SeqCap EZ Library SR
User’s Guide

Version 4.1
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Editions

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Preface

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

SeqCap EZ Library
SeqCap EZ Library is a solution-based capture method that enables enrichment of the whole exome or customer regions of interest in a single tube.

Contact Information

Technical Support
If you have questions, contact your local Roche Technical Support. Go to www.nimblegen.com/arraysupport for contact information.

Manufacturer and Distribution

Manufacturer  Roche NimbleGen, Inc.
Madison, WI USA

Distribution  Roche Diagnostics GmbH
Mannheim, Germany

Distribution in USA  Roche Diagnostics Corporation
Indianapolis, IN USA
Conventions Used in This Manual

Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Exclamation Mark]</td>
<td>Important Note: Information critical to the success of the procedure or use of the product. Failure to follow these instructions could result in compromised data.</td>
</tr>
<tr>
<td>![Magnifying Glass]</td>
<td>Information Note: Designates a note that provides additional information concerning the current topic or procedure.</td>
</tr>
</tbody>
</table>

Text

<table>
<thead>
<tr>
<th>Conventions</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Numbered listing</td>
<td>Indicates steps in a procedure that must be performed in the order listed.</td>
</tr>
<tr>
<td><em>Italic type, blue</em></td>
<td>Identifies a resource in a different area of this manual or on a web site.</td>
</tr>
<tr>
<td><em>Italic type</em></td>
<td>Identifies the names of dialog boxes, windows, tabs, panels, views, or message boxes in the software.</td>
</tr>
<tr>
<td><strong>Bold type</strong></td>
<td>Identifies names of menus and controls (buttons, checkboxes, etc.) in the software.</td>
</tr>
</tbody>
</table>
Chapter 1. Before You Begin

This User's Guide describes the process for the capture of genomic DNA (gDNA) using SeqCap EZ Libraries and the amplification of captured Multiplex DNA Sample Library Pool by ligation-mediated PCR (LM-PCR) (Figure 1). Specifically, this User's Guide describes a new Sequence Capture method that allows for the multiplexed capture of Illumina TruSeq DNA sample libraries using either SeqCap EZ Human Exome or SeqCap EZ Choice Libraries and the processing of the sample libraries and captured samples using the new Roche NimbleGen Accessory and Oligo Kits. This protocol starts with a gDNA library prepared using the Illumina TruSeq DNA Sample Preparation Kit and ends with captured gDNA fragments that can be sequenced directly using an Illumina HiSeq sequencing instrument.

Workflow

The SeqCap EZ Library protocols involve:

1. Preparing the gDNA library using the Illumina TruSeq DNA Sample Preparation Kit.
2. Sequencing the captured gDNA fragments using the Illumina Genome Analyzer II or HiSeq sequencing instruments.

Thermocyclers should be programmed before beginning this protocol to the required thermocycler reaction programs detailed on page 17 (Chapter 4, Step 3.2), page 31 (Chapter 7, Step 2.2), and page 37 (Chapter 8, Table 1).

Figure 1 lists the steps in the workflow for SeqCap EZ Libraries.

The corresponding estimated time for each step is based on processing one solution phase capture. When applicable, incubation times are indicated between process times in Figure 1.
Step | Processing Time
--- | ---
Library Preparation (Illumina TruSeq DNA Sample Preparation Kit) | 6h
Sample Library Amplification Using LM-PCR | 2 h
Mixing of Amplified Sample Libraries (Multiplex Sample Capture, only) | 0.5 h
Hybridization of Sample and SeqCap EZ Library | 1 h
Washing and Recovery of Captured DNA | 2 h
Captured DNA Amplification Using LM-PCR | 3 h
Measurement of Enrichment Using qPCR | 2 h
Proceed to Sequencing Using Illumina Sequencing Instrument and Associated Reagents | 3 day incubation

Figure 1: Workflow for SeqCap EZ Library Experiments Using Illumina Genome Analyzer II or HiSeq Sequencing Instruments

What’s New?

- Instructions are provided for using the new SeqCap EZ Pure Capture Bead Kit (includes DNA Purification Beads and Capture Beads) in conjunction with the SeqCap EZ Reagent and Accessory Kits.

- Note: This User’s Guide can be used with any SeqCap EZ Library.

To verify you are using the most up-to-date version of this User’s Guide to process your captures, go to www.nimblegen.com/lit/.

Terminology

LM-PCR: Ligation Mediated PCR. In the context of this document, PCR using primers complementary to the sequencing adapters.

Sequence Capture (or Capture): The process of enrichment of targeted regions from genomic DNA. In the context of this document, the hybridization of the amplified sample library and SeqCap EZ Library and subsequent washing steps.

SeqCap EZ Library: The complete set of biotinylated long oligonucleotide probes provided by Roche NimbleGen to perform sequence capture (SeqCap EZ Human Exome or SeqCap EZ Choice).
**Sample Library:** The initial shotgun library generated from genomic DNA by fragmentation and ligation of sequencing-platform-specific linkers. In the context of this document, the sample library before amplification by LM-PCR and before capture.

**Amplified Sample Library:** The sample library after amplification by LM-PCR but before capture.

**Captured Multiplex DNA Sample:** The enriched DNA population from the amplified sample library after the multiplex capture process but before another round of LM-PCR.

**Amplified, Captured Multiplex DNA Sample:** The captured DNA after LM-PCR amplification.

### Components Supplied

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SeqCap EZ Library</td>
<td>Available in 4, 12, 24, 48, 96, 384 or 960 reaction packs</td>
</tr>
<tr>
<td>Product CD/DVD</td>
<td>Design and annotation (.gff1/.bed1) files and user documentation are included.</td>
</tr>
</tbody>
</table>

2. View .bed files using the Internet-based UCSC Genome browser.

### Protocol Information & Safety

- Wear gloves and take precautions to avoid sample contamination.
- Perform all centrifugations at room temperature (+15 to +25°C) unless indicated otherwise.

### Storage of the SeqCap EZ Library

Aliquot and store the SeqCap EZ Library as described in Chapter 2 of this User’s Guide (page 12).

### Required Equipment, Labware & Consumables

You assume full responsibility when using the equipment, labware, and consumables described below. These protocols are designed for use with the specified equipment, labware, and consumables.

### Laboratory Equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier</th>
<th>Catalog No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Vacuum Concentrator (1.5 ml tubes)</td>
<td>Multiple Vendors</td>
<td></td>
</tr>
<tr>
<td>DynaMag-2 Magnet (16 x 1.5 ml tube holder)</td>
<td>Invitrogen</td>
<td>123-21D</td>
</tr>
<tr>
<td>Heat block</td>
<td>Multiple Vendors</td>
<td></td>
</tr>
<tr>
<td>Water bath</td>
<td>Multiple Vendors</td>
<td></td>
</tr>
<tr>
<td>Microcentrifuge with Multiplate Adaptors (16,000 x g capability)</td>
<td>Multiple Vendors</td>
<td></td>
</tr>
<tr>
<td>Spectrophotometer</td>
<td>NanoDrop</td>
<td>ND-1000</td>
</tr>
<tr>
<td>Bioanalyzer 2100</td>
<td>Agilent</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 1. Before You Begin

**Equipment**

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier</th>
<th>Catalog No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LightCycler® 480 Instrument II</td>
<td>Roche Applied Science</td>
<td>05 015 243 001 (384-well)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-or-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>05 015 278 001 (96-well)</td>
</tr>
<tr>
<td>Thermocycler (capable of maintaining +47°C for 64 - 72 hours; programmable heated lid preferred)</td>
<td>Multiple Vendors</td>
<td></td>
</tr>
<tr>
<td>Vortex mixer</td>
<td>Multiple Vendors</td>
<td></td>
</tr>
</tbody>
</table>

**Consumables Available from Roche Applied Science**

The package sizes listed provide sufficient material to perform a minimum of 24 Sequence Capture experiments.

<table>
<thead>
<tr>
<th>Component</th>
<th>Package Size/Contents</th>
<th>Catalog No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LightCycler® 480 Multiwell Plate 384 (with sealing foils)</td>
<td>5 x 10 plates</td>
<td>04 729 749 001</td>
</tr>
<tr>
<td>LightCycler® 480 SYBR Green I Master (2X Mix)</td>
<td>5 x 1 ml</td>
<td>04 707 516 001</td>
</tr>
<tr>
<td>PCR Grade Water</td>
<td>4 x 25 ml</td>
<td>03 315 843 001</td>
</tr>
<tr>
<td>NimbleGen SeqCap EZ Hybridization and Wash Kit¹</td>
<td>24 reactions</td>
<td>05 634 261 001</td>
</tr>
<tr>
<td>SeqCap EZ Accessory Kit¹</td>
<td>24 reactions</td>
<td>06 776 302 001</td>
</tr>
<tr>
<td>SeqCap EZ HE-Oligo Kit A¹</td>
<td>96 reactions</td>
<td>06 777 287 001</td>
</tr>
<tr>
<td>SeqCap EZ HE-Oligo Kit B</td>
<td>96 reactions</td>
<td>06 777 317 001</td>
</tr>
<tr>
<td>SeqCap EZ Pure Capture Bead Kit</td>
<td>24 reactions</td>
<td>06 977 952 001</td>
</tr>
</tbody>
</table>

¹ These items can be ordered together in the SeqCap EZ Reagent Kit, 24 reactions (Cat. No. 06 953 212 001)

**Consumables Purchased from Other Vendors**

<table>
<thead>
<tr>
<th>Component</th>
<th>Supplier</th>
<th>Package Size</th>
<th>Catalog No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Illumina TruSeq DNA Sample Prep Kit LT (48 samples)</td>
<td>Illumina</td>
<td>1 kit</td>
<td>FC-121-1001 (v1, Set A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- or -</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FC-121-2001 (v2, Set A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- or -</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FC-121-1002 (v1, Set B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- or -</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FC-121-2002 (v2, Set B)</td>
</tr>
<tr>
<td>Agilent DNA 1000 Kit</td>
<td>Agilent</td>
<td>1 kit</td>
<td>5067-1504</td>
</tr>
<tr>
<td>Ethanol, 200 proof (absolute), for molecular biology</td>
<td>Sigma-Aldrich</td>
<td>500 ml</td>
<td>E7023-500ML</td>
</tr>
</tbody>
</table>

Tubes: Multiple Vendors
- 0.2 ml PCR tubes
- 1.5 ml microcentrifuge tubes
## Custom Oligonucleotides Purchased from IDT or Another Vendor

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Sequence</th>
<th>Note(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>qPCR NSC-0237, forward, Oligo</td>
<td>2 µM</td>
<td>5' - CGC ATT CCT CAT CCC AGT ATG - 3'</td>
<td>These oligos are used in qPCR analysis of enrichment.</td>
</tr>
<tr>
<td>qPCR NSC-0237, reverse, Oligo</td>
<td>2 µM</td>
<td>5' - AAA GGA CTT GGT GCA GAG TTC AG - 3'</td>
<td></td>
</tr>
<tr>
<td>qPCR NSC-0247, forward, Oligo</td>
<td>2 µM</td>
<td>5' - CCC ACC GCC TTC GAC AT - 3'</td>
<td></td>
</tr>
<tr>
<td>qPCR NSC-0247, reverse, Oligo</td>
<td>2 µM</td>
<td>5' - CCT GCT TAC TGT GGG CTC TTG - 3'</td>
<td></td>
</tr>
<tr>
<td>qPCR NSC-0268, forward, Oligo</td>
<td>2 µM</td>
<td>5' - CTC GCT TAA CCA GAC TCA TCT ACT GT - 3'</td>
<td></td>
</tr>
<tr>
<td>qPCR NSC-0268, reverse, Oligo</td>
<td>2 µM</td>
<td>5' - ACT TGG CTC AGC TGT ATG AAG GT - 3'</td>
<td></td>
</tr>
<tr>
<td>qPCR NSC-0272, forward, Oligo</td>
<td>2 µM</td>
<td>5' - CAG CCC CAG CTC AGG TAC AG - 3'</td>
<td></td>
</tr>
<tr>
<td>qPCR NSC-0272, reverse, Oligo</td>
<td>2 µM</td>
<td>5' - ATG ATG CGA GTG CTG ATG ATG - 3'</td>
<td></td>
</tr>
</tbody>
</table>

Oligonucleotides can be resuspended in PCR grade water or TE buffer unless otherwise noted.
Chapter 2. Storing the SeqCap EZ Library, Accessory, Oligo, and Pure Capture Bead Kits

Chapter 2 describes the aliquoting of and storage conditions for the SeqCap EZ Library, SeqCap EZ Accessory Kit, and SeqCap EZ HE-Oligo Kits.

