3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

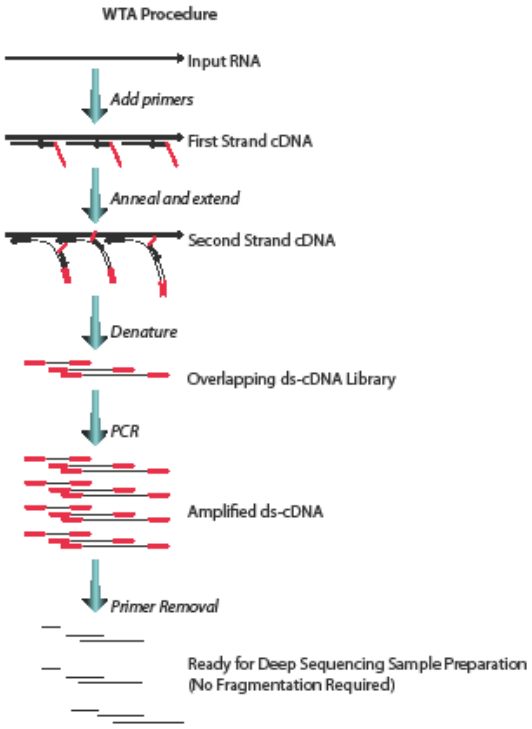
SeqPlex™ RNA Amplification Kit

Catalog Number **SEQR**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

The SeqPlex™ RNA Amplification kit provides a method for amplification of total RNA or isolated mRNA prior to entry into the workflows of the commonly used deep sequencing platforms. Microgram quantities of double-stranded cDNA are generated from low nanogram to picogram quantities of total RNA in eight hours or less. The SeqPlex RNA Amplification Kit amplifies RNA (including non-polyA-tailed RNA) isolated from tissue, cultured cells, formalin-fixed samples, or serum while maintaining patterns of differential expression found in the unamplified sample.



Preparation for sequencing involves three steps. First, sample RNA is reverse transcribed with primers having a semi-degenerate 3′-end and a defined universal 5′-end. As DNA polymerization proceeds, displaced single strands serve as new templates for primer annealing and extension. Next, the resultant double-stranded cDNA library, composed of random, overlapping fragments flanked by a single universal primer sequence is amplified under optimized PCR conditions to produce a WTA-product (whole transcriptome amplification). With nanogram quantities of intact input RNA, the bulk of the amplification product ranges in size from 200–400 base pairs. A size range of 150–250 base pairs is seen for damaged input RNA and picogram quantities of high quality RNA. Finally, integration of SeqPlex-amplified RNA with deep sequencing workflows requires the elimination of the primer sequences from each amplicon. An efficient primer removal step accomplishes this prior to sequencing sample preparation.

In addition to deep sequencing, SeqPlex-amplification product is suitable as microarray target or qPCR template, with or without primer removal.

SeqPlex RNA Amplification Kit Components

Description	Tube Label	10 RXN	50 RXN	500 RXN
Library Synthesis Solution	L8670	25 µL	125 µL	1.25 mL
Library Synthesis Buffer	L9418	25 µL	125 µL	1.25 mL
Library Synthesis Enzyme	L9543	20 µL	100 µL	1 mL
Nuclease-Free Water	W4502	5 mL	20 mL	20 ml & 100 mL
5× Amplification Mix	A5112	210 µL	1.05 mL	10.5 mL
Amplification Enzyme	A5237	10.5 µL	52.5 µL	525 µL
Primer Removal Solution	SR400	30 µL	150 µL	1.5 mL
10X Primer Removal Buffer	SR401	150 µL	750 µL	7.5 mL
Primer Removal Enzyme	SR402	37.5 µL	188 µL	1.88 mL

