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Molecular Research Center, Inc.

TRI REAGENT®BD - RNA / DNA / PROTEIN ISOLATION REAGENT for BLOOD DERIVATIVE

Cat. No. TB 126
Store at 25 C

PRODUCT DESCRIPTION

TRI Reagent® BD is a complete and ready to use reagent for isolation of total RNA or for the simultaneous isolation of RNA, DNA and proteins from serum, plasma or whole blood. TRI Reagent BD is an adaptation of the popular single-step method of total RNA isolation (1,2) permitting fast and efficient processing of blood derivatives. This highly reliable technique performs well with small and large sample volumes and permits simultaneous processing of a large number of samples. TRI Reagent BD and the single-step method are subjects of the US patents 4,843,155 and 5,346,994, other patents pending.

TRI Reagent BD combines phenol and guanidine thiocyanate in a mono-phase solution to facilitate the immediate and most effective inhibition of RNase. A sample of blood is lysed in TRI REAGENT BD and the lysate is separated into the aqueous and organic phases by the bromochloropropane or chloroform addition and centrifugation. RNA remains exclusively in the aqueous phase, DNA in the interphase and proteins remain in the organic phase. RNA is precipitated from the aqueous phase by isopropanol, washed with ethanol and solubilized. DNA and proteins are sequentially precipitated from the interphase and organic phase by ethanol and isopropanol, washed of remaining impurities and solubilized.

STABILITY: TRI Reagent BD is stable at 25 C for at least two years from the date of purchase (3).
SPECIAL HANDLING PRECAUTIONS
TRI REAGENT BD contains poison (phenol) and an irritant (guanidine thiocyanate). Causes burns. CAN BE FATAL. When working with TRI Reagent BD **use gloves and eye protection** (shield, safety goggles). Do not get on skin or clothing. Avoid breathing vapor. Read the warning note on the bottle and MSDS.

In case of contact: Immediately flush eyes or skin with a large amount of water for at least 15 minutes and seek immediate medical attention.

I. ISOLATION OF RNA

The isolation of total RNA by TRI Reagent BD from whole blood, plasma and serum **can be completed in 1 h, and the recovery of undegraded RNA is 30-150% higher than in other methods of RNA isolation.**TRI Reagent BD isolates a whole spectrum of RNA molecules rarely observed in RNA isolated by other methods. Typically, the column-based methods may artificially change the mRNA composition. TRI Reagent is especially useful for isolating viral RNA. The isolated RNA is can be used for northern analysis, dot blot hybridization, poly A^+ selection, in vitro translation, RNase protection assay, molecular cloning and for polymerase chain reaction (PCR*)

PROTOCOL

Reagents required, but not supplied: 1-bromo-3-chloropropane (BCP) or chloroform, isopropanol, ethanol and 5 N acetic acid.
We recommend the use of disposable polypropylene tubes provided by Molecular Research Center, Inc. Tubes from other suppliers should be tested to ensure integrity during centrifugation at 12,000 g with TRI Reagent BD.

The protocol includes the following steps:

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1. **LYSIS:** 0.75 ml TRI Reagent BD + 0.2 - 0.25 ml whole blood, plasma or serum.

2. **PHASE SEPARATION:** homogenate + 0.1 ml BCP or 0.2 ml chloroform.

3. RNA PRECIPITATION: aqueous phase + 0.5 ml isopropanol.

4. RNA WASH: 1 ml 75% ethanol.

5. RNA SOLUBILIZATION: FORMAzol®, 0.5% SDS, or water.

The procedure is performed at room temperature, unless stated otherwise.

1. LYSIS
A. SERUM. Add 0.25 ml of serum and 2 - 8 µl of Polyacryl Carrier (cat. no. PC 152) to 0.75 ml of TRI Reagent BD, close the tube and shake the resulting mixture well by hand or vortex.
B. WHOLE BLOOD or PLASMA. Add 0.2 ml of whole blood or plasma to 0.75 ml of TRI Reagent BD supplemented with 20 µl of 5 N acetic acid per 0.2 ml of whole blood or plasma. Close the tube and shake the resulting mixture well by hand or vortex.

