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Oestrogen receptor mutations and their influence on breast cancer growth

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CHAPTER FOUR

RESULTS

4.1 Analysis of clinical data

4.1.1 Patient population

Breast fine needle aspirates (FNAs) were obtained over a period of 5 years from 61 patients with confirmed breast carcinoma. Past carcinoma samples were obtained from 13 patients undergoing breast reduction. The mammographic characteristics with respect to menopausal status, clinical stage (Figure 1), tumour size, nodal involvement and presence of metastasis (Figure 2), tumour treatment and ER status (Figure 3) are listed in Table 5.1. Most (58 of 61, 95%) patients presented with infiltrating ductal carcinoma while 3 (5%) were diagnosed with lobular carcinoma. Information on tamoxifen treatment was available for 18 patients (13/18) responded favourably. At the end of the study only two patients died from the disease.

Table 5. Characteristics of Patients, Tumours and Treatmen

	Characteristic	Frequency (%
Patients	Total number	61
	Age in years	
	Mean	52.9
	Range	25-87
	Menopausal sta	
	Premenopausa	27
	Postmenopaus	34
Tumours	Size (cm)	
	T1 (<=2)	4(6)
	T2 (>2-5)	18(30)
	T3 (>5)	14(23)
	T4	23(38)
	Unknown	2(3)
	Nodal status	
	N0	20(33)
	N1	19(32)
	N2	18(31)
	Unknowm	3(4)
	Metastases	
	M0	43(71)
	M1	17(28)
	Unknown	1(1)
	Histology	
	Infiltrating duct	58(95)
	Lobular	3(5)
	Stage	
	I	4(7)
	II	16(26)
	III	22(36)
	IV	18(30)
	Unknown	1(1)
Hormone treatmen	Yes	22(36)
	No	39(64)
ERICA	Positive	26(43)
	Negative	24(39)
	Unknown	11(18)

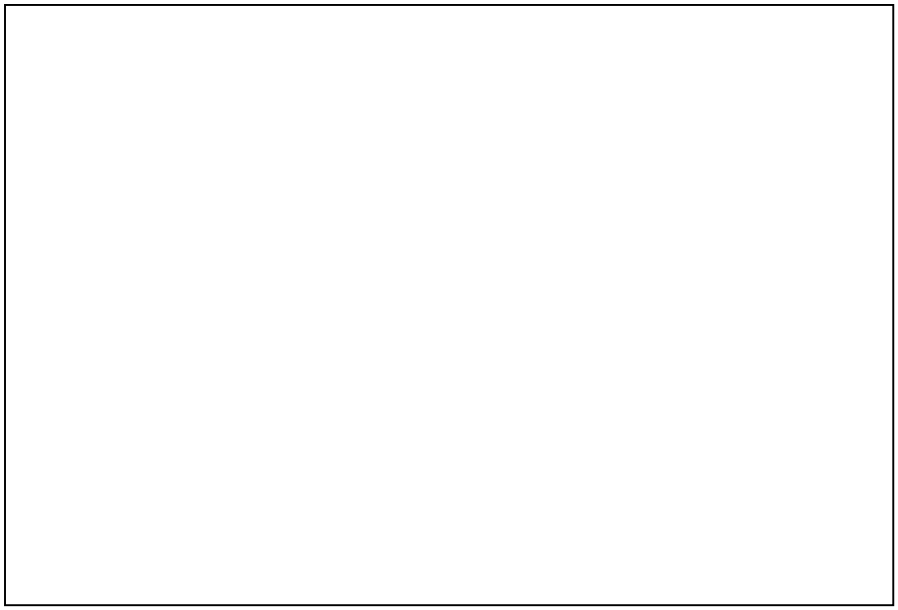


Figure 20. Distributions of Age of carcinoma patients in sample

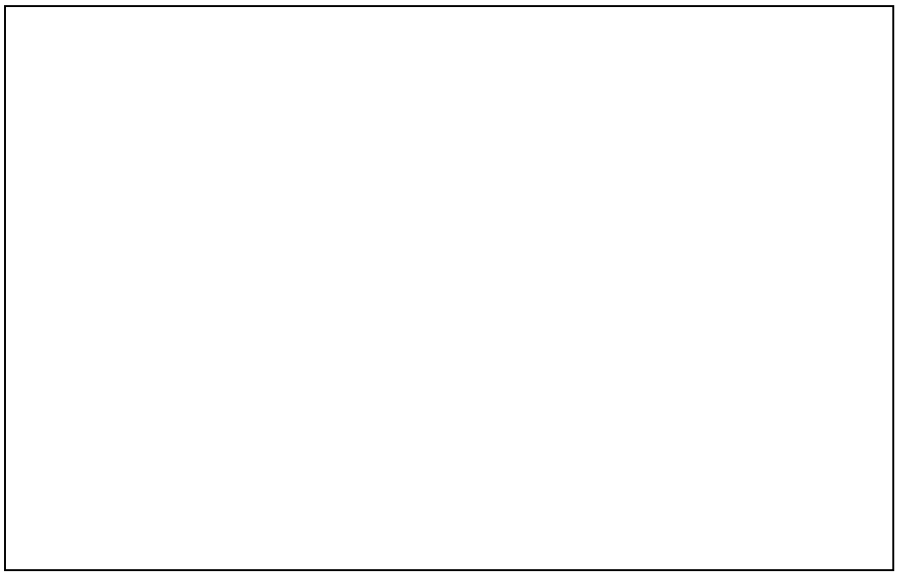


Figure 21. Histogram of clinical stage of disease in sample breast carcinoma patients

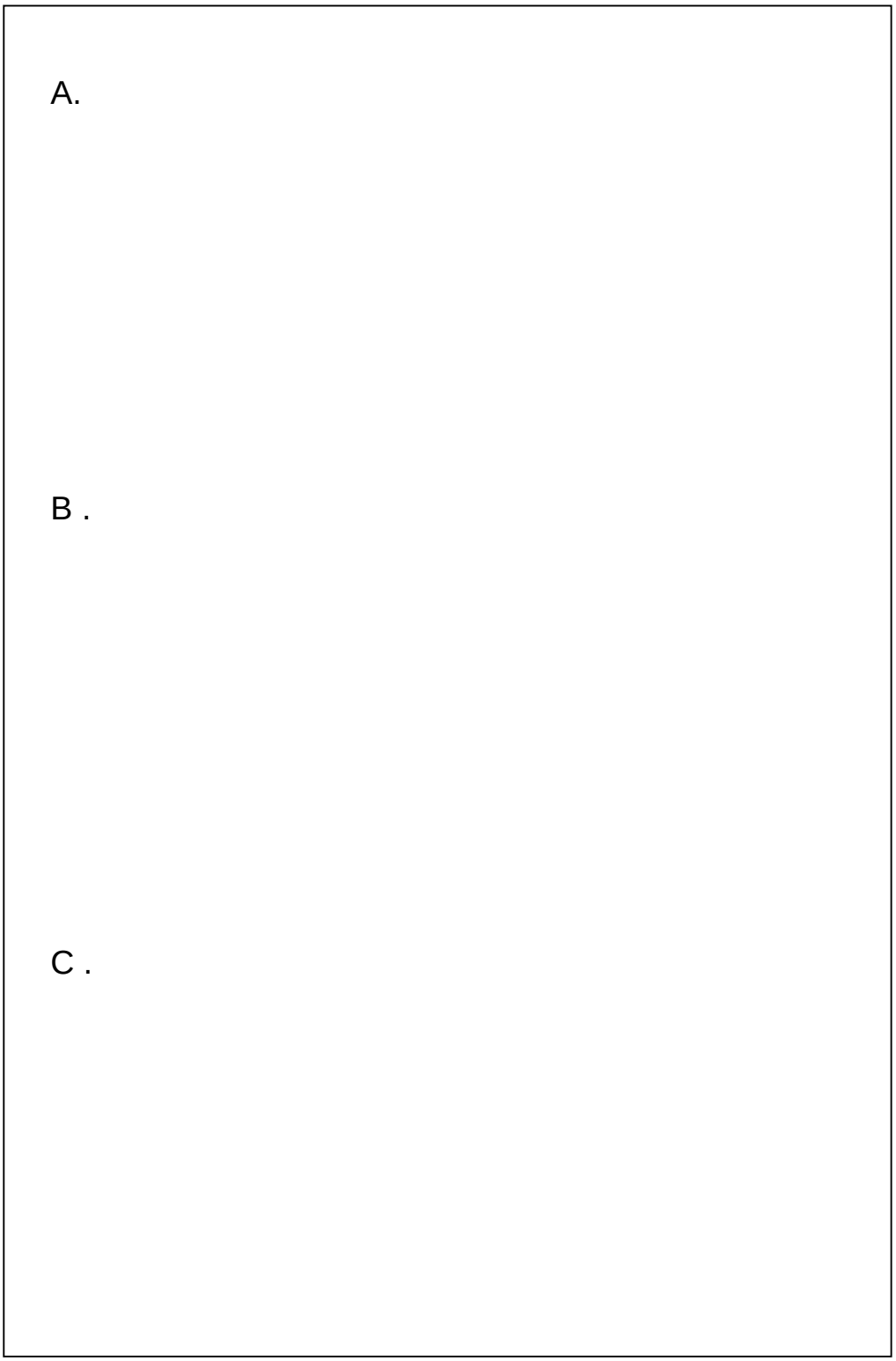


Figure 22. Histograms of distributions of (A) tumor size, (B) nodal involvement and (C) presence or absence of metastases in sample breast carcinoma patients.

All samples were tested by RT-PCR and sequencing for the presence of both ER² variants and the data were analyzed statistically using χ^2 (chi-square) analyses and Fisher's exact tests. Metastasis-free survival analysis was performed on 33 patients.

4.12. Nested PCR and sequence analysis of ER± exon variants in clinical samples

Breast tissue FNAs were reverse transcribed and PCR-amplified for the housekeeping gene in order to assess the integrity of the cDNA before further investigation. A total of 61 patients with breast carcinoma and 13 control samples were thus analyzed using RT-PCR. Each of the ER± exons and sequencing of each PCR product was performed as described.

4.12.1 Nested PCR and sequence analysis of ER± exon 2

PCR of ERα exon 2 revealed two bands on agarose gel electrophoresis (Figure 23). Each of these bands was purified and sequenced using automated sequencing (Applied Biosystems 310 Genetic Analyzer). Sequence analysis revealed the presence of the complete exon 2 (Figure 42, Appendix A1) as well as the deletion of the entire exon (Figure 42, Appendix A2). 11 (18%) of breast carcinomas exhibited only the deletion variant, 71 (82%)

exhibited only the complete exon 2 and 47.3% (9) exhibited both the presence
absence of exon 2 within the sample cells.

In the control samples, 3 (23%) samples exhibited presence of the
complete exon 2, lack of exon 2 (ie. only exhibited exon 1 and 3) and 8 (62%)
showed both the presence and absence of exon 2 within their cells.

χ^2 analysis was performed on the comparison of control and diseased tissu
ER±2 showed no significance with regard to tumour size
involvement (N) (Table 9), presence or absence of metast
stage of disease (Table 11) or menstrual status (Table 12).
complete ER±2, however, showed significance ($p<0.05$) with respect to ERI
status (Table 13). Fisher's exact were also performed between e
exon deletion results of diseased tissue. ER±2 was signifi
the presence of ER±4 (Fisher exact $p=0.005$) and the deletion variant
(Fisher exact $p=0.042$) (Table 6). ER±"2 was only significantly
ER±"6 (Fisher exact $p=0.017$) (Table 7).

4.1.2.2

Nested PCR and sequence analysis of ER± exon 3

Only one patient (2%) exhibited two bands on agarose gel el
25). The 205bp band includes the deletion of ER± exon
expected size of exon 3 is 117bp.

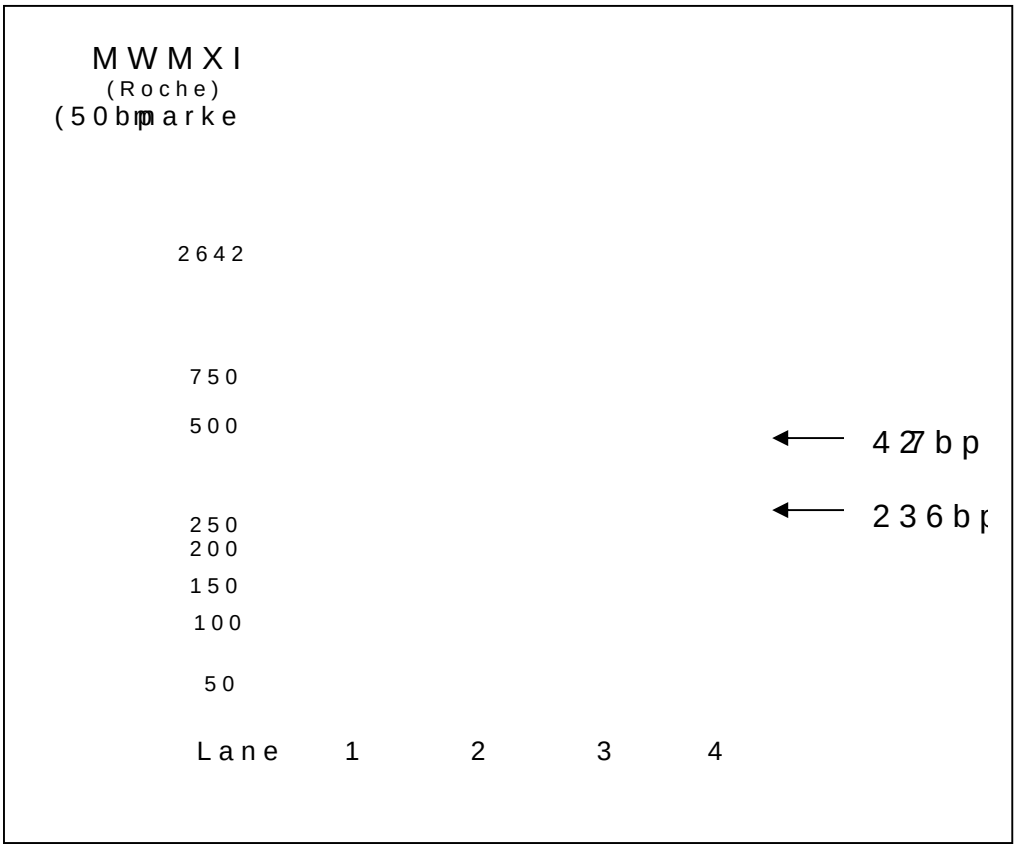


Figure 23. ER± exon 2 agarose electrophoresis gel

MWM XI (Roche, SA) is a 50bp (base pair) molecular weight marker (lane 1). ER±2 PCR product is represented by the 427 bp band in lanes 3 and 4. PCR product is represented by the 236 bp band in lanes 2 and 4.

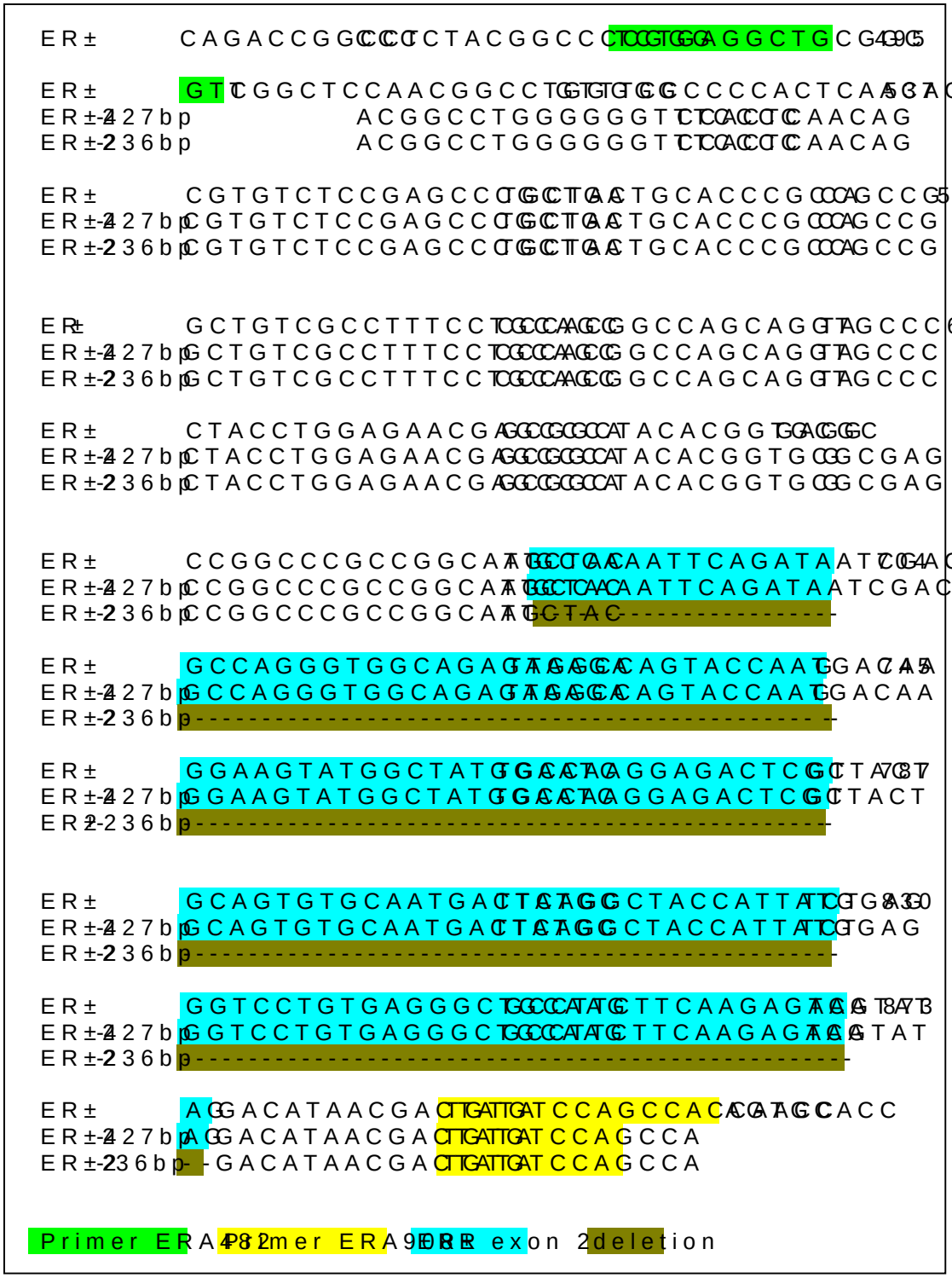


Figure 24. The gene alignment around the 427bp and 236bp PCR products with the ER± gene

The numbers on the right refer to the nucleotide number according to Pearson et al. 1988. Exon boundaries according to Ponglikitmongkol et al. 1988.