Materials and Reagents Required but Not Provided

- RNA to be amplified
- Thermocycler (preferably, "real-time") and tubes
- Vortex Mixer
- Microcentrifuge and microcentrifuge tubes
- Spectrophotometer or NanoDrop™
- Dedicated pipettes and PCR pipette tips
- Agilent Bioanalyzer
- 0.2-mL or 0.5-mL thin-walled PCR tubes or PCR multiwell plate
- Molecular Grade Water (Catalog No. W4502)
- DNase I, Amplification Grade (Catalog No. AMPD1)
- GenElute™ PCR Clean-up Kit (Catalog No. NA1020)
- SYBR® Green (Catalog No. S9430)
- SYBR Green Jump Start Taq ReadyMix (Catalog No. S4438)
- 1 M Tris-HCl, pH 8.0 (Catalog No. T3038)
- Sequencing Platform Sample Prep Reagents

Precautions and Disclaimer

This product is for research use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

All components should be stored at –20 °C. When thawed for use, components should be kept on ice.

RNA Handling Technique

The reagents in this kit have been tested to assure that RNases are absent. The user, however, must protect the integrity of experimental results by wearing basic protective equipment, including gloved hands and lab coat. All reagent transfers throughout this procedure should be performed in a laminar flow hood or dedicated clean room. Frozen RNA samples should be thawed on ice.

CAUTION:
Several components found in the Sigma Aldrich SeqPlex RNA Amplification kit (SEQR), SeqPlex DNA Amplification kit (SEQX), TransPlex® WTA kits, Complete Whole Transcriptome Amplification Kit (WTA2) and GenomePlex® WGA kits are similarly named. Though generally analogous in function, **they are not interchangeable**. Also, for users familiar with the WTA2 kit, please be aware that

- The Amplification Mix for the SeqPlex RNA Amplification kit is a 5× solution.
- The PCR amplification settings include final 30-minute 70 °C incubation.

Considerations for Downstream Sequencing

The SeqPlex RNA amplification kit provides for highly efficient removal of primer sequence following amplification. These primer sequences constitute 30–50% of amplification product mass and are removed by purification. Consequently, the quantity of amplification product used for input for the primer-removal reaction should be 2-fold of that required for sequencing.

The amount of nucleic acid required for sequencing is platform-specific, with Roche 454 minimally calling for ~1 µg of input nucleic acid. Illumina MiSeq and HiSeq platforms require as little as 50 ng, as does ABI SoLiD. Therefore, the procedure provided here is based on a final primer-removal reaction yield of 1 µg.

To be sure that you generate a sufficient quantity of amplified RNA, consult your sequencing service provider and scale the primer-removal reaction accordingly.

Procedure

An amplification reaction will produce 2–4 μg of amplified double-stranded cDNA when starting with 100 pg to 5 ng of high quality total RNA (RIN ≥8.0). Higher input quantities and higher quality of RNA template generally result in increased yields. For damaged RNA, such as RNA isolated from FFPE (formalin-fixed paraffin embedded) samples, 1–50 ng input RNA is recommended. Reaction volumes can be scaled up or down to accommodate preparation of desired quantities of final product.

Genomic DNA must be removed from the RNA sample prior to amplification. If this was not accomplished during RNA isolation, use Catalog No. AMPD-1 RNase-free DNase per kit instructions and the most convenient library synthesis scale.

The SeqPlex RNA Amplification Kit will amplify ribosomal RNA, though less efficiently than messenger RNA. Ribosomal RNA depletion should be employed when required and feasible.

Library synthesis having a final recommended reaction volume of 5 μL is described below. However, a volume range of 2.5 through 25 μL (while maintaining an amplification reaction volume of 75 μL) has been shown to have no effect on amplification as detected by Ct values during early exponential amplification, amplification product yields, or β-actin transcript levels (see "**Quality Control**, Product Retention"). Be sure to use the same library synthesis volume for all samples to be compared. Sufficient reagent has been supplied for the number of indicated kit reactions, at a library synthesis volume of 25 μL. **Optional set-up instructions for increased reaction scale are provided following the recommended procedure.**

Library Synthesis

1. Thaw the Library Synthesis Buffer, Library Synthesis Solution, and water. Mix thoroughly by inversion or brief vortexing. Dissolve any precipitate in the Library Synthesis Solution (L8670) by briefly heating at 37 °C, followed by thorough mixing. Keep on ice.

