

Illumina Sequencing Library Preparation for Highly Multiplexed Target Capture and Sequencing

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INTRODUCTION

The large amount of DNA sequence data generated by high-throughput sequencing technologies often allows multiple samples to be sequenced in parallel on a single sequencing run. This is particularly true if subsets of the genome are studied rather than complete genomes. In recent years, target capture from sequencing libraries has largely replaced polymerase chain reaction (PCR) as the preferred method of target enrichment. Parallelizing target capture and sequencing for multiple samples requires the incorporation of sample-specific barcodes into sequencing libraries, which is necessary to trace back the sample source of each sequence. This protocol describes a fast and reliable method for the preparation of barcoded ("indexed") sequencing libraries for Illumina's Genome Analyzer platform. The protocol avoids expensive commercial library preparation kits and can be performed in a 96-well plate setup using multi-channel pipettes, requiring not more than two or three days of lab work. Libraries can be prepared from any type of double-stranded DNA, even if present in subnanogram quantity.

RELATED INFORMATION

Illumina's "indexing" system differs from other sample barcoding methods for high-throughput sequencing in that the barcodes ("indexes") are placed within one of the adapters rather than being directly attached to the ends of template molecules (e.g., Craig et al. 2008; Meyer et al. 2008b). The barcode sequence is identified in a separate short sequencing read. This setup allows for a high degree of flexibility in experimental design, because libraries are first prepared with universal adapters and different indexes can repeatedly be added by amplification with tailed primers just before target capture or sequencing. The library preparation protocol described here (see Fig. 1 for an overview) is based on the general principle of library preparation originally developed for 454 sequencing (Margulies et al. 2005). By exchanging adapter sequences, removing and shortening several reaction steps, and introducing an amplification scheme, the protocol has been redesigned for rapid preparation of Illumina multiplex sequencing libraries using a 96-well plate format. In the example shown in Figure 2, the protocol was used to simultaneously capture and sequence target regions from 50 human samples using microarrays (HA Burbano, E Hodges, RE Green, AW Briggs, J Krause, M Meyer, JM Good, T Maricic, PLF Johnson, Z Xuan, et al., in prep.).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <I>, and recipes for reagents marked with <R>.

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Reagents

Agarose gel (2%) and reagents for agarose gel electrophoresis
AMPure XP 60 mL Kit (Agencourt-Beckman Coulter A63881)
ATP (100 mM) (Fermentas R0441)
Bst DNA polymerase, large fragment (supplied with 10X ThermoPol reaction buffer) (New England BioLabs M0275S)
DNA ladder (e.g., GeneRuler; Fermentas) (optional; see note before Step 6)
For unknown reasons, ladders from New England BioLabs do not work for this purpose.
dNTP mix (25 mM each) (Fermentas R1121)
<R>EBT buffer
Ethanol (70%, freshly prepared)
H₂O (HPLC grade)
Illumina reagents for DNA sequencing (Illumina, Inc.)
Cluster generation kit (e.g., GD-103-4001 [Standard Cluster Generation Kit v4], PE-203-4001 [Paired-End Cluster Generation Kit v4])
Multiplexing sequencing primer kit (PE-400-1002 [Multiplexing Sequencing Primers and PhiX Control Kit v1])
Alternatively, the following primers may be used for sequencing:
Read 1 Sequencing Primer: 5'-ACACTCTTCCCTACACGACGCTCTCCGATCT-3'
Index Read Sequencing Primer: 5'-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3'
Read 2 Sequencing Primer: 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'
Sequencing kit (FC-104-4002 [36 Cycle Sequencing Kit v4])
MinElute PCR Purification Kit (QIAGEN) (optional)
<R>Oligo hybridization buffer (10X)
Oligonucleotides (Sigma-Aldrich) (see Table 1)
Phusion Hot Start High-Fidelity DNA Polymerase (New England BioLabs F-540L) (supplied with 5X Phusion HF buffer)
Positive control DNA (200- to 300-bp fragment, generated via PCR using unmodified primers and a polymerase with terminal transferase activity, e.g., Taq DNA polymerase) (200-500 ng)
Sample DNA
This protocol works reliably with as little as 100 pg and up to 1 µg of double-stranded sample DNA (e.g., genomic DNA, long-range PCR products, or cDNA). The amount of starting material should be chosen so that the representation of target molecules in the final library is sufficient. The final yield of the library preparation process is ~10%-20%. Therefore, a library prepared from 1 ng of human genomic DNA (about 300 copies of the haploid genome), will contain 30 to 60 copies of the human genome.
Standard for quantitative PCR (qPCR) (see Steps 21.i-21.ii)
SYBR Green qPCR master mix (e.g., DyNAmo Flash SYBR Green qPCR Kit; New England BioLabs)
Tango buffer (10X; Fermentas BY5)
T4 DNA ligase (5 U/µL; Fermentas EL0011) (supplied with 10X T4 DNA ligase buffer and 50% PEG-4000 solution)
T4 DNA polymerase (5 U/µL; Fermentas EP0062)
T4 polynucleotide kinase (10 U/µL; Fermentas EK0032)
<R>TET buffer
Tween 20

Equipment

Centrifuge for 96-well plates
DNA shearing device (e.g., Bioruptor UCD-200 [Diagenode]; Covaris E210 [Covaris Inc]) (for high-molecular-weight DNA; see Step 3)
The Bioruptor UCD-200 can process 12 samples in parallel. Among the many alternative systems that are available for this step, the Covaris E210 system may be preferable, because it is compatible with the 96-well plate format.
Equipment for agarose gel electrophoresis

Equipment and reagents for target capture from sequencing libraries (optional)

Several systems are available; see e.g., Hodges et al. (2009) for a target capture approach using Agilent microarrays.

Ice

Multichannel pipettes

Multichannel reagent basins (e.g., Thermo Scientific 9510027)

PCR plates (96-well, 200- μ L capacity) and strip caps

Real-time PCR cycler (e.g., Mx3005P QPCR System; Agilent Technologies-Stratagene)

Sequencing machine (Genome Analyzer II/IIx/IIe or HiSeq2000; Illumina)

Spectrophotometer for DNA quantification (e.g., NanoDrop; Thermo Scientific)

SPRIPlate 96R-Ring Super Magnet Plate (Agencourt-Beckman Coulter A32782)

Thermal cycler

Tubes (microcentrifuge, 0.5-mL)

Tubes (PCR)

Vortex mixers for tubes and 96-well plates

METHOD

The protocol can be interrupted after Steps 3, 12, 16, 19, 24, and 26 by freezing the DNA at -20°C . Up to 94 samples can be processed in parallel on a 96-well reaction plate; two wells should be reserved for a blank and a positive control. Seal each reaction plate with strip caps and centrifuge to 2000g in a plate centrifuge after setting up each reaction in order to collect the liquid in the bottom of the wells. This prevents cross-contamination while removing the caps.

Preparation of Adapter Mix

This step produces sufficient adapter mix for 200 reactions. The adapter mix can be used repeatedly and stored at -20°C before and after usage.

