Final Protocol

Cloning

As far as cloning is concerned, we're using the NEBuilder HiFi Protocol.

Table1 Forward and reverse primers for HiFi of UVR8-COP1 construct for fragment 1 (neomycin resistance cassette), fragment 2 (35S CaMV-COP1-L-VP16-T2A), and fragment 3 (mphR(A)-L-UVR8-35S CaMV poly(A) terminator)

	Fragment 1	Fragment 2	Fragment 3
FWD primer	5'-ctttgacatttttggagtagatcggatcctgtcaaacactgatagt	5'-agtgtttgacaggatccgatctactccaaaaatgtcaaagatacagtctcagaag	5'-tagaggagaacccagggccaatgccccgtccaaagct
REV primer	5'-acattgcggacgtttttatgtactgaattaacgccgaattatcaagcttgacaggaggccc	5'-ttgagctttggacggggcattggccctgggttctcctc	5'-ttttcccagtcacgacgttgtaaaacgacggccagtgccaggtcactggattttggttttaggaattagaaat

Table2 Forward and reverse primers for HiFi of tasi-RNA construct for fragment 1(hygromycin resistance cassette) and fragment 2 (35S CaMV-tasi-RNA-NOS terminator).

	Fragment 1	Fragment 2
FWD primer	5'-GTCAGCGTctactccaaaaatgtcaaagatacagtctcagaag	5'-tggagtagACGCTGACGTATAGTCGATCGA
REV primer	5'-tactgaattaacgccgaattgatctggatttttagtactggattttggttttagga	5'cgacggccagtgccagatctagtaacatagatgacaccgcgc

Stable Transformation of *N. benthamiana* - link

Preparation of Agrobacterium suspension

- Streak the *Agrobacterium tumefaciens* (*A. tumefaciens;* GV3101 pMP90) strain carrying the transformation construct on YEB plates containing required antibiotics (Rifampicin, Gentamycin + construct-specific antibiotic).
- Two days prior to transformation: Inoculate 5 ml YEB liquid culture (with antibiotics); grow for 24 h with shaking at 30 °C.
- One day prior to transformation: Inoculate 100 ml culture with 1 ml of the pre-culture in an induction medium (containing antibiotics), shake overnight at 30 °C.
- Day of transformation: Pellet bacteria by centrifugation (40 ml in Falcon tubes, 20 min, 4000 x g. Resuspend bacteria in MMA medium (without antibiotics) to an OD_{600}=0.8 (50 ml).

Preparation of N. benthamiana leaf tissue for transformation

Try to injure plant tissue as little as possible! All steps following the surface-sterilization have to be carried out under sterile conditions.

- Harvest the 2-3 youngest, but fully expanded leaves of 3-5 weeks-old *Nicotiana benthamiana* plants (3-5 leaves are required for each transformation).
- Cut leaves into pieces, omitting main veins.
- Incubate leaf cuts for 30 sec in 1.2 % NaOCl (+0.01 % Tween).
- Wash twice in H_2O (+0.01 % Tween) to get rid of the bleach.
- Store leaf cuts in H_2O.

Transformation

- Further cut surface-sterilized leaves into smaller pieces (ca. 1x1 cm).
- Incubate leaf cuts in *A. tumefaciens* suspension: - Pour *A. tumefaciens* suspension in a square petri dish. - Place leaf cuts on the surface of the suspension. When the surface is covered, you will have more than enough leaf cuts for each transformation. - Incubate at least 30 min.
- Prepare a fresh square petri dish with a sterile piece of water-wetted Whatman paper.
- Transfer leaf cuts onto Whatman paper.
- Seal dishes with Leukopor tape and incubate for 2 days in the dark (24 °C, wrap in aluminium foil to protect from light).

Selection and shoot induction

- Prepare a square dish with 50 ml H_20 (containing Kanamycin (100 mg/l) and Cefotaxime (250 mg/l)) for washing of the leaf cuts.
- Pick up the Whatman paper with the leaf cuts using sterile forceps and place it upside-down onto the surface of the wash solution to release leaf cuts from the filter into the wash solution. Gently shake the petri dish, and incubate for > 3 min.
- Dry leaf cuts by gently swiping on a fresh, sterile Whatman filter.
- Place dried leaf cuts on shoot induction medium (MS-II). Use standard round petri dishes to minimize the risk of contamination. Place 8-10 leaf cuts on each plate.
- Seal plates with Leukopor tape. Incubate in a light cabinet until shoots occur (5-6 weeks under our conditions: 23°C, 24h light, intensity 105 – 125 µE $m^{-2}s^{-1}$).

- It may be necessary to transfer leaf cuts/ developing calli to new MS-II plates during the incubation if media start drying out

Root induction and transfer to soil

Shoots start developing from calli on MS-II plates and need to be transferred to MS-III plates to induce rooting. Since calli do not develop homogeneously, shoots may be cut and transferred at different time points.

- Cut well-developed shoots with a sterile blade. Try to cut as low as possible, but avoid transferring any callus tissue, as this will prevent further development of the shoot.
- Stick shoots with the cut surface into MS-III medium, and incubates under the same conditions as before for further development of the shoot and rooting. Note: Red light will prevent rooting!
- We use 0.5 l Weck glasses for rooting and place max. 3 shoots in each jar.
- Shoots originating from a single callus are, in general, originating from the same transgenic event. Thus, to only sample independent transformants, we only transfer one single shoot per callus to rooting medium.
- Calli can be kept as a back-up on MS-II medium, as shoots will continue to develop.
- Shoots will further develop in MS-III media, and will eventually form roots (2-3 weeks).
- Transfer well-developed shoots to soil. Remove MS medium from shoots/ roots to avoid contamination. We transfer shoots directly into potting soil that had been passed through a sieve. Again, shoot development is not homogeneous, and some shoots will not start rooting in a timely manner. Indeed, even rather small shoots without any roots will, in many cases, develop well once transferred to soil.
- It is important to keep plants under high humidity after transfer to soil. Place respective plants in a tray covered with a lid for ~ 2 weeks. Once plantlets appear sufficiently vigorous, reduce humidity in the course of 2-3 days by first displacing the lid before completely removing it.
- Grow plants as usual to obtain seeds. In our hands (GV3101 pMP90, pVS1 origin for transformation constructs), most plants will contain multiple insertions of the T-DNA.

