SEQUENTIFY

DEMOCRATIZING NEXT-GENERATION SEQUENCING

InfiniSeq™ Protocol v1.0.6

Compatible with Illumina™ platforms

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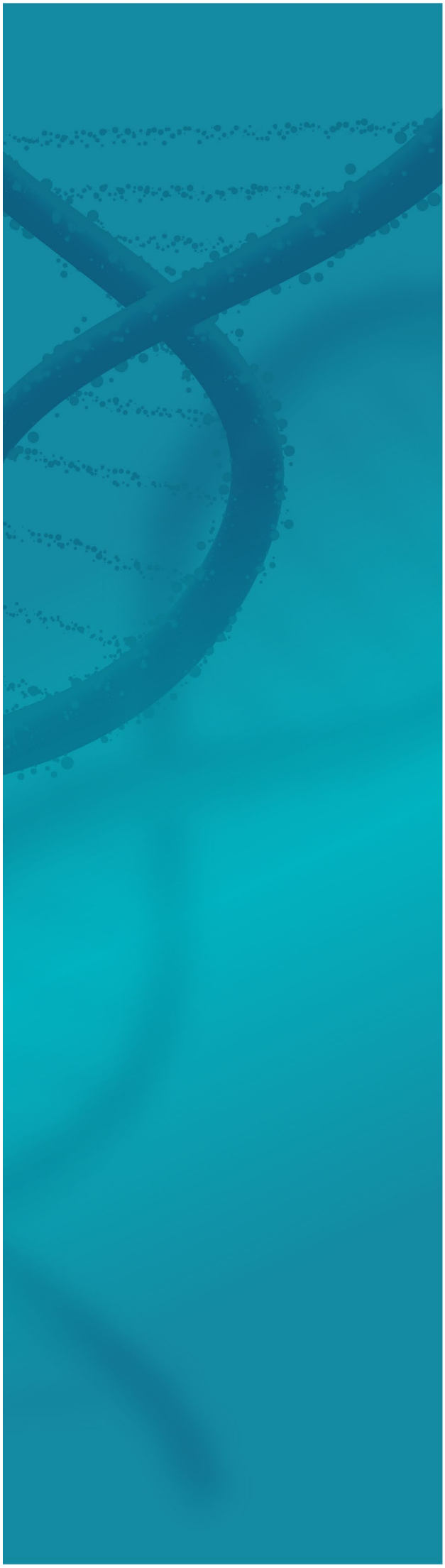

INFINISEQ REACTION OVERVIEW

This kit includes all reagents required for InfiniSeq, Sequentify's targeted sequencing library preparation protocol. The manual describes the 4 steps required to generate an Illumina NGS library in ~3.5 hours (Figure 1). Different InfiniSeq panels yield a different NGS library size. The final product of the protocol is compatible with Illumina sequencing machines. Sequencing requires custom primers (supplied in the kit) and libraries can be mixed with standard Illumina libraries (see Appendix A & Appendix B).

Figure 1: Infiniseq protocol workflow. The colors correspond with those of the reagent tubes.

