Replenisher Reagents for TACS® 2 TdT-Apoptosis Detection Kits

30 samples

Cat#: 4810-30-R
Cat#: 4810-30-CK

Replenisher Reagents for TACS® 2 TdT-Apoptosis Detection Kits

Catalog #s: 4810-30-R; 4810-30-CK

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I. Quick Reference Procedure for TACS® 2 TdT-Replenisher (Cat# 4810-30-R):

Read through the complete *Instructions for Use* prior to using this kit. Some incubation times and washes must be optimized using the controls to maximize labeling. All steps are performed at room temperature unless otherwise noted.

This page is designed to be photocopied and used as a checklist:

- ☐ 1. Immerse hydrated, fixed, and immobilized sample in 1X PBS for 10 minutes.
- ☐ 2. Cover sample with 50 µl of Proteinase K Solution (page 2) for 15-30 minutes, **or** cover sample with 50 µl of Cytonin™ (Cat# 4876-05-01) for 30 minutes.
- ☐ 3. Wash two times in deionized water, 2 minutes each.
- ☐ 4. Immerse sample in Quenching Solution for 5 minutes (page 2).
- ☐ 5. Wash sample in 1X PBS for 1 minute.
- ☐ 6. Immerse sample in 1X TdT Labeling Buffer for 5 minutes (page 3).
- ☐ 7. Cover sample with 50 µl of Labeling Reaction Mix (page 3). Incubate for 60 minutes at 37 °C in a humidity chamber (page 8).
- ☐ 8. Immerse sample in 1X TdT Stop Buffer (page 3) for 5 minutes.
- ☐ 9. Wash two times in 1X PBS, 2 minutes each.

A. If staining with DAB:

- ☐ 10a. Cover sample with 50 µl of Strep-HRP in 1X PBS (page 3), and incubate for 10 minutes at 37 °C in a Humidity Chamber to avoid evaporation (page 8).
- ☐ 11a. Wash two times in 1X PBS, 2 minutes each.
- ☐ 12a. Immerse in DAB Solution for 2 to 7 minutes.
- ☐ 13a. Wash two times in deionized H_2O, 2 minutes each, counterstain, and mount according to the appropriate complete instructions for use.

B. If staining with TACS Blue Label™:

- ☐ 10a. Cover sample with Strep-HRP in Blue-Strep diluent Solution (page 3), and incubate for 10 minutes at 37 °C in a Humidity Chamber to avoid evaporation (page 8).
- ☐ 11b. Wash two times in 1X PBS, 2 minutes each.
- ☐ 12b. Cover sections with TACS Blue Label™, wash, counterstain and mount according to the appropriate complete instructions for use.

II. Product Description

Trevigen's **TACS® 2 TdT-Replinisher kit** provides only the reagents that are most rapidly exhausted from the complete TACS® 2 TdT kits. The replenisher is also useful when using alternative detection and counterstaining methods in double-labeling experiments. Please ensure familiarity with the complete "Instructions for Use" for the TACS® 2 TdT kit of interest before relying on this abbreviated version.

III. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical and toxicological properties of the provided products may not yet have been fully investigated, therefore, Trevigen recommends the use of gloves, lab coats and eye protection while using these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products.

IV. Materials Supplied

Kit*	Component	Quantity	Storage	Cat#
R	Proteinase K Solution	30 µl	-20 °C	4800-30-01
C	10X TdT Labeling Buffer	100 ml	4 °C	4810-30-02
C	10X TdT Stop Buffer	100 ml	4 °C	4810-30-03
R,C	TdT dNTP Mix	30 µl	-20 °C	4810-30-04
R,C	TdT Enzyme	30 µl	-20 °C	4810-30-05
R,C	Strep-HRP	30 µl	4 °C	4800-30-06
R,C	50X Co^{2+}	30 µl	-20 °C	4810-30-09
R,C	50X Mg^{2+}	30 µl	-20 °C	4810-30-10
R,C	50X Mn^{2+}	30 µl	-20 °C	4810-30-14

* R = Replenisher; C = Core

V. Reagent Preparation

Reagents marked with an asterisk (*) should be prepared immediately before use. The volumes given for each reagent are based on processing samples of up to 4 cm^2 immobilized on glass slides. Different configurations of chamber slides, culture plates, free floating sections, and the use of glass cover slips may require adjustments to the stated volumes.

