

> Glutathione Reductase Activity Kit

Catalog # 900-159

Sufficient Reagents for 500 tests with 5 x 96-well plates
For use with cell and tissue extracts



Store GR Standard and NADPH at -20°C. All other reagents should be stored at 4°C.



Check our website for additional protocols, technical notes and FAQs.



For proper performance, use the insert provided with each individual kit received.

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

Introduction

Assay Designs' Glutathione Reductase Activity Kit is a complete kit for the measurement of glutathione reductase activity in cell and tissue extracts.

Glutathione reductase (GR), a homodimeric flavoprotein disulfide oxidoreductase, plays an indirect but essential role in the prevention of oxidative damage within the cell by helping to maintain appropriate levels of intracellular reduced glutathione (GSH). Reduced glutathione is essential for maintaining the normal structure of red blood cells and for keeping hemoglobin in the ferrous state¹. Glutathione reductase together with its co-factor, NADPH, catalyzes the reduction of oxidized glutathione (glutathione disulfide, GSSG) to glutathione (Figure 1). GSH is also a reactant for glutathione peroxidase, which converts hydrogen peroxide (H₂O₂) into water.

In this assay the oxidation of NADPH to NADP⁺ (Figure 1) is monitored by the decrease in absorbance at 340 nm. This rate of decrease in absorbance at 340 nm is directly proportional to the glutathione reductase activity in the sample because the enzyme is present at rate limiting concentrations²⁻⁴. The unit definition for glutathione reductase activity may be expressed in terms of the oxidation of NADPH or the reduction of GSSG since their molar ratio is 1:1. One unit of glutathione reductase oxidizes 1 μmol of NADPH per minute at 25°C, pH 7.5.

Principle

1. Samples and standards are added to wells of a 96-well plate. GR Master mix and buffers are added.
2. NADPH solution is added to the wells to initiate the reaction.
3. The plate is transferred to a plate reader and absorbance readings are taken at 340 nm every minute for 10 minutes.

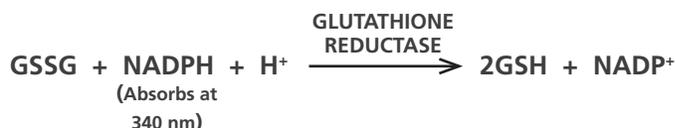


Figure 1. Reduction of glutathione disulfide (GSSG) by glutathione reductase and NADPH.

Materials Supplied

1. Clear Microtiter Plate
Five Plates of 96 Wells, Catalog No. 80-1639
Clear uncoated solid plates.
2. GR Standard
1 mL, Catalogue No. 80-1640
Glutathione reductase standard with an activity of 1 unit/mL. One unit of GR oxidizes 1 μ mole of NADPH per minute at 25°C, pH 7.5.
3. 10X GR Buffer
20 mL, Catalog No. 80-1637
4. NADPH
5 lyophilized vials, Catalog No. 80-1638
One vial is sufficient for one 96-well plate.
5. GSSG
3 mL, Catalog No. 80-1636
6. 20% Triton X-100
1 mL, Catalog No. 80-1641
7. Glutathione Reductase Assay Layout Sheet
1 each, Catalog No. 30-0239

Storage

The GR Standard and NADPH should be stored at -20°C. All other components of this kit are stable at 4°C. All kit components are stable at their recommended storage temperatures until the kit expiration date.

Materials Needed but Not Supplied

1. PBS
2. Distilled water
3. Protease inhibitors (optional) such as phenylmethylsulfonyl fluoride (PMSF), Sigma P7626 or equivalent
4. Reagents to determine protein concentration
5. Ficoll-Hypaque™ (erythrocyte, lymphocyte and monocyte preparations)
6. Microtubes, 0.5 and 1.5 mL
7. 15 mL conical tubes (adherent and suspension cell preparation)
8. 50 mL conical tubes (tissue preparation)
9. Precision pipettes for volumes between 5-1000 μ L
10. Multichannel pipettor for volumes between 1 - 50 μ L and 50 μ L – 200 μ L
11. Microplate reader capable of reading at 340 nm and taking readings every minute for ten minutes and exporting data to an Excel spreadsheet
12. Centrifuge and microfuge for processing samples



Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.



