TREVIGEN® **Instructions**

For Research Use Only. Not For Use In Diagnostic Procedures

FlowTACS™ Apoptosis Detection Kit

Detection of Apoptosis Using Flow Cytometry 60 samples

Catalog# 4817-60-K

FlowTACS™ Apoptosis Detection Kit

Catalog# 4817-60-K

60 Samples

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TACS: **T**revigen **A**poptotic **C**ell **S**ystem.

I. Quick Reference Procedure for FlowTACS™ Apoptosis Detection Kit (cat# 4817-60-K): Read through the complete *Instructions for Use* before following this protocol. Some incubation times and washes must be optimized by using the controls for determining optimal labeling conditions. **All steps are performed at room temperature (22-23 °C) unless otherwise specified**.

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

- ☐ 1. Pellet cells 1000 x g, for 5 minutes.
- ☐ 2. Resuspend in 1 ml 3.7% formaldehyde and incubate for 10 minutes.
- ☐ 3. Centrifuge 1500 x g, 5 minutes, discard fixative.
- ☐ 4. Resuspend in 100 ml Cytonin™. Incubate for 30 minutes.
- ☐ 5. Centrifuge as above and discard Cytonin™.
- ☐ 6. Wash in 1 ml 1X Labeling Buffer, centrifuge and discard buffer.
- ☐ 7. Resuspend in 25 µl Labeling Reaction mix (per 2 samples):
  - ☐ 1 µl 50X $MnCl_2$
  - ☐ 1 µl TdT dNTP mix
  - ☐ 1 µl TdT Enzyme
  - ☐ 50 µl 1X Labeling Buffer
- ☐ 8. Incubate 37 °C for 1 hour.
- ☐ 9. Wash in 1 ml 1X Stop Buffer, centrifuge and discard buffer.
- ☐ 10. Resuspend in 25 µl diluted Strep-Fluorescein (for 1-8 samples):
  - ☐ 200 µl 1X PBS
  - ☐ 1 µl Strep-Fluorescein
- ☐ 11. Incubate 10 minutes at room temperature in the dark.
- ☐ 12. Centrifuge and discard Strep-Fluorescein, resuspend in 500 µl PBS.
- ☐ 13. Add 10 ml Propidium Iodide/RNase solution.
- ☐ 14. Incubate for 5 minutes, analyze.

II. Background

Apoptosis is characterized by a number of intracellular phenomena such as membrane blebbing, chromatin condensation and nuclear DNA fragmentation. This DNA fragmentation provides the basis for several assays used to detect apoptosis *in situ*. During apoptosis, DNA cleavage occurs, most typically within the linker regions of DNA between nucleosomes. Extraction of DNA from apoptotic cells and analysis by agarose gel electrophoresis reveals a ladder pattern representing multiples of approximately 185 bp. This cleavage generates free 3'-hydroxyl residues that can be utilized by terminal deoxynucleotidyl transferase (TdT) in end-labeling reactions. Incorporation of biotinylated nucleotides into the DNA by TdT allows detection with streptavidin-linked conjugates.

Trevigen's **FlowTACS™ Apoptosis Detection Kit** is supplied with a streptavidin-FITC conjugate for detection of DNA fragmentation providing a fluorescent readout. The FlowTACS™ kit can be used for the detection of apoptosis using flow cytometry, direct visualization using a fluorescent microscope or for quantitation using a fluorometer. The protocol given here describes the use of the kit in flow cytometry and hints and tips for double labeling are provided in the appendix. Please see pages 9-11 for information on additional Trevigen products available for the study of apoptosis and cell death.

III. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical and toxicological properties of these 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.
3. The FlowTACS™ Apoptosis Detection Kit contains reagents that are harmful if swallowed or in contact with skin, and irritating to the eyes, respiratory system and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Material safety data sheets are available on request.

