

CHAPTER 7

DNA linear amplification

Chih Long Liu, Bradley E. Bernstein and Stuart L. Schreiber

Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford St., Cambridge, Massachusetts 02138, USA

1. INTRODUCTION

Amplification of nucleic acids has become a mainstay of molecular biology. It permits genomic and transcriptional analysis when the amount of tissue or number of cells being studied becomes limiting, and is invaluable in studies where the biology of the specimens being investigated severely limits the amount of nucleic acids available. It is also particularly important for large-scale, high-throughput genomic studies, since these studies typically use microarrays or other high-throughput assays that often require microgram amounts of nucleic acids. Furthermore, these studies may employ a large matrix of many different conditions and/or time points, which may make it prohibitively expensive to generate sufficient amounts of unamplified material.

One example of such a genomics application is the ChIP-chip method, where DNA recovered from chromatin immunoprecipitation (ChIP) of cell lysate is used for subsequent analysis on DNA microarrays. This method (for review, see 1) is typically used to identify transcription factor binding sites and to map histone variants, histone post-translational modification patterns, or any other interesting epitope within the genome. *Fig. 1* shows an example of the ChIP-chip method using spotted microarrays.

ChIP, however, typically yields DNA in the nanogram range, which is insufficient for most DNA microarray applications. Laboratories using the ChIP-chip technique typically employ an exponential amplification method, such as ligation-mediated PCR (LM-PCR) (2) or random PCR (R-PCR) (3–5), to obtain the quantities necessary for microarray analysis. R-PCR involves the annealing of primer adaptors with a 5' conserved end and a 3' degenerate end to the template DNA, followed by extension and subsequent PCR with primers complementary to the 5' conserved ends. These exponential amplification methods adequately fulfill the needs of ChIP-chip analysis and are still frequently used, particularly when amplification from subnanogram amounts are

78 ■ CHAPTER 7: DNA LINEAR AMPLIFICATION

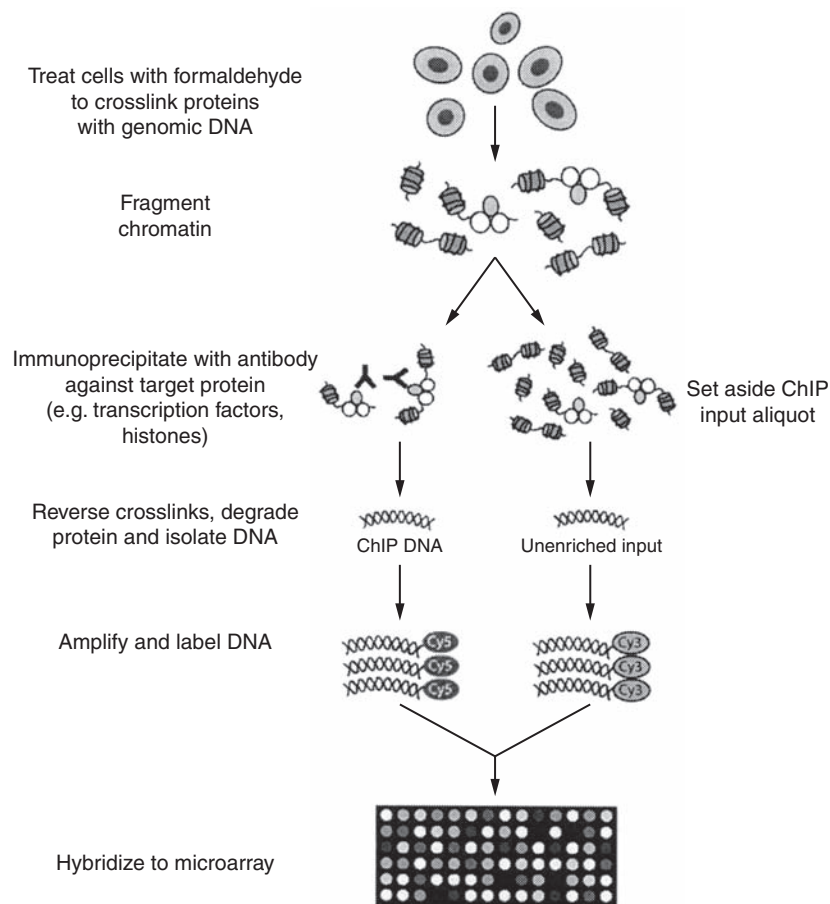


Figure 1. Schematic of the ChIP-chip method.

Cells are first crosslinked with formaldehyde prior to lysis and DNA fragmentation. Following fragmentation, chromatin is then incubated with antibodies and subsequently immunoprecipitated with Protein A or Protein G beads, which bind to the F_c segment of the antibodies. Following elution from the beads, reversal of crosslinks, and proteinase K digestion, ChIP samples are typically phenol-chloroform extracted, ethanol precipitated, and then treated with RNase A to eliminate RNA that has been carried over from the immunoprecipitation. The ChIP samples and the unenriched input material are then amplified and labeled with fluorescent dyes. The ChIP sample is subsequently hybridized, along with the unenriched input, on a spotted microarray.

desired. However, they have a number of shortcomings, in particular with regard to amplification fidelity and to a lesser extent with dynamic range compression, and with their inefficient amplification of short nucleic acids such as those less than 250 bp in length (6).

2. METHODS AND APPROACHES

2.1. DNA linear amplification

The DNA linear amplification method described here has been designed to address the shortcomings of exponential amplification. This method uses a linear amplification approach, based on the *in vitro* transcription (IVT) of template DNA by RNA polymerase from the T7 phage, a common strategy employed in a number of published RNA amplification protocols (7–9; for review, see 10). This linear amplification approach successfully addresses the amplification fidelity issues raised with an exponential amplification approach (6). The amplification method presented in this chapter was designed primarily to address shortcomings of the R-PCR protocol with the ChIP-chip method. Thus, optimizations made to this method have been done with this particular application in mind. For other applications that require DNA to be the end point, reverse transcription is a necessary step that increases the cost and complexity of necessary molecular biological manipulations to the sample, when compared with PCR. However, IVT amplification does offer improved fidelity and a much higher maximum yield per single reaction (6). Thus, other techniques that require amplification of complex mixtures of randomly fragmented genomic DNA can also benefit from this method.

2.2. General strategy

This section describes in detail the general strategy employed by this linear amplification method. This method takes nanogram quantities of dsDNA as the starting material and generates microgram amounts of amplified RNA. An overall schematic diagram is shown in *Fig. 2*. Briefly, the strategy is to add a 3' conserved end to the template dsDNA, using terminal deoxynucleotidyl transferase (TdT) tailing, which permits the addition of a T7 promoter sequence in the subsequent second-strand synthesis step. IVT can then utilize this newly appended T7 promoter and linearly amplify the template dsDNA, producing amplified RNA product.

2.3. Considerations for the starting dsDNA template

The ideal starting material for this method is dsDNA template in the 100–1000 bp size range with 3' protruding or 3' blunt ends. The 3' end must have a free hydroxyl (OH) group, since TdT does not add residues to template strands with 3' phosphate groups. Some restriction digests and other DNA fragmentation methods such as sonication and nuclease digestion may leave behind 3' phosphate groups on a significant proportion of the DNA molecules. These phosphate groups need to be removed prior to the tailing reaction; failure to do so may result in poor amplification.

