510(k) SUBSTANTIAL EQUIVALENCE DETERMINATION
DECISION SUMMARY

A. 510(k) Number:
k062694

B. Purpose for Submission:
New device

C. Measurand:
70 gene expression profile

D. Type of Test:
Expression microarray
Test service performed in a single laboratory in Agendia's Amsterdam facility.

E. Applicant:
Agendia BV

F. Proprietary and Established Names:
MammaPrint®

G. Regulatory Information:

1. Regulation section:
   21 CFR 866.6040 Gene expression profiling test system for breast cancer prognosis
2. Classification:
   Class II
3. Product code:
   NYI, Classifier, prognostic, recurrence risk assessment, RNA gene expression, breast cancer
4. Panel:
   Immunology (82)

H. Intended Use:

1. Intended use(s):
   MammaPrint® is a qualitative in vitro diagnostic test service, performed in a single laboratory, using the gene expression profile of fresh frozen breast cancer tissue samples to assess a patients' risk for distant metastasis.

   The test is performed for breast cancer patients who are less than 61 years old, with Stage I or Stage II disease, with tumor size ≤ 5.0 cm and who are lymph node negative. The MammaPrint® result is indicated for use by physicians as a prognostic marker only, along with other clinicopathological factors.
2. Indication(s) for use:
   Same as intended use
3. Special conditions for use statement(s):
   For prescription use only
   MammaPrint® is not intended for diagnosis, or to predict or detect response to therapy, or to help select the optimal therapy for patients.
4. Special instrument requirements:
   Agilent 2100 Bioanalyzer: Serial number DE54700497 en DE24802382
   Agilent DNA microarray scanner: Serial number us22502555

Note: The scanner and bioanalyzer are components of this assay and are cleared only for this assay and not for any other application. In addition, clearance is only limited to the bioanalyzer and scanner with the serial numbers as specified above.

I. Device Description:

The MammaPrint® test is performed and provided as a service by Agendia Laboratory. The test is a microarray based gene expression analysis of RNA extracted from breast tumor tissue. The test is a custom-designed array chip manufactured by Agilent Technologies using the Agilent oligonucleotide microarray platform which assesses the mRNA expression of the 70 genes in triplicate. The MammaPrint® microarray features eight 1900-feature subarrays per glass slide which can each be individually hybridized. Per subarray 232 reporter genes are printed in triplicate, including the 70 genes which make up the MammaPrint® prognostic profile. Each subarray additionally includes 915 normalization genes and 289 spots for hybridization and printing quality control.

The analysis is based on several processes: isolation of RNA from frozen tumor tissue sections, DNAse treatment of isolated RNA, linear amplification and labeling of DNAse treated RNA, cRNA purification, hybridization of the cRNA to the MammaPrint® microaray, scanning the MammaPrint® microarray and data acquisition (feature extraction), calculation and determination of the risk of recurrence in breast cancer patients.

The MammaPrint® analysis is designed to determine the gene activity of specific genes in a tissue sample compared to a reference standard. The result is an expression profile, or fingerprint, of the sample. The correlation of the sample expression profile to a template (the mean expression profile of 44 tumors with a known good clinical outcome) is calculated and the molecular profile of the sample is determined (Low Risk, High Risk, Low Risk Borderline, High Risk Borderline).

J. Substantial Equivalence Information:

1. Predicate device name(s):
   None
2. Predicate 510(k) number(s):
   Not applicable
3. Comparison with predicate:
   Not Applicable

K. Standard/Guidance Document Referenced (if applicable):

CDRH Guidance for Industry and FDA Reviewers: *Guidance for the content of premarket submissions for software contained in medical devices*, May 11, 2005

L. Test Principle:

The MammaPrint® service is a microarray based gene expression analysis of a tumor. To assess the gene activity in a sample, frozen tissue sections are made, using a freeze microtome, and are collected in a receptacle. Total RNA is extracted from the tissue sections using a standard commercially available isolation kit. The sample is purified from DNA by DNAse treatment using a standard commercially available kit. The purified RNA sample is amplified and labeled twice with a cyanine-CTP fluorescent dye, Cy3 and Cy5, using a standard commercially available kit. The Cy3-labeled cRNA of the sample, together with the Cy5-labeled cRNA from a reference sample, is subsequently distributed over the sub array.

