Summary of Safety and Effectiveness Data

I. General Information

Device Generic name(s):	*TOP2A* Gene amplification and deletion Kit
Device Trade name(s):	*TOP2A* FISH pharmDx™ Kit™
Product Code	NXG
Applicant's name and address	Dako Denmark A/S Produktionsvej 42 DK-2600 Glostrup Denmark Tel: 45 44 85 96 86
PMA number:	P050045
Date of Panel recommendation:	None
Date Of Notice Of Approval To The Applicant	January 11, 2008

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II. Indications for Use

For in vitro diagnostic use

TOP2A FISH pharmDx™ Kit is designed to detect amplifications and deletions (copy number changes) of the *TOP2A* gene using fluorescence in situ hybridization (FISH) technique on formalin-fixed, paraffin-embedded human breast cancer tissue specimens.

Deletions and amplifications of the *TOP2A* gene serve as a marker for poor prognosis in high-risk breast cancer patients.

Results from the *TOP2A* FISH pharmDx™ Kit are intended for use as an adjunct to existing clinical and pathological information.

III. Contraindications

There are no known contraindications for the Dako *TOP2A* FISH pharmDx™ Kit™.

IV. Warnings and Precautions

Warnings and precautions are stated in the product labeling.

V. Device Description

Summary and Explanation

TOP2A FISH pharmDx™ Kit is a laboratory test that uses fluorescent DNA probes to measure the number of copies of the TOP2A (Topoisomerase 2 alpha) gene on chromosome 17 in breast cancer cells. The TOP2A gene plays a role in cell division. Changes in the number of copies of TOP2A gene indicate an elevated risk of post-surgical recurrence of the breast cancer or decreased long term survival.

The clinical performance of Dako *TOP2A* FISH pharmDx™ Kit has been investigated in studies performed by the Danish Breast Cancer Cooperative Group (DBCG) (1-3). The performance characteristics and clinical utility have been established in a European population.

The data from the clinical validation studies demonstrate prognostic implications from *TOP2A* amplifications and deletions in breast cancer patients. Overall, patients with tumors showing *TOP2A* amplification have a significantly worse outcome than patients without such amplification. Patients with tumors showing *TOP2A* deletion have even poorer outcome. Prognostic implications with respect to overall survival are present among subgroups of patients treated with chemotherapy regimens that either include or do not include anthracyclines. The presence of predictive implications from *TOP2A* amplifications for optimal use of anthracycline-containing therapy is an area of active research with promising initial results that require grounding in a context of currently available chemotherapeutic options (1, 3-17).

The *TOP2A* gene codes for the enzyme topoisomerase IIα (topo IIα), which catalyzes the breakage and reunion of double-stranded DNA leading to relaxation of DNA supercoils.

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Type II topoisomerases are essential enzymes that interconvert topological forms of DNA by making transient double-stranded breaks in the DNA backbone (18). These enzymes play important roles in a number of fundamental nuclear processes (19) including DNA replication, transcription, chromosome structure, condensation and segregation (20). The topoisomerase IIα gene, *TOP2A*, is present in 2 copies in all normal diploid cells and is localized to chromosome 17q21 (21). The *TOP2A* gene spans an area of approximately 27.5 kb and contains 35 exons encoding a 170 kDa protein (22).

The topo IIα protein has been recognized as a proliferation marker and the expression of topo IIα varies during cell cycle both in normal and cancerous cells(23). The expression of topo IIα in breast tumors correlates with Ki-67 expression (24-27). No simple relationship has been found for topo IIα at the protein and gene level (24, 26, 27). Only 20% of the topo IIα protein overexpressed cases have *TOP2A* gene amplification but among the *TOP2A* gene amplified cases 93% had overexpression of topo IIα protein (28). Topo IIα overexpression seems to be composed of several contributing factors, both the cancer-specific amplification and the elevated cell proliferation rate. The Ki-67 and topo IIα proteins are expressed in parallel, which can be interpreted as a confirmation of the influence of cell proliferation rate on topo IIα expression, even in cases with *TOP2A* amplification (29).

Type II topoisomerase is a target for anthracyclines such as doxorubicin and epirubicin, which are also termed topoisomerase inhibitors (30-34). Both HER2 status (4, 8, 9) and *TOP2A* status (1, 3-16) have been studied as a marker for treatment with anthracyclines.

Some studies (1, 3) report *TOP2A* gene amplification in 12% of breast cancers and deletions with approximately equal frequency when both the HER2 positive and negative tumors are included in the studies. Initially, it was assumed that abnormal *TOP2A* gene copy numbers, as a result of amplification or deletion, were restricted to *HER2* amplified tumors (35, 4). More recently, copy number changes of the *TOP2A* gene have been detected in tumor samples with normal *HER2* gene status (1-3, 5-7, 36, 37).

Test Principles

The *TOP2A* FISH pharmDx™ Kit contains all key reagents required to complete a fluorescence in situ hybridization (FISH) procedure for routinely processed, formalin-fixed, paraffin-embedded tissue sections.

After deparaffinization and rehydration, specimens are heated in Pre-Treatment Solution for 10 minutes. The next step is a proteolytic digestion using ready-to-use Pepsin at room temperature for 5-15 minutes. Following the heating and the proteolytic pre-treatment, this kit employs a ready-to-use FISH Probe Mix based on a combination of PNA (peptide nucleic acid) (38) and DNA technology. This Probe Mix consists of a mixture of Texas Red-labeled DNA cosmid clones covering a total of 228 kb of the *TOP2A* amplicon, and a mixture of fluorescein-labeled PNA probes targeted at the centromeric region of chromosome 17. The specific hybridization to the two targets results in formation of a distinct red fluorescent signal at each *TOP2A* amplicon and a distinct green fluorescent signal at each centromeric region of chromosome 17. To diminish background staining, the Probe Mix also contains unlabeled PNA blocking probes. After a stringent wash, the

specimens are covered with fluorescence mounting medium containing DAPI and coverslipped. Results are interpreted using a fluorescence microscope equipped with appropriate filters (see Appendix 3 in product labeling). Cancer cells are located and then evaluated with regard to the *TOP2A*/CEN-17 signal ratio. Normal cells in the analyzed tissue section serve as an internal positive control of pre-treatment and hybridization efficiency. For details see the section below titled "Interpretation of Staining".

TOP2A FISH pharmDx™ Kit, Code K5333, is intended for use via a manual staining procedure.

Device Components

Materials provided

The materials listed below are sufficient for 20 tests (a test is defined as one 22 mm x 22 mm target area). The number of tests is based on the use of 250 μL per slide of Vial 2 (5-8 drops), 10 μL per slide of Vial 3, and 15 μL per slide of Vial 5. The solutions in Vial 3 and Vial 5 are viscous and may have to be centrifuged shortly in a microcentrifuge in order to be able to collect the entire provided reagent. The kit provides materials sufficient for 10 individual staining runs. The *TOP2A* FISH pharmDx™ Kit is shipped on dry ice.

Vial 1	**Pre-Treatment Solution (20x)** 75 mL, concentrated 20x, MES (2-[*N*-morpholino]ethanesulphonic acid) buffer.
Vial 2	**Pepsin** 5 mL, ready-to-use Pepsin solution, pH 2.0; contains stabilizer and an antimicrobial agent.
Vial 3	***TOP2A*/CEN-17 Probe Mix** 0.2 mL, ready-to-use, mix of Texas Red-labeled *TOP2A* DNA probes and fluorescein-labeled CEN-17 PNA probes; supplied in hybridization buffer with 45% formamide, stabilizer, and unlabeled PNA blocking probes.
Vial 4	**Stringent Wash Buffer (20x)** 150 mL, concentrated 20x SSC (saline-sodium citrate) buffer with detergent.
Vial 5	**Fluorescence Mounting Medium** 0.3 mL, ready-to-use fluorescence mounting medium with 100 μg/L DAPI (4',6-diamidine-2-phenylindole).
Vial 6	**Wash Buffer (20x)** 500 mL, concentrated 20x Tris/HCl buffer.
Coverslip Sealant	1 tube, ready-to-use solution for removable sealing of coverslips.

All reagents are stored at 2-8 °C in the dark and can tolerate frozen storage. Freezing and thawing the kit for each analysis does not affect performance. The ready-to-use Pepsin, *TOP2A*/CEN-17 Probe Mix, and Fluorescence Mounting Medium may be affected adversely if exposed to heat and should not be left at room temperature. The *TOP2A*/CEN-17 Probe Mix and Fluorescence Mounting Medium may be affected adversely if exposed to excessive light levels. For this reason, these reagents should not

be stored or used to perform the test in strong light, such as direct sunlight. If reagents are stored under conditions different from those specified in the package insert, the user must validate reagent performance (40). Since there are no obvious signs indicating instability of this product, it is important to evaluate normal cells in the analyzed tissue section.

VI. Alternate Practices and Procedures

Alternative practices and procedures for estimating the risk of breast cancer recurrence or survival include molecular testing of HER2/neu, radiological imaging, and clinical signs and symptoms (tumor growth rate).

VII. Marketing History

The Dako *TOP2A* FISH pharmDx™ Kit has not been marketed previously for clinical use.

VIII. Potential Adverse Effects of the Device on Health

A potential risk associated with false positive test results is selection of an unnecessarily aggressive follow-up or therapy regimen. Alternatively, a false negative test result may contribute to excluding a patient from more aggressive follow-up or therapy that might have been beneficial.

IX. Summary of Pre-clinical Studies

Hybridization Efficacy

Hybridization efficacy of the *TOP2A* FISH pharmDx™ Kit was investigated in a pathology laboratory. One hundred twenty-six (126) formalin-fixed, paraffin-embedded tissue sections were tested using the recommended procedure. Out of the 126 specimens, 124 were scored according to the product guideline, while 2 specimens could not be scored owing to technical reasons. Thus, the hybridization efficacy was 124/126= 98% (2).

Robustness Studies

The robustness of the *TOP2A* FISH pharmDx™ kit was tested by varying pre-treatment time and temperature, pepsin incubation time, denaturation temperature, hybridization time and temperature, and stringent wash time and temperature.

1. Pretreatment Solution Conditions
   a) pH
      Tris-buffered saline (TBS) was tested at pH 3.0, 6.0, 7.8 and 10.0. It was determined that a pH of 6.0 yielded the best morphological tissue preservation. This pH was bracketed using 2-[N-morpholino]ethanesulfonic acid (MES) buffer manufactured with pH values of 5.4, 6.4 and 7.4. Application of these formulas to tissues under the pretreatment conditions demonstrated that pH's of 5.4 and 6.4 were both acceptable, but pH 7.4 resulted in swollen nuclei and bad preservation of tissue morphology.
   b) Detergent
      Several detergent formulations of MES buffer of pH 6.4 were tried with no apparent effect.

c) Salt Concentration
Various concentrations of NaCl from 0 to 300mmol/L were added to the MES buffer at pH 6.4 and subjected to the pre-treatment conditions. No improvement in tissue morphology was seen.

d) Results
Based on results from the studies above, it was concluded that the formula for the pre-treatment solution should be 50 mmol/L MES at pH 6.4.

2. Incubation Time, and Temperature for Pretreatment
Pretreatment was tested at three incubation times of 7, 10 and 13 minutes. Incubation temperatures tested were 89°C, 92°C, and 95-97°C. Results showed that temperature at 89°C resulted in weaker signals in some of the tested sections (one tissue section out of four incubated for 10 minutes). The other testing conditions gave acceptable results. The package insert recommends pretreatment at 95-99°C for ± 10 minutes. The assay instructions required indirect heating of the pretreatment solution in an open water bath.

3. Pepsin Incubation Time
Pepsin Incubation Times of 2, 5, 10, 15, and 18 minutes were tested. No significant difference in results was observed under these conditions. The recommended Pepsin incubation time is at room temperature (20 – 25°C) for 5 – 15 minutes.

4. Denaturation Temperatures
Denaturation Temperatures of 72, 82, and 92°C were tested. No significant difference in results was observed under these conditions. The package insert recommends denaturation at 82°C for 5 minutes.

5. Hybridization Time
a) Hybridization time of 14 hours combined with each of the temperatures 40, 45, and 50°C were tested. No significant difference in results was observed under these conditions. The package insert recommends hybridization at 45(±2)°C.
b) Hybridization times of 10, 12, 14, 17 and 20 hours at a temperature of 45°C showed no significant difference in results under these conditions. The package insert recommends overnight (14-20 hours) hybridization at 45°C.

6. Stringent Wash

a) The Stringent Wash was tested for 10 minutes at 60, 65 and 70°C and 5, 10, 15 minutes at 65°C.

i. Stringent Wash for 10 minutes at 70°C resulted in loss of signals, whereas no significant difference in results was observed at the other time and temperature combinations. The package insert recommends using the Stringent Wash at 65 (± 2) °C for exactly 10 minutes.

ii. Additional temperature robustness testing with breast cancer tissue with (n=1) and without (n=1) TOP2A gene amplifications were rinsed with stringent wash at 63, 65 and 67°C for 10 minutes. The hybridization temperatures 65 ± 2 °C are recommended for the stringent wash..

b). Additions to the Stringent Wash Procedure.
Heating of only one of the Coplin staining jars to 65°C was acceptable. The Coplin jar filled with stringent wash buffer placed in the fume hood for the removal of the cover slip did not need to be heated to 65°C. It was acceptable to perform this step at room temperature and eliminated a potential source of error. The robustness study was performed following this procedure to validates it for further use. The final recommendations are to place one of the staining jars containing diluted Stringent Wash Buffer at room temperature in a fume hood and the other in a water bath. Heat the water bath and the diluted Stringent Wash Bath to 65 ± 2 °C.

7. Buffer Salt and Detergent Concentration for Stringent Wash
The following Dilutions of Stringent Wash were tested: 1:10, 1:15, 1:20, 1:30 and 1:40.

i. The 1:40 dilution of Stringent Wash Buffer resulted in loss of signals, whereas no significant difference in signal intensity was observed at the other dilutions.

ii. The Stringent Wash Buffer diluted at 1:20. From the dilution of 1:20, vial 4 tolerated dilutions from 1:10 to 1:30. The final recommendation in the package insert instructs users to dilute the provided Stringent Wash Buffer at 1:20.

iii. These results demonstrated a significant robustness towards varied dilutions of the Stringent Wash Buffer (vial 4).

