



Human TRAIL/Apo2L ELISA Kit

Catalog Number:

C5331-K01 (1 x 96 wells)

For Research Use Only. Not for use in diagnostic procedures.

v. 1.0

EAGLE BIOSCIENCES, INC.
20A Northwest Blvd., Suite 112, Nashua, NH 03063
Phone: 617-419-2019 Fax: 617-419-1110
WWW.EAGLEBIO.COM



1. Intended use

The Eagle Biosciences Human TRAIL/Apo2L ELISA is a solid phase sandwich ELISA for the *in-vitro* qualitative and quantitative determination of TRAIL in cell culture supernatants, buffered solutions or human serum, plasma, or other body fluids. This assay will recognize both natural and recombinant human TRAIL. The Eagle Biosciences Human TRAIL/Apo2L ELISA Assay kit is for Research Use Only.

2. Principle of the method

The TRAIL Kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for TRAIL has been coated onto the wells of the microtiter strips provided. Samples, including standards of known TRAIL concentrations and unknowns are pipetted into these wells. During the first incubation, the TRAIL antigen and a biotinylated monoclonal antibody specific for TRAIL are simultaneously incubated.

After washing, the enzyme (streptavidin-peroxydase) is added. After incubation and washing to remove all the unbound enzyme, a substrate solution which is acting on the bound enzyme is added to induce a coloured reaction product. The intensity of this coloured product is directly proportional to the concentration of TRAIL present in the samples.

3. Reagents provided and reconstitution

Reagents (Store@2-8°C)	Quantity 1x96 well kit	Reconstitution
96 well microtitre strip plate	1	Ready to use (Pre-coated)
Plastic plate covers	2	n/a
Standard: 3000pg/ml	2 vials	Reconstitute as directed on the vial (see Assay preparation, section 8)
Control	2 vials	Reconstitute as directed on the vial (see Assay preparation, section 8)
Standard Diluent (Buffer)	1 vial (25ml)	10x Concentrate, dilute in distilled water (see reagent preparation, section 8)
Biotinylated anti-TRAIL	1 vial (0.4ml)	Dilute in biotinylated antibody diluent (see Assay preparation, section 8)
Biotinylated Antibody diluent	1 vial (7ml)	Ready to use
Streptavidin-HRP	2 vials (5µl)	Add 0.5ml of HRP diluent prior to use (see Assay preparation, section 8)
HRP Diluent	1 vial (23ml)	Ready to use
Wash Buffer	1 vial (10ml)	200x Concentrate dilute in distilled water (see Assay preparation, section 8)
TMB Substrate	1 vial (11ml)	Ready to use
H ₂ SO ₄ stop reagent	1 vial (11ml)	Ready to use



4. Materials required but not provided

- Microtiter plate reader fitted with appropriate filters (450nm required with optional 630nm reference filter)
- Microplate washer or wash bottle
- 10, 50, 100, 200 and 1,000µl adjustable single channel micropipettes with disposable tips
- 50-300µl multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs
- Distilled water
- Vortex mixer
- Miscellaneous laboratory plastic and/or glass, if possible sterile

5. Storage Instructions

Store the TRAIL ELISA Assay kit reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2-8°C). Expiry of the kit and reagents is stated on box front labels. The expiry of the TRAIL ELISA Assay kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

- **Wash Buffer:** Once prepared store at 2-8°C for up to 1 week
- **Standard Diluent Buffer:** Once prepared store at 2-8°C for up to 1 week
- **Standards:** Once prepared use immediately and do not store
- **Controls:** Once prepared use immediately and do not store
- **Biotinylated Secondary Antibody:** Once prepared use immediately and do not store
- **Streptavidin-HRP:** Once prepared use immediately and do not store

6. Specimen collection, processing & storage

Cell culture supernatants, serum, plasma or other biological samples will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

Cell culture supernatants:

Remove particulates and aggregates by spinning at approximately 1000 x g for 10 min.

Serum:

Avoid any unintentional stimulation of the cells by the procedure. Use pyrogen/endotoxin free collecting tubes. Serum should be removed rapidly and carefully from the red cells after clotting. For that, after clotting, centrifuge at approximately 1000 x g for 10 min and remove serum.

Plasma:



EDTA, citrate and heparin plasma can be assayed. Spin samples at 1000 x g for 30 min to remove particulates. Harvest plasma.

Storage:

If not analyzed shortly after collection, samples should be aliquoted (250-500µl) to avoid repeated freeze-thaw cycles and stored frozen at -70°C. Avoid multiple freeze-thaw cycles of frozen specimens.

