

Protocol for 5' and 3' LongSAGE

Updated 18 January 2004 PN

Notes:

1. The entire procedure can be divided into the following subsections:

- (1) Isolation of mRNA
- (2) Isolation of cDNA (full-length cDNA in the case of 5' LongSAGE) containing Mmel flanking sites.
- (3) Formation of PCR-derived ditags
- (4) Restriction enzyme cleavage of PCR-derived ditags to give short (50bp) cohesive ditags used for concatemer formation.
- (5) Purification of concatemer fractions and their cloning into pZER0-1 for sequencing

2. Oligonucleotides and formation of double-stranded linker-adapters:

All oligonucleotides used for linker-adapter formation were purchased PAGE-purified, while other oligonucleotides were HPLC purified. Linker-adapters were made by annealing equimolar amounts of each component oligonucleotide in T₁₀N₅₀E_{0.1} buffer under the following conditions: 95°C x 2 min; 75°C x 2 min; 60°C x 2 min, 50°C x 2 min, 40°C x 2min; 20°C x 2min. All other oligonucleotides were resuspended in LoTE buffer (3 mM Tris-HCl (pH 8.0), 0.2 mM EDTA pH8.0) prior to use.

3. We have found it convenient to use Phase Lock Gel Light (Eppendorf) to facilitate the many phenol/chloroform extraction steps in the protocol.

Oligonucleotide and adapter sequences

| Name | DNA sequence |
|-----------------------|--|
| NotI-dT ₂₀ | 5' -GAGAGAGAGAGCGCCGCTTTTTTTTTTTTTTTTTTTTTTTVN- 3' |
| Adapter A(N5) | 5' -TTTGTAGGCCGATACTCCGTGTCATTACTAGGCTTAGGATCCGACGNNNNN- 3' 3' - Am -CATCCGGCTATGAGGCACAGTAATGATCCGAATCCTAGGCTG- Phos -5' |
| Adapter A(N6) | 5' -TTTGTAGGCCGATACTCCGTGTCATTACTAGGCTTAGGATCCGACGNNNNN- 3' 3' - Am -CATCCGGCTATGAGGCACAGTAATGATCCGAATCCTAGGCTG- Phos -5' |
| Adapter B(N5) | 5' -TTTTCACTCAGGCTCTAGCTCATCTATCACACATGGATCCGACGNNNNN- 3' 3' - Am -AGTGAGTCCGAGATCGAGTAGATAGTGTGTACCTAGGCTG- Phos -5' |
| Adapter B(N6) | 5' -TTTTCACTCAGGCTCTAGCTCATCTATCACACATGGATCCGACGNNNNN- 3' 3' - Am -AGTGAGTCCGAGATCGAGTAGATAGTGTGTACCTAGGCTG- Phos -5' |
| Adapter C | 5' - Phos -GGCCGCTCATAATCCAGAAGTAGTC- 3' 3' -CGAGTATTAGGTCTTCATCAG- Biot -5' |
| P1 | 5' - Biot -GTAGCCGATACTCCGTGTCATT- 3' |
| P2 | 5' - Biot -TCACTCAGGCTCTAGCTCATCTA- 3' |
| PMR011 | 5' -GATGTGCTGCAAGGCGATTAAG- 3' |
| PMR012 | 5' -AGCGGATAACAATTTACACAGG- 3' |
| PMR004 | 5' -GGAAACAGCTATGACCATG- 3' |
| GsuI-dT ₁₆ | 5' -GAGAGAGAGACTGGAGTTTTTTTTTTTTTTTTTTVN- 3' |
| SalI adapter | 5' - Biot -TCGACCCACGCGTCCG- 3' 3' -GGGTGCGCAGGC- Phos -5' |
| Mme/BamHI adapter A | 5' - Phos -GTCGGATCCTAAGCCTAGTAATGACACGGAGTATCGGCCTAC- 3' 3' -TTCAGCCTAGGATTCGGATCATTACTGTGCCTCATAGCCGGATGCC- 5' |
| Mme/BamHI adapter B | 5' - Phos - GTCGGATCCATGTGTGATAGATGAGCTAGAGCCTGAGTGA- 3' 3' -TTCAGCCTAGGTACACACTATCTACTCGATCTCGGACTCACTCCC- 5' |

