



Automation of xGen™ Hybridization Capture on the Sciclone® G3 NGS Workstation



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REVISION HISTORY

Document version	Date released	Description of changes
3	Oct. 2021	Updated xGen custom hybridization capture panel offering
2	March 2019	Added appendix to describe combining panels
1	March 2018	Initial release

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INTRODUCTION

IDT offers both predesigned and custom enrichment panels for targeted next generation sequencing (NGS) that consist of individually synthesized hybridization capture probes. The xGen™ Hybridization and Wash Kit includes buffers, xGen Human Cot DNA, and streptavidin beads required for hybridization capture of DNA. The automation of the hybrid capture workflow on the Sciclone® G3 NGS Workstation (Perkin Elmer) decreases variability in research experiments. The automated method outlined in this document was developed by IDT researchers and tested using sequencing panels of various sizes.

Methods

This protocol describes hybridization capture using either an IDT xGen Custom Hyb Panel, one of a variety of the xGen™ Predesigned Hyb Panels, or a combination of the two types, together with the xGen Hybridization and Wash Kit. The methods presented here have been developed for automation on the Perkin Elmer Sciclone G3 NGS Workstation. During the methodology development, libraries were prepared using the KAPA® Hyper Prep Kit with 1 µg of input DNA sheared to an average size of 330 bp, and were then quantified using a fluorescence-based quantification method (Qubit™ fluorometer; Thermo Fisher). Library DNA integrity and average fragment size were determined using a TapeStation® instrument (Agilent). PCR thermocycling was performed offline using a CT1000™ thermal cycler (BioRad). Samples were dried using a DNA 120 SpeedVac™ concentrator (Savant). All sequencing runs were performed on a NextSeq® system (Illumina), using either high-output or mid-output reagents and 2x150 read lengths. Five modules were developed to encompass the workflow from library normalization through post-capture PCR cleanup. The modules include:

- Normalization and pooling
- Blocking setup
- Hybridization setup
- Target capture
- Post-capture PCR/SPRI® bead-based purification (Beckman Coulter)

Before you start

At the start of each module, ensure the Sciclone G3 NGS Workstation, associated computer, and the Inheco® controller are turned on. All modules are associated with Excel® workbooks (Microsoft) that integrate directly with the Sciclone G3 NGS Workstation.

Because samples are processed by column, the scripts support sample numbers that are multiples of eight. The maximum number of samples that can be processed in a run is 96.

Use filtered 150 µL tips during this protocol. Use the following plate types:

- Hard-shell PCR plate, 96-well
- Polypropylene deep-well reservoir
- Polypropylene plate, 96–450 µL, v-bottom
- Polypropylene deep-well plate: 2 mL square well, v-bottom
- Universal lid

Reagents to use with the Sciclone scripts include:

- xGen Hybridization and Wash Kit (IDT; cat# 1080577 or 1080584)
- xGen Human Cot DNA (IDT; cat# 1080768 or 1080769)
- Vapor-Lock™ PCR encapsulation barrier (QIAGEN; cat# 981611)
- KAPA® HiFi HotStart® ReadyMix (2X) (Kapa Biosystems; cat# KK2601 or KK 2602)
- Agencourt® AMPure® XP beads (Beckman Coulter; cat# A63880)
- xGen Predesigned or Custom Hyb Panel (IDT)
- xGen Universal Blockers (IDT)
- xGen Library Amplification Primer Mix (IDT; cat# 1077675, 1077676, or 1077677)
- Ethanol

1	Normalization and pooling 	Total time: 2 min per sample
2	Blocking setup 	Total time: ~1 min per column of sample
3	Hybridization setup	Total time: ~5 min for every 2 columns of sample
4	Target capture	Total time*: ~2 hrs 25 min Breakdown: <ul style="list-style-type: none"> - From start of module to first pause for addition of sample: ~30 min  - From addition of samples to second pause for addition of PCR Master Mix: ~1 hr 45 min - From addition of PCR Master Mix until module completes: 10 min
5	Post-capture PCR SPRI	Total time: ~10 min

* Total time for target capture is based on using a 96-well plate.

