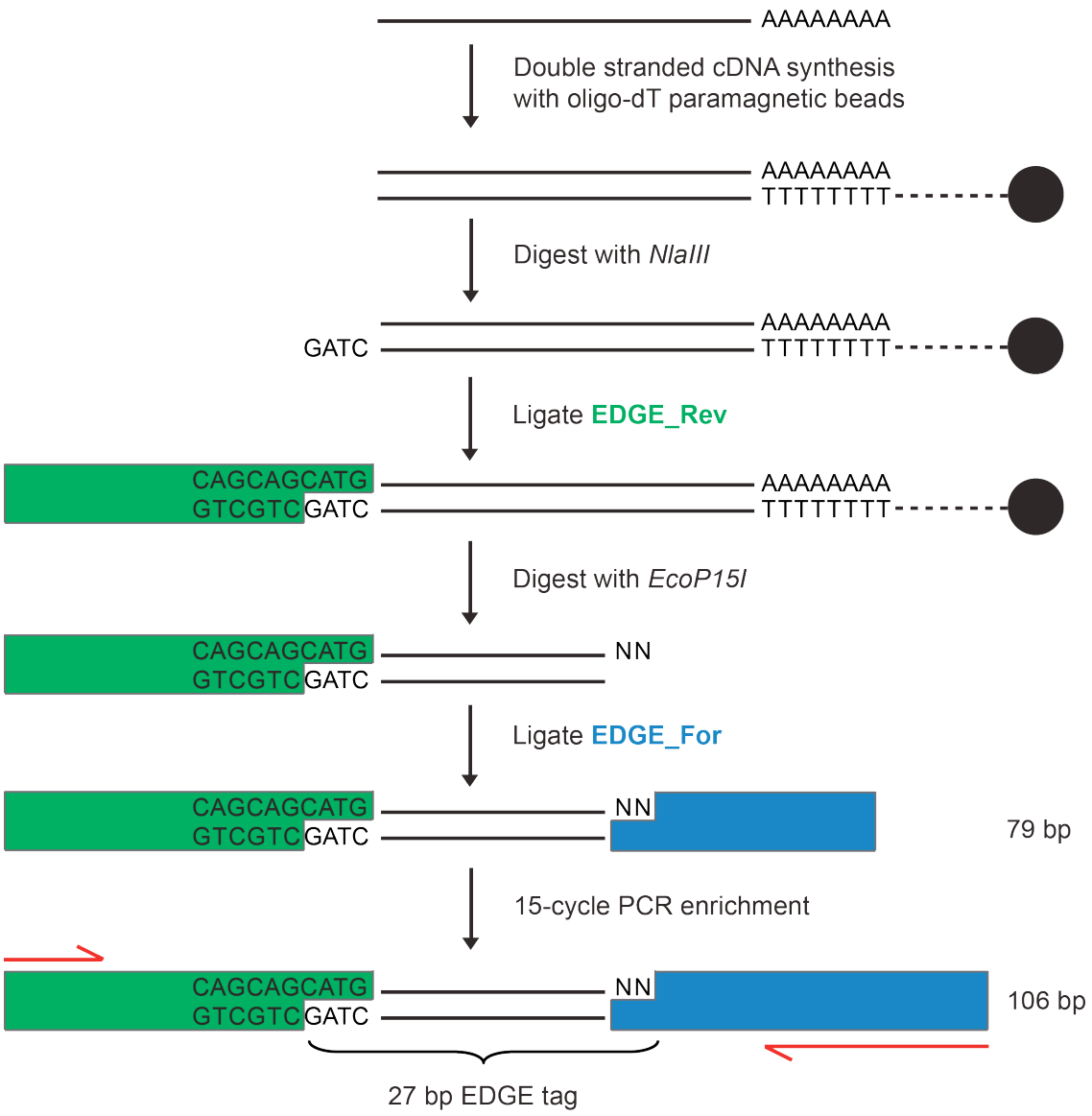
Barsh Lab EDGE protocol

EcoP15I-tagged Detection of Gene Expression

Last updated: 05/24/2011 by Lewis Hong

Contact information:
Greg Barsh
HudsonAlpha Institute for Biotechnology
601 Genome Way
Huntsville, AL 35806
Email: gbarsh@hudsonalpha.org

