

DNA Preparation and Linker Amplification for Pyrosequencing – June 7, 2010

(Adapted from the Broad's LASL Sanger Seq'g Protocol, and optimized for low template work)
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Ultrasonic shearing of DNA

Samples in 1x TE, pH7.5, <3µg in 120µl. Send to Covaris for shearing, turn around time about 1 week. Shear to 300-800 bp size. Service information at <http://www.covarisinc.com/services.htm>.

Concentrate sheared DNA

Centrifugal concentrators, Amicon Ultra-0.5 or Nanosep, with a 30kDa cut-off are used to concentrate the sample. Dispense up to 500µl into reservoir and centrifuge 14,000 g for 10 min 15°C (smaller starting volumes require less time). The concentrators should retain 10µl but sometimes look dry – can add back 10-15µl TE or flow through to membrane and rinse up and down. Invert unit and place into fresh tube. Centrifuge 1000 g for 3 min to elute concentrated sample. Should have 15-20µl.

End Repair DNA

Use End-Repair Kit (Epicentre Biotechnologies Cat. #ER0720 or ER81050, containing 20µl or 50µl enzyme, respectively). For each DNA sample prepare:

Sheared DNA	≤ 35µl	
10x End Repair Buffer	7.5µl	
2.5mM dNTP mix	7.5µl	
10mM ATP	7.5µl	
nuclease-free H ₂ O	to 72µl	
End-repair enzyme mix	3µl	(can perform 6 reactions per ER0720 kit)

Cycle 1:	4°C	2min
Cycle 2:	25°C	45min
Cycle 3:	4°C	Hold

When reaction is complete immediately purify the DNA using MinElute Reaction Clean Up kit protocol (Qiagen #28204) and elute samples with 33µl EB buffer.

MinElute Reaction Clean up using centrifuge

Label Min-Elute columns in collection vessel. Warm EB buffer to 55°C in heat block. Mix end-repaired DNA with 300µl ERC buffer. Apply to column. Centrifuge 16,100 g for 1min. Discard flow thru. Wash column with 750µl PE buffer (ethanol added); wait 3 min then centrifuge 16,100 g for 1 min. Discard flow thru and spin column to remove residual buffer for 2.5min at 16,100 g. Transfer column to fresh, labeled 1.5ml centrifuge tube. Elute sample using 33µl warm (55°C) EB buffer applied directly onto membrane. Wait 2 min. Centrifuge 1min at 16,100 g to collect DNA (caps open, so point caps inward toward rotor spindle and use cover if provided with centrifuge). Discard column. Note: EB buffer is 10mM TrisCl, pH8.5.

Anneal Adaptors (Linker A)

Purchase 100nm amount of forward and reverse linker A with forward 5' phosphorylated.

Linker A forward: 5'-phosphorylated-GTA TGC TTC GTG ATC TGT GTG GGT GT-3'

Linker A reverse: 5'-CCA CAC AGA TCA CGA AGC ATA C-3'

Resuspend each linker to 1.14mM concentration in 1xTE plus 50 mM NaCl. Mix in equal volumes. If your cycler has step-down function, cycle 93°C, 2min, ramp down to 4°C at 0.1°C/sec. Otherwise, use a boiling water bath: boil the mixture for 2 min in a 250-500ml beaker, then turn off the heat and allow the water bath to cool slowly to room temperature. Then place the annealed sample at 4°C or on ice for 5min. This stock annealed linker is now 570µM concentration for each linker.

Dilute stock annealed Adapter A to 10µM in nuclease-free water: dispense 5µl stock into 280µl water and mix.

Use Fast-Link Ligation kit (Epicentre Biotechnologies #LK0750H or LK6201H which contain 50µl or 100µl ligase, respectively).

End-repaired sheared DNA	30µl	
Adapter A (10pmol/µl=10 µM)	7.5µl	
10x Fast-Link Ligation buffer	5.0µl	
10mM ATP	2.5µl	
Fast-Link DNA Ligase (2U/µl)	<u>5.0µl</u>	(can perform 10 reactions per LK0750 kit)
Total Volume (µl)	50.0µl	

Cycle 1:	4°C	2min
Cycle2:	23.5°C	2hr
Cycle3:	4°C	Hold

Immediately remove sample from thermal cycler and add to 300µl ERC buffer (Qiagen kit 28204).

MinElute Reaction Clean up using centrifuge

Label Min-Elute columns in collection vessel. Warm 1x TE pH7.5 buffer to 55°C in heat block. Mix end-repaired DNA with 300µl ERC buffer. Apply to column. Centrifuge 16,100 g for 1min. Discard flow thru. Wash column with 750µl PE buffer (ethanol added); wait 3 min then centrifuge 16,100 g for 1 min. Discard flow thru and spin column to remove residual buffer for 2.5min at 16,100 g. Transfer column to fresh, labeled 1.5ml centrifuge tube. Elute sample using 20µl warm (55°C) 1x TE pH7.5 applied directly onto membrane. Wait 2 min. Centrifuge 1min at 16,100 g to collect DNA (caps open, so point caps inward toward rotor spindle and use cover if provided with centrifuge). Discard column. Note: final volume will be ~19µl.

