



## **TiterZyme® EIA**

### **human Total BRCA-1**

# **Enzyme Immunometric Assay Kit**

**Catalog No. 900-143**

**96 Well Kit**

#### Table of Contents

Description	Page	2
Introduction		2
Precautions		2
Materials Supplied		3
Storage		3
Materials Needed but Not Supplied		3
Sample Handling		4
Typical Stimulation Response		5
Procedural Notes		5
Reagent Preparation		6
Assay Procedure		6
Calculation of Results		7
Typical Results		8
Typical Standard Curve		8
Units of Measure		8
Performance Characteristics		9
Sample Dilution Recommendations		11
References		11
Limited Warranty		12

**FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

## **Description**

Assay Designs' human Total BRCA-1 TiterZyme® Enzyme Immunometric Assay (EIA) kit is a complete kit for the quantitative determination of BRCA-1 in cell lysates. Please read the complete kit insert before performing this assay. The kit uses a monoclonal antibody to human BRCA-1 immobilized on a microtiter plate to bind BRCA-1 in the standards or sample. A recombinant human BRCA-1 Standard is provided in the kit. After a short incubation, the excess sample or standard is washed out and a rabbit polyclonal antibody to BRCA-1 is added. This antibody binds to BRCA-1 captured on the plate. After a short incubation, the excess antibody is washed out and goat anti-rabbit IgG conjugated to Horseradish peroxidase is added, which binds to the rabbit polyclonal BRCA-1 antibody. Excess conjugate is washed out and substrate is added. After a short incubation, the enzyme reaction is stopped and the color generated is read at 450 nm. The measured optical density is directly proportional to the concentration of BRCA-1 in either standards or samples. For further explanation of the principles and practices of immunoassays please see the excellent books by Chard<sup>1</sup> or Tijssen<sup>2</sup>.

## **Introduction**

The human breast cancer type 1 (BRCA-1) susceptibility gene encodes a nuclear phosphoprotein of 1863 amino acids (MW= 220 kDa). This gene received considerable attention following the discovery that it is mutated in ~45-50% of hereditary breast cancers<sup>3,4</sup>. Additionally, BRCA1 has been implicated in cases of sporadic breast cancers<sup>5,6</sup>. It is now known that BRCA1 is ubiquitously expressed in adult tissues, participating in many critical cellular processes including DNA repair, cell cycle progression, transcriptional regulation, chromatin remodeling and ubiquitination<sup>7-9</sup>. All of these processes are essential to maintenance of proper cellular function and contribute to tumorigenesis when deregulated; thus, BRCA-1 has been proposed to function as a cellular caretaker<sup>10</sup>.

## **Precautions**

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

1. Stop Solution 2 is a 1N hydrochloric acid solution. This solution is caustic; care should be taken in use.
2. The activity of the Horseradish peroxidase conjugate is affected by nucleophiles such as azide, cyanide and hydroxylamine.
3. We test this kit's performance with a variety of buffers, however high levels of interfering substances may cause variation in assay results. **For best results, samples should be prepared in the buffers recommended and included in this kit.**
4. The human BRCA-1 Standard provided, Catalog No. 80-1306, should be handled with care because of the known and unknown effects of BRCA-1.
5. The human BRCA-1 Standard should not be left at room temperature for extended periods of time. A maximum of 10 minutes at room temperature is recommended.
6. Standards and samples must be prepared in polypropylene tubes. Preparation in glass will result in decreased protein stability.