Acetic acid can be added before or after mixing TRI Reagent BD with blood samples. Prepare 5 N acetic acid by mixing 1 ml of glacial acetic acid (>99%) with 2.48 ml of water. The ratio of the sample volume to the reagent volume should be always as indicated in the protocol above. Too large sample volume will result in DNA contamination and too small sample volume will lower the yield of RNA.

2. PHASE SEPARATION
Store the lysed samples for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Next, supplement the lysate with 0.1 ml BCP or 0.2 ml of chloroform per 0.75 ml of TRI Reagent BD, cover the samples tightly and shake vigorously for 15 seconds. Store the resulting mixture at room temperature for 2 - 5 minutes and centrifuge at 12,000 g for 15 minutes at 4 C. Following centrifugation, the mixture separates into a lower brownish phenol-chloroform phase, an interphase and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase whereas DNA and proteins are in the interphase and organic phase. The volume of the aqueous phase is about 60% of the volume of TRI Reagent BD used for lysis.

BCP is less toxic than chloroform and its use reduces the possibility of contaminating RNA with DNA (4). Chloroform used for phase separation should not contain isoamyl alcohol or any other additive.

It is important to perform centrifugation to separate aqueous and organic phases in the cold (4-10 C). If performed at elevated temperature, a residual amount of DNA may sequester in the aqueous phase. In this case, RNA can be used for northern analysis but it may not be suitable for PCR.

3. RNA PRECIPITATION
Transfer the aqueous phase to a fresh tube, and save the interphase and organic phase at 4 C for subsequent isolation of DNA and proteins. Precipitate RNA from the aqueous phase by mixing with isopropanol. Use 0.5 ml of isopropanol per 0.75 ml of TRI Reagent BD used for the initial lysis. Store samples at room temperature for 5-10 min and centrifuge at 12,000 g for 8 minutes at 4 - 25 C. RNA precipitate forms a gel-like or white pellet at the bottom of the tube.

4. RNA WASH
Remove the supernatant and mix the RNA pellet in 75% ethanol by vortexing. Add 1 ml of 75% ethanol per 0.75 ml of TRI Reagent BD. When the isolation is performed in large tubes (> 2 ml), add 1 ml of 75% ethanol to the RNA pellet and transfer the RNA-ethanol suspension to a microfuge tube. For the transfer, use a wide bore 1 ml pipette tip prepared by cutting 2- 3 mm from the end of a plastic tip. Centrifuge the RNA suspension at 7,500 g for 5 minutes at 4 - 25 C. If the RNA pellet accumulates on the side of a tube or has a tendency to float, perform the centrifugation at 12,000 g.

5. RNA SOLUBILIZATION
Remove the ethanol wash and briefly air-dry the RNA pellet for 5 min. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. **Do not dry RNA by centrifugation under vacuum.** Dissolve RNA in FORMAzol® stabilized formamide (cat. no. FO 121), water or 0.5% SDS by passing the solution a few times through a pipette tip and incubating for 10-15 minutes at 55-60 C. Water or the SDS solution used for RNA solubilization should be made RNase-free by diethyl pyrocarbonate (DEPC) treatment. RNA should be precipitated from FORMAzol with ethanol before using for RT-PCR. Drying of RNA is not necessary when FORMAzol is used for sample solubilization.

6. RESULTS
Ethidium bromide staining of RNA separated in an agarose gel or methylene blue staining of a hybridization membrane

after RNA transfer visualizes two predominant bands of small (2 kb) and large (5 kb) ribosomal RNA, low molecular weight (0.1 - 0.3 kb) RNA. The final preparation of total RNA is essentially free of DNA and proteins and has a 260/280 ratio 1.6 - 1.9. For RT-PCR analysis, DNase treatment may be necessary for optimal results. Typically, 15 - 20 µg of total RNA can be isolated from 1 ml of human whole blood. For optimal spectrophotometric measurements, RNA aliquots should be diluted with water or buffer with a pH > 7.5 such as Phosphate Buffer (cat. no. SP 130). Distilled water with a pH <7.0 falsely decreases the 260/280 ratio and impedes the detection of protein contamination in RNA samples (6).

