

**Onko-Sure<sup>®</sup>**  
**[AMDL- ELISA DR-70<sup>®</sup> (FDP)]**  
**PN: DR2101USA**

**The sale and distribution of this device is restricted by United States federal law to, by, or on the order of a physician. In addition, the use of this device is restricted to, by, or on the order of a physician. Because of differences in reagent specificity and assay methods, the concentration of DR-70<sup>®</sup> (FDP) in a given specimen may vary with devices from different manufacturers. Values obtained with different assay methods cannot be used interchangeably. It is mandatory that results reported by the laboratory to the physician include the identity of the assay used. If the assay method for DR-70<sup>®</sup> (FDP) is changed during the course of monitoring patients with serial DR-70<sup>®</sup> (FDP) levels, baseline values for the patients being serially monitored must be confirmed by additional sequential testing.**

*Onko-Sure<sup>®</sup> is a brand name of AMDL-ELISA DR-70<sup>®</sup> (FDP)*

**Intended Use**

The DR-70<sup>®</sup> (FDP) ELISA is designed for IN VITRO DIAGNOSTIC USE ONLY for the quantitative measurement of DR-70<sup>®</sup> (FDP) in human serum. Serial testing using the AMDL-ELISA DR-70<sup>®</sup> (FDP) is to be used as an aid in monitoring the disease progression in patients who have been diagnosed previously with colorectal cancer. Results of DR-70<sup>®</sup> (FDP) testing should be used in conjunction with other clinical modalities that are standard of care for monitoring disease progression in these patients.

**Summary and Explanation of Test**

Colorectal cancer (CRC) remains the second leading cause of cancer death in the United States<sup>1</sup>, despite a reported decrease in colorectal cancer mortality over the past forty years related to increased screening, intervention, and monitoring programs. In 2007, the Surveillance, Epidemiology and End Results (SEER) program estimated 153,760 new colorectal cancer patients<sup>2</sup> with a five-year survival prediction of 50%. CRC accounts for approximately 10.6% of all new cancer cases and approximately 10% of all cancer deaths in the United States. More than 56,000 patients died from CRC in 2005<sup>3</sup>. Although awareness of the importance of CRC screening<sup>4</sup> and early treatment has risen<sup>5</sup>, CRC is presently a significant health concern in the United States<sup>3</sup>.

Monitoring programs have emerged as an important tool in enhancing survival in post-operative CRC patients. According to Dr. Thomas Anthony, the survival benefit of post-operative monitoring programs “depends on the likelihood of recurrence, the availability of sensitive and specific tests to identify recurrences at a treatable stage, the availability of curative retreatment options, an aggressive application of retreatment whenever possible and the minimization of morbidity and mortality associated with retreatment”<sup>6</sup>. For CRC, approximately half of all patients treated will experience disease recurrence. Curative retreatment options exist and retreatment options are applied with a modest decrease in CRC mortality (approximately 10-15%)<sup>7-9</sup>. However, in order to enhance the survival benefit of CRC monitoring programs, “the availability of sensitive and specific tests to identify recurrences at a treatable stage” needs improvement.

The DR-70<sup>®</sup> (FDP) ELISA test (AMDL Diagnostics, Inc., Tustin, CA) provides physicians with an additional clinical tool for monitoring CRC patients. The DR-70<sup>®</sup> (FDP) ELISA measures both Fibrin and Fibrinogen Degradation Products in human serum samples. The FDP measured by the DR-70<sup>®</sup> (FDP) Immunoassay in serum from colorectal cancer patients was primarily produced by plasmin cleavage of either fibrinogen or fibrin. Cleavage of fibrinogen by plasmin produces fragments D and E as the primary end-products. Thrombin converts fibrinogen to fibrin in response to signals from the coagulation cascade. Cleavage of fibrin by plasmin produces D-dimer as a primary end-product. Depending upon the original substrate, the Fibrin/ogen Degradation Products detected by the DR-70<sup>®</sup> (FDP) immunoassay will include fragments D and E as well as D-dimer. Additional intermediate products from either cleavage may be detected by the assay. Measuring multiple FDP species prevents the DR-70<sup>®</sup> (FDP) Immunoassay from underestimating the cancer-related levels of FDP. The DR-70<sup>®</sup> (FDP) Immunoassay measures FDP generated from both of the major cancer induced FDP production pathways.

While the production of FDP is restricted in healthy individuals, FDP are over produced by proteolytic enzymes, such as plasmin and thrombin, released by cancer cells<sup>10</sup> and as a by-product of other cancer-related processes<sup>11-13</sup>. Current assays for FDP usually are restricted to measuring a specific FDP component, such as D-dimer, as a representative of this group; whereas the DR-70<sup>®</sup> (FDP) immunoassay detects the full complement of FDP components. Because FDP leaks from tumors into surrounding fluids<sup>14</sup>, elevated FDP levels can be measured in the urine of Subjects with bladder cancer, in the plasma of lung cancer Subjects<sup>15-18</sup>, and in the serum of other cancer patients. The utility of FDP measurements in cancer diagnostics has been suspected for years; however refined assays had not been developed that were able to quantitatively measure FDP with the sensitivity required. Recently, the FDA cleared a bladder cancer screening tests that measures FDP qualitatively in urine (K970353, Organon Teknika, Ltd.)<sup>16</sup>.