Size Fractionation

Prepare 1% SeaKem GTG agarose in 1x TAE buffer. For some samples, will only want to electrophorese one sample per gel, leaving 2-4 lanes in between ladders on either end and the sample. For other samples, can separate each sample by 2-3 lanes from other samples, being sure not to overload any of the samples. Gel trays, combs and unit should be treated with 10% bleach for 10min and rinsed prior to use.

In sterile bottle dispense enough agarose for the size gel tray you are using. Add appropriate volume of autoclaved 1x TAE buffer for 1% gel. Tare the bottle on the balance. Cover loosely and melt agarose in microwave until all particles are fully dissolved. Un-cap and place bottle on

tared balance. Add additional 1x TAE or R/O water to bring volume back to starting volume. Allow to cool 10-15min and then slowly pour into gel tray with combs in place. Once solidified, can load samples.

For lower MW DNA, use 100bp or 250bp DNA ladder in 6x Blue Orange Gel Loading Dye (Promega #G1881). This loading dye has a yellow marker dye that runs just in front of the 300bp fragments, making it easier to cut out 300-800bp sized DNA.

Mix 4µl 6x GLB to each DNA sample and load into 1-2 wells of the gel. Load the DNA ladder in the first and last well of the gel for sizing later. Run gel at 80V for 2hr.

Place gel tray on sheet of white paper with straight horizontal line drawn the length of the gel. Use this line to cut out the DNA marker lanes with a clean scalpel blade. Mark upper right and lower left edges of marker lane to help with orientation later/ Place the marker gel lanes into a stain solution (ethidium bromide, 5µl per 100ml 1x TAE, or SYBR green I gel stain, 30µl in 150ml ddH₂O). Stain for 10min. Cover remaining gel with Saran Wrap to keep from drying out and place at 4°C.

Observe stained marker gel pieces under UV transillumination and determine migration distance of 300-800bp markers with cm fluorescent ruler; use this distance to measure cutting distance of samples in the unstained gel that is placed on plastic wrap or UV-transparent gel carrier sheet. Use a fresh scalpel blade for excising each sample. Alternatively, can place the stained marker gel pieces back into the gel carrier and carefully slip the entire gel onto the UV-transparent sheet cut so that it will fit back into gel tray. Photograph the entire gel with marker lanes. Measure the total gel size in cm. In Adobe Photoshop, crop gel photo to include just the gel. Print with preview in landscape mode, negative, adjusting size of photo to exact size of gel. Can print a 20x20cm gel on a single sheet of 8 ½" x 11" paper. Draw a very straight line on the paper between the 300bp and the 800bp markers. Put the gel on the transparent sheet back into the carrier and place on top of the printed gel image so that the sides are completely aligned. Using a fresh scalpel blade for each sample, cut out the 300-800bp gel pieces (use the lower yellow dye to orient sample lane – it should be just below the 300bp line). Place gel slices into 2ml centrifuge tubes).

MinElute Gel Extraction

Elute DNA from gel slices using MinElute Gel Extraction kit (Qiagen #28604). Tare balance to empty 2ml tube. Weigh each sample and record (weight=volume). Add 3 volumes QG buffer to each sample. Warm at 55°C 10min until gel is completely dissolved. Add 1 volume isopropanol to each sample. Load 750µl per column and centrifuge 16,100 g, 1.5min. Discard flow thru and repeat until all of sample is loaded onto the column. Wash with 0.5ml QG buffer 16,100 g, 1.5min. Add 750µl buffer PE (to which ethanol has been added). Wait 3min, then centrifuge 2min at 16,100 g. Discard flow-thru and spin for 2.5min 16,100 g to remove residual wash buffers. Transfer column to fresh, labeled 1.5ml centrifuge tube. Elute sample using 35µl warm (55°C) EB buffer applied directly onto membrane. Wait 2 min. Centrifuge 1min at 16,100 g to collect DNA (caps open, so point caps inward toward rotor spindle and use cover if provided with centrifuge). Discard column.

Small-scale PCR Titration

Prepare PCR A primer: 5'-phosphorylated-CCA CAC AGA TCA CGA AGC ATA C-3'. This primer can contain a 5-10bp bar code at the 5' end using rules that there are no runs of nucleotides and the nucleotide at the 3' end of the bar code is not a C. Only one primer is needed as it primes off both the forward and reverse strand of the linker-ligated sheared DNA. Prepare 100 μ M stock of the primer in nuclease-free water. Dilute to 10 μ M in nuclease-free water for working dilution. Use LA Takara HS (Clontech #RR042 or RR042A) for high-fidelity PCR.