Transient Transformation of N. benthamiana - link

Time Frame

- Day 1: *Agrobacterium* transformation with binary vectors
- Day 3: Start *Agrobacterium* culture (including *Agrobacterium* containing p19 strain)
    - p19 is a suppressor of gene silencing from the tomato bushy stunt virus and must be infiltrated for proper gene expression
    - Water tobacco plants
- Day 6(?): Infiltration of transformed Agrobacterium in *N. benthamiana* leaves
- Day 6-10: Imaging of results

Infiltration

1. Prepare activation buffer
2. Measure the OD_{600} of overnight cultures
3. N. benthamiana will be infiltrated with a solution containing OD_{600} = 0.3 of each experimental Agro containing construct and OD_{600} = 0.1 of p19

   a. Calculate the volume of cultures ($V_{construct}$;V_{p19}) needed according to the formulas: $V_{construct} = V_{final} * 0.3/OD_{600}$; $V_{p19} = V_{final} * 0.1/OD_{600}$. One mL of infiltrate is often enough to complete a small experiment. Plan your final volume accordingly
4. Mix calculated volumes of constructs and p19 cultures into a 1.5 ml Eppendorf tube
5. Immediately centrifuge the mixtures at maximum speed for 30 seconds at room temperature
6. Discard the supernatant and resuspend the mixtures in V_{final} of activation buffer
7. Incubate the mixtures for at least 30 minutes at room temperature in dark
8. Infiltrate the mixtures using a 1 ml syringe without a needle into the abaxial side of *N. benthamiana* leaves
   a. Trace the infiltrated area with a marker
   b. Incubate the plants in the greenhouse for 2-6 days, depending on the level of protein expression. Two days of expression is often maximal

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Genotyping

Miniprep

Since there's little to we can add to the plenty of existing miniprep protocols, here are the protocols we'd use provided we could get into a lab:

E. coli - Standard QIAgen Miniprep protocol

Agrobacterium - Modified QIAgen Miniprep protocol

Primers

We have three pairs of PCR primers: two which are insert specific, and one which is orientation specific. The two insert specific primers check that the fragments are inserted into the backbone in the correct order. They flank the overlap regions of the COP1 and Neomycin (NOS-COP1) resistance cassettes as well as the COP1 and UVR8 cassettes. Failing proper insertion, no amplicons smaller than the backbone are expected.

The final orientation specific primer pair ensures that the insert produced from our three fragments is inserted correctly. It primes the 35s CaMV terminator (on the insert) and the right bound (on the backbone). Assuming the insert is in the correct orientation, an amplicon should be produced.

	35sT-RB	COP1-UVR8	NOS-35sP
FWD Primer	5'-cccagataagggaattagggtt	5'-acccgtcctgtcacttcacatcg	5'-cgtcatcggcgggggtcataac
REV Primer	5'-gtaacttaggacttgtgcgaca	5'-ccaagggatacatcggtcggcg	5'-ggaatccgaggaggtttcccga

Amplicon Size	600 bp	378 bp	200 bp

Note:

- All primers pairs have a ΔT_m of <2 C
- All primers have a ΔG of above -9 kcal
- All primers are 22 bp long, with the exception of COP1-UVR8 reverse which is 23 bp

Colony PCR

Our general approach centers around this E. coli colony PCR protocol. For *E. coli*, we will be following this protocol to the letter. For *A. tumefaciens*, we've added a few extra steps from this blogpost which should allow us to circumvent a lot of problems with *Agrobacterium* colony PCR.

E. coli Colony PCR

Note that this protocol will be using **all three** of the aforementioned primer pairs, but in separate reaction tubes:

1. Set up 4 x 50 µl reactions as follows. Tube 4 has no primers added to it:

Reagent	Volume
One*Taq* Master Mix	25 ul
35sT-RB FWD/REV (Tube 1)	200 nM
COP1-UVR8 (Tube 2 & 4)	
NOS-35sP (Tube 3)	
H_2O	to 50 ul

2. You should now have three tubes, each with a different primer pair. With the exception of Tube 4, use a sterile toothpick to pick up individual colonies and dip into each reaction tube.
    a. Place the unaltered backbone into tube 4 in place of the colonies. This tube will serve as a control.
3. As soon as the solution looks cloudy, remove the toothpick. To create a stock of each individual colony either:
    a. Dip the toothpick into 3 ml growth media with appropriate antibiotics and culture overnight.

b. Streak the toothpick onto another agar plate containing the appropriate antibiotics and grow overnight.

4. Transfer reactions to a PCR cycler, and perform PCR following the guidelines below for cycling conditions:

Initial Lysis Cycle	
94 C	2 minutes (*E. coli)*
For 30 cycles:	
1.) 94 C	15 - 30s
2.) 45 - 68 C	15 - 60s
3.) 68 C	1 minute per kb
Final Cycle:	
1.) 68 C	5 - 10 minutes
2.) 10 C	Hold

5. Load 4-6 µl of each PCR reaction directly onto an agarose gel, alongside an appropriate DNA ladder.

A. tumefaciens Colony PCR

A. tumefaciens requires a longer lysis cycle and subsequent cooling in an ice bath since it's difficult to lyse. Otherwise, the steps are the same as *E.coli*'s colony PCR (all three primer pairs are used, etc.):

1. Set up 4 x 50 µl reactions as follows:

Reagent	Volume
One*Taq* Master Mix	25 ul
COP1-UVR8 FWD/REV (Tube 1)	200 nM
35sT-RB FWD/REV (Tube 2 & 4)	

NOS-35sP (Tube 3)	
H_2O	to 50 ul

2. You should now have three tubes, each with a different primer pair. With the exception of Tube 4, use a sterile toothpick to pick up individual colonies and dip into each reaction tube.
    a. Place the unaltered backbone into tube 4 in place of the colonies. This tube will serve as a control.
3. As soon as the solution looks cloudy, remove the toothpick. To create a stock of each individual colony either:
    a. Dip the toothpick into 3 ml growth media with appropriate antibiotics and culture overnight.
    b. Streak the toothpick onto another agar plate containing the appropriate antibiotics and grow overnight.
4. Transfer the tubes to a PCR cycler and run an initial denaturation cycle for 30 minutes at 95 C to ensure complete lysis. While running, prepare an ice bath.
5. Place the tubes into the ice bath and allow them to cool for 3 minutes.
6. Transfer the tubes back to the PCR cycler, running with the below guidelines.

