xGen™ HS EGFR Pathway Amplicon Panel

For research use only. Not for use in diagnostic procedures. | Version 2

REVISION HISTORY

Version	Release date	Description of changes
2	February 2022	Updated UMI incorporation thermal cycler program settings
1	December 2021	Initial release

Table of contents

OVERVIEW

The xGen™ HS EGFR Pathway Amplicon Panel is a custom amplicon panel to prepare high quality, targeted, next generation sequencing (NGS) libraries from a variety of sample types, including formalin-fixed paraffin-embedded (FFPE) and circulating cell-free DNA (cfDNA). Indexing primers are included for combinatorial, dual indexing, and multiplexing up to 96 samples on a sequencing run (see Appendix A). The single tube workflow from DNA to library can be completed within three hours.

The xGen HS EGFR Pathway Amplicon Panel includes unique molecular identifiers (UMIs) that allow tracking of amplification products. The UMIs can be used to generate a consensus sequence to eliminate errors that may have originated during amplification or sequencing.

Figure 1. Overview of xGen HS EGFR Pathway Amplicon Panel UMI library structure. xGen HS technology utilizes a 10-base, random N sequence that enables more than one million UMIs. These are positioned at the start of Read 2. Each amplicon receives a UMI, which is assigned to each original, ssDNA (single-stranded DNA) template.

The xGen HS EGFR Pathway Amplicon Panel utilizes Illumina®-compatible adapter sequences and can be used with Illumina® platforms (see Appendix B).

Features	Specifications
Design coverage and panel information	xGen HS EGFR Pathway Amplicon Panel, 24 rxns 17 amplicons, sized 106–149 bp (average 134 bp)
Input material	10 ng for 0.5% detection; 20 ng for 0.25% detection
Time	3 hours DNA-to-library
Components	Target-specific multiplex primer pool PCR and library prep reagents Dual indexed primers
Multiplexing capability	Up to 96 combinatorial dual indexes (CDI)
Recommended depth	1M reads (0.5% from 10 ng), 1.5M reads (0.25% from 20 ng)

Supported applications and sample types

- **Applications:** Oncology research, variant discovery, sample tracking
- **Sample Types:**
  - gDNA
  - Freshly frozen DNA
  - Formalin-fixed, paraffin-embedded (FFPE) DNA
  - Cell-free DNA (cfDNA)
  - High molecular weight DNA

xGen HS EGFR PANEL WORKFLOW

This protocol contains three PCR steps: one for the incorporation of UMIs (PCR I), one for target amplification (PCR II), and one for the addition of combinatorial, dual indexed adapters (PCR III) (see Appendix A), enabling multiplexing of up to 96 unique libraries (see Appendix B).

Bead-based cleanups are used to purify the sample by removing unused oligonucleotides and changing buffer composition between steps.