One other consideration about 3' ends is their efficacy in serving as an efficient template for TdT-mediated polynucleotide tailing. The efficiency of the

80 ■ CHAPTER 7: DNA LINEAR AMPLIFICATION

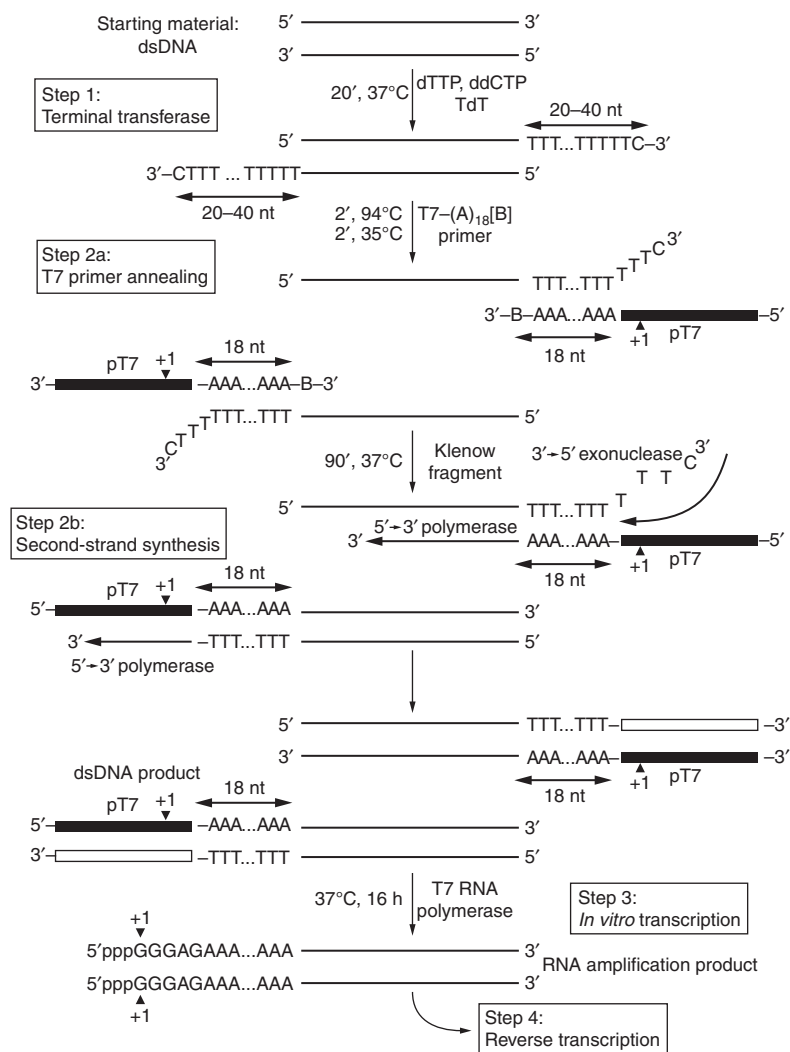


Figure 2. General strategy for the DNA linear amplification method.

Starting with dsDNA template, TdT is used to add a poly(dT) tail to the 3' ends of the template. This tail subsequently provides a conserved binding site for the annealing of T7 promoter (pT7)-poly(dA) primer adaptors. Following subsequent second-strand synthesis using the large fragment of DNA polymerase I (Klenow fragment), one pair of dsDNA templates, with each pair member representing one of the two complementary strands of the dsDNA, is generated, with a T7 promoter at the 5' end of the amplicon. In the subsequent IVT step, RNA is transcribed from this template in an isothermal reaction, producing an RNA amplification product consisting of both strands of the original dsDNA template in high microgram quantities. Note that each RNA strand will contain a short sequence from the T7 promoter and a poly(A) tract, 5' relative to the amplicon. Reprinted with permission from (6).

TdT enzyme is maximal on 3' protruding ends and good on 3' blunt ends. However, 3' recessed ends are only 'very reluctantly' tailed, and a mixture of templates containing all three types of 3' end will likely result in tailed products enriched with previously 3' protruding ends and depleted of previously 3' recessed ends (11). The end result is a larger spread in the size distribution of poly(dT) tail lengths, which may negatively impact subsequent amplification fidelity if the relationship between the template sequence and the nature of the 3' end is nonrandom. It is therefore recommended that, in applications that are sensitive to this issue, templates containing a significant proportion of 3' recessed ends are filled in with Klenow fragment DNA polymerase prior to the TdT tailing step.

The size range of approximately 100–1000 bp is considered to be optimal as this is the size range that was extensively tested with this method when it was developed. Amplification of fragments smaller than 100 bp is limited by the column-based purification steps, listed in *Protocols 5* and *6*, which rely on the Qiagen RNeasy columns and MinElute columns. The MinElute columns have a lower limit of 70 bp, while the RNeasy columns have a tested limit of 100 bp (which is lower than the 200 bp manufacturer-specified limit). While *Protocols 5* and *6* were developed with Qiagen columns, alternative reaction cleanup columns or other methods can be substituted.

Amplification of material smaller than 100 bp will also be less efficient in terms of mass. This is because the rate-limiting step in IVT is initiation of transcription (12), and because there is a greater molar ratio of template molecules to T7 RNA polymerase compared with an equivalent mass of a higher-molecular-weight template. Here, the low-molecular-weight template would require more initiation of transcription events per mass unit compared with a high-molecular-weight template, and the usual result is a significantly lower mass yield for the low-molecular-weight template (see Section 3.1 for details).

Amplification of fragments larger than 1000 bp has not been extensively tested. This should not be a particular problem for templates ranging up to 4–5 kb in size, since T7 RNA polymerase is highly processive (12). Extremely large fragments (>5 kb) are perhaps best amplified by other techniques (e.g. strand displacement, 13).

2.4. Using this method for ChIP–chip experiments

Since this method was originally designed to address the amplification needs of ChIP–chip experiments, this section will discuss in detail the considerations necessary to optimize the amplification fidelity and yield of the starting material. However, this approach will be appropriate for many applications that require several micrograms of DNA from nanogram quantities of starting DNA.

ChIPs frequently yield low amounts of DNA, which may be difficult to quantify via usual methods, such as absorbance at 260 nm with a spectrophotometer. Most commonly used spectrophotometers have a lower reliable detection limit of 0.01 absorbance units and are limited to a minimum cuvette volume of 50–100 μ l, translating to 0.5 ng/ μ l for dsDNA. Consequently, it becomes quite difficult to

82 ■ CHAPTER 7: DNA LINEAR AMPLIFICATION

detect and quantify reliably the presence of nucleic acid when the total yield is less than 250 ng, without sacrificing an excessive proportion of the total sample in the measurement. Reliable measurement of a ChIP mass yield is important for maximizing amplification yield for this method. Therefore, we recommend using a more sensitive instrument or a spectrophotometer designed to handle cuvettes with small volumes (<10 μ l). Since these instruments require five- to tenfold less sample per measurement, the lower limit then becomes 25–50 ng of ChIP yield for reliable measurement. For extremely low ChIP yields (<25 ng), quantification via fluorescence (e.g. using PicoGreen (Molecular Probes) with the Turner BioSystems TD-700 fluorometer) is the recommended method and should reliably measure samples down to a lower limit of 2.5 ng starting material.