## **Materials Supplied**

1. **human BRCA-1 Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-1305**  
A plate using break-apart strips coated with a mouse monoclonal antibody specific to human BRCA-1.
2. **Total BRCA-1 EIA Antibody, 11 mL, Catalog No. 80-1304**  
A yellow solution of rabbit polyclonal antibody to BRCA-1.
3. **Assay Buffer 17, 120 mL, Catalog No. 80-1622**  
Tris buffered saline containing proteins, detergents and protease inhibitor.
4. **Total BRCA-1 EIA Conjugate, 11 mL, Catalog No. 80-1310**  
A blue solution of goat anti-rabbit IgG conjugated to Horseradish peroxidase.
5. **Wash Buffer Concentrate, 20x, 100 mL, Catalog No. 80-1287**  
Tris buffered saline containing detergents.
6. **human BRCA-1 Standard, 2 vials, Catalog No. 80-1306**  
Two vials each containing 20,000 pg of lyophilized recombinant human BRCA-1.
7. **TMB Substrate, 11 mL, Catalog No. 80-0350**  
A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide. **Protect from prolonged exposure to light.**
8. **Stop Solution 2, 11 mL, Catalog No. 80-0377**  
A 1N solution of hydrochloric acid in water. Keep tightly capped. Caution: **Caustic.**
9. **RIPA Cell Lysis Buffer 2, 100 mL, Catalog No. 80-1284**  
50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% Sodium Deoxycholate and 0.1% SDS.
10. **human Total BRCA-1 Assay Layout Sheet, 1 each, Catalog No. 30-0221**
11. **Plate Sealer, 3 each, Catalog No. 30-0012**

## **Storage**

All components of this kit, **except the human BRCA-1 Standard**, are stable at 4 °C until the kit's expiration date. **The human BRCA-1 Standard must be stored at or below -20 °C.**

## **Materials Needed but Not Supplied**

1. Deionized or distilled water.
2. Phenylmethylsulfonyl fluoride (PMSF), Sigma #P7626 or equivalent.
3. Protease inhibitor cocktail (PIC), Sigma #P8340 or equivalent.
4. Sodium orthovanadate, Sigma #S6508 or equivalent.
5. Sodium pyrophosphate, Sigma #S6422 or equivalent.
6. Precision pipets for volumes between 100 µL and 1,000 µL.
7. Repeater pipet for dispensing 100 µL.
8. Disposable beakers for diluting buffer concentrates.
9. Graduated cylinders.
10. A microplate shaker.
11. Adsorbent paper for blotting.
12. Microplate reader capable of reading at 450 nm, preferably with correction between 570 nm and 590 nm.
13. Graph paper for plotting the standard curve.

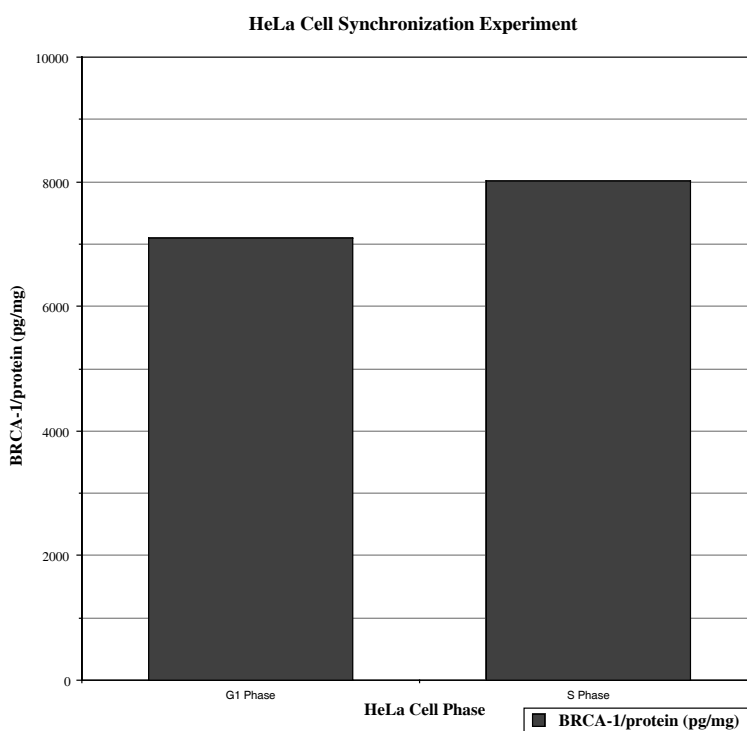
## Sample Handling

Assay Designs' TiterZyme® EIA is compatible with BRCA-1 samples in a wide range of cell lysates and buffers. Samples diluted sufficiently into Assay Buffer 17 plus Inhibitors (see Reagent Preparation, page 5, #2) can be read directly from a standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions. It is recommended that all samples be lysed with the provided RIPA Cell Lysis Buffer 2 modified by the addition of PIC and PMSF immediately prior to use (See Reagent Preparation, page 6, #4). Samples lysed in this modified RIPA Cell Lysis Buffer 2 must be diluted at least 1:12 with Assay Buffer 17 plus Inhibitors prior to assaying. Note that this dilution is based on the lysis of 28 million HeLa cells per mL. The 1:12 dilution contained 2.3 million cells per mL with a calculated recovery of 94.8%.

