iCLIP2 - Library preparation

iCLIP2 protocol with modifications from eCLIP + iiCLIP

Revised: 2022.03.25

1. Bead equilibration

1.1 Thaw iCLIP lysates on ice (~ 90 min)

- See here for lysate prep: https://docs.google.com/document/d/1VGXmCG3zFS5_dzDdBrxme8CXisnj-mbKejG3uVFVa2g/edit?usp=sharing

GFP-trap magnetic agarose beads

1.2 Wash 25-50 µl GFP-trap/Control beads (per sample) once in cold High salt wash buffer and twice in cold lysis buffer (w/ cOmplete 1tb/10ml + AEBSF 1:1000)

- DO NOT VORTEX beads, resuspend beads by pipetting
- Add 900 µl buffer, resuspend with pipette, attract beads on magnet

1.3 Resuspend in 50 µl lysis buffer per sample. Keep on ice.

Dynabeads with antibodies

1.4 Wash 50-100 µl Protein A Dynabeads (per sample) 2X in supplemented lysis buffer at RT

- Completely resuspend dynabeads using vortex/roller

1.5 Resuspend beads in 100 µl lysis buffer with 2-10 µg antibody per IP

1.6 Rotate tubes at RT for 30-60 mins

1.7 Wash 1X with High-salt wash buffer

1.8 Wash 2X with lysis buffer

1.9 Resuspend in 50 µl lysis buffer per sample. Keep on ice.

2. RNAse + DNAse treatment

2.1 Pre-warm thermoblock to 37 ˚C.

2.2 Dilute RNase I (Ambion) in cold PBS at appropriate dilution and keep on ice

- RNAse dilution series needs to be tested in pilot experiments

2.3 Add 2 µl Turbo DNase to the samples and vortex mix.

2.4 Add 10 µl (to 1 ml sample) diluted RNase I. Vortex mix and immediately proceed to the next step.

2.5 Incubate at 37˚C, 1,100 rpm for 3 min exactly → immediately place on ice for more than 3 min

2.6 (Optional) Add 5 µl RiboLock per ml of the samples - only with whole larval lysates

2.7 Take two 10 µl aliquots for size-matched input (SMInput) samples. Store at -80˚C.

3. Immunoprecipitation

3.1 Add equilibrated beads to the lysates

3.2 Incubate 2 hrs in the cold room with rotation.

3.3 Place a magnet rack on ice, attract beads and save s/n as Flow-through samples

3.4 Wash 2X with cold High-salt wash buffer (Incubate on ice for 2 min with 2nd wash) - all wash steps are done with 900 µl buffer

3.5 Wash 2X with cold PNK wash buffer. **Transfer to the new tubes after the first wash**.

4. On-bead chemistry

3'-end dephosphorylation

4.1 Pre-warm thermomixer to 37˚C

4.2 Resuspend beads in 40 µl PNK mix.

- 30 µl H2O
- 8 µl 5xPNK buffer pH 6.5 (1 mM DTT final)
- 1 µl PNK (M021L)
- 0.5 µl FastAP alkaline phosphatase (EF0654)
- 0.5 µl RNasin

4.3 Incubate 40 min at 37˚C in a thermomixer shaking at 1,100 rpm.

4.4 Add 500 µl cold PNK wash buffer

4.5 Wash 2X with cold High-salt wash buffer (Incubate on ice for 2 min for second wash)

4.6 Wash 2X with cold PNK wash buffer.

L3-IR-App adaptor ligation

4.7 Resuspend beads in 25 µl ligation mix (Add DMSO last when making the mix)

- 6 µl water
- 3 µl 10x ligation buffer (DTT free)
- 2.5 µl L3-IR-App adaptor (stock 1 µM)
- 2.5 µl T4 RNA ligase I High conc. (M0437M)
- 0.5 µl PNK enzyme
- 0.5 µl RNAsin
- 9 µl PEG8000
- 1 µl 100% DMSO

4.8 Incubate o/n at 16˚C shaking at 1,100 rpm (cover from light) ⏸ **Pause**

4.9 Add 500 µl cold PNK wash buffer

4.10 Wash 2X with cold High-salt wash buffer (Incubate on ice for 2 min for second wash)

4.11 Wash 2X with cold PNK wash buffer.

5. SDS-PAGE & Wet transfer

Elution

5.1 Prepare elution sample buffers and set the thermoblock to 70˚C

- IP samples: 1x NuPAGE sample buffer + 10% 1 M DTT
- SMInput samples: 2x NuPAGE sample buffer + 20% 1 M DTT

