Detection of Apoptotic Cells Using Immunohistochemistry

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Immunohistochemistry is commonly used to show the presence of apoptotic cells in situ. In this protocol, B-cell lymphoma cells are injected into recipient mice and, on tumor formation, the mice are treated with the apoptosis inducer vorinostat (a histone deacetylase inhibitor). Tumor samples are fixed and sectioned, and fragmented DNA (a feature of apoptotic cells) is end-labeled by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL). Immunohistochemical methods are then used to detect the labeled DNA and identify B-cell lymphoma cells in the last stage of apoptosis. Because the assay can lead to false-positive results, it is advisable to carry out an additional assay (e.g., immunohistochemistry for active caspase-3) to confirm the presence of apoptotic cells.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution’s Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore) or equivalent
The kit includes equilibration buffer, reaction buffer, TdT enzyme, stop/wash buffer, and horseradish peroxidase (HRP)-conjugated antidigoxigenin antibody. Prepare a working solution of TdT in reaction buffer according to the manufacturer’s directions.

Apoptosis inducer (e.g., vorinostat)
Prepare vorinostat by dissolving at 10 mM in DMSO. Store at −20°C, and avoid multiple freeze–thaw cycles.

C57BL/6 mice (8–10 wk of age)
Complete Anne Kelso medium <R>
Diaminobenzidine (DAB)

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Cite this protocol as Cold Spring Harb Protoc; doi:10.1101/pdb.prot082537
Dimethylsulfoxide (DMSO)
Ethanol (70%, 95%, and 100%)
Eμ-Myc B-cell lymphoma cells (cryopreserved)
Formalin, neutral-buffered (10%)
Hematoxylin
Hydrogen peroxide in PBS (3% solution)
Mounting medium
Paraffin
Phosphate-buffered saline (PBS) <R>
Proteinase K
Water (distilled)
Xylene

**Equipment**

Automated hematology analyzer (Advia 120 or equivalent)
Centrifuge (benchtop)
Conical tubes (50- and 15-mL)
Coplin jars
Coverslips
Dissection tools (small surgical scissors and curved microforceps)
Glass slides (Superfrost Plus)
Hemocytometer
Humidified chamber (at room temperature [for Step 19] and at 37°C [for Step 16])
Light microscope
Microtome
Pen (hydrophobic) for marking slides
Syringes (1-mL) with 25-gauge needles

**METHOD**

*Figure 1 summarizes the sequence of steps in this method. Perform all steps at room temperature unless otherwise stated.*

**Transplantation of B-Cell Lymphoma Cells and Induction of Apoptosis**

*Preparing the lymphoma cells and carrying out the injections will take ~60 min.*

1. Thaw cryopreserved Eμ-Myc B-cell lymphoma cells in complete Anne Kelso medium, wash the cells twice with PBS, and perform a cell count.

2. Resuspend $2 \times 10^6$ Eμ-Myc B-cell lymphoma cells in 1 mL of PBS and transfer 200 µL of the cell suspension ($4 \times 10^5$ cells) into syngeneic recipient mice by tail vein injection.

3. During tumor development over the next 7–14 d, monitor the disease burden by palpating the inguinal or brachial lymph nodes and by measuring the concentration of white blood cells (WBCs) in ~25 µL of blood from the tail vein using an Advia 120 automated hematology analyzer or equivalent.

4. Once lymph nodes are clearly palpable (corresponding to a peripheral WBC concentration of ~50 x 10^6 cells/mL or greater), weigh the mice and administer the apoptosis inducer (e.g., inject vorinostat intraperitoneally at 200 mg/kg). As a control, inject one mouse with an equal volume of vehicle only (e.g., for the vorinostat control, use DMSO).
Collection and Fixation of the Tumor Tissue

5. At each of several time points after administration of vorinostat (e.g., at 4, 8, 12, and 24 h), kill a set of the mice by cervical dislocation.

6. Using small scissors and curved forceps, collect lymph nodes (e.g., inguinal, brachial, auxiliary, and mesenteric) and/or spleen samples.

Collecting the tissues will take \( \sim 10 \text{ min per animal} \).

7. Transfer each tissue sample into a separate 15-mL Falcon tube filled with 10% neutral-buffered formalin, and fix the tissues for at least 12 h.

The fixation of tissue can require from 12 to 24 h.

8. Embed the formalin-fixed tissues in paraffin, prepare 4-μm sections, and transfer them onto Superfrost Plus glass slides.

9. Deparaffinize and rehydrate the formalin-fixed, paraffin-embedded tissue sections in a Coplin jar:
   i. Wash the slides in three changes of xylene for 5 min each time.

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**FIGURE 1.** Flowchart outlining the steps for detecting fragmented DNA in apoptotic cells using TUNEL. i.v., Intravenous; i.p., intraperitoneal.
ii. Wash the slides twice in 100% ethanol for 5 min each time.

iii. Wash the slides once in 95% ethanol and once in 70% ethanol for 3 min each time.

iv. Rinse the slides in one change of PBS for 5 min.

10. Mark the tissue sections on the slides by circling each section using a hydrophobic pen.

11. Prepare a freshly diluted solution of proteinase K (20 µg/mL). Expose the DNA by pretreating each tissue section with ~50 µL of the diluted enzyme for 15 min.

   The specific volume of diluted enzyme is not important. In this step and below, use a sufficient volume of the appropriate solution (~50–60 µL) to cover the section.

