Isothermal Amplification of RNA by Transcription-Mediated Amplification (TMA)

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Introduction

Conventional methods of amplifying an RNA target require a discrete reverse transcriptase step, followed by a separate DNA amplification method (e.g. PCR, LCR, SDA). Here, we describe transcription-mediated amplification (TMA), which amplifies RNA or DNA directly using reverse transcriptase and RNA polymerase (Kacian and Fultz, 1996). TMA has been used to amplify cellular mRNA, viral RNA and highly structured ribosomal RNA (McDonough, Bott and Giachetti, 1997; Jonas et al., 1993). Because RNA is an integral part of its amplification cycle, TMA has excellent sensitivity with these targets – typically <10 copies of mRNA and <50 copies of rRNA. TMA products are single-stranded RNAs, which do not need to be denatured prior to probe detection.

Although other amplification methods such as LCR and PCR require temperature cycling for denaturation and ligation or extension, TMA is isothermal and is performed at 37-42°C. TMA generally utilizes two primers which flank the region to be amplified: a promoter-primer, and a non-promoter primer that is the same sense as the target. The promoter-primer contains a 3'-region complementary to the RNA target, and a 5'-promoter sequence recognized by the RNA polymerase. In the examples described here, 21 bases of the wild type T7 promoter form the 5'-end of the promoter-primer.

A diagram depicting amplification of an RNA target by TMA is shown in Fig. 1. TMA is initiated by hybridization of the promoter-primer to the (+) sense target RNA. In steps 1 - 6 which follow, the RNA target is converted into a double-stranded (ds) transcription template. Reverse transcriptase extends the primers and its associated RNase H activity removes RNA

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from extension products. Hundreds of copies of (-) sense RNA amplicon are transcribed from this DNA intermediate (steps 7 and 8). Each of these amplicons can be similarly converted into a new dsDNA transcription template as shown in steps 9 - 12. The cycling reaction continues isothermally and exponentially, producing detectable amounts of (-) sense RNA amplicon in as little as 15 minutes.

TMA products may be analyzed in a variety of ways, including hybridization with radioactive or non-radioactive probes. We utilize the hybridization protection assay (HPA). HPA is a chemiluminescent, non-radioactive, solution-based homogeneous hybridization method that is highly sensitive, and simple and rapid to use (Arnold et al., 1989; Ou et al., 1990; Nelson et al., 1995). Oligonucleotide HPA probes are synthesized with an alkylamine linker arm to which an acridinium ester (AE) label is then attached. The AE label is resistant to hydrolysis when the probe is fully hybridized to a complementary target, but the AE label on an unhybridized probe is sensitive to alkaline hydrolysis, which renders it non-chemiluminescent. Thus,

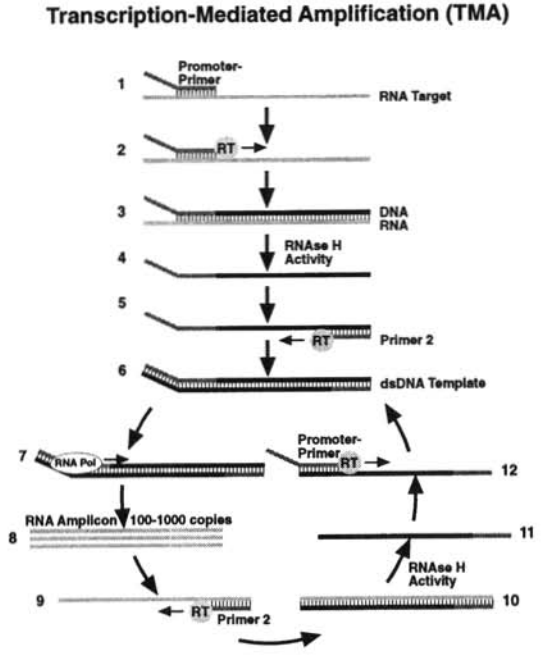

Fig. 1. Schematic presentation of TMA of an RNA target. In steps 1 through 6 the RNA target is converted into a dsDNA transcription template using reverse transcriptase and the two primers. Steps 7 through 12 depict the exponential cycling portion of the reaction in which RNA amplicon products are made

HPA signal is directly proportional to the amount of hybridized probe in the reaction tube. TMA coupled with HPA yields a consistent and quantitative result, which can be used to estimate input target levels. Amplification and detection are performed in the same tube, requiring only reagent additions. Thus the risk of amplicon contamination is minimized.

