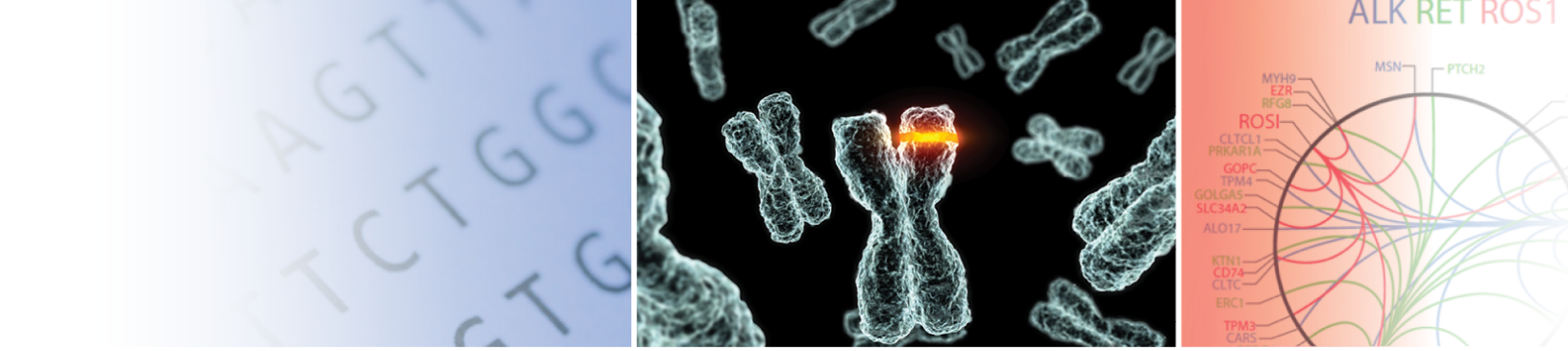

Instructions for Use

ARCHER™ by enzymatics

Archer™ Universal RNA Fusion Detection for Ion Torrent™ Platform

AK0025-8

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Product Description

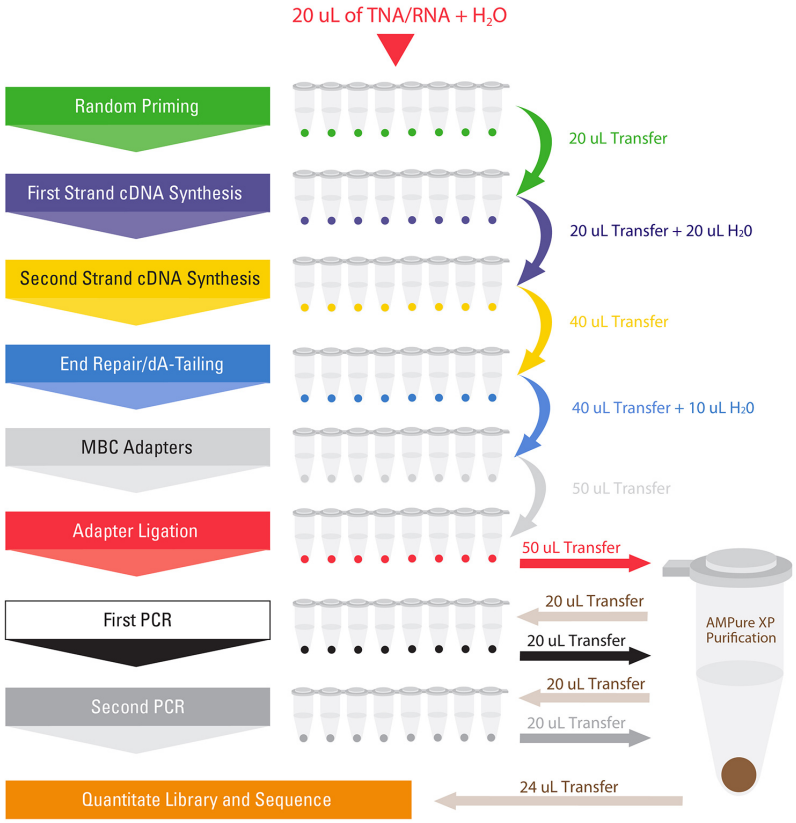
Gene fusions represent an important class of genomic rearrangements in translational research. The Archer™ Universal RNA Reagent Kits and FusionPlex™ assays utilize the power of next-generation sequencing to improve the detection of genomic rearrangements over traditional methods such as immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH).