There are a couple of additional technical considerations when this method is used with the ChIP-chip method. Calf intestinal alkaline phosphatase (CIP) treatment (*Protocol 1*) is presented as an optional protocol, but is necessary if the ChIP-chip method employs fragmentation methods (e.g. sonication or nuclease digestion) that leave behind a significant proportion of 3' phosphate groups in the template DNA. This is discussed in further detail in section 4.1.3. Additionally, since *Protocol 1* is typically carried out immediately after RNase A digestion in the ChIP-chip method, users may be concerned about sufficient elimination of RNase A for the remainder of *Protocol 1* and in *Protocols 2* and *3*, in time for the IVT step (*Protocol 4*). The three MinElute cleanup steps (*Protocol 6*, performed at the end of *Protocols 1*, *2*, and *3*), are usually sufficient to remove enough residual RNase A for it not to pose a problem. Furthermore, the Ambion IVT enzyme mix used in *Protocol 4* contains an RNase inhibitor. Care should be taken, however, to ensure that barrier tips are used, and ideally separate pipettes should be used when handling the RNase A enzyme to avoid RNase contamination. Troubleshooting RNase contamination is discussed in section 4.1.1.

2.5. Controls for new users

To maximize the success of this method, two positive controls are strongly suggested for new users. The troubleshooting section will discuss in greater detail a number of methods that can be used to resolve low-yield problems.

2.5.1. *Protocols 2 and 3*: positive amplification control

This control enables the user to distinguish sample-specific problems from protocol-implementation issues. Here, the starting material is 50 ng of blunt-ended PCR product in the 100–1000 bp range (preferably around 200–500 bp). If this control is used as part of a ChIP-chip experiment, the PCR product can be subject to RNase A and CIP treatment for troubleshooting purposes, although in practice these treatments are usually not necessary. Yields should typically range from 30 to 60 μ g (with a maximum of 80–100 μ g observed), depending on the size of the PCR product, protocol implementation, and quality of the reagents used (particularly the nucleotides used for IVT (in *Protocol 4*), which are highly sensitive to freeze-thaw cycles).

2.5.2. Protocol 4: positive IVT control

This control enables the user to distinguish sample-specific problems from IVT-specific problems (such as RNase contamination). Here, the IVT starting material is 250 ng of the pTRI-Xef linearized plasmid provided with the Ambion IVT kit (see *Protocol 4*). If not using the kit, an appropriate amount of a dsDNA template that contains the pT7 promoter, with prior history of use as a successful T7 RNA polymerase template, should be used. Yields should typically range from 100 to 140 μ g, limited by exhaustion of the nucleotides in the reaction mixture and the

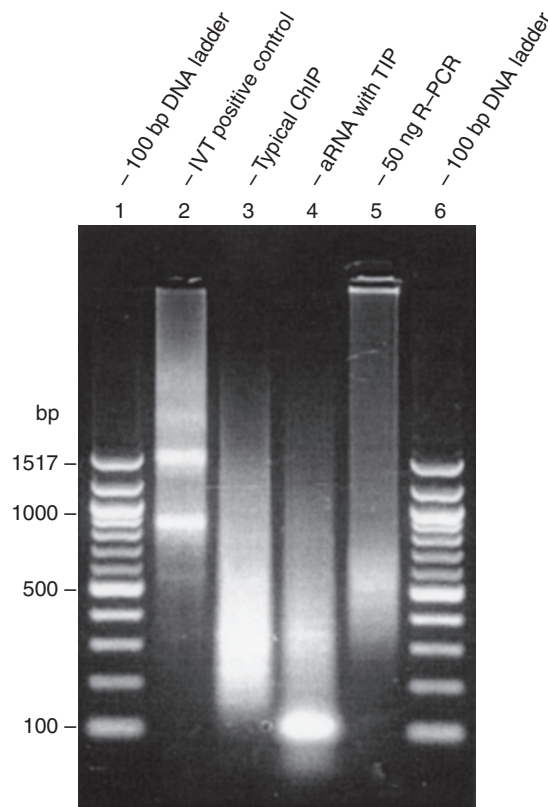


Figure 3. Amplification products on a non-denaturing 2% agarose gel in TAE buffer, stained with ethidium bromide.

A 100 bp ladder (500 ng, New England Biolabs) or 650 ng of samples were loaded. (Lanes 1 and 6) 100 bp ladder; (lanes 2–4) antisense RNA (aRNA) amplified using the method presented in this chapter, with the following templates: (lane 2) IVT positive control (pTRI-Xef) (see *Protocol 4*); (lane 3) a typical ChIP sample (*Saccharomyces cerevisiae* bis-acetyl histone H3 on lysine 9 and 14) from sonicated genomic DNA; (lane 4) amplification from 5 ng of a genomic digest of *S. cerevisiae* with *RsaI*, showing a strong band of template-independent product (TIP) of approximately 100 bp; (lane 5) the R-PCR product, for comparison, from a 50 ng amplification of the same template used to generate aRNA in lane 4. The most remarkable attribute of lane 5 is the near absence of DNA smaller than 250 bp.

rated 100 µg binding capacity of the Qiagen RNeasy column. Any yield somewhat less than this may suggest RNase contamination, evaporation of the reaction mixture during the long incubation period (due to the small reaction volume), or poor-quality reagents (particularly the nucleotides). Visualization on a non-denaturing agarose gel should yield two intense bands of approximately 0.9 and 1.5 kb, as shown in *Fig. 3 (lane 2)*.

3. RECOMMENDED PROTOCOLS

All protocols presented in this section are typically performed in the order presented when used in conjunction with the ChIP-chip method. *Protocol 1*, CIP treatment, is an optional but strongly recommended protocol for removing 3' phosphate groups, since most genomic DNA fragmentation methods (i.e. sonication, micrococcal nuclease digestion, and certain restriction digests) will produce a significant proportion of 3' phosphate groups within the mixture of fragmented genomic DNA. This protocol is compatible with the presence of RNase A and can be carried out immediately after RNase A digestion of RNA carried over from the ChIP, without any intermediate cleanup step.

The main linear amplification protocol begins with dsDNA and produces an RNA amplification product, as shown in *Fig. 2*. The procedure can be broken down into three main stages:

- (i) the tailing reaction with terminal transferase;
- (ii) second-strand synthesis with Klenow fragment polymerase; and
- (iii) IVT.

The tailing reaction involves the addition of a short (20–40 nt) poly(dT) tail to the template DNA. This poly(dT) tail provides a conserved 3' element that permits annealing of a T7 primer adaptor during second-strand synthesis, as described in *Protocol 3*. The inclusion of the dideoxynucleotide acts as a tail terminator in the reaction mixture and is necessary to maintain a tight size distribution of the poly(dT) tail.

Protocol 1

CIP treatment of samples with terminal 3' phosphate groups

Equipment and Reagents

- CIP (2.5 units/ μ l) (New England Biolabs)
- 10 \times NEB Buffer 3 (100 mM NaCl; 50 mM Tris-HCl (pH 7.9); 10 mM MgCl₂; 1 mM dithiothreitol (DTT)) (New England Biolabs). This is typically supplied with the CIP enzyme.
- TE (10 mM Tris-HCl (pH 8.0); 1 mM EDTA)
- Nuclease-free water (Ambion)
- Water bath or heat block set to 37°C

Method

1. Prepare the CIP reaction mixture by combining, for every 10 μ l^a:
 - 1 μ l of 10 \times NEB Buffer 3 (1 \times final concentration)
 - template DNA to a maximum of 50 ng/ μ l final concentration^{b,c,d}
 - 0.25 μ l of CIP
 - nuclease-free water up to a final volume of 10 μ l
2. Incubate the reaction at 37°C for 1 h^e.
3. Clean up the reaction with the Qiagen MinElute Kit (follow *Protocol 6*). Be sure to elute in 20 μ l, which is twice the manufacturer's suggested elution volume.