For each sample, prepare a master mix. For each 25 μ l {or 5x25 μ l} reactions:

Nuclease-free water	16.75 μ l {83.75 μ l }
LA Takara HS 10x Buffer	2.5 μ l {12.5 μ l }
2.5mM dNTP mix	4.0 μ l {30 μ l }
10 μ M PCR A primer	0.5 μ l {2.5 μ l }
5U/ μ l LA Takara HS	0.25 μ l {1.25 μ l }
Linker-ligated sheared DNA	<u>1.0μl {5.0 μl}</u> (may want to test several dilutions)
Total	25 μ l {125 μ l }

Dispense into 0.2ml thin-walled PCR tubes.

Cycle 1:	95°C	2min	
Cycle 2:	95°C	30sec	Set cycles to pause after 18, 22, 25, 28 & 30 cycles; remove one sample after each pause with last sample going through final 10min extension step.
	60°C	1min	
	72°C	1.5min	
Cycle3:	72°C	10min	

To determine optimal number of cycles (and dilution), mix 5 μ l of sample with 1 μ l 6x Blue Orange gel loading dye and examine in 1.2% agarose gel in 1xTAE. Look for the minimum number of cycles (and dilution) yielding maximum amount of desired size fragments, without too much lower MW products.

Large Scale PCR

For each sample, prepare a master mix. For each 25 μ l reaction:

Nuclease-free water	16.75 μ l
LA Takara HS 10x Buffer	2.5 μ l
2.5mM dNTP mix	4.0 μ l
10 μ M PCR A primer	0.5 μ l
5U/ μ l LA Takara HS	0.25 μ l
Linker-ligated sheared DNA	<u>1.0μl</u>
Total	25 μ l

Dispense into 0.2ml thin-walled PCR tubes.

Cycle as above with cycles set to determined optimum number followed by 10min extension step. Pool all replicate reactions and run 5 μ l in 1.2% agarose gel in 1x TAE to determine quality and yield.

Reconditioning PCR

For each large scale PCR reaction, prepare enough master mix for 10x reaction. Assuming 20 μ l large scale, prepare enough master mix for 200 μ l:

Nuclease-free water	15.5 μ l {155 μ l }
LA Takara HS 10x Buffer	2.5 μ l {25 μ l }
2.5mM dNTP mix	4.0 μ l {40 μ l }
10 μ M PCR A primer	0.5 μ l {5.0 μ l }
5U/ μ l LA Takara HS	0.25 μ l {2.5 μ l }
Large scale DNA product	<u>2.0μl {20μl }</u>
Total	25 μ l {200 μ l }

Dispense into 0.2ml thin-walled PCR tubes.

Cycle 1:	95°C	2min	
Cycle 2:	95°C	30sec	
	60°C	1min	3 Cycles (yes, only 3 cycles)
	72°C	1.5min	
Cycle3:	72°C	10min	

Pool all replicate reactions and run 5 μ l in 1.2% agarose gel in 1x TAE to determine quality and approximate yield.

PCR Clean-up

Use either MinElute PCR Clean-up kit (Qiagen #) or QiaQuick PCR Clean-up kit (Qiagen #28104). The MinElute columns hold a maximum of 5 μ g DNA and minimum elution volume of 10 μ l whereas QiaQuick columns hold a maximum of 10 μ g and minimum elution volume of 30 μ l.

Add 5 volumes of buffer PB to each pooled sample. Be sure to have pH indicator in the buffer as Takara PCR seems to be a bit alkaline and you may have to adjust pH with 3M sodium acetate pH5 as indicated in Qiagen protocol. Apply to column and centrifuge 16,100 g, 1min. Discard flow-through (can run it back over column one time and may want to save until final analysis is complete). Add 750 μ l buffer PE (with ethanol added), wait 3min, then centrifuge 1.5min at 16,100 g. Spin again to dry, 16,100 g for 2min. Add 10-40 μ l warm (55°C) buffer EB to each column (smaller amounts if using MinElute column), wait 2min, then spin 1min at 16,100 g. If you choose, you can elute a second time (can recover an additional 10%) using 10-30 μ l warm EB. Do not pool second elution with first until quantification.

Final Analysis

Quantify DNA using Quant-it PicoGreen dsDNA kit (Invitrogen). Run 1-2 μ l in 1.2% agarose gel in 1x TAE to determine quality. If desired, analyze sample using Agilent DNA 7500 kit and Agilent 2100 bioanalyzer to determine size range of PCR products.