Limit of Quantitation (Analytical Sensitivity)

1. Studies
To determine the limit of quantitation of the test, two separate studies were performed:

1) The TOP2A ratio was determined for one cell line that was TOP2A deleted, one that was normal and one with borderline amplification. The ratio between the number of TOP2A signals and CEN-17 signals was calculated based on the counting of 60 nuclei per cell line. The deleted cell line was scored as deleted with an average ratio of 0.31; the normal cell line was scored as normal with an average of 1.02 while the borderline amplified cell line was scored as border-line amplified with an average ratio of 1.99.

2) The TOP2A/CEN-17 ratio of 5 TOP2A non-amplified tissue sections from 5 different tissue blocks and the coefficient of variations (%CVs) were determined after scoring by 3 independent technicians scoring the signals of 60 cells (see Table 1). These 5 tissues were 3 breast cancer, a tonsil and a normal mammary tissue. All three technicians obtained non-amplified results with a mean TOP2A/CEN – 17 ratio close to 1.0 and % CVs ranging from 2% to 5%.

Table 1. Limit of Quantitation

	Tissue 1	**Tissue 2**	**Tissue 3**	**Tissue 4**	**Tissue 5**
Technician 1	0.95	0.94	0.92	1.03	0.99
Technician 2	1.05	0.94	0.97	1.01	1.04
Technician 3	1.03	0.99	0.96	1.05	1.04
Mean ratio	1.01	0.96	0.95	1.03	1.02

	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5
CV%	5	3	3	2	3
N	3	3	3	3	3

Notes:

a) The sponsor reported that 60 nuclei were counted for each test result. Five (5) non-amplified/normal tissues were counted by three technicians. The 15 results were averaged to obtain a mean ratio of 0.99 with 4.5% CV.

b) Using the sample with the largest coefficient of variation (5% CV), the largest standard deviation of a non-amplified tissue is calculated to be 0.05. Multiplying this by 2 to obtain 2 standard deviations and subtracting it from the lowest normal value seen in the normal range study (1.0), is 0.90. Thus the cutoff chosen for "deleted" tissue (i.e. TOP2A/CEN-17 ratio below 0.8) is far below the lowest normal value seen.

c) On the upper side of normal, the sum of two standard deviations (i.e. 0.10) and the largest ratio of a normal breast tissue seen in the normal range study (1.20) is 1.30. Thus the cutoff chosen for "amplified" tissue (i.e. TOP2A/CEN-17 ratio at or above 2.0) is far above the highest normal value seen.

2. Imprecison at the limit of quantitation

The limit of quantitation (analytical sensitivity) is an average of about one red event (TOP2A gene) present in one tumor cell with two green (CEN-17) signals. This required evaluation of the imprecision around the lower cutoff of 0.8 (range from 0.5-0.9 is appropriate) of the TOP2A/CEN-17 ratio. The acceptance criterion is 10% CV.

The data for a specimen that had TOP2A/CEN17 ratio of 0.73, near the cutoff for TOP2A deletion, is presented as specimen D1 in Table 13d below. It was counted twice, both times using counts from 60 cells and 60 nuclei. The %CVs based on counting of 15 replicates were 8.8% and 9.8%, respectively, meeting the acceptance criteria

Specificity of the Labeled Probe

1. Validation of Texas-red labeled DNA Probe
   TOP2A DNA probes in the TOP2A/CEN-17 Probe Mix were end-sequenced and mapped to confirm a total coverage of 228 kb including TOP2A gene. A reference batch of the relevant clones was manufactured and used for the validation studies. Part of the DNA was analyzed by restriction enzyme analysis.

   To exclude cross-hybridization to chromosomes other than chromosome 17, studies were performed on metaphase spreads according to standard Dako QC procedures. A

total of 250 metaphase spreads were evaluated for specific hybridization of the *TOP2A* DNA and CEN-17 PNA probe mixes. In all 250 cases the hybridization was specific for chromosome 17. No cross-hybridization to loci on other chromosomes was observed in any of the 250 cases.

Each new lot is compared with the initial lot by means of restriction enzyme analysis. The size of the identified bands are also compared with a theoretical restriction analysis conducted on a computer using the sequence information and the target of the applied restriction enzyme. To limit potential effects of long-term culture, a frozen clone bank is established which contains a number of identical starting cultures.

2. Additional validation of FITC-labeled PNA probe
   The CEN-17 PNA probes in the TOP2A/CEN-17 Probe Mix are chemically synthesized molecules. Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) and High Pressure Liquid Chromatography (HPLC) confirmed the mass and purity of the respective molecules. They were tested individually and in combination to confirm their specific hybridization to the centromeric region of chromosome 17.

Interfering Substances
Not tested. The test is not intended to be carried out with body fluids. Interferences from other biologicals is unlikely, therefore the decision of not testing interference substances was acceptable.

Linearity
Not applicable

Signal Enumeration Methods – 60 nuclei vs 60 signals
A pilot study was performed to determine whether results obtained by counting 60 nuclei were equivalent to counting 60 red signals (TOP2A). In this comparison study, TOP2A/CEN-17 ratios were determined in 120 patients by counting either 60 nuclei or 60 red signals and the results were compared to the clinical outcome. Although the results when using either counting method were very similar, higher precision and narrower estimated gray zones for both amplification and deletion were observed for the 60 nuclei counting method than the 60 signal method. Hence, as expected there is a higher risk for misclassifying a patient when merely 60 TOP2A signals are counted. Results of the comparison study and the gray zone estimates are summarized in Tables 2a-b below.

Table 2a: Counting Method Comparison - 60 nuclei vs. 60 Signals

		60 Nuclei		
		Deletion	Normal	Amplification
60 Signals	Deletion	8	1	0
	Normal	0	98	1
	Amplification	0	2	10

Table 2b: Counting Method Comparison – Gray Zones

	Estimated Gray Zones	
	Deletion	Amplification
60 Nuclei	0.73 – 0.87	1.80 – 2.21
60 Signals	0.70 - 0.91	1.62 – 2.38

Precision

1. Repeatability

1) Within-Sample Repeatability

The repeatability of the TOP2A/CEN-17 ratio was investigated using 10 consecutive sections of 1 (one) normal breast tissue and 1 (one) breast carcinoma. The sections were hybridized according to the validation protocol. Thirty (30) nuclei of each specimen were enumerated from up to 3 signal-containing areas. The sums and averages of the red and green signals were counted from the 30 nuclei on each of the consecutive sectioning specimens.

For the normal breast tissue, the average TOP2A/CEN-17 ratio was 1.11 (SD = 0.06) with 5.4%CV (Table 3). For the breast cancer, the average TOP2A/CEN-17 Ratio was 1.82 (SD = 0.08) with 4.4%CV (Table 4).

Table 3: Repeatability Study Using Normal Human Breast Tissue (n=1)

Repeatability Study Using Normal Human Breast Tissue*					
Section No.	TOP2A (Sum)	CEN-17 (Sum)	TOP2A (Average)	CEN-17 (Average)	TOP2A/CEN-17 Ratio
1	54	50	1.80	1.67	1.08
2	56	55	1.87	1.83	1.02
3	58	57	1.93	1.90	1.02
4	55	49	1.83	1.63	1.12
5	66	57	2.20	1.90	1.16
6	65	57	2.17	1.90	1.14
7	58	48	1.93	1.60	1.21
8	60	52	2.00	1.73	1.15
9	61	55	2.03	1.83	1.11
10	56	50	1.87	1.67	1.12

*Sample: Tissue 90/93 Human Normal Breast Tissue

Table 4: Repeatability Study Using Human Breast Cancer Tissue (n=1)

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Repeatability Study Using Human Breast Cancer Tissue*					
Section Number	TOP2A (Sum)	CEN-17 (Sum)	TOP2A (Average)	CEN-17 (Average)	TOP2A/CEN-17 Ratio
1	152	83	5.07	2.77	1.83
2	168	94	5.60	3.13	1.79
3	161	83	5.37	2.77	1.94
4	156	85	5.20	2.83	1.84
5	146	85	4.87	2.83	1.72
6	169	91	5.63	3.03	1.86
7	151	83	5.03	2.77	1.82
8	154	90	5.13	3.00	1.71
9	153	88	5.10	2.93	1.74
10	164	84	5.47	2.80	1.95

*Sample: Tissue 59/97H Human Breast Cancer with HER2 and TOP2A Gene Amplification

2) Effects of Tissue Thickness on Repeatability

A study was performed to determine if tissue thickness could affect assay performance using 10 consecutive sections of breast cancer tissue with different thickness (duplicates of 3,4,5,6,and 7 µm). Results showed that the average TOP2A/CEN-17 Ratio was 1.07 (SD = 0.03) and the coefficient of variation of the TOP2A/CEN-17 ratio was 2.8% (Table 5). The TOP2A/CEN-17 ratio did not change significantly with section thickness.

Table 5: Repeatability Study Using Human Normal Breast Tissue with Variations in Thickness

Repeatability Study Using Human Normal Breast Tissue with Variationa in Thickness*						
Section Number	Thickness (µm)	TOP2A (Sum)	TOP2A (Sum)	TOP2A (Average)	CEN-17 (Average)	TOP2A/CEN-17 Ratio
1	3	52	48	1.73	1.60	1.08
2	3	55	50	1.83	1.67	1.10
3	4	57	52	1.90	1.73	1.10
4	4	56	55	1.87	1.83	1.02
5	5	56	54	1.87	1.80	1.04
6	5	59	54	1.97	1.80	1.09
7	6	59	55	1.97	1.83	1.07
8	6	61	56	2.03	1.87	1.09
9	7	57	56	1.90	1.87	1.02
10	7	59	55	1.97	1.83	1.07

*Sample: Tissue 90/93 Human Normal Breast Tissue

2. Reproducibility

The *TOP2A* FISH pharmDx™ Kit was tested for lot-to-lot, day-to-day and observer-to-observer variability using 4 different formalin-fixed and paraffin-embedded cell lines (the non-amplified MDA-231 and MDA-175, the borderline-amplified SKBR3 and the deleted MDA-361). The cell line blocks were cut into 5 μm thick sections, placed on glass slides and treated according to the standard staining protocol for tissue sections. Sections were evaluated by counting 30 nuclei per specimen. The use of cut sections from a cell line block allows for a homogeneous cell composition across numerous independent slides, thus eliminating tissue heterogeneity that could affect a study conducted on tissue sections. For the day-to-day reproducibility study, a set of slides was stained and scored for each of the four independent days. For the observer-to-observer study, a set of 15 slides was stained in the same run and split between the three independent observers. The greatest *TOP2A*/CEN-17 ratio variation (10%) was found in the observer-to-observer study on the borderline-amplified cell line. This might be expected and possibly reflects certain subjectivity in signal interpretation and enumeration. Results expressed as mean ratio, standard deviation, and coefficients of variation are presented in Tables 6-8.

1) Lot-to-Lot Reproducibility with Cell Lines

Table 6: Lot-to-Lot Reproducibility

Cell Line	TOP2A/CEN-17 Ratio	Kit Lot 1	Kit Lot 2	Kit Lot 3	Total
MDA-231	Mean	1.02	1.01	1.05	1.03
	SD	0.04	0.04	0.05	0.04
	CV%	4	4	5	4
	N	5	5	5	15
MDA-175	Mean	1.28	1.30	1.28	1.28
	SD	0.09	0.11	0.05	0.08
	CV%	7	8	4	6
	N	5	5	5	15
SKBR3	Mean	2.03	2.08	1.98	2.03
	SD	0.12	0.11	0.12	0.12
	CV%	6	5	6	6
	N	5	5	5	15
MDA-361	Mean	0.33	0.33	0.33	0.33
	SD	0.01	0.01	0.01	0.01
	CV%	4	3	2	3
	N	5	5	5	15

Note: Three of the used cell lines were the cell lines on the HercepTest control slide, hence they were not actually placed on individual slides. The last cell line was made specifically for this study, and was placed on a separate slide.

2) Day-to-Day Reproducibility with Cell Lines

Table 7: Day-to-Day Reproducibility

Cell Line	TOP2A/CEN-17 Ratio	Day1	Day 2	Day 3	Day 5	Total
MDA-231	Mean	1.04	1.03	1.03	1.02	1.03
	SD	0.04	0.05	0.02	0.02	0.03
	CV%	4	5	2	2	3
	N	5	5	5	5	20
MDA-175	Mean	1.24	1.26	1.18	1.19	1.22
	SD	0.06	0.09	0.03	0.05	0.06
	CV%	5	7	2	4	5
	N	5	5	5	5	20
SKBR3	Mean	2.01	1.94	2.08	2.00	2.01
	SD	0.17	0.14	0.14	0.08	0.14
	CV%	9	7	7	4	7
	N	5	5	5	5	20
MDA-361	Mean	0.33	0.32	0.34	0.33	0.33
	SD	0.01	0.02	0.00	0.01	0.01
	CV%	2	6	1	3	4
	N	5	5	5	5	20

3) Observer-to-Observer Reproducibility with Cell Lines

a) Experiment #1 with Cell Lines

Table 8: Observer-to-Observer Reproducibility

Cell Line	TOP2A/CEN-17 Ratio	Observer 1	Observer 2	Observer 3	Total
MDA-231	Mean	1.03	1.05	1.08	1.05
	SD	0.02	0.05	0.05	0.04
	CV%	2	5	5	4
	N	5	5	5	15
MDA-175	Mean	1.23	1.18	1.11	1.18
	SD	0.08	0.12	0.05	0.09
	CV%	7	10	5	8
	N	5	5	5	15
SKBR3	Mean	1.92	1.63	1.67	1.74
	SD	0.19	0.09	0.06	0.18
	CV%	10	5	4	10
	N	5	5	5	15
MDA-361	Mean	0.31	0.34	0.36	0.34
	SD	0.01	0.01	0.03	0.03
	CV%	4	3	8	8
	N	5	5	5	15

Note: For the observer-to-observer study, a set of 15 slides was stained in the same run for each control cell line and split between the three independent observers (N=5 for each observer).

b) Experiment #2 with TOP2A-amplified Tissue Sections

A second inter-observer study was conducted on archieved breast cancer tissue specimens selected to reflect a range of TOP2A amplification levels. Three observers counted red signals (events) in 20 nuclei for each of 26 breast cancer specimens. Concordance between observers with regard to amplification/non-amplification status was above 96% in all cases (Tables 9a-c).

