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RNA amplification strategies for small sample populations

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Abstract

Advances in high throughput cloning strategies have led to sequencing of the human genome as well as progress in the sequencing of the genome of several other species. Consequently, the field of molecular genetics is blossoming into a multidisciplinary entity that is revolutionizing the way researchers evaluate a myriad of critical concepts such as development, homeostasis, and disease pathogenesis. There is tremendous interest in the quantitative assessment of tissue-specific expression of both newly identified and well characterized specific genes and proteins. At present, an ideal approach is to assess gene expression in single elements recorded physiologically in living preparations or by immunocytochemical or histochemical methods in fixed cells in vitro or in vivo. The quantity of RNA harvested from individual cells is not sufficient for standard RNA extraction methods. Therefore, exponential polymerase-chain reaction based analyses, and linear RNA amplification including amplified antisense RNA amplification and a newly developed terminal continuation RNA amplification methodology have been developed for use in combination with microdissection procedures and cDNA/oligonucleotide microarray platforms. RNA amplification is a series of intricate procedures to amplify genetic signals from minute quantities of starting materials for microarray analysis and other downstream genetic methodologies. RNA amplification procedures effectively generate quantities of RNA through in vitro transcription. The present report illustrates practical usage of RNA amplification technologies within the context of regional, population cell, and single cell analyses in the brain.

Keywords: Expression profiling; In vitro transcription; Postmortem human brain; Microarray; Molecular fingerprint; Functional genomics

1. Introduction

Conventional molecular biology techniques have enabled gene expression analysis in a wide variety of paradigms. Experimental methods include Southern analysis (DNA detection), Northern analysis (RNA detection), polymerase-chain reaction (PCR; DNA detection), reverse-transcriptase-PCR (RT-PCR; RNA detection), ribonuclease (RNase) protection assay, and in situ hybridization, among others. The majority of these approaches typically quantitate the abundance of individual elements one at a time (or a few at a time). Recent developments in high-throughput genomic methodologies enable the assessment of dozens to hundreds to thousands of genes simultaneously in a coordinated manner.

Microarray analysis has emerged as a useful and relatively cost effective tool to assess transcript levels in a myriad of systems and paradigms. A disadvantage to these high throughput technologies is a requirement for significant amounts of high quality input sources of RNA for increased sensitivity and reproducibility. Whole organism and regional studies can generate significant input amounts of RNA species without any amplification procedures [1,2]. Unfortunately, the quantity of RNA harvested from a single cell, estimated to be approximately 0.1–1.0 pg, is not sufficient for standard RNA extraction procedures [3–5]. Therefore, methods have been developed to increase the amount of input starting material for downstream genetic analyses, including exponential PCR-based analyses and linear RNA amplification procedures. This report focuses

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on molecular and cellular processes that occur during RNA amplification.

2. Input sources of RNA: single cell assessments

An advantage of single cell or single population gene expression analysis is that different cell types can be elucidated based upon their molecular fingerprint. For example, specific neuronal populations can be identified by selective expression of relevant proteins such as cholinergic basal forebrain neurons [6,7] and midbrain dopaminergic neurons [8,9]. Moreover, cells that lack a distinct or selective signature phenotype can be analyzed using a variety of Nissl stains {e.g., cresyl violet (CV) and thionin} [10–12] for downstream genetic applications. For example, CV has been performed in our laboratory to identify CA1 and CA3 pyramidal neurons in postmortem human tissue sections using single cell cDNA array analysis and quantitative real-time PCR (qPCR) [10,13]. Identification of individual cell types is quite important because this enables the differentiation of neuronal subtypes. In addition, neurons can be discriminated from glia, vascular epithelia, and other nonneuronal cells within the brain.

Single cell assessment and analysis of a homogeneous group of cells (termed population cells analysis) requires precise, non-destructive isolation from optimally prepared tissue sections. Provided that labeling procedures are performed on well prepared tissue sections and RNase free conditions are employed, both immunocytochemical and histochemical procedures can be utilized to identify specific cell(s) of interest [10,14,15]. Several techniques are used to aspirate individual cells or populations of cells including single cell microaspiration and laser capture microdissection (LCM). Single cell microaspiration entails visualizing an individual cell (or cells) using an inverted microscope connected to a micromanipulator, vacuum source, and an imaging workstation on an air table. This methodology results in accurate dissection of the neurons of interest with minimal disruption of the surrounding neuropil, and has been used on a variety of tissues and cell types including normal samples as well as tissues undergoing various forms of degeneration [7,14,16–18]. Another high throughput microaspiration device, LCM, enables rapid accession of single cells and populations for downstream molecular and cellular analyses. LCM is a widely used technique that was developed originally at the NIH [19,20]. Positive extraction (a method used by the PixCell IIe, Arcturus) entails pulsing an infrared laser onto thermoplastic film embedded in a specialized microfuge cap to form a thin protrusion that bridges the gap between the cap and a tissue section, effectively adhering to target cell(s). Lifting the thermoplastic cap separates targeted cells from surrounding undisturbed tissue. Negative extraction (or non-contact laser extraction) procedures employ a laser source to cut around the area of interest within a tissue section, and the microdissected material is catapulted into a microfuge tube (a method utilized by the P.A.L.M. system, P.A.L.M. Microlaser Technologies). Positive and negative extraction methods enable captured cells to be examined microscopically, confirming the identity and quality of isolated cell population(s). Individual cells, dozens, hundreds, and/or thousands of cells can be collected rapidly by LCM. RNA, DNA, and protein extraction methods can be performed on microdissected cells [21–25]. At present, LCM is typically utilized for RNA extraction and subsequent application to cDNA microarrays [7,10,15,19,20,26].

