Genomic Signature Tags (GST) Protocol

Required reagents and recommended suppliers

T4 DNA ligase 350U/µl from Takara Biotechnology equals ~2.8 Weiss U/µl
T4 DNA ligase High Concentration 5 Weiss U/µl from Invitrogen/Gibco
*Nla*III 10U/µl from New England Bio Labs (NEB) - **Store at -80°C**.
We also obtain *Not*I and *Bam*HI from NEB and
Exonuclease I 20U/µl
*Mme*I 2U/µl is obtained from Centrum Transferu technologii, Ul. Grunwaldzka 529, 80-320 Gdansk, Poland (**sole source**)
Streptavidin coated Dynabeads M-280 from Dynal or equivalent
Dynal MPC magnet or other suitable device.
Oligonucleotides are purchased from Integrated DNA Technologies, Inc. Oligonucleotides are purified by PAGE or HPLC.
10 x OFA(One Phor All buffer, Amersham, Pharmacia)
GlycoBlue 15 mg/ml (Ambion)
Taq DNA polymerase 10x reaction buffer without $MgCl_2$-Promega catalogue #M190
Platinum Taq DNA Polymerase High Fidelity (Invitrogen)

Preparation of linker cassettes

Oligonucleotides for the linker cassettes are dissolved in ddH_2O to a concentration of 100 pmoles/µl
Pairs are annealed together in the following standard reaction:
P=phosphorylated ; *= 3' amino modified

36 µl top strand oligonucleotide
36 µl bottom strand oligonucleotide
10 µl 10xOFA
18 µl TEsl (10 mM TrisHCl, pH 8.0; 0.1 m M EDTA-Na_3)
100 µl

95°C 2 min
65°C 10 min
37°C 10 min
RT 20 min--check annealing by electrophoresis on a 10% polyacrylamide gel, use 20-40 pmol of each separate strand and annealed product, store at -20°C

Preparation of genomic DNA

DNA is digested with *Bam*HI or other suitable fragmenting enzyme. Typical reaction would be 10 µg DNA in a final vol. of 100 µl NEB#2 buffer plus 1xBSA with 1µl enzyme for 2 hrs at 37°
PC (Phenol/chloroform) extracted and EtOH ppt from 0.3 M NaOAc, pH 6.0, Collect by centrifugation and resuspend in 34 µl TEsl

Ligation of biotinylated matching enzyme linker cassette (*Bam*HI cassette is shown)

5'-CGAACCCCTTCG
BIOTIN -TGCTTGGGGAAGCCTAG**p**

To 34 µl enzyme digested genomic DNA, add
5 µl 10x T4 DNA ligase buffer (Takara)
8 µl matching enzyme linker cassette (~50 fold excess, ~288 pmoles)
3 µl T4 DNA ligase (Takara)
50 µl incubate at 16°C O/N

PC extract to remove / inactivate ligase, wash PC phase with 50 µl TEsl, pool aqueous phases then ethanol precipitate. Chill sample at -80°C for 1 hour or O/N at -20°C, then spin 30 min at 4°C , wash with cold 75% EtOH, dry.

1st *Nla*III digestion
Rehydrate pellet in 83 µl ddH_2O, add:
10 µl NEB#4
1.0 µl 100xBSA 10 mg/ml
4 µl 100mM spermidine$(HCl)_3$
add 2.0 µl *Nla*III**, *Nla*III is stored at -80°C**
100 µl Final vol. incubate 3 hrs. at 37°C

Bind biotinylated fragments to streptavidin beads.

Remove 100µl thoroughly resuspended Dynal M280 streptavidin beads from the stock into a clean 1.5 ml siliconized or low adhesion (Ambion) microcentrifµge tube and place tube on magnet. Remove supernatant and wash beads with 400µl 1x B&W buffer (binding and wash buffer 10 mM TrisHCl, pH 8.0, 1 M NaCl, 1 mM EDTA-Na_3)

Resuspend beads in 100µl **2x** B&W buffer
Add 100µl digest
Incubate at RT for 1 hr. with gentle mixing. **Do NOT vortex**, but make sure beads are fully resuspended.

