Published in final edited form as:

Methods Mol Biol. 2012 ; 851: 9–46. doi:10.1007/978-1-61779-561-9_2.

Genomic Methods for Clinical and Translational Pain Research

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Abstract

Pain is a complex sensory experience for which the molecular mechanisms are yet to be fully elucidated. Individual differences in pain sensitivity are mediated by a complex network of multiple gene polymorphisms, physiological and psychological processes, and environmental factors. Here, we present the methods for applying unbiased molecular-genetic approaches, genome-wide association study (GWAS), and global gene expression analysis, to help better understand the molecular basis of pain sensitivity in humans and variable responses to analgesic drugs.

Keywords

Pain; Genome-wide association study; Single nucleotide polymorphism; Whole-genome sampling analysis; Copy number variation; Global gene expression analysis; Microarray

1. Introduction

Human pain sensitivity varies widely among different individuals. Understanding the genetic contribution to molecular processes that contribute to variation in pain is critical for elucidating the mechanism of variable responses to analgesic drugs and may contribute to better prevention, medical diagnosis, and individualized treatment. Candidate gene studies have identified several genetic variations that produce interindividual difference in pain sensitivity and analgesic drug responses (1–8). However, like many other “hypothesis-driven” genetic approaches, the significant findings are sometimes difficult to replicate when followed up in subsequent association studies (9–11). This is not surprising considering our imperfect understating of the complexity of pain biology and the size of human genome. Inconsistent outcomes are often caused by variable study designs, sample heterogeneity, small samples sizes, phenotype complexity, and alternative statistical approaches. Therefore, it is critical to use appropriate methodologies to gain insight into genetic mechanisms of pain and eventually to apply this knowledge to pain treatment.

Genome-wide association studies (GWAS) catalog human genetic variation and correlate it with phenotypic difference by scanning millions of single nucleotide polymorphism (SNP) across the whole genome, and overcome the limitation of candidate gene association studies. GWAS published to date use various commercial genotyping platforms to assess differences in allele frequencies between cases and controls. Though rare variants are often overlooked, GWAS have successfully uncovered hundreds of genetic loci associated with many common diseases, such as obesity (12), diabetes (13), rheumatoid arthritis (14), and Crohn’s disease (15). Our recent report (16) suggests that GWAS are also powerful and unbiased for identifying novel genes implicated in a complex phenotype such as pain and responses to analgesic drugs.

Comprehensive analysis of global gene expression using DNA microarray is another hypothesis-free method to identify disease-related genetic variations and has become a widespread technology in genomics research. Thousands of transcripts can be assayed simultaneously, therefore providing data on a large and unbiased set of traits (17). Over the past few years, we have successfully applied this method to the field of clinical pain research and identified genes with dynamic changes in expression that appear to be functionally associated with acute inflammatory pain and inflammation (18–21).

While these powerful methods hold great promise for elucidating novel targets for intervention and may provide greater understanding of the molecular-genetic pathways associated with pain, inflammation and the transition for acute to chronic pain, they are fraught with significant pitfalls in their application to clinical and translational research. In Subheading 4, we suggest a systematic approach to reporting important variables associated with conducting genetic studies in humans that permit evaluation of the strength of the findings based on considerations considered axiomatic since the adoption of the Consolidated Standards of Reporting Trials (CONSORT) recommendations for reporting the results of clinical trials.

2. Materials

2.1. Genome-Wide Association Study

2.1.1. Genomic DNA Preparation

1. Reduced EDTA TE buffer (10 mM Tris-HCL, 0.1 mM EDTA, pH 8.0, TEKnova), stored at room temperature.
2. AccuGENE water, molecular biology grade (Cambrex), kept at 4°C.
3. Purified genomic DNA, stored at –20°C.
4. Reference Genomic DNA 103, included in Affymetrix Genome-Wide Human SNP Nsp/Sty Assay kit 5.0/6.0 (kit 1, see Note ¹).

2.1.2. Sty Restriction Enzyme Digestion, Ligation, and PCR

1. Sty I (10 U/μl), NE Buffer 3 (10×), and BSA (100×; 10 mg/ml) from New England Biolabs (NEB), stored at –20°C.
2. T4 DNA Ligase (400 U/μl) and T4 DNA Ligase Buffer (10×) from NEB, stored –20°C (see Note ²).
3. Adaptor, Sty (50 μM) and PCR primer 002 (100 μM) included in kit 1, stored at –20°C.
4. Clontech TITANIUM DNA Amplification kit (kit 2) stored at –20°C:
 - dNTPs (2.5 mM each).
 - GC-Melt (5 M).

¹The kits used in this protocol:

- Kit 1: Genome-Wide Human SNP Nsp/Sty Assay kit 5.0/6.0 (Affymetrix).
- Kit 2: TITANIUM DNA Amplification kit (Clontech).
- Kit 3: GeneChip 3' IVT Express Kit (Affymetrix).
- Kit 4: GeneChip Hybridization, Wash, and Stain Kit (Affymetrix).

²Materials and reagents are listed when first required.

- TITANIUM™ Taq DNA Polymerase (50×).
 - TITANIUM™ Taq PCR Buffer (10×).
5. All Purpose Hi-Lo DNA Marker (Bionexus), stored at room temperature.
 6. Gel, Reliant® Gel System, precast agarose gel (2% Seakem Gold, TBE, Cambrex), stored at room temperature (see Note ³).
 7. Gel Loading Buffer, 2× (Sigma-Aldrich), stored at room temperature.
 8. Cooling chambers, single and double gold block (Diversified Biotech).
 9. Thermal cyclers (pre-PCR lab and main lab).

2.1.3. Nsp Restriction Enzyme Digestion, Ligation, and PCR

1. Nsp I (10 U/μl), NE Buffer 2 (10×), and BSA (100×; 10 mg/ml) from NEB, stored at −20°C.
2. Adaptor, Nsp I (50 μM), included in kit 1 and stored at −20°C.

2.1.4. PCR Product Pooling, Purification, and Quantitation

1. 75% Ethanol (ACS grade ethanol diluted to 75% using AccuGENE water), kept at room temperature.
2. Elution Buffer (Buffer EB, Qiagen), kept at room temperature.
3. Magnetic Beads (Agencourt), kept at 4°C.
4. Deep-Well Storage Plate, 2.4 ml (pooling plate) (E&K Scientific).
5. Plate holders (USA Scientific).
6. Jitterbug Microplate Incubator Shaker.
7. Vacuum Manifold, MultiScreenHTS (Millipore).
8. Plate, 2 ml, 96-Well Format Filterplate (PES 0.45 μm), Hydrophillic, Long Drip Director (E&K Scientific).
9. Elution catch plate, 96-well, conical bottom.
10. Spectrophotometer, high-throughput microplate spectrophotometer or NanoDrop.

2.1.5. Fragmentation and Labeling

1. Fragmentation Buffer (10×) and Fragmentation Reagent (DNase I), included in kit 1, and stored at −20°C.
2. DNA labeling Reagent (30 mM), Terminal Deoxynucleotidyl Transferase (TdT; 30 U/μl) and Terminal Deoxynucleotidyl Transferase Buffer (TdT Buffer; 5×), included in kit 1 and stored at −20°C.
3. 4% NuSieve 3:1 Plus gel (Cambrex).

2.1.6. Target Hybridization

1. Denhardt's Solution (50×) (Sigma-Aldrich), stored at −20°C.
2. DMSO (100%), stored at room temperature in dark area. Toxic, handle with care.

³The gel contains ethidium bromide and should be placed in biohazard box after use.

3. EDTA (0.5 M), stored at room temperature.
4. Herring Sperm DNA (HSDNA; 10 mg/ml, Promega), stored at -20°C .
5. Human Cot-1 DNA (1 mg/ml) (Invitrogen), stored at -20°C .
6. 12 $\times$ MES Stock solution (1 L): 70.4 g of MES hydrate (Sigma-Aldrich), 193.3 g MES sodium salt (Sigma-Aldrich) dissolved in AccuGENE water, pH 6.5–6.7, filter-sterilized, and stored at 4°C .
7. Tetramethylammonium Chloride (TMACl; 5 M, Sigma-Aldrich), stored at room temperature. Toxic, handle with care.
8. Tween-20, 10%, stored at room temperature.
9. Oligo Control Reagent (OCR), 0100, included in kit 1 and stored at -20°C .
10. Genome-Wide Human SNP Array 6.0 (Affymetrix), stored at 4°C .
11. GeneChip Hybridization Oven 640 (Affymetrix).
12. Tough-Spots (1/2 and 3/8 in.).

