Chapter 33

The Gene Cluster Instability (GCI) Assay for Recombination

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Abstract

A newly developed method for quantitatively detecting genomic restructuring in cultured human cell lines as the result of recombination is presented: the "gene cluster instability" (GCI) assay. The assay is physiological in that it detects spontaneous restructuring without the need for exogenous recombination-initiating treatments such as DNA damage. As an assay for genotoxicity, the GCI assay is complementary to well-established sister chromatid exchange (SCE) methods. Analysis of the U-2 OS osteosarcoma cell line is presented as an illustration of the method.

Key words Genotoxicity, Recombination, Gene cluster, Genomic instability

1 Introduction

Homologous recombination (HR) is essential in the maintenance of the integrity of the human genome and is the primary mechanism for error-free DNA repair of double strand breaks. This form of repair utilizes non-broken homologous sequence located elsewhere in the genome, such as on a sister chromatid, to effectively replace damaged sequence through the process of gene conversion. However, dysregulated homologous recombination between the many non-allelic repetitive sequences in the human genome [1] can also have disastrous consequences for genomic stability [2] by providing a mechanism for generating physical alterations in the genomic architecture, including chromosomal translocations, inversions and deletions. Depending on the relative orientations of the recombining sequences, the formation of dicentric and acentric chromosomes is also possible [3, 4]. These structural anomalies may contribute to cellular cancer phenotypes [5]. This type of dysregulated homologous recombination is referred to as non-allelic homologous recombination (NAHR) and involves the physical exchange of genetic material through crossover between two different chromosomal loci with a high degree of sequence identity.

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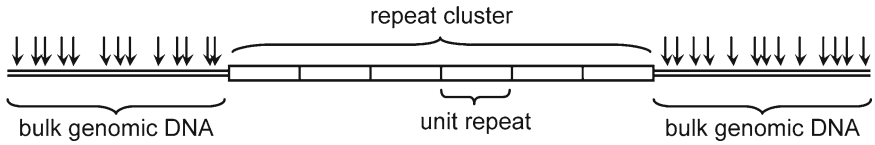

Fig. 1 Schematic of the restriction digestion procedure for liberating intact gene clusters from bulk genomic DNA. *Vertical arrows*: hypothetical restriction enzyme recognition sites; *Open Rectangles*: individual repeated genes in a gene cluster. Reproduced from [9]

Like error-free HR, NAHR is sequence similarity dependent and becomes more efficient when sequence similarity between the recombining sequences is greater than 98 % [6].

We describe here the Gene Cluster Instability (GCI) assay we developed for detecting spontaneous recombination-mediated genomic restructuring in human cells. We have successfully applied this technique to elucidate the genetics that regulates the NAHR reaction [7] and to show that NAHR is commonly associated with human adult solid tumors [8]. In principle the assay is also suitable for evaluating the genomic toxicity of drugs. The assay involves physical analysis of gene clusters, genomic loci in which the repeated genes have very high levels of sequence identity and are in very high local concentration, both factors in accelerating the rate at which they undergo recombination-mediated structural alteration. NAHR between similarly oriented repeats causes expansions and contractions of the number of repeats in the cluster. These length changes can be monitored by excising the gene clusters of interest from the genome enzymatically, resolving cluster lengths through pulsed-field electrophoresis and detecting the clusters by Southern hybridization [9].

The gene clusters we usually employ (and describe here) to monitor recombination are the clusters expressing the precursor transcript to the three largest of the four ribosomal RNA molecules. There are ten such gene clusters in the human genome found at both paternal and maternal 13p12, 14p12, 15p12, 21p12, and 22p12 chromosomal loci [10], each consisting of a tandemly repeated 43 kb gene (the "rDNA") with a variety of relative orientations [11] but commonly oriented such that transcription proceeds towards the centromere [12]. The individual rDNA clusters range from 1 to over 140 repeat copies representing overall lengths ranging from 43 kb to over 6 Mb with very strong variability demonstrated between individual humans [9]. We have found that assaying cluster length changes in the size range from 10 kb to 1 Mb combines good sensitivity for detecting recombination-mediated genomic structural alterations with relative technical ease.

A schematic of the procedure is shown in Fig. 1. High-molecular-weight genomic DNA from cells of interest is isolated in solid phase agarose to prevent mechanical shearing and subjected

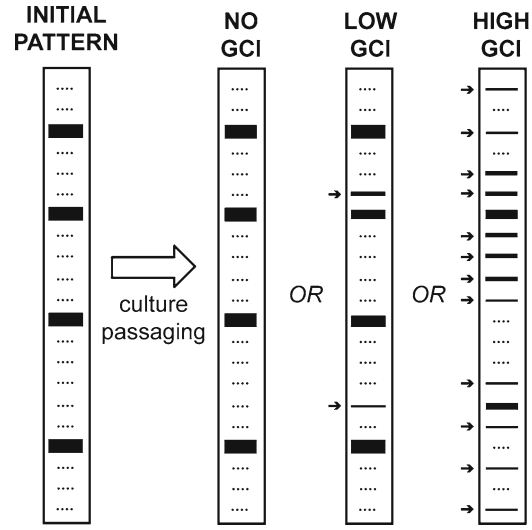

Fig. 2 Schematic of expected results. *Solid horizontal lines*: gene cluster bands detected by Southern hybridization following size separation by pulsed-field electrophoresis; *Dotted horizontal lines*: potentially allowable cluster lengths constrained by the requirement that recombination change cluster lengths by integer multiples of the unit repeat length; *Small horizontal arrows*: minor-intensity gene cluster bands indicative of subpopulations within the culture possessing cluster lengths that have been altered by non-allelic homologous recombination (NAHR). Adapted from [7]

to restriction digestion, also in the solid phase. Restriction enzymes are selected that do not have a recognition site in the gene cluster unit repeat. Since the gene repeats are nearly identical to each other, such an enzyme will likely not cut anywhere within the entire cluster, whereas non-repetitive flanking genomic DNA of essentially random sequence will be subject to digestion. So long as the cluster length is large relative to the cutting frequency of the enzyme in random sequence DNA, the cluster will be liberated with a relatively negligible length tail of non-cluster DNA on each end. For the rDNA, we find EcoRV to be an excellent enzyme: no recognition site in the rDNA clusters, frequent cutting in random sequence DNA, digests DNA in solid-phase agarose efficiently, and is affordable (*see* **Note 1**).