Overview of methodology



Introduction

This protocol is currently in use by the Barsh Lab at the HudsonAlpha Institute for Biotechnology and is adapted for the Illumina sequencing platform. *EcoP15I*-tagged Detection of Gene Expression (EDGE) is a method for ultra high-throughput sequencing of cDNA-tags and is particularly useful for profiling gene expression in non-model organisms. Briefly, double stranded cDNA synthesis is performed using paramagnetic oligo-dT beads to capture polyadenylated RNA. Next, each cDNA molecule is "anchored" by *NlaIII* restriction cleavage that exposes the 3'-most 'CATG' site within the transcript. Following this, the EDGE_Rev adaptor carrying an *EcoP15I* recognition site (5'CAGCAG-3') is ligated, and the resulting molecule is "tagged" by *EcoP15I* restriction digest, generating a 27 bp sequence tag. The sticky end is ligated to the EDGE_For adaptor. Finally, a 15-cycle PCR amplification using adaptor-specific primers is performed to add on the additional sequence required to complete the EDGE_For adaptor and to enrich for the desired final product. Following a cleanup step with AMPure XP beads, the library is ready to be sequenced on the Illumina Genome Analyzer.

Reagents

Dynabeads mRNA DIRECT Kit (Invitrogen 610-11)

SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen 18080-051)

Second-Strand Buffer (Invitrogen 10812-014)

E.coli DNA ligase (NEB M0205L)

E. coli DNA pol I (NEB M0209L)

Ribonuclease H (Invitrogen 18021-071)

NlaIII (NEB R0125S)

T4 NDA ligase (Invitrogen 15224-041)

EcoP15I (NEB R0646S)

Phusion High-Fidelity DNA Polymerase (NEB F-530S)

10mM ATP (NEB P0756S)

25 bp ladder (Invitrogen 10597011)

Glycogen (Roche 10901393001)

BSA (NEB B9001S)

MinElute PCR Purification kit (QIAGEN 28004)

MinElute Gel Extraction kit (QIAGEN 28604)

MinElute Reaction cleanup kit (QIAGEN 28204)

Agarose Nusieve GTG (Lonza 50084)

Agencourt AMPure XP (Beckman Coulter A63880)

Ultrapure DEPC-treated water (Invitrogen 750024)

Equipment

Magnetic stand; Dynal MPC-S (Invitrogen 120-21D)

Eppendorf thermomixer R (022670107) with 24 x 1.5 ml thermoblock (022670522)

Standard PCR thermocycler

Large gel electrophoresis box

Siliconized 1.5 ml microcentrifuge tubes (Fisher 02-681-331)

Oligos

- Resuspend the oligos in H_2O to create a 200 μM stock according to manufacturer's data sheet. The oligos were then quantitated by a Nanodrop and mixed at equimolar amounts (final molarity of ~100 μM each oligo).
- Add 1/10 volume of 10x T4 ligase buffer and mix thoroughly by vortexing.
- Hybridize the oligos using the following program:

95ºC	5 min
72ºC	5 min
65ºC	5 min
60ºC	5 min
50ºC	5 min
40ºC	5 min
20ºC	20 min
4ºC	forever

Oligos (from IDT, HPLC-purified):

EDGE_Rev_NlaIIIa: 5'- CAAGCAGAAGACGGCATACGACAGCAGCATG -3'

EDGE_Rev_NlaIIIb: 5'- /5Phos/CTGCTGTCGTATGCCGTCTTCTGCTTG -3'

EDGE_For_EcoP15a: 5'- TCCCTACACGACGCTCTTCCGATCT -3'

EDGE_For_EcoP15b: 5'- /5Phos/NNAGATCGGAAGAGCGTCGTGTAGGGA -3'

EDGE_Rev_NlaIIIa and EDGE_Rev_NlaIIIb are hybridized to form the EDGE_Rev_NlaIII adaptor.

EDGE_For_EcoP15b and EDGE_For_EcoP15b are hybridized to form the EDGE_For_EcoP15 adaptor.

