

Comprehensive Report on FFPE Extraction Methods

Mutation detection based on DNA or RNA sequencing is becoming increasingly important in both human disease research and clinical settings. Typically, tissue biopsies are taken and stored as formalin-fixed, paraffin-embedded (FFPE) blocks, so that tissue architecture is maintained in a low-cost and low-tech manner that can be stored at room temperature for prolonged periods. However, nucleic acid extraction from FFPE samples produces poorer-quality material than extractions from freshly frozen material. Additionally, this material is often limited, and so gains in extraction efficiency as it relates to NGS library construction are highly valuable.

Therefore, we set out to evaluate ways in which we could improve the performance of six popular commercially available FFPE extraction kits as it relates to Anchored Multiplex PCR (AMP™) chemistry performance. Here we describe the protocol modifications we tested, identify the ones we found to be beneficial, and compare the performance of these kits to each other in the context of an Archer® FusionPlex® Assay.

Below are recommendations for extraction kit-specific conditions for nucleic acid preparation from FFPE that are compatible with FusionPlex and VariantPlex® library preparation:

- Agencourt® FormaPure® Kit - proteinase K digestion at 55°C for 1 hour followed by crosslink reversal at 80°C for 1 hour
- Covaris® truXTRAC™ FFPE RNA Kit - crosslink reversal at 80°C for 1 hour
- QIAGEN® AllPrep® DNA/RNA FFPE Kit - crosslink reversal at 80°C for 15 minutes; mineral oil is interchangeable with the QIAGEN® Deparaffinization Solution for sample deparaffinization.
- Promega ReliaPrep™ FFPE Total RNA Miniprep System - crosslink reversal at 80°C for 1 hour
- Zymo Research Pinpoint™ Slide RNA Isolation System II - proteinase K digestion at 55°C for 1 hour followed by a crosslink reversal at 80°C for 1 hour; elute using 20µL water

Introduction

Nucleic acid extraction from formalin-fixed, paraffin-embedded (FFPE) tissues yields inferior-quality material for NGS analysis compared to nucleic acid extracted from fresh frozen tissue. This decrease in sample quality is due to several factors, most notably:

- Sample age
- Sample processing time
- Sample storage conditions
- Covalent nucleic acid modifications made by the formalin fixative

These factors manifest as fragmented material and inhibitory covalent marks on the DNA or RNA resultant from formaldehyde fixation. Despite the limitations these modifications place on NGS analysis, specimens stored as FFPE samples will continue to be part of the clinical laboratory for diagnostic as well as research purposes. In light of this reality, several companies have produced kits for nucleic acid extraction from FFPE specimens. Experiments were performed to optimize the extraction protocols and to expand the number of FFPE extraction kits that could be recommended for use with the FusionPlex and VariantPlex library preparation kits.

All FFPE sample extraction kits have the same basic steps:

1. Separate tissue from paraffin
2. Solubilize and digest the tissue to release nucleic acids
3. Reverse chemical crosslinking
4. Capture nucleic acids by affinity purification

The goal of this project was to determine which of these steps had the largest impact on purified sample quality in the context of AMP enrichment chemistry while not attempting to re-engineer the components of each kit. Based on a surveillance of the literature, the most fruitful aspects of the extraction process to investigate were:

- Crosslink reversal time and temperature
- Extraction pH
- Mode of paraffin removal

Materials and Methods

Six different FFPE sample extraction kits and methods were optimized and their performance compared. The kits used were:

- Agencourt Formapure Kit (cat # A33341)
- QIAGEN® AllPrep® DNA/RNA FFPE Kit (cat # 80234)
- Covaris truXTRAC FFPE RNA Kit (cat # 520161)
- Ambion® RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (cat # AM1975)
- Zymo Research Pinpoint™ Slide RNA Isolation System II (cat # R1007)
- Promega ReliaPrep™ FFPE Total RNA Miniprep System (cat # Z1001)
- Ambion® TRIzol® Reagent (cat # 15596-026)

For each kit, the manufacturer-suggested extraction protocol was compared to a modified protocol using technical replicate extractions of a control sample.

All extraction kits and protocol modifications were tested using a control FFPE sample comprised of a homogeneous mixture of four cell lines positive for EML4-ALK, CCDC6-RET and SLC34A2-ROS1 fusions derived from three different genomic rearrangements (further denoted as DX0535). This control was used for the majority of these analyses rather than more relevant real tissue samples, because a uniform product would provide greater insight into extraction effects. Real tissue samples have uneven fixation effects (e.g., position in the sample block, tissue architecture), and this variability could confuse data analysis.

Extraction success was assessed by fluorometric and qPCR-based methods. Nucleic acid mass was measured using the Invitrogen® Qubit® 3.0 Fluorometer (cat# Q33216) and either the Invitrogen® Qubit® dsDNA HS or RNA HS Assay Kits (cat# Q32851 and Q32852, respectively).

Amplifiable RNA was measured using the Archer PreSeq® RNA QC Assay, which targets a 113nt amplicon derived from the VCP gene transcript. The assay was designed such that one of the primers spans an exon-exon junction specific for cDNA. Extraction methods and kits were evaluated for their RNA extraction performance using PreSeq and quantified by comparing the level of the VCP prod-

uct relative to RNA standards, including BioChain® Total RNA, Human Adult Normal Lung Tissue (cat. #R1234152-50), Ambion® Human Lung Total RNA (cat. #AM7968), Clontech® Human Universal Reference Total RNA (cat. #636538) or according to a given kit's recommended protocol (e.g., 55°C for 16 hours for FormaPure) in cases where a RNA standard was not included in the analysis. Amplifiable DNA molecules were quantified using the Archer PreSeq DNA QC Assay, a qPCR assay that targets a 110nt amplicon of an intron in the GPI gene (reference DNA PreSeq white paper). Experimental DNA quantitation was performed relative to an intact genomic DNA (BioChain® Genomic DNA, Human Adult Normal Adrenal Tissue, cat. #D1234004). For both assays, equivalent DNA or RNA (as cDNA) masses as measured by Qubit fluorometry were compared (50ng RNA, 5ng DNA).

NGS libraries using cDNA input were prepared using the Archer Universal RNA Reagent Kit v2 for Illumina (cat #AK0040) and the Archer FusionPlex Solid Tumor Panel (Cat #AK0034). Libraries were sequenced on an Illumina® NextSeq® system, and the resulting sequences were subsampled to an equal depth to allow for comparisons. The sequences were analyzed using the Archer Analysis bioinformatics software. Library complexity was measured by the number of unique fragment start sites observed in the sequence data; a minimum of five unique fragments supporting a gene fusion was required for the fusion to be called by the software.

Results

Agencourt FormaPure Kit

Because the FormaPure kit is commonly used in-house for FFPE extractions and is recommended for Archer library preparation protocols, modifications to this protocol were tested to determine how nucleic acid quality was altered, as measured by PreSeq QC assay. These modifications are:

- Crosslinking reversal time
- Crosslinking temperature
- Crosslinking pH
- Divalent cation concentration
- Inclusion of a commercially available RNA storage reagent

A protocol modification that resulted in beneficial effects was reasoned to be broadly applicable across other kit manufacturers and tested using the other kits.

FormaPure: Crosslink reversal time and temperature optimization

Archer library preparation protocols currently recommend a modification to the FormaPure protocol that includes proteinase K digestion of the sample at 55°C for 16 hours (55°C/16h). Most other extraction kits require 15 minutes to 1 hour for proteinase K digestion; therefore, the increased incubation time was reasoned to remove the inhibitory covalent modifications to the nucleic acid (crosslink reversal).

The effect of changing the crosslink reversal time and temperature on nucleic acid quality was tested first. Samples were first incubated at 55°C for 1 hour (for proteinase K digestion) followed by varying crosslink reversal conditions as follows (all conditions were performed in triplicate):

- 55°C/15h (Archer-recommended protocol)
- 65°C/1h
- 65°C/15h
- 80°C/1h

In this experiment, the 80°C/1h method yielded RNA quality that was only marginally better than the recommended 55°C/15h method (Figure 1A). In other experiments, the 80°C/1h condition had been observed to be marginally worse (data not shown). These data indicate that there is no real difference in amplifiable RNA yield between crosslink reversal conditions.

However, the 80°C/1h method yielded almost twice as much amplifiable DNA (Figure 2) as the 55°C/15h method. For this reason, as well as the decreased time to complete the protocol, we recommend that proteinase K digestion at 55°C for 1 hour followed by crosslink reversal at 80°C for 1 hour for the FormaPure kit.

