Welcome!

Multiplex PCR for genotyping: technology overview and applications

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Part 1: Multiplex PCR for genotyping: technology overview and applications
August 13, 9:30 a.m. EDT, 2:30 p.m. GMT, 3:30 p.m. CEST

Part 2: Critical factors for successful end-point multiplex PCR

Part 3: Genotyping workflow: challenges and streamlined solutions with QIAxcel Systems
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Agenda

- Genotyping – analyzing genetic difference
- Sample handling and quality
- Multiplex PCR in genotyping
- Application Data
- Recommendations
- Summary and Q&A
Genotypes: DNA and genetic variations

Genotyping is a process of determining genetic variations among individuals in a species.
Genotyping – analyzing genetic differences

Sample to Insight

Genotyping applications
- Biomarker discovery
- Pharmacogenomics
- Genetic predisposition studies
- Disease association studies
- Population genetics
- Pathogen analysis
- Animal and plant breeding
- Species or individual identification
- Identity testing of animals, plants, GMOs
- Analysis of relationship

Genotyping

- Translocation
- InDels
- Microsatellite
- SNP
- CNV
- Transgene detection
- Microbes
- Samples
- Assays
- Insight
Genotyping – samples & assays to insights

**Samples**
- Sample collection & stabilization
- Genomic DNA purification
- Genomic DNA storage
- Whole genome amplification

**Assays**
- Genotyping analysis
- Detection

**Insights**
Challenges in genotyping: qualitative aspects

- Sample quantity and quality
  - Degraded nucleic acids – collection, storage, process
  - Limited specimens

- Sample purity (genetic heterogeneity)
  - Genomic mutations may be at low-frequency
  - Tumor cells may be a minor fraction of total sample
  - Multiple sub-clones of cancer may be present in one tumor sample

- Assay sensitivity, specificity and cost

- Fragmented workflow
The quality of the DNA sample material is critical for achieving accurate and reliable results in genotyping assays.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Starting material</th>
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</thead>
<tbody>
<tr>
<td>Human</td>
<td>Blood (whole, serum, plasma)</td>
</tr>
<tr>
<td>Animal</td>
<td>Other body fluids (urine, CSF, saliva, milk)</td>
</tr>
<tr>
<td>Plant</td>
<td>Tissues (fresh, frozen, FFPE)</td>
</tr>
<tr>
<td>Bacteria</td>
<td>Cells</td>
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<tr>
<td>Yeast</td>
<td>Stool, bone, tooth and other tough samples</td>
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<tr>
<td>Fungus</td>
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<tr>
<td>Viruses</td>
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</table>
Sample handing challenges: integrity

The integrity of the DNA sample material is critical

- Sample collection & stabilization
- Genomic DNA purification
- Genomic DNA storage
- Whole genome amplification

Ensure sample integrity and reduce variability in the process of collecting, transporting, and storage of samples, especially for blood samples.

May need simultaneous collection, fixation, stabilization of molecular content and preservation of morphology.
Sample handing challenges: DNA quality

Genomic DNA purification is a crucial step in all genotyping workflows

- Minimize the variables associated with sample collection and DNA purification
- Ensure high reproducibility
- Purity is critical, should be free of RNA contamination
Sample handing challenges: DNA quality

Genomic DNA purification is a crucial step in all genotyping workflows

Minimize the variables associated with sample collection and DNA purification

Ensure high reproducibility

Purity is critical, should be free of RNA contamination

DNA sample stability is extremely important
Sample handing and challenges: limited samples

Limited or insufficient DNA quantity can be a challenge in genotyping applications

- Sample collection & stabilization
- Genomic DNA purification
- Genomic DNA storage
- Whole genome amplification

Directly affect detection sensitivity and results

Traditional methods (such as PCR) may produce nonspecific amplification artifacts and give incomplete coverage of loci.

Amplified DNA may have high mutation rate
Pre-analytical considerations

- Sample collection and stabilization
- Genomic DNA extraction and isolation (yield and quality)
- Efficient removal/reduction of RNA
- Integrate all components into a seamless workflow

Sample technology for the pre-analytical phase
Sample collection and isolation: blood samples

PAXgene Blood Systems

Sample collection & stabilization → Genomic DNA purification

- Blood collection stability:
  - RT (14 days)
  - 2-4°C (28 days)
  - -80°C (long term storage)

- DNA purification:
  - Up to 200kb in size
  - Yield: 150-500 ug

Efficient, standardized system for blood collection, transport, and DNA purification in one system

- PAXgene Blood DNA Tubes
- PAXgene Blood DNA Kit

Consistent quality: sample to sample

<table>
<thead>
<tr>
<th>Donor</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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</table>

8 donors, 2 replicates, 300 ng gDNA per lane

No detectable RNA contamination

<table>
<thead>
<tr>
<th>Donor</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAXgene DNA</td>
<td></td>
<td></td>
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<td></td>
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<td>Commercial salting out</td>
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</tbody>
</table>