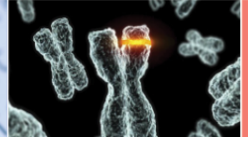
Modular Assay Format

The Universal RNA Reagent Kit, used in conjunction with Archer™ Assays and MBC Adapters, allows users to construct Ion Torrent™ sequencing platform ready libraries from total nucleic acid or RNA samples.

For Research Use Only. *Not for use in diagnostic procedures.*

Workflow Overview





Version Additions & Changes

- **Added multiplexing recommendations for the Archer™** FusionPlex™ Heme and Sarcoma Panels

Kit Contents

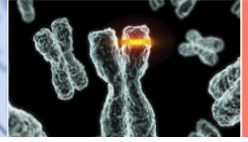
1. 500 mM Tris-HCl, pH 8.0 (SA0020)
2. Ultra Pure Water (SA0021)
3. Ultra Pure Water for Ethanol Dilution (SA0022)
4. Lyophilized Reagents:
    a. Step 1: Random Priming (SA0001)
    b. Step 2: First Strand cDNA Synthesis (SA0002)
    c. Step 3: Second Strand cDNA Synthesis (SA0003)
    d. Step 4: End repair/dA-tailing (SA0004)
    e. Step 5: Adapter Ligation (SA0005)
    f. Step 6: First PCR (SA0011)
    g. Step 7: Second PCR (SA0015)

Materials Required But Not Supplied

1. Archer™ MBC Adapters for Ion Torrent™
2. Archer™ FusionPlex™ Assay (Cat# AK0028-8, AK0028-9, AK0032-8)
3. Agencourt® AMPure® XP Beads (Cat# A63881)
4. Life Technologies® DynaMag™ (Cat# 12331D)
5. 100% ethanol (ACS grade)
6. KAPA Biosystems® Library Quantification Kit – Ion Torrent™/Universal (Cat# KK4827)
7. Custom Primer Panels, designed at http://assay.enzymatics.com
8. If nucleic acid is from FFPE tissue, it is recommended to use Agencourt® FormaPure® (A33342) for extraction

General Precautions

- Read the entire protocol before beginning.
- Take note of stopping points where samples can be frozen at -20°C and plan your workflow accordingly.
- Use good laboratory practices to minimize cross-contamination of nucleic acid products.
- Always use PCR tubes, microfuge tubes and pipette tips that are certified sterile, DNase- and RNase-free.
- Before starting, wipe down work area and pipettes with an RNase and DNA cleaning product such as RNase Away™ (Molecular BioProducts, Inc. San Diego, CA).
- For consistent library amplification, ensure the thermal cycler used in this protocol is in good working order and has been calibrated to within the manufacturer's specifications.



Storage

All components of Archer™ Universal RNA Reagent Kit (Part # AK0025-8) should be stored at 4°C.

Allow pouches to warm to room temperature before opening.

Sample Multiplexing

In order to efficiently utilize the throughput of the PGM™, multiple samples should be sequenced simultaneously. Samples can be identified through a unique nucleotide sequence that is part of the adapter attached to the nucleic acid molecule in a given sample during library construction, and which is subsequently read during the sequencing process. The unique nucleotide sequence is often termed an "index". Archer™ Universal RNA Reagent Kit for Ion Torrent™ Platform utilizes a single index to distinguish between samples. The index is added just before Step 5 (Adapter Ligation) and is embedded in the Ion Torrent™ Barcode Adapters.