3. RNA amplification strategies

An RNA amplification technique is often required to perform microarray analysis and related downstream genetic analyses when using small sample inputs as starting material. PCR-based amplification methods are not optimal, as exponential amplification can skew the original quantitative relationships between genes from an initial population [3,27]. Linear RNA amplification is another strategy that has been used successfully to generate input RNA for molecular profiling studies. One method of linear amplification termed amplified antisense RNA (aRNA) amplification [3,4,28,29] utilizes a T7 RNA polymerase based amplification procedure that allows quantitation of relative gene expression levels from microdissected cells and tissues (Fig. 1). aRNA maintains a proportional representation of the size and complexity of the initial mRNAs [29,30]. Briefly, aRNA amplification entails the hybridization of a 66 basepair oligonucleotide primer consisting of 24 thymidine triphosphates (TTPs) and a T7 RNA polymerase promoter sequence {oligo(dT)T7)} to mRNAs and conversion to an mRNA-cDNA hybrid by reverse transcriptase [30,31]. Upon conversion of the mRNA-cDNA hybrid to double stranded cDNA, a functional T7 RNA polymerase promoter is formed. aRNA synthesis occurs with the addition of T7 RNA polymerase and nucleotide triphosphates (NTPs). Relative mRNA expression levels can be quantified using cDNA microarrays or oligonucleotide arrays by probing with fluorescent and/or biotin tagged NTPs incorporated into aRNA products (Fig. 2A). Alternatively, radiolabeled NTPs can be incorporated into aRNA products and used for hybridization to membrane-based cDNA arrays via reverse Northern analysis to known cDNA clones/expressed sequence-tagged cDNAs (ESTs) (Fig. 2B). Each round of aRNA results in an approximate thousand fold amplification from the original amount of each polyadenylated {poly(A)$^+$} mRNA in the sample [28,29]. Two rounds of aRNA amplification are typically employed for microarray analysis. aRNA products are biased towards the 3′ end of the transcript because of the initial priming at the poly(A)$^+$ RNA tail [3,4]. This 3′ bias exists for all amplified aRNA products and relative levels of gene expression can be compared [4,27,32]. Thus, it is important to select clones with 3′ transcript representation or to assess multiple ESTs and/or oligonucleotides linked to the same gene [14,17,33]. aRNA products also tend not to be of full length [3,4,28]. Although aRNA is often a challenging procedure, successful results have been generated with microaspirated animal model and human tissues utilizing a