**If the end user chooses to use another lysis buffer or a greater number of cells, it is up to the end user to determine the appropriate dilution of samples and assay validation.** Only standard curves generated in Assay Buffer 17 plus Inhibitors should be used to calculate the concentration of BRCA-1. Samples must be stored frozen at or below -70 °C to avoid loss of bioactive BRCA-1. Excessive freeze/thaw cycles should be avoided. Prior to assay, frozen samples should be brought to 4 °C slowly and gently mixed.

## HeLa Cell Synchronization Experiment

HeLa cells were cultured in 100 mm dishes and grown to 60% confluency. Cells were rinsed with HBSS and treated with serum-free medium for 24 hrs. Cells were then rinsed with HBSS and treated with 200 µM mimosine in serum-free medium for 16 hrs. Fresh medium was added to release cells from G<sub>1</sub> block. Cells were harvested at t=0 (G<sub>1</sub> phase) and t=6.5 hours (S phase). Cells were pelleted at 1,500 rpm for 5 min. and the supernatant discarded. The resulting pellets were resuspended with modified RIPA Cell Lysis Buffer at a cellular concentration of 24 million cells per mL, placed on ice for 25 min., vortexed and centrifuged at 16,000 rpm for 20 min. at 4 °C. The supernatants were split for DC Lowry Assay and EIA. The soluble protein concentration was determined to be 9.3 and 12.4 mg/mL for the G<sub>1</sub> and S phase, respectively. Lysates were normalized for total cellular protein and run in the EIA to generate the data illustrated. Flow Cytometry was used to verify the shift in cell cycle from G<sub>1</sub> (t=0) to S phase (t=6.5 hrs).



## **Procedural Notes**

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents, except the human BRCA-1 Standard, to warm to room temperature for at least 30 minutes before opening.
3. The human BRCA-1 Standard should not be left at room temperature for extended periods of time. A maximum of 10 minutes at room temperature is recommended.
4. Standards must be made up in polypropylene tubes.
5. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
6. Pipet standards and samples to the bottom of the wells.
7. Add the reagents to the side of the well to avoid contamination.
8. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4 °C in the sealed bag provided. The wells should be used in the frame provided.
9. **Prior to addition of antibody, conjugate and substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**

## **Reagent Preparation**

### **1. Wash Buffer**

Prepare the Wash Buffer by diluting 50 mL of the supplied concentrate with 950 mL deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

### **2. Assay Buffer 17 plus Inhibitors**

Immediately prior to use in the assay, PMSF and PIC must be added to the buffer. If using Sigma Protease Inhibitor Cocktail (PIC) #P8340, add 0.5 µL/mL PIC or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #P7626, to a final concentration of 1 mM. **This modified Assay Buffer 17 must be used for standard reconstitution and all sample and standard dilutions to ensure optimal integrity of human BRCA-1. Fresh Assay Buffer 17 plus Inhibitors must be made for each assay.**

### **3. human BRCA-1 Standards**

Allow the lyophilized human BRCA-1 Standard to warm for no more than 10 minutes. Add 1 mL of Assay Buffer 17 plus Inhibitors to the lyophilized BRCA-1 vial and vortex. Wait 5 minutes and vortex again prior to use. Label the vial standard #1. Label five 12 x 75 mm polypropylene tubes #2 through #6. Pipet 500 µL of Assay Buffer 17 plus Inhibitors into tube #2 through #6. Add 500 µL of reconstituted standard #1 to tube #2 and vortex. Add 500 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #6.