- Recommended and validated genomic DNA template concentration: 50-200 ng/µl. <50 ng/µl samples were also demonstrated to work but may require protocol optimization by the user. For applications using DNA templates different than genomic DNA please consult Sequentify support.
- Recommended and validated genomic DNA template purity A260/280=1.8-2 A260/A230=2-2.2. DNA Integrity Number (DIN) score >3 was demonstrated to work for DNA extracted from FFPE samples. DNA samples of different purity scores were also demonstrated to work but may require protocol optimization by the user.
- Store all reagents at -20°C upon receipt.
- Before using in reactions:
  - Keep reagents marked "E" in -20°C freezer/cooling block until use.
  - Thaw reagents marked "R" and index primers on ice and keep cool until use. Avoid multiple freeze and thaw cycles. Prepare aliquots if necessary.
  - At any stage of the protocol, before opening the reaction tubes/unsealing the reaction plate, make sure to properly spin down (10-30 seconds) to collect evaporated liquid.
- It is recommended to add control reactions to each batch of experiments (supplied in the kit): Use control genomic DNA 50 ng/µl as a template for positive control and nuclease-free water as a template for negative control.
- Make sure your work area is clean: Clean pipettes and workbench using MBP DNA AWAY (Thermo Scientific, 7010) or an equivalent decontamination reagent.
- KAPA Pure Beads (Roche, KK8000, KK8001, KK8002) were validated for NGS library purification (See "Sample purification" section).
- Not supplied in the kit: Instruments/machines, consumables, sequencing kit and reagents, purification beads and magnet apparatus, DNA size and concentration measurement kits and reagents, water, Ethanol and DNA AWAY.
- We have confirmed that thawing and maintaining all reagents (including premixed Mix2) at a cooling temperature of 4°C throughout the entire reaction day, without any stops does not have any adverse impact on the yield
- Please refer to Appendix D for a comprehensive troubleshooting guideline addressing common issues that may arise during the process.
- Use is subject to Sequentify Terms and Conditions.

STEP 1 - Panel annealing

1. Thaw panel tube (marked in **green**) on ice. Keep on ice until use.
2. Immediately before use, gently vortex and spin-down the tube.
3. Distribute 3.5 µl of panel tube reagent to each reaction tube/well.
4. Add 6 µl of DNA template to each InfiniSeq reaction.
5. Carefully seal the plate/close tubes, gently vortex, spin-down and place in the thermal cycler.
6. Run the program described in **Table 1** with a heated lid (105°C).

Current total volume = 9.5 µl

Table 1: Step 1- Panel annealing - Thermal cycler program

Temperature	Minutes	Cycles
98°C	3	1
98°C - 56°C	Ramp rate -0.1°C/sec	1
56°C	120	1
56°C	**Hold until next step**	

Potential stop

Reaction plate/tubes can be stored at 56°C overnight.

STEP 2 - Target extension

⚠ E2 is very foamy. Do not vortex E2 and Mix2 until Mix 2 is dispensed in the rection tube.

1. Thaw **R2** tube (marked in **blue**) on ice. Keep on ice until use.
2. Keep **E2** (marked in **blue**) in -20°C until use.
3. Immediately before **Mix 2** preparation, spin-down both R2 and E2 tubes.
4. Prepare **Mix 2** according to **Table 2**. Mix by gently pipetting up and down, spin-down and keep **on ice** until distribution. Avoid Mix 2 freezing after preparation!
5. Remove reaction plate/tubes from the thermal cycler and spin down. Keep at room temperature until use.
6. Distribute **6 µl** of **Mix 2** to each InfiniSeq reaction.
7. Carefully seal the plate/close tubes, gently vortex, spin-down and place in the thermal cycler. Run the program described in **Table 3** with a heated lid (105°C).

Current total volume = 15.5 µl

Table 2: Preparation of Mix 2 per sample reaction. Suggested mix for 106 reactions (96 reactions plate +10% excess) added.

Reagent name	x1 (µl)	x106 (µl)
R2	4.8	508.8
E2	1.2	127.2
Total	**6**	**636**

Table 3: Step 2 – Target Extension - Thermal cycler program

Temperature	Minutes	Cycles
56°C	5	1
72°C	5	1
16°C	**Hold until next step**	

STEP 3 - Exonuclease

1. Keep **E3** tube (marked in **orange**) in -20°C. Spin-down immediately before distribution.
2. Remove reaction plate/tubes from the thermal cycler and spin down. Keep at room temperature until use.
3. Distribute **3 µl** of **E3** to each InfiniSeq reaction.
4. Carefully seal the plate/close tubes, vortex, spin-down and place in the thermal cycler.
5. Run the program as described in **Table 4** with a heated lid (105°C).

