Roche

Sequence Capture

NimbleGen

Technical Note

Targeted Sequencing with NimbleGen SeqCap EZ Libraries and Illumina TruSeq DNA Sample Preparation Kits

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Introduction

The instructions provided in this document are intended only as general guidelines for the use of Illumina TruSeq DNA libraries in NimbleGen Sequence Capture experiments. Because this draft protocol has not been comprehensively validated, results are not guaranteed.

Targeted sequencing has become an important tool for researchers in the areas of genetic disease and cancer to identify potentially causative mutations in disease associated regions or exomes. NimbleGen in-solution Sequence Capture technologies enable researchers to capture and efficiently sequence targeted regions in the human genome ranging from 100kb to 50Mb. Utilizing up to 2.1 million biotinylated oligonucleotide probes that are selected using a proprietary algorithm, SeqCap EZ Libraries offer highly specific capture and uniform coverage of target regions. SeqCap EZ Libraries have been fully evaluated for use with the 454 and Illumina sequencing platforms.

Current NimbleGen SeqCap EZ Library SR User's Guides require researchers to use Illumina Paired-End Genomic DNA Sample Preparation Kits in the sequence capture workflow. However, Illumina recently introduced TruSeq DNA Sample Preparation Kits, which provide several improvements:

- Indexed (bar-coded) sample DNA libraries are generated in a single step, and these libraries can be sequenced in single-end or paired-end fashion.
- A consolidated workflow that requires a lower amount of input DNA.
- Multiple libraries with different indices can be combined and sequenced together (because TruSeq libraries are indexed by default).

Roche NimbleGen has developed a Sequence Capture protocol that is compatible with Illumina's TruSeq DNA Sample Preparation Kits, and we present targeted sequencing results with the new protocol in this technical note.

Results

A SeqCap EZ Choice Library targeting a 250kb contiguous region plus 340kb of exons from human Chromosome 10 was used in this study. HapMap DNA sample NA12762, a male from the CEPH population (Utah residents with ancestry from northern and western Europe) was used for the experiments. This sample was selected because it had previously been analyzed by targeted enrichment on multiple sequencing platforms.

Two Illumina TruSeq sequencing libraries were each prepared from 1 µg of NA12762 genomic DNA, one with the index 5 adapter and the other with the index 6 adapter. SeqCap EZ Choice Library probes targeting the chr10 targets were used to perform sequence capture experiments according to an updated protocol (see Supplementary Information for details). Captured, amplified libraries were combined and sequenced using a single paired-end lane on an Illumina GAIIx sequencing instrument. Approximately equal numbers of raw reads (~ 40 million) were obtained for the two samples.

Unfiltered reads were mapped to the hg19 reference genome using SOAP2 v2.19. Reads that could not be unambiguously assigned to a single location in the genome were not mapped. Read pairs from possible PCR duplicated fragments were identified using both reads in a pair and only the highest quality pair was kept. SNP calling was performed using SoapSNP (v1.01 from Crossbow v0.1.3). Sequencing read coverage and SNP calling results are shown in Table 1.

About 63% of mapped reads were found to be unique, which is typical when over sampling reads from small target capture experiments. For the unique reads mapped to the hg19 genome, over 80% aligned to the targeted regions with at least one base of overlap, demonstrating high specificity. The mean and median coverage depths over the targeted bases were very high (~2000) due to the large amount of sequencing data generated and the high specificity of the capture. About 95.5% of the target bases were covered by at least one read, and about 94% of the target bases were covered by at least ten reads. The regions not covered were mostly repetitive regions where no capture probes were assigned. In fact, 88.5% of the initial target bases were covered by probes or located within 100bp of a probe, consistent with the final coverage data.

Mapping and Coverage

Platform	Capture 1 (TruSeq Library Index 5)	Captures 2 (TruSeq Library Index 6)
qPCR Measurement of Enrichment at Control Loci	603	732
Total Number of Raw Reads	40,347,884	39,926,838
Percent Raw Reads Mapped to Genome	89.6%	90.1%
Percent Mapped Reads that Are Unique	62.5%	62.6%
Number of Mapped and Unique Reads	22,591,564	22,542,044
Number of Reads on Target	18,305,033	18,318,033
Percent Reads on Target	81.0%	81.3%
Percent Bases with Coverage ≥ 1X	95.7%	95.5%
Percent Bases with Coverage ≥ 10X	94.0%	93.9%
Mean Fold Coverage Depth (X)	1,966	2,054
Median Fold Coverage Depth (X)	2,236	2,317
Sensitivity in Detecting Known SNPs	96.6% (230/239)	96.6% (230/239)
Specificity in Detecting Known SNPs	99.6%	99.6%

▲ **Figure 1: Mapping and coverage statistics of the two capture and sequencing experiments.**

SNP calls were compared to the 239 known HapMap SNPs in the target regions. We were able to detect 96.6% of known SNPs with a specificity of 99.6%.

When we looked at the per base coverage of exons, there was uniform coverage across nearly all the exons (Figure 1A). For the 250kb contig (Figure 1B), the coverage profile was consistent with probe locations ("capture_ target" track in the graph), and there was extended coverage for gap regions that were close to probes. The coverage of gap regions was due to the fact that DNA fragments in the TruSeq library were about 250-300bp in length, therefore successful capture of one end of the library fragment will generate useful sequence data up to 300bp away.