NOTES AND COMMENTS

1. To facilitate isolation of RNA from small volume of blood derivatives (<0.2 ml) perform lysis of samples in 0.75 ml of TRI Reagent BD supplemented with 2 - 8 µl of Polyacryl Carrier (cat. no. PC 152). For the sample volumes < 0.2 ml, adjust the volume to 0.2 ml with serum or use a smaller volume of the reagent. When isolating RNA from whole blood or plasma, add 10 µl of 5 N acetic acid per 0.1 ml of whole blood or plasma. Next, add BCP or chloroform and proceed with the phase separation and other steps of isolation as described above.
2. After lysis in TRI Reagent BD (before BCP or chloroform addition) samples can be stored at -70 C for several months. The RNA precipitate (step 4, RNA WASH) can be stored in 75% ethanol at 4 C for at least one week, or at least one year at - 20 C.
3. Cellular RNA degrades rapidly when blood samples are stored at 4 C. For isolation of undegraded RNA, samples have to be processed immediately after collection, or aliquoted and frozen at -70 with or without TRI Reagent BD. Isolation of viral RNA can be performed with samples stored at 4 C for several days.
4. Hands and dust may be the major source of the RNase contamination. Use gloves and keep tubes closed throughout the procedure.
5. See also poly A^+ RNA isolation and RT-PCR application notes and the Trouble Shooting Guide.

II. ISOLATION OF DNA BY TRI REAGENT BD

The protocol for DNA isolation with TRI Reagent BD is designed to overcome the heavy burden of proteins present in whole blood. DNA is precipitated from the interphase and the phenol phase obtained from the lysate as described in the RNA isolation protocol. Following precipitation, the DNA is solubilized in DNAzol®(cat. no. DN 127), re-precipitated and washed with ethanol. The final DNA preparation is solubilized in 8 mM NaOH, neutralized, and used for analysis. The isolated DNA can be used for PCR, restriction, Southern blotting, molecular cloning and other molecular biology applications.

PROTOCOL

Reagents required, but not supplied: isopropanol, ethanol, DNAzol and sodium hydroxide.

The following steps of the protocol describe isolation of DNA from the phenol phase and interphase of a sample lysed in 0.75 ml of TRI REAGENT BD.

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1. DNA PRECIPITATION: phenol phase and interphase + 0.4 ml ethanol (per 0.75 ml TRI REAGENT BD).

2. DNA SOLUBILIZATION: 0.25 ml DNAzol.

3. DNA PRECIPITATION: 0.125 ml ethanol, 2,000 g x 5 min.

4. DNA WASH: 1 ml 95% ethanol (2x).

5. DNA SOLUBILIZATION: 8 mM NaOH.

The procedure is carried out at room temperature, unless stated otherwise.

1. DNA PRECIPITATION

Remove the remaining aqueous phase overlying the interphase and precipitate DNA from the interphase and organic phase with ethanol. Add 0.4 ml of 100% ethanol per 0.75 ml of TRI Reagent BD used for the initial lysis and dissolve the interphase proteins by mixing samples thoroughly by inversion. Next, sediment DNA by centrifugation at 2,000 g for 5 minutes at 4 - 25 C. Careful removal of the aqueous phase is critical for the quality of the isolated DNA. *See Notes and Comments (4) for an alternative DNA isolation procedure.*

2. DNA SOLUBILIZATION
Remove the phenol-ethanol supernatant and save it at 4 C for the protein isolation. Add 0.25 ml of DNAzol and dissolve the DNA precipitate by agitating the tube. When the isolation is performed in large (> 2 ml) tubes, transfer the DNA solution to a microcentrifuge tube.

3. DNA PRECIPITATION
Precipitate DNA by adding 0.125 ml of 100% ethanol to 0.25 ml of DNAzol. Store tubes at room temperature for 2 - 5 minutes and sediment the DNA precipitate at 2,000 g for 3 minutes at 4 - 25 C.

4. DNA WASH
Wash the DNA precipitate twice with 0.8 - 1 ml of 95% ethanol. At each wash, suspend the precipitate by inverting the tubes and centrifuge at 2,000 g for 1 - 3 minutes at 4 - 25 C. If the DNA forms a compact pellet, no centrifugation is necessary and the ethanol wash can be removed by decanting.

