



DME

Admin file

Memorandum

P940036

Date AUG - 1 1996

From Director, Office of Device Evaluation (HFZ-400)  
Center for Devices and Radiological Health (CDRH)

Subject Premarket Approval of Bartels Prognostics Inc.  
Bartels ChemoResponse Assay - ACTION

To The Director, CDRH  
ORA \_\_\_\_\_

**ISSUE.** Publication of a notice announcing approval of the subject PMA.

**FACTS.** Tab A contains a FEDERAL REGISTER notice announcing:

- (1) a premarket approval order for the above referenced medical device (Tab B); and
- (2) the availability of a summary of safety and effectiveness data for the device (Tab C).

**RECOMMENDATION.** I recommend that the notice be signed and published.

*Susan Alpert*  
Susan Alpert, Ph.D., M.D.

- Attachments
- Tab A - Notice
  - Tab B - Order
  - Tab C - S & E Summary

**DECISION**

Approved \_\_\_\_\_ Disapproved \_\_\_\_\_ Date \_\_\_\_\_

**DRAFT**

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration

[DOCKET NO. \_\_\_\_\_]

Bartels Prognostics, Inc.; Premarket Approval of Bartels ChemoResponse Assay

AGENCY: Food and Drug Administration, HHS.

ACTION: Notice.

SUMMARY: The Food and Drug Administration (FDA) is announcing its approval of the application by Bartels Prognostics, Issaquah, WA, for premarket approval, under the Federal Food, Drug, and Cosmetic Act (the act), of Bartels ChemoResponse Assay. After reviewing the recommendation of the Microbiology Devices Panel, FDA's Center for Devices and Radiological Health (CDRH) notified the applicant, by letter on August 1, 1996, of the approval of the application.

DATES: Petitions for administrative review by (insert date 30 days after date of publication in the FEDERAL REGISTER).

ADDRESSES: Written requests for copies of the summary of safety and effectiveness data and petitions for administrative review, to the Dockets Management Branch (HFA-305), Food and Drug Administration, 12420 Parklawn Drive, Rm. 1-23, Rockville, MD 20857.

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## FOR FURTHER INFORMATION CONTACT:

Sharon L. Hansen, Ph.D.,  
Center for Devices and Radiological Health (HFZ-440),  
Food and Drug Administration,  
9200 Corporate Blvd.,  
Rockville, MD 20850,  
301-594-1293.

SUPPLEMENTARY INFORMATION: On November 23, 1994, Bartels Prognostics, Inc., Issaquah, WA 98027, submitted to CDRH an application for premarket approval of Bartels ChemoResponse Assay. The Bartels ChemoResponse Assay is an in vitro diagnostic device intended for use to determine resistance to 5-Fluorouracil (5-FU) of cells isolated from breast tumors. The ChemoResponse Assay is indicated for use to assist physicians in determining if 5-FU is an ineffective treatment for relapsed breast cancer patients.

On May 1, 1995, the Microbiology Devices Panel, an FDA advisory committee, reviewed and recommended approval of the application.

On August 1, 1996, CDRH approved the application by a letter to the applicant from the Director of the Office of Device Evaluation, CDRH.

A summary of the safety and effectiveness data on which CDRH based its approval is on file in the Dockets Management Branch (address above) and is available from that office upon written request. Requests should be identified with the name of the device and the docket number found in brackets in the heading of this document.

### Opportunity For Administrative Review

Section 515(d)(3) of the act (21 U.S.C. 360e(d)(3)) authorizes any interested person to petition, under section 515(g) of the act for administrative review of CDRH's decision to approve this application. A petitioner may request either a formal hearing under part 12 (21 CFR part 12) of FDA's administrative practices and procedures regulations or a review of the application and CDRH's action by an independent advisory committee of experts. A petition is to be in the form of a petition for reconsideration under 10.33(b) (21 CFR 10.33(b)). A petitioner shall identify the form of review requested (hearing or independent advisory committee) and shall submit with the petition supporting data and information showing that there is a genuine and substantial issue of material fact for resolution through administrative review. After reviewing the petition, FDA will decide whether to grant or deny the petition and will publish a notice of its decision in the FEDERAL REGISTER. If FDA grants the petition, the notice will state the issue to be reviewed, the form of the review to be used, the persons who may participate in the review, the time and place where the review will occur, and other details.

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Petitioners may, at any time on or before (insert date 30 days after date of publication in the FEDERAL REGISTER), file with the Dockets Management Branch (address above) two copies of each petition and supporting data and information, identified with the name of the device and the docket number found in brackets in the heading of this document. Received petitions may be seen in the office above between 9 a.m. and 4 p.m., Monday through Friday.



This notice is issued under the Federal Food, Drug, and Cosmetic Act (secs. 515(d), 520(h), (21 U.S.C. 360e(d), 360j(h))) and under authority delegated to the Commissioner of Food and Drugs (21 CFR 5.10) and redelegated to the Director, Center for Devices and Radiological Health (21 CFR 5.53).

Dated: \_\_\_\_\_.

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Food and Drug Administration  
2098 Gaither Road  
Rockville MD 20850

Yu Ping Maguire, Ph.D.  
Director of Clinical Oncology  
Laboratory Testing Program  
Bartels Prognostics, Inc.  
2005 NW Sammamish Road, Suite 107  
Issaquah, WA 98027

AUG 1 1996

Re: P940036  
Bartels ChemoResponse Assay  
Filed: November 23, 1994  
Amended: January 11, February 9, and 16, March 8, April 4,  
and December 21 1995; March 4, April 1, and June , 1996.

Dear Dr. Maguire:

The Center for Devices and Radiological Health (CDRH) of the Food and Drug Administration (FDA) has completed its review of your premarket approval application (PMA) for the Bartels ChemoResponse Assay. The Bartels ChemoResponse Assay is an *in vitro* diagnostic device intended for use to determine resistance to 5-Fluorouracil (5-FU) of cells isolated from breast tumors. The ChemoResponse Assay is indicated for use to assist physicians in determining if 5-FU is an ineffective treatment for relapsed breast cancer patients. We are pleased to inform you that the PMA is approved subject to the conditions described below and in the "Conditions of Approval" (enclosed). You may begin commercial distribution of the device upon receipt of this letter.

The sale, distribution, and use of this device are restricted to prescription use in accordance with 21 CFR 801.109 within the meaning of section 520(e) of the Federal Food, Drug, and Cosmetic Act (the act) under the authority of section 515(d)(1)(B)(ii) of the act. FDA has also determined that to ensure the safe and effective use of the device that the device is further restricted within the meaning of section 520(e) under the authority of section 515(d)(1)(B)(ii), (1) insofar as the labeling specify the requirements that apply to the training of practitioners who may use the device as approved in this order and (2) insofar as the sale, distribution, and use must not violate sections 502(q) and (r) of the act.

In addition to the postapproval requirements in the enclosure, the postapproval reports must include the following information and data to be collected on a minimum of 30 additional patients and submitted in accordance with a protocol reviewed by the FDA:

- a) Information on how the tests results are applied clinically, to provide added assurance of safety, effectiveness, and reliability of the device. This information should be documented as part of new customer auditing for a minimum of six months.

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- b) Data on a minimum of 30 additional patients to support the findings of the first study (30 evaluable patients) for its intended use, and to demonstrate confidence in the 29.1 percent control value cutoff.

These data are required to remove the limitations in the labeling section regarding the use of the median percent control value (40.5 percent) cutoff.

- c) Data on a minimum of 30 additional patients to further assess the correlation of *in-vitro* tumor resistance to *in-vivo* response or non-response.

Expiration dating for this device has been established and approved at 9 months when stored 2 - 8°C. This is to advise you that the protocol you used to establish this expiration dating is considered an approved protocol for the purpose of extending the expiration dating as provided by 21 CFR 814.39(a)(8).

CDRH will publish a notice of its decision to approve your PMA in the FEDERAL REGISTER. The notice will state that a summary of the safety and effectiveness data upon which the approval is based is available to the public upon request. Within 30 days of publication of the notice of approval in the FEDERAL REGISTER, any interested person may seek review of this decision by requesting an opportunity for administrative review, either through a hearing or review by an independent advisory committee, under section 515(g) of the Federal Food, Drug, and Cosmetic Act (the act).

Failure to comply with the conditions of approval invalidates this approval order. Commercial distribution of a device that is not in compliance with these conditions is a violation of the act.

You are reminded that as soon as possible, and before commercial distribution of your device, that you must submit an amendment to this PMA submission with copies of all approved labeling in final printed form.

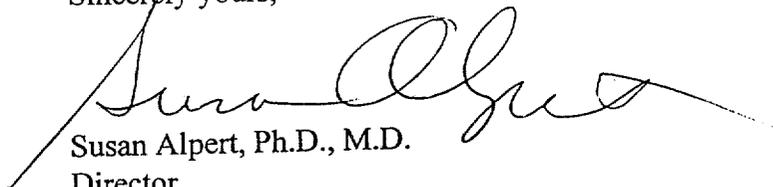
All required documents should be submitted in triplicate, unless otherwise specified, to the address below and should reference the above PMA number to facilitate processing.

PMA Document Mail Center (HFZ-401)  
Center for Devices and Radiological Health  
Food and Drug Administration  
9200 Corporate Blvd.  
Rockville, Maryland 20850

Page 3 - Yu Ping Maguire, Ph.D.

If you have any questions concerning this approval order, please contact Sharon L. Hansen, Ph.D.  
at (301) 594-2096.

Sincerely yours,

A handwritten signature in black ink, appearing to read "Susan Alpert", with a long horizontal flourish extending to the right.

Susan Alpert, Ph.D., M.D.

Director

Office of Device Evaluation

Center for Devices and

Radiological Health

Enclosure

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## SUMMARY OF SAFETY AND EFFECTIVENESS

### I. GENERAL INFORMATION

Generic Name: An *in vitro* assay intended for the determination of resistance of human tumor cells to chemotherapeutic agents.

Trade Name: Bartels ChemoResponse Assay

Applicant's Name and Address:  
Bartels Prognostics, Inc.  
2005 NW Sammamish Rd., Suite 107  
Issaquah, WA 98027

Premarket Approval Application (PMA) Number: P940036

An expedited review dated January 18, 1995, was granted because it is believed that clinically important information for the management protocols of patients who have failed previous chemotherapy be available to assist the clinician. The Bartels ChemoResponse Assay may offer significant advances in safety and effectiveness over other existing modalities in assisting the physician in identifying refractory breast cancer patients who did not respond to 5-FU, thereby potentially reducing the risk of drug toxicity and resulting side effects.

Date of Panel Recommendation: May 1, 1995

Date of Notice of Approval to the Applicant: AUG - 1 1996

### II. INDICATION FOR USE

The Bartels ChemoResponse Assay is an *in vitro* diagnostic device intended for use to determine resistance to 5-Fluorouracil (5-FU) of cells isolated from breast tumors. The ChemoResponse Assay is indicated for use to assist physicians in determining if 5-FU is an ineffective treatment for relapsed breast cancer patients.

#### **Background:**

Chemotherapy is the treatment of choice for patients with disseminated neoplasms and malignant tumors, who are not candidates for surgery. Due to the heterogenous nature of malignant tumors, the response of individual patients to chemotherapeutics is highly variable and difficult to predict.

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The current standard of chemotherapy is empirical and patients are treated based on historical drug information.

*In vitro* methods for predicting patients' clinical response have been reported in scientific literature. These methods include measurement of cell proliferation such as cloning, radionucleotide incorporation, measurement of metabolic activity, and the exclusion of vital dye by living cells. (1,2,3,4) Results of the clinical correlation studies in the past 15 years on more than 3000 specimens are similar: the prediction for drug sensitivity is 79-94 percent and the prediction for drug resistance is 67-86 percent. (5, 6) This data is comparable to the estrogen/progesterone receptor assay, an assay commonly employed by clinicians to aid in the treatment of patients with breast cancer. (5)

The ChemoResponse Assay has evolved from the above cited investigational methods. The device is an automated microwell assay with a radionucleotide incorporation endpoint. This device utilizes cell harvesting, and <sup>3</sup>H-uridine uptake which is measured by beta counters.

