Quantitative DNA Fiber Mapping

Chun-Mei Lu[1], Mei Wang[2], Karin M. Greulich-Bode[3], Jingly F. Weier[4] and Heinz-Ulli G. Weier

Life Sciences Division, University of California, E.O. Lawrence Berkeley National Laboratory (LBNL), Berkeley, CA, USA.

[1]Present address: Department of Chemical Engineering, National Chin-Yi University of Technology, 35 Chungshan Road, Section 1, Tai Ping, Taichung, Taiwan 411, ROC

[2]Present address: US DOE Joint Genome Institute//E.O. LBNL, 2800 Mitchell Drive, Walnut Creek CA 94598

[3]Present address: Division Genetics of Skin Carcinogenesis, DKFZ, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany

[4]Present address: Reprogenetics, LLC, 400 Oyster Point Blvd., South San Francisco, CA 94080.

Correspondence to:

Ulli Weier, Life Sciences Division, MS 74-157, University of California, E.O. Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, CA 94720, USA.

Phone: (510) 486 4000, fax: (510) 486 6746, e-mail: ugweier@lbl.gov

Key Words: Physical mapping, DNA molecules, DNA fibers, fluorescence in situ hybridization (FISH), digital image analysis

Prepared for: Fluorescence in situ hybridization (FISH) – Application Guide (Springer Verlag)

Abstract

Several hybridization-based methods used to delineate single copy or repeated DNA sequences in larger genomic intervals take advantage of the increased resolution and sensitivity of free chromatin, i.e., chromatin released from interphase cell nuclei. Quantitative DNA fiber mapping (QDFM) differs from the majority of these methods in that it applies FISH to purified, clonal DNA molecules which have been bound with at least one end to a solid substrate. The DNA molecules are then stretched by the action of a receding meniscus at the water-air interface resulting in DNA molecules stretched homogeneously to about 2.3 kb/μm. When non-isotopically, multicolor-labeled probes are hybridized to these stretched DNA fibers, their respective binding sites are visualized in the fluorescence microscope, their relative distance can be measured and converted into kilobasepairs (kb). The QDFM technique has found useful applications ranging from the detection and delineation of deletions or overlap between linked clones to the construction of high-resolution physical maps to studies of stalled DNA replication and transcription.

1 Introduction

High resolution physical map of genomes are indispensable for positional cloning of disease genes. Typically, such maps are based on ordered sets of clones from sources such as plasmid, cosmid, P1/PAC, bacterial (BAC) or yeast artificial chromosome (YAC) libraries. The assembly of such clones into contiguous (‘contig’) maps is greatly facilitated by application of fluorescence in situ hybridization (FISH). Hybridization of non-isotopically labeled probes on to preparations of DNA molecules called ‘DNA fibers‘, which were bound with at least one end to a solid substrate and stretched homogeneously has been termed ‘Quantitative DNA Fiber Mapping (QDFM)‘ technique. The QDFM DNA is readily accessible to probes and detection reagents. Thus, hybridization efficiencies are high and allow routine detection of DNA targets smaller than 500 bp (Weier 2001, Weier and Chu 2006). Importantly, QDFM experiments require only basic laboratory equipment with access to a fluorescence microscope. By hybridizing one clone onto another, the extent and orientation of overlaps can be quantitated with near kilobase (kb) resolution (Fig 1a). To measure the physical distance between non-overlapping DNA fragments, probes are hybridized to DNA fibers representing a larger genomic interval (Fig.1b). This also provides means for the mapping of expressed sequences (cDNAs) along DNA fibers representing genomic DNA, investigation of DNA replication (Breier et al. 2005) or the detection of rearrangements, especially deletions (Admire et al. 2006).

1.1 Outline of the procedure

- Select probe and QDFM targets from libraries or in-house resources
- Isolate DNA
- Pretreat microscope slides or coverslips
- Prepare DNA fibers on glass slides
- Select probe set
- Denature and hybridize probe set
- Remove unbound probe molecules
- Detect bound probes
- Acquire a sufficient images
- Analyze the images

1.2 Principles and applications

High resolution physical maps have proven indispensable for large-scale, cost-effective gene discovery. Knowledge about the extent of overlap between clones and the precise localization of cloned DNA fragments within much larger genomic fragments is needed to assemble such maps. As demonstrated in this chapter, FISH can provide this essential information.

Isolation of DNA from cell nuclei and preparation of so-called chromatin 'fibers' improves the accessibility of the DNA targets for probes as well as detection reagents (antibodies, avidin), thus increases the hybridization efficiency (Weier 2001). Furthermore, if the DNA molecules can be stretched in a linear fashion, they provide ideal templates for visual mapping. In the past, FISH has been applied to various types of crude DNA preparations allowing the visualization of probe overlap and providing some information about the existence and size of gaps between clones. However, none of those techniques provided sufficiently accurate information about the extent of clone overlap or the separation between elements in the map because the chromatin onto which clones were mapped was condensed to varying degrees from site to site.

We demonstrated that cloned DNA fragments can readily be mapped by FISH onto DNA molecules prepared by the hydrodynamic action of a receding meniscus and, referring to its quantitative nature, we termed the technique 'Quantitative DNA Fiber Mapping (QDFM)' (Weier et al. 1995; Weier and Chu 2006). In QDFM, a solution of DNA molecules is placed on a glass or mica surface prepared so that some DNA molecules attach at one or both ends. The DNA solution is then spread over a larger area by placing a coverslip on top, and additional DNA molecules are allowed to bind to the surface. During drying, the molecules are straightened and uniformly stretched by the hydrodynamic action of the receding meniscus. Molecules prepared in this manner are stretched with remarkable homogeneity to about ~2.3 kb/µm, i.e., approximately 30% over the length predicted for a double stranded DNA molecule of the same size (Weier et al. 1995; Wang et al. 1997). QDFM can be applied to DNA molecules ranging in size from a few kb to more than 1 Mbp, which allows to map small probes with near kilobase resolution onto entire yeast chromosomes and large (mega)YAC clones (Wang et al. 1996; Duell et al. 1997; Admire et al. 2006).

Applications of QDFM extend beyond map assembly and can provide valuable information for quality control, clone validation, definition of a minimal tiling path as well as for the sequence assembly process. Furthermore, due to its high hybridization efficiency obtained with DNA fibers, QDFM is also the method of choice for high resolution optical mapping of expressed sequences in genomic intervals defined by the DNA fibers.

2 Materials

2.1 Equipment

- Refrigerated Centrifuge
- Dry bath (hot plate)
- Fluorescence microscope equipped with 40x and 63x oil immersion lenses
- Incubator oven (set to 37℃)
- Pulsed Field Gel Electrophoresis (PFGE) system (Bio-Rad)
- Shaking Incubators: 30℃ for yeast cell culture, 37℃ for culture of E. coli
- Thermal cycler for in vitro DNA amplification
- Water bath
- Digital Imaging system (optional)

2.2 Reagents

Unless noted differently, all chemicals are from Sigma Chemicals.

- 3-Aminopropyltriethoxy silane (APS)
- β-Mercaptoethanol
- β-Agarase (New England Biolabs (NEB))
- Agarose (Invitrogen)
- Antibodies against digoxigenin, rhodamine-conjugated made in sheep (Roche Molecular Biochemicals), stock solution is 1 mg/ml in PNM, dilute 1:50 with PNM prior to use. Store at 4℃.
- Antibodies against FITC made in mouse (DAKO), stock solution is 1 mg/ml in PNM, dilute 1:50 or 1:100 prior to use. Store at 4℃.
- Anti-mouse antibodies, FITC conjugated, made in horse (Vector Labs), stock solution is 1 mg/ml in PNM, dilute 1:50 prior to use. Store at 4℃.
- Anti-avidin antibodies, biotinylated made in goat (Vector Labs), stock solution is 1 mg/ml in PNM, dilute 1:50 prior to use. Store at 4℃.