1. *Proteinase K Solution (included in replenisher kit only)

Use 50 µl of Proteinase K Solution per sample. Store on ice. Thaw provided Proteinase K (Cat# 4800-30-01) at room temperature, then place on ice. To prepare add:

	2 samples	10 samples	n samples
Deionized water	100 µl	500 µl	n x 50 µl
Proteinase K (Cat# 4800-30-01)	2 µl	10 µl	n x 1 µl

Under some circumstances, the Proteinase K may be used at a 1:200 dilution. For guidelines please refer to pages 5 and 6 (Appendices B and C).

2. *Quenching Solution

Use 50 ml of Quenching Solution to process 1 to 10 samples. To prepare add:

Methanol	45 ml
30% hydrogen peroxide	5 ml

Always use fresh 30% hydrogen peroxide. It is recommended that 6 ml aliquots of fresh 30% hydrogen peroxide be made and stored at 4 °C. For each labeling procedure, use a fresh 30% hydrogen peroxide aliquot then discard the unused portion.

3. 1X TdT Labeling Buffer (included in core kit only)

Dilute the 10X TdT Labeling Buffer (Cat# 4810-30-02) to 1X using distilled water. Leave at room temperature until use. Use 50 ml of 1X Labeling Buffer to process 1 to 10 samples. Remove an aliquot of 50 µl per sample for preparing the Labeling Reaction Mix (see below) and place on ice.

4. *Labeling Reaction Mix

Thaw TdT dNTP Mix (Cat# 4810-30-04) at room temperature, then place on ice. To maintain optimal enzyme activity, remove the TdT Enzyme (Cat# 4810-30-05) tube from freezer only long enough to pipette the required volume. Alternatively, place the TdT Enzyme in a -20 °C freezer block. Prepare the Labeling Reaction Mix just before use and keep the prepared reaction mix on ice. Prepare 50 µl per sample in the sequence given below:

	2 samples	10 samples	n sample
TdT dNTP Mix (Cat# 4828-30-04)	2 µl	10 µl	n x 1 µl
TdT Enzyme (Cat# 4810-30-05)	2 µl	10 µl	n x 1 µl
50X Cation Stock (see below)	2 µl	10 µl	n x 1 µl
1X TdT Labeling Buffer (item 3)	100 µl	500 µl	n x 50 µl

To select the proper 50X cation stock see Appendix B.

5. 1X TdT Stop Buffer (included in core kit only)

Dilute the 10X TdT Stop Buffer (Cat# 4810-30-03) to 1X using distilled water. Leave at room temperature until use. Use 50 ml of 1X TdT Stop Buffer to process 1 to 10 samples.

6a. *Strep-HRP Solution if staining with DAB

Use 50 µl of Strep-HRP Solution per sample. Store prepared Strep-HRP Solution at room temperature until use. To prepare add:

	2 samples	10 samples	n samples
1X PBS	100 µl	500 µl	n x 50 µl
Strep-HRP (Cat# 4800-30-06)	2 µl	10 µl	n x 1 µl

6b. *Strep-HRP Solution if staining with Blue Label

Use 50 µl of Strep-HRP Solution per sample. Store prepared Strep-HRP Solution at room temperature until use. To prepare add:

	Up to 15 samples
Blue Strep-Diluent (Cat# 4800-30-12)	500 µl
Strep-HRP (Cat# 4800-30-06)	1 µl

VI. References

1. Apoptosis: the molecular basis of cell death. Current communications in cell and molecular biology, Vol 3. Ed. L. D. Tomei and F. O. Cope (1991) Cold Spring Harbor Laboratory Press, New York.
2. Apoptosis II: the molecular basis of apoptosis in disease. Current communications in cell and molecular biology, Vol 8. Ed. L. D. Tomei and F. O. Cope (1994) Cold Spring Harbor Laboratory Press, New York.
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4. Gavrieli, Y., Y. Sherman, S.A. Ben-Sasson (1992) Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. J. Cell Biol.**119:**493-501.

