Product Information

SEQPLEX™-I WTA Kit

Whole Transcriptome Amplification, RNA Amplification

SeqRi
Storage Temperature −20 °C

Product Description

The SeqPlex™-I RNA Amplification Kit for whole transcriptome amplification (WTA) is designed to facilitate Illumina next generation sequencing (NGS) from extremely small quantities or degraded RNA. The SeqPlex™-I kit provides a ligation-free workflow for amplification of such samples for direct input onto Illumina next-generation sequencing (NGS) flow cells. The SeqPlex™-I process is comprised of three steps: Pre-amplification/Library Synthesis, Amplification 1 and Amplification 2. See Process Workflow chart on page 2.

First, template RNA is reverse transcribed using primers composed of a semi-degenerate 3′ and universal 5′-ends. As polymerization proceeds, displaced and RNaseH-generated single strands serve as new templates for additional primer annealing and extension producing random, overlapping cDNAs flanked by a universal primer (5′) and primer complement (3′) sequence.

Next, products from pre-amplification/library synthesis are amplified by single primer PCR via the universal end sequence. These amplification products typically range from 200 to 500+ base pairs. Amplicons from degraded RNA, such as Formalin Fixed Paraffin Embedded (FFPE), are typically shorter and dependent upon length of the starting RNA.

Last, single primer amplicons from amplification 1 are converted to dual Illumina primer PCR products ready for purification, quantification, and Illumina NGS. Amplification 1 amplicons are amplifiable with Illumina adapter-containing primers ending with 5′- …GCTCTTCCGATCT-3′ such as:

AATGATACGGCGACCACCGAGATCTACAC [i5 index]
ACACTCTTTCCCTACACGACGCTCTTCCGATCT

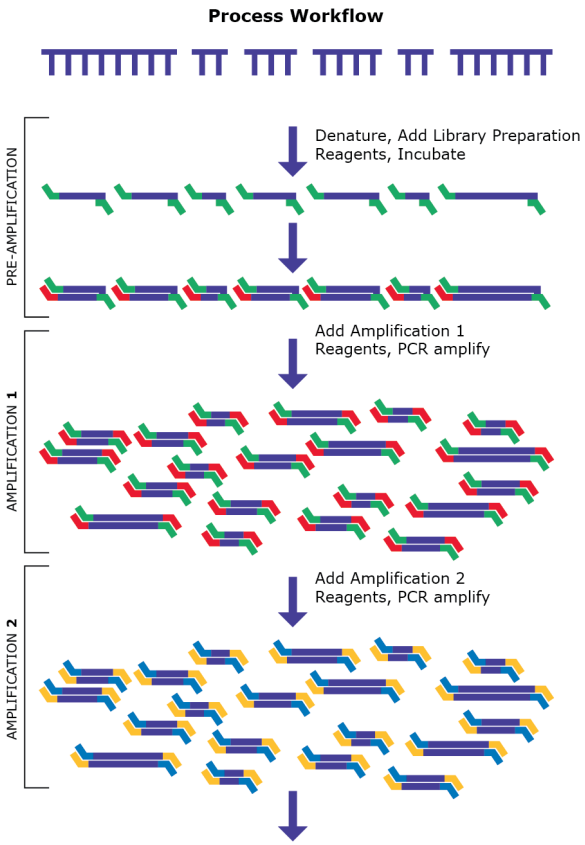
CAAGCAGAAGACGGCATACGAGAT [i7 index]
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

The example index sequences below are ready to be added to the adapter sequences as is, without reverse complement of the i7 indexes.

Examples of i5 indexes	Examples of i7 indexes
AP100 - AGCGCTAG	AP100 - CCGCGGTT
D501 – TATAGCCT	D701 – CGAGTAAT
D502 – ATAGAGGC	D702 – TCTCCGGA
D503 – CCTATCCT	D703 – AATGAGCG
D504 – GGCTCTGA	D704 – GGAATCTC
D505 – AGGCGAAG	D705 – TTCTGAAT

Note: Entering i5 and i7 index sequences into the Illumina sample sheet is machine specific. The table above shows examples of both index adapters that can be used in many combinations, in addition to the set included in the kit as AP100.