The Cy5-labeled cRNA of the sample, together with the Cy3-labeled cRNA from a reference sample, is subsequently distributed over a second subarray. This is called color reverse analysis. The hybridization chamber is composed of an 8-well gasket slide that fits over the microarray slide, and is held in place by a metal holder. During hybridization the slide is rotated, causing mixing by a bubble in the hybridization fluid. Hybridization is performed at 60°C for 17 hours in a rotation oven. By hybridization only complementary RNA will bind to a 60-mer oligo on the array.

For scanning the MammaPrint® microarray an Agilent microarray scanner is used. The Agilent DNA microarray scanner is a 48-slide scanning system that can read 1" x 3" glass slide microarrays. The result after scanning is a scan file (multi-page TIFF). This TIFF contains two pages, one page for each dye used (Cy3/Cy5). These are used by the feature extraction software.

Agilent Feature Extraction Software opens the multipage TIFF and combines those into one image which shows a pattern of differently colored spots. The Feature Extraction Software analyses the scan file (TIFF) by determining the intensities of the individual features, subtracting background signal, perform normalization, and calculate ratios, errors and p-values for each spot. The output is a data file (TXT) per subarray. The feature extraction software uses the MammaPrint® microarray chip design file as a template in order to identify control features, normalization features and reporter features. The fluorescent intensity of the features is a measure for the activity of that particular gene.

Data analysis is performed according to a specific MammaPrint® algorithm (MammaPrint® Index). The algorithm calculates the similarity ("cosinor correlation") of the sample expression profile to a template, (the mean expression profile of 44 tumors with a known good clinical outcome) and determines the molecular profile of the sample (Low Risk, High Risk). This algorithm is designed and programmed by Agendia and compiled into a standalone software program, "X-Print Analysis Software". The "X-Print Analysis Software" loads a data file (CSV) which is created by the laboratory technician by extracting specific information from the laboratory database. The CSV data file contains: external sample ID, internal sample ID, Technician name, Bio-analyzer ratio, RNA integrity number, location of straight and dye-swap data file (TXT), Chip Layout (8-pack) and additional comments by the technician.

The "X-Print Analysis Software" reads the CSV file, opens the Feature Extraction Software data files (TXT), performs quality control checks, determines the sample expression profile, calculates the correlation of sample profile to the "Low Risk" template profile on a scale of –1 to 1 (MammaPrint Index), compares the calculated correlation to a pre-defined cut-off value and determines the samples prognostic profile (Low Risk or High Risk). The analysis software output is an internal report (PDF) for every sample. In this report quality control values and analysis results are reported.

The MammaPrint® Index ranges from -1.0 to +1.0. Tumor samples with a MammaPrint® Index above the threshold of +0.4, are classified as low risk, and tumor samples with a MammaPrint® Index equal to or smaller than the threshold are classified as high risk.

If the MammaPrint® Index of a sample is between and including the borderline region of 0.365 and 0.435, this sample will be relabeled and rehybridized. After second analysis, the average MammaPrint® Index of both experiments will be calculated. If the MammaPrint® Index is still between the borderline region for two measurements (i.e. between and including 0.3775 and 0.4225), this sample will be reported as a borderline sample in the report form.

M. Performance Characteristics (if/when applicable):

1. Analytical performance:
   a. Precision/Reproducibility:
      1) Reproducibility starting from RNA pool:
      Three individual RNA samples, 1 Low Risk Control Pool (LRC), 1 high risk control pool (HRC) and 1 borderline sample (BLS) were used. LRC is a pool of 13 low risk samples and HRC is a pool of 12 high risk samples. For the LRC and HRC, there are 101 and 107 independent measurements respectively, performed from August 2005 till August 2006 and there are 37 independent measurements for BLS performed from April 2005 till August 2005.