Formamide gel, ~10 µg DNA per lane

Air dry pellet and resuspend DNA

Pure DNA
Sample isolation: genomic DNA from whole blood

QIAamp DNA Blood Kit

- **Maxi Kit**: 300–600 µg DNA from 3–10 ml blood
- **Midi Kit**: 20–60 µg DNA from 0.3–2 ml blood
- **Mini Kit**: 4–12 µg DNA from 50–200 µl blood

- Fresh and frozen whole blood or buffy coat
- Plasma or serum
- Bone marrow
- Lymphocytes
- Platelets
- Body fluids

4 steps: lyse, bind, wash, and elute

- Isolate genomic DNA, mitochondrial DNA, viral DNA
- DNA sized from 200 bp to 50 kb
- Removed contaminants and inhibitors

Paternity testing by RFLP analysis

|   | A | 1 | 2 | 3 | 4 | M | 1 | 2 | 3 | 4 | M |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
|   |   |   |   |   |   |   |   |   |   |   |   |   |

QIAamp Spin Column Procedure

- Sample
- Lyse
- Bind
- Wash
- Elute
- Pure DNA

- in microcentrifuges
- on vacuum manifolds
**QIAamp Circulating Nucleic Acid Kit**

4 steps: lyse, bind, wash, and elute

- Concentrate and purify free-circulating DNA and RNA from plasma or serum.
- No organic extraction or ethanol precipitation
- Removed contaminants and inhibitors
- For human plasma, serum and urine

**Improved recovery of fragmented DNA**

![Graph showing improved recovery of fragmented DNA](image)
Sample collection and isolation: tissues

PAXgene Tissue System

- A new standard in tissue fixation, stabilization, and purification.
- DNA without chemical modifications, can be used directly for downstream analysis
- Preserve tissue morphology
- High-quality DNA from tissues

<table>
<thead>
<tr>
<th>Intestine</th>
<th>Heart</th>
<th>Lung</th>
<th>Prostate</th>
<th>Brain</th>
</tr>
</thead>
</table>

-21 kb
Genotyping animal blood and tissues, and tough species

**DNeasy Blood & Tissue Kit**

- Animal blood and tissues
- Cells, yeast, bacteria, or viruses
- Tough samples, such as bones, teeth
- Can be automated on the QIAcube

Tissue disruption is critical

Protocols are available: including user developed protocols
Genotyping FFPE samples

QIAamp DNA FFPE Tissue Kit

Retrieve usable analytes from FFPE samples
- Deparaffinization – find the optimal condition
- Purify genomic DNA
- May need whole genome amplification

Download: unlocking your FFPE archive
Genotyping limited samples: whole genome amplification

QIAGEN’s REPLI-g technology for genotyping
REPLI-g FFPE Kit
REPLI-g Single Cell WGA Kit

For fast and accurate genomic analysis

Problem

- Incomplete or biased genome amplification with missing or underestimated sequence information

Solutions

- REPLI-g technology
  - Optimized Multiple Displacement Amplification (MDA) process, an innovative lysis process and an optimized form of Phi 29 DNA polymerase

Results

- High yield of high-molecular-weight DNA
- High fidelity amplification — minimal error rate
- Amplification of both DNA and RNA from a single sample for direct analysis with high accuracy and minimal amplification bias.

Read a white paper: “Genomic analysis of individual cells by NGS and real-time PCR”
Easy-to-use WGA – for FFPE sample

The REPLI-g FFPE Kit: for direct whole genome amplification of DNA from

- Novel DNA processing reaction that prepares and ligates fragmented DNA
- Long DNA strands can be created
- Standard yields of up to 40 µg, and scalable
Easy-to-use WGA – for single cells or limited samples

The REPLI-g WGA Single Cell Kit: for whole genome amplification (WGA) from samples as small as a single cell (1 tumor cell).

- Complete genome coverage
- Minimize sequence bias, allowing for discovery of rare variants
- Consistent yields of up to 40 μg from 1–1000 cells (average product length >10 kb)

Unbiased amplification of DNA

Analysis of 267 loci spread out over different chromosomes across the entire human genome

Learn about the technology: http://www.qiagen.com/us/resources/technologies/wga
Read a white paper: “Genomic analysis of individual cells by NGS and real-time PCR”
Sample isolation and storage: critical factors

Performance depends on your sample input!