Upon receipt, undertake the following steps to ensure the highest performance of the SeqCap EZ Library to avoid multiple freeze/thaw cycles or potential accidental contamination:

1. If frozen, thaw the tube of SeqCap EZ Library on ice.
2. Vortex the SeqCap EZ Library for 3 seconds.
3. Centrifuge the tube of SeqCap EZ Library at 10,000 x g for 30 seconds to ensure that the liquid is at the bottom of the tube before opening the tube.
4. Aliquot the SeqCap EZ Library into single-use aliquots (4.5 µl/aliquot) in 0.2 ml PCR tubes (or 96-well plates if following the higher throughput protocol described in Appendix B) and store at -15 to -25°C until use. The presence of some residual volume after dispensing all single-use aliquots is normal.
5. When ready to perform the experiment, thaw the required number of single-use SeqCap EZ Library aliquots on ice.

The SeqCap EZ Library should not undergo multiple freeze/thaw cycles. To help ensure the highest performance of the SeqCap EZ Library, Roche NimbleGen recommends aliquoting the SeqCap EZ Library into single-use volumes to prevent damage from successive freeze/thaw cycles.

Upon receipt, undertake the following steps to ensure the highest performance of the SeqCap EZ Accessory and HE-Oligo Kits:

1. Store the kits at -15 to -25°C until use.

Upon receipt, undertake the following steps to ensure the highest performance of the SeqCap EZ Pure Capture Bead Kit:

1. Store the SeqCap EZ Pure Capture Bead kit at +2 to +8°C until use.

The SeqCap EZ Pure Capture Bead Kit should not be frozen. This kit should be stored at +2 to +8°C.
Notes
Chapter 3. Preparing the Sample Library and Performing QA

Chapter 3 describes the sample library preparation method and how to assess the quality of the sample library before amplification using LM-PCR.

Sample library preparation using the Illumina TruSeq DNA Sample Prep Kit recommends the use of 1 µg of gDNA.

References

- Illumina TruSeq DNA Sample Preparation Guide (revision C, June 2012)
- Covaris E210 Water Bath Sonicator User’s Guide
- Agilent DNA 1000 Kit Guide

Step 1. Preparing the Sample Library

While this procedure omits the gel cut step for DNA fragment size selection, it is still possible to use the gel cut step for size selection if preferred.

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.”

   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. The user will also need to modify the AMPure XP 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.

2. Adjust the final volume of the Illumina TruSeq DNA Sample Library to 21 µl using the Resuspension Buffer supplied in the Illumina TruSeq DNA Sample Preparation Kit.

3. Proceed to Step 2. “Sample Library Quality Assessment”.
Step 2. Performing Sample Library Quality Assessment

1. Use 1 µl of the Illumina TruSeq DNA Sample Library from Step 1.3 above as input into the “Enrich DNA Fragments” protocol (page 80 or 116) in the Illumina “TruSeq DNA Sample Preparation Guide (June 2012).”

   The “Enrich DNA Fragments” protocol in the Illumina “TruSeq DNA Sample Preparation Guide” is used here for Sample Library quality assessment purposes only. The resulting amplified material is not used for any other purpose in this User’s Guide.

2. Following Step 13 of the “Enrich DNA Fragments – Clean Up PCR” protocol, run 1 µl of the amplified DNA on an Agilent DNA 1000 chip (Figure 2).

3. The DNA library should have a peak between 150 - 700 bp.

   ![Figure 2](image-url)

   Figure 2: Example of an Amplified TruSeq Library DNA Sample Run on an Agilent DNA 1000 Chip

4. If the library passes this quality assessment step, proceed to Chapter 4.
Chapter 4. Amplifying the Sample Library Using LM-PCR

This chapter describes how to amplify the sample library (prepared in Chapter 3) using LM-PCR in preparation for hybridization to the SeqCap EZ Library.

References
- Thermocycler Manual
- Agilent DNA 1000 Kit Guide

Sample Requirements
For each sample library to be captured, the entire sample library from Chapter 3, Step 2 (a total of 20 µl after removal of 1 µl for sample library quality assessment), is amplified via Pre-Capture LM-PCR.

Step 1. Resuspend the TS-PCR Oligos
1. Spin the lyophilized primers briefly to allow the contents to pellet at the bottom of the tube. Please note that both primers are contained within a single tube.
2. Add 400 µl PCR-grade water to the tube labeled "TS-PCR Oligo 1 & 2".
3. Briefly vortex the primers plus PCR-grade water and spin down the resuspended oligo tube.
4. The resuspended oligo tube should be stored at -15 to -25°C.

Step 2. Preparing the LM-PCR

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

We recommend the inclusion of negative (water) and positive (previously amplified library) controls in the Pre-Capture LM-PCR step.

1. Prepare the LM-PCR Master Mix in a 1.5 ml microcentrifuge (or 15 ml conical) tube. The amount of each reagent needed is listed below (if desired, increase all Master Mix volumes by 10% to account for pipetting variance):

<table>
<thead>
<tr>
<th>Pre-Capture LM-PCR Master Mix</th>
<th>Per Individual Sample Library or Negative Control</th>
<th>24 Sample Libraries</th>
<th>96 Sample Libraries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roche High-Fidelity PCR Master Mix (2x)</td>
<td>50 µl</td>
<td>1,200 µl</td>
<td>4,800 µl</td>
</tr>
<tr>
<td>PCR grade water</td>
<td>25 µl</td>
<td>600 µl</td>
<td>2,400 µl</td>
</tr>
<tr>
<td>TS-PCR Oligo 1 &amp; 2, 6 µM</td>
<td>5 µl</td>
<td>120 µl</td>
<td>480 µl</td>
</tr>
<tr>
<td>(Final Conc.: 0.375 nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>80 µl</td>
<td>3,840 µl</td>
<td>7,680 µl</td>
</tr>
</tbody>
</table>
2. Pipette 80 µl of LM-PCR Master Mix into each PCR tube or well.

3. Add the 20 µl of sample library (or PCR grade water for negative control) to the PCR tube or each well of the 96-well plate containing the LM-PCR Master Mix. Mix well by pipetting up and down five times. Do not vortex.

“Sample library” refers to the DNA from Chapter 3, Step 1, of this User’s Guide, not the DNA from the “Enrich DNA Fragments” step, of the Illumina “TruSeq DNA Sample Prep Guide.” Refer to Chapter 3 for more details.

### Step 3. Performing PCR Amplification

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

2. Amplify the sample library using the following Pre-Capture LM-PCR program:
   - Step 1: 2 minutes @ +94°C
   - Step 2: 10 seconds @ +94°C
   - Step 3: 70 seconds @ +60°C
   - Step 4: 45 seconds @ +72°C
   - Step 5: Go to Step 2, repeat seven times
   - Step 6: 7 minutes @ +72°C
   - Step 7: Hold @ +4°C

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

### Step 4. Cleaning up the Amplified Sample Library using DNA Purification Beads

Alternatively, samples can be purified using the Qiagen QIAquick PCR Purification Kit. If this purification method is chosen instead of the DNA Purification Beads, follow the protocol detailed in the Qiagen QIAquick PCR Purification Kit guide with the following exception: Elute the sample library DNA using PCR grade water instead of buffer EB.
1. Allow the DNA Purification Beads to warm to room temperature for at least 30 minutes before use.

2. Transfer each amplified sample library into a separate 1.5 ml microcentrifuge tube (approximately 100 µl). Process the negative control in exactly the same way as the amplified sample library.

3. Vortex the beads for 10 seconds before use to ensure a homogenous mixture of beads.

4. Add 180 µl (or 1.8x volume) DNA Purification Beads to the 100 µl amplified sample library.

5. Vortex briefly and incubate at room temperature for 15 minutes to allow the DNA to bind to the beads.

6. Place the tube containing the bead bound DNA in a magnetic particle collector.

7. Allow the solution to clear.

8. Once clear, remove and discard the supernatant being careful not to disturb the beads.

9. Add 200 µl freshly prepared 80% ethanol to the tube containing the beads plus DNA. The tube should be left in the magnetic particle collector during this step.

10. Incubate at room temperature for 30 seconds.

11. Remove and discard the 80% ethanol and repeat Steps 4.9-4.10 for a total of two washes with 80% ethanol.

12. Following the second wash, remove and discard all of the 80% ethanol.

13. Allow the beads to dry at room temperature with the tube lid open for 15 minutes (or until dry).

   Over drying of the beads can result in yield loss.

14. Remove the tube from the magnetic particle collector and resuspend the DNA using 52 µl of PCR-grade water.

15. Pipet up and down ten times to mix to ensure that all of the beads are resuspended.

16. Incubate at room temperature for 2 minutes.

17. Place the tube back in the magnetic particle collector and allow the solution to clear.

18. Remove 50 µl of the supernatant that now contains the amplified sample library and aliquot into a new 1.5 ml tube.

   It is critical that the amplified sample library is eluted with PCR grade water and not buffer EB or 1X TE.
Step 5. Checking the Quality of the Amplified Sample Library

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

   When working with samples that will be pooled for hybridization (i.e. multiplex Sequence Capture), accurate quantitation is essential. Alternative quantitation methods, such as those that are fluorometry-based, should be used in place of, or in addition to, the NanoDrop spectrophotometer. Slight differences in the mass of each sample combined to form the 'Multiplex DNA Sample Library Pool' will result in variations in the total number of sequencing reads obtained for each sample the library pool.

   - 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. If this is not the case, the measurement may be high due to the presence of unincorporated primers carried over from the LM-PCR reaction (refer to Step 4.2, below) and not an indication of possible contamination between amplified sample libraries.