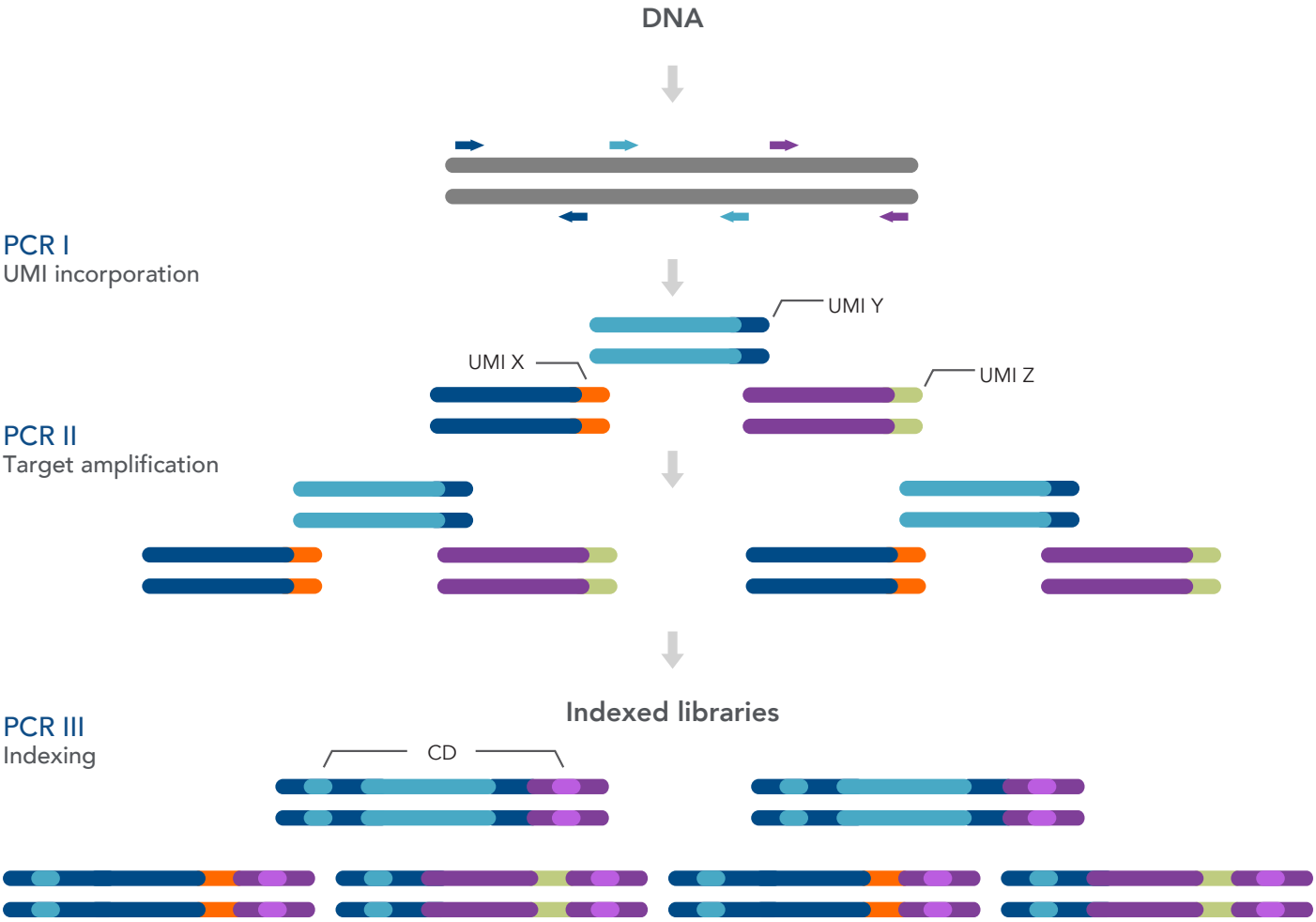

Figure 2. xGen HS EGFR Pathway Amplicon Panel workflow. There are three PCR steps to prepare dual indexed libraries for next generation sequencing, including 1) UMI incorporation, 2) target amplification, and 3) PCR indexing to add adapter sequences.

CONSUMABLES AND EQUIPMENT

These kits contain sufficient reagents for the preparation of 24 libraries (10% excess volume provided).

Consumables from IDT—Reagents

Workflow component	Product name	Catalog number
xGen HS Kit	xGen HS EGFR Pathway Amplicon Panel, 24 rxn	10009858

Consumables from IDT—Kit contents

Workflow stage	Component	24 rxn (µL)	Storage
UMI incorporation	• Reagent B1	53	–20°C
	• Enzyme B2	396	
	Pre-PCR TE	1000	Room temperature
Target amplification reagents	• Reagent G1	132	–20°C
	• Enzyme G2	660	
Indexing reagents	• Enzyme Y1	396	
	• IPA_D50X	25 each of D501-D508	
	• IPA_D7XX	18 each of D701-D712	
	Post-PCR TE	1000	Room temperature
	PEG NaCl	20 mL	

Consumables from other suppliers

Item	Supplier	Catalog number
SPRIselect® or AMPure® XP beads, or equivalent	Beckman Coulter	B23317/B23318/B23319 or A63880/A63881/A63882
Aerosol-resistant pipette tips ranging from 1 to 1000 µL	Various suppliers	Varies
0.2 mL PCR tubes or 96-well plates	Various suppliers	Varies
200 proof (absolute) ethanol (molecular biology grade)	Various suppliers	Varies
Nuclease-free water (molecular biology grade)	Various suppliers	Varies
Reagents for qPCR-, electrophoretic-, or fluorometric-based library quantification assay for Illumina® libraries	Various suppliers	Varies