2. Combine 100 pg to 5 ng of high quality intact total RNA or 1–50 ng damaged RNA (e.g., FFPE or laser capture sample RNA) with Library Synthesis Solution at the following single-reaction scale:

   0.5 μL Library Synthesis Solution (L8670)
   Add Nuclease-free water (W4502) to a total volume of 3.3 μL.

3. Mix by pipeting and incubate reactions in a thermocycler programmed for 70 °C for 5 minutes, then an 18 °C hold. Do not hold at 18 °C for more than 10 minutes. Remove reactions from thermocycler, and place at room temperature or maintain at 18 °C for the next steps, but do not place on ice.
   Note: To avoid RNA renaturation and possible degradation, perform steps 4 and 5, and setup for step 6 as rapidly as possible at 18 °C or room temperature.

4. Prepare the following premix during the incubation in the previous step and immediately combine 1.7 μL of following premix with denatured RNA in Library Synthesis Solution (step 3):

   0.5 μL Library Synthesis Buffer (L9418)
   0.8 μL Water
   0.4 μL Library Synthesis Enzyme (L9543)

5. Mix by pipeting and spin down residue from the sides of reaction tubes.

6. Incubate in a thermocycler using the following conditions:
   18 °C for 10 minutes
   25 °C for 10 minutes
   37 °C for 30 minutes
   42 °C for 10 minutes
   70 °C for 20 minutes
   4 °C hold

7. Spin down any condensation by centrifugation. Samples may be amplified immediately or stored at –20 °C for up to one month.

Amplification

8. Thaw the 5× Amplification Mix. Dissolve any precipitate by briefly heating at 37 °C, followed by thorough mixing. Keep on ice.

9. Transfer the following regents to the library synthesis reaction, using the following single-reaction scale*:
   53.50 μL Nuclease-free water
   15.00 μL 5X Amplification Mix (A5112)
   0.75 μL 1:1000 SYBR Green* in 10 mM Tris-HCl, pH 8.0 (Catalog No. T3038)
   0.75 μL Amplification Enzyme (A5237)
   75.00 μL Total reaction volume

* Addition of SYBR Green, Catalog No. S9430, not included in the kit, is optional, but strongly recommended for monitoring amplification. Prepare dilution and add immediately to the mix. Discard dilution after each experiment.

10. Mix thoroughly by pipeting or brief vortexing. Spin down residue from top and sides of reaction tubes.

11. Proceed with PCR using the following thermocycler program (real-time qPCR is strongly recommended):
    1 cycle
        94 °C for 2 minutes.
    17-19 cycles*
        94 °C for 30 seconds
        70 °C for 5 minutes (read)
    1 cycle
        70 °C for 30 minutes
    Note: The final 70 °C incubation for 30 minutes is critical for primer removal and downstream sequencing application.

    * – The optimal number of amplification cycles varies with RNA input quantity and quality. Optimal amplification is achieved by proceeding 2–3 cycles into the amplification "plateau", as observed with real-time quantitative PCR. Typically, ~19 cycles are required for 1–5 ng of high quality RNA or 10–50 ng of FFPE RNA. In this case, if amplifying without real-time monitoring, performing multiple reactions, or otherwise unable to practically monitor individual amplification reactions, set the number of amplification cycles at 20. RNA of lower quality or quantity may require higher input quantities and/or more amplification cycles. If amplifying less than 1 ng of RNA (RIN ≥8.0) or less than 10 ng of damaged RNA (RIN ≤8.0) without real-time monitoring, performing multiple reactions, or otherwise unable to practically monitor individual amplification reactions, set the number of amplification cycles at 25. **For best results, monitor amplification with Sybr Green.**

12. After cycling is complete, maintain the reactions at 4 °C or store at –20 °C until ready for purification.

13. To remove residual primers and nucleotides, purify with the GenElute PCR Cleanup Kit (Catalog No. NA1020) as described in the kit instructions. Elute with 50 µL nuclease-free water, not kit elution buffer. Eluate can be concentrated by vacuum centrifugation if necessary, but avoid heating and do not allow the sample to go to dryness. (Because the amplification product is in water alone, without counter-ions present, the sample will denature upon dryness. Denaturation will inhibit primer removal.) The capacity of the GenElute purification column is 10 µg, adequate for purification of a typical amplification reaction.