5.2 Remove s/n. Add pre-heated elution sample buffer to samples

- IP samples - Resuspend beads in 20 µl 1x Sample buffer
- SMInput - Add 10 µl 2x Sample buffer to 10 µl Input lysate

5.3 Heat samples for 10 min at 70˚C, shaking at 1,100 rpm. Place on ice for > 1 min.

5.4 Briefly spin and magnetically separate on ice. Load on gel immediately or store at -80˚C.

SDS-PAGE

5.5 Prepare 500 ml of running buffer (appropriate one for the gel type) and PAGE apparatus.

- Keep the running buffer cold
- Clean gel tank with 70% EtOH. Then rinse with MilliQ water
- Flush the wells before loading samples

5.6 Load the gel with 3 µl Protein ladder and 20 µl samples (alternating)

5.7 Run the gel until the dye-front reaches the bottom

- For 4-12% Bis-Tris gels - run at 180 V for 65 mins
- For 3-8% Tris-Acetate gels - run at 150 V for 65 mins

Wet transfer to nitrocellulose

5.8 Prepare transfer apparatus while the gel is running

- Cut out filter papers (8x) and nitrocellulose membrane (slightly smaller than the size of sponges)
- Prepare **cold** transfer buffer (500 ml) - 50 ml MeOH + 25 ml 20x Transfer buffer + 425 ml MilliQ water
- Pre-soak membrane in transfer buffer for several minutes just before transfer
- *Please make sure to use an eCLIP-validated nitrocellulose membrane for the transfer.*

5.9 Assemble transfer stack: (BOTTOM) 2x Sponge - 4x Filter paper - Gel - Membrane - 4x Filter paper - 2x Sponge (TOP)

5.10 Transfer at 30 V for 2 hours at RT (or overnight in the cold room)

5.11 Rinse the membrane in cold PBS and scan it at 700 channel for 30s + 800 channel for 2 min

- 700 channel is required to visualise the ladder, because the RNA signal might be too bright
- Do not let the membrane go dry
- (Optional) Also image the gel together to assess transfer efficiency

6. RNA extraction

Either perform a Zymo kit clean-up (preferred) or EtOH precipitation

6.1 Prepare PK-SDS solution (stable at room-temp for 6 months)

- 10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.2% SDS

6.2 Pre-heat thermoblock to 50˚C

6.3 Using the IR image as a guide, cut the lane from the RBP band to 75-100 kDa above. Transfer to a clean tube on ice.

- Cut into several fragments to be fully immersed in PK-SDS buffer later
- Excise the sample size-matched region for SMInput samples
- (Optional) Re-scan the membrane to confirm accuracy of cutting

6.4 Prepare PK-SDS mix. Add to membrane fragments. Incubate for 60 min at 50˚C with 1,100 rpm

- 20 µl Proteinase K + 180 µl PK-SDS solution (200 µl final)

6.5 Transfer s/n to a new tube. Rinse the membranes with another 50 µl PK-SDS buffer. Pool.

6.6 Add 1X volume (250 µl) of Phenol:Chloroform:Isoamyl Alcohol (pH 6.6-6.9, not acidic!)

6.7 Incubate for 10 min at 37˚C with 1,300 rpm. Do not vortex.

6.8 Pre-spin MaxTract tubes for 5 min, max speed, RT. Transfer RNA solution to MaxTract tubes.

6.9 Separate phases by centrifuging 16,000 g, 5 min, RT

Zymo RNA clean & concentrator cleanup

6.10 Transfer aqueous layer to a new tube and follow the kit protocol with 250 µl starting volume. Load the column 750 µl at a time.