Table 6. Statistical analyses of ER±2 and significant ER ex

associations.

	ER±2		% patient	Fisher exact p
	+	-		
ER±4 +	34	2	59.0	0.005
ER±4 -	16	9	41.0	
ER±”5 +	32	3	57.4	0.042
ER±”5 -	18	8	42.6	
% patients +/exon deletion	82.0	18.0		

(+-/refers to the presence or absence of the exon or exon

Table 7. Statistical analysis of ER±”2 and ER±”6.

	ER±2		% patien	Fisher exact p
	+	-		
+	5	4	14.8	0.007
ER±6 -	49	3	85.2	
% patients +/ exon/exon dele	88.5	11.5		

(+-/refers to the presence or absence of the exon or exon de

All patients and controls revealed a band of 322bp (Figure 26) w
purified and sequenced using an automated sequencer. Sequencing revealed
presence of the complete (AEPromega, Appendix VI
B1; Figure 26) within the lesions. The presence or rare absenc
ER±3 thus does not seem to play a role in the pathogenesis of the tumours studied.

4.1.2.3 Nested PCR and sequence analysis of ER± exon 4

Nested PCR of ER± exon 4 revealed up to three bands
electrophoresis (Figure 27). Sequence analysis of the excised and p
revealed that the 555bp band represented the entire ER± e
Spectrograph, Appendix VI C1; Figure 28). Sequencing of the 219bp b
revealed the deletion of exon 4 (ER±"4) (Appendix VI C2;
Appendix VI C1; Figure 29). A third band of approximately 425bp wa
usually in conjunction with the 555bp band and was ident
random mixture of partial exon 4 clones sequencing.

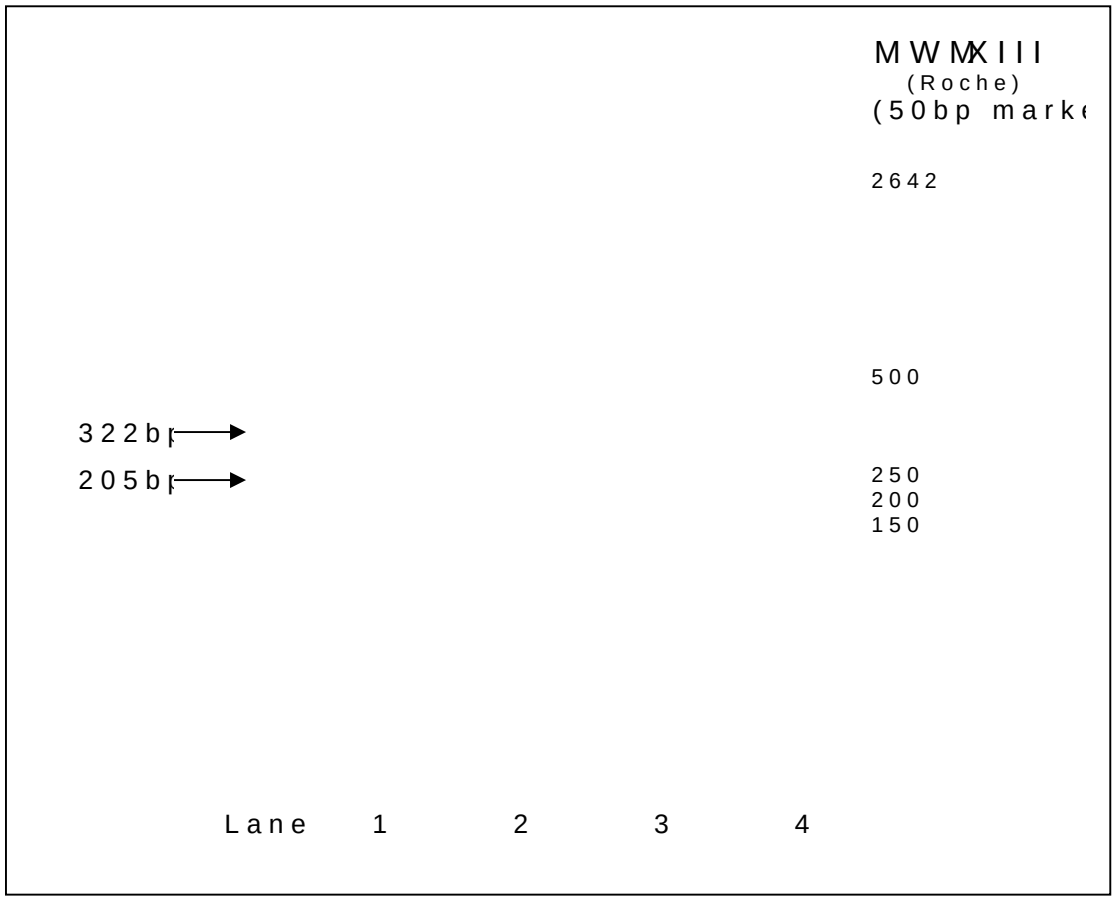


Figure 5.2 ER± exon 3 agarose electrophoresis gel

MWMXIII (Roche, SA) is a 50bp molecular weight marker (Lane 1). Lane 2 contains the PCR product including the 322bp band (Lane 5); ER±”3 PCR product represented by the 205bp band (Lanes 3, 4).

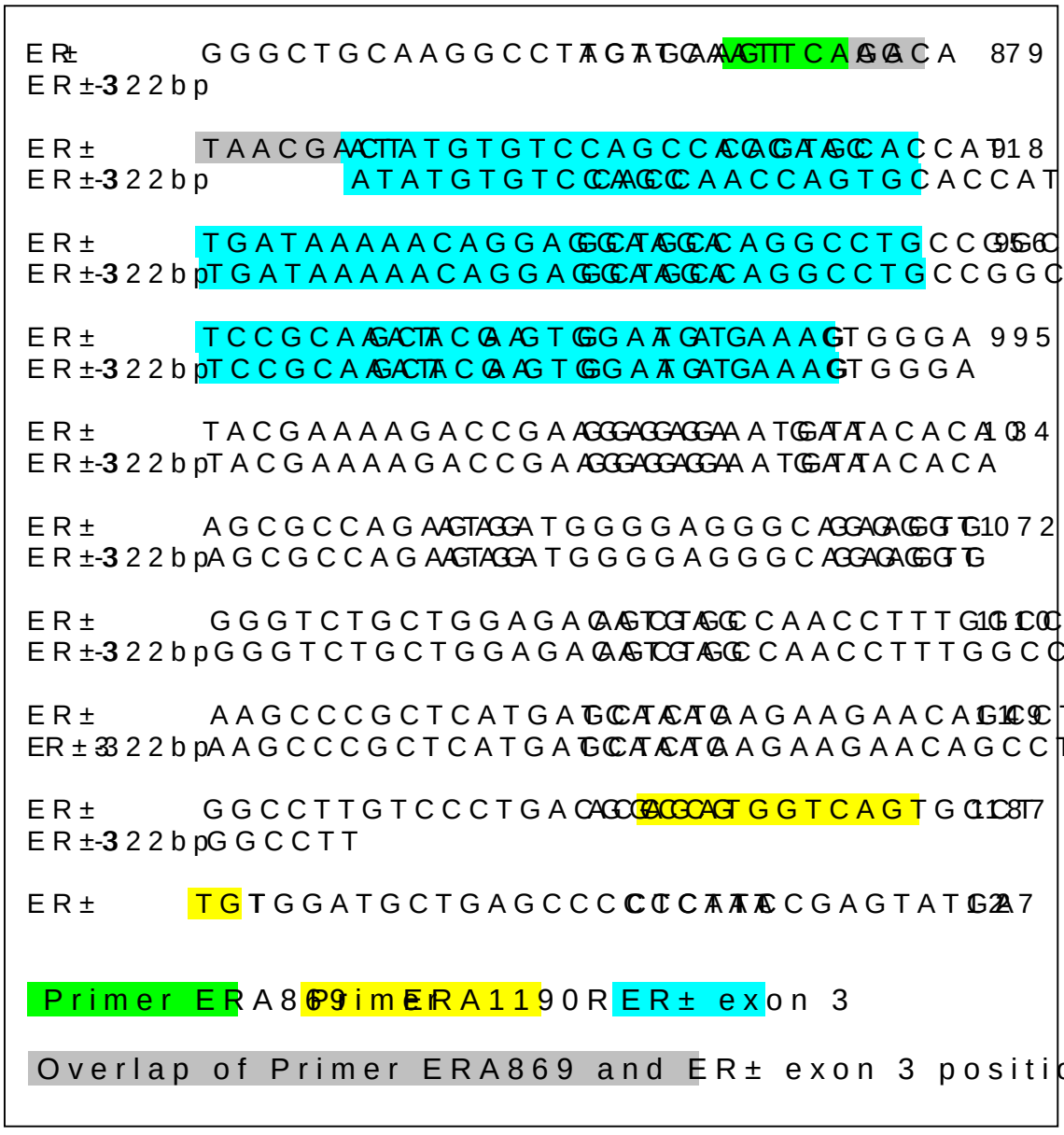


Figure 6.2 The gene alignment of ER± 322bp PCR product with the ER± gene

The numbers on the right indicate the nucleotide numbers according to Greene et al. 1986 Exon boundaries according to Ponglikitmongkol et al. 1988

Table 8. Associations between ER± exons.and tumour size (

EXON	TUMOUR SIZE (T)					% EXON (+-)	§² P
	T0	T1	T2	T3	T4		
ER±2	11	4	15	11	18	80.6	0.86
-	2	0	3	3	5	19.4	
ER±"2	10	3	14	14	21	86.1	0.27
-	3	1	4	0	2	13.9	
ER±3	13	4	18	14	23	100	
-	0	0	0	0	0	0	
ER±"3	0	0	1	0	0	1.4	0.55
-	13	4	17	14	23	98.6	
ER±4	3	4	10	9	12	52.8	0.06
-	10	0	8	5	11	47.2	
ER±"4	13	4	15	14	20	91.7	0.27
-	0	0	3	0	3	8.3	
ER±5	13	4	18	14	23	100	
-	0	0	0	0	0	0	
ER±"5	7	4	10	8	13	58.3	0.54
-	6	0	8	6	10	41.7	
ER±"5plu	0	1	2	2	4	12.5	0.55
-	13	3	16	12	19	87.5	
ER±6	13	3	18	14	23	98.6	0.00*
-	0	1	0	0	0	1.4	
ER±"6	0	0	3	1	4	11.1	0.43
-	13	4	15	13	19	88.9	
ER±7	12	4	10	11	21	80.6	0.02*
-	1	0	8	3	2	19.4	
ER±"7	11	4	16	11	22	88.9	0.50
-	2	0	2	3	1	111	
% patien	18.1	5.6	25.0	19.4	31.9		

(T0 refers to normal breast, +/- indicates presence or absence of the exon or exon deletions, * indicates significance).

Table 9. Associations between ER± exons and .Nodal involve

EXON	NODES			% EXON (+-/)	§[2] P
	0	1	2		
ER±2	15	17	15	81.0	0.50
-	5	2	4	19.0	
ER±"2	18	15	18	87.9	0.308
-	2	4	1	12.1	
ER±3	20	19	19	100	
-	0	0	0	0	
ER±"3	1	0	0	1.7	0.380
-	19	19	19	98.3	
ER±4	12	13	10	60.3	0.609
-	8	6	9	39.7	
ER±"4	19	17	16	89.7	0.542
-	1	2	3	10.3	
ER±5	20	19	19	100	
-	0	0	0	0	
ER±"5	12	10	12	58.6	0.795
-	8	9	7	41.4	
ER±"5plu	2	3	4	15.5	0.635
-	18	16	15	84.5	
ER±6	19	19	19	98.3	0.380
-	1	0	0	1.7	
ER±"6	2	4	2	13.8	0.534
-	18	15	17	86.2	
ER±7	15	13	17	77.6	0.281
-	5	6	2	22.4	
ER±"7	19	15	18	89.7	0.174
-	1	4	1	10.3	
% patien with nod	34.5	32.8	32.8		

(+-/refers to the presence or absence of the exon or exon de

Table 10. Associations between ER± exon absence or presence and the presence or absence of Metastases (M)

EXON	M		% EXON (+-/)	§[2] P
	M0	M1		
ER±2 -	38 5	11 6	81.7 18.3	0.078
ER±"2 -	37 6	16 1	88.3 11.7	0.666
ER±3 -	43 0	17 0	100	
ER±"3 -	0 43	1 16	1.7 98.3	0.628
ER±4 -	31 12	4 13	58.3 41.7	0.002*
ER±"4 -	37 6	17 0	90.0 10.0	0.252
ER±5 -	43 0	17 0	100	
ER±"5 -	27 16	8 9	58.3 41.7	0.410
ER±"5plus -	6 37	4 13	16.7 83.3	0.608
ER±6 -	42 1	16 1	96.7 3.3	0.915
ER±"6 -	3 40	6 11	15.0 85.0	0.018*
ER±7 -	32 11	15 2	78.3 21.7	0.411
ER±"7 -	39 4	15 2	90.0 10.0	0.849
% patients metastas	71.7	28.3		

(+-/refers to the presence or absence of the exon; * indicates statistical significance)

Table 11. Associations between ER± exons and Stage of dis

EXON	STAGE				% EXON (+-/)	§^{2} P
	1	2	3	4		
ER±2	4	14	19	12	81.7	0.232
-	0	2	3	6	18.3	
ER±”2	3	12	21	17	88.3	0.159
-	1	4	1	1	11.7	
ER±3	4	16	22	18	100	
-	0	0	0	0	0	
ER±”3	0	0	0	1	1.7	0.5
-	4	16	22	17	98.3	
ER±4	4	12	14	5	58.3	0.008*
-	0	4	8	13	41.7	
ER±”4	4	14	19	17	90.0	0.735
-	0	2	3	1	10.0	
ER±5	4	16	22	18	100	
-	0	0	0	0	0	
ER±”5	4	10	13	8	58.3	0.221
-	0	6	9	10	41.7	
ER±”5plu	3	15	18	14	83.3	0.596
-	1	1	4	4	16.7	
ER±6	3	16	22	17	96.7	0.06
-	1	0	0	1	3.3	
ER±”6	0	3	0	6	15.0	0.023*
-	4	13	22	12	85.0	
ER±7	4	11	16	16	78.3	0.313
-	0	5	2	2	21.7	
ER±”7	4	14	20	16	90.0	0.896
-	0	2	2	2	10.0	
% patient Stage	6.7	26.7	36.7	30.0		

(+-/refers to the presence or absence of the exon; * indicates exon de
significance)

Table 12. Associations between ER± exons and menstrual s

EXON	MENSTRUAL STAT		% EXON (+-/)	§[2] P
	Pre	Post		
ER±2	22	28	82.0	0.805
-	5	6	18.0	
ER±"2	23	31	88.5	0.745
-	4	3	11.5	
ER±3	27	34	100	
-	0	0		
ER±"3	1	0	1.6	0.907
-	26	34	98.4	
ER±4	15	21	59.0	0.820
-	12	13	41.0	
ER±"4	25	30	90.2	0.893
-	2	4	9.8	
ER±5	27	34	100	
-	0	0		
ER±"5	14	21	57.4	0.605
-	13	13	42.6	
ER±"5plu	3	7	16.4	0.519
-	24	27	83.6	
ER±6	26	33	96.7	0.577
-	1	1	3.3	
ER±"6	6	3	14.8	0.270
-	21	31	85.2	
ER±7 +	20	28	78.7	0.639
-	7	6	21.3	
ER±"7	24	31	90.2	0.893
-	3	3	9.8	
% patier with sta	44.3	55.7		

(+-/refers to the presence or absence of the exon or exon de

Table 13. Association between ER± exons and ER status.