14. Purified DNA is quantified by measuring absorbance. One A_{260} unit is equivalent to 50 ng/µL DNA.

Primer Removal

Primer removal results in little or no loss of amplification product. However, a 75-µL primer-removal reaction input of 2 µg of amplified product is recommended to yield 1 µg of final product for entering the deep sequencing workflow. (see "Considerations for Downstream Sequencing") This allows for loss due to primer removal plus any additional loss during reaction cleanup. A "no-enzyme" control reaction is also performed for each amplified sample. The control reaction is required to test for primer sequence removal (see **Quality Control** below).

Notes: The amplification product cannot be further amplified after primer removal.

Sufficient reagent has been included in the kit for a 75-µL "no-primer-removal-enzyme" control reaction. However, you may wish to reduce the scale of your "no enzyme control" to save reagent and amplified product, as shown below.

A reaction setup described in step 15 is a mix that accounts for both the primer removal reaction and (steps 16 and 17) the no- enzyme control.

15. Combine and mix the following reagents:

    8.50 µL - 10x Primer Removal Buffer (SR401)
    1.70 µL - Primer Removal Solution (SR400)
    X µL – **2.27** µg of purified SeqPlex-amplification product (**step 14**)
    Y µL - Water (W4502)
    80.75 µL Total reaction volume

16. Transfer 9.5 µL of the mix in **step 15** to a different reaction tube and add 0.5 µL water (W4502). This is the "no-enzyme" reaction. Mix thoroughly by pipeting. Spin down residue from top and sides of reaction tubes by brief centrifugation.

17. To the remaining 71.25 µL, add 3.75 µL Primer Removal Enzyme (SR402). This is the primer removal reaction. Mix thoroughly by pipeting. Spin down residue from top and sides of reaction tubes by brief centrifugation.

18. Incubate both primer removal and no-enzyme control reactions as follows:
    37 °C for 60 minutes
    65 °C for 20 minutes
    4 °C hold

19. Remove samples from thermocycler and centrifuge briefly.

20. Reserve 2 µL each of the primer removal reaction, and the entire no-enzyme control reaction for the **Quality Control** assays below. Keep on ice or store at –20 °C for up to one month.

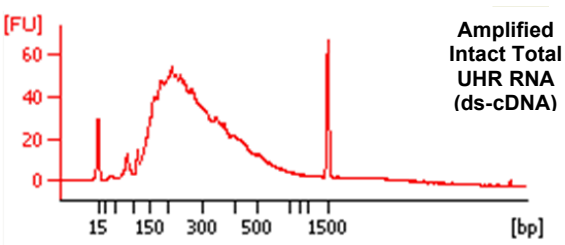
21. Purify the remaining primer removal reaction products using the GenElute PCR Clean-up Kit as described previously in **step 13**, or store unpurified samples at –20 °C for up to one month.

22. Primer removal reaction product yield and concentration is quantified by measuring absorbance. One A_{260} unit is equivalent to 50 ng/µL DNA.

Quality Control

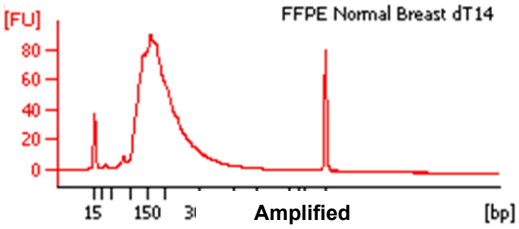
Amplification Product Size

Agilent Bioanalysis of amplified intact total RNA will show a smear, the bulk of which will range in size from 200–400 base pairs. Similar analysis of amplified damaged RNA or a low quantity input will range from 150–250 base pairs.

