

Digital Karyotyping

Detailed Protocol

**Tian-Li Wang, Bert Vogelstein,
Kenneth W. Kinzler and Victor E. Velculescu**

**The Howard Hughes Medical Institute
and The Sidney Kimmel Comprehensive Cancer Center,
The Johns Hopkins University Medical Institutions**

**1650 Orleans Street
Baltimore, MD 21231**

FAX (410) 955-0548

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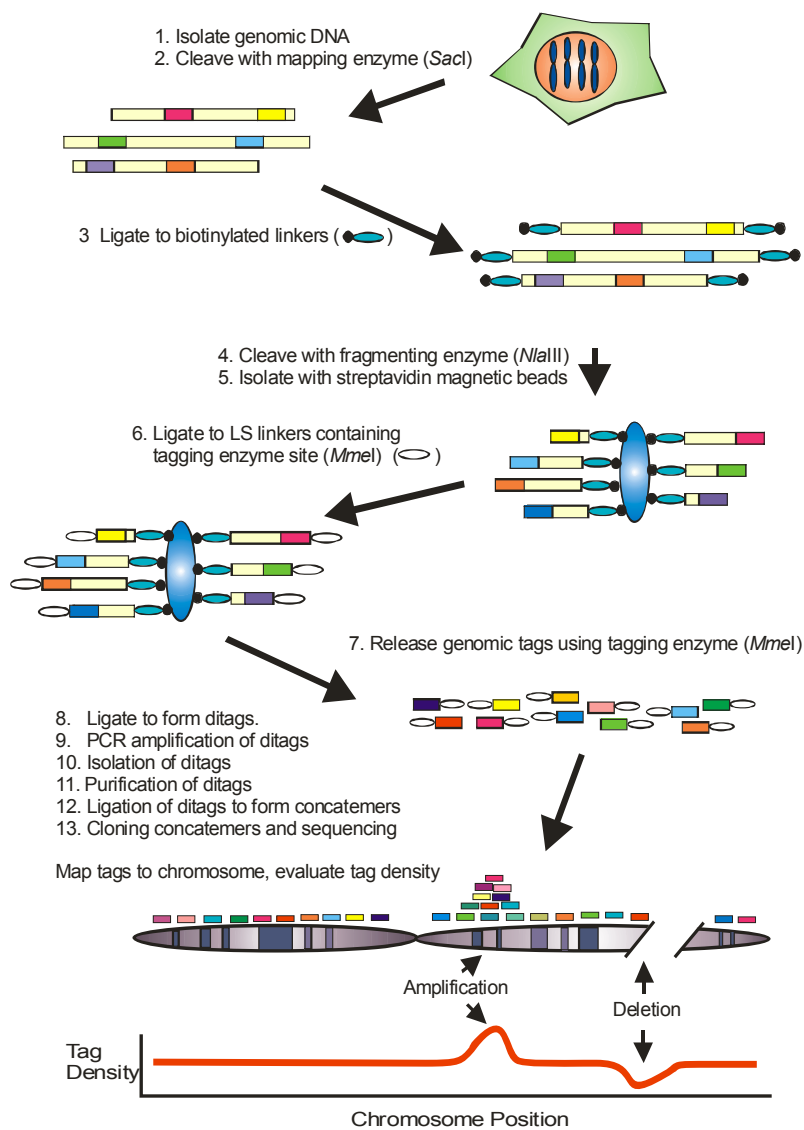
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Overview

Digital Karyotyping is an approach that permits comprehensive examination of cellular DNA content based on quantitative analysis of short fragments of genomic DNA (Wang et al, *PNAS* published online December 2, 2002, 10.1073/pnas.202610899). This method is based on two concepts. First, short sequence tags (21 bp each) can be obtained from specific locations in the genome. Second, populations of tags can be directly matched to the assembled genomic sequence, allowing observed tags to be sequentially ordered along each chromosome. Digital enumeration of tags observations along each chromosome can then be used to quantitatively evaluate DNA content with high resolution. For more information on Digital Karyotyping applications, visit the web site: <http://www.digitalkaryotyping.org/>. An outline of Digital Karyotyping with numbers corresponding to the steps in this protocol is presented below.



Methods

1. Isolate genomic DNA

- Prepare genomic DNA from tissue or cells of choice using DNeasy or QIAamp DNA blood kits (Qiagen, Chatsworth, CA) using the manufacturers' protocols
- Measure DNA concentration by method of choice, including OD 260, picoGreen assay, or Quantitative PCR. One microgram DNA is currently needed to construct each library.
- Be sure to check integrity of genomic DNA by gel electrophoresis or Southern hybridization.**

2. Cleave with mapping enzyme (*SacI*)

- Cleave genomic DNA with *SacI* (New England Biolabs, Beverly, MA).