In addition, the two independent capture experiments show almost identical coverage profiles, demonstrating a reproducible capture process.

Finally, we made two additional TruSeq libraries with indices 4 and 7, captured them using the same target design as described above, and combined the two captured samples for sequencing within one lane on a HiSeq2000 instrument. The results (data not shown) were very similar to the results from samples with indices 5 and 6 on GAIIx presented above.

▲ **Figure 2: Coverage profile for target regions.** Top panel (A): exon regions; bottom panel (B): contiguous region. The tracks from the top to bottom: per base coverage for the index 5 TruSeq library (purple), per base coverage for the index 6 TruSeq library (cyan), capture target (blue, regions covered by probes), and primary target regions (yellow, the initial targets we sought to capture). To generate this plot, 10 million randomly sampled raw sequencing reads were used for each analysis.

Conclusion

We demonstrated the use of the NimbleGen SeqCap EZ Choice Library and Illumina TruSeq DNA Sample Preparation Kits to enrich and sequence 590kb target regions in the human genome in solution from HapMap DNA sample NA12762. We were able to achieve high performance (~80% reads on target, >90% target base covered by at least 10 reads) for four different libraries (with indices 4, 5, 6, 7) when sequenced using either the Illumina GAIIx or the HiSeq2000 sequencing instruments.

Sequence Capture

Technical Note

For life science research only. Not for use in diagnostic procedures.

SeqCap EZ Ordering Information

SeqCap EZ Human Exome Library	Target Regions	Pack Size	Cat. No.
SeqCap EZ Human Exome Library v2.0, 4 reactions	Coding Exons and miRNA exon	4	05 860 482 001
SeqCap EZ Human Exome Library v2.0, 48 reactions		48	05 860 504 001

SeqCap EZ Choice Library	Target Regions	Pack Size	Cat. No.
SeqCap EZ Choice Library, 12 reactions	100 kb-7 Mb human genomic regions	12	06 266 282 001
SeqCap EZ Choice Library, 24 reactions		24	06 266 304 001
SeqCap EZ Choice Library, 48 reactions		48	06 266 312 001
SeqCap EZ Choice Library, 96 reactions		96	06 266 339 001
SeqCap EZ Choice Library, 384 reactions		384	06 266 347 001
SeqCap EZ Choice Library, 960 reactions		960	06 266 355 001
SeqCap EZ Choice XL Library, 12 reactions	7 Mb-50 Mb human genomic regions	12	06 266 363 001
SeqCap EZ Choice XL Library, 24 reactions		24	06 266 371 001
SeqCap EZ Choice XL Library, 48 reactions		48	06 266 380 001
SeqCap EZ Choice XL Library, 96 reactions		96	06 266 398 001
SeqCap EZ Choice XL Library, 384 reactions		384	06 266 401 001
SeqCap EZ Choice XL Library, 960 reactions		960	06 266 517 001

Accessories Ordering Information

Product	Pack Size	Cat. No.
NimbleGen SeqCap EZ Hybridization and Wash Kit, 24 reactions	24	05 634 261 001
NimbleGen SeqCap EZ Hybridization and Wash Kit, 96 reactions	96	05 634 253 001

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Technical Note Supplement: Targeted Sequencing with NimbleGen SeqCap EZ Libraries and Illumina TruSeq DNA Sample Preparation Kits

Instructions for using Illumina TruSeq DNA Libraries with SeqCap EZ Libraries

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Description

This protocol was used to generate the data shown in the Technical Note: ***Targeted Sequencing with NimbleGen SeqCap EZ Libraries and Illumina TruSeq DNA Sample Preparation Kits***. Please note that the performance of targeted enrichment methods is variable due to population variability among genomes and other properties intrinsic to the targeted region(s). When using a previously untested capture design and protocol, it is recommended to perform a small-scale experiment to determine the capture characteristics before beginning a larger study.

Use this protocol in conjunction with the *NimbleGen SeqCap EZ Choice Library SR User's Guide v1.0* or later (available at *www.nimblegen.com/products/lit/*, under *Sequence Capture → SeqCap EZ Library Documents*). The *NimbleGen SeqCap EZ Choice Library SR User's Guide v1.0* provides instructions for performing Sequence Capture from libraries prepared with the ***Illumina Paired-End Genomic DNA Sample Prep Kit***. The instructions provided below allow the user to adapt the *NimbleGen SeqCap EZ Choice Library SR User's Guide v1.0* to perform Sequence Capture from libraries prepared with the newer ***Illumina TruSeq DNA Sample Preparation Kits*** (revision A, November 2010). This includes important changes to the LM-PCR and Hybridization steps. Additionally, a strategy is presented in an appendix to this document for performing Sequence Capture of individual, indexed libraries with pooling performed immediately prior to sequencing (*Appendix A*).

The following table guides you through the Sequence Capture protocol. It identifies when to follow instructions in the User's Guide and when to follow instructions in this document.

