Protocols for siRNA Sequencing v2.0

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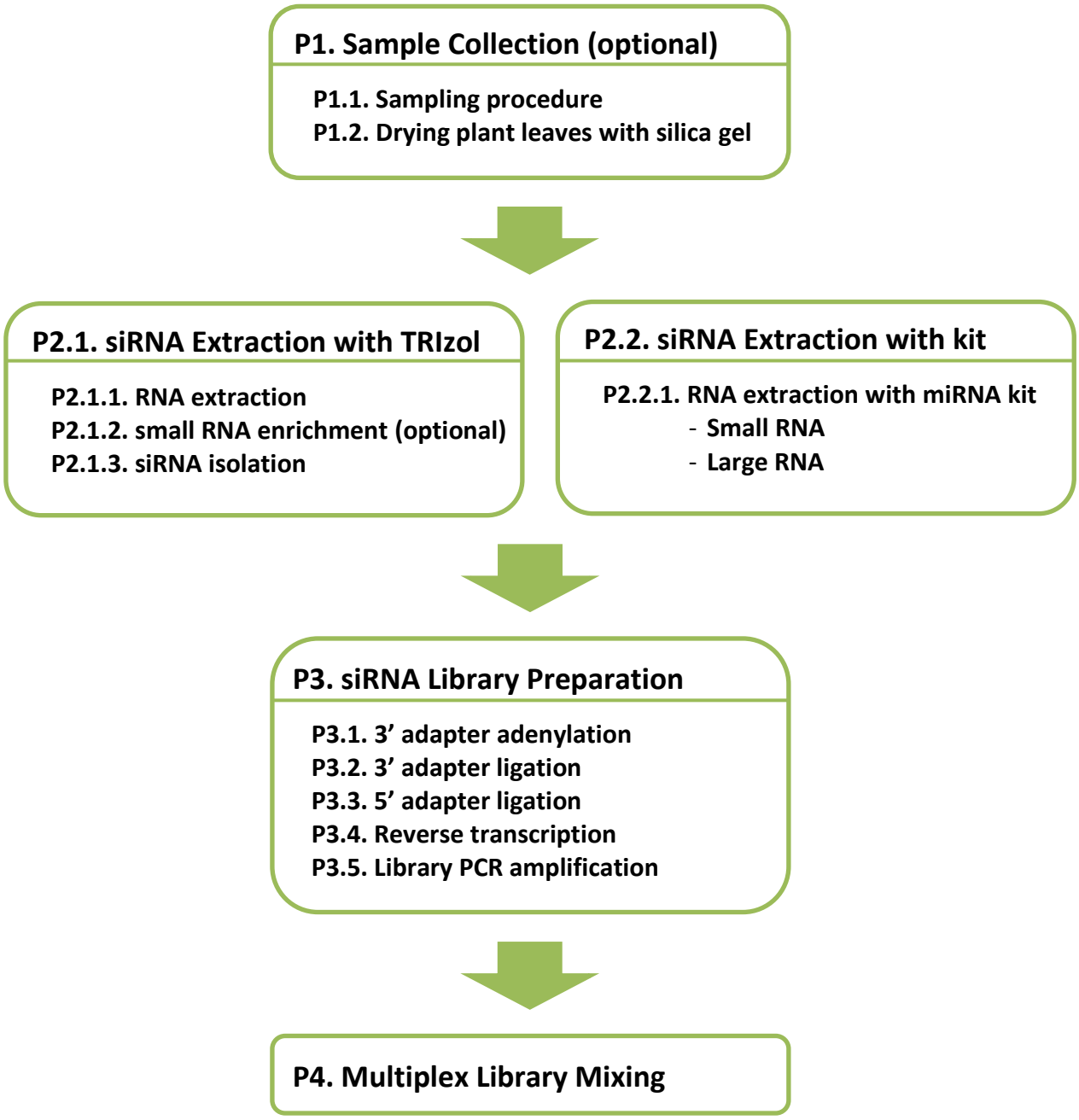


P1. Sample Collection (optional)

P1.1. Sampling procedure

In each field visited, record the GPS coordinates including latitude, longitude and altitude, do this by saving the waypoint on the GPS machine (use the saved waypoint as the field number, e.g. waypoint 01 is field 01, at the end of the survey transfer all waypoint data to a computer, these can be used to double check GPS data), and by noting it in the sampling sheet (Fig. 1) and on the barcode sticker. The same coordinates can be used for all the samples of the same field (no need to take new coordinates for each plant). Find 5-10 sweetpotato plants at random by walking a straight diagonal line through the field, if there are any plants with symptoms be sure to include them, but also include some symptomless plants. It is also permitted to take few samples (in addition to the sweetpotato samples) from other crops or Ipomoea weeds if they are grown together or in close proximity with sweetpotato crops. Before sampling, take an overview picture of each field (Fig. 2A) and note down the picture number (on the camera) on the sampling sheet (one picture per field is enough). Of each plant that you take a sample, take a picture of the entire plant (Fig. 2C) using a white umbrella to shade the plant (this diffuses the light and gives better picture results), and the grey background board with color chart, size measure and corresponding barcode (with GPS data on it, the stickers are removable and can be moved from the background board to the coffee filters, the color chart and size measure are attached to the board with a 'blue-tag' and can be removed to clean the board, which should be done at least at the end of each day, it gets dirty!). Then, take a close-up of the sampled leaves (three) on the background board (secures with painter tape) with the same barcode (Fig. 2D). Take the samples from the first and second fully expanded fresh looking leaves (usually 3rd or 4th leaf from the apex) and one old leaf with clear symptoms (if present). If the leaf is wet, use paper towel to dry it first, before taking the picture. Transfer the three leaves into coffee filters (one filter per leaf) securing them with staple and label with the same barcode sticker (with GPS data). Place the coffee filters into a Ziploc bag containing silica gel (~100 g). Keep the Ziploc bags in an airtight plastic container in a dry place (22-28°C). Record the data into the sampling sheets. After one or two days, replace the moisture saturated silica gel with another 100 g of dry silica gel. During the drying process, preserve the leaves as green as possible. When leaves are dry, replace the moisture saturated silica gel with a small amount of dry silica. Per each sample, send two dried leaves to Lima for further processing; the third dried leaf will be kept by our collaborator as a backup.