Separation of the clusters using pulsed-field electrophoresis and detection though Southern hybridization generates a characteristic electrophoretic karyotype of the gene clusters. For a clonal cell line with no recombinational instability, this pattern will be well defined with one band for each cluster, each band with a radioactive hybridization signal in proportion to the number of repeats in the cluster (Fig. 2: "Initial Pattern"). If there is no recombination-mediated gene cluster restructuring, this pattern

will be faithfully transmitted to all daughter cells. Recombination can, however, change the lengths of these clusters as cells are cultured. Thus, recombinational instability is manifested as subpopulations of cells in a culture with a different electrophoretic karyotype. Experimentally, these subpopulations can be detected by reduced-intensity bands that differ in length from the initial pattern superimposed upon the initial pattern (Fig. 2: "Low GCI"). In the case of extreme instability, such as when the Bloom syndrome protein is lost or inactivated, active restructuring generates a ladder-like pattern that essentially completely obscures the initial pattern (Fig. 2: "High GCI") [7]. The ladder-like banding is diagnostic for recombination-mediated changes, since recombination requires alignment of the repeated sequences and can thereby only change cluster lengths by integer multiples of the unit repeat length.

1.1 Expected Results

GCI analysis results for the human osteosarcoma line U-2 OS are illustrative. Figure 3a shows the electrophoretic karyotype for a stock culture of U-2 OS cells along with the SV40-transformed

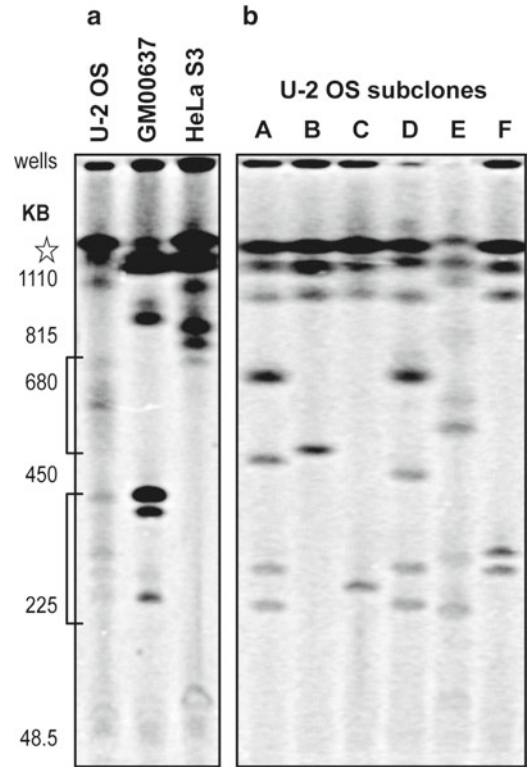

Fig. 3 GCI analysis. Panel (**a**): non-clonal cell lines. Panel (**b**): clonal isolates of the U-2 OS population from (**a**). *Brackets*: multiple bands of uneven intensity from mixed subpopulations in the U-2 OS parental population. *Star*: resolution limit of the pulsed field gel

wild-type fibroblast line GM00637 (Coriell) and the HeLa S3 cervical carcinoma line (ATCC). Since these three lines are derived from three different individuals, the initial pattern of bands is expected to be different between the three lines, as indeed it is; this attribute of the rDNA clusters is useful to ensure that cell lines do not become confused with each other. The U-2 OS culture shows a profusion of minor-intensity bands with no well-defined pattern, indicative of recombinational instability. In a non-clonal culture such as that shown for the U-2 OS cells in Fig. 3a, however, the total accumulated instability is a factor of three independent parameters: (1) the spontaneous per-cell-division recombination rate, (2) the number of cell divisions elapsed since clonality, and (3) the degree to which the culture has been subject to periodic genetic population bottlenecks due to repeated splitting of the culture. Usually it is the spontaneous recombination rate that is of interest. This can be determined directly by reinitiating a culture from single-cell-derived subclones to clear out all subpopulations, followed by free expansion without limit until genomic DNA is prepared. DNA isolated from such single-cell-derived subclones of the U-2 OS parental population yields the data shown in Fig. 3b. Unlike the mixed parental population, now the subclones reveal relatively recombination-stable initial patterns with few minor-intensity bands. The process can be reiterated by generating sub-subclonal lines from individual cells of the now well-defined subclonal populations. Figure 4 shows such analysis from sub-subclonal populations generated from the U-2 OS parental subclones A and B respectively. Recombinational instability is still present, as indicated by missing initial pattern bands and new minor-intensity bands, but of a similar rate to that seen with other wild-type immortalized cell lines [7]. Clearly the majority of the instability observed in the non-clonal parental U-2 OS culture (Fig. 3a) was due to extended time in culture and/or repeated high-dilution passaging.

2 Materials

2.1 Thawing Cryopreserved Cells

1. Latex or nitrile gloves.
2. 10-cm tissue culture plates (or tissue culture flasks for non-adherent cells).
3. Adherent or non-adherent human cells.
4. Humidified, CO_2 supplemented tissue culture incubator.
5. Laminar flow tissue culture biosafety hood with standard tissue culture setup, including serological pipettes, micropipettes, and vacuum aspiration apparatus.
6. Tissue culture medium appropriate for each cell type such as minimal essential medium (MEM) or RPMI 1640.