For 30 cycles:	
4.) 94 C	15 - 30s
5.) 45 - 68 C	15 - 60s
6.) 68 C	1 minute per kb
Final Cycle:	
3.) 68 C	5 - 10 minutes
4.) 10 C	Hold

7. Load 4-6 µl of each PCR reaction directly onto an agarose gel, alongside an appropriate DNA ladder.

Transformant Genotyping

DNA Extraction

Genotyping our putative transformants will involve qPCR. To get our DNA template, we first need to extract DNA from our plant tissue. We're following the QIAgen DNeasy Plant handbook's Mini protocol:

1. Before starting the extraction, ensure that buffers AW2 and AW1 have added to them the appropriate amount of 95-100% ethanol as indicated on their labels. A water bath or heating block needs to be preheated to 65 C
2. Cut leaf discs or otherwise extract plant tissue until a 100 mg sample is produced.
3. Place the sample into liquid nitrogen and grind thoroughly with a mortar and pestle. Decant the ground tissue and any remaining liquid nitrogen into an RNAse-free, liquid nitrogen-cooled 2 mL microcentrifuge tube. Let the tube stand until the liquid nitrogen has completely evaporated. Do not allow the sample to thaw.
4. Add 400 µl Buffer AP1 and 4 µl RNase A stock solution (100 mg/ml) to a maximum of 100 mg (wet weight) or 20 mg (dried) disrupted plant or fungal tissue and vortex vigorously.
    - No tissue clumps should be visible. Vortex or pipet further to remove any clumps. Clumps of tissue will not lyse properly and will therefore result in a lower yield of DNA. In rare cases, where clumps cannot be removed by pipetting and vortexing, a disposable micropestle may be used.
    - **Note:** Do not mix Buffer AP1 and RNase A before use!
5. Incubate the mixture for 10 min at 65°C. Mix 2–3 times during incubation by inverting the tube. This step lyses the cells.
6. Add 130 µl Buffer P3 to the lysate. Mix and incubate for 5 min on ice. This step precipitates detergent, proteins, and polysaccharides.
7. Centrifuge the lysate for 5 min at 20,000 x g (14,000 rpm). Some plant materials can generate very viscous lysates and large amounts of precipitates during this step. This can result in shearing of the DNA in the next step. In this case, optimal results are obtained if the majority of these precipitates are removed by centrifugation for 5 min at 20,000 x g (14,000 rpm). After centrifugation, apply supernatant to QIAshredder Mini spin column and continue with step 8.
8. Pipet the lysate into the QIAshredder Mini spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at 20,000 x g (14,000 rpm). It may be necessary to cut off the end of the pipette tip to apply the lysate to the QIAshredder Mini spin column. The QIAshredder Mini spin column removes most precipitates and cell debris, but a small amount will pass through and form a pellet in the collection tube. Be careful not to disturb this pellet in step 9.
9. Transfer the flow-through fraction from step 8 into a new tube (not supplied) without disturbing the cell-debris pellet. Typically 450 µl lysate is recovered. For some plant species less lysate is recovered. In this case, determine the volume for the next step.
10. Add 1.5 volumes of Buffer AW1 to the cleared lysate, and mix by pipetting. For example, to 450 µl lysate, add 675 µl Buffer AW1. Reduce the amount of Buffer AW1 accordingly if the volume of lysate is smaller. A precipitate may form after the addition of Buffer AW1, but this will not affect the DNeasy procedure.
    - **Note:** Ensure that ethanol has been added to Buffer AW1. Note: It is important to pipet Buffer AW1 directly onto the cleared lysate and to mix immediately.
11. Pipet 650 µl of the mixture from step 10, including any precipitate that may have formed, into the DNeasy Mini spin column placed in a 2 ml collection tube (supplied). Centrifuge for 1 min at ≥6000 x g (corresponds to ≥8000 rpm for most microcentrifuges), and discard the flow-through. Reuse the collection tube in step 12.
12. Repeat step 11 with the remaining sample. Discard flow-through and collection tube.
13. Place the DNeasy Mini spin column into a new 2 ml collection tube (supplied), add 500 µl Buffer AW2 and centrifuge for 1 min at ≥6000 x g (≥8000 rpm). Discard the flow-through and reuse the collection tube in step 17.

- **Note:** Ensure that ethanol is added to Buffer AW2.

14. Add 500 µl Buffer AW2 to the DNeasy Mini spin column, and centrifuge for 2 min at 20,000 x g (14,000 rpm) to dry the membrane.
    - It is important to dry the membrane of the DNeasy Mini spin column, because residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during elution. Discard flow-through and collection tube.
    - **Note:** After centrifugation, remove the DNeasy Mini spin column from the collection tube carefully so that the column does not come into contact with the flow-through, because that will result in carryover of ethanol.
15. Transfer the DNeasy Mini spin column to a 1.5 ml or 2 ml microcentrifuge tube (not supplied), and pipet 100 µl Buffer AE directly onto the DNeasy membrane. Incubate for 5 min at room temperature, and then centrifuge for 1 min at ≥6000 x g (≥8000 rpm) to elute.
    - Elution with 50 µl (instead of 100 µl) increases the final DNA concentration in the eluate significantly but also reduces overall DNA yield. If larger amounts of DNA (>20 µg) are loaded, eluting with 200 µl (instead of 100 µl) increases yield.
16. Repeat step 15 once.
    - A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, the microcentrifuge tube can be reused for the second elution step to combine the eluates.
    - **Note:** More than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

qPCR

Once we've got our DNA extracted, it's time to cycle. We used the NEB Luna® Universal qPCR Master Mix Protocol as a template:

1. Thaw Luna Universal qPCR Master Mix and other reaction components at room temperature, then place on ice. After thawing completely, briefly mix each of the following components by inversion, pipetting or gentle vortexing:

Reagent	Volume
Luna Universal qPCR Master Mix	10 uL
COP1-UVR8 FWD (10 uM)	0.5 uL
COP1-UVR8 REV (10 uM)	0.5 uL
Template DNA	Variable
Nuclease-free Water	To 20 uL

2. Determine the total volume for the appropriate number of reactions, plus 10% overage and prepare assay mix of all components except DNA template accordingly. Mix thoroughly but gently by pipetting or vortexing. Collect liquid to the bottom of the tube by brief centrifugation.
3. Aliquot assay mix into qPCR tubes or plate. For best results, ensure accurate and consistent pipetting volumes and minimize bubbles.
4. Add DNA templates to Tube 1. Do not add a template to Tube 2. Seal tubes with flat, optically transparent caps; seal plates with optically transparent film. Care should be taken to properly seal plate edges and corners to prevent artifacts caused by evaporation.
    a. Tube 2 will serve as a negative control or "no template" control when running gels later.
5. Spin tubes or plates briefly to remove bubbles and collect liquid (1 minute at 2,500–3,000 rpm).
6. Program real-time instrument with the below thermocycling protocol. Ensure a plate read is included at the end of the extension step.