EXON	ER STATUS		% EXON (+/-)	§^{2} P
	Positive	Negative		
ER±2	26	17	86.0	0.01*
-	0	7	14.0	
ER±"2	25	18	86.0	0.081
-	1	6	14.0	
ER±3 +	26	24	100	
-	0	0		
ER±"3	0	0		
-	26	24	100	
ER±4	21	12	66.0	0.046*
-	5	12	34.0	
ER±"4	22	22	88.0	0.741
-	4	2	12.0	
ER±5	26	24	100	
-	0	0		
ER±"5	20	9	58.0	0.011*
-	6	15	42.0	
ER±"5plus	3	3	12.0	0.741
-	23	21	88.0	
ER±6	25	24	98.0	0.968
-	1	0	2.0	
ER±"6	1	5	12.0	0.158
-	25	19	88.0	
ER±7	19	20	78.0	0.594
-	7	4	22.0	
ER±"7	24	20	88.0	0.589
-	2	4	12.0	
% patients ERICA sta	52.0	48.0		

(+-/refers to the presence or absence of the exon or exon indicates significance)

It was observed that 30 (49%) of breast carcinoma patien
presence and absence of ER± exon 4. Only 6 (10%) showe
alone and 25 (41%) exhibited ER±4 alone. All the control patients 3 (
had the complete ER± exon 4 combined with ER±"4 within
(77%) exhibited ER±"4 alone. None of the control patients exhib
exon 4 alone and the presence of intact ER±4 was analysed
accompanied by the artifact band (425bp) on PCR.

§^{2} analyses performed on results comparing normal and disc
that ER±4 is not significant with respect to tumour size
involvement (N) (Table 9) and ER±4 status (Table 12).

The presence of the complete ER±4 exon, however, has si
with the presence of metastasis (M) ($p=0.0025$) (Table 10), stage of di
($p=0.008$) (Table 11) and ER ($p=0.046$) status (Table 13). As alre
described, ER±4 has a significant association with ER ($p=0.025$) (Fishe
(Table 6). Statistical significance was also observed betw
(Fisher exact $p=0.025$) (Table 14).

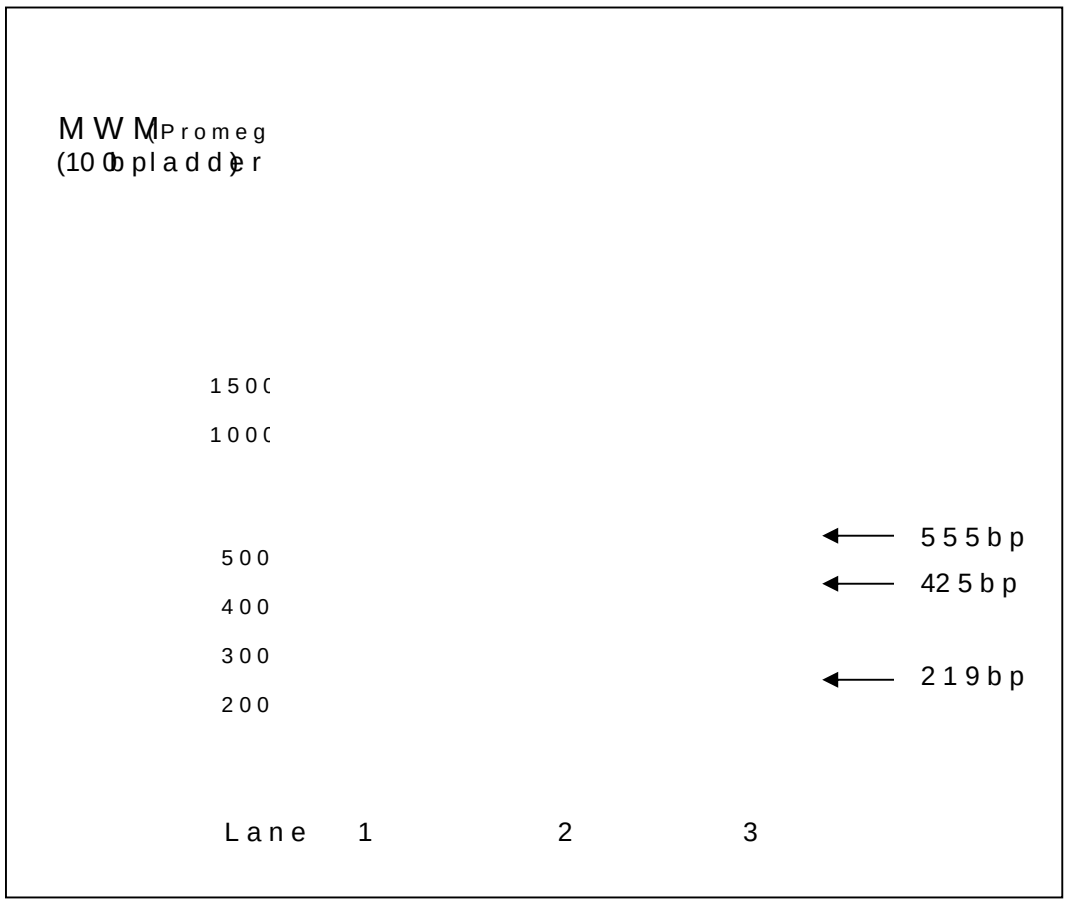


Figure 7.2 ER ± exon 4 agarose electrophoresis gel

100bp ladder (Promega, MWM) (lane 1); PCR product is inc
555bp band (lanes 2 and 3); PCR product is represented b
band (lane 3), the 425bp band (lanes 2 and 5) is a PC

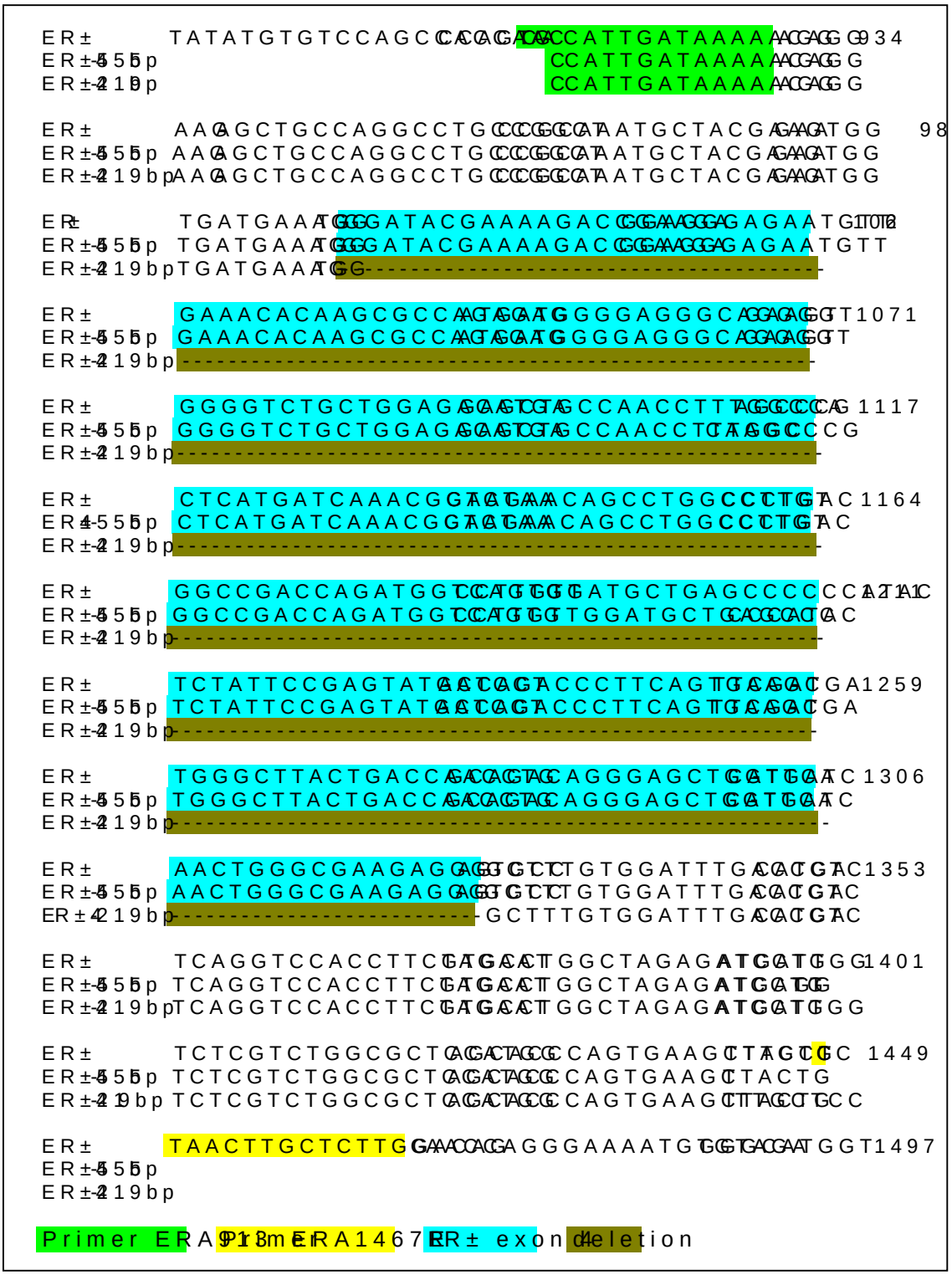


Figure 28. The gene alignment of ER± 555bp and 219bp PCR products with the ER± gene

The numbers on the right refer to the nucleotide numbers (according to Ponglikitmongkol 1988). Exon boundaries according to Green 1986.

Table 14. Statistical analysis of ER±4 and ER±”6.

	ER±4		% patien	Fishe exact p
	+	-		
+ ER±6 -	2 34	7 18	14.8 85.2	0.025
% patien-ts +/ exon/exon dele	59.0	41.0		

(+-/refers to the presence or absence of the exon or exon de

4.12.4

NesteRdCR and sequence analysis of ER± exon 5

Nested PCR of ER± exon 5 rethveeaeldbaumdstoon agarose g electrophoresis 2(9F.iSgeuqrueencing analysis of thepuerxifiiesd an bands indicated that the 544bp band included Fhiguecomplet 30;Appendix SIvectrographs D1aAappendDdiix;DV1). Sequencfing the 405bp band revealed the absence of theFiegnutree 3e0x,on 5 Appendix SIvectrographahsanDd2DA2bpp,endixDV2)and sequencing of the 80bp (fauntdher referred to asreEvRe±a’lapdsmsa)ll segment PCR product which amaPyCRbeartofiacttruncated ER± varia(Fnigurofe exon 30;Appendix SIvectrographAhppDe3n,dixDV3).

The entire ER± exon 5 wasalexpeasstscednicer patis(n5t4abmple band)ER±”5 (405bp band) was expressed pna3t5ns(57a%)nd o1f0th

(16%) exhibited the 80bp band. All the control patients als
of the entire sequence of ER±5 and 7 (54%) exhibited ER±"

§^{2} analyses of ER±5 and ER±"5 and clinical parameters indi
with respect to tumour size (T) (Table 8), nodal involve
presence or absence of metastases (M) (Table 10), stage o
menstrual status (Table 12). The deletion variant ER±"5, h
with regard to earlier (p=0.011) (Table 12). Fisher's exact tests perfor
on ER±5 and its variants revealed that ER±"5plus is sign
ER±"5 (Fisher p=0.034) and ER±6 (Fisher p=0.025) (Table 15).

4.1.2.5 Nested PCR and sequencing of ER± exon 6

Nested PCR of ER± exon 6 showed two bands (Figure 32, lane 3). The 388bp band
includes the complete ER± exon 6 and this band was obser
control (Figure 32, lane 2). Sequencing of the 388bp band (Appendix 3, Electrograph E1a; Appendix E1b; E1).
A faint second band of 254bp was observed simultaneously in
There was not sufficient PCR product to sequence this ban
an absence of ER± exon 6 (ER±"6).

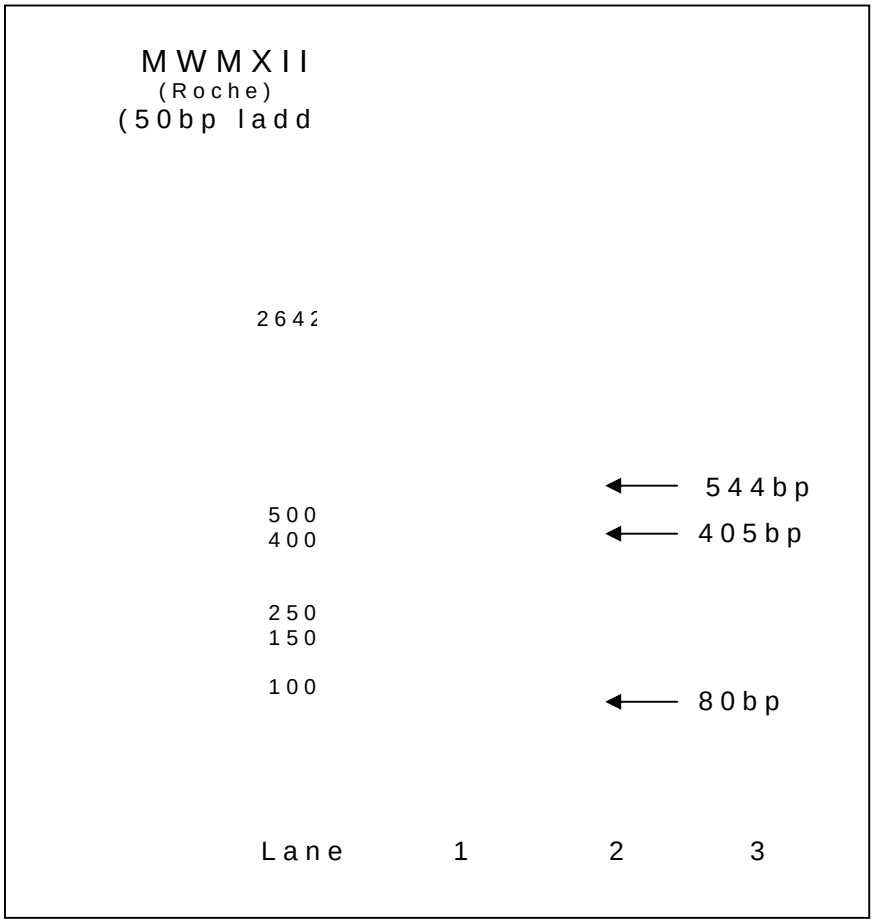


Figure 9 ER± exon 5 agarose electrophoresis gel

MWM XIII (Roche, SA) is a 50bp molecular weight marker. ER± PCR product is represented by the 544bp band (lane 2) and ER± PCR product is represented by the 405bp band (lane 2) and 80bp band (lane 3). Primer dimers artifacts are present (lanes 2 and 3).

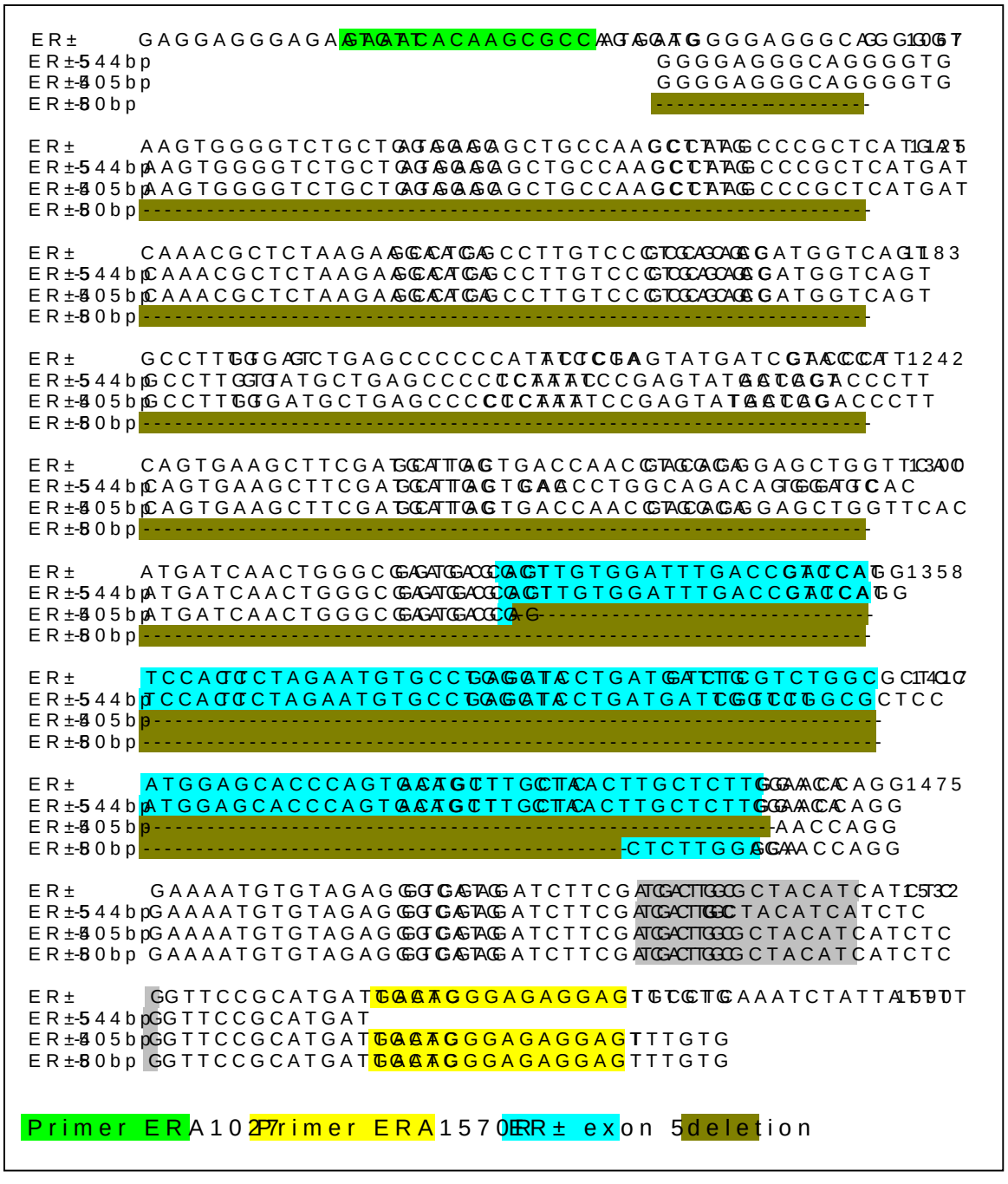


Figure 30 The gene alignment of 544bp, 405bp and 80bp second round PCR products with ERR± gene.