FormaPure: Effect of pH on crosslink reversal

The RNA phosphate backbone is susceptible to attack by hydroxide ions and is therefore less stable at higher pH. Thus, reducing the pH to decrease hydroxide ion concentration was hypothesized to stabilize RNA during crosslink reversal. In the For-

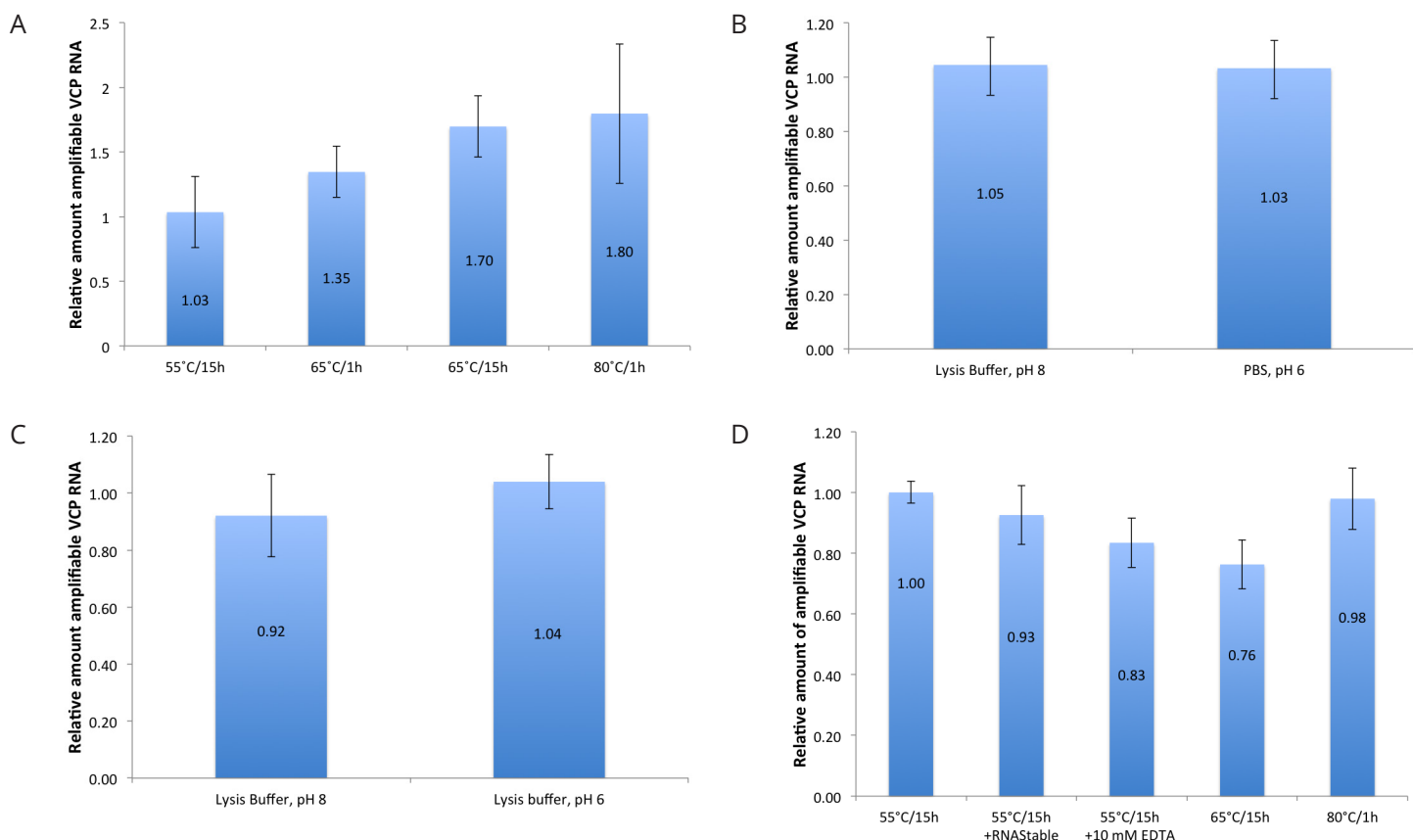


Figure 1. Shorter crosslink reversal incubation at higher temperature can replace longer incubation at lower temperature with the FormaPure kit. The relative amount of amplifiable RNA based on various crosslink reversal conditions was compared. (A) Varying crosslink reversal temperatures and incubation times are shown; data reveals no significant difference between crosslink reversal conditions. (B and C) Crosslink reversal at different pH conditions are shown. (B) Extractions were transferred to PBS pH 6 or remained in lysis buffer pH 8 prior to crosslink reversal; data reveals no difference in RNA quality between crosslink reversal conditions. (C) All extractions remained in lysis buffer; pH in one set of extractions adjusted to pH 6 prior to crosslink reversal; data shows no difference in RNA quality between conditions. (D) The addition of RNA-stabilizing reagents (RNAstable or EDTA) did not increase the proportion of amplifiable RNA.

maPure protocol, crosslink reversal is performed in lysis buffer that has a pH between 7 and 8. The lysis buffers from most kits are also in this pH range.

To test the effect of pH on RNA quality, extractions were buffer-exchanged into phosphate-buffered saline (PBS, pH 6) via desalting columns after proteinase K digestion for 1 hour, and 55°C/15h crosslink reversal was then performed. Results were compared to extractions that underwent identical digestion and crosslink reversal conditions while remaining in the FormaPure lysis buffer (pH 8). Each experimental condition was performed in triplicate. No significant difference between the amount of amplifiable RNA obtained using the two conditions was observed (Figure 1B). As an additional test, the pH of one set of extractions was adjusted to pH 6 after proteinase K digestion for 1 hour by adding 14µL 2.5M sodium monophosphate. Both pH 6 and pH 8

extraction conditions were incubated at 55°C for 15 hours. Each extraction condition was performed in triplicate. Again, no significant difference in the resulting RNA quality was observed (Figure 1C). Based on the apparent lack of effect in these two simple conditions, this line of experimentation was not pursued further with any other kit.

FormaPure: Effect of RNA-stabilizing additives on crosslink reversal

Biomatrixa® RNAstable® (cat # 93220-001) is a reagent that stabilizes RNA by preventing water from contacting the molecule. To test the impact of this RNA-stabilizing reagent on RNA quality, 0.1 volume RNAstable was added to one set of extractions after proteinase K digestion and prior to crosslink reversal at 55°C/15h. No significant difference between the RNAstable-containing extractions and the

standard extractions was observed (Figure 1D, see 55°C/15h vs. 55°C/15h + RNAsable). RNA crosslink reversal requires interaction with water, and the fact that RNAsable protects RNA by preventing this interaction might explain the inability of the reagent to improve the outcome of crosslink reversal.

EDTA can stabilize nucleic acids by sequestering magnesium, which, along with other divalent cations, coordinates the hydrolysis of RNA in aqueous solutions independent of its role as a co-factor for many different nucleases. EDTA is already present in the FormaPure lysis buffer, likely at a concentration of 1mM. To test if a higher lysis buffer EDTA concentration affected the outcome of crosslink reversal, the EDTA concentration in the lysis buffer was increased to a final concentration of 10mM prior to crosslink reversal at 55°C/15h. Consistent with the results of the RNAsable experiment, no difference in RNA quality was observed when the EDTA concentration was increased (Figure 1D, see 55°C/15h vs. 55°C/15h + EDTA). All extractions testing the impact of RNA stabilizing agents were performed in triplicate.

Based on these experiments, the FormaPure extraction protocol recommended for FusionPlex and VariantPlex library preparation should be modified as follows:

- Proteinase K digestion at 55°C for 1 hour, followed by
- Crosslink reversal at 80°C for 1 hour

This modification will decrease the time needed for FormaPure extractions by 14 hours and significantly improve the quality of DNA in the sample without any reproducible loss of amplifiable RNA yield.

Covaris truXTRAC FFPE RNA Kit

The truXTRAC FFPE RNA Kit differs from most other FFPE extraction kits in that deparaffinization is performed using Adaptive Focused Acoustics™. The proteinase K is then mixed into the sample along with a brief AFA treatment step. Crosslink reversal is performed at 80°C for 15 minutes, and nucleic acids are purified using a silica-based spin column. Our experiments with the truXTRAC kit were performed using an M220 AFA Ultrasonicator on loan from Covaris.

truXTRAC: Crosslink reversal time and temperature optimization

Nucleic acid extraction was performed using the truXTRAC protocol with varying crosslink reversal conditions as follows:

- 80°C/15m (manufacturer-recommended)
- 80°C/1h
- 55°C/16h

The two 80°C extractions were performed in triplicate and the 55°C/16h extraction was performed in duplicate.