#### Contraindications, Warnings, and Precautions

Bartels CRA is contraindicated for use in the determination of *in vitro* sensitivity of cells isolated from breast tumors to chemotherapeutic agents. The device was evaluated in this study with tumors obtained from relapsed breast cancer patients receiving continuous 5-FU (300 mg/m<sup>2</sup>/day) infusion, and the *in vitro* results were compared to the clinical response of these patients. The response of patients with primary breast cancer was not evaluated.

Warnings and precautions for use of the device are stated in the attached product labeling. (Attachment A)

### III. DEVICE DESCRIPTION

The Bartels ChemoResponse Assay (hereinafter referred to as CRA) consists of a defined culture supplement used in the presence of a 1 percent fetal bovine serum (FBS) in Roswell Park Institute (RPMI) 1640 medium and a biological matrix which promotes cell growth. The culture medium promotes the growth of tumor cells but suppresses normal stromal cells. The cell inoculation requirement is 1-2 X 10<sup>6</sup> cells/well. The device contains: a) 2 X 8 microwells coated with Bartels Extracellular Matrix™ (ECM) to be used as controls; b) 2 X 8 Bartels ECM coated microwells with four concentrations of 5-FU (100, 10, 1 and 0.1 ug/mL) dried on top of the Bartels ECM; and c) Cyto-Gro™ 289, a lyophilized growth factor and hormone culture supplement to be reconstituted with sterile distilled water. Each strip is individually packaged in an aluminum foil pouch containing one molecular sieve desiccant. Control strips are included.

The assay is performed as follows: ( See the Flow Chart on the following page)

- a) a cell suspension is prepared from tumor specimens;
- b) growth medium (Cyto-Gro™ 289, 1 percent FBS in RPMI) is added to the drug strips to reconstitute the drug;

- c) cells are plated at  $1-3 \times 10^4$  cells/well and incubated continuously with drugs for three days;
- d) radio-nucleotide is added and incubated overnight;
- e) cells are harvested with a cell harvester and incorporated radionucleotide is counted with a beta counter; and
- f) results are expressed as percent control (percent survival) and a graph is prepared.

#### IV. ALTERNATIVE PRACTICES AND PROCEDURES

Currently, there are no devices on the market for which FDA approved PMAs exist, to determine resistance of breast tumor cells to chemotherapeutic agents. However, there are various research methods described in the scientific literature including: a) a Cloning Assay, b) a Radionucleotide Incorporation Assay, c) a Metabolic Dye Conversion Assay, and d) a Dye Exclusion Assay. (1,2,3,8,9)

#### V. MARKETING HISTORY

The CRA has not been marketed in the United States or any other country.

#### VI. POTENTIAL ADVERSE EFFECT OF THE DEVICE ON HEALTH

##### A. Patients

Because the assay does not take into consideration an individual's physiological conditions, it is possible that a false-resistant result could lead to a medical decision depriving the patient of a potentially beneficial drug therapy.

Patients who have a surgical biopsy specimen taken exclusively for this assay can potentially experience infection(s) at the biopsy site

##### B. Laboratory Personnel

Chemotherapeutic agents are known carcinogens, and mutagens, and could impair fertility. Cyto-Gro™ 289 contains proteins isolated from human serum. If standard biohazard precautions are not practiced when handling this material, there is a minimum risk for transmission of infections from organisms not detected in the prescreening of the serum.

There are no other known potential adverse effects to the health of laboratory personnel if this device is used according to instruction.

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## VII. SUMMARY OF STUDIES

Nonclinical and Clinical studies were conducted to demonstrate the safety and effectiveness of the CRA device when used in accordance with the instructions provided.

Nonclinical or preclinical studies involved feasibility testing of the culture system used in the device for harvesting tumor cells. The ExtraCellular Matrix culture system was cleared as a premarket notification submission, K902726, on September 24, 1990. The reproducibility and stability of the device were also assessed in nonclinical studies.

The Clinical Study was conducted under an IDE (G910083). The device did not experience any failures during the study period. The patient complaints received concerned toxicity resulting from the administration of the 5-FU and not from the device. Two patients were removed from the protocol because of drug toxicity and one patient was admitted to a hospice because of declining health and was therefore discontinued from the protocol.

### A. NONCLINICAL LABORATORY STUDIES

Analytical studies were conducted to provide the following information: a) tumor cell growth in the ChemoResponse Assay Culture System (CRACS) b) normal stromal cell suppression in CRACS; c) the effects of specimen transport and processing of specimens on the response of cells to 5-FU; d) the effects of plating density and the length of incubation, and e) the stability of chemotherapeutic drugs and reagents.

In addition, reproducibility studies were conducted at three different sites, to demonstrate intra- and inter-lot, and intra- and inter-assay reproducibility.

#### 1. Analytical

##### a. Human Tumor Cell Growth in CRACS

In order to demonstrate that the cell culture medium used with this device can support the growth of tumor cells, fifteen primary tumors were cultured in Bartels Cyto-Gro™ 289 and another commercially available culture system. Cell proliferation was determined by a crystal violet staining process.

When Bartels Cyto-Gro™ was used in conjunction with Bartels Extracellular Matrix™, all 15 primary human tumors were satisfactorily harvested in CRACS.

##### b. Normal Cell Suppression in CRACS

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Because normal stromal cells may contaminate tumor specimens and alter its *in vitro* drug response, studies were performed to demonstrate that normal stromal cells are suppressed in CRACS. Cells cultured in CRACS for four days (day 4 preparation) and cells obtained from day 0 fresh tumors (day 0 preparation) were characterized and compared by immunohistological (IH) techniques and ploidy analysis by flow cytometry. The proliferating cells were identified by an autoradiography technique in conjunction with ploidy analysis.

IH techniques and ploidy analysis, are methods commonly used in cell culturing, and are accepted methods for demonstrating cell proliferation. These methods were also accepted for comparative use when the CRACS was cleared for use by the premarket notification (510(k)) process

Immunohistological (IH) analysis was performed on 19 breast specimens utilizing the streptavidin-biotin immunoperoxidase IH technique. A total of 68 paired day 0 and day 4 preparations were reacted with the tumor associated monoclonal antibodies (MAbs) B72.3, SM#, CEA, and p53. MAb Ber-Ep4 was also used to distinguish mesothelial from epithelial cells. The IH analysis demonstrated that only the tumor associated cells increased with culture.

Ploidy analysis was performed on 18 specimens (simultaneously with IH analysis), utilizing flow cytometry and tumor associated MAbs. A standard autoradiography technique with <sup>3</sup>H-thymidine was employed. Ploidy was analyzed for cells isolated from fresh specimens (day 0) and the same cells cultured in CRACS for four days (day 4). Comparison of the DNA histogram of both day 0 and day 4 cells indicated 15/18 (83 percent) had an increase in S-phase and the remaining three (17 percent) specimens showed no change. The aneuploid population for the majority of the specimens either remained the same or increased with culture. The percent S and G<sub>2</sub> phase of the diploid populations were either maintained or increased with culture in CRACS.

Autoradiography was conducted simultaneously with the ploidy analysis. Of the seven specimens tested, all had increases in the percent of cells scored with silver stains after culture (from a mean of 4.9 percent on day 0 to 32.6 percent on day 4). Five of the seven specimens exhibited greater than 20 percent of the cells in mitosis on day 4. Ploidy analysis was comparable to the autoradiography data. There was an increase in cells staining positive with the tumor associated MAbs.

The results of the IH and ploidy analysis, and the autoradiography, demonstrate that CRACS suppresses the growth of normal stromal cells.

c. The Effect of Specimen Processing and Transport

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To demonstrate that the procedures used to prepare specimens for the CRA do not adversely effect tumor cells and cause altered drug response, tumor cells were subjected to various stress conditions. CRA was conducted on test (treated) and control (untreated) cells.

Studies were also conducted to determine the optimal transport temperature and the effect of penicillin/streptomycin (P/S), (penicillin at 200 units/mL and streptomycin at 200 ug/mL) in transport medium on tumor drug response. Optimal transport temperature was demonstrated to be room temperature (22-24°C) and the presence of P/S in the transport medium did not alter drug response. P/S at 200 units/mL and 200 ug/mL, respectively did not significantly affect drug response when it was included in the growth medium during CRA.

The effect of the enzyme digestion step on drug susceptibility was conducted on 11 specimens. There was no significant difference due to enzyme treatment. Thus, enzyme treatment was recommended as the standard treatment for solid tumors.

Analytical studies were also conducted to determine if other factors such as plating density, and the length of time cells are incubated with the drug, would alter drug resistance. Incorrect plating densities result in nonlinear relationships of cell density versus radionucleotide uptake, which leads to altered drug sensitivity. The length of time that cells are exposed to drug can effect drug resistance for those drugs that are cell-cycle dependent. Results indicated that a 3-day incubation was optimal both for 5-FU to inhibit cell growth and for the control cells to remain in log phase growth for the duration of the assay in the microtiter format.

The results of these studies demonstrated that the procedures used to prepare tumor specimens for CRA did not adversely effect the tumor cells or alter drug sensitivity. The experimental conditions, as defined in the ChemoResponse Assay Operators Manual (Section VI, Appendix 2), resulted in a linear relationship between cpm and cell proliferation.

#### d. Reproducibility Studies

Reproducibility studies were conducted to demonstrate intra-lot, inter-lot, intra-lab, and inter-lab variation. Tumor specimens obtained from patients with breast cancer and two breast cancer cell lines (MCF-7 and T47D) were assayed in these studies and the CRA was performed using four concentrations of 5-FU (0.1, 1.0, 10, and 100 ug/mL). CRA results, for each concentration of 5-FU, were presented as percent control values. Percent control is defined as the average radionucleotide uptake (cpm) of four 5-FU-treated wells divided by the average radionucleotide uptake of 12 control wells. The Intraclass correlation coefficient

of percent control was calculated for each concentration of 5-FU. Intraclass correlation coefficients greater than 0.75 are considered high reproducibility, a value between 0.4 and 0.75 is moderate, and values below 0.4 are considered low reproducibility. (10) Ninety-five percent confidence intervals (CI) were determined for each coefficient using a bootstrap technique with 1000 replicates. The standard errors obtained from the bootstrap procedure agreed closely with those calculated using the delta method to approximate the variance of the estimates.

The intraclass correlation and the 95 percent CI for the intra-lot, inter-lot, inter-lab, and intra-lab reproducibility are presented in Table 1.

**TABLE 1**  
**SUMMARY OF REPRODUCIBILITY STUDIES**

5 FU Concentration ( $\mu\text{g/mL}$ )				
	0.1	1	10	100
<b>intra-lot IC<sup>1</sup> (95% CI<sup>2</sup>)</b>	<b>0.83 (.71 - .99)</b>	<b>0.93 (.86 - .997)</b>	<b>0.93 (.98 - .98)</b>	<b>0.97 (.95 - .997)</b>
<b>inter-lot IC (95% CI)</b>	<b>0.74 (.62 - .93)</b>	<b>0.97 (.94 - .99)</b>	<b>0.42 (.18 - .88)</b>	<b>0.89 (.73 - .97)</b>
<b>intra-lab IC (95% CI)</b>	<b>0.73 (.58 - .96)</b>	<b>0.61 (.40 - .95)</b>	<b>0.50 (.24 - .93)</b>	<b>0.48 (.21 - .95)</b>
<b>inter-lab IC (95% CI)</b>	<b>0.65 (.44 - .96)</b>	<b>0.88 (.82 - .97)</b>	<b>0.54 (.40 - .95)</b>	<b>0.22 (.08 - .87)</b>

<sup>1</sup> IC = Intraclass correlation coefficient

<sup>2</sup> CI = 95 percent confidence interval

**Conclusion:** The results of the intra-lot reproducibility demonstrated satisfactory reproducibility at all concentration of 5-FU. The lot to lot results were moderate to high at the 0.1, 1.0, and 100  $\mu\text{g/mL}$  concentrations but low at the 10  $\mu\text{g/mL}$  concentration. The variation in intraclass correlation coefficient for different 5-FU concentrations, inter and intra laboratory, was attributed to the lack of prior experience with tissue culture

techniques at some sites. Generally intraclass correlation was better at 0.1 and 1.0  $\mu\text{g/mL}$  for both inter and intra-laboratory.