Step	Temperature	Duration	Cycles
Initial Denaturation	95 C	60 s	1
Denaturation	95 C	15 s	40-45
Extension	60 C	30 s (and plate read)	
Melt Curve	60-95 C	Various	1

Gel Electrophoresis

Once Colony PCR and Transformant PCR runs have been completed, they need to be run on gels. We will be basing this portion of the protocol off of Addgene's Agarose Gel Electrophoresis page. The steps for preparing the gels are as follows:

1. Measure 1.5 g of agarose.
2. Mix agarose powder with 100 mL 1xTAE in a microwavable flask.
3. Microwave for 1-3 min until the agarose is completely dissolved (but do not overboil the solution, as some of the buffer will evaporate and thus alter the final percentage of agarose in the gel. Many people prefer to microwave in pulses, swirling the flask occasionally as the solution heats up.).
4. Let agarose solution cool down to about 50 °C (about when you can comfortably keep your hand on the flask), about 5 mins.
5. Add ethidium bromide (EtBr) to a final concentration of approximately 0.2-0.5 µg/mL (usually about 2-3 µl of lab stock solution per 100 mL gel). EtBr binds to the DNA and allows you to visualize the DNA under ultraviolet (UV) light.
6. Pour the agarose into a gel tray with the well comb in place

7. Place newly poured gel at 4 °C for 10-15 mins OR let sit at room temperature for 20-30 mins, until it has completely solidified.

Since we're looking to do this for the *E.coli/A. tumefaciens,* and transformant PCR runs, we will need to have two different loading procedures. They are as follows:

Loading E. coli/A.tumefaciens Samples

To each sample, a loading buffer is added as is called for by the NEB Protocol for All Loading Dyes. From left to right, samples should be loaded in the following order:

1. 100 bp DNA Ladder
2. Tube 1
3. Tube 2
4. Tube 3
5. Tube 4 (Negative control)

Loading Transformant Samples

To each sample, a loading buffer is added as is called for by the NEB Protocol for All Loading Dyes. From left to right, samples should be loaded in the following order:

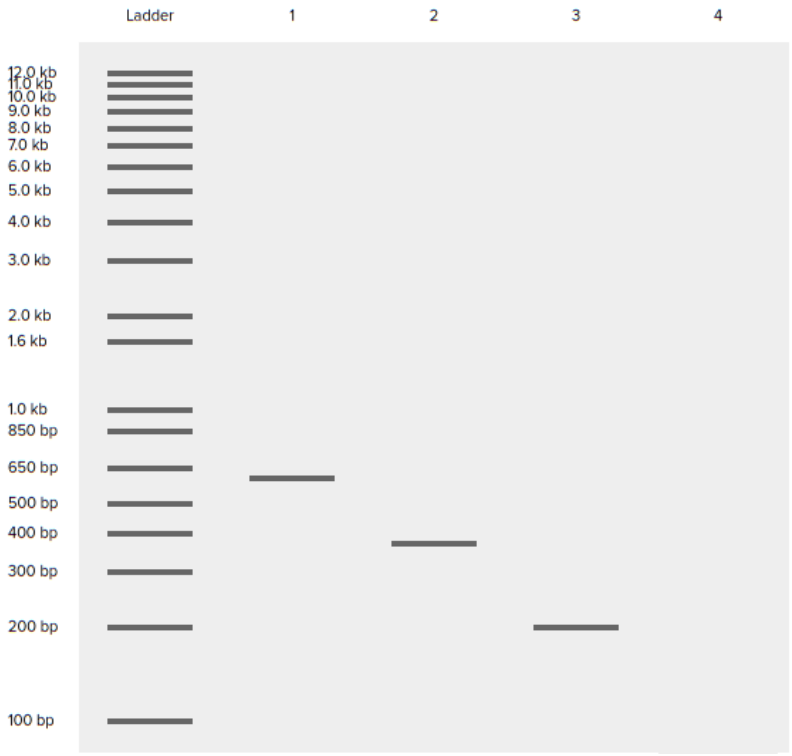
1. 100 bp DNA Ladder
2. Tube 1
3. Tube 2 (negative control)

Running the Gels

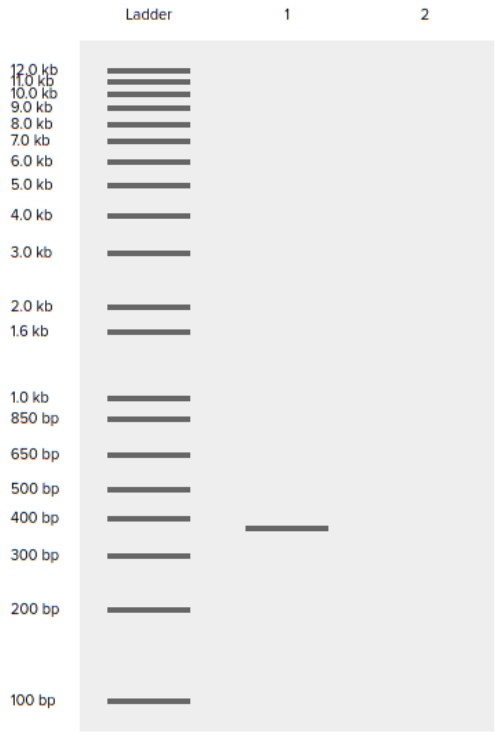
Since the gels will require the same conditions when run, run both of the prepared gels with the following steps:

1. Place the agarose gel into the gel box
2. Fill the gel box with 1xTAE (or TBE) until the gel is covered.
3. Load all samples as laid out above
4. Connect the gel box to the power supply, run the gel at 80-150 V for 1-1.5 hours or until the samples have migrated 75-80% of the length of the gel.
5. Turn OFF power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.
6. Using any device that has UV light, visualize your DNA fragments. The fragments of DNA are usually referred to as 'bands' due to their appearance on the gel.

If run correctly, the Colony PCR gels will look something like this:


Ladder
1
2
3
4
12.0 kb
11.0 kb
10.0 kb
9.0 kb
8.0 kb
7.0 kb
6.0 kb
5.0 kb
4.0 kb
3.0 kb
2.0 kb
1.6 kb
1.0 kb
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp

If run correctly, the Transformant samples should look something like this:



Phenotyping

Response to Antibiotics - E. coli - (link)

Day 1

1. Prepare kanamycin/neomycin to a concentration of 1mg/mL -- 4mg/mL in LB medium.
    a. The concentration of antibiotics required for effective over-agar selection has been empirically determined.
2. With a 6 cm diameter petri plate containing solidified LB-agar, pipette 150 µL of carbenicillin on top of the agar and gently spread over the surface until the liquid is mostly absorbed (there is a very small visible volume of pooled liquid remaining on the surface).
    a. We use the micropipette tip itself to do the spreading; the tip is gently bent to create an "L" shape, and then used like a cell spreader. Several other devices may be used for this purpose, provided that they fit your petri plate.
3. Incubate the plate at room temperature for at least 30 minutes with the lid on to give the antibiotic time to more fully absorb.