IV. Materials Supplied

Component	Quantity	Storage	Catalog #
Cytonin™	6 ml	4 °C	4876-05-01
10X TdT Labeling Buffer	20 ml	4 °C	4817-60-02
10X TdT Stop Buffer	20 ml	4 °C	4817-60-03
TdT dNTP Mix	35 µl	-20 °C	4810-30-04
TdT Enzyme	30 µl	-20 °C	4810-30-05
50X Mn^{2+}	50 µl	-20 °C	4810-30-14
Strep-Fluorescein	30 µl	-20°C In the dark	4800-30-14
Propidium Iodide/RNAse	1 ml	4 °C In the dark	4817-60-04
TACS-Nuclease™	15 µl	-20 °C	4800-30-15
TACS-Nuclease™ Buffer	1.5 ml	4 °C	4800-30-16

V. Materials/Equipment Required But Not Supplied

Equipment

1. 1 – 20 µl, 20 – 200 µl, and 200 – 1000 µl pipettors
2. 37 °C incubator
3. 50 and 500 ml graduated cylinders
4. -20 °C and 4 °C storage
5. ice bucket
6. Fluorescence microscope
7. Flow Cytometer
8. timer

Reagents

1. •Apoptosis Grade™ Water
2. •10X PBS
3. 37% formaldehyde

Disposables

1. •Treated Glass Microscope Slides (or alternative support)
2. 50 ml tubes
3. 1 – 200 µl and 200 – 1000 µl pipette tips
4. microcentrifuge tubes
5. ice
6. 1.5 and 10 ml serological pipettes
7. gloves
8. Flow Cytometry tubes
9. Aluminum Foil

•Available from Trevigen, Inc. (please see pages 9-11)

VI. Reagent Preparation

Reagents marked with an asterisk (*) should be prepared immediately before use.

1. 1X PBS

Please see page 11 for purchase or preparation of 10X PBS.
Approximately 500 ml of 1X PBS is used to process 1 to 10 slides. Dilute 10X PBS to 1X using distilled water. Store 1X PBS at room temperature.

2. *3.7% Buffered Formaldehyde

If required, 20 ml of freshly prepared fixative is used to process 20 samples. To prepare add:

37% formaldehyde	2 ml
10X PBS	2 ml
ddH_2O	16 ml

Wear gloves and exercise caution when handling formaldehyde solutions. Refer to Appendix B (page 11) for alternative fixation methods.

3. **Cytonin**™ (cat# 4876-05-01) is provided ready to use. If required, 100 µl of Cytonin™ is used per sample. Store at 4 °C. Discard if solution is cloudy.

4. 1X TdT Labeling Buffer

Dilute the 10X TdT Labeling Buffer (cat# 4817-60-02) to 1X using distilled water. Leave at room temperature until use. Remove an aliquot of 25 µl per sample for preparing the Labeling Reaction Mix (see below) and place on ice.

5. *Labeling Reaction Mix

Thaw **50X Mn^{2+}** (cat# 4810-30-14) and **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 ^{o}C freezer block. Prepare the Labeling Reaction Mix just before use and keep the prepared reaction mix on ice. Prepare 25 µl per sample in the sequence given below:

	2 samples	10 samples	n samples
1. TdT dNTP mix (cat# 4810-30-05)	1 µl	5 µl	n x 0.5 µl
2. 50X Mn2+ (cat# 4810-30-14)	1 µl	5 µl	n x 0.5 µl
3. TdT enzyme (cat# 4810-30-04)	1 µl	5 µl	n x 0.5 µl
4. 1X TdT Labeling Buffer (item 5)	50 µl	250 µl	n x 25 µl

6. 1X TdT Stop Buffer

Dilute the 10X TdT Stop Buffer (cat# 4817-60-03) to 1X using distilled water. Leave at room temperature until use. Prepare 1 ml of 1X TdT Stop Buffer per sample.

7. Strep-Fluorescein

Use 25 µl of Strep-Fluorescein Solution per sample under dimmed lights. Store prepared Strep-Fluorescein on ice in the dark until use. To prepare add:

	1-4 samples	10 samples	n samples
1X PBS	200 µl	500 µl	n x 50 µl
Strep-Fluorescein (cat# 4800-30-14)	1 µl	2.5 µl	n x 0.25 µl

8. Propidium iodide/RNAse solution (cat# 4817-60-04)

The propidium iodide/RNAse solution can be added directly to the sample just prior to flow cytometry. Typically 10 µl per 500 µl sample is sufficient. Incubate at room temperature for 5 minutes then analyze.