Table 9a: Inter-Observer Comparison – Observer 1 vs. 2

Observer 1	Observer 2			
	TOP2A Deletion	TOP2A Normal	TOP2A Amplification	Total
TOP2A Deletion	6	0	0	6
TOP2A Normal	0	15	0	15
TOP2A Amplification	0	1	4	5
Total	6	16	4	26

Concordance: (6+15+4)/26 = 96%

Table 9b: Inter-Observer Comparison – Observer 3 vs. 2

Observer 3	Observer 2			
	TOP2A Deletion	TOP2A Normal	TOP2A Amplification	Total
TOP2A Deletion	6	0	0	6
TOP2A Normal	0	16	0	16
TOP2A Amplification	0	0	4	4
Total	6	16	4	26

Concordance: (6+16+4)/26 = 100%

Table 9c: Inter-Observer Comparison – Observer 3 vs. 1

Observer 3	Observer 1			
	TOP2A Deletion	TOP2A Normal	TOP2A Amplification	Total
TOP2A Deletion	6	0	0	6
TOP2A Normal	0	15	1	16
TOP2A Amplification	0	0	4	4
Total	6	15	5	26

Concordance: (6+15+4)/26 = 96%

c) Experiment #3 with TOP2A Non-Amplified Tissue Sections
A third inter-observer study was performed on five (5) TOP2A gene non-amplified tissue sections. Each tissue section was scored by three (3) independent technicians. Results of TOP2A/CEN-17 Ratios are presented in Table 10 and confirmed all 5 tissue sections to be non-amplified with a mean TOP2A/CEN-17 ratio close to 1.0.

Table 10: Inter-Observer Study on TOP2A Non-Amplified Tissue Sections

	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5
Technician 1	0.95	0.94	0.92	1.03	0.99
Technician 2	1.05	0.94	0.97	1.01	1.04
Technician 3	1.03	0.99	0.96	1.05	1.04
Mean Ratio	1.01	0.96	0.95	1.03	1.02
S.D.	0.05	0.03	0.03	0.02	0.03
CV%	5	3	3	2	3

4) Inter-laboratory Reproducibility (Assay Portability)

a) Study Design

To assess interlaboratory reproducibility, a three center, blinded, randomized, comparative study using formalin-fixed, paraffin-embedded (FFPE) human breast cancer specimens with different levels of TOP2A gene status (deletion, normal and amplification) was conducted. Each site stained and interpreted 6 FFPE specimens in three separate runs (a total 30 slides, 5 from each block, divided from staining as described below). A control was provided and included in each run. This control was supplied by DakoCytomation Denmark A/S from a collection of known positive tissue blocks. The control slide was from the same specimen in every run, and served as an indicator of a successful staining. The control was not scored. The study design and samples used are summarized in Table 11.

Table 11: Study Design and Samples

Comparison	Procedure
Intra-assay (Within procedure reproducibility)	18 slides (3 from each of 6 specimens) + 1 control slide on Day 1
Inter-assay (Compare staining from two previous staining runs)	6 slides (1 from each of 6 specimens) + 1 control slide on day 2,
Inter–technician (Compare staining results between technicians)	6 slides (1 from each of 6 specimens) + 1 control slide on day 2, done by a different technician
Inter-laboratories (Pair-wise comparisons of staining results between laboratories)	Comparison of Day 1 results between pairs of laboratories
Inter-counting methods (Compare results between 2 counting methods)	Comparison of ratio obtained by the 2 counting methods on all 90 slides

b) Protocols

Each site designated one primary and one secondary technician for the staining comparison and one pathologist for result evaluation. The enumeration method used was by counting 60 nuclei. However, one site (Site 1) counted only 6 nuclei on the slides that were highly amplified according to their customary counting technique.

The initial assay on the first day had 18 randomized tissue slides tested, using 3 replicates of each of the 6 slides at each of the three study sites. One set of the six tissues was stained with hematoxylin and eosin (H & E) by DakoCytomation and provided to each laboratory to use to identify areas of invasive carcinoma. Within each laboratory, one pathologist (or designee) determined the areas of interest using the H & E stained tissue sections, and marked the slides prior to the FISH evaluation.

i. Between-Day Staining Protocol

At each site, the 6 specimens were stained on two different days by the same technician. Statistical analysis comparing the ratio of the copy status was performed using the lowest ratio obtained on day 1 to the ratio observed on the second test day.

ii. Between Technician Staining Protocol

At each site, the 6 specimens were stained on one different day by a different technician. Statistical analysis comparing the ratio of the copy status was

performed using the ratio obtained on day 2 to the ratio observed from specimens processed by the second technician.

iii. Enumeration Methods

Each specimen was counted on 3 different days and by counting 60 nuclei vs. 60 signals.

c) Results

Slide identities were masked by a random number labeling scheme. The ratio of copy status for each slide tested was reported by slide ID and by site. Day and technician were specified for each of the specimens, and used in the statistical analysis for between day and technician evaluations.

i. On the first day of staining, a total of 54 slides were stained across three sites. Three replicates of each tissue were stained at each laboratory. Results are reported as TOP2A/CEN-17 ratios. The distribution of the ratios is presented in Table 12:

Table 12: Reported TOP2A/CEN-17 Ratios on the first day of staining

		Overall Ratio (Count of specimens with this score)		
Site	Total	<0.80	≥ 0.80, <2.0	≥ 2.0
1	18	3	9	6
2	18	2	10	6
3	18	3	9	6
Sum	54	8	28	18

Reproducibility was demonstrated for all assay comparisons across the three sites. Results showed that one tissue was read differently between deleted and normal status at Site 2.

ii. Summary results across all 90 tests performed on different days with two different technicians at each of the 3 sites

Across the 90 tests performed, there were differences in the ratio for 2 tissues. Analyses were concordant for 13 out of 15 results from tissue D1 (containing a deletion) and for 12 out of 15 analyses from tissue D2 with one result in the equivocal zone 0.7-0.9. Gene copy status remained concordant across all tests for the other 4 tissues (2 normal and 2 amplified tissues)(Table 13).

Table 13: Summary of ratios reported for all specimens (inter-laboratory reproducibility)

		Overall Ratio(Count of specimens with this score)		
Site	Total	<0.80	≥0.80, <2.0	≥ 2.0
1	30	5	15	10
2	30	6	14	10
3	30	5	15	10
Sum	90	16	44	30

iii. Two counting methods were used, and a high concordance between the 2 counting methods was demonstrated. (Refer to the following section, on the Correlation Study of 60 nuclei to 60 signals)

Correlation Study of 60 nuclei to 60 signals

Six (6) tissue sections enumerated by 60 nuclei in an inter-laboratory reproducibility study (also refer to Table 9 above) were recounted using the alternative method of counting 60 red signals (60 counts) in three (3) sites for determining within run (n=3) and within site (n=5) reproducibility by the alternative method.

1) Within-Run Reproducibility of 60 cells (nuclei) vs. 60 counts (Table 14a)
The imprecisions of the within-run reproducibility were from 5.4% to 10.4% for the 60 cells (nuclei) method with median CV of 8.2%; The imprecisions of the within-run reproducibility were from 6.9 % to12.6% for 60 counts method with median CV of 10.3%.

Table 14a: Within-Run Reproducibility for the 6 Individual Patient Tissues at Three (3) Test Sites by the Two Counting Methods

Tissue	A1		A2		N1		N2		D1		D2	
	60 Cells	60 Counts	60 Cells	60 Counts	60 Cells	60 Counts	60 Cells	60 Counts	60 Cells	60 Counts	60 Cells	60 Counts
Mean	3.18	2.89	8.17	8.08	1.35	1.44	1.35	1.38	0.74	0.75	0.95	0.96
SD	0.25	0.36	0.45	0.69	0.12	0.17	0.11	0.14	0.08	0.08	0.07	0.07
% CV	7.9	12.6	5.4	8.5	8.6	11.8	8.4	10.4	10.4	10.2	7.4	6.9
N	9	9	6	9	9	9	9	9	9	9	9	9

SD: Standard Deviation CV: Coefficient of Variation N. Number of slides

2) Within-Site reproducibility of 60 cells (nuclei) vs. 60 counts

Summary of Safety and Effectiveness Data

The results of within-site imprecisions were summarized in the following tables.

Table 14b: Within site tissue variability seen at Site 1

Tissue:	A1		A2		N1		N2		D1		D2	
	60 cells	60 counts	60 cells	60 counts	60 cells	60 counts	60 cells	60 counts	60 cells	60 counts	60 cells	60 counts
Mean	2.66	2.40	ND	7.77	1.45	1.48	1.36	1.44	0.73	0.75	1.00	1.04
SD	0.14	0.29	ND	0.50	0.14	.022	0.18	0.17	0.08	0.10	0.15	0.21
%CV	5.22	12.12	ND	6.39	9.72	14.50	13.51	11.81	10.88	12.80	15.25	19.71
N	5	5	ND	5	5	5	5	5	5	5	5	5

SD: Standard Deviation CV: Coefficient of Variation N. Number of slides ND: Not done

Table 14c: Within site, tissue variability seen at Site 2

Tissue:	A1		A2		N1		N2		D1		D2	
	60 cells	60 counts	60 cells	60 counts	60 cells	60 counts	60 cells	60 counts	60 cells	60 counts	60 cells	60 counts
Mean	3.54	3.62	6.46	7.06	1.35	1.48	1.32	1.27	0.71	0.69	0.87	0.87
SD	0.51	0.57	1.46	2.33	0.06	0.18	0.08	0.09	0.08	0.06	0.14	0.12
%CV	14.39	15.80	22.60	32.97	4.55	12.32	5.87	7.39	11.07	8.61	16.28	14.39
N	5	5	5	5	5	5	5	5	5	5	5	5

SD: Standard Deviation CV: Coefficient of Variation N. Number of slides

Table 14d: Within site, tissue variability seen at Site 3

Tissue:	A1		A2		N1		N2		D1		D2	
	60 cells	60 counts	60 cells	60 counts	60 cells	60 counts	60 cells	60 counts	60 cells	60 counts	60 cells	60 counts
Mean	3.88	3.19	8.47	9.02	1.22	1.48	1.24	1.27	0.74	0.74	0.83	0.86

SD	0.83	0.71	1.71	3.25	0.11	0.18	0.06	0.09	0.03	0.05	0.04	0.03
%CV	21.87	22.18	20.13	36.01	9.24	12.32	5.05	9.66	4.42	6.51	4.37	3.63
N	5	5	5	5	5	5	5	5	5	5	5	5

SD: Standard Deviation CV: Coefficient of Variation N. Number of slides

3) Between-Site Reproducibility of 60 cells (nuclei) vs. 60 counts (Table 14e)
The imprecisions of the between-site reproducibility were from 8.8% to 24.6% for 60 cells (nuclei) method with median ratio of 12.7%; The imprecisions of the between-site reproducibility were from 9.8 % to 29.0% for 60 counts method with median ratio of 15.8%.

Table 14e: Between-Site Reproducibility for the 6 Individual Patient Tissues at Three (3) Test Sites by the Two Counting Methods

Tissue	A1		A2		N1		N2		D1		D2	
	60 Cells	60 Counts	60 Cells	60 Counts	60 Cells	60 Counts	60 Cells	60 Counts	60 Cells	60 Counts	60 Cells	60 Counts
Mean	3.33	3.07	7.46	7.95	1.34	1.39	1.31	1.33	0.73	0.72	0.90	0.92
SD	0.73	0.73	1.84	2.31	0.14	0.20	0.12	0.15	0.06	0.07	0.14	0.16
% CV	21.9	23.8	24.6	29.0	10.3	14.6	9.5	11.0	8.8	9.8	15.1	16.9
N	15	15	10	15	15	15	15	15	15	15	15	15

SD: Standard Deviation CV: Coefficient of Variation N. Number of slides

4) Correlation of TOP2A Gene Status Counted by the Two Different Counting Method

Table 14f: Correlation of TOP2A Gene Status Counted by the Two Different Counting Methods

60 Signals	60 Nuclei Frequency			
Frequency	Deleted	Normal	Amplified	Total
Deleted	16	0	0	16
Normal	1	43	0	44
Amplified	0	0	30	30
Total	17	43	30	90

Kappa statistics (n = 90); Simple Kappa 0.98 (95% CI 0.95-1.02)

5) Notes

a. The analysis of the data showed that equivalent results were obtained with both counting methods. However, the 60 cells and 60 counts methods may not be interchangable between sites.

b. For a high percentage of patients, using the less time consuming method of counting 60 TOP2A signals will be sufficient to be able to make a classification.

c. The labeling cautions users to inspect carefully the results when they fall into the equivocal zones at TOP2A/CEN17 ratio of 0.7-0.9 and 1.8-2.2, for deletion and amplification, respectively.

d. If the ratio falls into either of the two equivocal zones, it is recommended that the score of the specimen should be verified by rescoring by a second person, counting of nuclei from 3 more tumor areas and/or by counting a total of 60 nuclei.

d) Conclusions

Reproducibility was substantial for all assay comparisons across the 3 sites.

There were 2 evaluations of specimen D1 with deletion status that indicated normal gene copy status and 3 evaluations of specimen D2 with an equivocal status that indicated a deleted status (Table 14e).

Evaluation of amplified versus non-amplified status across all specimens had full agreement across all study sites and all specimens.

When the alternative counting method (see Interpretation of Staining) was used, there was high concordance between the 2 counting methods. Across all 3 sites, 89 of 90 scorings were concordant.

The overall statistics including intra-assay, inter-assay and inter-observer across sites for all slides using the two counting methods is shown in Table 14e.

Reference Range

Interpretation of Staining

Assessable Tissue

Only specimens from patients with invasive carcinoma should be tested. In cases with carcinoma in situ and invasive carcinoma in the same specimen, only the invasive component should be scored. Avoid areas of necrosis and areas where the nuclear borders are ambiguous. Do not include nuclei that require subjective judgment. Skip nuclei with weak signal intensity and non-specific or high background. Use the DAPI-filter to check for even staining of the nuclei.

Summary of Safety and Effectiveness Data

Signal enumeration: Locate the tumor within the context of the H&E stained slide and evaluate the same area on the FISH stained slide. Scan several areas of tumor cells to account for possible heterogeneity. Select an area having good nuclei distribution. Begin analysis in the upper left quadrant of the selected area and, scanning from left to right, count the number of signals within the nuclear boundary of each evaluated nucleus according to the guidelines below in Table 15.

a) Focus up and down to find all of the signals in the individual nucleus.

b) Count two signals that are the same size and separated by a distance equal to or less than the diameter of the signal as only one signal.

c) In nuclei with high levels of *TOP2A* gene amplification, the *TOP2A* signals may be positioned very close to each other forming a cluster of signals. In these cases the number of *TOP2A* signals cannot be counted, but must be estimated. Special attention must be paid to the green signals, as clusters of *TOP2A* signals can cover the green signals making them impossible to see. In case of doubt, please check the green signals using a specific FITC filter.

d) Do not score nuclei with no green signals. Score only those nuclei with one or more green reference signals. The TOP2A/CEN-17 ratio is calculated as red signals/green signals and the denominator cannot be zero.

e) Record counts in a table as shown in Appendix 2 of the product labelling.