Notes

^aEach reaction can be scaled up to 100 μ l per tube. A typical reaction volume is 30–40 μ l.

^bTemplate dsDNA samples should be suspended in water, TE, or 1 \times NEB Buffer 3 (which will not interfere with RNase digestion). If suspended in NEB Buffer 3, addition of extra NEB Buffer 3 is unnecessary. Template dsDNA samples must not be denatured.

^cThere should be no more than 500 ng of template dsDNA per 10 μ l reaction volume.

^dSamples processed from the ChIP–chip method are typically quantified by one of several methods mentioned in section 2.4. The exact concentration of template dsDNA is not very critical for *Protocol 1*. However, it is extremely important that the concentration of the template DNA be determined accurately for *Protocol 2*.

^eSome users might consider combining CIP treatment and RNase digestion to save time and labor, when incorporating the ChIP–chip method with DNA linear amplification. This can be done, but may diminish yield, depending on the amount of undigested RNA present. If the amount of undigested RNA is large (see *Fig. 1*), the yield may drop by up to 50%. This is likely due to the undigested RNA competing with template DNA for CIP activity, resulting in less-efficient removal of the 3' phosphate groups. However, ChIP samples are unlikely to contain large amounts of carry-over RNA and are less likely to be affected by this issue.

Protocol 2

Tailing reaction with terminal transferase

Equipment and Reagents

- TdT^a (20 units/μl) (New England Biolabs)
- 5× TdT buffer^b (1 M potassium cacodylate^c; 125 mM Tris-HCl (pH 6.6); 1.25 mg/ml bovine serum albumin) (Roche)
- 8% dideoxynucleotide tailing solution^d (92 μM dTTP; 8 μM ddCTP (Invitrogen))
- 5 mM cobalt chloride (Roche)
- 0.5 M EDTA (pH 8.0)
- Mineral oil (molecular biology grade)
- Water bath or heat block set to 37°C

Method

1. Prepare the TdT reaction mixture by combining:
 - 2 μl of 5× TdT buffer (1× final concentration)
 - 0.5 μl of 8% dideoxynucleotide tailing solution
 - 1.5 μl of 5 mM cobalt chloride (0.75 mM final concentration)
 - 5 μl of template DNA^e (maximum 7.5 ng/μl final concentration)
 - 1 μl of TdT enzyme (2 units/μl final concentration). TdT should be the *last* reagent added to the mixture
2. Add one to two drops of mineral oil to the top of the mixture to prevent evaporation during incubation.
3. Incubate the reaction at 37°C for 20 min.
4. Stop the reaction by adding 2 μl (per 10 μl reaction volume) of 0.5 M EDTA (pH 8.0).
5. Clean up the reaction using the Qiagen MinElute Kit (follow *Protocol 6*). It is preferable to minimize the amount of mineral oil carried over from the reaction, although trace amounts are acceptable. When using a 10 μl reaction volume, add 10 μl of water to bring the volume up to 20 μl prior to following *Protocol 6*^f.

Notes

^aThe New England Biolabs (NEB) recombinant enzyme is the preferred enzyme source. Use a recombinant enzyme, since an enzyme derived from a natural source (e.g. Roche, typically derived from calf thymus) may have lot-dependent variation and may result in unpredictable or lowered IVT yields. Furthermore, the NEB recombinant enzyme is preferred over the Roche recombinant enzyme, because the latter typically yields 50% less amplification product with the volumes specified in *Protocol 2*.

^bNote that this is *not* the buffer supplied with the NEB enzyme. This is actually the buffer supplied with either of the Roche enzymes (see ^a). The NEB TdT enzyme comes supplied with NEB Buffer 4 (50 mM potassium acetate; 20 mM Tris-acetate; 10 mM magnesium acetate; 1 mM DTT) and 2.5 mM cobalt chloride. The DTT in this buffer will precipitate the cobalt chloride and inhibit the reaction.

^cCacodylate is a methylated form of arsenic. While this form is less toxic than other forms of arsenic, it should still be treated as a toxic reagent and handled accordingly. Employ waste disposal practices appropriate for your institution.

^dAvoid subjecting the 8% tailing solution to more than three freeze-thaw cycles, as we have found that additional freeze-thaw cycles will further degrade the nucleotides and reduce the efficiency of the reaction.

^cThe maximum amount of template DNA is approximately 1 pmol of template molecules. This corresponds to an approximate maximum of 75 ng for a mixture of template DNA with an average size range of 250 bp. The tested range is 2.5–75 ng of DNA per 10 μ l reaction volume. Scale up the reaction volume accordingly for higher starting amounts, typically to 20 μ l. For ChIP samples, an accurate concentration is critical – underestimation of the concentration will result in lowering the yield to as little as 5–10% of the amount that is typical for *Protocol 2*, because much of the template DNA will not be tailed. If you are not sure how much you have and cannot accurately quantify your samples, scale up to a 20 μ l volume.

^fBe sure to elute in 20 μ l, which is twice the manufacturer's suggested elution volume of 10 μ l, to achieve sufficient yields. Eluting in 10 μ l may reduce the yield by up to 50%, possibly because the MinElute columns have a decreased recovery yield for nanogram quantities of DNA.

Second-strand synthesis involves synthesis of the second strand of the template DNA. At this stage, the strand-displacement activity of the Klenow fragment polymerase separates the two strands of the template DNA, after which the enzyme performs fill-in 5'→3' polymerization. Its 3'→5' exonuclease activity may also remove the 3' overhanging poly(dT) tails (see *Fig. 2*), although the efficiency of this activity will vary based on the length of the poly(dT) tail. If template-independent product is generated, as mentioned in section 4.1.4 and shown in *Fig. 3 (lane 4)*, refer to *Table 1* for adjustments to the reaction volumes of this protocol.

The IVT step is the stage at which linear amplification is performed. Because the T7-based IVT proceeds as an isothermal reaction, it linearly amplifies the template DNA, producing antisense RNA (aRNA), i.e. each strand of RNA produced is antisense to the original template strand. Since both strands are amplified, this distinction is usually not important and is affected only by the location of the T7 promoter and poly(A) tract on the aRNA. Note that RNA is produced at the end of this protocol, so practice correct techniques to maintain an RNase-free environment.

Table 1. Second-strand synthesis with limiting primer amounts

DNA (ng)	T7 primer (μ l) ^a	NEB Buffer 2 (μ l)	5 mM dNTPs (μ l)	Water (μ l)	Tailed DNA (μ l)	Klenow (μ l)	Total volume (μ l)
>75	0.60 (25 μ M)	5.0	2.0	20.40	20.0	2.0	50
50–75	0.30 (25 μ M)	2.5	1.0	0.20	20.0	1.0	25
25	0.15 (25 μ M)	2.5	1.0	0.35	20.0	1.0	25
10 ^b	1.50 (1 μ M)	1.0	0.4	0.20	6.5	0.4	10
5 ^b	0.75 (1 μ M)	1.0	0.4	0.95	6.5	0.4	10
2.5 ^b	0.38 (1 μ M)	1.0	0.4	1.32	6.5	0.4	10

Using limiting amounts of primer is highly advisable when amplifying from very small amounts of starting material. Not only will this decrease the amount of primer-dimer product, but it may also increase the yield of the desired amplification product. The table gives the single reaction volumes to use for a suggested mass range of starting material.

