Supplementary Table 1. Breast cancer cell lines used in study.

Cell Line	Study Sample ID	HER2 IHC	HER2 FISH	Her2 amol/µg
MDA231	ctrl_1	+0	no ampl	0.0
MCF7	ctrl_2	+0	no ampl	0.0
T47D	ctrl_3	+2	no ampl	697.0
ZR75-1	ctrl_4	+2	no ampl	988.0
SKBR3	ctrl_5	+3	ampl	7717.0
HCC1954	ctrl_6	+3	ampl	10020.0
ZR 75-30	ctrl_7	+3	ampl	16964.0

Supplementary Table 2. Clinicopathological characteristics of patients included in the survival analysis.

Characteristics	Nº	%
Histological grade		
G1-G2	33	35
G3	62	65
Pathological stage T		
Tx-T1	50	53
T2-T4	42	44
Unknown	3	3
Pathological stage N		
Nx-N0	40	42
N1-N3	52	55
Unknown	3	3
Hormone receptor status (HR)		
Negative	27	28
Positive	68	72
HER2 amplification by central ISH		
No amplification	6	6
Amplification	87	92
Not determined	2	2
HER2/CEP17 ratio by central ISH		
<2	6	6
2-6.3	32	34
≥6.4	55	58
Not determined	2	2
Pattern of amplification		
Double Minutes	32	34
Homogeneous stained regions	37	39

Mixed	18	19
Not determined	8	8
Anti-HER2 treatment		
Trastuzumab (single agent)	71	75
Trastuzumab + lapatinib	7	7
Trastuzumab + pertuzumab	15	16
T-DM1	2	2
Anti-HER2 treatment setting		
Adjuvant	68	72
Metastatic	27	28

Disease/Progression-free survival, years	
Adjuvant, Mean (95% CI)	3.2 (2.8–3.6)
Metastatic, Mean (95% CI)	5.2 (3.5-6.8)
Overall survival, years	
Adjuvant, Mean (95% CI)	3.1 (2.7-3.4)
Metastatic, Mean (95% CI)	4.4 (3.3-5.3)

Supplementary Table 3. HER2 expression as measured by SRM-MS according to the different immunohistochemistry (IHC) scores.

SD, standard deviation. [1]Kruskal-Wallis rank sum test.

		Mean	SD	Min	Max	N total	P-value[1]
					Her2_amolµg		
	+0	189.1	324.2	0.0	1348.7	41	**<0.001**
HER2 IHC	**+1**	259.9	357.2	0.0	1748.0	49	
	+2	406.7	481.9	0.0	2731.5	51	
	+3	4214.1	3768.0	163.7	17446.7	136	

Supplementary Table 4. HER2 expression as measured by SRM-MS according to the HER2 gene amplification status by in situ hybridization (ISH).

No ampl, absence of amplification; Ampl, presence of amplification; SD, standard deviation; [1]Wilcoxon test.

		Her2 (amol/µg)					
		Mean	**SD**	**Min**	**Max**	**N total**	**P-value[1]**
HER2 ISH	**No ampl**	383.8	339.1	0.0	1748.0	60	**<0.001**
	Ampl	4151.2	3682.1	272.8	17446.7	124	

Supplementary Table 5. Average HER2 levels as detected by SRM-MS according to sample collection year by IHC category (cat)

SD, Standard deviation; *P* value calculated with ANOVA.

IHC cat	Sample year	n	Average	SD	*P* value
			Her2_amolµg		
+0	<2008	2	365,25	516,54	
	2008	5	232,76	323,61	
	2009	15	131,21	226,17	.22
	2010	11	353,70	457,82	
	2011	6	,00	,00	
+1	<2008	3	174,00	301,38	
	2008	3	424,68	391,54	
	2009	9	375,90	289,88	.17
	2010	23	310,03	425,25	
	2011	11	38,63	128,11	
+2	2008	4	534,75	392,33	
	2009	16	245,58	181,15	
	2010	18	555,78	713,87	.31
	2011	5	194,01	265,66	
	2012-13	6	336,28	177,04	
+3	<2008	25	3820,55	3366,02	
	2008	21	4570,58	3071,53	
	2009	25	5155,53	4751,08	.40
	2010	19	2879,73	2523,23	
	2011	15	3932,04	3646,72	
	2012-13	28	3726,41	3520,68	

Supplementary Table 6. Details of discordant cases between SRM-MS and IHC/ISH after central ISH retest.