5. DNA SOLUBILIZATION
Air dry the DNA pellet by keeping tubes open for 5 - 10 minutes at room temperature. Dissolve DNA in 8 mM NaOH by slowly passing the pellet through a pipet. Add an adequate amount of 8 mM NaOH to approach a DNA concentration of 0.1 - 0.3 µg/µl. Typically, add 0.1 ml of 8 mM NaOH to the DNA isolated from 1 ml of whole blood. The use of a mild alkaline solution assures full solubilization of the DNA precipitate.

QUANTITATION OF DNA
For optimal spectrophotometric measurements, DNA aliquots should be diluted with water or buffer with a pH > 7.5 such as Phosphate Buffer. Distilled water with a pH < 7.0 falsely decreases the 260/280 ratio and impedes the detection of protein in RNA samples (6). Calculate the DNA content assuming that one A_{260} unit equals 50 µg double-stranded DNA/ml. For calculation of the cell number in analyzed samples assume that the amount of DNA per 10^6 of diploid cells of human, rat and mouse origin equals: 7.1 µg, 6.5 µg and 5.8 µg, respectively (5). A preparation of DNA isolated from whole blood contains >80% of 60-100 kb DNA and <10% of 20 kb DNA. The isolated DNA is free of RNA and proteins and has a 260/280 ratio >1.7. Typical yield is 10 - 20 µg DNA/ml of human blood.

AMPLIFICATION OF DNA BY PCR
Following solubilization in 8 mM NaOH, adjust the pH of the DNA sample to 8.4 using HEPES (see Table). Add an aliquot of the sample (typically 0.1 - 1 µg DNA) to a PCR reaction mix and perform PCR according to your standard protocol.

DIGESTION OF DNA BY RESTRICTASES
Adjust the pH of the DNA solution to a required value using HEPES (see Table). Alternatively, dialyze samples against 1 mM EDTA, pH 7 - pH 8. Carry out the DNA restriction for 3 - 24 h under optimal conditions for a specific restriction enzyme using 3 - 5 units of the enzyme per µg DNA. In a typical assay, 90% - 100% of the DNA preparation is digested by restrictases.

Adjustment of pH in DNA samples solubilized in 8 mM NaOH

For 1 ml of 8 mM NaOH, use the following amounts of 0.1M or 1 M HEPES (free acid)

Final pH	0.1 M HEPES (ul)	Final pH	1 M HEPES (ul)
8.4	86	7.2	23
8.2	93	7.0	32
8.0	101		
7.8	117		
7.5	159		

NOTES AND COMMENTS

1. If necessary, the phenol phase and interphase can be stored at 4 C overnight. Samples suspended in 75% ethanol can be stored at 4 C for a long period of time (months). Samples solubilized in 8 mM NaOH can be stored overnight at 4 C; for prolonged storage, adjust samples to pH 7 - 8 and supplement with 1 mM EDTA.
2. The isolation protocol can be modified if the DNA is isolated only for quantitative purposes. The phenol phase and the interphase can be stored at 4 C for a few days or at -70 C for a few months.
3. If the expected yield of DNA is < 5 µg, the use of Polyacryl Carrier (cat. no. PC 152) is recommended to assure

full recovery of DNA. Add 5 µl of Polyacryl Carrier to the interphase/organic phase and precipitate DNA with ethanol as described in the protocol.

4. This alternative procedure replaces steps 1 - 3 of the DNA Isolation procedure and can be performed without the use of DNAzol. Prepare a back extraction buffer containing: 4 M guanidine thiocyanate, 50 mM sodium citrate and 1 M Tris (free base).
   Following phase separation (RNA Isolation Procedure Step 2), remove any remaining aqueous phase overlying the interphase and add back extraction buffer to the interphase - organic phase mixture. Use 0.5 ml of back extraction buffer per 0.75 ml of TRI Reagent BD used for the initial lysis. Vigorously mix the sample by inversion for 15 sec and store for 10 min at room temperature. Perform phase separation by centrifugation at 12,000 g for 15 min at 4 C. Transfer the upper aqueous phase containing DNA to a clean tube and save the interphase and organic phase at 4 C for subsequent protein isolation. Precipitate DNA from the aqueous phase by adding 0.4 ml of isopropanol per 0.75 ml of TRI Reagent BD used for the initial lysis. Mix the sample by inversion and store for 5 min at room temperature. If the expected DNA yield is less than 20 µg, add 2 - 8 µl of Polyacryl Carrier to the aqueous phase prior to isopropanol addition and mix. Sediment DNA by centrifugation at 12,000 g for 5 min at 4 - 25 C and remove the supernatant. Wash the pellet with ethanol as described in the DNA Wash Step 4 and proceed with DNA solubilization as described in Step 5.
5. Also see the Troubleshooting Guide.