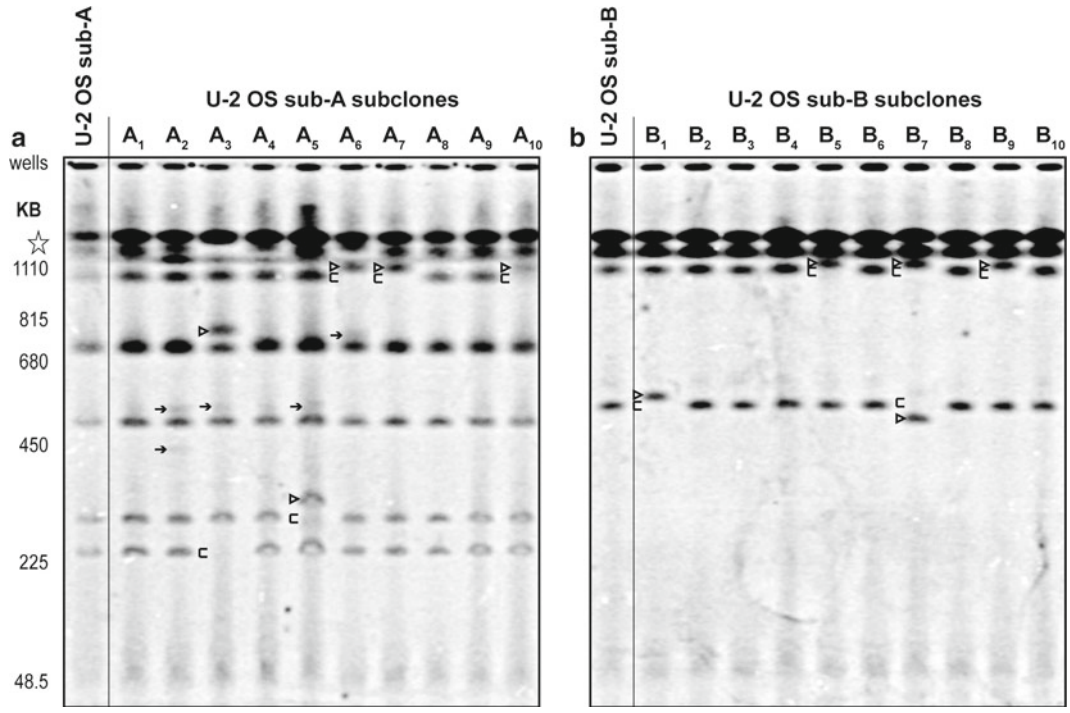

Fig. 4 GCI analysis of clonal cell lines: Panel (**a**): subclonal isolates from the clonal U-2 OS-A line shown in Fig. 3b. Panel (**b**): subclonal isolates from the clonal U-2 OS-B line shown in Fig. 3b. *Arrows*: minor intensity bands indicative of genomic restructuring during the expansion of the indicated sub-subclonal lines. *Open triangles*: gene clusters found in the sub-subclonal lines but not found in the parental subclonal lines. *Open brackets*: gene clusters not found in the sub-subclonal lines but present in the parental clonal lines. *Star*: resolution limit of the pulsed field gel

7. 95 % reagent grade ethanol.
8. Fetal bovine serum.
9. l-glutamine–Penicillin–Streptomycin 100× solution (10,000 U/ml penicillin; 10,000 μg/ml streptomycin; 200 mM *L*-gln).
10. Plasmocin 25 mg/ml (for mycoplasma prophylaxis).
11. Clinical centrifuge (e.g., Marathon model 3200, Fisher, Pittsburg, PA).
12. 15 ml conical centrifugation tubes.
13. Complete culture medium appropriate for tissue culture growth of cells: MEM or RPMI supplemented with 5–10 % fetal bovine serum, a 1:100 dilution of the stock Pen–Strep–*L-gln* solution, and a 1:10,000 dilution of the stock Plasmocin 25 mg/ml solution.
14. 70 % Ethanol: 95 % ethanol diluted to 70 % with sterile distilled water.

2.2 Subculturing Cells for GCI Analysis

1. Laminar flow hood with standard tissue culture setup, including serological pipettes, micropipettes, and vacuum aspiration apparatus.
2. Trypsin–EDTA solution (0.05 % Trypsin, 0.53 mM EDTA).
3. Tissue culture medium such as MEM or RPMI 1640, suitably supplemented with fetal bovine serum and antibiotics.
4. 10-cm tissue culture plates (or tissue culture flasks for non-adherent cells).

2.3 Deriving and Expanding Clonal Lines

1. 20 µl PIPETMAN (Gilson or equivalent) and sterile pipette tips.
2. 96-well flat bottom shaped well plates for adherent cells or V-bottom shaped well plates for non-adherent cells.
3. 24-well tissue culture plates.
4. 6-well tissue culture plates.
5. 10 cm tissue culture plates.
6. Hemocytometer or flow cytometer (e.g., Partec Analysis System PAS, Partec, Münster, Germany) for cell counting.

2.4 Preparing Subcultured Cells for DNA Extraction

1. Trypsin–EDTA solution (0.05 % Trypsin, 0.53 mM EDTA).
2. Tissue culture medium such as MEM or RPMI 1640, suitably supplemented with fetal bovine serum and antibiotics.
3. Clinical centrifuge.
4. 15 ml conical centrifugation tubes.
5. Sterile phosphate-buffered saline (also known as PBS or DPBS).
6. Hemocytometer or flow cytometer for cell counting.

2.5 Isolation of Solid-Phase High-Molecular-Weight Genomic DNA from Human Cells

1. Low-melting-point (LMP) agarose, analytical grade (cat. #V2111, Promega, Madison, WI).
2. DPBS (cat. #21-031-CV, Mediatech, Manassas, VA).
3. 1 ml syringes (cat. #309602, Becton, Dickinson, San Jose, CA).
4. Single-edged safety razor blades.
5. 8 ml flat bottom tubes.
6. Cell digestion buffer with proteinase K.
7. Environmental incubator shaker capable of maintaining 50 °C (e.g., G24 environmental incubator shaker, New Brunswick Scientific, Edison, NJ).
8. 50 ml conical tubes.
9. TE solution.
10. TE–glycerol solution.

11. Saturated phenylmethanesulfonylfluoride (PMSF) in isopropanol. *CAUTION*: very toxic!
12. Room-temperature shaker.
13. Proteinase K powder. Store at –20 °C.
14. Proteinase K solution: Dissolve in water to 15 mg/ml. Store in 160 µl aliquots at –20 °C. *CAUTION*: proteinase K fines are intensely irritating. Wear suitable respiratory protection when using powered proteinase K.
15. Cell digestion buffer with proteinase K: 500 mM EDTA pH 8.0, 1 % sarcosyl. Store at room temperature. Add 160 ml proteinase K per 5 ml digestion buffer for a final proteinase K concentration of approximately 0.5 mg/ml immediately before use.
16. TE: 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0.
17. TE–glycerol solution (10 mM Tris, 1 mM EDTA, 50 % glycerol(w/v), pH 8.0).
18. Saturated PMSF in isopropanol: Add isopropanol to PMSF crystals to make a saturated solution. Some PMSF crystals should remain undissolved in the bottom of the container. Store at room temperature.

2.6 Enzymatic Digestion of High-Molecular-Weight DNA for PFGE

1. Standard single edge safety razor blades.
2. 1.5 ml eppendorf tubes.
3. NEB buffer 3 (cat. #B7003S, New England Biolabs, Beverly, MA) or other suitable buffer for the restriction enzyme of choice.
4. EcoRV restriction enzyme (ca.# R0195L, New England Biolabs, Beverly, MA).
5. Warm room capable of maintaining 37 °C or environmental incubator shaker capable of maintaining 37 °C.
6. Mini-Labroller with 1.5 ml eppendorf tube holder attachment or equivalent (Labnet International).

2.7 Preparation of Agarose Gel for PFGE

1. Pulsed field certified (PFC) grade agarose (cat. #162-0137, Bio-Rad Laboratories, Hercules, CA).
2. 125 ml clean glass bottle with screw-cap lid.
3. 500 ml pyrex beaker.
4. Hot plate.
5. Nanopure H_2O.
6. Bio-Rad agarose gel casting system (cat. #107-3689, Bio-Rad Laboratories, Hercules, CA).
7. Bio-Rad universal comb holder and 15- or 20-well comb (cat. #170-3699; 170-3627; 170-4322, Bio-Rad Laboratories, Hercules, CA).
8. Bio-Rad leveling table (cat. #170-4046, Bio-Rad Laboratories, Hercules, CA).