1. Assemble the following hybridization reactions in separate PCR tubes:

Reagent	Volume (μL)	Final concentration in 100- μL reaction
Hybridization mix for adapter P5 (200 μM):		
IS1_adapter_P5.F (500 μM)	40	200 μM
IS3_adapter_P5+P7.R (500 μM)	40	200 μM
Oligo hybridization buffer (10X)	10	1X
H ₂ O	10	
Hybridization mix for adapter P7 (200 μM):		
IS2_adapter_P7.F (500 μM)	40	200 μM
IS3_adapter_P5+P7.R (500 μM)	40	200 μM
Oligo hybridization buffer (10X)	10	1X
H ₂ O	10	

2. Mix and incubate the reactions in a thermal cycler for 10 sec at 95°C , followed by a ramp from 95°C to 12°C at a rate of $0.1^{\circ}\text{C}/\text{sec}$. Combine both reactions to obtain a ready-to-use adapter mix (100 μM each adapter).

Fragmentation and Purification of Sample DNA

This step in the method is not always required. Prior to library preparation, high-molecular-weight sample DNA must be sheared into fragments of suitable size for Illumina sequencing (<600 bp). If samples other than high-molecular-weight DNA are used (e.g., short PCR products, highly degraded DNA, or short double-stranded cDNA), fragmentation may not be necessary. Step 3 describes DNA shearing by sonication using the Bioruptor UCD-200.

3. Shear the DNA as follows:

- i. Transfer the samples to 0.5-mL tubes, and add H₂O to reach final volumes of 50 μL .

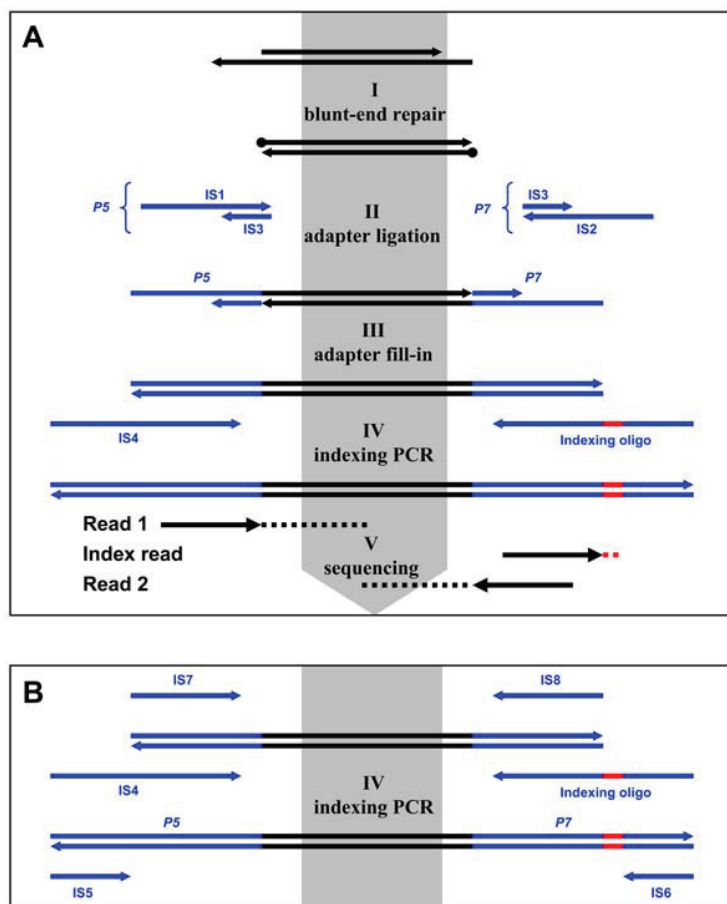


FIGURE 1. Schematic overview of the protocol and alternative amplification schemes. (A) Sample DNA is sheared into small fragments (not depicted). During blunt-end repair, overhanging 5'- and 3'-ends are filled in or removed by T4 DNA polymerase. 5'-phosphates are attached using T4 polynucleotide kinase (Steps 1-13). Two different adapters, P5 and P7, are ligated to both ends of the molecules using T4 DNA ligase (Steps 14-16). Ligation is nondirectional and also produces molecules which have the same adapters attached to both ends (*not depicted*). Such molecules do not interfere with sequencing and—due to the formation of hairpin structures—amplify very poorly during indexing PCR. Since the adapters do not carry 5'-phosphates, ligation joins only single strands. Nicks are removed in a fill-in reaction with Bst polymerase, which possesses strand-displacement activity (Steps 17-21). Indexes and full length adapter sequences are added by amplification with 5'-tailed primers (Steps 22-26). Indexed libraries are pooled in equimolar ratio. The pool is ready for target capture and/or sequencing on one of Illumina's sequencing platforms (Steps 27-28). Indexes are read in a separate sequencing read. Read 2, the paired end read, is optional. (B) Alternative amplification schemes can be used. Using the primers IS7 and IS8, libraries can be amplified prior to indexing. Using IS5 and IS6, single or pooled indexed libraries can be amplified, for example after target enrichment. (For color figure, see doi: 10.1101/pdb.prot5448 online at www.cshprotocols.org.)

- ii. Expose the DNA four times to sonication cycles of 7 min, using the energy setting "HIGH" and an "ON/OFF interval" of 30 sec. If liquid spills to the tube walls, shake it down to the bottom of the wells after each sonication cycle.

This produces a fragment size distribution between 100 bp and 400 bp, with a mean around 200 bp.

- iii. Transfer the sheared DNA samples to a 96-well PCR plate.

The fragment size distribution obtained from sonication is well-suited for sequencing. However, if a very narrow fragment size distribution is desired, the fragmented DNA may be separated on an agarose gel and isolated from a gel slice to obtain a more narrow distribution. In the example given in Figure 2, no gel excision was performed.

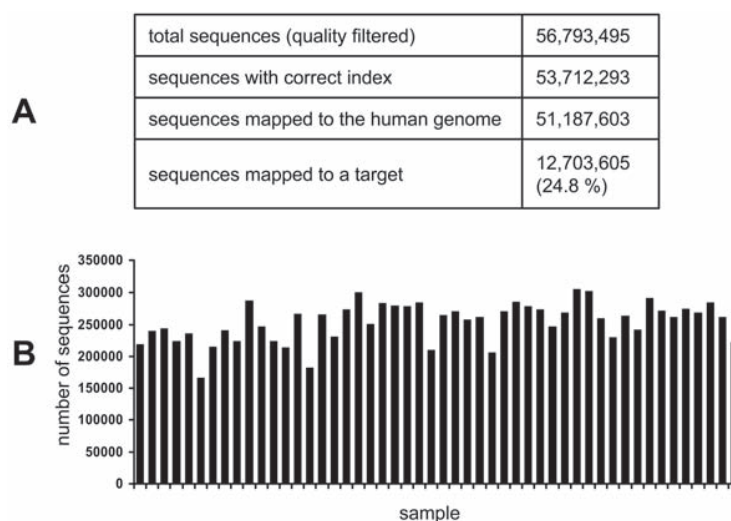


FIGURE 2. Example of a result from multiplex target capture and sequencing. Indexed libraries were prepared from 50 human samples from the CEPH human genome diversity panel as described in this protocol. Shearing was performed using the Bioruptor with no subsequent gel excision (see Step 3). The pool of libraries was loaded on a million-feature array from Agilent to capture 12,871 targets from the human genome with an average size of 232 bp (overall 2.9 million bp), following the protocol of Hodges et al. (2009). The array eluate was amplified for 12 cycles using primers IS5 and IS6 and sequenced on 5 lanes of the Illumina flow cell (2×100 cycles + 6 cycles index read). Shown are the results from mapping the sequences against the human genome (A) and the distribution of sequences among samples (B).