<table>
<thead>
<tr>
<th>Sample material</th>
<th>Manual</th>
<th>Automated systems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td></td>
<td>□</td>
</tr>
<tr>
<td>Buffy coat</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Plasma and serum</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Cell-free body fluids</td>
<td>□</td>
<td>□</td>
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<tr>
<td>Urine</td>
<td>□</td>
<td>□</td>
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<tr>
<td>Saliva</td>
<td>□</td>
<td>□</td>
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<tr>
<td>Bone marrow</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Dried blood spots</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Tissue</td>
<td>□</td>
<td>□</td>
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<tr>
<td>Fixed tissue</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Bones and tooth</td>
<td>□</td>
<td>□</td>
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<tr>
<td>Cultured cells</td>
<td>□</td>
<td>□</td>
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<tr>
<td>Swabs</td>
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<td>□</td>
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<tr>
<td>Forensic casework samples</td>
<td>□</td>
<td>□</td>
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<tr>
<td>Hair</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Stool</td>
<td>□</td>
<td>□</td>
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<tr>
<td>Insects</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Mouse tails</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Plant materials</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

Download app: [gDNA digital selection wheel](#)
Genotyping – samples & assays to insights

**Samples**

- Sample collection & stabilization
- Genomic DNA purification
- Genomic DNA storage
- Whole genome amplification

**Assays**

- Genotyping analysis
- Detection

**Insights**
Genotyping: assay technology

Multiplex PCR in genotyping

- More than one target in a sample is amplified and quantified in a single tube
- Enable cost savings and preservation of limited sample
- Simple, highly specific, sensitive, and amenable to full automation
- Successfully applied in:
  - Amplification of multiple DNA regions for SNP analysis
  - Amplification and analysis of microsatellites
  - Amplification and enrichment of targeted gene for NGS
  - Typing and detection of bacteria and viruses
  - Typing and analysis of transgenic organisms
  - Investigating relationship and paternity patterns
Genotyping technology: multiplex PCR

QIAGEN Multiplex PCR Kit
QIAGEN Multiplex PCR Plus Kit
Type-it Microsatellite PCR Kit
Type-it Mutation Detect PCR Kit

NGS – Target enrichment
GeneRead Gene Panels
Application data: multiplex SNP analysis

• Reported a novel multiplex T-ARMS-PCR method for genotyping six SNPs in a single reaction.

• **QIAGEN multiplex PCR kit** was used for multiplex PCR amplification

• **QIAxcel** was used for separating and detecting the multiplex PCR products

• Of the 186 samples, up to 11 amplicons can be produced in one single PCR and separated by capillary electrophoresis.

• The multiplex T-ARMS-PCR genotyping results were consistent with sequencing results

• Reliable, fast, and easy to perform

Application data: multiplex microsatellite or SSR

Reported a multiplex SSR genotyping method to analyze the populations and diversity of fungal plant pathogen

Easy-to-use, accurate, repeatable, economical, and fast

Automated and high-throughput (96-well plate)

**Sample**
- Fresh sporidia from cultures on malt-yeast agar MYA

**DNA extraction**
- Qiagen DNA ‘Biorobot 3000’ extraction robot
- Qiagen DNeasy Plant Maxi Kit

**Detection**
- Type-it microsatellite kit

Type-it Microsatellite PCR Kit
- For reliable microsatellite analysis
- Successful and specific coamplification of all fragments
- Optimized protocol for fast and reliable results without further optimization needed

Developed a method by detecting eDNA (environmental DNA) to survey rare and invasive stream species, Rocky Mountain tailed frog and Idaho giant salamander.

Tested 2 DNA extraction kits to isolate eDNA from water samples and 5 PCR protocols.

Results: The DNeasy Blood & Tissue kit and the Multiplex PCR kit improved species detection of both species in water filter samples, while the other kits failed.

Conclusion: Developed an efficient and cost-effective protocol for detecting targeted DNA sequences for two secretive amphibian species, Rocky Mountain tailed frog and Idaho giant salamander, even the populations are at low densities.
Genotyping: QIAGEN multiplex end-point PCR solutions

- No optimization is needed
- Ensure high specificity, sensitivity
- Ease of use and fast

<table>
<thead>
<tr>
<th>Application</th>
<th>Genotyping</th>
<th>Any multiplex PCR application</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Starting material</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gDNA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Application                      |           |                               |
| STR and microsatellite analysis  | ![ ]      | ![ ]                         |
| Mutation detection               | ![ ]      | ![ ]                         |
| SNP loci amplification           | ![ ]      | ![ ]                         |
| Single-cell PCR                  | ![ ]      | ![ ]                         |
| Proamplification                 | ![ ]      | ![ ]                         |

| PCR performance                  |           |                               |
| Hot-start (1.5 min activation)   | ![ ]      | ![ ]                         |
| Hot-start (5 min activation)     | ![ ]      | ![ ]                         |
| Q-Solution (PCR enhancer for difficult templates) | ![ ] | ![ ] |
| Maximum sensitivity and specificity | ![ ]      | ![ ]                         |
| Fidelity                         | ![ ]      | ![ ]                         |
| Amplification-product size       | ≤0.5 kb   | ≤3.5 kb                       |

| Ease of use and convenience      |           |                               |
| PCR buffer with tracking dyes (CoroLoad) | ![ ] | ![ ] |
| Room-temperature setup           | ![ ]      | ![ ]                         |
| Fridge storage                   | ![ ]*     | ![ ]*                        |
| Master mix format, including nucleotides | ![ ] | ![ ] |

* Up to 2 months.
† Up to 6 months.