Protocol Step	Refer to the following:*
A. Before You Begin	Chapter 1 in the *User's Guide** and "Additional Reagents, Consumables & Equipment" section in this document.
B. Sample Requirements and QC	Chapter 2 in the *User's Guide*.
C. Sample Library Preparation	"Sample Library Preparation" section in this document
D. Sample Library Amplification Using LM-PCR	"Pre-Capture Sample Library Amplification Using LM-PCR" section in this document.
E. Hybridization of Sample and EZ Libraries	"Hybridization of Amplified Sample and EZ Probe Libraries" section in this document.
F. Washing and Recovery of Captured DNA	Chapter 6 in the *User's Guide*.
G. Captured DNA Amplification Using LM-PCR	"Post-Capture Sample Library Amplification Using LM-PCR" section in this document.
H. Measurement of Enrichment Using qPCR	Chapter 8 in the *User's Guide*.

* *User's Guide = NimbleGen SeqCap EZ Choice Library SR User's Guide v1.0* or later

Additional Reagents, Consumables & Equipment

Refer to the most recent version of the Illumina *TruSeq DNA Sample Preparation Guide* for additional standard laboratory equipment and materials required for use with the Illumina TruSeq DNA Sample Prep Kit (Set A: Catalog No. FC-121-1001; Set B: Catalog No. FC-121-1002) in addition to the materials listed in *Chapter 1* of the *NimbleGen SeqCap EZ Choice Library SR User's Guide v1.0* or later.

Component	Supplier	Package Size	Catalog No.
Illumina TruSeq DNA Sample Prep Kits	Illumina	48 preps	FC-121-1001 (Set A) - or – FC-121-1002 (Set B)

Oligonucleotide Consumables

Important Note: NimbleGen SeqCap EZ enrichment experiments utilizing Illumina TruSeq libraries require the use of *two* Hybridization Enhancing (HE) Oligos. TS-HE Oligo 1 is a universal blocking oligo used for all experiments regardless of which TruSeq index sequence is present in the library adapter. The second HE Oligo should be selected from among the twelve TS-HE Index Oligos listed in the table below, and should correspond to the specific TruSeq index adapter used in the experiment (*e.g.* To perform SeqCap EZ enrichment using an Illumina TruSeq library constructed with adapter AD001, you will need to use ***TS-HE Oligo 1*** and ***TS-HE Index 1 Oligo***; to perform SeqCap EZ enrichment from an Illumina TruSeq library constructed with adapter AD007, you will need to use ***TS-HE Oligo 1*** and ***TS-HE Index 7 Oligo***; etc.). *Please read the footnotes at the end of the table (on page 4) which explain the purposes of the Oligos listed.*

Component	Concentration	Sequence	Note(s)
TS-PCR Oligo 1[A]	100 µM	5'- AAT GAT ACG GCG ACC ACC GAG A – 3'	These oligos are used for both Pre-Capture and Post-Capture LM-PCR.
TS-PCR Oligo 2[A]	100 µM	5' - CAA GCA GAA GAC GGC ATA CGA G – 3'	
TS-HE Oligo 1[B, C]	1000 µM	5' – AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T – 3'	These oligos must be resuspended in molecular grade water.
TS-HE Index 1 Oligo[B, D]	1000 µM	5' – GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC **ATC ACG** ATC TCG TAT GCC GTC TTC TGC TTG – 3'	
TS-HE Index 2 Oligo[B, D]	1000 µM	5' - GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC **CGA TGT** ATC TCG TAT GCC GTC TTC TGC TTG – 3'	
TS-HE Index 3 Oligo[B, D]	1000 µM	5' – GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC **TTA GGC** ATC TCG TAT GCC GTC TTC TGC TTG – 3'	
TS-HE Index 4 Oligo[B, D]	1000 µM	5' – GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC **TGA CCA** ATC TCG TAT GCC GTC TTC TGC TTG – 3'	
TS-HE Index 5 Oligo[B, D]	1000 µM	5' - GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC **ACA GTG** ATC TCG TAT GCC GTC TTC TGC TTG – 3'	
TS-HE Index 6 Oligo[B, D]	1000 µM	5' - GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC **GCC AAT** ATC TCG TAT GCC GTC TTC TGC TTG – 3'	
TS-HE Index 7 Oligo[B, D]	1000 µM	5' – GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC **CAG ATC** ATC TCG TAT GCC GTC TTC TGC TTG – 3'	
TS-HE Index 8 Oligo[B, D]	1000 µM	5' - GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC **ACT TGA** ATC TCG TAT GCC GTC TTC TGC TTG – 3'	
TS-HE Index 9 Oligo[B, D]	1000 µM	5' – GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC **GAT CAG** ATC TCG TAT GCC GTC TTC TGC TTG – 3'	
TS-HE Index 10 Oligo[B, D]	1000 µM	5' – GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC **TAG CTT** ATC TCG TAT GCC GTC TTC TGC TTG – 3'	
TS-HE Index 11 Oligo[B, D]	1000 µM	5' – GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC **GGC TAC** ATC TCG TAT GCC GTC TTC TGC TTG – 3'	
TS-HE Index 12 Oligo[B, D]	1000 µM	5' – GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC **CTT GTA** ATC TCG TAT GCC GTC TTC TGC TTG – 3'	
qPCR NSC-0237, forward, Oligo	2 µM	5' - CGC ATT CCT CAT CCC AGT ATG - 3'	These oligos are used in qPCR analysis of enrichment.
qPCR NSC-0237, reverse, Oligo	2 µM	5' - AAA GGA CTT GGT GCA GAG TTC AG - 3'	
qPCR NSC-0247, forward, Oligo	2 µM	5' - CCC ACC GCC TTC GAC AT - 3'	
qPCR NSC-0247, reverse, Oligo	2 µM	5' - CCT GCT TAC TGT GGG CTC TTG - 3'	
qPCR NSC-0268, forward, Oligo	2 µM	5' - CTC GCT TAA CCA GAC TCA TCT ACT GT - 3'	
qPCR NSC-0268, reverse, Oligo	2 µM	5' - ACT TGG CTC AGC TGT ATG AAG GT - 3'	
qPCR NSC-0272, forward, Oligo	2 µM	5' - CAG CCC CAG CTC AGG TAC AG - 3'	
qPCR NSC-0272, reverse, Oligo	2 µM	5' – ATG ATG CGA GTG CTG ATG ATG - 3'	