III. ISOLATION OF PROTEINS BY TRI REAGENT-BD

Revised protocol reduces extraction time and improves protein recovery!

Proteins are isolated from the phenol-ethanol supernatant obtained after precipitation of DNA with ethanol (step 1, DNA PRECIPITATION). The resulting preparation can be analyzed for the presence of specific proteins by Western blotting (2,7,8).

PROTOCOL

Reagents required but not supplied: guanidine hydrochloride, ethanol, isopropanol, acetone, glycerol, SDS, urea and tributylphosphine (Sigma T 7567).

The protocol includes the following steps:

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1. PROTEIN PRECIPITATION - 0.2-0.3 ml phenol-ethanol supernatant (1 volume) + acetone (3 volumes)

2. PROTEIN WASH - 1 ml of guanidine hydrochloride/ethanol/glycerol wash solution, 3 x 10 min.

- 1 ml ethanol/glycerol solution, 1 x 10 min.

3. PROTEIN SOLUBILIZATION - 1% SDS, 10M Urea or other suitable solvent.

The procedure is carried out at room temperature unless stated otherwise.

1. PROTEIN PRECIPITATION

Aliquot a portion of the phenol-ethanol supernatant (0.2 - 0.3 ml, 1 volume) into a microfuge tube. Precipitate proteins by adding 3 volumes of either acetone or isopropanol. Mix by inversion for 10-15 sec to obtain a homogeneous solution. Store samples for 10 min at room temperature and sediment the protein precipitate at 12,000 g for 10 min at 4 C (See Notes 1and 2).

2. PROTEIN WASH

Decant the phenol-ethanol supernatant and disperse the protein pellet in 0.5 ml of 0.3 M guanidine hydrochloride in 95% ethanol + 2.5 % glycerol (V:V). Disperse the pellet using a pipet tip, syringe needle or a small conical Teflon pestle (Fisher K749515-0000) attached to a mechanical stirrer (~30 sec @ 800-1000 RPM). After dispersing the pellet, add another 0.5 ml aliquot of the guanidine hydrochloride/ethanol/glycerol wash solution to the sample and store for 10 min at RT. Sediment the protein at 8,000 g for 5 min. Decant the wash solution and perform two more washes in 1 ml each of the guanidine/ethanol/glycerol wash solution. Disperse the pellet by vortexing after each wash to efficiently remove

residual phenol. Perform the final wash in 1 ml of ethanol containing 2.5 % glycerol (V:V). At the end of the 10 min ethanol wash, sediment the protein at 8,000 g for 5 minutes. Decant the alcohol, invert the tube and dry the pellet for 7-10 min at room temperature (See Note 3).

3. PROTEIN SOLUBILIZATION

Option 1. After briefly air-drying the protein pellet, add a suitable solvent such as 1% SDS, 10 M urea, or another suitable detergent-based solvent to the protein pellet (8). Use 0.8 ml of solvent per 300 ul of whole blood phenol-ethanol supernatant (See Note 4). Gently disperse and solubilize the pellet for 15-20 minutes by "flicking" the tube or pipetting as required. The addition of a suitable reducing agent such as tributylphosphine (2.5% of solution volume) will improve protein yield in most preparations. For immediate use in western analysis, heat the solution for 3 min at 100 C and sediment any insoluble material by centrifugation at 10,000 g for 5 min at RT. Transfer the supernatant to a clean tube and use the protein solution immediately for western blotting (See Note 5). Otherwise, store the solubilized proteins at -20 C and perform the heating (100 C, 3 min), centrifugation or other preparatory steps at the time of use.