The physical, chemical, and toxicological properties of the chemicals and reagents contained in this kit may not yet have been fully investigated. Therefore, we recommend the use of gloves, lab coats, and eye protection while using any of these chemical reagents.



Reagents require separate storage conditions.



All solutions must be prepared just before use.



Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.



Enzyme should be diluted just before use. Store enzyme on ice. Discard unused enzyme dilutions.

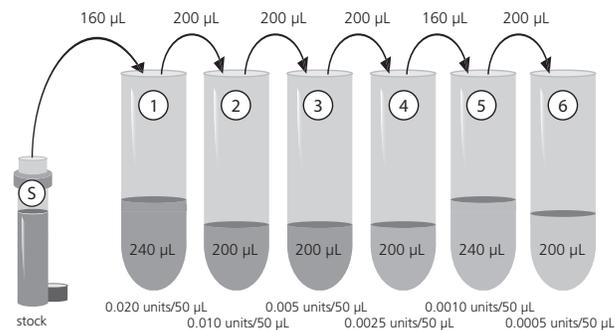


Store the reconstituted NADPH and NADPH solution on ice and use within 4 hours of preparation.

Reagent Preparation

- 1X GR Buffer
Dilute the 10X GR Buffer to 1X (1:10) with distilled water. The 1X GR Buffer is used to prepare dilutions of GR Standard Curve and to prepare the NADPH reagent. The 10X GR Buffer is used directly to prepare 1X Cell Extraction Buffer and Master Mix.

- GR Standard Curve



Thaw the 1 unit/mL GR Standard on ice. Label six 0.5 mL or 1.5 mL microtubes #1 through #6. Pipet 240 µL of 1X GR Buffer into tube #1. Pipet 200 µL of 1X GR Buffer into tubes #2, #3, #4, and #6. Pipet 240 µL of 1X GR Buffer into tube #5. Add 160 µL of GR Standard to tube #1 and vortex thoroughly. Add 200 µL of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 and #4. Add 160 µL of tube #4 to tube #5 and vortex thoroughly. Add 200 µL of tube #5 to tube #6 and vortex thoroughly.

Diluted standards may be kept at room temperature but must be used immediately. The concentrations of GR in the tubes are labeled above.

- 1X Cell Extraction Buffer
Prepare sufficient amount of Cell Extraction Buffer. Preparation for 10 mL is as follows:

10X GR Buffer	1.0 mL
20% (v/v) Triton X-100	0.2 mL
Distilled water	8.8 mL
200 mM PMSF (optional)	(10 µL)

- NADPH Solution
The kit contains 5 vials of lyophilized NADPH, each sufficient for 100 tests. Add 0.5 mL of 1X GR Buffer to dissolve the contents of the vial. Transfer the solution to a 15 mL conical tube. Wash the vial two more times with 0.5 mL of 1X GR Buffer and transfer to the 15 mL conical tube. Add 1X GR Buffer to the 15 mL conical tube to a final volume of 6.5 mL.

5. Master Mix

Count the total number of wells needed for the samples and add 21 (for the complete standard curve and Blank wells in triplicate). Use the following formula to calculate the volume of Master Mix required.

- A. Total volume required
[Total number of wells needed + 21] x 100 μ L = _____ μ L
- B. Volume of 10X GR Buffer required
[Total Volume Required (from A. above)] x 0.1 = _____ μ L
- C. Volume of GSSG Reagent required
[Total Volume required (from A. above)] x 0.04 = _____ μ L
- D. Volume of distilled water required
[Total Volume required (from A. above)] x 0.86 = _____ μ L

Prepare Master Mix by combining the appropriate reagent volumes calculated in B, C, and D above. For example, to prepare 100 μ L of Master Mix the following volumes are combined: 10 μ L 10X GR Buffer, 4 μ L GSSG reagent, and 86 μ L distilled water.

6. Biological Extracts

After preparing the samples as outlined in the Sample Handling section that follows, make serial dilutions of cell or tissue extracts with 1X GR Buffer. Initial concentrations between 0.5 μ g/50 μ L to 50 μ g/50 μ L are recommended.



Thaw the GSSG stock on ice. Keep the stock on ice while using.