SRM-MS cat: Negative, <740 amolµg; Positive, >740 amolµg. HER2 status local: Positive, IHC positive (3+) or ISH amplified (IHC equivocal). HER2 status central: Positive, ISH amplified; Negative, ISH non amplified. In **bold** discordant cases between SRM-MS and ISH after central re-test. DM, double minutes; MIX, mixed pattern. SR, surgical resection; RD, recurrent disease. IDC, invasive ductal carcinoma; HR, Hormone receptors. POS, positive; NEG, negative. [1]results based on literature data.

Oncoplex ID	HER2 amolµg	SRM-MS cat	HER2 IHC Local	HER2 status Local	HER2 status Central	Amplification pattern	PTN	Specimen	Diagnosis	Grade	HR
D0606	349.8	Negative	Equivocal	Positive	**Positive**	DM	T1N0	SR	IDC	3	POS
D0595	435.3	Negative	Equivocal	Positive	**Positive**	DM	T2N1	SR	IDC	2	POS
D0596	478.0	Negative	Equivocal	Positive	**Positive**	DM	T1N0	SR	IDC	3	NEG
C0593	163.8	Negative	Positive	Positive	Negative	-	T4NX	SR	IDC	2	NEG
D0188	272.8	Negative	Positive	Positive	**Positive**	DM	T2N1	SR	IDC	3	POS
C0568	283.3	Negative	Positive	Positive	Negative	-	T2N1	SR	IDC	3	POS
D0152	287.8	Negative	Positive	Positive	**Positive**	MIX	T1N0	SR	IDC	3	NEG
D0156	382.4	Negative	Positive	Positive	**Positive**	DM	T1N0	SR	IDC	1	POS
D0665	424.9	Negative	Positive	Positive	Negative	-	T1N1	SR	TC	1	POS
D0656	443.6	Negative	Positive	Positive	**Positive**	DM	T2N0	SR	IDC	2	POS
A0090	500.0	Negative	Positive	Positive	Negative	-	T1N1	SR	IDC	3	POS
C0594	522.8	Negative	Positive	Positive	Negative	-	TXN2	RD	IDC	3	NEG
D0165	535.3	Negative	Positive	Positive	**Positive**	DM	T3N3	RD	IDC	3	NEG
D0177	542.8	Negative	Positive	Positive	**Positive**	DM	T1N0	SR	IDC	2	POS
A0097	550.0	Negative	Positive	Positive	Negative	-	T2N1	SR	IDC	3	POS
A0091	580.0	Negative	Positive	Positive	Negative	-	T1N2	SR	IDC	2	POS
D0187	639.8	Negative	Positive	Positive	**Positive**	MIX	T2N0	SR	IDC	3	POS
D0574	1348.7	Positive	Negative	Negative	**Negative**	-	TXN0	SR	IDC	xx	POS
D0503	771.3	Positive	Negative	Negative	**Negative**	-	T1N0	SR	IDC	2	POS
D0547	834.5	Positive	Negative	Negative	**Negative**	-	T2N1	SR	IDC	3	POS
D0569	1748.0	Positive	Negative	Negative	**Negative**	-	T1N0	SR	PC	3	POS
Ctrl_4	988.0	Positive	Equivocal	Negative[1]	**Negative**[1]	-	xx	xx	xx	xx	xx
D0664	764.3	Positive	Positive	Positive	**Negative**	-	T4N2	SR	IDC	3	POS
D0173	959.8	Positive	Positive	Positive	**Negative**	-	T1N0	SR	IDC	2	POS

Supplementary Table 7. HER2 expression levels by SRM, HER2 gene copy number (GCN) and HER2/CEP17 ratio in ISH-positive breast cancer.

SD, standard deviation. ZR 75-30 cell line was not included in the analysis as central ISH re-test was not available.

