KH Richau 2011

Strand-specific cDNA library preparation

Throughout this protocol RNase/ DNase free H_2O, as well as low binding nucleotide free tubes and filter tips are used.

Isolation of total RNA via Trizol reagent procedure:

Input ~100mg of fresh weight
Pre- cool 4°C centrifuge

- Add 100µl Trizol reagent to the plant material in a low binding 1.5ml reaction vial
- Grind with a Destroy stick to homogenize tissue & rinse the stick with 900µl Trizol. Final volume ~1000µl. Vortex.
- Spin 5' at 14.000RPM at 4°C to remove the big chunks of tissue.
- Transfer the supernatant to a new vial & leave for 5' at RT.
- Add 200µl CHCl3 (Chlorophorm) vortex & leave for 5' RT
- Spin at 14.000RPM at 4°C for 15'. The top aqueous phase contains the RNA, the interface the DNA, and the organic phase in the bottom mainly contains proteins.
- Remove the upper aqueous phase (~600µl), transfer to a new vial& add 500µl isopropanol
- Let stand for 10' at RT, mix by inverting & spin for 10' at 14.000RPM at 4°C. RNA should form a pellet at the bottom of the tube.
- Pour of supernatant (dap on tissue paper) & wash with 1ml of 75% EtOH by vortexing. Spin 5' at 9.000RPM at 4°C
- Pour off EtOH (dap on tissue paper& let air dry for~ 10')
- Resuspend the pellet in 25µl RNase free water by gently pipetting up and down

NanoDrop measurements

- Proceed immediately with mRNA isolation or freeze in 80°C until further processing

STOPPING POINT

Purification of mRNA from total RNA via double Dynabead isolation procedure

Wash / calibrate Dynabeads

- Resuspend Dynabeads thoroughly in the vial to obtain a uniform brown suspension & transfer 200µl (1mg) of beads to a new vial
- Place the tube on a magnetic stand for 1-2 min & remove the supernatant

- Remove the tube from the stand and add 100µl of bead binding buffer to resuspend the beads
- Place again on a magnetic stand & remove the supernatant
- Finally resuspend the beads in 100µl binding buffer for further application

mRNA purification procedure

Preheat water bath to 65°C
Preheat heating block to 75-80°C

Input ~ 75µg of total RNA

- Adjust the volume of the 75µg total RNA sample to 100µl with distilled H_2O or 10mM Tris-HCL, pH 7.5
- Add 100µl of binding buffer & heat to 65°C for 2 min to disrupt secondary structure. Immediately place on ice!
- Add 200 µl of total RNA to the 100µl of washed beads (for every 75µg of total RNA use 1mg of beads resuspended in 100µl binding buffer)
- Mix thoroughly and anneal by continuously rotating for 5' on a mixer at RT
- Place the tube on magnetic stand and carefully remove supernatant
- Remove the tube from the magnetic stand and add 200µl washing buffer. Mix by pipetting carefully up & down
- Place the tube on magnetic stand again and carefully remove all the supernatant
- Repeat washing once more
- For first RNA elution add 50µl of cold Tris-HCL (pH7.5), heat the sample to 75-80°C for 2' and place the tube immediately on the magnetic stand to quickly remove the eluted mRNA into a new vial
- Wash the beads one time with 300µl binding buffer and resuspend in 100µl binding buffer to reuse them on the same sample again
- Add 50µl Tris-HCL & 100µl binding buffer to the eluted RNA & heat to 65°C for 2 min to disrupt secondary structure. Immediately place on ice!
- Add 200 µl of total RNA to the 100µl of washed beads
- Mix thoroughly and anneal by continuously rotating for 5' on a mixer at RT
- Place the tube on magnetic stand and carefully remove supernatant
- Remove the tube from the magnetic stand and add 200µl washing buffer. Mix by pipetting carefully up & down
- Place the tube on magnetic stand again and carefully remove all the supernatant
- Repeat washing once more
- If RNA elution is required, add the desired amount (20µl) of cold Tris-HCL (pH7.5), heat the sample to 75-80°C for 2' and place the tube immediately on the magnetic stand to quickly remove the eluted mRNA into a new vial.

