	Patient Trial Number	PSA Level micrograms/L	PSA Level ng/ml	Age Range	Gleason Score/ TNM
1	p-002w	70.3	70.3	5	Gleason score 10
2	p-003w	5.7	5.7	5	Benign
3	p-005w	0.3	0.3	5	Gleason 9 poorly diff
4	p-001	0.2	0.2	5	Benign
5	p-002	1.0	1.0	5	Benign
6	p-003	0.1	0.1	5	Gleason 7 pT3b pNX
7	p-004	9.9	9.9	5	Gleason 9 poorly diff
8	p-007	0.2	0.2	3	Benign
9	p-008	2.3	2.3	5	Benign
10	p-012	11.4	11.4	5	Gleason 6 pT2b
11	p-013	11.1	11.1	5	Gleason 6 TNM pT3a pNo
12	p-014	0.9	0.9	5	Benign
13	p-015	10.9	10.9	5	Gleason 6 adenoT2c pNX
14	p-016	0.1	0.1	3	Gleason 7 pT3b pNX
15	p-018	94.0	94.0	4	Gleason 10
16	p-019	4.0	4.0	5	Benign
17	p-021	1.5	1.5	5	Benign
18	p-022	2.1	2.1	5	Benign
19	p-023	11.4	11.4	5	Gleason 7
20	p-024	1.5	1.5	5	Benign
21	p-025	0.8	0.8	5	Gleason 9
22	p-026	0.9	0.9	5	Benign
23	p-028	1.5	1.5	5	Benign
24	p-032	4.8	4.8	5	Benign
25	p-035	2.8	2.8	5	Benign
26	p-031	33.4	33.4	5	Benign
27	p-041	8.0	8.0	5	Benign
28	p-043	2.1	2.1	5	Benign
29	p-034	0.4	0.4	5	Benign
30	p-033	5.0	5.0	5	Gleason 7 pT2b pNO
31	p-042	0.9	0.9	5	Gleason 7 pT2b pNO
32	p-037	7.9	7.9	5	Benign
33	p-039	3.6	3.6	5	Benign
34	p-048	4.5	4.5	5	Benign
35	p-049	15.1	15.1	5	Gleason 6 pT2b pNxMc
36	p-050	24.6	24.6	5	Gleason 10
37	p-047	4.8	4.8	5	Benign
38	p-051	1.8	1.8	5	Gleason 8
39	p-052	0.8	0.8	5	Benign
40	p-045	1.6	1.6	5	Gleason 6 T1=NxMx
41	p-061	29.6	29.6	5	Benign
42	p-074	0.3	0.3	5	Gleason 10 Post treatment
43	p-073	7.6	7.6	5	Benign
44	p-103	23.8	23.8	5	Benign
45	p-101	1.4	1.4	5	Benign
46	p-105	7.8	7.8	5	Gleason 8

No.	Patient Trial Number	PSA Level micrograms/L	PSA Level ng/ml	Age Range	Gleason Score/ TNM
48	p-093	3.7	3.7	5	Gleason 9 poorly diff
49	p-115	2.6	2.6	5	Benign
50	p-114	2.9	2.9	5	Benign
51	p-123	1.9	1.9	5	Benign
52	p-129	2.6	2.6	5	Benign
53	p-053	3.7	3.7	5	Gleason score 7
54	p-001a	13.7	13.7	5	Benign
55	p-005a	13.6	13.6	5	Gleason score 6
56	p-004a	56.2	56.2	5	Benign
57	p-003a	47.3	47.3	5	Gleason score 8
58	p-008a	0.6	0.6	4	Benign
59	p-007a	7.9	7.9	5	Gleason score 9
60	p-002a	5.6	5.6	5	Gleason score 9
61	p-012a	1.7	1.7	4	Benign
62	p-006a	4.5	4.5	5	Gleason score 9
63	p-009a	11.1	11.1	5	Gleason score 7
64	p-011a	16.1	16.1	5	Gleason score 8
65	p-015a	26.4	26.4	5	Gleason score 7
66	p-013a	6.7	6.7	5	Benign
67	p-016a	4.4	4.4	4	Gleason score 7
68	p-018a	15.5	15.5	5	Benign
69	p-019a	9.7	9.7	5	Benign
70	p-017a	9.3	9.3	5	Benign

Table f.2. Number of patients in the trial and the age range in which they fell into. Also shown is what the age range correlates to.