	N	Mean	SD	Min	Max
Her2 amolµg	123	4047.1	3508.9	272.8	17446.7
HER2 GCN	123	14.0	4.2	5.2	22.2
HER2/CEP17 Ratio	123	7.2	2.5	2.1	15.0

Supplementary Table 8. HER2 expression levels by SRM-MS according to amplification pattern.

DM, double minutes; HSR, homogeneously stained regions; MIX, mixed pattern. SD, standard deviation. [1]Kruskal-Wallis rank sum test.

		Her2 (amol/µg)					
		Mean	SD	Min	Max	N total	P-value[1]
Pattern of amplification	DM	2176.4	1908.1	272.8	8070.0	46	**<0.001**
	HSR	5462.9	3368.4	1099.3	17446.7	50	
	MIX	4612.1	4439.6	287.8	16303.3	27	

Supplementary Table 9. Distribution for pattern of HER2 amplification in SRM-MS negative (<740) and positive (>740) tumors.

DM, double minutes; HSR, homogeneously stained regions; MIX, mixed pattern.

		DM	MIX	HSR	
HER2 (amol/µg	<740	8	2	0	
	>740	38	25	50	
	Total	46	27	50	123

Supplementary Table 10. ROC analysis.

The optimal cutoff for HER2 levels by SRM-MS, HER2/CEP17 ratio and HER2 GCN discriminating between a positive or negative result, in terms of response to treatment and prolonged survival, was determined using ROC analysis. Patients were dichotomized into good responders (absence of disease progression at last follow up) or poor responders (presence of disease progression at last follow up) and into good survival (alive at last follow up) versus poor survival (dead at last follow up) groups. The cutoff values of HER2 amol/µg of 2200, HER2/CEP17 ratio of 6.4 and HER2 GCN of 12.50 were chosen as the optimal cutoff values for survival analyses. The HER SRM of 2200 amol/µg threshold represents an intermediate value between optimal thresholds distinguishing good versus poor survival (2110.25 amol/µg) and good versus poor responders (2302.25 amol/µg) in the adjuvant population. The sensitivity and specificity of this threshold in predicting survival was 100% and 65.57% in the adjuvant setting, and 56.25% and 100% in the metastatic setting. AUC: Area under ROC curve.

Oncoplex ID	HER2 amolµg	SRM-MS cat	HER2 IHC Local	HER2 status Local	HER2 status Central	Amplification pattern	PTN	Vital status	Specimen	Diagnosis	Grade	HR
D0606	349.8	Negative	Equivocal	Positive	**Positive**	DM	T1N0	-	SR	IDC	3	POS
D0595	435.3	Negative	Equivocal	Positive	**Positive**	DM	T2N1	-	SR	IDC	2	POS
D0596	478.0	Negative	Equivocal	Positive	**Positive**	DM	T1N0	ALIVE	SR	IDC	3	NEG
C0593	163.8	Negative	Positive	Positive	Negative	-	T4NX	DEAD	SR	IDC	2	NEG
D0188	272.8	Negative	Positive	Positive	**Positive**	DM	T2N1	-	SR	IDC	3	POS
C0568	283.3	Negative	Positive	Positive	Negative	-	T2N1	-	SR	IDC	3	POS
D0152	287.8	Negative	Positive	Positive	**Positive**	MIX	T1N0	-	SR	IDC	3	NEG
D0156	382.4	Negative	Positive	Positive	**Positive**	DM	T1N0	ALIVE	SR	IDC	1	POS
D0665	424.9	Negative	Positive	Positive	Negative	-	T1N1	-	SR	TC	1	POS
D0656	443.6	Negative	Positive	Positive	**Positive**	DM	T2N0	ALIVE	SR	IDC	2	POS
A0090	500.0	Negative	Positive	Positive	Negative	-	T1N1	DEAD	SR	IDC	3	POS
C0594	522.8	Negative	Positive	Positive	Negative	-	TXN2	DEAD	RD	IDC	3	NEG
D0165	535.3	Negative	Positive	Positive	**Positive**	DM	T3N3	DEAD	RD	IDC	3	NEG
D0177	542.8	Negative	Positive	Positive	**Positive**	DM	T1N0	ALIVE	SR	IDC	2	POS
A0097	550.0	Negative	Positive	Positive	Negative	-	T2N1	DEAD	SR	IDC	3	POS
A0091	580.0	Negative	Positive	Positive	Negative	-	T1N2	DEAD	SR	IDC	2	POS
D0187	639.8	Negative	Positive	Positive	**Positive**	MIX	T2N0	ALIVE	SR	IDC	3	POS
D0574	1348.7	Positive	Negative	Negative	**Negative**	-	TXN0	-	SR	IDC	xx	POS
D0503	771.3	Positive	Negative	Negative	**Negative**	-	T1N0	-	SR	IDC	2	POS
D0547	834.5	Positive	Negative	Negative	**Negative**	-	T2N1	-	SR	IDC	3	POS
D0569	1748.0	Positive	Negative	Negative	**Negative**	-	T1N0	-	SR	PC	3	POS
Ctrl_4	988.0	Positive	Equivocal	Negative[1]	**Negative**[1]	-	xx	xx	xx	xx	xx	xx
D0664	764.3	Positive	Positive	Positive	**Negative**	-	T4N2	DEAD	SR	IDC	3	POS
D0173	959.8	Positive	Positive	Positive	**Negative**	-	T1N0	ALIVE	SR	IDC	2	POS