6.11 Double elute the RNA in 11 µl H2O. Save RNA samples at -80˚C.

- 11 µl because about 1 µl is the retention volume of the zymo column

7. SMInput RNA processing (eCLIP-style)

In-solution chemistry of SMInput RNA

7.1 Pre-warm thermomixer to 37˚C

7.2 Add 10 µl PNK mix to 10 µl SMInput RNA

- 4.5 µl H2O
- 4 µl 5xPNK buffer pH 6.5 (Low DTT)
- 0.5 µl PNK (M021L)
- 0.5 µl FastAP alkaline phosphatase (EF0654)
- 0.5 µl RNasin

7.3 Incubate 40 min at 37˚C in thermomixer, 1100 rpm

7.4 RNA clean & concentrator-5 clean-up

- Add 40 µl RNA binding buffer. Pipette mix.
- Add 60 µl 100% EtOH. Pipette mix.
- Load on column. Spin (5000g, 30s). Discard flowthrough.
- Add 400 µl RNA prep buffer, spin and discard flowthrough.
- 2x Add 500 µl RNA wash buffer, spin and discard flowthrough.
- Spin 9000g for 2 min to dry the column.
- Add 11 µl H2O. Incubate for 1 min. Double elute (9000g 30s)
- Save 5 µl as backup

7.5 Add 15 µl L3-App adapter mix to 5 µl RNA

- 2 µl 10x NEB ligation buffer (with DTT)
- 0.3 µl DMSO
- 0.5 µl RNAsin
- 8 µl 50% PEG8000
- 2 µl L3-IR-App (1 µM stock)
- 1.5 µl RNA ligase high conc.
- 0.7 µl H2O

7.6 Flick mix and incubate for 75 min at RT with end-to-end rotation

7.7 MyONE clean-up. Wash 20 µl beads per sample with 500 µl RLT. Resuspend in 60 µl RLTW (RLT + 0.025% Tween-20) per sample.

- Add 60 µl bead-RLTW mix. Pipette mix.

- Add 70 µl 100% EtOH. Pipette mix.
- Incubate for 10 min. Pipette mix every 3-5 min.
- Separate, remove s/n
- Add 900 µl 80% EtOH and gently mix
- Separate, remove s/n. Wash 2X more.
- Separate, remove s/n, spin, remove s/n, air-dry 5 min
- Elute in 10.5 µl H2O. Incubate for 5 min.

7.8 Add **RecJf mix** (10 µl) to 10 µl RNA (*This step is required for SMInput but not IP samples because silane beads recover some of unattached L3-IR-App)*

- 2.5 µl H2O
- 2 µl NEB Buffer 2
- 0.5 µl 5' Deadenylase (M0331S)
- 0.5 µl RecJf endonuclease (M0264S)
- 0.5 µl RNAsin
- 4 µl 50% PEG8000 (supplied with NEB ligases)

7.9 Incubate 1 hour at 30˚C then 30 mins at 37˚C, 1,100 rpm

7.10 MyONE clean-up. Wash 20 µl beads per sample. Resuspend in 60 µl RLTW per sample.

- Add 60 µl bead-RLTW mix. Pipette mix.
- Add 70 µl 100% EtOH. Pipette mix.
- Incubate for 10 min. Pipette mix every 3-5 min.
- Separate, remove s/n
- Add 900 µl 80% EtOH and gently mix
- Separate, remove s/n. Wash 2X more.
- Separate, remove s/n, spin, remove s/n, air-dry 5 min
- Elute in 10.5 µl H2O. Incubate for 5 min.

N.B. SPRI beads could be used as an alternative to silane beads/columns in RNA clean-up steps

- SMInput PNK clean-up
  - Add 3x SPRI beads, mix well
  - Add 1.66x Isopropanol, mix well and incubate 5 min at room temp
  - Place on magnet for 2 min and remove s/n
  - Without removing beads from the magnet, wash 2X with 85% EtOH. Incubate 30s between each wash.
  - Remove s/n, spin down and remove remaining EtOH using smaller tips
  - Leave tubes open for 2 mins to dry
  - Resuspend in 6 µl nuclease-free water and elute for 5 min at room temp
  - Attract beads and collect cleaned-up RNA
- SMInput L3-IR-App & RecJ clean-up
  - Same protocol as PNK clean-up but use 0.5x water, 2.5x SPRI beads and 1.66x Isopropanol
  - Elute in 10.5 µl

8. Reverse transcription and cDNA purification

Reverse transcription using SuperScript IV

8.1 Add 1 µl RToligo (10 µM) + 1 µl dNTP mix (10 mM) to the 10 µl RNA

8.2 Run *'iCLIP2 Superscript IV RT Part 1'* programme

- 70˚C 5 min
- 25˚C Hold

8.3 Add RT mix - 8 µl

- 2 µl 50% PEG8000
- 4 µl 5x SSIV buffer
- 1 µl 0.1 M DTT
- 0.5 µl RNAsin
- 0.5 µl SuperScript IV

