INSTRUCTIONS

Controlled Protein-Protein Crosslinking Kit

23456

1298.3

Number	Description
23456	**Controlled Protein-Protein Crosslinking Kit**

Kit Contents:

Sulfo-SMCC Crosslinking Agent, 2mg
Molecular Weight: 436.37
Spacer Arm Length: 11.6Å

Activation/Conjugation Buffer (10X), 20mL, contains sodium phosphate and EDTA

BupH™ Phosphate Buffered Saline Pack, 2 packs, each pack yields 500mL of 0.1M phosphate, 0.15M sodium chloride; pH 7.2

2-Mercaptoethylamine•HCl, 6mg

Immobilized Reductant, 0.2mL

SATA, 2mg
Molecular Weight: 231.22
Spacer Arm Length: 2.8Å

Dimethylformamide, 1mL

Hydroxylamine•HCl, 5mg

Cysteine•HCl, 20mg
Molecular Weight: 175.6

Ellman's Reagent (5,5'-dithio-bis-(2-nitrobenzoic acid), 2mg

Dextran Desalting Column, 5K MWCO, 2 × 10mL, for sample volumes < 1.2mL

Column Extender, 2 each

Bottom Plugs, 2 each

Porous Discs, 2 each

Porous Disc Insertion Tool

Storage: Upon receipt store kit at 4°C. Kit is shipped at ambient temperature.

Contents

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Introduction

The Controlled Protein-Protein Crosslinking Kit provides a quick, convenient way to couple any two proteins through an amine (-NH_2) functional group on one protein and a sulfhydryl (-SH) group on the other using Sulfo-SMCC as the crosslinking agent. Sulfo-SMCC is a heterobifunctional crosslinker that contains an *N*-hydroxysuccinimide (NHS) ester and a maleimide group. NHS esters react with primary amines at pH 7-9 to form covalent amide bonds. Maleimides react with sulfhydryl groups at pH 6.5-7.5 to form stable thioether bonds. This kit contains all the necessary reagents for modifying proteins to contain sulfhydryl groups and for determining reaction efficiency. Additionally, instructions are designed to provide a successful crosslinking experience regardless of level of expertise. The crosslinking strategies incorporated in these instructions are outlined in Figure 1.

Note: Please review this document in its entirety before beginning any of the included procedures.

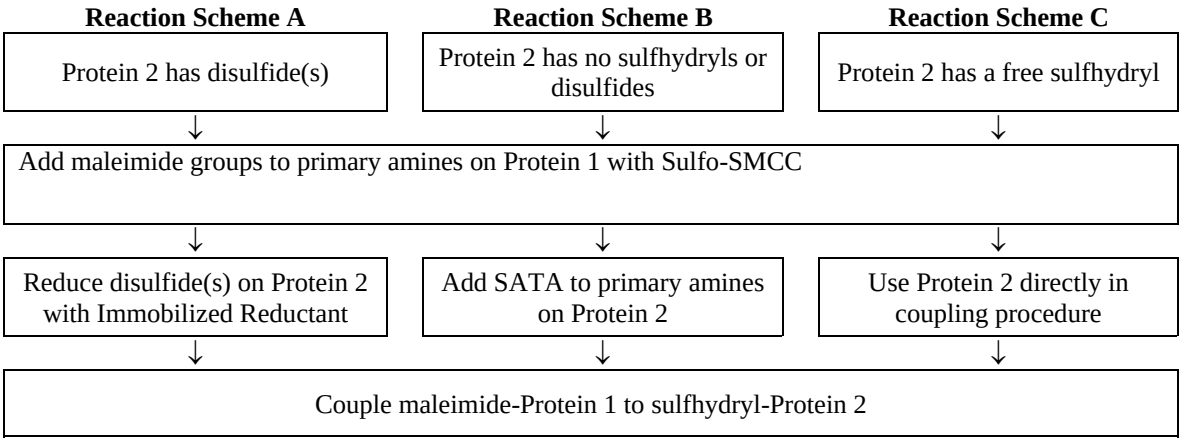

Figure 1. Coupling strategies for the Controlled Protein-Protein Crosslinking Kit. The steps necessary to couple two proteins depend on the structure of Protein 2. If Protein 2 does not have a free sulfhydryl group available, either reduce an existing disulfide, as in reaction scheme A or add SATA to a primary amine, as in reaction scheme B. If Protein 2 has a free sulfhydryl, then it can be directly conjugated, as in reaction scheme C.