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6. Migheli, A., A. Attanasio, W.-H. Lee, S.A. Bayer and B. Ghetti (1995) Detection of apoptosis in weaver cerebellum by electron microscopic *in situ* end-labeling of fragmented DNA. Neurosci. Lett. **199**:53-56
7. Negoescu, A. et al., *In situ* apoptotic cell labeling by the TUNEL method: improvements and evaluation on cell preparation. (1996) J. Histochem. Cytochem. **44**: 959-968.
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VII. Related Products Available from Trevigen

Kits

Catalog #	Description	Size
4810-30-K	TACS® 2 TdT-DAB *In Situ* Apoptosis Detection Kit	30 samples
4811-30-K	TACS® 2 TdT Blue Label *In Situ* Apoptosis Detection Kit	30 samples
4812-30-K	TACS® 2 TdT Fluorescein Apoptosis Detection Kit	30 samples
4817-60-K	FlowTACS™ Kit	60 samples
4822-96-K	HT TiterTACS™ Assay Kit	96 tests
4830-01-K	TACS® Annexin V FITC Kit	100 samples
4835-01-K	TACS® Annexin V Biotin Kit	100 samples
6300-100-K	DePsipher™ Mitochondrial Potential Assay Kit	100 tests
6305-100-K	MitoShift™ Mitochondrial Potential Assay Kit	100 tests
4815-30-K	TumorTACS™ *In Situ* Apoptosis Detection Kit	30 samples
4823-30-K	NeuroTACS™ *In Situ* Apoptosis Detection Kit	30 samples
4827-30-K	CardioTACS ™ *In Situ* Apoptosis Detection Kit	30 samples
4829-30-K	DermaTACS™ *In Situ* Apoptosis Detection Kit	30 samples
4826-30-K	VasoTACS™ *In Situ* Apoptosis Detection Kit	30 samples
4828-30-BK	TACS•XL® Blue Label *In Situ* Apoptosis Detection Kit	30 samples
4892-010-K	Cultrex® Calcein-AM Cell Viability Kit	1000 tests
4850-20-ET	TACS® Apoptotic DNA Laddering Kit EtBr	20 samples
4684-096-K	HT Colorimetric PARP Apoptosis Assay	96 samples
4685-096-K	HT Chemiluminescent PARP Apoptosis Assay	96 samples

Antibodies

Catalog #	Description	Size
4411-PC-100	Anti-Phosphorylated Histone-γ-H2AX polyclonal	100 µl
2291-MC-100	Anti-human-Bcl-2 mAb (clone YTH-8C8)	100 µg
2300-MC-100	Anti-human-Bcl-XL mAb (clone YTH-2H12)	100 µg
2280-MC-100	Anti-mouse-Bax mAb (clone YTH-5B7)	100 µg
2281-MC-100	Anti-human-Bax mAb (clone YTH-6A7)	100 µg
2282-MC-100	Anti-human-Bax mAb (clone YTH-2D2)	100 µg
2305-PC-100	Anti-cleaved human/mouse-Caspase 3 polyclonal	40 µg
6361-PC-100	Anti-human/mouse-PBR polyclonal	100 µl
6370-MC-100	Anti-human/murine-Cytochrome C	100 µg
6380-MC-100	Anti-human/murine-Holocytochrome C	100 µg
4335-MC-100	Anti-PAR polymer mAb (10HA)	100 µl
4336-BPC-100	Anti- PAR polymer polyclonal	100 µl
4338-MC-50	Anti-human/murine-PARP mAb (clone C2-10)	50 µg

Accessories

Catalog #	Description	Size
4800-30-40	**Tissue Control Slides**	**2 ea.**
4800-30-20	**Cell Culture Control Slides**	**2 ea.**
4864-100	**Treated Glass Microscope Slides w/3 sample Hydrophobic Barrier**	**100 ea.**
4867-100	**Hydrophobic Coverslips**	**100 ea.**
4862-10	**Coverslips 24 x 60 mm, No. 1.5**	**2 ea.**
4865-25	**Mounting Medium**	**25 ml**
4800-30-14	**Strep-Fluorescein**	**30 µl**
4830-01-3	**10X Propidium Iodide**	**1 ml**
4870-500-6	**10X PBS**	**6 x 500 ml**
4869-500-6	**Apoptosis Grade™ H_2O**	**6 x 500 ml**
4820-30-13	**Blue Counterstain**	**50 ml**
4825-30-RL	**Red Label**	**30 samples**
4878-05-02	**Cytonin™ IHC**	**2 x 5 ml**