Supplementary Table 11. DFS and OS HRs comparison using two different cut points (740 and 2200).

Adjuvant (n=68)		HR	CI$_{95\%}$	*p-value[1]*
DFS	**740**	0.705	0.09-5.514	0.738
	2200	0.219	0.059 - 0.811	0.0125
OS	**740**	0.274	0.031-2.375	0.209
	2200	NA	NA - NA	0.0014
Metastatic (n=27)		HR	CI$_{95\%}$	*p-value[1]*
DFS	**740**	0.331	0.104-1.056	0.05
	2200	0.561	0.243-1.294	0.169
OS	**740**	0.183	0.054-0.623	0.002
	2200	0.198	0.069 - 0.566	0.0008

NA, Not assessable; HR, hazard ratio; CI, confidence interval.

Supplementary Table 12. Correlation between HER2 status by SRM and ISH and clinicopathological characteristics in the adjuvant series.

[1]Chi square test; [2]Fisher exact test; [3]Wilcoxon test.

		HER2 (amol/ug)			HER2 Ratio			HER2 GCN		
		< 2200 (n=28, 41.2%)	>= 2200 (n=40, 58.8%)	*p-value*	< 6.4 (n=22, 33.3%)	>= 6.4 (n=44, 66.7%)	*p-value*	< 12.5 (n=30, 45.4%)	>= 12.5 (n=36, 54.6%)	*p-value*
HR	**Negative**	7 (10.3%)	10 (14.7%)	*0.968[1]*	7 (10.6%)	10 (15.2%)	*0.619[1]*	8 (12.1%))	9 (13.6%)	*0.898[1]*
	Positive	21 (30.89%)	30 (44.1%)		15 (22.7%)	34 (51.5%)		22 (33.3%)	27 (40.9%)	
Grade	**G1**	1 (1.5%)	0 (0%)	*0.023[2]*	1 (1.5%)	0 (0%)	*0.208[2]*	1 (1.5%)	0 (0%)	*0.695[2]*
	G2	15 (22.1%)	11 (16.2%)		10 (22.7%)	15 (16.7%)		12 (18.2%)	13 (19.7%)	
	G3-G4	12 (17.7%)	29 (42.7%)		11 (18.2%)	29 (43.9%)		17 (25.8%)	23 (34.9%)	
Pahological T	**TX - T1**	14 (20.6%)	28 (41.2%)	*0.157[1]*	14 (21.2)	26 (39.4)	*0.929[1]*	17 (25.8%)	23 (34.9%)	*0.730[1]*
	T2 - 4	14 (20.6%)	12 (17.7%)		8 (12.1%)	18 (27.3%)		13 (19.7%)	13 (19.7%)	
Pathological N	**NX – N0**	15 (22.1%)	20 (29.4%)	*0.965[1]*	12 (18.2%)	22 (33.3%)	*0.931[1]*	20 (30.3%)	14 (21.2%)	*0.045[1]*
	N1 – N3	13 (19.1%)	20 (29.4%)		10 (15.2%)	22 (33.3%)		10 (15.2%)	22 (33.3%)	
Follow up, Years Mean (sd)		3.5 (1.5)	3.4 (1.5)	*0.699[3]*	3.5 (1.4)	3.3 (1.5)	*0.414[3]*	3.3 (1.5)	3.4 (1.4)	*0.918[3]*
Disease progression	**No**	19 (27.9%)	37 (54.4%)	*0.021[2]*	16 (24.2%)	38 (57.6%)	*0.310[1]*	24 (36.4%)	30 (45.5%)	*0.977[1]*
	Yes	9 (13.2%)	3 (4.4%)		6 (9.1%)	6 (9.01%)		6 (9.1%)	6 (9.1%)	
Vital status	**Dead**	7 (10.3%)	0 (0%)	*0.001[2]*	4 (6.1%)	3 (4.6%)	*0.210[2]*	4 (6.1%)	3 (4.6%)	*0.693[2]*
	Alive	21 (30.9%)	40 (58.8%)		18 (27.3%)	41 (62.1%)		26 (39.4%)	33 (50%)	

