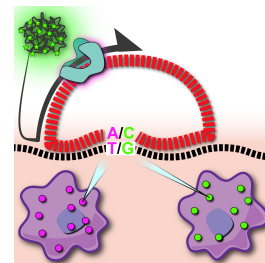


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Detection of single nucleotide polymorphisms with padlock probes

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Tomasz Krzywkowski¹, Mats Nilsson¹

¹Science for Life Laboratory, Department of Biochemistry and Biophysics, Stockholm University, Stockholm, Sweden

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Stockholm University

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Protocol status: Working

We use this protocol and it's working

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Keywords: Padlock probe, single nucleotide polymorphism, SNP, mRNA genotyping, in situ, single molecule, single-cell, detection of single nucleotide polymorphism, quantitation of target mrna, situ detection of mrna, target mrna, single nucleotide variant, mrna, physical link with the parent mrna molecule, conserved actb mrna, parent mrna molecule, desired mrna, mrna of interest, allele specific padlock probe, specific padlock probe, custom padlock probe, using padlock probe, cdna target, padlock probe, method for automated characterization, single cell

Abstract

This protocol is for in situ detection of mRNAs and single nucleotide polymorphisms using padlock probes and rolling circle amplification. In the accompanying publication, we take advantage of a single nucleotide variant within conserved ACTB mRNA to successfully differentiate human and mice co-cultured cells and apply following protocol to genotype PCDH X and Y homologs in human brain tissue sections. These are used as examples in the accompanying publication and custom padlock probes can be designed to allow for the targeting of own desired mRNA. We provide a method for automated characterization and quantitation of target mRNA in single cells or chosen tissue area.

mRNA of interest, harboring a polymorphism, is first reverse-transcribed to cDNA. Allele specific padlock probes are hybridized to the cDNA target and enzymatically circularized maintaining a physical link with the parent mRNA molecule. Lastly, circularized probes are replicated in situ, using rolling circle amplification mechanism to facilitate detection.

This protocol is taken directly from the accompanying publication. Further details and background information can be found in the cited published article.



Attachments



Krzywkowski-

Nilsson2...

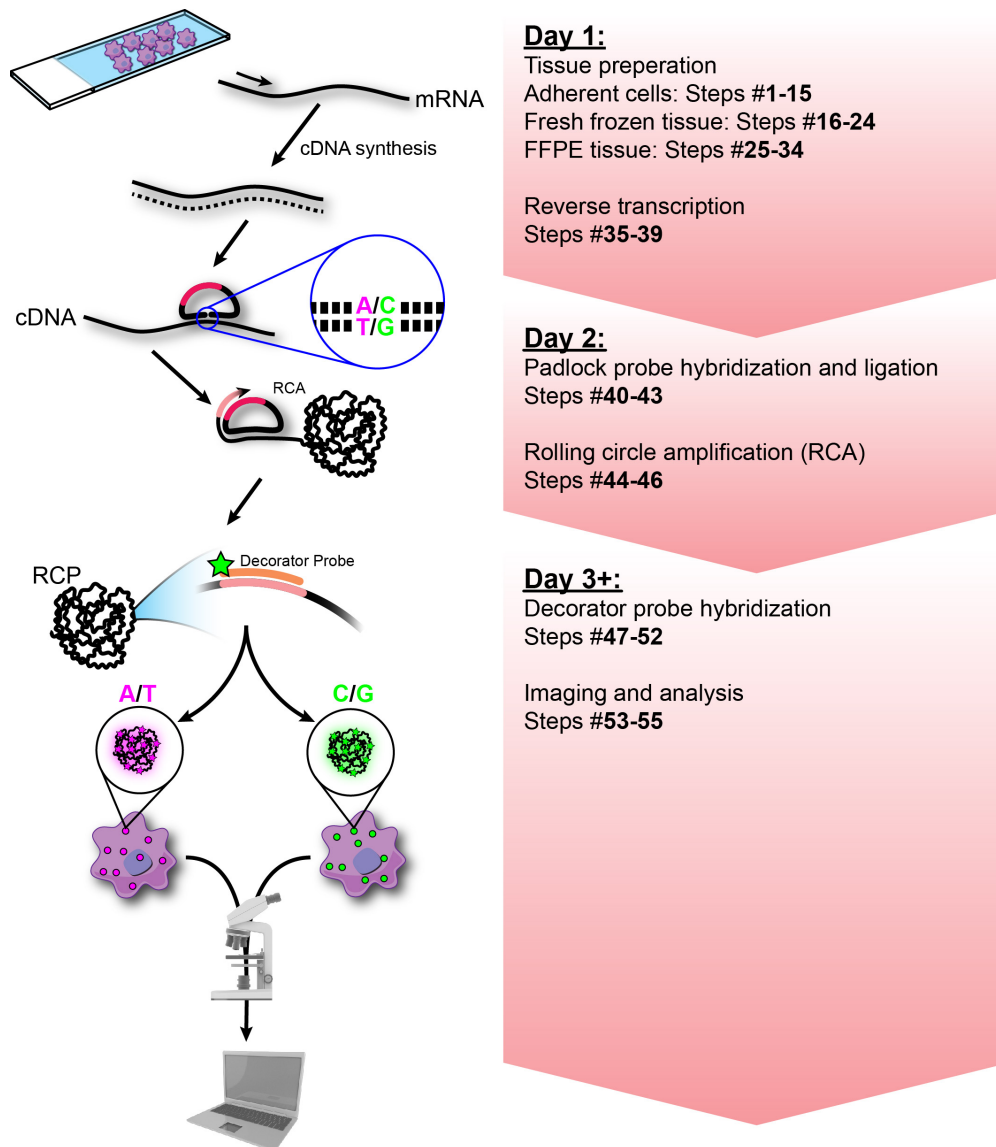
519KB

Guidelines

Protocol

This protocol follows the published protocol: **Padlock Probes to Detect Single Nucleotide Polymorphisms.** **Krzywkowski T et al. Methods Mol Biol. (2018)** and should be cited as such. Some changes have been applied for better comprehension and to fit the forming of protocols.io and therefore numbering may be different to published article.