2.1.7. Washing, Staining, and Scanning Arrays

1. 20 $\times$ SSPE (3 M NaCl, 0.2 M NaH_2PO_4 , 0.02 M EDTA; Cambrex).
2. Wash A (Nonstringent Wash Buffer): for 1 L, mix 300 ml of 20 $\times$ SSPE, 1.0 ml 10% Tween-20 and 699 ml of water, filtered through a 0.2- μm filter and stored at room temperature.
3. Wash B (Stringent Wash Buffer): for 1 L, mix 30 ml of 20 $\times$ SSPE, 1.0 ml of 10% Tween-20, and 969 ml of water, filtered through a 0.2- μm filter and stored at room temperature.
4. Stain Buffer: Add 360 μl of 20 $\times$ SSPE, 3.96 μl of Tween-20 (3%), and 24 μl of Denhardt's Solution (50 $\times$) to 800.04 μl of H_2O to make 1,188 μl of stain solution. Prepare the solution right before use.
5. SAPE stain solution: Remove Streptavidin Phycoerythrin (SAPE) from 4°C and tap the tube to mix well. Add 6 μl of 1 mg/ml SAPE to 594 μl of Stain buffer. Make fresh solution right before use.
6. Anti-streptavidin antibody (0.5 mg/ml): resuspend 0.5 mg antibody (goat, biotinylated from Vector laboratories) in 1 ml water and store at 4°C .
7. Array Holding Buffer (1 $\times$): Mix 8.3 ml of 12 $\times$ MES Stock Buffer, 18.5 ml of 5 M NaCl (RNase-free and DNase-free), 0.1 ml of 10% Tween-20, and 73.1 ml of water. Store at $2-8^{\circ}\text{C}$ and shield from light.
8. Distilled water.
9. Affymetrix GeneChip Fluidics Station 450.
10. Affymetrix GeneChip Scanner 3000 7G.

2.2. Global Gene Expression Analysis

2.2.1. Preparation of Total RNA

1. Purified total RNA sample.
2. Spectrophotometer (NanoDrop UV-vis Spectrophotometer).
3. Bioanalyzer (Agilent Technologies).

2.2.2. First-Strand cDNA Synthesis

1. First-strand Enzyme mix, included in GeneChip 3' In Vitro Transcription Express Kit (Kit 3, Affymetrix), and stored at -20°C .
2. First-strand Buffer mix (Kit 3, -20°C).
3. Poly-A Control stock and Poly-A Control Dilution Buffer (Kit 3, -20°C).
4. Thermal Cycler with heated lid (capable of holding 0.2-ml tubes for reaction incubation).
5. DNase and RNase-free water (Kit 3, -20°C).

2.2.3. Second-Strand cDNA Synthesis

1. Second-Strand Buffer mix (Kit 3, -20°C).
2. Second-Strand Enzyme mix (Kit 3, -20°C).

2.2.4. In Vitro Transcription to Synthesize Biotin-Labeled aRNA

1. IVT Biotin label (Kit 3, -20°C).
2. IVT Enzyme Mix (Kit 3, -20°C).
3. IVT Labeling Buffer (Kit 3, -20°C).

2.2.5. aRNA Purification

1. RNA binding beads (Kit 3, 4°C).
2. Magnetic Stand for 96-well plates (Ambion).
3. aRNA binding buffer concentrate (Kit 3, room temperature).
4. aRNA wash solution concentrate (Kit 3, room temperature).
5. 100% Ethanol, ACS reagent grade.
6. aRNA elution buffer (Kit 3, room temperature).
7. Orbital shaker for 96-well plates (or Jitterbug Microplate Incubator Shaker).

2.2.6. Evaluation and Fragmentation of aRNA

1. RNA 6000 Nano chip kit (Agilent Technologies).
2. $5\times$ Array Fragmentation Buffer (Kit 3, room temperature).

2.2.7. Target Hybridization

1. Control oligo B2 (3 nM, Kit 3, -20°C).
2. $20\times$ Hybridization Controls (Kit 3, -20°C).
3. $2\times$ Hybridization Mix (GeneChip Hybridization, Wash and Stain Kit, Affymetrix, Kit 4, kept at 4°C).
4. Prehybridization mix (Kit 4, 4°C).
5. DMSO (Kit 4, room temperature).
6. Nuclease-free water (Kit 4, 4°C).
7. GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix), stored at 4°C .

8. GeneChip Hybridization Oven 640 (Affymetrix).

2.2.8. Washing, Staining and Scanning Arrays

1. Stain Cocktail 1 (Kit 4, stored at 4°C).
2. Stain Cocktail 2 (Kit 4, 4°C).
3. Array Holding Buffer (Kit 4, 4°C).
4. Wash Buffer A (Kit 4, 4°C).
5. Wash Buffer B (Kit 4, 4°C).
6. GeneChip Fluidics Station 450.
7. Tough-Spots, label dots.
8. Affymetrix GeneChip Scanner 3000 7G.

3. Methods

3.1. Genome-Wide Association Study

GWAS use high-throughput genotyping technologies to assay at least 100,000 SNPs representing nearly 12 million unique human SNPs and relate them to clinical conditions and measurable traits (22). The method described here is based on the whole-genome sampling analysis (WGSa) for large-scale genotyping of complex DNA (23). The procedure uses restriction digestion to fractionate the genome, followed by PCR amplification of a selected subset of the genome, resulting reduction in genome complexity. The assay includes restriction digestion, ligation of adaptor, one primer PCR amplification in selected size range, fragmentation and labeling prior to hybridization to the oligo-nucleotide array.

The Affymetrix Genome-Wide Human SNP 6.0 is the sixth-generation product in genotyping SNPs based on the WGSa assay. A single array interrogates 906,600 SNPs by combining the Sty I and Nsp I PCR fractions and through reducing the absolute number of features associated with each SNP on the array. This array also contains more than 945,000 probes for the detection of copy number variations (CNVs) in the genome.

The following protocol is for processing 48 genomic DNA samples using the Affymetrix Genome-Wide Human SNP 6.0 Array and the recommended workflow is shown in Fig. 1. However, the actual workflow can be determined empirically with each possible stopping point presented in *italics*. To minimize possible cross-contamination, it is highly recommended to handle genomic DNA, perform restriction enzyme digestions and ligation, and set up PCR reactions in a separate room (pre-PCR room) from the rest of the procedures (main lab).

3.1.1. Genomic DNA Preparation

1. Dilute high quality genomic DNA from human peripheral blood or tissue to 50 ng/ μ l using reduced EDTA TE buffer (see Note ⁴).
2. Aliquot 5 μ l of each DNA to the corresponding wells of two 96-well plates (first four rows). Use Reference Genomic DNA 103 as positive control and water as negative control.

⁴DNA must be double-stranded and free of PCR inhibitors. We isolate human genomic DNA from white blood cells using the Puregene™ DNA isolation kit (Gentra Systems Inc.). Genomic DNA extracted with QIAamp DNA Blood Maxi kit (Qiagen) has also been tested successfully with the Assay.

3. Seal the plate and the *diluted genomic DNA can be stored at -20°C .*

3.1.2. Sty Restriction Enzyme Digestion, Ligation, and PCR—In this stage, the human genomic DNA will be first digested with Sty I restriction enzyme. The digested samples will be ligated to the Sty adaptor and finally the DNA will be amplified using the adaptor-specific primers. The PCR condition is optimized to preferentially amplify restriction fragments that are between 250 and 1,000 bp. PCR products will be confirmed by running 3 μl of the sample on a 2% TBE gel.

1. Take one genomic DNA plate out and keep it in a cooling chamber on ice (see Note ⁵).
2. Make Sty I digestion master mix in an Eppendorf tube (see Note ⁶):

	Per sample, μl	48 Samples (15% extra), μl
AccuGene Water	11.55	637.6
NE Buffer 3 (10 $\times$)	2	110.4
BSA (100 $\times$, 10 mg/ml)	0.2	11
Sty I (10 U/ μl)	1	55.2
Total	14.75	814.2

3. Aliquot 67 μl of Sty I digestion master mix to each tube of the 12-strip tubes.
4. Add 14.75 μl of Sty I digestion master mix to each DNA samples using a 12-channel P20 pipette. The total volume in each well is now 19.75 μl .
5. Seal the plate with adhesive film, mix thoroughly by vortexing the plate at high speed for 3 s (see Note ⁷) and centrifugation at $800 \times g$ for 30 s.
6. Incubate the plate on a thermal cycler (with preheated lid) and run the Digest program (see Note ⁸).