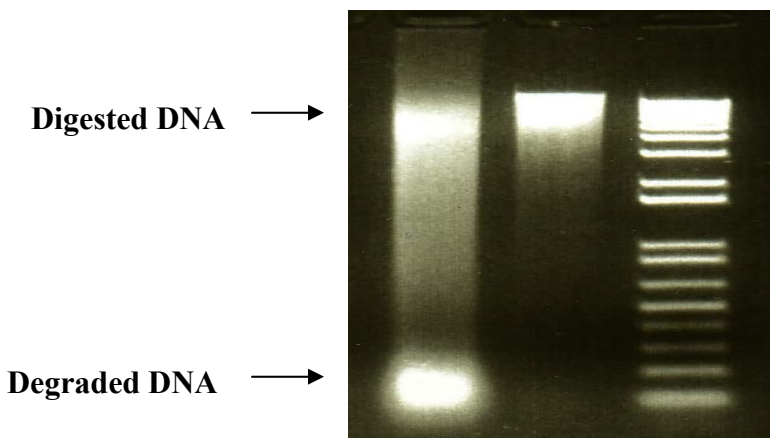
Contents	
Buffer 1 (10X, NEB)	20 ul
BSA. (100X, NEB)	2 ul
Genomic DNA	1 ug
DEPC treated dH2O	Add until total volume is 194 ul
Sac I (20 U/ml, NEB Cat no. R0156S)	6 ul

- Incubate at 37°C for 1 hour and 30 minutes.
- Evaluate genomic DNA after digestion, by analyzing 2 ul of digest using agarose gel electrophoresis. No degraded DNA should be present. If a portion of DNA is degraded, obtain new DNA sample and redigest. Do not proceed with degraded DNA as this will not allow for accurate analysis using digital karyotyping.

A. Degraded DNA

B. Undegraded DNA

A B



- Extract with equal volume PC8 and ethanol precipitate:
 - 200 ul sample
 - 133 ul 7.5M NH₄OAc
 - 3 ul glycogen
 - 1000 ul 100% EtOH
- Spin for 30 min at 4°C
- Wash twice with 70% ethanol, centrifuge and remove ethanol,
- Resuspend in 5 ul LoTE and incubate at 37°C for one minutes.

3. Ligate Biotinylated *SacI* linkers to digested DNA

⇒*SacI* Linker 1 (Appendix D) must be 5' biotinylated and the complementary *SacI* Linker 2 must be 5'-phosphorylated. We request such chemical modifications at the time of oligo synthesis (currently from Integrated DNA Technologies).

⇒ *SacI* Linkers should be obtained gel-purified after synthesis from oligo company

⇒Linker biotinylation and kinasing should be tested as described in Appendix C.

- Ligate *SacI* Linkers to the *SacI*-digested genomic DNA by adding the following to the sample tube:

Contents	
<i>SacI</i> Linker (35 ng.ul)	1.6 ul
<i>SacI</i> -digested genomic DNA (1 ug)	5 ul
Sterile dH ₂ O	2 ul
5X Ligation Buffer (Invitrogen).	2.4 ul
T4 DNA ligase (high conc: 5U/ul, Invitrogen Cat no.15224041)	1 ul

- Before adding T4 DNA Ligase, heat samples at 50°C for 2 minutes, let sit at room temperature for 10 minutes, then add ligase.
- Incubate three hours at 16°C.
- Add 188 ul LoTE to bring volume to 200 ul.
- Extract with equal volume PC8 and ethanol precipitate:
 - 200 ul sample
 - 133 ul 7.5M NH₄OAc
 - 3 ul glycogen
 - 1000 ul 100% EtOH
- Spin for 30 min at 4°C
- Wash twice with 70% ethanol, centrifuge and remove ethanol,
- Resuspend in 20 ul LoTE and incubate at 37°C for two minutes.

4. Cleave with fragmenting enzyme (*NlaIII*)

⇒Store *NlaIII* at -80°C

- Mix the following components:

Contents	Volume
LoTE	152 ul
DNA from previous step	20 ul
BSA (100X) (NEB)	2 ul
Buffer 4 (10X) (NEB)	20 ul
<i>Nla</i> III (10 U/ul) (NEB Cat. No. 125S)	6 ul

- Incubate at 37°C for 1 hour. Mix periodically.
- Extract with equal volume PC8 and ethanol precipitate:
 - 200 ul sample
 - 133 ul 7.5M NH₄OAc
 - 3 ul glycogen
 - 777 ul 100% EtOH
- Spin for 30 min at 4°C
- Wash twice with 70% ethanol, centrifuge and remove ethanol,
- Resuspend in 10 ul LoTE and incubate at 37°C for two minutes.

5. Isolate DNA fragments with strepavidin-magnetic beads

- Add 200ul Dynabead M-280 Streptavidin slurry (DynaL Cat. No. 112.05, 10 mg/ml) to each 1.5 ml microcentrifuge tubes.
- Use magnet to immobilize beads and remove supernatant.
- Wash beads once with 400ul Washing Buffer D, mix, magnet, remove supernatant.
- Mix 300 ul Wash Buffer D with 10 ul DNA from the previous step and add to the tube containing beads.
- Incubate 20 minutes at room temperature. Mix intermittently.
- Wash as above three times with 400ul Wash Buffer D, once with 300ul 1X Ligation Buffer, removing the wash each time.
- Resuspend the beads in 300 ul of 1X Ligase Buffer and divide the sample equally into two new tubes 1 and 2.
- Proceed immediately to step 4 below.