8.4 Run *'iCLIP2 Superscript IV RT Part 2'* programme

- 25˚C 5 min
- 50˚C 10 min
- 55˚C 10 min
- 80˚C 5 min
- 4˚C Hold

8.5 Add 2.5 µl 1 M NaOH. Mix and incubate at 85˚C for 15 min. Run *'Alkaline hydrolysis'* programme.

8.6 Add 2.5 µl 1 M HCl to neutralise pH (final volume = 25 µl)

cDNA purification (RT clean-up using MyONE silane beads)

8.7 Bring up MyONE beads to room temperature before opening. Vortex well.

8.8 Use 10 µl MyONE beads per sample. Discard s/n and wash beads with 500 µl RLT buffer. Resuspend in 3X volume of RLTW per sample. (e.g. if the RT reaction+NaOH/HCl is 25 µl, use 75 µl RLTW per sample)

8.9 Add 3X (75 µl) volume of beads-RLTW to each sample. Mix by pipetting.

8.10 Add 3.6X (90 µl) 100% EtOH to sample and mix carefully. Incubate for 5 min at RT.

8.11 Mix again by pipetting and repeat the 5 min incubation step

8.12 Attract beads on magnet for 1-2 min and remove s/n.

8.13 Wash 3X with 900 µl 80% EtOH. Incubate 30 s between resuspensions.

8.14 Spin down, remove s/n and air-dry the beads for 5 min at RT

8.15 Elute in 5 µl H2O. Incubate for 5 min at RT. Proceed to the next step <u>without removal of beads.</u>

9. Second adapter ligation and clean-up

Second adapter ligation

9.1 Pre-heat thermomixer to 75˚C

9.2 Add second adapter (different ones for multiplexed samples) and DMSO

- 2 µl L#clip2.0 oligo (10 µM)
- 1 µl 100% DMSO

9.3 Heat the mixture for 2 min at 75˚C and immediately place on ice

9.4 Add 13 µl ligation mix (prepared on ice) to cDNA-bead solution (ensure homogeneity of the mix)

- 0.3 µl 5' deadenylase
- 2 µl 10x NEB RNA ligase buffer (with DTT)
- 0.2 µl ATP (100 mM)
- 9 µl 50% PEG8000
- 1.5 µl High conc. RNA ligase

(N.B. 10x NEB buffer and ATP goes off quickly, aliquoting the initial vial recommended)

9.5 Flick mix well and incubate overnight at RT with 1100 rpm ⏸ **Pause**

Ligation reaction clean-up

9.6 Bring up MyONE beads to room temperature

9.7 Use 5 µl of fresh MyONE beads per sample. Discard s/n and wash with 500 µl RLT buffer. Resuspend in 60 µl RLTW buffer per sample (3X volume)

9.8 Add 60 µl beads-RLTW to the Second Ligation slurry. Mix by pipetting.

9.9 Add 60 µl 100% EtOH to sample and mix carefully. Incubate for 5 min at RT.

9.10 Mix again by pipetting and repeat the 5 min incubation step

9.11 Transfer to a new tube, attract beads on magnet and remove s/n

9.12 Wash 3X with 900 µl 80% EtOH. Incubate 30 s between resuspensions. (3X total)

9.13 Spin down, remove s/n and air-dry the beads for 5 min at RT

9.14 Elute the ligated cDNA in 23 µl H2O. Incubate for 5 min at RT.

10. Two-step PCR library amplification

First PCR (pre-amplification)

10.1 Prepare PCR mix

- 22.5 µl cDNA (entirety of the cDNA from the previous step)
- 2.5 µl Primer mix of **P5Solexa_s** and **P3Solexa_s** (10 µM each)
- 25 µl 2x Phusion HF Master mix

10.2 Run '*iCLIP2 First PCR'* programme

- 98˚C 30s
- 98˚C 10s I
- 65˚C 30s 6 cycles
- 72˚C 30s I
- 72˚C 3 min
- 16˚C Hold

First ProNex size selection

10.3 Equilibrate ProNex beads to room temperature for 30 mins

10.4 Transfer samples to a clean PCR-tube

10.5 Add **3X volumes** of ProNex Chemistry and mix by pipetting up and down 10 times

10.6 Incubate for 10 mins at RT

10.7 Place on magnetic stand for 2 min and discard s/n

10.8 **Leave the beads on the magnetic stand** and add 200 µl ProNex Wash Buffer. Incubate for 30-60s.