Equipment

Item	Supplier	Catalog number
Permagen® magnetic separator, or equivalent	Various suppliers	MSR812, MSP750
Instrument for qPCR- , electrophoretic-, or fluorometric-based library quantification assay for Illumina® libraries	Various suppliers	Varies
Microcentrifuge	Various suppliers	Varies
Vortex	Various suppliers	Varies
Programmable thermal cycler*	Various suppliers	Varies
Pipettes ranging from 1 to 1000 µL capacity	Various suppliers	Varies

* All xGen HS libraries are created with a Bio-Rad® T100.

GUIDELINES

Reagent handling

- Upon receipt, store the xGen HS Library Kit products at –20°C, except for the PEG solution and TE, which are stored at room temperature.
- Separate the UMI Incorporation and Target Amplification Reagents (keep in pre-PCR area) from the Indexing Reagents (keep in the post-PCR area). For more details, see Avoid cross-contamination.
- To maximize use of enzyme reagents, remove enzyme tubes from –20°C storage and place on ice for 10 minutes prior to pipetting. Attempting to pipette enzymes at –20°C may result in shortage of enzyme reagents.
- After thawing reagents on ice, briefly vortex (except enzymes) to mix well, then pulse-spin to collect contents before proceeding.
- Always add reagents to the master mix in the specified order as stated throughout the protocol. The indexing primers (xGen Amplicon CDI) are the only reagents that are added individually to each sample.
- Assemble all reagent master mixes and reactions ON ICE and scale volumes as appropriate, using 5% excess volume to compensate for pipetting loss. Neglecting to store master mixes and reagents on ice prior to incubations reduces yields and function of this product.

Avoid cross-contamination

To reduce the risk of DNA and library contamination, physically separate the laboratory space, equipment, and supplies where pre-PCR and post-PCR processes are performed, including appropriate reagent boxes for pre-PCR (UMI and target amplification) and post-PCR (indexing) reagents. Move samples to the post-PCR area before opening tubes. This workflow, like any amplicon enrichment technology, poses a risk of contamination to samples following the amplification step. Please use caution when opening your sample tubes after the multiplex PCR step (see Appendix B). Follow the instructions below to avoid cross-contamination:

- Clean lab areas using 0.5% sodium hypochlorite (10% bleach).
- Use barrier pipette tips to avoid exposure to potential contaminants.
- Always change tips between each sample.
- Perform pre-PCR reactions in a separate location from the post-PCR area, ideally in a PCR workstation.
- Separate the multiplex PCR reagents (keep in pre-PCR area) from the Indexing reagents (keep in post-PCR area).

Size selection during cleanups

SPRISelect beads from Beckman Coulter (B23317/B23318/B23319) are recommended; however, these can be substituted with Agencourt AMPure XP beads (Beckman Coulter, Cat. Nos. A63880/A63881/A63882). Make sure that the beads and samples are at room temperature before use. At no time should "with bead" samples be stored on ice, as this adversely affects DNA binding to the magnetic beads. Briefly vortex beads to homogenize before use. Ensure beads and samples never completely dry during processing.

Input material considerations

xGen HS panels enable the preparation of targeted NGS libraries from DNA templates.

The starting material should be quantified with the xGen Input DNA Quantification Primers (Cat. No. 10009856) for FFPE and cfDNA, high quality DNA from whole blood, freshly frozen, or cultured cells, as described in the xGen Input DNA Quant Primers protocol.