Use a tripod and a digital camera with at least 4 megapixels and zoom/macro options; save images in **raw or tiff format only**. Do not use flash. Photos should be taken in a perpendicular plane to avoid possible distortions, always check that the barcode and GPS data on the sticker are sharp after taking the picture.

Materials:

- Sampling sheet
- GPS
- Digital camera
- Tripod
- Big umbrella (preferably white)
- Grey background board
- Color chart
- Size measure
- Barcode sticker
- Blue tag
- Painter tape
- Paper towel
- Coffee filters
- Ziploc bags
- Airtight plastic container
- Silica gel

Microsoft Excel - Sampling Sheet-Ethiopia

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	
1	Sampling Protocol		ETHIOPIA	Sweetpotato	Person in charge of sampling:												
2	Sample number	Date dd/mm/yyyy	Region	District	Locality	Field number	Longitude	Latitude	Altitude (m)	Crop age (months)	Field size	Photo No. field	Photo No. plant	Photo No. leaf	Intercrop	Cultivar/ species	Notes
3	ET-001																
4	ET-002																
5	ET-003																
6	ET-004																
7	ET-005																
8	ET-006																
9	ET-007																
10	ET-008																
11	ET-009																
12	ET-010																
13	ET-011																
14	ET-012																
15	ET-013																
16	ET-014																
17	ET-015																
18	ET-016																

Figure 1. Sampling Sheet

Figure 2. Sweetpotato leaf collection in Ethiopia. A) Field picture, B) Taking plant and leaf pictures, C) Plant picture and D) Leaf picture.

P1.2. Drying plant leaves with silica gel

Collect fresh plant leaves and immediately transfer them to coffee filters (one coffee filter per leaf). If leaves are wet, use paper towel to dry them before transfer to the coffee filters. Place the coffee filters in a Ziploc bag containing approx. 100 g orange silica gel (Fig. 3). Keep the Ziploc bags in an airtight plastic container (Fig. 4) in a dry place (22-28°C). After one or two days, replace the moisture saturated silica gel (pale) (Fig. 5) with another 100 g of orange silica gel. During the drying process, preserve the leaves as green as possible, and the best way to keep them green is to dry the leaves quickly. For this reason, it is critical to replace the moisture saturated silica with orange silica gel as frequent as possible. When the leaves are dry, replace the moisture saturated silica gel with a small amount of the orange one.

Materials:

- Coffee filter #4: one coffee filter per leaf
- Ziploc bags (17.5x20 cm): One bag (9 coffee filters= 9 leaves per bag)
- Orange silica gel: approx. 100 g per bag
- Plastic containers

Figure 3. Orange silica gel

Figure 4. Airtight plastic container

Reactivating silica gel:

Spread the moisture saturated silica gel (pale) (Fig. 5) in a glass tray; place this in a warm oven at 100°C for several hours (approx. two days) and stir occasionally. When the silica gel returns to its original bright orange color (Fig. 3), it is dry. Store silica gel in an airtight container until it is used again.

Materials:

- Oven (at 100°C)
- Glass tray (pyrex)

Figure 5. Moisture saturated silica gel

P2.1. siRNA Extraction

P2.1.1. RNA extraction: TRIzol Reagent - Sweetpotato

1. Collect 250 mg fresh leaves (or 40 mg dried leaves) and place it in plastic bags
2. Add 1.5 ml TRIzol, grind tissues with a hand roller, incubate the samples at room temperature for 5 min, and then transfer to 2 ml microtubes
3. Centrifuge at 12,000 rpm, for 5 min at room temperature. Take 1 ml supernatant to a new 2 ml microtube
4. Add 500 µl Chloroform (per 1ml TRIzol), shake vigorously (do not vortex), and incubate for 3 min at room temperature (or at 4°C)
5. Centrifuge at 12,000 rpm, for 10 min at 4°C
6. Transfer the aqueous phase (500 µl) to a new 1.5 ml microtube, precipitate with isopropanol (1:1) (500 µl), and incubate for 10 min at room temperature (or at 4°C)
7. Centrifuge at 12,000 rpm, for 10 min at 4°C
8. Wash the pellet with 1 ml of 75% ethanol, and centrifuge at 12,000 rpm for 10 min at 4°C
9. Air-dry the pellet, and then keep it at -70°C

Materials:

- Plastic (crystal clear polyethylene) bags 4"x6"x6
- Hand roller
- Micropipettes
- Microcentrifuge
- Refrigerator/Freezer
- 2 ml microtubes
- 1.5 ml microtubes
- 1 ml tips
- TRIzol Reagent (Invitrogen, Cat.No. 15596018, 15596026)
- Chloroform
- Isopropanol (2-propanol)
- Ethanol

P2.1.2. small RNA enrichment (optional)

1. Suspend the total RNA pellet in 250 µl nuclease free water (NFW)

2. Precipitate the total RNA by adding 250 µl of 4 M LiCl (1:1). Incubate the samples overnight on ice in the cold room (~ 4°C)