A 20 µL Amplification 2 reaction will produce >100 ng of amplified double-stranded cDNA when starting with 100 pg to 5 ng of high-quality total RNA. Higher input quantities and higher quality RNA template generally result in increased yields. For damaged RNA, such as from FFPE, 1-50 ng input RNA is recommended. Reaction volumes may be scaled up or down to accommodate preparation of desired quantities of final product. Pre-amplification/Library synthesis reactions may be scaled to as low as 5 µL. Sufficient reagents have been supplied for the number of indicated kit reactions, at Pre-amplification/Library Synthesis, Amplification 1 and Amplification 2 volumes of 15, 30 and 20 µL respectively. Scaling up will reduce the overall number of reactions that the kit can accommodate. Use the same library synthesis volumes for all samples to be compared.



The SeqPlex-I WTA workflow consists of three phases.

Pre-Amplification

- RNA is reverse transcribed into cDNA using primers composed of a semi-degenerate 3'-end and a universal 5'-end that amplify overlapping fragments with comprehensive transcriptome coverage.
- Poly(A) tails are included in amplified library.
- Compatible with low-quantity ChIP or FFPE samples.

Amplification 1

- Pre-Amplification products are amplified by single primer PCR via the proprietary universal end sequence.
- Cycle numbers are variable, depending on quantity and quality of starting sample; amplification is monitored by SYBR green and stopped after signal plateau.

Amplification 2

- Amplification 1 products are converted to dual Illumina-adapted products using adapter primers.
- Adapter primers should be custom ordered with indexes for multiplex capability.
- Amplicon sizes average about 300 bp and are ideal for NGS.
- Following Amplification 2, samples are ready for clean up, quantification, and Illumina NGS.

Reagents Provided

Reagent	Reagent No.	24 RXN	96 RXN	384 RXN
10X Library Synthesis Solution for SeqRi	LP300	36 µL	144 µL	576 µL
10X Library Synthesis Buffer for SeqRi	L9700	36 µL	144 µL	576 µL
Library Preparation Enzyme for SeqRi	L9600	14.4 µL	57.6 µL	230.4 µL
2X Amplification 1 Mix for Seq-I	A8112	345.6 µL	1382.4 µL	5529.6 µL
Amplification 1 Polymerase for Seq-I	SP500	14.4 µL	57.6 µL	230.4 µL
5X Amplification 2 Mix for Seq-I	BA400	86.4 µL	345.6 µL	1382.4 µL
Amplification 2 Enzyme for Seq-I	BA500	12 µL	48 µL	192 µL
Dual Index Adapter Primers for Seq-I	AP100	48 µL	192 µL	768 µL
Water, Molecular Biology Reagent	W4502	5 mL	10 mL	20 mL

Materials and Reagents Required

(Required but not provided)

- Thermal cycler & qPCR instrument
- Sample RNA
- Spectrophotometer, NanoDrop™ or similar
- 0.2 mL Thin-Walled PCR tubes, strips or plates
- Pipetters and Pipette tips with aerosol barriers
- DNase I, Amplification grade, (Cat. No. AMPD1) (optional)
- SYBR® Green I, (Cat. No. S9430) (optional)
- GenElute™ PCR Clean-up Kit, (Cat. No. NA1020)
- Additional index primers if multiplexing samples (Cat. No. OLIGO)

Precautions and Disclaimer

The SeqPlex™-I RNA Amplification Kit for whole transcriptome amplification (WTA) 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. Dissolve any precipitate in these solutions by briefly heating at 37 °C, with thorough mixing. Stability of the L9600, SP500 and BA500 will be affected if stored above −20 °C or allowed to remain for long periods at temperatures over 4 °C.

RNA Handling Techniques

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.

Procedure

The following procedure has been used successfully to amplify and sequence from 100 pg to 5 ng of high-quality total RNA (RIN ≥ 8.0). Higher input quantities and higher quality RNA template generally result in increased yields. For damaged RNA, such as from FFPE, 1-50 ng input is recommended, depending on the degree of damage. Genomic DNA must be removed from the RNA sample prior to amplification. An RNase-free DNase (for example, AMPD-1 used per kit instructions) is recommended if DNA was not eliminated during RNA isolation. The SeqPlex™-I RNA Amplification Kit will amplify ribosomal RNA, though less efficiently than messenger RNA. Ribosomal RNA depletion may be employed when required but is not necessary for all sample types.