10.9 Discard s/n and repeat the wash. Incubate for 30-60s.

10.10 Discard s/n and allow samples to air-dry for 8-10 mins until cracking starts

10.11 Remove beads from the magnetic stand and elute in 23 µl ProNex Elution Buffer for 5 mins

10.12 Return to the magnetic stand, and transfer cDNA to a clean tube.

qPCR cycle optimisation

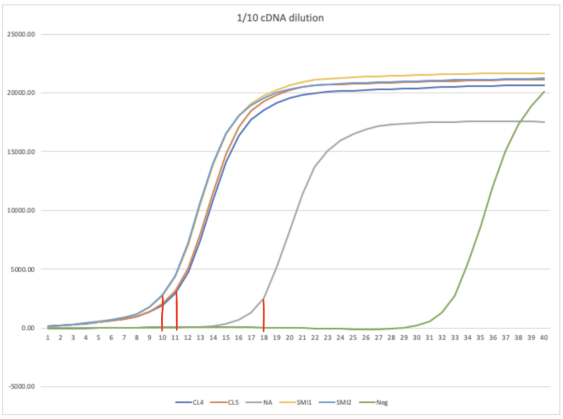
10.13 Prepare qPCR mix

- 1 µl cDNA diluted 1:10
- 3 µl H2O
- 0.5 µl EvaGreen (20x)
- 0.5 µl Primer mix of P5Solexa and P3Solexa (10 µM each)
- 5 µl 2x Phusion HF Master mix

10.14 Run '*iCLIP2 qPCR*' programme

- 98˚C 30s
- 98˚C 10s I
- 65˚C 30s 40 cycles (measure fluorescence after elongation steps)
- 72˚C 30s I
- 72˚C 3 min
- 16˚C Hold

10.15 Calculate the optimal number of cycles for each library = **Ct - 4** (It's better not to over-amplify!!)



Second PCR (Preparative)

Second PCR is performed in two half-experiments (10 µl cDNA each)

10.16 Prepare PCR mix

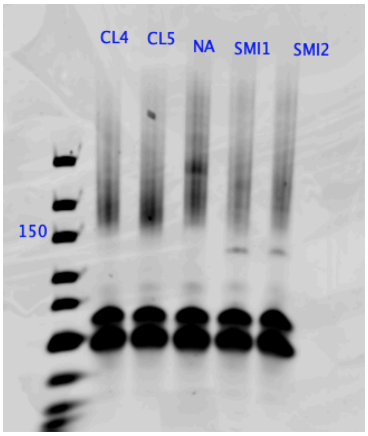
- 8 µl H2O
- 10 µl cDNA
- 2 µl Primer mix of **P5Solexa** and **P3Solexa** (10 µM each)
- 20 µl 2x Phusion HF Master mix

10.17 Run '*iCLIP2 Second PCR*' programme

- 98˚C 30s
- 98˚C 10s I
- 65˚C 30s adjusted # of cycles
- 72˚C 30s I
- 72˚C 3 min
- 16˚C Hold

10.18 Test 10 µl on 6% TBE gel (180 V, 25 min) + post-SyBrGold stain (1:10,000 in TBE, 15 mins, RT)

- Use 5 µl of ULR ladder (1:50 diluted in 1x TBE sample buffer)
- Look out for empty inserts (~150 bp, insufficient L3-IR removal) + High MW PCR artefacts (overamplified)



e.g. The last two lanes have empty insert artefacts (~ 150 bp).
If primer dimer/empty insert bands are present, do a low-melting point gel purification (see the eCLIP protocol)

10.19 If everything looks fine, amplify the second half of the library and combine with the first half

Second ProNex Size selection

10.20 Equilibrate ProNex chemistry to room temperature

10.21 Pool first and second halves of the preparative PCR products to a Lobind tube (30 + 40 = 70 µl)

10.22 Add 2.4X (168 µl) volume of ProNex Chemistry and mix by pipetting up and down 10 times

10.23 Incubate for 10 mins at RT

10.24 Place on magnetic stand for 2 min and discard s/n

10.25 **Leave the beads on the magnetic stand** and add 500 µl ProNex Wash Buffer. Incubate for 30-60s.