9. Parafilm M sealing film (cat. #PM-996, SPI supplies).
10. 0.5× TBE (45.5 mM Tris–45.5 mM Borate–2.0 mM EDTA): To 1,000 ml nanopure H_2O add 6.6 g Boric acid, 12.94 g TRIS base, and 4.8 ml of a 500 mM EDTA pH 8.0 solution. Bring volume up to 2,400 ml with water. Make fresh before each use.

2.8 Loading Digested DNA Samples into the Agarose Gel for PFGE

1. 0.5× TBE.
2. 200 µl PIPETMAN (Gilson or similar) and tips.
3. Low-melting-point (LMP) agarose, analytical grade (cat. #V2111, Promega, Madison, WI).
4. DPBS (cat. #21-031-CV, Mediatech, Manassas, VA).
5. Standard single edge razor blade.
6. Environmental incubator shaker capable of maintaining 50 °C.
7. *S. cerevisiae* chromosome molecular weight markers (New England Biolabs: cat. #N0345S). Store at –20 °C.

2.9 Loading and Running Pulsed Field Electrophoresis Gels

1. Bio-Rad CHEF MAPPER XA with cooling module system (Bio-Rad Laboratories).

2.10 Ethidium Staining and Preparing the Gel for In-Gel Hybridization of a Radiolabeled Probe

1. Gel imaging documentation system.
2. Flat stainless steel pan.
3. Hybridization oven (TECHNE).
4. Kimwipes (Kimberly-Clark).
5. Ethidium bromide: 1 % solution in water stored at 4 °C. *CAUTION*: Ethidium bromide is a known carcinogen. Wear suitable respiratory protection when manipulating ethidium bromide powder.
6. Ethidium bromide/glycerol solution. To 197 ml nanopure H_2O add 3 ml 50 % glycerol solution (w/v) and 15 µl of the 1 % stock solution of ethidium bromide.

2.11 PCR Reaction: Non-radiolabeled Southern Blot Probe Template Preparation and Radiolabeled 45S rDNA Southern Blot Probe Generation

1. Oligonucleotides: rDNA11-T: GGGCTCGAGATTTGGGA CGTCAGCTTCTG and rDNA11-B: GGGTCTAGAGTGCT CCC TTCCTCTGTGAG.
2. Thermocycler (e.g., Mastercycler gradient, Eppendorf Scientific).
3. dGATC-TP nucleotide mix, combined and diluted to a final concentration of 10 mM of each nucleotide (cat. #10297-018, Invitrogen, Carlsbad, CA).
4. dGCT-TP nucleotide mix, combined and diluted to a final concentration of 40 µM of each nucleotide (*see* **Note 2**).
5. dATP diluted to a final concentration of 20 µM.
6. α-^{32}P-dATP (50 uCi at 3,000 Ci/mmol) (Perkin Elmer).
7. TAQ polymerase (New England Biolabs).

8. 10× TAQ buffer (New England Biolabs).
9. Spin-50 mini-column (USA Scientific).
10. FlexiGene DNA Kit (Qiagen).
11. Illustra GFX PCR DNA and Gel Band Purification Kit (GE Biosciences).

2.12 Southern Blot Analysis Using In-Gel Hybridization of the Radiolabeled rDNA Probe

1. Hybridization tube (TECHNE).
2. Nylon mesh (PGC Scientifics).
3. Hybridization oven (TECHNE).
4. Polyvinyl chloride plastic wrap.
5. Molecular Probes Phosphor Screen (GE Lifesciences).
6. PhosphorImager (e.g., Storm 860, Molecular Dynamics).
7. SYBR® safe stain (Invitrogen).
8. Denaturation solution (0.4 N NaOH, 0.8 M NaCl): 1.6 g of NaOH pellets and 4.67 g NaCl dissolved in water to 100 ml. Make fresh before use.
9. Neutralization solution (0.5 M Tris pH 8.0, 0.8 M NaCl): Add 4.67 g NaCl to 50 ml of 1 M Tris pH 8.0 solution and add water to 100 ml final volume. Make fresh before use.
10. 20× SSC (3 M NaCl and 300 mM sodium citrate): Dissolve 175.4 g of NaCl and 88.2 g of sodium citrate dihydrate in 800 ml nanopure H_2O. Adjust volume to 1 l.
11. Hybridization solution (2× SSC, 7 % SDS, 0.5 % Hammersten casein): Add 100 ml 20× SSC to 700 ml nanopure H_2O. Warm to 65 °C. Dissolve 70 g SDS and 5 g Hammersten casein (USB corp. cat. #12840). Adjust volume to 1 l. Store at room temperature. *CAUTION*: SDS power fines are very irritating. Wear suitable respiratory protection.
12. Wash solution 1 (2× SSC and 0.1 % SDS): Add 10 ml of 20× SSC to 89 ml nanopure H_2O. Lastly add 1 ml of a 10 % SDS solution and mix well. Make fresh before each use.
13. Wash solution 2 (0.5× SSC and 0.1 % SDS) Add 2.5 ml of 20× SSC to 96.5 ml nanopure H_2O. Lastly add 1 ml of a 10 % SDS solution and mix well. Make fresh before each use.

3 Methods

3.1 Thawing Cryopreserved Cells

1. Prepare a tissue culture plate with pre-warmed culture medium at least an hour before thawing cells allowing for ample time for it to equilibrate to the correct pH and temperature in the tissue culture incubator at 37 °C and with 5 % CO_2.

2. Remove a vial of frozen cells from liquid nitrogen storage and wipe it down with 70 % EtOH in a laminar flow tissue culture hood.
3. Open the vial slightly to allow the gas pressure inside the cryovial to equalize with the ambient atmosphere. Close the vial of cells and continue thawing in your gloved hand until there is a still-frozen pellet in thawed liquid that is mobile when the tube is shaken.
4. Shake the vial vigorously, then remove the lid from the vial and pour the liquid and semi-frozen pellet of cells into the medium of the prepared tissue culture dish. Swirl the cells to thaw the residual frozen pellet and to get an even distribution of cells across the plate. Place the plate containing the cells immediately back in the tissue culture incubator overnight.
5. The next day the medium should be replaced to get remove any residual cryopreservative from the freezing media. For adherent cells the medium should be aspirated off and replaced with fresh pre-warmed medium. For non-adherent cells the cell suspension should be removed to a 15 ml conical tube and centrifuged at 200 × *g* for 5 min. The media can then be aspirated off and the resulting cell pellet be resuspended in pre-warmed culture medium and placed in a new tissue culture plate.