Note: Final yield after amplification varies significantly depending upon the quality of starting RNA. In most cases, > 100 ng can be expected. If larger quantities are needed, reactions can be scaled up to accommodate this need, but will limit the total reactions in the kit.

This procedure was developed using the specific reagents provided with, or recommended for use with, this kit. Substitutions may result in suboptimal results.

Pre-amplification/Library Synthesis

1. Thaw the 10X Library Synthesis Solution for SeqRi (LP300), 10X Library Synthesis Buffer for SeqRi (L9700) and water (W4502). Mix thoroughly before use.

   Combine 100 pg to 10 ng of quality RNA or 1-50 ng FFPE RNA with LP300 at the following Single-reaction scale. Combine as follows:

   1.5 µL of 10X Library Synthesis Solution for SeqRi (LP300)

   X µL RNA (for example: 1 ng)

   Y µL water (W4502)

   12.9 µL Total reaction volume

2. Mix thoroughly, centrifuge briefly, and incubate in a thermal cycler programmed for:

   70 °C for 5 minutes

   18 °C for 5 minutes

3. Add 1.5 µL of 10X Library Synthesis Buffer for SeqRi (L9700) and 0.6 µL of Library Preparation Enzyme for SeqRi (L9600) to the sample.

   1.5 µL of 10X Library Synthesis Buffer for SeqRi (L9700)

   0.6 µL of Library Preparation Enzyme for SeqRi (L9600)

   12.9 µL from previous step

   15 µL Total reaction volume

   Cap tube and mix thoroughly. Centrifuge briefly and immediately proceed to next step. (For multiple reactions, a master mix comprised of L9700 and L9600 may be prepared. Add 2.1 µL of the master mix to each sample).

4. Place reaction(s) in a thermal cycler and incubate:

   18 °C for 10 minutes

   25 °C for 10 minutes

   37 °C for 10 minutes

   18 °C for 10 minutes

   25 °C for 10 minutes

   37 °C for 10 minutes

   42 °C for 5 minutes

   95 °C for 1 minutes

   4 °C Hold

   Remove reaction(s) from thermal cycler and centrifuge briefly. Amplification 1 may be started immediately or store Pre-Amplification product at -20 °C for up to three days.

Caution—Experienced WTA and WGA users:

Several components found in the SeqPlex™ RNA Amplification kit (SEQR), SeqPlex™ Enhanced DNA Amplification kit (SEQXE), GenomePlex WGA kits, Transplex® WTA1 kit and Complete Whole Transcriptome Amplification Kit (WTA2) are similarly named. Though generally analogous in function, they are not interchangeable.

Amplification 1

5. Add the following reagents to the 15 µL of Pre-amplification/Library Synthesis product from Step 4. (For multiple reactions, a master mix comprised of reagents listed below may be prepared. Add 15/15.6 µL -/+ SYBR of the master mix to each reaction):

   14.4 µL 2X Amplification 1 Mix for Seq-I (A8112)

   0.6 µL Amplification 1 Polymerase for Seq-I (SP500)

   0.6 µL SYBR Green I (S9430) diluted 1/1000* (recommended)

   15.0 µL Pre-amp/Lib. Synthesis from Step 4

   - µL Instrument Specific Reference Dye (optional, if needed add to final total)

   30/30.6 µL (-/+ SYBR) Total reaction volume

* - For the best representation, real-time PCR with addition of freshly diluted SYBR Green I to the amplification reaction is strongly recommended to enable monitoring of the reaction progress. SYBR Green I (S9430) must be diluted 1,000-fold (1/1000) in water and 0.6 µL used per 30 µL of Amplification 1 reaction for a total of 30.6 µL to avoid inhibiting the amplification reaction. SYBR Green I formulations other than S9430 have not been tested and are not recommended.

Optimal results are achieved by not proceeding past the amplification "plateau" (See **Figure 1**). The optimal number of amplification cycles varies with starting RNA template quantity and quality.