Current total volume = 18.5 µl

Table 4: Step 3 – Exonuclease - Thermal cycler program

Temperature	Minutes	Cycles
37°C	10	1
95°C	5	1
16°C	**Hold until next step**	

Potential stop

Reaction plate/tubes can be stored at 4°C overnight.

STEP 4 - Barcoding PCR

This step will result in a dual index sequencing library (see scheme in Appendix B). The InfiniSeq index plates contain dual index combinations that allow for sample demultiplexing following the NGS run. Make sure to carefully document which index well was paired with which sample and avoid using the same index twice in the same experiment (i.e., for samples that will be pooled in the same NGS run). It is recommended to edit the sample and index information in the sample sheet before beginning the experiment. This information will be used for sample demultiplexing after sequencing is finished. All supported indexes and their respective location in the index plate are in the editable example sample sheet (single index/dual index). The order of index 1 barcodes in the index plate is by column: A1=D701, B1=D702, A2=D709, H12=D796. The order of index 2 barcodes (if applicable) is by plate: D501=Index plate 1, D502=Index plate 2.

1. Thaw **R4** tube (marked in **Yellow**) on ice. Keep on ice until use.
2. Remove reaction plate/tubes from the thermal cycler and spin down. Keep at room temperature until use.
3. Distribute **21 µl** of **R4** to each InfiniSeq reaction.
4. Distribute **3 µl** of index primers to each InfiniSeq reaction.
5. Carefully seal the plate/close tubes, vortex, spin-down and place in the thermal cycler. Run the program described in **Table 5** with a heated lid (105°C).

Current total volume = 42.5 µl

Table 5: Step 4 – Barcoding PCR - Thermal cycler program

Temperature	Seconds	Cycles
98°C	30	1
98°C	5	24
55°C	10	
72°C	15	
72°C	120	1
16°C	**Hold until next step**	

Potential stop

Reaction plate/tubes can be stored at 4°C overnight or -20°C for longer.

ADDITIONAL ACTIONS

Sample purification

Ensure the quality of your libraries by performing concentration and size measurements. Following purification, the expected concentration per sample should be between 1-30 ng/µl. To calculate the molarity of your sequencing pool/libraries, use a combination of concentration and sizing information. The library size will vary depending on your specific panel, and you can find this information in the COA of your kit or by contacting support@sequentify.com. Alternatively, you may measure molarity using dedicated qPCR kits for Illumina library concentration measurement.

There are two recommended approaches for pooling your samples. The standard method involves individually purifying each sample, pooling them based on QC measurements, and then purifying the entire pool once more (option 1). Alternatively, if your samples are expected to yield comparable concentrations, you can pool all samples together immediately after the Barcoding PCR step and perform purification directly on the pooled library (option 2). We have also successfully tested a workflow where individual library concentrations are measured without purification and the libraries are pooled based on their concentration, with purification only carried out on the final pool.

It's important to note that you can pool InfiniSeq libraries from previous experiments and/or libraries which originated from different panels as long as their barcodes do not overlap.

For purification, KAPA pure beads have been validated to work with the recommended protocol (APPENDIX C). If you choose to modify the bead purification protocol, particularly the bead volume ratio, it is advisable to consider the recommendations provided by the bead manufacturer.

Option 1

Individual sample purification > pooling > pool purification

1. Purify and concentrate samples using purification beads at 0.7X volumetric ratio. For example, when purifying the entire reaction (final volume **42.5 µl**), spin down reaction tube/plate before opening the cap/removing the seal. Add **29.7 µl** of purification beads. Prepare fresh ethanol according to the manufacturer's instructions. Elute in **15-20 µl** of nuclease-free water.
2. Perform QC as described above. Pool samples according to normalized molarity. This is done to achieve equal sample representation in the final pooled library.
3. Purify pooled samples as previously described using purification beads at **0.7X** volumetric ratio. Elute sample in **30-35 µl** of water. For example, if you have pooled **10 µl** from 10 different purified samples (total volume of **100 µl**), add 70 µl of beads to the purification reaction.
4. Perform QC to the final pool as described above.