Sample Handling

Choose the appropriate protocol in Section A to process sample before proceeding to Section B. Keep samples on ice to maintain enzyme activity.

Section A. Processing Samples

Suspension cells:

1. Centrifuge 2 to 6 x 10⁶ suspension cells at 250 x g for 10 minutes at 4°C. Discard the supernatant.
2. Suspend the cell pellet in 1 mL of ice-cold 1X PBS and transfer to a 1.5 mL microtube on ice. Centrifuge, discard supernatant, and place on ice.
3. Proceed to Section B. Preparation of Cytosolic Extracts

Adherent cells:

1. Wash 2 to 6 x 10⁶ adherent cells with 1X PBS. Adherent cells may be harvested by gentle trypsinization.
2. Transfer to a 15 mL tube on ice. Centrifuge at 250 x g for 10 minutes at 4°C and discard the supernatant.
3. Suspend the cell pellet in 1 mL of ice-cold 1X PBS and transfer to 1.5 mL microtube on ice. Centrifuge, discard supernatant, and place on ice..
4. Proceed to Section B. Preparation of Cytosolic Extracts.



Samples must be kept on ice to maintain enzyme activity.



Samples must be kept on ice to maintain enzyme activity.

Erythrocytes:

1. Dilute anticoagulated blood with an equal volume of PBS. Layer over Ficoll-Hypaque™ or similar reagent and centrifuge at 800 x g for 25 min at 12°C with the BRAKE OFF in a swinging bucket rotor.
2. Collect the mononuclear cells (lymphocytes and monocytes) at the interphase and transfer to another tube.
3. Remove the remaining liquid from above the red blood cell pellet. Wash the pellet with 10 cell volumes of PBS.
4. Determine the packed cell volume and add 10 cell volumes of cold distilled water. Mix well and incubate on ice for 10-15 minutes to lyse the red blood cells. Lysis occurs when the opaque solution changes to a brilliant clear red solution, indicating the release of hemoglobin.
5. Centrifuge at 10,000 x g for 10 minutes at 4°C to remove cell debris. Transfer the supernatant to a fresh tube and store on ice.

Lymphocytes and Monocytes:

1. Dilute anticoagulated blood with an equal volume of PBS. Layer over Ficoll-Hypaque™ or similar reagent and centrifuge at 800 x g for 25 min at 12°C with the BRAKE OFF in a swinging bucket rotor.
2. Collect the mononuclear cells (lymphocytes and monocytes) at the interphase and transfer to another tube.
3. Dilute the blood mononuclear cells with 5 volumes of PBS and centrifuge at 400 x g for 10 minutes at 4°C. Discard the supernatant
4. Suspend the cell pellet in 1 mL of ice-cold 1X PBS and transfer to a pre-chilled 1.5 mL microtube. Centrifuge, discard supernatant, and place on ice.
5. Proceed to Section B. Preparation of Cytosolic Extracts.

Tissue

1. Remove tissue and place in cold PBS in a 50 mL conical tube. Repeatedly wash the tissue with PBS to remove blood clots and other debris.
2. Transfer the tissue to a Petri dish on ice and mince the tissue to small pieces with surgical scissors.
3. Transfer the tissue pieces to a clean stainless steel sieve. Place the sieve with the tissue pieces in a Petri dish which contains about 20 mL of cold 1X PBS.
4. Create a single cell suspension of the tissue as follows: Using a pestle or a round bottom tube, grind the tissue pieces thoroughly until the bulk of the tissue passes through the sieve.
5. Transfer the PBS containing the single cell suspension to a 50 mL conical tube. Fill with cold PBS and mix by inverting the tube several times. Let the tube stand on ice for 1 minute to allow large aggregates of tissue to settle out of solution.
6. Carefully transfer the supernatant containing the single cell suspension to a clean 50 mL conical centrifuge tube. Centrifuge at 400 x g for 10 minutes at 4°C. Discard the supernatant. Suspend the cell pellet in 1 mL of ice-cold 1X PBS and transfer to a pre-chilled 1.5 mL microtube on ice. Centrifuge, discard the supernatant, and place on ice.
7. Proceed to Section B. Preparation of Cytosolic Extracts.