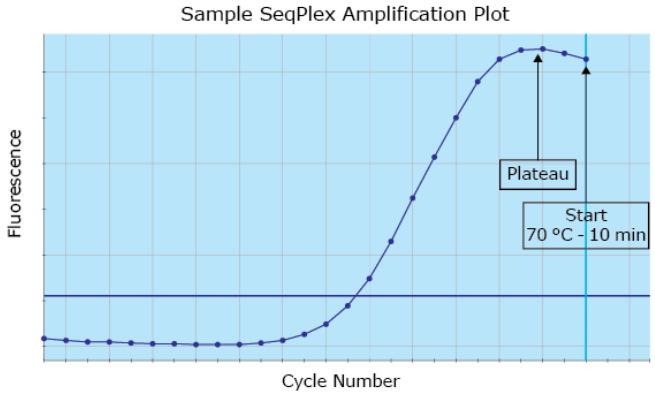
If amplification is performed without adding SYBR Green I, 17-22 cycles will usually give good results with 0.1-1.0 ng of high-quality RNA. Low quality RNA may require higher input quantities and/or more cycles. If input amounts are near 10 pg or lower, as many as 29 cycles may be required to reach amplification plateau.

RNA Input Amount and Typical # of Cycles to Reach Plateau Reference

Input Quality	# of Cycles
10 pg	25
100 pg	22
1 ng	17
10 ng	14

Note: If more than 29 cycles are required to achieve plateau, subsequent NGS results may be unsatisfactory. Consult the Troubleshooting Guide.

Figure 1



6. Cap tube(s) and mix thoroughly. Centrifuge briefly and cycle in a real-time thermal cycler:

   Initial Denaturation:

   94 °C for 2 minutes

   Cycle until plateau: (< 25)

   94 °C Denature for 15 seconds

   70 °C Anneal/Extend for 5 minutes (read fluorescence)

   After cycling:

   70 °C for 10 minutes

   4 °C Hold

After cycling is complete, remove reaction(s) and centrifuge briefly. Amplification 2 may be started immediately or store Amplification 1 product at -20 °C for up to three days.

Caution—Experienced WTA and WGA users:

- SeqPlex™-I uses a 2X Amplification Mix for Amplification 1.
- SYBR Green I (S9340) is recommended to monitor the amplification.
- Annealing/Extension temperature is 70 °C.
- A 10-min 70 °C post-amp 1 hold is required.

Amplification 2

7. Add the following reagents to a new tube or prepare a master mix for multiple reactions (Add 18 µL of the master mix to each tube). You will use 2 µL of the Amplification 1 product from Step 6 per reaction:

    3.6 µL 5X Amplification 2 Mix for Seq-I (BA400)

    0.5 µL Amplification 2 Enzyme for Seq-I (BA500)

    2.0 µL Dual Index Adapter Primers for Seq-I (AP100)

    11.5 µL Water, Molecular Biology Reagent (W4502)

    0.4 µL SYBR Green I (S9430) diluted 1/1000*

    2.0 µL Amplification 1 product from Step 6

    - µL Instrument Specific Reference Dye (optional, if needed add to final total)

    20.0 µL Total reaction volume

Note: The dual index adapter primers (AP100; see table on page 1 for i5 and i7 index sequences) provided in this kit will only work for one set of samples. If pooling samples for sequencing is required, the user must provide additional index primer sets. See example index primer sequences page 1. Using index primers at a concentration of 0.2 µM to 2 µM each is recommended. When used as instructed, the final concentrations of the provided dual index adapter primers (AP100) are 0.2 µM. Lower primer concentration results in higher single stranded hetero duplexes while higher concentrations produce more fully duplex product. Higher concentrations also produce more adapter dimers.

* - For the best representation, real-time PCR with addition of freshly diluted SYBR Green I to the amplification reaction is strongly recommended to enable monitoring of the reaction progress. SYBR Green I (S9430) must be diluted 1,000-fold (1/1000) in water and 0.4 µL used per 20 µL of Amplification 2 reaction to avoid inhibiting the amplification reaction. SYBR Green I formulations other than S9430 have not been tested and are not recommended.