6. Ligating LS linkers to bound genomic DNA

- ⇒ **LS Linkers (Appendix D) should be obtained gel-purified after synthesis from oligo company (we use Integrated DNA Technologies for linker syntheses).**
- ⇒ **LS Linkers 1B and 2B must be 5' kinased and annealed to their complementary linker 1A and 2A, respectively, before ligation. Kinasing can be performed chemically at the time of oligo synthesis.**
- ⇒ **Linker kinasing should be tested by self-ligation as described in Appendix C.**
- ⇒ **One tube for linker 1, the other for linker 2**

Remove the 1x Ligase Buffer from step 3 and process the two microcentrifuge tubes as follows:

Contents	Tube1	Tube2
Dynabeads bound to cDNA fragments	beads	beads
LoTE	23.5 ul	23.5 ul
Annealed LS Linker 1A,B (25 uM) (See Appendix D)	2 ul	0 ul
Annealed LS Linker 2A,B (25 uM) (See Appendix D)	0 ul	2 ul
5X Ligase Buffer (Invitrogen)	7 ul	7 ul

- Resuspend Dynabead slurry by mixing microcentrifuge tubes gently.
- Heat microcentrifuge tubes at 50°C for 2 minutes, then let sit at room temperature for 15 minutes.
- Add 2.5 ul T4 Ligase (High concentration 5U/ul; Invitrogen Cat. No. 15224041) to each microcentrifuge tube.
- Incubate for 2 hours at 16°C, gently mixing every 15 minutes.
- After ligation, wash each microcentrifuge tube 4 times with 600ul Washing Buffer D.
- Transfer contents to new tube
- Wash once with Washing Buffer D and once with 200 ul 1X Buffer 4 (NEB).
- Resuspend beads in 200 ul 1X Buffer 4 (NEB)

7. Release genomic tags using Tagging Enzyme (MmeI)

- Remove 1X Buffer 4 from each tube and immediately add the following components:

Contents	Tube1	Tube2
Beads sample 1 or 2	beads	beads
LoTE	118 ul	118 ul
10X Buffer 4 (NEB)	15 ul	15 ul
10X SAM (NEB)	15 ul	15 ul
MmeI (10 U/ul; NEB Cat. No. 572S)	3 ul	3 ul

- Incubate at 37°C for 1 hour and 10 minutes, gently mixing intermittently.
- Place on magnet, collect 100 ul supernatant from tube1 and tube 2 and combine them in a new tube (total volume will be 200 ul). This will be the Ligation group in the next step.
- Collect the remaining ~50 ul supernatant from tube1 and tube 2 and combine them in a new tube (total volume will be 100 ul). Add 100ul LoTE. This will be the No Ligase control in the next step.
- Extract each sample with equal volume PC8 and ethanol precipitate:
 - 200 ul sample
 - 133ul 7.5M NH₄OAc
 - 3ul glycogen
 - 1000ul 100% EtOH
- Spin for 30 min at 4°C
- Wash twice with 70% ethanol, centrifuge and remove ethanol,
- Resuspend each sample in 1.5 ul LoTE.

8. Ligate to form ditags

- Premixed, blunt-ended tags are ligated to each other in the following way:
- Using the ratios shown below make up an excess volume of “2X ligase mix” or “2X negative control mix”:

Contents	2X ligase mix	2X no ligase mix
3mM Tris HCl (pH 7.5)	5 ul	9 ul
5X Ligase buffer (GibcoBRL)	6ul	6 ul
T4 Ligase (high conc 5U/ul;Invitrogen Cat. No. 15224041)	4 ul	-

⇒The 2X ligase mix shown above, or the 2X no ligase mix, is combined with the premixed tags in a 1:1 ratio. The no ligase control serves as a negative control for contamination in later PCR steps.

- Add 1.5 ul of 2X ligase mix to tags resuspended in 1.5 ul LoTE (from step 7). Add 1.5 ul of 2X neg. control mix to the single no-ligase control tube.
- Incubate overnight 16°C.
- Add 14ul of LoTE to ligation mixture and proceed to PCR amplification below, or store at -20°C.

9. PCR amplification of ditags

⇒Amplify ditags using LS PCR primers 1 and 2 (Appendix D).

⇒Optimize amplification by using different dilutions of template (1ul of 1/20, 1/40 and 1/80 dilutions of ligation product per PCR reaction).

- Perform PCR using the following ingredients:

Contents	Per reaction	10 rxn mix
10X PCR Buffer (see appendix A)	5 ul	50 ul
DMSO (Sigma Cat. No. D-2650)	3 ul	30 ul
10 mM dNTPs (GibcoBRL)	7.5 ul	75 ul
LS PCR Primer 1 (350 ng/ul)	0.5 ul	5 ul
LS PCR Primer 2 (350 ng/ul)	0.5 ul	5 ul
dH ₂ O	32 ul	320 ul
Platinum Taq (5u/ul; Invitrogen Cat. No. 10966-034)	0.5 ul	5 ul
Ligation Product (at various dilutions)	1 ul	

- Aliquot 49 ul of each 10 reaction mix to each tube.
- Add 1 ul of each dilution to be tested to the reaction.
- Add 30 ul mineral oil to the reaction and perform PCR at following temperatures (optimized for HYBAID PCR machine):

Cycles	Temp / time
1 cycle	94°C 1 min
26-31 cycles	94°C 30"; 55°C 1 min; 70°C 1 min;
1 cycle	70°C 5 min at end.