37°C	120 min
65°C	20 min
4°C	Hold

7. Remove the plate and centrifuge at $800 \times g$ for 30 s.
8. *If not proceeding directly to the next step, store the plate at -20°C .*
9. Make Sty I ligation master mix in an Eppendorf tube (see Note ⁹):

⁵Always place 96-well plate in a cooling chamber (single or double based on the number of plate handled in the same time) on ice.

⁶When making master mix, keep all reagents (except enzymes) in a cooling chamber on ice and mix each component thoroughly by vortexing for 3 s and pulse-spin before adding to the tube. Leave the enzyme at -20°C until ready to use. Enzymes must not be vortexed. They should be spun down immediately before use. Mix the master mix thoroughly by vortexing and centrifugation. Always transport enzymes (and other reagents) in a Benchtop StrataCooler (Stratagene).

⁷When vortex a 96-well plate, always seal the plate tightly with adhesive film and vortex the plate at three different places for 1 s each.

⁸Preset all the programs for convenience.

⁹Aliquot the T4 DNA ligase Buffer (10 $\times$) after thawing on ice for the first time to avoid multiple freeze-thaw cycle.

	Per sample, μl	48 Samples (25% extra), μl
T4 Ligase Buffer (10 $\times$)	2.5	150
Adaptor Sty I (50 μM)	0.75	45
T4 DNA Ligase (400 U/ μl)	2	120
Total	5.25	315

10. Aliquot 25 μl of Sty I ligation master mix to each tube of the 12-strip tubes.
11. Adding 5.25 μl of Sty I ligation master mix to each DNA samples using a 12-channel P20 pipette. The total volume in each well is now 25 μl .
12. Seal the plate with adhesive film, mix thoroughly by vortexing the plate at high speed for 3 s and centrifugation at $800 \times g$ for 30 s.
13. Incubate the plate on a thermal cycler (with preheated lid) and run the Ligation program:

16°C	180 min
70°C	20 min
4°C	Hold

14. Remove the plate and spin it down at $800 \times g$ for 30 s. Place the plate in a cooling chamber.
15. Pour ~10 ml of precooled AccuGENE water into a solution basin and add 75 μl of the water to each reaction using a 12-channel P200 pipette. The total volume in each well is now 100 μl .
16. Seal the plate with adhesive film, mix thoroughly by vortexing the plate at high speed for 3 s and centrifugation at $800 \times g$ for 30 s. Label the plate as StyLig.
17. *If not proceeding directly to the next step, store the plate at -20°C .*
18. Take three 96-well reaction plates and label them StyP1, StyP2, and StyP3. Place the plates in two double cooling chambers on ice.
19. Transfer 10 μl of each Sty ligated sample to the corresponding well of each PCR plate. Seal the plates with adhesive films and leave them in cooling chambers on ice. Keep the StyLig plate (70 μl left) at -20°C .
20. Prepare the Sty PCR master mix in a 50-ml Falcon tube (see Note ¹⁰):

	Each reaction, μl	48 Samples $\times$ 3 (15% extra), ml
AccuGENE water	39.5	6.541
TITANIUM buffer (10 $\times$)	10	1.656
GC-Melt (5 M)	20	3.312

¹⁰Do not add TITANIUM Taq DNA Polymerase until ready to aliquot the master mix to ligated samples.

	Each reaction, μl	48 Samples $\times$ 3 (15% extra), ml
dNTP (2.5 mM each)	14	2.318
PCR primer 002 (100 μM)	4.5	0.745
TITANIUM Taq DNA		
Polymerase (50 $\times$)	2	0.331
Total	90	14.903

21. Vortex the master mix at high speed three times, 1 s each time.
22. Pour the mix into a solution basin on ice.
23. Add 90 μl of Sty PCR master mix to each sample using a 12-channel P200 pipette (see Note ¹¹). The total volume in each well is 100 μl .
24. Seal the plates tightly with adhesive films. Mix thoroughly by vortexing and centrifugation at $800 \times g$ for 30 s.
25. Transfer the plates to the main lab and incubate in a thermal cycler with preheated lid. Run the PCR program. The program varies depending on the thermal cyclers used in the lab.

For the GeneAmp PCR System 9700 (silver or gold-plated silver blocks)

94°C	3 min	1 $\times$
94°C	30 s	} 30 $\times$
60°C	45 s	
68°C	15 s	
68°C	7 min	
4°C	<i>Hold (or overnight)</i>	
Volume	100 μl	

For the Bio-Rad thermal cyclers (MJ Tetrad PTC-225)

94°C	3 min	1 $\times$
94°C	30 s	} 30 $\times$
60°C	30 s	
68°C	15 s	
68°C	7 min	
4°C	<i>Hold (or overnight)</i>	
Volume	100 μl	

26. Remove the plates from the thermal cycler and spin down at $800 \times g$ for 20 s. Keep the plates in the coolers on ice or at 4°C.

¹¹Change the pipette tips after each dispense when adding the PCR master mix to avoid cross-contamination.

27. Take one fresh 96-well plate and aliquot 3 μ l of Gel Loading Dye to each well in row A through D using a 12-channel P20 pipette. Label the plate "Pgel".
28. Transfer the first row of the amplified DNA in StyP1 plate (3 μ l) to the corresponding wells in Pgel plate.
29. Transfer the second row of the amplified DNA in StyP1 plate (3 μ l) to the corresponding wells in Pgel plate.
30. Transfer the third row of the amplified DNA in StyP2 plate (3 μ l) to the corresponding wells in Pgel plate.
31. Transfer the fourth row of the amplified DNA in StyP3 plate (3 μ l) to the corresponding wells in Pgel plate.
32. Seal the Pgel plate. Vortex for 3 s and spin down at $800 \times g$ for 30 s.
33. Load the total volume from each well of the Pgel plate onto 2% TBE gels along with the all purpose Hi-Lo DNA marker (see Note ¹²).
34. Run the gels at 120 V for 40–60 min. Verify that the PCR products are between ~250 and 1,100 bp.
35. Seal the Sty PCR plates (StyP1, StyP2, and StyP3) and store them at -20°C .

3.1.3. Nsp Restriction Enzyme Digestion, Ligation, and PCR—During this stage, the human genomic DNA will be first digested with Nsp I restriction enzyme. The digested samples will be ligated to the Nsp adaptor and the DNA will be amplified using the adaptor-specific primers. The PCR condition is optimized to preferentially amplify restriction fragments between 250 and 1,000 bp. PCR products will be confirmed by running 3 μ l of the sample on a 2% TBE gel.

1. Take the second genomic DNA plate out and keep it in a cooling chamber on ice.
2. Make Nsp I digestion master mix in an Eppendorf tube:

	Per sample, μ l	48 Samples (15% extra), μ l
AccuGene Water	11.55	637.6
NE Buffer 2 (10 $\times$)	2	110.4
BSA (100 $\times$, 10 mg/ml)	0.2	11
Nsp I (10 U/ μ l)	1	55.2
Total	14.75	814.2

3. Aliquot 67 μ l of Nsp I digestion master mix to each tube of the 12-strip tubes.
4. Add 14.75 μ l of Nsp I digestions master mix to each DNA samples using a 12-channel P20 pipette. The total volume in each well is now 19.75 μ l.
5. Seal the plate with adhesive film, mix thoroughly by vortexing the plate for 3 s and centrifugation at $800 \times g$ for 30 s.
6. Incubate the plate on a thermal cycler (with preheated lid) and run the Digest program:

¹²PCR products can also be verified using E-gel 48 2% agarose gel (Invitrogen).