Option 2

Pooling > pool purification X 2

1. Pool all samples together at an equal volume and purify the pooled sample twice using purification beads at **0.7X** volumetric ratio. For example, if you have pooled **20 µl** from 5 unpurified samples (total volume of **100 µl**), add **70 µl** of beads to the purification reaction, elute the reaction at **50 µl** of water 2. Purify again at **0.7X** volumetric ratio (add 35µl of beads). Elute the final reaction at **15 µl** of water.
2. Perform QC to the final pool as described above.

Potential stop

Reaction plate/tubes can be stored at 4°C overnight or -20°C for longer.

Sequencing

Sequencing InfiniSeq libraries requires custom primers that are supplied in the InfiniSeq kit. Custom primers are sequencing primers that are different from the original Illumina sequencing primers provided inside Illumina cartridges. Use of custom primers is only required during sequencing and not during the InfiniSeq protocol. The stock concentration of the custom primers P1, P2, and PI (custom sequencing primers for Read 1, Read 2, and index reads, respectively) is 100 µM. The PI tube contains both index primers and can be used in both single/dual index sequencing configurations (see Appendix B for InfiniSeq library scheme). Since sequencing is being performed on a 3rd party instrument (Illumina), Sequentify can only recommend the sequencing protocol and will not be held responsible for erroneous sequencing and Illumina kit/protocol modifications. Some Illumina instruments will not allow custom primer usage/spiking. Please validate that your instrument supports this feature. The sample sheet file prepared during step 4 (Barcoding PCR) documentation will be used for post-run demultiplexing*, either on board in supported Illumina instruments, or following the run via the bcl2fastq software, by Illumina.
There are two options to sequence InfiniSeq libraries:

Option 1

Use the standard custom primers protocol and the designated custom primer cartridge inlets.

Configure the machine to load custom primers from the appropriate inlet. Thaw and spin down custom primer tubes (P1, P2, PI), prepare working solutions per custom primer according to the machine-specific "Custom Primers Guide", provided on the Illumina website. Continue to library denaturation and loading.

Option 2 (Recommended)

Spike-in custom primers into standard Illumina cartridge inlets.

Custom primers spiking is simpler to operate and allows pooling of standard Illumina libraries into the same sequencing run, without any configuration modifications. Make sure to follow the specifications of Appendix A by the respective sequencing kit as it varies between kits. In addition, make sure to avoid contamination of the sequencing primers, as you will spike into inlets which already contain primers. Thaw and spin down custom primer tubes (P1, P2, PI). Spike-in custom primers according to the respective Illumina reagent kit (Appendix A) or use the standard custom primers protocol. Continue to library denaturation and loading.

Denaturation and loading of your library - Denature and load your library pool in accordance with the Illumina "Denature and Dilute Libraries Guide" that is unique to each Illumina instrument. We found that the best loading concentrations of InfiniSeq purified pools are: for Miniseq and Nextseq 500/550: 2.2 pM, for Novasseq 6000: 300pM . Loading concentration optimization may be required.

Run configuration - When sequencing libraries that originate from the same index plate, there is no need for index 2 sequencing In this case, recommended sequencing parameters are: Read 1: 151, Index 1: 8, Read 2: 151 (Miseq run configuration requires use of an edited single index sample sheet). When multiplexing samples from different plates, both index 1 and index 2 are required. Recommended sequencing parameters are: Read 1: 151, Index 1: 8, Index 2: 8, Read 2: 151 (for Miseq, use an edited dual index sample sheet).