2nd *Nla*III digestion
Collect beads, carefully remove supernatant and add premixed:
168 µl ddH_2O
20 µl 10xNEB #4
2 µl 100xBSA
8 µl 100mM spermidine$(HCl)_3$
2 µl *Nla*III

Incubate 2 hours 37°C with occasional mixing. Add an additional 2 µl *Nla*III and incubate for an additional 2 hours 37°C

LIGATION of 1ST LINKER CASSETTE (LINKER A) with site for *Mme*I tagging enzyme:

*Mme*I recognition sequence is :

```
5'  ... TCCRAC (N) 20 ... 3'
3'  ... AGGYTG (N) 18 ... 5'
```

Capture beads, rinse:

3 x w/ 200 µl TEsl

1 x w/ 200 µl 1x T4 DNA Ligase buffer (Takara)

Ligation of Linker A

Capture beads, remove wash, add (premixed):

38 µl ddH_2O

5 µl 10x T4 DNA Ligase Buffer (Takara)

4 µl *Mme*I linker cassette @ 10 pmoles/µl)

Heat @ 50°C 2 min, cool to RT 15 min, then add:

3 µl T4 DNA Ligase (Takara)

50µl

Incubate at 16°C for 2 hours with occasional gentle mixing

_Mme_I digestion to generate _Mme_I linker ligated tags

10x conc. *Mme*I Buffer

100 mM HEPES, pH 8.0

25 mM KOAc, pH 8.0

50 mM MgOAc, pH 8.0

20 mM DTT

plus

100x conc. SAM:

4 mM SAM (S-adenosylomethionine hydrochloride)

Wash bound ligation products to remove all unligated linkers

6 x w/ 400µl 1x B&W (can store at 4 °C O/N at this point)

Capture beads, remove wash,

wash beads 2 x with 200 µl 1x *Mme*I buffer

Capture beads, add the following premixed:

86µl	ddH_2O
10µl	10 x *Mme*I buffer
1µl	SAM (4 mM stock, final conc. = 40 µM) **THIS IS IMPORTANT**!
1µl	100xBSA
2µl	2U/µl *Mme*I
100µl	

Incubate @ 37°C 3 hours, mixing occasionally

*Bam*HI

```
5'-TTTGGATTTG CTGGTCGAGT ACAACTAGGC TTAATCCGACATGNNNNNNNNNNNNNNNNNN   NNNNNNN-LINKER
     *CCTAAAC GACCAGCTCA TGTTGATCCG AATTAGGCTGTACNNNNNNNNNNNNNNNNp  NNNNNNNNN-LINKER
```

Collect beads**. GST sample will be in supernatant**. Remove supernatant to clean tube.

Rinse beads with 100 µl TEsl and combine with 1st supernatant**.**

PC extract to remove / inactivate restriction enzyme, then ethanol precipitate tags:

200 µl	sample
133 µl	7.5 M NH_4OAc
3 µl	GlycoBlue
1.0 ml	100% Ethanol

Place at -80° C for 1 hour or O/N at -20° C, then spin 30 min at 4° C, wash with 70% EtOH in cold.

Degenerate Linker ligation:

Resuspend tags in 29.5µl TEsl plus 4µl 10 x T4 DNA ligase buffer (Takara).

Degenerate Linker

```
  pTTCATGGCGG AGACGTCCGC CACTAGTGTC GCAACTGACT A*
NNAAGTACCGCC TCTGCAGGCG GTGATCACAG CGTTGACTGA T
```

2nd Ligation:

33.5 µl	*Mme*I GST products in ligase buffer
3.5 µl	degenerate linker @ 10 pmol/µl=35 pmol)

Incubate at RT 15 minutes, then add:

3 µl	T4 DNA Ligase (Takara)
40µl	

Incubate 2 hours to O/N at 16°C

Product (slightly less than 100bp) with two *Nla*III sites (**CATG**):

```
5'-TTTGG –30-CCGACATGNNNNNNNNNNNNNNNNNTTCATGGCGG AGACGTCCGCCACTAGTGTCGCAACTGACTA*
3'-  *CC -30-GGCTGTACNNNNNNNNNNNNNNNNNAAGTACCGCC TCTGCAGGCGGTGATCACAGCGTTGACTGAT
```