VIII. Appendices

Appendix A. Fixation Methods

There are several fixation methods commonly used that are appropriate for the protocol described in the Instructions for Use. Formaldehyde is the recommended fixative based on laboratory testing. However, other fixatives that maintain DNA integrity may be used. These include other cross linking agents such as paraformaldehyde and glutaraldehyde. Bouin's fixative should be avoided due to the picric acid content. Regardless of the fixative used, it is important not to fix cells and tissues for extended periods of time. Your fixation method will likely be dictated by immunocytochemistry protocols in double labeling experiments (see Appendix C: *Double Labeling Hints and Tips*, page 6). Post fixation in acetone, ethanol, or methanol is common in preparation of tissues and is usually compatible with TACS® 2 TdT DAB kits.

To store the immobilized fixed cells, i.e. on slides, chamber slides or coverslips, post-fix in 100% methanol after fixation, wash in PBS and then store in Cytonin™ (Cat# 4876-05-01) at 4 °C for up to one week. After storage, wash in 1X Labeling buffer then continue with the labeling reaction at the Quenching step (please see the *Instructions for Use,* provided with the original kit).

> **Note**: If cells are fixed using alcohol, e.g. ethanol, there will be leakage of small DNA fragments from apoptotic cells during storage and labeling intensity of apoptotic cells will be reduced.

Appendix B. Suggestions for Assay Optimization

The following table shows examples of conditions that have been used for labeling the tissues listed and acts as a guide only. Actual incubation times and the permeabilization method selected will require empirical determination for optimal results. When using paraffin-embedded sections, permeabilization is often a limiting step, therefore, start with a 1:50 dilution of proteinase K for permeabilization at 37 °C for 15 minutes. Try manganese cation in the labeling reaction. If background is high, reduce the proteinase K concentration and incubation time and temperature. Use cobalt cation in the labeling reaction. If insufficient permeabilization is suspected, incubate the sample with a 1:50 dilution of proteinase K at 37 °C for 30 minutes. When using fresh frozen sections, nominal permeabi-lizatrion is required, therefore start with Cytonin™ (cat# 4876-05-01) for permeabilization. If there is insufficient labeling, permeabilize with proteinase K at 1:200 for 15 minutes at 37 ˚C.

	Cytonin™	Pro K (1:50)	Pro K (1:200)	Mg2+	Co2+	Mn2+
Brain	•		•			•
Heart		•			•	•
Lung	•		•		•	
Liver		•			•	•
Kidney		•				•
Spleen		•			•	
Duodenium		•			•	
Colon	•	•			•	
Small Intestine	•	•			•	•
Large Intestine	•	•			•	•
Skin		•				•
Bone/Cartilage	•	•				•
Tumor		•		•	•	
Epithelium		•	•			•
Endothelium		•	•			•
Cultured Cells (stored dry)	•	•			•	•
Cultured Cells (fresh)	•		•		•	•

Note: use only one Cation.

Appendix C. Double Labeling Hints and Tips

The *in situ* labeling protocol described here is useful for double labeling experiments when the occurrence of apoptosis can be correlated with cellular antigens against which antibodies are available. **Note**: The antibody must recognize the fixed form of the antigen of interest.

The key to double labeling experiments is determining fixation and permeabilization conditions under which both antigen and DNA integrity is maintained. Appropriate fixatives for DNA labeling are recommended in Appendix A (previous page). Post-treatments used in immunocytochemistry to permeabilize or expose antigenic determinants include treatment with proteases, acid or base, detergent and microwaving. Permeabilization with Cytonin™ IHC (Cat# 4878-05-02) may be sufficient for many antibodies and additional treatment may not be needed. Protease treatment is not recommended on most samples because the sample will often disintegrate later during immunocytochemistry or DNA labeling. Strong acid or base treatment should be avoided. Microwaving is an option that has given excellent results in double labeling experiments but requires careful empirical determination for correct wattage, time and cooling cycles for each sample.

Empirically determine optimal conditions for immunohistochemistry and *in situ* detection of apoptosis in separate experiments first. Combine the two methodologies only after optimizing separately on the same samples. Plan carefully and include controls to allow interpretation of double labeled samples. Controls for immunohistochemistry may include omission of primary antibodies to determine binding of the secondary antibody. In addition, blocking the primary antibody binding site with antigens may establish and demonstrate specificity.

