MGP Panel: a comprehensive targeted genomics panel for molecular profiling of multiple myeloma patients

Laboratory Protocol

Sample Considerations

For the lab and bioinformatic process there should be a tumor and matched non-tumor DNA sample for each person. This greatly reduces bioinformatic analysis time and increases specificity.

Tumor samples

- Bone marrow aspirates are used as a tumor sample source. For patients with plasma cell leukemia, peripheral blood can be used as well.
- Samples should undergo CD138+ selection to a purity typically greater than 70%. Samples with a lower purity can be used, but tumor purity directly affects the detection of abnormalities. Samples with a low purity will have lower mutation variant allele frequencies and copy number changes may appear sub-clonal.
- Samples can undergo CD138+ selection by many means, but the most common is magnetic bead sorting. Purity post-selection can be confirmed by flow cytometry (preferred) or cytospin.
- Approximately 250,000 cells can be used to easily extract nucleic acid. Column-based extraction can be used e.g. Qiagen AllPrep, to extract both RNA and DNA from tumor samples. If the number of cells is limiting, a direct DNA extraction from a cell pellet can be performed e.g. Qiagen Puregene. DNA should be eluted or resuspended in a low EDTA buffer such as EB (Qiagen) or water.
- DNA should be measured by a fluorometric method such as picogreen or Qubit to ensure that only DNA is measured. Optical methods (spectrophotometer, Nano-drop) will measure other nucleic acids as well.

Non-tumor samples

- The use of a matched non-tumor DNA sample is preferred for analysis.
- Peripheral blood mononuclear cells can be used if the patient has a low level of circulating plasma cells (usually for MGUS, SMM, newly diagnosed MM). If there are significant numbers of tumor plasma cells in the peripheral blood this will affect somatic calling of variants. DNA can be extracted directly from whole blood using commercial kits (e.g. Qiagen Puregene) and should be eluted in a low-EDTA buffer. Peripheral blood may also undergo red cell lysis to remove red cells, followed by centrifugation of the which cells and DNA extraction from the cell pellet.
- Peripheral blood should NOT be used as a control for plasma cell leukemia patients.
- Saliva can also be used as a source of non-tumor DNA. Patients should not eat or drink 30 minutes prior to providing the saliva sample. Saliva can be collected in various types of tubes including Oragene's OG-500. DNA can be extracted by magnetic purification, for example with Chemagic 360 kits (Perkin Elmer).

- DNA should be measured by a fluorometric method such as picogreen or Qubit to ensure that only DNA is measured. Optical methods (spectrophotometer, Nano-drop) will measure other nucleic acids as well.

Library prep for patient samples for targeted sequencing

This document describes the protocol for Next generation sequencing library preparation for patient samples for targeted sequencing using KAPA HyperPlus kit, KAPA Target enrichment probes and KAPA HyperCap kit. We start with 100 ng of genomic DNA from each sample. This protocol is run on the Pro-flex thermal cycler in the lab, if using any other thermal cycler please follow the appropriate protocol to set up and run the programs.

Library Preparation

Requirements

Reagents –

1. KAPA HyperPlus kit (Roche, #07 962 428 00)
   Note – KAPA HyperPlus kit includes Fragmentation Buffer, Fragmentation Enzyme, End Repair A tailing Buffer, End Repair A-Tailing enzyme mix, Ligation Buffer, Ligation Enzyme and KAPA HotStart PCR Mastermix (2x)
2. KAPA Universal adapters (Roche, #09 063 781 001)
3. KAPA UDI Primer mixes (Roche, #09 134 336 001)
4. BD AMPureXP beads (Becton, #A63881)
5. Qubit 1XdsDNA HS assay kit (Invitrogen, #Q33231)
6. Elution Buffer (Qiagen, #19086)
7. Nuclease free water (Ambion, #AM997)
8. 100% Ethanol for molecular biology

Equipment –

1. Thermal cycler
2. Microcentrifuge
3. Vortex mixer
4. Qubit 4.0 spectrophotometer
5. Alpaqua magnetic 96 well rack
6. PCR cooler

Consumables –

1. 0.2 ml 8-well tubes strip (cat no – 1402-2900)
2. MicroAmp™ TriFlex 3 x 32-Well PCR Reaction Plate (cat no – A32811)
3. Filter tips – 10 ul 200 ul and 1000 ul
4. Disposable serological pipettes
5. 50 ml tubes
6. 1.5 ml DNA LoBind tubes (cat no – 022431021)

Before you start-

1. All library preparation steps until dual size selection are to be performed in the PCR Prep hood or a designated pre-PCR room to prevent contamination from PCR products.
2. Wipe down the surface of the PCR prep hood with 70% ethanol.
3. If using for the first time Program the following on the thermal cycler:

Program name		**Fragmentation**
Lid temperature = 50 ° C, volume = 50 ul		
Step	**Temperature**	**Time**
1	4 ° C	hold
2	37 ° C	25 minutes
3	4 ° C	hold