PCR amplification of GSTs

primers:

forward is biotinylated and corresponds to a portion of *Mme*I linker's top strand

5'-Biotin-GGATTTGCTGGTCGAGTACA

reverse is biotinylated and corresponds to a portion of degenerate linker's bottom strand

5'-Biotin-TAGTCAGTTGCGACACTAGTGGC

GST PCR cycle

95°C 2 min
95°C 30 sec
58°C 30 sec **30 cycles steps 2-4**
72°C 30 sec
72°C 4 min
10°C hold

PCR Reaction: **use Promega buffer- Do not use a PCR buffer with ammonium sulfate as it is insoluble in EtOH which then causes problems with later steps.**

1 x µl	10 x µl	20 x µl	Stock Conc.	Reagent
18.55	185.5	371		ddH_2O
2.5	25	50	10x	Promega Buffer
1.0	10.0	20.0	50 mM	$MgSO_4$
0.75	7.5	15.0	10 mM each	dNTPs
1.0	10.0	20.0	10 µM	biotinyl forward primer
1.0	10.0	20.0	10 µM	biotinyl reverse primer
0.1	1.0	2.0		Platinum Taq polymerase mix
0.1	1.0	2.0		cDNA products*
25.0 µl	250 µl	500 µl	(five or ten tubes of 50µl)	

* We initially tried a range of concentrations, from 0.01µl to 1.0µl, but found that this produced the best yield - might need to optimize.
Pool products and then use 200µl in following reaction:

Linear amplification to resolve heteroduplexes (LARHD)

1 x µl	20 x µl	40 x µl	50 x µl	Stk Conc	Reagent
13.65	273	546	682.5		ddH_2O
2.5	50	100	125	10x	Promega buffer
1.0	20	40	50	50 mM	$MgSO_4$
0.75	15	30	37.5	10 mM each	dNTPs
1.0	20	40	50	10 µM	biotinyl forward primer
1.0	20	40	50	10 µM	biotinyl reverse primer
5.0	100	200	250		1^{st} round amp'd tags
0.1	2	4.0	5		Platinum Taq polymerase mix
25.0 µl	500µl	1000µl	1250µl	(10, 20 or 25 tubes of 50µl)	

LARHD cycle (only one cycle)

95°C 2.5 min
58°C 30 sec
72°C 5 min
10°C hold

Pool products, saving 10µl for gel analysis if desired. Products should be 94 bp in length

```
                                                [--------    GST ------------]
5′-GGATTTGCTGGTCGAGTACAACTAGGCTTAATCCGACATG NNNNNNNNNNNNNNNNNTTCATGGCGGAGA
3′-CCTAAACGACCAGCTCATGTTGATCCGAATTAGGCTGTAC NNNNNNNNNNNNNNNNNAAGTACCGCCTCT

CGTCCGCCACTAGTGTCGCAACTGACTA
GCAGGCGGTGATCACAGCGTTGACTGAT
```

Exonuclease I digestion of primers

To the pooled PCR products add:

10µl *E. coli* Exonuclease I, 10 U/µl, for 1000 µl of LARHD products

Incubate at 37°C 60 minutes, remove 5 µl for gel

PC extract to remove enzymes, wash PC with a small amount of TEsl, pool, then precipitate in as many tubes as needed:

1 tube = 270µl sample
30µl 3M NaOAc, pH 6.0
750µl 100% EtOH, Place at -80°C for 30 min.

spin down amplicons in **cold**, redissolve in **cold** O.3 M NaOAc and then reprecipitate in one tube with 2.5 vol. EtOH, wash with 70% EtOH, dry.

LARHD-2 25 cycles of linear amplification with biotinyl forward primer followed by **one** cycle of

amplification with biotinyl reverse primer.

