# *ESR1* Amplification in Breast Cancer – 25 Years of Debate

Supporting tables S1+S2 and supporting graphs S1-S4

| Tumor sample	Ratio *ESR1 / ESR2*	Ratio *ESR1 / SOD2*	FISH copy number increase *ESR1*-5'-adjacent	FISH copy number increase *ESR1*-3'-adjacent
N25	1.00	1.00	**-**	**-**
P1	13.03	2.53	yes	no
P2	2.63	0.73	no	no
P3	5.72	1.19	no	no
P4	1.94	0.77	yes	no
P5	3.26	1.41	yes	no
P6	2.84	1.22	no	no
P7	3.14	1.67	yes	no
P8	2.64	0.95	no	no
P9	2.92	1.79	yes	no
P10	1.87	1.35	?	no
P11	5.12	1.66	no	no
P12	4.5	1.14	no	no
P13	5.29	2.23	?	no
P14	8.18	1.85	yes	yes
P15	1.09	0.64	-	-
P16	1.09	0.66	-	-
P17	2.18	0.51	-	-
P18	1.52	1.04	-	-
P19	0.78	0.2	-	-
P20	1.62	0.46	-	-
P21	0.17	0.08	-	-
P22	0.78	0.34	-	-
P23	1.83	0.75	-	-
P24	3.69	1.56	-	-
Mean P1-P14	4.51	1.46	-	-
Median P1-P14	3.20	1.38		
Mean P15-P24	1.48	0.62	-	-
Median P1-P14	3.20	1.38	-	-
U-test (p-value. two sided) P1-P14 / P15-P24	0.0001	0.0003	-	-
U-test (p-value. two sided) P2+P3+P6+P8+P11+P12 / P15-P24	0.0017	0.015		

