TACS™ DNA Laddering Kit

Apoptosis Detection Kit

catalog number: TA4632

Chemiluminescent

20 tests

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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PRINCIPLE OF THE ASSAY

TACS™ Apoptotic DNA Laddering Kits are used to assay cells and tissues for apoptosis by detecting internucleosomal DNA fragmentation and displaying DNA laddering. DNA laddering is one of the hallmark events that confirms apoptosis. During apoptosis, endonucleases produce double-stranded breaks generating DNA fragments. These DNA fragments are isolated from cells or tissues using the optimized TACS DNA isolation reagents that inactivate endogenous nucleases and rapidly recover high and low molecular weight DNA. The isolated DNA is first labeled with biotinylated deoxynucleotides. The labeled DNA is then size-fractionated by gel electrophoresis. The biotinylated DNA is transferred to a membrane, bound by a streptavidin-HRP conjugate, and visualized using a chemiluminescent detection method.

REAGENTS PROVIDED

Product Code	Component	Size	Storage
4850-20-01	Lysis Solution 1	2 mL	18-24 °C
4850-20-02	Extraction Solution 2	20 mL	18-24 °C
4850-20-03	Extraction Buffer 3	8 mL	18-24 °C
4850-20-04	Sodium Acetate 4	1 mL	18-24 °C
4850-20-05	DNAse-free Water 5	2 mL	18-24 °C
4850-20-06	Sample Buffer	2 mL	18-24 °C
4850-20-07	DL 10X Klenow Buffer	0.5 mL	-20 °C
4850-20-08	DL Klenow Enzyme[1]	20 µL	-20 °C
4850-20-09	DL Klenow Water	1 mL	-20 °C
4850-20-10	Gel Loading Buffer	250 µL	18-24 °C
9804-006-P	TreviGel™ 500 Powder	6 g	18-24 °C
4855-20-11	DL dNTP Mix	40 µL	-20 °C
4855-20-12	DL Streptavidin-HRP[2]	40 µL	2-8 °C
4855-20-15	DL Control DNA	4 µL	-20 °C
4855-20-13	PeroxyGlow™ A	20 mL	2-8 °C
4855-20-14	PeroxyGlow™ B	20 mL	2-8 °C

[1] Do not store in a frost-free freezer

[2] Contains 0.05% thimerosal

MATERIALS REQUIRED BUT NOT PROVIDED

REAGENTS

- 2-propanol
- 70% ethanol
- DNase-free or molecular biology grade water or deionized, sterile water
- Microcentrifuge tubes
- 10X PBS (see page 9)
- 50X TAE (see page 9)
- X-ray film and supplies
- Non-fat dry milk or bovine serum albumin (BSA)
- Nitrocellulose membrane
- 20X SSC (see page 9)
- 0.4 M NaOH/0.8 M NaCl (see page 9)
- 0.25 M HCl (see page 9)
- 0.5 M Tris, pH 7.5/1.5 M NaCl (see page 9)
- Chloroform (optional)

EQUIPMENT

- Microcentrifuge
- Horizontal gel electrophoresis apparatus
- Vortex
- Adjustable pipettors (0-20 µL, 20-200 µL, 200-1000 µL) and tips
- Spectrophotometer

PROCEDURES

DNA ISOLATION: (*Note: For best results, process samples immediately after harvesting. For processing tissue samples, use the TA4634 Tissue Supplement kit available from R&D Systems.*)

1. Sample Preparation:

   Non-Adherent Cells - Collect 1 x 10^5-1 x 10^7 cells by centrifugation at 2,000 x g for 5 minutes at 2-8 °C. Resuspend cells in 5 mL ice cold 1X PBS. Repeat the centrifugation step. Resuspend cells in 100 µL Sample Buffer. Proceed to step 2.

   Adherent Cells - Decant the media and add 5 mL ice cold 1X PBS to the monolayer of 1 x 10^5-1 x 10^7 cells. Decant the PBS and lyse the cells by adding 100 µL Sample Buffer and 100 µL Lysis Solution 1 directly to the dish. Swirl gently to ensure complete lysis. Proceed to step 4.

   Tissues - See the Appendix, page 8.