37°C	120 min
65°C	20 min
4°C	Hold

7. Remove the plate and spin it down at $800 \times g$ for 30 s.
8. *If not proceeding directly to the next step, store the plate at -20°C .*
9. Make Nsp I ligation master mix in an Eppendorf tube:

	Per sample, μl	48 Samples (25% extra), μl
T4 Ligase buffer (10 $\times$)	2.5	150
Adaptor Nsp I (50 μM)	0.75	45
T4 DNA Ligase (400 U/ μl)	2	120
Total	5.25	315

10. Aliquot 25 μl of Nsp I ligation master mix to each tube of the 12-strip tubes.
11. Add 5.25 μl of Nsp I ligation master mix to each DNA samples using a 12-channel P20 pipette. The total volume in each well is now 25 μl .
12. Seal the plate with adhesive film, mix thoroughly by vortexing the plate for 3 s and centrifugation at $800 \times g$ for 30 s.
13. Incubate the plate on a thermal cycler (with preheated lid) and run the Ligation program:

16°C	180 min
70°C	20 min
4°C	Hold

14. Remove the plate and spin it down at $800 \times g$ for 30 s. Place the plate in a cooling chamber.
15. Pour ~10 ml of precooled AccuGENE water into a solution basin and add 75 μl of the water to each reaction using a 12-channel P200 pipette. The total volume in each well is now 100 μl .
16. Seal the plate with adhesive film, mix thoroughly by vortexing the plate at high speed for 3 s and centrifugation at $800 \times g$ for 30 s. Label the plate as NspLig.
17. *If not proceeding directly to the next step, store the plate at -20°C .*
18. Take four 96-well reaction plates and label them NspP1, NspP2, NspP3, and NspP4. Place the plates in three double cooling chambers on ice.
19. Transfer 10 μl of each Nsp ligated sample to the corresponding well of each PCR plate. Seal the plates with adhesive films and leave them in cooling chambers on ice. Keep the NspLig plate (60 μl left) at -20°C .
20. Prepare the Nsp PCR master mix in a 50 ml Falcon tube:

	Each reaction, μl	48 Samples $\times$ 4 (15% extra), ml
AccuGENE water	39.5	8.722
TITANIUM buffer (10 $\times$)	10	2.208
GC-Melt (5 M)	20	4.416
dNTP (2.5 mM each)	14	3.091
PCR primer 002 (100 μM)	4.5	0.994
TITANIUM Taq DNA		
Polymerase (50 $\times$)	2	0.442
Total	90	19.873

21. Vortex the master mix at high speed three times, 1 s each time.
22. Pour the mix into a solution basin on ice.
23. Adding 90 μl of Nsp PCR master mix to each sample in four PCR plates using a 12-channel P200 pipette. The total volume in each well is 100 μl .
24. Seal the plates tightly with adhesive films. Mix thoroughly by vortexing and centrifugation at $800 \times g$ for 30 s.
25. Transfer the plates to the main lab and incubate in a thermal cycler with preheated lid. Run the PCR program.
26. Remove the plates from the thermal cycler and spin down at $800 \times g$ for 30 s. Keep them in the coolers on ice or at 4°C .
27. Take one fresh 96-well plate and aliquot 3 μl of Gel Loading Dye to each well in row A through D using a 12-channel P20 pipette. Label the plate "Pgel".
28. Transfer the first row of the amplified DNA in NspP1 plate (3 μl) to the corresponding wells in Pgel plate.
29. Transfer the second row of the amplified DNA in NspP2 plate (3 μl) to the corresponding wells in Pgel plate.
30. Transfer the third row of the amplified DNA in NspP3 plate (3 μl) to the corresponding wells in Pgel plate.
31. Transfer the fourth row of the amplified DNA in NspP4 plate (3 μl) to the corresponding wells in Pgel plate.
32. Seal the Pgel plate. Vortex for 3 s and spin down at $800 \times g$ for 30 s.
33. Load the total volume from each well of the Pgel plate onto 2% TBE gels.
34. Run the gels at 120 V for 40–60 min. Verify that the PCR products are between ~250 and 1,100 bp.
35. Seal the Nsp PCR plates (NspP1, NspP2, NspP3, and NspP4) and store them at -20°C .

3.1.4. PCR Product Pooling, Purification and Quantitation—During this stage, the Sty and Nsp PCR products will be pooled and purified using magnetic beads in a deep well 96-well plate. The PCR products will be washed and eluted using a filter plate connected to a vacuum manifold. Finally, the purified PCR products will be quantitated using NanoDrop or spectrophotometer plate reader.

1. Thaw all the frozen PCR products (three Sty PCR plates and four Nsp PCR plates) to room temperature on the bench top.
2. Vortex each plate at high speed for 3 s and spin down at $800 \times g$ for 30 s. Cut the seal between each row so that it can be removed one row at a time.
3. Place a deep-well pooling plate on the bench top.
4. Remove the seal to expose row A on each PCR plate. Transfer the reactions from row A of each plate to the corresponding wells of row A on the pooling plate.
5. Remove the seal from each PCR plate to expose the next row.
6. Repeat step 4 and finish transferring the reactions from rows B to D.
7. When finished, the total volume of each well of pooling plate should be about 700 μ l.
8. Take the magnetic beads stock from the refrigerator and mix well by shaking the bottle gently but thoroughly.
9. Pipette ~50 ml of magnetic beads to a solution basin and add 1 ml of the beads to each well of pooled PCR product using a 12 channel P1200 pipette.
10. Mix well by pipetting up and down five times. To avoid overflow, move pipette up and down during the pipetting. Visually check each well. Change tips for each row.
11. Cover the plate to avoid contamination and incubate at room temperature for 10 min.
12. Set up the vacuum apparatus and connect the gauge and the pressure controller to the Millipore vacuum manifold. Place a filter plate in the Millipore vacuum manifold.
13. Transfer each reaction of the pooling plate to the corresponding wells of the filter plate using a 12-channel P1200 pipette.
14. Seal the unused wells with an adhesive film and turn on the vacuum to 20–24 in Hg (do not exceed 24 in Hg). If the vacuum power does not increase, press the plate against the manifold to secure the seal.
15. Cover the plate and allow the liquid to completely dry (~40–60 min).
16. Inspect each well using a flashlight to confirm complete dry (see Note ¹³) and turn off the vacuum.
17. Immediately add 0.9 ml of 75% ethanol to each well using a 12-channel P1200 pipette and turn the vacuum back on to 20–24 in Hg.
18. After the ethanol level is down, add another 0.9 ml of 75% ethanol to each well.
19. Allow the liquid to completely dry (10–20 min). Check with a flashlight.
20. Turn off the vacuum and remove the manifold from the apparatus.
21. Tap off excess ethanol from the bottom of the manifold on a large Kimwipe.
22. Return the manifold to the vacuum apparatus, turn on the vacuum to 20–24 in Hg and allow the plate to dry for additional 8 min.

¹³The surface changes from shiny to matte when dry. There should be no standing liquid in any of the wells.

23. Turn off the vacuum, remove the filter plate, and blot the bottom of the plate with Kimwipes to remove excess liquid. Take off the adhesive film.
24. Attach the elution plate to the bottom of the filter plate using two strips of tape (see Note ¹⁴). This assembly is now referred to as the plate stack.
25. Pipette 3 ml of Buffer EB to a solution basin and add 55 μ l of Buffer EB to each well using a 12-channel P200 pipette (see Note ¹⁵).
26. Tap the plate stack to move all the Buffer EB to the bottom of the wells.
27. Tightly seal the filter plate with an adhesive film.
28. Place the plate stack on Jitterbug for 10 min at setting 5. Inspect each well to make sure that the beads are thoroughly resuspended. Remove the adhesive film.
29. Put the plate stack on the vacuum manifold. Seal the empty wells with an adhesive film.
30. Place the deep well collar over the plate stack.
31. Turn the vacuum on to 20–24 mmHg and ensure that a seal is formed between the collar and the base of the manifold (see Note ¹⁶).
32. Leave the plate stack on the manifold for 5–15 min. Closely monitor the filter plate to ensure that the wells are completely dry.
33. Remove the plate and seal it with an adhesive film. Spin the plate down for 5 min at $1400 \times g$ in room temperature.
34. *Transfer 45 μ l of the eluate to a fresh PCR plate for fragmentation. Seal the plate and keep it at -20°C .*
35. For the rest of the eluate, check OD of each sample using NanoDrop or:
 - Add 198 AccuGENE water to rows A to D in an optical plate.
 - Transfer 2 μ l of the eluate to the corresponding wells of the optical plate.
 - Set a 12-channel P200 pipette to 180 μ l and mix each sample by pipetting up and down three times.
 - Add 200 μ l of AccuGENE water to row E (blank control).
 - Measure the OD of each PCR product at 260, 280 and 320 nm using a microplate spectrophotometer.
36. A final PCR product concentration should be between 4.0 and 6.0 $\mu\text{g}/\mu\text{l}$ and the OD₂₆₀/OD₂₈₀ ratio should be between 1.8 and 2.0 (see Note ¹⁷).

3.1.5. Fragmentation and Labeling—During this stage, the purified PCR products will be fragmented using Fragmentation reagent (DNase I), and the results will be confirmed by running 1.5 μ l of each reaction on a 4% TBE gel. The fragmented samples are then end-labeled with biotin using the DNA labeling Reagent.

¹⁴Do not completely seal the plate with tape.