Recommendation: Do not thaw by heating at 37°C or 56°C. Thaw at room temperature and make sure that sample is completely thawed and homogeneous before use. When possible avoid use of badly haemolysed or lipemic sera. If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

7. Safety & precautions for use

- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures , e.g.CDC/NIH Health manual : " Biosafety in Microbiological and Biomedical Laboratories" 1984
- Laboratory gloves should be worn at all times
- Avoid any skin contact with H₂SO₄ and TMB. In case of contact, wash thoroughly with water
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used
- Do not pipette by mouth
- When not in use, the Human TRAIL ELISA Assay kit components should be stored refrigerated or frozen as indicated on vials or bottles labels
- All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use
- Once the desired number of strips has been removed, immediately reseal the bag to protect the remaining strips from deterioration
- Cover or cap all reagents when not in use
- Do not mix or interchange reagents between different lots
- Do not use reagents beyond the expiration date of the Human TRAIL ELISA Assay kit
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of H₂SO₄ and substrate solution, avoid pipettes with metal parts
- Use a clean plastic container to prepare the washing solution
- Thoroughly mix the reagents and samples before use by agitation or swirling
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells
- The TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent colour development. Warning TMB is toxic avoid direct contact with hands. Dispose of properly
- If a dark blue colour develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded. Read absorbance's within 1 hour after completion of the assay
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells



- Follow incubation times described in the assay procedure
- Dispense the TMB solution within 15 min of the washing of the microtitre plate

8. Assay Preparation

Bring all reagents to room temperature before use

8.1. Assay Design

Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards. Each sample, standard and zero should be tested **in duplicate**. Remove sufficient Microwell Strips for testing from the pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8°C storage.

Example plate layout (example shown for a 6 point standard curve)

	Standards		Sample Wells									
	1	2	3	4	5	6	7	8	9	10	11	12
A	3000	3000										
B	1500	1500										
C	750	750										
D	375	375										
E	187.5	187.5										
F	93.75	93.75										
G	Zero	Zero										
H	CTRL	CTRL										

All remaining empty wells can be used to test samples in duplicate

8.2. Preparation of Wash Buffer

Dilute the (200x) wash buffer concentrate 200 fold with distilled water to give a 1x working solution. Pour entire contents (10 ml) of the Washing Buffer Concentrate into a clean 2,000 ml graduated cylinder. Bring final volume to 2,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. Transfer to a clean wash bottle and store at 2°-8°C for up to 1 week.

8.3. Preparation of Standard Diluent Buffer

Add the contents of the vial (10x concentrate) to 225ml of distilled water before use. This solution can be stored at 2-8°C for up to 1 week.



8.4. Preparation of Standard

Standard vials must be reconstituted with the volume of standard diluent shown on the vial immediately prior to use. This reconstitution gives a stock solution of 3000 pg/ml of TRAIL. **Mix the reconstituted standard gently by inversion only.** Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 3000 to 93.75pg/ml. A fresh standard curve should be produced for each new assay.

- Immediately after reconstitution add 200µl of the reconstituted standard to wells A1 and A2, which provides the highest concentration standard at 3000 pg/ml
- Add 100µl of Standard Diluent to the remaining standard wells B1 and B2 to F1 and F2
- Transfer 100µl from wells A1 and A2 to B1 and B2. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells
- Continue this 1:1 dilution using 100µl from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 3000pg/ml to 93.75pg/ml
- Discard 100µl from the final wells of the standard curve (F1 and F2)

Alternatively these dilutions can be performed in separate clean tubes and immediately transferred directly into the relevant wells.

8.5. Preparation of Controls

The supplied Controls must be reconstituted with the volume of Standard Diluent indicated on the vial. Reconstitution of the freeze-dried material with the indicated volume, will give a solution at the concentration stated on the vial. Do not store after use.

8.6. Preparation of Biotinylated anti-TRAIL

It is recommended this reagent is prepared immediately before use. Dilute the biotinylated anti TRAIL with the biotinylated antibody diluent in an appropriate clean glass vial using volumes appropriate to the number of required wells. Please see example volumes below:

Number of wells required	Biotinylated Antibody µl)	Biotinylated Antibody Diluent µl)
16	40	1060
24	60	1590
32	80	2120
48	120	3180
96	240	6360



8.7. Preparation of HRP Conjugate

It is recommended to centrifuge vial for a few seconds in a microcentrifuge to collect all the volume at the bottom. Dilute the 5∞l vial with 0.5ml of HRP diluent **immediately before use**. Do-not keep this diluted vial for future experiments. Further dilute the HRP solution to volumes appropriate for the number of required wells in a clean glass vial. Please see example volumes below:

Number of wells required	Streptavidin-HRP µl)	Streptavidin-HRP Diluent (ml)
16	30	2
24	45	3
32	60	4
48	75	5
96	150	10

9. Method

We strongly recommend that every vial is mixed thoroughly without foaming prior to use except the standard vial which must be mixed gently by inversion only.