- Avidin conjugated to AMCA (Vector Labs), stock solution is 2 mg/ml in PNM, dilute 1:500 prior to use. Store at 4℃.
- Blocking reagent (cat. #1096-176, Roche Molecular Biochemicals)
- Chloroform/isoamyl alcohol (24:1, vol:vol., Invitrogen)
- Dextran sulphate, average M_w >500,000
- 4,6-diamino-2-phenylindole (DAPI) (Calbiochem), 0.05 µg/ml in antifade solution. Store at -20℃.
- 10x dNTP mix: dATP, dCTP, dGTP, and dTTP, 10 mM each (Roche Molecular Biochemicals). Store at -20℃.
- Digoxigenin-11-dUTP: 1mM (Roche Molecular Biochemicals). Store at -20℃.
- EDTA (ethylenediamine tetraacetic acid): 0.5M (pH8.0, Invitrogen).
- Ethidium bromide, 10mg/ml (Invitrogen).
- Fluorescein avidin DCS (avidin-FITC, Vector), stock solution is 2 mg/ml in PNM, dilute 1:100 with PNM prior to use. Store at 4℃.
- Fluorescein-12-dUTP (1mM, Roche Molecular Biochemicals). Store at -20℃.
- Formamide (FA)(Invitrogen). Store at 4℃.
- Glycogen, 20mg/ml (Roche Molecular Biochemicals). Store at -20℃.
- Geneclean II kit (BIO 101).
- Human COT1™ DNA (1 mg/ml, Invitrogen). Store at -20°C.
- Lambda phage DNA (Roche or Invitrogen). Prepare 2.5 ng/µl in 2x SSC. Store at 4℃.
- Low melting point agarose (LMP, Bio-Rad).
- Lysozyme: prepare stock (50mg/ml in 10 mM Tris, pH 7.5), and store in aliquots at -20℃. Do not refreeze after thawing.
- 10x PCR buffer: 15 mM $MgCl_2$, 100mM Tris·HCl, 500 mM KCl, 0.01% gelatin.
- Phenol/Chloroform/Isoamyl alcohol (25:24:1, vol.:vol.:vol., Invitrogen). Store at 4℃.
- Random-Priming Kit (BioPrime kit, Invitrogen). Store at -20℃.

- Proteinase K (Roche Molecular Biochemicals): 20mg/ml in 10mM Tris·HCl, pH7.5. Store at -20℃.
- RNase (Roche Molecular Biochemicals), DNase-free: boil at 100℃ for 10 min., aliquot and store at -20℃.
- Salmon sperm DNA (3'-5', Boulder, CO; 20 mg/ml). Store at -20℃.
- Sodium dodecyl sulfate (SDS, Na salt): 10% in water.
- Thermus aquaticus (Tag) DNA polymerase (5 units/μl, Perkin Elmer). Store at -20℃.
- Ultrapure water (cat. # H453, Mallinckrodt).
- Yeast artificial chromosome (YAC) library (Invitrogen). Store at -80℃.
- YOYO-1 (Invitrogen): Stock is 1mM in DMSO. Dilute 1:1000 with water prior to use. Store at -20℃ and discard diluted dye after 1 week.
- Zymolase (70000 U/g): prepare 10mg/ml in 50mM KH_2PO_4, pH 7.8, 50% glycerol. Store at -20℃.

2.3 Buffers and other solutions

- AHC medium: add 36.7 g of AHC powder (BIO 101) per liter of purified water, autoclave at 121℃ for 15 min.
- AHC agar: add 53.7 g of AHC agar medium (BIO 101) per liter of purified water. Autoclave at 121℃ for 15 min. Cool to 50℃, mix and pour plates. Store plates at 4℃.
- Alkaline Lysis (AL) solutions sufficient for 12 preps at the level of 20 ml cell culture:
- AL Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0): add 4ml of 0.5M glucose, 0.8 ml of 0.5M EDTA and 1ml of 1M Tris-HCl to 35.2 ml water. Store at 4℃.
- AL Solution II (0.2 N NaOH, 1 % SDS): Add 1.4 ml of 10N NaOH, 7 ml of 10% SDS to 61.6 ml water.
- AL Solution III (3 M NaOAc, pH 4.8).

- Antifade solution: 1% p-phenylenediamine, 15 mM NaCl, 1 mM H_2PO_4, pH 8.0, 90% glycerol. Store at -80℃.
- Blocking solution: dissolve blocking reagent in maleic acid buffer (10% w/v) with shaking and heating. Autoclave stock solution and store in aliquots at 4℃.
- Cell fixative: acetic acid / methanol, 1:3 (vol.:vol.). Make fresh right before use.
- DB 0.5 solution: 0.5M EDTA (pH=8.0), 1.0% N- lauroyl sarcosine, 0.5 mg/ml Proteinase K (Roche Molecular Biochemicals).
- Denaturing solution: 70% FA, 2xSSC, pH 7.0. Prepare fresh at least every 2 weeks. Store at 4℃.
- ES Buffer: 0.5M EDTA (pH 8.0), 1% sarcosyl (N-lauroyl sarcosine, Na salt).
- Gel loading dye: 1% bromophenol blue in 30% glycerol.
- Hybridization master mix (MM): 14.3% w/vol dextran sulfate, 78.6% FA, 2.9 X SSC, pH 7.0. For 10 ml MM, mix 1.45 ml of 20X SSC with 0.7 ml ultrapure water, dissolve 1.43 g dextran sulfate (Calbiochem), incubate overnight, then add 7.86 ml formamide. Aliquot in 1.5 ml microcentrifuge tubes and store at -20℃. Seven microliters of MM in a 10 µl hybridization mixture results in 55% FA, 10% dextran sulphate in 2x SSC.
- Luria-Bertani (LB) broth (Maniatis et al. 1986): Dissolve 10g of tryptone, 5 g of yeast extract and 10 grams of sodium chloride in 1l of water. Adjust the pH to 7.4.
- Lysis buffer: 1% Triton X-100, 20mM Tris·HCl, 2 mM EDTA, pH 8.5.
- Maleic acid buffer: 100 mM maleic acid, 150 mM NaCl, adjust to pH 7.5 with concentrated NaOH.
- Modified deoxynucleoside triphosphate mix (10x,a total of 250 µl) for labeling with 1 mM dig-11-dUTP or FITC-12-dUTP: combine 5 µl each of 100 mM dATP, 100 mM dGTP and 100 mM dCTP with 2.5 µl of 1 M Tris-HCl (pH 7.5), 0.5 µl 0.5 M EDTA (pH 8.0) and 232 µl ultrapure water. Store at -20°C. The final concentration of nucleoside triphosphates is 2 mM each.

- PNM: Dissolve 5 g of non-fat dry milk in 100 ml of PN buffer (PN buffer is 0.1M sodium phosphate (pH 8.0), 0.1% nonidet-P40)), incubate at 50℃ overnight and add 1/50 vol. of sodium azide, spin at 1000g for 30 min, aliquot clear supernatant into 1.5 ml tubes and store at 4℃. Spin at 2000g for 20 sec prior to use.
- SCE: 1M sorbitol, 0.1 M Na citrate, 10mM EDTA, pH 7.8.
- Slide Blocking Solution (5x SSC containing 2% Blocking Reagent, 0.1% N-lauroyl sarcosine): combine 0.05g N-lauroyl sarcosine and 1g Blocking Reagent with 12.5ml of 20x SSC (pH7.0), add 30ml water, heat to 60℃ while stirring and bring the final volume to 50ml with ultrapure water, when the Blocking Reagent is dissolved. Aliquot into 1.5ml tubes, spin at 2000 rpm for 10 min. and store at 4℃.
- SSC: 20x SSC is 3 M NaCl, 0.3 M Na_3·citrate·$2H_2O$, pH 7.0.
- 10x Taq buffer: 500 mM KCl, 100 mM Tris·HCl, pH8.3, 10 mM $MgCl_2$.
- TBE (Tris/borate/EDTA) buffer, 10x is 890 mM Tris base, 890 mM boric acid, 20mM EDTA.
- TE (Tris/EDTA) buffer, 1x is 10mM Tris·HCl, 1 mM EDTA, pH 7.4, 7.5 or 8.0.
- TE 50 buffer: 10mM Tris·HCl, 50 mM EDTA, pH 7.8.
- Tris·HCl [tris(hydroxymethyl)aminomethane]: 1 M, pH7.5 or 8.0.