NanoDrop measurement

- Freeze in 20°C until further processing

STOPPING POINT

DNase –free: DNase treatment and removal reagent

Preaheat heating block to 37°C

- Add 2µl of 10X DNaseI buffer to 20µl isolated mRNA
- Use 0.5µl of DNase I (2U) for up to 10µg of RNA in a 20µl reaction volume. (These reaction conditions will remove up to 2µg of genomic DNA from total or mRNA in a 20µl reaction volume.)
- Incubate at 37°C for 20-30'
- Add 2µl resuspended DNase inactivation reagent (typically 0.1 volume) and mix well. (Use at least 2µl DNase inactivation reagent even it that is more than 0.1 volume of the reaction volume)
- Incubate 2' at RT with occasionally mixing
- Spin at 10.000RPM for ~1.5' & carefully transfer the RNA to a fresh tube.

  (NanoDrop measurement)

- Freeze in 20°C until further processing

STOPPING POINT

strand specific cDNA library preparation via the NEB Next kit

Input 100-500µg of purified mRNA

mRNA fragmentation

Pre-cool 4°C centrifuge

Mix the following components in a sterile PCR tube:

Purified mRNA	1-18µl
10X RNA Fragmentation buffer	2µl
Nuclease free water	variable
Total volume	20µl

- Incubate in a preheated thermal cycler for exactly 5' @ 94°C
- Transfer tube to ice
- Add 2µl 10X RNA stop solution

Ethanol precipitation

Mix the following components:

Fragmented mRNA	22µl
3M Sodium Acetate pH 5.2	2µl
linear acrylamide	1-2µl
100% EtOH	60µl
Total volume	85-86µl

- Freeze @ -80°C for 30' (or ON)
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STOPPING POINT

- Spin at 14.000RPM for 25' @ 4°C
- Carefully remove EtOH & wash pellet with 200µl 70% EtOH
- Spin again for 5' & carefully remove EtOH
- Air dry pellet for up to10' at RT to remove residual EtOH
- Resuspend in 13,5µl Nuclease-free H_2O

First strand cDNA synthesis

Mix the following components:

Fragmented mRNA	13.5µl
Random primers	1µl
Total volume	14.5µl

- Incubate for 5' @ 65°C
- Spin briefly & put on ice

To the fragmented mRNA and the random primer mix add:

5X first strand synthesis buffer	4µl
murine RNase inhibitor	0.5µl
Total volume	19µl

- Incubate for 2' @ 25°C
- Add 1µl SuperScript II reverse Transcriptase to the reaction
- Incubate sample as followed:

10' @ 25°C
50' @ 42°C
15' @ 70°C
hold @ 4°C

- Place tube on ice

Second strand synthesis for strand specific cDNA library

First remove dNTPs via PCIA (25:24:1)

Mix the following components in a 1.5ml tube:

RNA/DNA hybrid of the first strand reaction	20µl
H_2O	80µl
PCIA	100µl
Total volume	200µl

- Vortex for 15 seconds & and spin at 13.000 for 15'
- Transfer upper aqueous phase (~120µl) to new vial and precipitate RNA/DNA hybrid by adding

3M NaAc	10µl
EtOH 100%	250µl

- Freeze for at least 30' @ -80°C or ON

STOPPING POINT

Pre-cool 4°C centrifuge

- Spin at 14.000RPM for 25' @ 4°C
- Carefully remove EtOH
- Wash with 500µl 70% EtOH & spin again for 5'
- Carefully remove EtOH & air dry pellet for ~ 10min at RT
- Resuspend pellet in 59.7µl H_2O

Mix the following components in a PCR tube (Mastermix):

Purified RNA-DNA hybrid	59.7µl
5X FSS buffer	4µl
SSS buffer (-dNTPs)	8µl
dNTPS (10mM each)	2.3µl
DNA ligase (E. coli)	1µl (10U)
RNase H	1µl (2U)
DNA polymerase I (E. coli)	4µl (40U)
Total volume	80µl

- Incubate 2.5 h @ 16°C
- Purify cDNA using a PCR column purification kit & elute with 50µl H_2O

NanoDrop measurements

STOPPING POINT

End Repair of cDNA library

Mix the following components in a PCR tube:

Purified double-stranded cDNA	50µl
Nuclease-free H2O	25µl
10X Phosphorylation reaction buffer	10µl
dNTP solution mix	4µl
T4 polymerase	5µl
E.coli DNA polymerase I, large (Klenow) fragment	1µl
T4 Polynucleotide kinase	5µl
Total volume	100µl

