

Equinox Library Amplification Kits

Product Description

Equinox Library Amplification Kits are designed for high-efficiency, high-fidelity amplification of next generation sequencing (NGS) libraries. The ready-to-use mix contains an optimized PCR buffer and hot start enzyme formulation that enables library amplification with minimal bias and error across a broad range of input amounts (0.1 pg to 500 ng) and GC contents, and performance is maintained in the presence of a variety of paramagnetic beads. The hot start functionality inhibits 5' → 3' polymerase and 3' → 5' exonuclease activities, ensuring robust performance when amplification reactions are set up at room temperature.

Three different Equinox Library Amplification Kits are available, each provided with or without amplification primers:

1. Equinox Library Amplification Kit is provided at a 2X concentration, for standard library amplification reactions
2. Equinox High Concentration (HC) Library Amplification Kit is provided at 4X concentration, for library amplification reactions requiring higher concentration of master mix
3. Equinox Uracil Tolerant Library Amplification Kit for amplification of uracil-containing templates or primers.

Kit Contents

Kit	Kit Code	Description	Component Volume		
			24 rxn kit	96 rxn kit	384 rxn kit
Equinox Library Amplification Kit ¹	7K0014-024 (24 rxns)	Equinox Amplification Master Mix (2X) ²	690 µL	3 mL	12 mL
	7K0014-096 (96 rxns)				
	7K0014-384 (384 rxns)	P5/P7 Primer Mix (10X)	144 µL	600 µL	1.2 mL (x2)
Equinox Library Amplification Kit (w/o primers) ³	7K0021-024 (24 rxns)	Equinox Amplification Master Mix (2X) ²	690 µL	3.0 mL	12 mL
	7K0021-096 (96 rxns)				
	7K0021-384 (384 rxns)				
Equinox HC Library Amplification Kit	7K0094-096 (96 rxns)	Equinox Amplification Master Mix (4X)	N/A	1.5 mL	6 mL
	7K0094-384 (384 rxns)	P5/P7 Primer Mix (10X)	N/A	600 µL	1.2 mL (x2)
Equinox HC Library Amplification Kit (w/o primers)	7K0065-096 (96 rxns)	Equinox Amplification Master Mix (4X)	N/A	1.5 mL	6 mL
	7K0065-384 (384 rxns)				
Equinox Uracil Tolerant Library Amplification Kit	7K0023-024 (24 rxns)	Equinox Uracil Tolerant Amplification Master Mix (2X)	690 µL	3.0 mL	12 mL
	7K0023-096 (96 rxns)				
	7K0023-384 (384 rxns)	P5/P7 Primer Mix (10X)	144 µL	600 µL	1.2 mL (x2)
Equinox Uracil Tolerant Library Amplification Kit (w/o primers)	7K0028-024 (24 rxns)	Equinox Uracil Tolerant Amplification Master Mix (2X)	690 µL	3.0 mL	1.2 mL (x2)
	7K0028-096 (96 rxns)				
	7K0028-384 (384 rxns)				

¹Previously named **Library Amplification Kit for Illumina sequencing**

²Previously named **Library Amplification Hot Start Master Mix (2X)**

³Previously named **Library Amplification Master Mix**

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Product Applications

Equinox Library Amplification Kits were developed to address the stringent demands of NGS applications such as rare variant detection, circulating free DNA (cfDNA) analysis, single-cell analysis, and hybridization capture. In addition to uniform coverage, minimal bias, and the maintenance of library complexity, these applications require high yield, fidelity, and sensitivity.

Equinox Library Amplification Kits are ideally suited for:

- Efficient library amplification from a wide range of template amounts (0.1 pg – 500 ng) up to 1 kb in length
- Low-frequency variant detection NGS assays, including those utilizing challenging samples such as FFPE and cfDNA
- Hybridization capture workflows
- Single-cell analysis
- Whole genome sequencing (WGS)
- Amplicon sequencing
- Indexing PCR
- RNA-Seq
- ChIP-Seq, ATAC-Seq, and associated epigenetic applications
- Illumina and non-Illumina sample preparation workflows

Equinox Uracil Tolerant Library Amplification Kits are ideally suited for:

- Amplification of bisulfite-converted DNA
- Amplification of damaged DNA samples or templates containing modified bases
- Prevention of amplicon contamination with UDG

Storage and Handling

Equinox Library Amplification Kits are shipped on cold packs. Upon receipt, store all kit components at $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$.