2. Incubate the cell suspension at 18-24 °C for 10 minutes.
3. Lyse the sample and stabilize the DNA by adding 100 µL of Lysis Solution 1. Mix the resuspended cells with the lysis solution by inverting the tube gently several times. *The sample should be processed within the next two hours. If this is not possible, store samples at -80 °C; avoid freeze/thaw cycles.*
4. Transfer the sample to a 2 mL microcentrifuge tube. Shake Extraction Solution 2. Add 700 µL Extraction Solution 2 to the sample.
5. Add 400 µL of Extraction Buffer 3 to the sample. Vortex for 10 seconds.
6. Microcentrifuge at 12,000 x g for 5 minutes.
7. Transfer the upper (aqueous) layer to a new microcentrifuge tube. Avoid removing the darker, lower (organic) layer and interface layer. *If you accidentally transfer the interface, repeat the centrifugation step and carefully remove and save the upper layer. For any samples that are cloudy after transfer to the new tube, a chloroform extraction is required. Transfer upper layer to a new microcentrifuge tube. Add an equal volume of chloroform. Vortex for 10 seconds. Microcentrifuge for 30 seconds at 12,000 x g. Transfer the upper (aqueous) layer to a new microcentrifuge tube. Do not transfer the organic or interface layers.*
8. Add 0.1 volume of Sodium Acetate 4 to the aqueous DNA sample. Mix. (For example, if you have 400 µL of sample, add 40 µL of Sodium Acetate 4.)
9. To the total volume in the microcentrifuge tube, add an equal volume of 2-propanol. Mix. (For example, if you have a total of 440 µL, add 440 µL of 2-propanol.) *This is an optional stopping point. If desired, store samples at -20 °C overnight.*
10. Microcentrifuge at 12,000 x g for 10 minutes.
11. Carefully remove and discard the supernatant without disturbing the DNA pellet. Add 1 mL of 70% ethanol to the DNA pellet. Mix by inverting the tube gently several times.
12. Microcentrifuge at 12,000 x g for 5 minutes.
13. Carefully remove and discard the supernatant without disturbing the DNA pellet. Allow the DNA pellet to dry. The DNA pellet can be dried by inverting the tube on a laboratory tissue and allowing the liquid to evaporate. Alternatively, the DNA pellet can be dried with the aid of a vacuum centrifuge apparatus.

14. Resuspend the DNA in 100 µL of DNase-free Water 5.
15. Quantify the DNA using a spectrophotometer. Dilute 5 µL of the DNA in 995 µL of water. Read the optical density (OD) at a wavelength of 260 nm using a water blank. Determine the DNA concentration of the undiluted sample:

    Concentration (µg/µL) = OD_{260} x 9.88*

    **9.88 is a multiplication factor that accounts for the 1:200 dilution and the OD of the DNA in H_2O.*
16. Dilute the DNA to 1 µg/µL with sterile dH_2O. Store purified DNA at -20 °C.

CHEMILUMINESCENT LABELING AND DETECTION:

1. Prepare a reaction with 1 µg of DNA (1 µL) or 1 µL of DL Control DNA:

DNA	1 µL
DL 10X Klenow buffer	1 µL
DL Klenow water	5 µL
DL dNTP	2 µL
DL Klenow enzyme	1 µL
Total volume:	10 µL

***Note**: Return the enzyme to the freezer immediately. Do not keep the enzyme on ice.*

2. Incubate for 10 minutes at 18-24 °C.
3. Stop the reaction by adding 2 µL Gel Loading Buffer. Load onto a 1.5% TreviGel 500 gel in 1X TAE buffer (prepared according to the instructions on pages 8-9). Run until the dye front is close to the end of the gel. For a 15 cm gel at 100 V, this will be approximately 2 hours.
4. Transfer the DNA to a nitrocellulose membrane:

   ***Note**: For faster transfers, electroblotting using manufacturers' recommended conditions can be substituted. For orientation of the gel, cut one small corner from the gel before proceeding.*

   a. Depurinate by soaking the gel in 500 mL of 0.25 M HCl for 15 minutes at 18-24 °C.

   b. Rinse 2 times with dH_2O.

   c. Neutralize by soaking the gel in 500 mL of 0.4 M NaOH/0.8 M NaCl for 30 minutes at 18-24 °C.

   d. Soak gel in 500 mL 0.5 M Tris, pH 7.5/1.5 M NaCl for 30 minutes at 18-24 °C.

   e. Transfer the DNA from the gel onto a nitrocellulose membrane with 10X SSC overnight. *If you are not familiar with the technique of setting up Southern transfers, refer to Molecular Cloning, A Laboratory Manual, Sambrook et al., ed., Cold Spring Harbor Press, pp. 9.34-9.40 (1989) or any other basic molecular biology text book.*