9. *TACS-Nuclease and Buffer

For the preparation of a Nuclease-treated positive control sample, it is recommended that the DNA breaks be generated in a separate step (see SectionVIII, pg 6), in this case, TACS-Nuclease™ should be diluted 1:25 in TACS Nuclease buffer, as below:

	per control
TACS-Nuclease™ Buffer (cat# 4800-30-06)	24 µl
TACS-Nuclease™ (cat# 4800-30-15)	1 µl

VII. Assay Protocol

It is important to read through the *Instructions for Use* before preparing tissue or cell samples for labeling. There are key steps that are very important for successful labeling.

Step	Instructions	Notes
1	Pellet 10^6 cells at 1000 x g for 5 minutes at room temperature. Draw off media with a Pasteur pipette.	A small residual volume of medium may be left. Refer to Appendix D, page 13, Preparation of adherent cells.
2	Resuspend cells gently in 1 ml of 3.7% formaldehyde solution. Leave to stand at room temperature for 10 minutes. Tap the tube every two minutes to keep cells in suspension.	Refer to reagent preparation, page 3. See Appendix B (page 11) for alter-native fixation methods and storage of fixed cells. It is It is not necessary to Wash cells prior to fixation.
3	Pellet fixed cells at 1500 x g for 5 min-utes at room temperature. Draw off fixa-tive with a Pasteur pipette.	Do not disturb cell pellet. A small residual volume of fixative may be left (~50 µl).
4	Add 100 µl of Cytonin™ and tap tube several times to resuspend cells. Leave at room temperature for 30 minutes. Tap tube occasionally to keep cells in sus-pension.	Samples can be stored in Cytonin™ overnight at 4 °C. Prepare the 1X labeling buffers, labeling mixes and 1X Stop Buffer for the next steps. Refer to *Reagent Preparation* (page 3).
5	Pellet cells at 1500 x g for 5 minutes at room temperature. Draw off Cytonin™ with Pasteur pipette.	Do not disturb cell pellet. A small residual volume of Cytonin™ may Be left (~50 µl).
6	Wash cells by resuspending in 1 ml of 1 X Labeling buffer then repeating the centrifugation at 1500 x g for 5 minutes at room temperature. Draw off excess buffer with a Pasteur pipette.	Nuclease treat one sample after Cytonin™ treatment prior to step 6. The other samples can remain in 1X Labeling buffer for the additional 30 Minutes. Refer to section VIII for Controls, page 6.
7	Add 25 µl of labeling reaction mix and resuspend by gently tapping tube. Incu-bate in 37 °C water bath for 1 hour. Tap tube gently every 10 minutes to keep cells in suspension.	Shorter incubation times (10 to 30 minutes) may be applicable for some Samples. Remember to include the “no enzyme” and TACS Nuclease treated control. Refer to *Reagent Preparation* (page 4).
8	Add 1 ml of 1X Stop Buffer, tap tube to mix. Pellet at 1500 x g for 5 minutes at room temperature. Draw off Stop buffer with Pasteur Pipette.	Refer to *Reagent Preparation* (page 4).

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Step	Instructions	Notes
9	Add 25 µl of the diluted Strep-Fluorescein and incubate at room temperature in the dark for 10 minutes.	Refer to *Reagent Preparation*, page 4. Other streptavidin-fluorochrome conjugates may be substituted.
10	Pellet cells at 1500 x g for 5 minutes at room temperature. Remove the Strep-Fluorescein with a Pasteur pipette and resuspend cells in 500 µl of 1X PBS.	Additional washes may be required if there is high background fluorescence in the controls.
11	Add 10 µl of propidium iodide/RNAse solution, if required, incubate at room temperature for 5 minutes. Assay.	Place samples on ice in the dark until assayed. Perform flow cytometry within 2 hours.

VIII. Controls

The minimum number of controls that should be included when running your assay are:

a) Nuclease-treated Control

Treat one of your samples with TACS-Nuclease™ to generate DNA breaks in every cell. This sample will confirm that the labeling reaction has worked and can be used to determine the initial settings for flow cytometry.

i) After Step 5, page 5, wash cells in 1X PBS then resuspend in: 25 µl of Nuclease solution (see *Reagent Preparation*, page 4)
ii) Incubate at RT for 30 minutes.
iii) Add 1 ml of 1X PBS.
iv) Pellet cells at 1500 x g for 5 minutes at room temperature
v) Discard Nuclease solution and continue from Step 6 in the Assay Protocol, page 5.

b) Unlabeled experimental sample.