Keep all components and reaction mixes on ice or a cooled reagent block during routine use. Equinox Amplification Master Mixes and P5/P7 Primer Mix (10X) should be vortexed for 5 sec before use. The reactions may be set up at room temperature.

When stored and handled as indicated, the product will retain full performance until the expiry date printed on the kit box.

Required Materials not Included

- PCR-grade water
- Thermocycler
- Ampure® XP Beads (Beckman Coulter, Inc. #A63881) or equivalent (see **Prior to Starting** section)
- 80% Ethanol
- 200 μL thin-wall PCR tubes compatible with thermocycler, or 96-well 0.2 mL PCR Plates and Plate-Seals
- 10 mM Tris-HCl pH 8.0
- Magnetic rack compatible with 0.2 mL tubes and/or 96-well plate

These protocols are designed for use with the specified labware, consumables, and calibrated equipment.

Prior to Starting

Input DNA

Input Amount

This kit is compatible with a broad range of adapter-ligated library input amounts (0.1 pg – 500 ng) and GC content templates up to 1 kb in length.

Ethanol Contamination

Ethanol is a potent inhibitor of PCR. When using the Equinox Library Amplification Kit after a magnetic bead purification, care must be taken to ensure ethanol used in bead washes is not carried over into the PCR reaction, as even a few microliters of carryover can affect performance.

After ethanol washes, ensure all ethanol is aspirated from the bead pellet. Bead pellet drying time will vary depending on ambient condition, volume of beads, etc. A visual indication that beads are sufficiently dry is when cracking within the pellet begins to appear. A shiny pellet is not sufficiently dried. When eluting, ensure the pellet is fully homogenized to facilitate full yield recovery.

Amplification Primers

Storage and Handling

Use a buffered solution, such as 10 mM Tris-HCl, pH 8.0, to store and dilute primers. Limit the number of freeze-thaw cycles.

Primer Concentration

Always use equimolar concentrations of forward and reverse primers at a final concentration of 0.5 to 2 μM each. A final concentration of 0.5 μM is sufficient to yield 500 ng of library (approximately 40 nM in a 50 μL reaction for a library with an average fragment length of 400 bp), which is typically adequate for library QC, quantification, and sequencing. If the total library yield must exceed 500 ng or 40 nM, use 2 μM final concentration.

The P5/P7 Primer Mix (10X) is supplied at 20 μM (each primer) for a 2 μM final concentration.

Primer Design

Protection of the 3'-ends of amplification primers through the incorporation of one or more phosphorothioate bonds is highly recommended to achieve optimal specificity and library yields.

Library Amplification Optimization

Annealing Temperature

For user-supplied primers, the optimal annealing temperature in this proprietary buffer should be determined empirically in an annealing temperature gradient (55°C to 70°C) experiment.

Extension Time

Longer extension times may be employed to ensure efficient amplification of longer-insert libraries. A 30 sec extension is sufficient for libraries with a mode fragment size up to 500 bp. A 45 sec extension time is recommended for libraries with mode fragment sizes >500 bp. The optimal condition for each application may need to be determined empirically.

Cycle Number

Since bias and amplification artifacts are compounded with each cycle of PCR, it is desirable to use the lowest number of PCR cycles required for the application to maximize coverage uniformity and fidelity. Table 1 (page 4) provides a starting point for PCR cycle number optimization based on DNA input into library amplification. Note that this may be significantly less than the amount of DNA input into upstream library construction. Imperfect enzyme efficiencies, SPRI cleanups, and/or size selections result in a reduction of material available for library amplification. Adapter-ligated library may be quantified by qPCR or estimated by other means or methods to determine the optimal number of amplification cycles for the desired library yield.

SPRI Purification

Instructions are provided in this protocol for a bead cleanup to purify the amplified library. SPRI-to-sample bead ratios may be optimized for different applications. A 1X ratio is recommended as a starting point when using standard P5/P7 amplification primers.

The protocol outlined below assumes the use of AMPure XP (Beckman Coulter) reagent for bead purification steps.

Other SPRI bead brands may be used, assuming they are nuclease-free and deliver equivalent sizing performance at the given bead ratio (1X). Otherwise, bead purification ratios will need to be optimized for the bead brand used.