Table f.3. The number of healthy patients that took part in the trial and what age range they fell into. First column shows the total number of healthy patients and their age range, second two columns show the number of female and male patients and their age range. Percentage of patient/age ranges in brackets shown next to numbers of patients.

Table f.2

Table f.3

Age Range	Age	No. Patients	Age Range	Total Healthy	Female	Male
1	18 - 29	0	1	11 (58%)	7 (64%)	4 (50%)
2	30 - 39	0	2	4 (21%)	2 (18%)	2 (25%)
3	40 - 49	2	3	3 (16%)	1 (9%)	2 (25%)
4	50 - 59	4	4	1 (5%)	1 (9%)	0 (0%)
5	60 above	64	5	0 (0%)	0 (0%)	0 (0%)

$$\text{Sensitivity} = \frac{n \text{ with a disease and a positive test}}{n \text{ with a disease and either a positive or negative test}} \times 100$$

$$\text{Specificity} = \frac{n \text{ without a disease and a negative test}}{n \text{ without a disease and either a negative or positive test}} \times 100$$

Table f.4. Urine pellet cytospin Raw data. Results for ICC on MCM2, MCM5, MCM7 and PSA for half of the consented patients. Staining criteria used shown in Table 3.1.

Sample No.	Gleason score	MCM2	MCM5	MCM7	PSA
p-015	6	1	1	0	1
	6	1	1	0	1
p-012	6	2	1	0	0
	6	1	1	0	0
p-016a	7	2	2	1	2
	7	0	0	1	2
p-016	7	0	0	0	1
	7	1	2	1	1
p-003a	8	1	2	1	3
	8	2	2	0	3
p-105	8	1	1	0	1
	8	2	2	0	1
p-004	9	2	2	0	0
	9	1	1	1	1
p-006a	9	1	1	1	3
	9	2	2	0	3
p-074	10	2	2	0	3
	10	1	2	0	3
p-050	10	2	2	0	0
	10	1	2	0	0
p-007	Benign	0	0	0	1
	Benign	0	0	0	1
p-022	Benign	1	1	0	1
	Benign	0	1	0	1
p-035	Benign	0	0	0	3
	Benign	0	0	0	3
p-024	Benign	0	0	0	3
	Benign	0	0	0	3
p-002w	Benign	1	0	0	2
	Benign	0	0	0	2
p-019	Benign	1	1	0	2
	Benign	1	1	0	2
p-019a	Benign	0	1	0	1
	Benign	0	1	0	0
p-018a	Benign	0	0	0	3
	Benign	0	0	0	2
p-017a	Benign	1	2	0	2
	Benign	1	1	0	1
p-032	Benign	0	1	0	1
h-012 m	Healthy	0	1	0	1
	Healthy	1	1	0	1
h-003 f	Healthy	1	1	0	1
	Healthy	1	2	0	2
h-004 m	Healthy	0	1	0	2
	Healthy	0	1	0	2
h-002 f	Healthy	0	1	0	2
	Healthy	1	1	0	2

h-001 f	Healthy	0	0	0	2
	Healthy	1	2	0	2
h-007 m	Healthy	1	1	0	2
	Healthy	0	1	0	1
h-021 m	Healthy	1	1	0	1
	Healthy	1	1	0	1

Table f.5. Shows the mean Standard deviation obtained for Scores 6-10, BPH and healthy volunteers when the urine pellets were cytospun and IHC was performed for antibodies tot MCM2, 5, 7 and PSA. The grading system used is shown in *table 3.1*. Grades 6-10 n=4 (2 patients for each grade x 2 slides), BPH n= 20 (10 BPH patients 2X slides), Healthy Males n= 8 (4 healthy male volunteers x2 slides), and Healthy Females n= 6 (3 healthy female volunteers x2 slides).