Component	Concentration	Sequence	Note(s)

Oligonucleotides can be resuspended in PCR grade water or TE buffer unless otherwise noted.

[A] TS-PCR Oligo 1 and TS-PCR Oligo 2 are the same as the PE-POST 1 and PE-POST 2 oligos described in the SeqCap EZ Exome SR and SeqCap EZ Choice SR protocols.

[B] HE = "Hybridization Enhancing."

[C] TS-HE Oligo 1 is designed to block the universal portion of TruSeq library adapters during the sequence capture hybridization step. TS-HE Oligo 1 is the same as the PE-HE1 oligo described in the SeqCap EZ Exome SR and SeqCap EZ Choice SR protocols.

[D] TS-HE Index Oligos (1 through 12) are designed to block the indexed (variable) portion of the TruSeq library adapters during the sequence capture hybridization step. Nucleotides shown in red boldface text correspond to the variable region of adapters available in the current TruSeq DNA Sample Prep Kits (Kit A and Kit B): DNA Adapter Index 1 (AD001) through 12 (AD012).

Note: An alternative to the use of different specific TS-HE Index Oligos for capture from libraries prepared with different TruSeq library adapters is to use a generic HE oligo with deoxyinosine substituted for all six variable nucleotides in the TS-HE Index Oligos: 5' – GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC I*I*I* I*I*I* ATC TCG TAT GCC GTC TTC TGC TTG – 3' (I* indicates deoxyinosine). This generic HE oligo may be used as a substitute for any of the TS-HE Index Oligos in the hybridization step to decrease material costs and workflow complexity; however performance results may be reduced relative to experiments using specific TS-HE Index Oligos.

Sample Library Preparation

This section describes how to prepare the sample library. Sample library preparation using the Illumina TruSeq DNA Sample Preparation Kit requires 1 µg of genomic DNA input.

References

- Illumina TruSeq DNA Sample Preparation Guide (revision A, November 2010)

Step 1. Sample Library Preparation

1.1 Construct the sample library following the procedure described in the Illumina *TruSeq DNA Sample Preparation Guide*. Follow the protocol from "Fragment DNA" through "Ligate Adapters" with the following exceptions:

- The TruSeq library insert size range recommended by Illumina may not be optimal for all Sequence Capture applications. The user should consider the target type (i.e. small exons or larger contiguous regions) and the intended sequencing read length before selecting the optimal library insert size range for their experiment. Refer to Appendix A of the *NimbleGen SeqCap EZ Choice Library SR User's Guide v1.0* for an alternative sonication procedure for Illumina Paired-End Library Construction that can also be adapted for TruSeq library construction, however, note that results could vary if different amounts of DNA are used. The user will also need to modify the SPRI Bead volumes used when constructing their Illumina TruSeq libraries to obtain libraries either larger or smaller than recommended by the Illumina TruSeq protocol. Selecting a different insert size for your library construction may alter the expected size range of the Amplified Sample Library when examined with an Agilent DNA 1000 chip (Figure 1).
- Following the second purification step using Agencourt SPRI XP Beads, there is no need to perform the steps "Purify Ligation Products" or "Enrich DNA Fragments" prior to amplification via Pre-Capture LM-PCR.
- After the Illumina TruSeq DNA sample library has been constructed, proceed with 'Pre-Capture Sample Library Amplification Using LM-PCR' (this document).

Pre-Capture Sample Library Amplification Using LM-PCR

This section describes how to amplify the Illumina TruSeq adapter-ligated DNA library that was prepared in the previous "*Sample Library Preparation*" section, in preparation for hybridization to SeqCap EZ probe libraries. For each sample library to be captured, the entire sample library prepared in the "*Sample Library Preparation*" section is amplified via LM-PCR for use in capture (~20 µl total DNA).