A second reproducibility study was conducted by the sponsor at the recommendation of the FDA and the Microbiology Advisory Committee to validate the intra- and inter-laboratory studies. The study was conducted at three different laboratory sites, Bartels Prognostics (BP), Intracel (IC), and UCSF Mt. Zion Medical Center (MZ), using a different technologist at each site to perform the test. Duplicate testing was done using two different tumor cell suspensions prepared from the same tumor to determine whether the variation seen in the presubmission studies was a technical problem or a problem due to biological variation of the tumor itself. The technicians at the three sites had experience ranging from 3 months to 5 years. The SW-480 cell line was included as a daily quality control material.

The percent control values (the percentage of cells surviving incubation with 5-FU) for all 132 tests (duplicates/replicates) were statistically analyzed. The overall intraclass correlation was 0.84 with a 95 percent Confidence Interval of 0.70 - 0.94. Table 2 exhibits the site specific intraclass correlations for the intra- and inter-laboratory reproducibility. Table 3 summarizes the Intra-Lab variation as absolute replicate differences.

**TABLE 2**  
**INTRA-LABORATORY REPRODUCIBILITY**

<b>5-FU Concentration ( 1 <math>\mu\text{g/mL}</math>)</b>		
<b>Site</b>	<b>Intraclass Values</b>	<b>95% CI</b>
<b>BP</b>	<b>0.87</b>	<b>(.74 - .99)</b>
<b>IC</b>	<b>0.99</b>	<b>(.97 - 1.0)</b>
<b>MZ</b>	<b>0.94</b>	<b>(.89 - 1.0)</b>
<b>Totals</b>	<b>0.84</b>	<b>(.71 - .97)</b>

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**TABLE 3  
INTRA-LAB VARIATION**

<b>Absolute Replicate % Control Value Differences</b>		
<i>Site</i>	<i>Mean</i>	<i>Std. Dev.</i>
<b>BP</b>	<b>8.5</b>	<b>7.9</b>
<b>IC</b>	<b>5.1</b>	<b>4.9</b>
<b>MZ</b>	<b>3.6</b>	<b>2.8</b>

**Conclusion:** The results obtained in this study demonstrated satisfactory intra- and inter-laboratory reproducibility. Reproducibility of results however may vary with experience.

e. Stability

To assess the stability of the CRA device, stability studies were performed on various components of the device using three lots. The Cyto-Gro™ 289 was stable when stored at 2-8°C for three months. Bartels ECM was stable when stored unopened at 2-8°C for 12 months. The stability of the drugs on Bartels ECM was analyzed by High Performance Liquid Chromatography. The stability for ECM-drug strips was nine months. Each kit component will be labeled with the expiration dates.

2. Conclusions from the Nonclinical Studies

The Analytical studies demonstrated that CRA culture system can be used to grow tumor cells. Breast tumor cells were successfully grown in the CRACS. Normal stromal cells, benign or normal specimens did not actively proliferate in CRACS. Both the primary and metastatic tumor cells proliferated in CRA. The success rate for growth for specimens from breast cancer patients enrolled in the protocol was 96 percent.

Various procedures used to conduct the CRA and to process tumor specimens demonstrated a minimal effect on cell growth and did not appear to alter the tumor cell's response to 5-FU.

Intra-, inter-lot and intra-, inter-laboratory 5-FU CRA reproducibility studies were analyzed by intraclass correlation coefficient (ICC). The results demonstrated satisfactory intra-, inter-lot and inter-laboratory reproducibility

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Data from the stability studies supported a product shelf-life of nine months for the Bartels CRA drug strips when stored at 2-8 °C.

## B. SUMMARY OF CLINICAL STUDIES

A prospective, double masked clinical trial was conducted at four sites in geographically distinct areas of the United States with specimens obtained from patients with relapsed breast cancer to demonstrate the clinical utility of this device as an aid, to determine if 5-FU is an ineffective therapy for the treatment of relapsed breast cancer patients. The four sites were: Bartels Prognostics, Issaquah, Washington; Blount Diagnostic Laboratory, Maryville, TN; UCSF, Mt. Zion Medical Center, San Francisco, CA; and University of Texas Of San Antonio, San Antonio, TX.

Twenty-two clinical sites were recruited to maintain patients on the 5-FU continuous infusion protocol. Of these, only 17 sites maintained patients. Participating in this study were:

James Atkins, M.D. S. E. Medical Oncology Center Goldsboro, NC (2 patients)	Memorial Sloan-Kettering New York, NY (3 patients)
Robert Belt, M.D. Oncology Clinic of Kansas City Overland Park, KS (5 patients)	Stanley Leong, M.D. Mt. Zion, University of California San Francisco, CA (3 patients)
Walter Durkin, M.D. Halifax Medical Center Regional Oncology Center Daytona Beach, FL (1 patient)	Alvin Otsuka, M.D. Humana Hospital Mt. View Thorton, CO (1 patient)
Richard Elledge, M.D. University of Texas Health Science Center San Antonio, TX (9 patients)	Arthur Rossof, M.D. MacNeal Cancer Center Berwyn, IL (1 patient)
Rayna Kneuper-Hall, M.D. Medical University of South Ca. Charleston, SC (2 patients)	Major Don Shaffer, M.D. Department of the Army St. Luke's Lutheran Hospital Houston, TX (1 patient)
Jeremy Hon, M.D. Comprehensive Cancer Institute Huntsville, AL (10 patients)	Lon Smith, M.D. St. Luke's Lutheran Hospital San Antonio, TX (8 patients)
Clifford Hudis, M.D.	

Frederick Stutz, M.D.  
Skagit Valley Hospital and Health  
Center  
Mount Vernon, WA  
(1 patient)

Myo Thant, M.D.  
Franklin Square Medical Center  
Baltimore, MD  
(9 patients)

Ralph Vance, M.D.  
University of Mississippi  
Jackson, MS  
(1 patient)

Charles Vogel, M.D.  
Comprehensive Cancer Center  
North Miami Beach, FL  
(2 patients)

Charles Wiseman, M.D.  
St. Vincent Medical Center  
Los Angeles, CA  
(1 patient)

Patients with metastatic breast cancer, with tumors that were measurable and accessible to biopsy, were treated with continuous infusion of 5-FU by a pump at 300 mg/m<sup>2</sup>/day.

The *in vitro* results were not revealed to the treating physician to prevent bias of evaluating patient response, and the patient's clinical response was not available to the laboratory personnel conducting the CRA. At the end of the trial, clinical response was evaluated by the principal investigator, Richard Elledge, M.D., and corroborated by an independent panel of reviewers who were not previously involved in the trial.

The primary objective of the study was to demonstrate the correlation between *in vitro* tumor 5-FU resistance and a lack of clinical response to treatment with 5-FU in patients with metastatic breast cancer.

Sensitivity, specificity, 95 percent CI estimates, positive and negative predictive value, were calculated using the exact binomial distribution appropriate for rates and proportions. The patients were divided into two groups: those with *in vitro* resistant tumors, and those with *in vitro* indeterminate tumors. Clinical response rates were compared between these two groups using Fisher's Exact Test for rates and proportions.

The study was designed to have a minimum of 25 evaluable patients, with the expectation that approximately five would have a clinical response, and 20 would not have a clinical response to continuous infusion 5-FU. The patient sample size was selected using historical 5-FU information, and an 80 percent statistical power to detect a clinical response rate of less than 10 percent in patients with *in vitro* resistant tumors.

Specimens were obtained from patients enrolling in the protocol, and were tested for response to continuous 5-FU by the CRA. Patients determined to be eligible were treated with continuous 5-FU infusion by pump, set at 300 mg/m<sup>2</sup>/day. To meet the eligibility criteria for the study, only relapsed metastatic breast patients with previous chemotherapy, patients with specimens that could be biopsied, and patients with a measurable tumor were selected.

The Criteria used to measure response was developed by the Eastern Consortium of Oncology Groups (ECOG) and are as follows:

- CR: Complete response, complete disappearance of the measured tumor(s), for at least two measurements, four weeks apart.
- PR: Partial response, greater than 50 percent reduction in the size of the tumor(s), no progression of lesions or appearance of new lesions.
- PD: Progressive disease, greater than 25 percent increase in measurable tumor and/or developed new lesions.
- SD: Patients not falling into any of the other three categories.

Sixty patients were enrolled in the study. Of the 60, 15 were deemed ineligible for the 5-FU protocol because they had no measurable tumors or inadequate documentation of clinical history, and 15 were non-evaluable for the CRA because their specimens were insufficient or contaminated. Of the remaining 30 clinically evaluable patients only 25 had evaluable CRA. The reasons for the nonevaluable CRA were: two had insufficient viable cells, one was contaminated, one had technical problems, and one had no growth.

The study population consisted of female patients between the ages of 36 to 86 who presented with metastatic breast cancer. The median and mean age of the study population was 55.5 and 55.8 years of age respectively. The ethnic diversity groups of the women who participated in the study were; 7 African-American, 34 Caucasian, 3 Hispanic, and 1 Asian which totaled 45 eligible subjects. The performance status of the eligible subjects at entry into the study was from 0-3 using the ECOG rating scale. The sites of measurable disease that were followed during the course of therapy were: nine lymph nodes, three lungs, seven livers, and 26 skin or soft tissue (i.e., breast, chest walls, scalp, etc.). Fifteen subjects were ineligible to enter the 5-FU study. The ineligible patients were not included in the study demographics.

### 1. Determination of Assay Cutoff

To demonstrate the *in vitro* resistance of tumor cells to 5-FU, a cutoff point was identified. The cutoff point was determined by using a training set consisting of the 5-FU CRA results of specimens obtained from 34 patients with similar characteristics to those patients enrolled in the clinical trial. Based on historical 5-FU data, the *in vitro* response rate of these tumors to 5-FU was expected to be approximately 25 percent. The 25th percentile of percent control was calculated for each of the 5-FU concentrations (0.1, 1.0, 10, 100  $\mu\text{g}/\text{mL}$ ). The most appropriate concentration to define cutoff would be the concentration where the broadest percent control value gave the best separation of the 34 specimens into resistant or nonresistant categories. Of the four concentrations analyzed, the 1.0  $\mu\text{g}/\text{mL}$  concentration gave the broadest separation of specimens into different levels with a wider range of nonresistance. The 1.0  $\mu\text{g}/\text{mL}$  concentration was therefore selected as the concentration where the percent control value should be assessed.

### 2. *In Vitro* Results

A cutoff of 40.5 percent (the median percent control at 1  $\mu\text{g}/\text{mL}$  5-FU concentration) was used to determine *in vitro* resistance. At the 1  $\mu\text{g}/\text{mL}$  there were 12 patients with percent control values greater than 40.5 percent. No patients in this range should respond to therapy. Table 4 shows the *in vitro* result summary.