In order to maintain appropriate coverage depth, it is recommended to cap each PGM™ run at 2-10 samples per 318 chip. In general larger panels with more targets will require higher sequencing coverage depth, and should be run with fewer samples per chip. Below are some recommendations for panels of different sizes:

Archer™ Panel	# of Targets/Assay	Recommended # of samples/318 Chip
FusionPlex™ ALK, RET, ROS1 Panel v2	29	7-10
FusionPlex™ Heme Panel	132	2-3
FusionPlex™ Sarcoma Panel	134	2-3

Input Nucleic Acid Concentration and Purification

- Total nucleic acid is the preferred input for this assay.
- DO NOT treat the extracted total nucleic acid with DNase, as this will critically reduce the quality of RNA in the sample.
- If nucleic acid is from FFPE tissue, it is recommended to use Agencourt® FormaPure® (A33342) for extraction.
- When possible, it is recommended to increase the total nucleic acid input, which will increase library complexity and improve the sensitivity of the assay. If higher library complexity is desired, the assay can tolerate up to 250 ng of total nucleic acid.
- The minimum recommended input for the assay is 20 ng of total nucleic acid. Alternatively, 10 ng of RNA may be used.
- Efficient library preparation can be achieved with as little as 2 ng of total nucleic acid, provided that the starting material is of high quality and is not degraded. However, reduced input will decrease library complexity due to the restricted amount of starting unique target molecules. When using less than 10 ng of input material the PCR cycling conditions (Steps 6 and 7) may need to be altered.
- The use of EDTA-containing buffers in this protocol may result in lower library yields. Be sure to use buffers that do not contain EDTA (i.e. use Tris-HCl and not Tris-EDTA buffer).

Before You Begin

- **Make fresh 10 mM Tris-HCl.**
  - Mix 20 µL 500 mM Tris-HCl, pH 8.0 (SA0020) with 980 µL Ultra Pure Water (SA0021).
- **Make fresh 70% ethanol.**
  - Add 14 mL 100% ethanol (ACS grade; not included) to entire bottle containing Ultra Pure Water for Ethanol Dilution (SA0022).
  - Note the date on which ethanol is added, 70% ethanol is appropriate for use for one week after mixing. When not in use, tightly close the bottle cap to ensure minimal evaporation.

Instructions for Use

Step 1: Random Priming

1.1. Pre-heat thermal cycler to 65°C with a heated lid.

1.2. Gently open the **Random Priming** (SA0001) foil pouch by tearing along the indents located at the top of the silver package.

1.3. Remove the green 8-tube strip. Each tube in the strip provides a single reaction.

1.4. Centrifuge briefly to ensure lyophilized material is in the bottom of the tube.

1.5. If you would like to use fewer than eight reactions, detach the appropriate number of tubes carefully using clean scissors or a new razor blade. Store the remaining unused tubes in the sealed foil pouch with desiccant provided at 4°C.

1.6. Place the tubes on ice and to each add:

Ultra Pure Water (SA0021)	20 – X µL
Purified Total Nucleic Acid	X µL
Total	**20 µL**

1.7. After the lyophilized pellet dissolves, gently pipet up and down 6 – 8 times and briefly spin down.

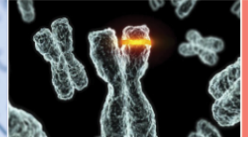
1.8. Transfer the tubes from ice to the thermal cycler and incubate at 65°C for 5 minutes.

1.9. Remove tubes from thermal cycler and place on ice for 2 minutes, then briefly centrifuge before proceeding with **First Strand DNA Synthesis.**

Step 2: First Strand cDNA Synthesis

2.1. Gently open the **First Strand cDNA Synthesis** (SA0002) foil pouch by tearing along the indents located at the top of the silver package.

2.2. Remove the purple 8-tube strip. Each tube in the strip provides a single reaction.



2.3. Centrifuge briefly to ensure lyophilized material is in the bottom of the tube.

2.4. If you would like to use fewer than eight reactions, detach the appropriate number of tubes carefully using clean scissors or a new razor blade. Store the remaining unused tubes in the sealed foil pouch with the desiccant provided at 4°C.