Table S1 qPCR ratios of *ESR1* to *ESR2* or *SOD2* reference normalized to sample N25. In tumors P1-P14 *ESR1* amplification has been detected by FISH. In tumors P15-P24 no *ESR1* copy number increase was determined by FISH. In tumors P2, P3, P6, P8, P11 and P12 *ESR1* amplification but no amplification of *ESR1* 5' or 3' flanking sequences was determined by FISH [1-3].
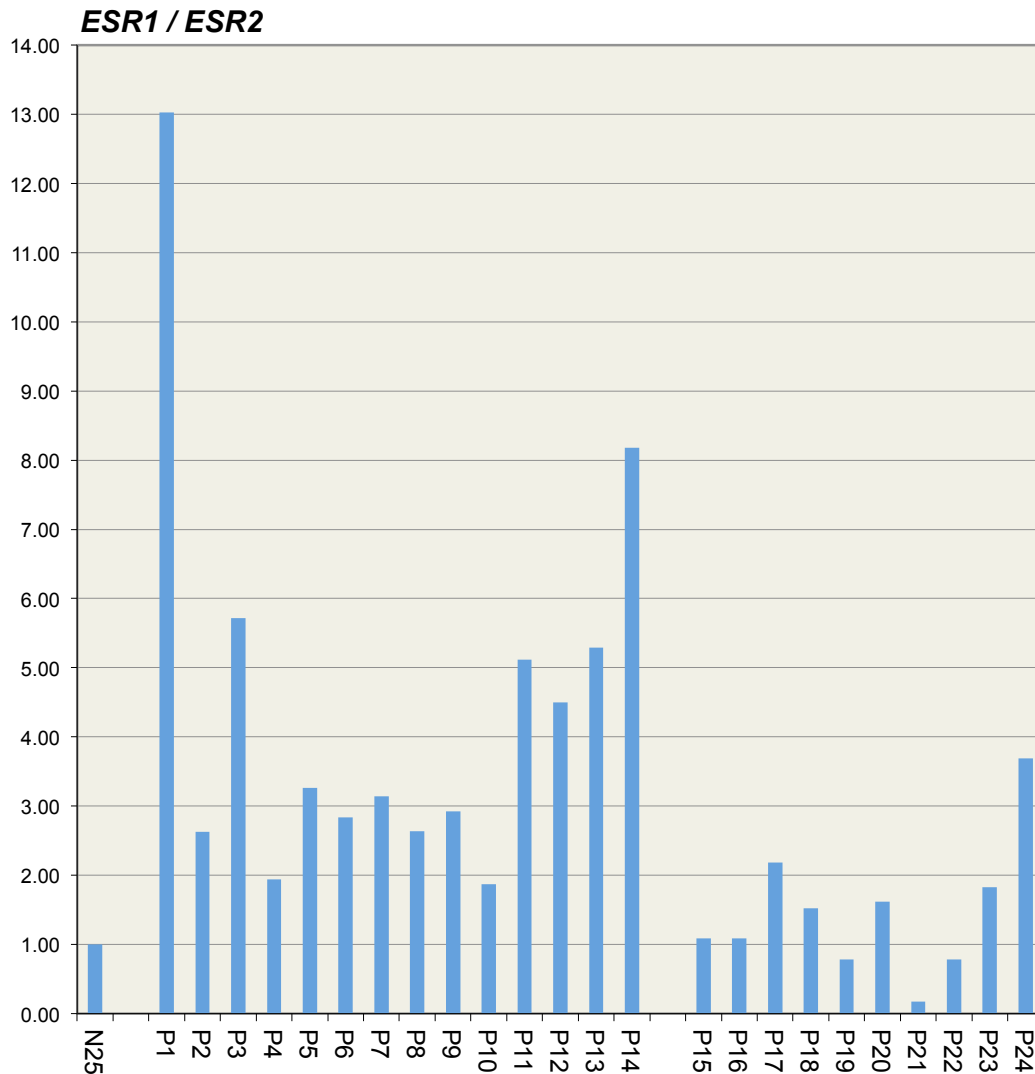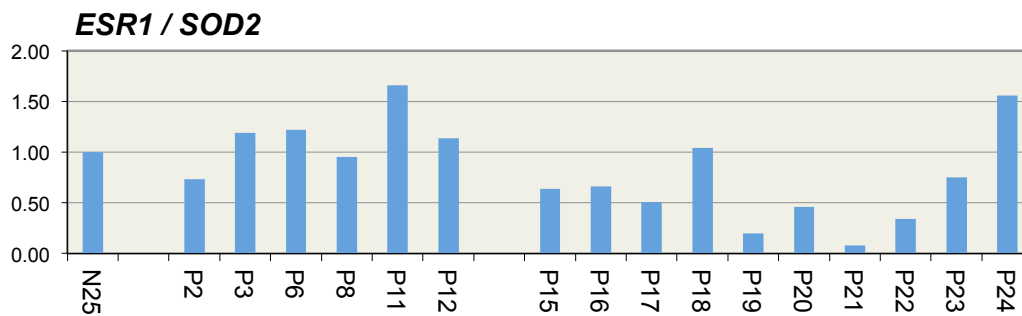
Graph S1a Bar chart for *ESR1* to *ESR2* qPCR ratios (y-axis) according to table S1. In tumors P1-P14 *ESR1* amplification has been detected by FISH. In tumors P15-P24 no *ESR1* amplification has been determined by FISH. The difference between tumor group P1-P14 and tumor group P15-P24 is statistically significant [1-3].
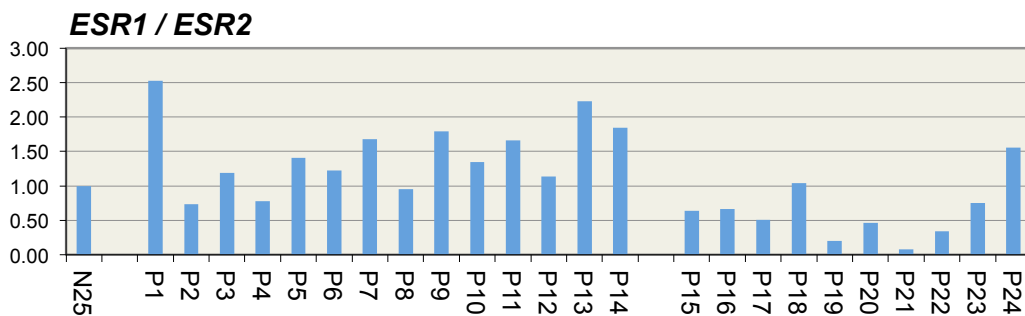
Graph S1b Bar chart for *ESR1* to *SOD2* qPCR ratios (y-axis) according to table S1. In tumors P1-P14 *ESR1* amplification has been detected by FISH. In tumors P15-P24 no *ESR1* amplification has been determined by FISH. The difference between tumor group P1-P14 and tumor group P15-P24 is statistically significant [1-3].