12. Rinse the slides twice in distilled water in a Coplin jar for 2 min each time.

13. Inactivate endogenous peroxidase by incubating the slides in 3% hydrogen peroxide in PBS in a Coplin jar for 5 min.

14. Rinse the slides twice with PBS in a Coplin jar for 5 min each time.

Labeling and Detection of Cells with Fragmented DNA

15. Gently tap the excess liquid off each slide, immediately apply ~60 µL of equilibration buffer to each section, and incubate at room temperature for at least for 30 sec.

16. Gently tap the excess liquid off each slide and immediately apply ~50 µL of the working solution of TdT to each section. Incubate the slides in a humidified chamber at 37°C for 1 h.

   Nucleotides in the reaction buffer (both digoxigenin-conjugated nucleotides and unlabeled nucleotides) are added to the DNA by TdT.

   We recommend including positive and negative controls. As a positive control for DNA fragmentation, DNase I-treated sections can be used; as a negative control, sections can be treated with reaction buffer only (by omitting TdT from the working solution) in this step.

17. Transfer the slides into a Coplin jar containing stop/wash buffer, agitate for 15 sec, and incubate at room temperature for another 10 min.

18. Rinse the slides three times in PBS for 2 min each time.

19. Gently tap the excess liquid off each slide and apply ~50 µL HRP-conjugated anti-digoxigenin antibody to each section. Incubate the slides in a humidified chamber at room temperature for 30 min.

20. In a Coplin jar, rinse the slides four times in PBS at room temperature for 2 min each time.

21. Apply ~60 µL of DAB (peroxidase substrate) to cover each of the sections and incubate for 1–3 min at room temperature.

22. Monitor color development by viewing the slides under a light microscope.

   To obtain optimal specific staining, develop the slides for the minimum time necessary.

23. Once the sections are optimally stained, rinse the slides three times in distilled water in a Coplin jar for 1 min each time.

24. Counterstain the sections in hematoxylin for 4–10 sec at room temperature and rinse the slides in water until the water is clear.

25. Dehydrate the sections in a Coplin jar:

   i. Wash the slides once in 95% ethanol for 5 min.

   ii. Wash the slides twice in 100% ethanol for 3 min each time.

   iii. Clear the slides twice in xylene for 5 min each time.

26. Mount the sections on the slides under a coverslip with mounting medium.
27. View the sections under a light microscope to identify areas of staining.

The labeled ends of DNA fragments are typically located in apoptotic nuclei. Therefore, it is advisable to focus on the nuclear areas of the cells when analyzing the staining patterns.

For an example of TUNEL staining in lymph nodes, see Figure 2.

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 27): The staining appears to be nonspecific and/or false-positive results are suspected.

Solution: The activity of endogenous nucleases may lead to false-positive results. These adverse effects can be diminished by fixing the tissue immediately after collecting it (Step 7) and/or by treating the sections with proteinase K (Step 11) for the minimum time necessary.

Nonspecific staining can be caused by endogenous peroxidase activity or by using a mixture of TdT that is too concentrated. When preparing the working solution of TdT in the reaction buffer, try lowering the concentration of TdT.

RELATED TECHNIQUES

In certain cases, the TUNEL assay may lead to false-positive results (Galluzzi et al. 2009). It is therefore advisable to carry out at least one additional immunohistochemistry assay (e.g., to detect active caspase-3) or a flow cytometry cell death assay. Flow cytometry assays that detect the binding of annexin V in combination with propidium iodide are described in Galluzzi et al. (2009) and Kepp et al. (2011); see also Protocol: Detection of Apoptotic Cells Using Propidium Iodide Staining (Newbold et al. 2014).

RECIPES

**Complete Anne Kelso Medium**

Prepare in high glucose Dulbecco’s modified Eagle’s medium (DMEM)
10% fetal calf serum (FCS)
100 units/mL penicillin G
100 µg/mL streptomycin
0.1 mM L-asparagine
50 µM 2-mercaptoethanol

Store at 4°C for up to several months.
Phosphate-Buffered Saline (PBS)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount to add (for 1× solution)</th>
<th>Final concentration (1×)</th>
<th>Amount to add (for 10× stock)</th>
<th>Final concentration (10×)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8 g</td>
<td>137 mM</td>
<td>80 g</td>
<td>1.37 M</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 g</td>
<td>2.7 mM</td>
<td>2 g</td>
<td>27 mM</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.44 g</td>
<td>10 mM</td>
<td>14.4 g</td>
<td>100 mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.24 g</td>
<td>1.8 mM</td>
<td>2.4 g</td>
<td>18 mM</td>
</tr>
</tbody>
</table>

If necessary, PBS may be supplemented with the following:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount to add (for 1× solution)</th>
<th>Final concentration (1×)</th>
<th>Amount to add (for 10× stock)</th>
<th>Final concentration (10×)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.133 g</td>
<td>1 mM</td>
<td>1.33 g</td>
<td>10 mM</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>0.10 g</td>
<td>0.5 mM</td>
<td>1.0 g</td>
<td>5 mM</td>
</tr>
</tbody>
</table>

PBS can be made as a 1× solution or as a 10× stock. To prepare 1 L of either 1× or 10× PBS, dissolve the reagents listed above in 800 mL of H₂O. Adjust the pH to 7.4 (or 7.2, if required) with HCl, and then add H₂O to 1 L. Dispense the solution into aliquots and sterilize them by autoclaving for 20 min at 15 psi (1.05 kg/cm²) on liquid cycle or by filter sterilization. Store PBS at room temperature.

ACKNOWLEDGMENTS

Work described here was supported by a grant from the Dutch Cancer Society to M.B. (AMC2009-4457) and a grant from Pfizer Inc. to C.C.

REFERENCES


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Cold Spring Harb Protoc; doi: 10.1101/pdb.prot082537