1 x µl	40 x µl	Stk Conc	Reagent
17.65	706		ddH_2O
2.5	100	10x	Promega buffer
1.0	40	50 mM	$MgSO_4$
0.75	30	10 mM each	dNTPs
1.0	40	10 µM	biotinyl forward primer
1.0	40		1st round amp'd tags
0.1	4.0		Platinum Taq polymerase mix
25.0 µl	1000 µl	(10 tubes of 100 µl)	

95°C 2 min
95°C 30 sec
58°C 30 sec **25 cycles steps 2-4**
72°C 30 sec
72°C 5 min
10°C hold

add 4 µl biotinyl B reverse primer and an additional 0.4 µl of Platinum Taq polymerase mix to each 100 µl reaction mix, followed by one cycle of denaturation and extension.

95°C 2.5 min
58°C 30 sec
72°C 5 min
10°C hold

Exonuclease I digestion of primers

To the pooled PCR products add:
10 µl *E. coli* Exonuclease I, for 1000µl of LARHD-2 products
Incubate at 37°C 60 minutes, remove 5µl for gel
PC extract to remove enzymes, wash PC with a small amount of TEsl, pool, then precipitate in as many tubes as needed:

1 tube = 270µl sample
30µl 3M NaOAc, pH 6.0
750µl 100% EtOH, Place at -80°C for 30 min.

spin down amplicons in **cold**, redissolve in **cold** O.3 M NaOAc, reprecipitate in one tube with 2.5 vol. EtOH, wash with 70% EtOH, dry.

***Nla*III digestion** *Nla*III is stored at -80°C

Digestion is performed at 37°C for 4 hrs in 400 µl 1 x NEB #4 buffer plus 1x BSA and 4 mM permidine$(HCl)_3$

*Nla*III (2 µl) is typically added twice, 2x 2 hr digestions for a total of 4 hrs.

Digestion products should be:

***Mme*I linker arm-**40mer

```
1 Biotin-GGATTTGCTG GTCGAGTACA ACTAGGCTTA ATCCGACATG
          CCTAAACGAC CAGCTCATGT TGATCCGAAT TAGGCT
```

plus GST - 23mer

```
      RRRRRR RRRRRRRRRR RTTCATG
   GTACYYYYYY YYYYYYYYYY YAA
```

plus degenerate linker arm -35mer

```
      GCGGAG ACGTCCGCCA CTAGTGTCGC AACTGACTA
   GTACCGCCTC TGCAGGCGGT GATCACAGCG TTGACTGAT-Biotin
```

PC extract on **ice**, EtOH ppt from 0.3 M NaOAc plus 2.5 µl GlycoBlue, chill at -80°C**,**
SPIN IN COLD ROOM (IMPORTANT)
wash pellet in **ice-cold** 70% EtOH, dry
Resuspend sample in 200 µl **cold** TEsl +25 mM NaCl

Bind biotinylated arms to Dynal streptavidin beads:

use 200µl Promega beads, prewashed in 1x B&W Buffer (1 M NaCl- no added BSA or glycogen)
resuspend washed beads in 200µl 2x B&W, add *Nla*III digest, mix at RT for 15-30 min,
Collect beads and save unbound fraction --**THESE ARE THE GSTs.**
Wash with 100µl 1x B&W buffer, pool and ppt with 2.5 vol. EtOH at -80°C, **SPIN IN COLD ROOM (IMPORTANT) wash pellet in ice-cold 70% EtOH,** dry

Self-ligation of cassette tags to form concatemers
Resuspend GST pellet in 12.5µl TEsl on ice, add
1.5µl 10 x T4 DNA ligase buffer (Takara)
1.0µl T4 DNA Ligase (Gibco-High Conc**)**
Incubate at 16°C 4-6 hours

Add 25 µl TEsl+25 mM NaCl, heat for 2.5 min at 65°C, quench on ice, add 4µl 80% glycerol, mix and apply to a single slot of an 8 slot 0.75% Low Melt agarose minigel bracketed by 100 and 500 bp ladders. Electrophorese and cut out concatemers.

Note in some cases we cut the gel to remove tags of < 100-250 bp, and reversed the gel's polarity to concentrate the DNA prior to elution.

Tags are purified using GFX Spin columns (Amersham, Pharmacia).