3. Centrifuge the sample at 12,000 rpm for 20 min at 4°C. Transfer the supernatant (containing small size RNAs) to a new 1.5 ml microtube (continue to step 4). Rinse the pellet (containing large size RNAs) with 75% Ethanol, centrifuge at maximum speed (14,000 rpm) for 10 min at 4°C. Rinse twice with ethanol (600 µl). Discard the supernatant, spin down and carefully remove the residual ethanol. Air-dry the pellet (downside, 10 min; lateral, 30 min) and store at -70 °C

4. Precipitate the supernatant (containing small size RNAs) by adding 1 volume of isopropanol (~ 500 µl). Incubate at room temperature (RT) for 10 min

5. Centrifuge the sample at maximum speed for 20 min at 4°C

6. Remove supernatant and rinse pellet with 75% ethanol (500 µl)

7. Centrifuge at maximum speed for 10 min at 4°C

8. Air-dry the pellet and store at -70°C

Materials:

- Micropipettes
- Microcentrifuge
- Refrigerator/Freezer
- 1.5 ml microtubes
- 1 ml tips
- 200 µl tips
- Nuclease free water (NFW)
- Lithium Chloride (LiCl)
- Isopropanol (2-propanol)
- Ethanol

P2.1.3. siRNA isolation

1. Run Total RNA (10 – 35 µg) or small RNA (10 – 17 µg) on a 3.5% agarose gel

3.5% Agarose Gel

- 14 g UltraPure Agarose (Invitrogen-Cat. 16500-500)
- 400 ml 1x TAE buffer (optional: prepare buffer with DEPC treated water)
- 2 µl GelRed (Precast) to be added to the molten agarose

Cleaver tray: 20x25 cm (multiSUB *maxi*)
No. samples: 12

	Upper Gel																			
20-well comb	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
(Red, 5mm, 1.5mm)		1			2			3			4			5			6			M
	Lower Gel																			
20-well comb	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
(Red, 5mm, 1.5mm)		7			8			9			10			11			12			M

Sample mix

- 32 µl sRNA sample
- 5 µl RNA formamide loading buffer (2x)
- 1 µl Diluted GelRed (1/10)

Marker mix (M)

- **1.5µl dsRNA marker**
- **5µl RNA formamide loading buffer 2X**
- **1µl Diluted GelRed (1/10)**
- **7.5µl NFW**

DNA loading buffer salB 10x

- 150 mg Bromophenol blue
- 150 mg Xylene Cyanol
- 200 mg Orange G
- 5 ml 10x TBE
- 60 g Sucrose
- Add sterile dH_2O up to 100 ml

RNA formamide loading buffer (2x)

- Formamide 3V
- DNA loading buffer salB 10x 1V
- DEPC treated water 1V

Diluted GelRed (1/10)

- 1.8 µl GelRed
- 16.2 µl Nuclease free water (NFW)

Running conditions

- 1x TAE buffer (optional: prepare buffer with DEPC treated water), 160 V, 265-380 mA, 2h

2. Excise the smallest band (Fig. 6) and place on a petri dish, chop into pieces and transfer to a Freeze N squeeze DNA gel extraction spin column

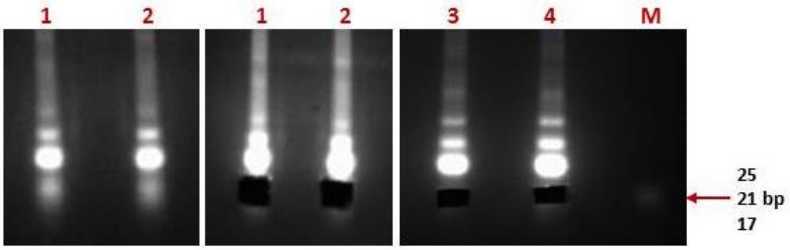


Figure 6. siRNA isolation on a 3.5% agarose gel in 1x TAE buffer. small RNA samples (1-4) and siRNA marker (M).

Note: The exposure of nucleic acids to UV light should be done rapidly because long exposures cause significant damage (degradation) to the sample.

3. Freeze the column at -20°C for no more than 20 min

4. Centrifuge at maximum speed (14,000 rpm) for 5 min at room temperature (RT)

5. Collect the flow-through and bring the volume up to 500 µl with nuclease free water (NFW). Add 0.5 volume (250 µl) of chloroform, mix vigorously and incubate on ice for 3 min. Centrifuge at 12,000 rpm for 10 min at 4°C

6. Transfer upper aqueous phase (400 µl) to a new 1.5 ml microtube and add 1 µl of glycogen (2 µg/µl), then precipitate with 2.5 volume (1 ml) of 100% ethanol and 0.1 volume (40 µl) of 3 M NaAc (pH 4.8-5.2). Incubate at -70°C for 1 h (or -20°C overnight)

7. Centrifuge at maximum speed for 20 min at 4°C

8. Remove supernatant and rinse pellet with cold 75% ethanol (600 µl)

9. Centrifuge at maximum speed for 10 min at 4°C

10. Carefully, remove the ethanol by pipetting and then spin down to remove residual ethanol

11. Air-dry the pellet at RT for ~20 min and store at -70°C

12. Resuspend the pellet in **15 µl** NFW just before library preparation

Materials:

- Micropipettes
- Microcentrifuge
- Refrigerator/Freezer
- Gel Doc/ UV transilluminator
- 1.5 ml microtubes
- 1 ml tips
- 200 µl tips
- 10 µl tips
- Blades (#11)
- Petri dishes
- Freeze N squeeze columns (BIORAD, Cat.No. 732-6166)
- Agarose Ultrapure (Invitrogen, Cat.No. 15510-027)
- 1x TAE buffer
- GelRed (Biotium, Inc. Cat.No. 41003)
- RNA formamide loading buffer (2x)
- dsRNA marker (NEB, Cat.No. N0363S)
- Nuclease free water (NFW)
- Chloroform
- Glycogen (Invitrogen, Cat.No. 10814-010)
- Ethanol
- Sodium Acetate (NaAc)

P3. siRNA Library Preparation

Modified from Chen et al., 2012.