References

- Phusion High-Fidelity PCR Master Mix with HF Buffer Kit protocol (New England Biolabs - Finnzymes)
- Thermocycler Manual
- QIAquick Spin Handbook (Qiagen)
- Agilent DNA 1000 Kit Guide

Step 1. Prepare the LM-PCR Master Mix

Important: *The LM-PCR Master Mix is temperature sensitive. Thawing of components and preparation of LM-PCR reactions must be performed on ice.*

1.1 Prepare the LM-PCR Master Mix in a 1.5 ml tube. The amount of each reagent needed for one reaction is listed below. Addition of a negative control reaction to monitor for reagent contamination or sample cross-contamination is optional but is recommended:

LM-PCR Master Mix	Amount
2x Phusion Master Mix with HF Buffer	50 µl
PCR grade water	26 µl
TS-PCR Oligo 1, 100 µM (Final Conc.: 2 µM)	2 µl
TS-PCR Oligo 2, 100 µM (Final Conc.: 2 µM)	2 µl
Total	**80 µl**

1.2 Pipette 80 µl of Pre-Capture LM-PCR Master Mix into each PCR tube or well.

1.3 Add the 20 µl of sample library (or PCR grade water for negative control) to the PCR tube or 96-well plate containing the LM-PCR Master Mix. Mix well by pipetting up and down 5 times.

Step 2. Perform PCR Amplification

2.1 Place the PCR tube (or 96-well PCR plate) in the thermocycler.

2.2 Amplify the sample library using the following LM-PCR program:

- Step 1: 30 seconds @ 98°C
- Step 2: 10 seconds @ 98°C
- Step 3: 30 seconds @ 60°C
- Step 4: 30 seconds @ 72°C
- Step 5: Go to Step 2, repeat 7 times
- Step 6: 5 minutes @ 72°C
- Step 7: Hold @ 4°C

2.3 Store the reaction at +2 to +8°C until ready for clean up, up to 72 hours.

Step 3. Clean up the Amplified Sample Library

3.1 Transfer each amplified sample library into one 1.5 ml microcentrifuge tube (approximately 100 µl). Process the negative control reaction, if included, in exactly the same way as the amplified sample library.

3.2 Follow the instructions provided with the Qiagen QIAquick PCR Purification Kit with the following modifications (listed below in Steps 3.3 - 3.9).

3.3 To each tube add 500 µl (5x) of Qiagen buffer PBI. Mix well by pipetting up and down 10 times.

3.4 Pipette the entire amplified sample library (in PBI) into a QIAquick PCR Purification Kit column.

3.5 Centrifuge at 10,000 x g for 30 - 60 seconds. Discard the flow-through.

3.6 Add 750 µl of PE buffer to the column. Centrifuge at 10,000 x g for 1 minute. Discard the flow-through.

3.7 Place the QIAquick column back in the same tube. Centrifuge the column for an additional minute.

3.8 Add 50 µl of PCR grade water directly to the column matrix. Transfer the column to a 1.5 ml microcentrifuge tube. Centrifuge at 10,000 x g for 1 minute to elute the DNA.

Important: *It is critical that the amplified sample library be eluted with PCR grade water and not buffer EB or 1X TE.*

Step 4. Check the Quality of the Amplified Sample Library

4.1 Measure the A_{260}/A_{280} ratio of the amplified sample library using a NanoDrop spectrophotometer to quantify the DNA concentration and determine the DNA quality.

- The A_{260}/A_{280} ratio should be 1.7 - 2.0.
- The sample library yield should be > 1.0 µg.
- The negative control yield should be negligible.

4.2 Run 1 µl of each amplified sample library, and the negative control, on an Agilent DNA 1000 chip. Run the chip according to manufacturer's instructions.

The Bioanalyzer should indicate that most of the fragments fall between 150 - 400 bp (Figure 2). The negative control should not show any significant signal, which could indicate contamination between amplified sample libraries.

4.3 If the amplified sample library meets these requirements, proceed to the "Hybridization of Amplified Sample and EZ Probe Libraries" section in this document. If the amplified sample library does not meet these requirements, reconstruct the library.

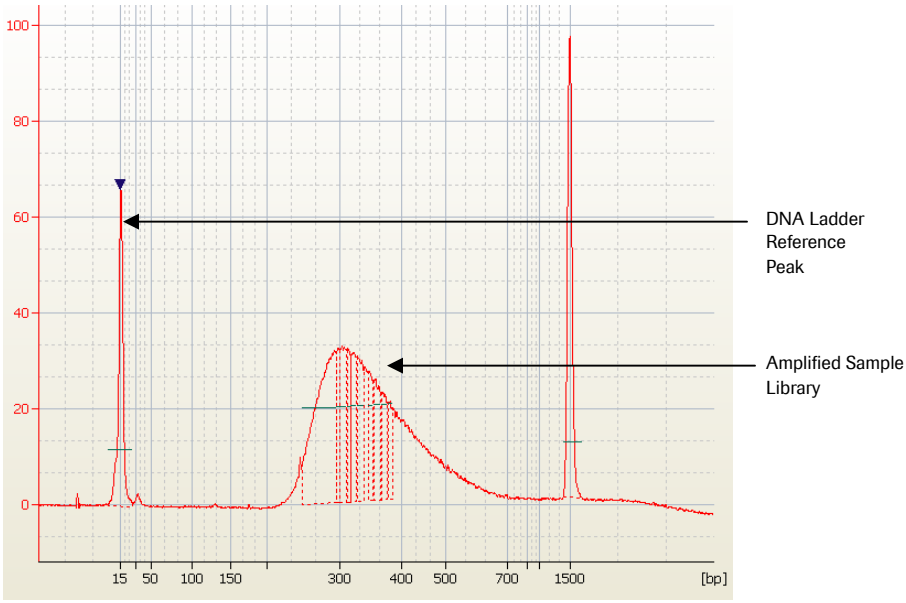


Figure 2: Example of an Amplified TruSeq Sample Library Analyzed Using an Agilent DNA 1000 Chip

Hybridization of Amplified Sample and EZ Probe Libraries

This section describes the NimbleGen protocol for hybridization of an Amplified Sample Library to an EZ Choice (or EZ Exome) Probe Library.