Researchers have established a strong link between increased FDP levels and cancer<sup>14, 19</sup>, which is based on multiple factors including: a cancer-caused redirection of the coagulation cascade and a cancer-related increase in proteolysis within tumors as they grow and metastasize. Clinical studies reveal that measuring FDP levels, either with the DR-70<sup>®</sup> (FDP) test or with other related tests, has significant diagnostic value for a variety of cancers<sup>20-28</sup>. Other studies demonstrate that FDP levels correlate with the cancer stage<sup>29-35</sup> and with the cancer progression<sup>33, 35, 36</sup>, as quantified by the number of lymph node metastases. Clinical research efforts have shown that pretreatment measurements of FDP levels have prognostic significance for post-treatment survival<sup>21, 34, 37-41</sup>. In addition to survival prognoses, pretreatment FDP values may be used to indicate when adjuvant systemic treatments are required for surgical Subjects<sup>39, 41</sup>.

**Principle of the Assay**

The DR-70<sup>®</sup> (FDP) assay is an ELISA based assay utilizing removable strips in a 96-well micro titer plate format. The wells are coated with affinity purified rabbit anti-DR-70<sup>®</sup> (FDP) polyclonal antibodies. The DR-70<sup>®</sup> (FDP) in diluted sera (1:200) is captured from the sera by these antibodies immobilized on the well of a micro titer plate. After a wash step, anti-DR-70<sup>®</sup> (FDP) antibodies conjugated to horseradish peroxidase are added to the wells. If the DR-70<sup>®</sup> (FDP) antigen is present, the anti-human fibrinogen peroxidase complex will bind to the captured tumor marker to form an immunological sandwich with the immobilized antibodies.

After a second wash step, the enzyme substrate 3,3',5,5'-tetramethylbenzidine (TMB) is added to the well. The end point is read in a micro plate reader at 450 nm once the reaction is stopped with 0.1N HCl. The intensity of the color formed is proportional to the amount of DR-70<sup>®</sup> (FDP) in the serum. The amount is quantified by interpolation from a standard curve using the calibrators provided with the kit.

**Materials Provided (DR-70<sup>®</sup> (FDP) Kit Components, Cat. No. DR2101)**

|           |                                                                                                                     |
|-----------|---------------------------------------------------------------------------------------------------------------------|
| DR2201    | DR-70 <sup>®</sup> (FDP) Antibody-Coated Wells, (96-well Plate)                                                     |
| DR2301    | Enzyme Antibody Conjugate: 1 vial (12 ml)                                                                           |
| DR2401A   | Low Control: 1 vial (500 µl)                                                                                        |
| DR2401B   | High Control: 1 vial (500 µl)                                                                                       |
| DR2501A-E | DR-70 <sup>®</sup> (FDP) calibrators: 5 vials (500 µl each) at concentrations of: 0, 0.625, 2.5, 5.0 and 10.0 µg/ml |
| DR2901A   | Diluent Buffer Concentrate (5X): 1 vial (40 ml)                                                                     |
| DR2091B   | Wash Buffer Concentrate (20X): 1 vial (50 ml)                                                                       |
| DR2601    | TMB Substrate: 1 vial (12 ml)                                                                                       |
| DR2701    | Stop Solution: 1 vial (12 ml)                                                                                       |
| N/A       | Dilution/Transfer Plate (96-well uncoated Plate)                                                                    |

**Materials Required But Not Provided**

The following materials are not provided but are required to perform DR-70<sup>®</sup> (FDP) analysis manually using the AMDL – ELISA DR-70<sup>®</sup> (FDP):

- 8-channel micropipettor that delivers 100 µl
- Micropipettor that delivers 10 µl
- Adjustable micropipettor that delivers 100-1000 µl
- Vortex mixer
- Plate washer
- Plate reader that reads the 450 nm (kinetic) wavelengths
- Computer with software to operate the reader and printer and for data reduction
- Solution basins

**Warnings and Precautions**

1. The DR-70<sup>®</sup> (FDP) assay contains human blood components. The starting materials were tested and were found to be negative for hepatitis B surface antigen (HBsAg) and for HIV-1 p24 (core) antigen. No known test method can offer complete assurance that products derived from human blood will not transmit infection. Therefore, all human blood derivatives should be handled as though they contain an infectious agent. Handle these reagents and human specimens using established good laboratory working practices.
2. **Do not** pipette by mouth.
3. Wear protective clothing, disposable gloves and goggles throughout the testing procedure.
4. All spills should be wiped up promptly and any surfaces contaminated with serum must also be sterilized with appropriate disinfectant such as a 10% v/v solution of sodium hypochlorite.
5. The reagents contain 50 µg/ml gentamicin and 2.5 µg/ml amphotericin B as preservatives. Therefore, caution should be taken in handling the reagents and in disposal of the spent reagents.
6. Kits should not be used beyond the expiration date.
7. Residual reagents should be discarded. They must not be pooled and reused.
8. Avoid exposing the TMB substrate to strong light during storage or prior to dispensing.
9. The AMDL-ELISA DR-70<sup>®</sup> (FDP) is intended for in vitro diagnostic use only.
10. The AMDL-ELISA DR-70<sup>®</sup> (FDP) assay has been designed so that the high dose “hook effect” is not a problem for the vast majority of samples. The “hook effect” phenomenon may occur at DR-70<sup>®</sup> (FDP) results exceeding 200 µg/ml.