Program name		**End Repair + A-tailing**
Lid temperature = 85 ° C, volume = 70 ul		
Step	**Temperature**	**Time**
1	65 ° C	30 minutes
2	4 ° C	hold

Program name		**Ligation**
Lid temperature = 50 ° C, volume = 100 ul		
Step	**Temperature**	**Time**
1	20° C	15 minutes
2	4 ° C	hold

Program name		**PreCap 6X**	
Lid temperature = 105 ° C, volume = 50 ul			
Step	**Temperature**	**Time**	**Number of cycles**
1	98 ° C	45 seconds	
2	98 ° C	15 seconds	6 cycles
3	60 ° C	30 seconds	
4	72 ° C	30 seconds	
5	72 ° C	1 minute	
6	4 ° C	hold	

Program name		**Hybridization**
Lid temperature = 50 ° C, volume = 50 ul		
Step	**Temperature**	**Time**
1	95° C	5 minutes
2	55 ° C	hold

Program name		**Post Cap 11X**	
Lid temperature = 105 ° C, volume = 50 ul			
Step	**Temperature**	**Time**	**Number of cycles**
1	98 ° C	45 seconds	
2	98 ° C	15 seconds	11 cycles
3	60 ° C	30 seconds	
4	72 ° C	30 seconds	

5	72 ° C	1 minute	
6	4 ° C	hold	

Reagent Preparation –

I. Preparing KAPA UDI Primer mixes.

1. Retrieve the KAPA UDI Primer Mixes plate from storage (+2 to +8°C).
2. Spin the KAPA UDI Primer Mixes plate at 280 x g for 1 minute to ensure the contents are at the bottom of the wells.
3. Before removing the foil cover, please ensure the plate is in the correct orientation before proceeding. In order to have well position A1 on the top left corner, the notched corner must be facing the user on the bottom left.
4. Carefully remove the foil cover on the plate ensuring to avoid cross contamination. Discard the original foil cover.
5. Using a multichannel pipette, add 11 µL of PCR Grade water directly to the bottom of each well and discard tips after dispensing PCR Grade water.

Note - A new pipette tip should be used for each well to avoid cross contamination. Be sure to dispense water slowly to the bottom of each well to avoid liquid splash over to adjacent wells.

6. Ensure every well contains 11 µL of PCR grade water and cover the plate with one of the adhesive foil seals provided in the kit.
7. Keep the plate upright. Use a roller or appropriate tool to ensure the foil seal is evenly applied.
8. Ensure wells at the corners of the plate are mixed well by vortexing the corners of the plate.
9. Spin the plate at 280 x g for 30 seconds to ensure the dispensed 10 µl is at the bottom of the well.
10. Thoroughly vortex the plate ensuring all wells are mixed well.
11. Spin the plate at 280 x g for 1 minute to ensure the contents are collected at the bottom of the wells.
12. The KAPA UDI Primer Mixes plate is now ready for use in the pre-capture PCR step.

13. Store any unused but already resuspended KAPA UDI Primer Mixes at -15 to -25°C. To avoid repeated freeze/thaw cycles you may transfer the resuspended primers to separate tubes or tube strips for storage

Procedure –

I. Fragmentation

1. Choose the fragmentation program on the thermal-cycler to cool the block to 4C.
2. Thaw KAPA Fragmentation enzyme and Buffer on ice. Briefly spin and return on ice.
3. Thaw the patient DNA samples. Briefly spin down. Samples should be prepared in low EDTA buffer, such as Elution Buffer (EB) or water. Use of TE will inhibit the enzymatic fragmentation step. If samples are in TE they can be re-purified using a DNA Clean & Concentrator kit (Zymo) and elute with EB or water.
4. Calculate the volume of each DNA sample required for 100 ng DNA, and the amount of elution buffer (EB) required to make to a final volume of 35 µl.
5. Place the tube strip or 32 well plate on the PCR cooler. Add the appropriate volume of elution buffer (EB) to the respective wells.
6. Add the appropriate volume of the patient sample DNA that corresponds to 100 ng to the respective wells.
7. Prepare Fragmentation master mix –
   (always prepare master mix for 10% additional reactions than the number of samples used in the experiment. For example, if preparing libraries for 8 individual samples, make master mix sufficient for 8.8 reactions)

Component	1X (ul)	For n number of reactions
KAPA Fragmentation Buffer	5	
KAPA Fragmentation Enzyme	10	

   Mix well by inverting and flicking the tube. Briefly spin. Return on ice.
8. Add 15 µl of the fragmentation master mix to the prepared DNA samples.
9. Close the lids or cover with sealing film. Mix well by inverting and flicking the tube. Briefly centrifuge. Return the strip/plate on the PCR cooler.
10. Transfer the strip/plate to the thermal cycler.
11. Choose the edit option on the thermal cycler. Select skip step to heat the thermal cycler to 37°C for fragmentation.