2. Run 1 µl of each amplified sample library (and any negative controls) on an Agilent DNA 1000 chip. Run the chip according to manufacturer’s instructions. The Bioanalyzer should indicate that average fragment size falls between 150 - 500 bp (Figure 3). The negative control should not show any significant signal within this size range, which could indicate contamination between amplified sample libraries. A sharp peak may be visible below 150 bp. This peak, which consists of unincorporated primers carried over from previous steps or the LM-PCR reaction, may be more prominent when the library preparation does not include the optional gel-cut step. The presence of this material will not interfere with the capture process.

   The negative control should not show any signal above baseline within the 150 - 400 bp size range, which could indicate contamination between amplified sample libraries, but it may exhibit sharp peaks visible below 150 bp. If the negative control reaction shows a positive signal by the NanoDrop spectrophotometer (refer to Step 4.1, earlier), but the Bioanalyzer trace indicates only the presence of a sharp peak below 150 bp in size, then the negative control should not be considered contaminated.
3. If the amplified sample library meets these requirements, proceed to Chapter 5. If the amplified sample library does not meet these requirements, reconstruct the library.

![Figure 3: Example of an Amplified Sample Library Analyzed Using an Agilent DNA 1000 Chip](image)

If there are any concerns at this stage regarding the quality of the amplified sample library, proceed to Appendix A.
Notes
Chapter 5. Hybridizing the Sample and SeqCap EZ Libraries

Chapter 5 describes the Roche NimbleGen protocol for hybridization of the amplified sample libraries and the SeqCap EZ Library.

The hybridization protocol requires a thermocycler capable of maintaining +47°C for 64 - 72 hours. A programmable heated lid is strongly recommended.

Note: Instructions for using SeqCap EZ Oligo Kits A & B with automated liquid handling instruments for setting up hybridizations is described in Appendix B: Automation & Oligo Kits A/B.

Step 1. Preparing for Hybridization

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

2. Remove the appropriate number of 4.5 µl SeqCap EZ Library aliquots (one per sample library) from the -15 to -25°C freezer and allow them to thaw on ice.

Step 2. Prepare the Multiplex DNA Sample Library Pool

1. Thaw on ice each of the differently-indexed amplified DNA sample libraries that will be included in the multiplex capture experiment (generated in Chapter 4).

2. Mix together equal amounts (by mass) of each of these amplified DNA sample libraries to obtain a single pool with a combined mass of at least 1.25 µg. This mixture will subsequently be referred to as the “Multiplex DNA Sample Library Pool”. One µg of the multiplex DNA sample library pool will be used in the sequence capture hybridization step, and 60 ng will be used for Measurement of Enrichment Using qPCR (Chapter 8).

   To obtain equal numbers of sequencing reads from each component libraries in the Multiplex DNA Sample Library Pool upon completion of the experiment, it is very important to combine identical amounts of each independently amplified DNA sample library at this step. Accurate quantification and pipetting are critical.

Note: Store remaining 250 ng of Multiplex DNA Sample Library Pool at -15 to -25°C until use in Measurement of Enrichment Using qPCR (Chapter 8).

Step 3. Resuspend the TS-HE Universal and TS-INV-HE Index Oligos

1. Spin the lyophilized oligo tubes briefly to allow the contents to pellet to the bottom of the tube.

2. Add 120 µl PCR-grade water to TS-HE Universal Oligo tube (1,000 µM final concentration).
3. Add 10 µl PCR-grade water to each TS-INV-HE Index Oligo tube (1,000 µM final concentration).

4. Vortex the primers plus PCR-grade water for five seconds and spin down the resuspended oligo tube.

5. The resuspended oligo tube should be stored at -15 to -25°C.

   To prevent damage to the Hybridization Enhancing (HE) oligos due to multiple freeze/thaw cycles, once resuspended the oligos can be aliquoted into smaller volumes to minimize the number of freeze/thaw cycles.

### Step 4. Prepare the Multiplex Hybridization Enhancing Oligo Pool

1. Thaw on ice the resuspended TS-HE Universal Oligo 1 (1,000 µM) and each resuspended TS-INV-HE Index oligo (1,000 µM) that matches a DNA Adapter Index included in the Multiplex DNA Sample Library Pool from Step 2 of this section.

2. Mix together the HE oligos so that the resulting Multiplex Hybridization Enhancing Oligo Pool contains, by mass, 50% TS-HE Universal Oligo 1 and 50% of a mixture of the appropriate TS-INV-HE Index oligos. The total combined mass of the Multiplex Hybridization Enhancing Oligo Pool should be 2,000 pmol, which is the amount required for a single Sequence Capture experiment.

**Example:** If a Multiplex DNA Sample Library Pool contains four DNA sample libraries prepared with Illumina TruSeq indexed adapters 2, 4, 6, and 8, respectively, then the Multiplex Hybridization Enhancing Oligo Pool would contain the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS-HE Universal Oligo 1</td>
<td>1,000 pmol (1 µl of 1,000 µM)</td>
</tr>
<tr>
<td>TS-INV-HE Index 2 Oligo</td>
<td>250 pmol (0.25 µl of 1,000 µM)</td>
</tr>
<tr>
<td>TS-INV-HE Index 4 Oligo</td>
<td>250 pmol (0.25 µl of 1,000 µM)</td>
</tr>
<tr>
<td>TS-INV-HE Index 6 Oligo</td>
<td>250 pmol (0.25 µl of 1,000 µM)</td>
</tr>
<tr>
<td>TS-INV-HE Index 8 Oligo</td>
<td>250 pmol (0.25 µl of 1,000 µM)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2,000 pmol (2 µl of 1,000 µM)</strong></td>
</tr>
</tbody>
</table>

Due to the difficulty of accurately pipetting small volumes, it is recommended to either prepare a larger volume of the Multiplex Hybridization Enhancing Oligo Pool using the 1,000 µM stocks or dilute the 1,000 µM stocks and then pool. These pools can be dispensed into individual single-use aliquots that can be stored at -15 to -25°C until needed.

For optimal results, it is important that the individual TS-INV-HE oligos contained in a Multiplex Hybridization Enhancing Oligo Pool are precisely matched with the adapter indexes present in the Multiplex DNA Sample Library Pool in a multiplexed Sequence Capture experiment.

### Step 5. Prepare the Hybridization Sample

1. Add 5 µl of COT Human DNA (1 mg/ml) to a new 1.5 ml tube.

2. Add 1 µg of Multiplex DNA Sample Library to the 1.5 ml tube containing 5 µl of COT Human DNA.
3. Add 2,000 pmol of Multiplex Hybridization Enhancing Oligo Pool (1,000 pmol TS-HE Universal Oligo 1 and 1,000 pmol TS-INV-HE Index Oligo pool) to the Multiplex DNA Sample Library Pool plus COT Human DNA.

4. Close the tube’s lid and make a hole in the top of the tube’s cap with an 18 - 20 gauge or smaller needle.
   
   The closed lid with a hole in the top of the tube’s cap is a precaution to suppress contamination in the DNA vacuum concentrator.

5. Dry the Multiplex DNA Sample Library Pool/COT Human DNA/Multiplex Hybridization Enhancing Oligo Pool in a DNA vacuum concentrator on high heat (+60°C).
   
   Denaturation of the DNA with high heat is not problematic after linker ligation because the hybridization utilizes single-stranded DNA.

6. To each dried-down Multiplex DNA Sample Library Pool/COT Human DNA/Multiplex Hybridization Enhancing Oligo Pool, add:
   
   - 7.5 µl of 2X Hybridization Buffer (vial 5)
   - 3 µl of Hybridization Component A (vial 6)

   The tube with the Multiplex DNA Sample Library Pool/COT Human DNA/Multiplex Hybridization Enhancing Oligo Pool should now contain the following components:

<table>
<thead>
<tr>
<th>Component</th>
<th>Solution Capture</th>
</tr>
</thead>
<tbody>
<tr>
<td>COT Human DNA</td>
<td>5 µg</td>
</tr>
<tr>
<td>Multiplex DNA Sample Library Pool</td>
<td>1 µg</td>
</tr>
<tr>
<td>Multiplex Hybridization Enhancing Oligo Pool</td>
<td>2,000 pmol*</td>
</tr>
<tr>
<td>2X Hybridization Buffer (vial 5)</td>
<td>7.5 µl</td>
</tr>
<tr>
<td>Hybridization Component A (vial 6)</td>
<td>3 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10.5 µl</strong></td>
</tr>
</tbody>
</table>

   *Composed of 50% (1,000 pmol) TS-HE Universal Oligo 1 and 50% (1,000 pmol) of a mixture of the appropriate TS-INV-HE Index oligos.

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

8. Vortex the Multiplex DNA Sample Library Pool/COT Human DNA/Multiplex Hybridization Enhancing Oligo Pool plus Hybridization Cocktail (2X Hybridization Buffer + Hybridization Component A) for 10 seconds and centrifuge at maximum speed for 10 seconds.

9. Place the Multiplex DNA Sample Library Pool/COT Human DNA/Multiplex Hybridization Enhancing Oligo Pool/Hybridization Cocktail in a +95°C heat block for 10 minutes to denature the DNA.

10. Centrifuge the Multiplex DNA Sample Library Pool/COT Human DNA/Multiplex Hybridization Enhancing Oligo Pool/Hybridization Cocktail at maximum speed for 10 seconds at room temperature.

11. Transfer the Multiplex DNA Sample Library Pool/COT Human DNA/Multiplex Hybridization Enhancing Oligo Pool/Hybridization Cocktail to the 4.5 µl aliquot of EZ Library in a 0.2 ml PCR tube prepared in Chapter 2 (the entire volume can also be transferred to one well of a 96-well PCR plate).

12. Vortex for 3 seconds and centrifuge at maximum speed for 10 seconds.
Chapter 5. Hybridizing the Sample and SeqCap EZ Libraries

The hybridization sample should now contain the following components:

<table>
<thead>
<tr>
<th>Component</th>
<th>Solution</th>
<th>Capture</th>
</tr>
</thead>
<tbody>
<tr>
<td>COT Human DNA</td>
<td>5 µg</td>
<td></td>
</tr>
<tr>
<td>Multiplex DNA Sample Library Pool</td>
<td>1 µg</td>
<td></td>
</tr>
<tr>
<td>Multiplex Hybridization Enhancing Oligo Pool</td>
<td>2,000 pmol*</td>
<td>2,000 pmol*</td>
</tr>
<tr>
<td>2X Hybridization Buffer (vial 5)</td>
<td>7.5 µl</td>
<td></td>
</tr>
<tr>
<td>Hybridization Component A (vial 6)</td>
<td>3 µl</td>
<td></td>
</tr>
<tr>
<td>EZ Library</td>
<td>4.5 µl</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>15 µl</strong></td>
<td></td>
</tr>
</tbody>
</table>

*Composed of 50% (1,000 pmol) TS-HE Universal Oligo 1 and 50% (1,000 pmol) of a mixture of the appropriate TS-INV-HE Index oligos.