2.5. Place the **First Strand cDNA Synthesis** tubes on ice and transfer 20 µL of the Random Priming mixture (**Step 1.9.**) to the lyophilized First Strand cDNA Synthesis pellet and mix well by pipetting up and down. Spin briefly to collect contents at the bottom of the tube.

2.6. Place the tubes into a thermal cycler with a heated lid set to ≥100°C and incubate as follows:

Step	Incubation Temperature	Incubation Time
1	25°C	10 min
2	42°C	30 min
3	80°C	20 min
4	4°C	Hold

2.7. Remove the PCR tubes from the thermal cycler and place on ice.

Step 3: Second Strand cDNA Synthesis

3.1. Gently open the Second Strand cDNA Synthesis (SA0003) foil pouch by tearing along the indents located at the top of the silver package.

3.2. Remove the **yellow** 8-tube strip. Each tube in the strip provides a single reaction.

3.3. Centrifuge briefly to ensure lyophilized material is in the bottom of the tube.

3.4. If you would like to use fewer than eight reactions, detach the appropriate number of tubes carefully using clean scissors or a new razor blade. Store the remaining unused tubes in the sealed foil pouch with the desiccant provided at 4°C.

3.5. To the Second Strand Synthesis tube on ice add:

Ultra Pure Water (SA0021)	20 µL
First Strand cDNA Synthesis reaction (**Step 2.7.**)	20 µL
Total	**40 µL**

3.6. Mix well by pipetting gently up and down 6 – 8 times. Spin briefly to collect contents at the bottom of the tube.

3.7. Incubate at 16°C for 1 hour. If a thermal cycler is used for the incubation do not use a heated lid or close the heated lid. Do not allow the temperature to rise above 16°C.

Stopping point: It is OK to stop and store the library at -20°C.

Step 4: End Repair/dA-Tailing

4.1. Gently open the **End Repair/dA-Tailing** (SA0004) foil pouch by tearing along the indents located at the top of the silver package.

4.2. Remove the blue 8-tube strip. Each tube in the strip provides a single reaction.

4.3. Centrifuge briefly to ensure lyophilized material is in the bottom of the tube.

4.4. If you would like to use fewer than eight reactions, detach the appropriate number of tubes carefully using clean scissors or a new razor blade. Store the remaining unused tubes in the sealed foil pouch with the desiccant provided at 4°C.

4.5. Transfer 40 µL of the Second Strand cDNA Synthesis reaction (Step 3.7.) into tube containing lyophilized **End Repair/dA-Tailing** (SA0004) reagents and mix well by pipetting up and down 6 – 8 times. Spin briefly to collect contents at the bottom of the tube.

4.6. Incubate the reaction in a thermal cycler with a heated lid set to ≥100°C and incubate as follows:

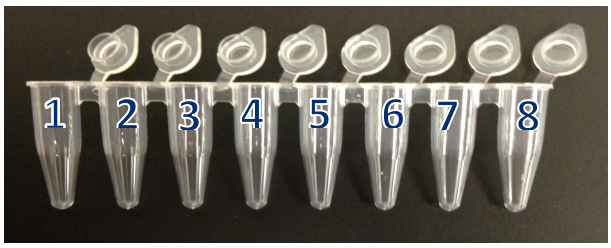
Step	Incubation Temperature	Incubation Time
1	12°C	15 min
2	37°C	15 min
3	72°C	15 min
4	4°C	Hold

4.7. Ensure the reaction cools to 4°C and briefly centrifuge End Repair reaction before proceeding.

4.8. **Gently open a pouch of Archer MBC Adapters for Ion Torrent™ by tearing along the indents located at the top of the silver package.**

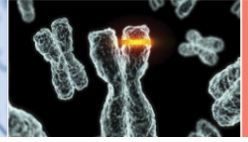
4.9. **Remove the clear 8-tube strip from the foil pouch. Each tube in the strip provides a single reaction and each tube contains a different Ion Torrent™ MBC Adapter or Ion Torrent™ Barcode Adapter. (For example, reactions 1 through 8 correspond to MBC Adapters 1 through 8).**

4.9.1. **CRITICAL:** Upon removing the 8-tube strip from the pouch, position the tubes with the hinges to the back and use a permanent marker to label the tubes 1 through 8 from left to right as shown below. Be sure to label and track the index number added to each sample from this point forward.