AUTOMATION PROTOCOL

Normalization and pooling module

This module uses 500 ng of each sample library and normalizes with IDTE to yield even volumes for optimal dry-down. Begin this module with a library concentration of 50–100 ng/μL.

 **Note:** Libraries of higher or lower concentration can be normalized and pooled with this module; however, other library concentrations were not tested during our protocol development. Therefore, manual normalization and pooling of libraries outside of the indicated range should be considered.

Duration

Approximately 2 min per sample

Procedure

1. Complete the Normalization and Pooling spreadsheet by entering your sample IDs, concentrations, source, final normalization sample volume, and destination wells.
2. Save the workbook.
 -  **Important:** When saving the workbook, never change the name of the workbook or the destination folder.
3. Launch the Sciclone software, open the normalization script, then press Play.

The software will prompt for confirmation that it is referencing the correct workbook.
4. Follow the on-screen instructions for preparation of the deck layout. The deck placement of the library plate, and clean PCR plate used for normalization, are indicated. Ensure a supply of clean tips are available.
5. After the Normalization and Pooling script completes, remove the original library plate from the deck, then seal and freeze for storage.
6. Remove the normalized library sample plate from the deck, then proceed to the Blocking setup module.
 -  **Safe stop:** At the end of this module, the plate can be safely stored per your established internal laboratory practices, or you can continue to the next module in the protocol.

Blocking setup module

This module adds a solution of blockers to the samples. At the end of this module, the plate will be ready to dry-down in the vacuum concentrator, such as the DNA 120 SpeedVac™ concentrator (Savant).

Duration

Approximately 1 min per column of samples

Procedure

1. Complete the Blocking Setup spreadsheet by indicating the number of columns where the Blocker Master Mix will be added.
2. Save the workbook.

Blocker Master Mix	1 Sample	8 Samples
Est-DNA	5	25.4
xGen Blockers	2	39.2
Total Volume	7	108.6

Figure 1. Workbook for the Blocking Setup module

3. Prepare the Blocker Master Mix based on the calculated volumes from the workbook.
4. Press Play on the script to start the module.

A window displays the number of columns that will be processed, as directed in the workbook.