Step 1. Prepare for Hybridization

1.1 Turn on a heat block to 95°C and let it equilibrate to the set temperature.

1.2 Remove the appropriate number of 4.5 µl Choice (or EZ Exome) Probe Library aliquots (1 per sample library) from the -15 to -25°C freezer and allow them to thaw on ice.

Step 2. Prepare the Hybridization Sample

2.1 Add 5 µl of 1 mg/ml Cot-1 DNA and 1 µg of amplified sample library to a new 1.5 ml tube.

2.2 Add 1 µl of TS-HE Oligo 1 (1,000 µM) and 1 µl of the appropriate (refer to table below) TS-HE Index Oligo (1,000 µM) to the amplified sample library plus Cot-1 DNA.

Illumina TruSeq DNA Adapter Index used for library construction	TS-HE Index Oligo to use in hybridization
AD001	TS-HE Index 1 Oligo
AD002	TS-HE Index 2 Oligo
AD003	TS-HE Index 3 Oligo
AD004	TS-HE Index 4 Oligo
AD005	TS-HE Index 5 Oligo
AD006	TS-HE Index 6 Oligo
AD007	TS-HE Index 7 Oligo
AD008	TS-HE Index 8 Oligo
AD009	TS-HE Index 9 Oligo
AD010	TS-HE Index 10 Oligo
AD011	TS-HE Index 11 Oligo
AD012	TS-HE Index 12 Oligo

2.3 Close each tube's lid and make a hole in the top of each tube's cap with an 18 - 20 gauge or smaller needle.

Note: *The hole in the top of each tube's cap is a precaution to suppress contamination in the DNA vacuum concentrator.*

2.4 Dry the amplified sample library/Cot-1 DNA/TS-HE Oligos in a DNA vacuum concentrator on high heat (60°C).

Note: *Denaturation of the DNA with high heat is not problematic after linker ligation because the hybridization utilizes single-stranded DNA.*

2.5 To each dried-down amplified sample library/Cot-1 DNA/TS-HE Oligos, add:

- 7.5 µl of 2X Hybridization Buffer (vial 5)
- 3 µl of Hybridization Component A (vial 6)

The tube with the amplified sample library/Cot-1 DNA/TS-HE Oligos should now contain the following components:

Component	Solution Capture
Cot-1 DNA	5 µg
Amplified Sample Library	1 µg
TS-HE Oligo 1	1,000 pmol
*TS-HE Index Oligo	1,000 pmol
2X Hybridization Buffer (vial 5)	7.5 µl
Hybridization Component A (vial 6)	3 µl
Total	**10.5 µl**

* Refer to the table in Step 2.2 above.

2.6 Cover the hole in the tube's cap with a sticker or small piece of laboratory tape.

2.7 Vortex the amplified sample library/Cot-1 DNA/TS-HE Oligos plus Hybridization Cocktail (2X Hybridization Buffer + Hybridization Component A) for 10 seconds and centrifuge at maximum speed for 10 seconds.

2.8 Place the amplified sample library/Cot-1 DNA/TS-HE Oligos/Hybridization Cocktail in a 95°C heat block for 10 minutes to denature the DNA.

2.9 Centrifuge the amplified sample library/Cot-1 DNA/TS-HE Oligos/Hybridization Cocktail at maximum speed for 10 seconds.

2.10 Transfer the amplified sample library/Cot-1 DNA/TS-HE Oligos/Hybridization Cocktail to the 4.5 µl aliquot of EZ Choice (or EZ Exome) Probe Library in a 0.2 ml PCR tube.

2.11 Vortex for 3 seconds and centrifuge at maximum speed for 10 seconds.

The hybridization sample should now contain the following components:

Component	Solution Capture
Cot-1 DNA	5 µg
Amplified Sample Library	1 µg
TS-HE Oligo 1	1,000 pmol
*TS-HE Index Oligo	1,000 pmol
2X Hybridization Buffer (vial 5)	7.5 µl
Hybridization Component A (vial 6)	3 µl
EZ Choice (or EZ Exome) Probe Library	4.5 µl
Total	**15 µl**

* Refer to the table in Step 2.2 above.

2.12 Incubate in a thermocycler at 47°C for 64 – 72 hours. The thermocycler's heated lid should be turned on and set to maintain 57°C (+10°C above the hybridization temperature).

2.13 Following hybridization, proceed to the "Chapter 6. Washing and Recovery of Captured DNA" section in the *NimbleGen SeqCap EZ Choice Library SR User's Guide v1.0* or later.

Post-Capture Sample Library Amplification Using LM-PCR

This section describes the amplification of captured DNA, bound to Streptavidin Dynabeads, using LM-PCR. A total of 2 reactions are performed per sample, and subsequently combined, to minimize PCR bias.

