Getting the Most from Your DNA Analysis from Purification to Downstream Analysis

Eric B. Vincent, Ph.D.

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Presentation Outline

Genomic Analysis

• Purification
• Quantitation
• Qualification
• Analysis Methods

• Key considerations at each step
• Ways to overcome major challenges
DNA Analysis
Each Step Affects the Quality of the Final Data

- Purify
- Quantify
- PCR Amplify
- qPCR
- Whole Genome Sequencing
- Microarray
- Cloning
## Downstream Assays

**Different Tools Based on Experimental Needs**

<table>
<thead>
<tr>
<th>PCR</th>
<th>qPCR</th>
<th>Sequencing</th>
<th>Arrays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloning</td>
<td>SNP Genotyping</td>
<td>SNP detection</td>
<td>SNP Detection</td>
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<tr>
<td>Sanger Sequencing:</td>
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<td>Comparative Genomic Hybridization</td>
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<tr>
<td>• Confirmation</td>
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<td>• Identification</td>
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<td>• Targeted</td>
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<tr>
<td>regions/application</td>
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<tr>
<td>Methylation Analysis</td>
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<tr>
<td>STR Analysis (ID)</td>
<td>Mutation detection</td>
<td>De Novo Genome Sequencing</td>
<td>Loss of Heterozygosity</td>
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<tr>
<td>Methylation Analysis</td>
<td>Pathogen identification</td>
<td>Microbiome Research</td>
<td>Pathogen Identification</td>
</tr>
<tr>
<td>Genotype Confirmation (KOs)</td>
<td></td>
<td>Chromatin Immoprecipitation</td>
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<tr>
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<td></td>
<td>Methylation Analysis</td>
<td></td>
</tr>
</tbody>
</table>
**Downstream Applications**  
**Importance of Input DNA Characteristics**

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<tr>
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<th>PCR</th>
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<th>Sequencing</th>
<th>Arrays</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Quantity of DNA</strong></td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Integrity of DNA</strong></td>
<td>+/-</td>
<td>-</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Depending on amplicon</td>
<td>Typically small amplicons</td>
<td>More important with longer read technologies, but many providers assume large fragments</td>
<td>Typically small fragments, but providers expect minimum fragment sizes</td>
</tr>
<tr>
<td><strong>Lack of Inhibitors</strong></td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Accurate Quantitation</strong></td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>
Purification - Setting the Stage for Downstream Success
DNA Purification Technologies
All Provide Advantages Depending on Specific Needs
# Purification, Manual
Low Investment and Scalability are Attractive

Manual columns and scalable solution-based purification are attractive low-throughput options for standard or difficult samples

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Reasons to Consider Other Options</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low initial investment vs. automation</td>
<td>Greater throughput desired</td>
</tr>
<tr>
<td>Flexibility in sample processing</td>
<td>Time constraints</td>
</tr>
<tr>
<td>Lower price per prep</td>
<td>Error reduction</td>
</tr>
<tr>
<td>Minimal set up time</td>
<td></td>
</tr>
</tbody>
</table>

Many sample types supported: Blood, Tissue, FFPE, Plant...
Small, dedicated purification instruments allow individuals to automate purification and increase productivity

<table>
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<tr>
<th>Advantages</th>
<th>Reasons to Consider Other Options</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimal initial investment</td>
<td>Not enough throughput to justify</td>
</tr>
<tr>
<td>Frees time for other activities</td>
<td>Even greater throughput desired</td>
</tr>
<tr>
<td>Fewer purification errors</td>
<td>Input sample volume incompatibility</td>
</tr>
<tr>
<td>Increases sample throughput</td>
<td></td>
</tr>
</tbody>
</table>

Maxwell® 16: 5 minute setup – 30-45 minutes to extract 1-16 samples
# DNA Purification, Manual 96 Well

Vacuum Purification Increases Sample Throughput

## Advantages

<table>
<thead>
<tr>
<th>Advantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low initial investment</td>
</tr>
<tr>
<td>High sample throughput</td>
</tr>
<tr>
<td>Offers performance equal to spin columns</td>
</tr>
</tbody>
</table>