The optimal coverage uniformity of this technology is achieved with qPCR-verified input amounts in the 10–50 ng range. Using less than 10 ng of DNA input may reduce the specificity of the assay and will affect variant calling for low frequency alleles. Consider the following example allele frequencies when determining the limit of detection:

Sample quantity (ng)	Human genome equivalents (total copies)	Example allele frequency (%)	Example allele equivalents (copies)	Feasibility of calling variant (high quality DNA)
50	15,000	0.2	30	√
50	15,000	0.1	15	√
50	15,000	0.05	7.5	X
20	6000	0.5	30	√
20	6000	0.25	15	√
20	6000	0.125	7.5	X
10	3000	1.0	30	√
10	3000	0.5	15	√
10	3000	0.25	7.5	X
1	300	5.0	15	√
1	300	1.0	3	X
1	300	0.5	1.5	X

Note: Inputs with less than 10 allele copies are not supported and may not even be detectable since the probability of detection follows a normal distribution. That is, the fewer the copies of an allele in the input material, the lower the chance that it will be present in the reaction.

Notes on automation

- This protocol is readily automatable. A 10% overage volume of reagents is supplied to accommodate automation. Contact us at applicationsupport@idtdna.com if you require additional reagent overage volume or would like to learn about our custom packaging options.
- While IDT does not supply automated liquid handling instruments or consumables, our automation team collaborates with automation solution providers and customers to develop and qualify automated scripts for use of our kits with liquid handling platforms routinely used in NGS library preparation. Contact us at applicationsupport@idtdna.com to discuss automating the xGen HS Kits with your automated liquid handling system.

PROTOCOL

Prepare the DNA libraries

1. Preprogram the thermal cycler for the UMI Incorporation, Target Amplification, and Indexing Reactions.

   Note: Cycling conditions and data quality can vary based on input quality and quantity.

PCR program name	Cycling conditions		
UMI Incorporation thermal cycler program	45 sec	98°C	3 cycles
	30 sec	98°C	
	2 min	64°C	
	2 min	62°C	
	4 min	60°C	
	2 min	58°C	
	1 min	65°C	
	1 min	65°C	
	Hold	4°C	
Target amplification thermal cycler program	45 sec	98°C	22 cycles*
	10 sec	98°C	
	15 sec	60°C	
	1 min	66°C	
	Hold	4°C	
Indexing Reaction thermal cycler program	45 sec	98°C	7 cycles
	10 sec	98°C	
	15 sec	60°C	
	1 min	66°C	
	Hold	4°C	

* For samples with <10 ng input, an additional 1 or 2 PCR cycles in the Target Amplification step can be used to increase yields.

Perform UMI incorporation

Important: Work in the designated pre-PCR area in your lab.

1. Start the UMI Incorporation PCR program and allow the block to reach 98°C before loading samples (confirm lid heading is turned ON and is set to reach 105°C).
2. Load 13 µL of sample DNA into each PCR tube. If there is less than 13 µL of sample DNA in a tube, adjust volumes by adding Pre-PCR TE to each tube to reach a total volume of 13 µL.
3. Make the Reaction Mix with the components described in the table below. Assemble the components on ice. Prepare enough volume of the B1 and B2 mixture for each sample.

Component	Volume (1 rxn) (µL)
• Reagent B1*	2
• Enzyme B2	15
Reaction mix	**17**

* Reagent B1 is the panel-specific set of multiplex amplification primers.

4. Mix well by pipetting up and down. Add 17 µL of the Reaction Mix to each 13 µL sample. Place tubes in the thermal cycler and run the program.

Perform DNA cleanup

1. Ensure beads and samples are at room temperature. Briefly vortex beads to homogenize before use.
2. Add 36 µL (ratio: 1.2X) of SPRIselect beads to each 30 µL sample. Mix by vortexing. Ensure no bead-sample suspension droplets are left on the sides of the tube. If droplets are visible, pulse-spin the samples in a microcentrifuge to collect contents.
3. Incubate the samples for 5 minutes at room temperature, off the magnet.
4. Pulse-spin the samples in a microcentrifuge. Place the sample tubes on a magnetic rack until the solution clears and a pellet is formed (~5 minutes).
5. While the sample is on the magnet, remove and discard the supernatant without disturbing the pellet (approximately 5 µL may be left behind). Leave tubes on the magnet.
6. Add 200 µL of freshly prepared ethanol solution to the pellet, while it is still on the magnet. Be careful that you do not disturb the pellet. Incubate for 30 seconds, then carefully remove the ethanol solution with a clean pipette tip.
7. Repeat step 6 for a second wash with the ethanol solution.
8. Pulse-spin the samples in a microcentrifuge, place back onto the magnet, and remove any residual ethanol solution from the bottom of the tube.
9. Add 20 µL of Pre-PCR TE buffer and resuspend the pellet, mixing well by pipetting up and down until homogenous. Incubate at room temperature for 2 minutes, off the magnet. Then, place the sample back on the magnet and transfer the clean 20 µL library eluate to a fresh tube.