4.10. Centrifuge briefly to ensure lyophilized material is in the bottom of the tube.

4.11. If you would like to use fewer than eight reactions, detach the appropriate number of tubes carefully using clean scissors or a new razor blade. Store the remaining unused tubes in the sealed foil pouch with



the desiccant provided at 4°C. Be sure to track which indices were used to ensure index compatibility when used in later experiments

4.12. To the **Archer™ MBC Adapters for Ion Torrent™** tube on ice add:

Ultra Pure Water (SA0021)	10 µL
End Repaired/dA-tailed DNA (**Step 4.7**)	40 µL
Total	**50 µL**

4.13. Allow the pellet to dissolve and then pipet up and down 6 – 8 times to mix. Spin briefly to collect contents at the bottom of the tube.

4.14. Immediately proceed to **Step 5**.

Step 5: Adapter Ligation

5.1. Gently open the **Adapter Ligation** (SA0005) foil pouch by tearing along the indents located at the top of the silver package.

5.2. Remove the red 8-tube strip. **Each tube in the strip provides a single reaction**.

5.3. Centrifuge briefly to ensure lyophilized material is in the bottom of the tube.

5.4. If you would like to use fewer than eight reactions, detach the appropriate number of tubes carefully using clean scissors or a new razor blade. Store the remaining unused tubes in the sealed foil pouch with the desiccant provided at 4°C.

5.5. Transfer 50 µL of the End Repaired/dA tailed DNA with the annealed Ion Torrent™ MBC Adapters (**Step 4.13.**) into the tube containing Adapter Ligation mix. Allow pellet to dissolve and then pipet up and down 6 – 8 times to mix. Spin briefly to collect contents at the bottom of the tube.

5.6. Incubate the reaction as follows. If a thermal cycler is used either set the thermal cycler lid to "off" or leave it open during the incubation.

Step	Incubation Temperature	Incubation Time
1	16°C	30 min
2	22°C	30 min

Post-Ligation AMPure® XP Beads Purification

5.7. Refer to manufacturer's protocol for details on methods of purification.

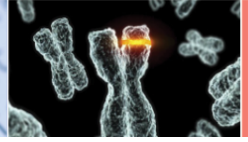
5.8. Add 40 µL of AMPure® XP beads to the 50 µL reaction for a ratio of 0.8X.

5.9. Vortex well or pipette 10 times to mix and incubate for 5 minutes at room temperature.

5.10. Collect beads with magnet for 2-4 minutes or until solution is clear.

5.11. Carefully pipette off and discard supernatant without disturbing the beads.

5.12. Wash twice with 200 µL of 70% ethanol while on the magnet. Spin down and carefully remove remaining supernatant while taking care to not resuspend beads.



5.13. After the second wash dry beads at room temperature for 5 minutes.

5.14. Elute cDNA in 24 µL of 10 mM Tris-HCl. Remove tubes from the magnet and thoroughly resuspend the beads with the 10 mM Tris-HCl.

5.15. Place cDNA bead solution back on magnet for 2 minutes.

5.16. Carefully transfer 22 µL of the purified library solution to a fresh 200 µL PCR tube or proceed directly to **Step 6**. Be sure to avoid transferring beads to the fresh tube.

Stopping point: It is OK to stop and store the library at -20°C.

Step 6: First PCR

NOTE:

The Archer™ Universal RNA Reagent Kits do **not** contain gene-specific primers (GSPs) in the reaction pellet.

6.1. Gently open the **First PCR** (SA0011) foil pouch by tearing along the indents located at the top of the silver package.