The selection of the color reaction products should be considered ahead of time. Trevigen offers Red Label (Cat# 4825-30-RL) for use with secondary antibodies conjugated to phosphatases. Red Label provides excellent contrast with DAB and Blue Counterstain (Cat# 4820-30-13).

A standard immunohistochemistry protocol is provided for using phosphatase-conjugated secondary antibody and color development with Red Label Solution. Antibody concen-trations, incubation times and temperatures and buffers may have to be optimized empirically for each system under study.

Note: Phosphatase-conjugated reagents are inhibited by PBS or other phosphate-containing buffers. Tris buffers should be substituted for PBS.

Method

1. After fixation, permeabilize with Cytonin™ IHC (Cat# 4878-05-02) at room temperature for 1 hour. Use Hydrophobic Coverslips and a humidity chamber (below) to prevent evaporation.
2. Wash 1 time in 100 mM Tris, pH 7.4.
3. Incubate at 4 °C overnight with primary antibody diluted in Cytonin™ IHC. Use Hydrophobic Coverslips and a humidity chamber to prevent evaporation.
4. Wash 3 times in 100 mM Tris, pH 7.4.
5. Incubate with phosphatase-conjugated secondary antibody diluted in Cytonin™ (Cat# 4876-05-01) at room temperature for 1 hour.
6. Wash 3 times in 100 mM Tris pH 7.4.
7. Prepare Red Label Solution:
   a. To a new microtube add 50 µl of deionized H_2O and 5 µl of Red Label Solution 1.
   b. To a new microtube add 1 µl each of Red Label Solution 2 and Red Label Solution 3, tap microtube to mix. Centrifuge briefly and let stand for 3 minutes at room temperature. Transfer this solution to microtube in Step 7a.
8. Cover sample with Prepared Red Label Solution and incubate for up to 30 minutes in the dark.
9. Wash in deionized water 3 times to stop reaction.
10. Proceed with *in situ* detection beginning with Quenching Solution step on page 11.

Many options are available for double labeling experiments. If the antigen is nuclear, carefully select the detection label and counterstains. Labeling nuclear antigens means the signal from the DNA labeling and immunocytochemistry will be in the same subcellar compartment and one signal may obscure the other. Similarly, many counterstains are not compatible with some color reaction products, e.g. Methyl Green cannot be used with Red Label. Some options are listed below.

- If a peroxidase-linked secondary antibody is preferred, use Quenching Solution prior to incubation with primary antibody and again prior to *in situ* detection of apoptosis. DAB Solution may be used for color reaction if alternative peroxidase based color development is used for detection of apoptosis.
- The Strep-HRP may be replaced with a streptavidin-phosphatase conjugate and developed using a phosphatase-based system such as Red Label.
- Similarly, fluorescent streptavidin conjugates and secondary antibodies may be used for a fluorescent read-out.

Appendix D. Electron Microscopy

The protocol given here can be adapted for electron microscopy. Both pre- and post-embedding labeling can be performed depending upon the system under study. For pre-embedding, fix sample and post-fix in osmium. After embedding and ultrathin sectioning, process sample for DNA labeling up to and including the washes of the labeling procedure prior to incubation with streptavidin. For detection of incorporated biotin use streptavidin conjugated to colloidal gold and incubate overnight at 4 °C. Stain with uranyl acetate.

For some samples post-embedding may be more convenient. Use fixed floating sections and process for *in situ* labeling up to and including the washes in the labeling procedure prior to streptavidin binding. Incubate in streptavidin conjugated to colloidal gold overnight at 4 °C. Wash, then proceed with standard embedding procedure and ultra thin sectioning. Contact Trevigen® for more details.

Appendix E. Humidity Chamber

To prevent evaporation it is recommended that incubations at 37 °C are carried out in a humidity chamber. A humidity chamber can be made using a plastic box with a tight fitting lid and two glass rods or other support. Place paper towel on the bottom of the box and wet thoroughly with water. Lay the glass rods parallel to each other and less than one slide length apart on the wet tissue. Position the slides on the glass rods and place the plastic box, with lid, in a 37 °C incubator. Ensure that the slides are horizontal.