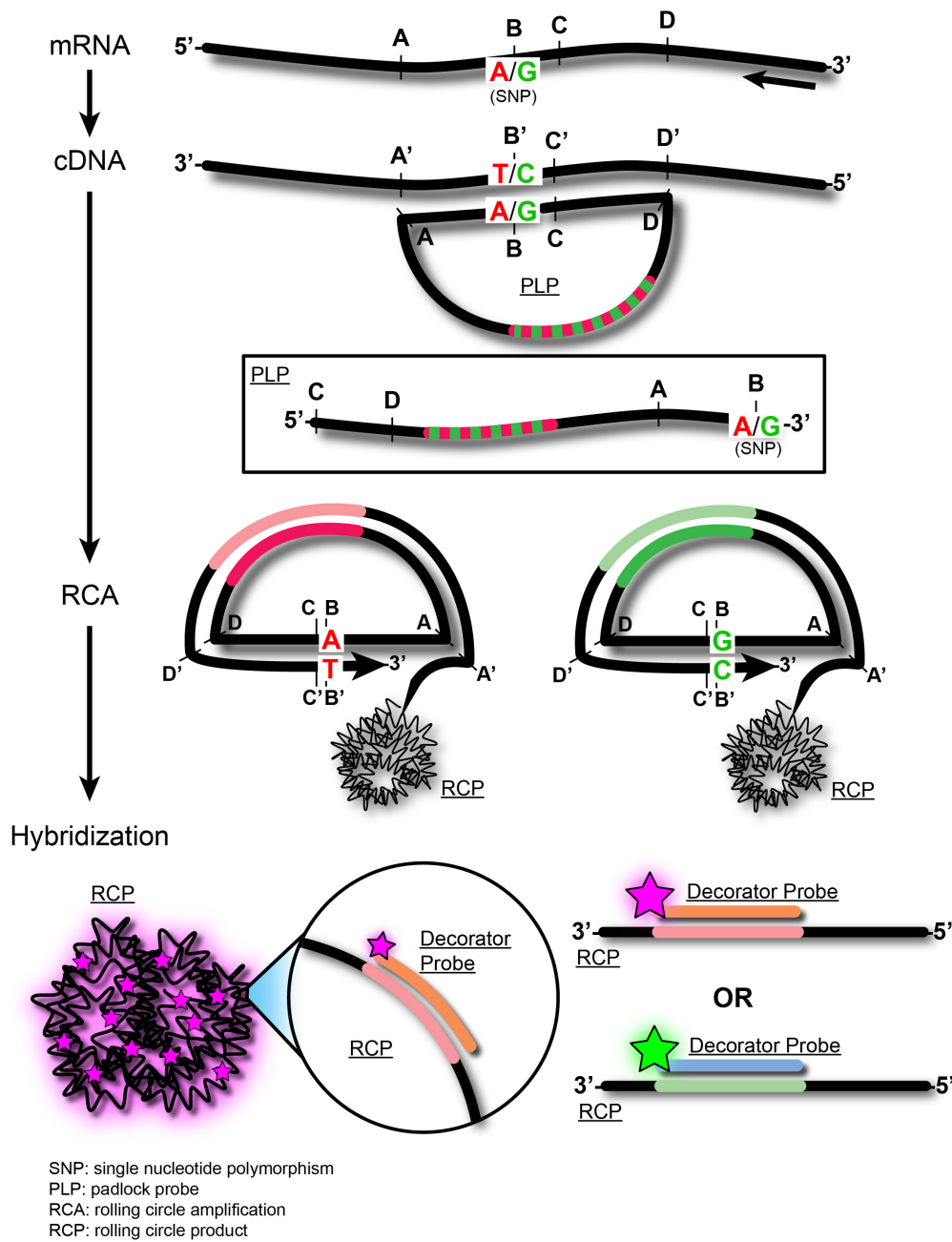
Protocol Workflow Overview



Schematic workflow of protocol. The times are estimations and can vary depending on modifications.

RCA: rolling circle amplification
RCP: rolling circle product

Padlock Probe Design



Schematic design of padlock probes. For more detail refer to publication. 'A, B, C, D' (black, bold) are used as reference points to aid in the visualization of how the padlock probe is designed. 'A/T' and 'C/G' (color) are used as an example of a site for SNP.

Abbreviations

PLP: padlock probe

RCA: rolling circle amplification

RCP: rolling circle product

General Guidelines and Controls

RNases can maintain enzymatic activity even after prolonged autoclaving. Therefore, special measures should be taken when working with RNA.