A greater proportion of amplifiable RNA was obtained with the 80°C/1h crosslink reversal condition than the 80°C/15m condition ($p=0.0023$; Figure 3A). Crosslink reversal performed at 80°C, however, resulted in 20-40% higher DNA yield than when performed at 55°C (Figure 3B). Based on these observations, crosslink reversal at 80°C for 1 hour is recommended after proteinase K digestion.

To confirm the known relationship between PreSeq QC assay differences and sequencing metrics, libraries were prepared according to the three crosslink reversal conditions using the Archer FusionPlex Solid Tumor Panel and the Archer RNA Universal Re-

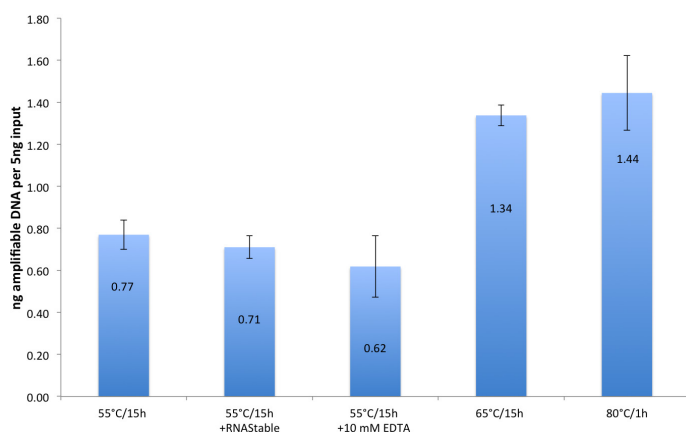
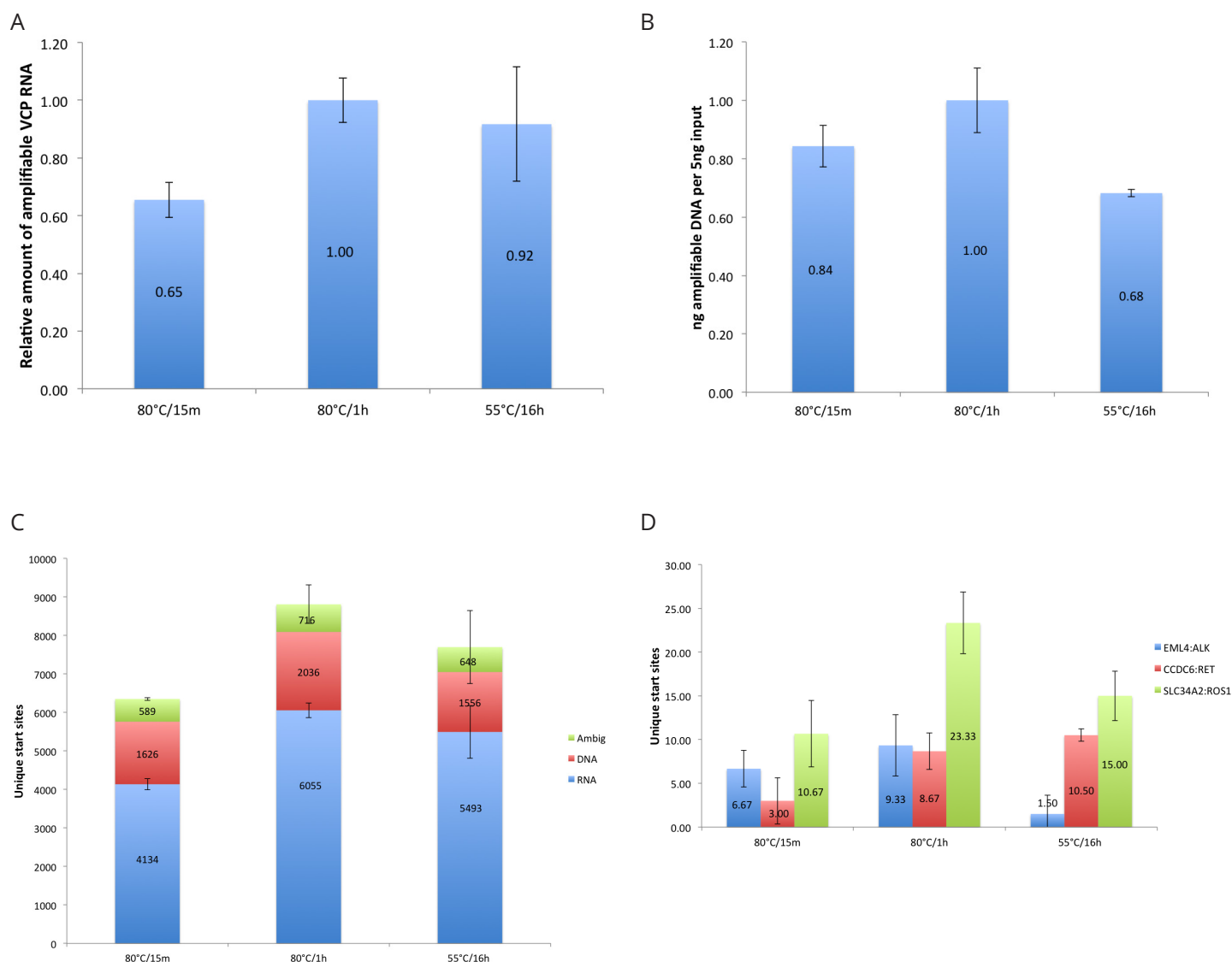


Figure 2. Higher-temperature crosslink reversal improves amplifiable DNA yield with the FormaPure kit. The quantity of amplifiable DNA per 5ng of total DNA is shown for FormaPure extractions performed using various crosslink reversal modifications as shown. The addition of RNAsable or EDTA to 10mM does not significantly improve DNA quality compared to 55°C/15h condition. Crosslink reversal at 65°C/15h or 80°C/1h results in greater DNA compared to 55°C/15h condition.



E

	EML4:ALK	CCDC6:RET	SLC34A2:ROS
truXTRACT 80°C 15 min	3 of 3	2 of 3	3 of 3
truXTRACT 80°C 1 hour	3 of 3	3 of 3	3 of 3
truXTRACT 55°C O.N.	1 of 2	2 of 2	2 of 2

Figure 3. Increased truXTRAC crosslink reversal time improves yield of amplifiable RNA and unique RNA molecules sequenced in Fusionplex Solid Tumor Assay. (A) The relative amount of amplifiable RNA based on the truXTRACT protocol and various crosslink reversal conditions was compared. The 80°C/1h condition yielded significantly higher RNA quality than the 80°C/15m method, while the 55°C/16h condition yielded a marginally significant increase compared to the 80°C/15m method. (B) The amount of amplifiable DNA (out of 5ng total input) is shown based on the truXTRACT protocol using various crosslink reversal conditions. Both the 80°C/15m and 80°C/1h methods yielded significantly greater DNA quality than the 55°C/16h condition. (C) The average number of unique start sites from libraries based on the truXTRACT protocol and various crosslink reversal conditions was compared. Results are broken down into different read types: RNA, DNA, and ambiguous (Ambig). Libraries prepared using the 80°C/1h or 55°C/16h crosslink reversal methods were significantly more complex than those prepared using the 80°C/15m method. (D) The number of unique start sites supporting each gene fusion found in libraries based on the truXTRACT protocol using various crosslink reversal conditions. (E) The number of libraries in which each fusion was called is shown. All three fusions were consistently called only in the libraries prepared using the 80°C/1h crosslink reversal method.

agent Kit v2 with 50ng input RNA. The libraries were then sequenced, and the FASTQ files were subsampled to a depth of 1.238 million reads to normalize read counts between samples. Libraries created from extractions that used the 2 longer crosslink reversal conditions (80°C/1h and 55°C/16h) both had significantly greater amounts of unique RNA and total start sites than the 80°C/15m condition (Figure 3C, blue bar components).