4. During the incubation, transform DH5α *E. coli* cells by heat shock with the plasmid of interest. See our transformation page for a detailed heat shock transformation protocol.
5. Plate 50 µL of transformed *E. coli*/rescue media suspension onto the agar and gently spread over the surface until the liquid is mostly absorbed.
    a. The spreading of cells can be done in the same way as the antibiotic, using either a bent micropipette tip or other cell spreading device that fits the plate.
6. Incubate plates at 37 ℃ for 18 hours.

Day 2

1. Observe plates for colony formation. Shown below are the results from an experiment optimizing the concentration of kanamycin, plated over-agar for selection of transformed *E. coli*.

Response to antibiotics - Modified for EHA105 Agrobacterium - (link)

Agrobacterium Competent Cell Preparation

Materials

- LB plates with 30 µg/ml
- Gen (2 plates is enough)
- 2 1 L culture flasks
- 2 sterile 500 ml centrifuge bottles
- 1.5 - 2 L sterile dH2O (should be cold)
- 50 ml sterile10% glycerol in dH2O (should be cold)

Steps

1. From a frozen glycerol stock grow a 20 ml culture of Agrobacterium overnight at room temperature
2. The following morning, dilute the culture 64,000 fold by adding 0.5 µl of culture to 2 ml of H2O making a 4000 fold dilution. From the 4000 fold dilution take 0.5 µl and add to 2 ml H2O making a 16,000 fold dilution, from the 16,000 fold dilution take 0.5 µl and add to 2 ml H2O making a 64,000 fold dilution
3. Note: 0.5 µl of culture into 50 ml H2O makes a 100,000 fold dilution and you can use 10 µl of this on a plate
4. Add 5 µl of the 64,000 fold dilution to an LB plate and thoroughly spread the culture on the plate, incubate at room temp or 28°C for 1 - 3 days
5. Once you have single colonies on a plate, pick a colony and start a 20 ml overnight culture in LB with Gen at 28°C , you can start this overnight culture early in the day as Agro grows slow and you want a thick culture for the next day. Make sure to take LB out of the fridge so that it is at room temperature for the 500 ml cultures the next day
6. Inoculate 2 500 ml flasks (use 1 liter flasks) of LB with 9 ml of the overnight culture, do not use antibiotic here, make sure to start these cultures early in the day (if possible < 8:00am) as Agro grows very slowly, a culture started around 8:00am will be ready hopefully by 3:00pm
7. Grow cells to an OD_{600} nm of 0.5 - 1
8. Precool Centrifuge with 500 ml bottle adaptors to 4°C
9. When cells are ready to harvest chill flasks on ice for 15 - 30 minutes
10. Centrifuge at 4000 g in bottles for 15 minutes at 4°C

11. Remove as much of the supernatant as possible and resuspended the pellet in each bottle in 500 ml of ice cold water, use graduations on side of centrifuge bottle to measure volume
12. Centrifuge at 4000 g for 15 min
13. Remove supernatant and resuspended the pellet in each bottle in 250 ml of ice cold water
14. Centrifuge at 4000 g for 15 min
15. Remove supernatant and resuspended the pellet in each bottle in 10 ml ice cold 10% glycerol
16. Combine contents of bottle into 1 50 ml Falcon tube (It will stand up the centrifugation force)
17. Centrifuge at 4000 g for 15 min Remove supernatant and resuspend the pellet in 2 - 3 ml ice cold 10% glycerol. The cell concentration should be about 1 - 3*1010 cells/ml
18. Store in 1.5 ml microfuge tubes in 50 µl aliquots, freeze in LN2, store in -80°C, cells are good for at last 6 months

Agrobacterium Transformation Reagents

- LB Liquid, 200 ml
  - In 200 ml dH2O + 5 g
  - LB Broth (Difco, Luria-Bertani)
  - Autoclave for 20 min
- LB Plates, 200 ml
  - In 200 ml dH2O
  - + 5 g LB Broth (Difco, Luria-Bertani)
  - + 3 g Agar (Fishger, BP 1423-500)
  - Autoclave for 20 min, swirl immediately, allow to cool to touch
  - Pour plates (35 ml) label, store at 4C
- 4 mg/ml Ampicillin, 10 ml
  - In 10 ml of sterile dH2O
  - + 0.04g Ampicillin (Roche 70043429)
  - Filter sterilize using 0.45 m syringe filter (Millipore Millex HA SLHA033SS)
  - Aliquot into sterile 1.5 ml microfuge tubes and store at -20C
- 10 mg/ml Kanamycin/Neomycin
  - 10 ml In 10 ml sterile dH2O
  - + 0.1 g Kanamycin/Neomycin Filter sterilize using 0.45 m syringe filter (Millipore Millex HA SLHA033SS)
  - Aliquot into sterile 1.5 ml microfuge tubes and store at -20C

Antibiotic	Stock (mg/ml)	Working (mg/ml)	ul of Stock in 5 ml	10 ml	20 ml	200 ml
Ampicillin	10	50	12.5	25	50	500
Kanamycin/Neomycin	10	100	50	100	200	2000
Gentamicin	10	30	15	30	60	600

- 10% Glycerol, 200 ml
  - In 180 ml dH2O
  - + 20 ml glycerol
  - Autoclave for 20 - 30 min, cool and store in cold room

Western blot for COP1/UVR8

Overview: A Western blot is composed of multiple steps that require careful consideration and planning. First, a protein sample is prepared, then subjected to gel electrophoresis allowing the separation of native or denatured proteins based on size. Subsequently, the separated molecules are transferred or blotted onto a second matrix, generally a nitrocellulose or polyvinylidene difluoride (PVDF) membrane. Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane. Most commonly, the transferred protein is then probed with a combination of antibodies: one antibody specific to the protein of interest (primary antibody) and another antibody specific to the host species of the primary antibody (secondary antibody).

The first step of a Western blot, the sample preparation, is most important because the success of later steps in immunoblotting depends on the initial preparation of the protein, which should yield a high amount of the target protein.