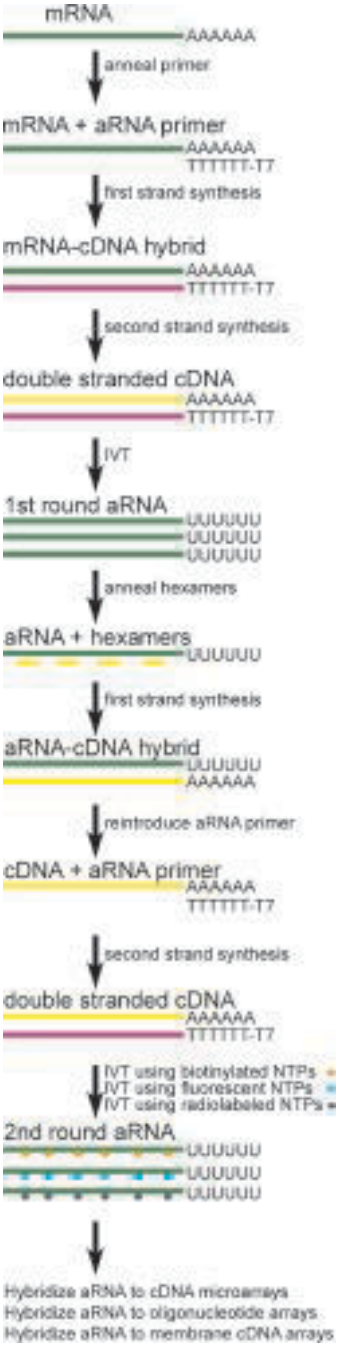

Fig. 1. aRNA amplification scheme. An aRNA primer {oligo(dT)T7} is hybridized directly to poly(A)$^+$ mRNAs. A double-stranded mRNA-cDNA hybrid is formed (first strand synthesis) by reverse transcribing the primed mRNAs with dNTPs and reverse transcriptase. The double stranded mRNA-cDNA hybrid is converted into double-stranded cDNA forming a functional T7 RNA polymerase promoter. A first round of aRNA synthesis occurs via IVT using T7 RNA polymerase and NTPs. A second round of aRNA amplification begins by annealing random hexamers to the newly formed aRNA, and performing first strand synthesis. The aRNA primer is then reintroduced which binds to the poly(A)$^+$ sequence on the newly synthesized cDNA strand. A double stranded cDNA template is formed by second strand synthesis. A second round of aRNA products is produced by IVT using biotinylated, fluorescent, or radiolabeled NTPs. Labeled second round aRNA products are used as probes to hybridize to cDNA microarrays, oligonucleotide arrays, or membrane-based cDNA arrays.

wide variety of microarray platforms [14,16–18,26]. Modifications of the initial aRNA procedure have been reported [34–36], and several aRNA kits are commercially available.

A variety of strategies have been developed by independent laboratories to improve RNA amplification including combining PCR with aRNA and isothermal RNA amplification, among others [35,37–42]. One obstacle is the problematic second strand cDNA synthesis. This impediment is not specific to the aRNA protocol. Rather, this issue is endemic to all current RNA amplification methods. Key factors to improving RNA amplification include increasing the efficiency of second strand cDNA synthesis and allowing for flexibility in the placement of bacteriophage transcriptional promoter sequences.

A new RNA amplification procedure has been developed in our laboratory that utilizes a method of terminal continuation (TC) (Fig. 3). TC RNA amplification entails synthesizing first strand cDNA complementary to the RNA template and generating second strand cDNA complementary to the first strand cDNA. RNA is amplified via in vitro transcription (IVT) using the newly formed double stranded cDNA as template [27,43]. Synthesis of the first strand cDNA complementary to template mRNA is performed by two oligonucleotide primers, a poly(dT) primer and a TC primer. The poly(dT) primer is similar to conventional primers that anneal to the poly(A)$^+$ sequence present on most mRNAs. The TC primer consists of an oligonucleotide sequence at the 5′ terminus and a short span of three cytidine triphosphates (CTPs) or guanosine triphosphates (GTPs) at the 3′ terminus. cDNA synthesis is initiated by annealing a second oligonucleotide primer complementary to the attached oligonucleotide [43]. By providing a known sequence at the 3′ region of first strand cDNA and a primer complementary to it, hairpin loops will not form. Moreover, second strand cDNA synthesis is performed with robust DNA polymerases, such as *Taq*. *Taq* polymerase has superb processivity as well as thermal stability. In addition, *Taq* polymerase synthesizes high fidelity DNA at high speed and at a high temperature that effectively reduces background that tends to manifest as nonspecific hybridization on array platforms. The TC RNA amplification method employs a hot start strategy for second strand cDNA synthesis, e.g., adding in *Taq* polymerase at the denaturing step when reaction temperature is at 95 °C [43,44]. One round of TC RNA amplification is sufficient for downstream genetic analyses [6,10,13,27,33,43]. TC RNA transcription can be driven using a promoter sequence attached to either the 3′ or 5′ oligonucleotide primers (Fig. 3). Therefore, transcript orientation can be in an antisense orientation (similar to conventional aRNA methods) when the bacteriophage promoter sequence is placed on the poly(dT) primer or in a sense orientation when the promoter sequence is attached to the TC primer, depending upon the design of the experimental paradigm (Fig. 3). A TC primer contains at least two or three dCTPs or dGTPs (CTPs and GTPs work as well (Fig. 4A)), which in turn can anneal to GC-rich regions at 5′-end of mRNAs. In this manner, a TC primer acts as an extended template for reverse transcriptase to continue. This results in long first-strand