⇒No ligase sample should be amplified for 35 cycles

⇒The appropriate cycle number is critical for isolating an adequate amount of DNA for Digital Karyotyping. Too few cycles will result in a low yield and may cause problems with subsequent steps. Too many cycles will give erratic results and can also result in low yields (see trouble shooting section). Therefore, we recommend trying various cycle numbers (e.g. 26, 28, 31) to determine the optimal number.

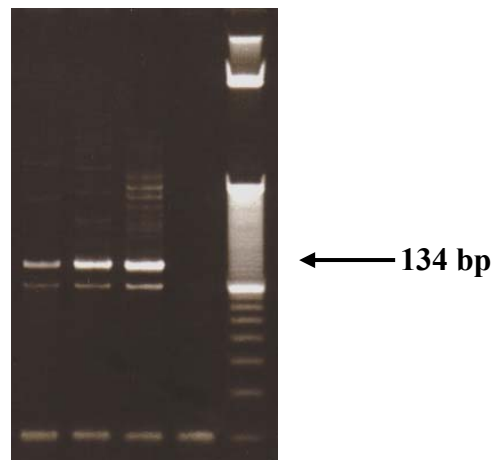
•Remove 10 ul from each reaction and run on pre-poured 4-20% gradient polyacrilamide gel (Novex Cat. No. EC62255), using a 10 bp ladder as a marker (Invirogen Cat. No. 10821-015).

⇒Amplified ditags should be 134 bp in size.

⇒A background band of equal or lower intensity occurs around 100 bp. All other background bands should be of substantially lower intensity.

⇒The no ligase samples should not contain any amplified product of the size of the ditags even at 35 cycles.

134 bp PCR Products



Dilution Fold 80 40 20

•After PCR conditions have been optimized, large scale PCR (two to three 96-well plates containing 50ul reactions/well) can be performed.

•We usually use a 200 reaction PCR premix which we aliquot into two 96 well plates

(Omniplate 96; Marsh Biomedical Products, Cat # J3-2031) of 50ul PCR reactions/well.

10. Isolation of ditags

- Pool PCR products into one 50ml conical tube.
- Add equal volume PC8 (approximately 10 ml), spin in swinging bucket rotor (SIGMA 4K15, rotor 11156/13115) at 5100 rpm for 10 minutes.
- Transfer aqueous phase to a new 50ml tube, and ethanol precipitate
 - 10 ml product,
 - 2.56 ml 7.5M ammonium acetate,
 - 57.6 ul glycogen,
 - 18.24 ml ethanol
- Incubate on dry ice for 30 minutes.
- Vortex vigorously, spin in fixed angle rotor (SIGMA 4K15, rotor 12169) at 10,000 rpm for 30 minutes.
- Wash two times with 12.5 ml 70% ethanol, vortex and spin for 5 minutes in swing bucket rotor at 5100 rpm for 5 minutes.
- Resuspend in 144 ul LoTE. Warm at 37°C for 5 minutes.
- Add 36 ul 5X sample buffer to this sample (180 ul total volume).
- Load 10ul of the sample into 18 wells of two 10 well 12% polyacrylamide gels (see Appendix C). Use 10ul of a 20 bp ladder as a marker on each gel.
- Run gel for 3 hours at 180V.
- Stain gel using ethidium bromide for 10 minutes.
- Visualize on UV box.

⇒ Amplified ditags should run at **134** bp while a background band runs at about 100 bp.

- Cut out only amplified ditags from gel, and place each band into each 0.5ml microcentrifuge tube (18 0.5ml tubes total) which bottom has been pierced with a 21 gauge needle to form a small hole of about 0.5mm diameter.
- Place 0.5ml microcentrifuge tubes in 2.0ml siliconized microcentrifuge tubes (Ambion Cat No. 12475) and spin in microfuge at full speed for 2 minutes (this serves to break up the cut-out-bands into small fragments at the bottom of the 2.0ml microcentrifuge tubes).
- Discard 0.5ml tubes, add 250 ul LoTE and 50ul 7.5M ammonium acetate to each 2.0ml tube.
- Vortex each tube and place at 65°C for 15 min.
- Transfer contents of each tube to each SpinX microcentrifuge tubes (18 SpinX microcentrifuge tubes).
- Spin each SpinX in microcentrifuge for 5 minutes at full speed.
- Consolidate eluates (300ul from each Spin X-tube) into a 50 ml conical tube.
- Ethanol precipitate eluates using the following mixture
 - per 300ul sample
 - 5ul glycogen

133ul 7.5M NH₄OAc
1000ul 100% ethanol

- Incubate on dry ice for 30 minutes.
 - Vortex vigorously, spin in fixed angle rotor (SIGMA 4K15, rotor 12169) at 10,000 rpm for 30 minutes.
 - Wash two times with 12.5 ml 70% ethanol, vortex and spin for 5 minutes in swing bucket rotor at 5100 rpm for 5 minutes.
 - Resuspend DNA in 80 ul LoTE.
- Digest PCR products (80 ul) in two tubes (each one with 40 ul DNA) with NlaIII.