3 Protocol

3.1 Preparation of APS-derivatized slides

The derivatization of glass substrates is one of the most critical steps of the procedure. The slides need to have the capacity to bind DNA molecules at one or both ends, but allow the molecules to stretch during the subsequent drying.

3.1.1 Slide preparation

a. Clean glass slides (Fisher Scientific) mechanically by repeated rubbing with wet cheesecloth to remove dust and glass particles.

b. Rinse thoroughly with ultrapure water.

c. Immerse slides in boiling ultrapure water for 10 minutes.

d. Air dry.

e. Immerse slides in 18 M sulfuric acid at 20℃ for at least 30 minutes to remove organic residues.

f. Immerse in boiling water for 2 minutes.

g. Air dry and store until further use.

3.1.2 Silane modification

a. Immerse precleaned dry slides in a solution of 0.1% APS in 95% ethanol for 10 minutes.

b. Remove slides from the silane solution.

c. Rinse several times with water, and immerse in ultrapure water for 2 minutes.

d. Dehydrate by immersing in absolute ethanol for 8 min.

e. Dry upright for 10 minutes at 65℃ on a hot plate.

f. Store slides for 2-6 weeks at 4℃ in a sealed box under nitrogen prior to use.

Coverslip silanation is performed similar to the procedure described above. Briefly, coverslips are rinsed with distilled water and dehydrated in 100% ethanol. Coverslips are derivatized with a 0.05-0.1% solution of APS, 95% ethanol for 2 min, rinsed and dried as described above (steps 3.1.2c-f).

3.2 Preparation of high molecular weight (hmw) DNA

The YAC clones used in the examples presented here were part of the CEPH/Genethon library (Cohen et al. 1993) commercially distributed by Research Genetics. Other clones might be used in their place without changing the protocols. Information for many of the CEPH clones including insert size, STS contents and radiation hybrid or genetic map position is available from the CEPH/Genethon server at URL http://www.genethon.fr/ genethon_en.html/ or the Massachusetts Institute of Technology (MIT) server (URL http://www-genome.wi.mit.edu/).

The agarose plug preparation and pulsed field gel electrophoresis using a CHEF electrophoresis system (Bio-Rad) follow standard protocols. Typically, five to fifteen individual YAC colonies are tested to account for deletions. In most cases, the largest clone carries the least deletion(s).

The P1/PAC/BAC clones (Invitrogen) typically show far fewer deletions, so that it often suffices to pick 2-3 colonies from a plate, grow the cells overnight in LB broth, and extract the DNA using an alkaline lysis protocol. The DNA can then be loaded directly onto the PFGE gel using a common gel loading dye.

The DNA is recovered from the low melting point agarose slab gel by excising the appropriate band using a knife or razor blade. High molecular weight DNA is then isolated by β-agarase digestion of the gel slices.

3.2.1 Pulsed field gel electrophoresis (PFGE)

3.2.1.1 Gel plugs containing YACs

a. Spin down cells from 5 ml AHC media at 400 rpm for 6 min. Resuspend cells in 0.5 ml of 0.125 M EDTA, pH 7.8. Spin again and remove supernatant.
b. Resuspend the ~ 70 µl cell pellet in 500 µl of SCE. Mix with an equal volume of 1.5% LMP agarose preheated to 43 ℃. Quickly pipet up and down, then vortex for 1-2 sec to mix. Pipet into plug molds (Bio-Rad) and allow to solidify at room temperature or on ice.
c. Remove plugs from molds, incubate samples in 2 ml SCE containing 100 µl of zymolase and shake at 150 rpm at 30 ℃ for 2.5 hrs to overnight.

d. Remove SCE and add 2 ml of ES containing 100 µl of proteinase K (20 mg/ml). Shake 5 hrs to overnight at 50℃.

e. Remove ES and rinse 5 times with 6 ml of TE50 for 30 min each rinse. Store at 4℃ until used in PFGE.

f. For P1/PAC/BAC's: growth cell overnight in LB medium containing the appropriate antibiotic and isolate DNA (see Sect. 3.4.1).

3.2.1.2 PFGE running conditions

a. YACs: voltage gradient, 6 V/cm; switching interval, 79 sec forward, 94 sec reverse; running time, 38 hrs; agarose concentration, 1.0% LMP agarose; temperature, 14 ℃; running buffer, 0.5x TBE.

b. P1/PAC/BAC clones: voltage gradient, 6 V/cm; switching interval, 2 sec forward, 12 sec reverse; running time, 18 hrs; agarose concentration, 1.0% LMP agarose; temperature, 14 ℃; running buffer, 0.5x TBE.

c. For probe production and PFGE optimization: stain the gel with ethidium bromide (EB, 0.5 µg/ml in water), cut a gel slice containing the target DNA band and transfer slice to a 14ml polystyrene tube (for example, cat.# AS-2264, Applied Scientific). Wash slice with ultrapure water for 30 minutes, and then incubate in 1x agarase buffer for 30 minutes.

d. For high molecular weight DNA isolates: run duplicate samples on the right and left side of the gel, respectively. After a predetermined run time, cut gel in half, and stain one half with EB. Measure the migrated distance on a UV transilluminator, cut out a gel slice at the corresponding position from the unstained half and proceed with the equilibration of the slice in agarose buffer (see Sect 3.2.1.2-c).

3.2.2 Recovery of hmw DNA from LMP agarose gels

a. Melt the gel slice completely by incubating it in a microcentrifuge tube for 10 min at 85℃.

b. Transfer tube to a 43℃ waterbath.

c. For every 25 µl of agarose, add 1µl β-agarase in buffer provided with the enzyme.

d. Incubate at 43℃ for 2 hrs.

e. Add an equal volume of 200 mM NaCl and store at 4℃ until use in QDFM experiments.

3.2.3 Genomic DNA

Genomic hmw DNA is isolated from exponentially growing human cells such as the C32 melanoma cell line (ATCC) or diploid fibroblast cells using standard procedures. Briefly, about $5x10^5$ cells are washed in PBS. The cells are then resuspended in 0.5 ml of PBS and mixed with 1.2% LMP agarose previously melted in PBS and allowed to cool down to 43℃. Aliquots of 100 μl are dispensed into plug molds and allowed to set for 30 min at 4℃. Agar ose plugs are then placed into DB 0.5 solution and incubated overnight at 50℃. Next, plugs are washe d 4-6 times for 30 min each in 50 mM Tris·HCl, 1 mM EDTA and stored at 4℃. The hmw DNA is rele ased by digestion of the plugs with β-agarase (see Sect 3.2.2).

3.3 Immobilization and stretching of DNA molecules

The correct immobilization of DNA molecules is important for the successful stretching as well as the minimization of DNA loss during denaturation and hybridization. We have used different methods for binding and stretching of DNA on APS-pretreated surfaces. The quality of the resulting DNA fibers on glass or mica surfaces (Hu et al. 1996) appears to be determined primarily by the DNA preparation and by properties of the modified surface rather than by the method of DNA stretching. In general, the ideal APS-surface binds the DNA molecules only at their ends, or in the case of circular DNA molecules, at the position of nicks. The remainder of the DNA molecule should be free in suspension. This can be observed in the fluorescence microscope after addition of 1 μM YOYO-1 to the DNA before immobilization.

One to two microliter of DNA are mixed with an equal amount of YOYO-1 (1 μM) and 8 μl water. One microliter of the diluted DNA is then applied to an untreated coverslip, which is placed DNA side down on the APS-treated slide or coverslip. The DNA concentration can be estimated in the fluorescence microscope using a filter set for FITC and adjusted if needed. After as little as 2 min of incubation at room temperature, the untreated coverslip can be removed slowly from one end,

allowing the receding meniscus to stretch bound DNA molecules in one direction (Hu et al. 1996). Alternatively, the slide or coverslip sandwich can be allowed to dry overnight at room temperature, after which the untreated coverslip is removed. Slides or coverslips carrying DNA fibers are then rinsed briefly with water, drained, allowed to dry at room temperature and aged in ambient air at 20℃ for 1 week before hybridization.

3.4 Probes generated from cloned DNA fragments

Most QDFM experiments use several different probes simultaneously. One probe is needed to counterstain the entire DNA fibers (Wang et al. 1996). This probe is usually prepared by labeling DNA from the same batch that was used to prepare the fibers. Probes for sequences to be mapped along the fibers are detected in a different color. Furthermore, it is recommended to include landmark probes that provide reference points by binding specifically to the vector part or the ends of DNA molecules (Hsieh et al. 2000).