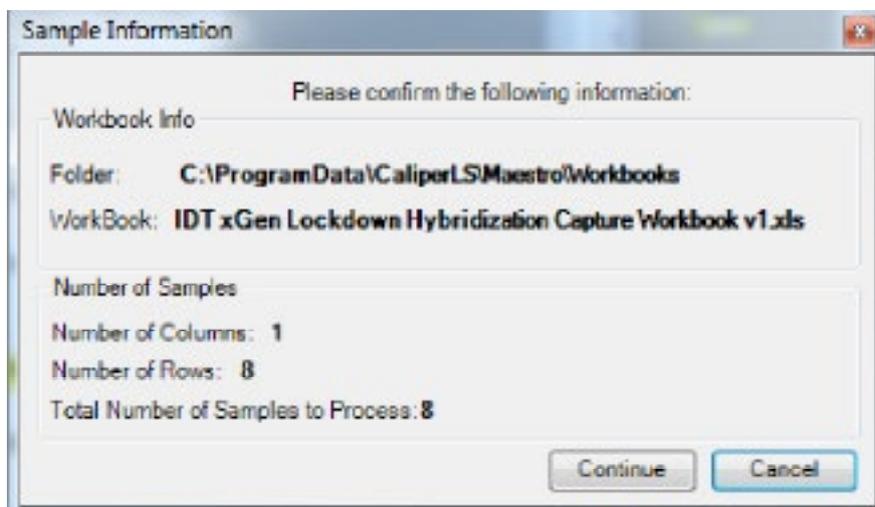


Figure 2. Sample information confirmation window

5. Follow the on-screen instructions to set up the deck, as shown in Figure 3.

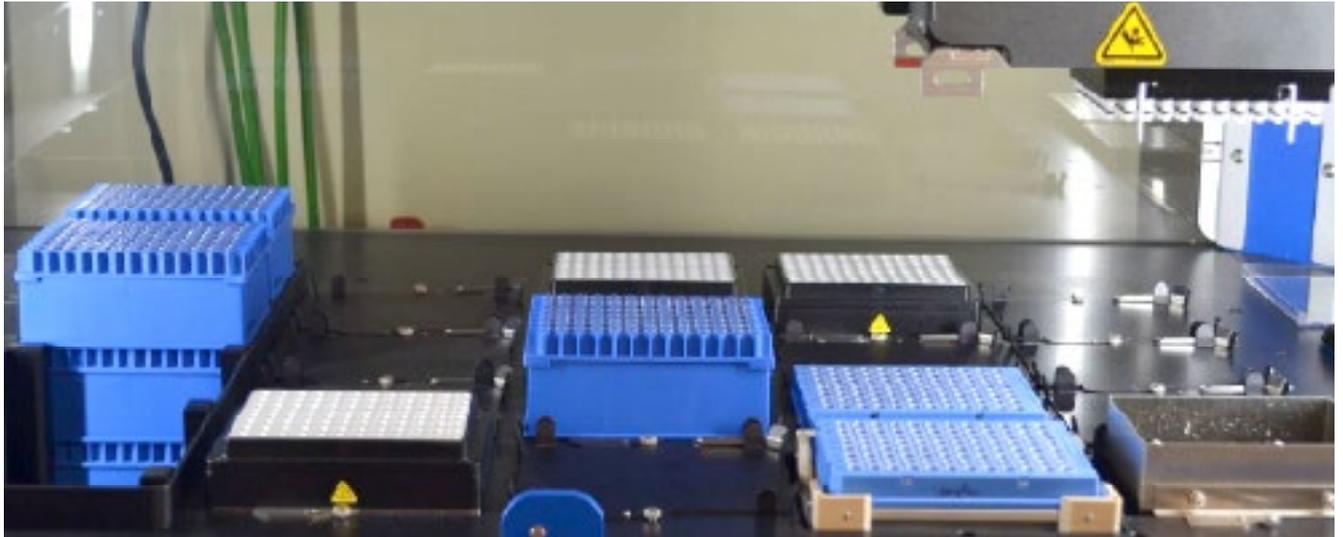


Figure 3. This is the correct deck layout.

6. Remove the sample plate from the deck, then transfer the plate with nominalized libraries and added blockers to the SpeedVac™ concentrator, or an equivalent instrument for dry-down.

When the dry-down is complete, proceed to the xGen Hybridization Setup module.



Safe Stop: If you are stopping here, seal and store the plate overnight per your established internal laboratory procedures. If you are not stopping, continue immediately to the next module.

IDT xGen Hybridization Setup module

This module adds the hybridization solution, including the xGen Hyb Panel. At the end of this module, the plate is ready for the hybridization incubation.

Duration

Approximately 5 min, based on 2 columns of samples

Procedure

1. If you are combining xGen Hyb Panels with additional xGen Probes, follow the recommendations as described in the Appendix.



Tip: Update the table shown in Figure 4 to allow the Maestro™ software to obtain the values directly from this workbook.

- Complete the Hybridization Setup spreadsheet by indication of columns where the Hybridization Master Mix is added

Requirement	Value
1. Buffer	100
2. Amount of total lanes	12
3. Amount of lanes	12
4. Total Amount of Samples	12
5. Hybridization Temperature (Index: All 100°C)	100

	1	2	3	4	5	6	7	8	9	10	11	12
20	100											
21	100											
22	100											
23	100											
24	100											
25	100											
26	100											
27	100											
28	100											
29	100											
30	100											
31	100											
32	100											
33	100											
34	100											

Figure 4. Workbook for the Hybridization Setup

- Save the workbook.
- Prepare the Hybridization Master Mix based on the calculated volumes from the workbook (Figure 4).
- Follow the on-screen instructions to set up the deck, as shown in Figure 5.



