Strand-specific cDNA library preparation

Throughout this protocol RNase/ DNase free H₂O, 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 H₂O 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

Preheat 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 if 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)

**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 to 10’ at RT to remove residual EtOH
• Resuspend in 13,5µl Nuclease-free H₂O

**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<sub>2</sub>O**: 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<sub>2</sub>O

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<sub>2</sub>O

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 H2O

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 H2O

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 H2O

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
  (optional 2nd fragment between 400-500bp)

• Purify gel slices using a gel extraction column purification kit & elute with 30μl H2O

**NanoDrop measurements**

**Second strand degradation**

Mix the following components in a PCR tube:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size selected cDNA</td>
<td>29μl</td>
</tr>
<tr>
<td>2U USER enzyme</td>
<td>2μl</td>
</tr>
</tbody>
</table>

• Incubate 15’ @ 37°C
• Stop reaction @ 95°C for 5’
• Purify cDNA with AmPURE beads
  • 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):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size selected cDNA</td>
<td>30μl</td>
</tr>
<tr>
<td>5X Phusion HF buffer</td>
<td>10μl</td>
</tr>
<tr>
<td>Primer 1 (25μM)(PE1)</td>
<td>1μl</td>
</tr>
<tr>
<td>Primer 2 (25μM)(PE2)</td>
<td>1μl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>1.5μl</td>
</tr>
<tr>
<td>H₂O</td>
<td>6μl</td>
</tr>
<tr>
<td>Phusion DNA polymerase</td>
<td>0.5μl</td>
</tr>
</tbody>
</table>

Total volume: 50μl

**PCR cycling conditions**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>98°C</td>
<td>10 sec</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98°C</td>
<td>10 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>65°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>5 sec</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td></td>
</tr>
</tbody>
</table>

- 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: amplify with PHUSION polymerase additional 7 cycles for Bio Analyzer high sensitivity chip.
**Verification of strand specificity of the library** by SANGER sequencing

Additional 30 cycles 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 40 µl H₂O

Mix the following components in a PCR tube:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified PCR fragments</td>
<td>15 µl</td>
</tr>
<tr>
<td>Tag polymerase buffer 10X</td>
<td>2 µl</td>
</tr>
<tr>
<td>dATP (25 mM)</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>2 µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>1.8 µl</td>
</tr>
</tbody>
</table>

Total volume 20 µl

- Incubate for 20-30’ @ 70°C

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

- Add 20% (4 µl) 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 30 µl H₂O

**Ligation with pGEM easy vector**

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

\[
\text{ng of vector} \times \frac{\text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}
\]
### Reaction Component Table

<table>
<thead>
<tr>
<th>Reaction Component</th>
<th>Standard Reaction</th>
<th>Positive Control</th>
<th>Background Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Rapid Ligation Buffer, T4 DNA Ligase</td>
<td>5µl</td>
<td>5µl</td>
<td>5µl</td>
</tr>
<tr>
<td>pGEM®-T or pGEM®-T Easy Vector (50ng)</td>
<td>1µl</td>
<td>1µl</td>
<td>1µl</td>
</tr>
<tr>
<td>PCR product</td>
<td>Xµl*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control Insert DNA</td>
<td>-</td>
<td>2µl</td>
<td>-</td>
</tr>
<tr>
<td>T4 DNA Ligase (3 Weiss units/µl)</td>
<td>1µl</td>
<td>1µl</td>
<td>1µl</td>
</tr>
<tr>
<td>nuclease-free water to a final volume of</td>
<td>10µl</td>
<td>10µl</td>
<td>10µl</td>
</tr>
</tbody>
</table>

*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 H₂O) & carefully transfer cells to each ligation mix tube (use 1µl for control plasmid)
- Electroporate at 1500Volts (2.2 V, 2000hm, 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 for 1.5h @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 H₂O

- Pick white colonies with a sterile toothpick (& blue ones as control) and dip the toothpick in 50µl H₂O
- 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:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA from colony suspension</td>
<td>5µl</td>
</tr>
<tr>
<td>10X Dream Taq buffer</td>
<td>5µl</td>
</tr>
<tr>
<td>primer 1 (10mM) (T7)</td>
<td>0.8µl</td>
</tr>
<tr>
<td>primer 2 (10mM) (SP6)</td>
<td>0.8µl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>0.75µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>8µl</td>
</tr>
<tr>
<td>Dream Taq DNA polymerase</td>
<td>0.25µl</td>
</tr>
</tbody>
</table>

**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.