References

- Phusion High-Fidelity PCR Master Mix with HF Buffer Kit protocol (New England Biolabs - Finnzymes)
- Thermocycler Manual
- Qiagen QIAquick Spin Handbook (Qiagen)
- Agilent DNA 1000 Kit Guide

Step 1. Prepare the LM-PCR Master Mix

Important: *The LM-PCR Master Mix and the individual PCR tubes must be prepared on ice.*

1.1 Prepare the LM-PCR Master Mix in a 1.5 ml tube. The amount of each reagent needed for two reactions (1 captured sample) is listed below:

LM-PCR Master Mix	2 Reactions (for 1 captured DNA sample or negative control)
2x Phusion Master Mix with HF Buffer	100 µl
PCR grade water	52 µl
TS-PCR Oligo 1, 100 µM (Final Conc.: 2 µM)	4 µl
TS-PCR Oligo 2, 100 µM (Final Conc.: 2 µM)	4 µl
Total	**160 µl**

1.2 Aliquot 20 µl of Captured DNA library as template into 2 PCR tubes.

1.3 Pipette 80 µl of LM-PCR Master Mix into the 2 reaction tubes. Mix well by pipetting up and down 5 times.

Step 2. Perform PCR Amplification

2.1 Place PCR tubes in the thermocycler.

2.2 Amplify samples using following LM-PCR program:

- Step 1: 30 seconds @ 98°C
- Step 2: 10 seconds @ 98°C
- Step 3: 30 seconds @ 60°C
- Step 4: 30 seconds @ 72°C
- Step 5: Repeat Steps 2-4, 17 times
- Step 6: 5 minutes @ 72°C
- Step 7: Hold @ 4°C

Note: *The pre-capture and post-capture LM-PCR thermal cycler conditions are different.*

2.3 Store reactions at +2 to +8°C until ready for purification.

Step 3. Clean up the Amplified Captured DNA

3.1 Pool the two reactions from each amplified captured DNA sample into one 1.5 ml microcentrifuge tube (approximately 200 µl). Process any negative control(s) in exactly the same way as the amplified sample library.

3.2 Follow the instructions provided with the Qiagen QIAquick PCR Purification Kit with the following modifications (Steps 3.3 - 3.9 below).

3.3 To each tube, add 1,000 µl (5x volume) of Qiagen buffer PBI. Mix well.

3.4 Pipette 750 µl of the sample into a QIAquick PCR Purification Kit column.

3.5 Centrifuge at ≥ 10,000 x g for 30 - 60 seconds. Discard the flow-through.

3.6 Load the rest of the amplified captured DNA into the same column and centrifuge at ≥ 10,000 x g for 30 - 60 seconds. Discard the flow-through.

3.7 Add 750 µl of buffer PE to the column. Centrifuge at ≥ 10,000 x g for 1 minute.

3.8 Discard the flow-through and place the column back in the same tube. Centrifuge the column for an additional minute.

3.9 Add 50 µl of EB buffer directly to the column matrix. Transfer the column to a 1.5 ml microcentrifuge tube. Let the column stand for 1 minute. Centrifuge at ≥ 10,000 x g for 1 minute to elute the DNA.

Step 4. Determine the Concentration, Size Distribution and Quality of the Sample

4.1 Analyze 1 µl of the amplified captured DNA and negative control using an Agilent DNA 1000 chip and measure the A_{260}/A_{280} ratio using a NanoDrop Spectrophotometer to quantify the concentration of DNA and to determine the DNA quality. The negative control should not show significant amplification, which could be indicative of contamination. Amplified captured DNA should exhibit the following characteristics:

- A_{260}/A_{280}: 1.7 - 2.0
- LM-PCR yield > 1.0 µg
- Average fragment length between 150 - 400 bp

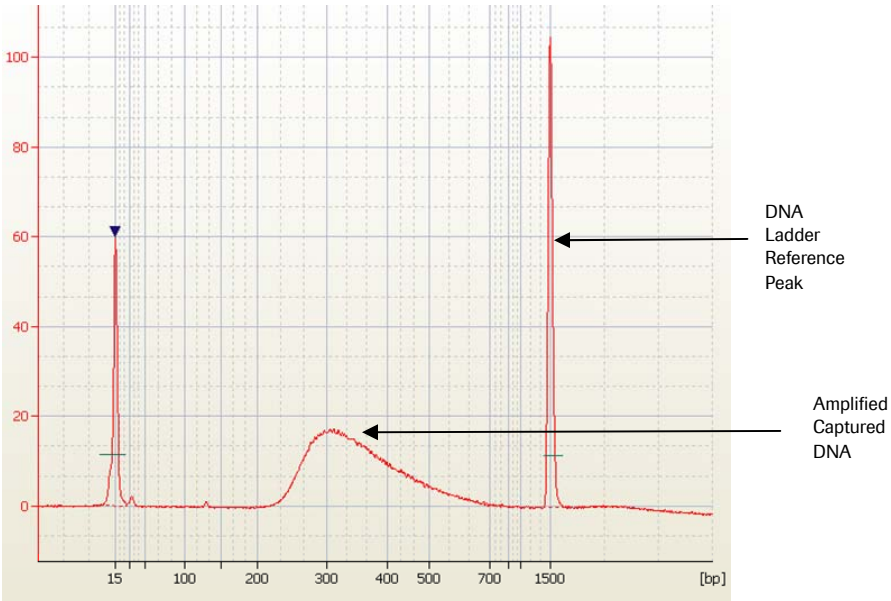


Figure 3: Example of Successfully Amplified Captured DNA Analyzed Using an Agilent DNA 1000 Chip