3.4.1 Alkaline lysis protocol and purification of DNA from P1, PAC or BAC clones

This protocol describes the isolation of hmw DNA from 20 ml overnight cultures using 40 ml volume Oakridge centrifugation tubes. The protocol can be scaled up or down to accommodate different volumes.

a. Grow culture overnight in ~30 ml LB medium containing the recommended amount of antibiotic.
b. Prepare Oakridge tubes. Write the clone ID on a piece of tape attached to the cap. Spin 18.5 ml of culture at 2000 g for 10 min at 4℃ and d iscard the supernatant.
c. Resuspend the pellet in 2340 µl of AL Solution I, then add 100 µl of lysozyme stock to each tube. Incubate tubes for 5 min at room temperature. Next, place the tubes on ice.
d. Add 5.2 ml of AL Solution II. The mixture should now become clear. Mix gently by inverting the tubes several times. Incubate for 5 min on ice.
e. Add 3.8 ml of AL Solution III and mix gently by inverting the tubes several times. Incubate for 10 min on ice.

f. Spin for 15 min at high speed (11,500 rpm / 14,000g).

g. Transfer 10.4 ml of each supernatant into a new Oakridge tube, add 5.8 ml of isopropanol and mix gently by inverting tubes several times. Use the old cap [with the ID sticker] on the new tube.

h. Spin for 5 min at ~10,000g and discard the supernatant. Watch the pellet!

i. Wash the pellet in cold 70% ethanol. Dry pellets briefly, i.e., at ~20-40min at 20℃ to 37℃.

3.4.1.1 Phenol/chloroform extraction of DNA

a. Resuspend each pellet in 0.8 ml of TE buffer and split the volume into two 1.5 ml microcentrifuge tubes.

b. Add 400 µl phenol/chloroform/isoamyl alcohol to each tube. All centrifugations during the following phenol/chloroform extraction are done at 12,000g.

c. Vortex for 15 sec and spin down for 3 min.

d. Remove most of the bottom layer and spin again for 3 min.

e. Transfer the top layer to new microcentrifuge tubes and add 400 µl chloroform/isoamyl alcohol (24:1, vol.:vol.)

f. Vortex well for 15 sec, spin down for 3 min and remove most of the bottom layer followed by a second centrifugation for 3 min.

g. Transfer top layer to a new tube, add 2.5 volumes, i.e., 1 ml of 100% ethanol and let the DNA precipitate for 30 min. at -20℃.

h. Spin down for 15 min, discard the supernatant and wash the pellet once in cold 70% ethanol, remove supernatant and air dry the pellet.

i. Resuspend the pellet in 20-40µl TE, pH 7.4 containing 10 µg/ml RNAse.

j. Incubate 30 min at 37℃ and store at -20℃ until used.

3.4.2 Preparation of DNA from YAC clones

Retrieve the desired the YAC-containing yeast clone from the library and grow it on AHC agar for 2-3 days at 30℃. Pick individual colonies from the plate and culture the cells in up to 35 ml AHC media at 30℃ for 2-3 days.

3.4.2.1 DNA extraction, phenol purification and alcohol precipitation:

a. Centrifuge cells (in ~35 ml AHC media) at 2000g at 4 ℃ for 5 min.
b. Decant the supernatant and resuspend cells in 3 ml total of 0.9 M sorbitol, 0.1 M EDTA, pH 7.5, containing 4 μl β-mercaptoethanol, followed by addition of 100 μl of zymolase (2.5 mg/ml), and then incubate at 37℃ for 60 min.
c. Pellet the cells at 2000g and 4 ℃ for 5 min and de cant supernatant.
d. Resuspend pellet in 5 ml of 50 mM Tris, pH 7.4, 20 mM EDTA. Add 0.5 ml of 10 % SDS and mix gently. Incubate at 65 ℃ for 30 min.
e. Add 1.5 ml of 5 M potassium acetate and place on ice for 60 min.
f. Spin at 12,000g for 15 min at 4 ℃, and transfer the supernatant to a new tube.
g. Mix the supernatant with 2 volumes of 100% ethanol by inverting the tube a few times. Spin in 5000 rpm (2000g) for 15 min at room temperature.
h. Prepare 12 sets of 1.5 ml microcentrifuge tubes.
i. Decant supernatant and air dry the pellet. Resuspend pellet in 3 ml of 1x TE, pH 7.5.
j. Transfer the DNA solution to four 1.5 ml microcentrifuge tubes.
k. Add an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0), vortex well and spin at high speed (10,000g) for 3 min.
l. Transfer the top layer to new 1.5 ml tubes and add an equal volume of chloroform/isoamyl alcohol (24:1). Vortex well and centrifuge at high speed (10,000g) for 3 min.
m. Transfer the top layer to new 1.5 ml tubes. Add 40 μl of RNAse (1 mg/ml, DNAse free) to each of the four tubes and incubate at 37℃ for 30 mi n.
n. Add 1 volume of isopropanol and gently mix by inversion. Centrifuge at high speed (10,000g) for 20 min.

o. Remove supernatant and wash pellet with 1 volume of cold 70% ethanol, and centrifuge at high speed (10,000g) for 3 min.

p. Remove the ethanol and air dry the pellet.

q. Resuspend pellet in 30 µl 1x TE, and measure DNA concentration after the pellet is completely dissolved.

3.5 Generation of probes by in vitro DNA amplification

In vitro DNA amplification using PCR is a very efficient method to synthesize probe DNA. It can be applied to amplify a particular DNA sequence, such as a part of the cloning vector, or with mixed-base primers to perform arbitrary amplification of almost any sequence of interest (Wang et al.1996). As illustrated below, the former amplification can be applied to prepare DNA landmark probes, while the latter allows the preparation of probes to counterstain the fibers.

3.5.1 Cloning vector-specific probes

Probes for P1/PAC-, BAC- and YAC-vectors DNA take advantage of the access to published vector sequences. PCR primers are typically designed to amplify fragments of 1100-1400 bp of vector sequence. Several such oligonucleotide pairs which have been designed are used in either single pairs or combinations (Hsieh et al. 2000). The PCR usually follows standard conditions, i.e., a Tris-HCl buffer containing 1.5 mM $MgCl_2$ and 1 unit Taq DNA polymerase per 50 µl reaction is used, annealing temperatures range from 50℃ to 60℃.

On the other hand, the YAC cloning vectors pJs97 and pJs98, cloned in plasmid vectors (Invitrogen), can be used to prepare probes useful to determine the orientation of the YAC insert (Duell et al. 1997). For this purpose, plasmid DNA is extracted using the alkaline lysis protocol and labeled by random priming as described below.

3.5.2 Mixed base oligonucleotide primed PCR

The DNA probes for counterstaining the YAC DNA fibers are generated by mixed base oligonucleotide primed PCR (also referred to as degenerate oligonucleotide primed PCR or ‘DOP-PCR’) (Cassel et al. 1997). An aliquot of the hmw DNA obtained by PFGE for fiber preparation is

PCR amplified for a total of 42 cycles with oligonucleotide primers that anneal about every 200-800 nucleotides. In our preferred scheme, we use two different DNA amplification programs. Initially we perform a few manual PCR cycles using T7 DNA polymerase to extend the oligonucleotide primers at a relatively low temperature. Next, DNA copies prepared in these first cycles are amplified using the thermostable Taq DNA polymerase and a rapid thermal cycling scheme.

In the first amplification stage, T7 DNA polymerase ('Sequenase II', Amersham Pharmacia Biotech) is used in 5-7 cycles to extend the mixed base primer JUN1 (5'-CCAAGCTTGCATGCGAATTCNNNNCAGG-3', N=ACGT) that is annealed at low temperature. Briefly, 2-3 µl of hmw DNA solution are removed from the bottom of each tube and PCR amplified using the following conditions: denaturation at 92℃ f or 3 min, primer annealing at 20℃ for 2 min and extension at 37℃ for 6 min. Since it is not hea t stable, Sequenase II must be added after each denaturation step.