1. We recommend designating bench area dedicated to RNA work only.
2. All bench surfaces, pipettes, or glassware should be treated with commercially available RNase- and DNase-inactivating agents. We usually wipe benches with 100% ethanol after such treatment.
3. Sterile, disposable plasticware (pipette tips, slide boxes, tubes, and flasks) work best in our hands and ensure RNase-free conditions.
4. We recommend validating efficiency and specificity of new PLPs on synthetic DNA targets.
5. Ligation fidelity can be monitored in vitro as a high molecular weight band on denaturing PAGE gel (linear PLPs and templates migrate faster than circularized PLPs).
6. RCA can be monitored in vitro (templates provide the 3'-OH group as a RCA primer, just as cDNA in regular protocol) by staining RCPs with either intercalating dyes (SYBR dyes) or decorator probes and visualized under a microscope (5-10 μ L of stained RCA mix can be mounted on a microscope slide) or q-PCR system.
7. As a biological positive and negative controls, cell lines with/without target of interest provide a good model to evaluate assay specificity and sensitivity.
8. In accordance with good research practice, different day replicates are recommended, since variation in handling slides or in cell lines growth may influence the final result.
9. Finally, we recommend frequent assessment of the whole genotyping procedure on well-established system (see Note 8) to ensure maximum detection sensitivity.

Equipment

- Diamond pen
- Forceps
- 37°C and 45°C incubators
- Humidity chamber to hold slides during incubations
- Fluorescence microscope
- Secure-Seal hybridization chambers (Grace Bio-Labs) (see Note 6)
- Cover slips (see Note 7)
- Coplin jars
- Microscopy slides (e.g., Menzel Gläser SuperFrost) (see Note 12)

Notes

1. Enzymes (including reverse transcriptase, RNase inhibitor, phi29 polymerase and RNaseH) are sold from multiple vendors and should be optimized but have performed equally well in our hands.
2. DEPC inhibits RNases present in water, buffers or labware. Following DEPC treatment, all solutions should be autoclaved to break down DEPC residue. Keep 0.1% (v/v) DEPC in PBS or ddH₂O for at least 1 hour at 37°C (or overnight at RT), followed by autoclaving. Purchasing and using RNase free solutions from the start is also an alternative.
3. We recommend using freshly prepared formaldehyde solutions in DEPC-PBS. Working solutions can be prepared from either 37% methanol-stabilized stock solution or from paraformaldehyde powder. Aliquots of 3.7% formaldehyde in DEPC-PBS in 1 mL (used during the experiment) and 15 mL (for cell fixation) can be stored at -20°C. Do not freeze and thaw.
4. Lyophilized pepsin batches may vary in activity, even from the same supplier. Every new batch of pepsin should be tested on established model. We typically detect housekeeping gene (ACTB or GAPDH) in the same tissue type to evaluate detection reproducibility.
5. Slow-Fade® Gold Antifade Mountant works best in our experience to prevent photobleaching.
6. Secure-Seal chambers come in different sizes, shapes and depths. For experiments performed on fixed cell lines, we typically use ~50 µL chambers (round, 9 mm diameter, and 0.8 mm deep). Other sizes are available to cover larger areas and then adjustments to volumes within protocol need to be done.
7. To achieve optimal optical resolution, cover glass thickness needs to be adjusted for the microscope setup used.
8. For low copy number transcripts we typically genotype KRAS codon 12 SNPs and for abundant mRNAs, ACTB in human/mouse fibroblasts can be detected.
9. Unlike in many in situ hybridization methods, where cells are grown on coverslips, we grow cells on the microscope slides directly. We found slides to be more resistant to breaking (especially when applying and peeling the silicone chambers off) and on the slides, multiple experiments can be run in parallel (up to eight), making the work more convenient. Special microscope culture slides can also be used in growing cells on slides. Also, multiple consecutive tissue section can be placed on a single slide to facilitate fast experimenting and imaging.
10. Optimal seeding conditions should be identified experimentally for every cell line. For cells with large cytoplasm, 3 mL suspension is usually enough to create homogenous cell layer on each slide. Cells with smaller cytoplasm can be seeded at higher density. In our experience, overnight incubation allows cells to adhere to slides efficiently. Extended incubation can result in cell proliferation on-slide (too dense or clumped cells are difficult to analyze by image analysis software).
11. Tween 20, as a surfactant, coats the chambers, facilitates buffer exchange and prevents formation of "dead spaces" inside the chamber. As a detergent, it can provoke bubble formation. We recommend adding a buffer into a chamber when slide is slightly tilted.
12. Any slides that enhance adhesion of tissue sections can be used. (SuperFrost Plus® from Menzel-Glaser work very well in our hands). Sections should be as thin as possible (preferably few cell layer). Thinner sections are more prone to break and fold during cutting. We commonly use 10 µm thick sections.
13. The fixation time needs to compromise optimal fixative diffusion and minimize loss of tissue content. We recommend pragmatic evaluation of fixation parameters (consecutive sections should be used for each condition). Fixation time may vary for different tissues and different specimen thicknesses. Housekeeping gene is a good candidate for such optimization studies. Use conditions showing maximal signal amount.