Gleason score	MCM2	MCM5	MCM7	PSA
6	0.50	0.00	0.00	0.50
7	0.96	1.15	0.75	1.50
8	0.58	0.50	0.25	2.00
9	0.58	0.58	0.50	1.75
10	0.58	0.00	0.00	1.50
BPH	0.47	0.60	0.00	0.91
Healthy Male	0.53	0.00	0.00	0.52
Healthy Female	0.52	0.75	0.00	0.41

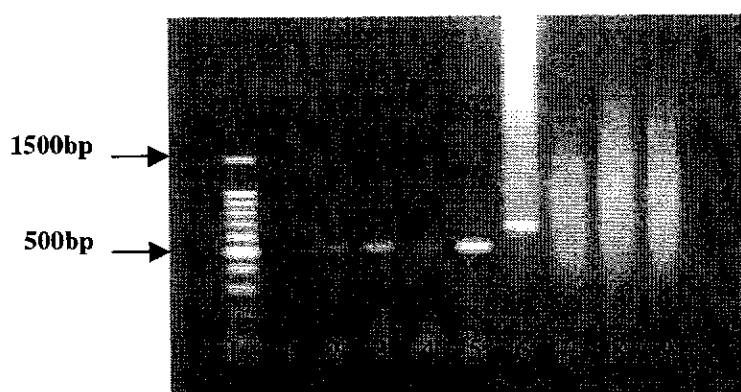


Figure f.1. Ethidium bromide gel of the RT-PCR optimisation results for MCM5 (500bp bands visible) using (100µg/µl) RNA extracted from a urine pellet. L = Ladder, 1 = 25 cycles, 2 = 30 Cycles, 3 = 35 cycles, 4 = 40 cycles, 5 = Positive control MCM5 expression extracted from (100µg/µl) PNT1A cells, 6 = Positive Control Beta Actin expression extracted from (100µg/µl) PNT1A cells, 7 = MCM5 negative control, 8 = BA negative control, 9 Negative control MCM5 PCR of RNA from urine pellets.

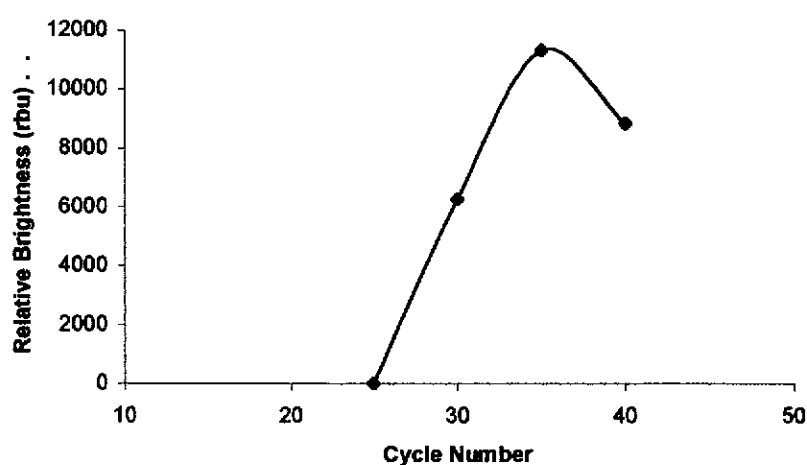


Figure f.2. A line graph of the relative brightness (rbu) reading for a urine pellet tested at different cycle numbers for MCM5 expression by RT-PCR.

Table f.6. RNA Concentrations for Urine pellets.