In the second amplification stage, 20 µl of the reaction product are resuspended in 200 µl Taq amplification reaction buffer and amplified with primer JUN15 (5'-CCCAAGCTTGCATGCGAATTC-3') with the following PCR conditions: denaturation for 1 min at 94℃, primer annealing for 1 min at 50℃, and extension at 72° for 2 min. This is repeat ed for a total of 35 cycles. After precipitation of the PCR products in 1.2 vol of isopropanol, the products are resuspended in 30 µl of TE buffer. Subsequently, 1.5 µl of this solution is labeled in a 25 µl random priming reaction incorporating FITC-12-dUTP.

3.6 Probe labeling via random priming and hybridization

Several manufacturers offer kits for labeling of DNA by random priming. Slight differences exist with regard to enzyme activity, amount of random primers and cost per reaction. The technique can be applied routinely to label DNA fragments from 100bp to several hundred kb. The procedure involves an initial thermal denaturation of the DNA to allow the random oligonucleotides ('primers') to anneal. Enzymatic restriction or hydrolysis of large molecules is not necessary.

3.6.1 Measurement of DNA concentration

The concentration of PCR products can be estimated from the agarose gels run to confirm target amplification. If a sufficient amount of clonal or genomic DNA is available, one or two microliters can be used to accurately determine the concentration using Hoechst 33258 fluorometry using a TK100 or similar fluorometer (Pharmacia).

3.6.2 Random Priming

a. Add about 250 ng of DNA to water to a final volume of 7μl in a 0.5 ml microcentrifuge tube.

b. Heat DNA for 5 min in boiling water, then quickly chill on ice.

c. For labeling with either dig-dUTP or FITC-dUTP, add:

2.5 μl 10x Modified Nucleotide Mixture

3.25 μl 1 mM dTTP

1.75 μl dig-11-dUTP or FITC-12-dUTP (1mM)

10 μl 2.5X Random primers (BioPrime kit, Invitrogen part# YO1393)

(For labeling the DNA with biotin, add 2.5 μl 10x dNTP mix provided with the BioPrime kit (containing biotin-14-dCTP), 5 μl water, and 10 μl 2.5x random primers).

d. Mix well, add 0.5μl DNA polymerase I (Klenow fragment, 40 units/μl, Invitrogen, part #YO1396) and incubate at 37 °C for 60-120 min.

e. Terminate the reaction by addition of 2.5 μl of 10X stop buffer (Invitrogen, part #YO1107, part of the BioPrime kit).

f. Store probe at –20 °C until used.

3.7 Fluorescence in situ hybridization (FISH)

All hybridizations are carried out overnight at 37℃ in a moisture chamber. Fiber hybridizations include a comparatively low concentration of a biotin- or FITC-labeled DNA probe prepared from the high molecular weight DNA that is used to prepare the fibers. This counterstains the otherwise invisible DNA fibers and allows competitive displacement by the probes to be mapped along the DNA fiber (Weier et al. 1995; Duell et al. 1997,1998; Weier and Chu 2006). One or more cloning vector-specific probes are included to allow a determination of the orientation of the insert.

3.7.1 Hybridization

The hybridization procedure is very similar to protocols to used on metaphase spreads:

a. Hybridization mix: combine 1 µl of each probe, 1 µl of human COT1™ DNA (optional), 1 µl of salmon or herring sperm DNA, and 7 µl of MM.
b. Apply the hybridization mixture to the slide and coverslip.
c. Denature the slide at 88-92 ℃ for 90 sec on a hot plate.
d. Transfer the slide to a moist chamber and incubate overnight at 37 ℃.

3.7.2 Post-hybridization steps

Wash and detection steps are similar to protocols used for FISH to interphase and metaphase cells that have been described (Wang et al. 1996):

a. After hybridization, wash the slide three times in 2x SSC at 20℃ for 10min each.
b. Incubated the slide with 100 µl PNM buffer or blocking stock solution under a plastic coverslip at 20℃ for 5 min.
c. The slide is then incubated at room temperature for 30 min with 100 µl PNM buffer containing AMCA-avidin, anti-digoxigenin-rhodamine and a mouse antibody against FITC. If only two labels are used, i.e., biotin and digoxigenin, bound probes are detected with avidin-FITC DCS and anti-digoxigenin-rhodamine, respectively.
d. The slide is washed two to three times in 2x SSC for 15 min each at 20 ℃ with constant motion on a shaking platform.
e. If necessary, signals are amplified using a biotinylated antibody against avidin raised in goat followed by another layer of AMCA-avidin, a Texas Red-labeled antibody against sheep raised in rabbit and a horse-anti-mouse antibody conjugated to FITC (Wang et al. 1996).
f. The slide is mounted in 8 µl of DAPI (0.05 µg/ml in antifade solution) and covered by a 22mm x 22mm coverslip.

3.8 Digital image acquisition and analysis

Although not a prerequisite for QDFM, digital image acquisition and computer-assisted analysis greatly facilitate the analysis of fiber FISH results. Since QDFM is based on simple measurements of distances between probe hybridization domains, the analysis can also be performed on images recorded on film.

Images are acquired using a standard fluorescence microscope equipped with 63x, 1.25 N.A. and 40x, 1.2 N.A. objectives, and a filter set for excitation and simultaneous observation of DAPI, Texas Red/rhodamine, FITC and CY5 fluorescence, respectively (ChromaTechnology, Brattleboro, VT). Current filters are capable of excitation in single bands centered on 360nm, 405 nm, 490 nm, 555nm, and 637nm, and visualization in multiple bands in the vicinities of 460 nm (blue), 520 nm (green), 600nm (red) and 680nm (infrared). Images are collected using a CCD camera connected to a computer workstation (Weier et al. 1995).

For determination of map positions, software is available for Apple Macintosh, IBM/PC or SUN computers that allows the user to trace DNA fibers by drawing a segmented line and then calculates the length of the line in pixels (Wang et al. 1996; Duell et al. 1997). The pixel spacing is known from the microscope objective used (use a 63x objective for molecules up to 100kb, a 40x objective for larger molecules) and is converted into µm (or kb using the factor of 2.3kb/µm). After all relevant distances along the DNA fibers in triplicate are measured, the results are imported into Microsoft Excel spreadsheets and used to calculate average values for each fiber and mean values and standard deviations for individual experiments.

4. Results

Solid substrates for QDFM are prepared in batches of 20-40 by derivatization of glass microscope slides, coverslips or sheets of mica with APS, which results in primary amino groups on the surface (Weier et al. 1995; Hu et al. 1996). For DNA fiber stretching, a solution of target DNA molecules on to which probes are to be mapped is placed on an untreated coverslip and spread by placing the coverslip upside-down on the derivatized glass or mica surface. Binding of DNA to the substrate and the stretching effect can be monitored in the fluorescence microscope after staining the DNA with YOYO-1 prior to deposition. This also allows rejecting slides that bind DNA too tightly. Following DNA binding and stretching, the coverslips are removed, slides are rinsed briefly with double-distilled water, air dried and stored at 4℃.

The DNAs from plasmid, cosmid, P1/PAC and BAC clones are isolated using the alkaline lysis protocol and inserts are sized by PFGE. Digestion of DNA with a rare cutting restriction enzyme can be used to produce linear hmw DNA molecules, but the alkaline lysis procedure typically provides sufficient amounts of nicked circular or randomly broken DNA suitable for QDFM (Wang et al. 1996). The DNA is loaded on a 1.0% LMP agarose gel and electrophoresed for up to 15 hr (or less for small molecules). The band containing the desired linear or circular DNA is then excised from the gel, and the gel slice is digested with agarase. Similarly, YAC DNA from various clones is purified by PFGE. The integrity of DNA molecules can be assessed by microscopic inspection of aliquots of DNA stained with 0.5 µM YOYO-1, before hmw DNAs are used for DNA fiber or FISH probe preparation or stored at 4℃ in 100mM NaCl.