Contents	Tube
PCR products in LoTE	40 ul
Sterile dH ₂ O	118 ul
10X NEB Buffer 4	20 ul
BSA (100X; NEB)	2 ul
Nla III (10 U/ul; NEB)	20 ul

- Incubate 1 hr and 10 minutes at 37°C.

11. Purification of ditags

⇒Biotinylation of Primer 1 and Primer 2 allows the removal of linker products following NlaIII digestion by use of streptavidin-linked magnetic beads. It is possible to remove the linkers solely by gel purification of ditags. However, we have found that addition of this extra purification step ensures complete removal of linkers which can poison the subsequent ditag concatamerization reaction by capping the concatamer ends, and rendering the concatamers unclonable. In our experience the combination of these two purifications techniques is better than the use of either alone. Removal of linkers by streptavidin beads can be done either before or after gel purification. We prefer the former because it allows us to visualize the purity of the ditags following removal of linkers by streptavidin-linked magnetic beads.

- Linker purification:

1. During the NlaIII digestion, 1600µl of Dynal streptavidin magnetic beads is placed into 2 tubes (800ul each) and each is prewashed 3 times with 800ul Washing Buffer D. After adding the last wash, aliquot 200µl into each of 8 tubes (2 for each of 4 purification cycle). Label four tubes A through D, do this twice for each set (e.g. A1,B1,C1, D1 and A2, B2, C2, D2).
2. To the completed NlaIII digest, add 400ul 2X B&W buffer and 4ul 100X BSA. Using a magnet, remove wash buffer from the first 2 tubes containing beads i.e. A1 & A2. Aliquot

the 800ul digest mix to both tubes (i.e. 400ul into A1 and A2) (purification cycle 1). Mix end over end at RT 15 min (Labquake rotator).

3. Place on magnet 2 min. At same time place second set 200µl aliquot of beads onto magnet. Remove wash buffer from second 200µl aliquot of beads i.e. B1 & B2. Transfer sup from first set of tubes i.e. A1 & A2 to B1 & B2 respectively.
4. Immediately add 200µl of rinse (1X B&W, 1X BSA) to first aliquot of beads (tubes A1 & A2). Pipette back and forth several times to remove residual ditags. Mix end over end A1, A2, B1 & B2 at room temp for 15 minutes.
5. Remove sup from C1 & C2. Transfer sup from B1 & B2 to C1 & C2 respectively. Transfer sup from A1 & A2 to B1 & B2 respectively. Mix end over end for 15 minutes.
6. Remove sup from D1 & D2. Transfer C1 & C2 to D1 & D2 respectively. Transfer B1 & B2 to C1 & C2. Mix end over end for 15minutes.

Collect sup from D1 & D2 and transfer to new tubes. Transfer sup from C1 & C2 to D1 & D2 respectively. Mix end over end for 15'. Consolidate supernatants (~550ul in each of 2 tubes).

⇒ We have noted that the ditags, although very stable in the presence of high salt, occasionally melt in the presence of low salt especially if the temperature rises above RT or DNA pellets are allowed to dry. Therefore, the following steps are performed on ice until samples are placed in the high salt TAE gel buffer. Also, pellets are resuspended in TE instead of LoTE.

- Extract with equal volume PC8.

- Pool aqueous phases and then ethanol precipitate in dry ice as follows:

200ul Sample
66ul 7.5 M NH₄OAc
5ul Glycogen
825ul 100% EtOH

- Vortex

- Place in dry-ice for 30 min

- Warm at RT for 2 min until solution has melted

- Spin at 4°C for 30 min

- Wash once with cold 75% ethanol, remove ethanol traces

- Resuspend pellet in each tube in 7 ul of cold **TE** (not LoTE)

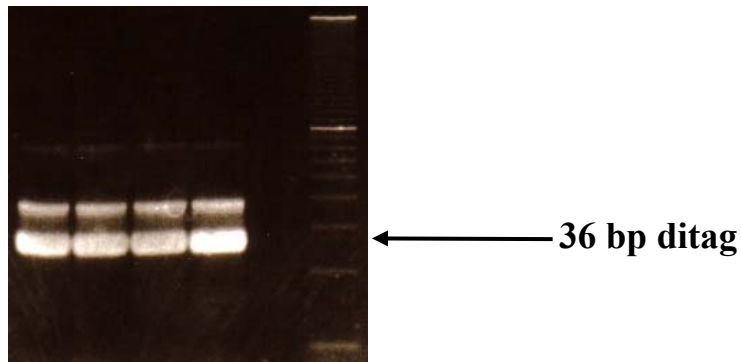
- Pool resuspended DNA into 1 tube (30ul total).

- On ice add 5X sample buffer.

- Load this sample into 4-5 lanes of a 12% polyacrylamide gel (10-well) and run at 180 V for 2 hrs.

- Stain gel using ethidium bromide.