**TABLE 4**  
**SUMMARY OF CLINICAL STUDY**  
***IN VITRO* RESULTS**

5- FU Concentration				
	0.1 $\mu\text{g}/\text{mL}$	1 $\mu\text{g}/\text{mL}$	10 $\mu\text{g}/\text{mL}$	100 $\mu\text{g}/\text{mL}$
<b># of Specimen Resistant</b>	<b>11</b>	<b>12</b>	<b>12</b>	<b>13</b>

### 3. *In Vitro-In Vivo* Correlation

Of the 25 patients that were evaluable *in vitro* and *in vivo*, 14 had PD disease and five had SD. Patients with PR and CR were considered to be responders in the correlation analysis and both PD and SD were considered to be non-responders.

A total of 25 patients were evaluable and also had a successful CRA. The results are exhibited in Tables 5.

**TABLE 5  
CLINICAL CORRELATION ANALYSIS**

<b>5-FU CRA %Control Values at 1<math>\mu</math>g/mL Based on 40.5% as Cutoff</b>			
<b>Clinical Response</b>	<b>&gt;40.5%</b>	<b>&lt;40.5 &gt;29.1</b>	<b>&lt;29.1</b>
<b>PR</b>	0	1	4
<b>CR</b>	0	0	1
<b>SD</b>	3	2	0
<b>PD</b>	9	3	2

**Conclusion:** There were two false negative specimens (specimen were non-resistant *in vitro* but the patients did not respond, PD); one was from a patient who had received only three weeks of 5-FU and the other was from a patient whose tumor measured for the study had shrunk but the patient developed a new lesion. There was one false positive specimen (the specimen was resistant *in vitro* but the patient responded, PR, to therapy).

The efficacy of the CRA to identify patients who will be clinically unresponsive to continuous infusion 5-FU was demonstrated using the definition of "extreme resistance" and a cutoff of 40.5 percent, as presented in Table 6.

**TABLE 6  
IN VITRO-IN VIVO CORRELATION  
Clinical**

<b>CRA</b>	<b>NO Response</b>	<b>Response</b>
<b>Resistant</b>	12	0
<b>Non Resistant</b>	7	6

Sensitivity = 12/19 (63%)	95% CI=38% - 84%
Specificity = 6/6 (100%)	95% CI=54% - 100%
Predictive Value + =12/12 (100%)	95% CI=74% - 100%
Predictive Value - = 6/13 (46%)	95% CI=19% - 75%

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**Conclusion:** The Sensitivity of the assay, calculated on resistance above the 40.5 percent cutoff was 63 percent and the Specificity was 100 percent. However, because of the small sample size, a wide 95 percent confidence interval was exhibited. This cutoff is the median percent control value of the 34 specimens employed in the training set, validated by the 25 evaluable patients.

## VIII. CONCLUSIONS DRAWN FROM THE NONCLINICAL AND CLINICAL STUDIES

The data presented in this PMA demonstrates that the ChemoResponse Assay is safe and effective for its intended use when used in accordance with the instructions provided.

Nonclinical studies indicate that breast tumor cells can be successfully cultured in the CRACS. The instructions provided for transport and processing of specimens should be adhered to for minimal effect on tumor cells. Plating density, and the length of incubation are critical for success of the assay. Intra-assay, inter-assay, and inter-lot reproducibility are within acceptable ranges, but is dependent on the experience of the user. Data from the stability studies supported the shelf-life of nine months at 2-8°C.

In clinical studies conducted at four sites in the United States, the CRA demonstrated sensitivity and specificity similar to those described in the literature. However, because of the small sample size, 95 percent confidence intervals were wide (38 to 84 percent with a 63 percent sensitivity and 54 to 100 percent with a 100 percent specificity). Correlations can not be made for use of this assay for determining response to 5-FU. The assay maybe clinically useful as an aid in determining if 5-FU would be ineffective for the treatment of relapsed breast cancer patients. Satisfactory correlations were obtained for patients not responding to 5-FU and the *in vitro* resistance of tumor cells obtained from these patients.

Selection of chemotherapeutic agents for treatment of metastatic breast cancer is dependent upon many factors. The current standard of chemotherapy is empirical and patients are treated based on historical drug information. The risk presented by the CRA to the United States market would be significant if a false resistant result were obtained. However, a true resistant result could identify patients who would most likely not respond to 5-FU therapy .

On the basis of the nonclinical and clinical data which have been presented, the CRA is safe and effective for the stated indications.

## IX. PANEL RECOMMENDATIONS

The Microbiology Devices Panel recommended at the panel meeting on May 1, 1995 that the PMA for ChemoResponse Assay be approved with conditions. These conditions included a validation of the inter- and intra-laboratory reproducibility data, a revision of the labeling to clarify the Intended Use of the device, the Instructions for Use, and the Interpretation of Results. The Panel also recommended that Post Approval studies be conducted to obtain additional information

to demonstrate confidence in the use of the 29.1 percent assay cutoff; to obtain data to assess the correlation between *in vitro* tumor susceptibility and resistance to clinical response and non-response; and to assess the clinical application of test results.

#### X. CDRH ACTION ON THE APPLICATION

CDRH concurred with the recommendations of the Panel. CDRH issued an approvable letter to the applicant on August 4, 1995, requesting the submission of an intra-laboratory reproducibility study to validate the original study; changes to the labeling related to the revision of Intended Use, clearer instructions for use, and the addition of a clinical data summary to the Performance Characteristics section; and a concurrence with the "Conditions of Approval."

The applicant satisfactorily responded to the request for additional reproducibility studies and changes to the product labeling.

CDRH issued a approval order for the applicant's PMA for the ChemoResponse Assay on  
AUG - 1 1996

The applicant's manufacturing and control facilities were inspected on April 21, 1995, for compliance with the Device Good Manufacturing Practice Regulations (GMPs) and the facilities were found to be in compliance.

The shelf life of the CRA has been established for the Cyto-Gro at three months, for 12 months for the ECM, and nine months for the ECM drug strips, when stored at 2-8°C.

#### XI. APPROVAL SPECIFICATIONS:

Directions for use: See attached labeling (Attachment A)

Conditions of Approval: CDRH approval of this PMA is subject to the conditions described in the approval order. (Attachment B)

Bartels ChemoResponse Assay is approved with the condition that the sponsor report to FDA periodically the safety, effectiveness, and reliability of the device for its intended use; and that Post Approval Studies be conducted to obtain additional information on the clinical application of the device.

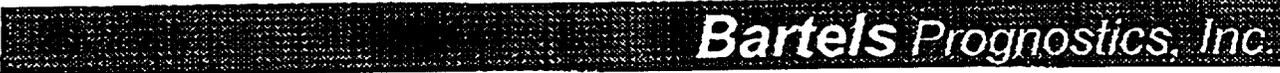


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***Bartels  
ChemoResponse Assay (CRA)  
System***  
US Patent Number 5242806

**5 specimen test kit, complete with controls, reagents and  
reference cell line.**

Store contents as specified on container labels.  
For *in vitro* diagnostic use only.

A handwritten signature or initials in the bottom right corner of the page.

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## *Bartels Prognostics, Inc.*

# ***Bartels ChemoResponse Assay System***

**Caution:** Federal law restricts this device to sale, distribution and use by or on the order of the physician, to clinical laboratories that have been trained by the manufacturer for the use of this device.

### **INTENDED USE**

The Bartels ChemoResponse Assay is an *in vitro* diagnostic device intended for use to determine resistance to 5-Fluorouracil (5-FU) of cells isolated from breast tumors. The result of the ChemoResponse Assay is indicated for use to assist physicians in determining if 5-FU is an ineffective treatment for relapsed breast cancer patients.

### **SUMMARY**

Approximately 130,900 women per year are diagnosed with breast cancer and 41,300 will eventually die from the disease (1). Chemotherapy is the treatment of choice in many of these cases. Unfortunately, a significant number of these patients relapse despite an initial response. Upon relapse, the response rate to subsequent chemotherapy diminishes and approximately 70% of these patients will demonstrate clinical resistance (2). Thus, a large portion of women gain no benefit from treatment yet experience the toxic side-effects of chemotherapy.

A variety of *in vitro* and *in vivo* methods have been developed to predict the patient's response in the laboratory setting prior to administering highly toxic drugs to patients (3-6). The traditional *in vitro* method developed by Salmon and Hamburger is referred to as the Human Tumor Cloning Assay (HTCA), in which single cell suspensions (exposed to drug and control) are plated in agar-containing plates (3). After two weeks of culture, the tumor cells are graded on their ability to form colonies *in vitro*. Other assays employ fluorescent cytoprinting, radionucleotide incorporation, and ATP-bioluminescence as endpoints (7-9).

The Bartels ChemoResponse Assay (CRA) System was developed to provide clinical laboratories with specified methods, reagents and drug strips to conduct *in vitro* predictive assays. This assay system is based on a simple microwell plate format which allows the analysis of specimens with cell counts of  $1 \times 10^6$  cells and tested over a 4-day assay period. The microwell format permits automated cell harvesting and result reporting. Included in the CRA system is a culture medium supplement which enhances tumor cell proliferation *in vitro*, increasing the percentage of specimens which can be successfully tested.

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The Bartels CRA was evaluated by a prospective clinical trial in predicting the response of 25 relapsed breast cancer patients to continuous 5-FU (300 mg/m<sup>2</sup>/day) infusion therapy (10).

### CONTRAINDICATIONS:

There are no known contraindications for use of the device.

### WARNINGS:

1. The ChemoResponse Assay is intended to determine *in vitro* tumor cell resistance only. Safety and effectiveness has not been established for *in vitro* tumor cell susceptibility.
2. The CRA was evaluated in this study with relapsed breast cancer patients receiving continuous 5-FU infusion. The response of primary breast cancer patients was not evaluated.
3. The cell population tested *in vitro* may not fully represent the *in vivo* cell population.
4. Training is required to conduct the CRA.
5. Inter-laboratory reproducibility studies showed lack of training results in reduced reliability of assay reproducibility.
6. The CRA has been evaluated for use only with breast tumor cells. The efficacy of this device for other tumor types has not been established.
7. **Microbial contamination** is a possibility with all primary tumor explants. Microbial contamination is known to invalidate CRA test results. To control for microbial contamination, Penicillin and Streptomycin have been added to CRA-TM and CRA-WM. If there is the possibility of microbial contamination, a microbiological smear of the final cell suspension can be examined with gram stain prior to inoculation of the CRA plates. Do not inoculate plates if microbial contamination is apparent in this smear. Contact Technical Services to determine the advisability of using the contaminated specimen in the CRA test.

### PRECAUTIONS:

1. Configured CRA plates should always be monitored visually on day 3 for signs of microbial contamination. If microbes were observed in the final cell suspension, more frequent observation is required. Visual signs of microbial growth include the following: a change in medium color to orange-yellow, cloudiness of the culture medium, filamentous strands in the culture medium, or mold floating on the surface of the medium. If contamination is suspected but not detected by visual observation, prepare a microbiological smear of one control well to confirm the absence of microbes. Do not add tritiated uridine to any wells that appear to be contaminated.
2. For *In Vitro* diagnostic use only.
3. Patient specimens must be transported in CRA-TM at room temperature. **DO NOT FREEZE or FIX SPECIMENS.**
4. Use aseptic technique for all procedures.
5. Each new ChemoResponse Assay System Kit must be tested once with the CRA CON-1 cell line prior to, or at the same time of testing tumor specimens. The CRA CON-1 (control cell line SW 480) must be tested within 48 hours of receipt.
6. All patient specimens must be considered potentially infectious.