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The numbers on the right refer to the nucleotide number according to Powell et al. 1987. Exon boundaries according to Powell et al. 1987.

Table 15. Statistical analyses of ER±"5 plus ER±"5 compared with ER±"6 and ER±6.

	ER±"5 plus		% patient	Fisher exact p
	+	-		
ER±"5 +	9	26	57.4	0.034
ER±"5 -	1	25	42.6	
ER±6 +	8	51	96.7	0.025
ER±6 -	2	0	3.3	
% patient-test exon/exon deletion	16.4	83.6		

(+/- refers to the presence or absence of the exon or exon deletion)

§[2] analyses performed on ER±6 results and clinical parame significance with regard to nodal involvement (N) (Table (Table 12) or ERICA status (Table 13). ER±6 is, however, tumour size (p=0.002) (Table 8) and ER±"6 is significant wit (Table 10, p=0.018) and stage of disease (Table 11). Fisher s tests performed on ER±6 indicated significance with rega variant ER±"5 plus, (Fisher p=0.025) as previously described (Table The ER±6 deletion variant, ER±"6, also showed significa ER±"2 (Fisher p=0.007) (Table 7) and ER±4 (Fisher p=0.025) (Table 14) as previously described.

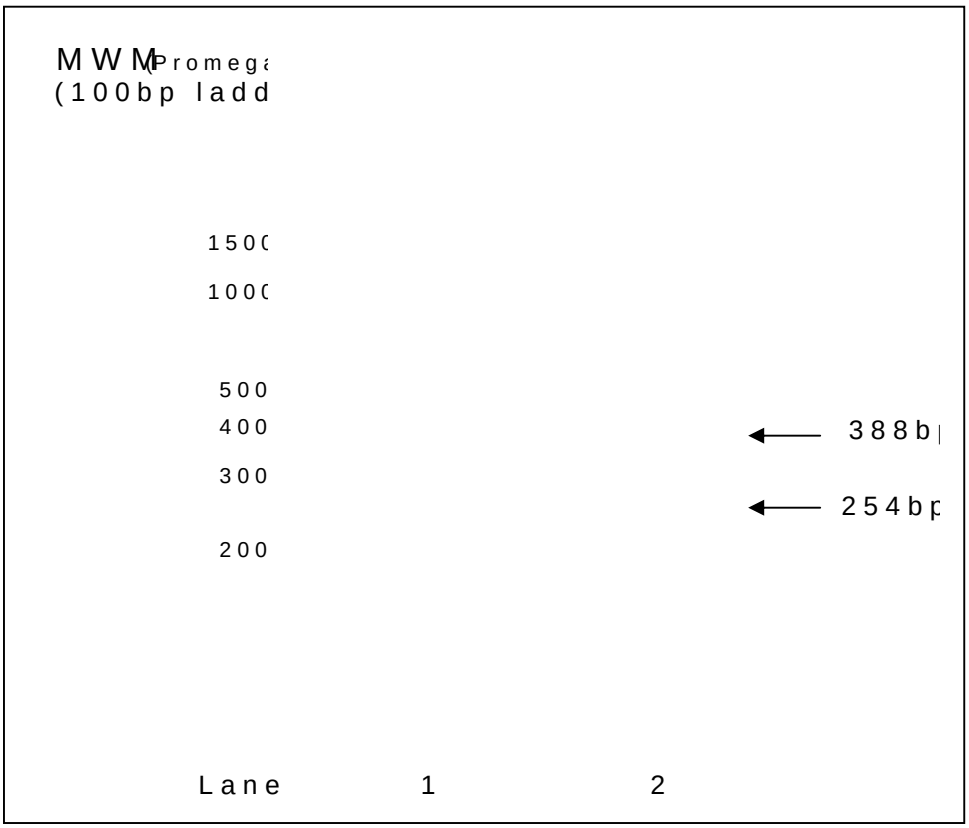


Figure 1.3 ER± exon 6 agarose electrophoresis gel

MWM 100bp ladder (Promega); Lane 1: ER± exon 6; PCR product is included i
388bp band; Lane 2: ER±” 6 PCR product presented by the 254bp ba

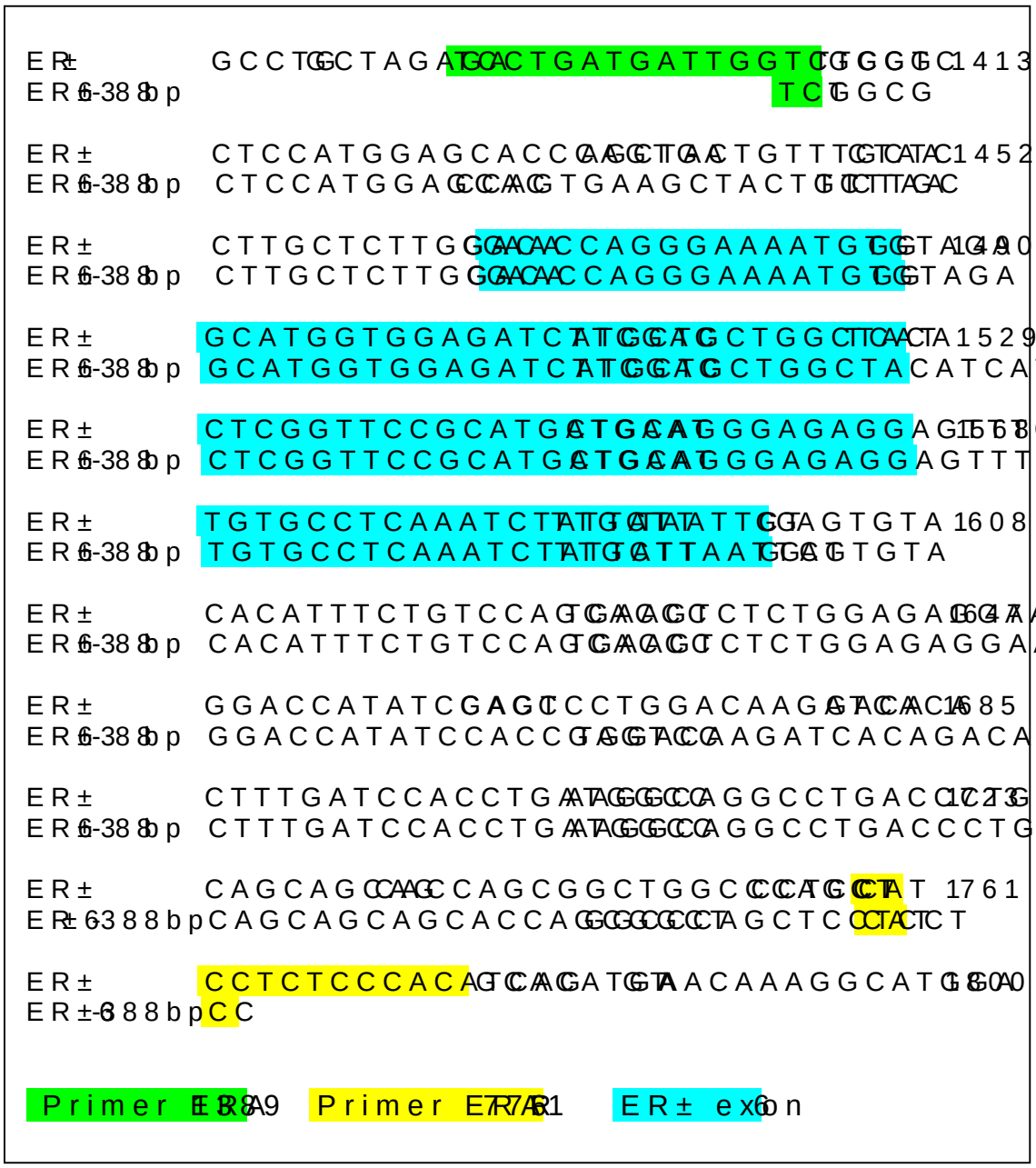


Figure 2 The gene alignment of ER± -6388bp second round PCR product with the ER± gene.

The numbers on the right indicate the nucleotide numbers according to Ponglikitmongkol et al, 1988. Exon boundaries according to Ponglikitmongkol et al, 1988.

4.12.6

Nested PCR and sequence analysis of ER± exon 7

Nested PCR of ER± exon 7 revealed up to three bands
electrophoresis (Fig. 3). Three size of the band
excised and purified bands revealed the 345bp band represented
presence of the ER± exon 7 (Figure 4A, Appendix IV, Spectrograph F1a
Appendix IV). This band was expressed in 48 (79%) of the p
(92%) of the controls. 6 (9%) patients and 2 (15%) controls
present without the 345bp band. The 310bp band repres
presence of ER±7 with random base pairs missing. This m
PCR or artifact and was usually associated with the expression of the 3
(Figure 4A, Appendix IV, Spectrograph F2a, Appendix IV).

Sequencing of the 161bp band indicated the complete abs
(ER±"7) (Figure 4A, Appendix IV, Spectrograph F3a, Appendix IV). This
band was expressed in 42 (69%) of patients and 345bp obtained wi
representing the complete ER± exon 7. In the 7% control
expressed both bands (22%) of patients and 1 control (8%) t
expressed alone.

χ^2 analyses of ER±7 and its variant ER±"7 indicated that ER
respect to tumour size ($p=0.026$) (Table 8) but none of the oth
parameters such as N, M, stage of disease, menstrual sta
(Tables 9, 10, 11, 12 and 13).

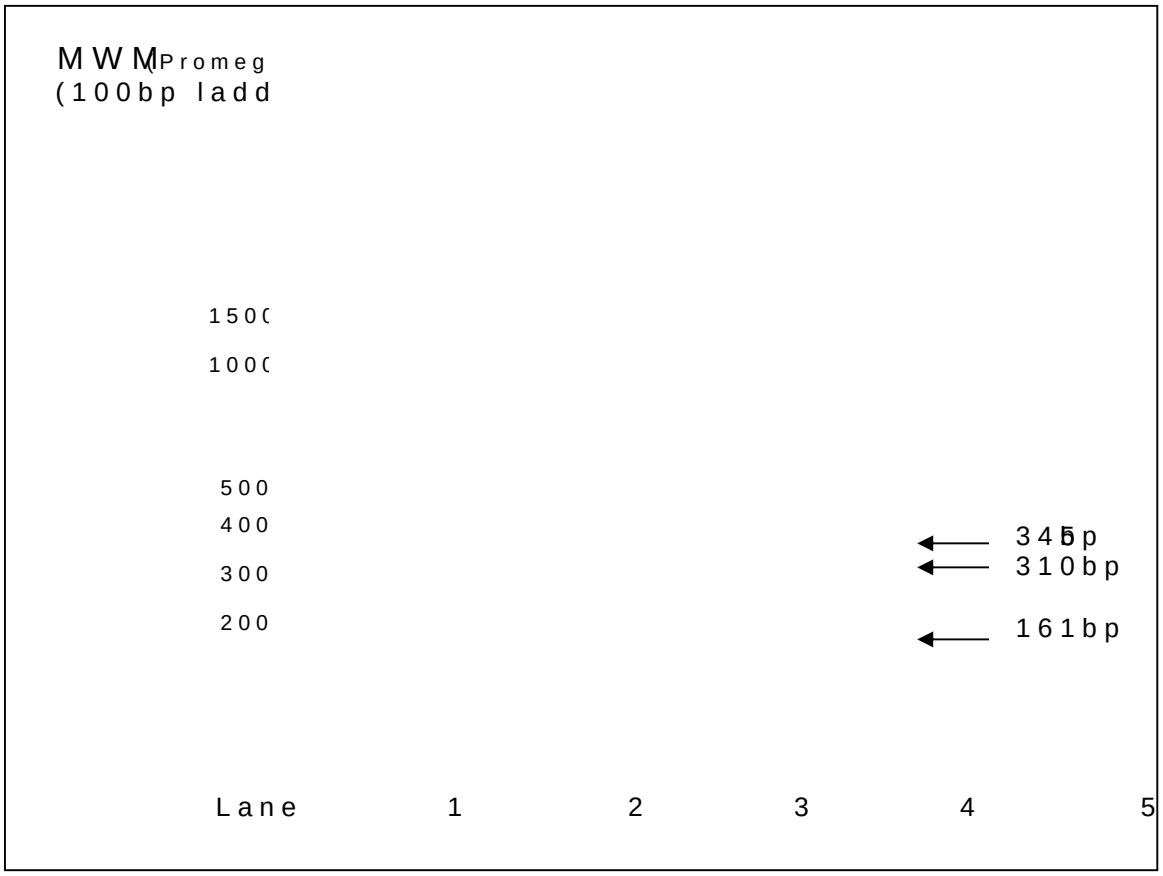


Figure 3.3 ER± exon 7 agarose electrophoresis gel

MWM 100bp ladder (Promega, RS±A7) PCR product is included i
345bp band (lanes 3 and 5); ER±"7 PCR product is represented by t
band (lanes 3, 5 and 6). 161bp band represents ER±7 with random base pairs
missing (PCR artifact).

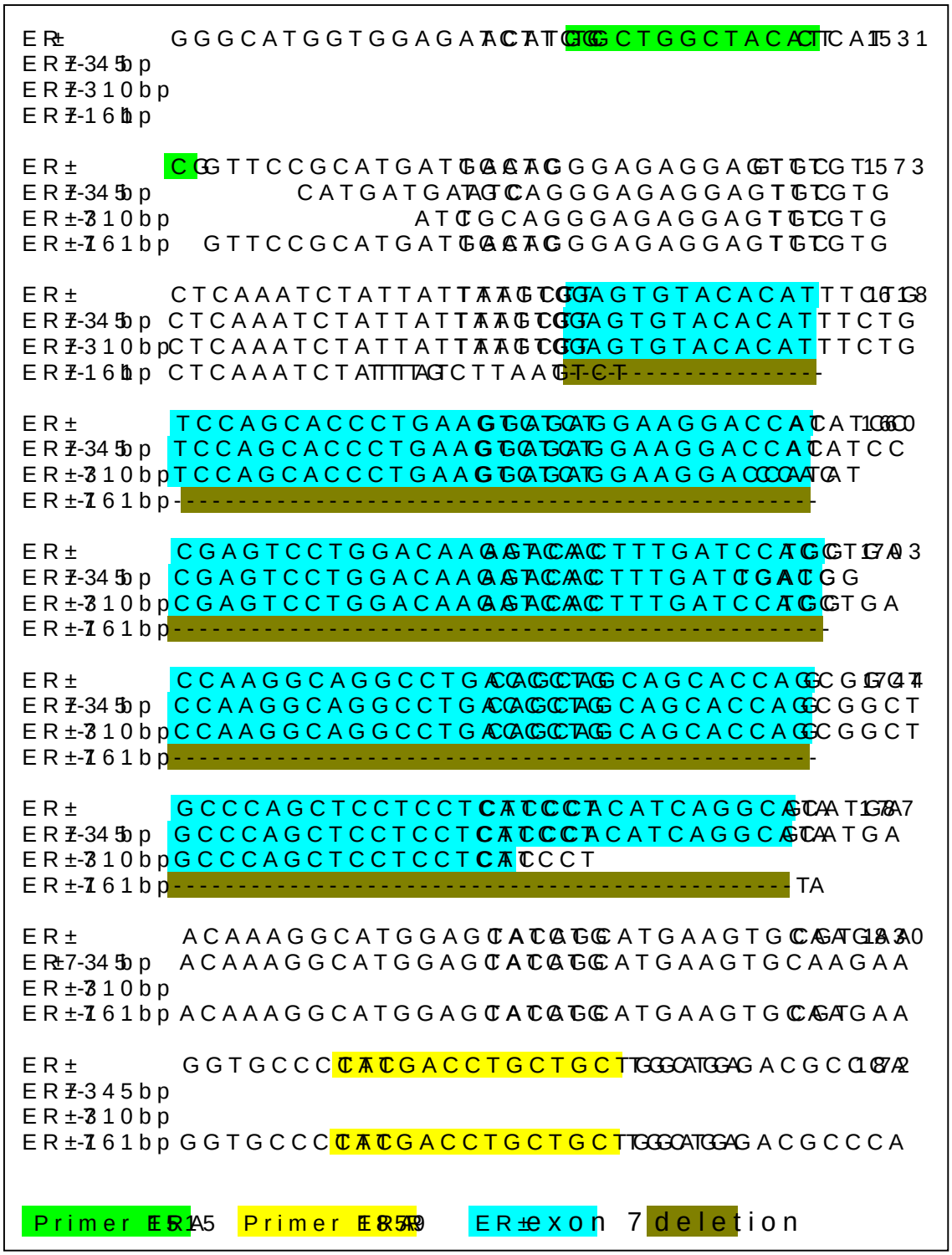


Figure 3.4 The gene alignment of 345bp, 310bp and 161bp nested PCR products with the ER+ gene.