Regarding fusion detection, libraries prepared using the 80°C/1h crosslink reversal condition identified all three fusions, whereas the libraries prepped using the 80°C/15m and 55°C/16h methods each missed a fusion call in one replicate (Figure 3E). Furthermore, the gene fusions were called with greater

confidence with crosslink reversal at 80°C/1h (Figure 3D, compare number of start sites for each fusion between crosslink reversal conditions).

truXTRAC: Crosslink reversal optimization when using FFPE tissue samples

All experiments thus far were performed using the DX0535 control specimen. Because the extraction kit manufacturer boasts higher deparaffinization efficiency from FFPE tissues, the conclusions made to date were tested using nucleic acid extracted from FFPE tissue samples (human liver or kidney sections) provided by Covaris. To better isolate the effects of crosslink reversal times, the extractions from each tissue type were pooled after proteinase K digestion

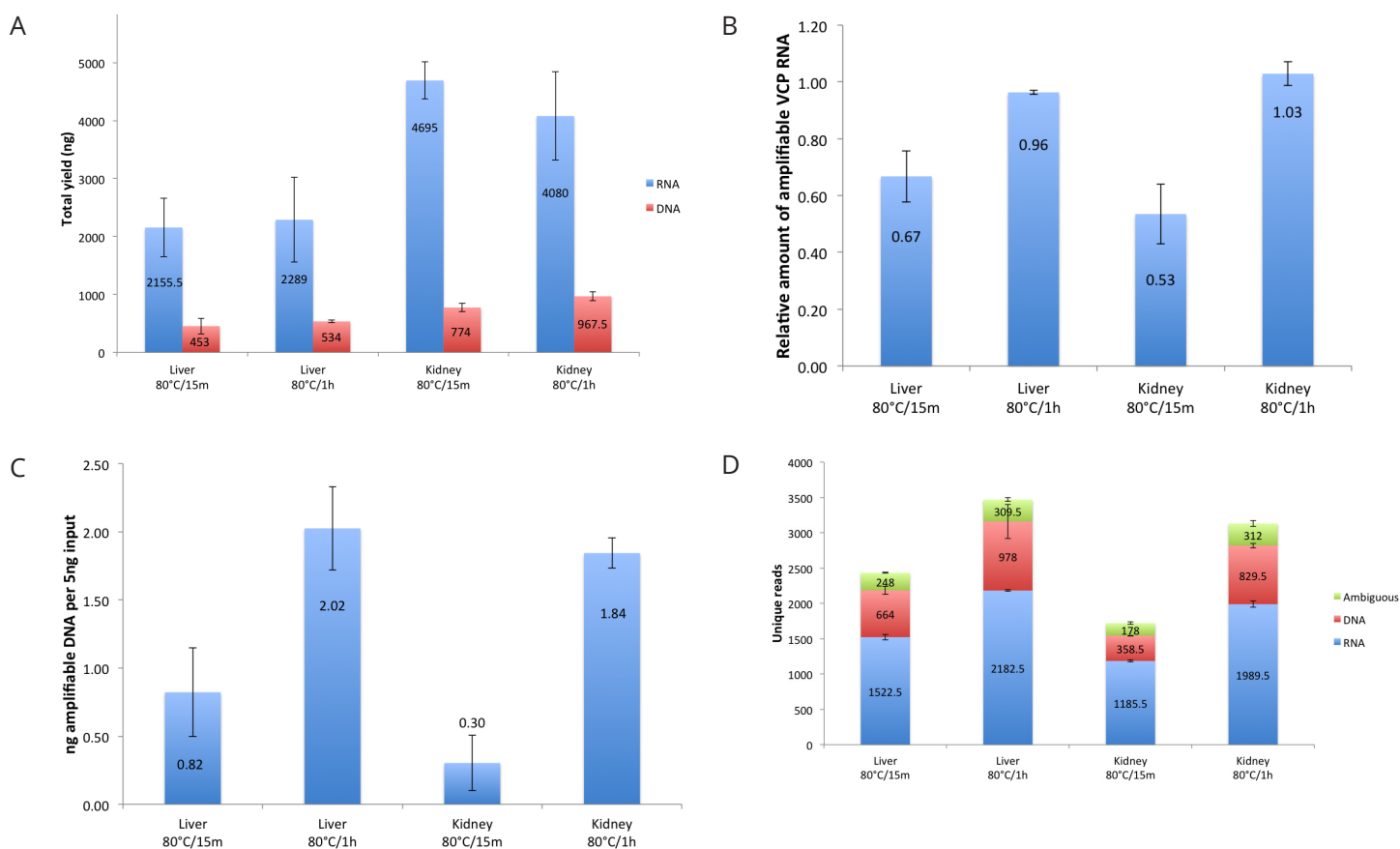


Figure 4. Increased crosslink reversal time improves amplifiable RNA and DNA yield from tissue sections using Covaris truXTRAC. Nucleic acid was extracted from liver and kidney FFPE sections using the Covaris truXTRAC protocol with crosslink reversal at 80°C for 15 minutes or 1 hour. (A) RNA and DNA yield based on crosslink reversal time and tissue type was compared. Greater RNA yield was obtained from kidney than liver independent of the crosslink reversal time. DNA yield was slightly greater in kidney tissue than liver, with the longer method yielding more than the shorter method. (B) Amplifiable RNA based on crosslink reversal time and tissue type was compared. The longer crosslink reversal method yielded greater amplifiable RNA independent of tissue type. (C) Amplifiable DNA (per 5ng total DNA input) based on crosslink reversal time and tissue type was compared. The longer crosslink reversal method yielded greater amplifiable DNA independent of tissue type. (D) The average number of unique start sites from libraries based on crosslink reversal time and tissue type was compared. Results are broken down into different read types: RNA, DNA, and ambiguous (Ambig). Libraries prepared using the 80°C/1h crosslink reversal method were significantly more complex than those prepared using the 80°C/15m method independent of tissue type.

and then equally aliquoted. The aliquots then underwent crosslink reversal at 80°C for 15 minutes or 1 hour prior to RNA and DNA QC analysis. Libraries were then prepared using the FusionPlex Solid Tumor Panel and Universal Reagent Kit v2 with 50ng RNA from each library. Both extraction conditions were performed in duplicate for each tissue type.

There was no significant difference in RNA or DNA mass yield between the two crosslink reversal conditions (Figure 4A). However, we recovered 20-30% more amplifiable RNA (Figure 4B) and 2-4 fold more amplifiable DNA (Figure 4C) with the 80°C/1h crosslink reversal compared to the shorter condition. The longer crosslink reversal also resulted in greater library complexity, as measured by unique start sites in the sequenced libraries (Figure 4D). Such improvements in library complexity are expected to produce gains in assay sensitivity. The results obtained using tissue samples are concordant with those obtained with DX0535, suggesting that valid conclusions can be drawn from experiments using DX0535 samples.

Based on these experiments, the Covaris truXTRAC RNA kit is recommended for FusionPlex library preparation. The crosslink reversal step should be modified to 80°C for 1 hour.

QIAGEN AllPrep DNA/RNA FFPE Kit

The QIAGEN AllPrep kit is intended as a dual-purpose DNA and RNA FFPE extraction kit. The extractions are performed separately and is thus more labor intensive, but the DNA and RNA can be extracted separately from the same material, enabling the user to conserve precious specimens with limiting material.

AllPrep: Deparaffinization optimization

The QIAGEN AllPrep kit protocol recommends deparaffinization with xylene, heptane or the QIAGEN Deparaffinization Solution (cat #19093). These deparaffinization agents are hazardous, so an innocuous but equally effective solution was investigated. The Promega ReliaPrep protocol recommends deparaffinization using mineral oil, which was tested as an alternative deparaffinization reagent in the context of the QIAGEN AllPrep kit. Nucleic acid was extracted from the DX0535 control sample using either mineral oil or QIAGEN Deparaffinization Solution, and amplifiable RNA (Figure 5A) and DNA were measured (Figure 5B). Each extraction condition was

performed in triplicate. No significant difference in amplifiable RNA yield was detected between the mineral oil-based and manufacturer-recommended deparaffinization conditions (Figure 5A).

Because the AllPrep kit has a sequential extraction workflow, DNA extraction was then performed for each condition to identify any possible downstream differences based on the deparaffinization method. Five nanograms of DNA from the DNA and RNA fractions were analyzed for the amount of amplifiable DNA. Data indicates that there was much more amplifiable DNA in the DNA fractions than in the RNA fractions independent of deparaffinization method (Figure 5B), demonstrating that downstream DNA extraction is not selectively affected by either deparaffinization solvent.

AllPrep: Crosslink reversal time optimization

The QIAGEN AllPrep protocol suggests crosslink reversal at 80°C for 15 minutes for RNA extraction. The experiments using the FormaPure and truXTRAC kits indicated that amplifiable RNA yield are improved with crosslink reversal at 80°C for 1 hour. To determine if the AllPrep protocol would yield higher-quality RNA with a longer crosslink reversal step, extractions were prepared with the AllPrep kit using mineral oil-based deparaffinization and crosslink reversal at 80°C for 15 minutes or 1 hour. The relative amount of amplifiable RNA was then determined. Both extraction types were performed in triplicate, but one of the 80°C/15m extractions failed, so the results for that condition are based on two replicates. Data revealed that the longer crosslink reversal time resulted in a significantly higher proportion of amplifiable RNA (Figure 5C).