Section B. Preparation of Cytosolic Extracts from Cells and Tissue

1. Measure the approximate volume of the cell pellets prepared above (except for erythrocytes) and suspend the cells in 5-10 volumes of cold 1X Cell Extraction Buffer. Incubate the cell suspensions on ice, with periodic vortexing, for 30 minutes.
2. Microcentrifuge the disrupted cell suspension at 10,000 x g for 10 minutes at 4°C to remove insoluble material. Recover the supernatant to a fresh tube pre-chilled on ice. Occasionally, the pellet may float and can easily be removed with a pipet tip.
3. Determine the protein concentration of the cleared cell lysate. Note that the 1X GR Buffer contains BSA at a concentration of 0.1 mg/mL and this should be subtracted from your observed protein concentration.
4. If not assaying for GR immediately, snap-freeze the cleared cell extract in 100 µL aliquots by immersing the aliquots in liquid nitrogen and store at -80°C. Avoid repeated freezing and thawing of the extract.

Assay Procedure

Refer to the Assay layout Sheet to determine the number of wells to be used.

1. Pipet 50 µL of the 1X GR Buffer to the bottom of the Blank wells.
2. Pipet 50 µL of the prepared GR Standards #1 through #6 to the bottom of the appropriate wells.
3. Pipet 50 µL of the diluted sample to the bottom of the appropriate wells.
4. Pipet 100 µL of Master Mix into each well.
5. Initiate the reaction by adding 50µL of NADPH Solution to all the wells using a multichannel pipet.
6. Immediately transfer the plate to a microtiter plate reader and take absorbance readings at 340 nm every minute for 10 minutes at room temperature. If possible, include a 10 second orbital shake prior to the first read.



All standards, controls, and samples should be run in triplicate.



Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.



Pipet the reagents to the sides of the wells to avoid possible contamination.

Calculation of Results

A. Time Course of the Change in Absorbance at 340 nm of the GR Standard

1. Plot the mean of the triplicate absorbance values at 340 nm versus time of the GR standard (Figure 2):

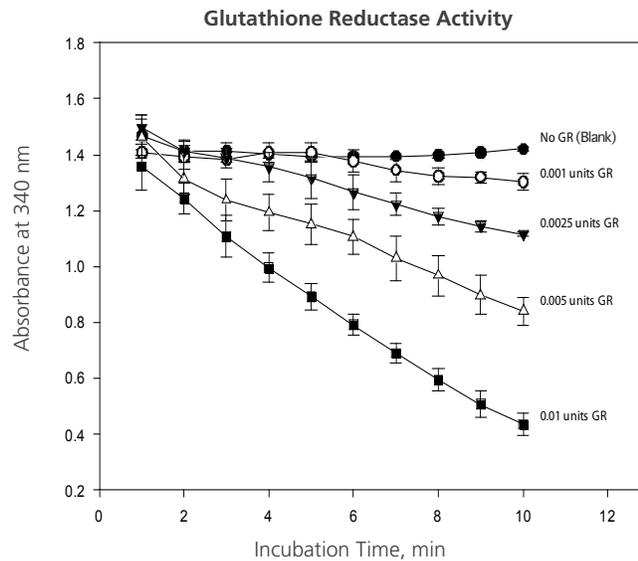


Figure 2: Change in absorbance at 340 nm with time for selected concentrations of the GR standard.

B. Determination of GR Activity in Experimental Samples from the GR Standard Curve

1. Determine the rate of decrease in absorbance at 340 nm per minute (ΔA_{340} nm/min) for each of the standards and the Blank wells (Buffer control):

$$\frac{(A_{340} \text{ nm @ 1 min.}) - (A_{340} \text{ nm @ 10 min.})}{9 \text{ min}} = \Delta A_{340} \text{ nm / min}$$

Note: Our plate reader takes absorbance readings beginning at the 1 minute timepoint and ends at 10 minutes.