**The concentrations of human BRCA-1 in standard vial #1 and tubes #2 through #6 will be 20,000, 10,000, 5,000, 2,500, 1,250 and 625 pg/mL, respectively. See human Total BRCA-1 Assay Layout Sheet for dilution details.**

**Reconstituted and diluted standards should be used within 60 minutes of preparation.**

**Discard any unused reconstituted standard and subsequent dilutions.**

#### 4. RIPA Cell Lysis Buffer 2

Allow buffer to come to room temperature. Ensure that it is completely in solution prior to use. Immediately prior to use in cell lysis, phosphatase inhibitors (Sodium orthovanadate and Sodium pyrophosphate) and protease inhibitors (PIC and PMSF) must be added to the buffer. If using Sigma Protease Inhibitor Cocktail (PIC) #P8340, add 0.5  $\mu\text{L}/\text{mL}$  PIC or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #7626, to a final concentration of 1 mM. Add Sodium orthovanadate, such as Sigma #S6508, to a final concentration of 2 mM and Sodium pyrophosphate, such as Sigma #S6422, to a final concentration of 20 mM. **Fresh modified RIPA Cell Lysis Buffer 2 plus Inhibitors must be made each time cells are lysed.**

### Assay Procedure

**Bring all reagents to room temperature for at least 30 minutes prior to opening.**

**All standards, controls and samples should be run in duplicate.**

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal. Store unused wells at 4 °C.
2. Pipet 100  $\mu\text{L}$  of Assay Buffer 17 plus Inhibitors into the S0 (0 pg/mL standard) wells.
3. Pipet 100  $\mu\text{L}$  of standards #1 through #6 into the appropriate wells.
4. Pipet 100  $\mu\text{L}$  of the samples into the appropriate wells.
5. Tap the plate gently to mix the contents.
6. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500 rpm.
7. Empty the contents of the wells and wash by adding 400  $\mu\text{L}$  of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
8. Pipet 100  $\mu\text{L}$  of yellow Antibody into each well, except the Blank.
9. Seal the plate and incubate at room temperature on a plate shaker for 2 hours at ~500 rpm.
10. Empty the contents of the wells and wash by adding 400  $\mu\text{L}$  of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
11. Add 100  $\mu\text{L}$  of blue Conjugate to each well, except the Blank.
12. Seal the plate and incubate at room temperature on a plate shaker for 30 minutes at ~500 rpm.
13. Empty the contents of the wells and wash by adding 400  $\mu\text{L}$  of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
14. Pipet 100  $\mu\text{L}$  of Substrate Solution into each well.
15. Incubate for 30 minutes at room temperature on a plate shaker at ~500 rpm.
16. Pipet 100  $\mu\text{L}$  Stop Solution to each well.
17. Blank the plate reader against the Blank wells, read the optical density at 450 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all the readings.

## **Calculation of Results**

Several options are available for the calculation of the concentration of BRCA-1 in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4-parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of BRCA-1 can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

$$\text{Average Net OD} = \text{Average OD} - \text{Average Blank OD}$$

2. Using linear graph paper, plot the Average Net OD for each standard versus human BRCA-1 concentration in each standard. Approximate a straight line through the points. The concentration of BRCA-1 in the unknowns can be determined by interpolation.