Chen, Y.; Zheng, Y.; Liu, B.; Zhong, S.; Giovannoni, J.; Fei, Z. 2012. A cost-effective method for Illumina small RNA-Seq library preparation using T4 RNA ligase 1 adenylated adapters. Plant Methods. 8:41. doi: 10.1186/1746-4811-8-41.

P3.1. 3’ adapter adenylation

1. Dissolve the 3' adapter oligo (5’ phosphorylated and 3’ blocked DNA oligo) in **Elution Buffer (10 mM Tris-HCl, pH 8.5)** to a final concentration of **100 µM**

2. Set up a reaction in 0.2 ml PCR tube containing:

No. of reactions:		1	2	
		Adenyl. Rx (µl)	Master Mix (µl)	
Components	**Volume:**	**100**	**100**	**Final Concentration**
3' adapter oligo (stock 100 µM)		10	20	10 µM 3' adapter oligo
10x T4 RNA ligase buffer		10	20	1x T4 RNA ligase buffer
10 mM ATP		10	20	1 mM ATP
Nuclease Free Water		20	40	---
50% PEG 8000		40	---	20% PEG 8000
T4 RNA ligase 1 (stock 10 U/µl)		10	---	1 U/µl T4 RNA ligase 1
			* Make aliquost of:	50 µl Master Mix
			To each aliquot add:	40 µl 50% PEG 8000
				10 µl T4 RNA ligase 1

*

Mix by pipetting and incubate overnight at 25°C in a thermal cycler (cover heating set at 50°C)

Note: In this reaction, T4 RNA ligase 1 will first adenylate the 5’ phosphorylated DNA oligo and attempt to join it with a suitable acceptor molecule with a 3’ hydroxyl group. The 3’ end of the oligo is block with amine and could not take part in the ligation reaction, leading to accumulation of the adenylated intermediate.

3. Optional: set up multiple reactions if more adenylated adapters are required

4. Purify the adenylated 3' adapter with Chloroform and Ethanol precipitation

   4.1. Bring the sample volume up to 600 µl with nuclease free water (NFW), mix by pipetting. Then, add 300 µl Chloroform. Mix vigorously (20 seconds)

   4.2. Centrifuge at maximum speed (14,000 rpm) for 5 min at 4°C

   4.3. Take 500 µl of the aqueous phase; add 500 µl isopropanol and 2 µl glycogen (2 µg/µl)

   4.4. Incubate at room temperature for 10 min, then, continue incubating at -20°C for 1 hour

4.5. Centrifuge at maximum speed for 30 min at 4°C

4.6. Rinse the pellet (twice) with 75% ethanol (800 µl)

4.7. Centrifuge at maximum speed for 10 min at 4°C

4.8. Remove the supernatant (by pipetting), spin down and carefully remove residual ethanol by pipetting

4.9. Air-dry the pellet (10 min-down side; 40 min-lateral)

4.10. Resuspend the pellet in 30 µl NFW (from a 100 µl Adenyl. reaction)

4.11. Determine the concentration in a nanodrop (ssDNA-33)

4.12. Adjust the adenylated 3' adapter concentration to 10 µM with NFW

MW:	6,451.20		Final Conc. (µM):	10
Conc. (ng/µl)*	Conc. (µM)	Volume (µl)	Final Volume (µl)	NFW (µl)
114.00	17.67	5.7	10.1	4.4
230.46	35.72	24	85.7	61.7
114.47	17.74	54	95.8	41.8
142.13	22.03	114	251.2	137.2

*Concentration determined by nanodrop _ ssDNA-33

$$\mu M = \frac{Conc.(\frac{ng}{\mu l})}{MW} x\, 10^3$$

$$Final\ volume(\mu l) = \frac{Conc.(uM)\ x\ volume\ (\mu l)}{Final\ conc.(10\ \mu M)}$$

Materials:

- 3' adapter oligo (20 bases, HPLC purification): 5'-/5Phos/CAG ATC GGA AGA GCA CAC GT/3AmMO/-3' (Integrated DNA Technologies-IDT)

- Micropipettes
- Microcentrifuge
- Refrigerator/Freezer
- Thermal cycler
- Nanodrop
- 1.5 ml microtubes
- 0.2 ml PCR tubes & caps (8-strip)
- 1 ml tips
- 200 µl tips
- 10 µl tips
- Nuclease free water (NFW)
- T4 RNA ligase 1 (NEB, Cat.No. M0204L)
- Chloroform
- Isopropanol (2-propanol)
- Glycogen (Invitrogen, Cat.No. 10814-010)
- Ethanol

P3.2. 3’ adapter ligation

5. Denature the siRNA sample (112-530 ng in **10 µl** – out of 15 **µl** from step 12 on page 8) at 65°C for 45 seconds, immediately chill on ice

6. Denature the adenylated 3’ adapter (stock 10 µM, in aliquots of 10 µl) at 65°C for 45 seconds, immediately chill on ice