Figure 5. Deck layout for the Hybridization Setup

- Remove the sample plate from the deck and seal the plate with a Microseal B seal.
- Briefly centrifuge the plate.
- Incubate the plate in a thermal cycler using the following program with a lid set at 100°C:

Temperature	Time
95°C	30 sec
65°C	4 hrs
65°C	Hold

IDT xGen Target Capture module

This module will wash the streptavidin beads, bind the captured target duplexes onto the beads, then wash the beads again to remove any non-specific DNA. As a final step, the PCR Master Mix is added to the resuspended beads. At the end of this module, the plate is ready to load onto a thermal cycler for PCR amplification. H3_Avenir Book 12/17pt, 80 pt tracking .1875" space before, .0625" space below. Ocean

Duration

Based on a 96-well sample plate:

- From start of module to the first pause for addition of samples: approximately 30 min
- From addition of samples to the second pause for addition of PCR Master Mix: 1 hr and 45 min
- From the addition of PCR Master Mix until completion of the module: 10 min

Procedure

1. Complete the Target Capture spreadsheet by indicating the number of columns that contain samples to be processed.
2. Save the workbook.
3. Prepare the plates with the diluted buffers and the beads, based on the calculated volumes from the workbook. Do not prepare the PCR Master Mix.
4. Follow the on-screen instructions to set up the deck, as shown in Figure 6



Figure 6. Deck layout for the Target Capture

6. Start the protocol with 30 min remaining on the 65°C, 4 hr incubation.
7. During the first half of the module, the software will pause and direct you to load the plate of samples and the plate containing the Vapor-Lock PCR encapsulation barrier or a similar barrier product.
 - ! **Important:** Ensure that the seal has been removed from the sample plate before loading.
 - ⊘ **Safe Stop:** The reagents on the deck are at a safe stopping point. A delay between the completion of the bead wash, and adding the hybridization reaction plate on the deck will not impact the target capture results.
8. The module next binds the beads to the sample and washes the beads.
 - ☰ **Note:** The streptavidin bead plate will have residual beads left, which will not impact the results.
 - ➡ **Tip:** The script takes 1 hr and 45 min until the next pause point.
9. With approximately 20 min remaining in the script, prepare the PCR Master Mix as outlined in the workbook.

10. After the beads with captured DNA have been washed and resuspended in water, the instrument will pause to prompt you to load the plate of PCR Master Mix and a fresh plate.

Note: The “Wash 1” buffer plate must be removed before loading the PCR Master Mix plate, as shown in Figure 7.