5'LongSAGE

First strand cDNA synthesis and full-length (-)DNA selection

Total RNA is prepared using Trizol (Invitrogen), as per the manufacturer's protocols. mRNA (polyA RNA) is then purified from this total RNA using a magnetic-bead based system (μ MACS mRNA Isolation Kit for Total RNA, #130-075-102, Miltenyi Biotec). Typical yields are 20-50 ug mRNA from 1mg total RNA. We have experimented with mRNA purification kits from several different suppliers and have found this one to be an ideal compromise between time and quality. The "cap-trapper" procedure (1) was used to select for full-length cDNA.

In the first step, cDNA is synthesized by reverse transcription with a NotI-oligo dT primer. Then, the first strand cDNA/RNA hybrids are subjected to a full-length enrichment procedure by the biotinylation-based cap-trapper approach.

The following were mixed:

| | |
|-------------------------------------|------------|
| NotI-dT primer (7 μ g/ μ l) | 2 μ l |
| PolyA RNA (20 μ g) | 18 μ l |

The obtained solution is heated to 65°C for 10min and 42°C for 1min.

Then, spin tube in microfuge and the following substances are preheated and added to the RNA/primer mix:

| | |
|---|------------|
| 2X GC-I buffer (Takara LA-PCR kit) | 75 μ l |
| RNaseIN inhibitor (Promega) | 1 μ l |
| 10mM dNTP (with methyl-dCTP instead of dCTP) | 4 μ l |
| Saturated trehalose in DEPC-H ₂ O | 10 μ l |
| 4.9M sorbitol in DEPC-H ₂ O | 26 μ l |
| Superscript II RT (200U/ μ l) | 15 μ l |

The obtained solution is incubated at 42°C for 40min, 50°C for 20 min and 55°C for 20min. 2 μ l of proteinase K (20 mg/ml) is then added to degrade the RT: the

obtained solution is incubated at 45°C for 15 min followed by phenol/chloroform extraction and isopropanol precipitation (do not use glycogen as subsequent selection depends on diol oxidation). The RNA/(-)DNA heteroduplex is resuspended into 44.5 µl of ddH₂O.

3µl of 1.1M NaOAc pH 4.5 and 2.5µl of 100mM NaIO₄ are added to oxidize the diol structures of the mRNA: 50µl of the reaction solution is incubated on ice in the dark for 45min followed by adding 0.5µl of 10% SDS, 11µl of 5M NaCl and 61µL of isopropanol to precipitate the RNA/(-)DNA. The precipitated RNA/DNA is resuspended in 50µl of ddH₂O.

5µL 1M NaOAc (pH6.1), 5µL 10% SDS and 150µL 10mM long-arm biotin hydrazide (freshly prepared) are added to biotinylate the RNA: the reaction is incubated at room temperature in the dark overnight.

The biotinylated RNA/(-)DNA is precipitated by adding 5µL 5M NaCl, 75µL 1M RNase-free NaOAc (pH6.1), and 750µL 100% EtOH or 200µL of 100% isopropanol: incubate at -80°C for 30min, and spin 14krpm at 4°C for 30min. The pellet is washed with 70% EtOH (in DEPC-treated H₂O) and 14krpm spin is carried out at 4°C for 10min. The pellet is air-dried and resuspended in 400µL DEPC-H₂O.

RNaseONE digestion to select for full-length first-strand cDNA:

50µL 10x RNaseONE buffer and 25units RNaseONE (Promega) per µg of starting mRNA are added. The obtained solution is incubated at 37°C for 30min. 10µL of 10mg/mL Yeast tRNA (Ambion) and 150µL of 5M NaCl are added to stop the reaction. The volume at this stage is approximately 600µL.