Blunt-End Repair

If the sample DNA is not dissolved in H_2O , Tris-Cl buffer (e.g., QIAGEN's Buffer EB), or TE buffer, purify the DNA as described in Steps 6-13 prior to beginning Step 4. If the sample volume exceeds 50 μL , purification can be used for concentrating the DNA. We strongly recommend carrying a positive and a blank control through Steps 4-18 of the protocol. As a positive control, 200-500 ng of a purified PCR product with a discrete size of 200-300 bp may be used. The product should be generated using unmodified PCR primers and a polymerase with terminal transferase activity (e.g., Taq DNA polymerase).

4. Add a blank control (50 μL of H_2O) and a positive control to two empty wells of the reaction plate. Prepare a master mix as below for the required number of reactions. Mix carefully by flicking the tube with a finger. Avoid vortexing after addition of enzymes.

Reagent	Volume (μL) per sample	Final concentration in 70- μL reaction
H_2O	7.12	
Buffer Tango (10X)	7	1X
dNTPs (25 mM each)	0.28	100 μM each
ATP (100 mM)	0.7	1 mM
T4 polynucleotide kinase (10 U/ μL)	3.5	0.5 U/ μL
T4 DNA polymerase (5 U/ μL)	1.4	0.1 U/ μL

5. Using a multichannel pipette, add 20 μL of master mix to 50 μL of sample. Mix and incubate in a thermal cycler for 15 min at 25°C followed by 5 min at 12°C. Place plate on ice or immediately proceed to the next step.

Reaction Clean-Up Using Solid Phase Reversible Immobilization (SPRI)

Carboxyl-coated magnetic beads (SPRI beads) are ideally suited for reaction purification in a 96-well plate setup. However, under the conditions described here, SPRI purification does not retain molecules shorter than 100-150 bp. The exact size cutoff may vary among different batches of beads. If retention of short molecules is desired, the size cutoff can be adjusted by varying the volume of SPRI bead/buffer suspension added to the sample. The appropriate ratio of SPRI suspension to sample volume can be empirically determined using a DNA ladder (e.g., GeneRuler ladders). If retention of very short molecules is desired (30-80 bp), all SPRI purification steps should be replaced by spin column purification using the MinElute PCR Purification Kit.

Table 1. Oligonucleotides and sequences

Oligo ID	Sequence ^a
IS1_adapter.P5	A*C*A*C*TCTTCCCTACACGACGCTCTCCG*A*T*C*T
IS2_adapter.P7	G*T*G*A*CTGGAGTTCAGACGTGTGCTCTTCCG*A*T*C*T
IS3_adapter.P5+P7	A*G*A*T*CGGAA*G*A*G*C
IS4_indPCR.P5	AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTT
IS5_reamp.P5	AATGATACGGCGACCACCGA
IS6_reamp.P7	CAAGCAGAAGACGGCATACGA
IS7_short_amp.P5	ACACTCTTCCCTACACGAC
IS8_short_amp.P7	GTGACTGGAGTTCAGACGTGT
BO1.P5.F	AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT-Pho
BO2.P5.R	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT-Pho
BO3.P7.part1.F	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-Pho
BO4.P7.part1.R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-Pho
BO5.P7.part2.F	ATCTCGTATGCCGTCTTCTGCTTG-Pho
BO6.P7.part2.R	CAAGCAGAAGACGGCATACGAGAT-Pho

^a5'-3'; * indicates a PTO bond; Pho indicates a 3'-phosphate.

See Supplemental Material (Indexing_Oligo_Sequences.doc) for indexing oligo sequences.

All oligos (HPLC purified, 0.2 µmol synthesis scale) should be dissolved in TE or H₂O. Oligos 1-3 should be dissolved to 500 µM, oligos BO1-BO6 to 200 µM, and all other oligos to 10 µM. The indexing oligos should be transferred to a 96-well plate to allow for multichannel pipetting. HPLC purification can potentially introduce cross-contamination among indexing oligos. It is therefore important to (1) instruct the company to properly wash the HPLC column before loading a new oligo and (2) synthesize the oligos in a different order than listed here. This makes sure that cross-contamination induced during synthesis can be detected after sequencing by the appearance of index sequences that were not used in the experiment. When designing index sequences, the following criteria were taken into account: (1) Index sequences differ by at least three substitutions. This reduces the chance of converting one index into another by sequencing and amplification errors. (2) Indexes cannot be converted into one another by deleting the first base, which is the only insertion/deletion error common with Illumina sequencing. (3) Index sequences do not contain three or more identical bases in a row to ensure that they can be differentiated from artifact sequences. (4) Stretches of bases illuminated with the same laser (ACA, CAC, GTG, and TGT) are avoided. Software for designing alternative index sequences, for example, with a length of 6 or 8 nt, and for selecting appropriate subsets for pooling is provided at <http://bioinf.eva.mpg.de>.

6. Resuspend the stock solution of SPRI bead suspension (AMPure kit) by vortexing. To make subsequent pipetting easier, add Tween 20 to the bead suspension to a final concentration of 0.05% (i.e., add 1 µL of Tween 20 to 2 mL of bead suspension).
7. Add SPRI bead suspension to the reactions as follows:
 - i. Add a 1.8-fold volume of SPRI bead suspension to each reaction (e.g., add 126 µL of SPRI beads to a 70-µL sample or 72 µL of SPRI beads to a 40-µL sample).
 - ii. Seal the wells with caps and vortex for several seconds. Ensure the beads are properly suspended and repeat vortexing if necessary.
 - iii. Let the plate stand for 5 min at room temperature.
 - iv. Collect the liquid at the bottom of the wells by briefly centrifuging in a plate centrifuge to 2000g.
8. Place the plate on a 96-well ring magnetic plate, and let it stand for 5 min to separate the beads from the solution. Pipette off and discard the supernatant without removing the beads.
9. Leave the plate on the magnetic rack, and wash the beads by adding 150 µL of freshly prepared 70% ethanol. Let stand for 1 min and remove the supernatant.
10. Repeat Step 9.
11. Using a multichannel pipette, remove residual traces of ethanol. Let the beads air-dry for 20 min at room temperature without caps.

12. Elute as follows:

- i. Add 20 μL of EBT to the wells and seal the plate with caps.
- ii. Remove the plate from the magnetic rack, and resuspend the beads by repeated vortexing.
- iii. Let stand for 1 min, and then collect the liquid in the bottom of the wells by briefly centrifuging the plate to 2000g.

Occasionally the beads may appear clumpy after vortexing; this does not have a negative effect on DNA recovery.

13. Place the plate back on the magnetic rack, let stand for 1 min, and transfer the supernatant to a new 96-well reaction plate.

Carryover of small amounts of beads will not inhibit subsequent reactions.

Adapter Ligation

14. Prepare a master mix for the required number of ligation reactions as shown below. If white precipitate is present in the 10X DNA ligase buffer after thawing, warm the buffer to 37°C and vortex until the precipitate has dissolved. Since PEG is highly viscous, vortex the master mix before adding T4 DNA ligase and mix gently thereafter.