Perform target amplification

1. Start the Target Amplification thermal cycler program and allow the block to reach 98°C before loading.
2. Make the Reaction Mix with the components described in the table below. Assemble the components on ice. Prepare enough volume of the G1 and G2 mixture for each sample

Component	Volume (1 rxn) (µL)
• Reagent G1	5
• Enzyme G2	25
Reaction mix	**30**

3. Mix well and then add 30 µL of the Reaction Mix to each 20 µL sample. Place tubes in the thermal cycler and run the Target Amplification program.

   Important: Move samples to the designated post-PCR area in your lab before opening tubes.

4. Start the Indexing Reaction thermal cycler program and allow the block to reach 98°C before loading samples (confirm lid heating is turned ON).

Perform DNA cleanup

1. Ensure beads and samples are at room temperature. Briefly vortex beads to homogenize before use.
2. Add 60 µL (ratio: 1.2X) of SPRISelect beads to each 50 µL sample. Mix by vortexing. Ensure no bead-sample suspension droplets are left on the sides of the tube. If droplets are visible, pulse-spin the samples in a microcentrifuge to collect contents.
3. Incubate the samples for 5 minutes at room temperature, off the magnet.
4. Pulse-spin the samples in a microcentrifuge. Place the sample tubes on a magnetic rack until the solution clears and a pellet is formed (~5 minutes).
5. While sample is on the magnet, remove and discard the supernatant without disturbing the pellet (approximately 5 µL may be left behind). Leave tubes on the magnet.
6. Add 200 µL of freshly prepared ethanol solution to the pellet while it is still on the magnet. Be careful that you do not disturb the pellet. Incubate for 30 seconds, then carefully remove the ethanol solution with a clean pipette tip.
7. Repeat step 6 for a second wash with the ethanol solution.
8. Pulse-spin the samples in a microcentrifuge, place back onto the magnet, and remove any residual ethanol solution from the bottom of the tube.
9. Proceed to Perform indexing step without delay.

Perform indexing

Important: Continue working in the designated post-PCR area in your lab.

1. Add a unique combination of 7.5 µL IPA_D50X + 7.5 µL IPA_D7XX to each sample bead pellet. Make sure that the beads do not over-dry.
2. Add 15 µL of Enzyme Y1 to each sample and resuspend the pellet (total volume 30 µL).

Component	Volume (1 rxn) (µL)
• IPA_D50X	7.5
• IPA_D7XX	7.5
• Enzyme Y1	15
Reaction mix	**30**

3. Place samples in the thermal cycler and run the Indexing Reaction program.

Perform DNA cleanup

1. Ensure PEG NaCl solution is at room temperature. Briefly vortex the PEG NaCl solution to homogenize before use.
2. Add 26 µL (ratio: 0.85X) of PEG NaCl solution to each 30 µL sample. Mix by vortexing. Ensure no bead-sample suspension droplets are left on the sides of the tube. If droplets are visible, pulse-spin the samples in a microcentrifuge to collect contents.
3. Incubate the samples for 5 minutes at room temperature, off the magnet.
4. Pulse-spin the samples in a microcentrifuge. Place the sample tubes on a magnetic rack until the solution clears and a pellet is formed (~5 minutes).
5. While sample is on the magnet, remove and discard the supernatant without disturbing the pellet (approximately 5 µL may be left behind). Leave tubes on the magnet.