While biotinylating the RNA/(-)DNA heteroduplex, the streptavidin Dynabeads (Dyna) are prepared as follows: 400µL of M280 streptavidin beads are pipetted into an RNase-free Eppendorf tube, the beads placed on a magnet, left for at

least 30sec, then the supernatant is removed. The beads are re-suspended in 400µL 1x binding buffer (2M NaCl, 50 mM EDTA, pH 8.0). The tube is placed on a magnet, left for at least 30sec, then the supernatant is removed. The 1x binding buffer wash is repeated 2 more times. The beads are resuspended in 400µL 1x binding buffer with 100µg of Yeast tRNA, and then incubated at 4°C for 30min with occasional mixing, to block all non-specific binding sites. The tube is placed on a magnet, left for at least 30 seconds, and the supernatant is removed. The beads are washed with 1x binding buffer for 3 times, and the supernatant removed just before addition of the RNA/(-)DNA heteroduplex

The prepared beads and RNA/(-)DNA heteroduplex are mixed (the binding condition is approximately 1M NaCl). The mixture is rotated at room temperature for 30min to allow binding. The tube is placed on a magnet stand, left at least 30 seconds, and the supernatant removed (the supernatant was saved as “unbound”).

The beads are washed 2 times with 400µL of 1x binding buffer. This is followed by a wash with 400µL of 0.4%(w/v) SDS, plus 50µg/mL Yeast tRNA, and another wash with 400µL of 1x wash buffer (10mM Tris-HCl pH 7.5, 0.2mM EDTA, 10mM NaCl, 20%(v/v) glycerol, and 40µg/mL Yeast tRNA). Finally, they are washed with 400µL of 50µg/mL Yeast tRNA. For all washes the tube is placed on a magnet stand, left for at least 30 seconds, and the supernatant is removed.

The first strand cDNA is released by alkaline hydrolysis of RNA. After removing the supernatant, the following is added: 50µL alkaline hydrolysis mix (50mM NaOH and 5 mM EDTA pH8.0), and the tube is rotated at 65°C for 10min. The tube is then placed on a magnet stand, and the supernatant (now containing the eluted full-length first-strand cDNA) transferred to another tube containing 50µL 1M Tris-HCl pH7.5 for neutralization. The hydrolysis procedure is repeated 2 more times. The final volume of supernatant is approximately 300µL (containing the full-length first-strand cDNA (FL (-)cDNA)).

The cDNA is extracted by phenol/chloroform and precipitated using 1mL ethanol with glycogen, then resuspended in 10µL LoTE (LoTE is low salt Tris-EDTA buffer (3mM Tris-HCl pH 7.5 and 0.2mM EDTA pH7.5)). The resuspended FL (-) cDNA is then split into 2 aliquots "A" and "B" of 5µL each. The reason why 2 different adapters have to be used is that otherwise panhandle structures will form, and inhibit subsequent PCR.

Second strand cDNA synthesis

The following reagents were added on ice:

| | |
|------------------------------------|------|
| FL (-) cDNA aliquot A | 5µL |
| 1.6µg Adapter A(N5) | 4µL |
| 0.4µg Adapter A(N6) | 1µL |
| Soln II (Takara ligation kit ver2) | 10µL |
| Soln I (Takara ligation kit ver2) | 20µL |

And in a separate tube:

| | |
|------------------------------------|------|
| FL (-) cDNA aliquot B | 5µL |
| 1.6µg Adapter B(N5) | 4µL |
| 0.4µg Adapter B(N6) | 1µL |
| Soln II (Takara ligation kit ver2) | 10µL |
| Soln I (Takara ligation kit ver2) | 20µL |

From this step onwards, each aliquot A and B is processed identically and in parallel. The cDNA and linker-adapter mixture is incubated at 16°C overnight, and then the following are added to each of tubes A and B on ice:

| | |
|---------------------------|------|
| dH ₂ O | 20µL |
| 10XExTaq buffer (Takara) | 8µL |
| 2.5mM dNTP (Takara) | 8µL |
| ExTaq polymerase (Takara) | 4µL |

The mixture is incubated in a preheated thermocycler 65°C, 5min; 68°C, 30min; 72°C, 10min, followed by phenol/chloroform extraction and ethanol precipitation with glycogen, and resuspended in 40µL dH₂O.