**Storage and Stability**

All unopened materials are stable until the expiration date on the label when stored at the specified 2-8° C.

**Specimen Collection and Handling**

Serum is required for the assay. No other specimen types should be used. The sample volume required for analysis is 10 µl.

No special patient preparation is necessary. Results are not affected by fasting status or time of sample collection. A venous blood sample is collected aseptically directly into a serum separator tube. Store upright at room temperature until a clot has formed (usually 30 minutes), then centrifuge to obtain the serum specimen for assay. Do not leave it more than 3 hours before centrifugation.

Once the serum is separated from the clot, the serum fraction should be transferred to storage vials and stored at 2-8° C for up to 24 hours prior to analysis. If the analysis cannot be done within 24 hours, the sample should be stored frozen at -20° C, or below, for up to one year. If the samples are not assayed within 24 hours after collection, they should be frozen at -20°C. Repeated freeze-thaw cycles should be avoided.

Turbid serum samples or samples containing particulate matter should be centrifuged prior to testing. Do not test grossly hemolyzed or lipemic specimens.

During shipping, samples must be packaged and labeled in compliance with applicable federal and international regulations. Prior to assay, slowly bring frozen samples to room temperature (18-23°C) and mix thoroughly to ensure consistency in the results.

## Procedure

### I. Reagent Preparation

#### A. DILUENT BUFFER

Bring all reagents to room temperature (18-23°C) before preparing the working reagent. Add the entire contents of the DILUENT BUFFER CONCENTRATE (5X) (40 ml) to an additional 160 ml of deionized or distilled water. Mix thoroughly and label appropriately.

#### B. WASH BUFFER

Add the entire content of the WASH BUFFER CONCENTRATE (20X) (50 ml) to an additional 950 ml of deionized or distilled water. Mix well and label appropriately.

#### C. CALIBRATORS AND CONTROLS

Kit calibrators and controls are treated in an identical fashion and must be run with each and every run. They must be diluted 1:200 with the DILUENT BUFFER prior to testing. The procedure to be followed is:

- Label one 12x75 mm glass test tube for each of the two controls and each of the five calibrators.
- Dispense 2 ml of DILUENT BUFFER into each one of the tubes.
- Vortex the controls and the calibrators.
- Dispense 10 µl of each into the correspondingly labeled tubes.
- Mix these tubes again.

### II. Serum Sample Dilutions

**Note: Only serum samples can be used in this assay, not whole blood or plasma.**

Dilute each patient serum specimen 1:200 as follows:

- Label a 12 x 75 mm glass tube for each serum sample.
- Dispense 2 ml of DILUENT BUFFER into each of the tubes.
- Vortex the serum samples and dispense 10 µl of each into the correspondingly labeled tubes.

### III. Low and High Controls

**Note: Two levels of control, normal and abnormally elevated, are included in the AMDL-ELISA DR-70® (FDP).**

- Assay control specimens should be run as if they were patient samples.
- Quality control material to be run with this assay is defined by individual laboratory policy and may include reference controls in addition to the quality control materials provided in the DR-70 assay kit. Those should be run with each assay even if other QC materials are used.

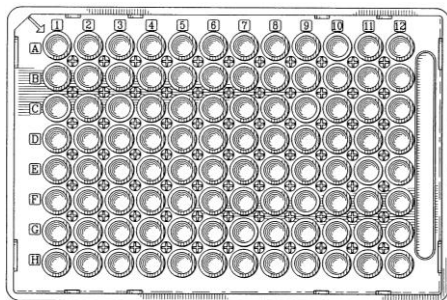
### IV. Procedural Notes

- All specimens should be tested in duplicate.
- All kit components and serum to be tested must be allowed to come to room temperature.
- The optimal lab space temperature should be 21-23°C (70-73F).
- Do not use expired reagents or mix reagents from different lots.
- Break off the number of strips needed for the test and place in a micro-titer plate holder.
- Unused micro-titer plate strips should be kept in a sealed mylar bag with desiccants (2-8°C).
- Water should be tested at least once per month and should be free of particulate matter, including bacteria. The pH of the water should also be routinely tested. For further information, consult the NCCLS document "Preparation and Testing of Reagent Water in the Clinical Laboratory," NCCLS Document C3-A2, Volume 11 No. 13, originally approved as a guideline by NCCLS in August 1991.**

### V. Assay Preparation

- Vortex each of the tubes of diluted controls, calibrators and patient serum.
- Using the transfer plate (uncoated), transfer 300 µl of each sample to the wells (leave one column of wells empty between the samples) (Fig 1).

Figure 1: The transfer plate (uncoated).



- This step is to increase the speed of the assay.

#### 11. For Column 1:

- Well A1: 300 µ Calibrator A
- Well B1: 300 µ Calibrator B
- Well C1: 300 µ Calibrator C
- Well D1: 300 µ Calibrator D
- Well E1: 300 µ Calibrator E
- Well F1: 300 µ Low Control
- Well G1: 300 µ High Control
- Well H1: 300 µ Patient Sample 1

- Leave Column 2 empty.