- Protein extraction from *N.benthamiana* leaves

Total protein is extracted by grinding plants in microextraction buffer (100 mM Tris-HCl, pH 7.5; 100 mM potassium fluoride; 5 mM EDTA; 250 mM sucrose; 40 mM sodium ascorbate; 0.2% BSA), 1% polyvinylpolypyrrolidone (PVPP) and 1% (v/v) plant protease inhibitor cocktail.) Homogenization is followed by a freeze-thaw treatment (10 s on dry ice followed by 10 s at 37°C) repeated three times. The homogenate is centrifuged for 10 min at 16,100g in a microcentrifuge at 4°C, and the supernatant is collected.

- **SDS-PAGE protocol**

Materials for 5X Sample Buffer

- 10% w/v SDS
- 10 mM Dithiothreitol, or beta-mercapto-ethanol
- 20% v/v glycerol
- 0.2M Tris-HCl, pH 6.8
- 0.05% w/v Bromphenolblue

Sample Preparation

1. Prepare a suitable amount of sample buffer (25 µl per reaction).
2. Determine protein concentration with NanoDrop.
3. Calculate required dilutions needed to get 150 µg protein in 75 µl of solution.

4. In the fume hood, add 25 µl of the sample buffer (blue) to 75 µl of protein samples (total volume of 100 µl). Centrifuge samples at 13000 rpm for 60 seconds.
5. Incubate samples for 5 minutes in a dry heating block at 90 ◦C.
6. Centrifuge samples at 13000 rpm for 60 sec.
7. Store all samples at −20 ◦C.

Gel Preparation

Separating gel

1. Prepare the gel casts in holders. Fill the holder with water to check for leakage after 5 min.After leakage check, pour all water from gel casts and dry residual with paper towel.
2. To prepare separation-gel, ix the reagents listed in the table below with the proper volumes to receive a gel with desired composition.
3. NOTE! Add TEMED and APS last, solidification of the gel will occur when these reagents are added.
4. Pipette gel mixture into gel casts up to a height approximately 1 cm below the gels comb.
5. Fill the remaining cast with isopropanol (if not available use distilled water).
6. Allow to harden for 30-60 min.
7. Pour all isopropanol (or water) from gel casts and dry residual with a paper towel.

Stacking gel

1. To prepare stacking-gel, in a tube mix the reagents listed in table 4 below with the proper volumes to receive a gel with desired composition.
2. NOTE! Add TEMED and APS last, solidification of the gel will occur when these reagents are added.
3. Pipette gel mixture on top of separating gel to fill cast and remove all air bubbles.
4. Fill the remaining cast with isopropanol (if not available use distilled water).
5. Insert the comb whilst the gel is still in liquid form. The comb will form wells to load samples when the gel turns solid.
6. Allow to harden for 45-60 min.

Electrophoresis

Running the gel

1. Mount the gels into the tank, remove combs and fill the inner chamber of the tank with 1x running buffer to the top. Fill a third of the volume in the outer chamber of the tank with 1x running buffer.
2. Pipette 2 µl ladder and 10 µl sample into each well.
3. Connect the power pack (red-red, black-black) and run at 100 V, 60 min or until the blue line almost reaches the bottom of the gel.
4. Once electrophoresis has finished, remove gels from the glass plates.
5. Carefully wash all equipment used for electrophoresis with water.

Coomassie Blue Staining

1. Prepare the staining solution containing 0.1 % Coomassie Blue in 40 % ethanol, 10 % acetic acid.
2. After electrophoresis, incubate 1 or 2 gels in a staining container containing 100 ml Coomassie Blue staining solution.

3. Incubate at room temperature for 1 hour until bands are visible.
4. Decant the stain and rinse the gel once with deionized water.
5. Prepare 100 ml containing a solution containing 10 % ethanol and 7.5 % acetic acid.
6. Gently shake the gel at room temperature on an orbital shaker until the desired background is achieved.

Western Blot Chemiluminescent Detection

Materials

- Nitrocellulose or PVDF transfer membrane
- Transfer buffer
- Wash buffer
- Blocking buffer
- Incubation trays and containers
- Primary antibodies
- Secondary antibodies

Procedure

Step	Directions
1	Prepare transfer buffer for wet and semi-dry transfers based on gel chemistry.
2	Prepare transfer membrane (semi-dry or wet transfers). Follow manufacturer instructions for dry membrane preparations. ● PVDF: pre-wet in methanol or ethanol (100%) for 30 seconds, briefly rinse in deionized water, and equilibrate in the transfer buffer for 5 minutes. ● Nitrocellulose: equilibrate directly in the transfer buffer for 5 minutes.
3	Follow manufacturer instructions for wet, semi-dry, or dry transfer.
4	After protein transfer, wash the membrane in deionized water 4 times for 5 minutes each with agitation to remove all transfer buffer.
5	Incubate the membrane with a sufficient volume of blocking buffer for 30–60 minutes at room temperature with agitation.
6	Dilute the primary antibody per supplier recommendations in the blocking buffer.
7	Incubate the membrane protein-side up in the primary antibody solution with agitation, for 1 hour at room temperature or overnight at 2–8°C. Ensure the volume of the antibody solution is enough to fully cover the membrane.
8	Wash the membrane 3 times with agitation for 10 minutes each in the wash buffer.
9	Prepare dilutions of the conjugated secondary antibody to 0.4 to 0.1 µg/mL in appropriate volume of wash buffer or alternatively in blocking buffer. From a 2 mg/mL antibody stock,

	dilute 1:5,000 to 1:20,000: ● 1:5,000: 3 µL of secondary antibody in 15 mL wash buffer ● 1:10,000: 1.5 µL of secondary antibody in 15 mL wash buffer ● 1:20,000: 0.75 µL of secondary antibody in 15 mL wash buffer
10	Incubate the membrane protein-side up in the secondary antibody solution for 1 hour with agitation at room temperature. Ensure the volume of the antibody solution is enough to fully cover the membrane and protect the membrane from bright light to prevent photobleaching of the fluorescent dyes.
11	Wash the membrane 6 times with agitation for 5 minutes each in the wash buffer to remove any unbound secondary antibodies. It is crucial to thoroughly wash the membrane at this step.
12	Blots can be imaged immediately while still wet, or alternatively may be dried prior to imaging. Place each blot in a sheet protector or on a clean surface prior to imaging to prevent contamination
13	Image the blot using an appropriate imaging system.