3.2 Subculturing Cells

1. Once the cells are growing and semi-confluent in their tissue culture dish, remove the cells from the incubator and place them in a laminar flow tissue culture hood. *For non-adherent cell lines, skip to* step 6.
2. Aspirate the medium completely from the plate.
3. Add an appropriate amount of trypsin–EDTA solution to the plate making sure that the entire bottom surface of the plate is evenly covered. For a 10-cm dish 2 ml is typically sufficient.
4. Allow cells to sit for several minutes while gently rocking the plate to maintain an even distribution of the solution and monitor the cells as they start to detach from the plate. The amount of time it takes for the cells to detach will vary widely depending on the cell type; however, this is easily determined by careful observation.
5. Once most of the cells have detached add 2× the volume of culture medium containing at least 5 % FBS. The FBS in the culture medium inactivates the trypsin and prevents it from damaging the cells through prolonged exposure. If the trypsin is not inactivated, prolonged exposure can cause the cells to lyse.
6. Pipette the cells up and down vigorously in order to break up any clumps and to ensure all cells have detached from the plate and are homogenously suspended.

7. Once you have a single cell suspension distribute the suspension among new culture dishes with pre-warmed medium in a ratio that is ideal for the cell line of interest. This can typically range from a 1:3 dilution for slower growing cells to a 1:10 dilution for faster growing cells such as HeLa.

3.3 Deriving and Expanding Clonal Lines

When the GCI assay is used to determine ongoing genomic instability in cultured cell lines it requires the development of clonal lines derived from a given cell line.

1. Start by preparing a single cell suspension in the same fashion as you would above as if subculturing the cells. The single cell suspension is then used to prepare a limiting dilution series and derive colonies grown from a single cell.

2a. *For adherent cells* set up a dilution series and plate the cells in 10-cm plates at 1:10; 1:50; 1:250; 1:500; 1:1,000; 1:2,000; and 1:4,000 dilutions.

or

2b. *For non-adherent cells* determine the cell density with a hemocytometer or flow cytometer, then dilute cells suitably with medium and aliquot cells into separate 96-well V-bottom plates at dilutions of 100 cells/well; 10 cells/well; 5cells/well; 1cell/well; and 1cell/5wells. Place cells into all 96 wells for each of the plates used (*see* **Note 3**).

Over the next 8–14 days colonies from begin to form either on plates for adherent cells or in wells for non-adherent cells.

3. *For adherent cells only*: once colonies are 2–3 mm in diameter they can be picked from the plate with a 20 μl pipette and moved to a single well in a 96-well tissue culture plate. For fragile cells, partial trypsinization by treatment with trypsin–EDTA solution diluted 10:1 with DPBS will help ensure cell integrity in the colony transfer process. Henceforth, treat adherent and non-adherent cultures similarly.

4. *For all cells*: clones should be expanded from 96-well plates to 24-well plates, 6-well plates, and finally 10 cm dishes successively. Cells should be allowed to grow to confluency before each expansion step. Once confluent they are subcultured as described above using smaller volumes of trypsin–EDTA solution for adherent cells: 50 μl per each well of a 96-well plate, 200 μl per well for 24-well plates, 500 μl per well for 6-well plates. Each step represents approximately a 1:4 expansion of the cells, which in our hands works well for almost any line chosen.

3.4 Preparing Cultured Cells for High-Molecular Weight Solid-Phase DNA Isolation

1. Melt certified nuclease-free low-melting-point (LMP) agarose at 1.2 % weight/volume in DPBS (phosphate-buffered saline without Ca^{2+} or Mg^{2+}, tissue culture grade) at 70 °C, place the melted gel solution in a water bath or oven at 42 °C and allow the temperature to equilibrate.

2. With adherent cells, treat with trypsin–EDTA to detach cells followed by addition of a 2× volume of culture medium and pipeting up and down in a serological pipette to achieve a single cell suspension. Likewise non-adherent cells can simply be pipetted up and down to break up clumps and create a single cell suspension. From this point on the cells are stored on ice at all times except during centrifugation which can be performed at room temperature.
3. Remove the suspension to a 15-ml conical vial and centrifuge at $200 \times g$ for 5 min.
4. Resuspend the cells in 10 ml DPBS to rinse.
5. Determine the concentration of cells with either a hemocytometer or flow cytometer.
6. Centrifuge the cells at $200 \times g$ for 5 min and aspirate the DPBS rinse solution.
7. Resuspend the cells in DPBS to a final concentration of 3×10^7 cells/ml, taking into account the non-zero volume of the pelleted cells themselves. Store the cells on ice temporarily if necessary.
8. Add two volumes of melted 1.2 % LMP agarose solution to the 3×10^7 cells/ml cell suspension for a final concentration of 1×10^7 cell/ml in 0.8 % LMP agarose and mix thoroughly by vortexing.
9. Draw the cell–gel suspension into a 1 ml syringe and immediately place the syringe on ice and cover with ice to solidify the agarose before the cells have a chance to settle.
10. After the agarose solution has solidified cut the end off the syringe with a single edged razor blade and extrude the DNA–agarose "worm" carefully into an 8 ml flat-bottomed tube.
11. Add 5 ml of digestion buffer with proteinase K, invert gently several times to mix and incubate overnight at 50 °C with gentle agitation (*see* **Note 4**).
12. After suitable digestion has cleared the appearance of the DNA–agarose, remove the DNA–agarose "worm" to a fresh 50 ml tube, add 40 ml TE and agitate gently at room temperature for 30 min. Do this step twice.
13. Decant the TE and transfer the "worm" to a new 8 ml flat-bottomed tube.
14. Add 6 ml TE and 6 μl saturated PMSF in isopropanol. Mix well and gently agitate at room temperature for 60 min.
15. Remove the TE–PMSF solution and add 6 ml of fresh TE without PMSF to rinse with gentle agitation at room temperature for 30 min. Do this step twice.
16. Pour off the TE and add back chilled TE–glycerol and gently agitate at 4 °C for 30 min.

17. Remove the TE–glycerol and add back 6 ml fresh TE–glycerol. Gently agitate overnight at room temperature.
18. Cut off the tip of a fresh 1 ml syringe with a clean razor blade and transfer the TE–glycerol equilibrated agarose "worm" into this syringe. Seal the syringe with Parafilm and store indefinitely at –20 °C.