8. Decontamination of laboratory equipment, benches, and clothing can be accomplished with a 1:10 dilution of household bleach or a standard laboratory disinfectant.
9. Do not pipet CRA Reagents by mouth.
10. Wear protective equipment such as gloves, lab coat and eye protection when handling CRA-5-FU strips.
11. Discard all radioactive waste in accordance with local, state, and federal regulation.
12. Reagents must not be used beyond stated expiration dates.
13. Incubation under conditions not prescribed may give false results.
14. Do not substitute reagents supplied by other manufacturers.
15. Cell Culture: **IMPORTANT:** Living cells in microwell plates or culture flasks cannot be stored refrigerated (2-8°C), but must be maintained lid side up in a humidified 34-37°C, 4-6% CO<sub>2</sub> supplemented air environment. (Microwell plates in an upright [lid side up] position; culture flasks on the large flat side.)

**BIOLOGICAL PRINCIPLE OF THE TEST**

The Bartels CRA System is composed of two parts: one is a selective culture medium based on CytoGro 289™; the other is a microwell plate format. The medium is selective for tumor cell growth *in vitro* and the microwell system allows for analysis of specimens with low cell counts. The CRA test contains two types of extracellular matrix (ECM)-coated strips, one containing the chemotherapeutic drug 5-FU and one containing ECM only as a control. Both types of strips are inoculated with tumor cells from the same tumor specimen and then incubated at 37°C for 4 days. To observe the toxic endpoint of 5-FU, tritiated uridine is added to the culture. Incorporation of uridine into tumor cell RNA is determined by scintillation counting. The incorporation of uridine by 5-FU treated tumor cells compared to untreated tumor cells is an indication of tumor cell resistance to 5-FU.

**REAGENTS**

<b>#1</b>	<b>CRA-5-FU (5-FU Drug Strip)</b>
Common Name:	5-FU Strip
Reactive Ingredients:	5-Fluorouracil, 90 µg/strip
Quantity/Concentration:	Extra Cellular Matrix Protein, 1.6 µg/strip
Non-reactive Ingredients:	Tissue Culture Grade Plastic
Instructions for Mixing/Reconstitution:	Reconstitute with 100 µL of CRA-GM. Incubate at room temperature 5 min. Add 100 µL tumor cell suspension in CRA-GM.
Storage/Shelf Life:	9 months in unopened foil pouch stored at 2-8°C. 24 hours in open pouch at 2-8°C.
Purification Treatment Required:	None
Indications of Instability:	None. However, CRA-5-FU Control is validated once per test kit with CRA-CON-1 cells.

<b>#2</b>	<b>CRA-5-FU Control (5-FU Control Strip)</b>
Common Name:	Control Strip, ECM Strip
Reactive Ingredients:	Extracellular Matrix 1.6 ug/strip

Quantity/Concentration:	
Instructions for Mixing/Reconstitution:	Reconstitute with 100 $\mu$ L CRA-GM. Incubate 5 min. Add 100 $\mu$ L of the tumor cell suspension in CRA-GM.
Storage/Shelf Life:	9 months in unopened foil pouch stored at 2-8°C. 24 hours in opened foil pouch at 2-8°C.
Indications of Instability:	None. However, CRA-5-FU Control is validated with CRA-CON-1 cells once per test kit.

<b>#3</b>	<b>CRA Frame/Lid (Plate Frame and Lid)</b>
Common Name:	Microwell plate
Reactive Ingredients:	None
Quantity/Concentration:	
Non-reactive Ingredients:	Tissue Culture Grade Plastic
Instructions for Mixing/Reconstitution:	Insert CRA-5-FU and 5-FU control strips into designated slots of the frame, cover with lid.
Storage/Shelf Life:	N/A
Purification/Treatment Required:	None
Indications of Instability:	None

<b>#4</b>	<b>CRA-TM (Transport Media)</b>
Common Name:	RPMI-1640 + 10% FBS
Reactive Ingredients:	RPMI-1640 90%
Quantity/Concentration:	FBS 10%, Penicillin 100 units/mL, Streptomycin 100 $\mu$ g/mL
Non-reactive Ingredients:	Water
<b>WARNINGS/PRECAUTIONS:</b>	<b>DO NOT FREEZE</b>
Instructions for Mixing/Reconstitution:	None
Storage/Shelf Life:	6 months at 2-8°C
Purification/Treatment Required:	None
Indications of Instability:	Cloudiness or color change to yellow-orange indicate contamination

<b>#5</b>	<b>CRA-WM (Wash Media 10X)</b>
Common Name:	10X RPMI-1640 Culture Medium
Reactive Ingredients:	RPMI-1640 Medium
Quantity/Concentration:	
Non-reactive Ingredients:	Water
<b>WARNINGS/PRECAUTIONS:</b>	<b>DO NOT FREEZE</b>
Instructions for Mixing/Reconstitution:	Add 900 mL sterile distilled water, 32.4 mL CRA-BICARB, 4.0 mL CRA-NAOH, 24 mL CRA-WM-ADD. Dilute to 1200 mL with sterile distilled water.
Storage/Shelf Life:	Undiluted CRA-WM = 9 months at 2-8°C. Mixed CRA-WM = 2 weeks at 2-8°C.
Treatment Required:	Filtration, if reconstituted with non-sterile distilled water.
Indications of Instability:	Cloudiness is an indication of bacterial contamination.

<b>#6</b>	<b>CRA-WM-ADD (Wash Media Additive)</b>
Common Name:	Sodium Pyruvate/Penicillin/Streptomycin/Fetal Bovine Serum
Reactive Ingredients:	Sodium Pyruvate 11mg/mL, Penicillin 5000 units/mL, Streptomycin 5

Quantity/Concentration	mg/mL, Fetal Bovine Serum 50% (v/v)
Non-reactive Ingredients	Water
Instructions for Mixing/Reconstitution	Add one vial to each bottle of CRA-WM.
Storage/Shelf Life	3 months at < -10°C. 2 weeks after mixing at 2-8°C
Purification Treatment Required	None
Indications of Instability	None
<b>#7</b>	<b>CRA-GM (Growth Media 10X)</b>
Common Name	10X RPMI-1640 Culture Medium
Reactive Ingredients	RPMI-1640 Medium
Quantity/Concentration	
Non-reactive Ingredients	Water
<b>WARNINGS/PRECAUTIONS</b>	<b>DO NOT FREEZE</b>
Instructions for Mixing/Reconstitution	Add 80 mL sterile distilled water, 3.4 mL CRA-BICARB, 0.4 mL CRA-NAOH, 2.5 mL CRA-GM-ADD, 28.7 mL sterile distilled water to each bottle of CRA-GM.
Storage/Shelf Life	Undiluted CRA-GM = 9 months at 2-8°C. Mixed CRA-GM = 2 weeks at 2-8°C.
Purification Treatment Required	Filtration, if reconstituted with non-sterile distilled water.
Indications of Instability	Cloudiness is an indication of bacterial contamination.

<b>#8</b>	<b>CRA-GM-ADD (Growth Media Additive)</b>
Common Name	Cyto-Gro 289™
Reactive Ingredients	Cyto-Gro 289 0.05% (v/v), Sodium Pyruvate 11 mg/mL, Asparagine
Quantity/Concentration	4.95 mg/mL. FBS 50% (v/v)
Non-reactive Ingredients	Water
Instructions for Mixing/Reconstitution	Add 7.5 mL to each 12.5 mL of undiluted CRA-GM.
Storage/Shelf Life	CRA-GM-ADD shelf life is 3 months at < -10°C. Mixed with CRA-GM shelf life is 2 weeks at 2-8°C storage.
Purification Treatment Required	None
Indications of Instability	None

<b>#9</b>	<b>CRA-DC-ENZ (DNase/Collagenase Solution)</b>
Common Name	DNase/Collagenase Enzyme Solution
Reactive Ingredients	DNase 0.002% (w/v), Collagenase 0.08% (w/v), Fetal Bovine Serum
Quantity/Concentration	10% (v/v), Penicillin 100 units/mL, Streptomycin 100µg/mL
Non-reactive Ingredients	RPMI Medium/Water
<b>WARNINGS/PRECAUTIONS</b>	Reagent supplied at working strength. Any dilution may decrease activity. Do not use beyond expiration date.
Instructions for Mixing/Reconstitution	None
Storage/Shelf Life	6 months at < -10°C, 24 hours at 2-8°C, 6 hours at room temperature
Purification Treatment Required	None
Indications of Instability	Loss of activity on tumor material.

<b>#10</b>	<b>CRA-TB (Trypan Blue)</b>
Common Name	Trypan Blue Stain Solution
Reactive Ingredients	Trypan Blue 0.02% (w/v)
Quantity/Concentration	
Non-reactive Ingredients	Phosphate Buffered Saline

<b>WARNINGS/PRECAUTIONS</b>	Carcinogen. Use standard personal protective equipment when handling. Do not freeze. Do not dilute.
<b>Instructions for Mixing/Reconstitution</b>	Mix 1:1 with cell suspension for counting live/dead cells.
<b>Storage/Shelf Life</b>	6 months at room temperature
<b>Purification Treatment Required</b>	None
<b>Indications of Instability</b>	Loss of blue color

<b>#11: CRA-T-ENZ (Trypsin Solution)</b>	
<b>Common Name</b>	Trypsin/EDTA
<b>Reactive Ingredients Quantity/Concentration</b>	Trypsin 0.05% (w/v), EDTA 0.02% (w/v)
<b>Non-reactive Ingredients</b>	PBS/Water
<b>WARNINGS/PRECAUTIONS</b>	Reagent supplied at working strength. Any dilution may decrease activity. Do not use beyond expiration date.
<b>Instructions for Mixing/Reconstitution</b>	None
<b>Storage/Shelf Life</b>	6 months at < -10°C, 2 weeks at 2-8°C
<b>Purification Treatment Required</b>	None
<b>Indications of Instability</b>	Loss of activity on cell monolayer.

<b>#12: CRA-CON 1 (Control Cell Line)</b>	
<b>Common Name</b>	SW 480 Cells
<b>Reactive Ingredients Quantity/Concentration</b>	SW 480 cells
<b>Non-reactive Ingredients</b>	RPMI-1640 Medium + 10% FBS
<b>WARNINGS/PRECAUTIONS</b>	Use cells within stated shelf life. Incubation at different temperatures or times will invalidate test result. Do not refrigerate living cells. Cells stored at greater than 38°C or less than 34°C or without 4-5% CO <sub>2</sub> are suboptimal for CRA testing.
<b>Instructions for Mixing/Reconstitution</b>	Remove cells from culture flask with CRA-T-ENZ. Dilute to 15,000 cells per mL and dispense at 0.1 mL/well into CRA 5-FU & 5-FU control strips.
<b>Storage/Shelf Life</b>	Store no more than 2 days at 34-38°C before use. Contact Bartels Prognostics Technical Service for further information.
<b>Purification Treatment Required</b>	Trypsinization from culture flask.
<b>Indications of Instability</b>	Medium color of orange-yellow or cloudiness of culture medium.

<b>#13: CRA-LB (Lysing Buffer)</b>	
<b>Common Name</b>	Lysing Buffer
<b>Reactive Ingredients Quantity/Concentration</b>	Ammonium Hydroxide 0.135% (w/v) Triton X-100 0.05% (v/v)
<b>Non-reactive Ingredients</b>	Water
<b>WARNINGS/PRECAUTIONS</b>	Do not freeze. Do not heat over 37°C. Do not dilute.
<b>Instructions for Mixing/Reconstitution</b>	None
<b>Storage/Shelf Life</b>	2 months at room temperature.
<b>Purification Treatment Required</b>	None
<b>Indications of Instability</b>	Loss of ability to lyse cells.