12. Fragmentation program

Temperature (°C)	Time
4	hold
37	25 minutes
4	hold

Lid temperature = +50°C

13. Place on PCR cooler and immediately proceed to the next step.

II. End-repair and A-tailing

1. While the fragmentation is in process, thaw KAPA End Repair buffer and end repair enzyme mix on ice. Vortex buffer to ensure any precipitates are fully dissolved.
2. With a few minutes left for fragmentation, prepare end repair and A-tailing master mix –

   Briefly centrifuge the reagent tubes to collect any liquid in the lid.

   (always prepare master mix for 10% extra than the number of samples used in the experiment. For example, if preparing libraries for 8 individual samples, make master mix sufficient for 8.8 reactions)

component	1X (µl)	(nx1.1)X (µl)
End repair and A- tailing Buffer	7	
End Repair and A-tailing enzyme mix	3	

Mix well by inverting and flicking the tube. Briefly spin. Return on ice.

3. Add 10 µl of the end repair and a tailing master mix to each well.
4. Close the lids or cover with sealing film. Mix well by inverting and flicking the tube. Briefly centrifuge. Return the strip/plate to the PCR cooler.
5. Choose the end repair program on the thermal cycler.
6. Transfer the strip/plate to the thermal cycler.
7. End-repair and A-tailing program

temperature	time

65 C	30 minutes
4 C	hold

Lid temperature = +85 ° C

8. Place on ice and immediately proceed to the next step.

III. Adapter Ligation

1. While the end-repair and A-tailing is in process, thaw Ligation buffer, KAPA DNA Ligase and KAPA Universal adapters. Vortex the ligase buffer and adapter to fully resuspend any precipitates. Briefly centrifuge and return the tubes to ice.
2. Prepare Ligation master mix

component	1X (µl)	(nx1.1)X
Ligation Buffer	30	
KAPA DNA Ligase enzyme	10	

Note – The ligation buffer and Ligase enzyme are viscous, carefully aspirate these reagents.

Mix well by inverting and flicking the tube. Briefly centrifuge. Return to ice.

3. Add 10 µl of KAPA Universal adapters to the DNA from step II.8.

   Note- add KAPA Universal adapters before adding the ligation master mix.
4. Add 40 µl of ligation mix to the DNA from the previous step.
5. Close the lids or cover with sealing film. Mix well by inverting and flicking the tube. Briefly centrifuge. Return the strip/plate to the PCR cooler.
6. Choose the ligation program under HyperCap v3 folder on the thermal-cycler.
7. Transfer the strip/well to the thermal cycler.
8. Adapter ligation program

Temperature (°C)	time
20	15 minutes
4	hold

Lid temperature = 85 C

9. Place at room temperature and immediately proceed to the next step.

IV. Post Ligation Cleanup

1. While the ligation reaction is in process, bring the KAPA HyperPure beads up to room temperature. Vortex the beads equilibrated to room temperature for 30 seconds.
2. Make 80 % ethanol (for example – Mix 8 ml 100 % ethanol and 2 ml nuclease free water)
3. After ligation, briefly centrifuge the tubes and transfer the tubes / well to the hood.
4. Add 88 µl of KAPA HyperPure beads to the DNA from the above step.
5. Close the tubes / seal the plate and mix well by vortexing for 10 seconds. Briefly centrifuge. Be careful to not settle down the beads during centrifugation.
6. Incubate at room temperature for 5 minutes.
7. After incubation, place the tubes on the Alpaqua magnetic rack for 3 minutes.
8. Aspirate the supernatant by pipetting.
9. While still on the magnet, add 200 µl of 80 % ethanol to the tubes. Incubate at room temperature for 30 seconds. Discard the ethanol.
10. Repeat step 9 once more.
11. Discard any remaining ethanol using a 10 µl pipette.
12. Dry the beads at room temperature for 5 minutes.
    Note – Be careful not to over-dry the beads as this may cause significant loss in yield. If any traces of ethanol remain after 5 minutes, allow the beads to dry for 2 – 3 more minutes.
13. While on the magnet, add 22 µl of EB to the tubes. Close the tubes / seal the plate. Remove from the magnet. Vortex the tubes / well for 10 seconds. Briefly centrifuge being careful to not settle down the beads.
14. Incubate at room temperature for 2 minutes. Incubation is not on the magnetic rack.
15. Place the tubes / plate on the magnetic rack for 3 minutes.
16. Transfer 20 µl of the supernatant to a new set of tubes.

V. Pre-capture Amplification using KAPA UDI Primer mixes

1. Thaw KAPA UDI Primer mixes and KAPA 2X HiFi PCR master mix.
2. Briefly centrifuge the KAPA UDI primer mixes plate.

3. Place the DNA from step IV.16 on the PCR cooler.
4. Arrange the KAPA UDI primer mixes plate so that the notch faces the bottom left.
5. Add 5 µl of the primer mixes from the appropriate wells of the KAPA UDI Primer mixes plate to the respective wells.

   Note the well number that goes into each sample – this information is required to know the barcode of the sample at the demultiplexing step after sequencing and is unique to each sample.