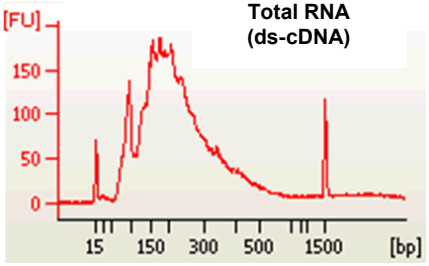


Amplified Intact Total UHR RNA (ds-cDNA)

Amplified FFPE Human Breast Total RNA (ds-cDNA)



Amplified HEK293 30-Cell Total RNA (ds-cDNA)



Primer Removal

The efficiency of primer removal can be estimated by qPCR using the 5× Amplification Mix and Amplification Enzyme. Use unpurified primer removal reaction and corresponding control reaction for these assays (**step 20**). Sufficient amplification mix and enzyme are provided in the kit for a 15-µL qPCR test reaction for both the primer removal reaction and "no-primer-removal-enzyme" control reaction. A 1/1,000,000 dilution of each primer-removal and control reaction is used for this assay. Expect primer removal to be greater than 90%.

For the Primer Removal QC, combine reagents at following scale per single qPCR reaction:

3 µL 5× Amplification Mix (A5112)
0.15 µL Amplification Enzyme (5237)
1.85 µL 1/10,000 dilution, SYBR Green in 10 mM Tris-HCl, pH 8.0 (Catalog No. T3038)
10 µL 1/1,000,000 dilution cDNA (from primer removal reaction or no enzyme control)

Amplification conditions:

1 cycle
    94 °C, 2.5 minutes
40 cycles
    94 °C for 30 seconds
    70 °C for 5 minutes (read)

Expect a ΔCt of 3–7 as an estimate of successful primer removal:

$$(Ct)_{\text{no primer removal enzyme}} - (Ct)_{\text{plus primer removal enzyme}}$$

Notes: Individual Primer Removal QC test reactions can be performed when necessary, as well as multiple reactions where a reagent mix is typically prepared (recommended). To pipet the small quantities indicated, use a pipette having the appropriate volume range and use siliconized or low-retention pipette tips. Prior to dispensing reagents, be sure any condensation that may have occurred during storage is spun down by centrifugation and appropriately mixed: by vortexing, pipeting or inversion. It has been demonstrated that the estimated percent primer removal determined with this assay correlates well with sequencing results.

Amplification Product Retention (Optional)
To demonstrate retention of amplification product during primer removal, an additional qPCR reaction(s) is performed using a primer pair(s), which encodes for a transcript(s) known to be expressed in the respective source sample. Include the no-enzyme control reaction in your experimental setup (see Primer Removal QC).

(For human RNA samples where β-actin is known to be expressed, the following primers can be used.)

5'-CGGGACCTGACTGACTACCTC-3'
5'-GAAGGAAGGCTGGAAGAGTGC-3'

1. **Do not** design primer pairs
   a. Where one of the primer pair aligns with any portion of the 3'-end of the upper strand of the double-stranded sequence shown immediately below.

   b. Or, where the primer pair amplifies a double-stranded DNA fragment that contains the following internal sequence:

2. Prepare 100 μM primer stock solutions.

3. Prepare a mix comprising a selected primer pair and 2× SYBR Green Jumpstart Taq ReadyMix (Catalog No. S4438).
   a. For 10 or more reactions:
      i. Determine the total quantity of 2× SYBR Green Jumpstart Taq ReadyMix required (10 μL per test reaction), equal to *x*.
      ii. Divide *x* by 200, equal to *y*.
      iii. Add *y* μL of each of the two primers directly to x μL 2× SYBR Green Jumpstart Taq and mix thoroughly by pipeting or vortexing.
      iv. Combine
         10 μL 2× SYBR Green Jumpstart Taq ReadyMix + primers
         10 μL 1/10,000 dilution of primer-removal reaction or control
   b. Alternatively, **for less than 10 reactions**:
      i. Divide *x* by 20, equal to *z*.
      ii. Add *z* μL of each of the two primers, diluted to 10 μM, directly to x μL 2X SYBR Green Jumpstart Taq and mix thoroughly by pipeting or vortexing.
      iii. Setup qPCR reactions as follows:
         Combine
            11 μL 2X SYBR Green Jumpstart Taq ReadyMix + primers
            9 μL 1/10,000 dilution of primer-removal reaction product or control

4. Perform qPCR using the following amplification conditions:

   1 cycle
      94 °C, 2.5 minutes
   40 cycles:
      94 °C, 30 seconds
      __ °C, 30 seconds*
      72 °C, 30 seconds (read)
   Melt Curve, 55–95 °C, 1 °C per second, reading every second.