Optimal results are achieved by not proceeding past the amplification "plateau". Proceeding past 1-2 cycles after "plateau" may increase bias and decrease amplicon representation". The optimal number of amplification cycles varies with starting DNA template quantity and quality.

If amplification is performed without adding SYBR Green I, 8-12 cycles will usually yield sufficient product. Low quality RNA may require higher input quantities and/or more cycles as observed in the Amplification 1 cycling.

8. Cap tube(s) and mix thoroughly. Centrifuge briefly and cycle in a real-time thermal cycler:

    Initial Incubation/Denaturation:

    37 °C for 5 minutes

    94 °C for 2 minutes

    Two Cycles:

    94 °C for 30 seconds

    60 °C for 5 minutes

    70 °C for 1 minutes

    Cycle until plateau: (< 12)

    94 °C Denature for 15 seconds

    70 °C Anneal/Extend for 1 minutes (read fluorescence)

    After cycling:

    70 °C for 10 minutes

    4 °C Hold

    Note: The extended incubation at 70 °C after cycling is essential for maximal duplex synthesis.

    After cycling is complete, remove reaction(s) and centrifuge briefly. Reactions can be purified now or stored at -20 °C for up to three days.

Caution—Experienced WTA and WGA users:

- SeqPlex™-I uses a 5X Amplification Mix for Amplification 2.
- SYBR Green I (S9340) is recommended to monitor the amplification.
- Annealing/Extension temperature is 70 °C.
- A 10-minute 70 °C post-amp 2 hold is required.

Purification

The final Amplification 2 reaction(s) can be purified using the GenElute PCR Clean-Up Kit (Cat. No. NA1020). Follow the Technical Bulletin for the kit. Elute in 50 µL nuclease-free water, instead of the Elution Solution provided in the kit. Alternatively, the reactions can be purified with SPRI magnetic beads. In order to minimize sequencing of primer dimers, use a clean up method that removes fragments less than 200 bp in length. The purified reaction(s) can be used immediately or stored at −20 °C for several weeks.

Yield Determination

The amplification product is generally a mixture of single- and double-stranded amplicons. Yield will vary depending on the quality of starting DNA. Concentration can be measured by:

- Traditional A260 absorbance can be used to measure concentration. One A_{260} unit is equivalent to 50 ng/µL dsDNA.
- Fluorescent dyes such as PicoGreen®. Duplex-specific dyes may underestimate the actual DNA yield.
- Quantitative PCR-based library quantification methods are recommended, as quantification is independent of strandedness.

Optional: Amplification quality may be assessed by capillary electrophoresis. Typically, 1 µL of crude or purified amplification product is sufficient for capillary chips, such as those for Agilent's Bioanalyzer or TapeStation. The amplification product is generally a mixture of single and double stranded amplicons.

Sequencing

The SeqPlex™-I DNA generated library is now ready to enter Illumina Next-Generation Sequencing (NGS). The first nine bases of each read should be disregarded, as these bases result from the pre-amplification/library synthesis primers used in that section of the kit.

Frequently Asked Questions

1. Is SeqPlex™-I WTA Kit compatible with microarrays and qPCR?

   Yes, libraries made using SeqPlex™-I WTA Kit can be used in these applications like genomic DNA or existing GenomePlex products.

2. Are there advantages to SeqPlex™-I over GenomePlex?

   SeqPlex™-I Pre-Amplification primers have been designed to target more frequently than existing GenomePlex WTA primers and therefore may provide the advantage of superior genome coverage in some regions.

3. Will reducing cycles during amplification improve representation?

   No, you need to reach "plateau" for optimum representation. Proceeding past 1-2 cycles will not negate the reactions, but best representation is achieved around "plateau". Insufficient cycling leads to a significant reduction in representation/coverage.

4. Will SeqPlex™-I libraries require special NGS sequencing protocols?

   No, libraries made with SeqPlex™-I WTA Kit fit directly into NGS workflows. Sequencing instrument operators should be notified of running libraries created with SeqPlex™-I WTA Kit and to expect a slight signal from any remaining primers in the IVC plots.