Reagent	Volume (μL) per sample	Final concentration in 40- μL reaction
H ₂ O	10	
T4 DNA ligase buffer (10X)	4	1X
PEG-4000 (50%)	4	5%
adapter mix from Step 2 (100 μM each)	1	2.5 μM each
T4 DNA ligase (5 U / μL)	1	0.125 U / μL

When starting from low template quantities (50 ng or less), the amount of adapter mix can be reduced to 0.2 μL per reaction.

15. Add 20 μL of master mix to each eluate from Step 13 to obtain reaction volumes of 40 μL . Mix and incubate for 30 min at 22°C in a thermal cycler.
16. Perform reaction purification exactly as described in Steps 6-13. Elute in 20 μL of EBT.

Adapter Fill-In

17. Prepare a master mix for the required number of reactions.

Reagent	Volume (μL) per sample	Final concentration in 40- μL reaction
H ₂ O	14.1	
ThermoPol reaction buffer (10X)	4	1X
dNTPs (25 mM each)	0.4	250 μM each
Bst polymerase, large fragment (8 U/ μL)	1.5	0.3 U/ μL

18. Add 20 μL of master mix to each eluate from Step 16 to obtain reaction volumes of 40 μL . Mix well and incubate in a thermal cycler for 20 min at 37°C.
19. Perform reaction purification exactly as described in Steps 6-13. Elute the library in 20 μL of EBT.

Library Characterization

In addition to agarose gel electrophoresis (Step 20), performance of qPCR (Step 21) prior to indexing PCR (Steps 22-24) is strongly recommended, particularly if little sample DNA was used for library preparation. This is the only option to directly measure the number of molecules in the library. If the mean average fragment length and the size of the genome are known, this number can be used to determine whether the average coverage of genomic targets in the library is sufficiently high for subsequent target capture or direct sequencing. Step 21 describes a qPCR assay using SYBR Green (for more details, see Meyer et al. 2008a).

20. To verify the success of the library preparation, load 10 μL of the positive control library side-by-side with 100 ng of the original positive control sample and a size marker on a 2% agarose gel and perform electrophoresis.

If all enzymatic reactions worked properly, the band produced by the control library should be shifted upward by 67 bp.

See Troubleshooting.

21. Measure the number of molecules by qPCR:

- i. Prepare a standard dilution series by incrementally diluting an indexed sequencing library of known molecular concentration 10-fold in TET buffer.
- ii. If no such library is available, amplify 0.5 μ L of the positive control in an indexing PCR (see Step 22). Purify the PCR product as described in Steps 6-13, determine its mass concentration on a spectrophotometer, calculate the molecular concentration, and use it as a standard as described in Step 21.i.
- iii. In a real-time PCR machine, amplify in parallel 1 μ L of each standard dilution and each sample using primer IS4 and one of the indexing oligos; we recommend using a commercial PCR master mix containing SYBR Green (e.g., DyNAmo Flash SYBR Green qPCR kit). Set the annealing temperature to 60°C, and otherwise follow the instructions provided by the manufacturers of the kit and the real-time PCR machine.

The concentration of molecules in the blank library (adapter dimers) should be at least one order of magnitude lower than in the sample libraries.

It is often necessary to measure dilutions of the samples (e.g., 1000-fold in EBT) to obtain values within the detection range of the qPCR system.

Indexing PCR and Pooling

To avoid a downstream failure of Illumina's image analysis software, subsets of indexes must be chosen in a way that prevents unbalanced usage of the four nucleotides or the two laser channels during any cycle of index sequencing. The indexes provided with this protocol (see Supplemental Material [Indexing_Oligo_Sequences.doc]) are in an appropriate order to fulfill these requirements and should be used accordingly. For example, the first 22 indexes should be used if 22 indexes are needed. Fewer than four indexes should never be used in any experiment. Additional sets of indexes with different length and varying edit distance between indexes are provided on <http://bioinf.eva.mpg.de>. It will often not be necessary to use the entire library as template for indexing PCR. In this case, it is advisable to keep a backup that can be later used to add a different barcode to the sample.

Note that Phusion polymerase has proofreading activity. If this property is not desired (e.g., if deoxyuracil is present in the template DNA), another polymerase can be chosen for indexing PCR.

22. Prepare a PCR master mix for the required number of reactions. Dispense the master mix into a 96-well reaction plate, and then add template DNA and a different indexing primer to each well using a multichannel pipette.

Reagent	Volume (μ L) per sample	Final concentration in 50- μ L reaction
Master mix:		
H ₂ O	37.1 – x	
Phusion HF buffer (5X)	10	1X
dNTPs (25 mM each)	0.4	200 μ M each
Primer IS4 (10 μ M)	1	200 nM
Phusion Hot Start High-Fidelity DNA Polymerase (2 U/ μ L)	0.5	0.02 U/ μ L
Add separately to each well:		
Indexing primer (10 μ M)	1	200 nM
Template DNA (library)	x	

If large amounts of sample DNA were used for library preparation (>100 ng), only a fraction of the library containing the equivalent of ~100 ng of starting material should be used for indexing PCR in order to prevent saturation of the PCR with template DNA.

23. Mix and perform cycling using the following temperature profile:

Initial denaturation	98°C	30 sec
Denaturation/cycle	98°C	10 sec
Annealing/cycle	60°C	20 sec
Elongation/cycle	72°C	20 sec
Final extension	72°C	10 min

The optimal number of PCR cycles, that is, the number of cycles required to reach PCR plateau, will depend on the amount and concentration of template DNA and can be directly inferred from the amplification plots of the qPCR (Step 21). The cycle number can also be adjusted by rule of thumb according to the lowest amount of sample DNA that was used for library preparation: >100 ng → 12 cycles; >10 ng → 16 cycles, >1 ng → 20 cycles, >100 pg → 24 cycles.

24. Perform reaction purification exactly as described in Steps 6-13. Elute the indexed libraries in 25 µL of EBT.

25. Load 3 µL of some of the PCR products on a 2% agarose gel to verify amplification success.

Indexed libraries prepared from sheared DNA should produce a smear. Due to the formation of heteroduplexes in the plateau phase of PCR (Ruano and Kidd 1992), the fragment size distribution inferred from the agarose gel may deviate slightly from the true distribution. However, no low-molecular-weight artifacts, such as primer dimers or adapter dimers, should be visible in the indexed sample libraries.

See Troubleshooting.

26. Determine the DNA concentration, and pool the indexed libraries in equimolar ratios.

The pool of indexed libraries is now ready for target capture or direct sequencing on one of Illumina's sequencing platforms.

Due to the presence of heteroduplexes, qPCR is the only means of exactly determining the DNA concentrations in indexed libraries. However, concentration estimates derived from measurements with a spectrophotometer are sufficient in this step and more convenient. End product yield of indexing PCR is usually similar for all samples, particularly if there are no major differences in fragment size distribution. If this is the case, as can be confirmed by measuring DNA concentrations in a subset of indexed libraries, pooling equal volumes of all libraries will be sufficient.

Target Capture and/or Sequencing on the Illumina Platform

27. For target capture on microarrays, follow, for example, the exact procedure given in the protocol of Hodges et al. (2009) with the following modifications:

- i. Use a different set of blocking oligos (BO1-BO6).
- ii. Use primers IS5 and IS6 at an annealing temperature of 60°C for amplifying the library pool after capture.

28. For sequencing and data analysis, use the recipes, kits, and analysis tools for multiplex sequencing provided by Illumina.

