



UV Crosslinking Protocol

UV-Cross-linking in decapping extracts

1. Set up the reaction in a 1.7ml tube:
 - 7-8 μ l extract (15-20 mg/ml) (or recombinant protein)
 - 1 μ l 20 mM MgCl₂ (optional)
 - 1 μ l 1 mM ATP (optional)
 - 1 μ l hot RNA (50-100 kcpm/ μ l)
2. Incubate at 30 ° C for 5-10 min.
3. Transfer to a small microtiter dish at 4 ° C. Cross-link with 254nm UV light for 10 min.

The light should be 2-3 cm from the dish. Make sure it all lines up well.
4. Transfer to a 1.7 ml tube containing 2 μ l RNase A (10mg/ml) and incubate at 37 ° C for 10 min.
5. Add an equal volume of 2 x SDS loading dye. Boil 4 min and load on 0.8 mm thick SDS/PA gel.
6. Transfer to Whatman 3MM paper and vacuum dry for 1 hr.
7. Expose to film at -80 ° C.

UV-cross-linking to radio-labeled RNAs.

1. Incubate *in vitro* transcribed RNAs with the extract or protein of interest for 5 min at 30 °C. A 10 ml reaction volume is ideal. Buffer conditions can be varied.

50-100 µg of extract or 100ng-1µg of recombinant protein.
50-100 kcpm of ³²P-labeled RNA (internally labeled or cap-labeled)
2. Transfer each reaction to a 50 well microtiter dish on ice. Generally, the UV light will efficiently radiate only two rows so bear this in mind when picking which wells to use.
3. Line the UV light up with the wells and switch on for 10 min. The light should be 1-2 cm from the dish. Make sure to use the *short* wave bulb (254nm).
4. Transfer the reactions back to 1.7ml tubes containing 1.5µl of RNase A (and 0.3µl RNase One if the substrate contains poly(A)). Incubate at 37 °C for 10 min to degrade the input RNA.
5. Add an equal volume of 2 x SDS loading dye. Boil and load on an SDS-gel. Dry the gel and expose.

Immunoprecipitation of cross-linked proteins.

Follow the protocol described above up to step 4.

6. Add 400µl of NET-2 buffer and 1-4µl of antibody to the reaction. Incubate on ice for 1 hour.
7. Spin for 2 min to pellet any precipitated proteins. Transfer the supernatant to a fresh tube.
8. Add 20µl of a 50% slurry of protein A sepharose (pre-washed in NET-2). Incubate with gentle rocking at 4 °C for 20 min.
9. Spin down the protein-A beads and remove the supernatant.
10. Wash the pellet 3-5 times with RIPA buffer.
11. Resuspend the pellet in 25µl of 2 x SDS-loading dye. Boil and load on SDS-gel.

NET-2 buffer

50 mM Tris-Cl (pH7.6)

150 mM NaCl

.01% NP40

RIPA buffer

50mM Tris-Cl (pH7.6)

150mM NaCl

0.1% SDS

1% NP40

0.5% deoxycholate

Partial V8 proteolysis of cross-linked proteins.

This protocol allows comparison of cross-linked proteins in extracts with recombinant proteins or allows bands of the same size to be distinguished.

Follow the protocol described above, up to step 5. Either load 4 lanes for each sample or use an extra wide well comb. It is important to use a 0.75mm thick gel for this step. *Do not dry the gel!*

12. Expose the wet gel to film or phosphorimager, and excise the band of interest. Insert each gel slice into the well of a 1.5 mm thick 15% SDS gel with a long stacking gel (7-8cm).
13. Overlay each gel slice with 40 μ l of 2xSDS loading dye containing V8 protease. There should be four lanes for each sample so add increasing amounts of protease – e.g. 0 μ g, 0.05 μ g, 0.5 μ g and 5 μ g.
14. Run the dye two-thirds through the stacking gel. Turn off the power for 30 min to allow the digestion to proceed.
15. Turn the power back on and run the dye front to the bottom of the gel as normal. Dry and expose.