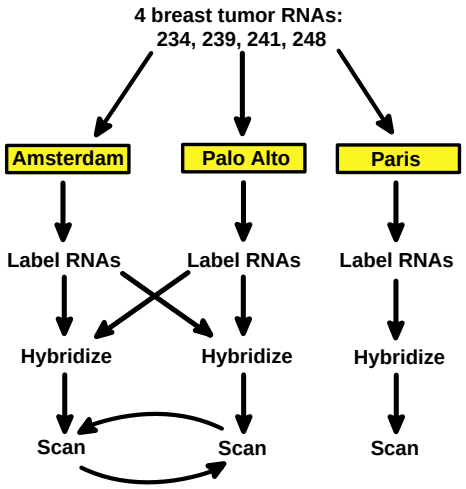
Samples	Mean Index	SD
LRC	0.689	0.026
HRC	-0.50428	0.02747
BLS	0.433	0.031

      2) Repeatability
         a) Starting from individual patients:
         Forty-six (46) different patient samples were amplified and hybridized a second time (on the same day). The intra-class correlation coefficient (ICC) was 0.9953.
         b) Starting from one labeled sample with multiple hybridizations:
         To determine whether the hybridization introduces variation, one labeled samples was hybridized 8 times. Results are summarized below:

	Hybridizations									
	1	2	3	4	5	6	7	8	Average	SD
Index	0.254	0.269	0.276	0.298	0.278	0.280	0.311	0.308	0.284	0.020

      3) Site-to-site reproducibility study (Note that this device is only cleared as a service to be run within a single clinical laboratory):
      Four breast tumor samples (one high risk and three low risks) were distributed to 3 independent laboratories (US, France, Netherlands) to validate the Classifier and to assess hybridization and scanner reproducibility. The individual laboratories processed the samples and performed the MammaPrint® assay. In addition, labeled RNAs were exchanged between the laboratories in the US and the Netherlands for hybridization and scanning (see study design below).

Scheduled Exchange of Labeled RNA and Hybridized Arrays.



Each tumor was labeled 2-3 times per lab with a total of eleven hybridizations and for each tumor the minimum and maximum index values are shown below.

Tumor	Minimum	Maximum
234	0.044	0.162
239	0.641	0.770
241	0.399	0.476
248	0.453	0.526

4) Reproducibility of multiple isolations starting from tissue sample:
In order to determine the reproducibility of the complete MammaPrint® device process, from tissue processing to the end result, five previously analyzed tumor samples (one borderline, two high risk and two low risk) were isolated in duplicate. Over multiple days, the ten isolations from five tumors were processed according to standard MammaPrint® protocols.

Sample	Original index	Result	Index from first isolation	Index from second isolation
S1	0.376	High risk (borderline)	0.254	0.374
S2	0.608	Low risk	0.564	0.553
S3	0.659	Low risk	0.639	0.680
S4	-0.105	High risk	0.068	0.067
S5	-0.305	High risk	-0.171	-0.337

No statistically significant difference in MammaPrint® risk group assignment or MammaPrint® index was observed between two separate RNA isolations.

b. Linearity/assay reportable range:
Linearity is not applicable for this type of assay. The correlation coefficient to the good profile (MammaPrint® Index) is reported on a scale of -1.000 to +1.000. Since cutoff for MammaPrint® Index is set at +0.40, risk assessment is reported as Low Risk (MammaPrint® Index > +0.40), or High Risk (MammaPrint® Index ≤ +0.40).

c. *Traceability, Stability, Expected values (controls, calibrators, or methods):*

Quality control materials

Agendia has two reference samples as quality controls to monitor experiment to experiment quality and temporal stability. Both reference samples (HRC and LRC) comprise of a pool of >10 previously analyzed patient samples with a similar MammaPrint® Index. HRC is a pool of High Risk patients; LRC is a pool of Low Risk patients. To monitor the experiment quality, the two reference samples are labeled and hybridized in each round of experiments. The MammaPrint® Index for these control samples have to be within set limits, if they are outside the limits, all samples for that assay run will be rejected. To check for temporal stability, MammaPrint® Index is plotted over time, and is monitored for trends on a 3 monthly basis. Agendia also has a reference sample called MammaReference Pool (MRP) which is used in color reverse analysis. The MRP is a RNA pool of tumors from 100 patients which were used in the NEJM publication, the balance between good and poor outcome is equally chosen as in the publication.

Device stability

The chip is stable for at least one year. The Cy Dyes are stable for up to 3 months at 2-8°C.

d. *Detection limit:*

Fifty percent (50%) tumor cell content in a sample was the detection limit used in the original MammaPrint manuscripts. Therefore 50% was used as cutoff for this validation experiment.