## Reasons to Consider Other Options

<table>
<thead>
<tr>
<th>Reason</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 well processing can be tedious</td>
</tr>
<tr>
<td>Desire to reduce errors</td>
</tr>
<tr>
<td>Staff time has become rate limiting</td>
</tr>
<tr>
<td>Greater throughput desired</td>
</tr>
<tr>
<td>Input sample volume incompatibility</td>
</tr>
</tbody>
</table>

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The Wizard® SV 96 Genomic system can isolate gDNA from many sample types in less than 60 minutes.
**DNA Purification, Automated 96 Well**

*Increases Laboratory Throughput and Lowers Costs*

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Reasons to Consider Other Options</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increases laboratory productivity</td>
<td>High initial cost</td>
</tr>
<tr>
<td>Aids in sample tracking</td>
<td>Not enough throughput to justify</td>
</tr>
<tr>
<td>Increases consistency of results</td>
<td></td>
</tr>
<tr>
<td>Can automate many activities</td>
<td></td>
</tr>
</tbody>
</table>
Automated Purification Reduces Errors by Removing Repetitive Manual Processes

- Process small samples in 96 well plates rapidly – including sample transfer!

The ReliaPrep™ SV 96 HT gDNA Isolation System can process 96 samples with no operator intervention

No Cross-contamination is Observed

- Consistent Purification Regardless of Input Sample Volume
  - R² = 0.9903

- No male DNA detected in female samples
DNA Purification
Yield, Integrity & Purity are Critical to Success

Key Challenges

• Purifying sufficient DNA from:
  • Small samples
  • Difficult samples
  • Degraded samples

• DNA integrity

• Isolating pure DNA
  • No enzyme inhibitors to affect downstream applications
Genomic DNA purified from a single 10µm mouse brain section using either the ReliaPrep™ FFPE gDNA Miniprep System or competitor kit.

More Sensitive qPCR Assays

Amplification of Large Targets

10µl purified gDNA used as template in multiplex PCR (GoTaq® Hot Start). Fragments ranging from 100-400bp detected.

Maxwell® 16: The same level of performance is available in an easy-to-use format.
Quantitation - A Simple but Critical Step in DNA Analysis
DNA Quantitation and Quality Determination
Important but Often Underappreciated

➢ UV Absorbance
  • Spectrophotometer
  • NanoDrop®/NanoVue™

➢ Fluorescent Dye-based Quantitation
  • Plate Reader
  • Hand-held Instruments

➢ Gel Electrophoresis
  • Agarose
  • Pulse Field Agarose Electrophoresis

➢ Agilent 2100 Bioanalyzer

What information does each method give us......

What information does each method not give us......

What are the advantages and disadvantages of each method.....
UV Absorbance Spectroscopy
Rapid Assessment of Concentration and Purity

Spectrophotometers
(various manufacturers)

NanoDrop®
(Thermo Scientific)

NanoVue™
(GE Healthcare)

Measure nucleic acid:
✓ Concentration
✓ Purity
### UV Absorbance

Each Wavelength Measures Different Parameters

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>Measurement</th>
</tr>
</thead>
</table>
| 260nm      | Amount of nucleic acid present in a sample  
A260nm of 1.0 = 50μg/ml for dsDNA  
40μg/ml for RNA  
33μg/ml for ssDNA |
| 280nm      | Amount of protein present in a sample |
| 230nm      | Amount of other contaminants present in a sample |
| 320nm      | Amount of light scattering components present in a sample; used for background subtraction |
## Purity Determination by UV Absorbance

Guanidine Thiocyanate Absorbs at 230nm

<table>
<thead>
<tr>
<th>Purity Measurement</th>
<th>Acceptable Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{260}/A_{280}$</td>
<td>Generally 1.8 – 2.2</td>
</tr>
<tr>
<td>$A_{260}/A_{230}$</td>
<td>Generally &gt;1.7</td>
</tr>
</tbody>
</table>

**EDTA, carbohydrates and phenol also absorb at 230nm**

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**Pure DNA**

$A_{260}/A_{280} = 1.8$

$A_{260}/A_{230} = 2.2$

**DNA with 0.01% Guanidine ITC**

$A_{260}/A_{280} = 1.88$

$A_{260}/A_{230} = 1.16$

UV Absorbance - NanoDrop®
Measures Small Volumes with Good Sensitivity

Features:

✓ Measures the absorbance of small volume samples
✓ 0.5 – 2μl of sample required
✓ 190nm – 840nm wavelength range
✓ Wide detection range
  • 2ng/μl minimum (DNA)
  • 15,000ng/μl maximum (DNA)
✓ Measurements in less than 30 seconds
✓ No other reagents or accessories required
Choose Read Type: DNA

Read:
- Water
- Blank
- Sample

Output:
- Spectra
- Table
  - Yields
  - Purity ratios
  - ABS readings
UV Absorbance – Disadvantages
Detects all Nucleic Acid with no specificity

- Lack of Specificity
  - Cannot distinguish between dsDNA, RNA or ssDNA
  - RNA contamination cannot be determined
- Overestimation of DNA concentration due to contaminants
  - Many contaminants absorb at 260nm
- No information on integrity
  - Nucleotides and small DNA fragments still contribute to the 260nm reading
Fluorescent Dye Quantification
The Most Sensitive Approach

- Dye binds dsDNA – the resulting conformation shift produces in fluorescence when excited
- Fluorescence is directly proportional to the amount of dsDNA in the sample
- Higher signal = more DNA present
- Unbound dye does not fluoresce
- Low background increases sensitivity

Easy Protocol: Add, Mix, Measure

Incubate at room temp for 5 minutes

Excitation @ 504nm

Emits @ 531nm

Unbound dye
Fluorescent Dye Assay Formats
Assay in Tubes or Plates to Match Your Workflow

1. Dilute Dye to make working solution (1:200)
2. Prepare standards and unknown samples
3. Add dye to samples and standards – incubate
4. Measure fluorescence

The QuantiFluor™ dsDNA System can be used in single tube as well as 96 well plate formats
Fluorescent Dye-based Quantification
Wide Dynamic Range

Quantitate from 50pg/ml to 1µg/ml with QuantiFluor™ dsDNA System

The QuantiFluor™ dsDNA System Provides sensitive quantitation of dsDNA
Fluorescent Dye Quantitation – Disadvantages
No Information on Purity or Integrity

✓ Must create high or low concentration standards
✓ Does not detect ssDNA; which can be problematic for some assays
✓ No information on purity
  • Separate dye-based quantification systems are available for RNA and protein
✓ No information on integrity
✓ Fluorescent dyes are potentially hazardous
Gel Electrophoresis
Most Common Analysis Method

• Agarose and sometimes acrylamide gel electrophoresis

• Nucleic acid fragments separated by size

• Fragments are visualized by staining with a fluorescent dye that binds DNA, commonly excited by a UV light
  - Ethidium Bromide, SYBR® Green, SYBR® Gold and Diamond™ Nucleic Acid Dyes

• Amount of DNA can be estimated
  - Estimate relative intensity of fluorescence compared to a standard(s)
  - Gel densitometry
Gel Electrophoresis
Assess Integrity and RNA/oligo Contamination

Qualitative Analysis

• DNA integrity (minimal smearing)
  • Intact gDNA may be:
    • Viewed as a slow migrating band in standard agarose gels
    • Separated on pulsed field gel apparatus designed to separate very large DNA molecules
  • RNA contamination can sometimes be observed

- RNase

+ RNase
DNA Purification Methods Affect Final gDNA Size Assessed by Gel Electrophoresis

Similar gDNA Size Estimation Using a 0.8% Agarose Gel

<table>
<thead>
<tr>
<th></th>
<th>ReliaPrep™ gDNA Purification</th>
<th>Precipitation Method</th>
<th>Silica Column Method</th>
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</thead>
<tbody>
<tr>
<td>23.1kb</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>9.4kb</td>
<td></td>
<td></td>
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<tr>
<td>6.6kb</td>
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<td>4.4kb</td>
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<td>2.3kb</td>
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<tr>
<td>2.0kb</td>
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0.8% Agarose Gel

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<td>4.4kb</td>
<td>2.3kb</td>
</tr>
<tr>
<td>2.0kb</td>
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</table>

Expands to

CHEF Gel Reveals Greater Size Range

<table>
<thead>
<tr>
<th>ReliaPrep™ Large Volume</th>
<th>Precipitation-based method</th>
<th>Silica Column method</th>
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<tbody>
<tr>
<td>350kb</td>
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</table>
Gel Electrophoresis
Drawbacks and Alternatives to Ethidium Bromide