The enzyme should be omitted from the labeling reaction mix. This control will indicate the level of background fluorescence associated with non-specific binding of the Strep-Fluorescein or if increased washes may be needed. This sample can be used to determine the settings for flow cytometry.

c) Control cells.

An appropriate experimental control should be included in each experiment.

d) Propidium Iodide/RNAse Solution.

If propidium iodide is used perform each of the above controls in duplicate so that the samples can be assayed with and without propidium iodide.

IX. Data Interpretation

Apoptosis is often defined by morphological criteria. It is important to consider the data obtained from standard microscopy and histochemistry in conjunction with biochemical assays used for confirmation of apoptosis.

Using FITC Label only
Apoptosis is indicated by positive fluorescence an order of magnitude greater than the unlabeled control sample (enzyme omitted from labeling reaction mix). There may be some spread in the degree of fluorescence of positive cells due to variability in the number of DNA breaks per cell. Different cell types may exhibit different degrees of fluorescence, therefore comparison with an unlabeled control cell of the same type under study is important for interpretation of data. The nuclease-treated labeled sample confirms satisfactory labeling. Note that the nuclease-treated cells will not necessarily be condensed and a slightly different profile on flow cytometry is to be expected.

Using FITC and Propidium Iodide
All cells are labeled by propidium iodide, after fixation and permeabilization, allowing the entire cell population to be visualized by flow cytometry. Propidium iodide fluorescence can also be used to aid in distinguishing between apoptotic (typically condensed and fragmented nuclei) and necrotic (swollen nuclei) cells. Propidium iodide may allow distinction to be made between these two modes of cell death when used in conjunction with FITC fluorescence and analysis of size by flow cytometry. The labeling by FITC should not be affected by the inclusion of propidium iodide if the flow cytometry apparatus settings are appropriately compensated. Typically the FITC is recorded on the FL1 channel and the propidium iodide on the FL2 channel. Use a TACS-Nuclease™ treated control or a positive (i.e. a known apoptotic) experimental sample without propidium iodide to set the FL1 compensation and an unlabeled (enzyme omitted) experimental sample with propidium iodide to set the FL2 compensation. If any of the controls generate unexpected results please refer to the trouble shooting guide (below).

X. Troubleshooting

Rule out major problems by checking the control samples first.

Problem	Cause	Action
No FITC labeling of positive control (Nuclease-treated or known apoptotic sample)	Enzyme inactive (most labile component)	Enzyme must be stored at -20 °C in manual defrost freezer. Do not bring enzyme up to ice temperature. Place in -20 °C freezer block or remove aliquot from tube directly at freezer.
	Inadequate TACS-Nuclease™ treatment	Increase incubation time.

Problem	Cause	Action
No FITC labeling of positive control (Nuclease-treated or known apoptotic sample) cont.	Strep-Fluorescein was frozen Inadequate permea-bilization, or over fixation.	Ensure that on arrival the Strep-Fluorescein was placed at 4 °C for storage in the dark. Time of Cytonin™ treatment can be increased up to 24 hours (perform at 4 °C for incubations of 2 hours or more). Avoid extensive fixation in strong cross-linking agents.
Labeling of majority of cells in negative control (i.e. enzyme omitted from labeling reaction).	Non-specific binding of Strep-Fluorescein.	Add in an additional wash with PBS before and after labeling with Strep-Fluorescein. Include 1% BSA in the Strep-Fluorescein labeling mix. Dilute the Strep-Fluorescein to 1:500 instead of 1:200 in PBS.
All cells exhibit low level fluorescence.	Autofluorescence Cells damaged during procedure	Gate flow cytometry channels appropriately to compensate. Avoid paraformaldehyde fixation. Avoid aggressive resuspension and perform only low speed centrifugations.
No labeling in experimental sample (positive controls label).	No apoptosis (or necrosis) occurring in sample.	Check morphology of cells prior to assay for characteristics of apoptosis.
Extensive labeling in experimental control i.e. healthy cells	High rate of cell death (apoptosis or necrosis).	Check culture conditions. Reduce time of assay.
Overlap of relative fluorescence of FITC between controls and experimental sample	Incorrect or inadequate compensation settings/ gates	Use the appropriate controls to set gates and compensation on FL1 and FL2 channels.

XI. References

Flow Cytometry: a practical approach, 2nd edition, ed. M.G. Ormerod (1994) IRL Oxford University Press Inc. New York.