- https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2002606/ (scroll to protein extraction and protein blot gel analysis)
    - "A polyclonal antibody made against a C-terminal peptide of UVR8 (VPDETGLTDGSSKGN) and an anti-GFP monoclonal antibody (632375; Clontech) were used to detect UVR8 and GFP-UVR8, respectively."
    - "Antibodies were used at 1:3000 dilution and detected using secondary anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase (Promega)."
    - It would be ideal to use an antibody made against the C-terminal peptide of UVR8, however, this antibody could potentially detect native UVR8 proteins, so we would have to overcome that. A better way of doing things would be to tag our UVR8 and COP1 fusion proteins with fluorescent markers, instead of utilizing an antibiotic resistance cassette to select for our final transformed plant. Antibiotic resistance made sense as a selection marker previously when we were going to do a floral dip for *Arabidopsis thaliana* because we could plate the seeds on agar plates w/ the appropriate antibiotic, however, we don't need to do this anymore if we use FP. Furthermore, FP allows for better visualization.

Northern blot for anti-WUS siRNA

RNA Extraction

We're following the QIAgen RNeasy plant extraction protocol for both RNA extraction and cleanup:

1. Cut leaf discs or otherwise extract plant tissue until a 100 mg sample is produced.
2. Place the sample into liquid nitrogen and grind thoroughly with a mortar and pestle. Decant the ground tissue and any remaining liquid nitrogen into an RNAse-free, liquid nitrogen-cooled 2 mL microcentrifuge tube. Let the tube stand until the liquid nitrogen has completely evaporated. Do not allow the sample to thaw.
3. Prepare Buffer RLT by adding 10 uL β-Mercaptoethanol (BME) to every 1 mL of RLT solution. Add 450 uL of Buffer RLT with BME to the sample. Vortex vigorously.
    a. A short 1-3 minute incubation at 56 C may also help to disrupt the tissue. Do not incubate samples with high starch contents, as swelling of the sample will occur.
4. Transfer the lysate to a QIAshredder spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Carefully transfer the supernatant of the flow-through to a new microcentrifuge tube (not supplied) without disturbing the cell-debris pellet in the collection tube. Use only this supernatant in subsequent steps.
    a. It may be necessary to cut off the end of the pipette tip to facilitate pipetting of the lysate into the QIAshredder spin column. Centrifugation through the QIAshredder spin column removes cell debris and simultaneously homogenizes the lysate. While most of the cell debris is retained on the QIAshredder spin column, a very small amount of cell debris RNeasy Mini Handbook 10/2019 65 will pass through and form a pellet in the collection tube. Be careful not to disturb this pellet when transferring the lysate to the new microcentrifuge tube.
5. Add 0.5 volume of ethanol (96–100%) to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 6.
    a. The lysate volume may be less than 450 uL due to loss during homogenization.
    b. Precipitates may appear after the addition of ethanol. This does not affect the procedure.
6. Transfer the sample (and any precipitate formed in the previous step) to an RNeasy spin column (pink) inside of a 2 mL collection tube (supplied in the kit). Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through. Reuse the collection tube in step 7.
7. Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 8.
8. Prepare buffer RPE by adding four volumes of 95-100% ethanol to the buffer RPE concentrate. Add 500 µl of the resulting diluted Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 9.
9. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.
    a. Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min. Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 9

10. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNAse-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.
11. If the expected RNA yield is >30 µg, repeat step 10 using another 30–50 µl RNAse-free water, or using the eluate from step 10 (if high RNA concentration is required). Reuse the collection tube from step 10. If using the eluate from step 11, the RNA yield will be 15–30% less than that obtained using a second volume of RNAse-free water, but the final RNA concentration will be higher.

RNA Cleanup

As RT-PCR is very sensitive to DNA contamination, it's recommended that you clean up the RNA extract before cycling. The steps to do this are as follows:

1. Adjust the sample to a volume of 100 µl with RNAse-free water. Add 350 µl Buffer RLT, and mix well.
2. Add 250 µl ethanol (96–100%) to the diluted RNA, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 3.
3. Transfer the sample (700 µl) to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.* Reuse the collection tube in step 4.
    a. After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.
4. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 5.
5. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.
    a. Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.
6. Optionally, you can place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min. Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 5.
7. 7. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNAse-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.
8. If the expected RNA yield is >30 µg, repeat step 7 using another 30–50 µl RNAse-free water, or using the eluate from step 7 (if high RNA concentration is required). Reuse the collection tube from step 7. If using the eluate from step 7, the RNA yield will be 15–30% less than that

obtained using a second volume of RNAse-free water, but the final RNA concentration will be higher

If at this point you are still concerned about DNA contamination, you may want to follow the optional on-column DNAse digestion as is laid out in appendix D of the RNeasy extraction kit.

Northern Blots for Small RNAs and MicroRNAs

Materials

Reagents

- Electrophoretic transfer buffer (20×)
    - Dilute the 20× stock to 1× in H_2O and precool to 4°C before use. Alternatively, use TBE electrophoresis buffer (10×), diluted to 0.5×. See Steps 4 and 5.
- Formamide gel-loading buffer
- Polyacrylamide-urea gel (10%–15%) and electrophoresis reagents
- Prehybridization/hybridization mix for northerns
- Probe DNA or RNA, labeled at 2×10^8 to 5×10^8 cpm/µg
    - The probe is typically a DNA oligonucleotide, end-labeled by kinasing, or an antisense in vitro RNA transcript, labeled internally.
- RNA marker ladder (10–150 nucleotides; e.g., U.S. Biochemicals Low Molecular Weight Marker or Ambion Decade Marker System)
    - The marker can be radiolabeled by kinasing and run in parallel with the unlabeled RNA samples in Step 2. It also serves as a control for electrophoretic transfer from the gel.
- RNA samples (each ~10–15 µg of total RNA)
- SSC for northerns (20×)
- SDS (10%)

Equipment

- Autoradiography or phosphorimaging equipment
- Ballpoint ink pen for marking the filter
- Containers for hybridization (polyethylene heat-seal bags or cylindrical glass hybridization bottles)
    - If heat-seal bags are used, a heat sealer (e.g., National Instruments, Model 310) will be required.