6. Add 200 µL of freshly prepared ethanol solution to the pellet while it is still on the magnet. Be careful that you do not disturb the pellet. Incubate for 30 seconds, then carefully remove the ethanol solution with a clean pipette tip.
7. Repeat step 6 for a second wash with the ethanol solution.
8. Pulse-spin the samples in a microcentrifuge, place back onto the magnet, and remove any residual ethanol solution from the bottom of the tube.
9. Add 20 µL of post-PCR TE buffer and resuspend the pellet, mixing well by pipetting up and down until homogenous. Incubate at room temperature for 2 minutes off the magnet.
10. Place the sample back on the magnet and transfer the clean 20 µL library eluate to a fresh tube. Make sure that the eluate does not contain magnetic beads (indicated by brown coloration in eluate). If magnetic beads are present, place the tubes back on the magnet, wait for a pellet to form, then transfer eluate to a fresh tube.

LIBRARY QUANTIFICATION

Library quantification can be performed by qPCR, a fluorometric method such as Qubit®, Bioanalyzer®, or similar. Use a library size of 275 bp for quantification calculations. When working with Illumina® instruments, refer to their user guides for specific recommendations regarding reagents and loading.

PhiX spike-in

Libraries prepared from xGen Amplicon Panels with at least 17 amplicons do not require a PhiX spike-in when sequencing on either the MiSeq® or MiniSeq® (Illumina®) instruments.

Contact applicationsupport@idtdna.com if you plan to sequence these libraries without the addition of higher complexity samples such as PhiX on any other Illumina® instruments.

DATA ANALYSIS AND BIOINFORMATICS

Adapter and primer trimming

xGen HS Panels are designed with overlapping amplicons to allow for contiguous regions of coverage in a single-tube format. Therefore, synthetic primer sequences will be encountered both at the beginning and end of some reads. These primer sequences must be trimmed during the data analysis. This can be done using IDT's tool, Primerclip. Make sure that adapter trimming is enabled while setting up the sequencing run.

Note: Download panel-specific files through our website. A target BED file is provided with purchase of the xGen HS EGFR Pathway Amplicon Panel.

Tip: Alternatively, adapter trimming can be performed bioinformatically before analysis.

There are a few key considerations when analyzing sequencing data generated from the xGen HS Panels with UMIs:

- The first 10 bases in front of Read 2 constitute a UMI (see Figure 1). Hence, trim (CROP) these first 10 bases from Read 2 with Trimmomatic to make a UMI .fastq file for use with the UMI pipeline, based on the fgbio package (Fulcrum Genomics).
- Prior to aligning the reads, make sure that the 10 bp UMI (which contains random bases) has been trimmed off from the 5′ end of Read 2. If additional informatics pipeline advice is needed, contact applicationsupport@idtdna.com.

APPENDIX A: LIBRARY MULTIPLEXING OPTIONS FOR MiSeq®

Use the following equation to determine the possible number of libraries to multiplex per sequencing run:

Level of multiplexing = (number of paired-end reads)/(number of amplicons * intended average read depth)

MiSeq® multiplexing guidelines for the xGen HS EGFR Pathway Amplicon Panel are shown in this table.

Input	LOD (%)	Minimum # recommended reads	v2 Nano (300 cycles) (2M paired-end reads)	v2 Micro (300 cycles) (8M paired-end reads)	v2 (300 cycles) (30M paired-end reads)	v3 (600 cycles) (50M paired-end reads)
10 ng	1	0.5M	4	16	60	96*
	0.5	1M	2	8	30	50
20 ng	1	0.75M	2	10	40	66
	0.5	1M	2	8	30	50
	0.25	1.5M	1	5	20	33

* Higher level of multiplexing is possible with a custom solution. Contact us at applicationsupport@idtdna.com.

APPENDIX B: INDEXED ADAPTER SEQUENCES

The full-length indexing primer sequences are below. Underlined text indicates the location of the index sequences, which are 8 bases for CDI. These sequences represent the adapter sequences following completion of the Indexing PCR step.

Index 1 (i7) Adapter:

5′ – GATCGGAAGAGCACACGTCTGAACTCCAGTCAC<u>XXXXXXXX</u>ATCTCGTATGCCGTCTTCTGCTTG – 3′

Index 2 (i5) Adapter:

5′ – AATGATACGGCGACCACCGAGATCTACAC<u>YYYYYYYY</u>ACACTCTTTCCCTACACGACGCTCTTCCGATCT – 3′

Refer to the Index Master List for index sequences in preparing your Illumina® sequencing sample sheet on the instrument of your choice. Contact applicationsupport@idtdna.com for assistance in confirming compatibility of your own primers with the xGen HS workflow, or contact your local sales representative or distributor.