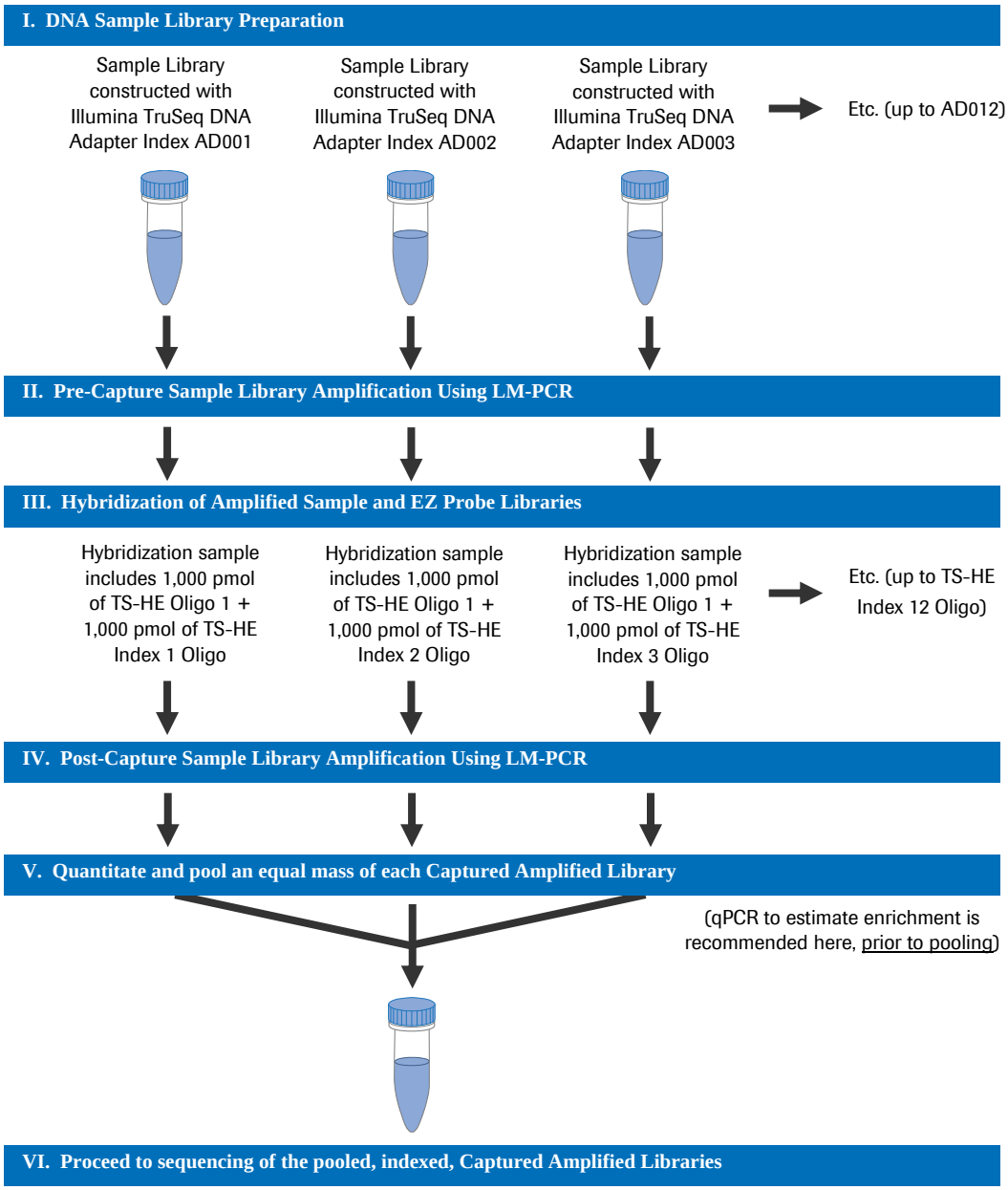
4.2 If samples meet the requirements, proceed to "Chapter 8. Measurement of Enrichment Using qPCR" in the *NimbleGen SeqCap EZ Choice Library SR User's Guide v1.0* or later.

If samples do not meet the A_{260}/A_{280} ratio requirement, purify the sample again using a second QIAquick PCR Purification Kit column (Qiagen).

Appendix A. Pooling Individually Captured Amplified Illumina TruSeq Libraries Prior to Sequencing

This appendix provides a recommendation for Pooling Captured Amplified Illumina TruSeq Libraries immediately prior to sequencing. Each DNA Sample Library contains a different DNA Adapter Index and is captured independently. Following the *post-capture* LM-PCR amplification step, the different Captured Amplified libraries are quantitated and pooled so that the pool contains equivalent amounts (by mass) of each library (Figure 4). To determine the appropriate number of libraries to pool, consider the capture target size, the capture specificity (*i.e.* on-target read rate) for the design, and your desired coverage depth.

Figure 4: Workflow for pooling Captured Amplified Indexed Illumina TruSeq Libraries prior to sequencing.



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