14. RNase H has the highest activity at 37°C. It degrades RNA from mRNA/cDNA heteroduplex during the first 37°C incubation. After 30 min, sample is transferred to 45°C which is the optimal temperature for the Tth ligase. Addition of formamide into the mix lowers dsDNA stability (T_m of PLP arms/cDNA duplex). This enables extension of PLP arms that strengthens probe “locking” on cDNA and gives a good balance between arms melting and specific binding.
15. The optimal temperature for phi29 polymerase is 37°C. We typically conduct RCA for 1 hour. If RCA is performed for several hours (or overnight) at 37°C, RCPs may start to fragment what could interfere with accurate signal counting. If large RCPs are desired (thick tissue sections or those with high autofluorescence), we advise doing RCA at RT overnight. Such approach will generate very large but compact RCPs.
16. In a multiplexed reaction (when more than one decorator probe is used), we recommend incubation at 37°C for 30 min (to minimize nonspecific binding of the oligonucleotides). In such case, cover the chamber inlets to prevent mix evaporation.
17. A double edge razor can be used to facilitate complete removal of the Secure-Seal chamber.
18. We typically apply 5–7 μ L of mounting medium for single, 50 μ L Secure-Seal chamber. Remove excess of the medium by gently pressing the slide and coverslip against a paper towel (excess of medium will be absorbed by the paper towel).
19. We use a Zeiss Axioplan II Epifluorescence microscope equipped with either a metal halide lamp or LED light source (Lumencor SpectraX) and a Hamamatsu, sCMOS camera. The following filter setup provides good wavelength separation and minimal crosstalk between different channels. 38HE (Zeiss) for imaging GFP/FITC/FAM dyes; SP102v2 (Chroma) for imaging Cy3 (minimal crosstalk with 38HE filter); SP103v2 (Chroma) for imaging Cy3.5/TexasRed; SP104v2 (Chroma) for imaging Cy5; 49007 (Chroma) for imaging Cy7/Alexa 7.5 dyes.
20. CellProfiler is a great, user-friendly tool to aid biologists in image processing and analysis. With respect to presented protocol, CellProfiler offers scripts for cell segmentation (definition of the nucleus and the cytoplasm), RCP segmentation, and assignment of RCPs to individual cells or fluorescence measurements. All scripts can be implemented in automated pipeline, allowing for batch image processing. An example script for cell and RCP identification is available at CellProfiler website <http://www.cellprofiler.org>. Briefly, gray scale TIFF images (offering highest resolution, JPEG images are processed faster and can also be used) from individual fluorescence channels are loaded into the pipeline. Cells are segmented to nuclei and cytoplasm and RCPs are identified and related to neighboring cells. Finally, number of RCPs for each cell is exported as a .csv file, which can be used for post-analysis processing.



Materials

MATERIALS

☒ BSA-Molecular Biology Grade - 12 mg **New England Biolabs Catalog #B9000S**

☒ Ethanol

☒ Tween 20 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P9416-50ML**

☒ nuclease free water

☒ PBS

☒ Trypsin-EDTA (0.25%), phenol red **Thermofisher Catalog #25200-056**

☒ Diethyl pyrocarbonate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D5758**

☒ phi29 DNA Polymerase (10 U/μL) **Thermo Scientific Catalog #EP0091**

☒ TRANSCRIPTME Reverse Transcriptase **BLIRT Catalog #RT32**

☒ RNase H **BLIRT Catalog #RT34**

☒ SlowFade™ Gold Antifade Mountant **Invitrogen - Thermo Fisher Catalog #S36936**

☒ RIBOPROTECT Hu RNase Inhibitor **BLIRT Catalog #RT35**

☒ dNTPs mix **BLIRT Catalog #RP65**

☒ Formamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #F9037**

☒ Formaldehyde solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #252549**

☒ Hydrochloric acid (HCl) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #258148**

☒ Glycerol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G5516**

☒ DAPI **Biotium Catalog #40043**

☒ Tth DNA Ligase **GeneCraft Catalog #GC-040**

☒ Pepsin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P7012**

Other common solutions needed that can be obtained from various vendors (see Note 1):

Xylene (safety precautions when using)

RNase and DNase inactivators for decontamination of working area and lab equipment.

20X SSC (3 M NaCl and 300 mM trisodium citrate in DEPC-H₂O, pH 7)

Safety warnings

! See safety data sheets for proper chemical handling, precautionary measures and waste disposal.

Formamide:

Handle with proper attire including gloves and eye protection. Work under fume hood when handling solution and dispose of waste appropriately.

Suspected of causing cancer.

May damage fertility or the unborn child.

May cause damage to organs (Blood) through prolonged or repeated exposure if swallowed.

Hydrochloric acid (HCl):

Handle with proper attire including gloves and eye protection. Work under fume hood when handling solution and dispose of waste appropriately.

May be corrosive to metals.

Causes severe skin burns and eye damage.

May cause respiratory irritation.

Formaldehyde/paraformaldehyde (PFA):

Handle with proper attire including gloves and eye protection. Work under fume hood when handling solution and dispose of waste appropriately.

May cause cancer.

Toxic if swallowed, in contact with skin or if inhaled.

Causes severe skin burns and eye damage.

May cause an allergic skin reaction.

May cause respiratory irritation.

Suspected of causing genetic defects.

Causes damage to organs (Eyes).

Xylene:

Work under fume hood with protective clothing when handling solution and dispose of waste appropriately

Flammable liquid and vapour.

May be fatal if swallowed and enters airways.

Causes skin irritation.

Harmful if inhaled.

May cause respiratory irritation.

May cause damage to organs (Central nervous system, Liver, Kidney) through prolonged or repeated exposure if inhaled.