7. Set up the ligation reaction:

No. of reactions:	1		8	
	Rx (µl)		Master Mix (µl)	
Components	**Volume:**	**21**	**80**	**Final Concentration**
siRNA sample	10	*1		112-530 ng siRNA
Adenylated 3' adapter (stock 10 µM)	1			0.5 µM adenyl. 3' adapter
10x T4 RNA ligase buffer (without ATP)	2	*2	16	1x T4 RNA ligase buffer
50% PEG 8000	6		48	15% PEG 8000
RNase inhibitor (RNaseOUT, 40 U/µl)	1		8	2 U/µl RNase inhibitor
T4 RNA ligase 2, truncated (stock 200 U/µl)	1		8	10 U/µl ligase 2, truncated

*1: Mix both components first

*2: Mix well the 10x T4 RNA ligase buffer with the 50% PEG 8000, and then add the other components. Add 10 µl Master Mix to each sample.

Mix by pipetting and incubate overnight at 18°C in a thermal cycler (cover heating set at 50°C)

Note: ligation reaction must be performed in the absence of ATP to prevent self-ligation of the siRNA that has a 5’ phosphate. Successfully ligated siRNA could be visualized on a 10% Urea-PAGE gel.

8. Add 0.5 µL reverse transcription primer (**20 µM**) to the ligation reaction, heat inactivate the reaction at 65°C for 15 min and incubate at 25°C for 10 min, in a thermal cycler

9. Optional: to minimize adapter dimer formation in the subsequent PCR enrichment, the ligated siRNA could be PAGE purified before proceeding to the following 5’ adapter ligation steps

Materials:

- Reverse transcription primer (multiplex) – Integrated DNA Technologies (IDT)
  RT_primer (20 bases, HPLC purification): 5'-/5AmMC6/ACG TGT GCT CTT CCG ATC TG-3'

- Micropipettes
- Microcentrifuge
- Refrigerator/Freezer
- Thermal cycler
- 1.5 ml microtubes
- 0.2 ml PCR tubes & caps (8-strip)
- 200 µl tips
- 10 µl tips
- Adenylated 3' adapter oligo
- Nuclease free water (NFW)
- T4 RNA ligase 2, truncated (NEB, Cat.No. M0242L)
- RNaseOUT (Invitrogen, Cat.No. 10777-019)

P3.3. 5’ adapter ligation

10. Denature the 5’ adapter (stock 10 µM) at 65°C for 45 seconds, immediately chill on ice

11. Set up the 5' ligation reaction:

No. of reactions:		1		8	
		Rx (µl)		Master Mix (µl)	
Components	**Volume:**	**31**		**68**	**Final Concentration**
3' ligation product		21.5			---
5' adapter (stock 10 µM)		1			0.33 µM 5' adapter
10x T4 RNA ligase buffer (without ATP)		1		8	1x T4 RNA ligase buffer
ATP (stock 10 mM)		3		24	1 mM ATP
Nuclease free water		1.5	*1	12	---
RNase inhibitor (RNaseOUT, 40 U/µl)		1		8	1.3 U/µl RNase inhibitor
T4 RNA ligase 1 (stock 10 U/µl)		2		16	0.65 U/µl T4 RNA ligase 1
*1: Make a master mix with these components, and then add 8.5 µl to each sample					

Mix by pipetting and incubate at 25°C for 4 hours in a thermal cycler (cover heating set at 50°C)

Note: the 3’ end of the small RNA has already been ligated to the 3’ adapter that has an amine group at the 3’ end, and could no longer take part in the ligation reaction; thus its 5’ end could be ligated to an RNA oligo in the presence of ATP.

Materials:

- 5' Adapter (RNA oligo, 26 bases, RNase Free HPLC purification):
  5'-GUU CAG AGU UCU ACA GUC CGA CGA UC-3' (Integrated DNA Technologies-IDT)

- Micropipettes
- Microcentrifuge
- Refrigerator/Freezer
- Thermal cycler
- 1.5 ml microtubes
- 0.2 ml PCR tubes & caps (8-strip)
- 200 µl tips
- 10 µl tips
- Nuclease free water (NFW)
- T4 RNA ligase 1 (NEB, Cat.No. M0204L)
- RNaseOUT (Invitrogen, Cat.No. 10777-019)

P3.4. Reverse transcription

12. Set up the reverse transcription reaction:

No. of reactions:	1		8	
		Rx (µl)	Master Mix (µl)	
Components	**Volume:**	**46.5**	**124**	**Final Concentration**
5' ligation reaction		31		---
dNTP (stock 10 mM)		2	16	0.44 mM dNTP
DTT (stock 100 mM)		3	24	6.53 mM DTT
5x SuperScript III First strand reaction buffer		9 *1	72	1x First strand buffer
RNase inhibitor (RNaseOUT, 40 U/µl)		0.5	4	0.44 U/µl RNase inhibitor
SuperScript III rev-transcriptase (200 U/µl)		1	8	4.35 U/µl rev-transcriptase
*1: Make a master mix with these components, and then add 15.5 µl to each sample				

13. Mix by pipetting and incubate the reaction at 50°C for 1 hour, and heat inactivate the reverse transcriptase at 75°C for 15 min; in a thermal cycler

Materials:

- Micropipettes
- Microcentrifuge
- Refrigerator/Freezer
- Thermal cycler
- 1.5 ml microtubes
- 0.2 ml PCR tubes & caps (8-strip)
- 200 µl tips
- 10 µl tips
- dNTPs (Invitrogen, Cat.No. 10297-018)
- RNaseOUT (Invitrogen, Cat.No. 10777-019)
- Superscript III Rev Transcript (Invitrogen, Cat.No. 18080-044)