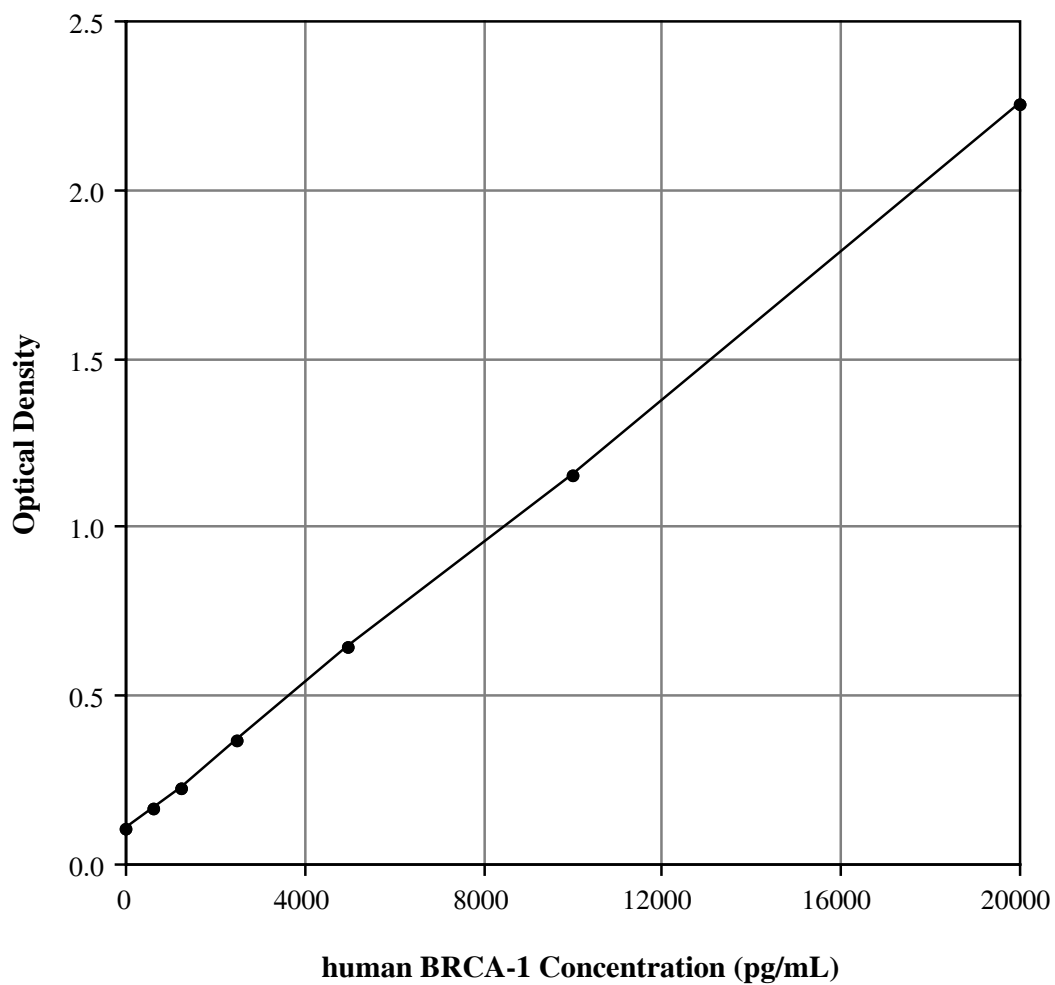
## **Typical Results**

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

<u>Sample</u>	<u>Average OD</u>	<u>Net OD</u>	<b>human BRCA-1 (pg/mL)</b>
Blank	(0.080)		
S0	0.184	0.104	<b>0</b>
S1	2.330	2.250	<b>20,000</b>
S2	1.234	1.154	<b>10,000</b>
S3	0.722	0.642	<b>5,000</b>
S4	0.444	0.364	<b>2,500</b>
S5	0.300	0.220	<b>1,250</b>
S6	0.245	0.165	<b>625</b>
Unknown 1	1.846	1.766	<b>15,567</b>
Unknown 2	0.477	0.397	<b>2,828</b>

### Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate BRCA-1 concentrations; each user must run a standard curve for each assay.



## **Performance Characteristics**

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols<sup>11</sup>.

### **Sensitivity**

Sensitivity was calculated by determining the average optical density bound for twenty (20) wells run with 0 pg/mL Standard, and comparing to the average optical density for twenty (20) wells run with Standard #6. The detection limit was determined as the concentration of BRCA-1 measured at two (2) standard deviations from the 0 pg/mL Standard along the standard curve.

Mean OD for S0 = 0.114 ± 0.011 (9.54%)

Mean OD for Standard #6 = 0.184 ± 0.018 (9.62%)

Delta Optical Density (625 - 0 pg/mL) = 0.184 - 0.114 = 0.070

2 SD's of 0 pg/mL Standard = 2 x 0.011 = 0.016

Sensitivity =  $\frac{0.022}{0.070} \times 625 \text{ pg/mL} = \mathbf{196.4 \text{ pg/mL}}$

### **Linearity**

A sample containing 15,969 pg/mL of human BRCA-1 was serially diluted 4 times 1:2 in the Assay Buffer 17 plus Inhibitors and measured in the assay. The data was plotted graphically as actual BRCA-1 concentration versus measured BRCA-1 concentration.