2. Calculate the net rate for each standard by subtracting the rate obtained for the Blank (the Blank measures the spontaneous oxidation of NADPH. This value is usually small and may be negligible compared to sample values).
3. Plot the number of units of GR/well versus these new ΔA_{340} nm/min values (Figure 3):

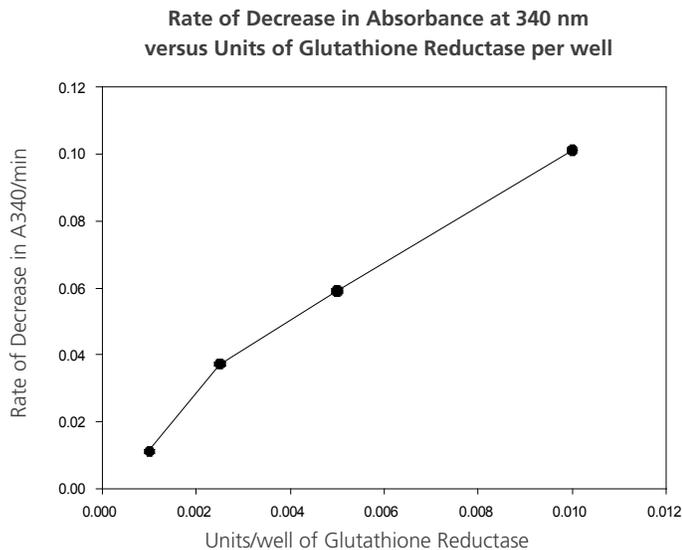


Figure 3: Graph of the rate of decrease in absorbance at 340 nm versus the units of GR in each well.

4. Plot the absorbance values of samples versus time as shown in Figure 2. Take the slope of the linear portion of the curves.
5. Calculate the net rate for each level of the samples by subtracting the rate obtained for the blank (the blank measures the spontaneous oxidation of NADPH. (This value is usually small and may be negligible compared to sample values).
6. Determine the number of units/well of GR in your samples from the linear portion of the GR standard curve of Figure 3.

C. Determination of GR Activity in Experimental Samples from the Molar Extinction Coefficient of NADPH

The GR activity in the sample(s) may be calculated by using the extinction coefficient of NADPH:

1. Determine the rate of decrease in absorbance per minute for both samples and the Blank as described above.
2. Calculate the net rate of the samples by subtracting the Blank rate from the sample rate.
3. Using the Beer-Lambert Law one can determine the concentration of NADPH in solution. Convert the net rate ($\Delta A_{340} \text{ nm/ min}$) to concentration of NADPH consumed, which is equal to the activity of GR in mU/ mL. The molar extinction coefficient (E^M) for NADPH is $6220 \text{ M}^{-1}\text{cm}^{-1}$ and $E^M = 6.22 \times 10^{-3} \text{ nmol/mL}$ if the pathlength is 1 cm.

One unit of glutathione reductase is defined as the amount of enzyme required to catalyze the reduction of one μmole of GSSG per minute at pH 7.5 and 25°C . One molecule of NADPH is oxidized per molecule of GSSG reduced. Therefore, the oxidation of NADPH (measured by loss of A340 nm) directly correlates with GSSG reduction.

$$\begin{aligned} 1 \text{ U of GR} &= 1 \mu\text{mol GSSG reduced/min} \\ &= 1 \mu\text{mol NADPH oxidized/min} \end{aligned}$$

$$\begin{aligned} 1 \text{ mU of GR} &= 1 \times 10^{-3} \mu\text{mol NADPH oxidized/min} \\ 1 \text{ mU of GR} &= 1 \text{ nmol NADPH oxidized/min} \end{aligned}$$

If E^M is the molar extinction coefficient for NADPH at 340 nm,

$$\begin{aligned} E^M &= 6220 \text{ M}^{-1}\text{cm}^{-1} \\ &= 6220 \times 10^{-6} \mu\text{M}^{-1}\text{cm}^{-1} \\ &= 6.22 \times 10^{-3} \mu\text{M}^{-1}\text{cm}^{-1} \\ &= 6.22 \times 10^{-3} \text{ L}/\mu\text{mol}/\text{cm} \\ &= 6.22 \times 10^{-3} \text{ mL}/\text{nmol}/\text{cm} \end{aligned}$$

Note: The path length for 200 μL of reaction volume in the 96 well plate is 0.6 cm.