Outline

RNA targets are prepared for TMA using standard protocols. The TMA reaction is typically performed in a 100-µl volume using the following steps:

- RNA target and amplification reagent are mixed in a 12x75 mm or 1.5 ml conical tube, and overlaid with oil to prevent evaporation.
- RNA target is denatured, then cooled to the amplification temperature.
- Amplification is initiated by addition of the enzyme reagent.

Following amplification, detection is performed by hybridization. For HPA:

- AE-probe reagent is added directly to the completed amplification reaction.
- Probe is hybridized to the RNA amplicon produced by TMA.
- AE label on unhybridized probe is inactivated in the alkaline hydrolysis selection step.
- Samples are cooled to room temperature, then read in a suitable luminometer. Results are quantitative within the dynamic range of the instrument.

Materials

Equipment

- Luminometer. Recommend LEADER 1, LEADER 50, or LEADER 450i (Gen-Probe Incorporated, San Diego, CA). Luminometer must be equipped with automatic injection of 0.2 ml each of two reagents.
- Eppendorf or other repeating pipettors recommended.
- Reactions are typically performed in Elkay 12 x 75 mm polypropylene reaction tubes.

Reagents

- Oligonucleotides: promoter-primer 1: 5'-AATTTAATAC GACTCACTAT AGGGAGACCA CACCTTGTCT TATGTCCAGA ATGCT-3', non-promoter primer 2: 5'-ATAATCCACC TATCCCAGTA GGAGAAAT-3', probe 1: 5'-GATGGATAAT CCTGGGATTA AATAAAATAG TAAGAATGTA TAGC-3', probe 2: 5'-ATCCTGGGAT TAAATAAAAT AGTAAGAATG TATAGCCCTA CCA-3'

T7 promoter and non-target sequences are underlined on promoter-primer 1. AE-labeled probes 1 and 2 are from the Gen-Probe HIV-1 *gag*-1 and 2 AccuSearch kit (Gen-Probe Incorporated, San Diego, CA).

- Amplification reagent (AR): 160 mM Tris-HCl, pH 7.5, 70 mM KCl, 92 mM $MgCl_2$, 16 mM ATP, 16 mM CTP, 20 mM GTP, 16 mM UTP, 6 mM dATP, 6 mM dCTP, 6 mM dGTP, 6 mM dTTP, 600-1200 nM each of promoter-primer and non-promoter primer. Store frozen. It is convenient to prepare AR without primers and to add the desired primers just before use.
- Enzyme reagent (ER): 140 mM Tris-HCl, 36 mM HEPES, pH 7.5, 70 mM potassium chloride, 4 mM DTT, 10% glycerol, 4% Triton X-100. Store frozen. Add 500 u MMLV RT and 400 U T7 RNA polymerase per 25 µl just prior to use.
- Probe reagent (PR): 1.2 M lithium chloride, 20 mM EDTA, 20 mM EGTA, 0.1 M lithium succinate, pH 5.1, 2% (w/v) lithium lauryl sulfate, 15 mM aldrithiol-2, 0.5 - 1 nM acridinium ester-labeled probe. Aldrithiol is relatively insoluble in aqueous solution, so a 500-mM stock is prepared in 100% EtOH, which is slowly added after everything else. A stock PR without AE-probe may be prepared and stored at room temperature. Following addition of the appropriate AE-probe, PR should be stored at 4°C to -20°C.
- Selection reagent (SR): 150 mM sodium tetraborate, pH 8.5, 1% Triton X-102. Store at room temperature.
- Detection reagent I: 1 mM nitric acid, 0.1% hydrogen peroxide, store at room temperature.
- Detection reagent II: Contains 1 N NaOH and a surfactant. Store at room temperature. NaOH is caustic. Care must be taken to avoid contact with skin. Detection reagents are available from Gen-Probe Incorporated, San Diego, CA.

Procedure

Amplification

1. Dilute purified RNA in water to appropriate concentration. Store on ice.
2. Add 25 µl of AR to 12 x 75 mm polypropylene tubes.
3. Overlay with 200 µl of oil.
4. Add RNA target in 50 µl to AR through oil. Mix by shaking.
5. Heat to 60°C for 5 - 10 minutes, cool in 42°C water bath 5 - 10 minutes.

6. Add 25 µl of enzyme reagent. Mix gently, incubate at 42°C for 60 minutes. Samples should not be allowed to cool during mixing.
7. Amplification reactions may be analyzed directly or stored frozen or refrigerated. A portion of the reaction may be removed and analyzed separately.