¹⁵Dispense as close to the beads as possible without touching them. If using Rainin tips, rest the ridge of the pipette tip on the lip of the plate when pipetting. Buffer EB should be applied directly on top of the beads.

¹⁶This process may take several tries before the pressure catches on.

¹⁷Do not proceed if the number falls outside of the range.

1. Take out the PCR plate with the purified DNA from -20°C and leave it on the cooling chamber on ice to thaw.
2. Thaw fragmentation buffer on ice (see Note ¹⁸).
3. Vortex and spin the Fragmentation buffer to mix thoroughly. Add 28 μl to each tube of the 12-strip tubes.
4. Add 5 μl of the Fragmentation Buffer to each of the purified DNA in the PCR plate. The total volume in each well is now 50 μl .
5. In an Eppendorf tube, add 309.6 μl of AccuGENE water (if the Fragmentation reagent is 2.5 U/ μl , see Note ¹⁹) and 36 μl of the Fragmentation Buffer.
6. Spin down the Fragmentation Reagent (do not vortex) and add 14.4 μl of the reagent to tube to make fragmentation master mix (see Note ²⁰).
7. Vortex the tube and spin down.
8. Add 28 μl of the fragmentation master mix to each well of the 12-strip tubes.
9. Add 5 μl of the fragmentation master mix to each of the four rows of the PCR plate. Change tips after each dispense. The total volume in each well is now 55 μl .
10. Seal the plate with an adhesive film. Vortex thoroughly but quickly. Spin down at 4°C at $800 \times g$ for 30 s.
11. Immediately load the plate onto the preheated block of the thermal cycler (preheat to 37°C for at least 10 min) and run the Fragment program:

37°C	35 min
95°C	15 min
4°C	Hold

12. While waiting, take out a standard non-PCR 96-well plate.
13. Add loading dye into a solution basin. Using a 12-channel P10 pipette, add 4 μl of the loading dye to the four rows of the 96-well plate.
14. Remove the PCR plate from the thermal cycler and spin down the plate at $800 \times g$ for 30 s.
15. Add 1.5 μl of each fragmented PCR product into 4 μl of loading dye in the standard 96-well plate.
16. Run on 4% TBE Gel with the BioNexus All purpose Hi-L ladder at 120 V for 30–60 min. Inspect the gel to verify that the average fragment size is <180 bp (see Note ²¹).
17. Thaw $5\times$ TdT Buffer and DNA Labeling Reagent on ice, and prepare Labeling master mix in an Eppendorf tube:

¹⁸Do not take out the fragmentation reagent. It is extremely temperature sensitive. Handle the tube by the cap only. Dilute immediately prior to use. Transport and hold in a -20°C cooler.

¹⁹The concentration of the Fragmentation Reagent varies. Check the tube label before diluting the reagent to 0.1 U/ μl .

²⁰The reagent is viscous. Pipette slowly and avoid excess solution on the outside of the pipette tip.

²¹The success of fragmentation is confirmed by the presence of a smear of less than 200 bp in size.

	Each sample, μl	48 Sample (15% extra), μl
TdT buffer (5 $\times$)	14	772.8
DNA Labeling Reagent (30 mM)	2	110.4
TdT enzyme (30 U/ μl)	3.5	193.2
Total	19.5	1,076.4

18. Aliquot 89 μl of the Labeling master mix to each well of 12-strip tubes.
19. Add 19.5 μl of Labeling master mix to each sample of the fragmented DNA. The total volume is now 73 μl .
20. Seal the plate with adhesive film. Vortex for 3 s and spin down at $800 \times g$ for 30 s.
21. Place the plate onto the preheated block of the thermal cycler (37°C, at least 10 min) and run the Label program:

37°C	4 h
95°C	15 min
4°C	Hold

22. Freeze the samples at -20°C .

3.1.6. Target Hybridization—During this stage, a Hybridization master mix will be added to each sample. After being denatured on a thermal cycler, each sample will be loaded onto a Genome-Wide Human SNP Array 6.0. The arrays are then placed into a hybridization oven and the samples are left to hybridize at 50°C for 16–18 h.

1. Turn on the thermal cycler to preheat the lid. Leave the block at room temperature.
2. Turn on the hybridization oven and set the temperature to 50°C with 60 rpm rotation. Preheat for at least 1 h.
3. Unwrap the arrays and place them on bench top, septa side up.
4. Thaw the label plate in cooling chamber on ice.
5. In a 50-ml centrifuge tube, make the Hybridization master mix (see Note ²²):

	One array, μl	48 Arrays (15% extra), μl
MES (12 $\times$; 1.25 M)	12	660
Denhardt's solution (50 $\times$)	13	715
EDTA (0.5 M)	3	165
HSDNA (10 mg/ml)	3	165
OCR, 0100	2	110

²²Add TMCAL first. Pipette DMSO to the solution to avoid touching the side wall of the tube. Hybridization master mix can be made ahead of time in a larger volume, aliquoted out 11 ml and stored for up to 1 week at -20°C .

	One array, μl	48 Arrays (15% extra), μl
H Cot-1 DNA (1 mg/ml)	3	165
Tween-20 (3%)	1	55
DMSO (100%)	13	715
TMACL (5 M)	140	7,700
Total	190	10,450

6. Vortex the Hybridization master mix until no precipitates (up to 5 min) and pulse-spin for 3 s.
7. Pour 11 ml of Hybridization master mix into a solution basin.
8. Using 12-channel P200 pipette, add 190 μl of the hybridization master mix to each sample on the label plate. The total volume in each well is now 263 μl .
9. Seal the plate tightly with adhesive film and vortex the center of the plate for at least 10 s.
10. Spin down the plate at $800 \times g$ for 30 s.
11. Cut the adhesive film between each row of samples. Do not remove the film.
12. Place the plate onto the thermal cycler and close the lid. Run the Hybridization program:

95°C	10 min
49°C	Hold

13. Turn on the computer and register all the samples in Affymetrix GeneChip Console (AGCC):
 - Click on Affymetrix Launcher and start the AGCC portal.
 - Select “Quick registration” and enter the number of samples.
 - Assign the samples to an existing project or name a new project.
 - Select the array type (SNP6.0) and put the array in a barcode reader to enter the barcode automatically.
 - Enter the sample file name for each array.
 - Turn off the computer.
14. During the denaturation, prepare the arrays: label each array and allow the arrays to warm to room temperature for 10–15 min. Attach two Tough-Spots to each array. Insert a 200 μl pipette tip into the upper right septum of each array for ventilation.
15. When the thermal cycler block reaches 49°C, slide back the lid to expose the first row of the samples only.
16. Remove the film from the first row.
17. Using P200 pipette, mix the denatured sample by pipetting up and down once, and remove 200 μl from the first well.

18. Immediately inject the sample into an Array from the lower left septum. Cover the septa with the Tough-Spots (1/2 in., see Note ²³).
19. Repeat the process on next sample. Put two arrays at a time into the oven and make sure that the oven is balanced (see Note ²⁴).
20. After the entire row is loaded, place a fresh adhesive film over the completed row.
21. Repeat steps 15–20 with the next row of samples.
22. Store the remaining samples and *any samples not yet hybridized in a tightly sealed plate at –20°C*.
23. Allow the Arrays to rotate at 50°C, 60 rpm for 16–18 h.

3.1.7. Washing, Staining, and Scanning Arrays—During this step, the hybridized arrays will be washed and stained with streptavidin–phycoerythrin conjugate (SAPE) using an automated protocol on the GeneChip Fluidics Station 450 which has been primed. Once stained, each array is filled with Array Holding Buffer before being scanned with the GeneChip Scanner 3700 7G.

1. Turn on the Fluidics Station. In the AGCC Launcher, click the AGCC Fluidics Control Icon.
2. Prime the Fluidics Station (see Note ²⁵):
 - Select protocol Prime_450 for each module.
 - Fill the intake buffer reservoir with Wash buffer A.
 - Fill the intake buffer reservoir B with Wash buffer B.
 - Empty the waste bottle and fill the water reservoir with deionized water.
 - Place three empty 1.5 ml tubes into the stain holder positions 1, 2, and 3.
 - Place the washblock lever into the engaged/closed position. Push the needle lever into the down position.
 - Run the Prime_450 maintenance protocol.
3. Remove the arrays from the oven. Take off the Tough Spots.
4. Insert a 200 µl pipette tip into the upper right septum of each array for ventilation.
5. Extract the hybridization cocktail from each array through lower left septum and transfer it to the corresponding well of the hybridization plate. Store on ice or at –20°C for long term storage.