A tool for splitting up the qseq sequence files according to indexes is available in CASAVA 1.6 and later versions (demultiplex.pl). However, when using the 7-nt index sequences given in this protocol, the —qseq-mask parameter must be set to seven (the default is six). No modifications to the recipes provided by the Illumina machine control software (SCS) are required, because seven cycles of index sequencing are carried out by default. Additional software for data analysis on FastQ files (SplitFastQIndex.py), a file format created for example by the alternative base caller Ibis (Kircher et al. 2009), is provided on <http://bioinf.eva.mpg.de>. If single mismatches are allowed during index identification, the fraction of unidentified index sequences typically reduces to ~5%, as compared to ~15% when a perfect match is required. Using alternative base callers like Alta-Cyclic (Erich et al. 2008), BayesCall (Kao et al. 2009), or IBIS (Kircher et al. 2009) may also increase the fraction of correctly identified indexes.

Indexed sequencing libraries are compatible with all capture methods requiring sequencing libraries. It is recommended to carry the blank library all the way through target capture and/or sequencing. To avoid cross-contamination of samples through jumping PCR (Meyerhans et al. 1990), pools of indexed libraries should be amplified with a minimum number of PCR cycles or sequenced without amplification if possible.

See Troubleshooting.

TROUBLESHOOTING

Problem: No size shift of the positive control library is visible on the agarose gel, or the size shift is incomplete.

[Step 20]

Solution: Consider the following:

1. Make sure the positive control PCR was generated using primers with unmodified 5'-ends.
2. One of the enzymes may have degraded. Replace all the enzymes and repeat.

Problem: The positive control library shows no band on the agarose gel.

[Step 20]

Solution: Verify that the SPRI bead suspension is functional and the size cutoff is appropriate, for example, by purifying a DNA ladder as described in Steps 6-13.

Problem: Artifact bands are visible on the agarose gel after indexing PCR.

[Step 25]

Solution: If enough sample DNA was used for library preparation, artifact bands are only expected from the blank control. Repeat library preparation using more sample DNA or reduce the amount of adapters to 0.2 µL per reaction. Make sure a hot start polymerase was used for the indexing PCR.

Problem: Sequencing results in a low percentage of reads with correct index sequences.

[Step 28]

Solution: On the Genome Analyzer II, up to 5% of the raw sequences can generally be expected to be artifacts and up to 25% of low quality. If “N” base calls are more frequent in the index read than the other sequencing read(s), image analysis and downstream base calling partially or completely failed due to unbalanced usage of nucleotides or laser channels. Prepare a new pool of libraries with a more balanced composition of indexes (see Step 22) and repeat sequencing.

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Appendix 1: Recipes

[NOTE: This print edition of *CSH Protocols* contains only recipes for reagents requiring multiple components or non-obvious critical steps. Recipes for reagents marked with the <R> symbol not listed below can be found online at <http://www.cshprotocols.org/recipes>.]

Acrylamide stock for RNA gels (40%)

Reagent	Quantity (for 500 mL)	Final concentration
<R>Acrylamide (electrophoresis grade)	190 g	38% (w/v)
<R>Bisacrylamide (electrophoresis grade)	10 g	2% (w/v)
H ₂ O, RNase-free	to 500 mL	—

Filter with a 0.45-μm filter and store at 4°C in a dark bottle.
Final solution is 19:1 acrylamide:bisacrylamide.

Ammonium sulfate cut dilution buffer (ASCDB)

<R>20 mM Tris-Cl (pH 7.2)
<R>1 mM DTT (dithiothreitol)
Prepare fresh and keep at 4°C.

Buffer A (FPLC Mono Q)

<R>1 mM DTT (dithiothreitol)
<R>50 mM Tris-Cl (pH 7.2)
Prepare fresh and keep at 4°C.

Buffer B (FPLC Mono Q)

<R>1 mM DTT (dithiothreitol)
1 M NaCl
<R>50 mM Tris-Cl (pH 7.2)
Prepare fresh and keep at 4°C.

Cell disruption buffer

Reagent	Quantity (for 500 mL)	Final concentration
KCl (2 M)	2.5 mL	10 mM
<R><R>MgCl ₂ (1 M)	0.75 mL	1.5 mM
<R>Tris-Cl (1 M, pH 7.5)	10 mL	20 mM
H ₂ O, RNase-free	486.75 mL	—

<R>Immediately before use, for each 10 mL of buffer needed, add 10 μL of DTT (dithiothreitol; 1 M) to give a final concentration of 1 mM. Discard any unused DTT-containing buffer.
Prepare all reagents used to make this buffer with RNase-free H₂O.

CsCl gradient solutions

<I>CsCl

<R>0.02 M Tris-Cl (pH 7.4)

For a 1.28 g/mL solution, dissolve 30 g of solid CsCl in 70 mL of 0.02 M Tris-Cl (pH 7.4).

For a 1.50 g/mL solution, dissolve 40 g of solid CsCl in 54 mL of 0.02 M Tris-Cl (pH 7.4).

Weigh 1-mL aliquots of each solution on an analytical balance to confirm densities. Filter-sterilize through a 0.22- μ m filter.

Disruption buffer

<I>5 μ g/mL chymostatin

<I>2 mM DTT (dithiothreitol)

<R>10 mM EDTA

40 mM HEPES (pH 7.2)

<I>10 μ g/mL leupeptin

5 μ g/mL pepstatin

Prepare fresh and keep at 4°C.

DNase inactivation buffer (2X)

Reagent	Quantity (for 10 mL)	Final concentration (for 2X)
<I>SDS (10%)	1 mL	1%
<R>EDTA (0.5 M, pH 8)	20 μ L	1 mM
<R>Tris-Cl (1 M, pH 7.5)	200 μ L	20 mM
H ₂ O, RNase-free	8.78 mL	—

All reagents used to make this buffer should be prepared with RNase-free H₂O. Store indefinitely at room temperature, but remake if cloudy (bacteria or mold contamination) or there is visible dust.

Double detergent

<I>5% deoxycholate

<I>10% Triton X-100

EBT buffer

<R>10 mM Tris-Cl, pH 8.0 or 8.5 (identical to QIAGEN's EB buffer)

0.05% Tween 20

EDTA

<I>EDTA (ethylenediaminetetraacetic acid)

<I>NaOH

To prepare EDTA at 0.5 M (pH 8.0): Add 186.1 g of disodium EDTA•2H₂O to 800 mL of H₂O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ~8.0 by the addition of NaOH.

FPLC gel filtration buffer

<1 mM DTT (dithiothreitol)
50 mM HEPES (pH 7.2)
250 mM KCl
0.2 mg/mL rcm-BSA (bovine serum albumin, reduced, carboxymethylated)
Prepare fresh and keep at 4°C.

FPLC wash buffer

<1 mM DTT (dithiothreitol)
10% glycerol
50 mM HEPES (pH 7.2)
Prepare fresh and keep at 4°C.

Growth medium (pH 7.4) (DMEM-10)

Dulbecco's modified Eagle's medium (DMEM; GIBCO/Invitrogen)
10% normal calf serum
<1% penicillin/streptomycin (Cellgro)
1% L-glutamine (Cellgro)
Filter-sterilize through a 0.22-μm filter.

HEPES-buffered saline (HEBS; 2X)

Dextrose (12 mM)
HEPES (50 mM)
KCl (10 mM)
NaCl (280 mM)
Na₂HPO₄•2H₂O (1.5 mM)
Adjust the pH to 7.05 with 10 N NaOH; accurate pH is critical for efficient transfection. Sterilize by filtration through a 0.45-μm nitrocellulose filter. Store at -20°C.