13. 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).
Chapter 6. Washing and Recovering Captured Multiplex DNA Sample

Chapter 6 describes the process for the washing and recovery of the captured multiplex DNA sample from the hybridization of the Multiplex DNA Sample Library Pool and SeqCap EZ Library. (Refer to Appendix C for instructions for increased throughput applications.)

It is extremely important that the water bath temperature be closely monitored and remains at +47°C. Because the displayed temperatures on many water baths are often imprecise, Roche NimbleGen recommends that you place an external, calibrated thermometer in the water bath.

Equilibrate buffers at +47°C for at least 2 hours before washing the captured Multiplex DNA sample.

Step 1. Preparing Sequence Capture and Bead Wash Buffers

1. Dilute 10X Wash Buffers (I, II, III and Stringent) and 2.5X Bead Wash Buffer to create 1X working solutions.

<table>
<thead>
<tr>
<th>Amount of Concentrated Buffer</th>
<th>Amount of PCR Grade Water</th>
<th>Total Volume of 1X Buffer*</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 µl – 10X Stringent Wash Buffer (vial 4)</td>
<td>360 µl</td>
<td>400 µl</td>
</tr>
<tr>
<td>30 µl – 10X Wash Buffer I (vial 1)</td>
<td>270 µl</td>
<td>300 µl</td>
</tr>
<tr>
<td>20 µl – 10X Wash Buffer II (vial 2)</td>
<td>180 µl</td>
<td>200 µl</td>
</tr>
<tr>
<td>20 µl – 10X Wash Buffer III (vial 3)</td>
<td>180 µl</td>
<td>200 µl</td>
</tr>
<tr>
<td>200 µl – 2.5X Bead Wash Buffer (vial 7)</td>
<td>300 µl</td>
<td>500 µl</td>
</tr>
</tbody>
</table>

* Store working solutions at room temperature (+15 to +25°C) for up to 2 weeks. The volumes in this table are calculated for a single experiment; scale up accordingly if multiple samples will be processed.

2. Preheat the following wash buffers to +47°C in a water bath:
   - 400 µl of 1X Stringent Wash Buffer
   - 100 µl of 1X Wash Buffer I.

Step 2. Preparing the Capture Beads

1. Allow the Capture Beads to warm to room temperature for 30 minutes prior to use.

2. Mix the beads thoroughly by vortexing for 15 seconds.

3. Aliquot 100 µl of beads for each capture into a single 1.5 ml tube (i.e. for one capture use 100 µl beads and for four captures use 400 µl beads, etc.). Enough beads for six captures can be prepared in a single tube.

4. Place the tube in a DynaMag-2 device. When the liquid becomes clear (should take less than 5 minutes), remove and discard the liquid being careful to leave all of the beads in the tube. Any remaining traces of liquid will be removed with subsequent wash steps.

5. While the tube is in the DynaMag-2 device, add twice the initial volume of beads of 1X Bead Wash Buffer (i.e. for one capture use 200 µl of buffer and for four captures use 800 µl buffer, etc.).
6. Remove the tube from the DynaMag-2 device and vortex for 10 seconds.
7. Place the tube back in the DynaMag-2 device to bind the beads. Once clear, remove and discard the liquid.
8. Repeat Steps 2.5 - 2.7 for a total of two washes.
9. After removing the buffer following the second wash, resuspend by vortexing the beads in 1x the original volume using the 1X Bead Wash Buffer (i.e. for one capture use 100 µl buffer and for four captures use 400 µl buffer, etc.).
10. Aliquot 100 µl of resuspended beads into new 0.2 ml tubes.
11. Place the tube in the DynaMag-2 device to bind the beads. Once clear, remove and discard the liquid.
12. The Capture Beads are now ready to bind the captured DNA. Proceed immediately to “Step 3. Binding DNA to the Capture Beads.”

   After Step 2.11, proceed to Step 3 as quickly as possible. Do not allow the Capture Beads to dry out. Small amounts of residual Bead Wash Buffer will not interfere with binding of DNA to the Capture Beads.

Step 3. Binding DNA to the Capture Beads
1. Transfer the hybridization samples to the Capture Beads prepared in Step 2.11 of this chapter.
2. Mix thoroughly by pipetting up and down ten times.
3. Bind the captured sample to the beads by placing the tubes containing the beads and DNA in a thermocycler set to +47°C for 45 minutes (heated lid set to +57°C). Mix the samples by vortexing for 3 seconds at 15 minute intervals to ensure that the beads remain in suspension. It is helpful to have a vortex mixer located close to the thermocycler for this step.

Step 4. Washing the Capture Beads Plus Bound DNA
1. After the 45-minute incubation, add 100 µl of 1X Wash Buffer I heated to +47°C to the 15 µl of Capture Beads Plus Bound DNA.
2. Mix by vortexing for 10 seconds.
3. Transfer the entire content of each 0.2 ml tube to a 1.5 ml tube.
4. Place the tubes in the DynaMag-2 device to bind the beads. Remove and discard the liquid once clear.
5. Remove the tubes from the DynaMag-2 device and add 200 µl of 1X Stringent Wash Buffer heated to +47°C. Pipette up and down ten times to mix. Work quickly so that the temperature does not drop much below +47°C.
6. Incubate at +47°C for 5 minutes.
7. Repeat Steps 4.4 - 4.6 for a total of two washes using 1X Stringent Wash Buffer heated to +47°C.
8. Place the tubes in the DynaMag-2 device to bind the beads. Remove and discard the liquid once clear.

9. Add 200 µl of room temperature 1X Wash Buffer I and mix by vortexing for 2 minutes. If liquid has collected in the tube's cap, tap the tube gently to collect the liquid into the tube's bottom before continuing to the next step.

10. Place the tubes in the DynaMag-2 device to bind the beads. Remove and discard the liquid once clear.

11. Add 200 µl of room temperature 1X Wash Buffer II and mix by vortexing for 1 minute.

12. Place the tubes in the DynaMag-2 device to bind the beads. Remove and discard the liquid once clear.

13. Add 200 µl of room temperature 1X Wash Buffer III and mix by vortexing for 30 seconds.

14. Place the tubes in the DynaMag-2 device to bind the beads. Remove and discard the liquid once clear.

15. Remove the tubes from the DynaMag-2 device and add 50 µl PCR grade water to each tube of bead-bound captured sample.

16. Store the beads plus captured samples at -15 to -25°C or proceed to Chapter 7.

There is no need to elute DNA off the beads. The beads plus captured DNA will be used as template in the LM-PCR as described in Chapter 7.
Chapter 6. Washing and Recovering Captured Multiplex DNA Sample

Notes

________________________________________________________________________________________

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Chapter 7. Amplifying Captured Multiplex DNA Sample Using LM-PCR

Chapter 7 describes the amplification of captured Multiplex DNA sample, bound to the Capture Beads, using LM-PCR. A total of two reactions are performed per sample, and subsequently combined, to minimize PCR bias.

References

- Thermocycler Manual
- Agilent DNA 1000 Kit Guide

Step 1. Preparing the LM-PCR

The Post-Capture LM-PCR Master Mix and the individual PCR tubes must be prepared on ice.

1. Prepare the Post-Capture LM-PCR Master Mix in a 1.5 ml tube. The amount of each reagent needed for two reactions (one captured DNA sample) is listed below (if desired, increase all Master Mix volumes by 10% to account for pipetting variance):

<table>
<thead>
<tr>
<th>Post-Capture LM-PCR Master Mix</th>
<th>Two Reactions (for one captured DNA sample or negative control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roche High-Fidelity PCR Master Mix (2x)</td>
<td>100 µl</td>
</tr>
<tr>
<td>PCR grade water</td>
<td>50 µl</td>
</tr>
<tr>
<td>TS-PCR Oligo 1 &amp; 2, 6 µM (Final Conc.: 0.375 nM)</td>
<td>10 µl</td>
</tr>
<tr>
<td>Total</td>
<td>160 µl</td>
</tr>
</tbody>
</table>

Two LM-PCR reactions will be performed for each captured multiplex DNA sample.

2. Pipette 80 µl of Post-Capture LM-PCR Master Mix into the reaction tubes or wells.

3. Vortex the bead-bound captured DNA to ensure a homogenous mixture of beads.

4. Aliquot 20 µl of bead-bound captured DNA as template into each of the two PCR tubes/wells. Add 20 µl of PCR grade water to the negative control. Mix well by pipetting up and down five times. Store the remaining bead bound captured DNA at -15 to -25°C.
Chapter 7. Amplifying Captured Multiplex DNA Sample Using LM-PCR

Step 2. Performing PCR Amplification

1. Place PCR tubes/plate in the thermocycler.

2. Amplify the captured DNA using the following Post-Capture LM-PCR program:
   - Step 1: 2 minutes @ +94°C
   - Step 2: 10 seconds @ +94°C
   - Step 3: 70 seconds @ +60°C
   - Step 4: 45 seconds @ +72°C
   - Step 5: Go to Step 2, repeat 17 times
   - Step 6: 7 minutes @ +72°C
   - Step 7: Hold @ +4°C

3. Store reactions at +2 to +8°C until ready for purification, up to 72 hours.

Step 3. Cleaning up the Amplified Captured Multiplex DNA Sample using DNA Purification Beads

Alternatively, samples can be purified using the Qiagen QIAquick PCR Purification Kit. If this purification method is chosen instead of the DNA Purification Beads, follow the protocol detailed in Appendix E.

1. Allow the DNA Purification Beads to warm to room temperature for at least 30 minutes before use.

2. Pool the like amplified captured Multiplex DNA Sample Libraries into a 1.5 ml microcentrifuge tube (approximately 200 µl). Process the negative control in exactly the same way as the amplified sample library.

3. Vortex the beads for 10 seconds before use to ensure a homogenous mixture of beads.

4. Add 360 µl (or 1.8x volume) DNA Purification Beads to the 200 µl pooled amplified captured Multiplex DNA Sample library.

5. Vortex briefly and incubate at room temperature for 15 minutes to allow the DNA to bind to the beads.

6. Place the tube containing the bead bound DNA in a magnetic particle collector.

7. Allow the solution to clear.

8. Once clear, remove and discard the supernatant being careful not to disturb the beads.

9. Add 200 µl freshly prepared 80% ethanol to the tube containing the beads plus DNA. The tube should be left in the magnetic particle collector during this step.

10. Incubate at room temperature for 30 seconds.

11. Remove and discard the 80% ethanol, and repeat Steps 3.9-3.10 for a total of two washes with 80% ethanol.

12. Following the second wash, remove and discard all of the 80% ethanol.

13. Allow the beads (with tube lid open) to dry at +37°C (approximately 5-10 minutes).

   Over drying of the beads can result in yield loss.
14. Remove the tube from the magnetic particle collector and resuspend the DNA using 52 µl of PCR-grade water.

15. Pipet up and down ten times to mix to ensure that all of the beads are resuspended.

16. Incubate at room temperature for 2 minutes.

17. Place the tube back in the magnetic particle collector and allow the solution to clear.

18. Remove 50 µl of the supernatant that now contains the amplified captured Multiplex DNA Sample Library Pool and aliquot into a new 1.5ml tube.