Formation of 5'LongSAGE tags

The full-length double-stranded cDNA (FL ds cDNA) is to be immobilized at the 3'-end so that MmeI digestion can be used to release 5'LongSAGE tags. NotI digestion is used to expose a cohesive site at the 3'-end of the ds cDNA, to which a biotinylated adapter can be ligated.

The following are added on ice:

| | |
|-------------------------|------|
| FL ds cDNA | 40µL |
| NEB Buffer 3 | 5µL |
| NotI (10units/µl) (NEB) | 4µL |

The obtained solution is incubated at 37°C for 2 hours, then 65°C for 15min. To remove unwanted digestion products and buffer salts before adapter ligation, Qiaquick-spin PCR purification column (Qiagen) is used as per the manufacturer's protocols, and the DNA eluted in 40 µL of EB buffer.

The following are added in a microfuge tube:

| | |
|--|------------|
| Purified, 3' NotI-cohesive ended ds cDNA | 40 μ L |
| Adapter C (1 μ g/ μ L) | 10 μ L |
| 10x ligation buffer (Invitrogen) | 6 μ L |
| T4 DNA ligase (5units/ μ l) | 4 μ L |

The reaction is incubated at 16°C overnight, then 65°C for 15min. It is important at this stage to remove all unligated Adapter C, as this will otherwise compete for binding to the streptavidin beads. To do this, and simultaneously select for larger-sized ds cDNA, cDNA size fractionation columns (Invitrogen) or ChromaSpin 400 columns (Clontech) are used.

In the meantime, 400 μ L MyOne streptavidin Dynabeads in kilobase binding buffer (Dyna) are prepared as per the manufacturer's protocols.

The purified, size-fractionated ds cDNA is immobilized via the 3'-end to the prepared streptavidin Dynabeads by rotating for 3hrs at room temperature. The bound cDNA is then equilibrated in 1x NEB Buffer 4 by washing 4x with 500 μ L buffer, then resuspended in 100 μ L 1x NEB Buffer 4 (with 40 μ M SAM). Two μ L (8units) of Mmel was added, and the reaction incubated at 37°C for 1hrs with occasional agitation. This Mmel digestion releases 5' terminal LongSAGE tags from A and B into solution, so the supernatant is recovered, The beads is washed with 100 μ l of ddH₂O and the supernatant is pooled and phenol/chloroform extracted and ethanol precipitated in the presence of glycogen and ammonium acetate. The DNA is resuspended in 5 μ L LoTE. To avoid denaturation, the tags should be kept on ice.

Formation of 5'LongSAGE ditags and scaling-up using PCR

| | |
|---------------------------------|-----------|
| 5'LongSAGE tags from A | 5 μ L |
| 5'LongSAGE tags from B | 5 μ L |
| 10x T4 DNA ligase buffer | 2 μ L |
| T4 DNA ligase (5units/ μ L) | 2 μ L |
| dH ₂ O | 6 μ L |

The ligation reaction is incubated at 16°C overnight; no heat inactivation.

To optimize the conditions needed to retrieve the ligated ditags using PCR, serial dilutions of the ligation reaction from 1:10 to 1:80 are made, and PCR reactions set-up including no-ligase and no DNA controls. Biotinylated PCR primers are used to facilitate subsequent purification of amplicons.

| | |
|------------------------------|-------------|
| Template | 1 μ L |
| Primer P1 (10 μ M stock) | 1 μ L |
| Primer P2 (10 μ M stock) | 1 μ L |
| 25 mM MgCl ₂ | 1.5 μ l |
| 10x PCR buffer (Invitrogen) | 5 μ L |
| 10mM dNTP | 1 μ L |
| Platinum Taq | 0.5 μ L |
| dH ₂ O | 39 μ L |

Thermocycling conditions:

| | |
|---|-------------|
| Step 1 | 94°C, 2min |
| Step 2 | 94°C, 30sec |
| Step 3 | 55°C, 1min |
| Step 4 | 72°C, 1min |
| Repeat steps 3-4, 30-40 cycles (to be determined empirically) | |
| Step 5 | 72°C, 8min |

To generate concatemers, the following is added to a 1.7ml microfuge tube:

| | |
|---------------------------------|---------------|
| 50bp cohesive ditag DNA | 100-400ng |
| 10x T4 DNA ligase buffer | 2 μ L |
| T4 DNA ligase (5units/ μ L) | 2 μ L |
| dH ₂ O | to 20 μ L |

The reaction is incubated at 16°C for 1-3hr.