Maintenance medium (pH 7.4) (DMEM-2)

Dulbecco's modified Eagle's medium (DMEM; GIBCO/Invitrogen)
2% fetal bovine serum (Cellgro)
<1% penicillin/streptomycin (Cellgro)
1% L-glutamine (Cellgro)
Filter-sterilize through a 0.22-μm filter.

MgCl₂ (Magnesium chloride)

To prepare 1 L of 1 M MgCl₂, dissolve 203.3 g of MgCl₂•6H₂O in 800 mL H₂O. Adjust the volume to 1 L using H₂O. Dispense into aliquots and sterilize by autoclaving.
Note: MgCl₂ is extremely hygroscopic. Buy small bottles and do not store opened bottles for long periods of time. Once the crystals become saturated with H₂O, dispose of the chemical properly.

MS medium (1X)

Component	Amount to add to make 1 L
MS salts	4.33 g
Bacto agar	7 g
H ₂ O	To make 1 L

Combine MS salts and H₂O, and stir to dissolve. Adjust pH to 5.7 with KOH. Add agar. Sterilize by autoclaving. Plants grow more quickly in MS containing 1% (w/v) sucrose, but so do microorganisms: Including sucrose in the medium significantly increases the chance of microbial contamination.

Neutral red staining solution

<1>10 mM 2-(N-morpholino)ethanesulfonic acid-Tris (MES-Tris; pH 6.5)
250 mM sucrose
<1>80 µM neutral red in H₂O

Nondenaturing loading dye (10X)

Reagent	For 10 mL	Final concentration
Ficoll 400	2.5 g	25%
<1>Bromophenol blue	25 mg	0.25%
H ₂ O, RNase-free	To 10 mL	—

Store at room temperature indefinitely.

Oligo hybridization buffer (10X)

500 mM NaCl
<R>10 mM Tris-Cl, pH 8.0
<R>1 mM EDTA, pH 8.0

Phosphate-buffered saline (PBS)

Reagent	Amount to add (for 1X solution)	Final concentration (1X)	Amount to add (for 10X stock)	Final concentration (10X)
NaCl	8 g	137 mM	80 g	1.37 M
<1>KCl	0.2 g	2.7 mM	2 g	27 mM
Na ₂ HPO ₄	1.44 g	10 mM	14.4 g	100 mM
KH ₂ PO ₄	0.24 g	1.8 mM	2.4 g	18 mM

If necessary, PBS may be supplemented with the following:

<1>CaCl ₂ •2H ₂ O	0.133 g	1 mM	1.33 g	10 mM
<1>MgCl ₂ •6H ₂ O	0.10 g	0.5 mM	1.0 g	5 mM

<1>PBS can be made as a 1X solution or as a 10X stock. To prepare 1 L of either 1X or 10X PBS, dissolve the reagents listed above in 800 mL of H₂O. Adjust the pH to 7.4 (or 7.2, if required) with HCl, and then add H₂O to 1 L. Dispense the solution into aliquots and sterilize them by autoclaving for 20 min at 15 psi (1.05 kg/cm²) on liquid cycle or by filter sterilization. Store PBS at room temperature.

Ringer's solution

0.125 M sodium chloride
<!--1.5 mM calcium chloride, dihydrate
5 mM potassium chloride
0.8 mM sodium phosphate, dibasic
Adjust the pH to 7.4. Filter-sterilize.

RNA gel-loading buffer (1.5X)

| Reagent | Quantity (for 10 mL of 1.5X) | Final concentration |
|----------------------------------|------------------------------|---------------------|
| <!--Formamide, ultrapure | 9.5 mL | 95% |
| <!--Bromophenol blue (2.5%, w/v) | 100 µL | 0.025% |
| <!--Xylene cyanol FF (2.5%, w/v) | 100 µL | 0.025% |
| <R>EDTA (0.25 M, pH 8.0) | 200 µL | 5 mM EDTA |

Use 5 µL for a 2.5-µL sample. Purchase a distilled, deionized preparation of formamide and the above loading dyes. Store in small aliquots at -20°C. This solution is available commercially (Ambion) and is recommended over homemade. Make 1-mL aliquots. Store for up to 1 yr at -20°C.

RQ1 DNase reaction buffer

400 mM Tris (pH 8.0)
<!--100 mM MgSO₄
<!--10 mM CaCl₂
Aliquot (1 µL of buffer is used per reaction) and store frozen.

SDS solubilization buffer (1X)

| Reagent | Quantity (for 500 mL) | Final concentration |
|---------------------------------------|-----------------------|---------------------|
| <R>EDTA (500 mM, pH 8.0) | 1 mL | 1 mM |
| <!--SDS (sodium dodecyl sulfate; 10%) | 25 mL | 0.5% |
| <R>Tris-Cl (1 M, pH 7.5) | 10 mL | 20 mM |
| H ₂ O, RNase-free | 464 mL | — |

Store for up to 1 yr at room temperature.

SDS solubilization buffer (10X)

| Reagent | Quantity (for 500 mL) | Final concentration |
|---------------------------------------|-----------------------|---------------------|
| <R>EDTA (500 mM, pH 8.0) | 10 mL | 10 mM |
| <!--SDS (sodium dodecyl sulfate; 10%) | 250 mL | 5% |
| <R>Tris-Cl (1 M, pH 7.5) | 100 mL | 200 mM |
| H ₂ O, RNase-free | 140 mL | — |

Store indefinitely at room temperature.

TBE electrophoresis buffer (10X)

| Reagent | Quantity (for 1 L) | Final concentration |
|----------------------|--------------------|---------------------|
| Tris base | 121.1 g | 1 M |
| Boric acid | 61.8 g | 1 M |
| EDTA (disodium salt) | 7.4 g | 0.02 M |

Prepare with RNase-free H₂O. Dilute 100 mL to 1 L to make gel running buffer. Store for up to 6 mo at room temperature.

TE buffer

| Reagent | Quantity (for 100 mL) | Final concentration |
|-----------------------|-----------------------|---------------------|
| EDTA (0.5 M, pH 8.0) | 0.2 mL | 1 mM |
| Tris-Cl (1 M, pH 8.0) | 1 mL | 10 mM |
| H ₂ O | to 100 mL | |

TE (pH 7.5)

| Reagent | Final concentration |
|------------------|---------------------|
| Tris-Cl (pH 7.5) | 10 mM |
| EDTA (pH 8.0) | 1 mM |

TET buffer

TE buffer
0.05% Tween 20

Tris-Cl

Tris base
HCl

To prepare a 1 M solution, dissolve 121.1 g of Tris base in 800 mL of H₂O. Adjust the pH to the desired value by adding concentrated HCl.

| pH | HCl |
|-----|-------|
| 7.4 | 70 mL |
| 7.6 | 60 mL |
| 8.0 | 42 mL |

Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 L with H₂O. Dispense into aliquots and sterilize by autoclaving.

If the 1 M solution has a yellow color, discard it and obtain Tris of better quality. The pH of Tris solutions is temperature-dependent and decreases ~0.03 pH units for each 1°C increase in temperature. For example, a 0.05 M solution has pH values of 9.5, 8.9, and 8.6 at 5°C, 25°C, and 37°C, respectively.