ESR1 / ESR2
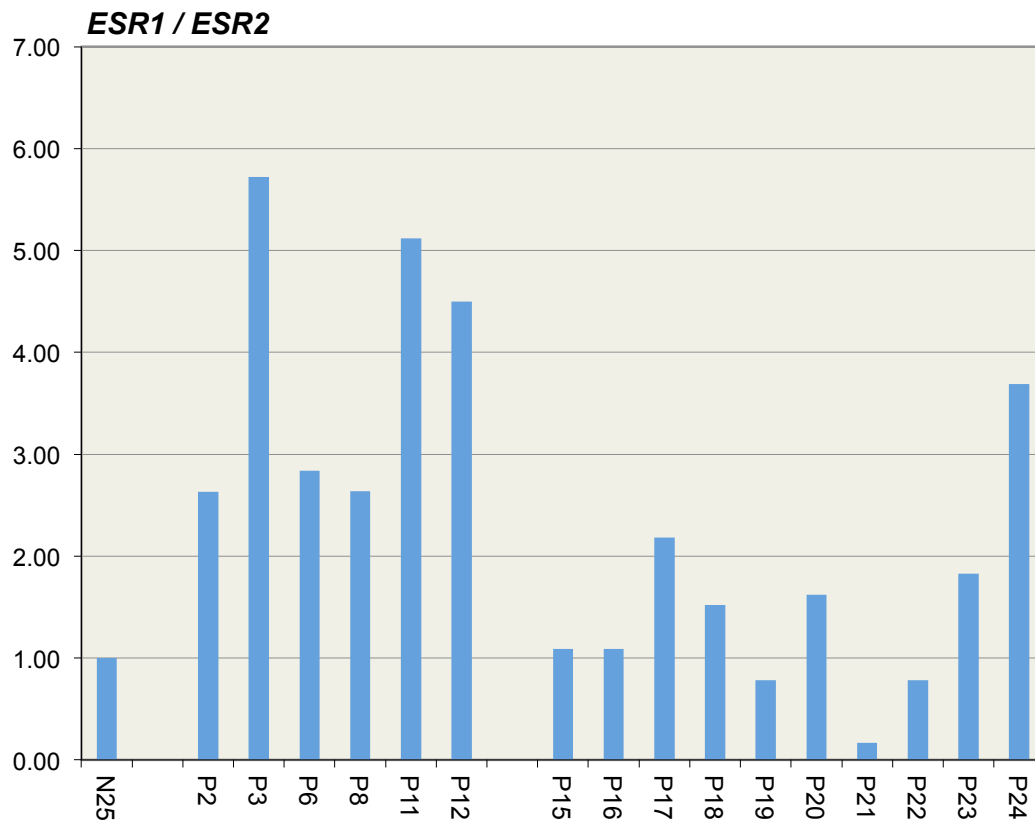
Graph S2a Bar chart for *ESR1* to *ESR2* qPCR ratios (y-axis) according to table S1 and graph S1a. In tumors P2. P3. P6. P8. P11 and P12 *ESR1* amplification but no amplification of *ESR1* 5' or 3' flanking DNA sequences has been determined by FISH. The difference between tumor group P2+P3+P6+P8+P11+P12 and tumor group P15-P24 is statistically significant [1-3].
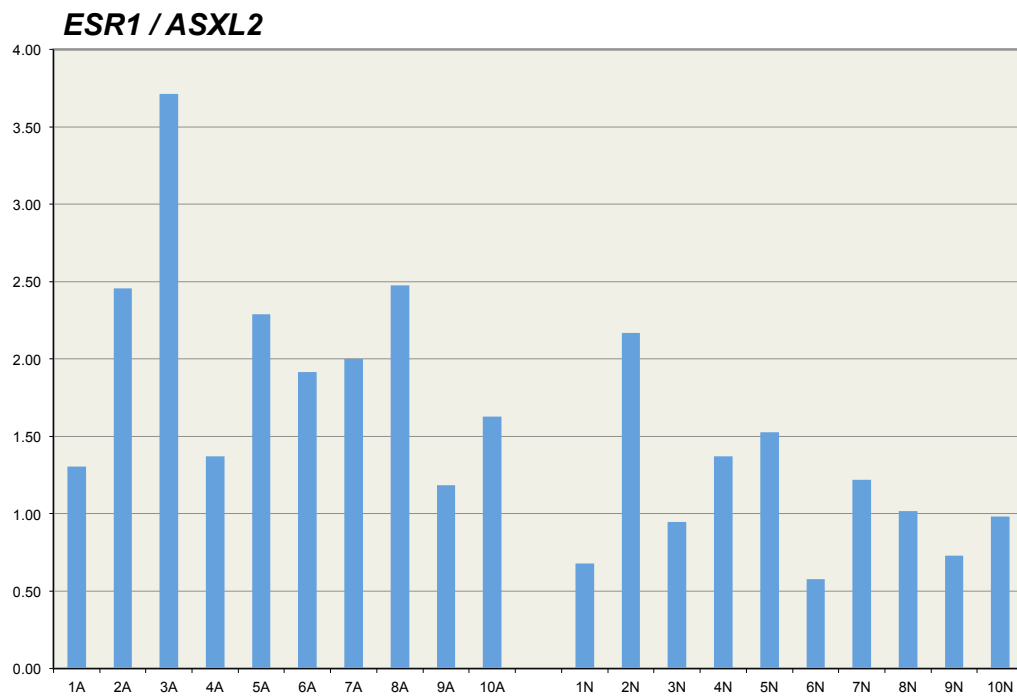
ESR1 / SOD2