Urine Samples	RNA Concentration micrograms/microlitre
P-005W	42
P-002A	27
P-049	99
P-007A	14
P-005A	37
P-011A	105
P-051	44
P-013	58
P-009A	32
P-004	97
P-033	198
P-123	17
P-048	80
P-043	83
P-004A	74
H-015	41
H-013	119
P-129	39
P-031	87
P012A	87
P-001A	25
P-037	7
P-112	77
H-011	113

P-073	147
H-006	68
P-101	34
H-019	105
P-008A	96
H-009	21
H-014	23
P-034	27
H-008	25
H-010	86
P-013A	-302

Table f.7. Results for the thirty-five urine pellets tested by RT-PCR on MCM5. Of these 12 were from patients with Gleason score 6–9, 14 were from BPH patients and 9 were from healthy volunteers of which 6 were female and 3 were male. The table also shows the RNA loaded for the RT-PCR reaction and the relative brightness (rbu) obtained.

Sample No.	Score	RNA Load ($\mu\text{g}/\mu\text{l}$)	Relative Brightness (rbu)	Relative Brightness 100 $\mu\text{g}/\mu\text{l}$
P-005w	9	100	1492.41	1492.41
P-002a	9	50	1103.34	2206.68
P-049	6	100	1561.24	1561.24
P-007a	9	40	1713.31	4283.28
P-005a	6	50	1849.29	3698.58
P-011a	8	100	1646.18	1646.18
P-051	8	100	1285.25	1285.25
P-013	6	50	1635.33	3270.66
P-023	7	40	0	0
P-009a	7	50	1458.46	2916.92
P-004	9	100	1403.46	1403.46
P-033	7	100	1337.81	1337.81
P-0132	B	100	1700.13	1700.13
P-048	B	100	0	0
P-043	B	100	0	0
P-004a	B	100	1460.9	1460.9
H-015	H F	100	1660.38	1660.38
H-013	H F	100	0	0
P-129	B	100	0	0
P-031	B	100	0	0
P-012a	B	100	0	0
P-001a	B	50	0	0
P-037	B	20	0	0
P-112	B	100	0	0
H-011	H F	100	0	0
P-073	B	100	0	0
H-006	H F	100	0	0
P-101	B	50	0	0
H-019	H M	100	0	0
P-008a	B	100	0	0
H-009	H F	50	0	0