10.26 Discard s/n and repeat the wash. Incubate for 30-60s.

10.27 Discard s/n and allow samples to air-dry for at least 10-15 mins with visible cracking

10.28 Remove beads from the magnetic stand and elute in 25 µl ProNex Elution Buffer for 5 mins

10.29 Return to the magnetic stand, attract beads and transfer s/n to a clean tube.

Final library clean-up

10.30 There are often residual primers left after the ProNex size selection. Perform another round of clean-up using either of the following:

- ProNex - 3X volumes
- Ampure XP or other SPRI beads - 1.8X volumes
- Low-melting point gel purification (see the eCLIP protocol)

10.31 Elute in 25 µl ProNex elution buffer and store the final libraries at -20˚C

11. Library quantification & Sequencing

11.1 qPCR with Kapa DNA standards - Concentration quantification

- Primer F: 5'-AAT GAT ACG GCG ACC ACC GA-3'
- Primer R: 5'-CAA GCA GAA GAC GGC ATA CGA-3'

11.2 High sensitivity DNA bioanalyser - Quality control + Median library size

- 1:2 dilution of each library usually runs fine on bioanalyser
- Manually integrate regions to get 50% coverage + note down the median cDNA size

11.3 Correct qPCR library concentration with the size factor (multiply 452/median library size)

11.4 Normalise libraries to 4 nM and pool according to the desired share of reads

- Ina Huppertz suggests adding a bit less of SMInput libraries since they are sequenced more efficiently
- Volumetric ratio used in Syp iCLIP [IP : SMInput : Control = 100 : 75 : 20] resulted in roughly equal share of reads between IP and SMInput libraries on Next-seq

11.5 Sequence single-end with no indexing (No custom primers)

- For Next-seq Basespace prep, custom library prep kit needs to be created with no adapter sequences
- Index needs to be assigned, then in the run tab, override default indexing reads to none
- 75 cycle High output kit can run 92 cycles in total

Appendix I - Reagents

- All buffers are made according to the original iCLIP2 protocol
- L3-IR-App is generated using the irCLIP protocol

Appendix II - Modifications from the original iCLIP2 protocol

- PNK dephosphorylation recipe altered to improve dephosphorylation efficiency
  - PNK + FastAP enzymes
  - Turbo DNAse added to remove residual DNA
- L3 ligation recipe altered to improve ligation efficiency
  - High conc. ligase
  - PEG8000 + DMSO to improve enzyme efficiency
  - PNK added
  - Using buffer without DTT for IP samples to prevent proteins eluting
- L3-IR-App is used to avoid radioactivity (from irCLIP)
- PK-SDS is used instead of PK-Urea due to improved RNA liberation from nitrocellulose (from irCLIP)
- Zymo Kit is used instead of EtOH precipitation for improved RNA quality
- **SMInput libraries are prepared for enrichment against cross-link-ome**
  - RecJf steps are required for SMInput samples
- SuperScript IV is used in place of SuperScript III
  - PEG8000 added for improved reverse-transcription efficiency
- qPCR is introduced to optimise second PCR amplification cycle #, instead of guessing

Appendix IV - L#clip2.0 barcodes (in the Davis lab)

Lclip adapters are ligated to the antisense cDNA - take reverse complement sequence for demultiplexing.
All 20 adapters have been tested to work well (March 2022 - JL)

L#clip2.0	Barcode	Barcode read from sequencing (rev-com)
L01	ATCACG	CGTGAT
L02	CGATGT	ACATCG
L03	TTAGGC	GCCTAA
L04	TGACCA	TGGTCA
L05	ACAGTG	CACTGT
L06	GCCAAT	ATTGGC

L07	CAGATC	GATCTG
L08	ACTTGA	TCAAGT
L09	GATCAG	CTGATC
L10	TAGCTT	AAGCTA
L11	ATGAGC	GCTCAT
L12	CTTGTA	TACAAG
L13	AGTCAA	TTGACT
L14	AGTTCC	GGAACT
L15	ATGTCA	TGACAT
L16	CCGTCC	GGACGG
L17	CAACTA	TAGTTG
L18	GTCCGC	GCGGAC
L19	GTGAAA	TTTCAC
L20	CACCGG	CCGGTG