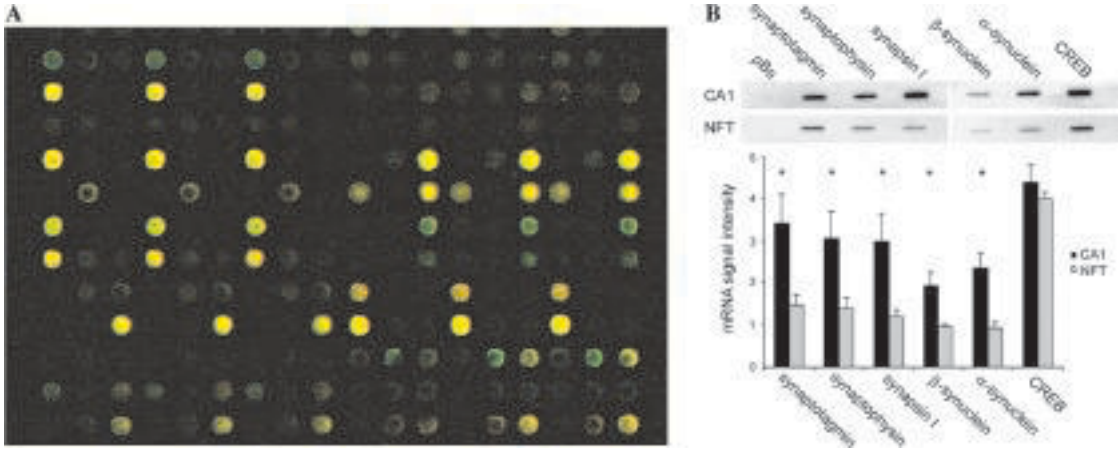

Fig. 2. Representative cDNA arrays hybridized with aRNA probes. (A) aRNA obtained from individual hippocampal CA1 pyramidal neurons from normal control brains and from neurofibrillary tangle (NFT) bearing CA1 neurons from Alzheimer's disease (AD) patients was hybridized to a spotted cDNA array platform. aRNA from normal CA1neurons (green), NFT bearing AD neurons (red), and overlay of both (yellow) is depicted. (B) Custom-designed membrane-based cDNA array and histogram illustrating the regulation of several genes in AD NFT bearing CA1 neurons as compared to normal CA1 neurons in control brains. Significant down regulation (asterisk denotes $p < 0.01$) is found for synaptic-related markers synaptotagmin, synaptophysin, synapsin I, β-synuclein, and α-synuclein. In contrast, no differential expression of cyclic AMP response element-binding protein (CREB) is found. Background hybridization is minimal as evidenced by the low hybridization signal of the empty vector Bluescript (pBs).

cDNA consisting of the poly(dT) primer, a sequence complementary to the original mRNA, and a sequence complementary to the TC primer present [43].

TC RNA amplification enables quantitative assessment of a large proportion of genes as evidenced by bioanalysis and microarray analysis in mouse and human postmortem brain tissues, including single cells and populations of homogeneous neurons (Fig. 4) [6,7,10,13,27,33,43]. Robust linear amplification is found. Amplification efficiency of approximately 2500- to 3000-fold is demonstrated with commercially available purified mRNAs and approximately 1000- to 1500-fold amplification is found after one round using biological samples of RNA extracted from a variety of brain sources [43]. The threshold of detection of genes with low hybridization signal intensity is increased, as many genes that are at the limit of detection using conventional aRNA can be readily observed with the TC method [43]. Importantly, increased sensitivity appears greatest for genes with relatively low abundance. Moreover, background hybridization is significantly attenuated when using TC RNA amplification [10,13,43].