^aSpin down the tubes every 30 min during the 37°C incubation step if using a thermal cycler that does not have a heated lid.

^bThe tailed DNA will have to be dried down in a vacuum centrifuge to the volume indicated for reaction volumes that total 10 μ l.

Protocol 3

Second-strand synthesis with Klenow fragment polymerase

Equipment and Reagents

- DNA polymerase I Klenow fragment (5000 units/ml) (New England Biolabs)
- NEB Buffer 2 (50 mM NaCl; 10 mM Tris-HCl (pH 7.9); 10 mM MgCl₂; 1 mM DTT) (New England Biolabs). This is typically supplied with the Klenow fragment enzyme.
- 25 μM T7-A₁₈B primer
(5'-GCATTAGCGGCCGCGAAATTAATACGACTCACTATAGGGAG(A)₁₈[B]-3')^a
- 5.0 mM dNTP mix^b (Invitrogen)
- 0.5 M EDTA (pH 8.0)
- Nuclease-free water (Ambion)
- Thermal cycler

Method

1. Prepare the second-strand reaction mixture in thermal-cycler-compatible tubes by combining^c:
 - 2.5 μl of 10× NEB Buffer 2^d (1× final concentration)
 - 1 μl of 5 mM dNTP solution (200 μM final concentration)
 - 0.3 μl of 25 mM T7-A₁₈B primer (300 nM final concentration)
 - 20 μl of poly(dT)-tailed template DNA (elution volume from *Protocol 6*)
 - nuclease-free water to a final volume of 24 μl (0.2 μl)

The volumes correspond to a typical reaction volume of 25 μl, taking into account the 1 μl of Klenow polymerase to be added in step 2. Do *not* use mineral oil. Trace amounts of mineral oil may interfere with the IVT reaction (see *Protocol 4*).
2. Incubate the reaction in the thermal cycler using the following program: 94°C for 2 min to denature; ramp -1°C/s to 35°C; hold at 35°C for 2 min to anneal primers; ramp -0.5°C/s to 25°C; hold for 45 s (pausing longer, up to 5 min, is permitted if the time is needed for adding Klenow to a large number of samples). Remove the tubes, add the amount of Klenow DNA polymerase indicated in *Table 1*, spin the tubes briefly, return them to the cycler, and incubate at 37°C for 90 min to fill in the second strand.
3. Stop the reaction by adding 0.5 M EDTA (pH 8.0) to 50 mM final concentration (2.5 μl for a 25 μl reaction volume).
4. Clean up the reaction with the Qiagen MinElute Kit (follow *Protocol 6*). Be sure to elute in 20 μl, which is twice the manufacturer's suggested elution volume.

Notes

^a[B] stands for any base other than A. The primer thus consists of a mix of primers that end in C, G or T. This primer adaptor should be obtained by high-pressure liquid chromatography, polyacrylamide gel electrophoresis, or an equivalent purification method.

^bThis is a deoxynucleotide mixture containing 5 mM each of dATP, dCTP, dTTP, and dUTP. We have found that additional freeze-thaw cycles will further degrade the dNTPs and reduce the reaction yield.

^cThe volume should be scaled up to 50 μl if *Protocol 2* was scaled up to 20 μl. The volumes correspond to a typical reaction volume of 25 μl. Refer to *Table 1* for any necessary adjustments.

^dIn early 2004, New England Biolabs switched the supplied buffer for the Klenow enzyme from EcoPol Buffer (10 mM Tris-HCl (pH 7.5); 5 mM magnesium chloride; 7.5 mM DTT) to NEB Buffer 2. The NEB Buffer 2 performs at least equivalently, if not better, than the EcoPol Buffer, which had to be pre-warmed to 37°C to dissolve any precipitated DTT.

Protocol 4

IVT

Equipment and Reagents

- T7 Megascript Kit (containing 75 mM each of ATP, CTP, GTP, and UTP nucleotide solutions^a; pTRI-Xef (0.5 mg/ml) control template (optional); nuclease-free water; 10× reaction buffer^b; 10× enzyme mix (T7 RNA polymerase and a proprietary RNase inhibitor) (Ambion))
- Air incubator set to 37°C

Method

1. If continuing from *Protocol 3*, dry down the eluate from 20 to 8 µl in a vacuum centrifuge at medium heat for 10–12 min.
2. Prepare the IVT reaction mixture in 0.2 ml RNase-free tubes by combining:
 - 8 µl of 75 mM NTP mix^a
 - 2 µl of 10× reaction buffer^b
 - 2 µl of enzyme mix^c
 - 8 µl of eluate from *Protocol 3*
3. Incubate at 37°C overnight (acceptable range is 5–20 h) in the air incubator or in a thermal cycler with a heated lid. Tubes of 0.2 ml are used to minimize vapor volume.

Notes

^aIf using a new kit, combine the NTP solutions into one tube, then aliquot back out into the four tubes. In the first three freeze–thaw cycles, yields drop approximately 10–15% after each cycle. If the NTPs go through more than three freeze–thaw cycles, we have found that each subsequent freeze–thaw cycle may additionally drop the yield by as much as 50%.

^bThe reaction buffer should be warmed to room temperature first. Adding cold buffer to the template DNA may risk precipitation of the DNA.

^cIf your template DNA is small (<300 bp), you can try boosting the reaction by increasing the enzyme mix to 2.4 µl and decreasing the NTP mix to 7.6 µl. The reaction yield may increase by 10–30% due to the more favorable stoichiometric ratio of enzyme to template DNA in the boosted reaction. Note, however, that this may lower your maximum theoretical yield, so this step is not recommended for larger DNA templates.

After the IVT reaction is complete, the aRNA product is cleaned up using the Qiagen RNeasy Kit. The following protocol is based on the manufacturer's protocol for cleaning up RNA reactions, with minor modifications.

If the aRNA is to be used for subsequent microarray experiments, the reverse transcription reaction should be primed with 5 µg pd(N)₆ random hexamer primers (Amersham Biosciences). The presence of oligo(dT) primers will not interfere with reverse transcription, but oligo(dT) primers will not prime the reaction correctly.

The Qiagen MinElute protocol is based on the manufacturer's protocol, except that the elution volume has been doubled to 20 µl, due to the small amounts of DNA being purified at each step. Without this increase in elution volume, yields may drop by as much as 50%.