Table 15. Interpretation Guide

1		Do not count. Nuclei are overlapping, not all areas of nuclei are visible
2		Count as two green signals
3		Two red signals, do not score nuclei with only red signals (denominator in the ratio cannot be 0)
4		Count as 3 green and 12 red signals (cluster estimation)
5		Count as 1 green and 1 red signal. Two signals of the same size and separated by a distance equal to or less than the diameter of one signal are counted as one
6	or	Do not count (over- or underdigested nuclei). Missing signals in the centre of nuclei (donut-shaped nuclei).

7		Count as 2 green and 3 red signals. Two signals of the same size and separated by a distance equal to or less than the diameter of one signal are counted as one
8		Count as 1 green and 5 red signals
9		Count as 3 green (1 green out of focus) and 3 red signals
10		Cluster of red signals hiding green signals, check the green signals with a specific FITC filter, or do not count

Note

The signals can be scored either by the conventional method (43) or by an alternative, time- and labor-reducing method (1, 2). Instead of the conventional method of counting signals in 60 nuclei, a total of 60 events are scored, where one event is a red gene signal. By this alternative counting method a variable number of nuclei are scored until 60 red *TOP2A* signals are reached. The corresponding green CEN-17 signals in the same nuclei are recorded. The minimum number of nuclei to score is 6. In normal specimens an average of 35 nuclei will be enough to reach 60 red signals. In amplified cases, 6-35 nuclei will be included. Even in cases with deletions, less than 60 nuclei will often be sufficient. The latter method has the advantage that the highest number of cells will be counted in the deleted and normal cases, while the lowest number of cells will be counted in the amplified cases. These cases are often obvious to identify just by looking in the microscope, but are quite tedious and time-demanding to count if 60 nuclei should be scored. The concordance between the two counting methods was high. In the reproducibility study (see Table 12) comparable ratios were obtained and 89/90 slides showed concordant results.

If possible count the nuclei from 3 distinct tumor areas (44). Calculate the *TOP2A*/CEN-17 ratio by dividing the total number of red *TOP2A* signals by the total number of green CEN-17 signals.

Specimens with a *TOP2A*/CEN-17 ratio above or equal to 2.00 should be considered as having *TOP2A* amplification and specimens with a *TOP2A*/CEN-17 ratio less than 0.80 should be considered as having *TOP2A* deletion (1, 2, 9).

Quality Control

1. Signals must be bright, distinct and easy to evaluate.

2. Normal cells allow for an internal control of the staining run.
   a) Normal cells should have 1-2 clearly visible green signals indicating that the CEN-17 PNA Probe has successfully hybridized to the centromeric region of chromosome 17.

   b) Normal cells should also have 1-2 clearly visible red signals indicating that the *TOP2A* DNA Probe has successfully hybridized to the *TOP2A* target region.

   c) Due to tissue sectioning, some normal cells will have less than the expected 2 signals of each color.

   d) Normal cells undergoing cell division may have more than the normal 1-2 signals of each color.

   e) Failure to detect signals in normal cells indicates assay failure, and results should be considered invalid.

3. Nuclear morphology must be intact when evaluated using a DAPI filter. Numerous ghost-like cells and a generally poor nuclear morphology indicate over-digestion of the specimen, resulting in loss or fragmentation of signals. Such specimens should be considered invalid.

4. Differences in tissue fixation, processing, and embedding in the user's laboratory may produce variability in results, necessitating regular evaluation of in-house controls.

Cut-off values and indeterminant zone
The TOP2A status was categorized into the following groups:
Deleted: when TOP2A/CEN-17 ratio <0.8 (equivocal zone between 0.7 – 0.9).
Normal: when TOP2A/CEN-17 ratio 0.8 - < 2.0
Amplified: when TOP2A/CEN-17 ratio ≥ 2.0 (equivocal zone between 1.9 – 2.1).

Results at or near the cut-off (1.9-2.1 for amplifications and 0.70-0.90 for deletions) should be interpreted with caution. It is recommended to check that the scorings do not include a high percentage of normal nuclei.

If the ratio on initial evaluation falls into either of the two equivocal zones, it is recommended that the score of the specimen should be verified by rescoring by a second person, by counting nuclei from 3 more tumor areas and/or counting a total of 60 nuclei. The final ratio should be recalculated based on all scorings. For borderline cases a consultation between the pathologist and the treating physician is warranted.

Expected values

Testing of normal tissue

To establish a range of results for normal tissue, a study was conducted that measured the distribution of *TOP2A*/CEN-17 ratios in normal breast tissue specimens using the recommended scoring method of 60 nuclei. In a sample set of 21 normal breast tissue specimens, the median *TOP2A*/CEN-17 ratio was 1.08 with the 2.5 and 97.5 percentiles forming an interval of 1.00 to 1.20.

Selection of cut-off

The reasons for using FISH ratio ≥ 2.0 as the cut-off for gene amplification are in line with those presented by Press and coworkers (32) 1) The established "cut-off" used for evaluating gene amplification with Southern Blot was originally a ratio of an index gene-to-control of 2.0 or greater. 2) The accepted FDA-approved FISH ratio for *HER2* gene amplification is ≥ 2.0 and there are no data establishing that another ratio should be chosen for *TOP2A*. 3) Because only a portion of a cell population is dividing at any one time, using a ratio of 2.0 or greater is not likely to lead to confusion with non-amplified actively dividing cell populations. A FISH ratio of ≤ 0.8 as indicative of *TOP2A* gene deletion has been selected because it allows identification of breast cancers that lose a single gene copy from a tetraploid or near-tetraploid, aneuploid breast cancer. In addition, this is the ratio that has been used by the majority of investigators as listed in Table 16 below.

Table 16. TOPA FISH Cutoff

Study	Cut-off *HER2* amplification	Cut-off *TOP2A* amplification	Cut-off *TOP2A* deletion	Reference
Järvinen, 1999	1.5	1.5	0.67	(35)
Järvinen, 2000	1.5	1.5	0.7	(4)
Di Leo, 2002	2.0	1.5	0.8	(9)
Coon, 2002	2.5	2.5	Ni	(8)
Park, 2003	4 (CISH)[1]	4 (CISH)[1]	Ni	(10)
Bofin, 2003	2.0	2.0	1.0	(37)
Olsen, 2004	2.0	2.0	0.8	(2)
Cardoso, 2004	2.0	1.5	ND	(29)
Durbecq, 2004	2.0	1.5	Ni	(26)
Hicks, 2005	2.0	2.0	0.7	(45)
Knoop, 2005	2.0	2.0	0.8	(1, 3)
Callagy, 2005	1.5	1.5	Ni	(28)
Press, 2005	2.0	2.0	0.8	(12, 13)
Tanner, 2005	CISH[1]	CISH[1]	Ni	(11)
O'Malley, 2007	2.0	2.0	0.8	(7)

Ni: Not investigated (requires FISH) ND: Not defined

[1] CISH (chromogen in situ hybridization) without CEN 17 does not allow detection of deletions

Note

Results at or near the cut-off (1.8-2.2 for amplifications and 0.70-0.90 for deletions) should be interpreted with caution. It is recommended to check that the scorings do not include a high percentage of normal nuclei.

If the ratio falls into either of the two equivocal zones, or in case of uncertainty, it is recommended that the score of the specimen should be verified by rescoring by a second person, by counting nuclei from 3 more tumor areas and/or counting a total of 60 nuclei. The final ratio should be recalculated based on all scorings. For borderline cases a consultation between the pathologist and the treating physician is warranted.

Stability Testing

1. Sample Stability

   Due to the greater surface and resulting exposure to oxygen, cut tissue sections are more prone to degradation. A retrospective analysis of the tissue sections was carried out to assess the stability of cut sections. The results indicated the tissue cut sections were stable throughout the test period of 29 months at 2-8°C. This is translated to a stability of 6.8 months at 25°C. The recommended sample storage conditions as follows:

Table 17: Tissue Cut Section Storage Conditions

Storage Condition	Duration
Room Temperature	4-6 months
+ 2-8°C	24 months
- 18°C	No data
Freeze-thaw	No data

2. Reagent and Component Stability

   Real time stability studies were conducted with the kit stored at 2-8°C, at 18°C and subjected to 15 freeze-thaw cycles. Stability was demonstrated, by consistent test results, for at least the conditions listed in Table 18.

Table 18: Reagent Storage Conditions

Storage Condition	Duration
Room Temperature	No Data
+ 2-8°C	12 months
- 18°C	18 months
Freeze-thaw	15 Cycles

Limitations of the Test

The following special measures or limitations apply to the test and are included in the Instructions for Use.

a) If the TOP2A/CEN-17 ratio falls into either of the two equivocal zones, the ratio of the specimen should be verified via rescoring by a second person who should count at least 60 nuclei from a minimum of 2-3 cancer cells-containing areas on the same slide.

b) FISH is a multi-step process that requires specialized training in the selection of the appropriate reagents, as well as in tissue selection, fixation, and processing, preparation of the FISH slide, and interpretation of the staining results.

c) FISH results are dependent on the handling and processing of the tissue prior to staining. Improper fixation, washing, drying, heating, sectioning, or contamination with other tissues or fluids may influence probe hybridization. Inconsistent results may be due to variations in fixation and embedding methods, or to inherent irregularities within the tissue.

d) For optimal and reproducible results, the tissue slides must be deparaffinized completely. The paraffin removal needs to be completed at the beginning of the staining process. (See INSTRUCTIONS FOR USE, section B.2 in product labeling).

e) Only temperature-calibrated water bath, heating block, and hybridization oven should be used. Use of other types of equipment must be validated by user to ensure that evaporation of *TOP2A*/CEN-17 Probe Mix during hybridization do not occur.

f) The clinical significance of the presence of cancer cells with chromosome 17 polysomy has not been determined in the scientific literature. The impact of chromosome 17 polysomy on the performance of the *TOP2A* assay has not been formally assessed and the analysis of chromosome 17 polysomy has not been included in the data presented below.

X. Summary of Clinical Investigations

Pilot study

The clinical performance of *TOP2A* FISH pharmDx™ Kit was evaluated in a pilot study on specimens from 120 breast cancer patients (2) in collaboration with the Danish Breast Cancer Cooperative Group (DBCG). A total of 20 tumors had *TOP2A* copy number changes, almost equally divided between amplifications (n=11) and deletions (n=9). The *TOP2A* changes were not exclusively found in HER2 positive tumors, as 4 tumors (20% of the *TOP2A* abnormal cases) were HER2 negative.

The pilot study showed that two different counting methods gave comparable results, i.e. either when the signals were counted in 60 nuclei or when a total of 60 red signals were counted along with the green signals in the same nuclei.

Pivotal study - DBCG 89D/*TOP2A*

In a larger scale study, also conducted in Europe, the clinical performance of *TOP2A* FISH pharmDx™ Kit was evaluated based on tumor samples prospectively collected (1, 3, 17) and retrospectively tested from the Danish Breast Cancer Group (DBCG) 89D adjuvant study (46).

Clinical Study Objective

Demonstration of the prognostic properties of *TOP2A* gene aberrations (amplifications or deletions) in high-risk breast cancer patients was a secondary objective of DBCG 89D and is the basis for FDA approval of the *TOP2A* FISH pharmDx™ Kit™.

Inclusion and Exclusion Criteria

The inclusion criteria in DBCG 89D protocol were:

a) Diagnosis of primary invasive breast cancer
b) The patient must either be:
   i. Premenopausal with tumor > 5 centimeters, or with positive axillary lymph nodes, and with steroid receptor negative or steroid receptor unknown tumor
   ii. Postmenopausal with tumor > 5 centimeters, or with positive axillary lymph nodes, and with steroid receptor negative tumor
   iii. Premenopausal with tumor ≤ 5 centimeters, and with negative axillary lymph nodes, and with ductal carcinoma with anaplasia degree II-III
c) Total mastectomy and dissection of the axilla level 1-2, or tumorectomy and dissection of axilla level 1-2.
d) The patient's acceptance of treatment/examination after being informed, verbally and in writing and have signed the informed consent form.

The exclusion criteria in the DBCG 89D protocol were:

a) Age ≥ 70 years
b) Signs of metastatic disease according to physical examination as well as x-ray examination of thorax, columna and pelvis, and ultrasound of the liver, if abnormal liver-biochemistry.
c) Other previous malignant disease, including previous breast cancer, and excluding skin cancer and cervix cancer in situ.
d) Other malignant breast tumors or invasive carcinoma.
e) Bilateral breast cancer.
f) Lobular carcinoma in situ and intra-ductal carcinoma.
g) Paget's disease of the nipple of in situ type.
h) Inflammatory breast cancer.

i) Contraindication for the use of the postoperative medical treatment, including coronary disease, which contraindicates treatment with epirubicin.

Study Overview

Protocols

The DBCG 89D study was designed as an open, prospective, randomized study. Following surgery, a total of 980 pre- and postmenopausal women with high-risk invasive breast cancer were randomized to CMF (cyclophosphamide/methotrexate/5-fluorouracil) or CEF (cyclophosphamide/epirubicin/5-fluorouracil). The primary efficacy outcome was RFS (recurrence free survival) with OS (overall survival) as a secondary endpoint. For the biological sub-study DBCG 89D/*TOP2A* tissue blocks from the patients who had participated in the DBCG 89D study were collected from the study sites and centrally analyzed retrospectively for *TOP2A* and *HER2* gene aberrations (Dako *HER2* FISH pharmDx™ Kit) as well as HER2 overexpression (Dako HercepTest™). Tissue blocks were available from 806 of the 962 patients included in the DBCG 89D study.

The issue of selection bias relative to patients participating in the randomized trial was addressed, comparing the 767 patients included in the multivariate analyses with the patients not included due to unavailability of tumor tissue (n=156), technical failure of the *TOP2A* test (n=33) or unknown covariates (n=6). The hypothesis of no difference in baseline values between the groups was investigated using contingency tables and χ^2–tests.