* TM is primer specific.
Expect to see the Ct value for your "+primer removal enzyme reaction" to be equivalent to your "–primer removal enzyme reaction" result, indicating full retention of amplification reaction product following primer-removal. However, expect to see a 30–50% mass loss by spectrophotometric detection due to primer removal.

Deep Sequencing
Samples are now ready to enter the deep sequencing work flow. The bulk of SeqPlex amplification product ranges in size from 200–400 base pairs. Though typically unnecessary, additional fragmentation can be accomplished using sonication, a DNA sheering instrument (e.g., Covaris), or enzymatic fragmentation.

The terminus of each amplicon possesses a 5'-phosphate and 2-base 3'-over-hang. Prior to ligation of sequencing primers, polish double-stranded fragment ends with T4 polymerase. Ligation products comprising sequence chimeras have been reported for Roche 454 library preparation. 3'-adenylation in the Illumina workflow largely prevents this from occurring. A second sizing step prior to sequencing also helps to reduce the incidence of chimeras in the case of ABI SoLID sequencing.

Amplification Scale-Up (Optional)
The 5-µL volume scale for "Library Synthesis", previously outlined, is recommended. For RNA sample volumes that cannot be adapted to this scale, the following reaction set-up instructions are provided.

Note: Input quantity is sometimes unknown, for example, when RNA is isolated from a small number of cells. Keep in mind that the number of amplification cycles required to reach plateau will vary as the quality and/or quantity of input RNA increases (fewer cycles) or decreases (more cycles).

The following table illustrates optional reaction volumes for **Library Synthesis**, **step 2.**

Table 1.

Input RNA Range	**100 pg - 5 ng Intact RNA 1 ng – 50 ng Damaged RNA**				
Library Synthesis Solution	**0.5 µL**	1 µL	1.5 µL	2 µL	2.5 µL
QS, Nuclease-Free Water	**__ µL**	__ µL	__ µL	__ µL	__ µL
Sub-Total	**3.3 µL**	6.6 µL	9.9 µL	13.2 µL	16.5 µL

Table 2 proceeds with the reaction scale volumes established in Table 1, corresponding to **Library Synthesis, step 4**.

Table 2.

Sub-Total	**3.3 µL**	6.6 µL	9.9 µL	13.2 µL	16.5 µL
Library Synthesis Buffer	**0.5 µL**	1 µL	1.5 µL	2 µL	2.5 µL
Nuclease-Free Water	**0.8 µL**	1.6 µL	2.4 µL	3.2 µL	4 µL
Library Synthesis Enzyme	**0.4 µL**	0.8 µL	1.2 µL	1.6 µL	2 µL
Total	**5 µL**	10 µL	15 µL	20 µL	25 µL

For **Amplification, step 9**, add the entirety of the library synthesis reaction to the 75-µL amplification reaction, adjusting water volume (Table 3).

Table 3

Library Synthesis Rxn Vol.	**5 µL**	10 µL	15 µL	20 µL	25 µL
Nuclease-Free Water	**53.5 µL**	48.5 µL	43.5 µL	38.5 µL	33.5 µL
5X Amplification Mix	**15 µL**	15 µL	15 µL	15 µL	15 µL
Amplification Enzyme	**0.75 µL**	0.75 µL	0.75 µL	0.75 µL	0.75 µL
Sybr Green	**0.75 µL**	0.75 µL	0.75 µL	0.75 µL	0.75 µL
Total	**75 µL**	75 µL	75 µL	75 µL	75 µL