Primers used for PCR enrichment (from IDT, PAGE-purified):

EDGE_For:

5'-AATGATACGGCGACCACCGACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'

EDGE_Rev:

5'-CAAGCAGAAGACGGCATACGACAGCAG-3'

Bead Washing

Please follow the steps below for washing the beads throughout the protocol:

1. Place the tube on a magnetic stand for 2 minutes. Place the pipette tip at the opposite side of the tube, away from the beads, and slowly slide the pipette tip to the bottom of the tube. Carefully remove the supernatant and discard. Do not disturb or remove any beads.
2. Remove the tube from the stand and add the appropriate volume of buffer.
3. Mix the contents of the tube by pipette mixing just enough to completely resuspend the beads.
4. Return the tube to the magnetic stand for 2 minutes and carefully remove the supernatant.
5. Repeat Steps 2–5 until all the washing steps are complete.
6. After the last wash, resuspend the beads in an appropriate buffer.

Step 1. Isolate total RNA

- Perform RNA isolation using your method of choice.

- Removal of DNA with DNaseI digest is necessary prior to building EDGE libraries if it was not done during RNA isolation.
-

Step 2. cDNA synthesis

- Prepare Dynabeads (from mRNA Direct kit), washing buffers, 1st strand mix (keep on ice) while RNA is thawing.

- Prepare fresh solutions per library.
  Glycogen or BSA is used in the following solutions to prevent clumping of Dynabeads.
  2x BW buffer = 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 2.0 M NaCl
  The volumes for each buffer represent the exact requirements for 1 library.

  - Buffer A: Washing Buffer A (from mRNA Direct kit) + 20 µg/ml glycogen → 2 ml
  - Buffer B: Washing Buffer B (from mRNA Direct kit) + 20 µg/ml glycogen → 1 ml
  - Buffer C: 1x BW + 1% SDS + 20 µg/ml glycogen → 2.5 ml
  - Buffer D: 1x BW + 200 µg/ml BSA → 7 ml
  - 1x 1st strand Buffer → 400 µl (dilute with DEPC H_20)
  - Buffer 4: 1x NEBuffer 4 + 200 µg/ml BSA → 400 µl

- Thoroughly resuspend Dynabeads oligo $(dT)_{25}$, transfer 150 µl to an RNase-free 1.5 ml tube, and place on magnet. After 30 seconds (or when the suspension is clear), remove the supernatant. Care should be used to prevent disturbing the beads (e.g., place pipette tips at the opposite side of the tube, lower to bottom of tube and pipette very slowly).

- Wash beads twice with 200 µl of lysis/binding buffer. In all washing steps add solutions while the tube is still on the magnetic stand to minimize "drying out" of the beads. Resuspend beads by pipette mixing. Remove buffer just prior to adding total RNA.

- Add the appropriate volume of lysis/binding buffer to 2-15 µg of total RNA to obtain a total volume of 500 µl.

- Rotate the tube in a tube rotator at room temperature for 30 min.

- Place the tube on the magnet for 2 min, and then carefully remove the supernatant.

- Wash 2 times with 1 ml of Buffer A.

Wash once with 1 ml of Buffer B.
Wash 4 times with 100 µl of 1x 1st strand buffer.
[Do not remove supernatant on the fourth wash before preparing 1st strand synthesis mix.]

- Resuspend beads in 1st strand synthesis mix:

DEPC H_2O	45.5 µl
10 mM dNTP	4.5 µl
10x RT buffer	9.0 µl
25 mM $MgCl_2$	18.0 µl
0.1 M DTT	9.0 µl
RNaseOUT (40U/µl)	1.0 µl
	87.0 µl

- Preheat the tube in the thermomixer at 50ºC for 2 min, then add 3 µl of Superscript III Reverse Transcriptase (200U/µl; Invitrogen). Incubate at 50ºC for 1 hr in the thermomixer that is constantly mixing at 1400 rpm.

- Incubate the tube at 85ºC in the thermomixer that is programmed to mix at 1400 rpm for 15 sec and then standing for 2 min, for a total of 6 min. Place the tube on ice.

- Transfer the samples to new tubes.

- On ice add the components of the 2nd strand synthesis (using reagents from Invitrogen and NEB), in the order shown, to the first strand reaction:

First strand reaction products	90 µl
DEPC H_2O (pre-chilled)	265 µl
5x 2nd strand buffer	100 µl
10 mM dNTP	15 µl
E. coli DNA ligase (10U/µl)	5 µl
E. coli DNA pol I (10U/µl)	20 µl
E. coli RNAse H (2U/µl)	5 µl
	500 µl

- Incubate at 16ºC in the thermomixer, programmed to mix at 1400 rpm for 15 sec and stand for 2 min, for a total of 2.5 hrs.