#### 13. For Column 3:

- Well A3: 300 µ Patient Sample 2
- Well B3: 300 µ Patient Sample 3
- Well C3: 300 µ Patient Sample 4
- Well D3: 300 µ Patient Sample 5
- Well E3: 300 µ Patient Sample 6
- Well F3: 300 µ Patient Sample 7
- Well G3: 300 µ Patient Sample 8
- Well H3: 300 µ Patient Sample 9

- Continue the same way to the end of the samples (You can test 41 samples in duplicates per kit).

- Open the DR-70® (FDP) Antibody coated plate in the aluminum pouch.

- Using a 8-channel pipettor, dispense 100 µl from each row of the transfer plate into two rows on the DR-70® (FDP) Antibody coated plate, so that each sample will then be tested in duplicate (two adjacent wells).

- For example, using an 8-channel pipettor, dispense 100 µl of diluted calibrators and controls and first sample (the whole column 1) from the column 1 transfer plate into the DR-70® (FDP) Antibody coated plate.

- Repeat this step and transfer another 100 µl of diluted calibrators and controls and first sample (the whole column 1) from the transfer plate into column 2 in the DR-70® (FDP) Antibody coated plate.

### VI. Incubation

- Incubate at room temperature (22-25°C) for 30 minutes.
- Wash the plate 6 times (each with 300 µl per well of wash buffer).
- Invert and blot plate onto clean absorbent paper.
- Dispense 100 µl of the antibody-enzyme conjugate per well using an 8-channel pipettor.
- Incubate at room temperature (22-25°C) for 30 minutes.
- Wash the plate 6 times (each with 300 µl per well of wash buffer).
- Dispense 100 µl of the TMB substrate per well using an 8-channel pipettor.
- Cover the plate with foil.
- Incubate in a dark area at room temperature (22-25°C) for 15 minutes.
- Stop the reaction by dispensing 100 µl of stop solution per well, using an 8-channel pipettor.
- Read immediately at 450 nm.

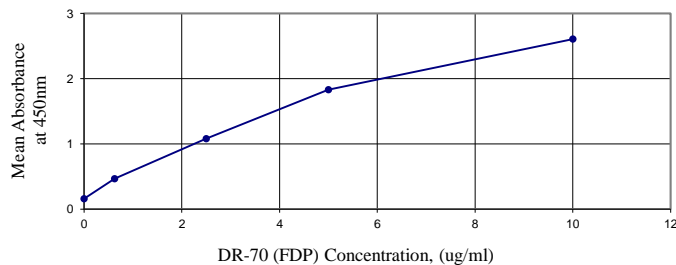
### VII. Calculation

- Plot the standard curve with the optical density on the Y axis and the µg/ml calibrator values on the X axis.
- Interpolate the average value for each test specimen from the standard curve. See example below.
- If data reduction software is used, use a linear curve or a quadratic or Four (4) Parameter algorithm to define the standard curve and any of the software programs available to interpolate the sample values from the standard curve.
- If a serum value is greater than the 10 µg/ml calibrator, the sample should be diluted with diluent buffer and then retested. The resultant value must then be corrected for the dilution factor.
- An example of a typical DR-70® (FDP) Standard Curve is presented in Table 1 & Figure 2.

Table 1: Typical DR-70® (FDP) Standard Curve numbers.

| Calibrator | Value (µg/ml) | Well | O.D.  | Mean  | S.D.  | %CV |
|------------|---------------|------|-------|-------|-------|-----|
| A          | 0.000         | A1   | 0.166 | 0.158 | 0.011 | 7.2 |
|            |               | B1   | 0.150 |       |       |     |
| B          | 0.625         | C1   | 0.472 | 0.465 | 0.010 | 2.1 |
|            |               | D1   | 0.458 |       |       |     |
| C          | 2.500         | E1   | 1.080 | 1.079 | 0.002 | 0.2 |
|            |               | F1   | 1.077 |       |       |     |
| D          | 5.000         | G1   | 1.823 | 1.830 | 0.010 | 0.5 |
|            |               | H1   | 1.837 |       |       |     |
| E          | 10.000        | A2   | 2.631 | 2.607 | 0.033 | 1.3 |
|            |               | B2   | 2.584 |       |       |     |

Figure 2: Typical DR-70® (FDP) Standard Curve.



## Evaluation of Results

### 1. Quality Control

- The DR-70<sup>®</sup> (FDP) control values must fall within the following concentration ranges:
  - Low Control Range 0.14 – 0.51 µg/ml
  - High Control Range 1.40 – 5.20 µg/ml
- If one or more control sample value(s) is out of the acceptable range, it will be necessary to investigate the validity of the calibration curve before reporting patient results. Standard laboratory procedures should be followed in accordance with the regulatory agency under which the laboratory operates.
- In order to monitor and evaluate the precision of the analytical performance, it is recommended that control samples with known values be assayed daily.

