4. Nonradioactive RNase Protection Assay

The ribonuclease protection assay is a powerful tool for:
- Detecting and quantifying specific RNAs
- Mapping transcription starts or termination sites of genes
- Determining the intron-exon structure of genes
- Detecting mutational alterations such as deletions, insertions, or rearrangements

The nonradioactive version of the RNase protection assay involves hybridization of a DIG-labeled antisense RNA probe to target sense RNA. After a single-strand specific RNase digests the areas of the probe that are not hybridized, the “protected” parts of the probe are determined by standard blotting techniques. This method offers the following advantages over techniques that rely on DNA:RNA hybridization (such as S1 mapping):
- Large amounts of labeled RNA are easily produced by transcription kits, reducing the time required for the assay.
- RNA:RNA hybrids are more stable than DNA:RNA hybrids, thus increasing the sensitivity and specificity of the assay.
- Digestion with RNases produces fewer artifacts than digestion with DNases, which reduces the occurrence of false or misleading results.

Key Products for This Assay

<table>
<thead>
<tr>
<th>Product</th>
<th>Catalog Number</th>
<th>Reagents included</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIG RNA Labeling Kit (SP6/T7)</td>
<td>1 175 025</td>
<td>- DNA templates, Control DNA and RNA</td>
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<td></td>
<td></td>
<td>- NTP labeling mixture, Transcription buffer</td>
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<td></td>
<td></td>
<td>- DNase I, RNase inhibitor</td>
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<td></td>
<td></td>
<td>- SP6 and T7 RNA Polymerases</td>
</tr>
<tr>
<td>RNase Protection Kit</td>
<td>1 427 580</td>
<td>- Buffers for hybridization, RNase digestion, and preparation of electrophoresis samples</td>
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<td></td>
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<td>- RNase T1, RNase A</td>
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<td>- SDS and proteinase K (for inactivating RNases)</td>
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<td>- Carrier RNA for ethanol precipitation of the hybrid RNAs</td>
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<td>- Control RNA and DNA</td>
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</table>

Also Required

<table>
<thead>
<tr>
<th>Product</th>
<th>Catalog Number</th>
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</thead>
<tbody>
<tr>
<td>Anti-DIG-AP, Fab fragments</td>
<td>1 093 274</td>
</tr>
<tr>
<td>Nylon Membranes, positively charged</td>
<td>1 209 299</td>
</tr>
<tr>
<td>Ready-to-use CDP-Star</td>
<td>2 041 677</td>
</tr>
<tr>
<td>DIG Wash and Block Buffer Set</td>
<td>1 585 762</td>
</tr>
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1 For more information on these products, see Chapter 2 or the Ordering Information in Appendix E.
4.1 Overview of Assay

The nonradioactive RNase protection assay with the DIG RNA Labeling Kit and the RNase Protection Kit involves the following major stages:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Prepare transcriptional vector or PCR product containing the target DNA sequence to be transcribed and an SP6 or T7 promoter sequence.</td>
<td>Variable</td>
</tr>
</tbody>
</table>
| B     | - Transcribe and DIG-label antisense RNA probe with components of the DIG RNA Labeling Kit (SP6/T7)\(^1\).  
- Remove DNA template with RNase-free DNase I\(^1\).  
- Quantify the DIG-labeled probe by the direct detection procedure\(^1\). | 2 h  
20 min  
Approx. 2 h |
| C     | Prepare target (sense) RNA, i.e. either total RNA or mRNA, by standard methods for RNA isolation. | Variable |
| D     | Hybridize DIG-labeled RNA probe to target RNA with components of the RNase Protection Kit\(^2\). | 4–16 h |
| E     | - Digest hybrid RNA with RNase T1 to remove all non-hybridized (single-stranded) regions of the RNA mixture\(^2\).  
- Remove RNase T1 from hybrid mixture with SDS and proteinase K\(^2\). | 30 min  
15 min |
| F     | - Precipitate digested RNA with ethanol in the presence of glycogen (as carrier molecule).  
- Pellet the RNA by centrifugation and dry the pellet.  
- Resuspend the RNA in sample loading buffer and denature RNA at 95°C.  
- Run the sample on a denaturing acrylamide gel.  
**Note:** We recommend precast 6% QuickPoint gels (QP9731), QuickPoint sample loading buffer (QP9239), and QuickPoint Running Buffer (QP9732) from Novex. | 25 min  
30 min  
5 min  
25–30 min |
| G     | - Transfer separated components to a positively charged nylon membrane by contact blotting\(^1\).  
- Fix the RNA to the membrane by UV crosslinking or baking\(^1\). | 20 min  
10–30 min |
| H     | Detect\(^1\) “protected” part of DIG-labeled probe with alkaline phosphatase-conjugated anti-DIG antibody and CDP-Star chemiluminescent alkaline phosphatase substrate.  
**Note:** The detection procedure also uses components of the DIG Wash and Block Buffer Set. | Approx. 2.5 h |
| I     | Record the chemiluminescent signals with X-ray film or the Lumi-Imager F1 Workstation (Cat. No. 2 012 847). | 5–20 min |