Based on these experiments, the QIAGEN AllPrep kit is recommended for FusionPlex and VariantPlex library preparation. The crosslink reversal step should be modified to 80°C for 1 hour for maximum nucleic acid yield. Furthermore, mineral oil is as effective a deparaffinization solvent as the QIAGEN Deparaffinization Solution.

Promega ReliaPrep FFPE Total RNA Miniprep System

The Promega ReliaPrep protocol is very similar to the protocol for the Promega Maxwell® automated nucleic acid extraction instrument. Unfortunately, a

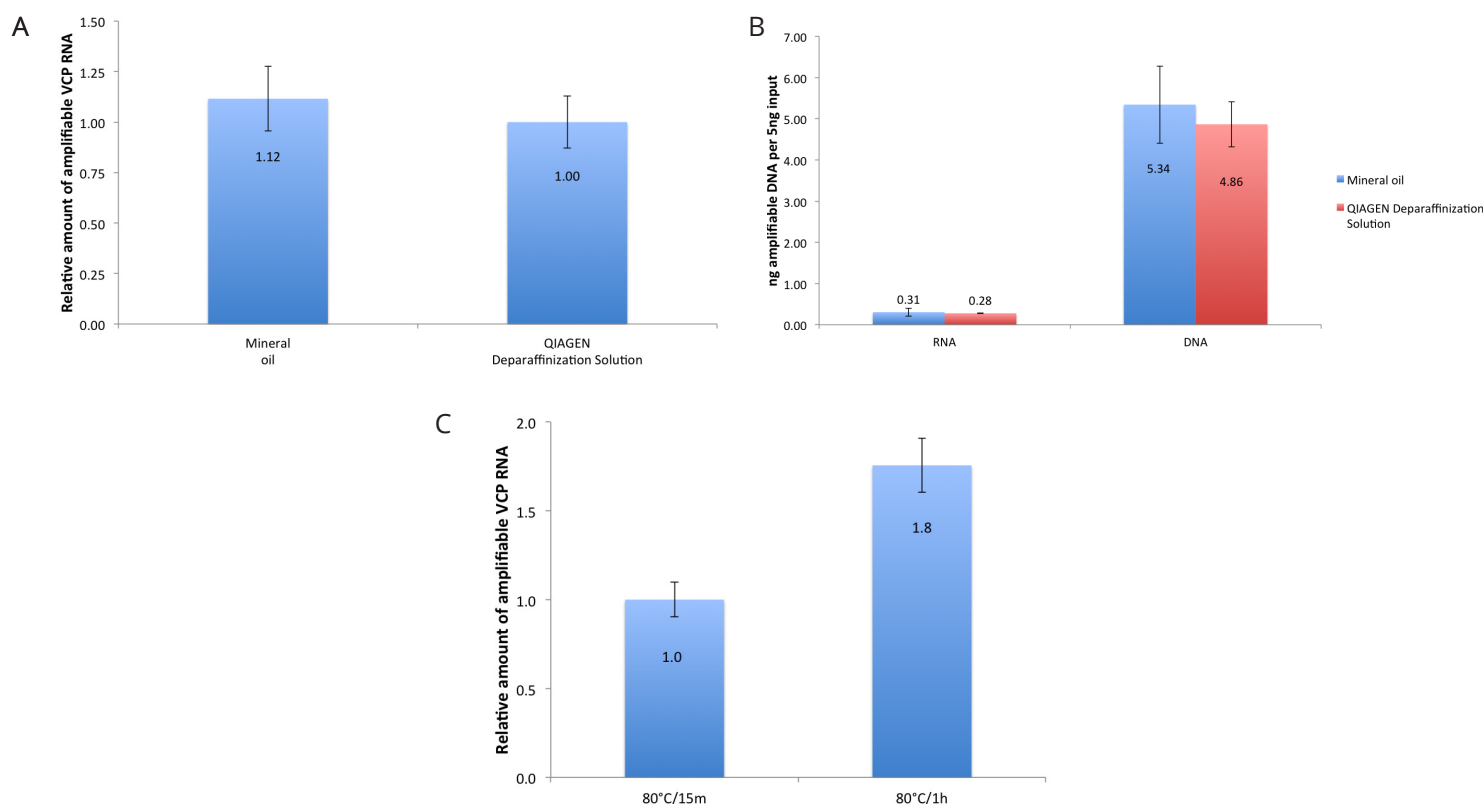


Figure 5. Mineral oil functions as well as QIAGEN Deparaffinization Solution for deparaffinization with the Qiagen AllPrep kit. (A-B) Nucleic acid extractions were performed using the QIAGEN AllPrep kit with either mineral oil or QIAGEN Deparaffinization Solution. (A) The relative amount of amplifiable RNA based on deparaffinization method (QIAGEN Deparaffinization Solution vs. mineral oil) was compared. No significant difference was observed between the two methods. (B) The amount of amplifiable DNA (in 5ng total DNA) based on deparaffinization methods was compared in both RNA and DNA fractions. Significantly greater-quality DNA was detected in the DNA fractions than the RNA fractions, and the increase was independent of deparaffinization method. (C) The relative amount of amplifiable RNA based on crosslink reversal time was compared. The longer crosslink reversal time yielded significantly greater amount of amplifiable RNA than the shorter time.

Maxwell instrument could not be obtained for these experiments. Nevertheless, the chemistry for extraction and crosslink reversal is identical between the ReliaPrep and Maxwell protocols, and these steps must be performed manually regardless. The difference between the two protocols is that purification using the ReliaPrep kit is performed by binding the sample to a silica column while the Maxwell protocol uses magnetic beads. Previous experiments using the FormaPure and truXTRAC kits (data not shown) demonstrate similar nucleic acid binding performance between the two media. Nevertheless, the protocol was further investigated to establish a recommended extraction protocol for automated FFPE extraction.

ReliaPrep: Crosslink reversal time optimization

The ReliaPrep protocol recommends crosslink reversal at 80°C for 15 minutes, whereas the Promega

Maxwell CSC RNA FFPE Kit (cat # AS1360) protocol recommends 80°C for 1 hour. To determine the optimal crosslink reversal time, the ReliaPrep protocol was performed using both crosslink reversal times, and the relative amount of amplifiable RNA and DNA were measured. Each extraction type was performed in triplicate. Increasing the crosslink reversal time from 15 minutes to 1 hour resulted in an ~70% increase in the proportion of amplifiable RNA (Figure 6A). The longer incubation time also resulted in an almost 7-fold increase in the proportion of amplifiable DNA (Figure 6B).

Based on these experiments, The Promega ReliaPrep kit is recommended for FusionPlex library preparation. The crosslink reversal step should be modified to 80°C for 1 hour. It is likely that the Promega CSC RNA FFPE kit will produce similar results for users interested in automated extraction.

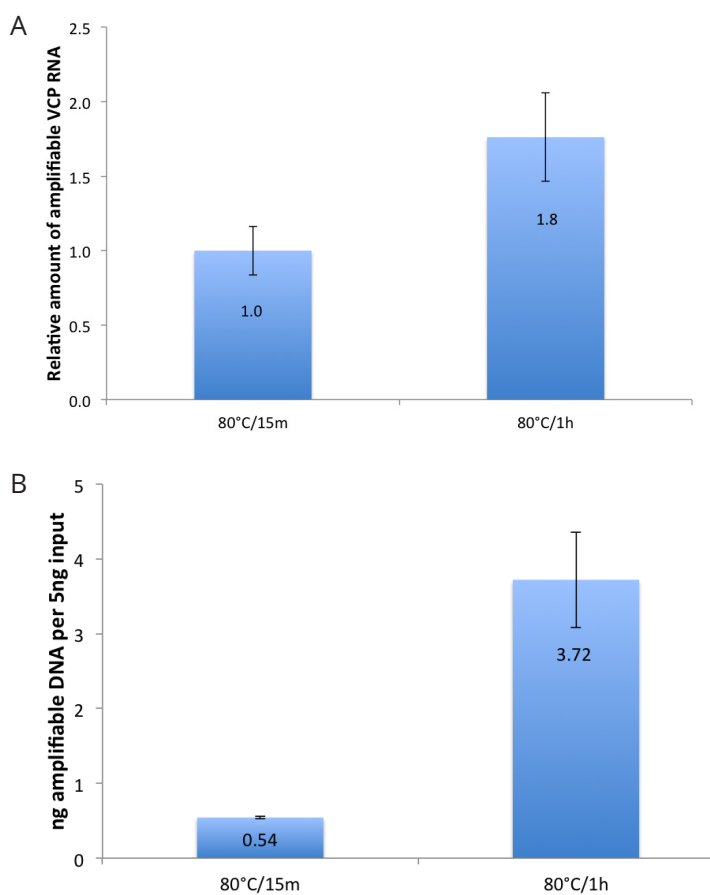


Figure 6. Increased crosslink reversal time improves amplifiable RNA and DNA yield with the Promega ReliaPrep extraction kit. Nucleic acid was extracted using the Promega ReliaPrep kit with crosslink reversal at 80°C for 15 minutes or 1 hour. The relative amount of amplifiable RNA (A) or DNA (B) based on crosslink reversal time was compared. The longer crosslink reversal time yielded more amplifiable RNA (A) and DNA (B) than the shorter time.