Prepare all reagents as shown in section 8.

Note: Final preparation of Biotinylated anti TRAIL (section 8.6) and Streptavidin-HRP (section 8.7) should occur immediately before use.

Assay Step		Details
1.	Addition	Prepare Standard curve as shown in section 8.4
2.	Addition	Add 100µl of each standard, control, sample and zero in duplicate to appropriate number of wells
3.	Addition	Add 50µl of diluted biotinylated anti TRAIL to all wells
4.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 3 hours
5.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.3 ml of 1x washing solution into each well c) Aspirate the contents of each well d) Repeat step b and c another two times
6.	Addition	Add 100µl of Streptavidin-HRP solution into all wells
7.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 30 min



8.	Wash	Repeat wash step 5.
9.	Addition	Add 100µl of ready-to-use TMB Substrate Solution into all wells
10.	Incubation	Incubate in the dark for 10-20 minutes* at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil
11.	Addition	Add 100µl of H₂SO₄:Stop Reagent into all wells
Read the absorbance value of each well (immediately after step 11.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 630 nm as the reference wave length (610 nm to 650 nm is acceptable).		

**Incubation time of the substrate solution is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the colour development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range*

10. Data Analysis

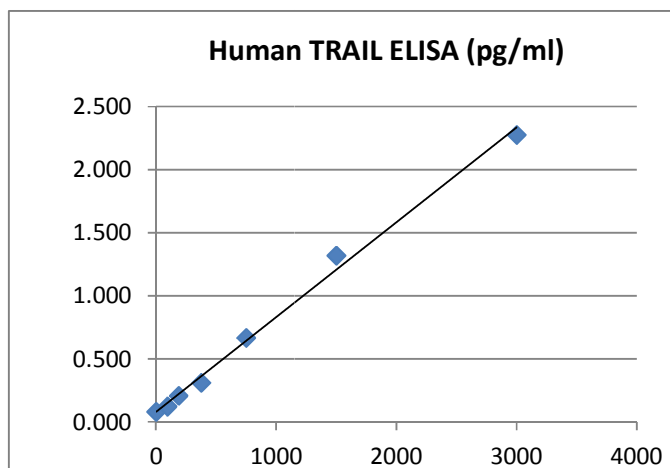
Calculate the average absorbance values for each set of duplicate standards and samples. Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding human TRAIL standard concentration on the horizontal axis.

The amount of TRAIL in each sample is determined by extrapolating OD values against TRAIL standard concentrations using the standard curve.

Example TRAIL Standard curve

Standard	TRAIL Conc	OD (450nm) Mean	CV (%)
1	3000	2.279	9.1
2	1500	1.322	5.4
3	750	0.669	1.2
4	375	0.313	1.5
5	187.5	0.210	1.5
6	93.75	0.125	5.7
Zero	0	0.083	-



Note; curve shown above should not be used to determine results. Every laboratory must produce a standard curve for each set of microwell strips assayed.



11. Assay limitations

Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard diluent or with your own sample buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration.

The influence of various drugs on end results has not been investigated. Bacterial or fungal contamination and laboratory cross-contamination may also cause irregular results.

Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Washing Buffer, fill with Washing Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

As with most biological assays conditions may vary from assay to assay therefore **a fresh standard curve must be prepared and run for every assay.**

12. Performance Characteristics

12.1. Sensitivity

The sensitivity, minimum detectable dose of human TRAIL using this Eagle Biosciences TRAIL ELISA kit was found to be **<64pg/ml**. This was determined by adding 3 standard deviations to the mean OD obtained when the zero standard was assayed 55 times.

12.2. Precision

Intra-Assay

Inter-Assay

Sample	n	Mean (pg/mL)	SD	CV%	Sample	n	Mean (pg/mL)	SD	CV%
A	40	1500	82.9	5.5	A	12	1619	62.8	3.8
B	33	116.3	8.7	7.4	B	12	162.8	13.1	8

12.3. Expected Serum Values

A panel of 50 human sera was tested for TRAIL. The detected level of TRAIL ranged from 430 to 1440 pg/ml with a mean of 973 +/- 263 pg/ml.