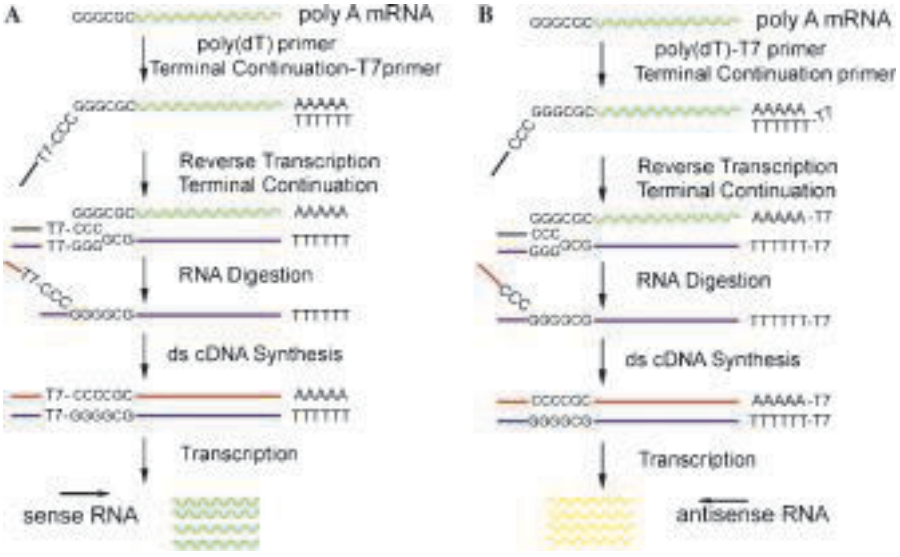

Fig. 3. Overview of the TC RNA amplification method. (A) A TC primer (containing a bacteriophage promoter sequence for sense orientation) and a poly(dT) primer are added to the mRNA population to be amplified (green rippled line). First-strand synthesis (blue line) occurs as an mRNA-cDNA hybrid is formed following reverse transcription and TC of the oligonucleotide primers. After an RNase H digestion step to remove the original mRNA template strand, second strand synthesis (red line) is performed using *Taq* polymerase. The resultant double stranded product is utilized as template for IVT, yielding high fidelity, linear RNA amplification of sense orientation (green rippled lines). (B) Schematic similar to (A), illustrating the TC RNA amplification procedure amplifying RNA in the antisense orientation (yellow rippled lines). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

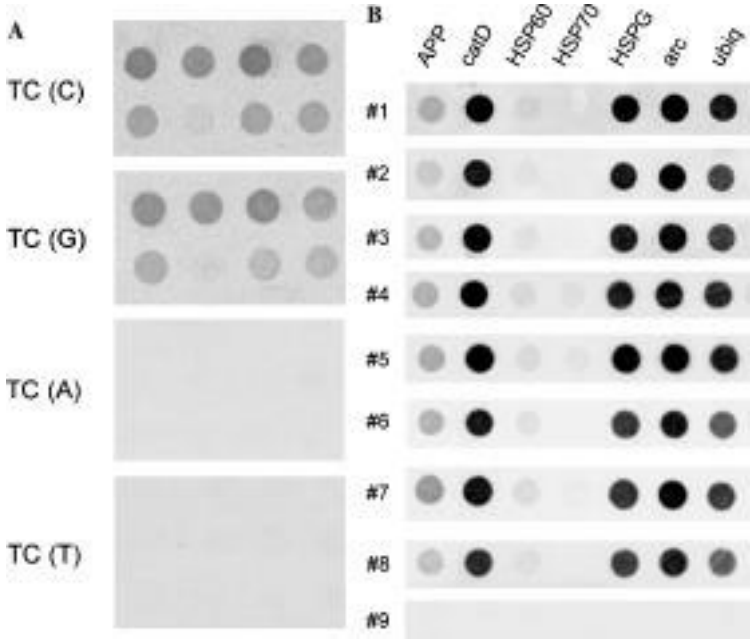

Fig. 4. Representative arrays depicting hybridization signal intensity using probes generated by TC RNA amplification. (A) cDNA array analysis of normal human postmortem hippocampus following TC RNA amplification with TC primers of differing nucleotide composition. The first and second arrays illustrate varying levels of hybridization signal intensity for eight transcripts using TC primers containing C or G at the 3′ terminus, respectively. In contrast, the third and fourth arrays illustrate a paucity of hybridization signal intensity using TC primers containing A or T at the 3′ terminus, respectively. (B) Single cell cDNA analysis of normal control CA1 pyramidal neurons obtained postmortem using custom-designed cDNA arrays and TC RNA amplification. A wide dynamic range of hybridization signal intensities for the eight cases (depicted as #1–#8). A negative control (#9) is a single CA1 pyramidal neuron from case #1 that does not have the primers necessary for TC RNA amplification. A moderate variation of gene level expression across the eight human cases is also observed, indicating the utility of using postmortem human samples for normative and neuropathological investigations. Key: amyloid-β precursor protein (APP), cathepsin D (catD), heat shock protein 60&70 (HSP60, HSP70), heparan sulfate proteoglycan (HSPG), activity regulated cytoskeletal-associated protein (arc), and ubiquitin (ubiq).

A mechanism for the TC primer to anneal preferentially to 5′ regions of transcripts is being investigated. Based upon sequence analysis of TC RNA amplified products from human and mouse brains, we hypothesize that the TC primer, with its span of CTPs (GTPs work as well [33]) anneals preferentially within CpG islands. CpG islands are nonmethylated GC-rich regions of the genome that tend to include the 5′ end of genes and are found at a significantly less frequency (CpGs are 25% less frequent than predicted) throughout the rest of the genome [45,46]. Estimates suggest that upwards of 60% of all human genes are located near CpG islands [45–47]. We hypothesize that the TC primer has to base pair with the complementary CTPs or GTPs at the termination site of the reverse transcription reaction in order to provide a short template for DNA synthesis to continue. Replacement of the CTPs/GTPs with ATPs or TTPs vastly diminishes the TC reaction [33]. In addition, random base pairing of ATPs and TTPs with complementary TTPs and ATPs in mRNAs may interrupt a proper reverse transcription process that is essential for generation of the first strand cDNA [33,43].