Protocol 5

Sample purification

Equipment and Reagents

- RNeasy Mini Kit (containing RNeasy columns; Buffer RLT; Buffer RPE (with 95 or 100% RNase-free ethanol added)^a; RNase-free water; 2 ml RNase-free collection tubes; 1.5 ml RNase-free collection tubes) (Qiagen)
- 14.2 M β -Mercaptoethanol (Sigma)
- 100% RNase-free ethanol (Sigma)

Method

1. Prepare the Buffer RLT master mix^b by combining the following in a 1.5 ml RNase-free microcentrifuge tube for each IVT reaction:
 - 350 μ l of Buffer RLT
 - 80 μ l of RNase-free water
 - 3.5 μ l of β -mercaptoethanol
2. Transfer the contents of the IVT reaction to the 1.5 ml tube and vortex briefly. Low-retention, aerosol-barrier, RNase-free pipette tips are highly recommended here, since the RNA concentration in the IVT reaction tubes may be as high as 5 μ g/ μ l.
3. Add 250 μ l of ethanol (95–100%) and mix well by pipetting. Do not spin the tubes down.
4. Apply the sample (~700 μ l) to an RNeasy mini spin column sitting in a collection tube. Centrifuge for 15 s at 8000 *g*. Discard flow-through.
5. Transfer the RNeasy column to a new 2 ml collection tube. Add 500 μ l of Buffer RPE (which must contain ethanol) and centrifuge for 15 s at 8000 *g*. Discard flow-through but re-use collection tube.
6. Pipette 500 μ l of Buffer RPE into the RNeasy column and centrifuge for 1 min at maximum speed in a microcentrifuge.
7. Remove flow-through and pipette another 500 μ l of Buffer RPE on to the column. Centrifuge for 2 min at maximum speed in a microcentrifuge to dry the column completely^c.
8. Transfer the RNeasy column into a new 1.5 ml RNase-free collection tube, taking care not to carry over any flow-through from the 2 ml collection tube.
9. Add 30 μ l of RNase-free water directly on to the membrane of the RNeasy column. Centrifuge for 1 min at 8000 *g* to elute. Repeat this step if the expected yield is ≥ 30 μ g.
10. Check RNA concentration and quality by measuring the absorbance at 260 nm and 260/280 nm, and by running a sample on a 1–2% agarose gel. A denaturing gel should only be used if the size distribution of the rRNA needs to be determined accurately. *Fig. 3 (lane 4)* indicates a typical 2% gel result.

Notes

^aThis protocol consumes 50% more Buffer RPE than the manufacturer's standard protocol. It may be necessary to order additional Buffer RPE separately for a sufficient supply. If necessary, 80% RNase-free ethanol may be substituted, although this has not been extensively tested.

^bThis mix can be prepared up to a week in advance and aliquoted into 1.5 ml microcentrifuge tubes.

^cThis is an additional wash that is not in the Qiagen protocol. If the RNA is to be used for microarray work or other applications involving fluorescence, we have found this additional wash to be necessary to remove remaining trace amounts of guanidine thiocyanate that would otherwise contaminate the eluted RNA and cause increased background noise in fluorescence applications such as microarrays.

Protocol 6

Qiagen MinElute Kit protocol^a

Equipment and Reagents

- MinElute Kit (containing MinElute columns; Buffer ERC; Buffer PE (with 95% or 100% ethanol added; Buffer EB) (Qiagen)
- 3 M Sodium acetate (pH 5.0) (optional)

Method

1. In a sample of volume 20–100 μl , add 300 μl of Buffer ERC and mix thoroughly. If the sample is in less than 20 μl , make it up to 20 μl with nuclease-free water. If the sample is in more than 100 μl , split the sample into aliquots smaller than 100 μl and process each aliquot in its own column.
2. If the buffer color is orange or purple (i.e. pH>7.5), add 10 μl of 3 M sodium acetate (pH 5.0). If the buffer is yellow, as is typically the case for *Protocols 1* and *2*, no additional sodium acetate is needed.
3. Apply the sample mixture to the MinElute spin column (sitting in a 2 ml collection tube) and spin for 1 min at maximum speed in a microcentrifuge.
4. Discard the flow-through and add 750 μl of Buffer PE (which must contain ethanol). Spin for 1 min at maximum speed in a microcentrifuge.
5. Discard the flow-through and spin for 1 min at maximum speed in a microcentrifuge to dry the column.
6. Transfer the column to a fresh 1.5 ml nuclease-free microcentrifuge tube. Pipette 20 μl of Buffer EB directly on to the column membrane. Leave it to stand for at least 1 min and then spin for 1 min at maximum speed in the microcentrifuge to elute.

Notes

^aThis is based on the MinElute Reaction Cleanup Kit protocol provided in the MinElute Handbook supplied with the kit.

3.1. Expected yields

Depending on the starting amount and size distribution of the starting material, the typical yield is 5–90 μg , as detailed in *Table 2*. The amplification yield for a given starting amount is represented by a range, which is dependent on the quality and size distribution of the starting material. A more direct determinant of amplification yield will be based on the number of picomoles of template used. Initiation of the reaction is the rate-limiting step for TdT tailing (see *Protocol 2*) and IVT (see *Protocol 4*), so the number of template molecules present will more directly determine the actual amplification yield. For example, amplifying 50 ng of a 200 bp PCR product is likely to produce a lower mass yield than amplifying 50 ng

Table 2. Typical amplification yields

Input (ng)	Yield (μg)	Fold amplification
75	60–100	800–1333
50	50–90	1000–1800
25	30–70	1200–2800
10	20–50	2000–5000
5	10–25	2000–5000
2.5	5–10	2000–4000
50 (R-PCR)	15	300

of an 800 bp PCR product, even though the number of aRNA molecules produced may actually be greater for the 200 bp product than for the 800 bp product. For comparison, the R-PCR yield (30 cycles) of a 50 ng starting amount is also shown (see *Fig. 3, lane 5*, and *Table 2*).

3.2. Composition of the amplification product

Typically, a 1–2% agarose gel, containing or stained with ethidium bromide, is used to assess the composition and quality of the amplified RNA. An example of such a gel is shown in *Fig. 3*. Unless size distribution is crucial, it is usually not necessary for the gel to be denaturing. Within the resolution limits of the agarose gel, the amplified product may shift higher on the gel, in the order of 20–40 bp. This shift is to be expected due to the addition and tightness of the size distribution of the poly(A) tail, plus the 5 nt sequence added by the T7 promoter. The size distribution of the poly(A) tail becomes particularly evident in amplification products produced from a single-size template, such as PCR products or a restriction-digested plasmid. Occasionally, a substantial amount of a low-molecular-weight band may also appear near the bottom of the gel, at around 100 bp (see *Fig. 3, lane 4*). This band has been observed under certain amplification conditions, usually when the concentration of starting material is significantly less than that of the primer during second-strand synthesis (see *Protocol 3*). Although the composition of this band has not been tested, it likely represents the amplification product produced from IVT-valid template synthesized through the formation of primer dimers during second-strand synthesis (see *Protocol 3*). This side reaction consumes reagents and activity of the RNA polymerase enzyme, reducing the true effective yield of the desired amplification product. Therefore, *Table 1* has been included in *Protocol 3*, in which optimum primer-to-template mass ratios (roughly 5:1) have been established to minimize this side reaction.

The Qiagen MinElute Kit is used a number of times in this method to remove buffer salts, spent enzymes, free nucleotides, and any DNA products shorter than 40 nt. Material between 40 and 70 nt may also be removed, but at a lower efficiency.