5. Remove the membrane from the transfer apparatus. Use a pencil to mark the orientation and position of the wells on the membrane. *If desired, crosslink the DNA to the membrane for 15 minutes using UV light. Verify the efficiency of DNA transfer to the membrane by ethidium bromide (0.5 µg/mL) staining the gel. DNA remaining in the gel will be stained by the ethidium bromide and can be visualized with a UV transilluminator. Typically, the transfer of higher molecular weight DNA is less efficient, but is sufficient for the detection of DNA laddering.*
6. Block the membrane by placing it in 100 mL of 1X PBS with 3% BSA or 5% non-fat dry milk. Shake/rock for 30 minutes at 18-24 °C.
7. Place the membrane in a plastic bag or dish. Add 20 mL 1X PBS. Add 10 µL of streptavidin-HRP conjugate. Shake/rock at 18-24 °C for 30 minutes.
8. Wash the membrane 3 times with 100 mL of 1X PBS at 18-24 °C.

Chemiluminescent Detection:

Transfer 5 mL of PeroxyGlow A to a fresh empty tube. Add 5 mL of PeroxyGlow B. Mix. This solution is stable for 1 hour. Add to the membrane. Shake/rock for 1 to 5 minutes to evenly distribute the PeroxyGlow solution over the membrane. Blot the membrane with paper towels, but do not completely dry the membrane. Wrap the membrane in plastic and expose to x-ray film. Typical exposure times are between 10 seconds and 5 minutes. The DL Control DNA should appear as a DNA ladder (see Results below).

RESULTS

DNA laddering is characteristic of apoptosis. During apoptosis, the activation of specific calcium-dependent endonucleases leads to DNA fragmentation. When size-fractionated using gel electrophoresis, extracted apoptotic DNA fragments appear as a DNA ladder consisting of multimers of 180-200 base pairs. The 180-200 base pair unit corresponds to the length of DNA around a nucleosome that is protected by histones. DNA from non-apoptotic populations of cells is high molecular weight and will not migrate very far into the gel. It is natural for control populations of cells to contain a small number of apoptotic cells.

TROUBLESHOOTING GUIDE

Problem	Possible Cause	Recommendation
DNA recovery is poor.	Inadequate lysis of sample because too much tissue is being processed.	Use a larger volume of Sample Buffer and Lysis Solution 1.
Phase inversion during DNA extraction.	Lysate is too viscous.	Use a larger volume of Sample Buffer and Lysis Solution 1.
Inadequate labeling of DNA.	Contamination of DNA with the organic phase during transfer following phase separation.	Do not transfer the organic phase, which inhibits the DL Klenow enzyme. A chloroform extraction can remove the organic contaminant.
	The DL Klenow enzyme may be inactive.	Use fresh enzyme. Do not use the enzyme if it is greater than one year old or stored improperly. Do not place the enzyme on ice. Remove the volume of enzyme required and return it to the freezer immediately. Do not store the enzyme in a frost-free freezer.
All samples show little or no DNA laddering.	Samples may not be undergoing apoptosis.	Conditions for inducing apoptosis may need to be changed. These conditions vary with cell type.
	A small percentage of cells are apoptotic.	Select a more sensitive detection method: Isotopic > Chemiluminescence > Colorimetric > Ethidium Bromide.
	Apoptotic cells may have been washed away.	For adherent cells, apoptotic cells may detach from plate. Harvest “floaters” in decanted media by centrifugation. Wash with ice cold 1X PBS and centrifuge to collect cells. Resuspend in the 100 µL sample buffer to be added to the plate.
	In rare instances, apoptosis may not be accompanied by DNA laddering.	
All samples, including the negative control, show extensive DNA degradation.	DNA is sheared during isolation because some cells lyse more easily than others.	Isolate DNA from fresh samples. Use caution and be gentle. Adhere to the protocol.
All samples, including the negative control, show extensive DNA laddering.	Negative control is inappropriate.	The negative control is undergoing apoptosis. Choose another negative control.

Problem	Possible Cause	Recommendation
Entire membrane contains signal.	Inadequate washing.	Increase the number of washes following the incubation with streptavidin-HRP conjugate.
	Non-fat dry milk may contain biotin.	Use 3% BSA to block the membrane.

APPENDICES

DNA Extraction from Tissue Samples

(For best results, process the tissue immediately after removal from the animal).