The density of DNA molecules after DNA fiber stretching can be set by adjusting the concentration of DNA prior to binding. Figure 2d shows the typical density of hybridized lambda DNA. In experiments depositing circular P1 and BAC DNA molecules, the fraction of intact DNA molecules can reach 70-80% (Fig.2f). While binding of DNA molecules in their circular form helps to maintain their integrity, it interferes with homogeneous DNA fiber stretching, and the molecules are found to be stretched to varying degrees (Fig.2f). Mapping on to circular molecules can thus be used

for rough estimation of overlap, and mapping on linear fibers for high precision measurements. This can be done in a single experiment, because some circular DNA molecules are sheared during deposition, thus providing randomly broken DNA molecules (Fig.2f).

Quantitative DNA Fiber Mapping can facilitate the construction of high resolution physical maps comprised of any combination of cosmid, P1, PAC or BAC clones in two ways: if a low resolution map is available, for example, in the form of a YAC contig, individual clones can be mapped directly on to DNA fibers prepared from the larger clones (Weier et al. 1995, Cheng and Weier 1997). Alternatively, a high resolution map can be constructed by measuring the extent and orientation of overlap between individual clones (Fig.1). In most experiments, the applied scheme will be determined by the sources of the clones and may combine the two schemes. Figure 2d shows a typical example of QDFM mapping a P1 clone (red) on to a colinear YAC clone (green). Precise localization of the region of overlap (OV) is facilitated by probes that mark specifically the ends of the YAC molecules (Fig.2d, red arrows). When plasmid libraries are prepared in preparation of shot-gun or directed sequencing projects, QDFM can help to identify chimeric clones (showing more than the expected one hybridization domain) as shown in the plasmid-on-to-P1 mapping example in Fig.2g.

Additionally, QDFM might also be applied to circular DNA molecules for which the preparation and purification is simple and fast. An example of mapping an exon specific plasmid clone onto circular BAC molecules is depicted in Fig.3a. The use of circular DNA molecules results in dense deposition of circular DNA molecules in the presence of linear fragments of different sizes (Fig.2f,3a). The largest circles are stretched to about ~2.3 kb/µm, but smaller, more condensed molecules can also be analyzed using the extent of the vector-specific green ~7kb domain on BACs as standard for normalization (Fig.3a). The linear fragments found on the same slides are stretched more homogeneously, thus providing DNA fibers without need for normalization (Fig.3a).

Expressed sequences can be mapped easily by QDFM, if each individual target extends for 500 bp or more. A common approach hybridizes small genomic DNA fragments of 1-2 kb that contain known exons onto larger genomic DNA molecules. If the cDNA sequence and some information

about intron-exon boundaries are available, such small DNA fragments can rapidly be generated from genomic DNA using PCR. Figure 3a demonstrates this by mapping exon 2 of the human Band 4.1 gene onto a homologous BAC molecule. This allowed the localization of the ~2 kb exon with near kilobase precision.

A rapid approach to study the genomic organization of genes is based on direct mapping of expressed sequences. The probe DNA is isolated from cDNA clones, labeled and hybridized on to DNA fibers prepared from genomic DNA or recombinant DNA clones. In the presence of blocking DNA, the cDNA-derived probes will bind specifically to their complementary DNA targets, i.e., exons and 5'- or 3'-untranslated regions (UTRs) along the DNA fiber leaving non-coding regions (introns, flanking DNA etc.) unstained (Fig.3b-d). Using FISH conditions similar to those applied to hybridization and probe detection on metaphase chromosomes, this system works well with cDNA probes of several kb.

Together, these results emphasize QDFM as a powerful tool offering unique opportunities which complement or might circumvent other time- or labor-intensive diagnostic approaches.

5. Troubleshooting

5.1 Slide pretreatment

Slides from different manufacturers or even of the same brand may produce very different qualities of fibers. Use a large batch of slides from one manufacturer; avoid slides that are painted on one end, since the paint might come off during pretreatment. Preferable are slides that have a sandblasted area at one end.

5.2 Homogenous stretching of DNA molecules

Different procedures have been described to stretch DNA molecules. In our hands, stretching involving a hydrodynamic force at 20℃ or 4℃ has prov en most reproducible. There is no need to wait until the preparation has completely dried. Once the DNA molecules have bound to the substrate, the coverslip can be lifted to exert the hydrodynamic stretching force (Hu et al. 1996).

5.3 Immunocytochemical signal amplification

Never let the slides or part of them dry out during the immunocytochemical signal amplification. It is important to just drain the liquids from the slides, and then rapidly apply the next solution such as a blocking solution or the antibodies. If the slides dry out, the level of background staining will increase to unacceptable levels.

5.4 Image acquisition

Since most fluorochromes fade very quickly, minimize the exposure of slides to the excitation light.

5.5 Image analysis

Always measure additional segments of the stained molecules such as the vector segment since these might provide additional information about the extent and homogeneity of DNA stretching.

5.6 Errors

Relative standard deviations are typically in the order of ~5%. Higher standard deviations signal to check the data analysis results for operator errors and undesirable images such as broken molecules or insufficiently stretched fibers.

6. Conclusion and Outlook

Quantitative DNA Fiber Mapping is a rapid technique for mapping cloned DNA fragments with near kilobase resolution. The technique has been applied for construction of physical maps, studies of the genomic organization of expressed sequences, stalled transcription and genomic rearrangements. Preparation of DNA fibers from genomic DNA allows the detection of deletions in large insert clones and validation of sequencing templates. Improvements in detection sensitivity and increased throughput will make QDFM the method of choice for quality control in production mapping and sequencing as well as in tumor research.

7. Acknowledgements

This work was supported by a grant from the Director, Office of Energy Research, Office of Health and Environmental Research, U. S. Department of Energy, under contract DE-AC02-05CH11231.

8. References

Admire A, Shanks L, Danzl N, Wang M, Weier U, Stevens W, Hunt E, Weinert T (2006) Cycles of chromosome instability are associated with a fragile site and are increased by defects in DNA replication and checkpoint controls in yeast. Genes Dev 20:159-173

Breier AM, Weier HU, Cozzarelli NR (2005) Independence of replisomes in Escherichia coli chromosomal replication. Proc Natl Acad Sci USA 102:3942-3947

Cassel MJ, Munné S, Fung J, Weier H-UG (1997) Carrier-specific breakpoint-spanning DNA probes for pre-implantation genetic diagnosis [PGD] in interphase cells. Hum Reprod 12:2019-2027

Cheng J-F, Weier H-UG (1997) Approaches to High Resolution Physical Mapping of the Human Genome. Biotechnology International, C. F. Fox and T.H. Connor, (Eds.), Universal Medical Press, San Francisco, pp. 149-157.

Cohen D, Chumakov I, Weissenbach J (1993) A first-generation physical map of the human genome. Nature 366:698-701

Duell T, Wang M, Wu J, Kim U-J, Weier H-UG (1997) High Resolution Physical Map of the Immuno-globulin Lambda Variant Gene Cluster Assembled by Quantitative DNA Fiber Mapping. Genomics 45:479-486

Duell T, Nielsen LB, Jones A, Wang M, Young SG, Weier H-UG (1998) Construction of two near-kilobase resolution restriction maps of the 5´ regulatory region of the human apolipoprotein B gene by Quantitative DNA Fiber Mapping (QDFM). Cytogenet Cell Genet 79:64-70

Hsieh HB, Wang M, Lersch RA, Kim U-J, Weier H-UG (2000) Rational design of landmark probes for quantitative DNA fiber mapping (QDFM). Nucl Acids Res 28:e30

Hu J, Wang M, Weier H-UG, Frantz P, Kolbe W, Olgletree DF, Salmeron M (1996) Imaging of single extended DNA molecules on flat (aminopropyl)triethoxysilane-mica by atomic force microscopy. Langmuir 12:1697-1700

Maniatis T, Fritsch EF, Sambrook J (1986) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Wang M, Duell T, Gray JW, Weier H-UG (1996) High sensitivity, high resolution physical mapping by fluorescence in situ hybridization [FISH] on to individual straightened DNA molecules. Bioimaging 4:1-11

Weier H-UG, Wang M, Mullikin JC, Zhu Y, Cheng J-F, Greulich KM, Bensimon A, Gray JW (1995) Quantitative DNA fiber mapping. Hum Mol Genet 4:1903-1910

Weier H-UG, Munné S, Fung J (1999) Patient-specific Probes for Preimplantation Genetic Diagnosis (PGD) of Structural and Numerical Aberrations in Interphase Cells. J Assist Reprod Genet 16:182-189