P3.5. Library PCR amplification

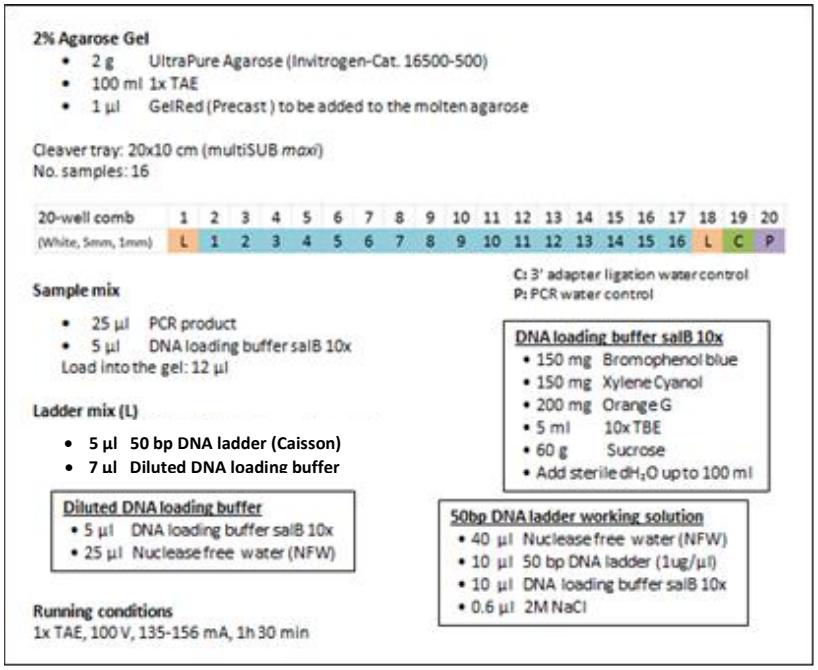
P3.5.1 PCR test

14.1. Set up the PCR reaction:

No. of reactions:	1	8	
	Rx (µl)	Master Mix (µl)	
Components	**Volume: 25**	**180**	**Final Concentration**
Forward primer: sRNA PCR 1 (stock 10 µM)	1	8	0.4 µM primer
Reverse primer: PCR2_1 (stock 10 µM)	1	8	0.4 µM primer
dNTP (stock 10 mM)	1	8	0.4 mM dNTP
5X Buffer HF	5	40	1x Buffer HF
Nuclease free water	14	112	---
$MgCl_2$ (stock 50 mM)	0.25	2	0.5 mM $MgCl_2$
Phusion DNA polymerase (2 U/µl)	0.25	2	0.02 U/µl DNA polymerase
RT product	2.5		
*1: Make a master mix with these components, and then add 22.5 µl to each sample			
PCR conditions:			
1 cycle	95 °C	2 min	
20 cycles	98 °C	11 seconds	
	65 °C	30 seconds	
	72 °C	30 seconds	
1 cycle	72 °C	2 min	
	10 °C	∞	

Run the PCR program in a thermal cycler

Separate the PCR product on a 2% agarose gel in 1x TAE buffer to confirm the amplification of siRNA libraries (Fig. 7)



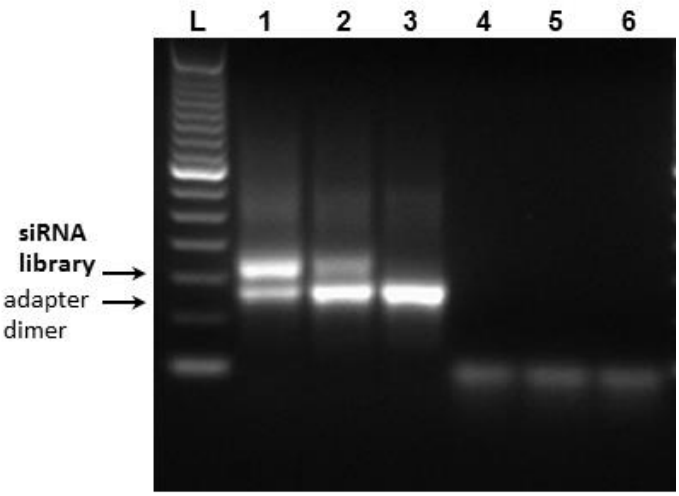


Figure 7. siRNA libraries on a 2% agarose gel in 1x TAE buffer. siRNA libraries (1-2), and water controls for 3' adapter ligation (3), 5' adapter ligation (4), reverse transcription (5) and PCR (6), 50 bp DNA ladder (L).

Materials:

- Primers (Integrated DNA Technologies-IDT):

  sRNA PCR 1 (60 bases, **standard desalting**):
  5'-AATGATACGGCGACCACCGAGATCTACACGACAGGTTCAGAGTTCTACAGTCCGACGA*T*C-3'

  PCR2_1 (64 bases, **standard desalting**)-multiplex:
  5'-CAAGCAGAAGACGGCATACGAGAT **ATCACG** GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3'

- Micropipettes
- Microcentrifuge
- Refrigerator/Freezer
- Thermal cycler
- Gel Doc/ UV transilluminator
- 1.5 ml microtubes
- 0.2 ml PCR tubes & caps (8-strip)
- 200 µl tips
- 10 µl tips
- dNTPs (Invitrogen, Cat.No. 10297-018)
- Nuclease free water (NFW)
- Phusion High-Fidelity DNA polymerase (NEB, Cat.No. M0530L)
- Agarose Ultrapure (Invitrogen, Cat.No. 15510-027)
- 1x TAE buffer
- GelRed (Biotium, Inc. Cat.No. 41003)
- DNA loading buffer salB 10x
- 50 bp DNA ladder (Caisson, Cat.No. DMR12-500UL)