*Reminder: If you have used custom primers "Option 1"- Set the custom primers check box during run configuration. If you have used custom primers "Option 2" - Do not check any custom primers check box**.*

* When using Miseq or Novaseq V1 dual index runs, index2 should undergo a reverse-complement conversion before analysis.
** For Miseq, follow the same principle: When using "Option 1" add the 3 custom primers required rows to the sample sheet, and when using "Option 2" delete them.

SUPPORT

We are here to assist :)

For any inquiry, please contact us at support@sequentify.com.

In your inquiry, kindly state the following:

1. The issue.
2. The targeted sequencing panel used, including LOT#.
3. QC results of the reactions, including positive and negative controls.
4. DNA template type and purity.
5. Any other relevant information.

APPENDIX A

Spiking custom primers guide

Based on Illumina's "Spiking custom primers into the Illumina sequencing primers", we have calculated the required volume for each supporting sequencing kit. The PI mix tube includes both index1 and index2 custom primers and it will work both for single index or dual index configuration.

Table 7: Examples for Illumina machines and their respective custom primers dilution ratios

	Kit version	Illumina Primer (name)	Cartridge Position	InfiniSeq custom primer tube name	Volume for custom primer (100uM stock,ul)	Total Volume (ul)	Custom primer final concentration (uM)
Miniseq	Rapid Run 100 Cycles	Read 1 (BP10)	24	P1	1.8	590	0.3
		Read 2 (BP11)	25	P2	1.6	530	0.3
		Index 1 (i7) and Index 2 (i5) (BP14)	28	PI	2.2	740	0.3
Miniseq	High Output 75 Cycles	Read 1 (BP10)	24	P1	1.7	550	0.3
		Read 2 (BP11)	25	P2	1.8	610	0.3
		Index 1 (i7) and Index 2 (i5) (BP14)	28	PI	2.5	820	0.3
Miniseq	High Output 150 Cycles	Read 1 (BP10)	24	P1	1.7	550	0.3
		Read 2 (BP11)	25	P2	1.8	610	0.3
		Index 1 (i7) and Index 2 (i5) (BP14)	28	PI	2.6	857	0.3
Miniseq	High Output 300 Cycles	Read 1 (BP10)	24	P1	1.7	550	0.3
		Read 2 (BP11)	25	P2	1.8	610	0.3
		Index 1 (i7) and Index 2 (i5) (BP14)	28	PI	2.5	820	0.3
Miniseq	Mid Output 300 Cycles	Read 1 (BP10)	24	P1	1.7	550	0.3
		Read 2 (BP11)	25	P2	1.8	610	0.3
		Index 1 (i7) and Index 2 (i5) (BP14)	28	PI	2.5	820	0.3

	Kit version	Illumina Primer (name)	Cartridge Position	InfiniSeq custom primer tube name	Volume for custom primer (100uM stock,ul)	Total Volume (ul)	Custom primer final concentration (uM)
Miseq	v2 and v3	Read 1 (HP10)	12	P1	3.4	680	0.5
		Index 1 (i7) (HP12)*	13	PI	3.4	680	0.5
		Read 2 (HP11)	14	P2	3.4	680	0.5

* PI mix includes both index1 and index2 custom primers. The miseq Index2 read does not require sequencing primer. Nevertheless, PI will work in Miseq runs, both for single index or dual index runs.