The TOP2A/CEN-17 ratio was calculated as the number of signals from the gene probes (*HER2* and *TOP2A* respectively) divided by the number of signals for the centromere 17. Cases were scored as *HER2* or *TOP2A* FISH amplified when the ratio was ≥ 2. A *TOP2A* deletion was considered present when the ratio was < 0.8. In the absence of a biologically well-defined ratio the cut-off points were based on the work of Di Leo et al.[21]

TOP2A status was categorized into the following groups:

a) Deleted: When *TOP2A/CEN-17* ratio < 0.8
b) Normal: When 0.8 ≤ *TOP2A/CEN-17* ratio < 2.0
c) Amplified: When *TOP2A*/CEN-17 ratio ≥ 2.0

All HercepTest™ 1+, 2+ and 3+ positive specimens were subjected to *HER2* FISH analysis. The scoring of HER2 positivity in the study was as following:

a) Positive: when HercepTest=3+, or (HercepTest=2+ and *HER2* FISH ratio ≥ 2.0)
b) Negative: when HercepTest=0,1+, or (HercepTest=2+ and *HER2* FISH ratio < 2.0)

The clinical study (DBCG 89D) (46) and the biological sub-study (DBCG 89D/*TOP2A*) (1, 3) were conducted according to the Helsinki declaration and approved by the local Ethical Committees.

Patients and Tissue Disposition

The study population consisted of 980 Danish patients randomized according to the DBCG 89D protocol (Figure 2):

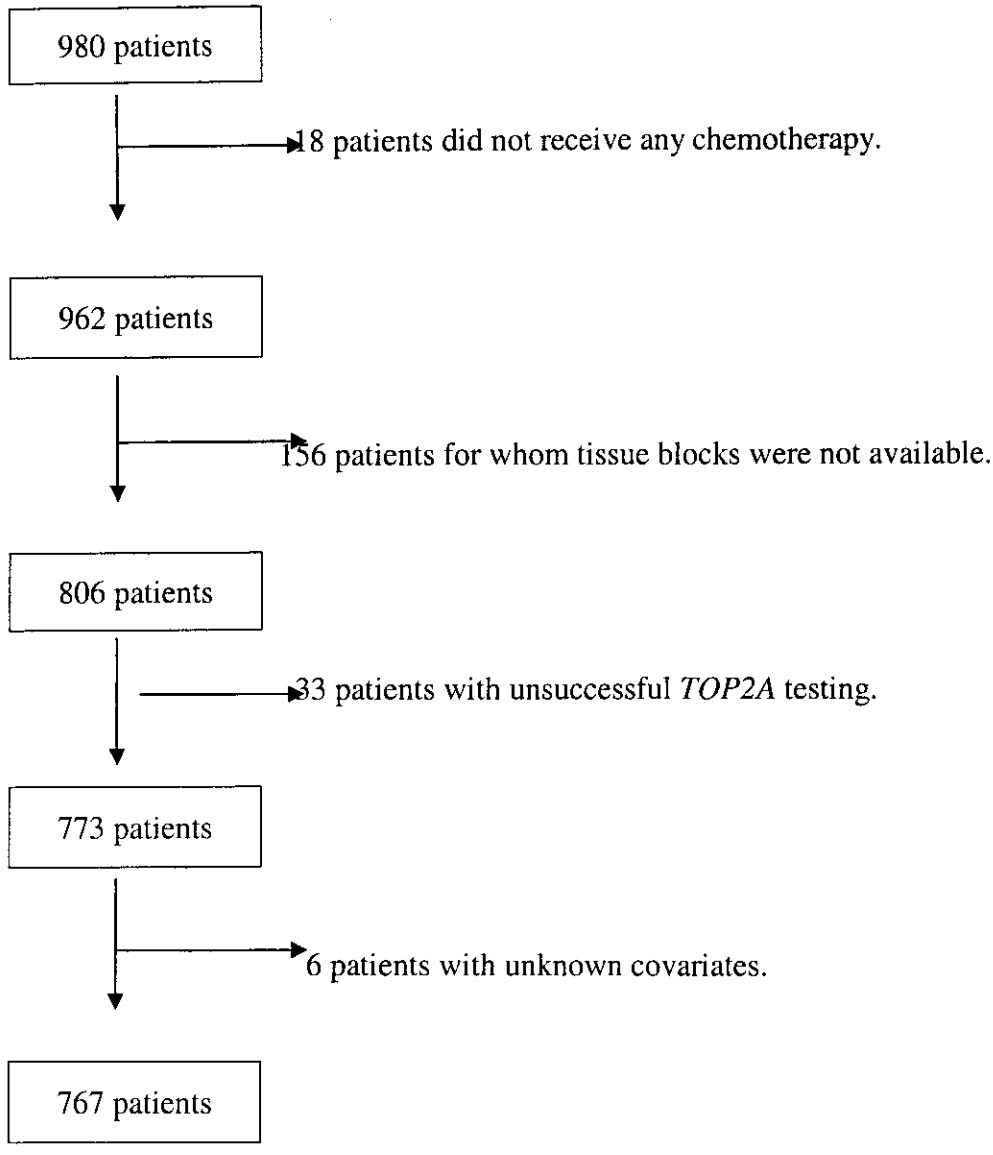


Figure 1. Patient Disposition

Missing Specimens

A tabulation by study site follows for patients not among the final 767 studied because they received no chemotherapy, had no tissue available for study, or had no valid *TOP2A* testing result.

Table 19. The distribution of available and missing samples across 21 study sites

Summary of Safety and Effectiveness Data

Distribution of number of patients and samples across study sites	Subpopulations of patients							
	No adjuvant chemo therapy		No tissue available		*TOP2A* testing unsuccessful		*TOP2A* testing successful	
	N	%	N	%	N	%	N	%
Randomization Center								
RIGSHOSPITALET	4	1.8	39	18.0	9	4.1	165	76.0
BISPEBJERG	.	.	2	6.9	2	6.9	25	86.2
HERLEV	5	3.7	30	22.1	2	1.5	99	72.8
ROSKILDE	1	1.8	4	7.3	1	1.8	49	89.1
HOLBÆK	.	.	.	.	.	.	1	100.0
SLAGELSE	.	.	1	50.0	.	.	1	50.0
NÆSTVED	1	2.1	13	27.7	2	4.3	31	66.0
NAKSKOV	1	5.0	2	10.0	.	.	17	85.0
RØNNE	.	.	.	.	.	.	10	100.0
ODENSE	3	2.8	14	13.0	.	.	91	84.3
SØNDERBORG	2	7.7	1	3.8	1	3.8	22	84.6
HADERSLEV	.	.	5	25.0	1	5.0	14	70.0
ÅBENRÅ	.	.	.	.	.	.	1	100.0
ESBJERG	.	.	17	22.7	.	.	58	77.3
VEJLE	1	2.4	5	11.9	10	23.8	26	61.9
HOLSTEBRO	.	.	2	20.0	.	.	8	80.0
HERNING	.	.	2	15.4	.	.	11	84.6
ÅRHUS KH	.	.	8	12.7	3	4.8	52	82.5
VIBORG	.	.	2	11.8	1	5.9	14	82.4
ÅLBORG	.	.	7	11.9	.	.	52	88.1
HJØRRING	.	.	2	6.9	1	3.4	26	89.7
All	18	1.8	156	15.9	33	3.4	773	78.9

Treatment Protocol

In the DBCG 89D study, patients received locoregional therapy and were randomized to one of four treatment arms: CMF, CEF, CMF plus Pamidronate, or CEF plus Pamidronate. Treatment details are presented in Table 20.

Table 20: Treatment Protocols

Adjuvant radiotherapy* after mastectomy:	– to patients ≤ 45 years with ≥ 4 positive axillary lymph nodes (against regional lymph nodes and chest wall) – to all patients with invasion of the profound resection surface (against the chest wall).
Adjuvant radiotherapy* after tumorectomy:	– to all patients (against the residual breast tissue). – to patients ≤ 45 years with ≥ 4 positive axillary lymph nodes (against regional lymph nodes).
CMF	– i.v. cyclophosphamide 600 mg/m^2 day 1 – i.v. methotrexate 40 mg/m^2 day 1 – i.v. 5-fluorouracil 600 mg/m^2 day 1 Treatment is repeated every three weeks, for a total of nine cycles. In case of simultaneous radiotherapy, only i.v. cyclophosphamide is given for 2 or 3 cycles, 850 mg/m^2.
CEF	– i.v. cyclophosphamide 600 mg/m2 day 1 – i.v. epirubicin 60 mg/m2 day 1 – i.v. 5-fluorouracil 600 mg/m2 day 1 Treatment is repeated every three weeks, a total of nine cycles. In case of simultaneous radiotherapy, only i.v. cyclophosphamide is given for 2 or 3 cycles, 850 mg/m^2.
Pamidronate	– p.o. 150 mg pamidronate b.i.d. for 4 years

Loco-regional radiotherapy was given after the first cycle of CMF or CEF given against the residual breast following lumpectomy (48 Gy + boost 10 Gy) or chest wall following mastectomy if the tumor was > 5 cm (48 Gy), and against regional nodes in node-positive disease (48 Gy). In all cases 2 Gy in 5 fractions per week.

Follow-up

After the initial medical treatment the patients were followed every 6 months for the first 5 years and subsequently every 12 months for a total of 10 years. Follow-up was discontinued earlier by one of the following reasons: Patients wants to stop, recurrence of the disease, any other malignant disease, lost to follow up or death. All patient records were updated as of December 31, 2004 with respect to recurrence free survival. All patient

records were updated with respect to mortality on December 31, 2004 and therefore all censored patients were known to be alive on January 1, 2005.

Table 21: Patient Selection and Follow-up

CMF	Randomly Assigned (n = 980)	CEF
Received as allocated (n=489) Receive CEF (n=6) No Chemotherapy (n =5) Total Randomized to CMF arm (n = 500)		Received as allocated (n=441) Receive CMF (n=26) No Chemotherapy (n = 13) Total Randomized to CEF arm (n= 480)
Follow-up (n = 515) Timing of primary and secondary outcomes (10 years)		Follow-up (n = 447) Timing of primary and secondary outcomes (10 years)
Tumor block Lost (n = 78) TOP-2A FISH unsuccessful (n = 16)	n = 773	Tumor block Lost (n = 78) TOP-2A FISH unsuccessful (n = 17)
n = 418 Missing Covariate (n = 3)	Total n = 767	n = 349 Missing Covariate (n = 3)

Assessments

The examination program included the following:

a) Physical examination.
b) Hemoglobin, leukocytes, and thrombocytes.
c) Serum creatinine, serum calcium (ionized), serum phosphate, alkaline phosphatase, ALAT, serum bilirubin.
d) X-ray of thorax.
e) Bone scintigraphy.
f) X-ray of columna and pelvis
g) Ultrasound of the liver

Examinations prior to study entry included a), b), c), d) and f). Item g) was done only if alkaline phosphates, ALAT or bilirubin was above upper normal level. During chemotherapy item b) was repeated at day one of each cycle. Item c) was repeated every 6 months. Bone scintigraphy e) was done after 6 months and then annually. If the bone scintigraphy was abnormal, x-ray was carried out for the corresponding region(s), and the

results of the x-ray was decisive for the diagnosis. Item f) was repeated annually. Other examinations were carried out upon indication or suspicion of residual disease.

Preparation of Tissue
Consecutive serial sections were cut at 4 µm from the available paraffin-embedded tumors for immunohistochemistry and FISH analyses and stored cold until staining was performed. A slide stained with haematoxylin and eosin, prepared from each block, was used for confirmation of invasive carcinoma.

HER2 IHC
The sections were stained within 5 days from cutting using a Techmate immunostainer (DakoCytomation, Glostrup, Denmark) according to the procedures for the HercepTest™ (DakoCytomation, Glostrup, Denmark). Positive controls as supplied with the kit were included as well as in house controls together with a negative control for each case. The results were scored 0, 1+, 2+, and 3+ as recommended for the HercepTest™.

TOP2A and HER2 FISH
Based on a list generated from the DBCG database, tissue blocks were collected at the Department of Pathology, Roskilde Hospital, Denmark, where the FISH-test of *TOP2A* and *HER2* was performed, together with the HER2 IHC test (HercepTest™). The *TOP2A* FISH pharmDx™ Kit and *HER2* FISH pharmDx™ Kit (DakoCytomation, Glostrup, Denmark) were each used on separate tissue slides according to the manufacturer's working procedure. In cases of ductal carcinoma with an in situ component, the negative control slide for HercepTest™ was used to mark the invasive areas avoiding any possibility of analyzing the in situ component. Up to 60 gene signals (or the number closest to 60+) were counted in nuclei with identifiable boundaries. Optimally, only signals distinctly separated from each other were included, but in case of clusters due to high levels of amplification the number was estimated.

As described above the ratio used in the analyses should be based on up to 60 or closest to 60+ *TOP2A* gene signals (or *HER2* gene signals). However, the number of cells counted varied, for some patients 60 cells were counted, for other patients the number of cells corresponding to 60 red gene signals, or more were counted. In a few patients less than 60 red gene signals were counted, due to the small amount of tumor tissue available. A minimum of 6 nuclei (=cells) was counted in this study.

The examination of the slides was done blinded, i.e. data concerning tumor size, malignancy grade, receptor status, number of positive lymph nodes, adjuvant therapy and clinical outcome data were unknown to the examiner.

Statistical Methodology

The primary endpoint for the study was RFS, defined as the time from randomization to an event or censoring. An event was defined as relapse local or distant, second malignancy or

death, whichever occurred first. Censoring was due to 'lost to follow up', 'patient will no longer participate' or 'alive without disease at end of follow-up'.

The secondary endpoint was OS, defined as the time from randomization to death (irrespective of cause) or censoring. Patients were considered as censored if patients were alive at end of follow-up.

The effect of TOP2A status within treatment groups is illustrated by figures of the univariate survival curves. The Cox proportional hazards model was adjusted according to the results of the goodness-of-fit procedures, defining the basic multivariate Cox model for analysis of RFS and OS. The hazards ratio (HR), the 95 % confidence interval and the p-value of the Wald test were calculated for each covariate in the Cox model.

Correlations between *TOP2A* status and clinical and pathological variables including HER2-status were tested by $\chi 2$-test.

Follow-up time was quantified in terms of a Kaplan-Meier estimate of potential follow-up. Analyses were performed for possible selection bias using the $\chi 2$-test and log-rank test. Patients with missing clinical covariates (N=6), except for receptor status, were excluded from the multivariate analyses in the Cox proportional hazard model.