- Preheat Buffer C to 75ºC.

- After incubation, place the reaction on ice and terminate reaction by adding 45 µl of 0.5 M EDTA.

- Draw off the supernatant, then add pre-heated 500 µl of Buffer C, mix well, and

incubate in the thermomixer at 75ºC, programmed to mix at 1400rpm for 15 sec and stand for 2 min, for a total of 12 min. This step is necessary to inactivate *E. coli* DNA polymerase.

- Remove supernatant and quickly wash again with 500 µl of Buffer C. Perform wash step quickly to prevent precipitation of SDS, which may trap the beads.

- Wash 4 times with 500 µl of Buffer D.

- Wash 2 times with 200 µl of Buffer 4 + BSA. Transfer to new tubes after the first wash.

- The tube can be stored in Buffer 4 overnight at 4ºC if not proceeding on to *NlaIII* digestion.

Step 3. Cleavage by anchoring enzyme *NlaIII*

- Resuspend beads in following mix:

LoTE	172 µl
100x BSA	2 µl
10x NEBuffer4	20 µl
NlaIII (NEB, 10U/µl)	6 µl
	200 µl

Incubate at 37ºC in the thermomixer, programmed to mix at 1400 rpm for 15 sec and stand for 2 min, for a total of 1 hr.

CAUTION: always store *NlaIII* at -80ºC. *NlaIII* is extremely sensitive to high temperatures, so avoid freeze-thaw cycles whenever possible.

- After incubation, wash beads with 2 times with 750 µl of Buffer C (preheated to 37ºC, wash quickly before SDS precipitates), then wash 4 times with 750 µl of Buffer D (beads can be stored overnight at 4ºC at this stage).

Step 4. Ligating EDGE_Rev adaptor to cDNA

- LoTE = 3 mM Tris-HCl; 0.2 mM EDTA (pH 7,5)

- Wash 2 times with 200 µl of 1x ligase buffer (Dilute 5x ligase buffer from Invitrogen with H_2O). At final rinse transfer into a new tube.

- Wash 1x 50 µl of 1x ligase buffer and leave on ice.

- Remove last wash and resuspend beads as follows:

LoTE	36.5 µl
5x ligase buffer	10.0 µl
EDGE_Rev_NlaIII	1.0 µl
	47.5 µl

  - Please refer to supplementary for information on how to make linkers.
  - This ligation reaction is performed in molar excess of linkers to minimize the formation of library-to-library dimers.

- Heat tubes in a thermomixer at 50ºC for 2 min then let sit at room temperature for 15 min, then chill the samples on ice. Add 2.5 µl of T4 DNA ligase to each tube and incubate at 16ºC in a thermomixer that is programmed to constantly mix at 1400 rpm for 2 hrs.

- Wash 4 times with 500 µl of Buffer D.

- Wash 2 times with 200 µl of 1x NEBuffer 3 + 200 µg/ml BSA (transfer to new tubes after first wash).

- Proceed directly to Step 5.

Step 5. Release of cDNA tags using tagging enzyme *EcoP15I*

- Resuspend beads in the following mix and incubate at 37ºC in a thermomixer that is constantly mixing at 1400 rpm for 6 hrs:

H_2O	92.5 µl
10X NEBuffer 3	15.0 µl
10mM ATP	25.0 µl
500 µM Sinefungin	15.0 µl
100x BSA	1.5 µl
EcoP15I (10U/µl)	1.0 µl
	150 µl

- After 6 hrs incubation, spike the digestion reaction with the following mix and continue incubation in a thermomixer that is constantly mixing at 1400 rpm for another 10 hrs:

H_2O	13.5 µl
10X NEBuffer 3	5.0 µl
10mM ATP	25.0 µl
500 µM Sinefungin	5.0 µl
100x BSA	0.5 µl
EcoP15I (10U/µl)	1.0 µl
	50 µl

- After incubation, place tubes on magnetic stand for 2 min. **Do not discard the supernatant!** Carefully remove the supernatant from each tube and transfer supernatant to a new microcentrifuge tube. Wash beads once with 100 µl of LoTE. Pool the supernatant together (300 µl total volume).