If using the Qiagen QIAquick PCR Purification Kit instead of the DNA Purification Beads to purify the Post-capture LM-PCR Amplified samples: Please note that the presence of the Capture Beads may make the Qiagen QIAquick column appear brown in color. The Capture Beads will not interfere with the action of the Qiagen QIAquick PCR Purification Kit column or buffers.
Step 4. Determining the Concentration, Size Distribution, and Quality of the Amplified Captured Multiplex DNA Sample

1. Analyze 1 µl of the amplified captured multiplex 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 multiplex DNA should exhibit the following characteristics:
   - The A_{260}/A_{280} ratio should be 1.7 - 2.0.
   - The LM-PCR yield should be ≥ 500 ng.
   - The average fragment length should be between 150 - 500 bp.

![Figure 4: Example of Successfully Amplified captured multiplex DNA Analyzed Using an Agilent DNA 1000 Chip](image)

2. If the amplified captured multiplex DNA meets the requirements, proceed to Chapter 8.
   If the amplified captured multiplex DNA does not meet the A_{260}/A_{280} ratio requirement, purify again using the DNA Purification Beads (or alternatively, a second Qiagen QIAquick PCR Purification column).

3. If the results of the qPCR assays in Chapter 8 indicate the enrichment was successful, the amplified captured multiplex DNA is ready for sequencing.
Notes
Chapter 8. Measuring Enrichment Using qPCR

Chapter 8 describes the qPCR assays used to estimate relative fold enrichment by measuring the relative abundance of control targets in amplified sample library and amplified captured multiplex DNA. These assays are an inexpensive way to determine whether the capture was successful prior to sequencing.

A standardized set of qPCR SYBR Green assays are employed as internal quality controls for NimbleGen Sequence Capture experiments performed with human total gDNA. The genomic loci recognized by these assays are included as capture targets in every SeqCap EZ Library. Comparison by qPCR of the relative DNA concentrations of these control loci in the amplified sample library and amplified captured multiplex DNA allows for the estimation of enrichment of a capture target before committing to expensive and/or time-consuming downstream applications.

The internal control region assays recommended in this chapter were selected due to their convenience for use across different species and because they produce consistent results. It is worth considering designing and evaluating locus-specific qPCR assays for your own region(s) of interest because capture results can vary from locus to locus. It is possible that your regions of interest may enrich differently than our internal control regions.

For more information regarding general PCR and qPCR methods, consult the PCR Applications Manual (3rd Edition), available from the Roche Applied Science website (www.roche-applied-science.com).

Step 1. Performing Advance Preparations

1. Determine the number of DNA samples to be analyzed.

A “DNA sample” in this chapter is defined as one Amplified Sample Library (Chapter 4) and the corresponding Amplified captured multiplex DNA (Chapter 7). Assuming a standard set of four NimbleGen Sequence Capture (NSC) control locus qPCR assays will be used and qPCR assays will be performed in triplicate, each DNA Sample will require 24 individual qPCR reactions for analysis. One negative control (i.e. no-template-control, NTC) should always be included to monitor for contamination in qPCR assay primers, other qPCR reagents, and the entire PCR process. One positive control template, ideally consisting of the original genomic DNA starting material, should always be included to verify assay function. The negative and positive controls will each require twelve additional qPCR reactions (refer to Figure 5).
4. The number of reaction wells required to perform a complete control locus qPCR analysis is determined by the formula, \( W = 24 \times (S + 1) \), where \( W \) is the total number of wells required and \( S \) is the number of original DNA samples in the experiment (one per capture). For example, analyzing one captured DNA sample, one negative control, and one positive control with four NSC assays performed in triplicate requires 48 individual qPCR reactions, \( W = 24 \times (1 + 1) \). Analyzing two captured DNA samples requires 72 qPCR reactions, \( W = 24 \times (2 + 1) \), etc.

5. Dilute the NSC assay forward and reverse primers to 2 µM.

6. Dilute sufficient amounts of amplified sample library and amplified captured multiplex DNA to a concentration of 5 ng/µl in PCR grade water for use as qPCR templates (approximately 100 µl each) using the following formula:

\[
\text{Sample Concentration} = \frac{(\text{sample A conc})(\text{sample A vol}) + (\text{sample B conc})(\text{sample B vol})}{\text{Final volume}}
\]

7. Add the following for a 15 µl NSC assay (for larger volume reactions, adjust the reagent amounts proportionally):

- 5.9 µl of PCR grade water
- 0.3 µl of NSC Assay forward primer (2 µM)
- 0.3 µl of NSC Assay reverse primer (2 µM)
- 7.5 µl of SYBR Green Master (2X)
- 1 µl of 5 ng/µl template (amplified sample library, amplified captured multiplex DNA, positive control genomic DNA, or negative control PCR grade water, templates. The final concentration of templates in the reaction should be 0.333 ng/µl, except for the negative control PCR grade water template.)

8. Program the qPCR instrument using the conditions specified in Table 1.

![Figure 5: qPCR Experimental Overview](image)

These conditions are optimized for use with the LightCycler®480 Instrument II and LightCycler®480 SYBR Green I 2X Master Mix. The use of a different thermocycler or reagents could require altering these conditions to achieve optimal results.
## Table 1: qPCR Instrument Cycling Conditions

<table>
<thead>
<tr>
<th>Program Name</th>
<th>Cycles</th>
<th>Analysis Mode</th>
<th>Target (°C)</th>
<th>Hold (hh:mm:ss)</th>
<th>Ramp Rate (°C/s)</th>
<th>Acquisitions (per °C)</th>
<th>Acquisition Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-incubation</td>
<td>1</td>
<td>None</td>
<td>95</td>
<td>00:10:00</td>
<td>4.8</td>
<td>---</td>
<td>None</td>
</tr>
<tr>
<td>Amplification</td>
<td>40</td>
<td>Quantification</td>
<td>95</td>
<td>00:00:10</td>
<td>4.8</td>
<td>---</td>
<td>None</td>
</tr>
<tr>
<td>Melting Curve</td>
<td>1</td>
<td>Melting Curves</td>
<td>95</td>
<td>00:00:10</td>
<td>4.8</td>
<td>---</td>
<td>None</td>
</tr>
<tr>
<td>Cooling</td>
<td>1</td>
<td>None</td>
<td>40</td>
<td>00:00:10</td>
<td>2</td>
<td>5</td>
<td>Continuous</td>
</tr>
</tbody>
</table>

**Table 2: Recommended NSC qPCR Assays.** Forward and reverse primer sequences for four different NSC control locus assays are shown in Table 2. All of these assays have been confirmed on the SeqCap EZ Library using human captured DNA.

Control Locus NSC qPCR assays enable you to measure the enrichment of a small set of standardized capture control loci that represent a range of known capture efficiencies. These assays act as a proxy for estimating the enrichment of larger populations of capture targets without a need for sequencing. If qPCR analysis using NSC assays indicates a successful capture of the control loci, it is likely that the experimental loci of interest were also successfully captured.

It is recommended that the four assays listed in Table 2 are the minimum number used for analysis of a SeqCap EZ Library experiment to obtain an adequate representation of the diverse population of capture target loci. However, analysis using additional assays that are custom designed by you to measure enrichment of your specific targets of interest could provide additional useful information.
Step 2. Setting up Control Locus qPCR Reactions

1. Using the information provided in Chapter 8, Steps 1.1 - 1.3, set up the required number of NSC assays with amplified sample library templates, amplified captured multiplex DNA templates, negative control templates (PCR grade water), and positive control templates (gDNA). Set up the reactions inside a PCR hood to minimize contamination.

2. Run the reactions in the thermocycler using the cycling conditions listed in Table 1.

3. Following raw data collection, run the Absolute Quantification Analysis Module within the LightCycler® 480 Software, Version 1.5, using the 2nd Derivative Maximum Method. It is not necessary to run a template standard curve for the analysis unless you want to measure the efficiency of the qPCR assays. The raw Cp values will be used to perform a simplified version of relative quantification comparing amplified sample library and amplified captured multiplex DNA.

4. Perform a melting curve (dissociation) analysis to verify that nonspecific amplification products, primer dimers, and other artifacts are not contributing to the Cp values for any samples.

5. Copy or export the Cp values to a spreadsheet program for further analysis.

   Cp (crossing point) values reported by the LightCycler® 480 Instrument II and software are analogous to the Ct (crossing threshold) values reported by other instruments and represent the cycle at which fluorescence signal in a reaction well rises above background fluorescence signals in that well. The Cp value measured for a sample is dependent on the initial concentration of template DNA in the reaction. Lower Cp values correspond to higher initial template concentrations.

   ![Fluorescence History](image)

   **Figure 6: Example of Sequence Capture qPCR Data for Two NSC Assays Generated Using the LightCycler® 480 Instrument II.** In a successful experiment, the Cp values from qPCR of amplified captured multiplex DNA templates (CAP) will be significantly lower than Cp values from amplified sample library templates (NON) for all assays.

Step 3. Analyzing Data

1. Calculate the average Cp values for all replicate reactions.

   Replicate Cp values obtained from qPCR of each different template should be similar. If the calculated standard deviation for three replicate Cp values is > 0.2 for a qPCR assay, you should consider repeating the assay to obtain more consistent results.
2. Confirm that the negative control reactions did not produce significant fluorescent signals, which might indicate a problem with PCR contamination resulting in difficulty interpreting experimental results.

Primer dimer amplification can occasionally produce an increase in fluorescence signal in a negative control reaction in the absence of target template molecules, but this typically occurs very late in the run (i.e. Cp approaching 35 cycles), and it is easy to differentiate primer dimer from target amplicon by melting curve analysis (refer to Step 2.4). Primer dimers are typically indicated by a peak melting temperature significantly lower than the peak melting temperature of the correct amplicon. Primer dimers in a negative control reaction are not usually a concern because they are an artifact of low-affinity annealing in the absence of competing template. However, fluorescence created from the presence of primer dimer (as indicated by a second, lower Tm peak in the melting curve) in an experimental sample reaction can lead to an erroneously high estimate of template concentration in that sample, and sample(s) with multiple melting curve peaks should be regarded as suspect and omitted from Cp value determinations.

3. For each different sample and NSC assay combination, subtract the average Cp value measured for the amplified captured multiplex DNA template from the average Cp value measured for the corresponding amplified sample library template. This value is the delta-Cp. A successfully enriched amplified captured multiplex DNA sample should generate a lower Cp value than its corresponding amplified sample library. Thus the delta-Cp calculated from an NSC assay should be positive if the capture process enriched the corresponding locus.

4. Calculate the fold enrichment for a NSC control locus by raising the PCR Efficiency (E) for that assay to the power of the delta-Cp measured for the corresponding control locus, or $E_{\Delta \text{Cp}}$.