Protein Requirements

- The two proteins to be crosslinked must be highly purified and the molecular weights must be known.
- Protein 1 must have an available primary amine (i.e., N-terminus or a lysine residue) to react with the Sulfo-SMCC (Figure 2).
- Protein 2 must have one of the following criteria: an available sulfhydryl group (-SH) as determined by Ellman's Reagent (see Procedure IV); a disulfide bond that can be reduced to generate free -SH groups (Figure 3); or an available primary amine so that the required -SH group can be added chemically (Figure 4).

Material Preparation

Phosphate Buffered Saline (PBS)	Dissolve the dry-blend buffer with 500mL of ultrapure water. For long-term storage of excess buffer, sterile-filter the solution or add sodium azide to a final concentration of 0.02% and store at 4°C.
Proteins	Dissolve 0.5-5.0mg of protein 1 and protein 2 with up to 1mL of PBS. For proteins already in solution, make a 1:1 dilution of the protein with PBS or dialyze against PBS. Reconstitute proteins containing free sulfhydryls immediately before performing the crosslinking procedure to minimize disulfide formation. **Note:** If proteins are in buffers that contains primary amines (e.g., Tris or glycine), these compounds must be thoroughly removed by dialysis or desalting, as they will quench the Sulfo-SMCC and SATA reactions.

I. Procedure for Maleimide Activation of Protein 1 (60 minutes)

Note: In the following reaction (Figure 2), five-fold molar excess of Sulfo-SMCC is added to the protein, which generally results in 1-3 moles of maleimide per mole of protein. See Table 1 in the Appendix for the volume of Sulfo-SMCC in PBS to add per milliliter of Protein 1 to achieve five-fold excess.

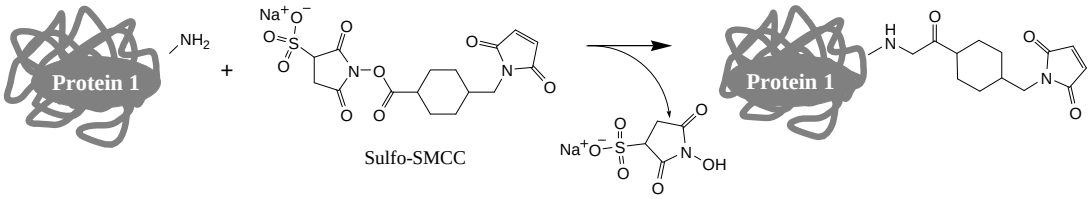

Figure 2. Reaction scheme for maleimide activation of protein 1. The Sulfo NHS ester of Sulfo-SMCC reacts with primary amines on lysine residues resulting in the protein containing an available sulfhydryl-reactive maleimide group.

1. Invert the desalting column several times to suspend the resin. Position the column upright in a test tube or clamp and allow the resin to settle for several minutes.
2. Remove the top cap from the column and carefully pipette the storage solution (contains 0.02% sodium azide) until 5-10mm of solution remains above the resin bed.
3. (Optional) Using the open end of the supplied porous disc insertion tool, insert and slide a porous disc to within 1mm of the resin bed. The top porous disc provides a stop-flow function that prevents disturbance and drying of the resin bed during use.
4. Twist off the column's bottom end tab.
5. Equilibrate column by adding 30mL of PBS and allowing it to flow through.
6. Add 2mL of PBS to the vial containing 2mg Sulfo-SMCC and mix thoroughly to dissolve.
7. Immediately add ~5-fold molar excess (see Table 1) of the Sulfo-SMCC solution to Protein 1 and incubate for 30 minutes at room temperature.
8. To remove nonreacted Sulfo-SMCC, apply ~1mL of the maleimide-Protein 1 reaction mixture to the equilibrated desalting column.
9. Add PBS to the desalting column. Collect 0.5mL fractions and measure the absorbance at 280nm of each fraction to locate the protein peak. Pool fractions that contain most of the protein.
10. Determine concentration of the maleimide-Protein 1 (i.e., the pooled fractions) by comparing its absorbance at 280mn with the absorbance of the original protein solution. Alternatively, determine protein concentration using the Thermo Scientific Coomassie Plus (Bradford) Assay Kit (Product No. 23236).
11. Use the maleimide-Protein 1 for the protein-protein crosslinking procedure (Procedure III, Figure 5). To determine the maleimide content of Protein 1, perform Procedure V.