	Kit version	Illumina Primer (name)	Cartridge Position	InfiniSeq custom primer tube name	Volume for custom primer (100uM stock,ul)	Total Volume (ul)	Custom primer final concentration (uM)
NextSeq 500/550	High Output 75 Cycles	Read 1 (BP10)	20	P1	5.2	1.72	0.3
		Read 2 (BP11)	21	P2	5.9	1.98	0.3
		Index 1 (i7) and Index 2 (i5) (BP14)	22	PI	8.5	2.83	0.3
NextSeq 500/550	High Output 150 Cycles	Read 1 (BP10)	20	P1	5.2	1.73	0.3
		Read 2 (BP11)	21	P2	5.9	1.98	0.3
		Index 1 (i7) and Index 2 (i5) (BP14)	22	PI	8.5	2.83	0.3
NextSeq 500/550	High Output 300 Cycles	Read 1 (BP10)	20	P1	5.2	1.73	0.3
		Read 2 (BP11)	21	P2	5.9	1.98	0.3
		Index 1 (i7) and Index 2 (i5) (BP14)	22	PI	8.5	2.83	0.3
NextSeq 500/550	Mid Output 150 Cycles	Read 1 (BP10)	20	P1	4	1.34	0.3
		Read 2 (BP11)	21	P2	4.5	1.51	0.3
		Index 1 (i7) and Index 2 (i5) (BP14)	22	PI	6.3	2.09	0.3
NextSeq 500/550	Mid Output 300 Cycles	Read 1 (BP10)	20	P1	4	1.33	0.3
		Read 2 (BP11)	21	P2	4.6	1.52	0.3
		Index 1 (i7) and Index 2 (i5) (BP14)	22	PI	6.3	2.09	0.3

	Kit version	Illumina Primer (name)	Cartridge Position	InfiniSeq custom primer tube name	Volume for custom primer (100uM stock,ul)	Total Volume (ul)	Custom primer final concentration (uM)
Novaseq 1.5V	SP 100/200/300 /500 cycles	Read 1 (VP10)	24	P1	12	4	0.3
		Index 1 (i7) and Index 2 (i5) (VP14)	23	PI	15	5	0.3
		Read 2 (VP11)	13	P2	6	2	0.3
Novaseq 1.5V	S1 and S2 100/200/300 cycles	Read 1 (VP10)	24	P1	12	4	0.3
		Index 1 (i7) and Index 2 (i5) (VP14)	23	PI	15	5	0.3
		Read 2 (VP11)	13	P2	6	2	0.3
Novaseq 1.5V	S4 200/300 cycles	Read 1 (VP10)	24	P1	21.9	7.3	0.3
		Index 1 (i7) and Index 2 (i5) (VP14)	23	PI	15	5	0.3
		Read 2 (VP11)	13	P2	10.5	3.5	0.3

APPENDIX B

InfiniSeq library molecular biology principle

Infiniseq is a 4-step targeted sequencing protocol that targets a DNA template using a set of target specific probes (Figure 2). The final product of the InfiniSeq protocol is a dual index, NGS library. Sequencing InfiniSeq libraries requires custom primers that target the insert (the region we would like to sequence) and the indexes (to enable pooling and post-NGS run sample data demultiplexing). Standard InfiniSeq index plates (used in step 4) contain primers that generate dual indexing libraries with combinatorial indexes. That means that when using a single index plate, there is no need to sequence index 2. For more information and for custom index requests, please contact support@sequentify.com.