Ensure beads are equilibrated to room temperature and thoroughly resuspended via vortexing prior to use.

Library Amplification Protocol

Note:

- If planning to bead purify the amplified library, ensure SPRI purification beads are fully equilibrated to room temperature and thoroughly resuspended via vortexing prior to use.
- If amplifying in the presence of paramagnetic beads, please review **Appendix A** prior to proceeding.

1. Library Amplification

- 1.1 Equilibrate the Equinox Amplification Master Mix (2X) and primers—either supplied P5/P7 Primer Mix (10X) or user-supplied—on ice.
- 1.2 Once thawed, vortex the Equinox Amplification Master Mix and primers for 5 sec and briefly centrifuge.
- 1.3 Program a thermocycler as indicated below:

Step	Temperature (°C)	Time (sec)	Cycles
Initial denaturation	98	45	1
Denaturation	98	15	See Table 1
Annealing ¹	60	30	
Extension ²	72	30	
Final extension	72	60	1
Final hold	4 – 12	Hold	–

¹An annealing temperature of 60°C is recommended for standard Illumina® “P5” and “P7” primers (P5: AATGATACGGCGACCACCGA; P7: CAAGCAGAAGACGGCATACGAGAT). For other amplification primers, the optimal annealing temperature should be determined empirically in an annealing temperature gradient (55°C – 70°C) experiment.

²Longer extension times may be employed to ensure efficient amplification of longer-insert libraries. A 30 sec extension is sufficient for libraries with a mode fragment size up to 500 bp; a 45 sec extension time is recommended for libraries with mode fragment sizes >500 bp. The optimal condition for each application may need to be determined empirically.

Table 1. Recommended PCR cycle numbers based on DNA input

Adapter-ligated DNA (ng)	PCR cycles ¹ to generate:	
	40 nM library	1 µg library
500	1	2
100	3	4
50	4	5
10	7	8
5	8	9
1	11	12
0.5	12	13
0.1	15	16
0.01	18	19
0.001	22	23
0.0001	26	27

¹Cycle number recommendations are appropriate for libraries with mean fragment length of approximately 300 – 600 bp. Cycle numbers may be adjusted accordingly if mean fragment length differs significantly from this range.

- 1.4 Assemble each amplification reaction according to the tables below, following the recipe that corresponds to primer source:

Equinox Library Amplification Kit or Equinox Uracil Tolerant Library Amplification Kit:

Reaction setup: P5/P7 Primer Mix (10X)

Component	Final Concentration	Volume (50 µL reaction)
Equinox Amplification Master Mix (2X) or Equinox Uracil Tolerant Amplification Master Mix (2X)	1X	25 µL
P5/P7 Primer Mix (10X)	2 µM	5 µL
Template DNA	0.1 pg – 500 ng	Variable
PCR-grade water	–	Up to 50 µL

Reaction setup: User-supplied primers

Component	Final Concentration	Volume (50 μ L reaction)
Equinox Amplification Master Mix (2X) or Equinox Uracil Tolerant Amplification Master Mix (2X)	1X	25 μ L
Each primer (Fwd/Rev) ¹	0.5 – 2 μ M	Variable
Template DNA	0.1 pg – 500 ng	Variable
PCR-grade water	–	Up to 50 μ L

¹See **Prior to Starting** for more information on user-supplied primers.

Equinox HC Library Amplification Kit:

Reaction setup: P5/P7 Primer Mix (10X)

Component	Final Concentration	Volume (50 μ L reaction)
Equinox Amplification Master Mix (4X)	1X	12.5 μ L
P5/P7 Primer Mix (10X)	2 μ M	5 μ L
Template DNA	0.1 pg – 500 ng	Variable
PCR-grade water	–	Up to 50 μ L

Reaction setup: User-supplied primers

Component	Final Concentration	Volume (50 μ L reaction)
Equinox Amplification Master Mix (4X)	1X	12.5 μ L
Each primer (Fwd/Rev) ¹	0.5 – 2 μ M	Variable
Template DNA	0.1 pg – 500 ng	Variable
PCR-grade water	–	Up to 50 μ L

¹See **Prior to Starting** for more information on user-supplied primers.

- Mix thoroughly by pipetting and centrifuge briefly.
- Place reactions in the thermocycler (programmed in **Step 1.3**) and run the program.
- If purifying amplified material, proceed into **Post-amplification Cleanup**.

