Technical Note

Locked Nucleic Acid

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Efficient poly(A)+ RNA selection using LNA oligo(T) capture

Introduction

This technical note describes a new method for the isolation of intact polyadenylated mRNA using LNA oligo(T) capture. The method enables efficient isolation of polyadenylated mRNA directly from lysed Guanidinium thiocyanate (GuSCN)-containing cell or tissue extracts by combining the design of biotinylated LNA oligo(T) capture probes with subsequent immobilization of the captured poly(A)+RNA onto streptavidin-coated magnetic particles. In contrast to DNA oligo-dT and poly T PNA based mRNA isolation techniques, the LNA oligo(T) capture method allows poly(A) selection in presence of 4 M GuSCN cell lysis buffer, which is needed for efficient inactivation of endogenous RNases. In addition, LNA oligo(T) facilitates highly efficient poly(A)+RNA isolation at elevated temperatures compared to standard DNA oligo(dT) technology. The successful use of the LNA oligo(T) capture method in the recovery of yeast in vitro synthesized polyadenylated ACT1 spike mRNA as well as in the isolation of intact poly(A)+RNA from C. elegans worms is presented.

Materials and methods

- Biotinylated LNA oligo-T capture probe (see Table 1)
- Solution D (4 M Guanidinium thiocyanate, 25 mM Na-citrate, pH 7.0, 0.5% (w/v) sodium N-lauroyl sarcosinate)
- Washing buffer (20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA (pH 7.5) 0.1% (w/v) lauryl sarcosinate)
- TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5)
- Quartz sand, baked at 220 (C for 12 hours
- Pestle
- Streptavidin-coated magnetic particles (e.g. Roche Cat. # 1 641 778)
- Magnetic separator (e.g. the PickPen system from BioNobile, Finland)
- Yeast RNA (e.g. Ambion, USA cat. # 7120G)
- Thermomixer
- Siliconized, RNase-free tubes (e.g. Ambion, USA cat # 12450)
- Sodium acetate pH 5.5 (e.g. Ambion, USA cat. # 9740)
- Glycogen Carrier (e.g. Ambion, USA cat. #9510)
- 70% ethanol
- DEPC-treated H2O

Name	Sequence
DNA_dT_{20} (control)	5'-Biotin-TTTTTTTTTTTTTTTTTTT-3'
LNA_4.T	5'-Biotin-$TT\mathbf{T}^LTTT\mathbf{T}^LTTT\mathbf{T}^LTTT\mathbf{T}^LTTT\mathbf{T}^LT$-3'
LNA_3.T	5'-Biotin-$\mathbf{T}^LTT\mathbf{T}^LTT\mathbf{T}^LTT\mathbf{T}^LTT\mathbf{T}^LTT\mathbf{T}^LTT\mathbf{T}^LT$-3'
LNA_2.T	5'-Biotin-$\mathbf{T}^LT\mathbf{T}^LT\mathbf{T}^LT\mathbf{T}^LT\mathbf{T}^LT\mathbf{T}^LT\mathbf{T}^LT\mathbf{T}^LT\mathbf{T}^LT\mathbf{T}^LT$-3'
LNA_T_{10}	5'-Biotin-$\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L$-3'
LNA_T_{15}	5'-Biotin-$\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L\mathbf{T}^L$-3'
LNA_T_{20}	5'-Biotin-$\mathbf{T}^L$-3'

$\mathbf{T}^L$= LNA thymidine

Table 1. LNA oligo(T) oligonucleotide capture probes.

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Procedure for direct poly(A)+RNA selection

Sample preparation

1. Thaw the cell or tissue sample (e.g. C. elegans worms stored in RNAlater, Ambion, USA cat. # 7020).
2. Spin 4000g 2 min and carefully remove the supernatant.
3. Add 200 µL solution D. Vortex briefly.
4. Add quartz sand and mix 2 min on ice using a pestle for homogenizing the sample.
5. Spin the tube briefly (e.g. 60 s at 16100g or at max speed.) and carefully remove the supernatant to a clean tube.
6. Heat the lysate for 30 min. at 65ºC on an Eppendorf Thermomixer (shaking 700 rpm).
7. Spin the tube briefly (e.g. 60 s at 16100g) and transfer the supernatant to a clean RNase-free tube.