Detection

1. If amplification reactions have been stored frozen or refrigerated, warm them to room temperature.
2. Add 100 µl PR containing AE-probes, mix, hybridize at 60°C for 15-30 minutes.
3. Remove rack from water bath, add 300 µl SR, vortex briefly and place at 60 °C for another 15 minutes. AE label on unhybridized probe is hydrolyzed at this step.
4. Cool samples to room temperature (1 to 5 minutes in a room temperature water bath) and read in a luminometer. The luminometer must inject 0.2 ml detection reagent I, delay 2 s, inject 0.2 ml of detection reagent II, then read immediately for two seconds. Chemiluminescent signal is expressed in relative light units (RLU).
5. A "positive" value from TMA reactions detected by HPA may be defined as some multiple of the average negative signal (often 20x), or as a cut-off value empirically determined.

Results

As an example illustrating the sensitivity and quantitative nature of TMA and HPA with RNA targets, part of the HIV-1 group-specific antigen (gag) region of HIV-1 (Ratner et al., 1985) was amplified and detected using primers and probes described in Materials. TMA/HPA results using the full-length HIV transcript RNA are shown in Figure 2. Negative reactions (n=8) produced an average of 804 RLU (not shown). Sensitivity in this assay was at least 50 copies of RNA transcript, which produced an average signal 200-fold over background. The quantitative capability of TMA is also evident, as the RLU signal increased linearly from 50 to 1600 copies of input RNA in this example. Above that level, TMA was still linear but the HPA signal began to saturate. In practice, the TMA/HPA combination can be linear over four orders of magnitude of target input. Unlabeled probe may be added to the HPA to lower the probe's specific activity or amplicon products may diluted to quantitate over this large dynamic range.

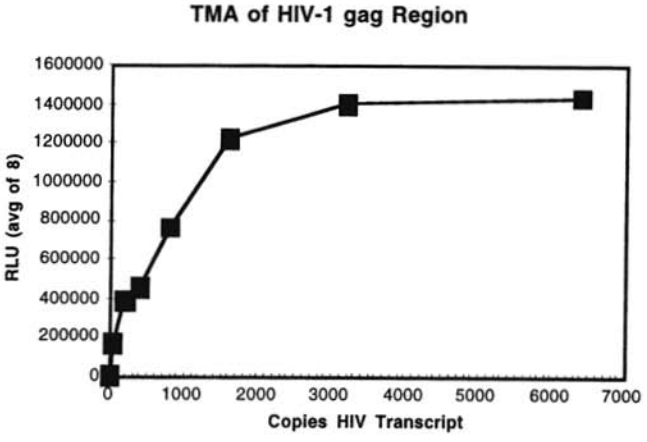

Fig. 2. Detection of HIV-1 *gag* RNA. Dilutions of HIV-1 RNA transcript were amplified and detected using the primers and probes described in Materials. Eight individual reactions were run at the RNA copy level indicated. Average RLU values are shown

Troubleshooting

- Primer design is a critical factor in TMA performance. Standard rules for primer design apply. Avoid regions with high secondary structure, runs of Gs or Cs on the 3' end, and primers which can hybridize to themselves or each other. Primers should have a calculated Tm in the range of 60 - 70 °C (for just the target binding region in the promoter-primer). Primer location and length must be optimized for best performance.
- Amplicons in the range of 100 - 250 bases are usually the most sensitive.
- RNA target denaturation prior to amplification is typically performed between 60°C and 95°C. The optimum temperature is dependent on secondary structure, and is empirically determined.
- Kinetics of product accumulation may vary with primer design, temperature of reaction, and nature of the target nucleic acid.
- Some parameters may need to be optimized for certain targets. In rough order of importance, these include: primer design, concentration and ratio of the two enzymes, $MgCl_2$ concentration, nucleotide concentration, and primer concentration.
- Optimum concentrations of RT and RNA polymerase from various vendors may differ.

Comments

TMA is a powerful yet easy way to amplify RNA targets. Once optimized it is a reliable and robust system. It works well with targets ranging from highly purified nucleic acids to bacterial or cell lysates. TMA itself is quantitative and does not require normalization to internal control RNAs. Although TMA products can be visualized by gel electrophoresis, the single-stranded RNA amplicons are well suited to detection in solution by oligonucleotide probes. Coupled with a specific and rapid detection method such as HPA, TMA can become the method of choice for routine detection and quantitation of RNA targets from a variety of samples.

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