6.2. Remove the clear 8-tube strip. Each tube in the strip provides a single reaction.

6.3. Centrifuge briefly to ensure lyophilized material is in the bottom of the tube.

6.4. If you would like to use fewer than eight reactions, detach the appropriate number of tubes carefully using clean scissors or a new razor blade. Store the remaining unused tubes in the sealed foil pouch at 4°C.

6.5. To the First PCR tube on ice add:

Purified library DNA (**Step 5.16.**)	18 µL
Liquid GSP1 Mix	2 µL
Total	**20 µL**

6.6. Allow the pellet to dissolve and then pipet up and down 6 – 8 times to mix. Spin briefly to collect contents at the bottom of the tube.

6.7. Incubate the reaction as follows. Note the ramp rate between 98°C and 68°C; consult your instrument user's manual to confirm that this setting is correct. Ensure the lid temperature tracks 5°C above the incubation temperature or set the lid to ≥100°C.

Incubation Temperature	Incubation Time	# of cycles
98°C	30 sec	1
98°C 68°C [ramp rate of 2.3°C/sec]	10 sec 30 sec	20*
72°C	3 min	1
4°C	HOLD	1

*NOTE: If library yields are too low, the cycle number can be increased up to 22 cycles. The number of unique molecules will be reduced when the PCR cycles are increased, and can be decreased based on user experience with different amount of input material and specific sample types.

Post-First PCR AMPure® XP Beads Purification

6.8. Refer to manufacturer's protocol for details on methods of purification.

6.9. Add 16 µL of AMPure®XP beads to the 20 µL reaction for a ratio of 0.8X.

6.10. Vortex well or pipette 10 times to mix and incubate for 5 minutes at room temperature.

6.11. Collect beads with magnet for 2-4 minutes or until solution is clear.

6.12. Carefully pipette off and discard supernatant without disturbing the beads.

6.13. Wash twice with 200 µL of 70% ethanol while on the magnet. Spin down and carefully remove remaining supernatant while taking care to not resuspend beads.

6.14. After the second wash dry beads at room temperature for 5 minutes.

6.15. Elute cDNA in 24 µL of 10 mM Tris-HCl. Remove tubes from the magnet and thoroughly resuspend the beads with the 10 mM Tris-HCl.

6.16. Place cDNA bead solution back on magnet for 2 minutes.

6.17. Carefully transfer 22 µL of the purified library solution to a fresh 200 µL PCR tube or proceed directly to **Step 7**. Be sure to avoid transferring beads to the fresh tube.

Stopping point: It is OK to stop and store the library at -20°C.

Step 7: Second PCR

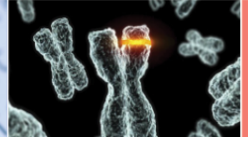
NOTE:

The Archer™ Universal RNA Reagent Kits for Ion Torrent™ Platform kits do not contain gene-specific primers (GSPs) in the reaction pellet.

7.1. Gently open the **Second PCR** (SA0015) foil pouch by tearing along the indents located at the top of the silver package.

7.2. Remove the **clear** 8-tube strip from the foil pouch. **Each tube in the strip provides a single reaction.**

7.2.1. **CRITICAL:** Upon removing the 8-tube strip from the pouch, position the tubes with the hinges to the back and use a permanent marker to label the tubes 1 through 8 from left to right as shown below. Be sure the label is placed where it will not be compromised when placed in a thermal cycler.



7.3. Centrifuge briefly to ensure lyophilized material is in the bottom of the tube.

7.4. If you would like to use fewer than eight reactions, detach the appropriate number of tubes, carefully using clean scissors or a new razor blade. Store the remaining unused tubes in the sealed foil pouch with the desiccant provided at 4°C.

7.5. To the Second PCR tube on ice add:

7.5.1.

Purified library DNA (**Step 6.17.**)	18 µL
Liquid GSP2 Mix	2 µL
Total	20 µL

7.6. Allow the pellet to dissolve and then pipet up and down 6 – 8 times to mix. Spin briefly to collect contents at the bottom of the tube.