Toxic to aquatic life.

Diethyl pyrocarbonate (DEPC):



Handle with proper attire including gloves and eye protection. Work under fume hood when handling solution and dispose of waste appropriately.

Combustible liquid.

Harmful if swallowed.

Before start

Below, we present an updated protocol for mRNA SNP genotyping in cell lines, fresh frozen and FFPE tissue sections.

All reaction volumes within the protocol are at 50 μ L and should be adjusted depending on chamber size (see Note 6).

To minimize introduction of contaminants into chambers, work in as clean environment as possible with clean RNase-free lab reagents and equipment. Decontaminate working area, use gloves, and fresh washing buffers. Incubations above room temperature, Secure-Seal chamber inlets should be covered with standard PCR film to prevent evaporation of reaction mix and slides should be contained within a humidified box during incubations.

Buffers and Solutions

All concentrated buffers are provided together with enzymes and stored according to vendor specification. Custom-made buffers should be prepared from DEPC-treated PBS or ddH₂O and can be kept at RT for up to one month. We also recommend aliquoting DEPC-ddH₂O and DEPC-PBS into smaller volumes (50 mL) to minimize contamination. (see Note 2)

- Phosphate buffered saline (PBS), can be purchased from numerous vendors:
 - 137 mM NaCl
 - 10 mM sodium phosphate
 - 2.7 mM KCl
 - DEPC-ddH₂O, pH 7.4
- Washing buffer (PBS-T):
 - 0.05% Tween 20
 - 1X PBS
- Saline-sodium citrate buffer (20X SSC), can be purchased from numerous vendors:
 - 3 M NaCl
 - 300 mM trisodium citrate
 - DEPC-ddH₂O, pH 7

Reaction mixes combined fresh during protocol procedure:

- RT-mix (50 μ L per chamber):
 - 5 μ L 10X RT-reverse transcriptase buffer,
 - 1 μ L 40 U/ μ L RNase Inhibitor
 - 0.5 μ L 20 μ g/ μ L BSA
 - 2.5 μ L 10 mM dNTPs mix
 - 0.5 μ L 100 μ M LNA primer or 2.5 μ L 100 μ M random decamers
 - TRANSCRIPTMereverse transcriptase (typically use 5 U/ μ L for cell lines and 20 U/ μ L for tissue sections)
 - Fill up to 50 μ L with DEPC-ddH₂O.



- Ligation mix (50 μ L per chamber):
 - 5 μ L 10X Tth ligase buffer
 - 2.5 μ L 2 μ M padlock probe(s)
 - 4 μ L 5 U/ μ L RNase H
 - 0.5 μ L 20 μ g/ μ L BSA
 - 2.5 μ L 1 M KCl
 - 10 μ L 100% formamide
 - 1.25 μ L 200 U/ μ L Tth ligase
 - Fill up to 50 μ L with DEPC-ddH₂O
- RCA mix (50 μ L per chamber):
 - 5 μ L 10X Phi29 DNA polymerase buffer
 - 1.25 μ L 10 mM dNTPs mix, 0.5 μ L 20 μ g/ μ L BSA
 - 5 μ L 50% glycerol
 - 5 μ L 10 U/ μ L Phi29 DNA polymerase
 - Fill up to 50 μ L with DEPC-ddH₂O.
- Hybridization mix (50 μ L per chamber):
 - 5 μ L 20X SSC
 - 10 μ L 100% formamide
 - 0.5 μ L 10 μ M Decorator probe(s)
 - 1.5 μ L, and
 - 0.25 μ l 100 μ g/ml DAPI
 - Fill up to 50 μ L with DEPC-ddH₂O.

Specimen Pretreatment: Adherent Cells

- 1 Culture cells in a flask until 80-90% confluence.
- 2 Wash cells twice with PBS, and dislodge cells by adding 1 mL/25 cm² of 0.25% Trypsin-EDTA for 1-2 min.

 00:01:00 Trypsin

- 3 Aspirate Trypsin-EDTA and monitor cells trypsinization under a brightfield microscope.
- 4 Resuspend the cells in appropriate culturing medium (FBS in a medium inactivates the trypsin).
- 5 Place sterile microscope slides in a 150 mm X 25 mm culture dish and add ~22 mL of medium to cover the slides. Cells adhere to most type of slides.

Note: You could also use specialized chambered culture slides that contain cell cultures to small volume directly on the slide.

- 6 Carefully, seed a total 3 mL of suspended cells directly on the slides (see Note 9).

Note

Note 9: Unlike in many in situ hybridization methods, where cells are grown on coverslips, we grow cells on the microscope slides directly. We found slides to be more resistant to breaking (especially when applying and peeling the silicone chambers off) and on the slides, multiple experiments can be run in parallel (up to eight), making the work more convenient. Special microscope culture slides can also be used in growing cells on slides. Also, multiple consecutive tissue section can be placed on a single slide to facilitate fast experimenting and imaging.

- 7 Carefully transfer the closed dish into the incubator and incubate the cells under appropriate conditions to allow them to adhere to the microscope slides (see Note 10).

**Note**

Note 10: Optimal seeding conditions should be identified experimentally for every cell line. For cells with large cytoplasm, 3 mL suspension is usually enough to create homogenous cell layer on each slide. Cells with smaller cytoplasm can be seeded at higher density. In our experience, overnight incubation allows cells to adhere to slides efficiently. Extended incubation can result in cell proliferation on-slide (too dense or clumped cells are difficult to analyze by image analysis software).