### 2. Interpretation of DR-70<sup>®</sup> (FDP) Patient Values

For each patient, a baseline reading should precede the evaluation of their DR-70<sup>®</sup> (FDP) levels. The DR-70<sup>®</sup> (FDP) value of the initial serum draw serves as the baseline reading. The value of successive serum draws are evaluated by constructing the following ratio (**R**); where:

$$a = \text{the initial DR-70}^{\circledR} \text{ (FDP) value}$$

$$b = \text{the current DR-70}^{\circledR} \text{ (FDP) value}$$

$$R = \frac{b}{a}$$

When the ratio of the current DR-70<sup>®</sup> (FDP) value relative to the baseline DR-70<sup>®</sup> (FDP) value is greater than 1.15, the patient is likely to have a progression of the disease, however, results of DR-70<sup>®</sup> (FDP) testing should be used in conjunction with other clinical modalities that are standard of care for monitoring disease progression in these patients.

### Limitations of the Procedure

#### AMDL-ELISA DR-70<sup>®</sup> (FDP) SHOULD NOT BE USED AS A SCREENING TEST.

The results obtained from this assay should be used considering the history of the patient and in conjunction with symptoms, results of other tests, clinical impressions, therapy, etc.

Using AMDL-ELISA DR-70<sup>®</sup> (FDP), the highest concentration of DR-70<sup>®</sup> (FDP) measurable (without dilution) is 10 µg/ml, and the lowest measurable concentration is 0.06 µg/ml (assay sensitivity).

Hemolysis has been shown to cause higher readings in the test results in some cases and results should be interpreted with caution.

Lipemia has an insignificant effect on the assay except in the case of gross lipemia where spatial interference may occur causing a falsely suppressed result. It is preferred that grossly lipemic samples be ultracentrifuged before any analysis.

An elevated DR-70<sup>®</sup> (FDP) may result from testing serum from patients who have pancreatic disease, heart disease, coagulation disorders, acute infection or trauma.

DR-70<sup>®</sup> (FDP) results below the upper reference limit of normal do not indicate the absence of malignancy because patients with histopathologic evidence of colorectal cancer may have DR-70<sup>®</sup> (FDP) assay values within the range of normal individuals.

Conversely, a DR-70<sup>®</sup> (FDP) assay value exceeding the upper limit reference limit of normal does not necessarily indicate the presence of colorectal malignancy since a small percentage of healthy individuals and individuals with non-malignant conditions as well as malignancy in other locations than the colon or rectum may have elevations in DR-70<sup>®</sup> (FDP) assay results.

A DR-70<sup>®</sup> (FDP) assay value should not be interpreted as absolute evidence for the presence or absence of malignant disease of the pancreas. It should be remembered that this assay is to be used as a monitoring aid, not as an aid in diagnosis.

For a more complete understanding of the limitations of this procedure, please refer to the Specimen Collection and Handling, Warnings and Precautions, Storage and Stability, and Procedural Notes sections in this insert.

### Expected Values

Each laboratory should determine a reference interval corresponding to the characteristics of their population. As with all diagnostic procedures, clinical results must be interpreted with regard to concomitant medications administered.

For the serial monitoring of patients previously diagnosed with colorectal cancer, a determination was made that a meaningful increase to determine evidence of progression was 15% increase or more. Thus, if the ratio of the current DR-70<sup>®</sup> (FDP) value relative to the baseline DR-70<sup>®</sup> (FDP) value is 1.15 or higher, the DR-70<sup>®</sup> (FDP) test is deemed to be positive, otherwise it is deemed to be negative.

A 15% increase from the previous visit was chosen as the threshold for significant % change for the determination of disease progression in the DR-70<sup>®</sup> (FDP) immunoassay based on the imprecision study where the total CV over all runs, days, and intra-assay was computed for each specimen analyzed.

**Note: Results of DR-70<sup>®</sup> (FDP) testing should be used in conjunction with other clinical modalities that are standard of care for monitoring disease progression in these patients.**

### I. Normal, Benign, Malignant Disease Studies

Values for DR-70<sup>®</sup> (FDP) were analyzed for the different DR-70<sup>®</sup> (FDP) concentration levels within each normal, benign and malignant disease cohort and are presented in Table 2.

This table is provided as a reference for informational purposes only.

### II. Serial Monitoring Studies

In summary, serial samples were taken from 112 colon cancer patients resulting in 445 paired observations (and 335 paired consecutive visits) in which a DR-70<sup>®</sup> (FDP) reading and a determination of disease progression were obtained.

The sequential draws covered an average longitudinal period of at least nine months or until disease recurrence. Progression of the DR-70<sup>®</sup> (FDP) value in the serial monitoring set was determined as a significant percentage change between the current and previous readings. Disease progression was determined by the Subject's physician based on procedures that were the standard of care during the time of the monitoring period.