3.5 Enzymatic Digestion of High-Molecular-Weight DNA for PFGE

1. Agarose–DNA slices of ~15 μl volume are cut from the high-molecular-weight DNA–agarose "worms" prepared and stored in 1 ml syringes with a standard single edge razor blade. Each slice should be approximately 0.8–0.9 mm thick. It is helpful to practice your slicing technique with an agarose "worm" that does not contain DNA, measuring slice volumes on an analytical balance until uniform thickness slices can be consistently achieved. Aim for a consistent slice weight of approximately 15 mg.
2. Place the DNA-containing agarose slice inside the lid of an inverted 1.5 ml eppendorf tube. The lid of an inverted 1.5 ml eppendorf tube provides a perfect sized container for the slice with a flat bottom that also holds 200 μl of buffer.
3. Remove the glycerol–TE from the slice by washing the slice in the lid of the 1.5 ml eppendorf tube three times with 200 μl of 1× NEB buffer 3 by pipetting gently up and down so the slice is agitated in the buffer solution and then pipetting off the wash solution. Take care not to damage the slice with the pipette tip during the washes.
4. Remove the last wash and replace with 200 μl of NEB buffer 3 containing 20 units of EcoRV restriction enzyme.
5. While keeping the eppendorf tube inverted, gently but firmly close the eppendorf tube lid. Mount the inverted eppendorf tube in the Mini-Labroller at 30° off vertical so that when the Labroller is turned on the tubes will be gently agitated while maintaining their inverted orientation. Place the Labroller with the inverted eppendorf tubes in a warm room at 37 °C overnight, and turn on the Labroller.
6. The next day, remove the Labroller apparatus with eppendorf tubes from the warm room. Remove the inverted eppendorf tubes from the Labroller, while maintaining their inverted orientation.
7. Rinse the agarose slices containing the now digested genomic DNA with 0.5× TBE by pipetting gently up and down and then pipetting off the rinse, taking care not to disrupt the delicate agarose slices with the pipette. Do this rinse twice. Additionally, slice off and likewise rinse a thin sample of *S. cerevisiae* chromosome molecular weight markers.
8. The slices are now ready to load into the pulsed-field gel. Leave the slices in the final rinse solution in sealed inverted eppendorf tubes until ready to load the samples.

3.6 Preparation of an Agarose Gel for PFGE

1. Make 2.4 l of 0.5× TBE.
2. Place 1 g of PFC grade agarose into a 125 ml glass bottle (e.g., Kimax) along with 100 ml of 0.5× TBE. Gently swirl to ensure the agarose is hydrated and non-clumpy. Try not to let agarose clumps adhere to the walls of the bottle above the liquid. Cap the bottle tightly.
3. Place 250 ml water in a 500 ml glass beaker and bring to a gentle boil on a hot plate. Melt the agarose in 0.5× TBE by placing the sealed bottle containing the agarose–0.5× TBE mixture into the boiling water bath. Heat with periodic gentle swirling until all the agarose has been completely homogenously melted. The melted agarose solution should be clear, colorless and featureless when swirled.
4. Allow the melted agarose to cool only slightly (less than 5 min) on the benchtop prior to pouring into the gel casting set up. This should be ample time to set up the casting apparatus. We have found the pouring the gel while it is hot produces optimal resolution of the gene cluster bands. The gel casting system should be set up with the universal comb holder and 15- or 20-well comb and placed flat on a Bio-Rad (or similar) leveling table.
5. Once the casting apparatus is prepared pour the melted agarose solution slowly into the casting tray trying to avoid forming any bubbles. Eliminate any bubbles that may arise while the agarose solidifies. Pay special attention to any bubbles that might form along the wells of the comb and the edges of the casting tray. Allow the gel to solidify at room temperature on the benchtop for 1 h. Then, place the solidified gel, still in the casting tray, in a refrigerator at 4 °C for an additional hour. The gel will then be ready for loading the prepared DNA samples (*see* **Note 5**).

3.7 Loading Digested DNA Samples into the Agarose Gel for PFGE

1. Place a mixture of 0.8 % LMP agarose suspended in 0.5× TBE into a tightly capped 50 ml plastic conical tube and heat in a boiling water bath until the agarose mixture is uniformly melted. Remove from the bath and place at room temperature (*see* **Note 6**).
2. Remove the solidified pulsed-field gel from the refrigerator and carefully remove the comb from the wells.
3. Remove the 0.5× TBE final buffer rinse from the first agarose slice in the lid of an inverted 1.5 ml eppendorf tube. Gently flick the lid of the eppendorf tube onto a clean razor blade so the gel slice lands flat on the razor blade. Remove excess liquid from the gel slice with a pipette tip or the corner of a Kimwipe (*see* **Note 7**).
4. Push the slice from the razor blade into the well of the gel with a clean pipette tip. Make sure that the agarose slice flatly contacts the front of the well facing the direction in which the gel will be run. Allowing the slice to stick to the back of the well instead will compromise the resolution and final location of resulting bands.

5. Gently push the slice down into the well until the bottom of the slice touches the bottom of the well, then fill the remaining space in the well carefully with melted 0.8 % LMP agarose.
6. Repeat this step for each sample to be loaded, including the *S. cerevisiae* chromosome molecular weight markers.
7. Once the samples are all loaded the gel should be allowed to sit at room temperature for 10 min to allow the sealing agarose to begin to set (*see* **Note 8**).
8. Next the casting stand should be removed taking care not to dislodge the gel from the black running platform.
9. Wipe away any accumulated gel waste on the bottom of the running platform.
10. The gel and platform should then be placed at 4 °C for 30 min. Again, this step is essential for optimal resolution.

3.8 Loading and Running the Bio-Rad CHEF MAPPER Apparatus for PFGE

We have found that the optimal range for GCI analysis of the human rDNA gene clusters is from 50 kb up to 1 Mb. The instructions and the algorithm provided here are optimized for this size range. If this methodology is used to look at gene clusters other than the rDNA or in order of size ranges of the rDNA clusters the conditions will require optimization for the desired size range. Consult Birren and Lai [13] for more details.

1. Once the gel is loaded and has been chilled at 4 °C, place the remaining 0.5× TBE buffer into the electrophoresis cell of the Bio-Rad CHEF MAPPER XA system.
2. The buffer pump should be turned on to circulate buffer and eliminate air bubbles from the system before the gel is placed into the cell.
3. Once this is done turn the pump off and place the gel into the cell making sure the retention bracket is in place to prevent the gel from moving and that the wells are oriented at the top of the gel relative to the direction the DNA is going to travel.
4. Close the lid and make sure the safety interlock is engaged and turn the buffer pump back on.
5. Engage the cooling module of the Bio-Rad CHEF MAPPER XA system and allow it to cool the buffer and the gel to 14 °C.
6. Enter a two state program such that the electrical field vector included angle is 120° and the electrical field strength for each vector is 6 V/cm. Set the run time for 24 h and the switch time from 3 to 90 s with a ramp factor of 0.357. Once you are sure the algorithm is entered correctly and the chiller has cooled the buffer and gel to 14 °C press “START” on the MAPPER. Look for bubbles from the electrodes to be sure the gel is running correctly.