When PCR assays operate at 100% theoretical efficiency (i.e. a perfect doubling of target sequences in every cycle), E = 2. The E values for the NSC assays listed in Table 2 were measured by Roche NimbleGen. However, because multiple parameters (PCR instrument, reagent lots, etc.) can affect the efficiency of a PCR assay, it is recommended to determine E values empirically in your own laboratory for each different NSC assay. The efficiency of NSC assays is determined in the same way as other PCR assays by applying linear regression analysis to amplification data from a template standard dilution series. The slope of the standard curve is used to determine E with the equation $E = 10^{(-1/\text{slope})}$. A slope of -3.3 indicates an E value of 2. Calculated E values less than two are common. The software provided with most qPCR instruments can perform calculations of E automatically, or they may be calculated using external mathematical or spreadsheet software.
Example: NSC-0268 assay
(assuming E = 1.78)
- Replicate Cp values for qPCR of amplified sample library = 28.3, 28.5, 28.4
- Replicate Cp values for qPCR of amplified captured multiplex DNA = 17.5, 17.3, 17.7
  - Average Cp_{amplified sample library} = 28.4
  - Average Cp_{amplified captured multiplex DNA} = 17.5
  - Delta-Cp = 10.9
  - Fold enrichment (E_{delta-Cp}) = (1.78)^{10.9} = 537

Example: NSC-0272 assay
(assuming E = 1.93)
- Replicate Cp values for qPCR of amplified sample library = 28.0, 28.2, 28.1
- Replicate Cp values for qPCR of amplified captured multiplex DNA = 18.2, 18.1, 18.0
  - Average Cp_{amplified sample library} = 28.1
  - Average Cp_{amplified captured multiplex DNA} = 18.1
  - Delta-Cp = 10
  - Fold enrichment (E_{delta-Cp}) = (1.93)^{10} = 717

Average Cp values for negative control (PCR grade water) assays should be negligible, or they may indicate the presence of cross-contamination among wells or reagent contamination. If this is observed, the qPCR experiment should be repeated.

Average Cp values for Positive Control (genomic DNA) assays should be similar (within approximately 1 Cp) to the average Cp values obtained for the qPCR of amplified sample library.

Interpreting qPCR Results – SeqCap EZ Library Captures
The theoretical maximum average fold enrichment of a SeqCap EZ experiment varies significantly based on target size, from 75-fold for a 40 Mb target to 12,000-fold for a 250 kb target. Actual average fold enrichment values are typically lower than this, and different targets of the same cumulative size can enrich to different levels depending on variation in the sequence makeup of the region(s) targeted for capture.

Probes targeting the NSC control loci were designed using different criteria than the SeqCap EZ Library probes, and so their reported qPCR values should not be interpreted as a literal estimate of the expected enrichment of other targeted loci. Rather, the NSC control assays are primarily intended as a screen for potentially poor enrichment results so that unproductive sequencing may be avoided. Based on experiments conducted at Roche NimbleGen, we recommend not sequencing the captured DNA from any SeqCap EZ experiment with calculated average fold enrichment values less than tenfold because of the potential that this indicates a failed enrichment. We recommend such experiments be repeated from the beginning.
The utility of these metrics for identifying poor enrichment results is strongly dependent on the use of good qPCR technique, correct qPCR efficiency ($E$) values and inclusion of appropriate experimental controls. A SeqCap EZ experiment producing control locus fold enrichment values lower than tenfold is not a guarantee of a failed enrichment, since genomic polymorphisms and other variables may affect qPCR amplification. Similarly, a SeqCap EZ experiment producing control locus fold enrichment values greater than tenfold is not a guarantee of a successful result.

**Data Analysis Considerations**

Careful consideration and data interpretation are necessary when deciding whether to sequence captured DNA. Consider the types of targets and how the control loci represent them, the region targeted for enrichment, and qPCR as an estimation platform.

The large difference between the fold enrichment values for the two example NSC assays (Step 3.4, above) demonstrates the importance of testing multiple NSC control loci to obtain values more likely to accurately represent the larger population of capture targets. This difference is primarily influenced by two parameters: 1) differences in capture efficiency of the different loci, and 2) amplification efficiency differences between the two qPCR assays. Without determination of the efficiency of the qPCR assays in each run, the local differences in capture efficiency cannot be determined.

The efficiency of the qPCR assays can be variable across runs, which means that strict determination requires a standard dilution curve for each locus during each run. This User’s Guide assumes that across a collection of loci, both the intrinsic locus capture efficiencies and the individual qPCR reaction efficiencies will settle in upon some common mean value. Therefore, employments of more loci yield better global estimates. It is not uncommon for highly efficient qPCR assays to demonstrate fold enrichment values on the order of 1,000 or more. However, benchmarking with such large values results in over-estimating global capture (i.e. pulls the average fold enrichment up too far). Also, platform error in qPCR measurements is $\pm$ 50% (or one cycle when comparing two measures) meaning that it takes many replicates to discriminate a 300-fold enrichment from a 600-fold enrichment. Consideration of this platform error means that twofold differences should be considered the same measure, rather than different measures of enrichment. Consider the size of your sequencing budget and establish qPCR benchmarks that fit your needs.

The average maximum enrichment level of capture targets within a large genome is dependent on the size of the capture target as a percentage of its genome. For example, if 50% of a genome were targeted for capture, an ideal result should yield no better than a twofold average enrichment for the targeted loci. Smaller capture targets in the same genome would have higher maximum average enrichment levels. Thus, the definition of a “successful” NimbleGen Sequence Capture experiment, as estimated by fold enrichment values for control loci, might differ substantially with different sized capture targets or different downstream applications for the captured DNA.
Appendix A. Using qPCR for Amplified Sample Library Quality Control

This appendix provides instructions for performing a qPCR-based procedure to measure the quality of the amplified sample library prior to hybridization. This quality control test is optional, and recommended for use particularly in these situations:

- The user is inexperienced with using the SeqCap EZ Library SR protocol.
- The user is concerned about the quality of the input genomic DNA sample.
- The user is concerned about the quality of the amplified sample library following the Pre-Capture LM-PCR step.

This procedure is not suitable for identifying amplified sample libraries of only slightly reduced quality but in a limited number of cases has been able to identify significantly flawed amplified sample libraries.

Step 1. Performing Advance Preparations & Setting up qPCR Reactions

1. Using the NanoDrop concentration of the amplified sample library obtained in Chapter 4, Step 4.1, dilute a small portion of the amplified sample library to 5 ng/µl using PCR grade water (approximately 50 µl final volume).

2. Dilute a genomic DNA control (unamplified) to 5 ng/µl using PCR grade water (approximately 50 µl final volume).

3. Follow the instructions in Chapter 8, Step 1, to set up and perform Control Locus qPCR assays using as template: amplified sample library, genomic DNA (positive control), and PCR grade water (negative control).

Step 2. Performing Data Analysis

The input material used for library construction is 100% genomic DNA. After fragmentation and ligation of adapters, the resulting library contains approximately 70% genomic DNA by mass, with the other 30% consisting of adapter DNA (the precise fraction depends on the distribution of DNA fragment sizes and the amount of adapter dimers present in the library preparation). Thus, assuming the same amount of DNA is used as template for qPCR, each amplification target in a genomic DNA template will have a slightly higher relative concentration than the same target in an LM-PCR amplified library template. Raw Cp values from qPCR analysis of various loci could reflect this difference, with LM-PCR amplified library templates generating slightly higher Cp values than control genomic DNA templates of the same mass. Furthermore, in a well-made genomic DNA library, all loci present in the starting genomic DNA will be present in the resulting library in the same proportion. Poor construction of a genomic DNA library, or poor LM-PCR amplification of a genomic DNA library, can alter these proportions significantly and result in allelic bias or even allele “drop out” in extreme cases.
Appendix A. Using qPCR for Amplified Sample Library Quality Control

Figure 7: Use of qPCR to Check Amplified Library Quality Prior to Hybridization. Based on experiments performed by Roche NimbleGen, an LM-PCR amplified library may not be suitable for hybridization if the amplified library’s C_p value is > 15% higher than the C_p value of the input genomic DNA in qPCR assays for two or more of the four standard control loci. The data was collected using the protocol and conditions described in Chapter 8. Error bars are ± 1 standard deviation. The % difference in C_p values from qPCR of genomic and amplified Pre-Capture library DNA is calculated using the formula: (Pre-Capture C_p - Genomic C_p) / Genomic C_p

Figure 7 shows summarized results from qPCR analysis of four NSC control loci in several LM-PCR amplified libraries and their respective input genomic DNAs. In a series of capture experiments that were considered successful (i.e. “Good Library”), the average C_p values from qPCR of the amplified libraries were typically less than 10% higher than the C_p values from the input genomic DNAs. A series of libraries that were intentionally poorly constructed or amplified (e.g. low genomic DNA amounts used for library construction, low library amounts used as template for LM-PCR reactions, polymerase stored at improper temperature prior to LM-PCR, or combinations of these) yielded amplified library C_p values that were often greater than 10% higher than the C_p values from the corresponding genomic DNAs. Two libraries used in capture experiments that were ultimately considered failures showed a C_p value difference of greater than 15% for all four NSC control loci.

Based on these observations, Roche NimbleGen recommends that you do not hybridize the amplified library if qPCR using two or more NSC control assays yield C_p values from the amplified library that are greater than 15% higher than C_p values from the input genomic DNA. Instead, you should reconstruct and test the library again before proceeding. Application of these guidelines is expected to reduce but not completely eliminate the risk of poor sequence capture results related to library quality.
Appendix B. Automation & Oligo Kits A/B

This appendix describes a procedure for using the SeqCap EZ Oligo Kits A and B with liquid handling instruments for setting up Hybridizations. This appendix can be used in place of Chapter 5, when working with a liquid handling instrument. Note: Instructions in this appendix will require additional PCR Grade Water from Roche Applied Science—see order information in Chapter 1.

Step 1. Prepare for Hybridization

1. Turn on a heat block to +95°C and let it equilibrate to the set temperature.
2. Remove the appropriate number of 4.5 µl EZ Library aliquots (one per sample library) from the -15 to -25°C freezer and allow them to thaw on ice.

Step 2. Prepare the Multiplex DNA Sample Library Pool

1. Thaw on ice each of the differently-indexed amplified DNA sample libraries that will be included in the multiplex capture experiment (generated in Chapter 4).
2. Mix together equal amounts (by mass) of each of these amplified DNA sample libraries to obtain a single pool with a combined mass of at least 1.1 µg. This mixture will subsequently be referred to as the “Multiplex DNA Sample Library Pool”. One µg of the multiplex DNA sample library pool will be used in the sequence capture hybridization step, and 60 ng will be used for Measurement of Enrichment Using qPCR (Chapter 8).

To obtain equal numbers of sequencing reads from each component libraries in the Multiplex DNA Sample Library Pool upon completion of the experiment, it is very important to combine identical amounts of each independently amplified DNA sample library at this step. Accurate quantification and pipetting are critical.

Step 3. Resuspend the TS-HE Universal and TS-INV-HE Index Oligos

1. Spin the lyophilized oligo tubes briefly to allow the contents to pellet to the bottom of the tube.
2. Add 1,200 µl PCR-grade water to TS-HE Universal Oligo tube (100 µM final concentration).
3. Add 100 µl PCR-grade water to each TS-INV-HE Index Oligo tube (100 µM final concentration).
4. Vortex the primers plus PCR-grade water for 5 seconds and spin down the resupended oligo tube.
5. The resuspended oligo tube should be stored at -20°C.

