Detection of bacterial pathogens and drug resistance markers using Rolling circle amplification

Malte Kühnemund[1], Ivan Hernandez[1], Mohd Istiaq Sharif[1], Matteo Cornaglia[2], Martin A.M. Gijs[2], Mats Nilsson[1]

[1]Stockholm University, Science for Life Laboratory, [2]EPFL - EPF Lausanne

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Working

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Molecular Diagnostics - Mats Nilsson Group

Ivan Hernandez

ABSTRACT

Here is described the protocol used to detect bacteria and antibiotic resistance markers using padlock probes and Rolling circle amplification. This method allows for single-molecule/digital quantification in a semi-quantitative way.

EXTERNAL LINK

https://academic.oup.com/nar/article/45/8/e59/2888443

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Sensitive and inexpensive digital DNA analysis by microfluidic enrichment of rolling circle amplified single-molecules

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

MATERIALS TEXT

Materials

Ampligase buffer 10x (Epicenter)

200 mM Tris-HCl (pH 8.3), 250 mM KCl, 100 mM $MgCl_2$, 5 mM NAD 0.1% Triton® X-100

Phi29 buffer 10x (ThermoScientific)

330 mM Tris-acetate pH-7.9, 100 mM Mg-acetate, 660 mM K-acetate, 1% (v/v) Tween 20, 10 mM DTT

Labeling buffer 2x

20 mM Tris-HCl pH-8.0, 20 mM EDTA, 0.1% Tween-20, 2 M NaCl

Padlock probes

5' phosphorylated padlock probes are obtained from IDT and resuspended in IDTE buffer (pH 8.0) to 100 µM for long storage at -20°C.

A working aliquot of 1 µM/each containing all the probes in ddH_2O. Storage at -20°C is recommended.

Detection oligos

Fluorophore-tagged oligos are obtained from IDT and resuspended in IDTE buffer (pH 8.0) to 100 µM for long storage at -20°C.

A working aliquot of 1 µM/each containing all the detection probes in ddH_2O. Ambar tubes and storage at -20°C is recommended.

dNTPs

Prepare a working aliquot of 2.5 mM/each in water.

BSA

Prepare a working aliquot of 2 µg/µl in water.

1 Dilute padlock probes to 10 nM. Is recommended to prepare a fresh dilution for each experiment.

2 Denature extracted DNA from culture isolate or clinical sample by heating at **95 °C** **00:05:00** .Put immediately on ice

3 Prepare ligation mix for the total number of reactions you require

	Initial conc.	Final conc.	x1 reaction
Probe mix	10 nM	100 pM	0.2
BSA	2 µg/µl	0.2 µg/µl	2
Ampligase Buffer	10 ×	1 ×	2
Ampligase	5 U/µl	0.25 U/µl	0.5
mQ H_2O	-		5.3
		Final mix volume (µl)	10

Add 10 µl of master mix to each assay tube and add 10 µl of the denatured DNA from Step 1.

Incubate the assay tubes at:

95 °C **00:00:30**

55 °C **00:40:00**

4 Prepare RCA mix for the total number of reactions you require

	Initial conc.	Final conc.	x1 reaction
dNTP's	2,5mM	125 µM	1.5
phi 29 buffer	10x	1 x	3
BSA	2 µg/µl	0.2 µg/µl	3
phi 29 polymerase	10 U/uL	400 mU/uL	1.2
mQ H_2O			1.3
		Final mix volume (µl)	10

Add 10 µL to each tube assay containing the ligation reaction

Incubate the assays tubes at:

37 °C **03:00:00**

65 °C **00:02:00**

Note 1. In principle the longer the amplification time the longer amplification products and thus stronger signals one can get. Phi29 polymerase is reported to be stable for up to 12 h. For quantifying rolling circle products spread on microscope slides 1 h amplification time is enough to get robust signals. For microfluidic enrichment ≥ 2 h amplification is recommended.

Note 2. Ampligase buffer can also be used in this step. Volumes need to be adjusted in that case

5 Prepare the labeling mix for the total number of reactions you require

	Initial conc.	Final conc.	x1 reaction
Labelling buffer	2x	1x	15
Detection oligo mix	1 µM	5nM	0.3
mQ H2O			14.4
		Final mix volume (µl)	30
		Total volume (µl)	60

Labeling master mix

Add 30 µL to each tube assay containing the Rreaction

Incubate at:

75 °C **00:02:00**

55 °C **00:15:00**

Note 3. For optimal results using microfluidic enrichment, filtering this mix through a 0.1 µmfilter (Millipore). This will filter out fluorophore aggregates or cristals that may cause background.

6 If the initial concentration of samples is higher than 100 femtomolar, pipette a 5-10 µL drop on a Superfrost Microscope Slide (Thermofisher) and put a 24x24 mm cover slip (Menzel) on top to spread it.

Incubate the slide at room temperature for **00:10:00** and you can proceed to image using fluorescence microscopy. 10x or 20x objectives are recommended.

If the concentration is lower than 100 femtomolar, microfluidic enrichment is required.