4. Conclusions

Single cell and population cell profiling techniques coupled with microarray platforms have the potential to quantify simultaneous expression levels of numerous genes in a given cell, thereby allowing for previously unobserved gene interactions, and ultimately protein interactions, to become more evident. RNA amplification is becoming a critical aspect of these paradigms, as input sources of RNA are becoming smaller and more focused upon individual cell types within the brain and other organs. Currently, there are several RNA amplification strategies that are now available to investigators, and recent improvements have led to greater reproducibility and flexibility in the laboratory. The brain is an obvious site for single cell/population cell RNA studies, due to the plethora of cell types and intricate connectivity of regions. However, RNA amplification procedures, notably the TC RNA amplification methodology, have much broader applications. Virtually any in vivo or in vitro paradigm can be employed for TC RNA amplification. Disciplines that will benefit from this technology include, but are not restricted to, cancer biology, development, drug discovery, and a myriad applications where input sources of RNA are limited and/or cell-type specific.

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Appendix A. Sample preparation and equipment

RNA amplification provides a powerful tool to overcome limitations in the amount of starting materials. However, these procedures do not diminish the importance of careful sample handling, as any RNA (especially minute quantities of RNAs) are vulnerable to RNase degradation [48]. An RNase-free environment is requisite. Long term storage of RNAs (especially amplified RNAs) is not recommended and repeated freeze-thaw should be avoided. Furthermore, dubious RNA handling can complicate any troubleshooting process when problems arise.

The equipment necessary to perform RNA amplification methodologies does not differ greatly from standard molecular and cellular based applications. An investigator should dedicate refrigerator, −20 °C freezer, −80 °C freezer, and bench space to RNA amplification, making sure that these areas are RNase-free and far away as possible from potential contaminants such as PCR primers and extraneous DNA and/or RNA sources. A refrigerated tabletop centrifuge with multiple rotors is desirable as well as repeater and digital pipettors, as these instruments enable precise delivery of small amounts of reagents and enzymes that are part of the RNA amplification procedure. Aerosol barrier tips are requisite. All solutions should be made with in 18.2 MΩ RNase-free water {or diethyl pyrocarbonate (DEPC) treated water}. Primers and enzymes should be aliquoted into single (or double) use microfuge tubes to avoid potential contamination. RNA amplification procedures are typically high-end molecular biological procedures performed in small volumes, so proper laboratory technique is critical for the success of the procedures.

Appendix B. TC RNA amplification protocol

This Appendix is a step-by-step protocol for TC RNA amplification as described originally by Che and Ginsberg [43]. For clarity, the protocol begins at the point where cells are captured (either by LCM or a microaspiration strategy) and follows through IVT using biotinylated or radioactive methodologies to label the TC RNA amplified products.

B.1. Isolation of RNA

1. Add 250 μL of Trizol reagent (Invitrogen) to 500 μl thin wall PCR tubes that will receive microdissected cells and keep on wet ice.
2. Invert tube so that Trizol reagent bathes microdissected cells and keep on wet ice. Samples can also be stored at −80 °C for future use.
3. Add 50 μL of chloroform to each sample. Vortex vigorously for 15 s and centrifuge samples at 12,000 RPM for 15 min at 4 °C.
4. The mixture separates into an upper aqueous phase (clear) and a lower organic phase (red). Collect the aqueous phase containing RNA by aspirating with a pipette.
5. Add 125 μL of 100% isopropyl alcohol and 5 μL of linear acrylamide (Ambion) to precipitate the RNA from the aqueous phase. Samples can be stored at −80 °C to precipitate RNAs if desired.
6. Vortex vigorously for 15 s and centrifuge samples at 12,000 RPM for 15 min at 4 °C.
7. Decant the supernatant, by inverting the tube, being careful not to dislodge the pellet.
8. Add 250 μL of 75% EtOH to each sample to wash RNA pellet. Vortex vigorously for 15 s and centrifuge samples at 7500 RPM for 5 min, at 4 °C.
9. Decant the supernatant, by inverting the tube, being careful not to dislodge the pellet.
10. Air dry the sample by inverting the tube for 5 min in the hood. Do not wait longer than 5 min, as the pellet becomes difficult to resuspend.
11. Resuspend pellet in 5 μL in 18.2 MΩ RNase-free water.