Elute with 180 µl ddH_2O
add 20 µl 3M NaOAc, pH 6.0,
2 µl GlycoBlue
500µl EtOH, chill spin, wash with 70% EtOH, dry,

up in 8 µl TEsl
plus 1 µl 10x T4 DNA ligase buffer Takara**,** mix, add
0.5 µl *Sph*1 cut pZero-BNL, heat at 65°C for 30 sec, quench on ice, add
1 µl T4 DNA ligase (Takara), mix, incubate at 16°C for several hrs.
dilute to 50 µl by adding 40 µl 1X T4 Ligation buffer plus 1 µl T4 DNA ligase, incubate O/N at 16°C,

PC extract, EtOH ppt from NaOAc + GlycoBlue

Sample up in 10 -15 µl ddH2O, Electroporate 5 µl sample into 50 µl TOP10 competent cells.

Phenotypically express each sample in 1 ml 2xYT for 1 hr at 37°C with shaking, pool = 3 ml.
Plate 200, 100, 50 and 25 µl onto prewarmed 2xYT plates + 50 µg/ml kanamycin.
Add 0.1 vol of 80% glycerol to the remaining cells and store at -80°C.
Incubate plates overnight. A good library should provide 100-200 colonies on the 25 µl to 50 µl platings.

GST concatemers in

pZero are sequenced with the m13 FORWARD primer

GSTs should have the following sequence where polarity is indicated by R and Y

```
     5'-RRRRRR RRRRRRRRRR RTTCATG-3'
3'-GTACYYYYYY YYYYYYYYYY YAA-5'
```

Concatemers should have the following type GST units which are extracted using software developed at BNL

```
  1      RRRRRR RRRRRRRRRR RTTCATGAAY YYYYYYYYYY YYYYYYCATG RRRRRRRRRR
    GTACYYYYYY YYYYYYYYYY YAAGTACTTR RRRRRRRRRR RRRRRRGTAC YYYYYYYYYY

 57  RRRRRRRTTC ATGAAYYYYY YYYYYYYYYY YYCATGRRRR RRRRRRRRRR RRRTTCATGR
     YYYYYYYAAG TACTTRRRRR RRRRRRRRRR RRGTACYYYY YYYYYYYYYY YYYAAGTACY

117  RRRRRRRRRR RRRRRRTTCA TGAAYYYYYY YYYYYYYYYY YCATGRRRRR RRRRRRRRRR
     YYYYYYYYYY YYYYYYAAGT ACTTRRRRRR RRRRRRRRRR RGTACYYYYY YYYYYYYYYY

177  RRTTCATGRR RRRRRRRRRR RRRRRTTCAT GRRRRRRRRR RRRRRRRRTT CATGAAYYYY
     YYAAGTACYY YYYYYYYYYY YYYYYAAGTA CYYYYYYYYY YYYYYYYYAA GTACTTRRRR

237  YYYYYYYYYY YYYCATGAAY YYYYYYYYYY YYYYYYCATG AAYYYYYYYY YYYYYYYYYC
     RRRRRRRRRR RRRGTACTTR RRRRRRRRRR RRRRRRGTAC TTRRRRRRRR RRRRRRRRRG

297  ATGAAYYYYY YYYYYYYYYY YYCATG
     TACTTRRRRR RRRRRRRRRR RR
```

*Mme*I Long SAGE Protocol

This method uses many of the same steps and reagents as in the GST protocol. The main difference is that it uses cDNA prepared by Reverse Transcription of poly(A)$^+$ mRNA as the starting material. The efficiency of this step is increased by capturing the poly(A)$^+$ mRNA on oligo $(dT)_{25}$ magnetic beads directly from a cell lysate and by repeating the first-strand synthesis step several times.

The following new reagents that are needed:

Reverse Transcriptase RNase H-free SuperScript II
DEPC treated dH2O
E. coli DNA ligase, DNA polymerase and RNase H
1st and 2nd strand buffers.
Glycogen 20 mg/ml

All of these reagents are obtained from Invitrogen

In addition, a Dynal Dynabeads mRNA Direct kit (catalogue #610.11) is recommended. This kit contains reagents for cell lysis and includes the oligo $(dT)_{25}$ beads.