   Note – reseal the KAPA UDI primer mixes plate with a fresh foil seal. Mark the last used well.
6. Add 25 ul of the KAPA 2X HiFi PCR master mix to each well.
7. Close the tubes / seal the plate. Mix well by inverting and flicking. Briefly centrifuge. Return the tubes to the PCR cooler.
8. Select the program titled pre-capture 6X PCR on the thermal-cycler.
9. Pre-capture 6X PCR cycling conditions –

Temperature (º C)	Time (seconds)	No of cycles
98	45	
98	15	6
60	30	
72	30	
72	60	
4	hold	

VI. Post – PCR cleanup

1. Equilibrate KAPA HyperPure beads to room temperature. Vortex KAPA HyperPure beads for 30 seconds.
2. Briefly spin the DNA from step V.9. Add 70 ul of KAPA HyperPure beads to each well.
3. Close the tubes / seal the plate. Vortex for 10 seconds. Briefly centrifuge the tubes being careful not to settle the beads.
4. Incubate at room temperature for 5 minutes. Incubation is not on the magnet.
5. Place the tubes on the magnetic rack. Allow the beads to settle for 3 minutes.
6. Discard the supernatant.

7. While still on the magnet, add 200 µl of 80 % ethanol to the tubes. Incubate at room temperature for 30 seconds. Discard the ethanol.
8. Repeat step 7 once more.
9. Discard any remaining ethanol using a 10 µl pipette.
10. Dry the beads at room temperature for 5 minutes.

    Note – Be careful not to over-dry the beads as this may cause significant loss in yield. If any traces of ethanol remain after 5 minutes, allow the beads to dry for 2 – 3 more minutes.
11. While on the magnet, add 52 µl of EB to the tubes. Close the tubes / seal the plate. Remove from the magnet. Vortex the tubes / well for 10 seconds. Briefly centrifuge being careful to not settle down the beads.
12. Incubate at room temperature for 2 minutes. Incubation is not on the magnetic rack.
13. Place the tubes / plate on the magnetic rack for 3 minutes.
14. Transfer 50 µl of the supernatant to a new set of tubes.

VII. Dual size selection

Important – Dual size selection is optional. If the libraries do not have an average fragment size of about 320 bp and you observe larger fragments, follow dual size selection as described below.

1. Equilibrate AMPure XP beads to room temperature. Vortex for 30 seconds.
2. Add 35 µl of AMPureXP beads to the libraries. Close the tubes / seal the plate. Mix well by vortexing for 10 seconds. Briefly centrifuge being careful not to settle the beads.
3. Incubate at room temperature for 15 minutes.
4. After incubation, place the tubes on the magnetic rack. Allow the beads to settle for 3 minutes.
5. Transfer 80 µl the supernatant to a fresh set of tubes.
6. Add 10 µl of AMPureXP beads to the supernatant. Close the tubes / seal the plate. Mix well by vortexing for 10 seconds. Briefly centrifuge being careful not to settle the beads.
7. Incubate at room temperature for 15 minutes.

8. While incubation is ongoing make 80 % ethanol (for example – Mix 8 ml 100 % ethanol with 2 ml nuclease free water).
9. After incubation, place the tubes on the magnetic rack. Allow beads to settle for 3 minutes.
10. Discard the supernatant.
11. While still on the magnet, add 200 µl of 80 % ethanol to the tubes. Incubate at room temperature for 30 seconds. Discard the ethanol.
12. Repeat step 7 once more.
13. Discard any remaining ethanol using 10 µl pipette.
14. Dry the beads at room temperature for 5 minutes.
    Note – Be careful not to over-dry the beads as this may cause significant loss in yield. If any traces of ethanol remain after 5 minutes, allow the beads to dry for 2 – 3 more minutes.
15. While on the magnet, add 22 µl of EB to the tubes. Close the tubes / seal the plate. Remove from the magnet. Vortex the tubes / well for 10 seconds. Briefly centrifuge being careful to not settle down the beads.
16. Incubate at room temperature for 2 minutes.
17. After incubation, place the tubes on the magnetic rack. Allow beads to settle for 3 minutes.
18. Transfer 20 µl of the supernatant to a fresh set of tubes.

VIII. Determining concentration and size distribution of the libraries

1. Dilute the eluted libraries from step VII.18 1 in 10 by adding 2 µl of the eluted libraries to 18 µl of nuclease free water.

2. Measure concentration of the diluted libraries using Qubit 1X dsDNA HS assay on Qubit 4.0 fluorometer. Add 5 µl of the diluted libraries to 195 µl of the assay buffer.

Prepare the standards as described in the kit protocol. Vortex and briefly centrifuge. Incubate for 2 minutes at room temperature. Measure the concentration using Qubit 4.0 fluorometer.

1. Send the diluted libraries for QC on Bioanalyzer or TapeStation using DNA high sensitivity assay with the range setting from 150 to 1000 bp.