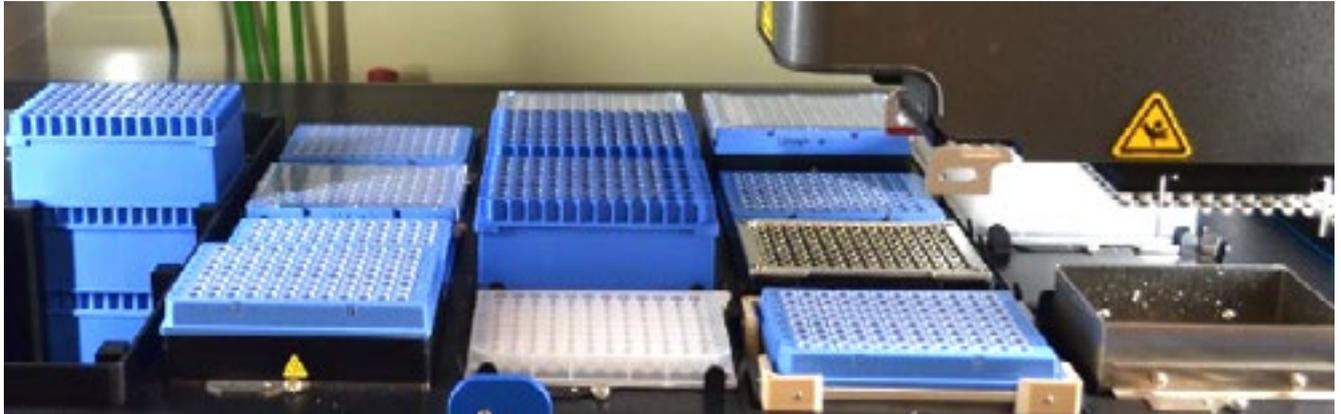


Figure 7. Deck layout when the PCR Master Mix is added to the deck.

11. Click OK to continue the protocol.

Note: After eluting the captured fragments, the beads were transferred to the fresh plate on the deck. Residual beads may remain in the sample plate but will not impact the results.

12. At the end of the module, remove the sample plate that contains the PCR Master Mix from the deck.

13. Seal the plate, then briefly centrifuge.

14. Place the plate in a thermal cycler, and run the following program with the heated lid set to 105°C:

Step	Number of cycles	Temperature	Time
Polymerase activation	1	98°C	45 sec
Amplification			
Denaturation	Variable—refer to the	98°C	15 sec
Annealing	Panel size table below	60°C	30 sec
Extension		72°C	30 sec
Final extension	1	72°C	1 min
Hold		4°C	Hold

Note: To ensure there is enough yield for sequencing, the number of PCR cycles should be optimized per panel size and the number of pooled libraries per capture.

We recommend starting optimization with the following:

Panel size Step	1-plex	4-plex	8-plex	12-plex
>100,000 probes (xGen Exome Hyb Panel)	10 cycles	8 cycles	7 cycles	6 cycles
10,001–100,000 probes	12 cycles	10 cycles	9 cycles	8 cycles
501–10,000 probes	13 cycles	11 cycles	10 cycles	10 cycles
1–500 probes	14 cycles	12 cycles	11 cycles	11 cycles

IDT xGen post-capture PCR bead-based cleanup module

This module performs the final cleanup of the PCR reaction using 1.5X AMPure XP bead volume (Beckman Coulter). At the end of this module, you can store the samples, or continue with qPCR quantification of the DNA.

Duration

Approximately 10 min

Procedure

1. Complete the Post-capture PCR SPRI (Bead cleanup) spreadsheet by indicating the number of columns that contain samples for the cleanup.
2. Save the workbook.
3. Follow the on-screen instructions to set up the deck, as shown in Figure 8.



Figure 8. Deck layout for the Post-Capture PCR SPRI cleanup

4. Start the Bead Cleanup module.
When initiated, the system will aspirate the supernatant of the PCR from the Dynabeads M-270 Streptavidin.
5. The software will pause to prompt you to remove the 96-well PCR plate. After you have removed the plate, click OK to continue the protocol.
6. After this module completes, remove the sample plate from the deck and seal for storage, or proceed immediately to library quantification and sequencing.

Three runs were performed on the SciClone G3 NGS workstation with 8 samples in each run for a total of 24 exome samples. Library samples with 1ug NA24385 (Coriell) were captured using an exome panel and xGen Universal Blockers- TS, together with xGen Human Cot DNA. The samples were sequenced on a NextSeq 550 and down-sampled to 50 million reads per exome sample. Sequencing data were aligned with BWA-MEM [1] to human reference hg19. Coverage statistics were compiled with BedTools [2].