<b>#14</b>	<b>CRA-NAOH (Sodium Hydroxide)</b>
Common Name:	Sodium Hydroxide, 10% Solution
Reactive Ingredients Quantity/Concentration:	Sodium Hydroxide 10% (w/v)
Non-reactive Ingredients:	Water
<b>WARNINGS/PRECAUTIONS:</b>	Corrosive Poison Handle with care. Strong bases may react violently with water and cause skin burns.
Instructions for Mixing/Reconstitution:	Reagent supplied at working strength.
Storage/ Shelf Life:	6 months at room temperature
Purification Treatment Required:	None
Indications of Instability:	None

<b>#15</b>	<b>CRA-BICARB (Sodium Bicarbonate)</b>
Common Name:	Sodium Bicarbonate
Reactive Ingredients Quantity/Concentration:	Sodium Bicarbonate 7.5% (w/v)
Non-reactive Ingredients:	Water
Instructions for Mixing/Reconstitution:	Reagent supplied at working strength
Storage/ Shelf Life:	6 months at room temperature
Purification Treatment Required:	None
Indications of Instability:	Loss of ability to buffer culture medium at pH 7.0 - 7.4.

**General Stability/Storage:**

CRA-WM and CRA-GM are stable for 2 weeks after reconstitution with distilled water and CRA Additives. All other CRA Reagents will retain potency until the expiration date imprinted on the label. Return reagents to proper storage condition promptly after use.

**Specimen Collection and Storage**

Fine needle aspirates or tumor tissue (trimmed of fatty material) not exceeding 10 grams can be placed directly into one tube of sterile CRA-TM. Fluid specimens can be collected in sterile vacuum containers containing 10 units/mL of preservative-free Heparin.

At least  $1 \times 10^6$  tumor cells are required for one CRA test. Since viable tumor cell number is difficult to determine by sample size, obtain as large a sample as possible.

Specimens should be transported to the laboratory at room temperature (20-28 °C) in leak-proof containers. Best results are obtained when the specimen is processed and plated in CRA format within 24 hours of collection. Solid specimens that cannot be processed immediately can be stored at room temperature in the dark for no more than 24 hours. Fluid specimens may be stored for up to 48 hours in the dark. Longer storage may result in invalid test. **Do not freeze or fix specimen.**

If shipment is required, place the specimen in a biohazard approved leak-proof container and transport on wet ice. Ship via overnight express service and process specimen within 6 hours of receipt. (Specific requirements for shipping specimens are found in CFR 42, Section 72.25)

### **Materials Supplied:**

	<b>Item Name</b>	<b>Description</b>	<b>Qty/Volume</b>
1.	CRA 5-FU	5-FU Drug Strips	6
2.	CRA 5-FU Control	5-FU Control Strips	6
3.	CRA Frame/Lids	Plate Frame and Lid	6
4.	CRA-TM	Transport Media	5x30 mL
5.	CRA-WM	Wash Media 10X	1x12 mL
6.	CRA-WM-ADD	Wash Media Additive	1x24 mL
7.	CRA-GM	Growth Media 10X	1x12.5 mL
8.	CRA-GM-ADD	Growth Media Additive	1x3 mL
9.	CRA-DC-ENZ	DNase/Collagenase Solution	5x10 mL
10.	CRA-TB	Trypan Blue	1x3 mL
11.	CRA-T-ENZ	Trypsin Solution	1x5 mL
12.	CRA-CON 1	Control Cell Line	1x25cm <sup>2</sup> flask
13.	CRA-LB	Lysing Buffer	6x10 mL
14.	CRA-NAOH	Sodium Hydroxide	1x5 mL
15.	CRA-BICARB	Sodium Bicarbonate	1x40 mL

Reagents and materials supplied are for five (5) specimen tests and one (1) CRA-CON 1 test. Inspect components of the CRA System to insure receipt of all listed components and undamaged condition of all containers.

### **Materials Required But Not Supplied**

1. Preservative-free Heparin, sterile (Sigma #H3125)\*
2. Tritiated Uridine, sterile (Specific Activity >27.5 Ci/mMol)
3. Ethanol 70%
4. Percoll, isotonic, sterile (Pharmacia/LKB #17-089)\*
5. Ficoll, sterile (Pel-Freeze, Isolymp #60002-2)\*
6. 6N HCl, sterile
7. Scalpel handle with blades, sterile
8. Hemacytometer
9. Forceps sterile
10. Micropipets (10 to 1000 µL) with sterile tips
11. Sterile petri dishes (100 mm<sup>2</sup>)
12. Sterile serological pipets (1,5,10 mL)
13. Sterile polypropylene centrifuge tubes (50 and 15 mL)

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14. Sterile Pasteur pipets
15. Sterile 10 cc syringe with 6" x 10 gauge cannula
16. Centrifuge capable of 1000g
17. Sterile Collector™ tissue sieve (Belco #1985-18500)\*
18. 2-8°C refrigerator
19. pH 6.9-7.6 litmus paper, with 0.1 pH unit increments
20. Brightfield microscope (10-20x objectives)
21. Inverted microscope (10-20x objectives)
22. Biological Safety Cabinet (Laminar Flow Hood)
23. 96-well cell harvester with vacuum pump
24.  $\beta$ -Counter
25. CO<sub>2</sub> Incubator
26. Glass fiber filters
27. Scintillation fluid and vials
28. Latex gloves
29. Sterile distilled water (pH 6.8 - 7.2)
30. Additional CRA-TM for specimen transport

\*Or equivalent

## Procedure

### Preparation of Reagents

*Prepare all reagents in a sterile environment, preferably a Laminar Flow Hood.*

#### A. CRA-WM -- Preparation of working strength solution.

- 1) CRA-WM is supplied as a 10X solution. Dilute to working strength by adding the following components in the specified order to the bottle containing the CRA-WM concentrate.
  - a) 900 mL sterile distilled water
  - b) 32.4 mL CRA-BICARB
  - c) 4.0 mL CRA-NAOH -- (Add slowly)
  - d) 24.0 mL CRA-WM-ADD
- 2) Mix and let stand 3 minutes. Spot a pH paper which will measure between 7.0 - 7.4 pH with the solution. The pH must be between 7.0 and 7.4. If pH is higher or lower, add sterile HCl or CRA-NAOH until pH is within the proper range.
- 3) Add 119.6 mL sterile distilled water.
- 4) Label bottle "Sterile CRA-WM Working Strength" with 2 week expiration date.

#### B. CRA-GM - Preparation of working strength solution.

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- 1) **CRA-GM** is supplied in the kit as a 10X solution. Dilute the solution to working strength by adding the following components in the specified order to the bottle containing the **CRA-GM** concentrate.
  - a) 80 mL sterile distilled water
  - b) 3.4 mL **CRA-BICARB**
  - c) 0.4 mL **CRA-NAOH** --(Add slowly)
  - d) 2.5 mL **CRA-GM-ADD**
- 2) Mix and let stand 3 minutes. Spot a pH paper which will measure between 7.0 - 7.4 pH with the solution. The pH must be between 7.0 and 7.4. If pH is higher or lower, add sterile HCl or **CRA-NAOH** until pH is within the proper range.
- 3) Add 28.7 mL sterile distilled water
- 4) Label bottle: "Sterile **CRA-GM** Working Strength" with 2 week expiration date.
- 5) NOTE: If required, **CRA-WM** and **CRA-GM** can be filter sterilized using a 0.22 $\mu$  filter.

**C. CRA-CON-1 (Control Cells) require refeeding upon receipt.**

- 1) Aspirate all medium from the 25cm<sup>2</sup> flask. Replace with 5 ml **CRA-GM**.
- 2) Observe cells under an inverted microscope for confluence (approximately 60%) and viability. Confluence is estimated visually with an inverted microscope at 100x magnification. Determine the "proportion" of cells vs. empty space in at least 5 visual fields and average.
- 3) Place in 34-38°C, 4-6% CO<sub>2</sub> incubator for at least 16 hours.
- 4) Perform CRA test on the control cells within 48 hours of receipt. After 48 hours, discard control cells and re-order.

**D. CRA-TM**

- 1) **CRA-TM** supplied is used for ficoll or percoll cell separations.
- 2) Additional **CRA-TM** is required for specimen transport. (Order additional tubes.)

**E. OTHER CRA REAGENTS**

The remaining reagents are supplied at working strength. Store as specified on container labels.

**F. Limitations**

See "Limitations" Section of product insert.

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**ChemoResponse Assay****A. Processing the Specimen**

**Work in an aseptic environment.**

- 1) Remove transport medium from tube and wash solid specimens twice in 8 mL of CRA-WM by repeatedly inverting the tubes 5-6 times to remove possible external interfering substances deposited during collection. Save wash each time and transport medium from the specimen in a 50 mL culture tube. If contamination is suspected, wash the specimen for 3 additional times and discard all supernatant.
- 2) Fluid specimens and needle biopsies should be concentrated by centrifugation at 200g for 10 minutes. Recover the cell pellet and wash by centrifugation in two changes of 20 mL CRA-WM. After the final wash, suspend the cell pellet in 3 mL of CRA-GM.

**Fluid specimens: proceed to #8.**

**Solid specimens: proceed to #3.**

- 3) Place solid specimens into a 100 cm<sup>2</sup> petri dish with 3-5 mL of CRA-WM.
- 4) Remove excess fatty and necrotic tissue and then macerate the remaining tissue with a sterile scalpel blade. Use sterile forceps to hold the tissue during this process.
- 5) Combine with the wash and transport medium from Step #1 and centrifuge. Discard the supernatant.
- 6) Thaw one vial of CRA-DC-ENZ and add to the cell pellet from Step #5. Mix well.
- 7) Combine fluid with the macerated tissue and transfer to a 25 cm<sup>2</sup> culture flask. Incubate at 37°C for 30-110 minutes. Check every 30 minutes for tissue dissociation.
- 8) After incubation, pass the mixture through a Collector sieve. Rinse the tissue in the Collector 3 times with 10 mL of CRA-WM each rinse. Collect rinse fluid in a sterile petri dish. Mix the tissue with a sterile pipet while rinsing to dislodge any adherent cells. Discard tissue residue.

- 9) Transfer the supernatant to a centrifuge tube and centrifuge the cell suspension at 200g for 10 minutes. Save the cell pellet and wash cells by centrifugation in 2 changes of 20 mL of CRA-WM. After the final wash, suspend the cell pellet in 3 mL of CRA-GM.
- 10) Observe washed cells with the aid of a microscope, from #1 (fluids) or #7 (solids). Mix 20  $\mu$ L of CRA-TB with 20  $\mu$ L of cell suspension. Count the number of live tumor cells (large non-stained cells). Determine the proportion of dead cells, red blood cells (RBC, small orange tinted cells), and lymphocytes (smooth-edged cells slightly larger than RBC) in the specimen. If the percentage of dead cells is greater than 50% or RBC's is greater than 200%, proceed to #11. If the percentage of lymphocytes is greater than 70, proceed to #12. If neither is true, proceed to #13.
- 11) **FICOLL PURIFICATION OF TUMOR CELLS:**  
~~CAUTION: 20-50% of the viable tumor cells will be lost during this procedure. All reagents must be at room temperature.~~  
Dilute the cell suspension to  $5 \times 10^6$  cells/mL in CRA-TM. In a 15 mL centrifuge tube, layer 10 mL of cell suspension over 4 mL Ficoll. Do not mix the two solutions. Use a sufficient number of tubes for the entire cell suspension. Centrifuge at 1,000g for 15 minutes at room temperature. Collect viable tumor cells from interface of the medium layer to the Ficoll layer in a 50 mL test tube and wash the combined cell pellets by centrifugation (200g for 10 minutes) in two (2) changes of 50 mL CRA-WM. After the final wash, suspend the pellet in 3 mL of CRA-GM. Repeat Step #10.
- 12) **PERCOLL PURIFICATION OF TUMOR CELLS:**  
~~CAUTION: 20-50% of the viable tumor cells will be lost during the Percoll purification process. All reagents must be at room temperature.~~  
Prepare a 10%/20% discontinuous Percoll gradient 4 mL each in a 15 mL centrifuge tube as directed by the manufacturer. Prepare a sufficient number of tubes for the entire sample. Use a 10 cc syringe and sterile 6" x 10 gauge cannula to place the 20% Percoll solution beneath the 10% solution. Dilute the cell suspension to  $5 \times 10^6$  cells/mL in CRA-TM and add 2-3 mL cell suspension to each gradient tube. Do not mix cell suspension with Percoll or the Percoll layers with each other. Centrifuge at 50-60g for 10 minutes at room temperature. After centrifugation, the tumor cells will pellet at the bottom of the centrifuge tube. Carefully collect these cells and wash by centrifugation in two (2) changes of 50 mL CRA-WM. After the final wash, suspend the pellet in 3 mL of CRA-GM. Repeat Step #10.
- 13) Calculate the number of viable tumor cells per mL. Prepare a solution of at least  $10^5$  viable tumor cells/mL in 5 mL of CRA-GM (at least  $5 \times 10^5$  cells total). Dispense cell suspension within 30 minutes of dilution.