The numbers on the right indicate the nucleotide numbers according to Exon boundaries according to Ponglikitmongkol et al, 1988.

ERα7 was not significant with respect to any of the param
and ERα7 were not observed as significant with regard to
or ERβ2.

4.1.3 Nested PCR and sequence analysis of ERβ1 in clinical samples

Nested PCR and sequence analysis of ERβ2 was performed on few
ERα variants due to the lack of sample material (FNA) after
ERα exons.

4.13.1 Nested PCR and sequence analysis of ERβ2 DBD

The number of clinical patient samples with sufficient material to assess ER
was 28 out of the original 56 (46%) samples (3.8% of 13 d(
sufficient material for a PCR analysis individual ERβ2 exons c
be analyzed as with the ERα exons

As described in Chapter 3, the first round of nested PCR of the ERβ2
RT-PCR of a fragment of ERβ2 located between exon 2 and
round of nested PCR utilized primers designed within exon
amplify the segment of the gene including the DNA binding domain of ERβ2

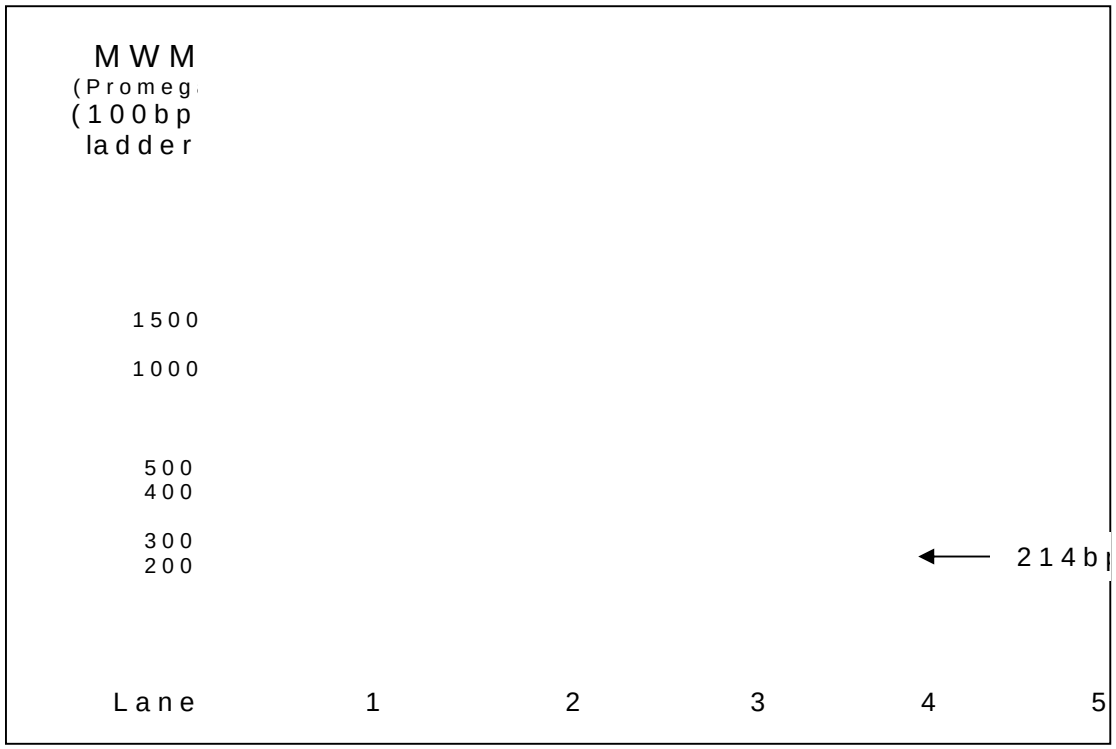


Figure 5 ER² DNA Binding Domain agarose electrophoresis g

MWM 100bp ladder (Promega); ER² DBD PCR product is inclu
the 214bp band (lanes 1, 2 and 5); lanes 3, 4, 6 and 7 are negative for ER²DB

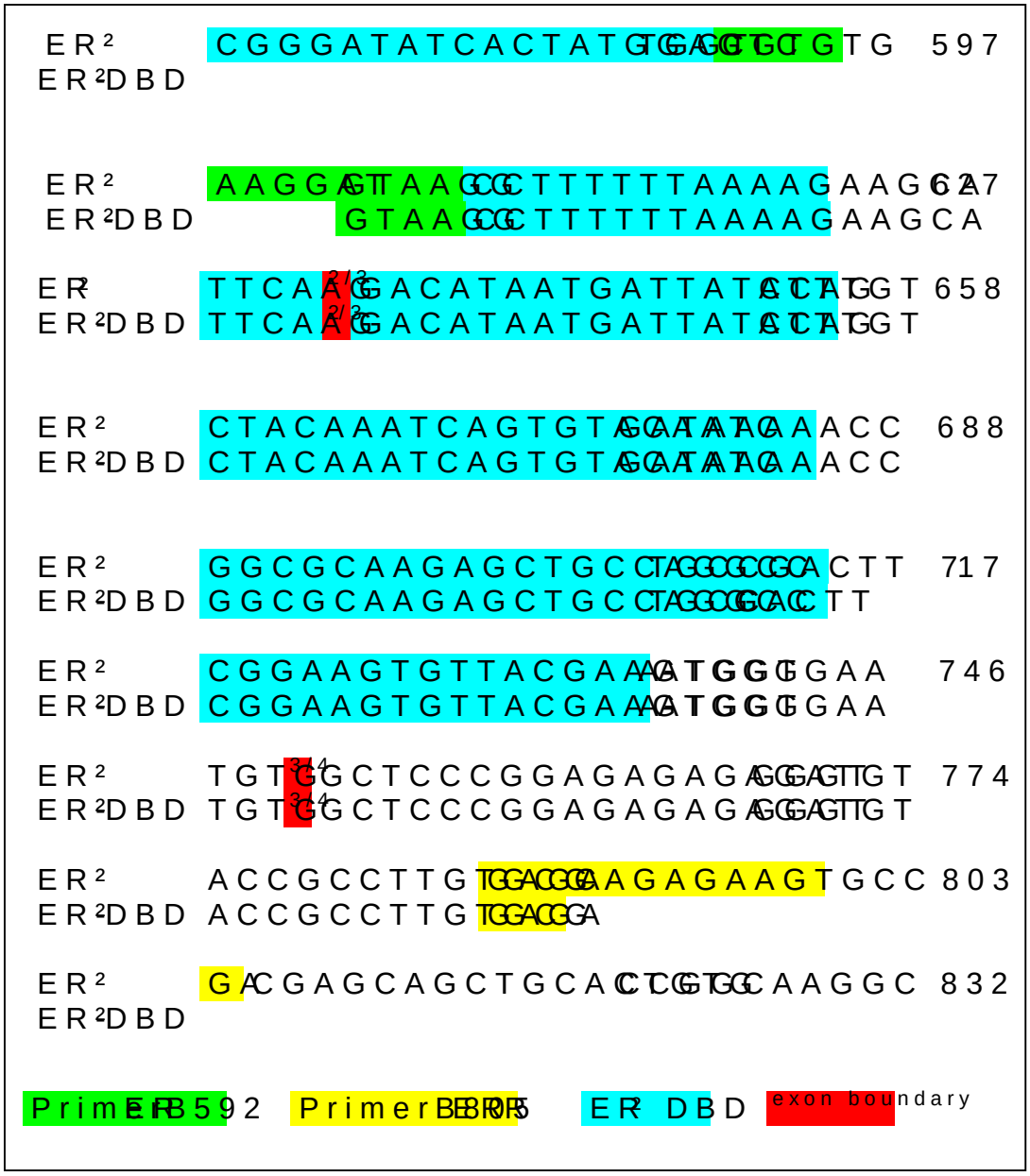


Figure 6 The gene alignment of DBD ERβ1 and second round PCR product with the ERβ gene

The numbers on the right refer to the nucleotide numbers
al. 1998a. Exon boundaries and position of LBD according t
2004.

In 24 (86%) of clinical patients a band of the expected size on agarose gel electrophoresis (Figure 35). In the contr revealed the presence of this fragment. The sequence o confirmed using automated sequencing (Figure 36, Appendix V A1a and A1b; Appendix VII).

§^{2} analysis of these results showed that ER²DBD expressio with respect to clinical parameters such as tumour size (T), involvement (N), presence or absence of metastases (M), or menstrual status of the patients studied.

Table 16. Associations between ER²DBD and ER²LBD and Tumour size (T).

EXON	TUMOUR SIZE (T)					% EXON (+/-)	§^{2} P
	T0	T1	T2	T3	T4		
ER²DBD+	23	20	70	62	82	78.1	0.145
-	3	0	0	2	2	21.9	
% patient for ER²DBD	15.6	6.2	21.9	25.0	31.2		
ER²LBD+	50	00	90	42	91	90	0.159
-	0	0	0	2	1	10	
% patient for ER²LBD	16.7	0	30.0	20.0	33.3		

(T0 refers to normal breast tissue sample; +/- refers to the presence or absence of the exon)

4.13.2

Nested PCR and sequence analysis of ER² LBD

The number of clinical patients with assessable ER² LBD RNA was 26 out of the original 61 (43%) and 5 control samples out of 13 material for analysis.

First round PCR of the ER² gene was performed as described in order to amplify the segment of the ER² gene encoding the ligand binding domain a second round utilizing PCR primer set designed within Exons 6 and 7 was performed. The resulting amplified fragment of 282bp (Figure 8) was observed in 23 (89%) of the clinical patient samples analyzed and in 1 (20%) of the control samples. The position of this fragment within the ER² gene was confirmed by automated sequencing (Figure 8) (Appendix V). Spectrographs B1a and B1b (Appendix B1).

Statistical analysis of the ER² LBD results showed that as ER² LBD expression was significant with respect to clinical parameters such as tumour size (T), nodal involvement (N), presence or absence of metastases (M), stage of disease or menstrual status of the patients studied.

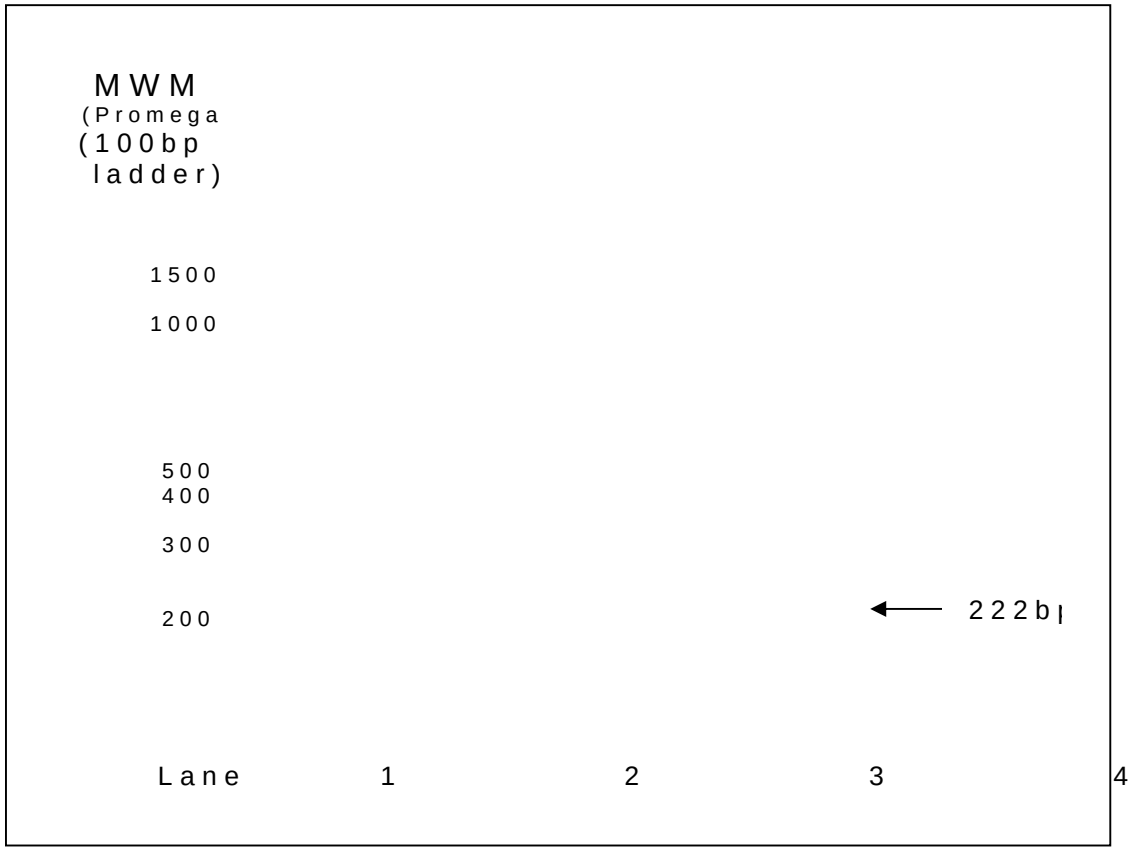


Figure 3.7. ERα Ligand Binding Domain agarose electrophoresis.

MWM 100bp ladder (Promega); lanes 1-5, ERα LBD PCR products. Lanes 2 to 5 include the 222bp band (lanes 2 to 5).

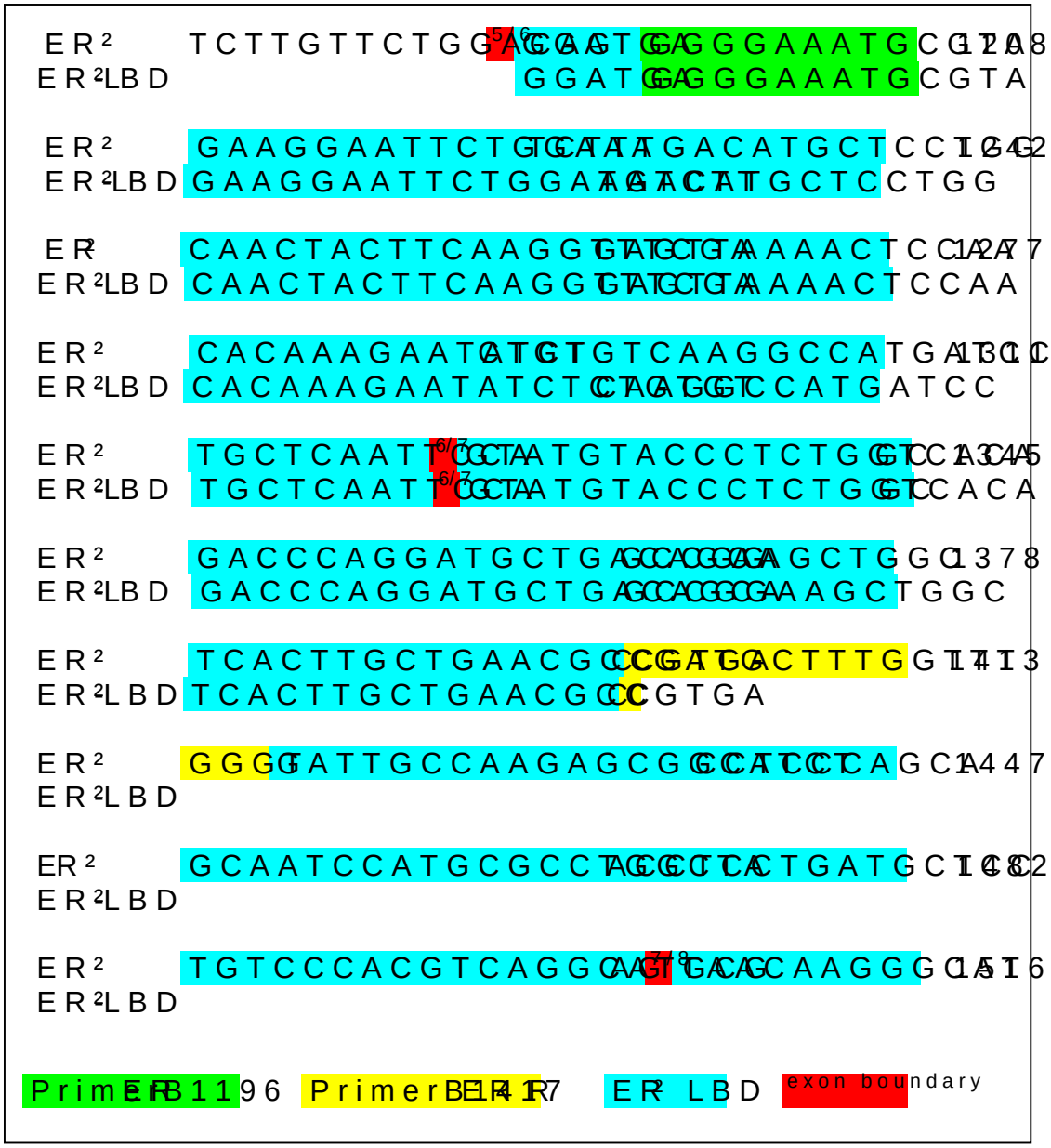


Figure 8. The generated alignment of ER2 LBD 222 second round PCR product with the ER2 gene

The numbers on the right refer to the nucleotide numbers
al 1998a. Exon boundaries and position of LBD according t
2004.