The analysis of percentage tumor epithelial cells is performed by the tumor nuclei method (i.e. percentage of tumor cells present). To determine the eligibility of a tumor specimen for MammaPrint® analysis, the first and last viable sections are used to determine the relative amount of tumor present in the biopsy. After embedding, the tumor is sectioned multiple times. The first and last viable sections are used for tumor percentage analysis. The mean of the two values is taken as the actual tumor cell content of a given specimen.

To determine the amount of tumor present, a pathologist inspects the H&E stained slides. An estimate is made of the tumor cell nuclei versus the total number of nuclei (i.e., tumor cell nuclei + lymphocytes + endothelial cells + other non tumor cells). Although the development of the original sample acceptance parameters were based on the original tumor samples with a tumor % cut-off of 50%. Subsequently, these parameters were adapted and validated to perform on tissue samples with a tumor % cut-off of 30%.

Specimen Requirements

Minimum amount of tissue material required to perform an acceptable assay:

- A 6 mm biopsy punch is used to excise the tumor from a surgical specimen. One standard biopsied tumor sample of 5 mm x 6 mm diameter is sufficient to perform 2500 MammaPrint® analysis.

- The amount of cRNA required for one reaction is 8 µl of labeled cRNA at 25 ng/µL.

e. Analytical specificity:
RNA specifications provided were adequate to exclude the presence of any effect from likely interfering substances.

f. Assay cut-off:
Mammaprint® Index cut-off is set at +0.40. The cut-off is based on clinical performance characteristics of MammaPrint® (10% clinical false negative) and not on a technical performance characteristic. The cut-off was determined prior to the independent validation trial described below.

2. Comparison studies:
   a. Method comparison with predicate device:
   Not applicable
   b. Matrix comparison:
   Not applicable
3. Clinical studies:
   MammaPrint® is a gene expression profiling that predicts the risk of metastasis in breast cancer patients. The test was developed based on research performed at the Netherlands Cancer Institute and the Antoni van Leeuwenhoek Hospital in Amsterdam, The Netherlands. The study data published in *Nature (Training set)* and the *New England Journal of Medicine (Test set)*, showed that the 70-gene signature can predict the development of distant metastases in lymph node negative primary breast cancer. The gene signature was subsequently developed into the diagnostic test, the MammaPrint® which was published in BioMed Central (BMC) Genomics. The MammaPrint® was further validated in an independent external study by the TRANSBIG consortium, which involved patients from five European centers, namely Institute Roussy (Villejuif, France), Karolinska Institute (Stockholm, Sweden), Centre Rene Hugenin (Saint-Cloud, France), Guy's Hospital (London, U.K.) and John Radcliffe Hospital (Oxford, U.K.). The data from this study was published in September 2006 issue of Journal of National Cancer Institute (JNCI). Patients were eligible for inclusion if they were younger than 61 years old at diagnosis, diagnosed before 1999 with node negative, T1-T2 (<5cm) breast cancer and had not received adjuvant systemic therapy. Patients with previous malignancies (except basal cell carcinoma) or with bilateral synchronous breast cancer were excluded.

   Initially there were 403 samples from eligible patients. Usefully RNA could be extracted for hybridization and analysis from 326 samples. Paraffin embedded tumor samples from this validation series were independently evaluated. Data on tumor size was missing for 3 patients and 16 others were found to be ineligible in the independent validation of the clinical data. Of the 307 patients, ER status was missing on 5 patients leaving 302 patients with complete data.

   Patient characteristics were summarized below:
   Age: 58 patients less than 41 years, 135 patients between 41 and 50 years and 99 patients between 51 and 60 years of age.

   Tumor size: 11 patients had tumors less than 1 cm, 99 patients had tumors between 1 and

2 cm, 192 patients had tumors exceeding 2 cm.

Tumor grade: 47 patients had good differentiation, 125 patients had intermediate differentiation, 124 had poor differentiation and one had an unknown tumor grade.

ER status: 90 patients were ER- and 212 patients were ER+.