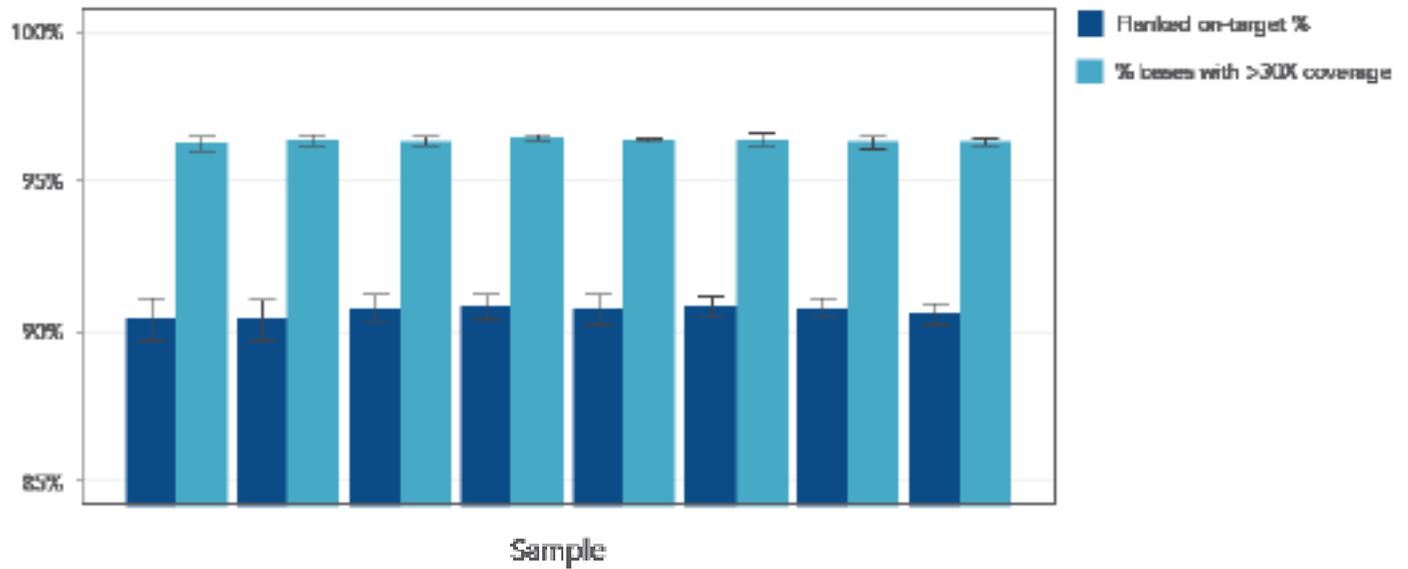


Figure 9. IDT Target Capture sample statistics

REFERENCES

1. Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv pre-print server. 2013. arxiv:1303.3997 [q-bio.GN].
2. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics (Oxford, England). 2010;26(6):841-842.

APPENDIX

Combining xGen Hyb Panels and xGen Probes

Combine panels at equimolar amounts to achieve uniform coverage. When combining panels (during the IDT xGen Hybridization Setup module), prepare the Hybridization Master Mix based on the recommendations in Table 1. Multiply by the number of samples, then add a 10% overfill (See Table 2).

For more information regarding spike-in panels, contact our technical support group at applicationsupport@idtdna.com.

Table 1. Spike -in volumes when combining two panels

	Spike-in panel types			
	xGen Custom Hyb Panel <20,000 probes		xGen Custom Hyb Panel >20,000 probes	
	Volume		Volume	
	Backbone	Spike-in	Backbone	Spike-in
xGen Custom Hyb Panel <20,000 probes	3 μ L	3 μ L	N/A	N/A
xGen Custom Hyb Panel >20,000 probes	4 μ L	1 μ L	3 μ L	3 μ L

Table 2. Volumes per component of the Hybridization Master Mix

Hybridization Master mix components	Volume per reaction (μ L)
xGen 2X Hybridization Buffer	8.5
xGen Hybridization Buffer Enhancer	2.7
Main panel	See Table 1
Spike-in panel	See Table 1
Nuclease-Free Water	0_0.8 (only if needed*)
Total volume*	17-17.2 μ L

* Depending on the spike-in panel used, your volume could be less than 17 μ L; if so, add a small amount of water to reach the total recommended volume (17–17.2 μ L).

Automation of xGen™ Hybridization Capture on the Sciclone® G3 NGS Workstation

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