Step 4. Prepare the Multiplex Hybridization Enhancing Oligo Pool

1. Thaw on ice the resuspended TS-HE Universal Oligo 1 (100 µM) and each resuspended TS-INV-HE Index oligo (100 µM) that matches a DNA Adapter Index included in the Multiplex DNA Sample Library Pool from Step 2 of this section.
2. Mix together the HE oligos so that the resulting Multiplex Hybridization Enhancing Oligo Pool contains, by mass, 50% TS-HE Universal Oligo 1 and 50% of a mixture of the appropriate TS-INV-HE Index oligos. The total combined mass of the Multiplex Hybridization Enhancing Oligo Pool should be 2,000 pmol, which is the amount required for a single Sequence Capture experiment.

**Example:** If a Multiplex DNA Sample Library Pool contains four DNA sample libraries prepared with Illumina TruSeq indexed adapters 2, 4, 6, and 8, respectively, then the Multiplex Hybridization Enhancing Oligo Pool would contain the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS-HE Universal Oligo 1</td>
<td>1,000 pmol (10 μl of 100 μM)</td>
</tr>
<tr>
<td>TS-INV-HE Index 2 Oligo</td>
<td>250 pmol (2.5 μl of 100 μM)</td>
</tr>
<tr>
<td>TS-INV-HE Index 4 Oligo</td>
<td>250 pmol (2.5 μl of 100 μM)</td>
</tr>
<tr>
<td>TS-INV-HE Index 6 Oligo</td>
<td>250 pmol (2.5 μl of 100 μM)</td>
</tr>
<tr>
<td>TS-INV-HE Index 8 Oligo</td>
<td>250 pmol (2.5 μl of 100 μM)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2,000 pmol (2 μl of 1,000 μM)</strong></td>
</tr>
</tbody>
</table>

Due to the difficulty in accurately pipetting small volumes, it will be helpful where possible to prepare a larger volume Multiplex Hybridization Enhancing Oligo Pool and then to dispense this into individual single-use aliquots that can be stored at -15 to -25°C until needed. For optimal results, it is important that the individual TS-INV-HE oligos contained in a Multiplex Hybridization Enhancing Oligo Pool are precisely matched with the adapter indexes present in the Multiplex DNA Sample Library Pool in a multiplexed Sequence Capture experiment.

**Step 5. Prepare the Hybridization Sample**

1. Add 5 μl of COT Human DNA (1 mg/ml) to a new 1.5 ml tube.

2. Add 1 μg of Multiplex DNA Sample Library to the 1.5 ml tube containing 5 μl of COT Human DNA.

3. Add 2,000 pmol of Multiplex Hybridization Enhancing Oligo Pool (1,000 pmol TS-HE Universal Oligo 1 and 1,000 pmol TS-INV-HE Index Oligo pool) to the Multiplex DNA Sample Library Pool plus COT Human DNA.

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

   The closed lid with a hole in the top of the tube’s cap is a precaution to suppress contamination in the DNA vacuum concentrator.

5. Dry the Multiplex DNA Sample Library Pool/COT Human DNA/Multiplex Hybridization Enhancing Oligo Pool in a DNA vacuum concentrator on high heat (60°C).

   Denaturation of the DNA with high heat is not problematic after linker ligation because the hybridization utilizes single-stranded DNA.
6. To each dried-down Multiplex DNA Sample Library Pool/COT Human DNA/Multiplex Hybridization Enhancing Oligo Pool, add:
   - 7.5 µl of 2X Hybridization Buffer (vial 5)
   - 3 µl of Hybridization Component A (vial 6)

   The tube with the Multiplex DNA Sample Library Pool/COT Human DNA/Multiplex Hybridization Enhancing Oligo Pool should now contain the following components:

<table>
<thead>
<tr>
<th>Component</th>
<th>Solution Capture</th>
</tr>
</thead>
<tbody>
<tr>
<td>COT Human DNA</td>
<td>5 µg</td>
</tr>
<tr>
<td>Multiplex DNA Sample Library Pool</td>
<td>1 µg</td>
</tr>
<tr>
<td>Multiplex Hybridization Enhancing Oligo Pool</td>
<td>2,000 pmol*</td>
</tr>
<tr>
<td>2X Hybridization Buffer (vial 5)</td>
<td>7.5 µl</td>
</tr>
<tr>
<td>Hybridization Component A (vial 6)</td>
<td>3 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10.5 µl</strong></td>
</tr>
</tbody>
</table>

   *Composed of 50% (1,000 pmol) TS-HE Universal Oligo 1 and 50% (1,000 pmol) of a mixture of the appropriate TS-INV-HE Index oligos.

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

8. Vortex the Multiplex DNA Sample Library Pool/COT Human DNA/Multiplex Hybridization Enhancing Oligo Pool plus Hybridization Cocktail (2X Hybridization Buffer + Hybridization Component A) for 10 seconds and centrifuge at maximum speed for 10 seconds.

9. Place the Multiplex DNA Sample Library Pool/COT Human DNA/Multiplex Hybridization Enhancing Oligo Pool/Hybridization Cocktail in a +95°C heat block for 10 minutes to denature the DNA.

10. Centrifuge the Multiplex DNA Sample Library Pool/COT Human DNA/Multiplex Hybridization Enhancing Oligo Pool/Hybridization Cocktail at maximum speed for 10 seconds at room temperature.

11. Transfer the Multiplex DNA Sample Library Pool/COT Human DNA/Multiplex Hybridization Enhancing Oligo Pool/Hybridization Cocktail to the 4.5 µl aliquot of EZ Library in a 0.2 ml PCR tube prepared in Chapter 2 (entire volume can also be transferred to a 96-well PCR plate).

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

   The hybridization sample should now contain the following components:

<table>
<thead>
<tr>
<th>Component</th>
<th>Solution Capture</th>
</tr>
</thead>
<tbody>
<tr>
<td>COT Human DNA</td>
<td>5 µg</td>
</tr>
<tr>
<td>Multiplex DNA Sample Library Pool</td>
<td>1 µg</td>
</tr>
<tr>
<td>Multiplex Hybridization Enhancing Oligo Pool</td>
<td>2,000 pmol*</td>
</tr>
<tr>
<td>2X Hybridization Buffer (vial 5)</td>
<td>7.5 µl</td>
</tr>
<tr>
<td>Hybridization Component A (vial 6)</td>
<td>3 µl</td>
</tr>
<tr>
<td><strong>EZ Library</strong></td>
<td><strong>4.5 µl</strong></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>15 µl</strong></td>
</tr>
</tbody>
</table>

   *Composed of 50% (1,000 pmol) TS-HE Universal Oligo 1 and 50% (1,000 pmol) of a mixture of the appropriate TS-INV-HE Index oligos.

13. 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).
Appendix C. Processing Higher Throughput, 96-Well Plates (Manual)

This appendix describes a procedure for an increased throughput method for SeqCap EZ Library washing and recovery of captured DNA. This appendix can be substituted for Chapter 6 of the protocol to process captured DNA in a 96-well plate format and to adapt the protocol onto a liquid handling instrument.

Additional Equipment, Labware & Consumables

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier</th>
<th>Item Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>MagnaBot II Magnetic Separation Device</td>
<td>Promega</td>
<td>V8351</td>
</tr>
<tr>
<td>Multichannel Pipettors (20 µl and 200 µl)</td>
<td>Multiple vendors</td>
<td></td>
</tr>
<tr>
<td>96-well PCR Plate (1/2 skirt)</td>
<td>Multiple Vendors</td>
<td></td>
</tr>
<tr>
<td>Note: Full skirt 96-well PCR plates will not work with the MagnaBot II Magnetic Separation Device.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 ml and 50 ml Conical Tubes</td>
<td>Multiple vendors</td>
<td></td>
</tr>
</tbody>
</table>

Step 1. Preparing Buffers

1. Prepare 50 ml of a 1X concentration buffer for each of the Sequence Capture Wash Buffers (enough of each buffer for a full 96-well plate) each in a separate 50 ml conical tube.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Stringent Wash Buffer</td>
<td>5 ml</td>
</tr>
<tr>
<td>PCR grade water</td>
<td>45 ml</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50 ml</strong></td>
</tr>
</tbody>
</table>

Preheat the buffer to +47°C in a water bath.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Wash Buffer I</td>
<td>5 ml</td>
</tr>
<tr>
<td>PCR grade water</td>
<td>45 ml</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50 ml</strong></td>
</tr>
</tbody>
</table>

Remove 10 ml of buffer to a separate 15 ml conical tube and preheat in a +47°C water bath. Store the remaining buffer at room temperature.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Wash Buffer II</td>
<td>5 ml</td>
</tr>
<tr>
<td>PCR grade water</td>
<td>45 ml</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50 ml</strong></td>
</tr>
</tbody>
</table>

Store at room temperature after preparation.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Wash Buffer III</td>
<td>5 ml</td>
</tr>
<tr>
<td>PCR grade water</td>
<td>45 ml</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50 ml</strong></td>
</tr>
</tbody>
</table>

Store at room temperature after preparation.
Step 2. Preparing the Capture Beads
1. Warm the Capture Beads to room temperature for 30 minutes prior to use.
2. Vortex the Capture Beads for 15 seconds to resuspend.
3. For each capture reaction, aliquot 100 µl of beads into a well of a 96-well PCR plate.
4. Place the PCR plate onto the MagnaBot II magnetic separation device.
5. Wait for 1 minute to capture the Capture Beads to the side of the tubes. Remove supernatant and discard to waste.
6. Remove the PCR plate from the MagnaBot II device.
7. Add 200 µl of 1X Bead Wash Buffer to each well of the 96-well plate and mix by pipetting up and down to thoroughly resuspend the Capture Beads into solution.
8. Place the PCR plate onto the MagnaBot II device. Wait for 1 minute to capture the Capture Beads to the side of the tubes. Remove the supernatant and discard to waste.
9. Remove the PCR plate from the MagnaBot II device.
10. Repeat Steps 2.7 - 2.9.
11. Add 100 µl of 1X Bead Wash Buffer (vial 7) to each well of the 96-well plate and mix by pipetting up and down to thoroughly resuspend the Capture Beads into solution.
12. Place the PCR plate onto the MagnaBot II device. Wait for 1 minute to capture the Capture Beads to the side of the tubes. Remove the supernatant and discard to waste.

Step 3. Binding DNA to the Capture Beads
1. Transfer each hybridization sample (approximately 15 µl) to a well of the 96-well plate containing the prepared Capture Beads.
2. Mix thoroughly by pipetting up and down ten times.
3. Place the 96-well plate containing the sample and beads in a thermocycler and incubate for 45 minutes at +47°C. Mix the samples by vortexing at 15-minute intervals.