Note: the incubation time has to be optimized, to ensure a smear from approximately 300 – 2000bp.

4 μ L of 6x loading buffer is then added and the entire sample heated at 65°C for 15min. The sample is then loaded in a single well of an 8% TBE-PAGE minigel and run at 200V for about 1 hr, or until Bromophenol Blue was about 2 cm from bottom.

The smear of ligation products is excised as 2 or more fractions, eg. 500-750bp; 750-1000bp; >1000bp.

Elution of DNA from the gel pieces is performed as described earlier. The eluate is extracted with phenol/chloroform, then ethanol precipitated. Resuspend the DNA pellet in 6 μ l LoTE.

Cloning of concatemers

The cloning vector is prepared by digesting 2 μ g of pZER0-1 plasmid DNA (Invitrogen) with 10units of BamHI for 3hrs at 37°C. The digested DNA is phenol/chloroform extracted and ethanol precipitated, then resuspended in LoTE at a concentration of approximately 50ng/ μ l.

The ligation reaction is performed as follows:

| | |
|-----------------------------|-----------|
| Concatemer DNA | 6 μ L |
| BamHI/pZErO-1 | 1 μ L |
| 10x ligase buffer | 1 μ L |
| T4 DNA ligase (5U/ μ L) | 1 μ L |
| dH ₂ O | 1 μ L |

Incubate at 16°C overnight, with vector self-ligation performed in parallel as a control.

The ligation products are purified before electroporation by phenol/chloroform extraction followed by ethanol precipitation; the pellet is washed 3 times with 75% ethanol before resuspending in 20 μ l LoTE. 2 μ l of this DNA is used to transform 50 μ l of electrocompetent TOP10 bacterial cells (Invitrogen). After recovery in 1mL LB media, 50 μ l is plated on a small (100mm) agar plate (containing Low Salt LB agar (Lennox L) plus Zeocin (25-50 μ g/ml) and incubated overnight at 37°C. As a background control, bacteria are plated out that have been similarly transformed with the vector self-ligation reaction above. The background is usually between 1-5%. (Note: IPTG is optional when using TOP10 cells but may reduce background).

5'LongSAGE library QC (Quality Check)

The following day, several (we usually pick 24-48 colonies) are picked to check for insert size by PCR. For each reaction, a single colony was picked into a PCR tube containing:

| | |
|-----------------------------------|--------------|
| 10x HiFi buffer | 2 μ L |
| 10mM dNTP | 0.4 μ L |
| PMR011 (10 μ M) | 1 μ L |
| PMR012 (10 μ M) | 1 μ L |
| Eppendorf TripleMaster polymerase | 0.2 μ L |
| dH ₂ O | 11.4 μ L |

Thermocycling conditions:

- Step1: 95°C x 2min
- Step 2: 95°C x 30sec
- Step 3: 55°C x 1min
- Step 4: 72°C x 3min
- Repeat steps (2-4), 24x
- Step 5: 72°C x 8min
- Hold at 16°C forever

The PCR products are visualized on a 1% agarose gel.

Note: the primer pair PMR011/PMR012 gives a band of approximately 300bp in the absence of any cloned insert. If the quality of the library thus produced appears good, the remaining transformation mixture can be plated out on large agar plates (we use 20cm x 20cm Q-trays, Genetix) in preparation for DNA sequencing analysis.

3'LongSAGE

First strand cDNA synthesis

Total RNA is prepared using Trizol (Invitrogen), as per the manufacturer's protocols. mRNA (polyA RNA) is then purified from this total RNA using a magnetic-bead based system (μ MACS mRNA Isolation Kit for Total RNA, #130-075-102, Miltenyi Biotec). Typical yields are 20-50 ug mRNA from 1mg total RNA. We have experimented with mRNA purification kits from several different suppliers and have found this one to be an ideal compromise between time and quality.