References

1. Cadwell, Ken, et al. Virus-Plus-Susceptibility Gene Interaction Determines Crohn's Disease Gene Atg16L1 Phenotypes in Intestine. *Cell* **141**, 1135–1145 (2010).
2. Gonzalez-Roca, et al. Eva Accurate Expression Profiling of Very Small Cell Populations. *PLoS ONE*, **5**, e14418. (2010).
3. Flynn, James M, et al. Single Cell Transcriptional Profiling of Adult Mouse Cardiomyocytes. The Journal of Visualized Experiments. http://www.jove.com/video/3302/single-cell-transcriptional-profiling-of-adult-mouse-cardiomyocytes (2011).
4. Rowley, Anne H., et al. Ultrastructural, Immunofluorescence, and RNA Evidence Support the Hypothesis of a “New” Virus Associated With Kawasaki Disease. J Infect Dis. **203**, 1021-1030 (2011).
5. Sachsenröder, Jana, et al. Simultaneous Identification of DNA and RNA Viruses Present in Pig Faeces Using Process-Controlled Deep Sequencing. PLoS ONE, **7**, 34631 (2012).

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Troubleshooting Guide

Observation	Cause	Recommended Solution
No fluorescence detected during real-time monitoring of amplification	SYBR Green is pH-sensitive.	SYBR Green, Catalog No. S9430 (not included with SeqPlex RNA Amplification kit) is not stable when diluted in water and kept at room temperature for long periods of time. Prepare the SYBR Green dilution, mixing SYBR Green in 10 mM Tris-HCl, pH 8 to improve stability. Diluted solutions should be kept on ice and protected from light.
	Incorrect qPCR conditions or programming	Check any calculations and program settings for error.
(1) No amplification product, or (2) Amplification plateau occurring at > 25 cycles, or (3) Yields < 3 μg per amplification reaction	Reaction impurities	Carefully follow GenElute RNA extraction and GenElute PCR Cleanup instructions to ensure removal of residual salts (or organics in the case of TRI Reagent®) that may interfere with downstream reactions.
	Inefficient RNA isolation method used for small samples (1-10 cell samples or laser capture)	Recommend GenElute™ Mammalian Total RNA Miniprep Kit (RTN), or Agencourt® RNAClean™ XP magnetic beads Agencourt® Part Number A63987 (see reference 5).
	Sample RNA degradation	An amplification curve may be detected at a high threshold Ct value as result of primer-dimer formation in the absence of amplifiable template or very low template levels. In the case of low RNA input or damaged RNA, yields of ≤ 3 μg per amplification reaction may be observed, also associated with high threshold Cts and yields similar to a no-RNA reaction. However, RNA having an RIN as low as 2.0 has been successfully amplified. Check amplification product size on an Agilent Bioanalyzer. Average amplification product should be larger that ~100 base pairs, though significant dimerization may occur at very low levels of input RNA. Also test the qualitative integrity of the amplification product by qPCR, confirming amplicon size by electrophoresis. Be sure to design your qPCR primers to reflect the size of the amplification product determined by Bioanalyzer analysis. Your qPCR product should not be larger than the average size of amplified RNA.
	Insufficient Input RNA	Repeat, if possible, starting with more input RNA.
	Incorrect qPCR conditions or programming	Too much Sybr Green will inhibit amplification.
		Check any calculations and program settings for error.
Primer removal, < 85%	Loss of Primer Removal Enzyme activity	If kit was recently purchased and enzyme not mishandled, request replacement of Primer Removal Enzyme or kit.
Low yield after primer removal	Excess single-stranded amplification product	Be sure to incubate at 70° C for an additional 30 minutes following amplification plateau.