Graph S2b Bar chart for *ESR1* to *SOD2* qPCR ratios (y-axis) according to table S1 and graph S1b. In tumors P2, P3, P6, P8, P11 and P12 *ESR1* amplification but no amplification of *ESR1* 5' or 3' flanking DNA sequences has been determined by FISH. The difference between tumor group P2+P3+P6+P8+P11+P12 and and tumor group P15-P24 is statistically significant [1-3].

Tumor sample	Ratio ESR1 / SOD2 (median=1)	Ratio ESR1 / SOD2 (mean=1)	Ratio ESR1 / ASXL2 (median=1)	Ratio ESR1 / ASXL2 (mean=1)	FISH amplification ESR1- 5'-adjacent	FISH amplification ESR1- 3'-adjacent
1A	1.01	1.10	1.31	1.16	no	?
2A	1.74	1.89	2.46	2.19	no	no
3A	1.68	1.82	3.71	3.31	?	?
4A	1.25	1.36	1.37	1.22	?	?
5A	1.11	1.21	2.29	2.04	no	no
6A	1.05	1.14	1.92	1.71	no	no
7A	1.13	1.23	2.00	1.78	no	no
8A	1.25	1.36	2.47	2.21	no	no
9A	1.37	1.49	1.19	1.06	no	no
10A	1.74	1.89	1.63	1.45	no	no
Median 1A-10A	1.25	1.36	2.00	1.74	-	-
Mean 1A-10A	1.34	1.45	1.99	1.81	-	-
1N	0.87	0.94	0.68	0.60	-	-
2N	1.03	1.12	2.17	1.93	-	-
3N	0.77	0.83	0.95	0.85	-	-
4N	1.01	1.10	1.37	1.22	-	-
5N	1.23	1.34	1.53	1.36	-	-
6N	0.46	0.50	0.58	0.51	-	-
7N	0.99	1.07	1.22	1.09	-	-
8N	1.03	1.12	1.02	0.91	-	-
9N	1.07	1.16	0.73	0.65	-	-
10N	0.75	0.81	0.98	0.88	-	-
Median 1N-10N	1.00	1.09	1.00	0.89	-	-
Mean 1N-10N	0.92	1.00	1.12	1.00	-	-
					-	-
U-test (p-value. two sided) 1A-10A / 1N-10N	0.0007	0.001	0.003	0.003	-	-
U-test (p-value. two sided) 2A+5A-10A / 1N-10N	0.001	0.001	0.005	0.005		