Why QIAGEN multiplex PCR kits offer instant success? Attend the Part 2 webinar: **Critical factors for successful end-point multiplex PCR**
Genotyping technology: multiplex PCR

QIAGEN Multiplex PCR kits offers instant success and guarantee efficient amplification and high PCR sensitivity and specificity

Multiplex PCR Kit
QIAGEN Multiplex PCR Plus Kit
Type-it Microsatellite PCR Kit
Type-it Mutation Detect PCR Kit

NGS – Target enrichment
GeneRead Gene Panels
Genotyping technology: multiple options for NGS

Why use targeted enrichment?
Choose the sequencing level that is appropriate for your application

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Whole Genome Sequencing (WGS)</th>
<th>Whole Exome Sequencing (WES)</th>
<th>Targeted DNA Sequencing (TDS)</th>
<th>Benefits of Targeted DNA Sequencing:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Information level</td>
<td>3 x 10⁹ bps</td>
<td>5 x 10⁷ bps</td>
<td>6 x 10⁴ bps#</td>
<td>• More relevant data (VUSs)</td>
</tr>
<tr>
<td>Cost per sample</td>
<td>$5000</td>
<td>$2000</td>
<td>$200</td>
<td>• More cost-effective</td>
</tr>
<tr>
<td>Coverage achieved</td>
<td>30x</td>
<td>100x</td>
<td>1000x</td>
<td>• Increased confidence in sequencing results</td>
</tr>
<tr>
<td>DNA input</td>
<td>1 µg</td>
<td>0.5 – 1 µg</td>
<td>10 ng</td>
<td>• Lower DNA requirements</td>
</tr>
<tr>
<td>No. of samples multiplexed</td>
<td>1^</td>
<td>2*</td>
<td>96*</td>
<td>• Higher multiplexing capabilities</td>
</tr>
</tbody>
</table>

# 30 genes; ^ 7.5 GB sequencing capacity
^ 95 GB sequencing capacity
VUSs – variants of uncertain significance
Genotyping technology: Why use multiplex PCR for target enrichment?

**Hybridization**
- **Workflow:**
  - Library construction (4 hrs)
  - Hybridization with probes (24-48 hrs)
  - PCR & Indexing (2 hrs)
- **DNA input:** 1-3 ug
- **Time from DNA sample to NGS library:** 2-3 day
- **Target size:** 2MB – Exome
- **Type of variants**
  - Point mutations
  - Large Indels
  - Structural variation

**Hybridization, ligation + PCR**
- **Workflow:**
  - Hybridization with probe (16 hrs)
  - Ligation (1 hr)
  - PCR & Indexing (2 hrs)
- **DNA input:** 200-400 ng
- **Time from DNA sample to NGS library:** 2 days
- **Target size:** 2 MB to Exome
- **Type of variants**
  - Point mutations
  - Large Indels
  - Structural variation

**Multiplex PCR**
- **Workflow:** (SIMPLE)
  - PCR amplification (3 hrs)
  - Library construction (4 hrs)
- **DNA input:** <100 ng (LOW)
- **Time from DNA sample to NGS library:** 1 day (RAPID)
- **Target size:** < 2 MB
- **Type of variants**
  - Somatic mutation
  - CNV
GeneRead DNAseq Gene Panels V2

- Multiplex PCR-enabled enrichment of genes of interest
- Cover all human exons (coding region + UTR), providing primer sets for any region, gene, or set of genes in the human genome
- Saves sample, time and money
- Largest collection of wet-bench verified gene panels
- Require just 10 ng per PCR reaction
- Custom Panel option available
- Can be used across any sequencing platform

Register for the 4-part NGS webinar series: [https://attendee.gotowebinar.com/register/725836371064017154](https://attendee.gotowebinar.com/register/725836371064017154)
Take away messages

- **Sample quantity and quality are very critical**
  - Choose appropriate sample collection and isolation kits based on sample type and sources
  - Minimize the variables in the process of sample collection, transportation, storage and DNA purification
  - Maximize the quality and purity of DNA
  - Scarce DNA quantity can be recovered using WGA

- **Detection assay sensitivity and specificity are important for accurate results**
  - Multiplex PCR kit
  - Multiplex-PCR based target enrichment for NGS
  - Validation of new tests are needed

- **Fragmented workflow**
  - Optimize every step of the workflow
  - Automate the workflow to ensure high standardization (QIAxcel)
Questions?

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