B.2. First-strand synthesis

1. To each RNA sample add 1 μl first strand synthesis primer {poly(dT); 10 ng/μl}. Quick spin 10 s.
2. Heat mixture for 2 min at 75 °C. Quick spin and place on ice. Total volume is 6 μl.
3. Prepare reverse transcription (RT) Master Mix (prepare enough mix for the total number of samples plus two extra). Calculate volumes of the mix for x # of samples + control + one.
4. *RT Master Mix* consists of (on wet ice):

5× first strand buffer (Invitrogen)	4 μl
dNTPs (10 mM)	1 μl
0.1 M DTT	1 μl
RNase inhibitor (Invitrogen; 10 U/μl)	0.5 μl
18.2 MΩ RNase-free water	5.5 μl

5. Warm the *RT Master Mix* (without primers or enzyme) for 2 min at 50 °C.
6. Take an aliquot of terminal continuation (TC) primer (10 ng/μl) (each sample in the *RT Master Mix* will get 1 μl; thus if you have eight samples, the *RT Master Mix* will be equivalent to 10 samples and you will add 10 μl of the TC primer to the *RT Master Mix* prior to adding to the sample) and heat denature for 2 min at 70 °C and then place on ice for several minutes and then add to the *RT Master Mix*.
7. Aliquot Superscript III (Invitrogen 200 U/μl) to the *RT Master Mix* being careful to not contaminate the vial of enzyme. Similar to the TC primer, each sample in the *RT Master Mix* will get 1 μl of RT; thus if you have eight samples, the *RT Master Mix* will be equivalent to 10 samples and you will add 10 μl of the Superscript III to the *RT Master Mix* prior to adding to the sample.
8. The *RT Master Mix* should comprise of 14 μl per reaction.

9. Add the *RT Master Mix* to the 6 μl of sample, pipette vigorously, and quick spin. Incubate the mixture for 60 min at 50 °C.
10. Inactivate the RT reaction by heating the sample an additional 15 min at 65 °C.
11. Quick spin the reaction mixture and cool immediately on ice. Samples can be stored at −20 °C for short term or −80 °C for longer term storage.

B.3. Second-strand synthesis

1. Prepare *Second Strand Master Mix* (prepare enough mix for the total number of samples plus two extra). Calculate volumes of the mix for x # of samples + control + one.
2. *Second Strand Master Mix* consists of (on wet ice):

10× PCR buffer (PE Biosystems w/15 mM $MgCl_2$)	10 μl
RNase H (Invitrogen; 2 U/μl)	0.5 μl
18.2 MΩ RNase-free water	69 μl

3. Mix well with pipette tip and quick spin.
4. Distribute 79.5 μl of *Second Strand Master Mix* to the appropriate number of 0.5 ml capacity thin walled PCR tubes.
5. Add the 20 μl of sample to the *Second Strand Master Mix* and mix thoroughly with a pipette tip.
6. Place samples in a thermal cycler. It is a good idea to create a specific program for the second strand synthesis procedure using final volume of 100 μl.
7. Once the samples are in the thermal cycler, degrade the RNA for 10 min at 37 °C. As soon as the block temperature reaches 95 °C, *Pause* the reaction. Using a dedicated PCR pipette add into each sample.

Taq polymerase (PE Biosystems; 5 U/μl)	0.5 μl

8. Mix thoroughly with a pipette tip (this is a critical step!).
9. Continue the second strand synthesis program by pushing the *Continue* function.
10. *Second Strand Synthesis* program consists of:

Hot start denaturation	95 °C for 3 min
Annealing	60 °C for 3 min
Elongation	75 °C for 30 min

11. Remove samples, quick spin and store at −20 °C for short term or −80 °C for longer term storage.

B.4. Double stranded cDNA preparation

1. To extract each sample add:

5 M ammonium acetate (NH_4OAc)	100 μl
Phenol:chloroform:isoamyl alcohol (25:24:1)	170 μl

2. Vortex vigorously for 30 s. Spin in microfuge at 14,000 RPM for 5 min at 22 °C and collect aqueous phase into fresh 1.7 ml microfuge tubes.
3. To precipitate each sample add:

100% cold ethanol	1 ml

4. Centrifuge at 14,000 RPM for 30 min at 4 °C.
5. Carefully discard the supernatant by inverting the tube to pour off and blot. Air-dry the pellet, inverted, for 5 min in the hood.
6. Samples are drop dialyzed prior to IVT for maximum efficiency. Resuspend the double stranded cDNA pellet in 20 μl of 18.2 MΩ RNase-free water.
7. Fill 50 ml conical tubes with 18.2 MΩ RNase-free water and float one 0.025 μm Millipore membrane filter (VWR; Appendix C), shiny side up, on the surface of the water using a dedicated RNase-free tissue forceps. Make sure not to sink the membrane.
8. Load each sample onto the center of one of the floating membranes and carefully place the cap back on the conical tube.
9. Let stand undisturbed for 4 h at 22 °C.
10. Carefully remove the dialyzed sample into a new microfuge tube using a pipette tip.
11. Quick spin and store at −20 °C for short term or −80 °C for longer term storage.