The results of the computed per-visit analysis from 335 per visit evaluations was as following: (CI = bootstrap lower 95% confidence interval)

|                                           |             |                         |
|-------------------------------------------|-------------|-------------------------|
| Sensitivity = (100×88)/135 = 65.19        | (SD = 2.58) | (lower 95% CI = 60.13)  |
| Specificity = (100×134)/199 = 67.34       | (SD = 2.94) | (lower 95% CI = 61.58)  |
| Sum of Sensitivity & Specificity = 132.53 | (SD = 3.91) | (lower 95% CI = 124.87) |
| PPV = (100×88)/153 = 57.52                | (SD = 1.63) | (lower 95% CI = 54.33)  |
| NPV = (100×134)/181 = 74.03               | (SD = 2.44) | (lower 95% CI = 69.25)  |

The results of the computed per-patient analysis from 112 per patient evaluations was as following: (CI = bootstrap lower 95% confidence interval)

|                                           |             |                         |
|-------------------------------------------|-------------|-------------------------|
| Sensitivity = (100×45.68)/69 = 66.21      | (SD = 2.58) | (lower 95% CI = 57.28)  |
| Specificity = (100×58.63)/86 = 68.18      | (SD = 2.94) | (lower 95% CI = 61.46)  |
| Sum of Sensitivity & Specificity = 134.39 | (SD = 3.91) | (lower 95% CI = 121.82) |
| PPV = (100×51.83)/97 = 53.44              | (SD = 1.63) | (lower 95% CI = 44.19)  |
| NPV = (100×71.67)/103 = 69.58             | (SD = 2.44) | (lower 95% CI = 61.17)  |

Since the bootstrap lower 95% confidence interval for the sum of sensitivity and specificity is greater than 100 in both the per-visit analysis and the per-patient analysis, the data and analyses demonstrate that the DR-70 test when taken as a 15% or greater change from the previous visit, yields informative data regarding colon cancer progression. The DR-70<sup>®</sup> (FDP) immunoassay results must be used in conjunction with standard of care procedures for monitoring colorectal cancer patients.

**Table 2. Distribution in percent of serum DR-70<sup>®</sup> (FDP) values \*\***

| Disease               | # of subjects | Percent (%), 95% CI (lower-upper %)* |                   |                   |                   |
|-----------------------|---------------|--------------------------------------|-------------------|-------------------|-------------------|
|                       |               | 0-1.4 µg/ml                          | 1.5-2.4 µg/ml     | 2.5-4.9µg/ml      | ≥ 5.0 µg/ml       |
| <b>Normal</b>         | 420           | 94.5 (91.9, 96.5)                    | 5.0 (3.1, 7.5)    | 0.5 (0.1, 1.7)    | 0.0 (0.0, 0.9)    |
| <b>&lt; 65 years</b>  | 337           | 96.4 (93.9, 98.2)                    | 3.3 (1.6, 5.8)    | 0.3 (0.0, 1.6)    | 0.0 (0.0, 1.1)    |
| <b>≥ 65 years</b>     | 83            | 86.8 (77.5, 93.2)                    | 12.1 (5.9, 21.0)  | 1.2 (0.0, 6.5)    | 0.0 (0.0, 4.4)    |
| <b>Benign</b>         | 326           | 75.5 (70.4, 80.0)                    | 6.8 (4.3, 10.0)   | 0.6 (0.1, 2.2)    | 17.2 (13.2, 21.7) |
| <b>GU Disease</b>     | 94            | 94.7 (88.0, 98.3)                    | 4.3 (1.2, 10.5)   | 0.0 (0.0, 3.9)    | 1.1 (0.0, 5.8)    |
| <b>GI Disease</b>     | 61            | 90.2 (79.8, 96.3)                    | 3.3 (0.4, 11.4)   | 0.0 (0.0, 5.9)    | 6.6 (1.8, 16.0)   |
| <b>Pancreas</b>       | 84            | 60.7 (49.5, 71.2)                    | 15.5 (8.5, 25.0)  | 2.4 (0.3, 8.3)    | 21.4 (13.2, 31.7) |
| <b>Heart Disease</b>  | 87            | 58.6 (47.6, 69.1)                    | 3.5 (0.7, 9.8)    | 0.0 (0.0, 4.2)    | 37.9 (27.7, 49.0) |
| <b>Malignant</b>      | 439           | 44.0 (39.3, 48.8)                    | 24.2 (20.2, 28.4) | 19.6 (16.0, 23.6) | 12.3 (9.4, 15.7)  |
| <b>Colon</b>          | 187           | 55.6 (48.2, 62.9)                    | 21.4 (15.7, 28.0) | 15.0 (10.2, 20.9) | 8.0 (4.6, 12.9)   |
| <b>Lung</b>           | 44            | 34.1 (20.5, 49.9)                    | 38.6 (24.4, 54.5) | 18.2 (8.2, 32.7)  | 9.1 (2.5, 21.7)   |
| <b>Liver</b>          | 44            | 31.8 (18.6, 47.6)                    | 27.3 (15.0, 42.8) | 22.7 (11.5, 37.8) | 18.2 (8.2, 32.7)  |
| <b>Breast</b>         | 31            | 54.8 (36.0, 72.7)                    | 25.8 (11.9, 44.6) | 12.9 (3.6, 29.8)  | 6.5 (0.8, 21.4)   |
| <b>Ovarian</b>        | 31            | 25.8 (11.9, 44.6)                    | 6.5 (0.8, 21.4)   | 32.3 (16.7, 51.4) | 35.5 (19.2, 54.6) |
| <b>Cervical</b>       | 28            | 50.0 (30.7, 69.4)                    | 28.6 (13.2, 48.7) | 7.1 (0.9, 23.5)   | 14.3 (4.0, 32.7)  |
| <b>Gall Bladder</b>   | 19            | 42.1 (20.3, 66.5)                    | 26.3 (9.2, 51.2)  | 31.6 (12.6, 56.6) | 0.0 (0.0, 17.7)   |
| <b>Pancreas</b>       | 28            | 25.0 (10.7, 44.9)                    | 17.9 (6.1, 36.9)  | 35.7 (18.6, 55.9) | 21.4 (8.3, 41.0)  |
| <b>Gastric/ Other</b> | 27            | 22.2 (8.6, 42.3)                     | 33.3 (16.5, 54.0) | 29.6 (13.8, 50.2) | 14.8 (4.2, 33.7)  |

\* Exact binomial confidence limits.