8 Wash the slides twice with cold PBS and transfer the slides to a sterile Coplin jar or sterile slides transport box.

9 Fix the cells with 3.7% formaldehyde at room temperature (RT) for 20 min.

 00:20:00 Fixation

Safety information

Safety precautions: Formaldehyde

10 Discard formaldehyde appropriately and wash the slides twice with PBS.

11 Gradually dehydrate and pre-permeabilize the cells in ethanol series (70, 85, and 99.5% each step for 3 min).

 00:03:00 70% EtOH

 00:03:00 85% EtOH

 00:03:00 99.5% EtOH

12 Air dry the slides.

For future use, slides can be stored at -80°C (long-term storage) or -20°C (up to 2 weeks). Make sure slides are labeled appropriately.

For stored frozen slides, allow to thaw to RT before continuing.



- 13 Attach Secure-Seal chamber of appropriate size and rehydrate the cells by adding PBS-T into the chamber (see Note 11).

Note

Note 11: Tween 20, as a surfactant, coats the chambers, facilitates buffer exchange and prevents formation of "dead spaces" inside the chamber. As a detergent, it can provoke bubble formation. We recommend adding a buffer into a chamber when slide is slightly tilted.

- 14 Remove PBS-T and permeabilize the cells by adding 0.1 M HCl for 5 min.

 00:05:00 0.1 M HCl

Safety information

Safety precautions: Hydrochloric acid

- 15 Remove HCl and wash the cells twice with PBS-T

Specimen Pretreatment: Freshly Frozen Tissue

- 16 Tissue is cryosectioned and mounted on microscope slides (see Note 12) and can be stored at -80°C until use.

Note

Note 12: Any slides that enhance adhesion of tissue sections can be used. (SuperFrost Plus® from Menzel-Glaser work very well in our hands). Sections should be as thin as possible (preferably few cell layer). Thinner sections are more prone to break and fold during cutting. We commonly use 10 µm thick sections.



17 Thaw slides at RT for a few minutes.

 00:03:00 RT Thaw

18 Fix the tissue in 3.7% formaldehyde for 45 min. Use a sterile Coplin Jar or perform fixation directly on tissue sections on flat laying slides.

 00:45:00 Fixation

Safety information

Safety precautions: Formaldehyde

19 Wash once with PBS for 5 min.

 00:05:00 PBS wash

20 (Optional)

Permeabilize the tissue by incubating with prewarmed pepsin in 0.1 M HCl at 37°C. Digestion with 250 U/mL (0.1 mg/mL) pepsin for 5 min is a good starting point in our experience. Optimal conditions need to be identified for the respective specimen (see Notes 4 and 13).

 00:05:00 HCl  37 °C

Safety information

Safety precautions: Hydrochloric acid

**Note**

Note 4: Lyophilized pepsin batches may vary in activity, even from the same supplier. Every new batch of pepsin should be tested on established model. We typically detect housekeeping gene (ACTB or GAPDH) in the same tissue type to evaluate detection reproducibility.

Note 13: The fixation time needs to compromise optimal fixative diffusion and minimize loss of tissue content. We recommend pragmatic evaluation of fixation parameters (consecutive sections should be used for each condition). Fixation time may vary for different tissues and different specimen thicknesses. Housekeeping gene is a good candidate for such optimization studies. Use conditions showing maximal signal amount.

- 21 Wash once with PBS for 5 min.

 00:05:00 Wash

- 22 Dehydrate the tissue section in the ethanol series (70, 85, and 99.5% ethanol, 3 min each).

 00:03:00 70% EtOH

 00:03:00 85% EtOH

 00:03:00 99.5% EtOH

- 23 Let slide air dry and mount appropriate size Secure-Seal chamber.

- 24 Rehydrate the tissue with PBS-T.

Specimen Pretreatment: Formalin-Fixed, Paraffin-Embedded (FFPE) Tissue

- 25 Sectioned tissues are mounted on microscope slides (see Note 12) and can be stored at -80°C until use.

**Note**

Note 12: Any slides that enhance adhesion of tissue sections can be used. (SuperFrost Plus® from Menzel-Glaser work very well in our hands). Sections should be as thin as possible (preferably few cell layer). Thinner sections are more prone to break and fold during cutting. We commonly use 10 µm thick sections.

26 Frozen samples are thawed at RT.

27 Dewax samples by submerging slides in a solvent series in separate Coplin jars:

15 min xylene  00:15:00 Xylene (1)

10 min xylene  00:10:00 Xylene (2)

2X 2 min 100% ethanol  00:02:00 100% EtOH (1)  00:02:00 100% EtOH (2)

2X 2 min 95% ethanol  00:02:00 95% EtOH (1)  00:02:00 95% EtOH (2)

2X 2 min 70% ethanol  00:02:00 70% EtOH (1)  00:02:00 70% EtOH (2)

5 min DEPC-H₂O  00:05:00 H₂O

5 min PBS  00:05:00 PBS

Safety information

Safety precautions: Xylene

28 Permeabilize the tissue by incubating with pre-warmed pepsin in 0.1 M HCl at 37°C for 5 min (see Notes 4 and 13).

 00:05:00 HCl  37 °C

Safety information

Safety precautions: Hydrochloric acid

**Note**

Note 4: Lyophilized pepsin batches may vary in activity, even from the same supplier. Every new batch of pepsin should be tested on established model. We typically detect housekeeping gene (ACTB or GAPDH) in the same tissue type to evaluate detection reproducibility.