- Incubate 30'h @ 20°C
- Purify end-repaired cDNA using a PCR column purification kit & elute with 32µl H_2O

dA-tailing of the library

Mix the following components in a 1.5ml tube:

Purified, end-repaired cDNA	32µl
10X NEB buffer 2	5µl
dATP (1mM)	10µl
Klenow fragment (3'-> 5' exo-)	3µl
Total volume	50µl

- Incubate 30'h @ 37°C
- Purify end-repaired cDNA using a PCR column purification kit & elute with 23µl H_2O

Adaptor ligation

Mix the following components in a PCR tube:

Purified dA-tailed cDNA	23µl
2X Quick ligation reaction buffer	25µl
DNA adaptor (15µM)	1µl
Quick T4 DNA ligase	1µl
Total volume	50µl

- Incubate 15'h @ RT
- Purify adaptor- ligated cDNA using a PCR column purification kit & elute with 10µl H_2O

Stopping point

Size selection of cDNa library

Soak gel tray & comb for 30' in 400mM NaOH for cleaning

Pre-heat heating block to 50°C

- Run adaptor ligated cDNA library (10µl sample + 2.5µl Orange G loading dye) on a 3% agarose gel
- Cut desired cDNA fragments size from the gel

1st fragment between 200-300bp
(optinal 2nd fragment between 400-500bp)

- Purify gel slices using a gel extraction column purification kit & elute with 30µl H_2O

NanoDrop measurements

Second strand degradation

Mix the following components in a PCR tube:

Size selected cDNA	29µl
2U USER enzyme	2µl

- Incubate 15' @ 37°C
- Stop reaction @ 95°C for 5'
- Purify cDNA with AmPURE beads
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- Mix beads (reaction volume * 1,8 = beads volume) with PCR product in a PCR tube by thoroughly pipetting 10x up& down & incubate for 5' at RT
- Place the tube on magnetic plate for 2' & discard supernatant
- ON the magnetic plate: wash the beads with 200µl 80% EtOH incubate for 30 seconds at RT
- Repeat wash for a total of two washes
- Air dry pellet for up to10' at RT to remove residual EtOH
- OFF the magnetic plate: add 30µl Resuspension buffer (TRIS Acetate buffer pH 8.0 r TE buffer) & mix thoroughly by 10x pipetting up & down
- Place on the magnetic plate for 1' & elute with 30µl Resuspension Buffer (10mM Tris-Acetate pH 8.0 or TE buffer)

NanoDrop measurements

PCR enrichment of adaptor ligated cDNA library

Mix the following components in a PCR tube (Mastermix):

Size selected cDNA	30µl
5X Phusion HF buffer	10µl
Primer 1 (25µM)(PE1)	1µl
Primer 2 (25µM)(PE2)	1µl
dNTP mix	1.5µl
H_2O	6µl
Phusion DNA polymerase	0.5µl
Total volume	50µl

PCR cycling conditions

Initial denaturation	98°C	10 seconds	
Denaturation	98°C	10 seconds	
Annealing	65°C	30 seconds	15 cycles
Extension	72°C	30 seconds	
Final extension	72°C	5'	
Hold	4°C		

- Purify PCR enriched cDNA with 90µl AMPure beads (reaction volume * 1,8 = beads volume)& elute in 30µl Resuspension Buffer (10mM Tris-Acetate pH 8.0 or TE buffer)

Library is ready for BIOANALYZER check (DNA1000 or high sensitivity chip) subsequent to sequencing event

Optional

Check product on an agarose gel
Therefore amplify 1µl library additional 35 cycles with GoTAQ polymerase & check for visible band

If library concentration is low: amplifiy with PHUSION polymerase additional 7 cycles for Bio Analyzer high sensitivity chip.