**Released Ditags
(With linker purification)**



- Cut out 36 bp band from 4 lanes, and place each cut-out-bands in each 0.5ml microcentrifuge tube.
- Pierce the bottom of 0.5ml tube with a 21 gauge needle, and place the tubes in 2.0ml siliconized microcentrifuge tubes and spin in microfuge at full speed for 2 min.
- Discard 0.5ml tubes, add 250ul **TE** and 50ul 7.5M ammonium acetate to 2.0ml tubes.
- Vortex the tubes, and place at 37°C (**not 65°C!**) for 20 min.
- Transfer content from each tube to each SpinX tubes as above and spin to isolate eluate.
- Consolidate eluates (300ul from each Spin X-tube) into a 50 ml conical tube.
- Ethanol precipitate eluates using the following mixture
per 200ul sample
3 ul glycogen
66 ul 7.5M NH₄OAc
825 ul 100% ethanol
- Incubate on dry ice for 30 minutes.
- Vortex vigorously, spin in fixed angle rotor (SIGMA 4K15, rotor 12169) at 10,000 rpm for 30 minutes.
- Wash two times with 12.5 ml 70% ethanol, vortex and spin for 5 minutes in swing bucket rotor at 5100 rpm for 5 minutes.
- Air dry on ice for 5 minutes.
- Resuspend DNA in 10.5 ul cold LoTE.

12. Ligation of ditags to form concatemers

- ⇒Length of ligation time depends on quantity and purity of ditags.
⇒Typically, several hundred nanograms of ditags are isolated and produce large concatemers when the ligation reaction is carried for 1 to 3 hours at 16°C (lower quantities or less pure ditags will require longer ligations).
- Mix the following:

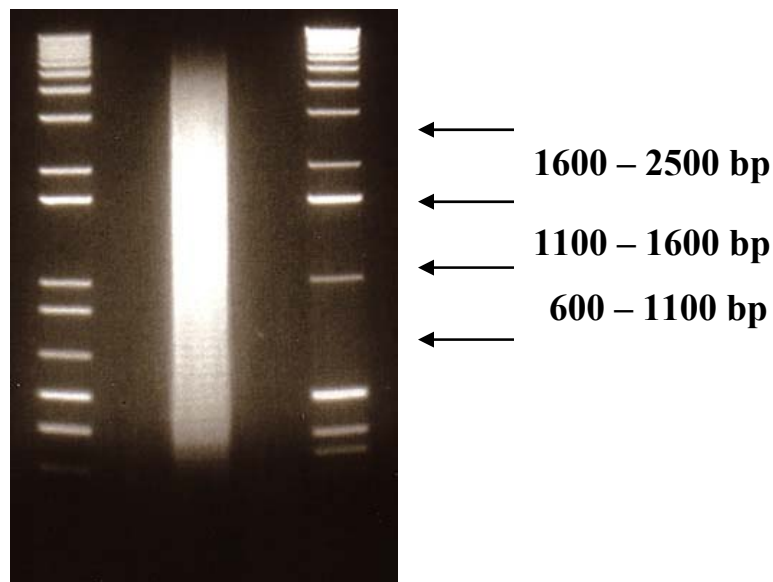
Contents	Tube
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Pooled purified ditags	10.5 ul
5x Ligation Buffer (BRL)	3 ul
T4 Ligase (high concentration 5U/ul; GibcoBRL Cat. No. 15224-041)	1.5 ul

- Incubate 2-3 hours at 16 °C.
- Afterwards, add 2.5ul 5X sample buffer to ligation reaction.
- Heat sample 65°C 5 min then place on ice.

⇒Concatemers will form a smear on gel with a range from about one hundred base pairs to several kilobases. We usually isolate region 600-1200bp and 1200bp to 2500bp.

Concatemer



⇒Use agarose gel electrophoresis to separate concatemers.

- Prepare a 13 cm long 1.5% agarose gel (we use rigs from Owl Scientific Cat. No. B2) .
- In the first lane of the gel load 30 ul 1 kb plus ladder (25ng/ul) as a marker.
- After ligation reaction and subsequent heating and ice step detailed above, load entire concatemer mix onto 1 lane of a 1.5 mm 12 well gel.
- Run at 125 V for 2 hours.
- Stain for 10 minutes with ethidium bromide (0.5 ug/ml), and visualize the DNA under UV light. Excise desired fractions.

⇒Concatemers will form a smear on gel with a range from about one hundred base pairs to several kilobases. We usually isolate three regions 650-1100 bp and 1100 bp to 1600 bp, and 1600 bp to 2500 bp.

- Extract DNA with QIAquick gel extraction kit (Qiagen, Cat. No. 28704) following manufacturer's protocol, except that step 5 on page 25 should be skipped.
- Elute two times with 50 ul elution buffer. Combine two elutes into one tube.

- Extract with PC8, and ethanol precipitate
 - 100 ul sample
 - 66.5 ul 7.5M ammonium acetate,
 - 3 ul glycogen,
 - 388.5 ul 100% ethanol.
- Wash twice with 70% ethanol, and centrifuge and remove ethanol.
- Resuspend in 6ul LoTE.