7.7. Incubate the reaction as follows. Note the ramp rate between 98°C and 68°C; consult your instrument user's manual to confirm that this setting is correct. Ensure the lid temperature tracks 5°C above the incubation temperature or set the lid to 100°C.

Incubation Temperature	Incubation Time	# of cycles
98°C	30 sec	1
98°C 68°C [ramp rate of 2.3°C/sec]	10 sec 30 sec	24*
72°C	3 min	1
4°C	HOLD	1

*NOTE: The number of unique molecules will be reduced when the PCR cycles are increased, and can be decreased based on user experience with different amount of input material and specific sample types.

Post-Second PCR AMPure® XP Beads Purification

7.8. Refer to manufacturer's protocol for details on methods of purification.

7.9. Add 16 µL of AMPure®XP beads to the reaction for a ratio of 0.8X.

7.10. Vortex well or pipette 10 times to mix and incubate for 5 minutes at room temperature.

7.11. Collect beads with magnet for 2-4 minutes or until solution is clear.

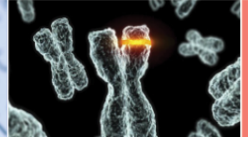
7.12. Carefully pipette off and discard supernatant without disturbing the beads.

7.13. Wash twice with 200 µL of 70% ethanol while on the magnet. Spin down and carefully remove remaining supernatant while taking care not to resuspend beads.

7.14. After the second wash dry beads at room temperature for 5 minutes.

7.15. Elute cDNA in 24 µL of 10 mM Tris-HCl. Remove tubes from the magnet and thoroughly resuspend the beads with the 10 mM Tris-HCl.

7.16. Place cDNA bead solution back on magnet for 2 minutes.



7.17. Carefully transfer 24 µL of the purified cDNA solution to a fresh 200 µL PCR tube or proceed directly to Step 8. Be sure to avoid transferring beads to the fresh tube.

Stopping point: It is OK to stop and store the library at -20°C

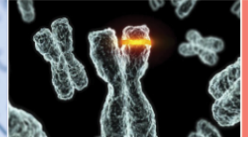
Step 8: Quantify Library and Sequence

8.1. Use the KAPA Biosystems® qPCR kit (KK4827) for Ion Torrent™ to quantify the concentration of each library. Assume a 250 bp fragment length. After quantification, pool the barcoded libraries at equimolar concentrations and perform template preparation following the manufacture's protocol. Sequence on an Ion Torrent™ PGM™ 318 chip. A generic sequencing template can be created on the Torrent™ Server after uploading the index tag files and this template can be used to plan the run. In order to maintain the appropriate read coverage per target it is suggested to limit input to 10 samples per 318 chip.

8.1.1. Samples within the pool should be demulitplexed using the Torrent Server with the appropriate barcode sequence. In addition the index tag file can be downloaded from our site for convenience http://www.enzymatics.com/archer.

8.2. This assay workflow leads to constructs with highly efficient ePCR amplification during PGM™ template preparation. In order to achieve 10-30% unenriched template positive ISPs, library should be loaded into the ePCR at 13 pM using the Ion Torrent™ PGM™ Template OT2 200 kit. Typically Ion Torrent™ recommends using ~20 pM of library into ePCR. However, with our more efficient amplified constructs, loading at a lower concentration is necessary to avoid a high percentage of polyclonal ISPs. After the first chip the loading concentration can be adjusted accordingly to achieve the optimal percentage of unenriched template positive ISPs.

8.3. Upon completion of the run the data should be analyzed using the Archer™ Analysis Pipeline http://archer.enzymatics.com



Limitations of Use

For Research Use Only. Not for use in diagnostic procedures.

This product was developed, manufactured, and sold for in vitro use only. The product is not suitable for administration to humans or animals. SDS sheets relevant to this product are available upon request.

For more information please visit http://www.enzymatics.com/archer

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