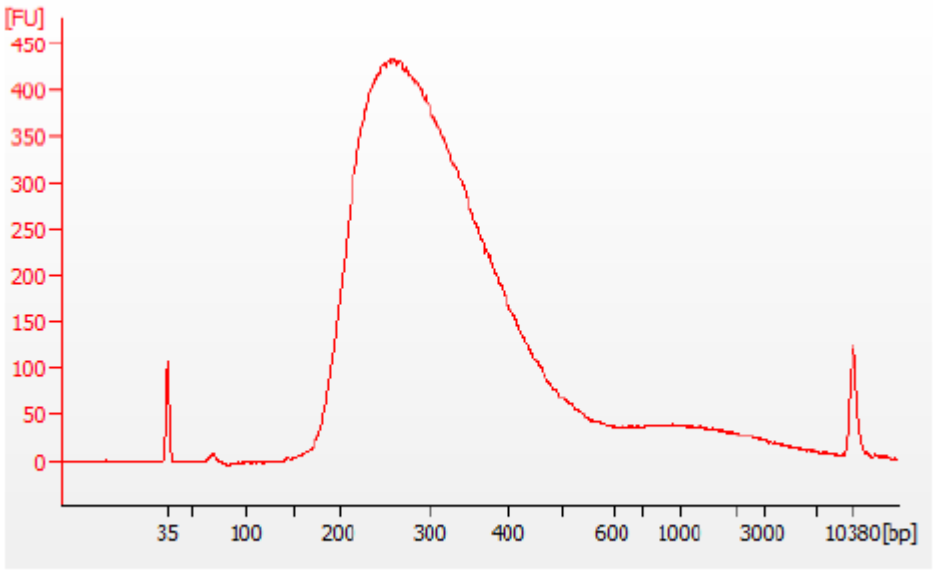

Figure 3: Example of an amplified HyperPlus sample library analyzed using the Agilent Bioanalyzer High Sensitivity DNA assay

2. If the libraries' size distribution looks like above (average size approx. 320 bp) and the undiluted libraries have >1000 ng of total DNA, proceed to hybridization.

Expected Library Concentrations

The concentration of the libraries after dual size selection is around 30 to 50 ng/µl.

Hybridize libraries to KAPA Target enrichment probes

This chapter describes the protocol for hybridization of the libraries from the previous step to KAPA target enrichment probes. This document describes the protocol for hybridization of unique index libraries with target enrichment probes with capture target size ≤ 40 Mbp.

Materials

Reagents -

1. KAPA HyperCapture bead kit (Roche, #09 075 780 001)
2. KAPA HyperCapture reagent kit (Roche, #09 075 810 001)
3. KAPA Target enrichment probes (see publication or Github site for IRNs of capture probes)
4. Molecular Biology grade ethanol
5. PCR grade water (Ambion,#AM9937)
6. Qubit 1X dsDNA HS assay kit

Equipment –

1. Thermal-cycler
2. Alpaqua magnetic rack
3. Qubit 4.0 fluorometer

Consumables –

1. 10 ul, 200 ul and 1000 ul filter tips
2. disposable pipettes
3. Qubit assay tubes
4. DNA LoBind tubes (cat no – 022431021)
5. 0.2 ml 8-well tubes strip (cat no – 1402-2900)
6. MicroAmp™ TriFlex 3 x 32-Well PCR Reaction Plate (cat no – A32811)

Before you start –

1. Make sure that no two samples with the same barcode are included in the same hybridization reaction.

Reagent Preparation –

1. If using a new vial of target enrichment probes, first verify the probe reaction number and the resuspension volume on the vial label. Spin the vial at 10,000 x g for 30 seconds. Add the recommended volume of resuspension buffer to the target enrichment probes. Vortex the tube and centrifuge at 10,000 x g for 30 seconds. Aliquot the target enrichment probes into single use aliquots of 4 µl and store at -20 ° C until use. Remove the sticker from the vial and attach it to the container with the aliquots.
2. If using the post-capture PCR oligos for the first time, briefly spin the vial to collect the contents. Add 480 µl of PCR grade water to the tube. Vortex for 10 seconds. Briefly spin the tube to collect the contents. Resuspended post- capture PCR Oligos must be stored at -20 ° C.
3. Prepare 80 % ethanol by adding 16 ml Molecular Biology grade 200 proof ethanol to 4 ml PCR grade water.

Procedure –

I. Prepare hybridization sample

1. Thaw the required number of aliquots of target enrichment probes (both mutation and translocation panel) on ice. One aliquot per hybridization reaction.
2. Thaw the libraries to be included in the hybridization reactions on ice. Give a brief spin.
3. Thaw COT human DNA, Universal enhancing oligos, Hybridization buffer on ice.
4. Thaw component H at room temperature.
5. For multiplexed samples, mix together equal amounts of each uniquely indexed libraries to obtain a combined mass of 1.5 ug. We pool 16 uniquely barcoded libraries in one hybridization pool, so 94 ng of each sample is required.

   This mixture is here-after referred to as 'Multiplex DNA sample library pool'
6. Add PCR grade water to make the volume to 45 µl.
7. Add 20 µl of human COT DNA to the DNA sample library pool.
8. Add 130 µl of KAPA HyperPure beads to the mixture from step 8.