Step 4. Washing the Capture Beads plus bound DNA
1. After the 45-minute incubation at +47°C, place the PCR plate onto the MagnaBot II device. Wait for 1 minute to capture the Capture Beads to the side of the tubes. Remove the supernatant to waste.
2. Add 100 µl of preheated (+47°C) 1X Wash Buffer I to each well of the 96-well PCR plate. Mix by pipetting up and down to thoroughly resuspend the Capture Beads into solution.
   Alternatively, it is fine to add 100 µl of preheated (+47°C) 1X Wash Buffer I to each well of the 96-well PCR plate prior to Step 4.1 if there is sufficient room in the individual PCR wells to accommodate the additional volume of the buffer.
3. Place the PCR plate onto the MagnaBot II device. Wait for 1 minute to capture the Capture Beads to the side of the tubes. Remove the supernatant and discard to waste.
4. Add 200 µl of 1X Stringent Wash Buffer heated to +47°C to each well of the 96-well plate. Mix by pipetting up and down to thoroughly resuspend the Capture Beads into solution.

5. Place the PCR plate into a thermocycler and incubate for five minutes at +47°C.

6. Place the PCR plate onto the MagnaBot II device. Wait for 1 minute to capture the Capture Beads to the side of the tubes. Remove the supernatant and discard to waste.

7. Repeat Steps 4.4 - 4.6 for a total of two washes with the 1X Stringent Wash Buffer heated to +47°C.

8. Add 200 µl of room temperature 1X Wash Buffer I to each well of the 96-well PCR plate. Mix by pipetting up and down to thoroughly resuspend the Capture Beads into solution.

9. Place the PCR plate onto the MagnaBot II device. Wait for 1 minute to capture the Capture Beads to the side of the tubes. Remove the supernatant and discard to waste.

10. Add 200 µl of room temperature 1X Wash Buffer II to each well of the 96-well PCR plate. Mix by pipetting up and down to thoroughly resuspend the Capture Beads into solution.

11. Place the PCR plate onto the MagnaBot II device. Wait for 1 minute to capture the Capture Beads to the side of the tubes. Remove the supernatant and discard to waste.

12. Add 200 µl of room temperature 1X Wash Buffer III to each well of the 96-well PCR plate. Mix by pipetting up and down to thoroughly resuspend the Capture Beads into solution.

13. Place the PCR plate onto the MagnaBot II device. Wait for 1 minute to capture the Capture Beads to the side of the tubes. Remove the supernatant and discard to waste.

14. Remove the plate from the MagnaBot II device and add 50 µl of PCR grade water to each well of the 96-well PCR plate. Mix by pipetting up and down to thoroughly resuspend the Capture Beads into solution.

15. Store the beads plus captured samples at -15 to -25°C or proceed to Chapter 7.

There is no need to elute DNA off the beads. The beads plus captured DNA will be used as template in the LM-PCR as described in Chapter 7.
Notes


## Appendix D. Multiplexed (Post-Capture) for Sequencing

This appendix provides a recommended workflow 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. 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.

### I. Preparing the DNA Sample Library

<table>
<thead>
<tr>
<th>Sample Library</th>
<th>Sample Library</th>
<th>Sample Library</th>
</tr>
</thead>
<tbody>
<tr>
<td>constructed with Illumina TruSeq DNA Adapter Index AD002</td>
<td>constructed with Illumina TruSeq DNA Adapter Index AD004</td>
<td>constructed with Illumina TruSeq DNA Adapter Index AD005</td>
</tr>
</tbody>
</table>

### II. Amplifying Pre-Capture Sample Library Using LM-PCR

### III. Hybridizing Amplified Sample and EZ Probe Libraries

- Hybridization sample includes 1,000 pmol of TS-HE Universal Oligo 1 + 1,000 pmol of TS-INV-HE Index 2 Oligo
- Hybridization sample includes 1,000 pmol of TS-HE Universal Oligo 1 + 1,000 pmol of TS-INV-HE Index 4 Oligo
- Hybridization sample includes 1,000 pmol of TS-HE Universal Oligo 1 + 1,000 pmol of TS-INV-HE Index 5 Oligo

### IV. Amplifying Post-Capture Sample Library Using LM-PCR

### V. Quantitating and pooling an equal mass of each Captured Amplified Library

(qPCR to estimate enrichment is recommended here, prior to pooling)

* Pooling unequal amounts of Captured Amplified Libraries will result in skewed ratios of sequence reads representing the component indexes following step VI.

### VI. Proceed to sequencing of the pooled, indexed, Captured Amplified Libraries
Appendix E. Cleaning up the Amplified Captured DNA using Qiagen QIAquick PCR Purification Kit

This appendix provides instructions for the use of Qiagen QIAquick PCR Purification Kit in place of DNA Purification Beads for the purification of the PCR products resulting prior to and after hybridization.

References

- Microcentrifuge Manual
- Qiagen QIAquick PCR Purification Kit Protocol

1. Pool the two reactions from each amplified captured DNA sample into a single 1.5 ml microcentrifuge tube (approximately 200 µl). Process the negative control in exactly the same way as the amplified captured DNA.

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

3. To each tube of amplified captured DNA (plus Capture Beads), add 1,000 µl (5x volume) of Qiagen buffer PBI. Mix well.

4. Pipette 750 µl of the amplified captured DNA plus beads in PBI into a QIAquick PCR Purification column.

5. Centrifuge at 16,000 x g for 1 minute. Discard the flow-through.

6. Load the remainder of the amplified captured DNA in PBI into the same column and centrifuge at 16,000 x g for 1 minute. Discard the flow-through.

7. Add 750 µl of buffer PE to the column. Centrifuge at 16,000 x g for 1 minute.

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

9. Add 50 µl of buffer EB directly to the column matrix. Transfer the column to a 1.5 ml microcentrifuge tube. Let the column stand for 1 minute. Centrifuge at 16,000 x g for 1 minute to elute the DNA.

Due to the presence of the Capture Beads, the Qiagen QIAquick column might appear brown in color. The Capture Beads will not interfere with the action of the Qiagen QIAquick PCR Purification Kit column or buffers.
Appendix F. Troubleshooting

This appendix provides guidance for interpreting unexpected results and recommendations for implementing corrective action if problems occur. For technical questions, contact your local Roche Technical Support. Go to www.nimblegen.com/arraysupport for contact information.

<table>
<thead>
<tr>
<th>Observation</th>
<th>Cause(s) / Recommendation(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample Library Preparation</strong></td>
<td></td>
</tr>
<tr>
<td>The Sample Library Quality Assessment step (Chapter 3, Step 2) indicates the DNA library peak is not within the optimal size range of 150 - 400 bp.</td>
<td>Repeat the Sample Library Quality Assessment step to confirm the result. Repeat library preparation if necessary.</td>
</tr>
<tr>
<td>The test in Appendix A indicates amplified library Cp value is &gt; 15% higher than the Cp value of the input genomic DNA in qPCR assays for two or more of the four standard control loci.</td>
<td>The LM-PCR amplified sample library may not be suitable for hybridization. Repeat the test in Appendix A to confirm the result. Repeat library preparation if necessary.</td>
</tr>
</tbody>
</table>

**Amplified Sample Library (Pre-Capture LM-PCR Product)**

<table>
<thead>
<tr>
<th>Observation</th>
<th>Cause(s) / Recommendation(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield is &lt; 1 µg (yield should be e 1 µg).</td>
<td>Possible error occurred during library preparation or damaged reagents were used. Use an evaluated sample library as a positive control for LM-PCR reagents. Repeat library preparation if necessary.</td>
</tr>
<tr>
<td>Fragment distribution (analyzed using a Agilent DNA 1000 chip) shows that the average amplified fragment size is not within the size range of 150 - 500 bp.</td>
<td>Poor fragmentation occurred. Repeat library preparation. Consider implementing the gel-cut size selection option.</td>
</tr>
<tr>
<td>$A_{agg}/A_{raw}$ is &lt; 1.7 (ratio should be 1.7 - 2.0).</td>
<td>Inefficient sample cleanup was performed. Repeat cleanup.</td>
</tr>
<tr>
<td>The negative control yield measured by the NanoDrop spectrophotometer is non-negligible.</td>
<td>The measurement may be high due to the presence of oligonucleotides carried over from previous steps/LM-PCR. This carryover will be apparent as one or more sharp peaks visible less than 150 bp in size when examining the data from the Agilent Bioanalyzer DNA 1000 chip. This carryover is not a sign of contamination and will not interfere with the capture process.</td>
</tr>
<tr>
<td>The Agilent Bioanalyzer DNA 1000 chip indicates one or more visible sharp peaks that are &lt; 150 bp in size.</td>
<td>These peaks, which represent oligonucleotides carried over from previous steps/LM-PCR, may be especially prominent when the library preparation procedure omits the optional gel-cut step. This carryover will not interfere with the capture process but could lead to overestimation of the amplified library yield when interpreting the data from NanoDrop spectrophotometer.</td>
</tr>
<tr>
<td>The Agilent Bioanalyzer DNA 1000 chip indicates that the average amplified material is 150 - 500 bp in size in the negative control for sample library amplification.</td>
<td>This material could represent cross-contamination between amplified sample libraries. Test reagents for contamination and replace if necessary. Repeat library construction using fresh genomic DNA.</td>
</tr>
</tbody>
</table>
## Observation | Cause(s) / Recommendation(s)
--- | ---
**Amplified captured multiplex DNA (Post-Capture LM-PCR Product)**
Yield is < 500 ng (yield should be e 500 ng). | Incorrect hybridization or wash temperatures was used. Make sure the correct hybridization and wash temperatures were used. If temperatures were not correct, repeat the experiment from hybridization. PCR reagents are damaged. Verify that the positive control worked. If the positive control did not work, repeat hybridization and re-amplify using fresh PCR reagents. **Note:** Experiments designed to capture less genomic DNA (*i.e.* a smaller cumulative target size) may be successful even though they can generate lower LM-PCR yields than experiments designed to capture larger targets. Target size and enrichment qPCR results ([Chapter 8](#)) should be taken into consideration when evaluating low Post-Capture LM-PCR yield.

Fragment distribution (analyzed using a Agilent DNA 1000 chip) shows that the average amplified fragment size is not within the size range of 150 - 500 bp. | Poor fragmentation occurred. Repeat library preparation. Consider implementing the gel-cut size selection option.

A<sub>260</sub>/A<sub>280</sub> is < 1.7 (ratio should be 1.7 to 2.0). | Inefficient sample cleanup was performed. Repeat cleanup.

### Measurement of Enrichment Using qPCR
Standard deviation of triplicate qPCR reactions is > 0.5. | Pipetting error may have occurred. Repeat qPCR assays.
Average Cp values for negative control assays are not negligible. | Possible cross contamination across wells or reagent contamination occurred. Repeat qPCR assays.
One or more calculated NSC control locus fold enrichment values, or the average of all four, are less than the recommended value of tenfold ([Chapter 8](#)). | Low qPCR values often correlate with low capture specificity, or a reduced “on target” rate, for captured fragments. Repeat qPCR assays. If the result is confirmed, repeat the experiment from the beginning.

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⚠️ The Illumina library preparation and sequencing workflows are not supported by Roche NimbleGen Technical Support.
Appendix G. Limited Warranty

ROCHE NIMBLEGEN, INC.
NIMBLEGEN PRODUCTS

1. Limited Warranty

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Evidence of original purchase is required. It is important to save your sales receipt or packaging slip to verify purchase.