Notice

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SeqRi Experienced User Protocol

Pre-Amplification/Library Synthesis

Reagent	Step 1	Step 2
RNA	1 µL to 10 µL	Incubate 70 °C — 5 min 18 °C — 5 min Add reagents in Step 3 to samples
10X Library Synthesis Solution (LP300)	1.5 µL	
Water (W4502)	10.4 µL to 1.4 µL	
Total	12.9 µL	

Reagent	Step 3	Step 4
Reaction from Step 2	(12.9 µL)	PCR 18 °C — 10 min 25 °C — 10 min } 2 cycles 37 °C — 10 min 42 °C — 5 min 95 °C — 1 min 4 °C — hold
10X Library Synthesis Buffer (L9700)	1.5 µL	
Library Preparation Enzyme (L9600)	0.6 µL	
Total	15 µL	

Amplification 1

Reagent	Step 5	Step 6
2X Amplification 1 Mix for Seq-I (A8112)	14.4 µL	PCR 94 °C — 2 min 94 °C — 15 sec } < 25 cycles (monitor) 70 °C — 5 min 70 °C — 10 min 4 °C — hold
Amplification 1 Polymerase for Seq-I (SP500)	0.6 µL	
SYBR® Green (diluted 1:1000) *(S9430, not included in kit, but recommended for monitoring cycling)*	(0.6 µL)	
Pre-Amplification/Library Synthesis product from Step 4	15 µL	
Total	30.0 (30.6) µL	

Amplification 2

Reagent	Step 7	Step 8
5X Amplification 2 Mix for Seq-I (BA400)	3.6 µL	PCR 37 °C — 5 min 94 °C — 2 min 94 °C — 30 sec 60 °C — 5 min } 2 cycles 70 °C — 1 min 94 °C — 15 sec 70 °C — 1 min } < 12 cycles (monitor) 70 °C — 10 min 4 °C — hold
Amplification 2 Enzyme for Seq-I (BA500)	0.5 µL	
Dual Index Adapter Primers for Seq-I (AP100; see table on page 1 for i5 and i7 index sequences)	2 µL	
Water (W4502)	11.5 or 11.9 µL	
SYBR® Green (diluted 1:1000) *(S9430, not included in kit, but recommended for monitoring cycling)*	(0.4 µL)	
Amplification 1 Reaction (undiluted) from Step 6	2 µL	
Total	20 µL	

Troubleshooting Guide

Observation	Cause	Recommended Solution
No product was detected after amplification.	Incorrect Annealing/Extension temperature or time	Perform Amp 1 reaction again with 70 °C Annealing/Extension for 5 minutes each cycle.
	Too few PCR cycles during Amplification	Perform reaction again with more cycles (up to 29) and monitor amplification with SYBR® Green in a real-time thermal cycler.
	Starting RNA was insufficient or too severely degraded	Perform reaction again with more starting RNA.
	DNA may be single stranded	Use a non-duplex-dependent endpoint method (such as qPCR) to detect DNA amplification.
No amplification curve was seen during real-time PCR (qPCR) monitoring	SYBR® Green was not added	SYBR® Green I, Cat. No. S9430, is not included with SeqPlex™-I reagents, but must be added to monitor real-time PCR.
	A reference dye specific to the qPCR instrument may be required	Add instrument specific reference dye. If adding reference dye is not possible, excess cycles should be completed to ensure complete cycling.
Presence of enriched small product(s), particularly 176 bp, after Amplification 2	Primer dimers	Decrease concentration of index adapter primers used in amplification 2. It is recommended to use primers at a final concentration of 0.2 uM to 2 uM; high concentrations increase risk of primer dimer formation. Increase template concentration; primer dimer formation decreases with additional template. If primer dimer cannot be eliminated by decreasing adapter primer concentration or increasing template concentration, primers can be removed using a left side size selection or double size selection with SPRI beads.
Poor representation after NGS	Not enough cycles during SeqPlex™ Amplification	Monitor amplification cycling and cycle until plateau is reached for both Amp1 and Amp 2. If monitoring completely fails, a default 29 cycles may be used for Amp 1 and 12 cycles for Amp 2.
NGS IVC abnormalities	IVC plots show identical sequence (primer) for the first few nucleotides in multiple reads	Optimum instrument cluster calling may be achieved by normalizing the run to a lane that does not contain SeqPlex™-I DNA.