High molecular weight (HMW) DNA analysis is required for physical mapping, contiguous map construction and generating large insert libraries in cosmid, yeast artificial chromosome (YAC) or bacterial artificial chromosome (BAC) vectors (6,7). Procedures of HMW DNA isolation and gel-blot analysis using multi-copy probes have been described for wheat (5). Most of the current HMW DNA isolation procedures for wheat or other plants involve protoplast isolation, embedding them in low-melting agarose and lysis in agarose plugs (1,5). The protoplast-based procedures yield good quality DNA, but require expensive cell wall degrading enzymes and expertise in protoplast handling. Guidet et al. (4) described a direct method of HMW DNA isolation that involved embedding powdered leaf tissue in agarose, followed by cell lysis. Recently, a procedure for HMW DNA isolation from nuclei has been described for sorghum that is also suitable for wheat (7). We attempted unsuccessfully to use the previously described methods of HMW DNA isolation for gel-blot DNA hybridization analysis using single-copy probes. In this paper, we standardized or modified HMW DNA analysis methods to make them cost-effective, safer, reproducible and suitable for single-copy probe analysis.

To isolate HMW DNA, young leaves were ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was transferred to a tube, kept at 60°C and quickly mixed with 20 mM Tris-HCl, pH 8.0, 30 mM NaCl, 300 mM EDTA, pH 9.0, and 1.5% low-melting agarose (CHEF grade from Bio-Rad, Hercules, CA, USA) at a concentration of 0.5 g leaf tissue/mL. An equal volume of hot mineral oil (50°C) was mixed with the slurry, and the mixture was immediately pipetted into 100–200 mL of ice-cold stirring TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The agarose beads were then allowed to settle, and the TE was replaced with 10 volumes (of beads) of lysis buffer (10 mM Tris-HCl, pH 8.0, 500 mM EDTA, pH 9.0, 1% Sarkosyl and 0.1 mg/mL proteinase K) for 36–48 h with one change of the buffer. The beads were washed with three to five changes of TE at 50°C for 2–4 h. The DNA in beads was stored in TE buffer at 4°C for more than one year without any degradation.

The HMW DNA contained in the agarose beads was purified as follows: a 1% high-melting-point agarose gel (Bio-Rad) was prepared in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0). A two-inch-wide piece of gel, including the wells, was cut out and replaced by 1% CHEF-grade low-melting-point agarose (Bio-Rad) solution prepared in 0.5× TBE. A 0.5-cm slot was made across the low-melting agarose gel by using a comb that had Scotch® tape on its teeth. The agarose beads containing DNA were loaded in the slot that was then sealed with 1% high-melting-point agarose. The gel was run using a HULA® gel apparatus (Hoefer Pharmacia Biotech, San Francisco, CA, USA) at the following conditions: 0.5x TBE running buffer, for 40 h at 180 V, 60–120-s ramped pulse, 120° angle and 11°C. The gel was stained in ethidium bromide solution, and the HMW DNA band (ca. 0.5 cm wide) was cut out and dialyzed in two to three changes of TE at 50°C for 2–4 h. The gel piece containing the purified DNA was cut into approximately 0.5× 1.0-cm sized pieces (plugs). The purified DNA was stored at 4°C in TE for more than two years without any degradation.

For restriction enzyme digestion, each plug containing purified HMW DNA was cut into four to six pieces and incubated in 300 μL of restriction enzyme buffer (plug volume was approximately 80 μL with 30 μL 10× buffer and 190 μL sterile distilled water) on ice for 1–3 h. The buffer was then replaced with 75 μL of enzyme solution (7.5 μL 10× buffer,30–40 U of enzyme and sterile distilled water to the volume) and incubated at recommended temperature for 8–12 h. For partial digestion, the buffer solution was replaced with 60 μL of enzyme solution.
(6 µL 10× buffer, 6 µL bovine serum albumin [BSA], 1.6 µL of 100 mM spermidine and 2 U of enzyme), and the plugs were incubated on ice for 20 min. The plug pieces were then incubated at 37°C for 5 min. The amount of enzyme and the incubation time varied with the DNA concentration of the plugs and the desired average size of the partially digested DNA. The restriction digestion reaction was stopped by adding 10 µL of 0.5 M EDTA. The digested DNA was size-separated on a 1% agarose gel in 0.5× TBE running buffer for 40 h at 180 V, 60–120-s ramped pulse, 120° angle and 11°C. When partially digested DNA was to be used for cloning, the DNA was size-separated on low-melting-point agarose.

For blotting, the gel was stained in ethidium bromide solution for 30 min, destained in distilled water for 10 min and photographed. The gel was then exposed to 100 mJoules of ultraviolet (UV) light using Stratalinker® (Stratagene, La Jolla, CA, USA). Denaturation, neutralization and the transfer steps were performed following membrane manufacturer’s recommendations (Micron Separations Inc. [MSI], Westboro, MA, USA). Probe labeling and hybridization were performed as previously described (2).