Table S2 qPCR ratios of *ESR1* to *SOD2* or *ASXL2* reference. Ratios have been normalized to median or mean of samples 1N-10N. In tumors 1A-10A *ESR1* amplification has been detected by FISH. In tumors 1N-10N no *ESR1* copy number increase was determined by FISH. In tumors 2A and 5A-10A *ESR1* amplification but no amplification of *ESR1* 5' or 3' flanking sequences was determined by FISH [1, 3, 4].
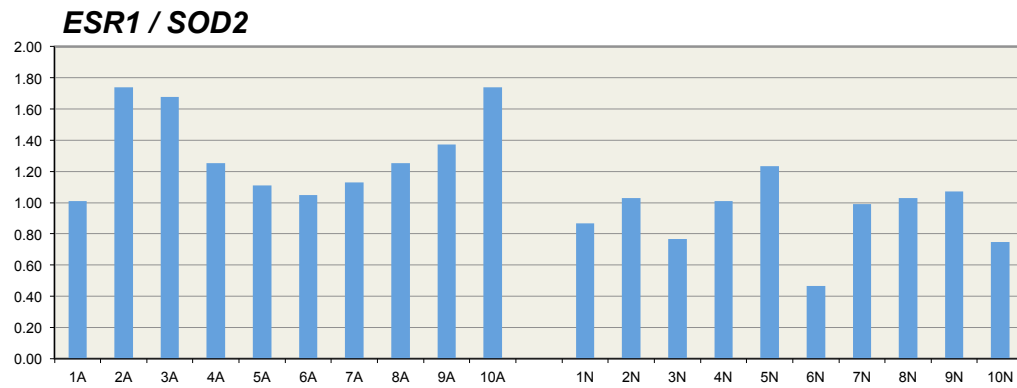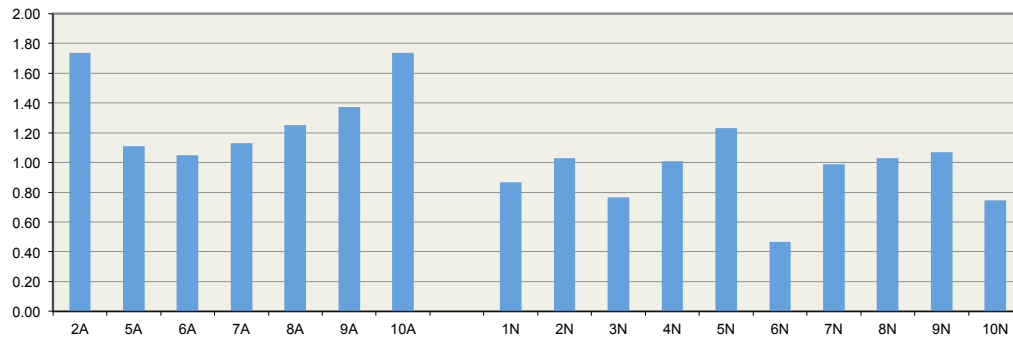
ESR1 / SOD2

Graph S3a Bar chart for *ESR1* to *SOD2* qPCR ratios (y-axis) according to table S2. In tumors 1A-10A *ESR1* amplification has been detected by FISH. In tumors 1N-10N no no *ESR1* amplification has been determined by FISH. The difference between tumor group 1A-10A and tumor group 1N-10N is statistically significant [1-4].

ESR1 / ASXL2

Graph S3a Bar chart for *ESR1* to *SOD2* qPCR ratios (y-axis) according to table S2. In tumors 1A-10A *ESR1* amplification has been detected by FISH. In tumors 1N-10N no *ESR1* amplification has been determined by FISH. The difference between tumor group 1A-10A and tumor group 1N-10N is statistically significant [1-4].
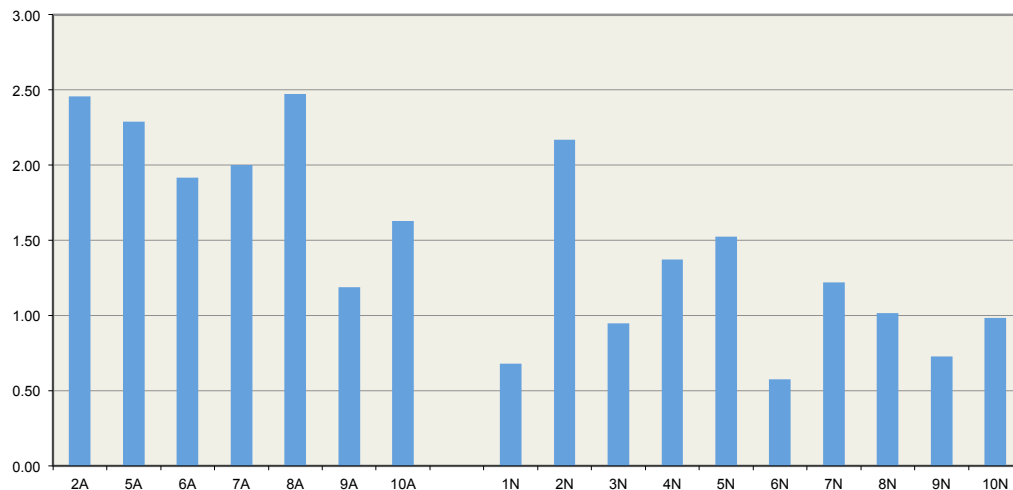
ESR1 / SOD2

Graph S4a Bar chart for *ESR1* to *SOD2* qPCR ratios (y-axis) according to table S2 and graph S3a. In tumors 2A, 5A, 6A, 7a, 8A, 9A and 10A *ESR1* amplification but no amplification of *ESR1* 5' or 3' flanking DNA sequences has been determined by FISH. In tumors 2A and 5A-10A *ESR1* amplification but no amplification of *ESR1* 5' or 3' flanking sequences was determined by FISH. The difference between tumor group 2A+5A-10A and tumor group 1N-10N is statistically significant [1-4].