Supplementary Table 13. Correlation between HER2 status by SRM and ISH and clinicopathological characteristics in metastatic series.

[1]Chi square test; [2]Fisher exact test; [3]Wilcoxon test.

		HER2 (amol/ug)			HER2 Ratio			HER2 GCN		
		< 2200 (n=9, 33.3%)	>= 2200 (n=18, 66.7%)	*p-value*	< 6.4 (n=16, 59.3%)	>= 6.4 (n=11, 40.7%)	*p-value*	< 12.5 (n=13, 48.1%)	>= 12.5 (n=14, 51.9%)	*p-value*
Follow up, Years	Mean (sd)	6.0 (4.7)	8.5 (5.5)	*0.232[3]*	6.2 (4.3)	9.8 (6.0)	*0.056[3]*	5.8 (4.2)	9.4 (5.7)	*0.105[3]*
Disease progression	No	0 (0%)	2 (7.4%)	*0.539[2]*	1 (3.7%)	1 (3.7%)	*0.987[2]*	0 (0%)	2 (7.4%)	*0.482[2]*
	Yes	9 (33.3%)	16 (59.3%)		15 (55.6%)	10 (31.0%)		13 (48.1%)	12 (44.4%)	
Vital status	Dead	9 (33.3%)	7 (25.9%)	*0.003[2]*	11 (40.7%)	5 (18.5%)	*0.264[2]*	11 (40.7%)	5 (18.5%)	*0.018[2]*
	Alive	0 (0%)	11 (40.7%)		5 (18.5%)	6 (22.2%)		2 (7.4%)	9 (33.3%)	

Supplementary Table 14. Disease free survival and overall survival hazard ratios according to HER2 pattern of amplification. DM, doble minutes, HSR, homogeneously stained regions; MIX, mixed; HR, hazard ratio; CI, confidence interval.

Adjuvant (n=63)		HR	CI$_{95}$	*p-value*
DFS	DM/HSR	1.59	0.35-7.14	0.546
	DM/MIX	0.83	0.18-3.70	0.804
	HSR/MIX	0.52	0.10-2.60	0.427
OS	DM/HSR	2.62	0.23-29.80	0.438
	DM/MIX	0.96	0.10-8.93	0.973
	HSR/MIX	0.37	0.03-4.44	0.431
Metastatic (n=24)		HR	CI$_{95}$	*p-value*
DFS	DM/HSR	0.50	0.13-1.85	0.297
	DM/MIX	1.13	0.21-6.01	0.883
	HSR/MIX	2.28	0.39-13.42	0.362
OS	DM/HSR	4.14	0.89-8.41	0.067
	DM/MIX	5.63	0.71-44.45	0.101
	HSR/MIX	1.36	0.12-15.56	0.804