P3.5.2 PCR Enrichment

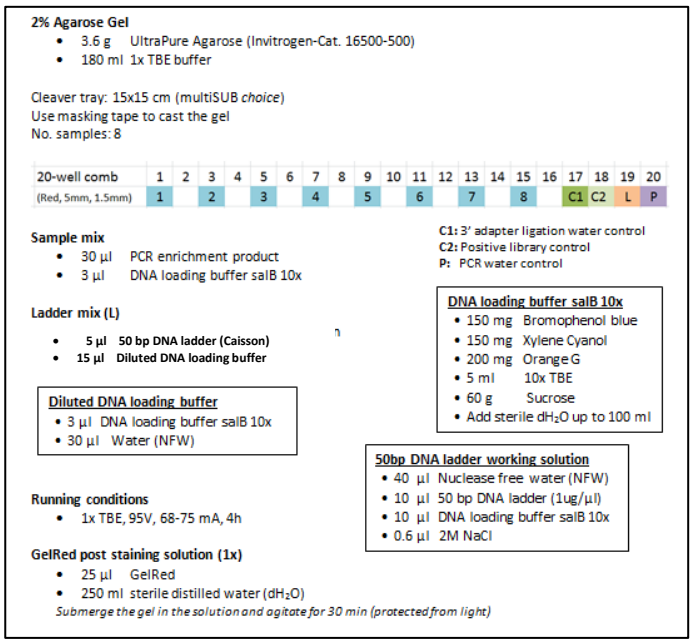
14.2. Set up the PCR reaction

No. of reactions:		1		8	
		Rx (µl)		Master Mix (µl)	
Components	**Volume:**	**30**		**152**	**Final Concentration**
Forward primer: sRNA PCR 1 (stock 10 µM)		1	*1	8	0.33 µM primer
dNTP (stock 10 mM)		1		8	0.33 mM dNTP
5X Buffer HF		6		48	1x Buffer HF
Nuclease free water		10.2		81.6	---
$MgCl_2$ (stock 50 mM)		0.3		2.4	0.5 mM $MgCl_2$
Phusion DNA polymerase (2 U/µl)		0.5		4	0.04 U/µl DNA polymerase
Reverse primer: PCR2_* (stock 10 µM)		1			0.33 µM primer
RT product		10			
*1: Make a master mix with these components, and then add 19 µl to each sample					
PCR conditions:					
1 cycle		94 °C		2 min	
15 cycles		98 °C		12 seconds	
		65 °C		30 seconds	
		72 °C		30 seconds	
1 cycle		72 °C		2 min	
		10 °C		∞	

Run the program for PCR conditions in a thermal cycler

Note: PCR enrichment products need to be processed (or cleaned) as soon as the PCR cycling finish; otherwise, the nucleic acids may be affected.

Separate the PCR product on a 2% agarose gel in 1x TBE buffer



Excise the band corresponding to the siRNA library (Fig. 8) and proceed with the Gel Extraction Kit, according to manufacturer's instructions.

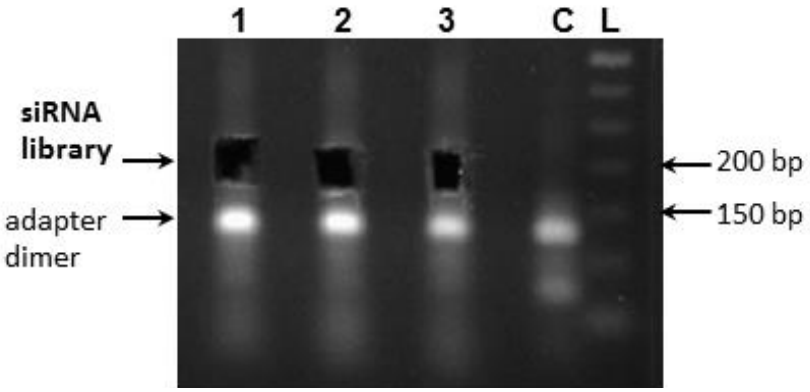


Figure 8. siRNA libraries on a 2% agarose gel in 1x TBE buffer. siRNA libraries (1-3), water control for 3' adapter ligation (C), and 50 bp DNA ladder (L).

Note: The exposure of nucleic acids to UV light should be done rapidly because long exposures cause significant damage (degradation) to the sample.

Materials:

- Primers (Integrated DNA Technologies-IDT):

  sRNA PCR 1 (60 bases, HPLC purification):
  5'-AATGATACGGCGACCACCGAGATCTACACGACAGGTTCAGAGTTCTACAGTCCGACGA*T*C-3'

  PCR2_* (64 bases, HPLC purification)-multiplex:
  5'-CAAGCAGAAGACGGCATACGAGAT **NNNNNN** GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3'