**B. Inoculation of CRA Strips**

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Each **CRA FRAME** (microwell plate frame) contains 12 slots labeled 1-12 across the top of the frame and 8 rows labeled A-H down the left-hand side of the plate. The **CRA-5-FU** and **CRA-5-FU Control** strip are packaged individually in foil pouches. One **CRA 5-FU** and one **CRA-5-FU Control** are required for each test.

The **CRA-5-FU** strip is marked with yellow paint at the top. Four concentrations of 5-FU have been processed into **CRA-5-FU strips**. The four wells at the top (yellow, blunt end) contain 100 µg/mL, the next four wells 10µg/mL, the next four wells 1 µg/mL, and the bottom four wells 0.1µg/mL 5-FU.

The **CRA-5-FU Control** strip is clear. The top edge of this strip contains a rectangular tab (blunt end) and the lower end a semi-circular tab. The entire unit of **CRA-Frame/Lid & strips** is called the **CRA-Assembly** (see figure below).

*NOTE: CRA-5-FU and CRA-5-FU Control should be at room temperature prior to opening. Wear protective equipment such as gloves, lab coat and eye protection when handling CRA-5-FU strips.*

- 1) Place one sterile **CRA Frame/Lid** into the aseptic work space. Open the package. Position the open frame so that the letters A-H are on the left.
- 2) Open one **CRA-5-FU Control** package; remove the strip from the package. Align the strip with the first two slots (#s 1 & 2) with the blunt end toward the top of the plate and push in. **DO NOT TOUCH THE OPENINGS OF THE WELLS DURING THIS PROCESS.** Handle the strip at the top (blunt end) and bottom. Open one **CRA-5-FU** package; insert into frame slots 3 & 4, as described above.
- 3) Place the lid on the frame and mark the location of each **CRA strip** on the lid. Include specimen number and date. Mark the location of four (4) negative control wells and twelve (12) positive control wells on the lid for the **CRA-5-FU Control**.
- 4) Five (5) minutes prior to adding the cell suspension, measure 3 mL of **CRA-GM** into a sterile tube and then transfer 100 µL **CRA-GM** to the twelve (12) positive control wells and to all wells of the **CRA-5-FU** strip. **DO NOT ADD MEDIUM TO THE NEGATIVE CONTROL WELLS.** Use a micropipet (which has been cleaned with an alcohol wipe) with a sterile tip. Incubate at room temperature for five (5) minutes.
- 5) Using a sterile pipet, thoroughly mix the cell suspension prepared in Step A,13. While agitating the tube, transfer 100 µL to the twelve (12) positive control wells and to each well of the **CRA-5-FU** strip. **DO NOT ADD CELLS TO THE NEGATIVE CONTROL WELLS.**

- 6) Inspect several wells with an inverted microscope at 100X magnification to insure the inoculation of tumor cells.
- 7) Place the **CRA Assembly** lid side up into a 34-38°C, 4-6% CO<sub>2</sub> incubator for 3 days.

**C. Endpoint -- After 3 days of incubation.**

**IMPORTANT: Discard all radioactive waste in accordance with local, state, and federal regulation.**

- 1) Prepare a sterile solution of tritiated uridine in **CRA-GM** as follows:  
Add 20 µL of tritiated uridine (1 mCi/mL) to 2 mL **CRA-GM**.
- 2) Remove **CRA Assembly** from the incubator. Inspect wells for signs of microbial contamination, such as cloudiness of medium or yellow-orange medium. ( See Quality Control below). Mark the lid with the location of any contaminated wells and do not add tritiated uridine to these wells.
- 3) Aseptically transfer 50 µL of the tritiated uridine solution to each well of the **CRA Assembly**. Use a micropipet with a sterile tip.
- 4) Return the **CRA Assembly** to 34-38°C, 4-6% CO<sub>2</sub> incubator for 16-24 hours.
- 5) Remove **CRA Assembly** from incubator and harvest cellular RNA onto glass filter mats with a cell harvester.

Harvest Procedure: Wash each well of the **CRA Assembly** three (3) times by first aspirating the culture medium from the well and then alternately filling the wells with distilled water and aspirating the wells to dryness. Do not overfill wells while washing. Leave the wells empty after the final rinse. Add 50 µL **CRA-LB** and incubate for at least 1.5 minutes. Repeat distilled water wash sequence 3 times, leaving wells empty. Add 50 µL **CRA-LB** and incubate for at least 1.5 minutes. Repeat distilled water wash sequence 6 times. Then aspirate 30 ml of 70% Ethanol through the glass fiber filter. Leave suction on for at least 2 minutes to aid in drying the filter.

- 6) To determine the amount of tritiated uridine incorporated into RNA, the individual filter discs corresponding to each **CRA-5-FU Control** and **CRA 5-FU** well must be analyzed with a β-Counter. Obtain cpm as directed in the β-Counter operators manual.
- 7) To ensure accurate results, filter discs must be counted within 3 days of processing. Store filter in room temperature until counting.

## Quality Control

### A. Positive and Negative Control

**Negative Control.** The negative control is comprised of four (4) **CRA-5-FU Control** wells which were not inoculated with tumor cells. There should be no incorporation of tritiated uridine into RNA in these wells. The negative control is used to determine the efficiency of filter mat washing by the cell harvester. The mean negative control is subtracted from each of the other test values. The cpm must not exceed 500 and a majority of wells must be included in the calculation of the mean.

**Positive Control:** The positive control is comprised of twelve (12) **CRA-5-FU Control** wells which contain untreated tumor cells from the same specimen used in the **CRA-5-FU** strip. The positive control represents the maximal incorporation of tritiated uridine into RNA by untreated tumor cells. The positive control is used as a reference for treated tumor cells, i.e. to determine the degree of tritiated uridine incorporation by the treated cells. The positive control must be at least three (3) times the negative control, and the coefficient of variation between the twelve positive control observations must be less than 50%. Data from at least eight (8) wells must be included in the calculation of the mean.

### B. Quality Control For CRA System - CRA Control-1 Cells (CRA CON-1)

The CRA CON-1 cells are used with CRA-5FU as quality control material for the various components of the CRA system and for instruments used to conduct the test.

**NOTE:** The CRA CON-1 cells must be tested upon receiving each new Chemo Response Assay System Kit or with the first set of tumor specimen tested. Use cells within stated shelf life. Incubation at different temperatures or times will invalidate test results. Do not refrigerate living cells. Cells stored at greater than 38°C or less than 54°C or without 4-6% CO<sub>2</sub> are suboptimal for CRA testing. Microbial contamination will invalidate test results.

#### 1. Preparation of Control Cells

- a. Use CRA CON-1 cells at 60-80% confluence. Confluence is estimated visually with an inverted microscope at 100x magnification. Determine the "proportion" of cells vs. empty space in at least 5 visual fields and average.
- b. To remove cells from the 25 cm<sup>2</sup> culture flask, remove all medium from the flask with a pipet. Wash the flask once with 2 mL CRA-T-ENZ. Remove fluid and add 1 mL CRA-T-ENZ. Incubate at 34-38°C for 2-5 minutes until cells have detached from the surface.

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- c. Add 5 ml **CRA-GM** to the flask, rinse, and then transfer to a 15 mL centrifuge tube. Add 5 mL of **CRA-GM** and centrifuge at 200g for 10 minutes. Aspirate medium from the tube and resuspend the cell pellet in 3 mL **CRA-GM**.
- d. Mix cells thoroughly and then dilute 20  $\mu$ L of the cell suspension with 20  $\mu$ L **CRA-TB**. Count number of viable cells (unstained) with a hemacytometer and brightfield microscope (200X magnification).
- e. Adjust cell concentration to 15,000 cells per mL with **CRA-GM**.

## 2. Inoculation of CRA Strips

Each **CRA FRAME** (microwell plate frame) contains 12 slots labeled 1-12 across the top of the frame and 8 rows labeled A-H down the left-hand side of the plate. The **CRA-5-FU** and **CRA-5-FU Control** strips are packaged individually in foil pouches. One **CRA 5-FU** and one **CRA-5-FU Control** are required for each test.

The **CRA-5-FU** strip is marked with yellow paint at the top. Four concentrations of 5-FU have been processed into **CRA-5-FU strips**. The four wells at the top (yellow, blunt end) contain 100  $\mu$ g/mL, the next four wells 10 $\mu$ g/mL, the next four wells 1  $\mu$ g/mL, and the bottom four wells 0.1 $\mu$ g/mL 5-FU.

The **CRA-5-FU Control** strip is clear. The top edge of this strip contains a rectangular tab (blunt end) and the lower end a semi-circular tab. **CRA-5-FU** and **CRA-5-FU Control** should be at room temperature prior to opening.

*NOTE: Wear protective equipment such as gloves, lab coat and eye protection when handling CRA-5-FU strips.*

- a. Place one sterile **CRA Frame/Lid** into the aseptic work space. Open the package. Position the open frame so that the letters A-H are on the left.
- b. Open one **CRA-5-FU Control** package; remove the strip from the package. Align the strip with the first two slots (#s 1 & 2) with the blunt end toward the top of the plate and push in. **DO NOT TOUCH THE OPENINGS OF THE WELLS DURING THIS PROCESS.** Handle the strip at the top (blunt end) and bottom. Open one **CRA-5-FU** package; insert into frame slots 3 & 4, as described above.
- c. Place the lid on the frame and mark the location of each **CRA strip** on the lid. Include lot number and date. Mark the location of four (4) negative control wells and twelve (12) positive control wells on the lid for the **CRA-5-FU Control**.
- d. Five (5) minutes prior to adding the cell suspension, measure 3 mL of **CRA-GM** into a sterile tube and then transfer 100  $\mu$ L **CRA-GM** to the twelve

(12) positive control wells and to all wells of the CRA-5-FU strip. DO NOT ADD MEDIUM TO THE NEGATIVE CONTROL WELLS. Use a micropipet with a sterile tip. Incubate at room temperature for five (5) minutes.

e. Thoroughly mix CRA-CON-1 cell suspension (15,000/mL) and use a micropipet with sterile tip to add 100  $\mu$ L to each of the twelve (12) positive control and all of the 5-FU wells. DO NOT ADD CELLS TO THE FOUR (4) NEGATIVE CONTROL WELLS.

f. Place the CRA-Assembly lid side up into a 34-38°C, 4-6% CO<sub>2</sub> incubator for 3 days.

### 3. Endpoint -- After 3 days of incubation.

**IMPORTANT: Discard all radioactive waste in accordance with local, state, and federal regulation.**

a. Prepare a sterile solution of tritiated uridine in CRA-GM as follows: Add 20  $\mu$ L of tritiated uridine (1 mCi/mL) to 2 mL CRA-GM.

b. Remove CRA Assembly from the incubator. Inspect wells for signs of microbial contamination, such as cloudiness of medium or yellow-orange medium. If contamination is suspected, follow Quality Control Procedure for confirmation of microbial contamination. Mark the lid with the location of any contaminated wells and do not add tritiated uridine to these wells.

c. Aseptically transfer 50  $\mu$ L of the tritiated uridine solution to each well of the CRA Assembly. Use a micropipet with a sterile tip.

d. Return the CRA Assembly to 34-38°C, 4-6% CO<sub>2</sub> incubator for 16-24 hours.

e. Remove CRA Assembly from incubator and harvest cellular RNA onto glass filter mats with a 96-well cell harvester.