13. References

- Wiley S. et al. (1995) : Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* 6 : 673-682.
- Griffith T et al. (1999) : Functional analysis of TRAIL receptors using monoclonal antibodies. *J. Immunol.* 162 : 2597-2605.
- Martinez-Lorenzo et al. (1999) : Activated human T cells release bioactive Fas ligand and Apo2 ligand in microvesicles. *J. Immunol.* 163 : 1274-1281.
- Ashkenazi A. et al. (1999) : Safety and antitumor activity of recombinant soluble Apo2 ligand. *J. Clin. Invest.* 104 : 155-162.
- Fanger N. et al. (1999) : Human dendritic cells mediate cellular apoptosis via Tumor Necrosis Factor-related apoptosis-inducing ligand. *J. Exp. Med.* 190 : 1155-1164.
- Kashii Y. et al. (1999) : Constitutive expression and role of the TNF family ligands in apoptotic killing of tumor cells by human NK cells. *J. Immunol.* 163 : 5358-5366.
- Walczak H. et al. (1999) : Tumoricidal activity of tumor necrosis factor-related apoptosis inducing ligand in-vivo. *Nat. Med.* 5 : 157-163.
- Kayagaki N. et al. (1999) : Involvement of TNF-related Apoptosis-inducing Ligand in Human CD4+ T cell-mediated Cytotoxicity. *J. Immunol.* 162 : 2639-2647.
- Kayagaki et al. (1999) : Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells : A novel mechanism for the antitumor effects of type I IFNs. *J. Exp. Med.* 189 : 1451-1460.
- Phillips T. et al. (1999) : TRAIL (Apo2L) and TRAIL receptors in human placentas : implications for immune privilege. *J. Immunol.* 16 : 6053-6059.
- Hymowitz S. et al. (2000) : A unique zinc-binding site revealed by a high-resolution X-ray structure of homotrimeric Apo2L/TRAIL. *Biochemistry* 39 : 633-640.
- Audige, A. et al., (2006) : Anti-HIV state but not apoptosis depends on IFN signature in CD4+ T cells. *J Immunol.* : 177(9): 6227-37
- Bremer E. et al., (2005) : Target Cell-Restricted Apoptosis Induction of Acute Leukemic T Cells by a Recombinant Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Fusion Protein with Specificity for Human CD7. *Cancer Res.*, 2005 : 65(8): 3380 – 3388
- Cassatella, M. A. et al., *J Leukoc Biol.*, 2006; 79(1): 123-32. Interferon-activated neutrophils store a TNF-related apoptosis-inducing ligand (TRAIL/Apo-2 ligand) intracellular pool that is readily mobilizable following exposure to proinflammatory mediators
- Castellino, G. et al., *Lupus*, 2007; 16(7): 479-482. Elevated levels of TRAIL in systemic lupus erythematosus are associated to the presence of anti-SSA/SSB antibodies



Chehimi, J. et al., *J. Virol.*, 2010; 84(6): 2762-2773. Inability of Plasmacytoid Dendritic Cells To Directly Lyse HIV-Infected Autologous CD4⁺ T Cells despite Induction of Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand

de Bruyn, M et al., *Clin. Cancer Res.*, 2011; 17: 5626 – 5637. Cell Surface Delivery of TRAIL Strongly Augments the Tumoricidal Activity of T Cells.

Gasper-Smith, N. et al., *J. Virol.*, 2008; 82:7700-7710. Induction of Plasma (TRAIL), TNFR-2, Fas Ligand and Plasma Microparticles After HIV-1 Transmission: Implications for HIV-1 Vaccine Design.

Ghorbel, M. T. et al., *J. Thorac. Cardiovasc. Surg.*, 2010; 140(2): 337-345. Transcriptomic analysis of patients with tetralogy of Fallot reveals the effect of chronic hypoxia on myocardial gene expression

Herbeuval J-P. et al., *Blood*, 2005; 105(6): 2458 – 2464. TNF-related apoptosis-inducing ligand (TRAIL) in HIV-1-infected patients and its in vitro production by antigen-presenting cells

Herbeuval, J. P. et al., *J Natl Cancer Inst.*, 2003; 95(8): 611-21. Macrophages from cancer patients: analysis of TRAIL, TRAIL receptors, and colon tumor cell apoptosis

Indraccolo, S. et al., *J Immunol.*, 2007; 178(2): 1122-35. Identification of genes selectively regulated by IFNs in endothelial cells