    Stopping Point: The maleimide-activated protein may be stored frozen before or after desalting provided the protein is not adversely affected by freezing.

II. Procedures for Sulfhydryl Modification of Protein 2

Protocol for Reaction Scheme A: Reduce Disulfide Bonds (90 minutes)

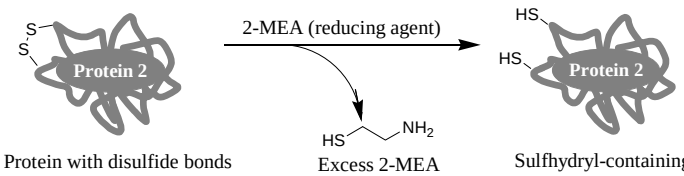

Figure 3. Reaction scheme for disulfide reduction of Protein 2. Immobilized reductant containing 2-MEA reduces disulfide bonds resulting in available sulfhydryl groups able to react with maleimides.

1. Prepare PBS-EDTA by mixing 90mL PBS, pH 7.2, with 10mL Conjugation Buffer (10X).

2. Add 100µL Conjugation Buffer (10X) to the 6mg of 2-Mercaptoethylamine•HCl (MEA). Mix thoroughly to dissolve.
3. To activate the Immobilized Reductant, add 25µL of the MEA solution to the vial of Immobilized Reductant and mix thoroughly. Incubate the mixture for 15 minutes.
4. Centrifuge the mixture to pellet the activated Immobilized Reductant. Remove and discard the supernatant.
5. Suspend and wash the activated Immobilized Reductant four times using 1mL of PBS-EDTA for each wash. Remove the wash buffer by centrifugation.
6. Add 1mL of Protein 2 and 50µL of Conjugation Buffer (10X) to the activated Immobilized Reductant and mix thoroughly. Incubate the mixture for 60 minutes at 37°C.

   Note: Higher temperature (up to 65°C) may be required to unfold protein and completely reduce buried disulfide bonds. When incubating at 65°C, shorten incubation time to 10-20 minutes.
7. Briefly centrifuge mixture to pellet the Immobilized Reductant.
8. Remove and retain supernatant containing sulfhydryl-Protein 2 for the crosslinking procedure outlined in Procedure III. Sulfhydryl groups are unstable, therefore, once sulfhydryls are generated, initiate the crosslinking procedure within 1 hour. Refrigerate the reduced sulfhydryl-Protein 2 until ready to initiate crosslinking. To determine sulfhydryl content, perform Procedure IV.

Protocol for Reaction Scheme B: Add Sulfhydryl Groups by Reaction with SATA (180 minutes)

Note: In the following reaction (Figure 4), 10-fold molar excess of SATA is added to Protein 2, which generally results in 1-5 sulfhydryl groups per protein. See Table 2 in the Appendix for the volume of SATA in DMF to add to Protein 2 to achieve 10-fold excess.

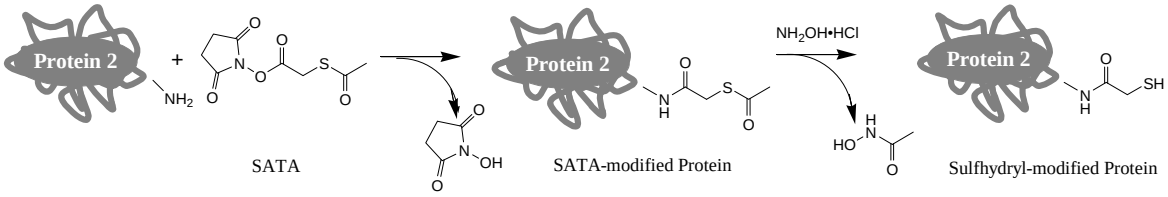

Figure 4. Reaction scheme for sulfhydryl modification of Protein 2. The NHS ester of SATA reacts with primary amines on lysine residues. Hydroxylamine de-protects the latent sulfhydryl groups, which are able to react with maleimides.