Study Results

Patient Characteristics

Of the 773 patients with successful *TOP2A* FISH, 421(54%) were treated with CMF and 352 (46%) with CEF. With respect to loco-regional radiotherapy, 206 (40.0%) patients in CMF group and 173 (38.7%) in CEF group received radiotherapy. No significant differences between the two treatments arms were seen concerning the baseline characteristics. The baseline characteristics of the 773 patients divided into the two treatment arms are shown in Table 22.

Table 22: Patients Characteristic (N = 773)

Patients characteristic	**CMF** N (%)	**CEF** N (%)
Age, Years		
-39	70 (16.6)	57 (16.2)
40-49	201 (47.7)	167 (47.4)
50-59	92 (21.9)	78 (22.2)
60-69	58 (13.8)	50 (14.2)
Menopausal status		
Premenopausal	294 (69.8)	241 (68.5)
Postmenopausal	127 (30.2)	111 (31.5)
Removed lymph nodes		
0-9	166 (39.4)	125 (35.5)
10-	255 (60.6)	227 (64.5)
Nodal status		
0	150 (35.6)	133 (37.8)

Summary of Safety and Effectiveness Data

Patients characteristic	CMF N (%)	CEF N (%)
1-3	140 (33.3)	104 (29.5)
3-	131 (31.3)	115 (32.7)
Tumor size		
1-20 mm	178 (42.4)	138 (39.3)
21-50 mm	208 (49.5)	184 (52.4)
50- mm	34 (8.1)	29 (8.3)
ER Receptor status		
Positive	114 (27.1)	88 (25.0)
Negative	281 (66.7)	240 (68.2)
Unknown	26 (6.2)	24 (6.8)
Malignancy grade		
Grade I	32 (7.6)	21 (6.0)
Grade II	195 (46.3)	166 (47.2)
Grade III	166 (39.4)	143 (40.6)
Non-ductal	26 (6.2)	20 (5.7)

Distribution of Test Results

Out of the 980 Danish patients included in the study 806 had tissue blocks available for testing of *TOP2A* status with the *TOP2A* FISH pharmDx™ Kit. The distribution of the 806 patients with respect to HercepTest, *HER2* FISH and *TOP2A* FISH test results are summarized in Table 23 below.

Table 23. Distribution of HercepTest, *HER2* FISH and *TOP2A* FISH tests (N=806)

HercepTest	*HER2* FISH	*TOP2A* FISH				Total
		Deleted	Normal	Amplified	Unsuccessful	
0	Normal	11	85	5	2	103
	Amplified					
	Unknown		107	1	4	112
1+	Normal	12	222	5	1	240
	Amplified		7	1		8
	Unknown	1	9		5	15
2+	Normal	2	56	0	1	59
	Amplified	1	8	8	0	17
	Unknown	0	1	2	2	5
3+	Normal	5	13	2	2	22
	Amplified	55	86	68	11	220
	Unknown	0	0	0	4	4
Unknown	Normal	0	0	0	1	1
Total		87	594	92	33	806

The distribution of HER2 and *TOP2A* results for the 773 patients in the two treatment groups are shown in Table 24. No significant differences were seen between the two treatments arm with respect to HER2 and *TOP2A* test results.

Table 24. HER2 and *TOP2A* results (N=773)

Test result	CMF N (%)	CEF N (%)
HercepTest		
0	118 (28.0)	91 (25.9)
1+	141 (33.5)	116 (33.0)
2+	37 (8.8)	41 (11.6)
3+	125 (29.7)	104 (29.5)
HER2 FISH ratio		
‹ 2.0	222 (52.7)	196 (55.7)
≥2.0	124 (29.5)	110 (31.2)
not performed	75 (17.8)	46 (13.1)
HER2 status *		
Positive	135 (32.1)	111 (31.5)
Negative	286 (67.9)	241 (68.5)
TOP2A status		
Deleted	50 (11.9)	37 (10.5)
Normal	325 (77.2)	269 (76.4)
Amplified	46 (10.9)	46 (13.1)

*HER2 status was positive when Hercep test was 3+ or Hercep test 2+ and HER2 FISH ≥2.0..

Distribution of *TOP2A* Status

The overall distribution of *TOP2A* status among the eligible patients are shown in Table 25. Amplification of *TOP2A* was seen in 92 (11.9%) of the 773 eligible patients and deletion in 87 (11.3%).

Table 25. Distribution of *TOP2A* Status

TOP2A status	N (%)
Deleted	87 (11.3)
Normal	594 (76.8)
Amplified	92 (11.9)
Total	773

Of the 773 study subjects, 72 patients (9.3%) had TOP2A/CEN-17 ratios within 0.1 of the cut-off value (0.8) for deletions, and 19 patients (2.5%) had TOP2A/CEN-17 ratios within 0.1 of the cut-off value (2.0) for amplifications.

Summary of Safety and Effectiveness Data

The distribution of *TOP2A* status in relation to clinical and pathological characteristics was investigated using contingency tables and χ^2 –tests and the results are shown in Table 26 below.

Table 26. Clinical and pathological characteristics in relation to *TOP2A* status

Clinical and Pathological characteristics		N	Deleted N (%)	Normal N (%)	Amplified N (%)	p-value χ^2
All		773				
Treatment	CEF	352	37 (10.5)	269 (76.4)	46 (13.1)	0.59
	CMF	421	50 (11.9)	325 (77.2)	46 (10.9)	
Menopause	Pre	535	51 (9.5)	433 (80.9)	51 (9.5)	0.0003
	Post	238	36 (15.1)	161 (67.7)	41 (17.2)	
Age at surgery (yrs.)	-39	127	7 (5.5)	109 (85.8)	11(8.7)	0.0066
	40-49	368	43 (11.7)	289 (78.5)	36 (9.8)	
	50-59	170	19 (11.2)	126 (74.1)	25 (14.7)	
	60-69	108	18 (16.7)	70 (64.8)	20 (18.5)	
Size mm	0-20	316	25 (7.9)	265 (83.9)	26 (8.2)	0.003
	21-50	392	53 (13.5)	285 (72.7)	54 (13.8)	
	-51	63	8 (12.7)	43 (68.3)	12 (19.0)	
	Unknown	2	1 (50.0)	1 (50.0)	0 (0.0)	
No. of positive nodes	None	283	16 (5.7)	246 (86.9)	21 (7.4)	< 0.0001
	1-3	244	28 (11.5)	181 (74.2)	35 (14.3)	
	-4	246	43 (17.5)	167 (67.9)	36 (14.6)	
Removed lymph nodes	0-3	9	2 (22.2)	5 (55.6)	2 (22.2)	0.97
	4-9	282	30 (10.6)	220 (78.0)	32 (11.4)	
	10-	482	55 (11.4)	369 (76.6)	58 (12.0)	
Malignancy grade	I	53	5 (9.4)	42 (79.3)	6 (11.3)	0.83
	II	361	47 (13.0)	270 (74.8)	44 (12.2)	
	III	309	32 (10.4)	240 (77.7)	37 (12.0)	
	Non-ductal	46	3 (6.5)	38 (82.6)	5 (10.9)	
	Unknown	4	0 (0.0)	4 (100.0)	0 (0.0)	
ER status	Positive	202	14 (6.9)	168 (83.2)	20 (9.9)	0.12
	Negative	521	68 (13.1)	388 (74.5)	65 (12.5)	
	Unknown	50	5 (10.0)	38 (76.0)	7 (14.0)	
ER status*	Positive	202	14 (6.9)	168 (83.2)	20 (9.9)	0.032
	Negative/Unknown	571	73 (12.8)	426 (74.6)	72 (12.6)	

* ER Receptor negative and unknown as one group.

The proportion of women with *TOP2A* aberrations, amplifications or deletions, increased with age and therefore was higher in the postmenopausal than in the pre-menopausal women. The proportion of women with *TOP2A* aberrations increased with tumor size and number of positive lymph nodes. *TOP2A* aberrations were more frequent among tumors

that were hormone receptor negative or that had unknown hormone receptor status than among receptor positive tumors.

Association of *TOP2A* Status and the HercepTest Score
There was a highly significant association between the *TOP2A* and the HercepTest score with more *TOP2A* aberrations among the HercepTest 3+ tumors. *TOP2A* aberrations were seen in 130 (56.8%) of the 229 HercepTest 3+ patients and 49 (9.0%) of the 544 patients with a HercepTest score < 3+. The distribution of *TOP2A* status in relation to the HercepTest score is summarized in Table 27 below.

Table 27. Distribution of HercepTest score in relation to *TOP2A* status

	TOP2A	N	Deleted N (%)	Normal N (%)	Amplified N (%)	p-value χ^2
HercepTest	0	209	11 (5.3)	192 (91.9)	6 (2.9)	p< 0.0001
	1+	257	13 (5.1)	238 (92.6)	6 (2.3)	
	2+	78	3 (3.9)	65 (83.3)	10 (12.8)	
	3+	229	60 (26.2)	99 (43.3)	70 (30.6)	
	Total	773	87 (11.3)	594 (76.8)	92 (11.9)	

Association of *TOP2A* Status and the *HER2* FISH
A highly significant association between the *TOP2A* and *HER2* gene aberrations was found with more *TOP2A* aberrations among the *HER2* amplified tumors. *TOP2A* aberrations were seen in 133 (56.8%) of the 234 *HER2* amplified patients and 46 (8.5%) of the 539 *HER2* normal patients. In addition, 15 patients with *TOP2A* amplifications were found among the *HER2* normal patients (see table 28 below).

Table 28. Distribution *HER2* FISH status in relation to *TOP2A* FISH status

	TOP2A	N	Deleted N (%)	Normal N (%)	Amplified N (%)	p-value χ^2
***HER2* FISH**	Normal	539	31 (5.8)	493 (91.5)	15 (2.8)	p< 0.0001
	Amplified	234	56 (23.9)	101 (43.2)	77(32.9)	
	Total	773	87 (11.3)	594 (76.8)	92 (11.9)	

HER2 FISH ratio ≥ 2.0 is defined as amplified and normal if *HER2* FISH ratio < 2.0

Association of *TOP2A* Score and HER2 Status
The distribution of *TOP2A* amplifications and deletions in relation to the HER2 status is shown in table 29 below.

Table 29. Distribution of HER2 status in relation to *TOP2A* status

	TOP2A	N	Deleted N (%)	Normal N (%)	Amplified N (%)	p-value χ^2
HER2 Status	Negative	527	26 (4.9)	487 (92.4)	14 (2.7)	p< 0.0001
	Positive	246	61 (24.8)	107 (43.5)	78 (31.7)	
	Total	773	87 (11.3)	594 (76.8)	92 (11.9)	

HER2 status was defined as positive if HercepTest=3+ or HercepTest=2+ and FISH *HER2* ratio ≥ 2.0 and negative if HercepTest=0, 1+, or HercepTest=2+ and FISH *HER2* ratio < 2.0

As for the HercepTest and *HER2* FISH data, not surprisingly, a highly significant association was found between *TOP2A* status and HER2 status, with more *TOP2A* aberrations among the HER2 positive tumors. *TOP2A* aberrations were seen in 139 (56.5%) of the 246 HER2 positive tumors and 40 (7.6%) of the 527 HER2 negative tumors.

Selection Bias

Table 30. Distribution of Baseline Characteristic vs. analyses groups

		N	– Multivariate Analyses N (%)	+ Multivariate Analyses N (%)	p-value
All		962	195[1]	767	-
HER2 Status	HER2 negative	542	19 (50.0)	523 (68.2)	0.02[2]
	HER2 positive	263	19 (50.0)	244 (31.8)	
***TOP2A* Status**[4]	Deletion	87	1 (16.7)	86 (11.2)	
	Normal	594	5 (83.3)	589 (76.8)	
	Amplification	92	0 (0.0)	92 (12.0)	
Events	No event	508	108 (55.4)	400 (52.2)	0.42[2]/0.32[3]
	Event	454	87 (44.6)	367 (47.9)	
Death	Alive	546	112 (57.4)	434 (56.6)	0.83[2]/0.4[3]
	Dead	416	83 (42.6)	333 (43.4)	
Treatment	CEF	447	98 (50.3)	349 (45.5)	0.232[2]
	CMF	515	97 (49.7)	418 (54.5)	
Menopause	Pre	688	156 (80.0)	532 (69.4)	0.0033[2]
	Post	274	39 (20.0)	235 (30.6)	
Age at surgery (yrs.)	-39	163	39 (20.0)	124 (16.2)	0.075[2]
	40-49	472	105 (53.9)	357 (47.9)	
	50-59	201	31 (15.9)	170 (22.2)	

	60-69	126	20 (16.7)	106 (13.8)	
Tumor size (mm)	0-20	422	107 (56.6)	315 (41.1))	< 0.001[2]
	21-50	461	72 (38.1)	389 (50.7)	
	-51	73	10 (5.3)	63 (8.2)	
No. of positive nodes	None	352	70 (35.9)	282 (36,8)	0.075[2]
	1-3	321	77 (39.5)	244 (31.8)	
	-4	289	48 (24.6)	241 (31.4)	
Malignancy grade	I	128	29 (15.7)	99 (12.9)	0.002[2]
	II	468	108 (58.4)	360 (47.0)	
	III	356	48 (26.0)	308 (40.2)	
Receptor status	Positive/Unknown	250	48 (24.6)	202 (26.3)	0.62[2]
	Negative	712	147 (75.4)	565 (73.7)	

[1]195 patients were not included in the multivariate analyses due to unavailability of tumor tissue (n=156), unsuccessful test of *TOP2A* (n=33) or unknown covariates (n=6).

[2] χ^2-test

[3]Log-rank test in univariate survival analysis

[4]No test performed due to the small number, however no bias observed in the estimated frequencies,

Selection Bias due to Unavailability of Tissue
The issue of bias by selection was addressed, since 156 patients, who received chemotherapy were excluded from the analyses due to unavailability of tumor tissue. The distribution of clinical and pathological variables was given for each of the two populations (patients with tissue blocks available, N=806 vs. patients with no tissue blocks available, N=156). The hypothesis of no difference in baseline values between the groups was investigated using contingency tables and χ^2–tests. Difference in RFS and OS was investigated using Kaplan-Meier plots and log-rank tests.

The conclusions were that outcomes (RFS and OS) showed no association with tissue availability. In contrast, such association was detected for menopausal status, number of positive lymph nodes, tumor size, receptor status and grade, as the tissue was more often available when the prognostic factors were worse (higher age, more positive lymph nodes, larger tumor size, higher grade).