Option 2. Dialyze the phenol-ethanol supernatant (II DNA Isolation Step 1, DNA Precipitate) in a suitable, regenerated cellulose dialysis tubing against three changes of 0.1% SDS at 4 C. Centrifuge the dialysate at 10,000 g for 10 min at 4 C and use the clear supernatant for Western blotting.

NOTES

1. Isopropanol may replace acetone during protein precipitation. Blood proteins that have been precipitated with isopropanol sidperse more easily.

2. Limiting the volume of phenol - ethanol supernatant to 0.2 - 0.3 ml per tube will produce a smaller, more manageable protein pellet and improve protein yield. TRI Reagent-BD protein extracts will yield 130-170 ug of protein / ul of human whole blood.

3. In general, protein pellets suspended in 0.3 M guanidine hydrochloride/ethanol/glycerol wash solution or in ethanol/glycerol wash solution can be stored for at least one month at 4 C or one year -20 C. Individual proteins may display different sensitivity to long-term storage and optimal storage conditions should be established for sensitive and labile proteins.

4. The solubility and stability of specific proteins can be influenced by different detergent solutions (8). To obtain optimal results in various experimental applications, investigators may solubilize small amounts of protein in different solvents and determine which solution best addresses their unique experimental objectives. Generally, the best yield of total recovered protein from human whole blood is obtained with a urea-based solvent

5. Solubilized protein may form insoluble aggregates during storage at -20 C. Prior to western analysis, thaw the samples at 25 C for 10-15 minutes. Heat the solubilized protein sample for 3 min at 100 C, pipette the solution and remove insoluble protein by centrifugation as outlined in the protocol.

IV. TROUBLESHOOTING GUIDE

RNA ISOLATION

- Low yield. a) incomplete solubilization of the final RNA pellet.
- 260/280 ratio < 1.6. a) contamination of the aqueous phase with phenol phase, b) too small volume of the reagent used for sample lysis.
- RNA degradation. a) samples were not immediately processed or frozen after collection, b) samples used for isolation or the isolated RNA preparations were stored at -20 C instead of at -70 C, c) aqueous solutions or tubes used for solubilization of RNA were not RNase-free, e) formaldehyde used for agarose-gel electrophoresis had a pH below 3.5.
- DNA contamination. a) too small volume of the reagent was used for sample lysis. Phase separation was performed at temps above 10 C.

DNA ISOLATION

- Low yield. a) incomplete solubilization of the final DNA pellet.
- 260/280 ratio < 1.7. a) incomplete solubilization of the final DNA pellet. b) if insoluble particles remain in the

DNA preparation, remove the particles by centrifugation at 10,000 g for 5 minutes at 4 - 25 C.

- DNA degradation. a) blood samples were stored at room temperature. Too much mechanical force was applied during the procedure.
- RNA contamination. a) too large of a volume of aqueous phase remained with the interphase and organic phase.

PROTEIN ISOLATION

- Low yield. a) incomplete lysis of samples, b) incomplete solubilization of the final protein pellet.
- Protein degradation. Samples were not immediately processed or frozen after collection.
- Band deformation in PAGE. Insufficient wash of the protein pellet.

V. ISOLATION OF POLY A$^+$ RNA

Following RNA precipitation with isopropanol (step 3), the RNA pellet can be dissolved in a binding buffer and selection of poly A$^+$ RNA can be performed on an oligo-dT column according to a standard protocol of Aviv and Leder (Proc Natl Acad Sci USA, 1972, 69, 1408-1412). For best results, use MRC oligo-dT columns (cat. no. OT 125). Small amounts of total RNA (<0.5 mg) can be processed by a direct application of the aqueous phase mixed with isopropanol on the MRC oligo-dT columns.

VI. RT-PCR AND PCR APPLICATION NOTE

A more complete evaporation of ethanol is required when RNA or DNA samples (5-20 µl) are prepared for RT-PCR and PCR, respectively. If not dried sufficiently, the small samples may contain a relatively high level of ethanol.

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The use of TRI REAGENT can be cited by referring to this brochure: TRI Reagent®BD - RNA, DNA, protein isolation reagent. Manufacturer's protocol (1995), Molecular Research Center, Inc. Cincinnati, OH, or to references 1 or 2.

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