Ubiquitination mix

50 mg/mL creatine phosphokinase
1 mM DTT (dithiothreitol)
5 mM MgCl₂
1 pmol E1

5 pmol E2-C
<1>1-2 pmol (~10⁵ cpm) of ¹²⁵I-labeled cyclin B (13-91)/protein A
10 mM phosphocreatine
1 mg/mL rcM-BSA (bovine serum albumin, reduced, carboxymethylated)
<R>40 mM Tris-Cl (pH 7.6)
<1>50 μM ubiquitin
<1>1 μM ubiquitin aldehyde
Prepare fresh and keep at 4°C.

E1 was purified from human cells utilizing the method described in Hershko et al. (Hershko A, Heller H, Elias S, Ciechanover A. 1983. Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. *J Biol Chem* **258**: 8206-8214).

Recombinant E2-C was expressed in bacteria as described in Aristarkhov et al. (Aristarkhov A, Eytan E, Moghe A, Admon A, Hershko A, Ruderman JV. 1996. E2-C, a cyclin-selective ubiquitin carrier protein required for the destruction of mitotic cyclins. *Proc Natl Acad Sci* **93**: 4294-4299) and purified by gel filtration on a Superdex FPLC column.

Sea urchin cyclin B derivative cyclin B(13-91)/protein A was obtained and iodinated as described by Glotzer et al. (Glotzer M, Murray AW, Kirschner MW. 1991. Cyclin is degraded by the ubiquitin pathway. *Nature* **349**: 132-138).

Appendix 2: Cautions

[NOTE: For reagents marked with the <!> symbol not listed below, please consult the manufacturer's Material Safety Data Sheet for further information. Researchers using the procedures in these protocols contained in this issue of *CSH Protocols* do so at their own risk. Cold Spring Harbor Laboratory makes no representations or warranties with respect to the material set forth in these protocols and has no liability in connection with the use of these materials. Materials used in these protocols may be considered hazardous and should be used with caution.]

Ammonium persulfate ($(\text{NH}_4)_2\text{S}_2\text{O}_8$) is extremely destructive to tissue of the mucous membranes and upper respiratory tract, eyes, and skin. Inhalation may be fatal. Wear appropriate gloves, safety glasses, and protective clothing. Always use in a chemical fume hood. Wash thoroughly after handling.

Bisacrylamide is a potent neurotoxin and is absorbed through the skin (the effects are cumulative). Avoid breathing the dust. Wear appropriate gloves and a face mask when weighing powdered acrylamide and methylene-bisacrylamide.

Boric acid (H_3BO_3) may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and goggles.

Bromophenol blue may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses and use in a chemical fume hood.

CaCl_2 (Calcium chloride) is hygroscopic and may cause cardiac disturbances. It may be harmful by inhalation, ingestion, or skin absorption. Do not breathe the dust. Wear appropriate gloves and safety goggles.

Chloroform (CHCl_3) is irritating to the skin, eyes, mucous membranes, and respiratory tract. It is a carcinogen and may damage the liver and kidneys. It is also volatile. Avoid breathing the vapors. Wear appropriate gloves and safety glasses. Always use in a chemical fume hood.

Chloroform:isoamyl alcohol Chloroform (CHCl_3) is irritating to the skin, eyes, mucous membranes, and respiratory tract. It is a carcinogen and may damage the liver and kidneys. It is also volatile. Avoid breathing the vapors. Wear appropriate gloves and safety glasses. Always use in a chemical fume hood.

Isoamyl alcohol may be harmful by inhalation, ingestion, or skin absorption and presents a risk of serious damage to the eyes. Wear appropriate gloves and safety goggles. Keep away from heat, sparks, and open flame.

Chymostatin is an irritant and may be harmful by inhalation, ingestion, or skin absorption. Do not breathe the dust.

CsCl (Cesium chloride) may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses.

Deoxycholate (DOC) may be harmful by inhalation, ingestion, or skin absorption. Do not breathe the dust. Wear appropriate gloves and safety glasses.

DMSO (Dimethyl sulfoxide) may be harmful by inhalation or skin absorption. Wear appropriate gloves and safety glasses. Use in a chemical fume hood. DMSO is also combustible. Store in a tightly closed container. Keep away from heat, sparks, and open flame.

Dry ice (Carbon dioxide; CO_2) CO_2 (carbon dioxide; dry ice) in all forms may be fatal by inhalation, ingestion, or skin absorption. In high concentrations, it can paralyze the respiratory center and cause suffocation. Use only in well-ventilated areas. In the form of dry ice, contact with carbon dioxide can also cause frostbite. Do not place large quantities of dry ice in enclosed areas such as cold rooms. Wear appropriate gloves and safety goggles.

DTT (Dithiothreitol) is a strong reducing agent that emits a foul odor. It may be harmful by inhalation, ingestion, or skin absorption. When working with the solid form or highly concentrated stocks, wear appropriate gloves and safety glasses and use in a chemical fume hood.

EDTA (Ethylenediaminetetraacetic acid) may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses. Severe overexposure can result in death.

Emetine is very toxic and may be fatal if inhaled or swallowed. It is harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety goggles. Do not breathe the dust.

Ethidium bromide is a powerful mutagen and is toxic. Consult the local institutional safety officer for specific handling and disposal procedures. Avoid breathing the dust. Wear appropriate gloves when working with solutions that contain this dye.

Fixatives Follow manufacturer's safety guidelines.

Formamide is teratogenic. The vapor is irritating to the eyes, skin, mucous membranes, and upper respiratory tract. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses. Always use in a chemical fume hood when working with concentrated solutions of formamide. Keep working solutions covered as much as possible.

HCl (Hydrochloric acid, Hydrochloride) is volatile and may be fatal if inhaled, ingested, or absorbed through the skin. It

is extremely destructive to mucous membranes, upper respiratory tract, eyes, and skin. Wear appropriate gloves and safety glasses. Use with great care in a chemical fume hood. Wear goggles when handling large quantities.

Isopropanol (Isopropyl alcohol, 2-Propanol) is flammable and irritating. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses. Do not breathe the vapor. Keep away from heat, sparks, and open flame.

Isotope ^{125}I accumulates in the thyroid and is a potential health hazard. Consult the local radiation safety office for further guidance in the appropriate use and disposal of radioactive materials. Wear appropriate gloves when handling radioactive substances. The $^{125}\text{I}_2$ formed during oxidation of Na^{125}I is volatile. Work in an approved chemical fume hood with a charcoal filter when exposing the Na^{125}I to oxidizing reagents such as chloramine-T, IODO-GEN, or acids. Because the oxidation proceeds very rapidly and releases large amounts of volatile $^{125}\text{I}_2$ when chloramine-T is used, it is important to be well prepared for each step of the reaction, so that the danger of contamination from volatile radiation can be minimized. Shield all forms of the isotope with lead. When handling the isotope, wear one or two pairs of appropriate gloves, depending on the amount of isotope being used and the difficulty of the manipulation required.

Leupeptin (or its hemisulfate) may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses and use in a chemical fume hood.

Liquid nitrogen (LN_2) can cause severe damage due to its extreme temperature. Handle frozen samples with extreme caution. Do not breathe the vapors. Seepage of liquid nitrogen into frozen vials that are immersed in liquid nitrogen can cause the vials to explode when they are removed. Use vials with O-rings when possible. Wear cryo-mitts and a face mask. Do not allow the liquid nitrogen to spill onto clothing. Do not breathe the vapors.