\*\* It is recommended that each laboratory establish its own expected reference range for the population of interest.

### Performance Testing

To determine the analytic validity of the DR-70<sup>®</sup> (FDP) immunoassay, the following performance tests were conducted.

#### I. Precision

Imprecision was tested on the AMDL-ELISA DR-70<sup>®</sup> (FDP) using three serum pools and two quality control materials with concentrations of DR-70<sup>®</sup> (FDP) across the linear range of the assay run in quadruplicate in a randomized manner in two runs per day for twenty days at three sites using three manufactured lots. The results are presented in the table 3 below:

**Table 3. Total and Components of Assay Variance and Percentage by Source.**

| Specimen      | Site Mean (µg/mL) | Site Variance (%) | Day Variance (%) | Lot Variance (%) | Run Variance (%) | Residual Variance (%) | Total Variance |
|---------------|-------------------|-------------------|------------------|------------------|------------------|-----------------------|----------------|
| <b>Pool 1</b> | 0.315             | 0.001336 (23.53)  | 0.000417 (8.28)  | 0.000437 (8.68)  | 0.000303 (6.02)  | 0.002543 (50.50)      | 0.005036       |
| <b>Pool 2</b> | 1.389             | 0.002385 (11.00)  | 0.003585 (16.53) | 0.000132 (0.61)  | 0.001810 (8.34)  | 0.01378 (63.53)       | 0.021692       |
| <b>Pool 3</b> | 2.739             | 0.01483 (20.11)   | 0.008803 (11.94) | 0.000431 (0.58)  | 0.005631 (7.64)  | 0.04405 (59.73)       | 0.073745       |
| <b>QC1</b>    | 0.240             | 0.001088 (23.74)  | 0.000424 (9.25)  | 0.000701 (15.29) | 0.000135 (2.95)  | 0.002236 (48.78)      | 0.004584       |
| <b>QC2</b>    | 2.994             | 0.02246 (20.03)   | 0.01255 (11.19)  | 0.008363 (7.46)  | 0.001438 (1.28)  | 0.06731 (60.03)       | 0.112121       |

#### II. Spike Recovery

Serums from three normal subjects having DR-70<sup>®</sup> (FDP) values ranging from 0.3-0.6 µg/ml and a control diluent buffer were spiked with a DR-70<sup>®</sup> (FDP) antibody solution to obtain expected levels ranging from 1.5-10 µg/ml to represent the range of the DR-70<sup>®</sup> (FDP) calibrators. The values of DR-70<sup>®</sup> (FDP) in the spiked serum were measured and compared to the theoretical values and to values obtained for the control diluent buffer. The experiment was designed to compare responses of the analyte in a biological sample versus the standard diluent to assess for any difference in assay

response. Based on the overall analysis of results, the DR-70<sup>®</sup> (FDP) immunoassay kit is a quantitative test without concerns of sample matrix affects (Table 4).

**Table 4. Spike Recovery Matrix.**

| Sample              | DR-70 concentration value (µg/ml) |                      |                      |                      |                      |                     |
|---------------------|-----------------------------------|----------------------|----------------------|----------------------|----------------------|---------------------|
|                     | No spike                          | Spike 1<br>1.5 µg/ml | Spike 2<br>2.5 µg/ml | Spike 3<br>5.0 µg/ml | Spike 4<br>7.0 µg/ml | Spike 5<br>10 µg/ml |
| Diluent buffer (5x) | 0                                 | 1.517                | 2.649                | 4.586                | 6.983                | 10.94               |
| Patient 1           | 0.428                             | 1.743                | 2.908                | 4.839                | 7.057                | 13.11               |
| Patient 2           | 0.576                             | 1.520                | 2.680                | 4.848                | 7.050                | 11.95               |
| Patient 3           | 0.464                             | 1.598                | 2.967                | 5.193                | 6.701                | 10.88               |
| Patient mean value  | 0.489                             | 1.620                | 2.852                | 4.960                | 6.936                | 11.98               |
| % Mean Recovery     | -----                             | 107%                 | 108%                 | 108%                 | 99%                  | 110%                |

### III. Linearity

Serums from 5 colorectal cancer patients with DR-70<sup>®</sup> assay values in the range of 19.7 to 22.2 µg/ml were diluted with assay diluent buffer in a two-fold serial dilution series. For each CRC patient serum sample, a total of nine DR-70<sup>®</sup> (FDP) dilution samples were tested.

The following table lists the percent difference between the actual DR-70<sup>®</sup> (FDP) concentrations and the estimated DR-70<sup>®</sup> (FDP) concentrations (1<sup>st</sup> column in table below) for each patient at each dilution.