A. Negoescu *et al.*, In situ apoptotic cell labeling by the TUNEL method: improvements and evaluation on cell preparation. *J. Histochem. Cytochem.* **44**: 959-968 (1996)

S.-R. Shi et al. Antigen retrieval immunocytochemistry: practice and development. *J. Histotechnology*, **20**: 145-154 (1997)

XII. Related products available from Trevigen.

Apoptosis Kits

Catalog #	Description	Size
4815-30-K	TumorTACS™ *In Situ* Apoptosis Detection Kit	30 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
4823-30-K	NeuroTACS™ II *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-DK	TACS•XL® DAB *In Situ* Apoptosis Detection Kit	30 samples
4828-30-BK	TACS•XL® Blue Label *In Situ* Apoptosis Detection Kit	30 samples
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
4850-20-ET	TACS® Apoptotic DNA Laddering Kit EtBr	20 samples
4892-010-K	Cultrex® Calcein-AM Cell Viability Kit	1000 tests
4684-096-K	HT Colorimetric PARP Apoptosis Assay	96 samples
4685-096-K	HT Chemiluminescent PARP Apoptosis Assay	96 samples

Antibodies

Catalog #	Description	Size
2281-MC-100	Anti-Bax Monoclonal Antibody (Clone YTH-6A7)	100 µg
2291-MC-100	Anti-Bcl-2 Monoclonal Antibody (Clone YTH-8C8)	100 µg
4411-PC-100	Anti-Phosphorylated Histone-γ-H2AX polyclonal	100 µl
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.

Catalog #	Description	Size
4864-100	Treated Glass Microscope Slides w/3 sample	100 ea.

	Hydrophobic Barrier	
4862-10	**Coverslips 24 x 60 mm, No. 1.5**	**2 ea.**
4867-100	**Hydrophobic Coverslips**	**100 ea.**
4865-25	**Mounting Medium**	**25 ml**
4800-30-14	**Strep-Fluorescein**	**30 µl**
4830-010-03	**Propidium Iodide**	**1 ml**
4820-30-13	**Blue Counterstain**	**50 ml**
4825-30-RL	**Red Label**	**30 samples**
4870-500-6	**10X PBS**	**6 x 500 ml**
4869-500-6	**Apoptosis Grade™ H_2O**	**6 x 500 ml**
4878-05-02	**Cytonin™ IHC**	**2 x 5 ml**

XIII. Appendices

Appendix A. Reagent and Buffer Composition

10X PBS, pH 7.4 (cat# 4870-500-6) – Sold Separately:
75 mM disodium hydrogen phosphate (Na_2HPO_4)
25 mM sodium dihydrogen phosphate (NaH_2PO_4)
1.45 M sodium chloride (NaCl)

Apoptosis Grade™ Water (cat# 4869-500-6) – Sold Separately:
Deionized (18 mΩ) sterile water
Distilled autoclaved water can be used

Cytonin™ (cat# 4876-05-01):
Proprietary permeabilization and blocking agent

10X TdT Labeling Buffer (cat# 4810-30-02):
1 M TACS Safe-TdT™ Buffer
0.5 mg/ml BSA (RIA Grade)
0.6 mM 2-mercaptoethanesulfonic acid (MESNA)

TdT dNTP Mix (cat# 4810-30-04):
0.25 mM biotinylated dNTP

50X Manganese cation (cat#4810-30-14):
20 mM manganese cation

Strep-Fluorescein (cat #4800-30-14):
1 mg/ml Strep-Fluorescein 0.1% Sodium azide

TACS-Nuclease™ (cat #4800-30-15):
Proprietary endonuclease. Avoid repeated freeze-thaw.

TACS-Nuclease™ Buffer (cat #4800-30-16):
50 mM Tris-Cl, pH 8.0 1 mM MgCl2 100 mg/ml BSA

Propidium Iodide/ RNase Solution (cat #4817-60-04):
1 mg/ml propidium iodide; 50 mg/ml RNase A

Appendix B.

Fixation Methods

There are several common fixation methods that are appropriate for use with the protocol described here. Formaldehyde is the recommended fixative based on laboratory testing and data obtained from our laboratory start-up service. However, other fixatives that maintain DNA integrity may be used also. These include alcohol fixatives such as ethanol, methanol or acetone. Paraformaldehyde is not recommended due to autofluorescence of paraformaldehyde-fixed samples. Glutaraldehyde has poor penetration but remains as an alternative to formaldehyde. Fixation method will likely be dictated by immunocytochemistry protocols in double labeling experiments.