• Low cost, but requires significant amount of handling and time
• Typically requires a few nanograms of DNA
  • Minimum detectable mass varies by stain
• SYBR® Green II, SYBR® Gold and Diamond™ dyes are more sensitive than ethidium bromide
• SYBR® Green II, SYBR® Gold, and Diamond™ dyes can be viewed as safer alternatives to ethidium bromide, a known carcinogen
Agilent 2100 Bioanalyzer
Microfluidics to Analyze 1μl of Sample

• Uses microfluidics to analyze DNA, RNA, protein, and cells using sample specific chips
• Samples are combined with a fluorescent dye and added into wells in the chip
• Size separated by electrophoresis

• The samples are detected by fluorescence, and electropherograms & gel-like images are provided for sizing and quantification
• 1μl of sample is required
• 11-12 samples are run per chip
• Analysis is complete in 30-40 minutes
Agilent 2100 Bioanalyzer DNA Analysis
High Sensitivity Fragment Analysis

DNA Analysis Kits

- High Sensitivity DNA Kit - dsDNA fragments from 50 - 7000bp (5-500 pg/µl)
- DNA 12000 - dsDNA fragments from 100 to 12000bp (0.5-50 ng/µl)
- DNA 7500 Kit - dsDNA fragments from 100 to 7500bp (0.5-50 ng/µl)
- DNA 1000 Kit - dsDNA fragments from 25 to 1000bp (0.5-50 ng/µl)

Information provided:

- Sizes of DNA fragments
- Concentration of each fragment
- Gel-like image
Agilent 2100 Bioanalyzer – Disadvantages
No Assessment of Purity and Limited Size Range

- Intact gDNA will not be detected (may clog the chip)
- No Information on purity
  - Does not detect contaminants
- High costs of instruments and consumables
**Summary: Each Method Provides Different Information**

<table>
<thead>
<tr>
<th>Data</th>
<th>UV Spec</th>
<th>Fluorescent Dye</th>
<th>Agilent Bioanalyzer</th>
<th>Gel Electrophoresis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>Y</td>
<td>Y</td>
<td>Y - but only smaller sizes</td>
<td>Qualitative</td>
</tr>
<tr>
<td>Integrity</td>
<td>N</td>
<td>N</td>
<td>N but can view</td>
<td>N but can view</td>
</tr>
<tr>
<td>Specificity</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Presence of Inhibitors</td>
<td>Y</td>
<td>N</td>
<td>Most expensive</td>
<td>Low cost</td>
</tr>
<tr>
<td>Cost</td>
<td>Instrument can be $$$ but no reagent cost</td>
<td>Instrument and reagent</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
DNA Analysis Methods
Choose a Method that Best Matches Your Needs
DNA Analysis Methods
Multiple Options Available

Key questions to ask yourself:

• How many targets do I want to measure?
• Am I interested in whole genome analysis?
• Am I interested in identifying new mutations?
• Do I want information on target size, integrity?
• Do I want highly quantitative data or is qualitative assessment OK?

PCR Amplify

qPCR

Whole Genome Sequencing

Microarray

Cloning
Southern Blot Provides Information on Fragment Size & Copy Number

Advantages:
- Provides fragment size and can provide copy number
- May help identifying possible pseudogenes

Disadvantages:
- Requires a lot of sample
- Time consuming
- Throughput
**PCR**
A Basic Technique Enabling Many Types of Analysis

- Widely used laboratory tool
  - More advanced methods are available
- Rapid and cost effective analysis and preparation of nucleic acids

**Genotyping** – mice and transgenic animals

**Cloning** - elements for genetic study (creation of cassettes for reporter gene assays)

**Mutation Detection** – AMP-FLP

http://www.promega.com/resources/articles/pubhub/enotes/gotaq-hot-start-polymerase-for-mouse-genotyping/
Taq is Taq, Right?
Enzyme Choice & Rxn Conditions Make a Difference!