Verification of strand specificity of the library by SANGER sequencing

Additional 30cycles PCR (see above) for cloning into pGEM vector (AT cloning)

Therefore, an A-tailing of amplified cDNA libraries is necessary, since proof reading polymerase produces blunt end DNA

A- tailing of amplified cDNa libraries

- Purify PCR enriched cDNA using a PCR column purification kit & elute with 40µl H_2O

Mix the following components in a PCR tube:

Purified PCR fragments	15µl
Tag polymerase buffer 10X	2µl
dATP (25mM)	0.2µl
Taq polymerase	2µl
H_2O	1.8µl
Total volume	20µl

- Incubate for 20-30' @ 70°C

Size selection of the extra amplified, A-tailed cDNA library

- Add 20% (4µl) orange-G loading dye (Orange –G in 50% glycerol =6x) to the amplified cDNA & run A-tailed cDNA library on a 2% agarose gel and isolate desired cDNA fragments size from the gel
- Purify gel slices using a gel extraction column purification kit & elute with 30µl H_2O

Ligation with pGEM easy vector

- Calculate insert: vector ration (3:1) according to the following equation;

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

Reaction Component	Standard Reaction	Positive Control	Background Control
2X Rapid Ligation Buffer, T4 DNA Ligase	5µl	5µl	5µl
pGEM®-T or pGEM®-T Easy Vector (50ng)	1µl	1µl	1µl
PCR product	Xµl*	–	–
Control Insert DNA	–	2µl	–
T4 DNA Ligase (3 Weiss units/µl)	1µl	1µl	1µl
nuclease-free water to a final volume of	**10µl**	**10µl**	**10µl**
*Molar ratio of PCR product:vector may require optimization.			

- Incubate the reactions for 1h @ RT or ON at 4°C
- Prepare LB/Carbenicillin plates (1:100 delution of Carb)

Next day

Preheat water bath at exactly 42°C for chemical transformation

Prepare LB/Carb/IPTG/X-Gal plates
- Spread on each plate 100µl IPTG & X-Gal and 8µl Carb

Chemical transformation

- Spin ligation mix briefly
- Add 2µl ligation mix to a sterile 1.5ml tube
- Place chemical competent cells on ice until thawed (5') &mix them by gently flicking
- Carefully transfer 50µl of cells to each ligation mix tube (use 100µl for DNA control)
- Incubate for 20' on ice
- Heat shock cells for 45-50 sec at exactly 42°C & the immediately chill transformation mix on ice for 2'
- Add 950µl RT LB medium to transformation mix (900µl for DNA control) & incubate for 1.5h @37°C with mild shaking (~150rpm)

Electro transformation
- Spin ligation mix briefly
- Add 1µl ligation mix to a sterile 1.5ml tube
- Place electro competent cells on ice until thawed (5') & mix them by gently flicking
- Dilute cells 1:5 (5µl cells: 20µl ice cold H_2O) & carefully transfer cells to each ligation mix tube (use 1µl for control plasmid)
- Electroporate at 1500Volts (2.2 V, 200Ohm, 25µF) in 1mm cuvette (time

constant should be 4-5 msec for successful transformation)
- Rinse cuvette with 450µl SOC medium to the transformation mix & incubate for1.5h @37°C with mild shaking (~150rpm)

- Plate out 100µl of each transformation mix onto LB/Carb/IPTG/X-Gal plates (from the DNA control plate out a 1:10 dilution in SOC medium)
- Incubate the plate @37°C ON

(optional keep transformation mix on ice ON for additional plating next day if yield is low)

Next day

Screen for transformants via blue/white selection

Prepare PCR tubes with 50µl H_2O

- Pick white colonies with a sterile toothpick (& blue ones as control) and dip the toothpick in 50µl H_2O
- Vortex & heat shock 5' @95°C
- Spin 5' @ 13000rpm

Split transformation reaction into two parts;
1st part for colony PCR (5µl) &
2nd part (15µl) for OV cultures of the transformants

Colony PCR

Mix the following components in a PCR tube:

cDNA from colony suspension	5µl
10X Dream Taq buffer	5µl
primer 1 (10mM) (T7)	0.8µl
primer 2 (10mM) (SP6)	0.8µl
dNTP mix	0.75µl
H_2O	8µl
Dream Taq DNA polymerase	0.25µl
Total volume	15µl

Colony PCR conditions

Initial denaturation	94°C	10 seconds	
Denaturation	98°C	10 seconds	
Annealing	52°C	30 seconds	35 cycles
Extension	72°C	30 seconds	
Final extension	72°C	5’	
Hold	4°C		

- add 20% 6x Orange- G loading dye to the enriched DNA
- load on a 2% agarose gel to check for desired fragments

Positive bands give indication for right transformation, meaning that the associated ON culture can be used for MiniPrep application to gain DNA for sequencing of the strand specific library in direct comparison to the non-strand specific one to verify the specificity.