H-014	HM	50	0	0
P-034	B	50	0	0
H-008	HM	50	0	0
H-010	HF	100	0	0

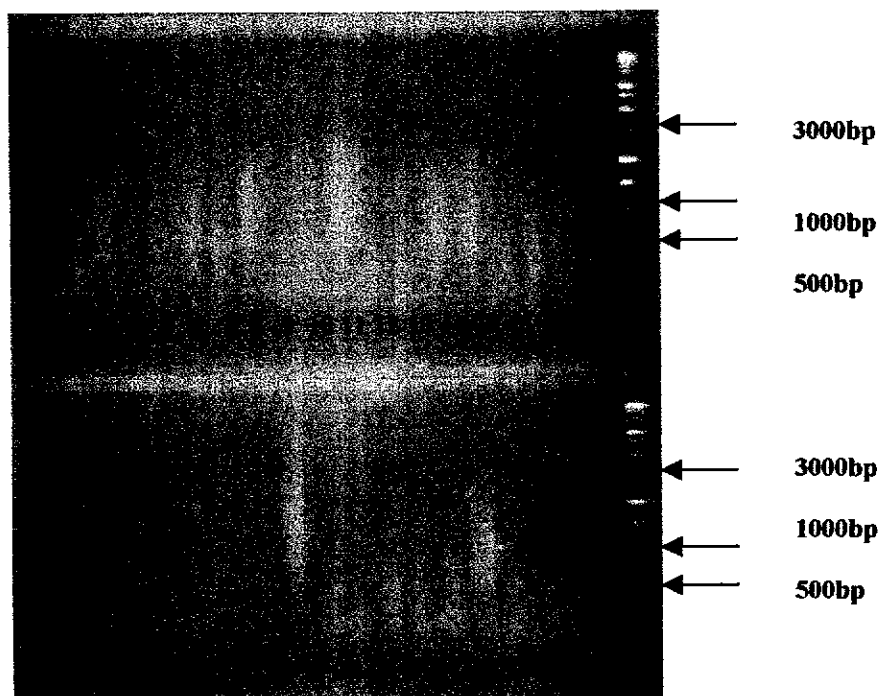


Figure f.3. Ethidium bromide gel of the PCR results for MCM5 (No RT) on RNA extracted from the urine pellets. L = 1Kb pair Ladder 1 = p-031 2 = p-129, 3 = H-013, 4 = H-015, 5 = p-004a, 6 = p-043, 7 = p-048, 8 = p-123, 9 = p-033, 10 = p-004, 11 = p-09a, 12 = p-023, 13 = p-013, 14 = p-051, 15 = p011a, 16 = p-005a, 17 = p-049, 18 = p-002a, 19 = p-005w, 20 = Negative Control, 21 = H-010, 22 = H-008, 23 = p-034, 24 = H-014, 25 = H-009, 26 = p-008a, 27 = H-019, 28 = p-101, 29 = H-006, 30 = p-073, 31 = H-011, 32 = p-112, 33 = p-037, 34 = p-001a, 35 = p-012a .

Table f.7. The mean Relative Brightness (rbu) reading obtained for different cancerous conditions, benign, and healthy volunteers. Sample numbers for each grade also shown in the table.

Gleason score	Number Samples	Reading (rbu)
G-9	4	2346.46
G-8	2	1465.72
G-7	3	1418.24
G-6	6	2843.49
BPH	14	225.79
HF	6	276.73
HM	3	0.00

Table f.8. The Statistical results obtained when a One-Way Anova is performed on the Relative Brightness (rbu) reading for MCM5 urine pellet results. If a value less than 0.05 is recorded this means there is a significant difference is seen. A *P-value* <0.05 is considered significant.

Table f.9. The statistical results obtained when a Bonferroni test is performed on the Relative Brightness (rbu) reading for MCM5 urine pellet results at different scores (BPH – Gleason 9). If a value less than 0.05 is recorded this means there is a significant difference between the groups. Grades homogeneous shown by ****.

Table f.8 Univariate Tests of Significance for ABSORB 100µg/µlSigma-restricted parameterization Effective hypothesis decomposition

	SS	Degree. Of freedom	MS	F	P
Intercept	36997775	1	36997775	54.85603	0.000000
Score	32199937	6	5366656	7.95706	0.000046
Error	18884663	28	674452		

Table f.9 Bonferroni test; variable ABSORB 100µg/µl Homogenous Groups, alpha = .05000
Error: Between MS = 6745E2, df = 28.000

Score	ABSORB 100µg/µl	1	2
H.Males	0.000	****	
Benign	225.788	****	
H.Females	276.730	****	
7	1418.243	****	****
8	1465.715	****	****
9	2346.458		****
6	2843.493		****

Table f.10. The number of cells obtained from 15mls of urine that was processed by two means. Urine Process 1:- samples left at 4°C and then processed within 1, 8, 24 and 48hrs, Urine Process 2:- samples left at RT for 1hr/8hrs after which it was stored at 4°C until processing at 24 and 48 hrs. Also shown is the percent remianing for Urine Process 1 = Percent remaining 1 and Urine Process 2 = Percent remaining 2.

Hours	Urine Process 1	Urine Process 2	Percent remaining 1	Percent remaining 2
1	84000	84000	100%	100%
8	66000	80000	79%	96%
24	52000	46000	62%	55%
48	46000	36000	55%	43%

Table f.11. Blood samples RNA concentrations.