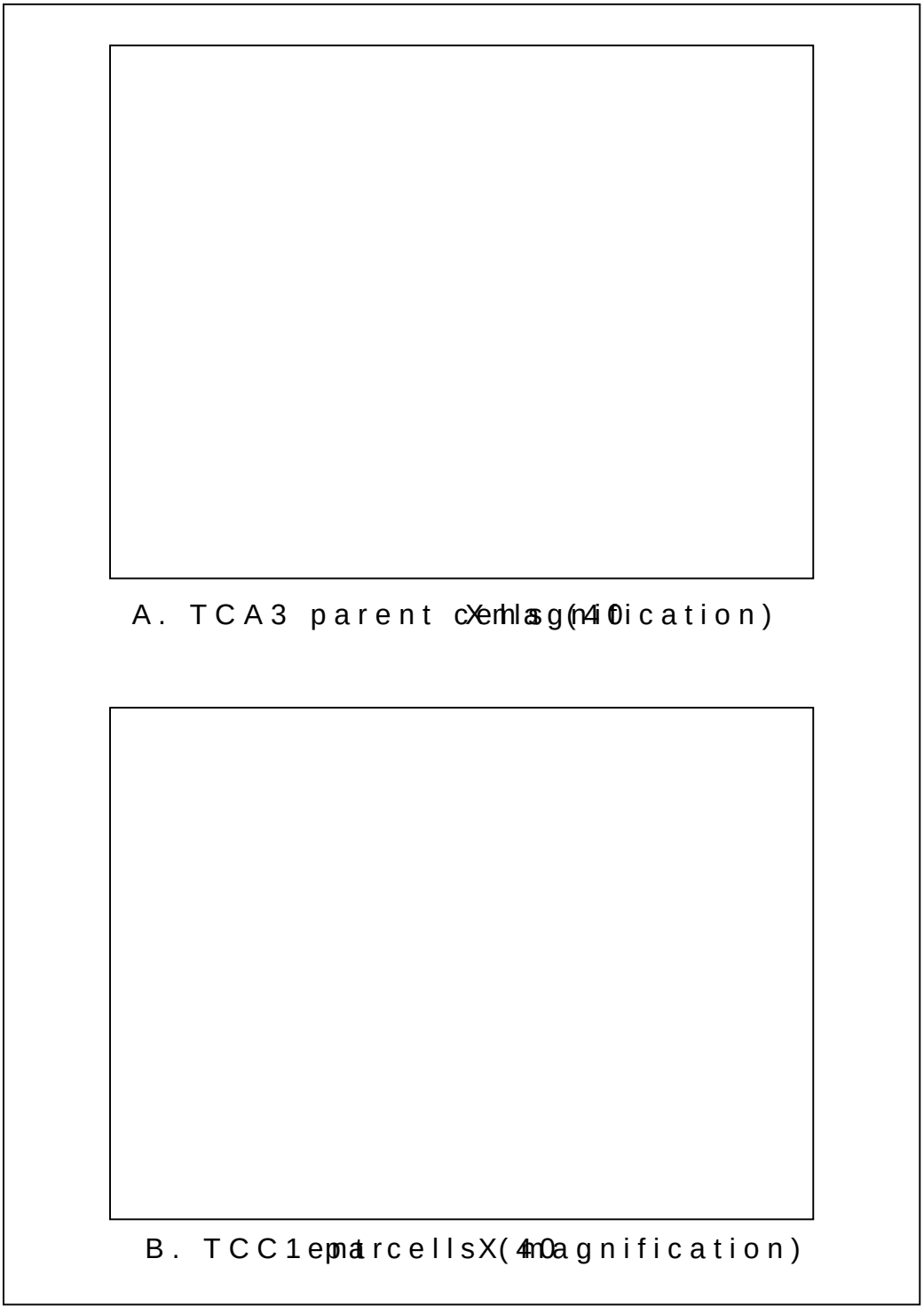
4.1.4 Response to Hormone Treatment and Survival Analysis

Fisher's exact tests performed comparing each of the ER± patient responses to tamoxifen treatment in the cohort and the ER± variant played a significant role. Similarly, Kaplan-Meier survival analysis in each case revealed no significance with respect to any exon variant.

4.2 The Cloning of T-47D cells

In a pilot study, MCF-7 cells were cloned as described in Chapter 3. These MCF-7 cells, however, showed no response to oestrogen in response testing and therefore T-47D cells were adopted as the model for studies.

T-47D cells were cloned as described in Chapter 3. Initial oestrogen response testing indicated that T-47D cells are oestrogen responsive. Five colonies were selected for evaluation using oestrogen response RT-PCR testing (Figure 4.9). Two clones were selected, namely C4 and C13, which was observed to be oestrogen and tamoxifen responsive (Figure 4.10A) and C1, which showed resistance to tamoxifen (Figure 4.10B).



A. TCA3 parent cells (40X magnification)

B. TCC1 parent cells (40X magnification)

Figure 39. Slides of parent cells of TCA3 and TCC1 (40X magnification)

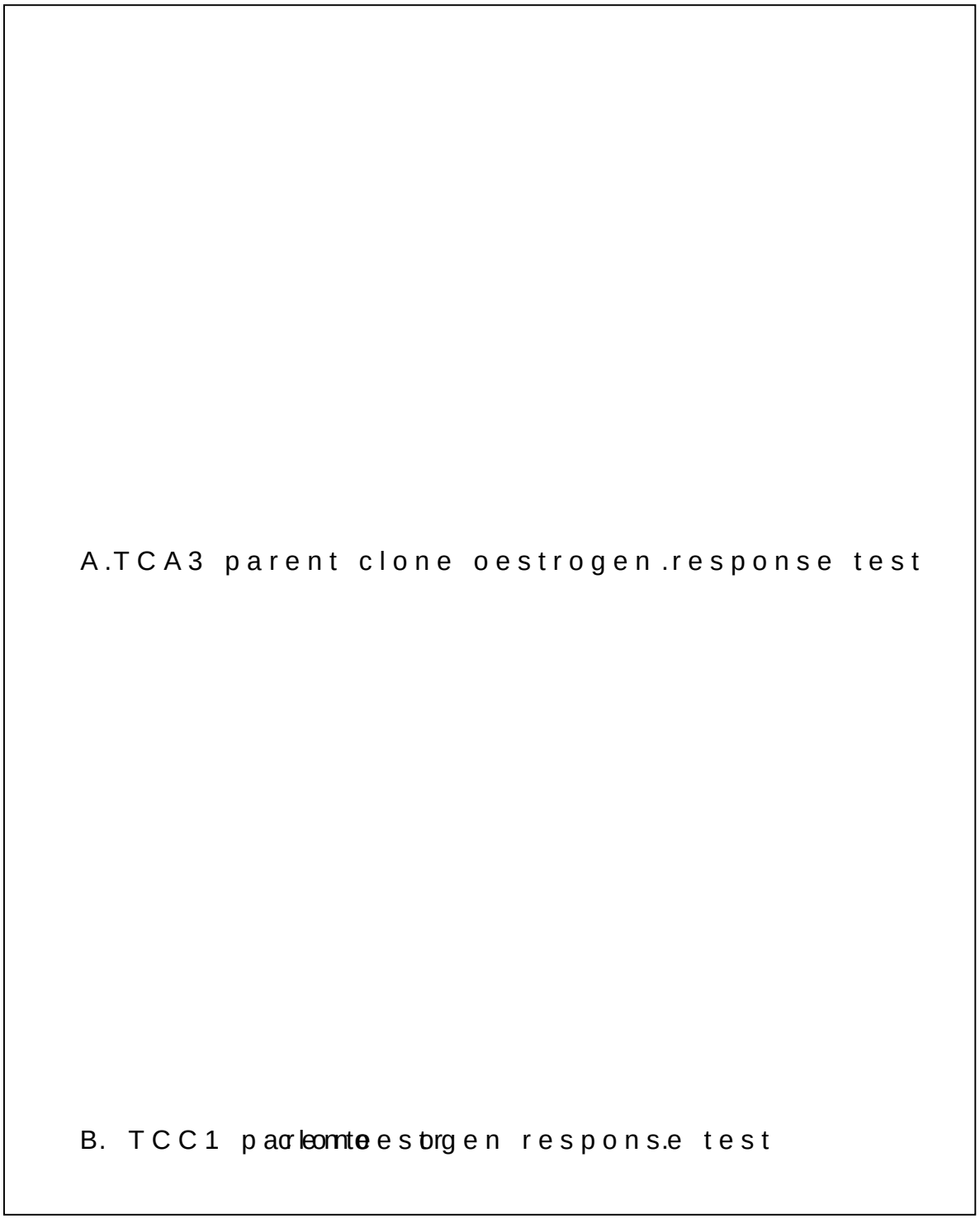


Figure 4.0 Graphs of oestrogen response tests of parent lines T-47D clones TCA3 and TCC1
(red arrow indicates time of oestradiol supplementation see Chapter 3.10)

Slides of parent cells of clone were stained and viewed at 400X magnification as described in Chapter 3.11 (Figure 39).

Both clones expressed the complete ER± gene, as confirmed by RT-PCR. The TCA3 parent clone also expressed ER² mRNA (Figure 40), as did ER"4, ER"5 and ER"7 (Table 17). The TCA3 parent clone and the entire PCR regions of both ER²DBD and ER²LBD as described

4.3

Induction of ER mutation by environmental manipul

T-47D parent clones TCCCA13 were treated per trial as follows: Each clone was grown in plates containing DMEM (control plates) as well as petri dishes containing the following respective concentrations: 17²-oestradiol, 10^{-8}M; 4-hydroxytamoxifen, 10^{-8}M; anoglutethimide, 10^{-8}M; androstenedione, 10^{-8}M; oestriol, 10^{-8}M and cholesterol, 10^{-8}M. Hormone response tests were performed after the tenth passage and RNA was extracted for RT-PCR analysis of ER± and ER².

4 3. 1

TCA3 clone responses

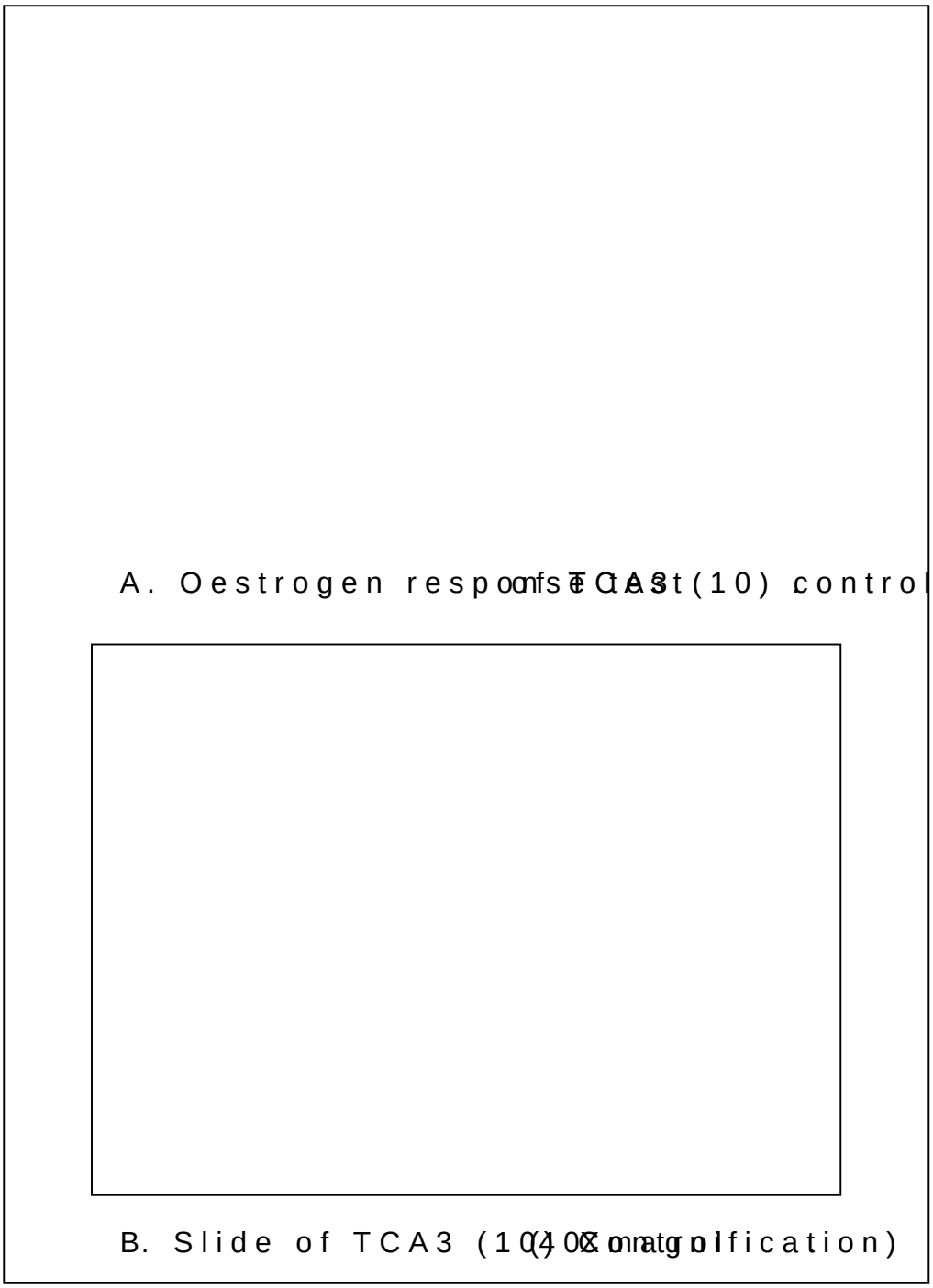
The TCA3 clone was observed to grow faster than both the TCC1 clone grew very slowly in medium containing 10^{-8}M tamoxifen. RNA was extracted from TCA3 cells grown in 10^{-8}M tamoxifen for 10 passages distributed to cover up and declining the oestrogen response upon the separation of the slides. Similarly, TCA3 cells grown in 10^{-8}M oestrogen survived for passages and although RNA was extracted they did not to the substrate during the oestrogen response. The two conditions were therefore not available.

The TCA3 control cells for 10 passages (TCA3 [10]) the same ER± and ER² profiles and the same hormone responsiveness clone (Figure 4, Tables 17 and 18). The hormone response profi conditions also remained the same as the parent clone (Figures 2, 3 and 4). The ER±3, ER±6 (Tables 17 and 18) and ER²LBD profiles in all were observed as complete and unchanged from the parent and ER±7 had both their complete exons and their deleted simultaneously as observed in the parent clone. In addition, in 10^{-8}M aminoglutethimide, the TCA3 clone expressed both the exon as well as ER±"4. 10^{-8}M aminoglutethimide revealed ER alone thus deviating from the parent clone profile (Table 18

Table 17. Tabulation of hormone responses and ER± and ER² exon profiles of MCF7 parent clones TCA3 and TCC1

Clon	Hormon Respon Test	ER±"	ER±3	ER±"	ER±"	ER±"	ER±"	ER² DBD	ER² LBD
TCA	Strongl oestrog respons Tamoxi respons	+	-	+	+	-	+	+	+
TCC	Slightly oestrog respons Tamoxi resistar	-	-	+	-	-	-	+	+

(+ orrefers to the presence or absence of the exon deletion simultaneous presence of the complete exon)



A. Oestrogen response of TCA3 (10) control

B. Slide of TCA3 (10) control (40X magnification)

Figure 1.4 TCA3 clone at passage 10

A. Graph of oestrogen response of TCA3 (10) control cells (red arrow indicates time of withdrawal/tamoxifen supplementation as described in Chapter 3.10)