²³Slightly cover the middle window to make it easy for later removal.

²⁴Do not allow loaded arrays to sit at room temperature for more than 1.5 min. Load no more than 32 Arrays in one hybridization oven at a time. Do not place arrays next to each other without spacing at least one section.

²⁵Priming ensures the lines of the fluidics station are filled with the appropriate buffers and should be done:

- When the fluidics station is first started.
- When wash solutions are changed.
- Before washing, if a shutdown has been performed.
- If the LCD window instructs to do so.

6. Fill each array completely with ~270 μ l of 1 $\times$ Array Holding Buffer (see Note ²⁶). Allow the arrays to equilibrate to room temperature. Pipetting back and forth may be needed to remove bubbles.
7. Start the AGCC Fluidics Control software.
8. Select the correct sample file name.
9. Select the protocol GenomeWideSNP6_450.
10. Start the protocol and follow the instructions in the LCD on the fluidics station.
11. Insert an array into the designated module of the fluidics station.
12. When “Load Vials 1–2–3” is shown on the LCD of the fluidics station, place the three vials into the sample holders 1, 2 and 3:
 - Place one vial (600 μ l) of SAPE stain solution in sample holder 1.
 - Place one vial (600 μ l) of anti-streptavidin biotinylated antibody stain solution in sample holder 2.
 - Place one vial (1 ml) of Array holding buffer in sample holder 3.
13. Press down on the needle lever to start the run. The dialog box at the workstation terminal and LCD of the fluidics station will display the status of the washing and staining steps.
14. When staining is finished, remove the three vials containing stain and replace with three empty Eppendorf tubes as prompted.
15. Remove the array from the fluidics station.
16. Check the array window to make sure that there is no bubbles or air pockets (see Note ²⁷).
17. If the array has no large bubble, it is ready for scanning. Arrays can be stored at 4°C in the dark for up to 24 h. Allow them to warm up to room temperature before scanning to avoid breakage.
18. After all the arrays have been stained, shut down the Fluidics station:
 - When the last array is removed, gently lift up the cartridge lever to engage the washblock. The instrument automatically performs a Cleanout procedure.
 - When REMOVE VIALS is shown in the LCD, remove the Eppendorf tubes from the sample holders.
 - Place the wash lines into a bottle filled with dH₂O.
 - Using AGCC, choose the Shutdown_450 protocol for all modules and run the protocol.
 - Turn off the Fluidics Station.
 - Place the wash lines in a different bottle of dH₂O than the one used for the shutdown protocol.

²⁶Arrays can be stored in the Array Holding Buffer at 4°C for up to 3 h before washing and staining.

²⁷If a bubble is present, do not return the array to array holder. Manually fill the array with Array Holding Buffer.

19. If necessary, clean the glass surface of the array with Kimwipe. On the back of the array, clean excess fluid around the septa.
20. Cover both septa with Tough Spots (3/8 in., see Note ²⁸).
21. Turn on the scanner for at least 10 min before use. Click the AGCC Scan Control icon in the Affymetric Launcher.
22. Insert an array into the scanner.
23. Follow AGCC instructions and begin the scan.

3.2. Global Gene Expression Analysis

This protocol is based on the linear RNA amplification using T7 in vitro transcription technology with as little as 50 ng of total RNA. Total RNA first undergoes reverse transcription to synthesize first-strand cDNA. This cDNA is then converted to a double-stranded cDNA which is used as template for in vitro transcription. In vitro transcription synthesizes biotin-labeled aRNA when biotin-labeled ribonucleotides are included in the reaction. After purification and fragmentation, biotin labeled aRNA is hybridized to a probe array. The hybridized probe array is stained with streptavidin–phycoerythrin conjugate and scanned by the GeneChip Scanner 3000 7G. The amount of light emitted at 570 nm is proportional to the bound target at each location on the probe array.

We use Human Genome U133 Plus 2.0 Array which provides comprehensive analysis of the relative expression level of more than 47,000 transcripts and variants, representing more than 38,500 well-characterized human genes.

This protocol is written for processing 16 samples using 100 ng of total RNA and the recommended workflow is shown in Fig. 2. Actual sample number, total RNA amount and stopping point (presented in *italics*) can be determined empirically.

3.2.1. Preparation of Total RNA—RNA quality is the most important factor affecting the outcome of the experiment. The purified total RNA (see Note ²⁹) is quantitated using a NanoDrop spectrophotometer. The ratio of A260–280 values should fall in the range of 1.9–2.1. It is highly recommended to check RNA integrity using the Agilent 2100 bioanalyzer with an RNA LabChip kit, following manufacturer's guide.

3.2.2. First-Strand cDNA Synthesis—During this step, total RNA is primed with engineered primers containing a T7 promoter sequence. The reaction synthesizes single-stranded cDNA containing a T7 promoter sequence using SuperScript reverse transcriptase. The poly-A RNA controls is diluted and added to the starting samples so that the whole processes can be monitored independently from the quality of the starting total RNA.

1. Prepare Poly-A control RNA (*Bacillus subtilis* genes *lys*, *phe*, *thr*, and *dap*) dilution:
 - Add 2 µl of the Poly-A Control stock to 38 µl of Poly-A Control Dilution Buffer to make the first dilution (1:20). Mix thoroughly.
 - Add 2 µl of the first dilution to 98 µl of Poly-A Control Dilution Buffer to make the second dilution (1:50). Mix thoroughly.

²⁸The Tough Spots used here are smaller than the previous ones so that they will not interfere with the scanning process.

²⁹Total RNA can be isolated from tissues or blood samples using Trizol Reagent (Invitrogen), RNeasy Mini kit (Qiagen), or PAXgene Blood RNA kit (Qiagen).

- Add 2 μ l of the second dilution to 98 μ l of Poly-A Control Dilution Buffer to make the third dilution (1:50). Mix thoroughly.
 - Add 2 μ l of the third dilution to 18 μ l of Poly-A Control Dilution Buffer to make the fourth dilution (1:10). Mix thoroughly.
2. Place the supplied PCR tubes on ice. Add 2 μ l of the fourth dilution and 100 ng of total RNA to the tubes (see Note ³⁰):

Total RNA sample (100 ng)	Variable
Diluted Poly-A RNA controls	2 μ l (fourth dilution)
Nuclease-free water	To 5 μ l

3. Thaw first-strand synthesis reagents on ice. Mix the reagents well by vortexing and pulse spin.
4. Make first-strand master mix in a nuclease-free microfuge tube on ice (see Note ³¹):

	Per sample, μ l	16 Samples (10% extra), μ l
First-Strand Buffer mix	4	70.4
First-Strand Enzyme mix	1	17.6
Total Volume	5	88

5. Mix well by gently vortexing and pulse-spin for 3 s.
6. Add 5 μ l first-strand master mix to each of the total RNA/Poly-A control mixture for a final volume of 10 μ l.
7. Mix thoroughly by gently vortexing. Spin the tubes down for 3 s.
8. Place the tubes on a thermal cycler and run the First-strand cDNA synthesis program (see Note ³²):

42°C	2 h
4°C	Hold

9. Centrifuge for 5 s to collect the samples at the bottom of the tubes. Place the tubes on ice and immediately proceed to next step.

3.2.3. Second-Strand cDNA Synthesis—During this step, single-stranded cDNA is converted to double-stranded cDNA, which will be used later as template for transcription.

³⁰Use 50–500 ng of total RNA. If necessary, concentrate the total RNA by vacuum centrifugation.

³¹First-Strand cDNA Synthesis mix includes T7-(N)₆ primers and SuperScript reverse transcriptase which can also be purchased separately (Affymetrix and Invitrogen).

³²Do not use water bath or heat block. Incubate all reactions in a thermal cycler. Preset all the programs for convenience.

The reaction uses DNA polymerase I and RNaseH to simultaneously synthesize second-strand cDNA and degrade the RNA.

1. Thaw second-strand synthesis reagents on ice. Mix the reagents well by vortexing and pulse spin.
2. Make second-strand master mix on ice:

	Per sample, μl	16 Samples (10% extra), μl
Nuclease-free water	13	228.8
Second-strand buffer mix	5	88
Second-strand enzyme mix	2	35.2
Total	20	352

3. Mix well by gently vortexing and pulse-spin for 3 s.
4. Add 20 μl of Second-strand master mix to each First-strand cDNA sample. Mix thoroughly by flicking the tubes 3–4 times. Centrifuge briefly to collect the reaction at the bottom of the tubes. The total volume is now 30 μl . Place the tubes on ice.
5. Precool the thermal cycler block to 16°C (see Note ³³). Place the tubes on the thermal cycler and run the second-strand cDNA synthesis program:

16°C	1 h
65°C	10 min
4°C	Hold

6. After the reaction, centrifuge briefly and place the tubes on ice.
7. Immediately proceed to Subheading 3.2.4 or store the samples at -20°C .