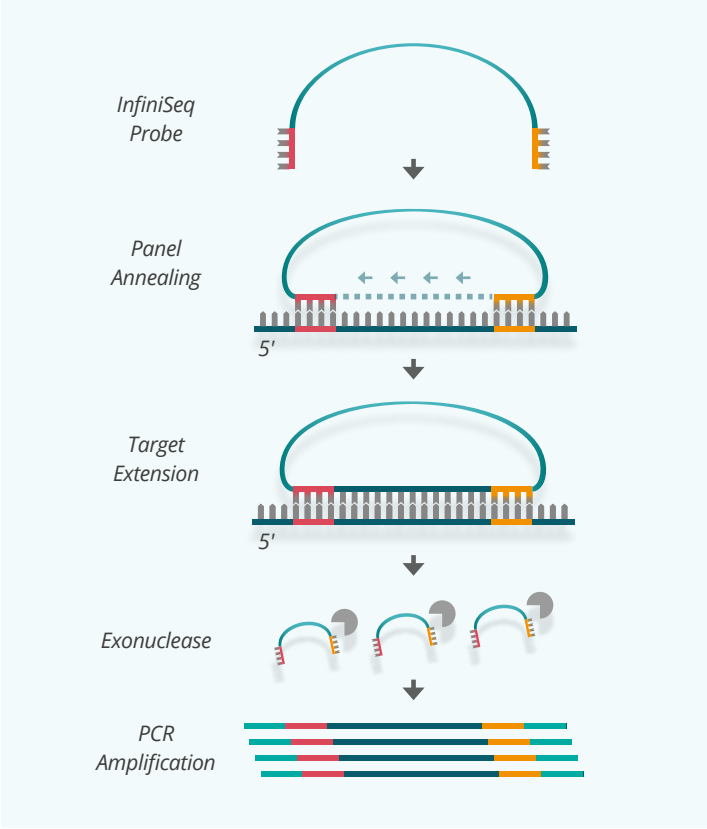

Figure 2: Infiniseq is a 4-step targeted sequencing protocol that targets a DNA template using a set of target specific probes.

The following scheme describes the key segments that form the final InfiniSeq library and the respective custom primers' sites and directionalities.

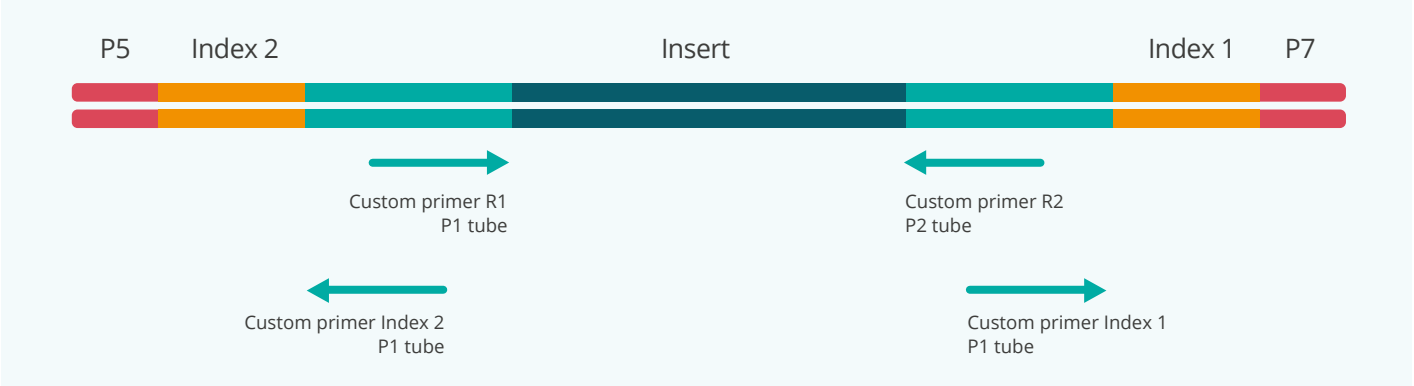

Figure 3: An example of a final library TapeStation result.

APPENDIX C

KAPA Pure InfiniSeq recommended protocol

This section provides a modified KAPA Pure Beads protocol that our team has validated and optimized for time and efficiency. The basis of the protocol is the KAPA Pure beads manufacturer protocol.