   Note – If the volume of the DNA sample library pool is greater than 45 µl, transfer the multiplex DNA sample library to a 1.5 ml DNA LoBind tube. Add 20 µl of COT human DNA. Add 2X of the volume of KAPA HyperPure beads to the mixture. For example – combined volume of multiplex DNA sample

library and human COT DNA is 80 µl (60 ul of library + 20 ul of human COT DNA), add 160 µl of KAPA HyperPure beads. Proceed with the next step

10. Mix thoroughly by vortexing for 10 seconds. Give a quick spin.
11. Incubate at room temperature for 10 minutes.
12. After incubation, place the sample on the magnetic rack. Incubate until the liquid is clear.
13. Carefully remove and discard the supernatant.
14. While the sample is still on the magnet, add 200 µl of freshly prepared 80 % ethanol.
15. Incubate at room temperature for 30 secs, carefully remove and discard the ethanol.
16. Remove any residual ethanol with a 10 µl pipette.
17. Dry the beads for 5 minutes at room temperature. If any residual ethanol remains, dry for 2-3 more minutes.
18. Add 13.4 µl of Universal Enhancing Oligos to the bead – bound DNA sample.
19. Remove the tube from the magnet and vortex for 10 seconds. Give a brief spin.

Note – it is important to achieve a uniform suspension at this stage.

20. Prepare Hybridization master mix as follows –

    Briefly spin all the components before preparing the master mix. Note that all the volumes are for a single hybridization reaction. If preparing more than one hybridization reaction, multiply these volumes by the number of hybridization reactions.

component	Volume (µl)
Hybridization Buffer	28
Component H	12
PCR grade water	3
Total	43

Mix well by flicking and inverting the tube. Briefly spin.

21. Add 43 µl of Hybridization master mix to the bead bound DNA mixture resuspended in Universal Enhancing oligos.
22. Mix thoroughly by inverting and flicking the tube. Briefly spin.
23. Incubate at room temperature for 2 minutes.

24. Place the sample on the magnet. Allow the liquid to clear.
25. Pool the capture probes in a 0.2 ml tube by mixing together 3.5 µl of mutations panel v21 and 0.5 µl of translocation panel v21. Mix thoroughly by vortexing and give a brief spin.
26. Transfer 56.4 µl of the supernatant to the tube containing aliquots of target enrichment probe.
27. Mix thoroughly by vortexing for 10 seconds. Give a brief spin.
28. Place the tube in the thermal cycler. Choose the "hybridization" program on the thermal-cycler.
29. Perform hybridization with the following conditions –

    +95 ° C for 5 minutes

    +55 ° C for 16-20 hours

    Lid temperature = +105 ° C

Note – the sample must remain at 55 ° C until it is transferred to capture beads.

II. Wash and recover captured multiplex DNA sample

1. Thaw the wash buffers included in the KAPA HyperCapture reagent kit at room temperature. If any precipitate is observed, incubate the buffers at 37 °C and thoroughly vortex.

2. Dilute the wash buffers as described below.

Note- These volumes are for one hybridization reaction. If performing more than one hybridization reaction, multiply the volumes by the number of hybridizations.

Concentrated buffer	Volume of concentrated buffer (µl)	Volume of PCR grade water (µl)	Total volume of 1X Buffer (ul)	Temperature
10X Stringent Wash Buffer	40	360	400	55
10X Wash Buffer I	10	90	100	55
	20	180	200	Room temperature
10X Wash Buffer II	20	180	200	Room temperature
10X Wash Buffer III	20	180	200	Room temperature
2.5X bead wash buffer	120	180	300	Room temperature

Aliquot the 1X stringent wash buffers into two aliquots of 200 µl. Place the tubes in the heat block heated to 55 C.

Place the 100 µl aliquot if 1X Wash Buffer I in the heat blocked heated to 55 ° C.

The buffers need to be pre-warmed for at least 15 minutes.

III. Prepare capture beads

1. Equilibrate the capture beads to room temperature.
2. Vortex the Capture Beads for 15 seconds to get a homogenous mixture.

3. Aliquot 50 µl of Capture beads into a 0.2 ml tube or 1.5 ml DNA LoBind tubes depending on the number of hybridization reactions.
   For example – for one hybridization reaction use 50 µl of Capture beads, for 4 reactions use 200 µl of Capture beads)
4. Place the tube on the magnet to collect the beads. Once the liquid id clear, carefully remove and discard the supernatant.
5. While the tube is still on the magnet add 2X the initial volume of the beads of 1X bead wash buffer (for 1 hybridization reaction – add 100 µl of bead wash buffer). Remove the tube from the magnet and mix by vortexing for 10 seconds. Give a brief spin.
6. Place the tube back on the magnet. Allow the beads to collect. Once the liquid is clear, carefully remove and discard the supernatant.
7. While the tube is still on the magnet, add 2X the initial volume of the capture beads of 1X bead wash buffer (for 1 hybridization reaction – add 100 µl of 1X bead wash buffer). Remove the tube from the magnet and mix thoroughly by vortexing. Give a brief spin.
8. Place the tube back on the magnet. Allow the beads to collect, once the liquid is clear, carefully remove and discard the supernatant.
9. While the tube is on the magnet, add 1X of the initial volume of capture beads of 1X bead wash buffer (for 1 reaction – add 50 µl of 1X bead wash buffer). Mix thoroughly by vortexing. Give a brief spin.