Weier HU (2001) DNA fiber mapping techniques for the assembly of high-resolution physical maps. J Histochem Cytochem 49:939-948

Weier HUG, Chu L (2006) Quantitative DNA fiber mapping in genome research and construction of physical maps. Methods Mol Biol 338:31-57

9. Suppliers

5 Prime, 5603 Arapahoe, Boulder, CO 80303, USA; Phone: 800-533-5703-or-303-440-3705; http://www.5prime.com/

Amersham Pharmacia Biotech, Inc., 800 Centennial Avenue, P.O. Box 1327, Piscataway, NJ 08855-1327, USA; phone (732)-457-8000, fax: 1-732-457-0557; http://www.amersham.com.au/

Applied Spectral Imaging Inc., 1497 Poinsettia Avenue, Suite 158, Vista, CA 92081, USA; phone (800) (760) 929-2840, fax (760) 929-2842 (760); http://www.spectral-imaging.com/

BIO 101, Inc., 1070 Joshua Way, Vista, CA 92083 USA; phone (760) 598-7299; http://www.bio101.com/index-main.html

BIO-RAD Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547-9980, USA; phone (510) 741-1000, fax (510) 741-5800, http://www.bio-rad.com/index1.html

Calbiochem, 10394 Pacific Center Court, San Diego, CA 92121 USA; phone (858) 450 9600, fax (858) 453 3552; http://www.emdbiosciences.com/html/CBC/home.html

Fisher Scientific Company, LLC, 9999 Veterans Memorial Drive, Houston, TX 77038-2499, USA; phone (800) 766-7000, fax (800) 926-1166; http://www.fishersci.com

Invitrogen, P.O. Box 9418, Gaithersburg, MD 20898, USA; phone (301) 840-8000, fax (301) 670-8539; http://www.invitrogen.com/

Labline Instruments, 15th and Bloomingdale Ave., Melrose Park, IL 60160-1491, USA; phone (800) 522-5463, fax (708) 450-0943; http://www.labline.com/

New Brunswick Co., Inc., 44 Talmadge Rd., Edison, NJ 08818-4005, phone (908) 287 1200, fax (908) 287 4222, http://www.nbsc.com/

New England Biolabs, Inc., 32 Tozer Road, Beverly, MA 01915-5599, USA; phone (800) 632-5227, fax (978) 921-1350, http://www.neb.com/

Perkin Elmer, 850 Lincoln Center Drive, Foster City, CA94404, USA; phone (800) 345-5224, fax (415) 572-2743; http://www.perkinelmer.com/

Pharmacia Biotech Inc., 800 Centennial Ave., P.O. Box 1327 Piscataway, Nj 08855-1327, USA; phone (800) 526 3593, fax (800) 329 3593, http://www.biotech.pharmacia.com

Roche Molecular Biochemicals, 9115 Hague Rd., P.O. Box 50414, Indianapolis, IN 46250-0414, USA; phone (800) 428-5433; fax (800) 428-5433; http://www.roche.com/

Sigma, P.O. Box 14508, St. Louis, MO 63178, USA; phone (800) 325-3010, fax (800)325-5052; http://www.sigmaaldrich.com/

Thermatron Industries, Inc., 291 Kollen Park Drive, Holland, MI49423, USA; phone (616) 393-4580, fax (616)392-5643; http://www.thermatron.com/

Vector Labpratories, Inc., 30 Ingold Road, Burlingame, CA 94010, USA; phone (415) 697-3600, fax (415)697-0339; http://www.vectorlabs.com/

VWR Scientific, 1310 Goshen Pkwy., West Chester, PA 19380, USA; phone (800)932-5000, fax (610) 436-1761; email solutions@vwrsp.com; http://www.vwrsp.com/

Abbott Molecular (formerly Vysis, Inc.), 3100 Woodcreek Drive, Downers Grove, IL, USA: phone (800) 553-7042; http://www.thermatron.com/

10. Abbreviations

BAC – bacterial artificial chromosome

CEPH - Centre des Études du Polymorphisms Humain, Paris, France

LMP – low melting point

PCR – polymerase chain reaction

PFGE - pulsed field gel electrophoresis

QDFM – quantitative DNA fiber mapping

UTR – untranslated region

YAC – yeast artificial chromosome

11. Figure legends

Figure 1: Physical mapping strategies using QDFM.

a. Mapping of overlap between the inserts of clone A and clone B by pairwise hybridization

b. Mapping two smaller DNA molecules (clone A,B) on to a larger, co-linear DNA molecule. One hybridization allows the measurement of the size and relative distance of the clones as well as distances from the end of the larger DNA molecule. The 'gap' indicates the distance between clones A and B.

Figure 2: Quantitative DNA Fiber Mapping (QDFM) using phage, YAC or P1 DNA molecules.

a. – c. Schematic representation of the action of the receding meniscus during drying. DNA molecules attached with one or both ends are pulled in the direction if the moving air-water interface. Drying typically starts on one side of the slide (a), progresses (b) and leads to more-or-less complete drying (c).

d. Lambda DNA molecules immobilized on APS-derivatized glass slides were hybridized with a mixture of biotin- and digoxigenin-labeled lambda DNA restriction fragments. The molecules show specific labeling after incubation with avidin-FITC and rhodamine-labeled antibodies against digoxigenin.

e. Mapping of overlap (OV) between a YAC clone (green) and a P1 DNA molecule (red). The stretched YAC molecule is delineated by hybridization with a DNA probe prepared from the same DNA (green), while the P1 clone-specific signals are shown in red. Small red signals at the ends of the YAC molecule (arrows) represent probes targeting the YAC cloning vector and indicate a complete YAC molecule.

f. Circular DNA molecules excised from a PFGE gel and stained with YOYO-1 revealed closed circular DNA molecules in the presence of randomly broken, linear molecules of different lengths.

g. Detection of chimerism in DNA sequencing templates. A probe prepared from a plasmid clone (H65_2D8) shows two red hybridization domains on a co-linear circular, blue P1 molecule (H65) indicating chimerism. The vector part of the P1 molecule is highlighted with a combination

of PCR-generated red and green probe signals, so that the orientation of the insert becomes readily visible.

Figure 3: Mapping the location and genomic organization of expressed sequences.

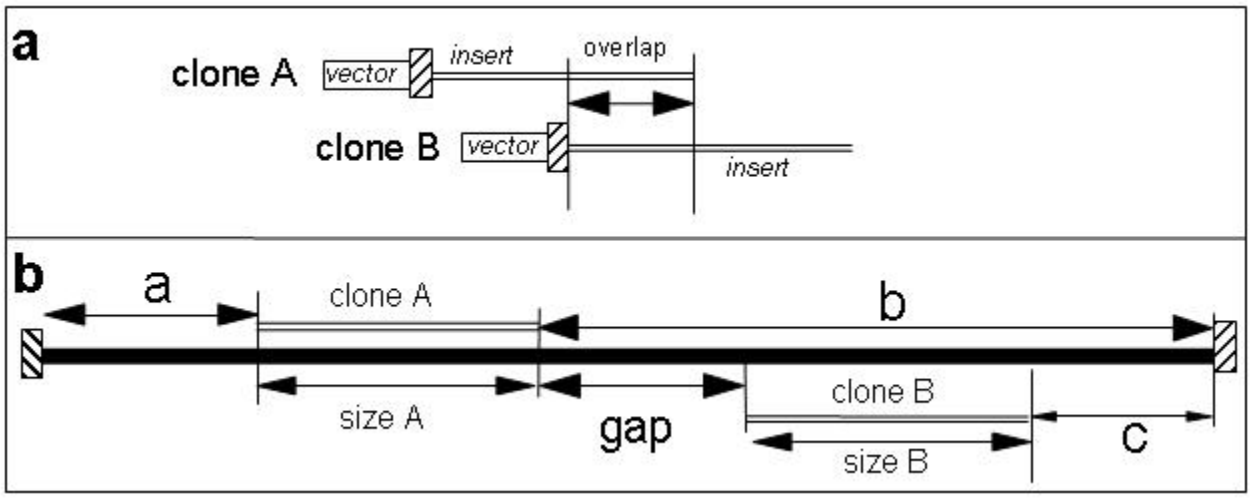
a. Mapping of a gene-specific plasmid clone along circular P1 molecules. The short area of overlap is near the cloning vector part and visible as strong red signals next to the green-red-green vector part. The circular P1 molecules were counterstained using a probe detected in blue. Please note the different degree of stretching of the circular molecules.