Bias due to Unsuccessful TOP2A *test*
Tissue samples for testing of the *TOP2A* status using *TOP2A* FISH pharmDx™ Kit were available from 806 patients. However, the testing was unsuccessful for 33 patients (4%), and it was investigated whether success of the *TOP2A* tests was independent of the

prognostic factors. This was done using contingency tables and χ^2–tests. A comparison of RFS and OS in the two groups was carried out using log-rank tests.

Based on the analyses it was concluded, that the patients with unsuccessful *TOP2A* testing, displayed the same characteristics as patients with successful *TOP2A* testing, except with regard to HER2 status. The proportion of patients with unsuccessful *TOP2A* tests was greater among the HER2 positive patients. No difference was seen in either RFS or OS between the two groups. However, as only few of the patients had an unsuccessful *TOP2A* test, the statistical power for finding such a difference was low.

Summary of Clinical Performance

Data Sets Analyzed

According to section under Patient Disposition, 6 of the 773 patients with *TOP2A* data available had missing data that excluded them from the multivariate analyses. Among the remaining 767 patients, 49 of them had unknown receptor status. To verify whether it was appropriate to include this group in the further analyses, it was investigated if there were differences in RFS and OS between patients with unknown receptor status in comparison to patients with negative and positive receptor status. This comparison was performed using the Kaplan-Meier method and log-rank tests. The result of this testing showed that there was no difference between patients with receptor unknown and receptor negative and positive tumors as regard to both RFS and OS. For further analyses the data were pooled and regarded as one group. With respect to the remaining 767 patients, 3 of them with a HercepTest 2+, the *HER2* FISH test results were lacking. The HER2 status for these 3 patients was regarded as negative.

Follow-up time

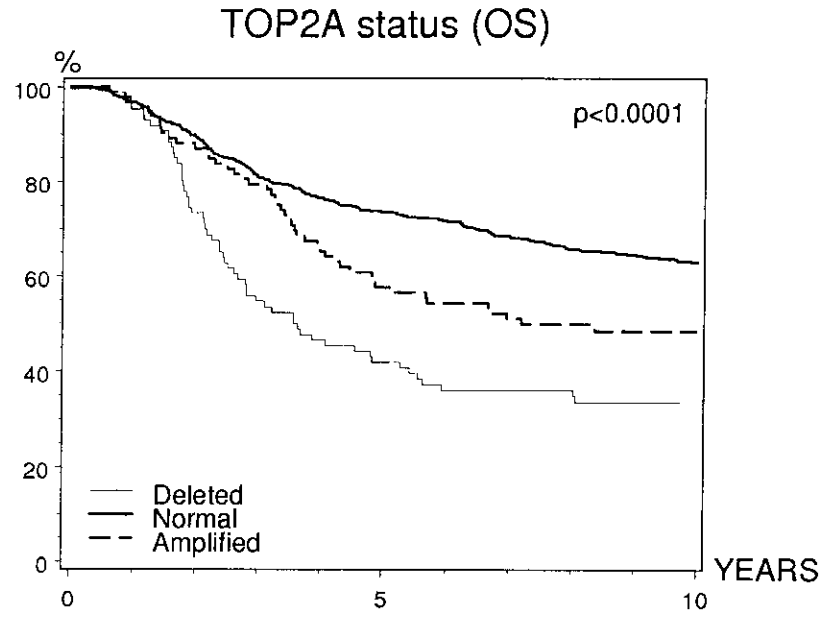
Among the patients where *TOP2A* tests were available the potential follow-up time with respect to RFS had a median value of 9.4 years and the numbers of events were 371. The median of the potential follow-up time with respect to OS was 11.1 years, and the numbers of events were 336. The median potential follow-up time in each of the *TOP2A* groups are shown in following Table 31.

Table 31. Median potential follow-up time for the *TOP2A* patients

Median potential follow up	*TOP2A* status		
	Deleted	**Normal**	**Amplified**
RFS	9.4 year	9.5 year	8.6 year
OS	10.6 year	11.3 year	10.4 year

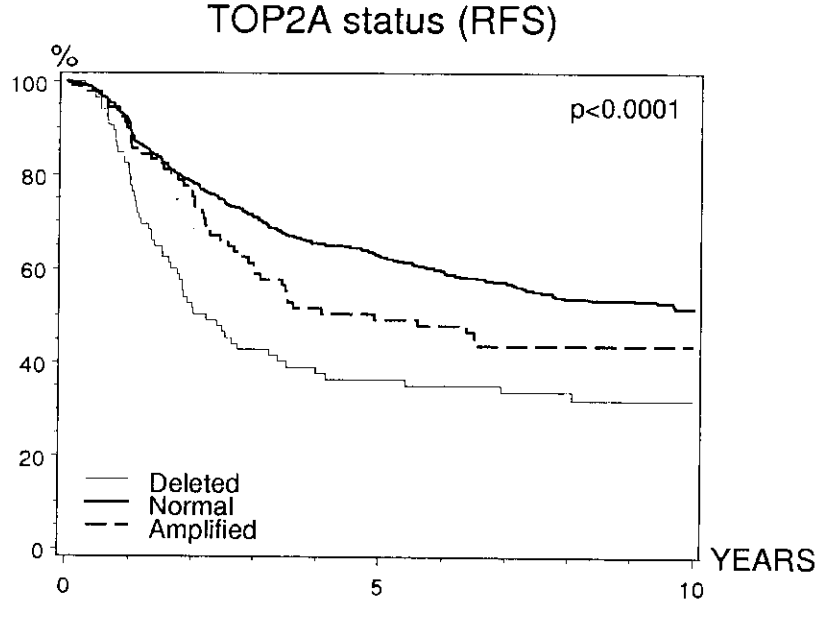
Univariate Survival Analyses

Kaplan-Meier plots and log-rank tests were used to examine the effect of TOP2A status on RFS and OS for all patients, for patients treated with CMF and for patients treated with CEF. The Kaplan-Meier plots of RFS and OS, by *TOP2A* status within each patient group, are in Figs. 2-4.



Patients at risk

Amplified	92	53	26
Normal	589	433	239
Deleted	86	36	18

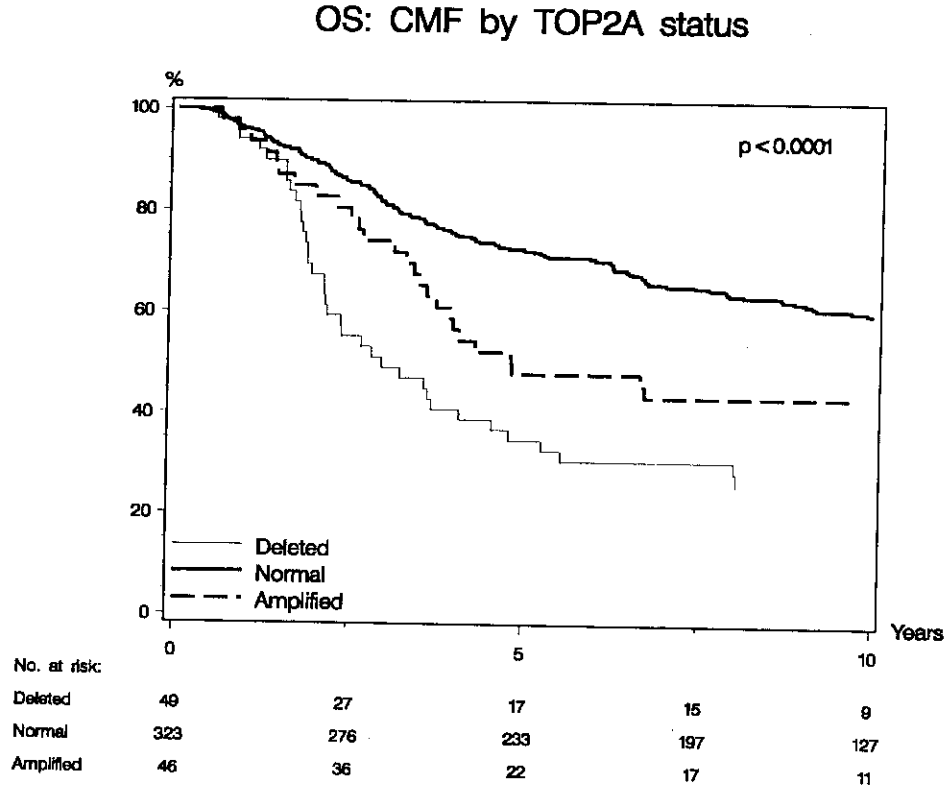


Patients at risk

Amplified	92	40	8
Normal	589	339	77
Deleted	86	28	6

50

Figure 2a and 2b. Overall survival (OS, upper panel) and Recurrence free survival (RFS, lower panel) for patients in the 3 *TOP2A* groups (normal, amplified and deleted *TOP2A* status) showing a significant worse outcome for patients with *TOP2A* amplification and even poorer for patients with *TOP2A* deletion. Results were combined across treatment arms.



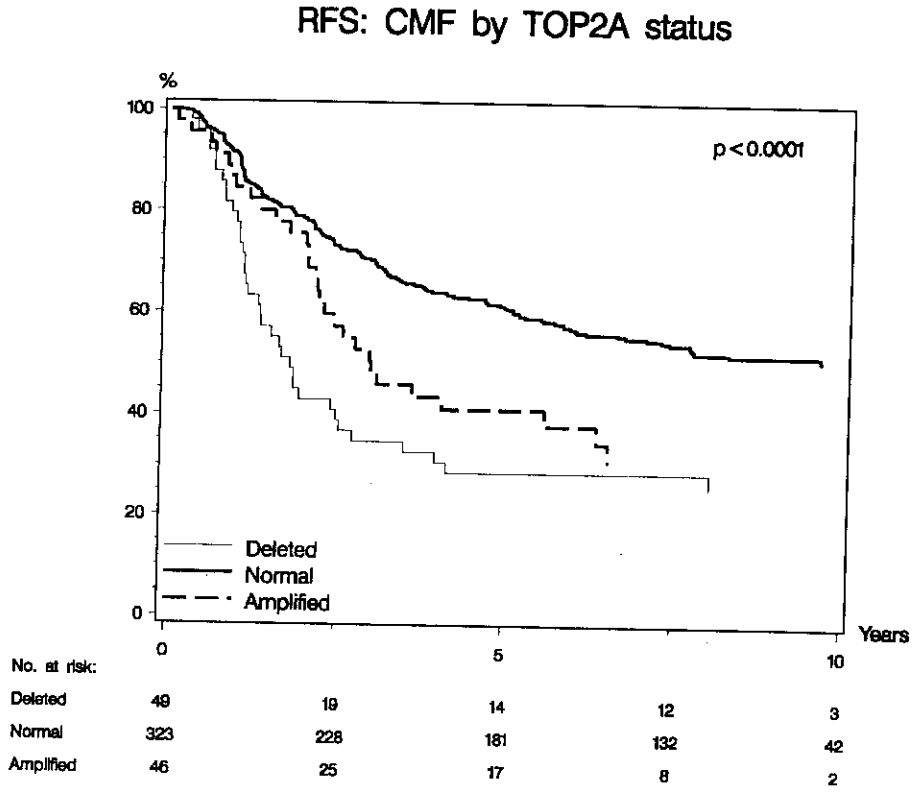
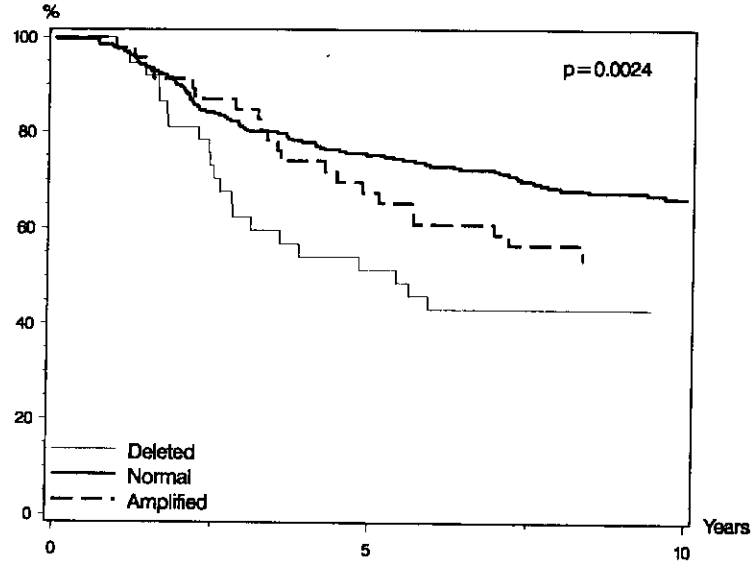


Figure 3a and 3b. Overall survival (OS, upper panel) and Recurrence free survival (RFS, lower panel) for patients treated with CMF. Patients with normal *TOP2A* status have a significant better outcome than patients with *TOP2A* amplification or deletion.

OS: CEF by TOP2A status



No. at risk:					
Deleted	37	27	19	15	9
Normal	266	224	200	166	112
Amplified	46	40	31	22	15

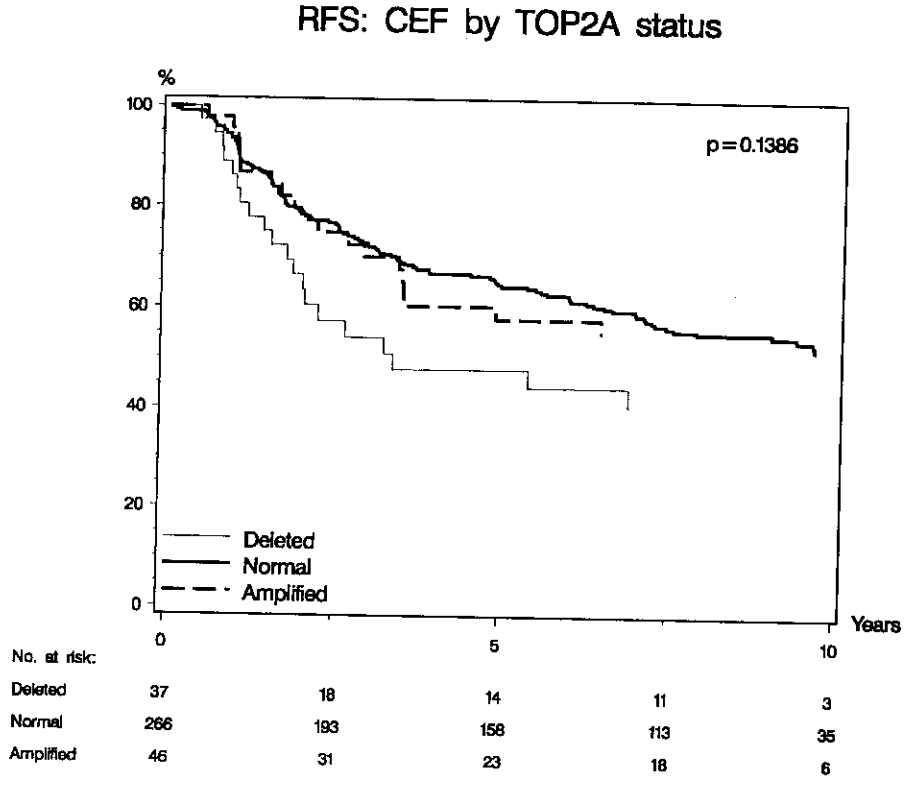


Figure 4a and 4b. Overall survival (OS, upper panel) and Recurrence free survival (RFS, lower panel) for patients treated with CEF. For OS patients with *TOP2A* amplifications and deletions had a significant worse outcome than patients with *TOP2A* normal status. For RFS, patients with *TOP2A* amplification had outcome comparable to patients with *TOP2A* normal status.