\(^1\) For details of these procedures, see Sections 2.3, 2.6, 3.2, and 4.1 of Chapter 2.  
\(^2\) For details of the nonradioactive RNase protection assay procedure, see the package insert for the RNase Protection Kit, Cat. No. 1 427 580, available at our Web site (http://biochem.roche.com). Reagents are included in RNase Protection Kit.
4.2 Typical Result from the Nonradioactive RNase Protection Assay

Figure 33. Detection of the housekeeping genes GAPDH and β-actin with the nonradioactive RNase Protection Assay.

Two antisense RNA probes were transcribed and DIG labeled with T7 RNA polymerase. For detection of GAPDH, a 403-nucleotide antisense RNA probe was transcribed from the plasmid pTRI-GAPDH human (Ambion). For β-actin, a 255-nucleotide antisense RNA probe was transcribed from the control DNA template supplied in the RNase Protection Assay Kit (linearized pA544 DNA containing an insert from the human β-actin gene). These antisense RNA-probes (1 ng/assay) were hybridized to target RNAs (0.5 µg to 5 µg samples of total RNA from human whole brain) overnight at 45°C. Probe-target hybrids were subjected to an RNase protection assay (as described in “Overview of Assay”) and detected chemiluminescently (5 min exposure in the Lumi-Imager).

The blot above shows the results of the assay with the following labeled probes and target RNAs:
- Lane 1: probe, GAPDH; target, 5.0 µg total RNA from human brain
- Lane 2: probe, GAPDH; target, 2.5 µg total RNA from human brain
- Lane 3: probe, GAPDH; target, 1.0 µg total RNA from human brain
- Lane 4: probe, GAPDH; target, 0.5 µg total RNA from human brain
- Lane 5: probe, GAPDH; negative control (20 µg yeast tRNA)
- Lane 6: probe, GAPDH; positive control (untreated), only 1/10 of probe was used.
- Lane 7: probe, β-actin; target, 5.0 µg total RNA from human brain
- Lane 8: probe, β-actin; target, 2.5 µg total RNA from human brain
- Lane 9: probe, β-actin; target, 1.0 µg total RNA from human brain
- Lane 10: probe, β-actin; target, 0.5 µg total RNA from human brain
- Lane 11: probe, β-actin; negative control (20 µg yeast tRNA)
- Lane 12: probe, β-actin; positive control (untreated), only 1/10 of probe was used.
- Lane 13: probe, β-actin; kit control (0.2 pg human β-actin sense transcript, mixed with yeast RNA)
- Lane 14: probe, β-actin; kit control (0.1 pg human β-actin sense transcript, mixed with yeast RNA)

Result: The size of the original, undigested probes can be seen in positive control lanes 6 (GAPDH, 403 nucleotides) and 12 (β-actin, 255 nucleotides). No signals are detectable in the negative controls (lanes 5 and 11). The protecting effects of as little as 0.5 µg experimental target RNA (lanes 4, 10) can be seen after only a 5 min exposure in the Lumi-Imager. All concentrations of the experimental target RNA from human whole brain (lanes 1–4 and 7–10) protect fragments of the antisense RNA probes. As little as 0.1 pg control RNA transcript (protecting 210 nucleotides) is effective in the assay (lane 14). By contrast, an RNase protection experiment with a radioactively labeled probe would require 10 µg of target RNA.