Sample No.	Concentration in micrograms/microlitre	Sample No.	Concentration in micrograms/microlitre
P-016	51.00	P-049	39.27
P-003W	52.00	P-129	50.67
P-002	86.00	P-052	54.00
P-002W	99.00	H-013	360.00
P-032	110.00	P-025	69.00
P-073	54.00	P-074	157.00
P-123	93.00	P-047	85.00
P-005W	355.00	H-001	108.00
P-003W	65.00	H-015	134.00
P-031	71.00	P-103	94.00
P-012	184.00	P-039	14.00
P-013	180.00	H-009	14.00
P-004	89.00	H-007	56.00
P-115	88.00	P-014	87.00
P-033	139.00	P-008	45.00
P-114	124.00	P-156	363.00
P-026	63.00	H-010	363.00
P-014	100.00	H-014	146.00
P-037	104.67	P-013	52.00
H-019	193.33	P-023	16.00
H-004	144.67	P-061	53.00
H-005	254.00	P-028	94.00
P-021	94.67	H-021	608.00
P-093	103.33	P-048	28.00
P-112	117.33	H-003	39.00
H-008	33.33	P-022	152.00
P-051	126.00	P-024	50.00
H-006	279.00	H-012	55.00
P-050	23.00		

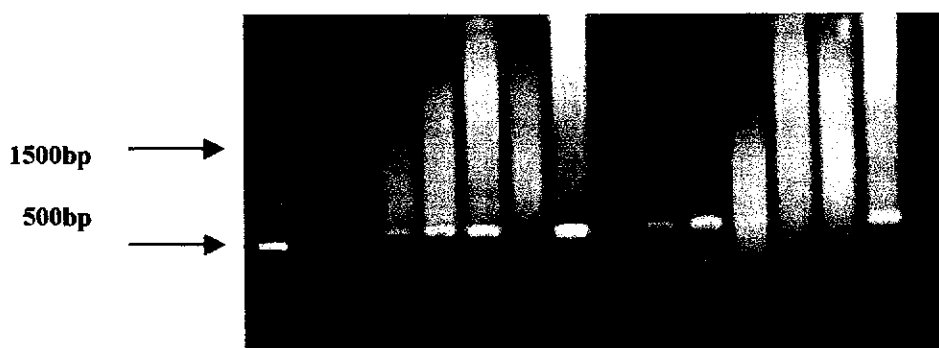


Figure f.4. Ethidium bromide gel showing RT-PCR blood optimisation results for MCM5 (500bp bands visible) and β -Actin (680bp bands visible) on 100 μ g/ μ l of RNA from a blood sample. 1 = MCM5 reading after 25 cycles, 2 = MCM5 reading after 30 cycles, 3 = MCM5 reading after 35 cycles, 4 = MCM5 reading after 40 Cycles, 5 = Negative Control MCM5, 6 = Positive Control 100 μ g/ μ l of RNA from PNT1A cell line, 7 = β -actin reading after 25 cycles, 8 = β -actin reading after 30 cycles, 9 = β -actin reading after 35 cycles, 10 = β -actin reading after 40 Cycles, 11 = β -actin negative control, 12 = β -actin Positive control 100 μ g/ μ l of RNA from cell line PNT1A.

Table f.12. RT-PCR blood optimisation Relative Brightness (rbu) reading for MCM5 and β -Actin on 100 μ g/ μ l of RNA from a blood sample after different cycle times.

Cycle Number	MCM5	Beta Actin
25	0	1267.46
30	1841.76	2254.78
35	2891.54	4521.87
40	4418.45	4121.32