1. Prepare PBS-EDTA by mixing 90mL PBS, pH 7.2, with 10mL Conjugation Buffer (10X).
2. Invert the desalting column several times to suspend the resin, then position the column upright in a test tube or clamp and allow the resin to settle for several minutes.
3. Remove the top cap from the column and carefully pipette the storage solution (contains 0.02% sodium azide) until 5-10mm of solution remains above the resin bed.
4. (Optional) Using the open end of the supplied porous disc insertion tool, insert and slide a porous disc to within 1mm of the resin bed. A top porous disc provides a “stop-flow” function that prevents disturbance and drying of the resin bed during use.
5. Twist off column bottom end tab. Equilibrate column by adding 30mL of PBS-EDTA and allowing it to flow through.
6. Add 500µL of DMF to the vial containing 2mg of SATA and mix thoroughly to dissolve.
7. Add a 10-fold molar excess of SATA/DMF solution to 1mL of Protein 2 solution (see Table 2). Incubate reaction for 30 minutes at room temperature.

   Note: The SATA-modified protein may be stored at -20°C provided protein is not adversely affected by freezing.
8. Add 100µL of Conjugation Buffer (10X) to the vial containing 5mg Hydroxylamine•HCl and mix thoroughly to dissolve.
9. To de-protect the latent sulfhydryl, add 100µL of hydroxylamine solution to the SATA-modified Protein 2. Incubate mixture for 2 hours at room temperature.
10. To remove nonreacted reagents, apply ~1mL of de-protected sulfhydryl-Protein 2 to the equilibrated desalting column.

11. Add PBS-EDTA to the Desalting Column. Collect 1mL fractions and measure the absorbance at 280nm of each fraction to locate the protein. Pool fractions containing most of the protein.
12. Determine concentration of sulfhydryl-Protein 2 (i.e., the pooled protein sample) by comparing its absorbance at 280 nm with the absorbance of the original protein solution. Alternatively, determine protein concentration using the Coomassie Plus (Bradford) Assay Kit (Product No. 23236).
13. Use sulfhydryl-Protein 2 for crosslinking as outlined in Procedure III. Sulfhydryl groups are unstable, therefore, initiate the crosslinking procedure within 1 hour of sulfhydryl modification. Refrigerate the sulfhydryl-Protein 2 until ready to initiate the crosslinking reaction. To determine sulfhydryl content, perform Procedure IV.

III. Procedure for Crosslinking Maleimide-Protein 1 to Sulfhydryl-Protein 2 (60 minutes)

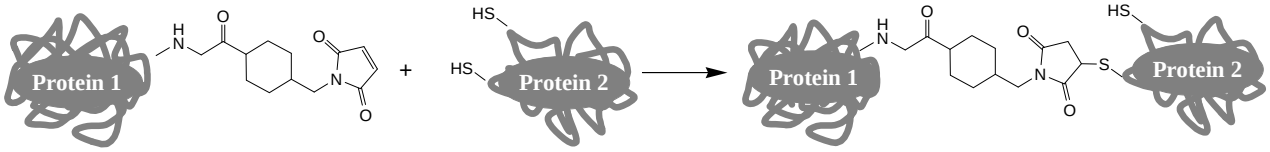

Figure 5. Reaction scheme for crosslinking maleimide-activated Protein 1 to sulfhydryl-containing Protein 2. The double bond of the maleimide reacts with sulfhydryl groups at pH 6.5-7.5 to form a stable thioether bond.

1. Mix maleimide-Protein 1 and sulfhydryl-Protein 2 in approximately equal molar amounts. Generally, if the concentrations of the proteins are approximately equal, reacting equal mass amounts of the two proteins will achieve sufficient crosslinking.
2. Incubate mixture for 60 minutes at room temperature.
3. Store conjugate at 4°C. For long-term storage (i.e., > 1 month), mix conjugate with an equal volume of 50% ethylene glycol or 100% glycerol and store at -20°C.