- Proceed directly to Step 6.

Step 6. Ligating EDGE_For adaptor

- Add 900 µl of Buffer ERC to the supernatant and mix. Use 400 µl per MinElute column and perform reaction cleanup using the QIAGEN protocol.

- Elute the first column with 15 µl of EB, and perform successive elutions on the second and third columns using the same eluate. The final volume should be ~13µl after the final elution.

- Prepare mixes on ice as follows:

Eluate from column	~13 µl
5x Ligase Buffer	4 µl
EDGE_For_EcoP15	1 µl
T4 DNA Ligase	2 µl
	20 µl

- Incubate at 16ºC for 16 hrs. Incubation should be performed in a thermocycler with heated lid.

- Dilute 20 µl ligation product with 10 µl LoTE + 6 µl 6x loading buffer. Divide sample into 2 lanes (18 µl each) and run on 3% TAE Nusieve gel (with EtBr added) at 125V for 2.5 hrs. Leave a blank lane between samples to avoid cross contamination. Load 1.5 µg/lane of the 25 bp ladder.

- View the gel on a Dark Reader transilluminator. Excise the 79 bp band and place the gel slice from each lane into one tube (two tubes per library). The band at 79 bp is usually not visible, but just cut out the ~75 to 90 bp region of the gel anyway. (A small proportion of dimers will migrate as a 50-55 bp band. Be careful to avoid excising the linker band at this step.)

- Perform gel-purification using the MinElute Gel Extraction kit. Dissolve gel by rotating in QG buffer for ~10 minutes at room temperature (not 50°C). Perform the final elution with 20 µl of EB.

Step 7. Enrichment of the library using PCR

- PCR is performed at this stage to enrich for the library and to attach the additional sequence that is required at the end of both linkers.

- To check if the library is built properly up to this point, perform PCR as described below with 20 cycles of amplification and run out on a 1.5% agarose gel to verify presence of a 106 bp library product. A larger band at ~140 bp or a thick band from 110 to 140 bp might be observed if library yield is high.

- Following successful verification, set up the enrichment PCR as follows:

Ligation product (from Step 6)	2.0 µl
H_20	31.5 µl
5x Phusion HF buffer	10.0 µl
10 mM dNTP mix	2.0 µl
10 µM EDGE_For primer	2.0 µl
10 µM EDGE_Rev primer	2.0 µl
Phusion DNA polymerase (2U/ul)	0.5 µl
	50 µl

Run the following PCR program:

30 sec at 98ºC
10 sec at 98ºC
30 sec at 63ºC } 15 cycles
15 sec at 72ºC
10 min at 72ºC
4ºC forever

- Purify the final library product using Agencourt AMPure XL beads, as follows:

1) Combine PCR products (150 µl total) in a regular 1.5 ml microcentrifuge tube.
2) Gently shake the Agencourt AMPure XL bottle to resuspend any magnetic particles that have settled. Do not vortex.
3) Add 270 µl of AMPure beads per tube and mix thoroughly by pipette mixing 10 times. Let the mixed sample incubate at room temperature for 5 min.
4) Place the tubes on the magnetic stand for 2 min.
5) Discard the cleared solution. Do not disturb the separated magnetic beads. If beads are drawn out, leave a few microliters behind.
6) With the tubes still sitting on the magnetic stand, wash with 500 µl of 70% ethanol, incubating for 30 sec each time. Do not disturb the magnetic beads.
7) Wash again with 500 µl of 70% ethanol. Be sure to remove all the ethanol from the tube, including the sides.
8) Let beads dry out for 5 min on the magnetic stand. Be careful not to exceed 5 min as excessive drying will significantly reduce elution efficiency.

9) Elute with 20 µl of water and mix thoroughly by pipette mixing 10 times. Do not scrape the beads off the sides of the tube. Avoid ethanol droplets on the sides of the tube that haven't dried out.
10) Place tubes on magnetic stand for 1 min.
11) Transfer the eluate to a new tube. Be very careful not to remove any beads.

- Finally, quantify the library concentration using a Bioanalyzer or a Quant-iT DNA assay kit (Invitrogen).