1. Mince the tissue into small pieces and freeze in liquid nitrogen.
2. Grind 0.2-1.0 g of the frozen tissue into a powder using either a hammer or mortar and pestle (these should be pre-chilled to -80 °C). Add additional liquid nitrogen as required to keep the sample frozen. *An excess of tissue is ground*.
3. Resuspend 0.1 g of frozen powdered tissue in 200 µL of Sample Buffer.
4. Add 20 µL of 10X Tissue Buffer. *Verify that all of the tissue is in suspension.*
5. Incubate at 50 °C for 12-18 hours. Gentle shaking is recommended.
6. Transfer 100 µL of the tissue suspension to a new microcentrifuge tube. Add 100 µL of Lysis Solution 1 from the TACS Apoptotic DNA Laddering Kit and mix well. Proceed to step 4 in the DNA Isolation Protocol (page 4).

Casting TreviGel™ 500

Note: The following method for TAE gel casting gives consistent, high resolution results. For high efficiency transfer out of gels, thinner gels are recommended (Transfer efficiency = 1/distance^2). Use ½ of the typical volume of gel.

1. Prepare enough 1X TAE buffer (prepared according to the directions on page 9) for both casting and running of the gel.
2. Prepare a 1.5% TreviGel 500 gel (% [w/v]) in 1X TAE:
   a. Add the correct, measured quantity of 1X TAE.
   b. Add the correct amount of TreviGel 500 powder to a clean, dust-free glass Erlenmeyer flask.
   c. Weigh the flask and record its weight.
   d. Heat the flask in a microwave oven on a medium setting until no particulates are visible in the molten gel. The time required will vary because microwave ovens vary in power. In general, TreviGel 500 requires one minute longer than agarose. GENTLY swirl the flask to mix. HANDLE WITH CARE as the molten TreviGel 500 is very hot.
   e. Replace the water that evaporated during heating by adding distilled water until the previously recorded weight is reached. GENTLY swirl the flask to mix.
   f. Once bubbles have stopped forming, wait an additional minute and pour the gel into a casting tray. Allow the gel to cool and set for 30 minutes.
   g. For optimal results, place the gel at 2-8 °C for 30 minutes before loading the samples.

Buffer Compositions

50X TAE: 242 g Tris base 57.1 mL glacial acetic acid 100 mL 0.5 M EDTA (pH 8.0) Add dH_2O to 1 liter.	**0.25 M HCl:** 10.8 mL concentrated HCl Add dH_2O to 500 mL.
20X SSC: 175.32 g NaCl 88.23 g sodium citrate Add dH_2O to 1 liter.	**0.4 M NaOH/0.8 M NaCl:** 40 mL 5 M NaOH 80 mL 5 M NaCl Add dH_2O to 500 mL.
10X PBS: 85 g NaCl 10.7 g Na_2HPO_4 (anhydrous) 3.9 g $NaH_2PO_4 \bullet 2H_2O$ Add dH_2O to 1 liter. The pH should be approximately 7.2.	**0.5 M Tris, pH 7.5/1.5 M NaCl:** 250 mL 1 M Tris, pH 7.5 150 mL 5 M NaCl Add dH_2O to 500 mL.

WARNINGS

HAZARDOUS INGREDIENTS

The acute and chronic effects of overexposure to reagents of this kit are unknown. Some kit reagents contain minute amounts of NaCl, guanidine thiocyanate, organic solvents, and thimerosal, which as concentrated solutions are poisonous and may be fatal if swallowed, absorbed through the skin, or inhaled. We recommend following the handling, emergency, and disposal procedures indicated below for all kit reagents.

HANDLING PRECAUTIONS

Safe laboratory procedures should be followed when handling all kit reagents. It is recommended that protective laboratory clothing (gloves, laboratory coat, glasses) be worn when handling kit reagents.

EMERGENCY EXPOSURE PROCEDURES

In case of exposure to reagent solutions, we recommend following these emergency first-aid procedures:

Skin or eye contact
Wash with water for at least 15 minutes. Remove any contaminated clothes.

Inhalation
Remove individual to fresh air. If breathing is difficult, give oxygen and call a physician.

Ingestion
Rinse mouth with copious amounts of water and call a physician.

REACTIVITY DATA

Extraction Solution 2 and Extraction Buffer 3 contain flammable ingredients. Keep them away from excessive heat, sparks and flame. Do not mix these reagents with strong oxidizing agents. Lysis Solution 1 contains guanidine thiocyanate, which is a toxic irritant.

NOTES