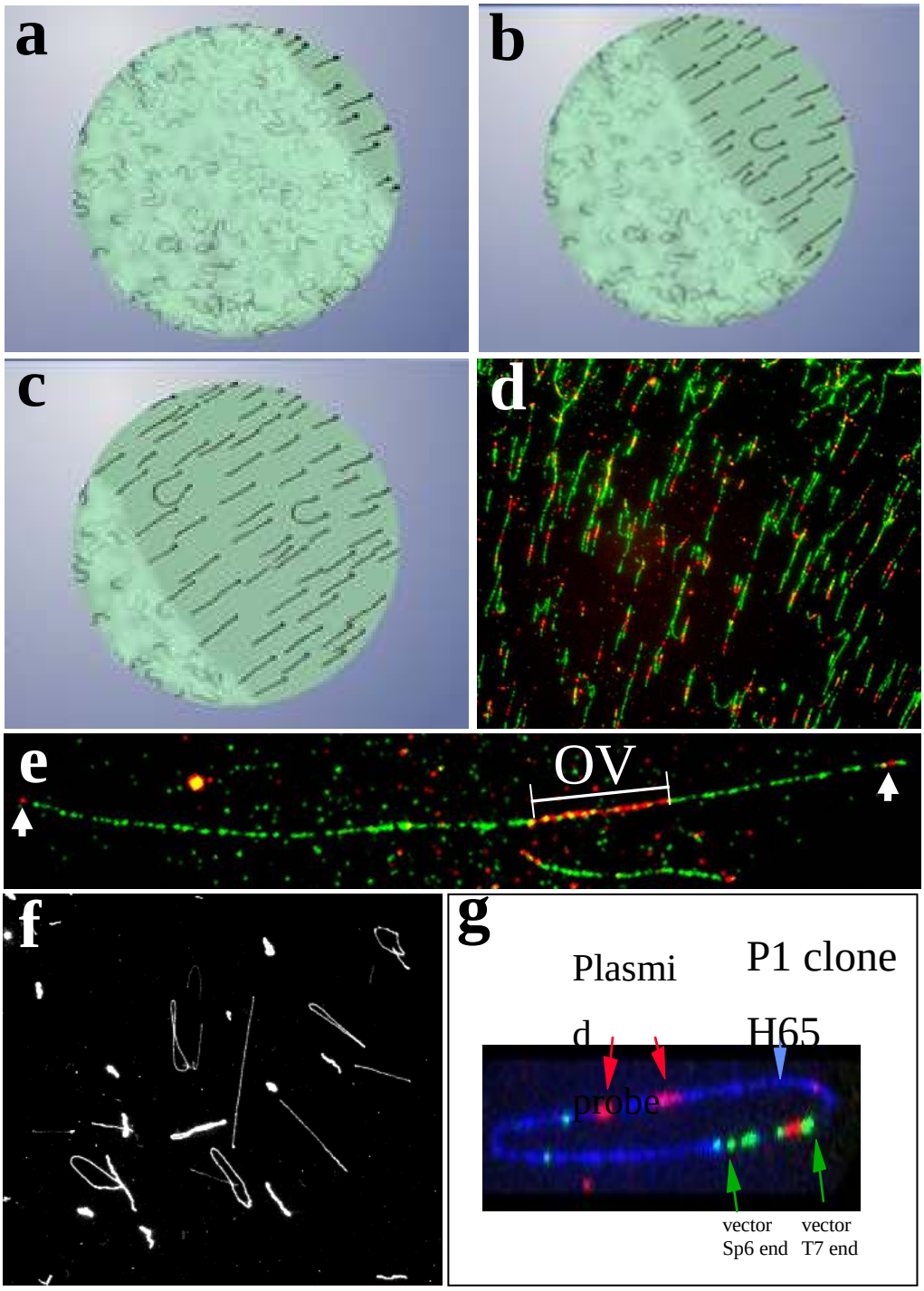
b. Physical mapping of small cDNA clones in larger genomic intervals. Here, linearized DNA fibers (blue) prepared from a BAC clone were hybridized with a probe prepared from a ~5 kb insert of a cDNA clone (red).

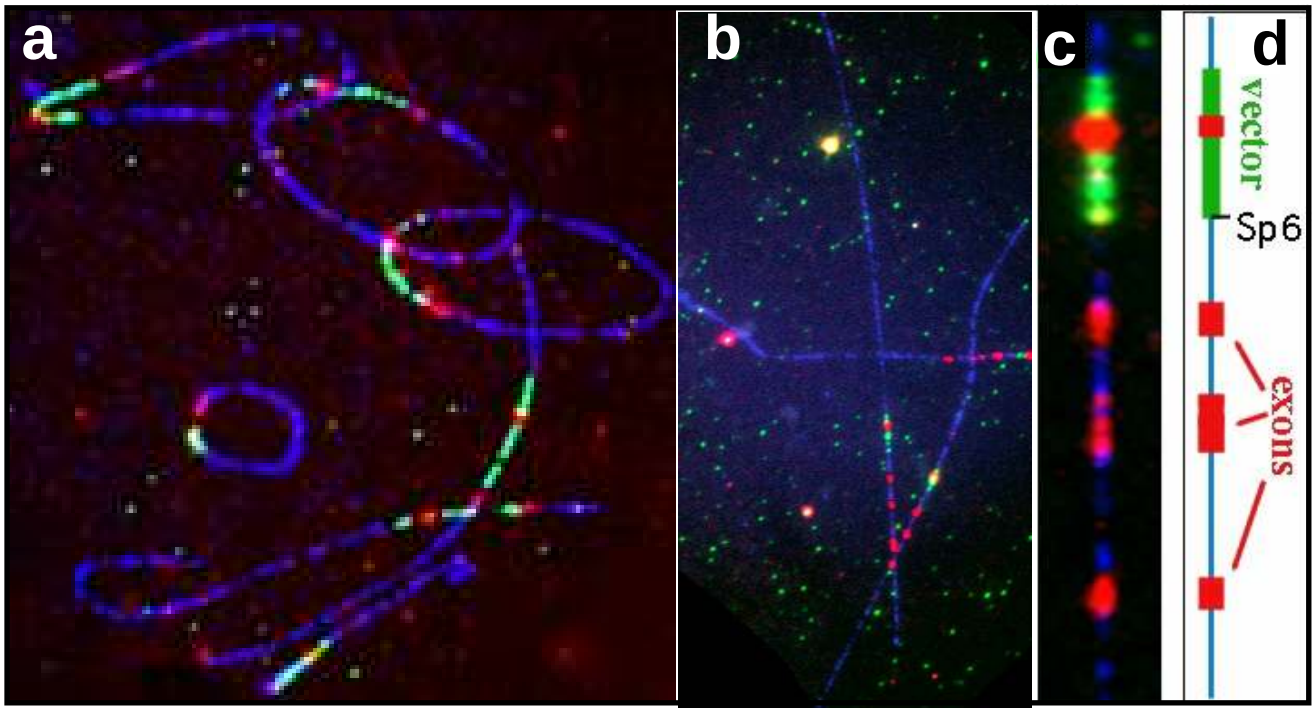
c. Enlarged view of BAC DNA fibers (blue) hybridized with the ~5kb cDNA probe (red). Three hybridization domains representing larger exons and the 3‘-UTR were detected on the DNA fibers. The green probes in b and c delineate the BAC vector; a red, vector-specific probe binds away from the Sp6 promotor and towards the T7 RNA polymerase promoter. The coding region of interest is located on the BAC molecule near the Sp6 promotor.

d. Schematic representation of the ODFM results shown in c.

12. DISCLAIMER


a
clone A
vector
insert
overlap
clone B
vector
insert
b
a
clone A
b
size A
gap
clone B
size B
c


a
b
c
d
e
OV
f
g
Plasmi
d
probe
P1 clone
H65
vector
Sp6 end
vector
T7 end


a
b
c
d
vector
Sp6
exons