In this step, the first cDNA is synthesized with a Gsul-oligo dT₁₆ primer. Then, the first strand cDNA/RNA hybrids are done by Superscript II RT.

The following were mixed:

| | |
|---|-----------|
| Gsul-dT ₁₆ primer (0.5 μ g/ μ l) | 1 μ l |
| PolyA RNA (5 μ g) | 7 μ l |

The obtained solution is heated to 65°C for 10min and put on ice.

Then, spin tube in microfuge and the following substances are added to the RNA/primer mix:

| | |
|--|-----------|
| 5X 1 st strand cDNA synthesis buffer (Invitrogen) | 4 μ l |
| 0.1M DTT | 2 μ l |
| 10mM dNTP (with methyl-dCTP instead of dCTP) | 1 μ l |
| Superscript II RT (200U/ μ l) | 5 μ l |

The obtained solution is incubated at 37°C for 1 hr then put on ice. The following reagents are added to carry out second strand synthesis.

Second strand cDNA synthesis

The following reagents were added on ice:

| | |
|---|-------|
| DEPC ddH ₂ O | 91 μL |
| 5X second strand buffer | 30 μL |
| 10 mM dNTP | 3 μL |
| <i>E. coli</i> DNA ligase (10 units/μl) | 1 μL |
| <i>E. coli</i> DNA polymerase (10 units/μl) | 4 μL |
| <i>E. coli</i> RNaseH (2 units/μl) | 1 μL |

Final reaction volume is 150 μL. Incubate at 16°C for 2 hours followed by added 2 μl of T4 DNA polymerase (10 units) and leave at 16°C for 5 more minutes.

Place the reaction on ice and add 10 μl of EDTA (0.5 M). The ds cDNA is phenol chloroform extraction and ethanol ppt. The pellet is resuspended in 25 μl of water and ligated with biotinylated Sall adaptor at 16°C for overnight.

Biotinylated Sall adaptor is used to ligate at the 5'-end of the ds cDNA. The double-stranded cDNA can be immobilized at the 5'-end so that Mmel digestion can be used to release 3'LongSAGE tags.

| | |
|-------------------------------------|-------|
| ds cDNA | 25 μL |
| Biotinylated Sall adaptor (1 μg/μl) | 10 μl |
| 5x Ligase buffer | 10 μl |
| T4 DNA ligase | 5 μl |

To remove excess adaptors, Qiaquick-spin PCR purification column (Qiagen) is used as per the manufacturer's protocols, and the DNA eluted in 80 μL EB buffer. The DNA is digested with Gsul to expose the AA dinucleotides for adaptor A and B ligation.

| | |
|--------------------|-------|
| cDNA | 80 μL |
| Gsul | 8 μL |
| 10x Buffer B | 10 μL |
| ddH ₂ O | 2 μL |

Total volume: 100 μL at 30°C for 2 hours.

The cDNA is purified by Qiaquick column to remove the enzyme, salt and then resuspended in 50µL LoTE (LoTE is low salt Tris-EDTA buffer (3mM Tris-HCl pH 7.5 and 0.2mM EDTA pH7.5)). The resuspended cDNA is then split into 2 aliquots "A" and "B" of 25µL each. The reason why 2 different adapters have to be used is that otherwise panhandle structures will form, and inhibit subsequent PCR. From this step onwards, each aliquot A and B is processed identically and in parallel.

The following reagents were added on ice:

| | |
|----------------------------|------|
| cDNA aliquot A | 25µL |
| A linker adaptor (1 µg/µl) | 10µL |
| 5x ligation buffer | 10µL |
| T4 DNA ligase | 5µL |

And in a separate tube:

| | |
|----------------------------|------|
| cDNA aliquot B | 25µL |
| B linker adaptor (1 µg/µl) | 10µL |
| 5x ligation buffer | 10µL |
| T4 DNA ligase | 5µL |

Ligation is done at 16°C for overnight. The next day, dilute 1:1 with 2 X kilobase binding buffer

In the meantime, 400µL MyOne streptavidin Dynabeads in kilobase binding buffer (Dyna) are prepared as per the manufacturer's protocols.