3.3. Results

3.3.1. Summary of amplification fidelity

A detailed discussion of the validation procedure for amplification fidelity and reproducibility will not be repeated here, since it has been previously described in detail (6). However, key points from this discussion will be briefly mentioned. First, within the 100–700 bp range tested for this method, the size distribution of the amplification product is effectively preserved compared with R-PCR, which loses material below 250 bp and subsequently has reduced fidelity (see *Fig. 3, lane 5*). IVT amplification also produces an increased dynamic range that does not appear to impact negatively on its fidelity, improving the prospects of separating biological trends from the noise inherent in microarray experiments. This is another improvement over R-PCR, which often suffers from dynamic range compression. Furthermore, IVT amplification retains fidelity and representation of the starting material when compared with an unamplified Klenow-labeled standard.

3.3.2. Updates to this method

Since the initial publication of this method (6), a number of updates have been incorporated, as presented in this chapter. While most of the changes are minor, a number of more significant changes have been implemented. This method was optimized with the NEB Klenow enzyme for the second-strand synthesis step (see *Protocol 3*). Recently, NEB switched from supplying EcoPol buffer with this enzyme to Buffer 2. This switch has now been tested, with results indicating no change or a slight improvement (5–15%) in yields. Furthermore, the second-strand synthesis step now contains a detailed table (see *Table 1*) of optimized primer amounts and recommended final reaction volumes for a given starting amount. These measures combine to reduce the side reaction of low-molecular-weight material generated from speculated primer-dimer formation from excess primer (6, 7). Moreover, the default reaction volume has been reduced from 50 to 25 μ l for the recommended starting amount of 50–75 ng, which permits the reduction of reagent costs without affecting yield. Finally, for the IVT step, amplification of low-molecular-weight material (i.e. <300 bp) has been optimized by boosting the amount of T7 enzyme used per reaction. The boost typically results in a 10–30% yield improvement, likely due to a more favorable stoichiometric ratio of the T7 enzyme to the DNA template.

3.4. ChIP-chip results

The method presented in this chapter has already been used in a number of published studies (14–17). An example of the results obtained from the ChIP-chip method, when used in conjunction with DNA linear amplification, is shown in *Fig. 4*. Here, the ChIP-chip method was performed on *Saccharomyces cerevisiae* histone H3 and FLAG-H2B, carried through the DNA linear amplification method

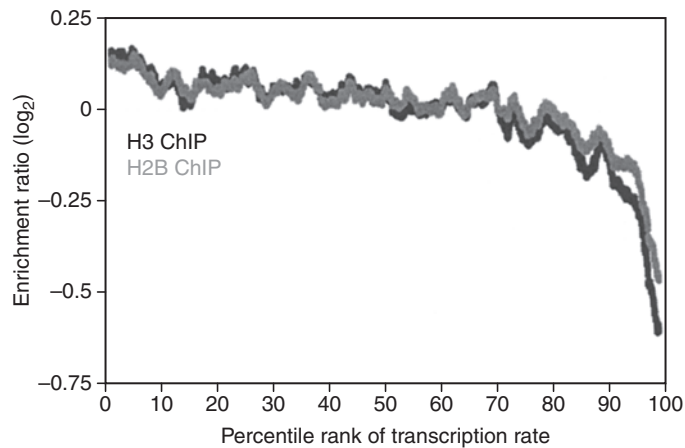


Figure 4. Example of ChIP-chip results.

Results from ChIPs of *S. cerevisiae* histone H3 and FLAG-H2B, plotted by intergenic genomic loci as a function of the percentile rank of the transcription rate in the downstream genes (14). An inverse relationship can be seen between nucleosome occupancy (as represented by enrichment for histone H3 and H2B) and promoter strength, as indicated by the transcription rate. These ChIP samples were amplified with the DNA linear amplification protocol presented in this chapter. Reprinted with permission from (14).

presented in this chapter, reverse transcribed, labeled, and hybridized on to *S. cerevisiae* genome arrays consisting of intergenic regions. This was plotted against the transcription rate of downstream genes; one can observe the inverse relationship between nucleosome occupancy and promoter strength.

4. TROUBLESHOOTING

These protocols routinely work well when the samples used are within the recommended starting amount range, and particularly when users new to these protocols use the controls described in section 2.5. Poor aRNA yield is the most frequently encountered problem. Occasionally, one may encounter more subtle problems, either stemming from the nature and composition of the template DNA used, or in ensuring compatibility with downstream applications such as microarrays.

4.1. Poor amplification yield

If aRNA yield is poor, examination of the controls can quickly pinpoint the likely cause of the problem.

4.1.1. RNase contamination

The IVT control provides a good way of determining whether there are any problems associated with handling RNA and maintaining an RNase-free environment. If the IVT control produces a poor yield, this may be due to contamination with RNases. This can be confirmed by running a 2% non-denaturing agarose gel in Tris-acetate-EDTA (TAE) and ethidium bromide. An RNase-contaminated IVT sample will yield a smear of low-molecular-weight material. If RNase contamination is determined to be the cause, ensure that aerosol-barrier, RNase-free pipette tips are used, and that working surfaces are treated with RNaseZap (Ambion) or other RNase-decontaminating agents. This is particularly important if working with ChIP samples. A way to verify that the RNase A used to digest RNA carried over from the ChIP is completely eliminated is to use the pTRI-Xef linear plasmid supplied with the Ambion IVT kit, add RNase A to the amount typically used for post-ChIP RNA digestion, and then carry it through three successive cleanups using *Protocol 6*.

4.1.2. Suboptimal IVT conditions

If there is no RNase contamination detected, either via the A_{260}/A_{280} ratio from a UV absorbance reading of the sample, or from analyzing the sample by gel electrophoresis, it is likely that there are problems with the IVT reaction conditions:

- The NTP mix may have gone through too many freeze-thaw cycles. As described in *Protocol 4*, NTPs are very sensitive to freeze-thaw cycles and each one decreases the yield. If this is the case, use a fresh IVT kit and aliquot the NTP mix (as described in *Protocol 4*, note ^a; into more than four aliquots if necessary) before use.
- Excessive evaporation of the reaction volume may have occurred during incubation. *Protocol 4* describes the proper incubation conditions for the IVT reactions. These conditions have been designed to limit evaporation and vapor volume during the long incubation period. Follow the conditions described in *Protocol 4*. Using mineral oil is not recommended, since it may interfere with either the IVT reaction (see *Protocol 4*) or the aRNA cleanup (see *Protocol 5*), or both.

You may also consult the troubleshooting section in the manufacturer's manual accompanying the IVT kit.

4.1.3. Poor yield with the positive-amplification control (*Protocols 2 and 3*)

This is likely to occur if the considerations mentioned in section 2.5 were not followed.

- If the template DNA has a large proportion of 3' recessed ends, this template will not tail efficiently. A fill-in reaction using Klenow enzyme is recommended. ChIP samples from sheared genomic DNA typically have approximately half of their ends 3' recessed; this correspondingly reduces the

yield by half. Double the starting amount or use Klenow fill-in to obtain yields comparable to those indicated in *Table 2*.

- If the template DNA has a large proportion of 3' phosphate groups, the template will not tail efficiently. Treat the template DNA with an alkaline phosphatase, as suggested in *Protocol 1*. Note that some DNA fragmentation methods such as restriction digestion may leave behind 3' phosphate groups. Because this information is not always readily available for a given restriction enzyme, try *Protocol 1* on the template DNA and see whether this solves the yield problem.
- Check the recovery yield of the template DNA at the end of *Protocol 6* (Qiagen MinElute column cleanup). From 50 ng of input DNA, a yield of 50–80% is typical. If the yields obtained are significantly less than this, verify that the column membrane is completely wetted by Buffer EB during step 6 of *Protocol 6*. Furthermore, verify that the columns are viable – starting from 2004, Qiagen required that the MinElute columns be stored at 4°C when not in use.