3.9 Ethidium Staining and Drying the Gel in Preparation for In-Gel Hybridization with a Radio-Labeled Probe

The next day, after the PFGE program has completed running, the gel can be stained with ethidium bromide and visualized to determine the outcome. The gel also needs to be prepared for in-gel hybridization of the radiolabeled probe. The in-gel hybridization involves drying the gel so that it is thin enough to permit the radiolabeled probe to easily diffuse into and out of the gel yet not so thin as to shatter. This is accomplished by equilibrating the gel to a final concentration of 0.5 % glycerol and then drying the gel at 65 °C (*see* **Note 9**).

1. After the PFGE program has finished its run carefully remove the gel and place it in 200 ml of ethidium bromide/glycerol solution. Including the 100 ml volume of the gel, the final concentration of glycerol will be 0.5 %. Incubate at room temperature for 30 min with gentle agitation.
2. After incubation the gel can be placed on the imaging system so that the ethidium stained gel can be documented. Here it is important to minimize the amount of UV exposure as it can degrade the DNA especially in the presence of EtBr.
3. After the ethidium stained gel has been documented the surfaces of the gel are dried with a Kimwipe. Place the gel upside-down on a flat surface such that the flat side of the gel is on top. Fold up two Kimwipes together, then partially wet one edge of the folded Kimwipes with 0.5 % glycerol solution and use this pre-wet edge of the Kimwipe "sandwich" to wick liquid from the top surface of the gel. Using the partially wet edge of the Kimwipes will prevent the Kimwipes from adhering to the gel surface. Similarly wick liquid away from all sides of the gel. Now invert the gel so the flat wiped-dry face points down and place the gel in a metal pan. Wick any remaining liquid from the top surface of the gel using Kimwipes (*see* **Note 10**).
4. Place the gel and pan in the hybridization over at 65 °C until the gel appears homogenously dry and flat. The gel will dry to a thickness similar to a sheet of paper. The gel can be left overnight to dry but we have found that removal of the gel immediately after it finishing drying results in better hybridization of the radiolabeled probe and thereby better results.
5. Once the gel is dry it can be processed immediately or stored covered with PVC wrap (e.g., Saran Wrap) for up to 2 weeks in a dark dry area.

3.10 Preparing the Template DNA for PCR Radiolabeling

Template DNA is prepared by PCR-amplifying a region of the rDNA gene from human genomic DNA using primers 5′-GGG CTCGAGATTTGGGACGTCAGCTTCTG and 5′-GGGTCTAG AGTGCTCCC TTCCTCTGTGAG, to yield a 532-bp fragment.

The PCR product can be subsequently digested with XhoI and XbaI and subcloned into pBluescript II SK—or other suitable cloning vector for long term propagation. It is also possible to simply use the PCR product as a template in the subsequent radiolabeling PCR reaction directly without subcloning, although this is not recommended.

1. Isolate human genomic DNA from any human cell line using a Qiagen FlexiGene DNA kit. Dissolve the DNA to a final concentration of 1 μg/μl in water.
2. Set up the following PCR reaction:

2.0 μl	10× TAQ buffer with $MgCl_2$
1.0 μl	Genomic DNA at 1 μg/μl
0.4 μl	dGATC-TP nucleotide mix at 10 mM each nucleotide
2.0 μl	rDNA11-T primer at 1 μM concentration
2.0 μl	rDNA11-B primer at 1 μM concentration
0.4 μl	TAQ DNA polymerase (2 U total)
12.2 μl	Water
20 μl	*FINAL VOLUME*

3. Run the following PCR program with lid temperature set to 105 °C:

#	Instructions	Comment
1.	94 °C for 3 min	Initial denaturation
2.	94 °C for 30 s	Denature
3.	45 °C for 30 s	Anneal
4.	72 °C for 1 min, plus 2 s per cycle	Extend
5.	Go to **step 2**, repeat 29 times	30 Cycles total
6.	72 °C for 7 min	Polish
7.	Hold at 4 °C	Store indefinitely

4. Run the complete PCR reaction on a 1 % agarose gel with suitable size makers.
5. Stain with ethidium bromide, visualize with UV light and excise the 523 bp band with a clean razor blade.
6. Purify the template DNA from the gel slice using the GFX Kit.
7. Quantify the recovered DNA using Hoechst 33258 fluorimetry or similar methodology. Store at –20 °C.

8. *Optional, but recommended:* Subclone the PCR product into a convenient cloning vector and have sequenced to ensure the rDNA probe is correct.

3.11 Preparing the Radiolabeled 45S rDNA Probe

The radiolabeled probe is prepared by PCR using α^{32}P radiolabeled dATP. This method yields a probe that has very high specific activity (*see* **Note 11**).

1. If using plasmid as a template, dilute an aliquot of concentrated plasmid stock solution to 50 pg/μl. If using isolated PCR product as a template, dilute an aliquot of the PCR product to 10 pg/μl (*see* **Note 12**).
2. Set up the following PCR reaction:

2.0 μl	10× TAQ buffer with $MgCl_2$
2.0 μl	rDNA containing plasmid at 50 pg/μl *or* PCR product at 10 pg/μl
2.0 μl	rDNA11-T primer at 1 μM concentration
2.0 μl	rDNA11-B primer at 1 μM concentration
5.0 μl	α^{32}P dATP (50 μCi at 3,000 Ci/mmol)
2.0 μl	dGTC-TP at 40 μM concentration each
1.0 μl	dATP at 20 μM concentration—*not* radioactive
0.4 μl	TAQ DNA polymerase (2 U total)
3.6 μl	Water
20 μl	*FINAL VOLUME OF THE REACTION*

3. Run the following PCR program with lid temperature set to 105 °C:

#	Instructions	Comment
1.	94 °C for 3 min	Initial denaturation
2.	94 °C for 30 s	Denature
3.	45 °C for 30 s	Anneal
4.	72 °C for 1 min, plus 2 s per cycle	Extend
5.	Go to **step 2**, repeat 39 times	40 Cycles total
6.	72 °C for 7 min	Polish
7.	Hold at 4 °C	Store indefinitely

4. After the PCR reaction the probe is separated from the unincorporated nucleotides and primers with a USA scientific spin-50 mini-column. The radiolabeled probe is in the flow-through non-bound liquid phase (*see* **Note 13**).