12. Cloning concatemers and sequencing

⇒Concatemers can be cloned and sequenced in a vector of choice. We currently clone concatemers into an SphI cleaved pZero (Invitrogen Cat. No. K2500-01).

- For this cloning, mix the following:

Contents	Sample	Vector Alone Control	No Ligase Control
purified concatemer	6ul		
dH2O		6ul	7ul
pZero cut with SphI (25ng/ul)	1ul	1ul	1ul
5X Ligase buffer (GibcoBRL)	2ul	2ul	2ul
T4 ligase (1U/ul; GibcoBRL Cat. No. 15224-017)	1ul	1ul	0

- Incubate 3 hours at 16°C.
 - Bring sample volume to 200ul with LoTE.
 - Extract with equal volume PC8, ethanol precipitate,
 - 200 ul sample
 - 133 ul 7.5M ammonium acetate,
 - 5 ul glycogen,
 - 777 ul 100% ethanol.
 - wash four times with 70% ethanol,
 - centrifuge and remove ethanol, and resuspend in 10 ul LoTE.
 - Transfect 1 ul DNA into ElectroMAX DH10Bs (GibcoBRL Cat. No. 18290-015) by electroporation,
 - Plate 1/100, 1/50, 1/20 of transfected bacteria onto each 10 cm zeocin-containing plate, analyze 12-16hrs later.
- ⇒Insert containing plates should have hundreds to thousands of colonies while control plates should have 0 to tens of colonies
- Save all plates for each concatemer ligation reaction since, if insert size appears appropriate these may be used for sequencing as described below.
 - Check insert sizes by PCR 96 bacteria colonies. Set up 25 ul PCR reactions using the following

conditions:

Contents	Tube
10X PCR Buffer (see appendix A)	2.5 ul
DMSO (Sigma Cat. No. D-2650)	1.25 ul
10 mM dNTPs (GibcoBRL)	1.25 ul
M13F (350 ng/ul)	0.5 ul
M13R (350 ng/ul)	0.5 ul
dH ₂ O	19 ul
Platinum Taq (5 U/ul; GibcoBRL Cat. No. 10966-034)	0.2 ul

- Add PCR components to wells of a 96-well PCR plate (e.g. Omniplate 96; Marsh Biomedical Products, Cat # J3-2031),
- Use a sterile tip to gently touch colony and then dip tip into PCR mix.
- Add one drop of oil over PCR mix.
- Perform PCR at following temperatures (optimized for HYBAID PCR machine) :

1 cycle	95°C 2 min at start;
35 cycles	95°C 30"; 56°C 1 min; 72°C 30";
1 cycle	70°C 5 min at end.

- Run 4ul on a 1.5% agarose gel.
- Check insert sizes and determine the cloning efficiency.

⇒ Purify remaining PCR reactions that contain concatemers comprised of at least ~15 ditags (> 750 bp [226bp vector + 36bp per ditag x 15 ditags]) by isopropanol precipitation.

- Mix 17ul of PCR reaction with 75ul of the following premixed isopropanol solution:

dH ₂ O	28ul
2M NaClO ₄	15ul
2-Propanol	33ul
Total	75ul

- Spin at ~6000g in a centrifuge containing plate bucket rotor.
- Decant and soak up residual precipitation mix by placing upside-down on paper towel.
- Rinse once by adding 100ul 70% ethanol to wells.
- Spin 5 min at maximum speed.
- Decant, blot on paper towel, dry samples (e.g. under laminar flow or in an immobile speed vacuum) and resuspend in 25ul dH₂O.
- Store at -20°C.

⇒ Sequencing can be performed manually or on automated sequencers. We currently sequence using ABI -21M13 Dye Primer FS sequencing kit (Perkin Elmer Cat. No. 402111) following

Perkin Elmer's protocol. We use about 1/5 of the purified PCR product per sequencing reaction. Load and run on ABI 373 or 377 automated sequencer.

Appendix A: Special Reagents to Order

Magnetic Beads

Dynabeads M-280 Streptavidin Slurry (Dynal Cat. No. 112.05)

Magnet (Dynal Cat No. 120.04)

Enzymes

SacI (NEB Cat no. R0156S)

MmeI (University of Gdansk Center for Technology Transfer, Gdansk, Poland or NEB Cat. No. 572S)

NlaIII (NEB Cat. No. 125S) – ship on dry ice and store at –80°C

Sph I (NEB Cat. No. 182S)

T4 Ligase High Concentration (5U/ul) (Invitrogen Cat. No. 15224-041)

Platinum Taq (Invitrogen Cat. No.10966-034)

Miscellaneous

Glycogen (Boehringer Mannheim Cat. No. 901-393)

pZERO-1 plasmid (Invitrogen Cat No. K2500-01)

10mM dNTP mix (GibcoBRL Cat. No. 18427-013)

DMSO (Sigma Cat. No. D2650)

7.5 M NH₄OAc (Sigma Cat. No. A2706)

Primers and linkers (Appendix D)

Appendix B: Solutions

Washing Buffer D

5 mM Tris-HCl pH7.5
0.5 mM EDTA
1M NaCl
200 ug/ml bovine serum albumin

2X B+W Buffer

10mM Tris-HCl (pH 7.5)
1mM EDTA
2.0 M NaCl
store at room temperature

TE

10 mM Tris-HCl (pH 7.4)
1 mM EDTA (pH 8.0)
store at room temperature

LoTE

3mM Tris-HCl (pH 7.5)
0.2mM EDTA (pH 7.5)
in dH₂O
store at 4°C

PC8

480ml Phenol (warm to 65°C)
320ml 0.5M Tris-HCl (pH 8.0)
640ml Chloroform

Add in sequence, shake, and place at 4°C. After 2-3hours shake again. After another 2-3 hours, aspirate aqueous layer. Aliquot and store at -20°C.