Since none of these studies involved any US patients, it is clearly stated in the MammaPrint® patient report that "its performance characteristics and clinical utility in the United States Population have not been established" and "the metastasis free survival data is from an independent external patient group in Europe".

a. *Clinical Sensitivity:*
No clinical sensitivity for this type of submission. Positive predictive value is calculated using the data from the TRANSBIG trial. Positive predictive value (PPV) is the probability that a condition occurs (e.g. metastatic disease occurs within a given time frame) given the device output for that patient is high risk.

b. *Clinical specificity:*
No clinical specificity for this type of submission. Negative predictive value is calculated using the data from the TRANSBIG trial. Negative predictive value (NPV) is the probability that a condition does not occur (i.e. metastatic disease does not occur within a given time frame) given the device output for that patient is low risk.

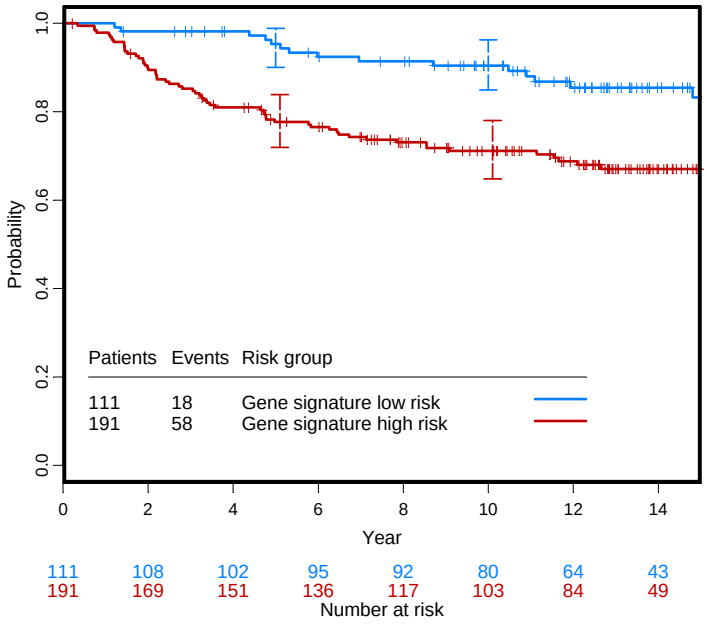
c. *Other clinical supportive data (when a. and b. are not applicable):*
TRANSBIG study
The TRANSBIG study was an independent European validation of the MammaPrint® 70-gene signature on 302 node negative patients who were less than 61 years of age and did not have adjuvant therapy. Results of the study are summarized below:

PPV and NPV may vary with prevalence of gene signature high risk or low risk. In this study there were 191/302 = 63.2% high risk patients.

Metastatic disease within 5 yrs
PPV = 0.22 (0.16-0.28)
NPV = 0.95 (0.91-0.99)

Metastatic disease within 10 years
PPV = 0.29 (0.22-0.35)
NPV = 0.90 (0.85-0.96)

Time to distant metastases



Using the information provided in the Kaplan-Meier survival curves:
5-year time to distant metastases (no metastatic disease within 5 year)
Low risk group: 0.95 (0.91-0.99)
High risk group: 0.78 (0.72-0.84)

10-year time to distant metastases (no metastatic disease within 10 year)
Low risk group: 0.90 (0.85-0.96)
High risk group: 0.71 (0.65-0.78)

In summary, study showed that at 5 years Low risk patients have a probability of 95% of metastasis free survival whereas High Risk patients have a probability of 78% metastasis free survival. At 10 years, Low risk patients have a probability of 90% of metastasis free survival and High Risk patients have a probability of 71% metastasis free survival.

The sponsor reported an unadjusted hazard ratio (this could be interpreted as stand alone performance) for time to distant metastases of 2.32 (95% CI: 1.35 to 4.00). A hazard significantly above one in this analysis signifies that the gene signature is predictive of the time to distant metastases. Adjustment for being at high risk of metastases via the Adjuvant on-line still resulted in a hazard ratio of 2.13 (95% CI: 1.19 to 3.82). The modest drop in the hazard probably reflects that the genomic signature may be related to the clinical factors but is clearly providing additional information. Many more details of the study using exactly these reported patients are in the manuscript by Buyse et al. 2006, JNCI 98: 1183-1192.