B. Slide photograph of TCA3 (10) control cells

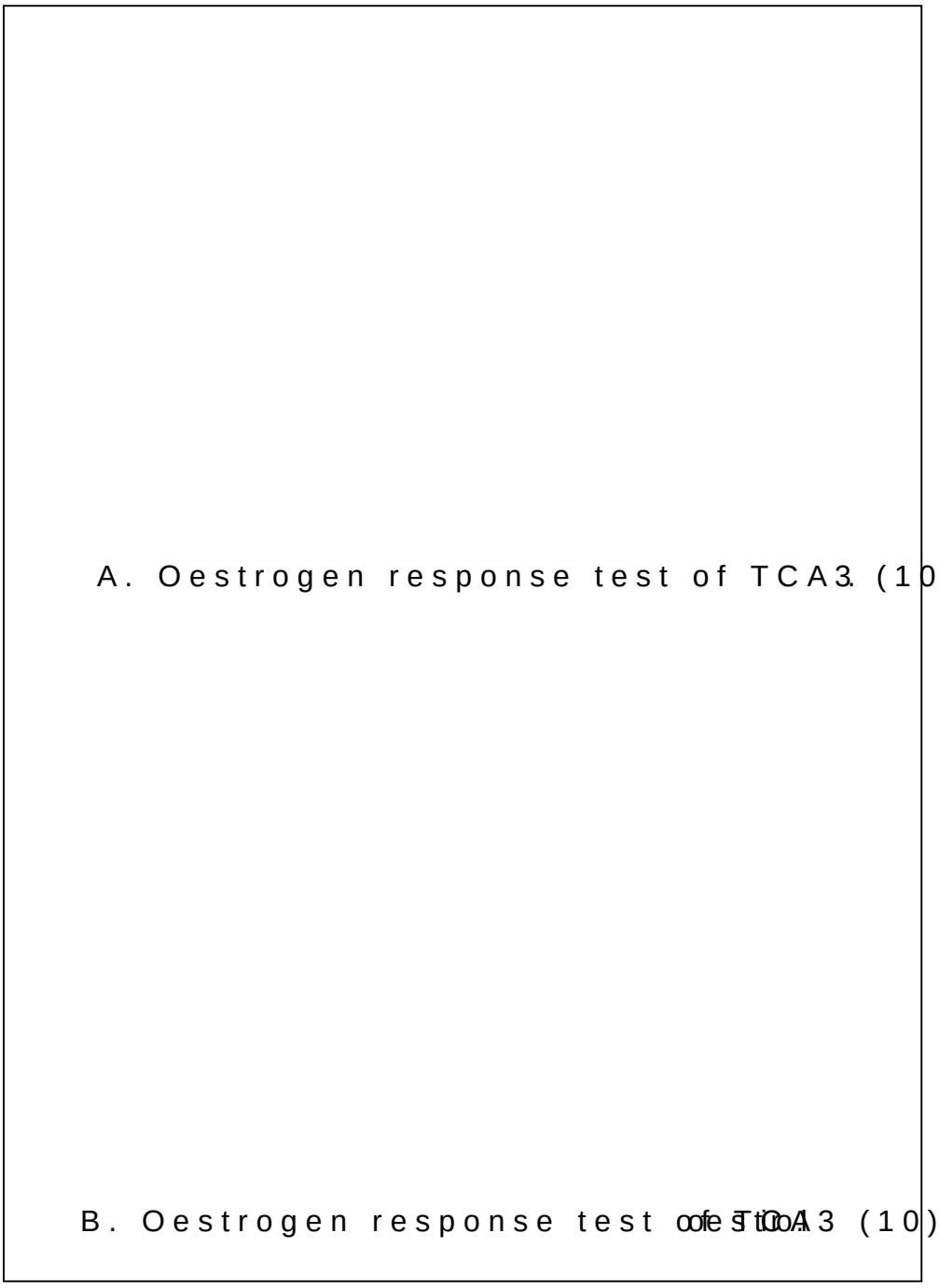


Figure 2.4 A. Graph of oestrogen response test of TCA3 clone at passage (10) grown in 10^{-8}M 10 oestradiol)
B. Graph of oestrogen response test of TCA3 clone a passage (10) grown in 10^{-8}M 10 oestriol)
(red arrow indicates time of oestradiol/tamoxifen su as described in Chapter 3.10)

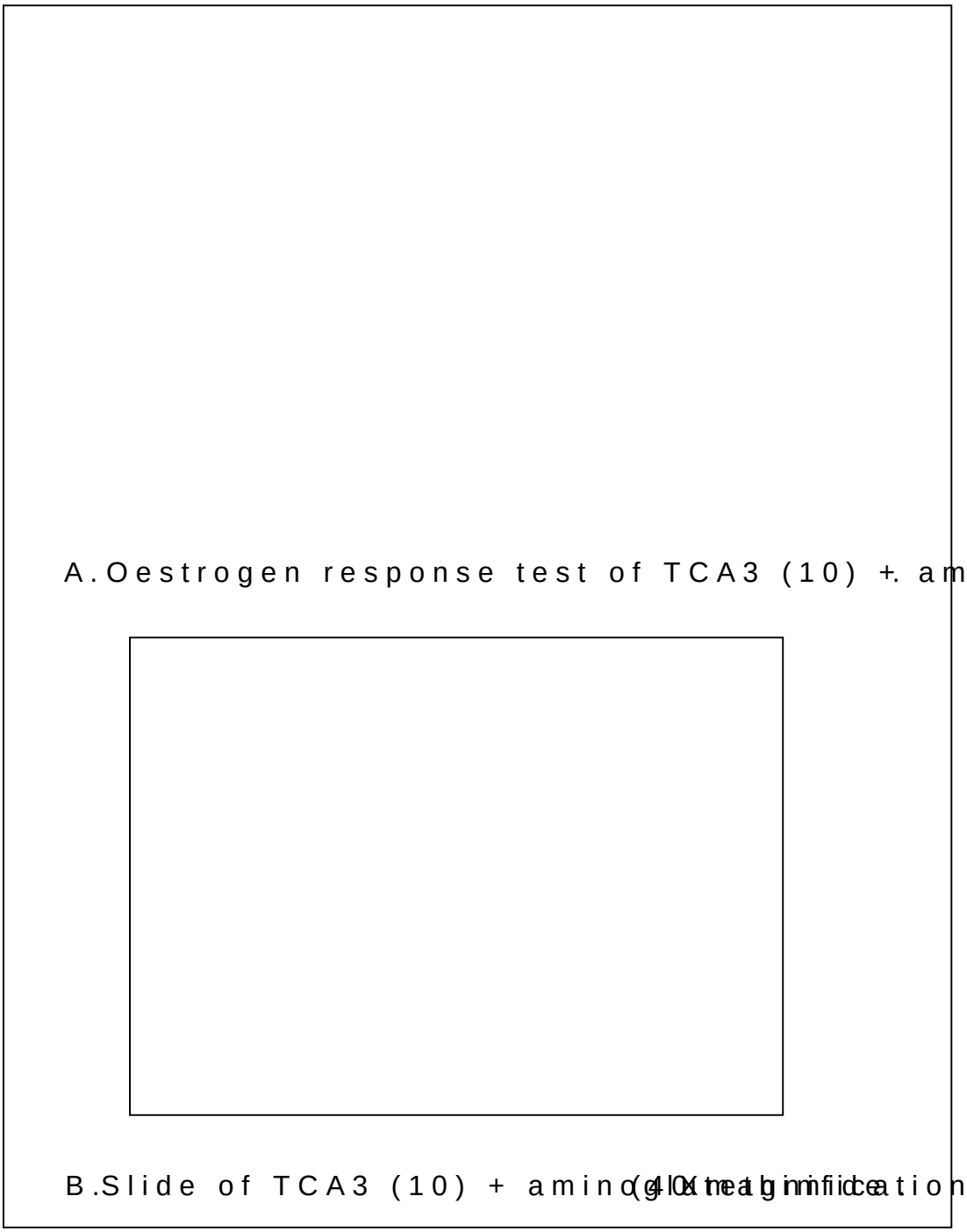


Figure 3.4 TCA3 clone at passage 10 grown in 10^{-8} M 10 aminoglutethimide.

A. Graph of oestrogen response test of TCA3 clone at passage 10 (+ aminoglutethimide)
(red arrow indicates time of oestrogen/anti-oestrogen supplementation as described in Chapter 3.10)

B. Slide photograph of TCA3 (10) (+ aminoglutethimide)

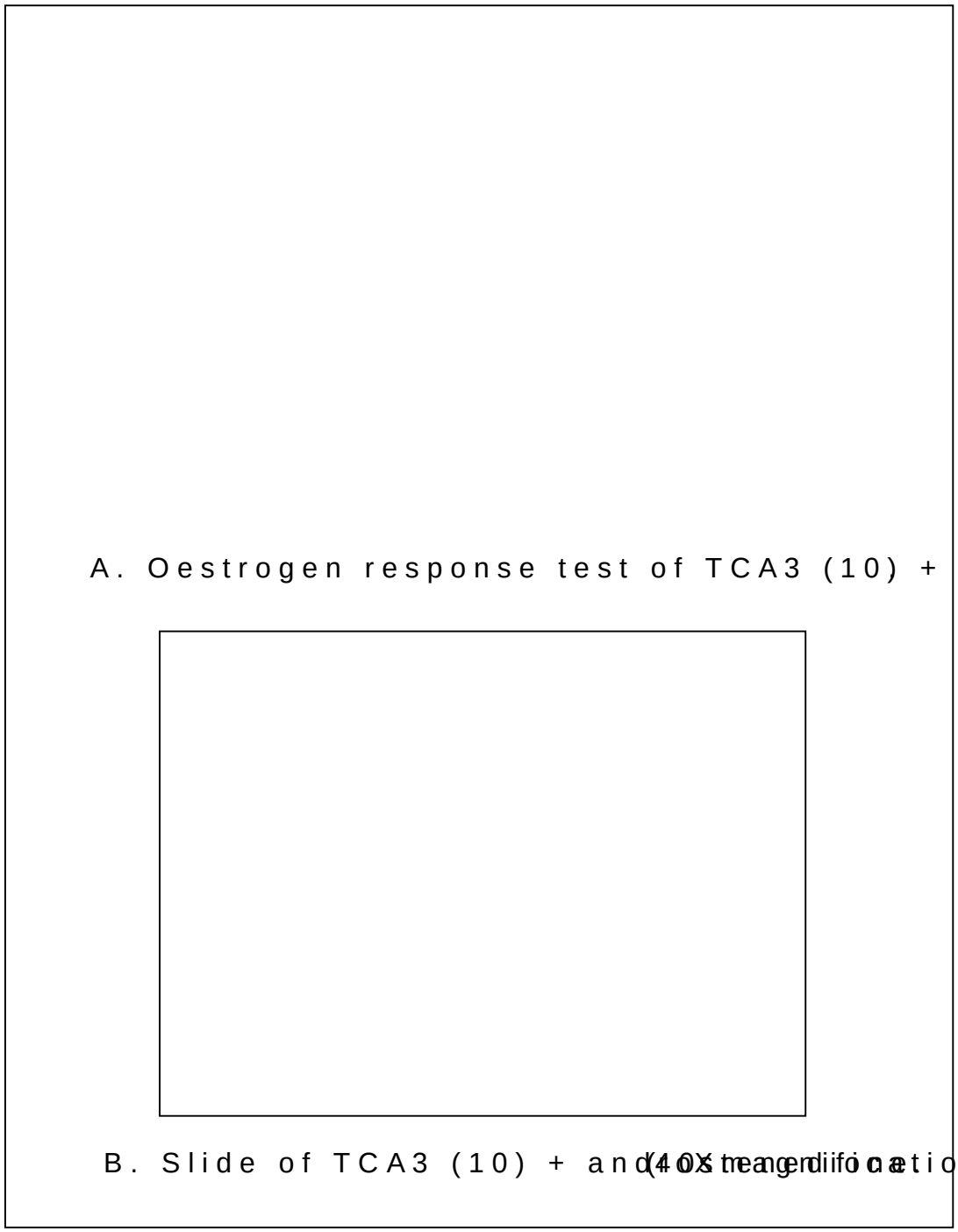


Figure 4. TCA3 clone at passage 10 (10^{-8}M androstenedione)

A. Graph of oestrogen response test of TCA3 clone at passage 10 (+ androstenedione)
(red arrow indicates time of oestrogen supplementation/tamoxifen withdrawal described in Chapter 3.10)

B. Slide photograph of TCA3(10) cells (+androstenedione)

Table 18. Table of hormone and ER± exon profiles of T-47D clone TCA3 under various growth conditions.

Condition (10^{-8}M)	Hormone Response Test	ER±"1	ER±"3	ER±"4	ER±"5	ER±"6	ER±"7
Control	Oestrogen response Tamoxifen response	+	-	+	+	-	+
Oestradiol	Oestrogen response Tamoxifen response	+	-	+	+	-	+
Tamoxifen		+	-	+	+	-	+
Aminoglutethimide	Oestrogen response Tamoxifen response	+	-	+*	+	-	+
Androstenedione	Oestrogen response Tamoxifen response	+	-	+	+	-	+
Oestriol	Oestrogen response Tamoxifen response	+	-	+	+	-	+
Cholesterol		+	-	+	+	-	+

(+ or - refers to presence or absence of simultaneous use with the presence of the complete exon, * refers to the presence of ER±"4 al

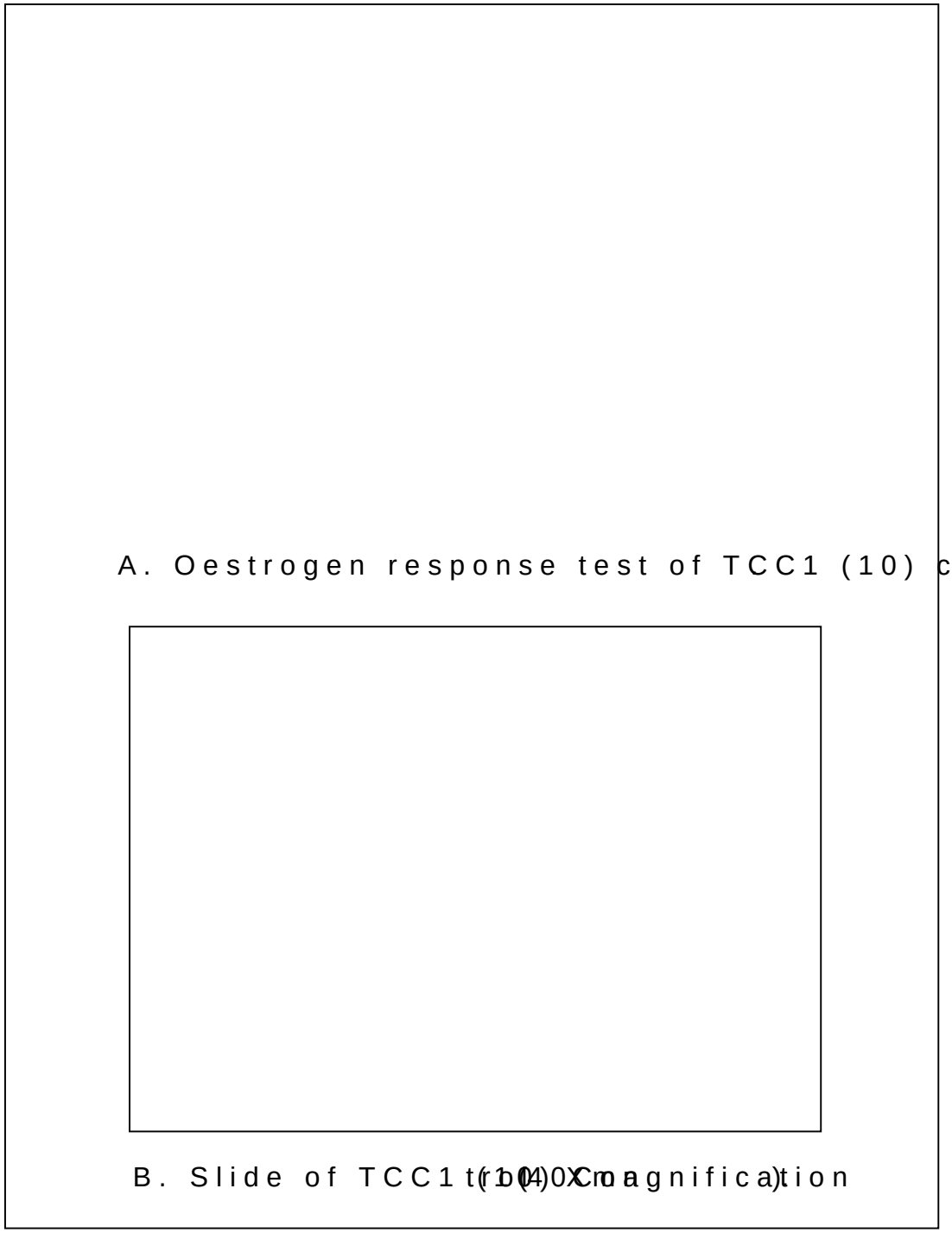
4 3.2

TCC1 clone responses

The clone TCC1 cells grown in 10^{-8}M tamoxifen and 10^{-8}M cholesterol for 10 passages did not show oestrogen responsiveness and therefore further investigation into the hormone response profiles were not available under the two conditions. RNA was extracted after the tenth passage and PCR was performed on these extracts as described.

The TCC1 (10) control cells showed greater tamoxifen sensitivity than the parent clone (Figure 45) with ER±2, ER±5 and ER±7 deletions also becoming apparent after the tenth passage under control conditions (Table 19). ER²DBD and ER²LBD were present under all conditions of growth. The ER²DBD under all growth conditions mirrored the control sample at the tenth passage with complete exon deletions in exons ER±2, ER±4 and ER±5 still present. The ER²LBD, however, although expressed with ER±7 in the control showed no deletions with the other variants (Table 19).

Hormone responsiveness varied between growth conditions with 10^{-8}M oestradiol showing slight tamoxifen response (Figure 46), 10^{-8}M 10 aminoglutethimide (Figure 48), in 10^{-8}M androstenedione (Figure 49), in 10^{-8}M oestriol (Figure 47) and in 10^{-8}M 10 cholesterol (Figure 50) all indicated tamoxifen resistance.