Figure 1 shows unpurified HMW DNA of hexaploid (Triticum aestivum) wheat cultivar Chinese Spring. The HMW DNA band was higher than that of the 2.5-Mb yeast chromosome. Besides the HMW DNA band, a smear was observed with a size range of 2.5 Mb to less than 100 kb. The smear probably contained organelle and sheared genomic DNA. The unpurified DNA was suitable for partial and complete restriction digestion and long-range mapping using repetitive DNA probes. Lanes 3 and 4 of Figure 1 show PstI- and EcoRI-digested, respectively, wheat DNA. As expected, the average fragment size of PstI-digested DNA was higher than that of EcoRI. The PstI enzyme is a rare-cutter for wheat because of its sensitivity to C-methylation (3). The restriction digestion with both enzymes was complete. Gel-blot DNA hybridization of the digested and undigested DNA (Figure 1) using pTa71(ribosomal DNA probe) showed sharp fragment bands (data not shown). We were not successful in using unpurified DNA to analyze single-copy probes. Therefore, the DNA purification step was incorporated.
The purified DNA was at a high concentration and devoid of low molecular weight DNA smear (Figure 2). Using the purified DNA, we were able to analyze both single and multiple copy probes by the gel-blot DNA hybridization procedure. Figure 2 shows an autoradiograph of uncut and PstI-digested DNA of Chinese Spring wheat hybridized with pTa71. In the uncut DNA lane (Figure 2, lane 5), a hybridization band was observed corresponding to the HMW DNA band observed on the ethidium bromide-stained gel (Figure 2, lane 6). No smear was observed in either lane showing that most of the DNA was of high molecular weight with no subsequent degradation during handling or storage. Figure 3 shows a gel-blot DNA hybridization pattern of *Triticum tauschii* (TA1695), the D genome progenitor species of wheat, using a single-copy DNA probe (xksus1). The probe xksus1 detected single locus on each of the wheat genomes (2,3). Using HMW DNA, the probe detected one fragment band each with enzyme *Nol*I, *Sal*I and *Sfi*I and two with *Srf*I (Figure 3). Similarly, we successfully analyzed HMW DNA of tetraploid and hexaploid wheat by gel-blot DNA hybridization using single-copy probes.

Both purified and unpurified DNA were suitable for partial DNA digestion. However, the purified DNA was better as there was less variation in the fragment size, and a higher proportion of DNA was in the range of the desired size. We are using the partially digested purified DNA to generate a wheat BAC library (our unpublished results).

In summary, we report a high molecular weight (HMW) DNA analysis method in wheat that is suitable for long-range mapping and BAC library construction. A direct, cost-effective procedure of HMW DNA isolation was devised that yielded only megabase size DNA. The gel-blot DNA hybridization procedure was standardized to analyze both single- and multiple-copy probes. The cost was reduced by eliminating protoplast isolation and decreasing the amount of proteinase K to 10% of the original concentration. The quality of DNA was improved by incorporating a gel purification step. The procedure was made safer by eliminating the use of phenylmethylsulfonyl fluoride (PMSF). The procedure is suitable for diploid, tetraploid and hexaploid wheat species.

**REFERENCES**


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**ImmunoglobulinVariable-Region mRNA Direct Sequencing: A Method to Bypass Aberrant Myeloma Light-Chain Transcripts**

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Direct sequencing of immunoglobulin (Ig) variable-region mRNA is a rapid method to obtain nucleotide sequence information from hybridomas. Several protocols have been described. Recently, we have developed a rapid procedure that combines amplification of reverse-transcribed, first-strand cDNA, anchored polymerase chain reaction (PCR) followed by semi-nested PCR and direct sequencing of the second-round PCR products (1). However, when this procedure is used for κ light-chain variable region (Vκ) sequencing, overlapping sequence information may interfere due to the concomitant expression of aberrant light-chain mRNA (Vκ21E) present in SP2/O or P3-X63-Ag8.653 and the functional light-chain mRNA (3). To separate these light-chain mRNAs from each other, the PCR products must be cloned, and many colonies must be screened to obtain the functional Vκ sequence. This step is time-consuming, even with automated sequencing, especially when the functional light-chain mRNA is weakly expressed. Therefore, we have developed an efficient colony screening test that enables the functional Vκ to be distinguished from the aberrant light-chain mRNA when the functional light-chain mRNA does not belong to the Vκ21E gene family. This method adds only a PCR step to the direct-sequencing protocol and avoids sequencing plasmids containing aberrant light-chain variable-region inserts. Direct sequencing of Ig Vκ mRNA was performed as described previously (1). Briefly, cDNA was prepared using an oligo(dT) primer and 10 μg of total purified RNA, and a poly(dG) homopolymeric tail was added to the 3’ end of this first-strand cDNA. The 3’ poly(dG) products were purified, and two rounds of PCR amplification were