Zymo Research Pinpoint Slide RNA Isolation System II

A key feature of the Pinpoint kit is that it is designed to remove FFPE tissues from slides while leaving the paraffin behind or for selectively removing regions from a biopsy for extraction (e.g., a region of high tumor cellularity). This study uses DX0535 sections that are already removed from slides, so investigating the selective removal feature of this kit is beyond the scope of this study.

Pinpoint: Deparaffinization optimization

Because no significant difference between deparaffinization solvents were observed with the QIA-

GEN AllPrep kit, the Pinpoint kit was tested to determine if the same result would hold true. Sections were dissolved in 1mL xylene (Xy), 320µL mineral oil (M.O) or 320µL QIAGEN Deparaffinization Solution (D.S.) and then incubated at room temperature, at 80°C for 1 minute, or at 56°C for 3 minutes, respectively. Five separate extractions were performed to test each condition. Steps I.3 and I.4 of the Pinpoint II protocol were completed for the xylene samples; the M.O. samples were cooled and separated by centrifugation; and the D.S. samples were cooled, separated by centrifugation and dried at 37°C for 10 minutes. Section III onward of the protocol was completed per manufacturer's recommendations for all conditions with the following exceptions:

- The aqueous layers of the M.O. samples were transferred to new tubes prior to the addition of RNA Extraction Buffer
- All samples were eluted in 15µL water

The three deparaffinization methods showed only small differences in the amount of amplifiable RNA (Figure 7A).

A note on user experience:

While the kit was fast and simple, the columns supplied with the kit often experienced wash or elution solution retention, resulting in 2 of 14 tubes yielding ~2x the expected eluate volume and another two yielding ~0.5x the expected eluate volume. In addition, raw RNA and DNA yields were variable, and some samples saw low raw RNA yields (<5ng/µL). However, low raw yield was not experienced for Experiment 2.

Pinpoint: Crosslink reversal optimization

The Pinpoint protocol suggests incubation at 55°C for 4 hours after proteinase K is added to the solution, which acts as a combination proteinase K digestion/crosslink reversal step. Because the FormaPure experiments demonstrated improved yields with crosslink reversal at 80°C for 1 hour or 55°C for 16 hours, three variations of the Pinpoint protocol were tested after deparaffinization with mineral oil as described in the previous section:

- 55°C/4h proteinase K digestion (as per manufacturer's protocol)
- 55°C/16h proteinase K digestion
- 55°C/1h proteinase K digestion + 80°C/1h crosslink reversal

Five separate extractions were performed to test each condition, and all samples were eluted in 20µL water to ensure sufficient material for quantification and library preparation

No significant change in RNA quality was observed between the 55°C/4h and 55°C/16h conditions; but by reducing the proteinase K digestion time to 1 hour at 55°C and performing an additional crosslink reversal step at 80°C for 1 hour, the proportion of amplifiable RNA obtained with this kit was increased 1.8 fold (Figure 7B). The RNA obtained with the modified protocol was among the highest quality RNA recovered from the FFPE extraction kits tested in this study (Figure 10A). Furthermore, more than twice as much amplifiable DNA was recovered with the 55°C/1h + 80°C/1h method than the other two conditions tested (Figure 7C).

Based on these experiments, the Zymo Research Pinpoint kit is recommended for FusionPlex library preparation. The protocol should be modified by reducing proteinase K digestion at 55° to 1 hour and adding a crosslink reversal incubation at 80°C for 1 hour. Purified nucleic acid should also be eluted with 20µL water to ensure sufficient sample is available for quantification and library preparation.

Ambion RecoverAll Total Nucleic Acid Isolation Kit for FFPE

The RecoverAll kit is designed to isolate both RNA and DNA from the same sample with sequential RNA and DNA steps similar to the QIAGEN AllPrep kit.

RecoverAll: Deparaffinization optimization and crosslink reversal optimization

The RecoverAll protocol recommends deparaffinization with xylene, so mineral oil was tested as an alternative deparaffinization solvent. Samples were treated with xylene according to the manufacturer's protocol. For the other samples, 300µL mineral oil was added to the section, vortexed, and incubated at 80°C for 1 minute. Then 100µL digestion buffer was added to these latter samples, and after mixing, the aqueous layer was collected before continuing with the protocol.

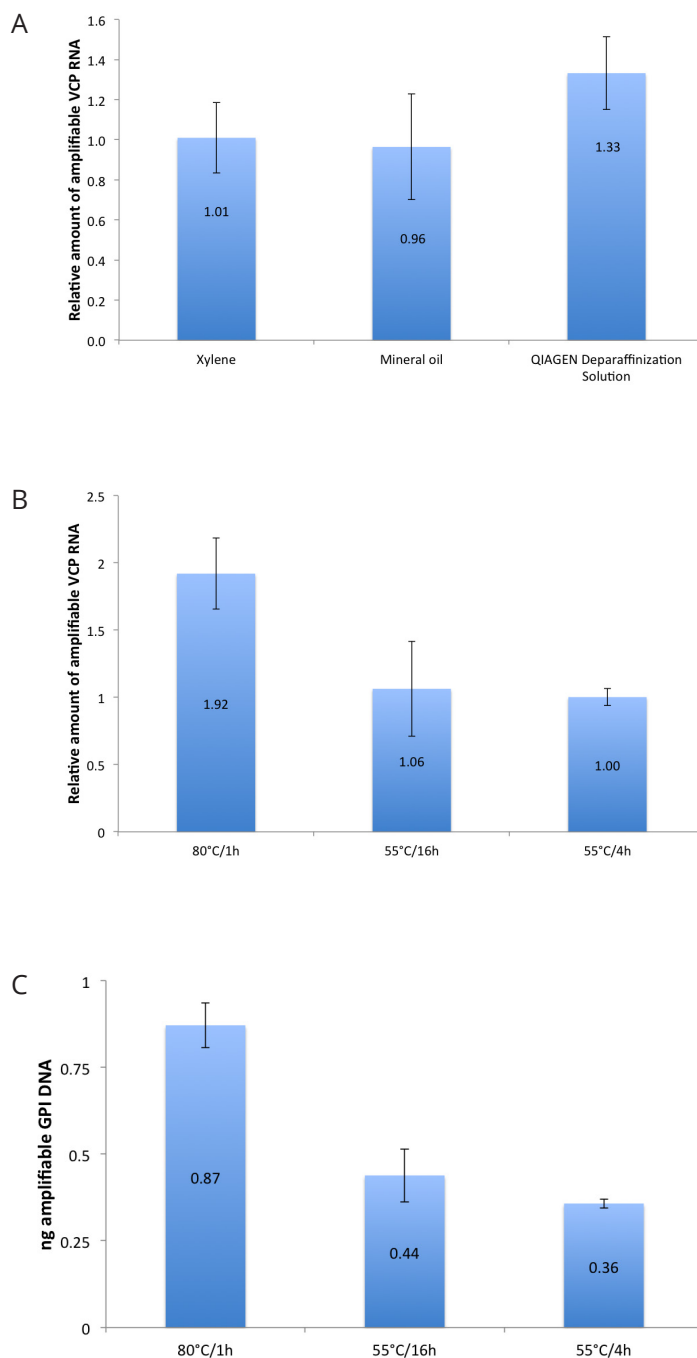


Figure 7. RNA and DNA yields using the Pinpoint kit are improved with increased crosslink reversal temperature. Nucleic acid extraction was performed using the Pinpoint kit and varying extraction conditions. (A) The relative amount of amplifiable RNA based on deparaffinization solvents was compared. No significant difference was observed between xylene, mineral oil and the QIAGEN Deparaffinization Solution. (B and C) The relative amount of amplifiable RNA (B) or DNA (C) based on digestion/crosslink reversal conditions was compared, with mineral oil used for deparaffinization. A significant increase in both RNA (B) and DNA (C) was observed using the 55°C/1h proteinase K digestion + 80°C/1h crosslink reversal method compared to 55°C/4h or 55°C/16h methods.