5. Boil the probe for 2 min in a boiling water bath and then snap cool on water-saturated ice with periodic agitation for 2 min.
6. The probe should be kept on ice until it is used for hybridization. The probe should be used immediately rather than stored long-term to avoid radiolytic degradation.

3.12 In-Gel Hybridization of the Radiolabeled rDNA Probe and Southern Analysis

While the radiolabeled probe is being prepared the dried gel can be processed to prepare it for hybridization.

1. Place the dried gel into H_2O for 10 min with gentle agitation and allow it to rehydrate.
2. After that the gel should be easily removable from the surface of the pan and can be placed in a deeper stainless steel pan or glass dish for the subsequent washes.
3. Wash the gel with 100 ml of H_2O for 5 min. Do this step twice.
4. Remove the water and replace it with 100 ml of denaturation solution, incubate for 30 min with gentle agitation.
5. Remove the denaturation solution and replace it with 100 ml of neutralization solution, incubate for 30 min with gentle agitation.
6. Remove the gel from the neutralization solution and transfer it to a hybridization roller tube placing nylon mesh between the gel and the glass surface. The mesh allows the gel to get an even exposure to hybridization solution on both sides.
7. Pre-hybridize the gel for 3 h in 35 ml of hybridization solution at 65 °C in the hybridization oven.
8. After 3 h replace the hybridization solution with 25 ml of fresh preheated hybridization (65 °C) solution.
9. Add the radiolabeled probe directly into the hybridization solution and close the end caps on the bottle. Place the hybridization tube back in the hybridization oven at 65 °C and allow the gel to hybridize for at least 12 h (*see* **Note 14**).
10. Following hybridization, remove the cap from the hybridization tube and discard the radioactive solution appropriately. Add 50 ml of wash solution 1. Place the tube back in the hybridization oven and incubate for 30 min at 65 °C.
11. Discard the wash solution 1 from the hybridization tube and add 50 ml of fresh wash solution 1 for an additional 60 min at 65 °C.
12. Discard the wash solution 1 and rinse the gel twice with wash solution 2. Each wash uses 50 ml solution 2 and a 2 h incubation at 65 °C.
13. Remove the gel from the hybridization tube and rinse briefly in 2× SSC.

14. Place the washed gel in all-purpose polyvinyl chloride plastic wrap (Saran Wrap or equivalent) so that it does not dry out, being careful to blot up excess liquid with a paper towel or Kimwipe.
15. Expose the gel on a PhosphorImager screen overnight and detect the following day on a Molecular Dynamics Storm PhosphorImager or similar (*see* **Note 15**).
16. After data is acquired the gel can be stained in a 1:10,000 dilution of SYBR® safe dye. This fluorescently stained gel can be scanned on the Molecular Probes Storm PhosphorImager in fluorescent mode to give an accurate representation of the location of the size markers on the dehydrated gel.
17. Process the gel image using suitable image processing software. We prefer Adobe Photoshop and the following algorithm:

1.	Convert the image to 8-bit grayscale and save in TIFF format					
2.	Crop the image suitably					
3.	Adjust the levels of the grayscale to maximize the difference between the white background and the darkest band present					
4.	Blur using the following horizontal band-enhancing custom filter with a scale factor of 105 and zero offset to leave overall intensities unchanged:	1 5 9 5 1	1 5 9 5 1	1 5 9 5 1	1 5 9 5 1	1 5 9 5 1
5.	Unsharp mask: 200 % with 5.0 pixel radius and zero threshold					
6.	Unsharp mask again: 50 % with 3.0 pixel radius and zero threshold					

4 Notes

1. The radiolabeled probe sequence and restriction enzymes described herein are specific for the human rDNA sequence. Gene cluster analysis in other species and/or with non-rDNA clusters will require a different suitable choice of Southern hybridization probe and restriction enzymes.
2. There is no dATP in this solution. Low concentration dATP will be added separately.
3. The V-bottoms on the 96-well plates tend to concentrate the diluted cells and drastically improve non-adherent cell plating efficiencies at these low dilutions. In most cases use of these V-bottom plates is indispensable. Single plates are usually

sufficient for the higher dilutions, but it is usually necessary to prepare up to ten plates at the two lowest dilutions. This way of deriving single cell colonies relies on a probability distribution and on the plating efficiency of the cell line. To be reasonably confident that all the clones at a given dilution level arose from a single cell, the dilution needs to produce no more than 10 wells showing growth per 96-well plate. Colonies are only expanded from this lowest dilution plate.

4. Incubation in digestion buffer should be allowed to proceed until the initial cloudy appearance of the agarose "worm" has cleared. For some cell types, this may take 2 days rather than overnight. It is permissible to incubate the DNA–agarose at 50 °C in digestion buffer for longer periods of time if convenient.
5. Placing the gel on the benchtop for an hour and then leaving it at 4 °C for an hour may seem trivial, however, in our hands this procedure gives reproducibly sharp bands when the gel is run.
6. The 0.8 % LMP agarose will be used to seal the digested agarose slices into the wells of the pulsed-field gel. The 0.8 % LMP agarose usually takes at least 30 min to solidify at room temperature in the 50 ml plastic tube after removal from the boiling water bath, which is easily sufficient time for loading the pulsed-field gel.
7. Sometimes, particularly with extra thin agarose slices, the slice may flick out of the eppendorf tube and land on the razor blade folded in half. If this should happen, the slice can be gently unfolded by pipetting 100 μl 0.5× TBE buffer onto the folded slice in a manner that swirls the liquid. After the slice has unfolded, wick away the excess liquid with a Kimwipe.
8. Since the gel was at 4 °C prior to loading, the 0.8 % agarose will solidify rapidly after addition to the wells.
9. Never run PFGE with gels that have ethidium bromide already in them—the ethidium bromide intercalates the DNA and will drastically effect run time and results.
10. Be sure that there is no liquid trapped under the gel between the gel and the pan. Gels will shatter upon drying if there is residual trapped liquid.
11. To avoid chain termination by misincorporation of unlabeled dNTPs, it is necessary to decrease the concentration of the unlabeled dNTPs to be similar to that of the limiting radiolabeled dATP [14].
12. We have found it helpful to store the template in concentrated form and to dilute aliquots suitably for each labeling reaction. Storing very dilute solutions of DNA long-term is not recommended as adherence to the container of the DNA solution

can dramatically reduce the DNA concentration and the final yield of radiolabeled product.

13. The degree of radiolabeling can be roughly measured by using a Geiger counter to observe the amount of radioactivity in the liquid flow-through *vs* that trapped in the spin-column. For a successful radiolabeling reaction, the liquid should have at least twice as much radioactivity as the column.
14. Longer hybridization times make no difference.
15. We often reexpose the gel on the screen for an additional 3-day exposure to give a good reduction in background noise and sharper bands.

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