Safe stopping point. Samples can be stored at 4°C overnight and at -20°C for up to 1 month.

2. Post-amplification Cleanup

- Freshly prepare at least 0.4 mL of an 80% ethanol solution for each reaction.

- Vortex room-temperature SPRI beads to thoroughly mix. Add 50 μ L (1X) of beads to each amplification reaction and mix thoroughly by pipetting.

NOTE: SPRI-to-sample bead ratios may be optimized for different applications. A 1X ratio is recommended as a starting point when using standard P5/P7 amplification primers.

- Incubate the library-bead mixtures at room temperature for at least 5 min to maximize library recovery.
- Place sample tubes on a magnet for at least 5 min, or until all beads have collected on the tube wall and the solution is clear.
- Carefully remove and discard the supernatant from each tube.
- Add 200 μ L of freshly prepared 80% ethanol to each tube, taking care to not disturb the bead pellet on the tube wall.
- Incubate tubes at room temperature for at least 30 sec without removing the tube from the magnet or disturbing the bead pellet. Carefully remove and discard the ethanol.
- Repeat **Steps 2.6 – 2.7** for a total of two washes.
- Allow remaining ethanol to evaporate by allowing the pellets to air dry for 3 – 5 min.

NOTE: Beads have been sufficiently dried when cracks appear in the surface of the bead pellet and no liquid is visible in the tube.

- Remove sample tubes from the magnet and carefully resuspend each bead pellet in 22 μ L of 10 mM Tris-HCl pH 8.0. Pipetting carefully will minimize bubbling and allow for greater library recovery.
- Incubate tubes at room temperature for at least 2 min before placing back on the magnet.
- Leave sample tubes on the magnet for at least 2 min, or until all beads have collected on the tube wall and the solution is clear.
- Carefully transfer each library-containing supernatant to a new tube.
- At this point, libraries are ready for quantification, normalization, pooling, hybridization capture, and/or sequencing.

NOTE: We recommend quantifying libraries using qPCR and analyzing quality and sizing using capillary electrophoresis prior to preparing the libraries for sequencing.

Appendix A: Amplification with Paramagnetic Purification Beads

For some library preparation workflows, it may be preferred or advantageous to perform PCR in the presence of paramagnetic purification beads. For example, in hybridization capture workflows, post-capture amplification may be performed by adding PCR mix directly to the capture beads with bound library, eliminating an elution step. Alternatively, libraries may be eluted directly from SPRI beads in the PCR reaction mixture.

We have found paramagnetic purification beads to fall into three categories of compatibility with PCR and sequencing based on their surface chemistry: Group I—fully compatible, Group II—potentially inhibitory, and Group III—incompatible. While Group II beads are inhibitory to non-optimized PCR systems, the Equinox Amplification Master Mix has been optimized to allow amplification in the presence of both Group I and Group II beads, with no observable loss in performance (e.g., efficiency, uniformity, or fidelity). The Equinox Amplification Master Mix and other library amplification systems are not compatible with the extreme inhibition characterized by Group III beads. Table 2 details the various paramagnetic bead types evaluated.

Table 2. Paramagnetic purification bead types

Bead type	Vendor	Catalog number	Compatibility/ amount tested ¹
Group I (Tosyl-activated beads)			
Dynabeads™ M280 Streptavidin	Thermo Fisher	11205D	500 µg
Dynabeads MyOne™ Streptavidin T1	Thermo Fisher	65601	500 µg
Group II (Carboxylic acid-activated beads)			
Dynabeads M270 Streptavidin ²	Thermo Fisher	65305	500 µg
SPRI	Various, incl. Beckman Coulter	A63882	100 µL
Dynabeads MyOne Streptavidin C1	Thermo Fisher	65001	500 µg
Group III (Not compatible with PCR)			
Dynabeads M270 Carboxylic Acid	Thermo Fisher	14305D	500 µg

¹Volume of slurry or mass of beads per 50 µL amplification reaction.

²Used in xGen Hybridization and Wash Kit (Integrated DNA Technologies).

Revision History

Version	Description	Date
1.0	• First protocol release	11/2021
2.0	• Additional Equinox Library Preparation Kits commercialized and added to the User Guide.	01/2023



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