If you wish to store fixed cells, Trevigen recommends fixing in formaldehyde followed by a 20 minute postfix in 100% methanol. Wash in PBS then store in Cytonin at 4 °C for up to 1 month. Proceed with a wash in 1X Labeling Buffer then continue with the labeling reaction. Note that if cells are fixed using alcohol e.g. ethanol, there will be leakage of small DNA fragments from apoptotic cells and the propidium iodide profile will be different compared to cells labeled without prolonged storage times. The cross-linking fixatives such as formaldehyde limit DNA loss.

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. The rapid assay for apoptosis detection using Annexin V-FITC or Annexin V-Biotin uses unfixed and unpermeabilized cells and is therefore only applicable for double labeling when native cell surface markers are being detected along with DNA fragmentation.

The key to double labeling experiments is determining fixation and permeabilization conditions under which both antigen and DNA integrity are maintained. Appropriate fixatives for DNA labeling are provided in Appendix B on page 11. Post-treatments used in immunocytochemistry to permeabilize or expose antigenic determinants include treatment with proteases, acid or base, detergent and microwaving. Permeabilization with Cytonin may be sufficient for many antibodies and additional treatment may not be needed. Protease treatment may be performed but should be kept to a minimum to avoid complete cell destruction. Acid or base treatment should be avoided. Microwaving is an option that has given excellent results in double labeling experiments but requires careful experimentation to determine the correct wattage, time and cooling cycles for each experimental sample (Shi *et al.*, 1997).

It is recommended that conditions for DNA labeling and immunocytochemistry are optimized in separate experiments using conditions of fixation and permeabilization that will be appropriate for both methods. Once each procedure has been successful independently they can be combined as follows:

1. Fix and permeabilize (maintain DNA and antigen integrity)
2. Bind primary antibody, wash
3. Perform DNA labeling procedure (start with wash in 1X Labeling) up to wash, with 1X Stop buffer prior to labeling with Strep-Fluorescein
4. Combine Strep-Fluorescein and secondary tagged antibody (fluorochrome other than FITC) and incubate with cells for 30 minutes
5. Wash with PBS and perform flow cytometry

Times and temperature of incubations should be optimized for the system under study. In tricolor experiments (*e.g.* propidium iodide and FITC for FlowTACS™ plus fluorochrome for antigen identification) select a chromophore that is compatible with FITC and propidium iodide and with the filters you have available on the flow cytometry equipment. Phycoerythrin in combination with propidium iodide and FITC provides good contrast.

Remember to carefully plan and include controls in addition to the ones recommended for detection of apoptosis to allow interpretation of double-labeled samples.

Appendix D.

Preparation of Adherent Cells.

Detection of apoptosis using flow cytometric analysis of a fluorescent tag is most convenient for suspension cells. Adherent cells require lifting from the substrate on which they are growing prior to fixation and permeabilization. Adherent cells can be treated with trypsin or pepsin. The inclusion of EDTA will improve the efficiency of trypsinization. Most cells can be trypsinized in about 5 minutes at 37˚C in 0.5% trypsin, 2 mM EDTA. The trypsinization should be stopped by the addition of complete medium (containing fetal calf serum) followed by two washes in 1X PBS. Use of proteinase K or trypsin may destroy certain antigens and should therefore not be used if immunocytochemistry is also to be performed, unless antigen retrieval using proteases is recommended for the antigen of interest.

As an alternative to proteases, treat the cells with 2 mM EDTA in PBS for 5 minutes at 37 °C then scrape the cells from the culture vessel using a rubber policeman. Do not treat cells for extensive periods of time with EDTA because the chelation of calcium by EDTA may alter cell morphology etc. prior to fixation. Cells may also be scraped directly without EDTA treatment but this method tends to generate clumps of cells that are not easily dispersed into single cell suspension and increases the amount of cell debris.

Alternative methods for detection of apoptosis in adherent cells are available that allow direct visualization of apoptotic cells *in situ.* Contact Trevigen Technical Services for advice on alternative methods available.

The product accompanying this document is intended for research use only and is not intended for diagnostic purposes or for use in humans.

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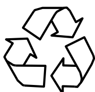

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