IV. Procedure for Determining Sulfhydryl Content

1. Prepare PBS-EDTA by mixing 90mL PBS, pH 7.2, with 10mL Conjugation Buffer (10X).
2. Add 1mL PBS-EDTA solution to the vial containing 2mg Ellman's Reagent and mix for 5-10 minutes to dissolve completely. This 2mg/mL stock solution of Ellman's Reagent is stable for up to 1 week stored frozen.
3. Dilute a portion of the stock Ellman's Reagent 1:2 with PBS-EDTA (i.e., add 1 part Ellman's Reagent to 2 parts PBS-EDTA) to make the 1.0mg/mL working Ellman's Reagent.
4. To prepare the cysteine (-SH group) standards, add 2mL PBS-EDTA solution to the vial containing 20mg cysteine and mix thoroughly to dissolve. This 10mg/mL stock cysteine solution is stable for up to 1 week stored frozen.
5. Dilute a portion of the stock cysteine 1:200 with PBS-EDTA to make a 0.05mg/mL working cysteine solution (285nmol/mL).
6. Prepare 10 serial dilutions of the working cysteine solution (i.e., dilute the 0.05mg/mL working cysteine 1:1 with PBS-EDTA solution, then dilute that 0.025mg/mL solution 1:1, etc.). These 10 dilutions will be used as cysteine standards.
7. In separate wells of a microplate, pipette duplicate samples containing 100µL of each of the following:
   - PBS-EDTA solution (reagent blank)
   - Each of the 10 cysteine standards
   - Sulfhydryl-Protein 2

   Note: If the concentration of sulfhydryl-Protein 2 is low (i.e., 0.5-1.0mg/mL), use 200µL samples rather than 100µL. Also use 200µL of PBS-EDTA solution and 200µL of each standard.
8. To each 100µL sample in the microplate, add 10µL of Ellman's Reagent (0.5mg/mL) and mix well.

   Note: If using 200µL samples, add 20µL working Ellman's Reagent to each sample.
9. Use a plate reader to measure the absorbance of each well at 405 nm. Absorbance measurement at 412nm or 420nm is also acceptable.

10. Calculate sulfhydryl content by either of the following methods:
    - Plot the absorbance values of the cysteine standards. Determine slope of the curve and the Y-intercept and determine sulfhydryl content as follows:

$$\text{sulfhydryl content (moles)} = \frac{A_{405} \text{ of the sample - Y-intercept}}{\text{slope}}$$

    - Alternatively, divide the absorbance value of each cysteine standard by its cysteine content (mole cysteine) then calculate the average absorbance units (average AU per mole of cysteine) for all the standards. Determine sulfhydryl content as follows:

$$\text{sulfhydryl content (moles)} = \frac{A_{405} \text{ of the sample}}{\text{average AU per mole cysteine}}$$

11. Using the results from Step 10 and the molecular weight of the protein, calculate the number of sulfhydryls per protein molecule as follows:

$$\text{sulfhydryl/protein} = \frac{\text{moles of cysteine in sample}}{\text{moles of protein}}$$

V. Procedure for Indirectly Determining Maleimide Content

1. Prepare PBS-EDTA by mixing 90mL PBS with 10mL Conjugation Buffer (10X).
2. Prepare cysteine standards and reagent blank as in Steps 2-6 of Procedure IV. Use 200µL of each standard and blank per well.
3. In a separate well, mix 100µL of working cysteine (0.05mg/mL) reagent and 100µL of maleimide-Protein 1. Incubate plate for 1 hour at room temperature.
4. In separate wells, prepare the following two controls:
    - 200µL of maleimide-Protein 1
    - 100µL of working cysteine (0.05mg/mL) + 100µL PBS-EDTA
5. Incubate plate for 1 hour at room temperature. Add 20µL working Ellman's Reagent (0.5mg/mL) to each well.
6. Measure the absorbance of each well at 405 nm. Divide the absorbance value of each cysteine standard by its cysteine content (mole cysteine) then calculate the average absorbance units (average AU per mole of cysteine) for all standards.
7. The maleimide content of the protein (moles of maleimide/mole of Protein 1) will equal the number of moles of cysteine bound to the protein. Use the average AU per mole of cysteine to determine sulfhydryl content as follows:
    - *Calc 1*: Original moles of cysteine added to maleimide-Protein 1:

$$\frac{A_{405} \text{ of the cysteine control}}{\text{average AU per mole of cysteine}}$$

    - *Calc 2*: Moles of cysteine remaining after incubation with maleimide-Protein 1:

$$\frac{(A_{405} \text{ of the protein:cysteine mixture}) - (A_{405} \text{ of the protein})}{\text{average AU per mole of cysteine}}$$

    - Moles of maleimide/mole of Protein 1 = moles of cysteine bound to the protein sample = *Calc 1 – Calc 2*.

Appendix

Adding the amount of Sulfo-SMCC indicated in Table 1 generally results in 1-3 maleimides per protein (mole/mole). Actual maleimide incorporation depends on the number of accessible amines on the protein.