- Electroblot transfer tank and power supply (e.g., Bio-Rad Trans-Blot cell with wire electrodes and PowerPac HC Power Supply)
- Nylon transfer membrane (positively charged; e.g., GE Healthcare, Hybond-N+)
    - The membrane should be cut to the same size as the gel.
- Oven with shaker or hybridization oven with a rotating rack for glass hybridization bottles, set at 42°C
- Parafilm
- Polyacrylamide gel electrophoresis system and power supply
    - The gel is typically ~18 × 18-cm, 1.5 mm thick, set with a 20-well comb. For details, see Polyacrylamide Gel Electrophoresis of RNA (Rio et al. 2010).
- Stratalinker (Stratagene) with 254-nm bulbs
    - Alternatively, use a 254-nm UV transilluminator or vacuum oven set at 80°C (see Step 7).
- Water bath or temperature block (90°C)
- Whatman 3MM chromatography paper (four sheets must be the same size as the gel; see Steps 3 and 4)

Denaturing Polyacrylamide–Urea Gel and Electrophoretic Transfer

1	Resuspend each RNA sample (~10–15 µg of total RNA) in 8–12 µL of formamide gel-loading buffer. Heat the samples for 2 min at 90°C.
2	Load the samples and the RNA marker ladder onto a high percentage (10%–15%) denaturing polyacrylamide–urea gel. Run the gel between 15 and 20 W on constant power, usually for 3–4 h, or until the bromophenol blue (in the formamide gel-loading buffer) is near the bottom of the gel. For details, see Polyacrylamide Gel Electrophoresis of RNA (Rio et al. 2010).
3	Remove the gel from the tank and separate the plates. Wet two sheets of Whatman 3MM paper with 20× SSC and place them on top of the gel.
4	Set up the electroblot transfer: i. Soak a sponge from the electroblot apparatus in electrophoretic transfer buffer until wet. ii. Transfer the gel with the paper onto the sponge, such that the paper is on top of the sponge. iii. Float the nylon transfer membrane in H_2O until wet, then immerse it in 20× SSC. iv. Place the nylon membrane on top of the gel and remove any bubbles.

	v. Wet two more sheets of Whatman 3MM paper with 20× SSC, and place them on top of the membrane. vi. Add the second sponge on top of the Whatman paper sheets and clamp the cassette closed. The layers of the sandwich are as follows, starting with the negative (−) pole: sponge, two sheets of Whatman 3MM paper, gel, nylon membrane, two sheets of Whatman 3MM paper, sponge. The RNA will migrate to the positive (+) pole.
5	Place the assembled gel cassette into the electroblot chamber and fill the chamber with 1× electrophoretic transfer buffer, precooled to 4°C. Carry out the transfer at 250 mA for 2 h at 4°C and then at 350 mA for a further 2 h at 4°C.

Cross-Linking, Prehybridization, and Hybridization

6	Prepare the membrane for cross-linking: i. Disassemble the apparatus. Remove and save the nylon membrane. ii. Carefully remove any gel fragments from the membrane and mark the orientation of the membrane with a ballpoint ink pen. iii. Wrap the membrane in plastic wrap and place it on a sheet of Whatman 3MM paper.
7	To cross-link the RNA to the nylon transfer membrane, place the membrane in a Stratalinker with the RNA surface facing up. Expose it to short-wave UV light (254 nm; the 1200 mJ auto-cross-linking setting in a Stratalinker) for 1 min. Alternatively, use a 254-nm UV transilluminator, exposing the membrane with the RNA surface facing down against the glass surface; or bake the membrane in a vacuum oven for 1 h at 80°C. For microRNAs (miRNAs; RNAs <50 nucleotides), covalent chemical cross-linking (Pall and Hamilton 2008) may be used to fix the small RNAs to the nylon membrane.
8	Place the membrane in a heat-seal bag or cylindrical glass hybridization bottle and cover with prehybridization/hybridization mix, using 10 mL for a heat-seal bag or ∼3–5 mL for a small hybridization tube. Prehybridize the membrane with shaking or rotation for at least 2 h at 42°C. 9. Replace the buffer with an appropriate volume of fresh prehybridization/hybridization mix containing radiolabeled probe at 10^6cpm/mL, and hybridize overnight at 42°C.
9	Replace the buffer with an appropriate volume of fresh prehybridization/hybridization mix containing radiolabeled probe at 10^6cpm/mL, and hybridize overnight at 42°C.

Washing and Detection[1]

10	Remove and safely dispose of the radioactive hybridization buffer.
11	Prepare three wash solutions: 2× SSC/1% SDS, 1× SSC/1% SDS, and 0.1× SSC.
12	Perform the following series of 15-min washes on the membrane, each at 42°C with agitation: i. 2× SSC/0.1% SDS ii. 1× SSC/0.1% SDS iii. 0.1× SSC
13	Air-dry the membrane, cover it with parafilm and expose it to a phosphor screen for phosphorimaging or to X-ray film for autoradiography. Exposure for several hours to overnight may be necessary.
14	Image on a phosphorimager or develop the X-ray film.

Troubleshooting

Problem: A high background is observed on the membrane.	Solutions: High background may be reduced by one of the following approaches. 1. Try prehybridizing for a longer period of time (Step 8). 2. When preparing the prehybridization/hybridization mix, include tRNA and/or poly(A) at a concentration of 200 µg/mL. 3. Polyacrylamide sticking to the membrane can cause high background by retaining the excess probe or unincorporated label. After transfer and while the membrane is still wet (Step 6.ii), "wash" the side of the membrane in contact with the gel in 1× electrophoretic transfer buffer using gloved fingers.

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Problem: The bands are faint or absent.	Solution: For the detection of miRNAs and other 20- to 30-nucleotide RNAs, it may be useful to try using low-stringency hybridization conditions (e.g., a low-stringency Church and Gilbert hybridization buffer containing 10 mg/mL BSA, 0.5 M sodium phosphate [pH 7.2], 1 mM EDTA, and 7% SDS) and to omit the high-stringency wash in Step 12.iii.

ANNEX

RT-PCR

Finally!

1. Mix RNA sample and primer d(T)23VN in two sterile RNase-free microfuge tubes. Prepare the tube as follows:

Reagent	Volume
Extracted RNA	1-6 uL
d(T)23VN (50 µM)	2 uL
Nuclease-Free Water	Variable, fill to 8 uL as needed
Total volume	8 uL

2. Denature RNA for 5 minutes at 70°C. Spin briefly and put promptly on ice. This step is optional. However, it improves the cDNA yield for long messenger RNAs and GC-rich RNA regions.
3. For the template lane, add the following components to one tube:

Reagent	Volume
M-MuLV Reaction Mix	10 uL
M-MuLV Enzyme Mix	2 uL

4. For the control lane, add the following components to one tube:

Reagent	Volume
M-MuLV Reaction Mix	10 uL
H_2O	2 uL

5. Incubate the 20 µl cDNA synthesis reaction at 42°C for one hour. If Random Primer Mix is used, an incubation step at 25°C for 5 min is recommended before the 42°C incubation.
6. Inactivate the enzyme at 80°C for 5 minutes. Dilute reaction to 50 µl with 30 µl H2O for PCR. The cDNA product should be stored at -20°C. For downstream PCR amplification, the volume of cDNA products should not exceed 1/10 of the PCR reaction volume.