Note 13: The fixation time needs to compromise optimal fixative diffusion and minimize loss of tissue content. We recommend pragmatic evaluation of fixation parameters (consecutive sections should be used for each condition). Fixation time may vary for different tissues and different specimen thicknesses. Housekeeping gene is a good candidate for such optimization studies. Use conditions showing maximal signal amount.

29 Wash with PBS for 5 min.

 00:05:00 PBS Wash

30 Postfix the tissue in 3.7% formaldehyde for 10 min at RT.

 00:10:00 Postfix

31 Wash twice with PBS for 5 min.

 00:05:00 PBS Wash (1)

 00:05:00 PBS Wash (2)

32 Dehydrate the tissue section in the ethanol series (70, 85, and 99.5% ethanol, each step for 3 min).

 00:03:00 70% EtOH

 00:03:00 85% EtOH

 00:03:00 99.5% EtOH

33 Air dry the slides and mount Secure-Seal chambers.

34 Rehydrate the tissue with PBS-T.

SNP Detection Protocol: Reverse Transcription



- 35 Combine reagents in table below and then remove PBS-T from chamber and apply the RT mix to the chamber.μ

Reagent	Conc.	Volume (μl)
RT-reverse transcriptase buffer	10X	5
RNase Inhibitor	40 U/μl	1
BSA	20 μg/μl	0.5
dNTP mix	10 mM	2.5
LNA primer/random decamers	100 μM	0.5/2.5
TRANSCRIPTME reverse transcriptase	200 U/μl	5 U/μl for cell lines and 20 U/μl for tissue sections
DEPC-ddH2O		Up to 50

Note: Water is used fill up to 50 ul. This could change depending on initial concentration of starting reagents.

- 36 Cover the chamber inlets and incubate the slides at 37°C.
The optimal incubation time needs to be determined experimentally.

With LNA-modified target-specific primers, 1 hour is sufficient.

 37 °C  01:00:00 LNA primers

With random decamers, reverse transcription is typically conducted overnight.

 37 °C Decamers overnight.

SNP Detection Protocol: Postfixation

- 37 Remove reverse transcription reagents and wash the chamber once with PBS-T.

38 Fix cells with 3.7% formaldehyde. (See Note 3)

For cultured cells, 10 min.

⌚ 00:10:00 Cell postfixation

For tissue sections, up to 45 min.

⌚ 00:45:00 Tissue postfixation

Note

Note 3: We recommend using freshly prepared formaldehyde solutions in DEPC-PBS. Working solutions can be prepared from either 37% methanol-stabilized stock solution or from paraformaldehyde powder. Aliquots of 3.7% formaldehyde in DEPC-PBS in 1 mL (used during the experiment) and 15 mL (for cell fixation) can be stored at -20°C. Do not freeze and thaw.

Safety information

Safety precautions: Formaldehyde

39 Wash the chamber twice with PBS-T.

SNP Detection Protocol: mRNA Degradation, Padlock Probe Hybridization and Ligation

40 Combine reagents in the table below for the padlock probe ligation mix.

	Reagent	Conc.	Volume (μl)
	Tth Ligase Buffer	10X	5
	Padlock Probe(s)	2 μM	2.5

	RNase H	5 U/ μ l	4
	BSA	20 μ g/ μ l	0.5
	KCl	1 M	2.5
	Formamide	100%	10
	Tth Ligase	200 U/ μ l	1.25
	DEPC-H ₂ O		up to 50

Note: amount of water needed will depend on initial concentration of reagents and amount of padlock probes used in assay.

Safety information

Safety precautions: Formamide

- 41 Remove PBS-T and add combined ligation mix reagents to the chamber. Cover the chamber inlets and incubate the slides in a humidity chamber at 37°C for 30 min.

 37 °C  00:30:00 PLP hybridization (1)

- 42 Transfer humidity chamber to 45°C incubator for 45 min. (see Note 14)

 45 °C  00:45:00 PLP hybridization (2)

Note

Note 14: RNase H has the highest activity at 37°C. It degrades RNA from mRNA/cDNA heteroduplex during the first 37°C incubation. After 30 min, sample is transferred to 45°C which is the optimal temperature for the Tth ligase. Addition of formamide into the mix lowers dsDNA stability (T_m of PLP arms/cDNA duplex). This enables extension of PLP arms that strengthens probe “locking” on cDNA and gives a good balance between arms melting and specific binding.

43 Remove reagents and wash the chamber twice with PBS-T.

SNP Detection Protocol: Rolling Circle Amplification

44 Combine the reagents in the table below for the RCA mix.

Reagents	Conc.	Volume (μl)
Phi29 DNA polymerase buffer	10X	5
dNTP mix	10 mM	1.25
BSA	20 μg/μl	0.5
Glycerol	50%	5
Phi29 DNA polymerase	10 U/μl	5
DEPC-ddH2O		Up to 50

Note: amount of water needed will depend on initial concentration of reagents.