ESR1 / ASXL2

Graph S4b Bar chart for *ESR1* to *ASXL2* qPCR ratios (y-axis) according to table S2 and graph S3b. In tumors 2A, 5A, 6A, 7A, 8A, 9A and 10A *ESR1* amplification but no amplification of *ESR1* 5' or 3' flanking DNA sequences has been determined by FISH. In tumors 2A and 5A-10A *ESR1* amplification but no amplification of *ESR1* 5' or 3' flanking sequences was determined by FISH. The difference between tumor group 2A+5A-10A and tumor group 1N-10N is statistically significant [1-4].

An *ESR1* FISH analysis documentation of cases P1-P24 (graph S1a-S2b) has been published [3, 5, 6]. Accordant case identification numbers are listed below:

Tumor sample ID (Holst 2012)	Case ID (Moelans et al. 2013)
P1	1
P2	2
P3	5
P4	7
P5	9
P6	10
P7	12
P8	13
P9	n.a.
P10	16
P11	17
P12	19
P13	21
P14	23
P15	25
P16	26
P17	27
P18	28
P19	29
P20	30
P21	31
P22	32
P23	33
P24	34

Appendix A
Frequeny of ESR1 Amplification (by ERBB2 Testing Threshold definition) in Literature

Method	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Southern Blot	27.3	2.7															
Gene Chips	9.7	9.6	7.0	4.5	4.3	3.7	3.4	3.0	2.3	1.0	0.9	0.7	0.0	0.0	0.0	0.0	0.0
qPCR / MLPA	38.1	16.0	11.7	5.7	2.8	2.4	2.4		2.2	2.0	0.0	0.0					
FISH / CISH	23.7	22.6	20.6	14.3	13.6	13.3	4.2	4.0	1.4	1.2	0.0	0.0					

Legend: ● Southern Blot   ◆ Gene Chips   ▲ qPCR / MLPA   ■ FISH / CISH
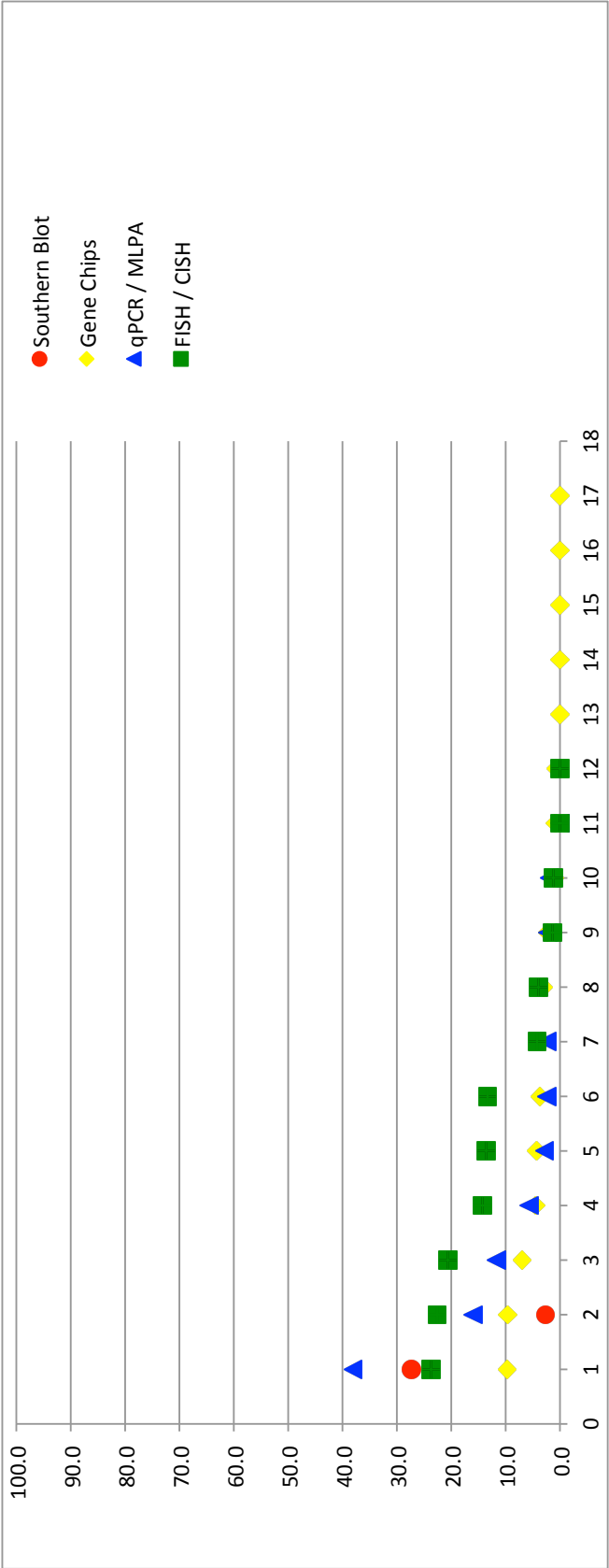
Appendix B
Frequeny of ESR1 Copy Number Increase (Amplification+Gain) in Literature