Table 1. Volume (µL) of Sulfo-SMCC in PBS to add per milliliter of a protein of known concentration and molecular weight (MW). For example, for a 50 kDa protein at 2mg/mL, add 88µL of the Sulfo-SMCC solution to 1mL of protein.

Protein	Protein Concentration (mg/mL)					
MW × 10^3	0.5	1	2	3	4	5
5	220	440	880	1,200	–	–
10	110	220	440	660	880	1,100
25	44	88	176	264	352	868
50	22	44	88	132	176	434
100	11	22	44	66	88	217
200	6	11	22	33	44	109

Adding the amount of SATA (10-fold molar excess) indicated in Table 2 generally results in 1-5 sulfhydryl groups per protein (mole/mole). Actual sulfhydryl incorporation depends on the number of accessible amines on the protein.

Table 2. Volume (µL) of SATA in DMF to add per milliliter of protein of known concentration and molecular weight (MW). For example, for a 50 kDa protein at a concentration of 2mg/mL, add 22µL of SATA/DMF to 1mL of protein.

Protein	Protein Concentration (mg/mL)					
MW × 10^3	0.5	1	2	3	4	5
5	56	110	220	280	440	–
10	28	55	110	170	220	280
25	11	22	44	68	88	112
50	6	11	22	34	44	56
100	3	6	11	17	22	28
200	1.4	2.8	6	9	11	14

Additional Information

Please visit the web site for additional information relating to this product including the following items:

- Tech Tip: Protein stability and storage
- Tech Tip: Determine reactivity of NHS ester biotinylation and crosslinking reagents
- Tech Tip: Attach an antibody onto glass, silica or quartz surface

Related Thermo Scientific Products

22322 **Sulfo-SMCC** (sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate), 50mg

26102 **SATA** (*N*-succinimidyl S-acetylthioacetate), 50mg

22582 **Ellman's Reagent** (5,5'-dithio-bis-(2-nitrobenzoic acid), 5 g

17904 **Glycerol,** 1 L

29810 **Ethylene Glycol (50% aqueous),** 200mL

References

Hermanson, Greg. T. (1996). *Bioconjugate Techniques*. Academic Press: London.

Riddle, P.W. *et al*, (1983). Reassessment of Ellman's reagent. *Methods Enzymol.* **91(8):**49-60.

Duncan, R.J.S., *et al.* (1983). A new reagent which may be used to introduce sulfhydryl groups into proteins, and its use in the preparation of conjugates for immunoassay. *Anal. Biochem.* **132:**68-73.

Jocelyn, P.C. (1972). *Biochemistry of the SH Group*. Academic Press: London, 40-42.

Imagawa, M., *et al.* (1982). Characteristics and evaluation of antibody-horseradish peroxidase conjugates prepared by using a maleimide compound, glutaraldehyde, and periodate. *J. Appl. Biochem.* **4:**41-57.

Hashida, S., *et al.* (1984). More useful maleimide compounds for the conjugation of Fab' to horseradish peroxidase through thiol groups in the hinge. *J. Appl. Biochem.* **6:**56-63.

Products described herein containing one or more Sulfo-NHS ester moiety is covered by one or both of the following US Patents: 5,872,261 and 5,892,057.

This product ("Product") is warranted to operate or perform substantially in conformance with published Product specifications in effect at the time of sale, as set forth in the Product documentation, specifications and/or accompanying package inserts ("Documentation") and to be free from defects in material and workmanship. Unless otherwise expressly authorized in writing, Products are supplied for research use only. No claim of suitability for use in applications regulated by FDA is made. The warranty provided herein is valid only when used by properly trained individuals. Unless otherwise stated in the Documentation, this warranty is limited to one year from date of shipment when the Product is subjected to normal, proper and intended usage. This warranty does not extend to anyone other than the original purchaser of the Product ("Buyer").

No other warranties, express or implied, are granted, including without limitation, implied warranties of merchantability, fitness for any particular purpose, or non infringement. Buyer's exclusive remedy for non-conforming Products during the warranty period is limited to replacement of or refund for the non-conforming Product(s).

There is no obligation to replace Products as the result of (i) accident, disaster or event of force majeure, (ii) misuse, fault or negligence of or by Buyer, (iii) use of the Products in a manner for which they were not designed, or (iv) improper storage and handling of the Products.

Current versions of product instructions are available at *www.thermo.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.