APPENDIX C: STRUCTURE AND MIGRATION BEHAVIOR OF xGen HS LIBRARIES

If using high quality DNA as input for the xGen HS libraries, "extended amplicons" can be observed on an Agilent Bioanalyzer™ or TapeStation™. These are formed from the forward primer and the reverse primer of two adjacent amplicons. These extended amplicons are not usually formed when using fragmented or cross-linked (FFPE) DNA, or cell-free DNA. Coverage uniformity should not be affected by the presence or absence of extended amplicons.

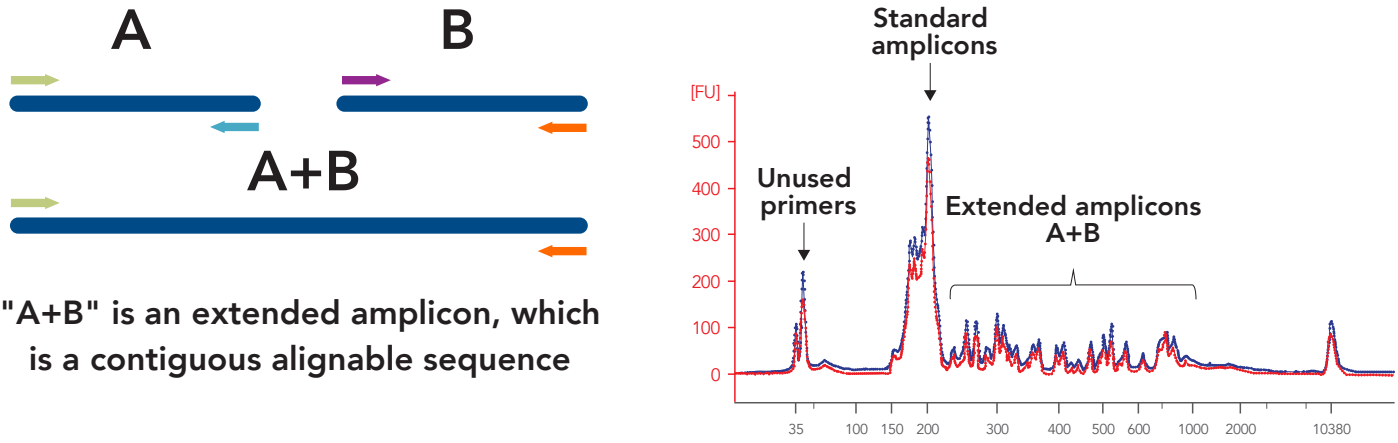

Figure 3. Extended amplicons when using high quality DNA. The forward and reverse primers of two adjacent amplicons can cause "extended amplicons" (left) that are detected on an Agilent Bioanalyzer™ or TapeStation™ (right). The extended amplicons usually produce peaks in the data around 300–600 bp.

APPENDIX D: TROUBLESHOOTING

Issue	Possible cause	Suggested remedy
Lower than expected yields	Inadequate sample quality and/or quantity, incorrect input quantification method, or incorrect SPRI methods	Use qPCR-quantified input. Perform SPRI carefully to avoid overdrying or cracking of beads.
Incomplete resuspension of beads after ethanol wash during SPRI steps	Overdrying of beads	Continue pipetting the liquid over the beads for complete resuspension.
Lower than expected cluster density	Error in library quantification	Quantify library with a qPCR-based method.
Precipitates in Enzymes B2, G2, or Y1	Salt precipitation	Allow the vial to reach room temperature and gently rock until precipitate dissolves. Place on ice for remainder of use.

Note: If you experience problems with your library prep, contact applicationsupport@idtdna.com.

xGen™ HS EGFR Pathway Amplicon Panel

Technical support: applicationsupport@idtdna.com

Doc ID: RUO21-0489_002 02/22