When CEF was compared to CMF using all 767 patients, the better outcome was observed in CEF. The difference was statistically marginally significant in both recurrence-free survival and overall survival (p=0.0627 for REF, p=0.0535 for OS). In terms of risk reduction in which CEF is compared to CMF, 18% risk reduction was observed in CEF for recurrence-free survival, 19% risk reduction was observed in CEF in overall survival.

TOP2A Status as a Risk Factor Compared within Each Treatment Arm

a) For CMF (n=418), the three TOP2A groups were analyzed. For both OS and RFS, *p*-values were <0.0001. In both analyses, the normal TOP2A resulted in the highest overall survival/recurrence-free survival and the deleted TOP2A resulted in the worst overall survival/recurrence-free survival (Fig. 3a and 3b).

b) For CEF (n=349), the three TOP2A groups were analyzed. For OS, p=0.0024. For RFS, p=0.1386. In both analyses, the normal TOP2A resulted in the highest overall survival/recurrence-free survival and the deleted TOP2A resulted in the worst overall survival/recurrence-free survival (Fig. 4a and 4b).

5-year overall survival and recurrence-free survival by TOP2A status

The five year OS and RFS estimates based on current study dada are presented in Table 32-35. The TOP2A deleted results in the worst 5-year outcomes. The TOP2A normal results in the best 5-year outcomes regardless of treatment.

Table 32. 5-year overall survival estimates for patients treated with CMF (N=418)

TOP2A status	Proportion of survival	95% CI	No. Deaths	No. Alive
Deleted (N=49)	0.35	0.21-0.48	32	17
Normal (N=323)	0.72	0.67-0.77	90	233
Amplified (N=46)	0.48	0.33-0.62	24	22

Table33. 5-year recurrence-free survival estimates for patients treated with CMF (N=418)

TOP2A status	Proportion of recurrence-free survival	95% CI	No. Recurrences	No. Recurrence-free
Deleted (N=49)	0.29	0.16-0.41	35	14
Normal (N=323)	0.61	0.56-0.67	122	182
Amplified (N=46)	0.41	0.26-0.56	26	17

Table 34. 5-year overall survival estimates for patients treated with CEF (N=349)

TOP2A status	Proportion of survival	95% CI	No. Deaths	No. Alive
Deleted (N=37)	0.51	0.35-0.67	18	19
Normal (N=266)	0.75	0.70-0.80	66	200
Amplified (N=46)	0.67	0.54-0.81	15	31

Table 35. 5-year recurrence-free survival estimates for patients treated with CEF (N=349)

TOP2A status	Proportion of recurrence-free survival	95% CI	No. Recurrences	No. Recurrence-free
Deleted (N=37)	0.48	0.31-0.65	18	15
Normal (N=266)	0.64	0.58-0.70	91	158
Amplified (N=46)	0.58	0.43-0.73	18	23

Summary

The univariate log-rank tests showed no overall significant effect of treatment despite a clear trend of better effect of CEF over CMF for RFS (p=0.063) and OS (p=0.054). Looking at the treatment outcome according to the patients *TOP2A* status, again the same trend was seen, and for the *TOP2A* amplified group, CEF showed to be statistically significantly superior to CMF with regards to RFS (p=0.037), however, not significantly superior to CMF with regards to OS (p=0.151).

With respect to *TOP2A* status a clear significant effect was demonstrated for both RFS (p<0.0001) and OS (p<0.0001). Patients with amplifications and deletions had a significant reduction in survival compared to patients with a normal *TOP2A* status. Survival curves also showed that patients with deletions had an even poorer prognosis than patients with an amplified or normal *TOP2A* tumor.

Multivariate Survival Analyses

Besides the association with the established clinical prognostic factors it was shown that *TOP2A* aberrations had an independent prognostic value. Using the Cox proportional hazard model it was demonstrated that a *TOP2A* gene aberration was associated with a significantly worse prognosis in the CMF treatment arm both with respect to RFS (P=0.0209 and OS (P=0.0102). The HR (Hazard Ratio) and the 95% confidence limits based on Cox model for RFS and OS are shown in Table 36 and 37.

The basic multivariate Cox model included the prognostic parameters: menopausal status, tumor size, number of positive lymph nodes, HER2 and *TOP2A* status, malignancy grade and receptor status. Malignancy grade and receptor status were included as stratification variables. The prognostic value of positive lymph node status and TOP2A varied within treatment arms and thus separate coefficients are provided for each. The poor prognosis associated with deleted or amplified status within the CMF arm remains statistically significant after considering other clinical variables but is no longer significant in the CEF arm. This was consistent for both RFS and OS.

Table 36. Hazard Ratio (HR) for Overall Survival (OS)

Variable	P-value	HR	95% CI
Menopause	0.0135		
Pre		1	
Post		1.36	(1.07-1.75)
Tumor size	<0.0001		
pr. increasing cm		1.17	(1.10-1.24)
Treatment	0.0059		
CMF		1	
CEF		0.39	(0.20-0.76)
Positive lymph nodes within treatment CEF :	<0.0001		
0		1	
1-3		5.51	(2.76- 11.02)
4-		9.77	(4.96-19.25)
Positive lymph nodes within treatment CMF :	<0.0001		
0		1	
1-3		1.74	(1.06-2.85)
4-		4.09	(2.53-6.62)
TOP2A status within treatment CMF :	0.0102		
Deleted		1.84	(1.23-2.75)
Normal		1	
Amplified		1.40	(0.89-2.21)
TOP2A status within treatment CEF	0.1305		
Deleted		1.28	(0.78-2.10)
Normal		1	
Amplified		0.70	(0.42-1.16)
HER2 status	0.0414		
Negative		1	
Positive		1.33	(1.01-1.75)

Table 37. Hazard Ratio (HR) for Recurrence Free Survival (RFS)

Variable	P-value	HR	95% CI
Menopause	0.0673		
Pre		1	
Post		1.25	(0.98-1.59)
Tumor size pr. increasing cm	<0.0001	1.15	(1.09-1.22)
Treatment	0.4173		
CMF		1	
CEF		0.82	(0.51-1.32)
Positive lymph nodes within treatment CEF :	<0.0001		
0		1	
1-3		2.56	(1.53-4.28)
4-		4.21	(2.56-6.92)
Positive lymph nodes within treatment CMF :	<0.0001		
0		1	
1-3		1.71	(1.09-2.67)
4-		4.16	(2.69-6.43)
TOP2A status within treatment CMF :	0.0209		
Deleted		1.63	(1.10-2.42)
Normal		1	
Amplified		1.55	(1.00-2.41)
TOP2A status within treatment CEF	0.1923		
Deleted		1.07	(0.64-1.77)
Normal		1	
Amplified		0.64	(0.38-1.08)
HER2 status	0.2309		
Negative		1	
Positive		1.17	(0.90 -1.53)

The estimates of the effect due to *TOP2A* status with respect to OS are similar to the estimates of the TOP2A effect with respect to RFS.

The prognostic value of the HER2 status was also investigated and the univariate survival analyses indicated a significant negative effect on both OS and RFS, as HER2 positive patients had a reduction in survival compared to patients with a normal HER2 status. When repeating the analysis with respect to OS the positive HER2 status came out having a significant negative impact on survival. The primary analysis of RFS using the Cox proportional hazard regression analysis showed no significant effect of the HER2 status.

The effect of the interaction between *TOP2A* status and HER2 status, investigated by using a multivariate Cox-model in HER2-positive patients only, showed significant effect of *TOP2A* status (P=0.011) for RFS and (P=0.048) for OS. However, because of the limited number of patients that were TOP2A amplified but HER2 negative the relationship of these two markers as prognostic factors could not be fully explored.

Discussion and Conclusion

The DBCG 89D/*TOP2A* study has demonstrated significant prognostic value of *TOP2A* gene amplifications and deletions. Based on the comparisons to HER2 status it can be concluded that the HER2 status and *TOP2A* status are not interchangeable for the prognostic value.

TOP2A is a molecular target for the pharmacological action of anthracyclines. Anthracycline-based chemotherapy with doxorubicin or epirubicin is among the most active regimens in breast cancer (25, 26). However, these compounds possess significant acute and long-term serious side effects, such as cardiotoxicity and leukemia. The presence of predictive implications from *TOP2A* amplifications for optimal use of anthracycline-containing therapy is an area of active research with promising initial results that require confirmation and extension in a context of currently available chemotherapeutic options. Whether *TOP2A* amplification is an independent predictive marker of response to any type of treatment (chemotherapy or immune-based) remains to be established.

<u>Safety and Effectiveness</u>

Safety

The Dako *TOP2A* FISH pharmDx™ Kit is an *in vitro* diagnostic test and does not transfer energy to the patient. Instructions for the safe use of the product are included in the package insert. As a diagnostic test, the *TOP2A* FISH pharmDx™ Kit involves testing on formalin-fixed, paraffin-embedded human breast cancer tissue sections. The test, therefore, presents no additional safety hazard to the patient being tested.

Effectiveness

Prognostic utility

Summary of Safety and Effectiveness Data

An objective of the DBCG 89D/P265.01 study was to investigate the prognostic value of *TOP2A* aberration in breast cancer patients. The univariate survival analyses illustrated a significant negative effect on both OS and RFS, as patients with amplifications and deletions had a significant reduction in survival compared to patients with a normal *TOP2A* status. The survival curves also indicated that patients with deletions had an even worse prognosis than patients with an amplified or normal *TOP2A* status.

Analysis of the distribution of the *TOP2A* aberrations with respect to the usual clinico-pathological characteristics, showed a significant association with several of the established histopathological prognostic parameters, such as tumor size, number of positive lymph nodes, ER/PR receptor status and HER2 status. Further, the data demonstrated that the proportion of women with *TOP2A* aberrations increased with age resulting in a higher frequency among postmenopausal than premenopausal women. Beside the association with the established clinical prognostic factors, it was shown that *TOP2A* aberrations had an independent prognostic value. Using the Cox proportional hazard model it was found that a *TOP2A* gene aberration was associated with a significantly worse prognosis both with respect to OS (p=0.01) and RFS (p=0.02).

Risk Benefit Analysis

Patients falsely characterized by the Dako *TOP2A* FISH pharmDx™ Kit as positive for abnormal *TOP2A* status might overestimate their risk of breast cancer recurrence or decreased longevity.

Patients falsely characterized by the Dako *TOP2A* FISH pharmDx™ Kit as negative for abnormal TOP2A status might underestimate their risk of breast cancer recurrence or decreased longevity.

The assay is approved for prognostic estimation, and not to predict the likelihood of benefit from any therapy or differential benefit between therapies. Risk/benefit effects with uses other than the approved intended use have not been determined.

The *TOP2A* FISH pharmDx™ Kit is safe and effective when used according to the manufacturer's instructions and for its approved intended use (together with other clinical and pathological information) as a marker for poor prognosis in high-risk breast cancer patients similar to those studied in DBCG 89D. Any false test results pose minimal additional risk to patients.

Limitation of Clinical Studies

Clinical study design

The study was observational in nature, and did not integrate the marker-based randomization design feature. In the DBCG89D/P265.01 study the tissue samples and data were collected and analyzed retrospectively. Studies with a retrospective design possess the possibility of an increased risk of bias compared to the prospective design. The main

design risk that was apparent for the DBCG89D/P265.01 study was that missing blocks created a potential for unrepresentative results due to selection-bias. Among the patients randomized in the DBCG 89D study, tissue blocks from 156 patients were missing. Statistically significant associations with tissue availability were detected for menopausal status, number of positive lymph nodes, tumor size, receptor status and tumor grade. The tissue was more often available when the prognostic factors were worse (higher age, more positive lymph nodes, larger tumor size, higher grade). In addition, some TOP2A or HER2 assay results were absent for some patients. However, analyses showed no association between any of these missing variates and outcomes (RFS and OS). The likelihood that missing variates confound the prognostic conclusions is negligible.

XI. Conclusions of the clinical Studies

The DBCG 89D/*TOP2A* study demonstrated prognostic value of *TOP2A* gene aberrations. A significant univariate association between TOP2A aberrations and OS and RFS was shown by comparing *TOP2A* gene amplification, normal and deletion cohorts within the CMF and CEF arms, respectively. In multivariate analyses, TOP2A had prognostic value for OS and RFS among CMF treated patients.

Scientific Evidence
This evaluation was a controlled investigation conducted in accordance with the existing FDA Guidance for Industry E6 Good Clinical Practice Consolidated Guidance; International Conference on Harmonization and FDA 21 CFR 58 Good Laboratory Practice. Results are believed to be valid scientific evidence of the clinical utility of *TOP2A* FISH assay as a prognostic marker.

Safety and Effectiveness
Based upon the results of the pre-clinical and clinical studies, the *TOP2A* FISH assay, when used according to the provided directions and in conjunction with other clinical and laboratory information, is safe and effective for the stated intended use.

XI. Panel Recommendation - None

XII. CDRH Decision

FDA issued an approval order on January 11, 2008.
The applicant's manufacturing facility was inspected on March 9, 2006 at Dako Denmark A/S and was found to be in compliance with the Quality Systems Regulation (21 CFR 820).

XIII. Approval Specifications

Directions for use: See the labeling.

Conditions of Approval: CDRH Approval of this PMA is subject to full compliance with the conditions described in the Approval Order.

Postapproval Requirements and Restrictions: See Approval Order.

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