The RecoverAll protocol suggests proteinase K/crosslink reversal at 65°C for 4 hours or 50°C for 16 hours, both followed by 80°C for 15 minutes. Because crosslink reversal at 80°C/1h consistently outperformed 55°C/15h and 80°C/15m in other experiments in this study, the following crosslink reversal incubations were tested:

- 80°C/1h
- 50°C/16h

Deparaffinization conditions were incorporated into

the crosslink reversal condition experiments. Five separate extractions were performed for each condition.

Consistent with other kit modifications, a 80°C crosslink reversal step yielded significantly greater amplifiable RNA compared to the 50°C/16h method (Figure 8A). This conditional increase was also observed with DNA, although the magnitude increased differed between RNA and DNA (Figure 8B). As with the QIAGEN and Zymo Research kits, the quality and yield changes occurred independent of deparaffinization methods tested. The RecoverAll kit resulted in poor library complexity compared to other kits in this study (Figure 10A).

Based on these experiments, the Ambion RecoverAll kit is not recommended for FusionPlex library preparation. The high quality of DNA obtained from this kit suggests it could be ideal for extraction prior to VariantPlex library prep, but this was not tested.

Ambion TRIzol Reagent

FFPE extraction using TRIzol reagent was recommended to ArcherDx by a customer to yield high-quality nucleic acid, and so TRIzol was tested as a viable extraction method prior to library preparation.

TRIzol: Deparaffinization optimization

The TRIzol protocol begins with deparaffinization using Protocol™ SafeClear II Xylene Substitute, after which the sample is dried with ethanol and digested with proteinase K at 55°C for 15h (which doubles as the crosslink reversal step). The nucleic acids are then purified by TRIzol extraction and isopropanol precipitation. Because the xylene substitute could not be obtained in volumes small enough to minimize environmental health impact, the deparaffinization protocol was modified using xylene or mineral oil.

TRIzol extraction was predicted to result a relatively low RNA yield, because ~40% of the product is lost during the extraction in order to avoid disturbing the aqueous/organic interface. Therefore, the amount of input material was increased (3 DX0535 sections for each extraction rather than 1 section) to compensate for this loss.

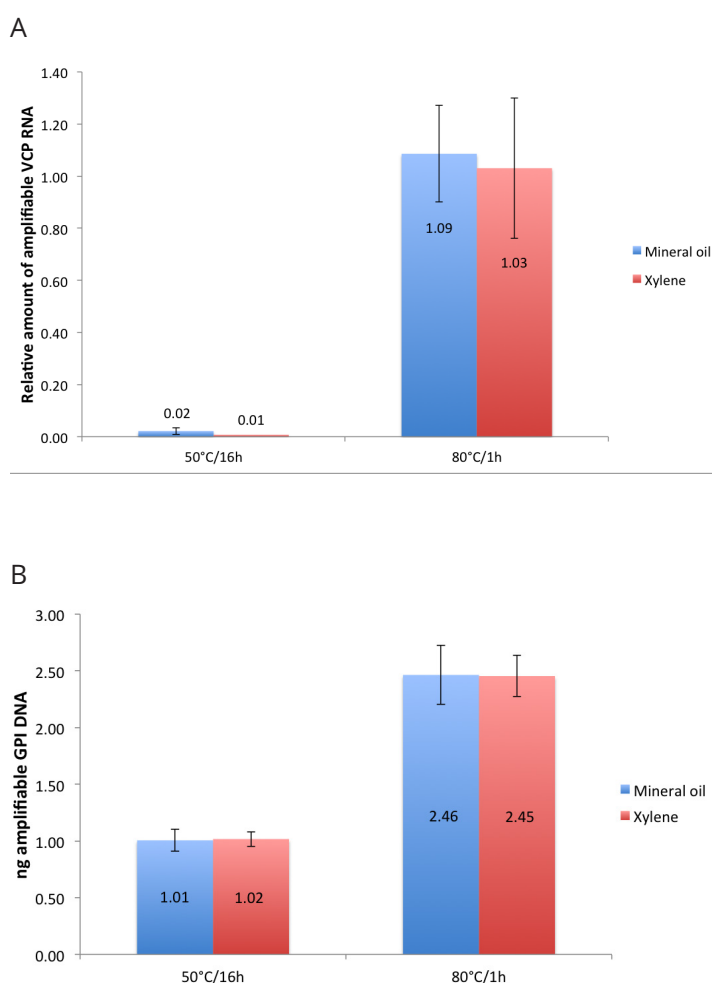


Figure 8. Increasing crosslink reversal time substantially improves performance of the RecoverAll kit. Nucleic acid was extracted using the RecoverAll kit with varying deparaffinization and crosslink reversal conditions, and the relative amount of amplifiable RNA (A) or DNA (B) based on these conditions was compared. A significant increase in both RNA (A) and DNA (B) was observed using the 80°C/1h crosslink reversal method compared to the 50°C/16h method independent of deparaffinization solvent (xylene vs. mineral oil).

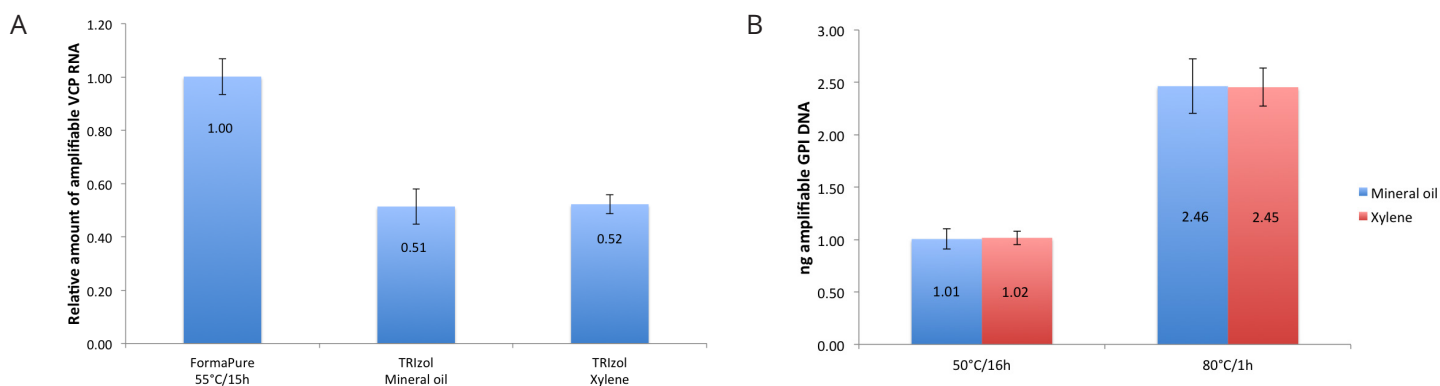


Figure 9. TRIzol can be used to isolate RNA from FFPE samples, but the recovery is reduced compared to the standard FormaPure extraction protocol. Nucleic acid was extracted using TRIzol with varying deparaffinization and crosslink reversal conditions, and the relative amount of amplifiable RNA (A) or DNA (B) based on these conditions was compared. TRIzol extraction resulted in significantly less RNA (A) and DNA (B) compared to FormaPure extraction. The results occurred independent of deparaffinization solvent (xylene vs. mineral oil).

For the mineral oil extraction, 300µL mineral oil was added to the sample and the sample was incubated at 80°C for 1 minute. Then 230µL digestion buffer (20mM Tris pH 7.5, 20mM EDTA, 1% SDS) and 20µL proteinase K (20mg/µL) was added to the mixture once it cooled. After the proteinase K digestion, the aqueous layer was transferred to a new tube and TRIzol extraction was performed according to the manufacturer's protocol. As a control, FormaPure extractions using three DX0535 sections per extraction and a 55°C/16h proteinase K digestion and crosslink reversal step were performed. All extraction conditions were tested in triplicate.

As expected, the TRIzol extractions yielded substantially less amplifiable RNA than the FormaPure extractions independent of deparaffinization condition used (Figure 9A). The proportion of amplifiable DNA in the TRIzol extractions was also considerably less than that in the FormaPure extractions (Figure 9B). This last result is not surprising given that the DNA fraction is expected to reside in the interphase of the TRIzol extraction. If the user is not averse to recovering DNA in their extractions, then the entire aqueous phase could possibly be recovered and thus much more DNA could be recovered using the DNA recovery protocol.

Based on these experiments, TRIzol is not recommended for extraction prior to FusionPlex or VariantPlex library preparation due to the relatively low quality nucleic acid obtained compared to other extraction methods.