Pre-blocking of Streptavidin-coated magnetic particles

8. Pipette 60 µL of Streptavidin-coated magnetic particles into a tube for each sample preparation.
9. Use a magnetic separator and remove the supernatant
10. Add 100 µL 1 µg/mL yeast RNA diluted in TE buffer.
11. Pre-block for 5 min at room temperature
12. Wash the particles in 100 µL TE buffer

Pipette into tubes for each sample preparation:
13. 100 µL solution D
14. 3 aliquots of 100 µL Washing buffer
15. 50 µL DEPC-treated H2O

Poly(A)+RNA isolation

16. To each cell-free extract, add 200 pmol biotinylated LNA oligo-T capture probe (2 µL of a 100 µM stock) and transfer the pre-blocked Streptavidin-coated particles to the tube.
17. Incubate 10 min at 37°C (or optionally at 55ºC) in an Eppendorf Thermomixer (shaking 700 rpm). The poly(A)+RNA is bound to the particles.
18. Particles are recollected and released into the washing buffer
19. Collect the particles from the washing buffer and repeat the washing step twice
(= three washing steps)
20. Transfer the particles to a tube containing DEPC-H2O.
21. Incubate at 65ºC 10 min to elute the poly(A)+RNA from the particles and quench on ice for 5 min
22. Recollect the particles twice from the eluted poly(A)+RNA sample
23. Spin the eluted poly(A)+RNA sample briefly (e.g. 60 s 16100g) and transfer to a clean siliconized tube to avoid any remaining magnetic particles

Ethanol precipitation

24. Add 1/10 vol. of 3 M sodium acetate pH 5.5, 150 µg/mL Glycogen Carrier and 2.5 vols of 96% ethanol to the tube.
25. Pecipitate at -20(C overnight
26. Spin 30 min 16100g at 4(C
27. Remove the supernatant and wash the pellet with ice-cold 70% ethanol prepared in DEPC-water
28. Dry the pellet at room temperature
29. Dissolve in a small volume of DEPC-treated H2O (e.g. 5-10 µL)
30. Spin briefly (e.g. 60s 16100g)
31. Quantify the purified polyadenylated RNA.

Results

Recovery assay for in vitro synthesized yeast ACT1 spike mRNA

The recovery of in vitro synthesized polyadenylated ACT1 spike mRNA from 4M GuSCN cell lysis buffer was assessed using the DNA-dT20, LNA_2.T, LNA_T10 or LNA_T15 at various hybridization temperatures. As shown in Fig. 1, the use of LNA oligo(T) capture results in 40-fold higher recovery of the ACT1 spike mRNA at 37°C compared to the DNA oligo(dT) control. When the hybridization temperature is increased to 55°C, only the LNA oligo(T) capture oligos are capable of binding the spike mRNA, thus demonstrating the usefulness of LNA capture under high stringency conditions.