GoTaq® Long PCR Master Mix

Improved Results with a Specialized Enzyme
Standard Taq (L) vs. GoTaq®(r) DNA polymerase with different targets

Amplifying Targets up to 30kb

Improvement by Optimizing Conditions
• Annealing temperature
• Mg+ concentration
• PCR additives...
PCR
Rapid but Requires Gel Analysis

Advantages:
• Rapid analysis
• Inexpensive
• Relatively sensitive

Disadvantages:
• Time consuming
• Throughput
• Requires gel analysis which is labor intensive

Sensitive Amplification of a gDNA Target
End-Point vs. Real-Time PCR
Low vs. Highly Quantitative Analysis

- End-Point PCR is detected in the variable plateau phase.
- Real-Time PCR can detect product in exponential phase allowing more precise quantitation.
Robust enzyme and buffer systems in GoTaq® qPCR Systems enables sensitive detection of downstream targets

**GoTaq® qPCR Mastermix can provide earlier C_q values compared to other qPCR mixes**

**GoTaq® qPCR master mix allows sensitive detection of targets down to one copy**
**Fluorescence**

Brighter Signal Produces More Sensitive Assays

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**Graph 1:**

- **Delta Rn vs. Cycle**
  - GoTaq® Master Mix
  - Company A
  - Higher Fluorescence
  - Earlier Cq's

**Graph 2:**

- **Comparison of Normalized Fluorescence (dRn)**
  - Brighter Fluorescence
  - Promega, B, Q, IP*, IE, R, A

---

GoTaq® qPCR shows significantly higher fluorescence and earlier Cq at all template levels.

GAPDH from human gDNA was amplified using GoTaq® qPCR and Master Mix A using 100ng, 10ng, 0.1ng and 0.01ng of template on the same plate.

GoTaq® qPCR shows brighter fluorescent signal than leading competitors. GAPDH was amplified from 1ng of human genomic using GoTaq® qPCR Master Mix and six other commercially available dye-based master mixes using manufacturer protocols.
Advantages:
• Sensitivity
• Highly quantitative measurements
• Some multiplexing is possible
• Wide Dynamic range
• Can be often used with degraded samples

Disadvantages:
• Limited number of targets assessed per reaction
Microarrays
“Multiplex Fragment Detection”

- DNA samples are labeled, probes on arrays are unlabeled
- One-color microarrays are also performed (Affymetrix arrays)
- Compare one array to another to determine differences between samples
Microarray Applications
Well Suited for Structural Studies

- Comparative genomic hybridization (CGH): analysis of copy number changes
- Gene ID: can be used to rapidly ID unknown organisms
- Chromatin Immunoprecipitation: ID protein binding sites
- SNP detection: Can screen samples for millions of sites at a time
Microarrays
Good Genomic Coverage but Low Sample Throughput

Advantages:
• Can provide good coverage
• Can be quite flexible (if you can make your own arrays)
• Reasonable data analysis

Disadvantages:
• Requires a lot of sample
• CGH cannot detect changes in small regions
• Time consuming
• Cost – some technologies are expensive
• Low throughput

aCGH Profile of the IMR32 Neuroblastoma Cell Line
Next-Gen Sequencing
A New “Gold Standard”?

Many methods, many applications, many considerations

Several technologies exist and additional technologies are in development for large scale sequencing of genomes with many applications:

• SNP discovery
• Mutation discovery/detection
• Identity
• Methylation

Ultimately, sequencing data is dependent on the integrity and quality of the starting material
While the DNA is fragmented in the first step in preparation, protocols assume certain degrees of intactness.

Inhibitors of downstream enzymes can effect the production of libraries – best to use a purification kit that provides pure, intact DNA.
Whole Genome Sequencing
The Most Data per Experiment but at a High Cost

Advantages
- Provides the most information in a single experiment
- Absolute quantitation of sequence changes
- Absolute quantitation of previously undiscovered genes

Disadvantages
- Expensive
- Requires extensive bioinformatic support = $$$
- Also requires large data storage capabilities
Summary

- ReliaPrep™ FFPE gDNA Miniprep System
- Wizard® Genomic DNA Miniprep System
- Maxwell 16® Instrument and Kits

Purify

- GoTaq® DNA Polymerase
- GoTaq® Hot Start DNA Polymerase
- GoTaq® Long PCR Master Mix

Quantify

- QuantiFluor™ dsDNA System

PCR Amplify

- GoTaq® qPCR Master Mixes
- GoTaq qPCR Probe qPCR Master Mixes

Whole Genome Sequencing

Cloning

- pGEM T-Easy Vector
- Wizard® SV Gel and PCR Clean up System

Microarray