The linker adaptor ligated ds cDNA is immobilized via the 5'-end to the prepared streptavidin Dynabeads by rotating for 3hrs at room temperature. The bound cDNA is then equilibrated in 1x NEB Buffer 4 by washing 4x with 500µL buffer, then resuspended in 100µL 1x NEB Buffer 4 (40µM SAM). Two µL (8units) of Mmel was added, and the reaction incubated at 37°C for 1hrs with occasional agitation. This Mmel digestion releases 3' terminal LongSAGE tags from A and B

into solution, so the supernatant is recovered, The beads is washed with 100 μ l of ddH₂O and the supernatant is pooled and phenol/chloroform extracted and ethanol precipitated in the presence of glycogen and ammonium acetate. The DNA is resuspended in 5 μ L LoTE. To avoid denaturation, the tags should be kept on ice.

Formation of 3'LongSAGE ditags and scaling-up using PCR

| | |
|---------------------------------|-----------|
| 3'LongSAGE tags from A | 5 μ L |
| 3'LongSAGE tags from B | 5 μ L |
| 10x T4 DNA ligase buffer | 2 μ L |
| T4 DNA ligase (5units/ μ L) | 2 μ L |
| dH ₂ O | 6 μ L |

The ligation reaction is incubated at 16°C for overnight; no heat inactivation.

To optimize the conditions needed to retrieve the ligated ditags using PCR, serial dilutions of the ligation reaction from 1:10 to 1:80 are made, and PCR reactions set-up including no-ligase and no DNA controls. Biotinylated PCR primers are used to facilitate subsequent purification of amplicons.

| | |
|------------------------------|-------------|
| Template | 1 μ L |
| Primer P1 (10 μ M stock) | 1 μ L |
| Primer P2 (10 μ M stock) | 1 μ L |
| 25 mM MgCl ₂ | 1.5 μ l |
| 10x PCR buffer (Invitrogen) | 5 μ L |
| 10mM dNTP | 1 μ L |
| Platinum Taq | 0.5 μ L |
| dH ₂ O | 39 μ L |

Thermocycling conditions:

| | |
|--------|------------|
| Step 1 | 94°C, 2min |
|--------|------------|

| | |
|---|-------------|
| Step 2 | 94°C, 30sec |
| Step 3 | 55°C, 1min |
| Step 4 | 72°C, 1min |
| Repeat steps 3-4, 30-40 cycles (to be determined empirically) | |
| Step 5 | 72°C, 8min |
| Hold at 16°C | |

The PCR products are electrophoresed on 2% agarose, and the conditions that produce the most specific result are selected for large-scale amplification of the 3'LongSAGE ditags, usually 4 x 96well reactions. After PCR, the 4 x96 reactions are pooled, phenol/chloroform extracted and ethanol precipitated, then resuspended in 200µL LoTE buffer.

The required amplicons corresponding to the 120bp ditags are gel-purified by electrophoresis on a large 15cm x 17cm 12% TBE-PAGE gel, and the 120bp ditag band excised after staining in SYBRGreen I (Molecular Probes). Note: to prevent overloading, we do not load more than 5 µg DNA per lane. To prevent DNA damage, we have found it convenient to use a blue light transilluminator (DarkReader, Clare Chemical Research) instead of a standard ultraviolet one.

An easy way to elute the excised DNA is to place each gel slice into a 600µL microfuge tube, the bottom of which has been pierced with a 21G needle. Each tube is then placed into a larger 1.7mL tube, and the tubes centrifuged at 14krpm for 10min at 4°C. This effectively homogenizes the gel slices, which are collected in the bottom of each 1.7mL tube. For elution, 200µL LoTE buffer was added to each sample, which is then incubated at 65°C for 2hrs. Eluted DNA is separated from gel residue using spin-filter units (SpinX, Corning or Mermaid, Qbiogene). The collected, purified DNA is ethanol precipitated and resuspended in 100µL LoTE.

30min at room temperature, the supernatant is removed, ethanol precipitated, and resuspended in 40 μ L LoTE.

To ensure complete removal of all biotinylated termini (that would otherwise inhibit subsequent concatenation), the purified 50bp cohesive ditag DNA above is gel-purified using a large 12% TBE-PAGE gel. The 50bp band is excised and purified as mentioned previously.