45 Remove PBS-T and add combined ligation mix reagents to the chamber. Cover the chamber inlets and incubate the slides in a humidity chamber at 37°C for ≥1 hour. (see Note 15)

🔥 37 °C ⌚ 01:00:00 RCA

Note

Note 15: The optimal temperature for phi29 polymerase is 37°C. We typically conduct RCA for 1 hour. If RCA is performed for several hours (or overnight) at 37°C, RCPs may start to fragment what could interfere with accurate signal counting. If large RCPs are desired (thick tissue sections or those with high autofluorescence), we advise doing RCA at RT overnight. Such approach will generate very large but compact RCPs.

46 Remove RCA reagents and wash the chamber twice with PBS-T.



SNP Detection Protocol: Decorator Probe Hybridization and Nuclei Counterstaining

- 47 Combine the reagents in the table below for the hybridization mix.

	Reagent	Conc.	Volume (μl)
	SSC	20X	5
	Formamide	100%	10
	Decorator probes(s)	10 μM	0.5
	DAPI	100 μg/ml	0.25
	DEPC-ddH2O		up to 50

Safety information

Safety precautions: Formamide

- 48 Remove PBS-T and apply combined reagents to chamber and incubate the slide at RT for ~20 min. (see Note 16)

 00:20:00 Decorator probe

Note

Note 16: In a multiplexed reaction (when more than one decorator probe is used), we recommend incubation at 37°C for 30 min (to minimize nonspecific binding of the oligonucleotides). In such case, cover the chamber inlets to prevent mix evaporation.

- 49 Remove reagents and wash the chamber twice with PBS-T.
- 50 Mark the position of the chamber on the backside of the slide with a diamond pen and remove the Secure-Seal chamber. (see Note 17)

**Note**

Note 17: A double edge razor can be used to facilitate complete removal of the Secure-Seal chamber.

- 51 Dehydrate the slides through an ethanol series (70, 85, and 99.5% ethanol, each for 3 min).

⌚ 00:03:00 70% EtOH

⌚ 00:03:00 85% EtOH

⌚ 00:03:00 99.5% EtOH

Allow slide to air dry.

- 52 Apply Slow-Fade medium to the specimen and gently, mount the specimen. (see Note 5 and 18)

Note

Note 5: Slow-Fade® Gold Antifade Mountant works best in our experience to prevent photobleaching.

Note 18: We typically apply 5-7 μ L of mounting medium for single, 50 μ L Secure-Seal chamber. Remove excess of the medium by gently pressing the slide and coverslip against a paper towel (excess of medium will be absorbed by the paper towel).

SNP Detection Protocol: Image Acquisition and Analysis

- 53 Mounted slides can be imaged immediately. Conventional wide-field epifluorescence microscopes are usually sufficient to image RCA products in tissue sections and cells. (see Note 19)

Note

Note 19: We use a Zeiss Axioplan II Epifluorescence microscope equipped with either a metal halide lamp or LED light source (Lumencor SpectraX) and a Hamamatsu, sCMOS camera. The following filter setup provides good wavelength separation and minimal crosstalk between different channels. 38HE (Zeiss) for imaging GFP/FITC/FAM dyes; SP102v2 (Chroma) for imaging Cy3 (minimal cross talk with 38HE filter); SP103v2 (Chroma) for imaging Cy3.5/TexasRed; SP104v2 (Chroma) for imaging Cy5; 49007 (Chroma) for imaging Cy7/Alexa 7.5 dyes.

Note

Make sure to use filters appropriate for nuclear staining and fluorophores used in the experiment.

Depending on the level of detail desired, use appropriate objective (we typically use 10X, 20X and 40X high numerical aperture objectives).

To ensure accurate signal quantitation during image analysis, carefully adjust exposure time for a signal channels (abundant RCPs might be hard to segment if overexposed).

We typically acquire 5-10 μm thick z-stack of images to capture RCPs in all focal planes (if possible, preview the specimen to define "first" and "last" stack corresponding to RCPs present in the lowest and highest stack and capture all images within this range).

- 54 Z-Stacks with multiple focal planes are combined to a single 2D maximum intensity projection (MIP) image that can be used in image analysis. We recommend taking images from several positions for each experiment (depending on cell density) to account for cell-to-cell expression variations.
- 55 Open-source programs such as CellProfiler or ImageJ can be used for image analysis. (see Note 20)



Note

Note 20: CellProfiler is a great, user-friendly tool to aid biologists in image processing and analysis. With respect to presented protocol, CellProfiler offers scripts for cell segmentation (definition of the nucleus and the cytoplasm), RCP segmentation, and assignment of RCPs to individual cells or fluorescence measurements. All scripts can be implemented in automated pipeline, allowing for batch image processing. An example script for cell and RCP identification is available at CellProfiler website <http://www.cellprofiler.org>. Briefly, gray scale TIFF images (offering highest resolution, JPEG images are processed faster and can also be used) from individual fluorescence channels are loaded into the pipeline. Cells are segmented to nuclei and cytoplasm and RCPs are identified and related to neighboring cells. Finally, number of RCPs for each cell is exported as a .csv file, which can be used for post-analysis processing.

Anticipated Results

- 56 Every discrete, fluorescent detected signal originates from a single, successful detection of the SNP

All RCPs should be localized to the cytoplasm, however RCPs can occasionally dissociate out of cells and generate RCPs on the glass slide (such signals should be disregarded).

Expected result

For anticipated results, we refer to the accompanying publication.

https://link.springer.com/protocol/10.1007%2F978-1-4939-7213-5_14