4.1.4. Formation of template-independent product

The amplification product may contain a substantial amount of template-independent product when the mass ratio of T7 primer to template DNA significantly exceeds 5:1. This can easily occur when the starting amount of the template DNA is significantly overestimated. An example of this template-independent product is shown in *Fig. 3 (lane 4)*. We speculate that excess T7 primer during *Protocol 3* produces primer dimers, which yields an IVT template that produces the band of low-molecular-weight material shown in *Fig. 3*. This IVT side reaction diminishes the yield of the true amplification product. To prevent this from happening, follow *Table 1* in *Protocol 3*, which indicates the proper volumes and concentrations of T7 primer and template DNA to use for this step. If necessary, follow the recommendations discussed in section 2.4 for accurate measurement of the starting amount of template DNA.

4.2. Poly(A) tracts in the amplified RNA

The TdT step (see *Protocol 2*) produces poly(dT) tails within a size range of 20–40 nt. While we speculate that overhanging 3' regions of the poly(dT) tails in the second-strand synthesis step (see *Protocol 3*) are removed via the 3' exonuclease activity of the Klenow fragment enzyme, there exists the possibility that the T7 primer may anneal in such a way that the 3' anchor of the primer (denoted by base designation '[B]' in *Fig. 2*) may not be base paired, and that the rest of the poly(dA) region of the primer is base paired anywhere along the length of the poly(dT) tail. The resulting size distribution may thus be larger than the original template, usually in the order of 20–40 bp, and would appear as a gel shift and a broadening of the gel bands corresponding to that size range. This issue has not been examined carefully because the potential variability in tail length does not appear to affect amplification efficiency and fidelity when used on microarrays. However, this issue may be important for consideration of

applications that are sensitive to this potential variability in tail lengths and to the poly(A) tracts that will appear in the final amplification product.

4.3. DsRNA formation in the amplified RNA product

Theoretically, dsRNA can conceivably form from the amplification products, since aRNA based on both strands of the original template is produced. However, we have not tested whether dsRNA actually forms under the conditions outlined in this method. For spotted microarray experiments, if DNA probe produced from the aRNA product undergoes some degree of self-hybridization, the end result could potentially be a decrease in net signal intensity or compression of the dynamic range in the ratiometric data obtained, or both. We have observed this in one case with a yeast open reading frame microarray where, in a low-complexity mixture containing less than 300 unique DNA species, amplification of both strands compressed the dynamic range by ~60–70% when compared with that obtained with a single-strand amplification (Rebecca Butcher, Harvard University, MA, USA, personal communication). We were able to make this determination via single-strand amplification because the starting material already had conserved sequences that were different on each end of the amplicon. Note that the protocols as described in this chapter normally do not provide that opportunity.

We believe this issue, however, should not significantly impact on most studies that amplify highly complex mixtures of DNA, such as randomly fragmented genomic DNA. The amplification fidelity and signal quality have already been demonstrated to be at least as good as direct, unamplified Klenow labeling and better than R-PCR (6). We speculate that in a microarray hybridization, a DNA probe synthesized from a high-complexity mixture of aRNA (such as from amplification of sheared genomic DNA) is less likely to be affected by probe self-hybridization than probe synthesized from a low-complexity mixture (such as from amplification of a transcription factor ChIP that localizes to a small number of locations within the genome). The reason is that, during the hybridization process, a given probe strand in a highly complex mixture is more likely to hybridize to its complementary target on the microarray than to its complementary probe strand floating free in solution. This is because the complementary target on the microarray is fixed in location, while the complementary probe strand is free-floating and migrating throughout the hybridization solution. Thus, we believe that only in the case where probe composition is of low complexity should the user be concerned about probe self-hybridization.

If dsRNA does form in significant proportions, it may also reduce the efficiency and yield of reverse transcription either by slowing down the reverse transcriptase enzyme in the reverse transcription step or by causing insufficient denaturing of the dsRNA, leading to less efficient primer annealing. To compensate, we suggest using 50% more RNA than the amount typically used for microarray probe labeling. Nevertheless, we have not found it necessary to investigate carefully the potential impact of aRNA self-hybridization on reverse transcriptase efficiency, since high amplification fidelity is typically obtained. Furthermore, we have found

in many cases that foregoing this increase will still yield a lower but usable net signal intensity for most spotted microarray hybridizations.

Acknowledgements

C.L.L. is supported by a Graduate Research Fellowship from the National Science Foundation. S.L.S. is an investigator at the Howard Hughes Medical Institute. B.E.B. is supported by a K08 Development Award from the National Cancer Institute. This work was supported by a grant from the National Institute for General Medical Sciences.

5. REFERENCES

- ★★ 1. Buck MJ & Lieb JD (2004) *Genomics*, **83**, 349–360. – *An excellent review of the ChIP–chip method.*
2. Ren B, Robert F, Wyrick JJ, *et al.* (2000) *Science*, **290**, 2306–2309.
3. Bohlander SK, Espinosa R, III, Le Beau MM, Rowley JD & Diaz MO (1992) *Genomics*, **13**, 1322–1324.
4. Gerton JL, DeRisi J, Shroff R, Lichten M, Brown PO & Petes TD (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 11383–11390.
5. Iyer VR, Horak CE, Scafe CS, Botstein D, Snyder M & Brown PO (2001) *Nature*, **409**, 533–538.
- ★★★ 6. Liu CL, Schreiber SL & Bernstein BE (2003) *BMC Genomics*, **4**, 19. – *Original publication describing the DNA linear amplification method presented in this chapter.*
- ★ 7. Baugh LR, Hill AA, Brown EL & Hunter CP (2001) *Nucleic Acids Res.* **29**, e29. – *First detailed description of elimination of template-independent amplification product.*
- ★ 8. Phillips J & Eberwine JH (1996) *Methods*, **10**, 283–288. – *Description of the first RNA linear amplification method.*
9. Wang E, Miller LD, Ohnmacht GA, Liu ET & Marincola FM (2000) *Nat. Biotechnol.* **18**, 457–459.
- ★ 10. Marko NF, Frank B, Quackenbush J & Lee NH (2005) *BMC Genomics*, **6**, 27. – *The background introduction provides an excellent summary of the state of the RNA amplification field at the time of publication of this chapter.*
11. Sambrook J & Russell DW (2001) *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
12. Martin CT, Muller DK & Coleman JE (1988) *Biochemistry*, **27**, 3966–3974.
13. Lage JM, Leamon JH, Pejovic T, *et al.* (2003) *Genome Res.* **13**, 294–307.
14. Bernstein BE, Liu CL, Humphrey EL, Perlstein EO & Schreiber SL (2004) *Genome Biol.* **5**, R62.
15. Bernstein BE, Kamal M, Lindblad-Toh K, *et al.* (2005) *Cell*, **120**, 169–181.
16. Humphrey EL, Shamji AF, Bernstein BE & Schreiber SL (2004) *Chem. Biol.* **11**, 295–299.
17. Lee CK, Shibata Y, Rao B, Strahl BD & Lieb JD (2004) *Nat. Genet.* **36**, 900–905.