Outline of Procedure

Library Synthesis

RNA Denaturation and Primer Annealing

__ µL RNA sample (100 pg - 5 ng intact RNA; 1-50ng damaged RNA)
0.5 µL Library Synthesis Solution (L8670)
__ µL Nuclease-free water (W4502)
3.3 µL

70° C for 5 minutes
18° C hold (No more than 10 minutes)

Optional Volume Scale

RNA	**___ µL**	___ µL	___ µL	___ µL	___ µL
L8670	**0.5 µL**	1 µL	1.5 µL	2 µL	2.5 µL
Water	**___ µL**	___ µL	___ µL	___ µL	___ µL
Sub-Total	**3.3 µL**	6.6 µL	9.9 µL	13.2 µL	16.5 µL

First/Second-Strand Synthesis (with Strand-Displacement)

0.5 µL Library Synthesis Buffer (L9418)

0.8 µL Water
0.4 µL Library Synthesis Enzyme (L9543)
5.0 µL

18° C for 10 minutes
25° C for 10 minutes
37° C for 30 minutes
42° C for 10 minutes
70° C for 20 minutes
4° C hold

Optional Volume Scale

Sub-Total	**3.3 µL**	6.6 µL	9.9 µL	13.2 µL	16.5 µL
L9418	**0.5 µL**	1 µL	1.5 µL	2 µL	2.5 µL
Water	**0.8 µL**	1.6 µL	2.4 µL	3.2 µL	4 µL
L9543	**0.4 µL**	0.8 µL	1.2 µL	1.6 µL	2 µL
Total	**5 µL**	10 µL	15 µL	20 µL	25 µL

Amplification

53.5 µL Nuclease-free water
15.0 µL 5X Amplification Mix (A5112)
0.75 µL Amplification Enzyme (A5237)
0.75 µL SybrGreen (1:1000 in 10 mM Tris-HCl, pH 8.0)
75.00 µL

1 cycle
94° C for 2 minutes.
17-21 cycles (2-3 cycle into plateau)
94° C for 30 seconds
70° C for 5 minutes (read)
1 cycle
70° C for 30 minutes

Optional Volume Scale

Lib. Syn. Rxn Vol.	**5 µL**	10 µL	15 µL	20 µL	25 µL
Water	**53.5 µL**	48.5 µL	43.5 µL	38.5 µL	33.5 µL
A5112	**15 µL**	15 µL	15 µL	15 µL	15 µL
A5237	**0.75 µL**	0.75 µL	0.75 µL	0.75 µL	0.75 µL
Sybr Green	**0.75 µL**	0.75 µL	0.75 µL	0.75 µL	0.75 µL
Total	**75 µL**	75 µL	75 µL	75 µL	75 µL

Amplification Reaction Purification

GenElute PCR Cleanup Kit (Sigma Catalog Number NA1020)

Primer Removal (see detailed procedure for setup of no-enzyme control)

7.5 µL 10x Primer Removal Buffer (SR401)
1.5 µL Primer Removal Solution (SR400)
3.75 µL Primer Removal Enzyme (SR402) or water (for no enzyme control)
X µL 2 µg of purified SeqPlex-amplification Product (see detailed procedure for scaling this step, up or down as needed.)
Y ___ µL Water
75 µL

37° C for 60 minutes
65° C for 20 minutes
4° C hold

Quality Control

Efficiency of Primer Removal

3.0 µL 5X Amplification Mix (A5112)
0.15 µL Amplification Enzyme (5237)
1.85 µL 1/10,000 dilution, SYBR Green in 10 mM Tris-HCl, pH 8.0 (Catalog No. T3038)
10.0 µL 1/1,000,000 dilution cDNA (from primer removal reaction or no enzyme control)
15.0 µL

1 cycle
94° C for 2 minutes.
40 cycles
94° C for 30 seconds
70° C for 5 minutes (read)

Retention of Amp Product (OPTIONAL)

10.0 µL 2X SYBR Green Jumpstart Taq ReadyMix + 0.5 µM β-actin primers
10.0 µL 1/10,000 dilution cDNA (from primer removal reaction or no enzyme control)
20.0 µL

1 cycle
94° C for 1.5 minutes.
40 cycles
94° C for 30 seconds
60° C for 30 seconds
72° C for 1.5 minutes (read)
Melt Curve, 55° - 95°

Primer Removal Reaction Purification

GenElute PCR Cleanup Kit (Sigma Catalog Number NA1020)