A. Oestrogen response test of TCC1 (10) control

B. Slide of TCC1 trol (10) X magnification

Figure 5. TCC1 clone at passage 10 (Control)

A. Graph of oestrogen response test of TCC1 (10) control cells (red arrow indicates time of addition of testosterone supplementation described in Chap 10)

B. Slide photograph of TCC1 (10) control cells

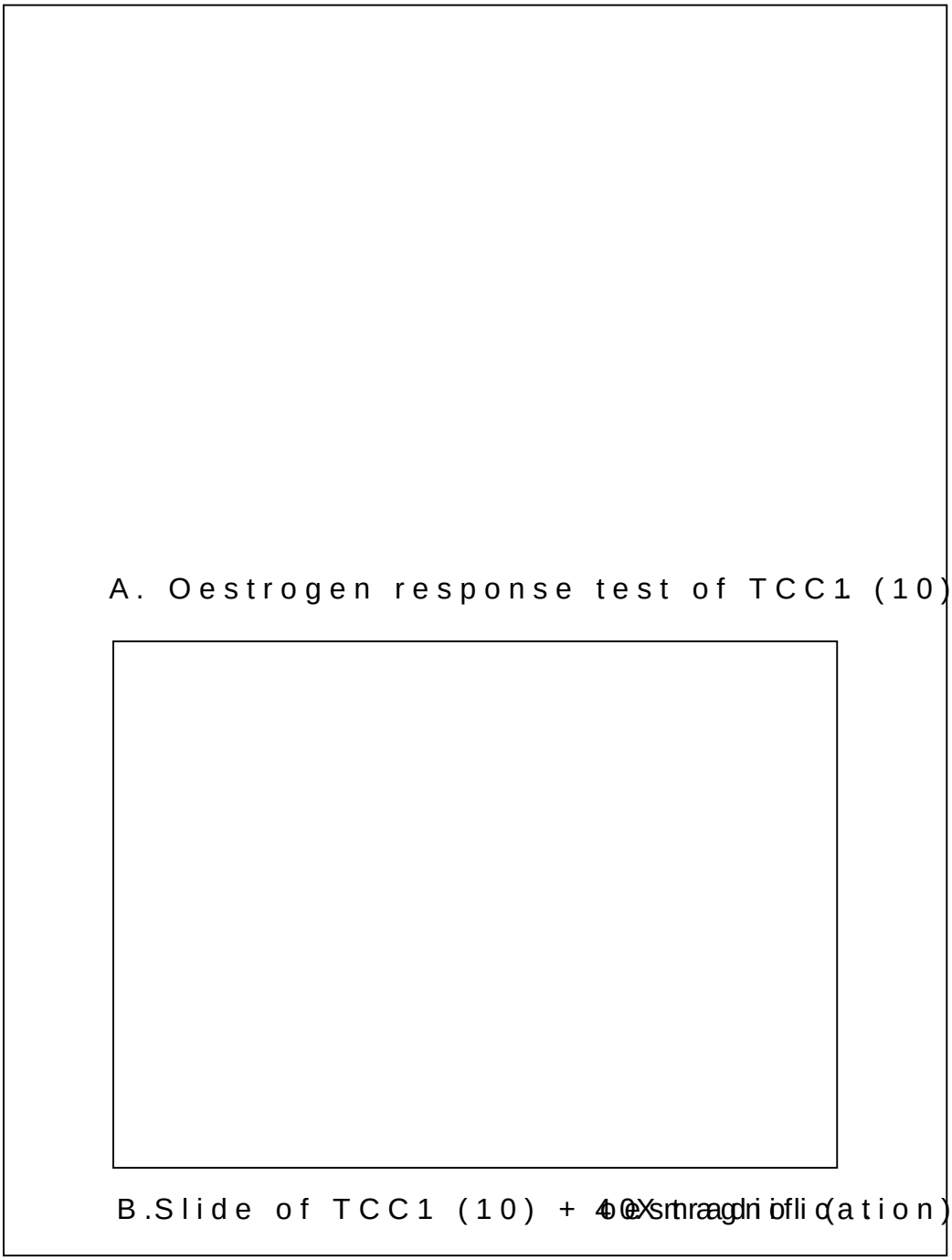
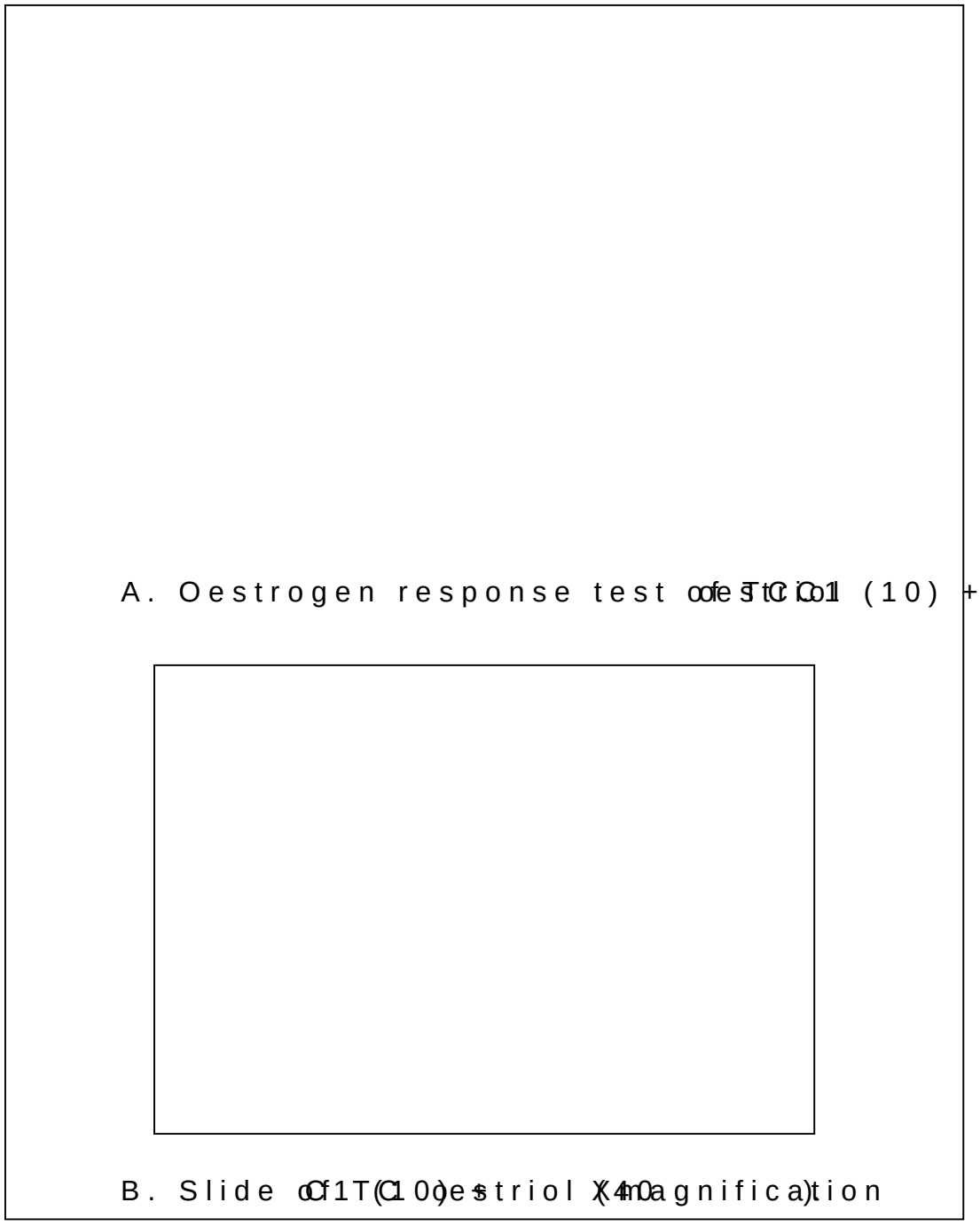


Figure 46. TCC1 clone at passage 110-8 (+ oestradiol)

A. Graph of oestrogen response test of TCC1 at passage 10 (+ oestradiol)
(red arrow indicates time of oestrogen withdrawal/addition of tamoxifen described in chapter 3.10)

B. Slide photograph of TCC1 (10) cells (+ oestra



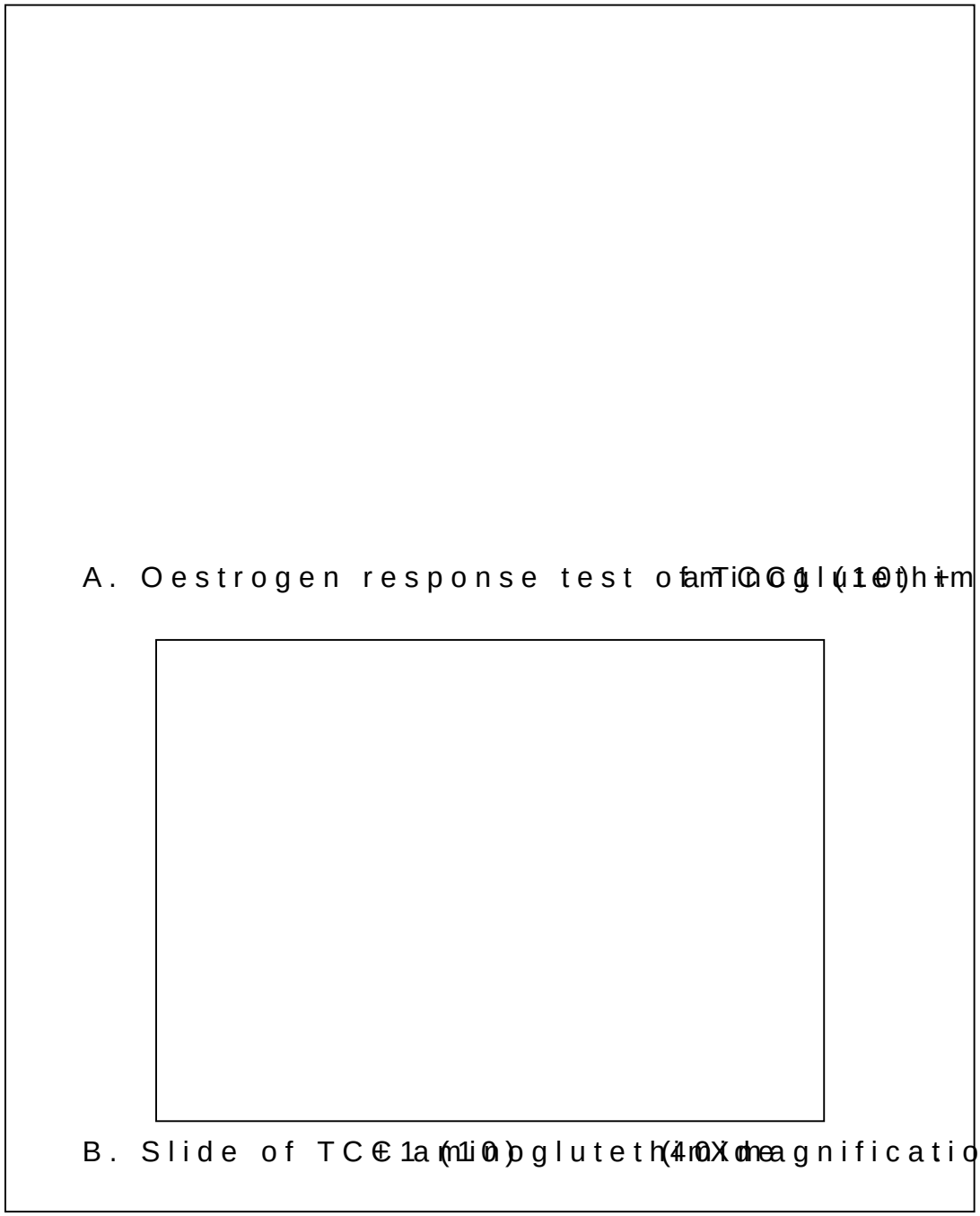
A. Oestrogen response test of TCC1 (10) +

B. Slide of TCC1 (10) + oestriol X400 magnification

Figure 47. TCC1 clone at passage 10^{8} M (oestriol) in 10

A. Graph of oestrogen response test of TCC1 clone at passage 10 (+ oestriol)
(red arrow indicates time of addition/tamoxifen treatment as described in chapter 3.10)

B. Slide photograph of TCC1 (10) + oestriol cells



A. Oestrogen response test of TCC1 clone at passage 10 (+ aminoglutethimide)

B. Slide of TCC1 clone at passage 10 (+ aminoglutethimide) (40X magnification)

Figure 48. TCC1 clone at passage 10 (10^{-8}M aminoglutethimide)

A. Graph of oestrogen response test of TCC1 clone at pass
(+ aminoglutethimide)
(red arrow indicates time of oestrogen withdrawal/ treatment as described in Chap 10)

B. Slide photograph of TCC1 cells (+ aminoglutethimide)

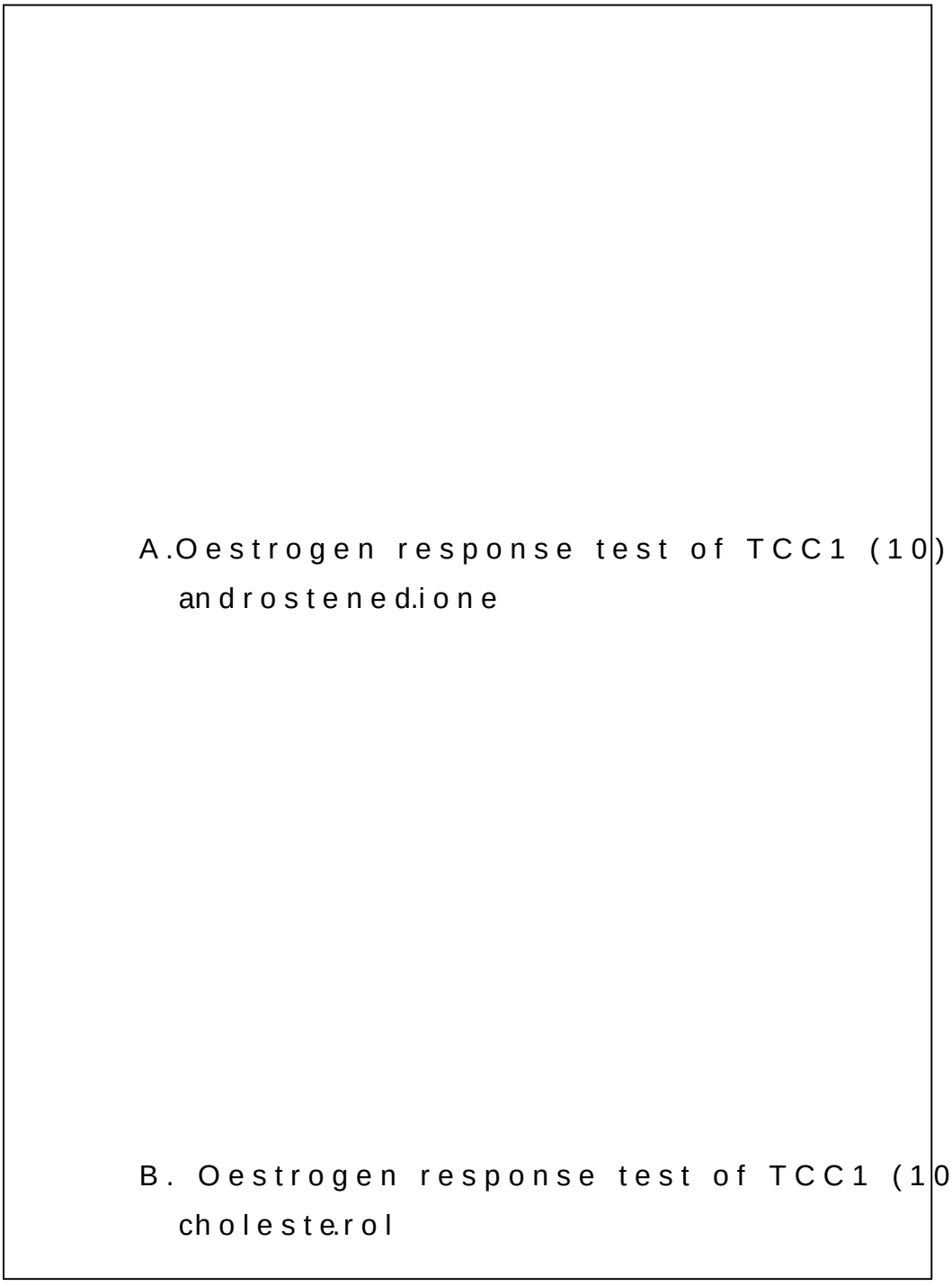


Figure 49. A. Graph of oestrogen response test of TCC1 clone at passage (10) grown in 10^{-8}M androstenedione.
B. Graph of oestrogen response test of TCC1 clone at passage (10) grown in 10^{-8}M cholesterol (red arrow indicates time of oestradiol/tamoxifen supplementation as described in Chapter 3.10)

Table 19. Tabulation of hormone responses and ER± exon profiles of T-47D clone TCC1 under various growth conditions.

Condition (10^{-8}M)	Hormone Response Test	ER±"2	ER±"3	ER±"4	ER±"5	ER±"6	ER±"7
Control	Oestroge responsiv Tamoxife responsiv	+	-	+	+	-	+
Oestradiol	Oestroge Responsi Tamoxife responsiv (slightly)	+	-	+	+	-	-
Tamoxifen		+	-	+	+	-	-
Aminogluteth	Oestroge responsiv Tamoxife resistant	+	-	+	+	-	-
Androstenedi	Oestroge responsive Tamoxife resistant	+	-	+	+	-	-
Oestriol	Oestroge responsiv Tamoxife resistant	+	-	+	+	-	-
Cholesterol	Oestroge responsiv Tamoxife resistant	+	-	+	+	-	-

(+ or - refers to presence or absence of the exon as simultaneous with the presence of the complete exon)