KCl (Potassium chloride) may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses.

MES (2-[N-morpholino]ethanesulfonic acid) may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses.

MgCl_2 (Magnesium chloride) may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses and use in a chemical fume hood.

MgSO_4 (Magnesium sulfate) presents chronic health hazards and affects the central nervous system and the gastrointestinal tract. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses and use in a chemical fume hood.

N,N,N',N'-Tetramethylethylenediamine (TEMED) is highly caustic to the eyes and mucus membranes and may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and tightly sealed safety goggles.

NaOH (Sodium hydroxide) and solutions containing NaOH are highly toxic and caustic and should be handled with great care. Wear appropriate gloves and a face mask. All concentrated bases should be handled in a similar manner.

Neutral red may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses.

Nitric acid (HNO_3) is volatile and must be handled with great care. It is toxic by inhalation, ingestion, and skin absorption. Wear appropriate gloves and safety goggles. Use in a chemical fume hood. Do not breathe the vapors. Keep away from heat, sparks, and open flame.

Nitrogen (gaseous or liquid) may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses. Consult your local safety office for proper precautions.

Nocodazole is a possible mutagen. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety goggles.

Phenol is extremely toxic, highly corrosive, and can cause severe burns. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves, goggles, and protective clothing. Always use in a chemical fume hood. Rinse any areas of skin that come in contact with phenol with a large volume of water and wash with soap and water; do not use ethanol!

Phenol:chloroform Phenol is extremely toxic, highly corrosive, and can cause severe burns. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves, goggles, and protective clothing. Always use in a chemical fume hood. Rinse any areas of skin that come in contact with phenol with a large volume of water and wash with soap and water; do not use ethanol!

Chloroform (CHCl_3) is irritating to the skin, eyes, mucous membranes, and respiratory tract. It is a carcinogen and may damage the liver and kidneys. It is also volatile. Avoid breathing the vapors. Wear appropriate gloves and safety glasses. Always use in a chemical fume hood.

Phenol:chloroform:isoamyl alcohol Phenol is extremely toxic, highly corrosive, and can cause severe burns. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves, goggles and protective clothing. Always use in a chemical fume hood. Rinse any areas of skin that come in contact with phenol with a large volume of water and wash with soap and water; do not use ethanol!

Chloroform (CHCl_3) is irritating to the skin, eyes, mucous membranes, and respiratory tract. It is a carcinogen and may

damage the liver and kidneys. It is also volatile. Avoid breathing the vapors. Wear appropriate gloves and safety glasses. Always use in a chemical fume hood.

Isoamyl alcohol may be harmful by inhalation, ingestion, or skin absorption and presents a risk of serious damage to the eyes. Wear appropriate gloves and safety goggles. Keep away from heat, sparks, and open flame.

Proteinase K is an irritant and may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses.

SDS (Sodium dodecyl sulfate) is toxic, an irritant, and poses a risk of severe damage to the eyes. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety goggles. Do not breathe the dust.

Streptomycin is toxic and a suspected carcinogen and mutagen. It may cause allergic reactions. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses.

SYBR Gold is supplied by the manufacturer as a 10,000-fold concentrate in DMSO which transports chemicals across the skin and other tissues. Wear appropriate gloves and safety glasses and decontaminate according to Safety Office guidelines. See DMSO.

Tris base may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses.

Triton X-100 causes severe eye irritation and burns. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety goggles.

Trizol may be fatal if absorbed through the skin, inhaled, or swallowed. It can also cause severe burns. Wear appropriate gloves, safety goggles, protective clothing, and always use in a chemical fume hood. Rinse any areas of skin that come in contact with trizol with a large volume of water and wash with soap and water; do not use ethanol!

Trypsin may cause an allergic respiratory reaction. It may be harmful by inhalation, ingestion, or skin absorption. Do not breathe the dust. Wear appropriate gloves and safety goggles. Use with adequate ventilation.

Ubiquitin (Ubiquitin aldehyde) is an irritant and may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses.

Urea may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses.

UV light and/or UV radiation is dangerous and can damage the retina of the eyes. Never look at an unshielded UV light source with naked eyes. Examples of UV light sources that are common in the laboratory include handheld lamps and transilluminators. View only through a filter or safety glasses that absorb harmful wavelengths. UV radiation is also mutagenic and carcinogenic. To minimize exposure, make sure that the UV light source is adequately shielded. Wear protective appropriate gloves when holding materials under the UV light source.

Xylene cyanol is an irritant and may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses. Do not breathe the dust.

| Oligo ID | Oligo sequence (5'-3')
(index marked with small letters) | Index sequence
(5'-3') |
|------------|---|---------------------------|
| indexing1 | CAAGCAGAAGACGGCATAACGAGATcctgcgaGTGACTGGAGTTCAGACGTGT | TCGCAGG |
| indexing2 | CAAGCAGAAGACGGCATAACGAGATtgcagagGTGACTGGAGTTCAGACGTGT | CTCTGCA |
| indexing3 | CAAGCAGAAGACGGCATAACGAGATacctaggGTGACTGGAGTTCAGACGTGT | CCTAGGT |
| indexing4 | CAAGCAGAAGACGGCATAACGAGATttgatccGTGACTGGAGTTCAGACGTGT | GGATCAA |
| indexing5 | CAAGCAGAAGACGGCATAACGAGATatcttgcGTGACTGGAGTTCAGACGTGT | GCAAGAT |
| indexing6 | CAAGCAGAAGACGGCATAACGAGATtctccatGTGACTGGAGTTCAGACGTGT | ATGGAGA |
| indexing7 | CAAGCAGAAGACGGCATAACGAGATcatcgagGTGACTGGAGTTCAGACGTGT | CTCGATG |
| indexing8 | CAAGCAGAAGACGGCATAACGAGATttcgagcGTGACTGGAGTTCAGACGTGT | GCTCGAA |
| indexing9 | CAAGCAGAAGACGGCATAACGAGATagtgtgtGTGACTGGAGTTCAGACGTGT | ACCAACT |
| indexing10 | CAAGCAGAAGACGGCATAACGAGATgtaccggGTGACTGGAGTTCAGACGTGT | CCGGTAC |
| indexing11 | CAAGCAGAAGACGGCATAACGAGATcggagttGTGACTGGAGTTCAGACGTGT | AACTCCG |
| indexing12 | CAAGCAGAAGACGGCATAACGAGATactcaaGTGACTGGAGTTCAGACGTGT | TTGAAGT |
| indexing13 | CAAGCAGAAGACGGCATAACGAGATtgatagtGTGACTGGAGTTCAGACGTGT | ACTATCA |
| indexing14 | CAAGCAGAAGACGGCATAACGAGATgatccaaGTGACTGGAGTTCAGACGTGT | TTGGATC |
| indexing15 | CAAGCAGAAGACGGCATAACGAGATcaggtcgGTGACTGGAGTTCAGACGTGT | CGACCTG |
| indexing16 | CAAGCAGAAGACGGCATAACGAGATcgcattaGTGACTGGAGTTCAGACGTGT | TAATGCG |
| indexing17 | CAAGCAGAAGACGGCATAACGAGATggtacctGTGACTGGAGTTCAGACGTGT | AGGTACC |
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