B.5. IVT for TC RNA amplification: biotinylated/fluorescent probe labeling

1. Prepare *IVT Master Mix* (prepare enough mix for the total number of samples plus two extra). Calculate volumes of the mix for x # of samples + control + one. This protocol is based upon using the BioArray RNA transcript labeling kit (Enzo), although any biotinylated and/or fluorescent labeling protocol is suitable (minor modifications may apply).
2. *IVT Master Mix* consists of (on wet ice):

10× Hybridization reaction buffer	4 μl
10× Biotin labeled ribonucleotides	4 μl
10× DTT	4 μl
10× RNase inhibitor mix	4 μl
18.2 MΩ RNase-free water	12 μl
20× T7 RNA polymerase	2 μl
(or T7 RNA polymerase from Epicentre; Appendix C	2 μl)

3. The *IVT Master Mix* should comprise of 30 μl per reaction.
4. Add 10 μl of double stranded TC sample to the *IVT Master Mix* (final volume of the reaction is 40 μl).
5. Mix thoroughly (very important!!). Quick spin and incubate for 5 h at 37 °C.

6. TC RNA amplified products are now ready for purification (if desired), fragmentation (if desired), and application to cDNA or oligonucleotide arrays. There are numerous published protocols for hybridization of labeled probes to microarray platforms, subsequent washing, and imaging protocols (e.g., www.affymetrix.com, www.enzo.com, [49]).

B.6. IVT for TC RNA amplification: radioactive probe labeling

1. Prepare *IVT Master Mix* (prepare enough mix for the total number of samples plus two extra). Calculate volumes of the mix for x # of samples + control + one.
2. *IVT Master Mix* consists of (on wet ice):

10× RNA amplification buffer	2 μl
0.1 M DTT	1 μl
3NTPs (A,C,G; 2.5 mM each)	2 μl
UTP (100 μM)	2 μl
RNase inhibitor (20 U)	0.5 μl

3. The *IVT Master Mix* should comprise of 7.5 μl per reaction.
4. Quick spin and add 7.5 μl of double stranded TC sample to the *IVT Master Mix (on wet ice)*.
5. To each sample add ^{33}P-UTP (GE-Health care; 20 mCi/ml) 4 μl
6. Add T7 RNA polymerase (Epicentre # TH950K) 1 μl
7. Mix thoroughly (very important!!). Quick spin and incubate for 4 h at 37 °C.
8. TC RNA amplified products are now ready to be hybridized to membrane-based array platforms. Procedures for membrane synthesis, prehybridization, hybridization, washing conditions, and imaging are described in detail elsewhere (e.g. [10,27,33,43]).

Appendix C. Reagent list

ATP	Invitrogen	18330-019
CTP	Invitrogen	18331-017
GTP	Invitrogen	18332-015
UTP	Invitrogen	18333-013
dNTPs	Invitrogen	10297-018
RNase H	Invitrogen	18021-071
Ammonium acetate	Ambion	9071
Linear acrylamide	Ambion	9520
10× RNA transcription buffer	Ambion	8151G

or to prepare in the lab for 5 ml (aliquot into 5 × 1 ml tubes)

400 mM Tris-base pH 7.5 (*from 1 M stock*)	2000 μl
70 mM $MgCl_2$ (*from 1 M stock*)	350 μl
100 mM NaCl (*from 5 M stock*)	100 μl
20 mM spermidine (*from 1 M stock*)	100 μl
18.2 MΩ RNase-free water	2450 μl

Taq polymerase	PE Biosystems	N-808166
10× PCR buffer	PE Biosystems w/ 15 mM $MgCl_2$	N-808166
Phenol:chloroform: isoamyl	Invitrogen	15593031
UTP P^{33}	GE-Health care	BF10021MCI
T7 RNA polymerase	Epicentre Technologies	TH950K
RNase inhibitor	Invitrogen	15518-012
0.025 μm membrane filters	VWR	VSWP 01300
RNA transcript labeling kit	Enzo Life Sciences	900182

Appendix D

Representative oligonucleotide sequences utilized for the poly(dT) and TC primers for the TC RNA amplification method.D.

D.1. Antisense RNA orientation

poly(dT)-T7 primer (66 bp):	3′-AAA CGA CGG CCA GTG AAT TGT AAT AC G ACT CAC TAT AGG CGC TTT TTT TTT TTT TTT TTT TTT TTT-5′
TC primer (17 bp):	5′-TAT CAA CGC AGA GTC CC-3′

D.2. Sense RNA orientation

poly(dT) primer (18 bp):	3′-TTT TTT TTT TTT TTT TTT-5′
TC-T7 primer (51 bp):	5′-AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG CGC GAG AGC CCC-3′

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