NOTES:

Amicon Ultra-0.5-30K (Millipore UFC503024) or Nanosep-30K (OD030C33), 1 per sample (Note: Microcon YM concentrators have been discontinued by the manufacturer).

1x TE, pH 7.5

End-It DNA End Repair Kit (3ul enzyme per reaction) Epicentre #ER0720 (contains 20ul enzyme; available through Fisher as NC9807549) or ER81050 (contains 50ul enzyme)

Nuclease-free Water Promega #P1193 (available through Fisher as PRP1193))

Minelute Reaction Clean-up kit (2 columns per sample) Qiagen 28204

Minelute Gel Extraction Kit (1-2 columns per sample) Qiagen 28604

QiaQuick PCR Purification Kit (1 column per sample) Qiagen 28104

Fast Link DNA Ligation Kit (5ul enzyme per sample) Epicentre #LK0750H (contains 50ul enzyme; available through Fisher as NC9804176) or LK6201H (contains 100ul enzyme; available through Fisher as NC9527092)

SeaKem GTG Agarose Cambrex #50074 or from Fisher #138319 (which is Lonza #50071)

Blue Orange 6X Loading Dye Promega #G1881 (this is a great loading dye as the yellow marker migrates just ahead of the 300bp size; available through Fisher as PRG1881)

DNA ladder, but we found the 250bp or 100bp ladder to be better for sizing the fractions: 1Kbp Invitrogen #15615-06, 250 bp Invitrogen #10596-013, 100bp Invitrogen #15628-019

Takara LS Taq HS, sold by Fisher for Clontech as RR042 (25µl) and RR042A (4x25µl). Also order additional dNTP mix as you will run out at these reaction conditions, Clontech #TAK4030

Quant-it PicoGreen dsDNA kit, Invitrogen #P7589

Broad also recommends using Sybr Green Gel Stain (Invitrogen 40412) but ethidium bromide was sensitive enough for our samples

E Gels as suggested with Sybr Safe (not very sensitive; regular gel worked fine) - first time order needs starter kit which has the electrical hookup (not available alone) Starter kit Invitrogen G620601; 1.2% gels only with Sybr Safe G521801. These E-gels also come with EtBr stain.

In addition to all of this you also need to order the linker and the primer:

Linker A forward: 5'-phosphorylated -GTA TGC TTC GTG ATC TGT GTG GGT GT-3'

Linker A reverse: 5'-CCA CAC AGA TCA CGA AGC ATA C-3'

PCR-A Primer: 5'-phosphorylated -[5bp bar code] CCA CAC AGA TCA CGA AGC ATA C-3'

Order 100nmol amount of each oligo. For the Linker A annealing, the lyophilized material was diluted to 1.14mM in 1xTE plus 50mM NaCl. Equal amounts of the forward and reverse oligo were mixed, boiled it for 2 min and removed the beaker from the heat but the tube was left in the water until it cooled down to room temperature then placed at 4C. This stock should be 570uM concentration which is then diluted to 10uM for the working dilution.

The PCR-A primer was diluted to 100uM in nuclease-free water and the working stock was diluted to 10uM in nuclease-free water. You only need one primer since it primes off of both strands.

Based on first 454 sequencing results, we have decided to use DNase treatment of the resuspended iron chloride precipitated viral preparation, followed by CsCl gradient purification and subsequent Wizard Prep DNA extraction prior to shearing.

The new additions to version 3 are use of LS Takara HS enzyme system, testing both # PCR cycles and template dilution, and use of the “reconditioning step” to obtain final DNA for sequencing. Reasons:

- a. Higher product yield with LS Takara HS than with PFU Turbo. Takara Taq has proof-reading ability. Our protocol uses higher amount of dNTP and reducing that has not yet been tested. We have been able to amplify <100fg DNA/25ul reaction with this enzyme.**
- b. Experiments comparing Takara and PFU demonstrated 2 things: less template in the reaction tends to yield more higher MW products so diluting templates (eg, 1:10, 1:100) when testing optimal number of cycles can improve percent of higher MW products and reduce amount of linkered DNA need to get enough product for sequencing; and as cycle number increases, the MW of the products tends to decrease so we test routinely from 20-30 cycles and no more unless template simply will not amplify with fewer cycles. We chose both dilution of template and # of PCR cycles for final PCR based on titration results.**
- c. Reconditioning step is taken from J.R. Thompson et al, 2002 (Nucleic Acid Res. 30(9):2083-2088; <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC113844/>): this step not only increases product yield significantly but should also reduce heteroduplex formation that occurs with mixed template samples.**

Version 4 has been modified to account for discontinued Microcon YM-100 centrifugal concentrators. The alternative products, Amico Ultra and Nanosep, both recommend a 30kDa cut-off for DNA concentration, therefore, they are included in this version.