similar to classical approaches such as primer extension. The minimal requirement for mRNA template permits mapping of multiple promoters even when the mRNA source is limited. The mapping procedure includes sequencing of the 5' end of the transcript, thus providing the information on the exon/intron structure of the gene of interest. This feature is very useful for analysis of sequenced eukaryotic genomes because computerized gene prediction often yields erroneous gene structure, especially in the 5'-regions. PCR amplification, purification of RACE products, and sequencing are easily adapted to a standard microplate format for high-throughput application. Thus, we report here an approach for large-scale transcript mapping that, in combination with ample gene expression data available from the microarray experiments, may lead to rapid identification of regulatory sequences in eukaryotic promoters.

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Use of the Formazan Dye Zincon for Staining Proteins in Polyacrylamide Gels

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The formazan dye 2-carboxy-2'-hydroxy-5'-sulformazylbenzene (zincon) has been used previously as a colorimetric indicator of calcium in serum (1) and, histologically, for the differentiation of zinc and copper in brain tissue (2). Zincon has been shown to inhibit insulin binding to its receptor (3) and to attenuate the activity of ATPase (4) and other enzymes (5), presumably because of the chelating properties of the dye. Zincon forms blue complexes with zinc at alkaline pH and with copper at neutral or acidic pH; it forms other colored complexes characteristic of the specific transition metal ion with which it coordinates (6,7).

The finding that zincon nonspecifically binds proteins at acidic pH prompted further investigation into the use of the chelating agent as a polyacrylamide gel stain. Preliminary studies showed that in this capacity, zincon staining of SDS protein derivatives was (i) more rapid than Coomassie Brilliant Blue® (CBB) staining, (ii) reversible, and (iii) only slightly less sensitive than the CBB dyes. Moreover, (iv) the sensitivity of zincon staining could be increased by coordination of the dye with metals such as copper, cobalt, and nickel.

A saturated zincon solution was prepared by dissolving 200 mg monosodium salt (Sigma, St. Louis, MO, USA) in 100 mL absolute ethanol. This solution was filtered through Whatman® chromatography paper and was stable at room temperature for several months. SDS-PAGE was performed as described by Laemmli (8) and later modified by Shainoff et al. (9). Representative protein samples included human plasma, purified human ceruloplasmin, and protein standards from New England Biolabs (Beverly, MA, USA) and Promega (Madison, WI, USA). Concentrations of the protein standards were provided by the manufacturer. The concentration of purified ceruloplasmin was determined spectrophotometrically as described by

Ehrenwald and Fox (10). Polyacrylamide gels were cast in $10 \times 10 \times 0.1$ cm disposable glass cassettes (ZAXIS, Hudson, OH, USA). SDS-PAGE was performed in a SE-260B apparatus with extended lower buffer chamber (Amersham Biosciences, Piscataway, NJ, USA). Immediately following electrophoresis, the gels were washed 3×10 min in 45% ethanol to facilitate the removal of SDS (11).

Two methods are described for staining proteins in polyacrylamide gels with zincon. The first method uses a molecular dispersion of the dye. A 0.09% zincon solution in 45% ethanol, 10% acetic acid is prepared by admixing 45 mL saturated zincon solution with 10 mL acetic acid and 45 mL water. Maximum sensitivity was achieved following overnight staining in the dispersed dye but was concomitant with

Figure 1. Negative staining of proteins in 10% polyacrylamide gel using zincon. Serial 1:2 dilutions of human plasma and purified human ceruloplasmin (Cp) following SDS-PAGE and staining for 12 h with zincon. Gel was destained for 10 min in water. Bands x, y, and z correspond to 58%, 28%, and 12%, respectively, of the total protein in the purified ceruloplasmin. In the 0.5 μ g lane, bands x, y, and z contain an estimated 290, 140, and 60 ng protein, respectively. Molecular weight (MW) is indicated on the left.