PCR2_*	Barcode sequence: NNNNNN		
PCR2_1	5'-CAA...GAT	ATCACG	GTG...TC*T-3'
PCR2_2	5'-CAA...GAT	CGATGT	GTG...TC*T-3'
PCR2_3	5'-CAA...GAT	TTAGGC	GTG...TC*T-3'
PCR2_4	5'-CAA...GAT	TGACCA	GTG...TC*T-3'
PCR2_5	5'-CAA...GAT	ACAGTG	GTG...TC*T-3'
PCR2_6	5'-CAA...GAT	GCCAAT	GTG...TC*T-3'
PCR2_7	5'-CAA...GAT	CAGATC	GTG...TC*T-3'
PCR2_8	5'-CAA...GAT	ACTTGA	GTG...TC*T-3'
PCR2_9	5'-CAA...GAT	GATCAG	GTG...TC*T-3'
PCR2_10	5'-CAA...GAT	TAGCTT	GTG...TC*T-3'
PCR2_11	5'-CAA...GAT	GGCTAC	GTG...TC*T-3'
PCR2_12	5'-CAA...GAT	CTTGTA	GTG...TC*T-3'
PCR2_13	5'-CAA...GAT	AGTCAA	GTG...TC*T-3'
PCR2_14	5'-CAA...GAT	AGTTCC	GTG...TC*T-3'
PCR2_15	5'-CAA...GAT	ATGTCA	GTG...TC*T-3'
PCR2_16	5'-CAA...GAT	CCGTCC	GTG...TC*T-3'
PCR2_17	5'-CAA...GAT	GTAGAG	GTG...TC*T-3'
PCR2_18	5'-CAA...GAT	GTCCGC	GTG...TC*T-3'
PCR2_19	5'-CAA...GAT	GTGAAA	GTG...TC*T-3'
PCR2_20	5'-CAA...GAT	GTGGCC	GTG...TC*T-3'
PCR2_21	5'-CAA...GAT	GTTTCG	GTG...TC*T-3'
PCR2_22	5'-CAA...GAT	CGTACG	GTG...TC*T-3'
PCR2_23	5'-CAA...GAT	GAGTGG	GTG...TC*T-3'
PCR2_24	5'-CAA...GAT	GGTAGC	GTG...TC*T-3'

PCR2_*	Barcode sequence: NNNNNN		
PCR2_25	5'-CAA...GAT	ACTGAT	GTG...TC*T-3'
PCR2_26	5'-CAA...GAT	ATGAGC	GTG...TC*T-3'
PCR2_27	5'-CAA...GAT	ATTCCT	GTG...TC*T-3'
PCR2_28	5'-CAA...GAT	CAAAAG	GTG...TC*T-3'
PCR2_29	5'-CAA...GAT	CAACTA	GTG...TC*T-3'
PCR2_30	5'-CAA...GAT	CACCGG	GTG...TC*T-3'
PCR2_31	5'-CAA...GAT	CACGAT	GTG...TC*T-3'
PCR2_32	5'-CAA...GAT	CACTCA	GTG...TC*T-3'
PCR2_33	5'-CAA...GAT	CAGGCG	GTG...TC*T-3'
PCR2_34	5'-CAA...GAT	CATGGC	GTG...TC*T-3'
PCR2_35	5'-CAA...GAT	CATTTT	GTG...TC*T-3'
PCR2_36	5'-CAA...GAT	CCAACA	GTG...TC*T-3'
PCR2_37	5'-CAA...GAT	CGGAAT	GTG...TC*T-3'
PCR2_38	5'-CAA...GAT	CTAGCT	GTG...TC*T-3'
PCR2_39	5'-CAA...GAT	CTATAC	GTG...TC*T-3'
PCR2_40	5'-CAA...GAT	CTCAGA	GTG...TC*T-3'
PCR2_41	5'-CAA...GAT	GACGAC	GTG...TC*T-3'
PCR2_42	5'-CAA...GAT	TAATCG	GTG...TC*T-3'
PCR2_43	5'-CAA...GAT	TACAGC	GTG...TC*T-3'
PCR2_44	5'-CAA...GAT	TATAAT	GTG...TC*T-3'
PCR2_45	5'-CAA...GAT	TCATTC	GTG...TC*T-3'
PCR2_46	5'-CAA...GAT	TCCCGA	GTG...TC*T-3'
PCR2_47	5'-CAA...GAT	TCGAAG	GTG...TC*T-3'
PCR2_48	5'-CAA...GAT	TCGGCA	GTG...TC*T-3'

Materials (continue):

- Micropipettes
- Microcentrifuge
- Refrigerator/Freezer
- Thermal cycler
- Gel Doc/ UV transilluminator
- 2 ml microtubes
- 1.5 ml microtubes
- 0.2 ml PCR tubes & caps (8-strip)
- 200 µl tips
- 10 µl tips
- Blades (#11)
- dNTPs (Invitrogen, Cat.No. 10297-018)
- Nuclease free water (NFW)
- Isopropanol (2-propanol)
- Phusion High-Fidelity DNA polymerase (NEB, Cat.No. M0530L)
- Agarose Ultrapure (Invitrogen, Cat.No. 15510-027)
- 1x TBE buffer
- GelRed (Biotium, Inc. Cat.No. 41003)
- DNA loading buffer SALB 10X
- 50 bp DNA ladder (Caisson, Cat.No. DMR12-500UL)
- Gel Extraction Kit
  (High Pure PCR product Purification kit from ROCHE, Cat. No. 11732676001 - or another available in your lab.)

P4. Multiplex Library Mixing

1. Quantify the purified siRNA library using a Quant-iT PicoGreen dsDNA Assay Kit and read in a Qubit 3 fluorometer

2. Mix equal amount (12 ng) of each siRNA library and concentrate in a speed vac

3. Use 10 µl libraries with a final concentration no less than 2 ng/µl for each lane of Illumina sequencing

Materials:

- Micropipettes
- Refrigerator
- Qubit 3 fluorometer (Invitrogen, Cat.No. Q33216)
- Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Cat.No. P7589)
- Nuclease free water (NFW)
- 0.5 ml PCR tubes
- 1 ml tips
- 200 µl tips
- 10 µl tips

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