In addition to the TRANSBIG study, clinical performance was supported by three other studies. All four studies are summarized below. However, device performance is

calculated using data from the TRANSBIG study.

Study	*Purpose*	*Time Frame*	*Comments*
Nature Paper (1)	Development of breast cancer prognosis 70-gene profile (LNO, <55y)	2002, 78 patients, 6.4% adjuvant treatment	Within 5 year metastasis risk by profile multivariate OR 18
NEJM Paper (2)	Validation of the 70-gene profile in consecutive series of breast cancer patients (LNO, <53y)	2002, 151 patients, 5.2% adjuvant treatment	Metastasis-free survival by profile at 10 yrs: low risk profile 87%, high risk profile 44% (at 5 yrs: 93% and 56% respectively)
MammaPrint Paper (3)	Development of MammaPrint	2006,reproducibility of (1) and (2) on MammaPrint	Highly reproducible MammaPrint as diagnostic tool
Transbig Paper (4)	Independent European validation of 70-gene signature (LN0, <61y)	2006, 302 patients, no adjuvant treatment	Metastasis-free survival by profile at 10 yrs: low risk profile 88%, high risk profile 71% (at 5 yrs: 96% and 83% respectively)

References:

(1) van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH. Gene expression profiling predicts clinical outcome of breast cancer. Nature. 2002 Jan 31;415(6871):530-6.

(2) van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L, van der Velde T, Bartelink H, Rodenhuis S, Rutgers ET, Friend SH, Bernards R. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med. 2002 Dec 19;347(25):1999-2009.

(3) Glas AM, Floore A, Delahaye LJ, Witteveen AT, Pover RC, Bakx N, Lahti-Domenici JS, Bruinsma TJ, Warmoes MO, Bernards R, Wessels LF, Van't Veer LJ. Converting a breast cancer microarray signature into a high-throughput diagnostic test. BMC Genomics. 2006 Oct 30;7:278.

(4) Buyse M, Loi S, van't Veer L, Viale G, Delorenzi M, Glas AM, d'Assignies MS, Bergh J, Lidereau R, Ellis P, Harris A, Bogaerts J, Therasse P, Floore A, Amakrane M, Piette F, Rutgers E, Sotiriou C, Cardoso F, Piccart MJ; TRANSBIG Consortium. Validation and clinical utility of a 70-gene prognostic signature for women with node-negative breast cancer. J Natl Cancer Inst. 2006 Sep 6;98(17):1183-92.

4. Clinical cut-off**:**
   Same as Assay cut-off
5. Expected values/Reference range:
   Risk assessment is reported as Low Risk (MammaPrint Index >+0.40), High Risk (MammaPrint Index <= +0.40), or High Risk – borderline or Low Risk – borderline (if the index is between and including 0.3775 and 0.4225)

N. Instrument Name:

Agilent DNA microarray scanner (This scanner is not cleared for any other application.)

O. System Descriptions:

1. Modes of Operation:
   Automated
2. Software:
   MammaPrint analysis involves the use of scanner software, feature extraction software, and data analysis software. The scanner- and feature extraction software are off the shelf software (OTS) developed by Agilent Technologies. The data analysis software (X-Print analysis software) is custom software developed by Agendia.

   FDA has reviewed applicant's Hazard Analysis and software development processes for this line of product types:
   Yes __x_____ or No _____
3. Specimen Identification:
   Barcode
4. Specimen Sampling and Handling:
   Batch
5. Calibration:
   Installation and calibration are performed by the instrument manufacturer. No user calibration required.
6. Quality Control:
   QC protocol uses a fluorescently labeled reference sample complimentary to every oligo on the QC microarray.

P. Other Supportive Instrument Performance Characteristics Data Not Covered In The "Performance Characteristics" Section above:

None.

Q. Proposed Labeling:

The labeling is sufficient and it satisfies the requirements of 21 CFR Part 809.10.

R. Conclusion:

The petition for Evaluation of Automatic Class III Designation for this device is accepted. The device is classified as Class II under regulation 21 CFR 866.6040 with special controls. The special control guidance document "Class II Special Controls Guidance Document: Gene expression profiling test system for breast cancer prognosis" is available at http://www.fda.gov/cdrh/oivd/guidance/1627.html.