Method	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Southern Blot	27.3	2.7															
Gene Chips	25.8	20.5	12.0	11.4	9.6	9.0	7.4	7.0	5.6	5.4	4.5	4.0	3.7	3.0	2.1	0.9	0.0
qPCR / MLPA	38.1	20.0	16.0	16.0	11.7	11.4	8.1	5.9	2.8	2.4	2.4						
FISH / CISH	74.7	58.8	47.0	42.4	35.9	33.9	21.6	13.6	12.7	8.6	5.9	1.4	1.2				
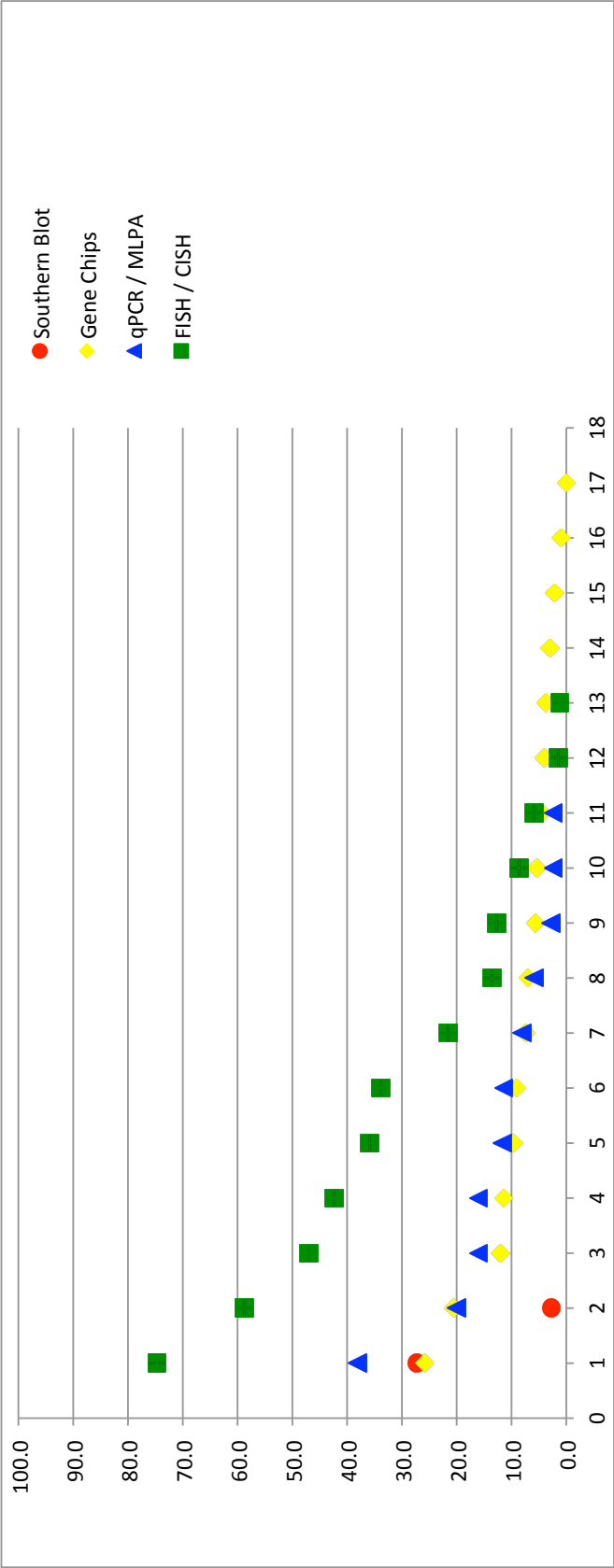
Appendix C
Different *ESR1* amplification frequencies found by different studies:

Southern Blot	Amplification (%)	Increase (%)	n
Nembrot *et al.* 1990	27.3	27.3	22
Watts *et al.* 1992	2.7	2.7	37

aCGH / Gene Chips	Amplification (%)	Increase (%)	n
Nessling *et al.* 2005	9.7	25.8	31
Holst *et al.* 2007	9.6	9.6	21
Hicks *et al.* 2006	7.0	7.0	99
Horlings *et al.* 2008	4.5	4.5	112
Reis-Filho *et al.* 2008	4.3	11.4	70
Fridlyand *et al.* 2006	3.7	3.7	54
Horlings *et al.* 2008	3.4	5.6	89
Brown *et al.* 2008	3.0	12.0	77
Dunbier *et al.* 2011	2.3	20.5	44
Brown *et al.* 2008	1.0	9.0	171
Vincent-Salomon *et al.* 2008	0.9	0.9	341
Horlings *et al.* 2008	0.7	2.1	148
Horlings *et al.* 2008	0.0	7.4	68
Brown *et al.* 2008	0.0	3.0	143
Horlings *et al.* 2008	0.0	5.4	37
Horlings *et al.* 2008	0.0	4.0	50
Adélaïde *et al.*, 2008	0.0	0.0	274

qPCR / MLPA	Amplification (%)	Increase (%)	n
Schuur *et al.* 2000	38.1	38.1	21
Moelans *et al.* 2010 (3)	?	20.0	39
Brown et al. 2008	16.0	16.0	125
Markiewicz *et al.* 2013	11.7	11.7	281
Reis-Filho *et al.* 2008	5.7	11.4	35
Reis-Filho *et al.* 2008	2.8	2.8	35
Vincent-Salomon *et al.* 2008	2.4	2.4	168
Brown *et al.* 2008	2.4	2.4	125
Moelans *et al.* 2010 (1)	2.2	8.1	135
Moelans *et al.* 2010 (2)	2.0	16.0	104
Ooi *et al.* 2012	0.0	5.9	51

FISH / CISH	Amplification (%)	Increase (%)	n
Singer *et al.* 2012	?	47.0	
Laenkholm *et al.* 2012	23.7	42.4	220
Tomita *et al.* 2009	22.6	33.9	133
Holst *et al.* 2007	20.6	35.9	1739
Vang Nielsen *et al.* 2011	14.3	74.7	91
Ejlertsen *et al.* 2011	13.6	13.6	1129
Tsiambas *et al.* 2010	13.3	21.6	60
Pentheroudakis *et al.* 2013	4.2	58.8	1010
Lin *et al.* 2012	4.0	12.7	150
Reis-Filho *et al.* 2008	1.4	1.4	245
Brown *et al.* 2008	1.2	1.2	334
Chen *et al.* 2014	0.0	8.6	301
Ooi *et al.* 2012	0.0	5.9	51

References

1   Holst F. ESR1-Amplifikationen in humanen gynäkologischen Tumoren. Department of Biology. Hamburg: University of Hamburg, 2012.

2   Holst F, Stahl PR, Ruiz C, Hellwinkel O, Jehan Z, Wendland M, Lebeau A, Terracciano L, Al-Kuraya K, Janicke F, Sauter G, Simon R. Estrogen receptor alpha (ESR1) gene amplification is frequent in breast cancer. *Nat Genet* 2007; **39**(5): 655-660 [PMID: 17417639 DOI: ng2006 [pii] 10.1038/ng2006]

3   Moelans CB, Holst F, Hellwinkel O, Simon R, van Diest PJ. ESR1 Amplification in Breast Cancer by Optimized RNase FISH: Frequent but Low-Level and Heterogeneous. *PloS one* 2013; **8**(12): e84189 [PMID: 24367641 PMCID: 3867473 DOI: 10.1371/journal.pone.0084189]

4   Holst F, Stahl P, Hellwinkel O, Dancau A, Krohn A, Wuth L, Heupel C, Lebeau A, Terracciano L, Al-Kuraya K, Jänicke F, Sauter G, Simon R. ESR1 gene amplification in breast cancer: a common phenomenon? *Nat Genet* 2008; **40**(7): 810-812 [PMID: 18583966 DOI: ng0708-809b [pii] 10.1038/ng0708-809b]

5   Moelans CB, Holst F, Hellwinkel O, Simon R, van Diest PJ. ESR1 Amplification in Breast Cancer by Optimized RNase FISH: Frequent but Low-Level and Heterogeneous. PLoS ONE 8(12): e84189. Optical Dataset S1. figshare. 2013 [DOI: 10.6084/m9.figshare.864194]

6   Moelans CB, Holst F, Hellwinkel O, Simon R, van Diest PJ. ESR1 Amplification in Breast Cancer by Optimized RNase FISH: Frequent but Low-Level and Heterogeneous. PLoS ONE 8(12): e84189. Optical Dataset S2. figshare., 2013.