For each dilution, the average percent difference is listed as well as the average percent recovery. Grey boxes contain percent differences per CRC patient at dilutions whose values were statistically non-linear. Values below the lowest calibrator included in the DR-70<sup>®</sup> (FDP) assay kit are in the non-linear portion of the DR-70<sup>®</sup> (FDP) assay curve (Table 5).

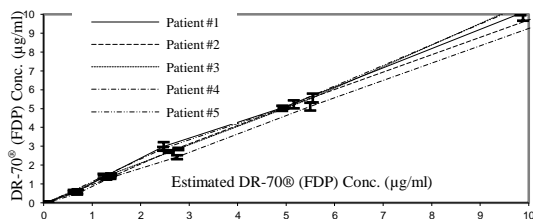
**Table 5. Percent Difference Between Actual and Estimated DR-70<sup>®</sup> (FDP) Values in Linearity Study.**

| Estimated DR-70 <sup>®</sup> (FDP) Conc. | Dilution Ratio | % Difference per CRC Patient |       |       |      |        | Average % Difference | Average % Recovery |
|------------------------------------------|----------------|------------------------------|-------|-------|------|--------|----------------------|--------------------|
|                                          |                | 1                            | 2     | 3     | 4    | 5      |                      |                    |
| 20                                       | 1              | (14.5)                       | (0.1) | (6.9) | 2.6  | (3.2)  | (4.4)                | 96                 |
| 10                                       | 1/2            | (2.3)                        | 3.1   | (7.4) | 7.5  | (5.7)  | (1.0)                | 99                 |
| 5                                        | 1/4            | (2.6)                        | (1.4) | (0.3) | 7.0  | (2.1)  | 0.1                  | 100                |
| 2.5                                      | 1/8            | (21.2)                       | (5.8) | (3.2) | 11.8 | (16.8) | (7.0)                | 93                 |
| 1.25                                     | 1/16           | (11.0)                       | 2.0   | (8.6) | 3.6  | (14.5) | (5.7)                | 94                 |
| 1.125                                    | 1/32           | 18.1                         | 5.6   | (0.1) | 20.1 | (5.8)  | 7.6                  | 108                |
| 0.625                                    | 1/64           | 13.9                         | 11.8  | 12.6  | 12.9 | 16.7   | 13.6                 | 114                |

— indicates that these dilutions were statistically non-linear

The results of the linearity study are presented in graphic form on the following page. For each of the five CRC patient serums, the estimated DR-70<sup>®</sup> (FDP) Conc. (µg/ml) is graphed against the actual DR-70<sup>®</sup> (FDP) Conc. (µg/ml) with the standard deviation among the five replicates at each point represented by the Y-axis error bars. For all of these patients, the DR-70<sup>®</sup> (FDP) concentrations were statistically found to be linearly related; except for those dilutions below a DR-70<sup>®</sup> (FDP) concentration of 0.625 µg/ml.

**Table 5. Linear Regression of DR-70<sup>®</sup> (FDP) and Estimated Diluted Fractions for DR-70<sup>®</sup> (FDP) Assay Range 0.625 - 10 µg/ml.**



### IV. Analytical Sensitivity

The minimal detectable concentration (MDC) of DR-70<sup>®</sup> (FDP) is estimated to be 0.06 µg/ml. The MDC is defined as that concentration of DR-70<sup>®</sup> (FDP) corresponding to the absorbance that is two standard deviations from the mean rate of absorbance of 20 replicate determinations of a zero calibrator.

### V. Functional Sensitivity

The functional sensitivity was determined by diluting the lowest non-zero calibrator serially, measuring the DR-70<sup>®</sup> (FDP) concentration and extrapolating to the point where the CV% = 20%. Functional sensitivity for the AMDL-ELISA DR-70<sup>®</sup> (FDP) is calculated as being 0.063 µg/ml. This compares well to the Analytical Sensitivity.

### VI. Interference

Interference is defined to be recovery outside of 10% of the known specimen mean concentration for purposes of this study.

- Added hemoglobin (up to 500 mg/dl) does not interfere with the assay.
- Added bilirubin (up to 30 mg/dl) do not interfere with the assay.
- Lipemia (indicated by added triglyceride up to 1000 mg/dl) does not interfere with the assay.
- Heparin (at concentrations of 500 U/ml) does not interfere with the assay.
- The following pharmaceutical agents were tested at levels indicated and found not to cause analyte recovery outside 10%: 5'-fluorouracil (Aducril) at 1.0 mg/ml, acetaminophen at 0.2 mg/ml, adriamycin (Doxorubicin HCl) at 0.10 mg/ml, coumarin at 1.4 mg/ml, cyclophosphamide (Cytosan) at 0.25 mg/ml, Paclitaxel at 3.5 x 10<sup>-6</sup> g/m<sup>2</sup>, amethopterin hydrate (Methotrexate) at 4.5 mg/ml, mitoxantrone (Novatrone) at 0.5 mg/ml, folic acid (Leucovorin) at 1.10 mg/ml, Mitomycin C at 0.06 mg/ml, Cisplatin at 0.10 mg/ml.

### VII. Hook Effect

Studies were performed testing for hook effect in the AMDL-ELISA DR-70<sup>®</sup> (FDP). No evidence of a hook effect was found up to a concentration of 250 µg/ml.

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