Jungel, A. et al., *Ann Rheum Dis.*, 2006; 65(7): 910-2. Trichostatin A sensitises rheumatoid arthritis synovial fibroblasts for TRAIL-induced apoptosis

Koga Y. et al., *Cancer Res.*, 2004; 64(3): 1037 – 1043. Neutrophil-Derived TNF-Related Apoptosis-Inducing Ligand (TRAIL): A Novel Mechanism of Antitumor Effect by Neutrophils

Lub-De Hooge M.N., *Shock*, 2004; 22(2): 186 – 188. Endotoxin increases plasma soluble tumor necrosis factor-related apoptosis-inducing ligand level mediated by the p38 mitogen-activated protein kinase signaling pathway

Lub-de Hooge M.N. et al., *Ann. Rheum. Dis*, 2005; 64(6): 854 -858. Soluble TRAIL concentrations are raised in patients with systemic lupus erythematosus

Ludwig A.T. et al., *Cancer Res.*, 2004; 64(10): 3386 – 3390. Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand: A Novel Mechanism for Bacillus Calmette-Guerin-Induced Antitumor Activity

Lunemann JD. et al., *J. Immunol.*, 2002; 168(10): 4881 -4888. Death ligand TRAIL induces no apoptosis but inhibits activation of human (auto)antigen-specific T cells

Merino, D. et al., *Mol Cell Biol.*, 2006; 26(19): 7046-55. Differential inhibition of TRAIL-mediated DR5-DISC formation by decoy receptors 1 and 2



Potu, H. et al., *Cancer Res.*, 2010; 70(2): 655-665. Identification of USP18 as an Important Regulator of the Susceptibility to IFN- α and Drug-Induced Apoptosis

Simons, M. P. et al., *Infect Immun.*, 2007; 75(3): 1265-71. Identification of the mycobacterial subcomponents involved in the release of tumor necrosis factor-related apoptosis-inducing ligand from human neutrophils

Tecchio C. et al., *Blood*, 2004; 103(10): 3837 – 3844. IFN α -stimulated neutrophils and monocytes release a soluble form of TNF-related apoptosis-inducing ligand (TRAIL/Apo-2 ligand) displaying apoptotic activity on leukemic cells

Vermot-Desroches C. et al., *Cell Immunol.*, 2005; 236(1-2): 86-91. Characterization of monoclonal antibodies directed against TRAIL or TRAIL receptors

Wandinger KP et al., *Lancet*, 2003; 361(9374) : 2036 – 2043. TNF-related apoptosis inducing ligand (TRAIL) as a potential response marker for interferon-beta treatment in multiple sclerosis

Wegrzynowska, M. et al., *Poster Session 3 Europace* 13 (suppl_3): 10.1093/europace/eur229. Apoptosis and lipid peroxidation after sinus rhythm restoration in patients with persistent atrial fibrillation

Yang, L. et al., *Blood*, 2010; 116(20): 4307-4316. Contributions of TRAIL-mediated megakaryocyte apoptosis to impaired megakaryocyte and platelet production in immune thrombocytopenia



14. Assay Summary

Total procedure length: 3h45mn

Add 100µl sample and diluted standard and 50µl Biotinylated anti-TRAIL



Incubate 3 hours at room temperature



Wash three times



Add 100µl of Streptavidin-HRP



Incubate 30min at room temperature



Wash three times



Add 100 µl of ready-to-use TMB
Protect from light. Let the color develop for 10-20 mn.



Add 100 H₂SO₄



Read Absorbance at 450 nm



Warranty Information

Eagle Biosciences, Inc. warrants its Product(s) to operate or perform substantially in conformance with its specifications, as set forth in the accompanying package insert. This warranty is expressly limited to the refund of the price of any defective Product or the replacement of any defective Product with new Product. This warranty applies only when the Buyer gives written notice to the Eagle Biosciences within the expiration period of the Product(s) by the Buyer. In addition, Eagle Biosciences has no obligation to replace Product(s) as result of a) Buyer negligence, fault, or misuse, b) improper use, c) improper storage and handling, d) intentional damage, or e) event of force majeure, acts of God, or accident.

Eagle Biosciences makes no warranties, either expressed or implied, except as provided herein, including without limitation thereof, warranties as to marketability, merchantability, fitness for a particular purpose or use, or non-infringement of any intellectual property rights. In no event shall the company be liable for any indirect, incidental, or consequential damages of any nature, or losses or expenses resulting from any defective product or the use of any product. Product(s) may not be resold, modified, or altered for resale without prior written approval from Eagle Biosciences, Inc.

For further information about this kit, its application or the procedures in this kit, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.