Therefore,

$$\begin{aligned} E^M &= 6.22 \times 10^{-3} \text{ mL}/\text{nmol}/\text{cm} \times 0.6 \text{ cm} \\ &= 3.732 \times 10^{-3} \text{ mL}/\text{nmole} \end{aligned}$$

$$\begin{aligned} \Delta A_{340} \text{ nm}/\text{min} &= \Delta A_{340} \text{ nm}/\text{min} \\ &= 3.732 \times 10^{-3} \text{ mL}/\text{nmole NADPH} \\ &= Y \text{ nmole NADPH}/\text{min}/\text{mL} \\ &= Y \text{ mU}/\text{mL GR} \end{aligned}$$

- Correct for the sample dilution in the assay and for the sample dilution performed prior to the assay. For example: If the sample volume was $50 \mu\text{L}$ and was diluted 1/50 prior to the assay:

$$\text{Mean } \Delta A_{340} \text{ nm}/\text{min (Sample)} = 0.0325/\text{min}$$

$$\text{Mean } \Delta A_{340} \text{ nm}/\text{min (Blank)} = 0.0005/\text{min}$$

$$\text{Net Rate, } \Delta A_{340} \text{ nm}/\text{min} = 0.0320/\text{min}$$

$$\begin{aligned} \text{Glutathione Reductase Activity} &= 0.0320/\text{min}/3.732 \times 10^{-3} \text{ mL}/\text{nmol NADPH}/\text{min} \\ &= 8.57 \text{ mU}/\text{mL} \end{aligned}$$

$$\begin{aligned} \text{Assay Dilution Correction} &= 200 \mu\text{L}/50 \mu\text{L} \times 8.57 \text{ mU}/\text{mL} \\ &= 34.30 \text{ mU}/\text{mL} \end{aligned}$$

$$\begin{aligned} \text{Sample Dilution Correction} &= 50 \times 34.30 \text{ mU}/\text{mL} \\ &= 1715 \text{ mU}/\text{mL} \end{aligned}$$

- Divide the GR activity (mU/mL) by the protein concentration to determine the specific activity of GR in your sample (mU GR/mg protein).

Troubleshooting

PROBLEM	CAUSE	SOLUTION
No change in absorbance at 340 nm with time in all the wells	Failure to add GSSG to the Master Mix	Add the GSSG reagent to the Master Mix
Absorbance at 340 nm in wells with 1X GR Buffer alone is less than 0.5	Failure to add NADPH to the wells	Follow protocol for making the NADPH reagent and add 50 μ L to each well
	NADPH has degraded	Contact Customer Service
Change in absorbance with the GR standard is satisfactory, but no apparent change in absorbance observed in your sample	GR activity in cells and tissues very low	Extend reaction to 20 minutes
		Reduce the amount of distilled water in the Master Mix. Add 50 μ L of this modified Master Mix and 100 μ L of your extract to each well
Change in absorbance with the GR standard is satisfactory, but absorbance with sample drops to baseline within one or two minutes	GR activity in cells and tissues very high	Increase the dilution of the sample in order to reduce the amount of sample added to the wells

References

1. Berg JM, Tymoczko JL, Stryer L. 2002. Biochemistry, 5th Edition, WH Freeman and Company, New York.
2. Tietze, F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal. Biochem., **27**, 502-522 (1969).
3. Smith IK, Vierheller TL, Thorne CA. Assay of glutathione reductase in crude tissue homogenates using 5,5'-dithiobis(2-nitrobenzoic acid). Anal. Biochem., **175**, 408-413 (1988).
4. Dolphin, D, Poulson, R, Avramovic, O. (Eds.), Glutathione: Chemical, Biochemical, and Metabolic Aspects, Volumes A and B, Wiley and Sons (1989).
5. Dringen R, Gutterer JM. Glutathione reductase from bovine brain. Methods Enzymol. **348**, 281-288 (2002).



MSDS (Material Safety Data Sheet) available online

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.

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Kits for Charity™

Assay Designs has always been an active contributor to a number of local, national and international charities. We have broadened our charitable contributions by implementing a program called Kits for Charity™.

Each quarter, Assay Designs will feature a different non-religious and non-political charitable organization on our website. For each kit sold during this time period, we will make a monetary contribution to the featured charity. Please check our website for the current quarter's charity to see what organization your purchases are helping to support.

If you have any suggestions for future Kits for Charity™ recipients, please contact us at 800.833.8651 or 734.668.6113.

Contact Us

For more details concerning the information within this kit insert, or to order any of the 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.

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