3.2.4. In Vitro Transcription (IVT) to Synthesize Biotin-Labeled aRNA—During this step, antisense biotin-labeled aRNA is synthesized and amplified by in vitro transcription of the second-strand cDNA template using T7 RNA polymerase and biotin-labeled ribonucleotides.

1. Thaw the IVT reagents on ice. Mix the reagents well by vortexing and pulse spin.
2. At room temperature, prepare IVT master mix in a nuclease-free tube:

	Per sample, μl	16 Samples (10% extra), μl
IVT Biotin Label	4	70.4
IVT Labeling Buffer	20	352

³³Subjecting the reaction to temperatures over 16°C will affect aRNA yield. Disable the heated lid or keep the lid off during the second-strand cDNA synthesis.

	Per sample, μl	16 Samples (10% extra), μl
IVT Enzyme Mix	6	105.6
Total	30	528

3. Mix well by gently vortexing and pulse-spin for 3 s.
4. Add 30 μl of the IVT master mix to each double-stranded cDNA sample. Mix thoroughly by gently vortexing. Centrifuge briefly to collect the reaction at the bottom of the tubes. The total volume in each tube is now 60 μl .
5. Place the tubes on a thermal cycler and run the IVT program (see Note ³⁴)

40°C	16 h
4°C	Hold

6. Place the tubes on ice briefly and proceed to the aRNA purification or *immediately freeze at -20°C for overnight storage.*

3.2.5. aRNA Purification—In this procedure, aRNA will be mixed with and bind to RNA Binding Beads. Enzymes, salt, inorganic phosphate, and unincorporated NTPs will be removed. After wash, purified aRNA will be eluted in aRNA Elution solution to improve its stability.

1. Preheat the bottle of aRNA Elution Solution to 50–60°C for at least 10 min.
2. Make sure to add 100% ethanol to the bottle of aRNA Wash Solution.
3. Vortex the RNA Binding beads and prepare aRNA Binding Mix in a nuclease-free tube:

	Per sample, μl	16 Samples (10% extra), μl
RNA Binding beads	10	176
aRNA Binding Buffer concentrate	50	880
Total	60	1,056

4. Add 60 μl of aRNA Binding Mix to each sample and transfer each sample to a well of a U-Bottom plate. Mix well by pipetting up and down several times.
5. Using an 8-channel P200 pipette, add 120 μl of 100% ethanol to each sample. Mix by pipetting up and down several times.
6. Put the plate on an orbital shaker and shake gently (300–500 rpm, see Note ³⁵) for at least 2 min.

³⁴Optimal IVT incubation time is sample-type and RNA-amount dependent, and should be determined empirically. Overnight IVT reaction time has been shown to maximize the labeled cRNA yield. If a shorter incubation time (4 h) is desired, 1 μl (200 U) of T7 RNA polymerase (Ambion) can be added to each reaction.

³⁵Setting 4 on the Barnstead/Lab-line Titer Plate Shaker (Model # 4625) or setting 1 on Boekel “Jitterbug” Plate Shaker (Model # 130000).

7. Move the plate to a magnetic stand and capture the magnetic beads for about 5 min (see Note ³⁶).
8. Carefully aspirate the supernatant without disturbing the beads.
9. Remove the plate from the magnetic stand. Add 100 µl of aRNA Wash Solution to each sample and shake the plate at moderate speed (700–900 rpm, see Note ³⁷) for 1 min.
10. Capture the beads on the magnetic stand for 5 min.
11. Carefully aspirate the supernatant without disturbing the beads.
12. Repeat steps 9 – 11 to do another wash.
13. Move the plate to the shaker and shake the plate vigorously (1,000–1,200 rpm, see Note ³⁸) for 1 min to evaporate residual ethanol from the beads.
14. Add 50 µl of preheated aRNA Elution Solution to each sample.
15. Vigorously shake (1,000–1,200 rpm) for 3 min (see Note ³⁹).
16. Move the plate to the magnetic stand and capture the beads for 5 min.
17. Carefully transfer the supernatant to nuclease-free PCR tubes.
18. Place the tubes on ice to proceed with quantitation and fragmentation.
Alternatively, store aRNA at –20 or –80°C (see Note ⁴⁰).

3.2.6. Evaluation and Fragmentation of aRNA—During this step, the yield of the purified aRNA will be assessed by UV absorbance and the sizes of the unfragmented aRNA will be assessed using an Agilent bioanalyzer. aRNA will then be fragmented by incubating in Fragmentation buffer at 94°C.

1. Measure 1.5 µl of the aRNA sample on NanoDrop Spectrophotometer using aRNA Elution Solution as blank (see Note ⁴¹).
2. Determine the yield of aRNA (see Note ⁴²).
3. Analyzing aRNA size distribution using an Agilent bioanalyzer and RNA 6000 Nano chip kit following the manufacturer's instructions. Unfragmented aRNA should distribute between 250 and 5,500 nt with most between 600 and 1,200 nt.
4. Add 15 µg of each aRNA to fresh nuclease-free tubes. Add Nuclease-free water to each tube so that the sample volume is 32 µl.
5. Add 8 µl of 5× Array Fragmentation Buffer to each tube. Mixing thoroughly by vortexing and pulse spin. The total volume of each reaction is now 40 µl.
6. Place the tubes on the thermal cycler and run the Fragmentation program:

³⁶When capture is complete, the mixture becomes transparent and the RNA Binding Beads will form pellets.

³⁷Setting 7 on the Barnstead/Lab-line Titer Plate Shaker or setting 4 on Boekel Jitterbug Plate Shaker.

³⁸Setting 10 on the Barnstead/Lab-line Titer Plate Shaker or setting 7 on Boekel Jitterbug Plate Shaker.

³⁹Check to make sure that the RNA Binding Beads are fully dispersed.

⁴⁰Purified aRNA can be stored at –20°C for up to 1 year, but the number of freeze–thaw cycles should be minimized to maintain aRNA integrity.

⁴¹We use NanoDrop for its convenience. No dilutions or cuvettes are needed. aRNA concentration can also be determined by using traditional spectrophotometer (reading at 260 nm) or by using RiboGreen (Invitrogen). Follow the manufacturer's instructions for using RiboGreen.

⁴²aRNA yield from equal amounts of total RNA from different sources may vary considerably. If necessary, concentrate the aRNA by vacuum centrifugation using medium or low heating setting.

94°C	35 min
4°C	Hold

- Place the tubes on ice (see Note ⁴³). Use fragmented aRNA immediately or *store at -20°C*.

3.2.7. Target Hybridization—During this step, the fragmented aRNA will be diluted with a Hybridization mix. After being denatured on a thermal cycler, each sample will be loaded onto a Human Genome U133 Plus 2.0 Array. The arrays are then placed into a hybridization oven and the samples are left to hybridize at 45°C for 16 h.

- Thaw Control oligo B2 and 20× Hybridization controls on ice. Heat the 20× Hybridization controls to 65°C for 5 min to completely resuspend the aRNA. Take other hybridization reagents from the refrigerator.
- Equilibrate the arrays to room temperature immediately before use. Label the arrays with corresponding sample names.
- Turn on the hybridization oven and set the temperature to 45°C (see Note ⁴⁴). Preheat for at least 1 h.
- Prepare Hybridization master mix in a nuclease-free bottle provided in Kit 4 (see Note ⁴⁵):

	Per sample, µl	16 Samples (10% extra), µl
Control oligo B2 (3 nM)	4.2	73.9
20× Hybridization controls (<i>bioB</i> , <i>bioC</i> , <i>bioD</i> , and <i>cre</i>)	12.5	220
2× Hybridization mix	125	2,200
DMSO	25	440
Nuclease-free water	50	880
Total	216.7	3,813.9

- Vortex the tube and centrifuge at 1000 × *g* for 30 s.
- Aliquot 216.7 µl Hybridization master mix to each of 16 nuclease-free tubes. Add 33.3 µl (12.5 µg) of each fragmented aRNA to the tubes. Mix thoroughly. The total volume of each reaction is now 250 µl.
- Turn on the computer and register all the samples in AGCC (project name, array name, barcode, and sample name) as detailed in Subheading 3.1 Turn off the computer.
- Load the hybridization cocktails (fragmented aRNA + Hybridization master mix) to the arrays:

⁴³The success of the fragmentation can be confirmed by assessing 300 ng of fragmented aRNA using an Agilent bioanalyzer and RNA 6000 Nano chip kit. The reaction should produce a distribution of 35–200 nt aRNA fragment with a peak at approximately 100–120 nt.