Note – If performing more than one hybridization reaction, aliquot 50 µl of capture beads per reaction in 0.2 ml tubes.

10. Place the tube on the magnet. Allow the beads to collect. Carefully remove and discard the supernatant.
11. Immediately proceed to the next step so that the Capture beads do not dry.

IV. Bind Hybridized DNA to Capture beads

1. Remove the hybridization reaction tube from the thermal -cycler

Note – Do not turn off the thermal-cycler.

2. Give a brief spin.

3. Transfer the entire volume of the hybridization reaction to the Capture beads prepared as above**.**

4. Incubate the hybridization reaction at 55 C in the thermal-cycler for 15 minutes. The thermal cycler lid temperature is to be set at 105 °C.
5. Proceed immediately to the next step.

V. Wash the bead bound DNA

1. Remove the reaction tube from the thermal-cycler.

Note - Do not turn off the thermal-cycler.

2. Add 100 µl of pre-warmed Wash buffer I to the hybridization reaction. Mix well by vortexing for 10 seconds. Perform a quick spin.
3. Place the sample on the magnet. Allow the beads to collect. Once the liquid is clear, carefully remove and discard the supernatant.
4. Add 200 µl of pre-warmed 1X stringent wash buffer. Mix well by vortexing for 10 seconds. Briefly spin.
5. Place the sample in the thermal-cycler heated to 55 C and incubate for 5 minutes.
6. After the incubation, remove the tube from the thermal-cycler. Place the sample on the magnet and allow the beads to collect. Carefully remove and discard the supernatant.
7. Add 200 µl of pre-warmed 1X stringent wash buffer. Mix well by vortexing for 10 seconds. Briefly spin.
8. Place the sample in the thermal-cycler heated to 55 C and incubate for 5 minutes.
9. After the incubation, remove the tube from the thermal-cycler. Place the sample on the magnet and allow the beads to collect. Carefully remove and discard the supernatant.
10. Add 200 µl of room-temperature 1X wash buffer I. Mix thoroughly by vortexing for 10 seconds. Briefly spin. Incubate at room temperature for 1 minute.
11. Place the sample on the magnet. Allow the beads to collect. Carefully remove and discard the supernatant.
12. Add 200 µl of 1X Wash Buffer II. Mix thoroughly by vortexing for 10 seconds. Briefly spin. Incubate at room temperature for 1 minute.
13. Place the sample on the magnet. Allow the beads to collect. Carefully remove and discard the supernatant.
14. Add 200 µl of 1X Wash Buffer III. Mix thoroughly by vortexing for 10 seconds. Briefly spin. Incubate at room temperature for 1 minute.
15. Place the sample on the magnet. Allow the beads to collect. Carefully remove and discard the supernatant.

16. Remove the tube from the magnet and add 20 µl of PCR grade water.

VI. Post – capture Amplification of Enriched Multiplex DNA sample

1. Equilibrate KAPA HyperPure beads to room temperature.
2. Make the post-capture PCR master mix as below

Note – The volumes described here are for 1 hybridization reaction. If preparing the master mix for more than one hybridization reaction, multiply the volumes by the number of hybridization reactions and one additional reaction accounting for pipetting error. Include a negative control as well when making the post-capture PCR master mix.

component	Volume/reaction (µl)
KAPA HiFi HotStart ReadyMix (2X)	25
Post capture PCR Oligos	5
total	30

3. Add 30 µl of the post-capture PCR Master mix to the respective hybridization reaction. To the negative control, add 20 µl of the PCR grade water used to elute the beads in step V.16.
4. Mix thoroughly by inverting and flicking the tube. Briefly spin.
5. Place the tube in the thermal cycler and choose the program “Post-cap PCR” on the thermal-cycler.
6. Perform the post capture PCR amplification as described below

Note – These conditions are for amplifying capture target size > 2 Mb to 40 Mb.

Lid temperature should be set at 105 °C.

Temperature (°C)	Time (seconds)	Number of cycles
98	45	
98	15	11
60	30	
72	30	
72	60	
4	hold	

VII. Purification of amplified enriched multiplex DNA sample

1. Remove the PCR reaction tube from the thermal-cycler. Briefly spin.

2. Add 70 µl of KAPA HyperPure beads equilibrated to room temperature to 50 µl of amplified enriched multiples DNA sample library. Mix thoroughly by vortexing for 10 seconds. Briefly spin.