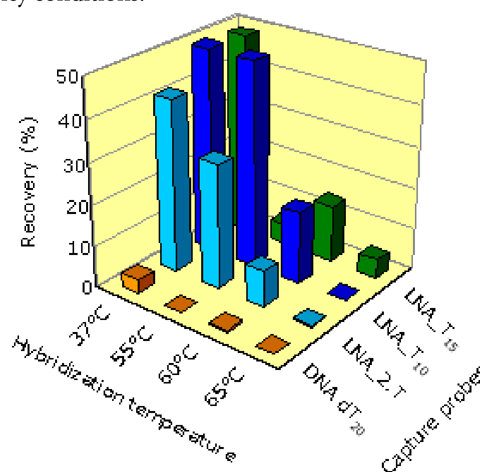
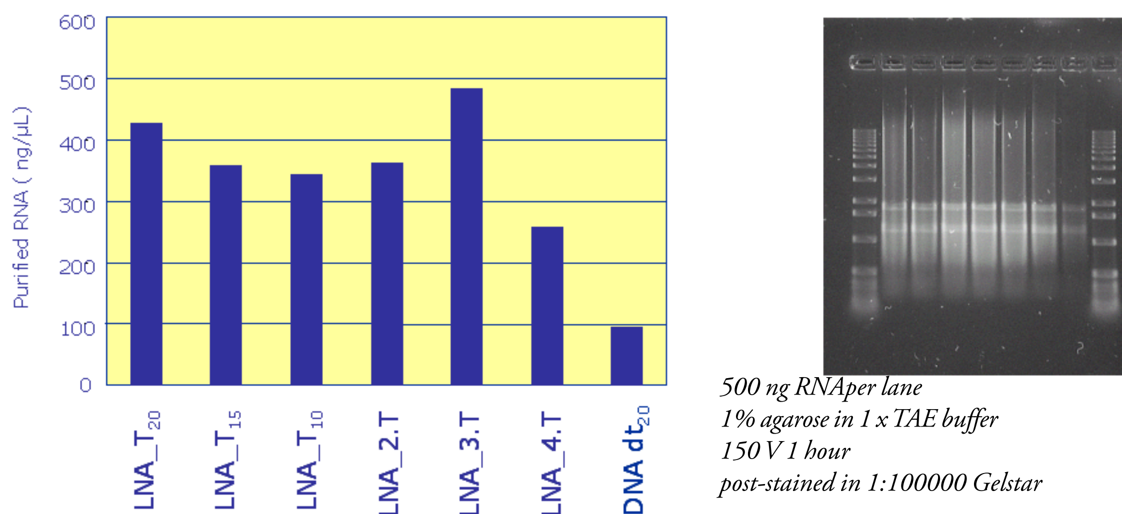


Fig. 1. Recovery of polyadenylated yeast ACT1 spike mRNA in 4 M guanidinium thiocyanate buffer at different temperatures using LNA oligo(T)capture.

Isolation of poly(A)+RNA from C. elegans worms

Polyadenylated mRNA was isolated from C. elegans mixed stage worms according to the one-step method as described above. The use of LNA_3.T in the poly(A) selection resulted in a 5-fold mRNA yield increase compared to the DNA control (Figure 2). All worm poly(A)+RNA samples were intact as judged by gel electrophoresis on a 1 % agarose gel (Fig. 2).



500 ng RNAper lane
1% agarose in 1 x TAE buffer
150 V 1 hour
post-stained in 1:100000 Gelstar

Fig. 2. Isolation of intact poly(A)+RNA from C. elegans worms using LNA oligo(T) capture.

Conclusions

A common problem for all current mRNA isolation methods is that they do not allow poly(A) selection in 4 M guanidinium thiocyanate, which is needed for efficient inactivation of endogenous RNases. In contrast, an astonishing feature of LNA oligonucleotides is that they are capable of forming duplexes in strong chaotrophic buffers, and can thus readily be applied to poly(A)+RNA selection from 4 M GuSCN cell extracts. The benefits of using LNA oligo(T) in poly(A)+RNA selection are:

- Significantly improved poly(A)+RNA isolation in the presence of 4 M guanidinium thiocyanate lysis buffer compared to DNA oligo-dT and poly T PNA
- One-step poly(A)+RNA isolation from tissue and cell extracts in 4M GuSCN extraction buffer
- Up to 40-fold higher recovery of mRNA from 4M GuSCN cell lysis buffer at elevated temperatures compared to the DNA oligo-dT control
- Intact poly(A)+RNA due to efficient inactivation of endogenous RNases dusing mRNA isolation

Trademarks and patents

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