Harvest Procedure: Wash each well of the CRA Assembly three (3) times by first aspirating the culture medium from the wells and then alternately filling the wells with distilled water and aspirating the wells to dryness. Do not overflow wells while washing. Leave the wells empty after the final rinse. Add 50  $\mu$ L CRA-LB and incubate for at least 1.5 minutes. Repeat distilled water wash sequence 3 times, leaving wells empty. Add 50  $\mu$ L CRA-LB and incubate for at least 1.5 minutes. Repeat distilled water wash sequence 6 times. Then aspirate 30 mL of 70% Ethanol through the glass fiber filter. Leave suction on for at least 2 minutes to aid in drying the filter.

f. To determine the amount of tritiated uridine incorporated into RNA, the individual filter discs corresponding to each CRA-5-FU Control and CRA 5-FU well must be analyzed with a  $\beta$ -Counter. For scintillation counting, each disc must be placed into a separate scintillation vial with 1 ml of scintillation fluid. Keep track of the order from the plate (i.e. Slot 1A, Slot 1B... Slot 5H) so that the CRA Lid diagram (B,3) can be used to determine treatment group. Obtain cpm as directed in the  $\beta$ -Counter operators manual

g. To ensure accurate results, filter discs should be counted within 3 days of processing. Store filter in room temperature until counting.

4. **Results:**

a. Organize raw data from the printout of the beta counter to negative, positive controls and 5-FU treatment groups. Discard any obvious outliers which are plus or minus three (3) times the standard deviation (S.D.) from the mean of the rest of the group.

b. After discard the outliers, re-calculate the mean and S.D. for the negative control. Discard any observations which are plus or minus two (2) times the S.D. from the mean of the rest of the group. Recalculate the mean. The negative control must be less than 500 cpm and contain the data from a majority of wells to be considered valid.

c. Subtract the mean of the negative control from all of the values (positive control and 5-FU treated groups). Calculate the mean and S.D. for each group. Discard any data points which are plus or minus two (2) times the S.D. from the mean of the rest of the group. Recalculate the mean for each group.

For the positive control group, calculate the C.V. The C.V. of the positive control must be less than 50% and the mean cpm must be three (3) times the negative control or the test is invalid. In addition, the mean of the positive control must contain at least 8 values (after discarding outliers and data points  $> \pm 2$  S.D. from the mean) and the 5-FU treatment groups must contain a majority of values or the test is invalid. See example below:

	Control cpm		5-FU $\mu\text{g/mL}$		
Negative	71000 <sup>1</sup>	85	991038 <sup>1</sup>	1255	100 $\mu\text{g/mL}$
Negative	52	69	1238	1091	
Positive	52663	1015	411	411	10 $\mu\text{g/mL}$
Positive	6098	65303	378	433	
Positive	58010	58900	1520	1600	1 $\mu\text{g/mL}$
Positive	39092	32200	14988	15725	
Positive	52772	58902	20528	361 <sup>2</sup>	.01 $\mu\text{g/mL}$
Positive	42400	462100 <sup>1</sup>	34723	27963	

<sup>1</sup> Outliers, discard. Extremely high cpm is indicative of microbial contamination. Extremely low cpm may indicate failure to add cells or radioactive nucleotide to the well.

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<sup>2</sup> These values are outside of two (2) S. D. from the mean of the rest of the group and are excluded from calculation.

**Steps for calculation:**

**Mean Negative Control cpm:**  $(100 + 85 + 52)/3 = 79$ , S.D. 20

Subtract the mean negative control value 79 from all the values.

**Mean Positive Control cpm:**  $(43019 + 37071 + 39011 + 25911 + 42421 + 52586 + 65424 + 58821 + 32121 + 58821)/10 = 48275$ , S.D. 1516, C.V. 21%

(All data points are within  $\pm 2$ SD from the mean, therefore no further data exclusion) The C.V. of positive control is  $\leq 50\%$ , therefore the assay is valid.

Proceed to the next calculation step.

**Mean % control values for 5-FU treated group:**

Divide the mean of each 5-FU treatment group by the mean of the positive control.

The result will be a percent control for each 5-FU treatment. 5-FU values should be less than 120% control or the result is invalid.

**100 ug/ml** - mean =  $(1248 + 1159 + 1176)/3 = 1194$  (SD 39)

% control =  $1194/48275 = 2.5\%$

**10 ug/ml** - mean =  $(3095 + 1109 + 3679 + 4109)/4 = 3061$  (SD 197)

% control =  $3061/48275 = 6.3\%$

**1 ug/ml** - mean =  $(7193 + 11401 + 11825 + 5792)/4 = 6193$  (SD 1197)

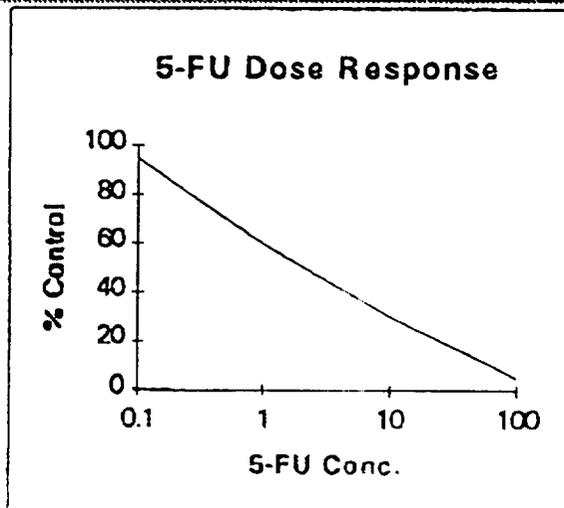
% control =  $6193/48275 = 12.8\%$

**0.1 ug/ml** -  $(20449 + 34644 + 27884)/3 = 27659$  (SD 5792)

% control =  $27659/48275 = 57.3\%$

d. Plot the percent control of the CRA-CON-1 against 5-FU concentration. See example below:

**EXAMPLE**



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## e. Expected Results for CRA-CON-1 Cells:

The expected values for the CRA-CON 1 cells are specified in the labeling for each lot of shipment. Each lot of control cells have different expected results. An example of control cells tested on the same lot of 5-FU done in ten separate experiments is presented below:

5-FU Concentration	Range of Expected Value (% Control)	Mean (% Control)
100 µg/mL	0.1-3.2	0.36
10 µg/mL	2.5-17.1	7.70
1 µg/mL	14.5-36.6	20.40
0.1 µg/mL	63.4-91.2	77.50

**Caution:** The expected results are lot specific. Do not substitute CRA-CON-1 cells from other lots.

Please contact Bartels Prognostics, Inc. Technical Services (206-392-1013) if values are outside of the range of the expected values specified in the product insert.

## CRA RESULTS

### A. Data Calculation

To calculate the result, follow directions outlined for result calculation for CRA CON-1 (control cells) in the previous section. The % control value for 5-FU treated wells should not be above 120% control or the results are invalid.

### B. Interpretation of Results

Percent control values of 40.5% or higher at the 1 µg/mL concentration indicate that the tumor has a 100% probability of resistance to monotherapy with continuous infusion 5-FU at 300 mg/m<sup>2</sup>/day. Specimens with a <40.5% control value at 1 µg/mL of 5-FU concentration are considered non-resistant.

## Limitations

1. Microbial contamination is known to invalidate CRA test results.
2. The test requires an adequate number of viable tumor cells.
3. The cell population tested *in vitro* may not fully represent the *in vivo* cell population.
4. Any deviation from the CRA Protocol may result in a false prediction of resistance.
5. Assay reproducibility may be adversely affected by lack of training.

**Expected Values**

Expected values were derived from a prospective clinical trial with 25 relapsed breast cancer patients treated with 5-FU by continuous infusion. Clinical response was based on standard criteria for measurable disease (10). To determine the cutoff point (drug concentration and % control value) for *in vitro* response, a training set was constructed with 34 specimens obtained from patients with similar disease and chemotherapy history as eligible patients entering the clinical trial. The cutoff was determined from the 5-FU dose/response curves done at the 100, 10, 1, and 0.1 ug/mL concentration for the 34 specimens. The 1 ug/mL concentration was selected because of superior resolution; ie, broadest separation of specimens into different categories of responses (10). At the 1 ug/mL concentration, the % control values for the 34 specimens were ranked and the median value of 40.5% at the 1 ug/mL concentration was selected to determine *in vitro* resistance. Using this cutoff, the expected % control values for the 25 specimens from eligible patients and the 34 training set specimens were:

	% of Resistant Specimen	Mean (Median)	Range
Clinical Patients	48% (12/25)	65.2 (60.4)	41.3-99.9
Training Set	50% (17/34)	55.4 (54.2)	40.5-90.8

**Specific Performance Characteristics**

**A. Non-Clinical Laboratory Studies**

The CRA culture system is designed for culturing primary tumor cells. Studies were conducted to analyze the effects of following treatments and procedures on CRA results: enzyme dissociation and anti-microbial reagents, storage and transport, plating density and assay duration. No adverse effect was found if CRA procedures were strictly followed. Additional studies indicated normal stromal cells and benign specimens grew poorly in the CRA culture and had minimal effects on results. Ploidy analysis and immunohistochemistry studies demonstrated the efficacy of CRA culture for growing tumor cells.

**B. Reproducibility**

The intra-, inter-laboratory and the intra-, inter-lot reproducibility studies were conducted with breast tumor specimens and breast cell lines. The results at the 1 ug/mL 5-FU concentration were analyzed with intraclass correlation coefficient (ICC):

	Intra-Lab (7 specimens - 1 cell line)	Inter-Lab (19 specimens - 2 cell lines)	Intra-Lot (7 specimens - 1 cell line)	Inter-Lot (6 specimens - 1 cell line)
ICC	0.61	0.65	0.93	0.97
95% CI	0.31- 0.93	0.40 - 0.84	0.79 - 0.99	0.90 - 0.99

A separate inter-laboratory study was conducted with three sites and eleven tumor specimens. The reproducibility at the 1 ug/mL 5-FU concentration was ICC = 0.84 (95% CI 0.70-0.94). The higher ICC value was in part contributed by standardized reagents and drug strips, the use of control cells, strict adherence to the assay protocol and improved training.

**C. Clinical Correlation Study**

A prospective double blind clinical trial with 25 relapsed breast cancer patients treated with continuous infusion 5-FU (300 mg/m<sup>2</sup>/day) was conducted to determine the clinical accuracy of CRA. Eligibility criteria included measurable tumor to monitor response and tumor accessible to biopsy for CRA. Tumor specimen must contain sufficient number of viable cells for CRA. Sixty patients were enrolled and 25 were eligible both *in vivo* and *in vitro*. Clinical response was determined by standard criteria for measurable disease (10). Patients with complete or partial responses were classified as non-resistant and stable or progressive disease were considered resistant. The cutoff point to specify *in vitro* resistance was determined from a training set of 34 specimens. The median value for the 34 specimens analyzed at the 1 ug/mL concentration was 40.5%. Based on the median value, the assay accuracy for resistance determination was 100% (see the following table).

Specificity:	6/6	100%	(95% CI = 54-100)
Sensitivity:	12/19	63%	(95% CI = 38-84)
Predictive Value Positive:	12/12	100%	(95% CI = 74-100)
Predictive Value Negative:	6/13	46%	(95% CI = 19-75)

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**Technical Information**

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