Figure 2. Comparison of zincon versus CBB staining. Serial 1:2 dilutions of protein molecular weight standards in 8%–18% polyacrylamide gel stained with either colloidal zincon for 20 min (left) or colloidal CBB for 12 h (right). Based on serial dilutions of BSA (arrow), CBB was approximately four times more sensitive than zincon but required prolonged staining and destaining times. Staining with colloidal zincon was more rapid and did not require destaining. The BSA concentrations were 600, 300, 150, 75, and 38 ng for lanes 1–5, respectively.

high background staining. Gels were destained in 100 mM EDTA or water. A negative staining effect was observed where minor bands became visible within a clear "halo" produced as the dye is rapidly shed from the peripheries of the protein bands as proteins become deprotonated and repel the dye. Bands corresponding to 60 ng ceruloplasmin were detectable (Figure 1).

The second method uses a colloidal form of the dye that stains more rapidly and with minimal background staining but is less sensitive. Immediately before use, a 0.06% zincon solution in 30% ethanol was prepared by admixing 30 mL saturated zincon solution with 70 mL water. The gel was equilibrated for 5 min in this solution, followed by the addition of 10 mL acetic acid. Upon addition of the acetic acid, major bands developed instantaneously. Staining with colloidal zincon was complete in 20 min, as the dye shifted to its colloidal form and precipitated. Colloidal zincon detected 150 ng BSA against a clear background after only 20 min of staining (Figure 2, left, lane 3). By comparison, colloidal CBB G-250 stain (Gradipore, North Ryde, NSW, Australia) detected 38 ng BSA but required overnight staining and destaining in water for several hours (Figure 2, right, lane 5).

Following staining with colloidal zincon, the gels were washed 3×5 min in water. Protein bands were intensified by incubating the gels for 10 min in 0.4 M CuSO_4 , CoCl_2 , or NiCl_2 dissolved in water. Sensitivity was increased approximately 2-fold when zincon stained gels were enhanced with these metals. Enhancement with Cu^{2+} ions was accompanied by a shift in band color, from magenta to blue, whereas Co^{2+} and Ni^{2+} ions produced violet and green color transitions, respectively (6). No enhancement was observed in gels incubated in 0.1 M ZnCl_2 because the zincon- Zn^{2+} complex is formed at alkaline pH at which most of the dye has been released from the protein bands. For this reason, destaining of gels is greatly accelerated in alkaline pH buffers. SnCl_4 caused the magenta-colored zincon stained gel to become instantaneously "erased", presumably because of the oxidation of the formazan to produce a colorless tetrazolium.

Under these conditions, metalloproteins were not stained differentially. Coordinated copper was released from ceruloplasmin in the presence of SDS such that the three ceruloplasmin bands stained similarly to other plasma proteins (Figure 1, 136-, 116-, and 20-kDa bands). The metalloproteases alcohol dehydrogenase and carbonic anhydrase also did not stain preferentially with zincon.

Like the CBB dyes, zincon forms salt bridges with the primary amines of proteins via the sulfonate group of the dye. At acidic pH, the protonation of protein amines promotes such interaction, whereas, at neutral or alkaline pH, zincon failed to stain proteins in polyacrylamide gels. By comparison, the CBB dyes contain two sulfonate groups such that a single dye molecule can simultaneously bind two amines and form stable protein-dye-protein complexes (12), whereas the single sulfonate in zincon forms a weak interaction that is relatively unstable, as evidenced by the rapid and total destaining in water. Unlike the dyes Nile Red (13) and SYPRO Orange® (Molecular Probes, Eugene, OR, USA) (14), zincon apparently does not bind proteins via hydrophobic interaction with SDS or the hydrophobic domains of proteins (15).

Summarily, zincon stains proteins with less sensitivity than CBB but stains and destains more rapidly. More importantly, zincon staining is reversible, suggesting it might be applicable to 2-D PAGE and downstream analyses by mass spectrometry.

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