To generate concatemers, the following is added to a 1.7ml microfuge tube:

| | |
|---------------------------------|---------------|
| 50bp cohesive ditag DNA | 100-400ng |
| 10x T4 DNA ligase buffer | 2 μ L |
| T4 DNA ligase (5units/ μ L) | 2 μ L |
| dH ₂ O | to 20 μ L |

The reaction is incubated at 16°C for 1-3hr.

Note: the incubation time has to be optimized, to ensure a smear from approximately 300 – 2000bp.

4 μ L of 6x loading buffer is then added and the entire sample heated at 65°C for 15min. The sample is then loaded in a single well of an 8% TBE-PAGE minigel and run at 200V for about 1 hr, or until Bromophenol Blue was about 2 cm from bottom.

The smear of ligation products is excised as 2 or more fractions, eg. 500-750bp; 750-1000bp; >1000bp.

Elution of DNA from the gel pieces is performed as described earlier. The eluate is extracted with phenol/chloroform, then ethanol precipitated. Resuspend the DNA pellet in 6 μ L LoTE.

Cloning of concatemers

The cloning vector is prepared by digesting 2µg of pZErO-1 plasmid DNA (Invitrogen) with 10units of BamHI for 3hrs at 37°C. The digested DNA is phenol/chloroform extracted and ethanol precipitated, then resuspended in LoTE at a concentration of approximately 50ng/µl.

The ligation reaction is performed as follows:

| | |
|-----------------------|-----|
| Concatemer DNA | 6µL |
| BamHI/pZErO-1 | 1µL |
| 10x ligase buffer | 1µL |
| T4 DNA ligase (5U/µL) | 1µL |
| dH2O | 1µL |

Incubate at 16°C overnight, with vector self-ligation performed in parallel as a control.

The ligation products are purified before electroporation by phenol/chloroform extraction followed by ethanol precipitation; the pellet is washed 3 times with 75% ethanol before resuspending in 20µl LoTE. 2µl of this DNA is used to transform 50µl of electrocompetent TOP10 bacterial cells (Invitrogen). After recovery in 1mL LB media, 50µl is plated on a small (100mm) agar plate (containing Low Salt LB agar (Lennox L) plus Zeocin (25-50µg/ml) and incubated overnight at 37°C. As a background control, bacteria are plated out that have been similarly transformed with the vector self-ligation reaction above. The background is usually between 1-5%. (Note: IPTG is optional when using TOP10 cells but may reduce background).

3'LongSAGE library QC (Quality Check)

The following day, several (we usually pick 24-48 colonies) are picked to check for insert size by PCR. For each reaction, a single colony was picked into a PCR tube containing:

| | |
|-----------------------------------|--------------|
| 10x HiFi buffer | 2 μ L |
| 10mM dNTP | 0.4 μ L |
| PMR011 (10 μ M) | 1 μ L |
| PMR012 (10 μ M) | 1 μ L |
| Eppendorf TripleMaster polymerase | 0.2 μ L |
| dH ₂ O | 11.4 μ L |

Thermocycling conditions:

- Step1: 95°C x 2min
- Step 2: 95°C x 30sec
- Step 3: 55°C x 1min
- Step 4: 72°C x 3min
- Repeat steps (2-4), 24x
- Step 5: 72°C x 8min
- Hold at 16°C forever

The PCR products are visualized on a 1% agarose gel.

Note: the primer pair PMR011/PMR012 gives a band of approximately 300bp in the absence of any cloned insert. If the quality of the library thus produced appears good, the remaining transformation mixture can be plated out on large agar plates (we use 20cm x 20cm Q-trays, Genetix) in preparation for DNA sequencing analysis.

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References

1. Carninci, P. & Hayashizaki, Y. (1999) *Methods Enzymol* 303, 19-44.
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2. Shiraki, T., Kondo, S., Katayama, S., Waki, K., Kasukawa, T., Kawaji, H., Kodzius, R., Watahiki, A., Nakamura, M., Arakawa, T., et al. (2003) *Proc Natl Acad Sci U S A* 100, 15776-15781.