The line obtained had a slope of 0.933 with a correlation coefficient of 0.999.

## **Precision**

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of BRCA-1 and running these samples multiple times (n=24) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of BRCA-1 in multiple assays (n=13).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of BRCA-1 determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	human BRCA-1 (pg/mL)	Intra-assay <u>% CV</u>	Inter-assay <u>% CV</u>
Low	2,946	3.4	
Medium	7,649	3.4	
High	14,751	2.6	
Low	3,021		7.4
Medium	7,470		4.4
High	14,816		9.9

## **Cross Reactivities**

The cross reactivities for a number of related compounds were determined by dissolving cross reactants in assay buffer at a concentration of 400,000 pg/mL. These samples were then measured in the assay.

<u>Compound</u>	<u>Cross Reactivity</u>
human BRCA-1	100%
RAD51	<0.03%
Aurora A	<0.03%

## **Sample Recoveries**

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

BRCA-1 concentrations were measured in modified RIPA Cell Lysis Buffer 2 and cell lysates. BRCA-1 was spiked into the undiluted modified RIPA Cell Lysis Buffer 2. The cells were run unspiked. These samples were then diluted with Assay Buffer 17 plus Inhibitors and assayed in the kit. The following results were obtained:

<b><u>Sample</u></b>	<b><u>% Recovery*</u></b>	<b><u>Recommended Dilution*</u></b>
RIPA Cell Lysis Buffer 2	93.0%	≥1:12
28 million HeLa cells per mL	94.8%	≥1:12

**WARNING: If the end user chooses to not use the provided Cell Lysis Buffer 2, it is up to the end user to determine the appropriate dilution of samples and assay validation for their chosen cell lysis buffer.**

\* See Sample Handling instructions on page 4 for details.

## **References**

1. T. Chard, "An Introduction to Radioimmunoassay & Related Techniques 4th Edition", (1990) Amsterdam: Elsevier.
2. P. Tijssen, "Practice & Theory of Enzyme Immunoassays", (1985) Amsterdam: Elsevier.
3. P.A. Futreal, et al., Science, (1994) 266: 120-122.
4. D. Ford, et al., Breast Cancer Linkage Consortium. Lancet, (1994) 343: 692-695.
5. M.E. Thompson, et al., Nat. Genet., (1995) 9: 444-450.
6. C.A. Wilson, et al., Nat. Genet., (1999) 21: 236-240.
7. Q. Wang, et al., Oncogene, (2000) 19: 6152-6158.
8. A.R. Venkitaraman, Cell, (2002) 108: 171-82.
9. L.M. Starita and J.D. Parvin, Curr. Opin. Cell Biol., (2003) 15: 345-350.
10. K.W. Kinzler and B. Vogelstein, Nature, (1997) 386: 761-763.
11. National Committee for Clinical Laboratory Standards Evaluation Protocols, SC1, (1989) Villanova, PA: NCCLS.

## **LIMITED WARRANTY**

Assay Designs, Inc. warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

Assay Designs must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if Assay Designs is not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.



**For more details concerning the information within this kit insert, or to order any of Assay Designs' products, please call (734) 668-6113 between 8:30 a.m. and 5:30 p.m. EST. Orders or technical questions can also be transmitted by fax or e-mail 24 hours a day.**

**Material Safety Data Sheet (MSDS) available on our website or by fax.**

**Assay Designs, Inc.  
5777 Hines Drive  
Ann Arbor, MI 48108  
U.S.A.**

**Telephone: (734) 668-6113  
(800) 833-8651 (USA & Canada only)  
Fax: (734) 668-2793  
e-mail: [info@assaydesigns.com](mailto:info@assaydesigns.com)  
website: [www.assaydesigns.com](http://www.assaydesigns.com)**

*Simplify Your Science®*

Catalog No. 25-0553

© 2005



September 13, 2006