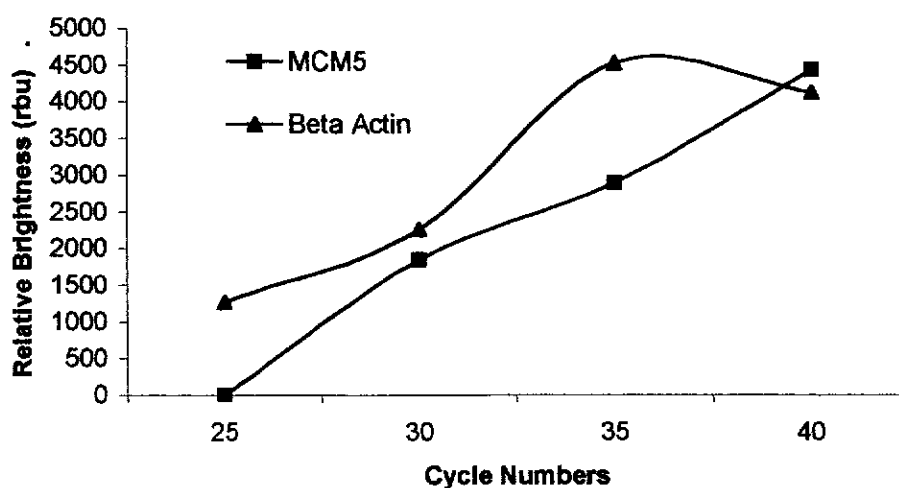


Figure f.5. RT-PCR blood optimisation Relative Brightness (rbu) reading for MCM5 and β -Actin on 100 μ g/ μ l of RNA from a blood sample after different cycle times.

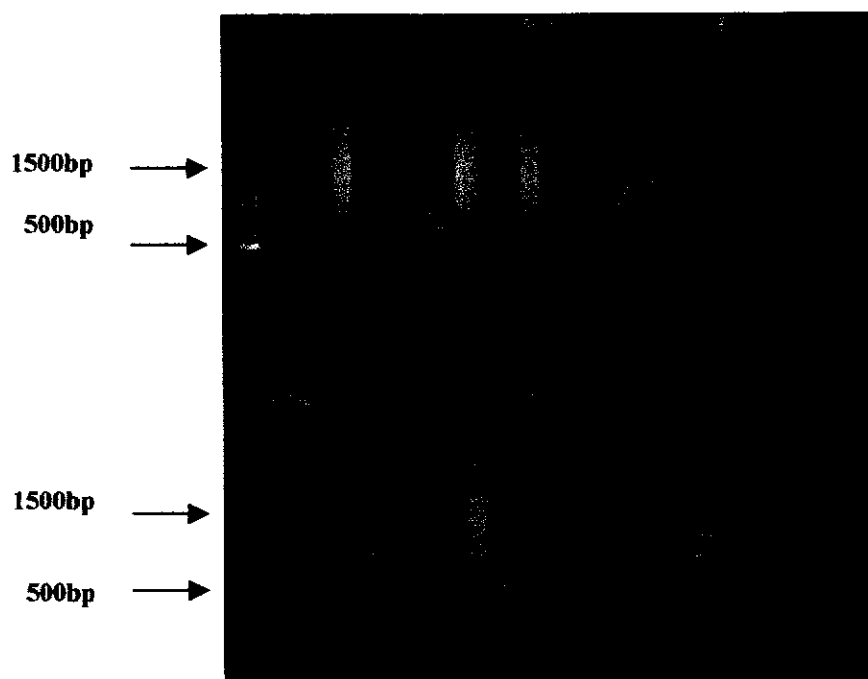


Figure f.6. Ethidium bromide gel showing PCR results for β -actin on 50-150 μ g/ μ l of RNA extracted from blood (Negative Controls No RT). L = Ladder, 1 = P-016, 2 = P-003W, 3 = P-002, 4 = P-002W, 5 = P-032, 6 = P-073, 7 = P-123, 8 = P-005W, 9 = P-031, 10 = P-012, 11 = P-013, 12 = P-004, 13 = P-115, 14 = P-033, 15 = P-114, 16 = P-026, 17 = P-014, 18 = P-037, 19 = H-019, 20 = H-004, 21 = H-005, 22 = P-021, 23 = P-093, 24 = P-112, 25 = H-008, 26 = P-051, 27 = P-049, 28 = P-129, 29 = P-052, 30 = H-013, 31 = P-025, 32 = P-074, 33 = P-047, 34 = H-001, 35 = H-015, 36 = P-103, 37 = P-039, 38 = H-009.