Library complexity based on the extraction kits tested

To determine which kits produce nucleic acid suitable for FusionPlex library preparation, libraries were prepared using the FusionPlex Solid Tumor Panel and Universal RNA Reagent Kit v2 with 50ng input nucleic acid extracted using each kit. The random priming and first- and second-strand synthesis steps were not necessarily performed at the same time or with the same lots of reagents for all of these libraries. However, the remaining preparation steps were performed in parallel with the same lots of reagents. The extractions were performed according to the manufacturer's instructions except as indicated below:

- Agencourt FormaPure Kit: 55°C/16h crosslink reversal (6 replicates)
- Agencourt FormaPure Kit: 80°C/1h crosslink reversal (6 replicates)
- Ambion RecoverAll Kit: 80°C/1h crosslink reversal (5 replicates)
- Zymo Research Pinpoint Kit: 80°C/1h crosslink reversal (5 replicates)
- Promega ReliaPrep Kit: 80°C/1h crosslink reversal (3 replicates)
- QIAGEN AllPrep Kit: 80°C/1h crosslink reversal (3 replicates)
- Covaris truXTRAC Kit: 80°C/1h crosslink reversal (2 replicates)
- Universal RNA (Clontech, cat# 636690) as a control (3 replicates)

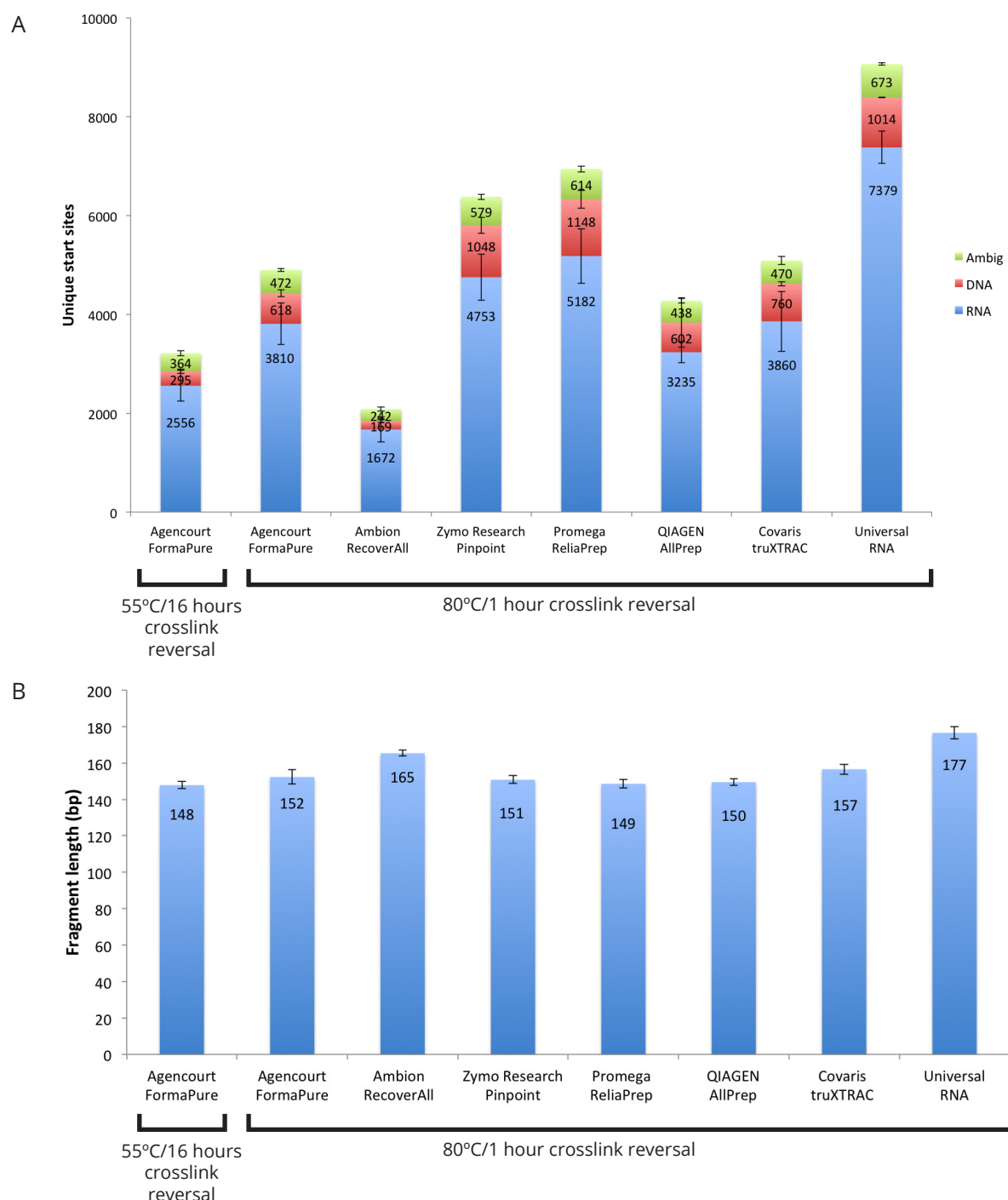


Figure 10. Commercially available FFPE extraction kits result in variable library complexity. Libraries were prepared from RNA extracted from each of the indicated extraction kits (with modified protocols). Library complexity was measured as a function of unique start sites (A) and mean unique fragment length (B).

Libraries were then sequenced on an Illumina NextSeq 500 system, and the resulting FASTQ files were subsampled to a depth of 1.19M reads.

Figure 10A shows the average number of unique start sites of each type (RNA, DNA and ambiguous) obtained from each library. The Agencourt FormaPure 80°C/1h approach yielded more complex li-

braries (as measured by the number of unique start sites per library) than the 55°C/16h approach. The Zymo Research Pinpoint and Promega ReliaPrep methods yielded more complex libraries than the FormaPure 80°C/1h protocol. The Ambion RecoverAll method resulted in libraries with significantly less complexity than any other extraction method. Both the QIAGEN AllPrep and Covaris truXTRAC ex-

traction methods both resulted in library complexity that was not significantly different from that attained using the FormaPure 80°C/1h approach.

All of the extraction methods generated libraries with similar average fragment lengths (Figure 10B). The mean fragment length using the RecoverAll method was significantly higher than that from all of the other extraction methods, but the difference was minimal (10-15bp).

Conclusions

Multiple extraction kits are compatible with the FusionPlex library preparation workflow

The results of this study demonstrate that high-complexity FusionPlex libraries can be produced using six different RNA extraction methods from FFPE material. The quality of RNA obtained from these kits (as measured by the number of amplifiable molecules or unique RNA start sites) varied between kits and methods, but all of them produced RNA compatible with our library preparation chemistry.

Crosslink reversal temperature and time impacts extraction yield

Increasing the crosslink reversal incubation time or the temperature increases the amount of amplifiable RNA and DNA molecules. For every RNA extraction kit or method that was tested, the yield of useful RNA and DNA molecules (when applicable) was increased when an 80°C/1h incubation step was performed after proteinase K digestion. This step not only increased the yield of useful RNA and DNA molecules per mass but also saved considerable time in extraction procedures without any measurable loss in sample quality or fragment length.

Mineral oil is an effective and safe deparaffinization solvent substitute

There was no significant difference in amplifiable RNA yield or DNA yield (when applicable) between methods when deparaffinization was performed using mineral oil compared to a hazardous solvent. Previous reports demonstrate that amplifiable RNA and DNA yields are strongly correlative to library complexity (reference RNA and DNA input QC white papers). So it stands to reason that substituting mineral oil in protocols that use xylene or other hazardous organics will generate libraries of equal complexity and compatibility with the FusionPlex or VariantPlex workflow.

For more information, visit <http://archerdx.com/support/faqs>

Limitations of Use:

For Research Use Only. Not for use in diagnostic procedures.

©2017 ArcherDX, Inc. All rights reserved. Archer®, FusionPlex®, VariantPlex®, PreSeq® and AMP™ are trademarks of ArcherDX, Inc. RecoverAll™ and Protocol™ are trademarks, and TRIzol®, Invitrogen®, Qubit®, and Ambion® are registered trademarks of Thermo Fisher Scientific, Inc. Agencourt® and FormaPure® are registered trademarks of Beckman Coulter, Inc. truXTRACT™ is a trademark and Covaris® is a registered trademark of Covaris, Inc. QIAGEN® and AllPrep® are registered trademarks of QIAGEN, Inc. ReliaPrep™ is a trademark and Maxwell® is a registered trademark of Promega, Inc. Pinpoint™ is a trademark of Zymo Research, Inc. Biochain® is a registered trademark of BioChain Institute, Inc. ClonTech® is a registered trademark of the Takara Bio company. Illumina® and NextSeq® are registered trademarks of Illumina, Inc. Biomatrix® and RNastable® are registered trademarks of Biomatrix.