1. Allow the KAPA Pure Beads to equilibrate to room temperature and vortex to ensure complete resuspension.
2. Add 0.7X volume of KAPA Pure Beads to the sample or pool.
3. Thoroughly mix the mixture by vortexing and/or pipetting up and down 10 times.
4. Incubate the plate or tube(s) at room temperature for 5 minutes to allow DNA binding to the beads.
5. Place the plate or tube(s) on a magnet to capture the beads and incubate until the liquid becomes clear.
6. Carefully remove and discard the supernatant, leaving behind 5 µL of supernatant to prevent bead loss.
7. While keeping the plate or tube(s) on the magnet, add 100 µL of 80% ethanol.
8. Incubate the plate or tube(s) on the magnet at room temperature for at least 30 seconds, then remove and discard the ethanol, ensuring all residual ethanol is removed without disturbing the beads.
9. Repeat the previous two steps once.
10. Allow the beads to dry at room temperature for 1-3 minutes or until all of the ethanol has evaporated. Note: Be cautious not to over-dry the beads, as it may reduce yield.
11. Remove the plate or tube(s) from the magnet.
12. Add 20 µl of elution buffer or ultra-pure water and pipette up and down to resuspend the beads.
13. Incubate the plate or tube(s) for 2 minutes to elute the DNA from the beads.
14. Place the plate or tube(s) on a magnet to capture the beads and incubate until the liquid becomes clear.
15. Transfer the clear supernatant to a new plate or tube(s).

APPENDIX D

Troubleshooting Guide

We have created a handy reference below to assist you in resolving any issues that may arise during the InfiniSeq protocol. This troubleshooting table presents common challenges, their potential causes, and effective solutions to help you navigate the process with ease. Don't worry, we've got you covered every step of the way. For any further issues, feel free to contact us at support@sequentify.com.

Issue	Possible cause	Solution
Sample evaporation during the hybridization step.	During the hybridization step, it's important to be mindful of certain PCR machines that might not effectively seal the plate. This can potentially lead to evaporation issues, especially in the corners and/or wells located at the periphery of the plate.	To prevent evaporation in the problematic wells, consider adding additional sealing tapes that cover the sides of the plate.
		Some PCR machines have a supplemented sealing rubber to prevent evaporation.
	Sealing tape does not cover the wells appropriately.	Replace sealing tape and/or ensure adequate sealing.
The absence of a library, poor library quality, or an unclear main library peak.	Erroneous InfiniSeq protocol runs (user/ machine issue).	Run a positive control template as an internal quality control.
	One of the reagents in steps 1-4 was not inserted into the reaction tube.	After adding the reagents in each step of the protocol, vortex and spin down, make sure the reagent was added by looking at the volume of the well.
	Missing DNA template.	Ensure proper dispensing of template into the probe mix (R1).
	Low concentration DNA template.	InfiniSeq protocol providesyields libraries from as little as 1.5 ng of total DNA. However, library quality is reduced when DNA concentration is less than 50 ng/uL. If possible, increase DNA concentration.
	Low quality DNA.	Fragmented DNA or FFPE DNA may yield a poor library and even no library at all. If most of your samples are with these properties, contact our team for a modified kit.
	DNA contains inhibitors. Some sample types are known to contain inhibitors that can affect the InfiniSeq reaction. Among them are samples from blood, urine and feces. Possible inhibitors are ethanol, Isopropanol, KCl, NaCl, sodium deoxycholate and SDS.	Perform an additional DNA purification step prior to the protocol beginning. Perform QC using a spectrophotometer to ensure sample quality.
Library is not in the correct size according to the bioanalyzer.	Product is not clean.	There might be up to ±10% sizing accuracy for TapeStation assays. Accuracy tends to be lower when samples are not clean.
A large peak around 100 bp.	This peak is likely a primer dimer.	This peak is usually removed by library beads purification, as recommended in the protocol.
Drop in library yield after beads cleanup.	Over drying of the beads.	Avoid bead overdrying as it affects DNA yield.
	Washing the beads with the wrong reagent - water instead of ethanol, ethanol was not prepared properly or is not fresh.	Mistakes happen. Make sure to prepare fresh ethanol wash solution in accordance with the protocol. Label the prepared solution properly.
	Improper bead volume taken/dispensed.	Ensure accurate dispensing of the desired volume by carefully inspecting and confirming the correct volume in the pipette tip. Take note that beads may adhere to the pipette tip, so be cautious not to pick up any unintended beads from the tip circumference. Additionally, ensure that you dispense the entire intended volume without leaving any residue.