⁴⁴The hybridization temperature is 45°C instead of 50°C which is used in GWAS SNP array.

⁴⁵This is the standard format. For midi, mini or micro array format, please refer to manufacturer's guide. Make sure that DMSO is completely thawed when first used. Store DMSO at room temperature after the first use.

- Heat the hybridization cocktails to 99°C for 5 min in a heat block.
 - While waiting, insert a 200 µl pipette tip into the upper right septum of each array. Wet the arrays with 200 µl of prehybridization mix by filling it through the lower left septum.
 - Incubate the arrays in the hybridization oven at 45°C for 10 min. Rotate at 60 rpm.
 - Transfer the hybridization cocktails from 99°C heat block to 45°C heat block. Keep at 45°C for 5 min.
 - Spin the hybridization cocktails at maximum speed for 10 min to precipitate any insoluble material.
 - Remove the arrays from the hybridization oven. Vent the array with a clean P200 pipette tip and extract the prehybridization mix from the array with a micropipettor. Refill the array with 200 µl of the clarified hybridization cocktail.
 - Cover the septa with the Tough-Spots (1/2 in.).
9. Put the arrays into the oven and make sure that the oven is balanced.
 10. Allow the arrays to rotate at 45°C, 60 rpm for 16 h.

3.2.8. Washing, Staining, and Scanning Arrays—In this procedure, hybridized arrays are washed and stained with streptavidin–phycoerythrin conjugate using an automated protocol on the GeneChip Fluidics Station 450, followed by scanning on GeneChip Scanner 3000 7G.

1. Turn on the Fluidics Station and the computer.
2. Prime the station as in Subheading 3.1. Use Wash Buffer A and B in kit 4.
3. Remove the arrays from the hybridization oven. Vent the array by inserting a clean pipette tip into top right septum and extract the hybridization cocktail with a micropipettor through the remaining septum (see Note ⁴⁶).
4. Refill the arrays with 250 µl of Wash Buffer A (see Note ⁴⁷).
5. Take Stain Cocktail 1, Stain Cocktail 2, and Array Holding Buffer from kit 4 at 4°C.
6. Gently tap the bottles to mix well.
7. Add 600 µl of Stain Cocktail 1 into a 1.5-ml amber microfuge tube (see Note ⁴⁸).
8. Add 600 µl of Stain Cocktail 2 into a 1.5-ml microfuge tube.
9. Add 800 µl of Array Holding Buffer into a 1.5-ml microfuge tube.
10. Spin down all vials to get rid of air bubbles.
11. Start the AGCC Fluidics Control Software.
12. Select the correct sample name. Select the protocol FS 450_0001.

⁴⁶The used hybridization cocktails can be rehybridized on another array if necessary. Store the cocktail at –20°C.

⁴⁷The arrays in Wash Buffer A can be store at 4°C for up to 3 h before proceeding to washing and staining. Equilibrate the arrays to room temperature before washing and staining.

⁴⁸Stain Cocktail 1 is light-sensitive.

13. Start the protocol and follow the instructions in the LCD on the fluidics station.
14. Insert an array into the designated module of the fluidics station.
15. When “Load Vials 1–2–3” is shown on the LCD of the fluidics station, place the three vials into the sample holders 1, 2, and 3:
 - Place one vial containing 600 µl Stain Cocktail 1 in sample holder 1.
 - Place one vial containing 600 µl Stain Cocktail 2 in sample holder 2.
 - Place one vial containing 800 µl Array holding buffer in sample holder 3.
16. Press down on the needle lever to start the run. The dialog box at the workstation terminal and LCD of the fluidics station will display the status of the washing and staining steps.
17. When staining is finished, removed the three vials containing stain and replace with three empty 1.5 ml tubes as prompted.
18. Remove the array from the fluidics stain.
19. Check the array window to make sure that there is no bubbles or air pockets.
20. If the array has no large bubble, it is ready for scanning. Arrays can be stored at 4°C in the dark for up to 24 h. Allow them warm up to room temperature before scanning.
21. After all the arrays have been stained, shut down the Fluidics station as detailed in Subheading 3.1.
22. If necessary, clean the glass surface of the array with Kimwipe. On the back of the array, clean excess fluid around the septa.
23. Cover both septa with Tough Spots (3/8 in.).
24. Turn on the scanner for 30 min before use. Click the AGCC Scan Control icon in the Affymetric Launcher.
25. Insert an array into the scanner.
26. Follow AGCC instructions and begin the scan.

4. Clinical Protocols for Genetic Pain Research

4.1. Limitations and Pitfalls of Genetic Association Studies

The molecular methods described above are powerful means for identifying common variants influencing complex traits, genome wide screening to identifying SNPs that may be associated with a trait, or evaluating changes in the expression of these genes that may differ over time, across diseases and symptoms or contribute to interindividual variability. Candidate gene association studies (CGAS) rely heavily on choosing genes on the basis of already-revealed biological processes. Owing to this limitation, CGAS can only identify genetic risk factors in which the pathophysiology is relatively well understood. However, most published CGAS for pain and analgesic responses show only weak associations and often fail to replicate in subsequent studies (11). This suggests that either the rationale for choosing the genes of interest or the particular SNPs has been biased by this prior knowledge of the pathophysiology, that the total number of comparisons made in performing the statistical analyses was not taken into account and resulted in a false positive association or other pitfalls inherent in association studies have biased the outcome (for discussion, see ref. 11).

The availability of high-throughput genotyping technology, as described in Subheading 3, allows whole-genome screening in GWAS. Currently available platforms allow the genotyping of more than one million SNPs, rapidly assessing a very large percentage of the genetic differences between individual samples. The sample sizes required are increased by the large number of hypotheses that are tested and create additional pressure to loosen criteria for subject enrollment but possibly confound the genetic association due to population stratification and enrollment of subjects who may not be fully comparable to others in the same phenotypic classification. Even if multistage association studies are done to narrow down the candidate genomic regions, a few thousand cases and controls may be needed, but collecting several thousand patients diagnosed with a single painful condition to result in a homogeneous population is very challenging.

Despite these powerful techniques, the availability of high-quality human reference genome and the ability to carefully characterize a clinical pain phenotype with quantitative sensory testing, there are still many potential pitfalls. Even for highly heritable phenotypes such as height, estimated as approximately 80%, the contribution of genetic variability for phenotypic expression of the characteristic suggests that phenotypic expression stems from multiple genetic and environmental factors. Pain has a lower degree of heritability and is likely a more complex phenomenon than height, making it unlikely that the influence of a single SNP on a pain phenotype may be extremely small. Statistical approaches vary highly across published studies (11) based on the nature of the outcome measured and reporting of corrections for multiple-test corrections. Population stratification can also produce false associations since the pattern of genetic variations and the rating of pain may not be uniform between ethnic populations. The potential for multiple factors that may produce spurious results and misleading conclusions in genetic studies of pain in humans suggests that only studies with careful designs followed by replication from independent groups and supportive laboratory data can be considered as well validated.

4.1.1. Recommendations for Reporting Clinical Genetic Pain Research

Findings—Population stratification, lack of adequate sample size, inappropriate choice of genetic variations, inevitable type 1 and 2 errors, and problematic statistical analysis along with publication bias are recognized problems in genetics research. The heterogeneity of pain and the interpretation of pain models also add complexity to study design and interpretation. Figure 3 presents a schematic approach to reporting possible sources of error in conducting genetic studies of pain modified from the CONSORT guidelines for reporting clinical trials data. Special attention to the steps unique to genetic studies that should be considered in evaluating genetic findings in clinical pain research includes the following:

- Size of the sample relative to the expected genetic effect.
- Definition of the phenotype and genotype (SNP versus haplotype).
- Stratification, Hardy–Weinberg equilibrium, and other sample population characteristics.
- Plausibility of the genes under study affecting the phenotype.
- Replication studies.
- Statistical issues associated with multiple comparisons.

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Fig. 1.
Recommended workflow for Affymetrix Genome-Wide Human SNP 6.0 Array.

Fig. 2.
Recommended workflow for GeneChip Expression Analysis.

Fig. 3.
CONSORT-like guideline for reporting genetic association data. Reproduced from ref. 11
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