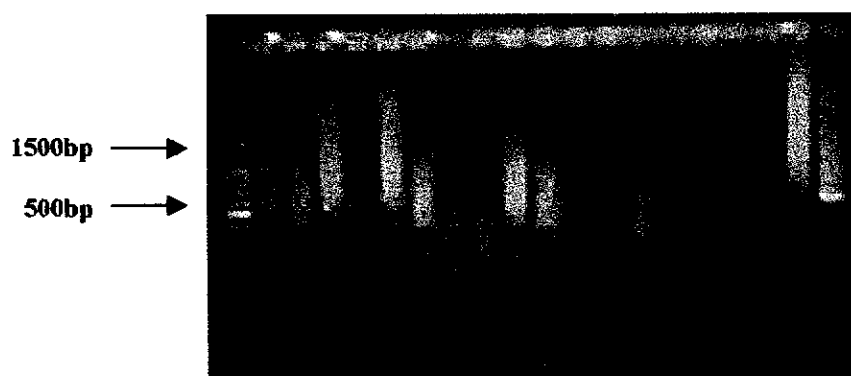


Figure f.7. Ethidium bromide gel showing PCR results for β -Actin on 50-150 μ g/ μ l of RNA extracted from blood (Negative controls No RT). L = Ladder, 39 = H-007, 40 = P-008, 41 = H-010, 42 = H-014, 43 = P-001, 44 = P-023, 45 = P-061, 46 = P-028, 47 = H-021, 48 = P-048, 49 = H-003, 50 = P-022, 51 = P-024, 52 = H-006, 53 = H-012, 54 = P-050, - = Negative Control, 55 = Positive Control 150 μ g/ μ l of RNA from PNT1A cells.

Table f.13. The blood samples that expressed either 150µg/µl MCM5 or Beta actin after RT-PCR is performed. Readings recorded are Relative brightness (rbu). Score = Gleason score Number , B – BPH, HF - Healthy Female and HM - Healthy Male, Consent No. = Patients ID Number.

No.	Consent No.	Conc (µg/µl)	Score	MCM5	Beta Actin
1	P-016	100	7	-	-
2	P-003W	100	B	-	-
3	P-002	150	B	-	-
4	P-002W	150	10	-	-
5	P-032	150	B	-	-
6	P-073	100	B	-	-
7	P-123	150	B	-	8594.11
8	P-005W	150	9	2851.64	10318.14
9	P-031	150	B	-	5971.90
10	P-012	150	6	4632.36	9588.50
11	P-013	150	6	2523.37	6932.98
12	P-004	150	9	-	4971.99
13	P-115	150	B	4778.50	8975.24
14	P-033	150	7	7949.35	8303.31
15	P-114	150	B	-	-
16	P-026	100	B	-	-
17	P-014	150	B	-	-
18	P-037	150	B	1646.82	4881.81
19	H-019	150	HM	-	8550.19
20	H-004	150	HM	2362.52	6063.43
21	H-005	150	HF	-	-
22	P-021	150	B	-	4728.76
23	P-093	150	9	1681.88	-
24	P-112	150	B	-	-
25	H-008	50	HF	-	4432.10
26	P-051	150	8	-	-
27	P-049	50	6	3402.09	19534.2
28	P-129	100	B	-	-
29	P-052	100	B	5069.87	9600.13
30	H-013	150	HF	2123.05	-
31	P-025	100	9	-	-
32	P-074	150	10	-	-
33	P-047	150	B	-	-
34	H-001	150	HF	-	-
35	H-015	150	HF	2940.12	-
36	P-103	150	B	-	-
37	P-039	30	B	-	-
38	H-009	30	HF	-	-
39	H-007	100	HM	-	-
40	P-008	100	B	-	-
41	H-010	100	HF	3368.41	5292.65
42	H-014	100	HM	-	5870.82
43	P-001	100	B	-	6102.93
44	P-023	30	7	-	-
45	P-061	100	B	-	-
46	P-028	150	B	-	-
47	H-021	150	HF	-	-
48	P-048	30	B	-	-
49	H-003	30	HF	-	-
50	P-022	150	B	-	-

51	P-024	100	B	-	-
52	H-006	150	HF	-	-
53	H-012	100	HM	-	-
54	P-050	30	10	-	-