3. Incubate at room temperature for 5 minutes.

4. Place the tube on the magnet. Incubate for 3 minutes to allow the beads to collect.

5. Carefully remove and discard the supernatant.

6. Add 200 µl of freshly prepared 80 % ethanol. Incubate at room temperature for 30 seconds.

7. Carefully remove and discard the ethanol.

8. Add 200 µl of freshly prepared 80 % ethanol. Incubate at room temperature for 30 seconds.

9. Carefully remove and discard the ethanol. Remove any residual ethanol with a 10 µl pipette.

10. Dry the beads at room temperature for 5 minutes. If any residual ethanol is observed after 5 minutes, dry the beads for 2-3 minutes until all traces of ethanol have evaporated.

11. While on the magnet. Add 22 µl of Elution Buffer to the beads. Mix thoroughly by vortexing. Briefly spin.

12. Incubate at room temperature for 2 minutes.

Place the tube on the magnet for 3 minutes until the beads have collected and the liquid is clear.

13. Transfer the supernatant to a new tube.

VIII. Determining concentration, size distribution and quality of the amplified enriched multiplex DNA sample library

1. Make a 1/10 dilution of the amplified enriched multiplex DNA sample library by adding 2 µl of the elute from step VII.13. to 18 µl of PCR grade water. Mix thoroughly by vortexing. Briefly spin.
2. Add 5 µl of the diluted library to 195 µl of Qubit 1X dsDNA HS assay buffer. Quantify the diluted library using Qubit 4.0 fluorometer.

3. Analyze the size distribution with Agilent Bioanalyzer or TapeStation DNA high sensitivity assay.
4. The average size distribution should be approximately 320 bp. The amplified enriched multiplex DNA sample library yield should be ≥ 100 ng and the negative control should have a not -significant yield. If the amplified enriched multiplex library meets these criteria, the library is ready to be sequenced.

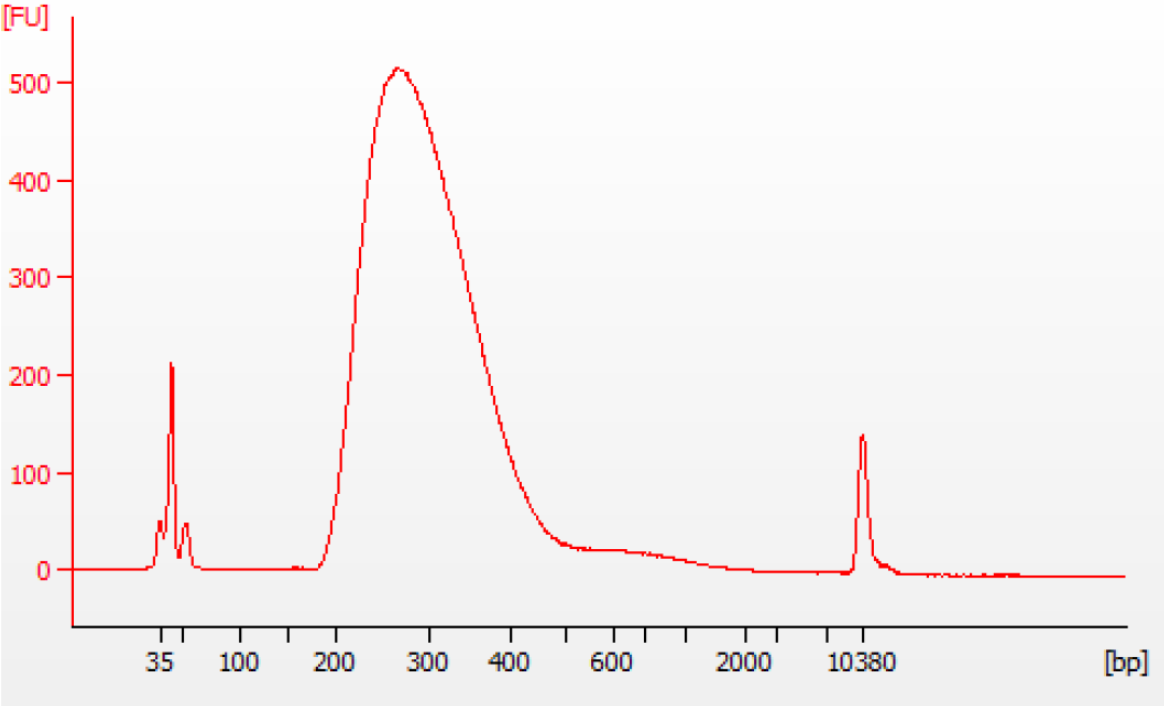

Figure 5: Example of a successfully amplified enriched Multiplex DNA Sample using the KAPA HyperPlus Kit and analyzed using an Agilent Bioanalyzer High Sensitivity DNA chip.

Expected Concentration of the amplified enriched multiplex DNA sample

The expected concentration of the amplified enriched multiplex DNA sample is between 30 – 50 ng/µl

Next Generation Sequencing

DNA libraries can be sequenced on any Illumina sequencer. One hybridized pool (16 samples) on a Mid output NextSeq run with 75 bp paired end reads will generate a mean target coverage for the mutation panel of 450x and for the translocation panel of 150x. Longer reads can be used, as long as they are paired end to enable translocation detection.