10X PCR Buffer

166mM (NH₄)₂SO₄
670mM Tris pH 8.8
67mM MgCl₂
100mM Beta-mercaptoethanol
distribute into 0.5ml aliquots and store at -20°C

Appendix C: Gel Electrophoresis and miscellaneous methods

12% PAGE (for isolating PCR products and ditags)

40% Polyacrylamide (19:1 acrylamide:bis)(Bio-Rad Cat No. 161-0144)	10.5ml
dH ₂ O	23.5ml
50X Tris Acetate Buffer (Quality Biological Cat. No. 330-008-161)	700ul
10% APS	350ul
TEMED	30ul

Mix above and add to vertical gel apparatus. We currently use Owl Scientific Products (Model No. P9DS) with 1.5mm spacers. Add comb and let gel sit at least 30 minutes to polymeize. Run gel at 160 V for 2 - 2.5 hours (see text for details).

PC8 extraction

Add equal volume PC8 to sample.
Vortex for several seconds.
Spin for 2 minutes at full speed in microcentrifuge.
Transfer aqueous (top) layer to a new microcentrifuge tube.

Anneling primers to form linkers

Mix 9ul Linker 1A to 20ul kinased Linker 1B (final conc 200 ng/ul)
9ul Linker 2A to 20ul kinased Linker 2B (final conc 200 ng/ul)
To anneal linkers, heat to 95°C for 2 min, then place at 65°C for 10 min, 37°C for 10 min and room temp for 20 minutes, store at -20°C.

Testing 5' phosphorylation of ordered linkers

Kinasing should be tested by self ligating about 200ng of each linker pair and running on 20% Novex gel (Invitrogen, Cat. No. EC6315). Kinased linkers should allow linker-linker dimers (80-100 bp) to form after ligation, while unkinased linkers will prevent self-ligation. Only linker pairs that self-ligate >70% should be used in further steps.

Testing biotinylation of ordered oligos

Obtain biotin-modified, gel purified oligos from oligo synthesis company. Test biotinylation by adding to several hundred nanograms of biotin-oligo to 1ug streptavidin (Sigma Cat No. S-4762). Incubate several minutes at room temperature. Both the oligo alone and the one bound to streptavidin are run on a 20% Novex gel (Cat. No. EC6315). If the oligo is well biotinylated, the the entire amount of oligo should be shifted to higher molecular weight in the lane containing the streptavidin. Alternatively, increasing amounts of oligo (from several hundred nanograms to several micrograms) can be incubated with and without separate aliquots of 100ul of Dynabeads (Dyna). After 15', the beads are separated from the supernatant using a magnet, the supernatant is removed, and DNA quantitation is performed at OD260. At low amounts of oligo, when bead binding capacity is not saturated, the ratio of unbound oligo to the total oligo will indicate the percent of oligo that is not biotinylated.

Appendix D: Linker & Primer Sequences

SacI Linker A (obtain gel purify)

5'-biotin-TTTGCAGAGGTTTCGTAATCGAGTTGGGTGAGCT-3'

SacI Linker B (obtain gel purify)

5'-phosphate-CACCCAACCTCGATTACGAACCTCTGC-3'

LS Linker 1 A (obtain gel-purified)

5'-TTTGGATTTGCTGGTGCAGTACAACCTAGGCTTAATATCCGACATG-3'

LS Linker 1 B (obtain gel-purified)

5'-Phosphate-TCGGATATTAAGCCTAGTTGTACTGCACCAGCAAATCC
AminoModified C7-3'

LS Linker 2 A (obtain gel-purified)

5'- TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGTCCGACATG-3'

LS Linker 2 B (obtain gel-purified)

5'-Phosphate-TCGGACGTACATCGTTAGAAGCTTGAATTCGAGCAG AminoModified
C7-3'

LS Primer 1 (obtain gel-purified)

5'-biotin-TTTTTTTTTGGATTTGCTGGTGCAGTACA-3'

LS Primer 2 (obtain gel-purified)

5'-biotin-TTTTTTTTTCTGCTCGAATTCAAGCTTCT-3'

M13 Forward

5'-GTAAAACGACGGCCAGT-3'

M13 Reverse

5'-GGAAACAGCTATGACCATG-3'

Note: High quality linkers are crucial to several steps in the Digital Karyotyping method. We now ask the modification of primers during oligo synthesis. Except M13 primers, all the primers should be obtained gel-purified from the oligo synthesis company. We routinely obtain these oligos from Integrated DNA Technologies (tel. 800-328-2661).