INTRODUCTION

Sirius Red specifically binds to the [Gly-X-Y]ₙ helical structure of fibrillar collagens such as type I to V collagen, and is used for detecting all types and species of collagen, whereas Fast Green binds to non-collagenous proteins. By exploiting the unique features of these two dyes, we provide a simple semi-quantitative assay kit for determining the amounts of collagen and non-collagenous proteins in cultured cell layers and tissue sections (1-2). Because this assay does not require collagen solubilization, it is widely used for the measurement of total collagen content in various tissues (3-7).

Since Sirius Red and Fast Green have absorptions at 540 nm and 605 nm respectively, the OD values of the extracted dyes can be used for the calculation of collagen and non-collagenous protein content in each section. For general histological studies in which tissue sections are 10-20 μm thick, the assay sensitivity for collagen and non-collagenous proteins is greater than 3 μg/section and 50 μg/section, respectively. This kit contains enough reagents to stain between 30-50 samples.

KIT COMPONENTS

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Amount</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dye Solution</td>
<td>1 bottle</td>
<td>10 ml</td>
<td>RT</td>
</tr>
<tr>
<td>Dye Extraction Buffer</td>
<td>2 bottles</td>
<td>50 ml</td>
<td>RT</td>
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</tbody>
</table>

ASSAY PROCEDURE

The following sample preparation methods are standard protocols. Therefore, these protocols may need to be optimized depending on the sample types.

Paraffin Embedded Tissue Sections

1. Prepare paraffin-embedded tissue sections (approximately 30-50 nm², 10-20 μm thick).
2. Deparaffinize the tissue section with the following steps below:
   - Xylene, 10 minutes
   - Xylene 1:1 with 100% ethanol, 10 minutes
   - 100% ethanol, 10 minutes
   - 50% ethanol/distilled water, 5 minutes
   - Distilled water, 5 minutes
3. Transfer individual slides to petri dishes.
4. Add 0.2-0.3 ml of Dye Solution to completely immerse the tissue section and incubate at room temperature for 30 minutes.

Note: To avoid evaporation of the Dye Solution, place a piece of wet filter paper underneath the slide and cover the petri dish with a lid.
5. Carefully remove the Dye Solution.

6. Rinse the stained tissue section with 0.5 ml of distilled water repeatedly until the fluid is colorless.
   
   Option: This tissue section may be observed with a microscope without the following extraction step. Dry the tissue section and mount with 50% glycerol/distilled water.

7. In a petri dish, add 1 ml of Dye Extraction Buffer and gently mix by pipetting until the color is eluted from the tissue section.

8. Collect the eluted Dye Solution and read the OD values at 540 nm and 605 nm with a spectrophotometer.

**Frozen Tissue Sections**

1. Prepare frozen tissue sections (approximately 30-50 nm², 10-20 μm thick) according to standard methods.

2. Wash with PBS.

   Optional: Fixing step

   Add 0.5 ml of Kahle fixative to completely immerse the tissue section and incubate for 10 minutes at room temperature.

   Kahle fixative recipe (please contact Customer Service if you would like to purchase this buffer):

   - 60 ml distilled water
   - 28 ml 96% ethanol
   - 10 ml 37% formaldehyde
   - 2 ml concentrated acetic acid

3. Remove the fixative and wash with PBS, then transfer individual slides to petri dishes.

4. Add 0.2-0.3 ml of Dye Solution to completely immerse the tissue section and incubate at room temperature for 30 minutes.

   Note: To avoid evaporation of the Dye Solution, place a piece of wet filter paper underneath the slide and cover the petri dish with a lid.

5. Carefully remove the Dye Solution.

6. Rinse the stained tissue section with 0.5 ml of distilled water repeatedly until the fluid is colorless.

   Option: This tissue section may be observed with a microscope without the following extraction step. Dry the tissue section and mount with 50% glycerol/distilled water.

7. In a petri dish, add 1 ml of Dye Extraction Buffer and gently mix by pipetting until the color is eluted from the tissue section.

8. Collect the eluted Dye Solution and read the OD values at 540 nm and 605 nm with a spectrophotometer.

**In Vitro Cultured Cell Layers**

1. If you plan to mount cell layers and observe with a microscope before proceeding with the dye extraction step, place a sterilized round glass slide at the bottom of each well of a 24-well culture plate. Otherwise, you may skip this step.

2. Culture cells in the 24-well culture plates for a desired period of time.

3. Remove the culture medium and wash the wells with PBS.
4. Add 0.5 ml of Kahle fixative to completely immerse the tissue section and incubate for 10 minutes at room temperature.

   Kahle fixative recipe (please contact Customer Service if you would like to purchase this buffer):
   - 60 ml distilled water
   - 28 ml 96% ethanol
   - 10 ml 37% formaldehyde
   - 2 ml concentrated acetic acid

5. Remove fixative and wash with PBS.

6. Add 0.2-0.3 ml of Dye Solution to completely immerse the fixed cell layers and incubate at room temperature for 30 minutes. If cells are on glass slides, remove the slides and place them in petri dishes for the staining.

   Note: To avoid evaporation of the Dye Solution, place a piece of wet filter paper underneath the slide and cover the petri dish with a lid.

7. Carefully remove the Dye Solution.

8. Rinse the stained tissue section with 0.5 ml of distilled water repeatedly until the fluid is colorless.

   Option: This tissue section may be observed with a microscope without the following extraction step. Dry the tissue section and mount with 50% glycerol/distilled water.

9. In a petri dish, add 1 ml of Dye Extraction Buffer and gently mix by pipetting until the color is eluted from the tissue section.

10. Collect the eluted dye solution and read the OD values at 540 nm and 605 nm with a spectrophotometer.

**CALCULATION**

To calculate the amount of collagen, first, correct the OD 540 value by subtracting the contribution of Fast Green at 540 nm, which is 29.1% of the OD 605 value. The color equivalence (OD values/μg protein) is 0.0378 for collagen and 0.00204 for non-collagenous protein at OD 540 and 605, respectively (2).

\[
\text{Collagen (μg/section)} = \frac{\text{OD 540 value} - (\text{OD 605 value} \times 0.291)}{0.0378}
\]

\[
\text{Non-collagenous proteins (μg/section)} = \frac{\text{OD 605 value}}{0.00204}
\]

**Note 1:** Thicker sections tend to detach from slides during washing steps. Please wash the slides carefully. Moreover, since the assay sensitivity depends on the surface area of the tissue on the slides, cutting thinner sections will not significantly increase the assay sensitivity.

**Note 2:** Non-collagenous protein values may be used to normalize the results of samples, since protein levels are relative to the surface area or cell density of the samples.
REFERENCES


