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Simultaneous detection of miRNA and mRNA at the single-cell level in plant tissues (v2) V.2

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Protocol status: Working
We use this protocol and it's working

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Abstract

Detecting the simultaneous presence of a microRNA (miRNA) and a mRNA in a specific tissue can provide support for the prediction that the miRNA regulates the mRNA. We develop a method that uses sequence-specific miRNA-locked nucleic acid (LNA) and mRNA-LNA probes. Moreover, it augments the detection signal by rolling circle amplification, achieving a high signal-noise ratio at the single-cell level. Dot signals are counted for determining the expression levels of mRNA and miRNA molecules in specific cells. We show a high sequence specificity of our miRNA-LNA probe, revealing that it can discriminate single-base mismatches. Numerical quantification by our method is tested in transgenic rice lines with different gene expression levels.

1 Section permeabilization

	A
	1. The slides with sections are taken out the freezer and equilibrated to RT for 40 mins.
	2. Permeabilized in 20 ug/ml proteinase k for proper duration
	3. Quickly wash in DEPC-PBS
	4. Quickly dehydrate the slides in EtOH (50, 70, 99 %) and then air dry
	5. Mount the secure seal reaction chambers onto the slides.
	6. Add 1x DEPC-PBS-tween 0.05 % (Wash buffer) into the chambers to keep the slides wet until RT reaction ready

2 Mixture for miRNA hybridization (50 ul)

	A	B	C
		Stock	Final
	Formamide	100%	50%
	SSC	20x%	5x
	tRNA	10 mg/ml	0.5 ug/ul
	Denhardt's	50x	1x
	LNA probe A	10 uM	2-3 pmole
	DEPC-H2O	-	

1. process

	A
	Hybridization below the predicted melting temperature of the probe, about 2 C, for an hour
	Wash with 0.1X SSC three times at the temperature set in the Step 1
	Wash with 2X SSC at RT once
	Wahs wtih the wash buffer (PBS, 0.05% Tween-20) once

3 Mixture for mRNA cDNA synthesis (50 ul)

Reagent	Stock	Final
NEB Tag DNA ligase	40U/ul	0.5 U/ul
Rnase H	5 U/ul	0.4 U/ul
Ribolock Rnase inhibitor	40 U/ul	1 U/ul
NEB Tag ligase buffer	10x	1x
BSA	20 ug/ul	0.2 ug/ul
KCl	1 M	0.05 M
Formamide	100%	20%
Pd_A	10 uM	0.1 uM
Pd_B	10 uM	0.1 uM
Pd_C	10 uM	0.1 uM
DEPC-H2O		

Process

1. Add ligation mixture in chambers, seal with adhesive film
2. Incubate for 30 min at 37 C followed by 45 min at 48 C
3. Wash 2x, 1x DEPC-PBS-Tween 20, 0.05%

4 Mixture for miRNA and mRNA padlock probe hybridization and ligation (50 ul)

Reagent	Stock	Final
NEB Tag DNA ligase	40U/ul	0.5 U/ul
Rnase H	5 U/ul	0.4 U/ul
Ribolock Rnase inhibitor	40 U/ul	1 U/ul
NEB Tag ligase	10x	1x

buffer		
BSA	20 ug/ul	0.2 ug/ul
KCl	1 M	0.05 M
Formamide	100%	20%
Pd_A	10 uM	0.1 uM
Pd_B	10 uM	0.1 uM
Pd_C	10 uM	0.1 uM
DEPC-H2O		

process

A
1. Add ligation mixture in chambers, seal with adhesive film
2. Incubate for 30 min at 37 C followed by 45 min at 48 C
3. Wash 2x, 1x DEPC-PBS-Tween 20, 0.05%

5 Rolling circle amplification (50 ul)

Reagent	Stock	Final
Phi 29 polymerase	10 U/ul	1 U/ul
Ribolock Rnase inhibitor	40 U/ul	1 U/ul
10 X phi 29 buffer	10 x	1 x
dNTP	10 mM	0.25 mM
BSA	20 ug/ul	0.2 ug/ul
Glycerol	50%	5%
DEPC-H2O		

process

A
1. Add reaction mixture and seal chamber
2. Incubate for over night at 37 C
3. Wash 2x, DEPC-PBS-Tween 20, 0.05 %

6 Mixture for detection oligo hybridization (50 ul)

	A	B	C
	Reagent	Stock	Final
	Hyb mixture	4 x	2 x
	Detection oligo 1-FITC	1 uM	0.1 uM
	Detection oligo 2-Cy3	1 uM	0.1 uM
	Detection oligo 3-Cy5	1 uM	0.1 uM
	DEPC-H2O		

process

1. Add reaction mixture
2. Incubate for 30 min at 37 C
3. Wash 2x, DEPC-PBS-Tween 20, 0.05 %
4. Dehydrated by EtOH 50, 70, 99 %; then air dry.
5. Mount cover slips