SuperRNasin is obtained from Ambion

Thoughly resuspend and remove 100µl suspended Dynal oligo(dT) beads from the stock into a clean 1.5 ml siliconized or low adhesion Ambion microcentrifµge tube and place tube on magnet. Remove supernatant and wash beads with 400µl lysis /binding buffer from the Dynal Dynabeads mRNA Direct kit.

Mix RNA with 500µl Dynal Lysis/Binding buffer supplemented with 10 µg/ml glycogen.

Collect washed beads, add RNA solution to beads, mix, heat to 60 °C for 5 min, cool to room temp for 10 min on bench with occasional mixing (about 1x per min)

Collect beads, wash 2 x with 400µl Dynal wash buffer A (with LiDS)
supplemented with 20 µg/ml glycogen.
Wash 3 x with 400µl Dynal wash buffer B (w/o LiDS) + 20 µg / ml glycogen.
Move beads to new tube after1st wash.
Wash 2 x with 400µl RT 1st strand buffer + 20 µg/ml glycogen + 2µl SuperRNasin.

First Strand cDNA synthesis

a) Resuspend beads in 25µl RT 1st strand buffer (+SuperRNasin. + glycogen as above).
b) Incubate at 42°C 2 minutes
c) Incubate at 37°C 2 minutes. Scrape down beads off sides if necessary, add a mixed containing:

9.0µl	DEPC treated water
1.0µl	SuperRNasin
5.0µl	5x 1st strand buffer
2.5µl	dNTPs (10 mM each) then add RT b/f adding to beads)
5.0µl	DTT
2.5µl	Superscript II Reverse Transcriptase

Incubate at 37°C 1 hr with gentle mixing.

Heat to 60°C 3 minutes, incubate at 37° 2 minutes, then add an additional 2 µl RT. Incubate at 37°C an additional 1 hr. Repeat one more time. Total of 6.5 µl RT is used for 3 cycles of cDNA synthesis.

Collect beads, carefully remove 1st stand reagents.

Second Strand cDNA synthesis

Add to beads (premixed)

253.5µl	dH_2O
70µl	5x 2nd strand buffer
8µl	dNTPs (10 mM each)
2.5µl	E. coli DNA ligase
10µl	E. coli DNA polymerase
2.5µl	E.coli RNAse H
3.5µl	glycogen @ 5 µg /µl
350µl	

Mix, incubate at 16°C O/N, with occasional mixing for the first 3 hrs.

a) Wash beads 6 x with 1x B&W buffer plus 1x BSA (0.1 mg/ml) using 500µl/wash
1st time: Resuspend in 1x B&W buffer / BSA and heat to 75°C for 10 minutes, cool
2nd time: Wash without heat
3-6 times: Quick rinse without heat. After 4th wash, transfer to clean tube.
(can store in 500µl 1x B&W buffer+BSA at 4°C)

Digestion with *Nla*III anchoring enzyme
Wash beads 3 x with 200µl 1x NEB #4 buffer
Resuspend in
200µl 1x NEB #4 buffer plus 1x BSA plus 4 mM spermidine$(HCl)_3$; add
2µl *Nla*III (10 U/µl from NEB) ***Nla*III is stored at -80°C**
Incubate at 37°C for 2 hours.
add an additional 2µl *Nla*III and incubate for an additional 2 hrs with occasional mixing

Capture beads, remove supernatant and EtOH ppt for gel analysis (digested cDNA should produce a visible smear of low MW fragments ~100 -500 bp)

Resuspend beads in 400µl 1x B&W buffer + BSA and heat at 65°C for 20 min to inactivate *Nla*III; cool and wash 4 more times with 400µl of 1x B&W buffer + BSA. EtOH ppt these washes as well for gel analysis

Store beads in 400µl 1x B&W buffer+ BSA at 4°C.

Proceed to

LIGATION of 1ST LINKER CASSETTE (LINKER A) with site for *Mme*I tagging enzyme.

All subsequent steps are the same as in the GST protocol.