Segment II:

- What do you need to set up MALDI-TOF/MS PGX assays?
- The challenges of implementing MALDI-TOF/MS PGx tests

Select Gene-Drug Pairs for Clinical Service Implementation

Examples:

Gene	Drug
CYP2D6	Codeine, fluvoxamine, clomipramine (with CYP2C19)
CYP3A5	Tacrolimus
TPMT	Azathioprine; Mercaptopurine, thioguanine
HLA-B57:01	Abacavir

MJ Arwood, et.al., Clin Transl Sci (2016) 9, 233–245;

Lab Space

- Three separate (non-contiguous) work areas to prevent contamination of PCR products.

Lab Area	Activities
1	Isolate and dilute DNA
2	Pre-PCR preparation and addition of DNA template to the PCR cocktail
3	Post PCR processing

Equipment & Labware

- Liquid handler for the Pre-PCR sample set up (optional)
- Centrifuge
- Standard thermocycler
- Sample dispenser (e.g. MassARRAY Nano dispenser)
- MALDI plates
- MALID-TOF/MS (e.g. MassARRAY analyzer or MALDI-TOF from other vendors)

- Microplates
- Plate Seals
- Pressure Pads to minimize evaporation
- Single and multi-channel pipettors
- Repeater pipettes
- Filtered and Non-filtered tips
- Microtubes

DNA Extraction

Some examples of commercially available kits:

- QIAamp DNA Mini Kit (Qiagen Inc., Valencia,CA), based on solid-phase extraction technology,
- MagMax™ kit (Applied Biosystems, Foster City, CA)
- Maxwell® 16 system (Promega Corporation, Madison, WI), employed magnetic bead technology to extract DNA from the sample.

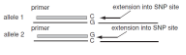
DNA quality is critical for successful genotyping regardless of the platform or methodology.

Required: A260/280 ratios between 1.7 and 1.9

PCR primers and Single Base Extension Probes

- Forward and reverse PCR primers

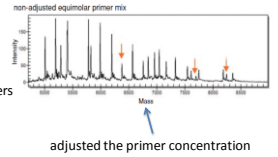
- SBE Probes or " mini-sequencing" primers



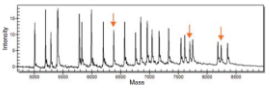
Primer pool

IF you use MassARRAY system, then you can use

MassARRAY® Assay Design Software



adjusted the primer concentration



Figures adapted from sequenom's iPLex application guide

Optimization of Multiplex PCR conditions

Post-PCR cleanup (SAP Reaction)

Basic PCR protocol:

# of cycle	Time (s)	T (°C)	
1	5	94	initial denaturation
45 cycles	20	94	denaturation
	30	56	annealing
	60	72	extension
1 cycle	180	72	Final extension
Final step	indefinitely	4	hold

Basic thermocycler program:

37 °C	40 min
85 °C	5 min

Single Base Extension

You need:

- ddNTP mixture
- dNTP mixture
- Extension primer
- Thermosequenase
- Ultrapure water
- 5 µL of SAP-treated PCR product

IF you use MassARRAY system, then you need:

- iPLEX enzyme
- iPLEX buffer
- Extension mix
- SpectroCHIP 96

PCR reagent kit by Agena

Basic SBE program as follows:

# of cycle	Time (s)	T (°C)	
1	30	94	Initial denaturation
40 cycles	5	94	denaturation
	5	52	annealing
	5	80	extension
1 cycle	180	72	Final extension
Final step	indefinitely	4	hold

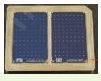
Store the base extension product at -20 °C if MALDI-TOF MS analysis cannot be carried out immediately.

Sample Cleanup

Example:

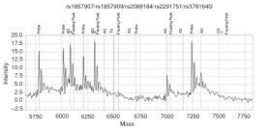
Add ultrapure water to resin; mix well before use

Agena Nanodispenser



Spectrochips with pre-spotted matrix

IF SBE cleanup failed, then----



spectrum with excessive back noise

http://www.nature.com/protocolexchange/protocols/73#/equipment

Detection of Primer Extension Products by MALDI-TOF/MS

Bruker AutoflexSpeed MALDI-TOF

MassARRAY analyzer Manufactured by Bruker

Mass range of the MS instrument: 4000-9000 Da

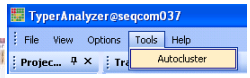
How about using MALDI-TOF at the microbiology lab in your institution?

Anticipated Problems---

1. Agreement from the microbiology laboratory
2. Cross-contamination
3. Middleware adjustment

Software for Genotyping Calls

- e.g. MassArray Typer

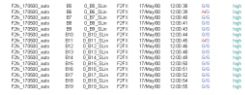


- Automated cluster-based genotype assignment functionality
- Report tables containing
  - Allele calls
  - Mutation frequency
  - A confidence score

Software for Genotyping Calls

Bruker Daltonics genotools SNP manager

- Calculates expected masses from given primer and product sequences
- Performs genotype analysis
- Graphically displays results.



http://bruker.poznan.pl/images/stories/Daltonics/noty/mt55.pdf

Current Procedural Terminology (CPT) Codes

CPT code	Test	Description of Test
81225	CYP2C19 genotyping	Detects genetic variants of CYP2C19 associated with variable drug metabolism
81226	CYP2D6 genotyping	Detects genetic variants of CYP2D6 associated with variable drug metabolism
81350	UGT1A1 genotyping	Detects genetic variants of UGT1A1 associated with irinotecan toxicity

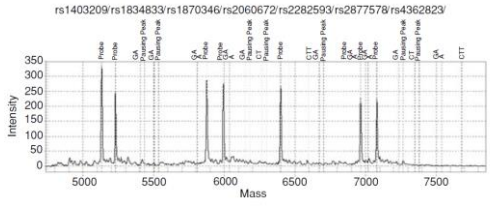
"Stacked Code"--- Example	
83891	Extraction of highly purified nucleic acid
83900	Mutliplex PCR
83914	Mutation identification by MALDI-TOF
83912	Interpretation and Reporting
83912-26	Pathologist interpretation if performed

J AHIMA. 2016 January ; 87(1): 56–59.

Factors to be considered to "home brew" MALDI-TOF assays in clinical labs

- Perform the multi-step preparation in a single vessel to minimize sample contamination and labor steps
- Keep reaction volumes as low as possible to reduce cost
- Automatic process (e.g. robotic spotting of MALDI Plates, laser targeting)
- Barcoding of both sample and MALDI Plate to assist in tracking sample
- Automate interpretation of mass spectra and genotype calling

IF lack of primer extension (#1)

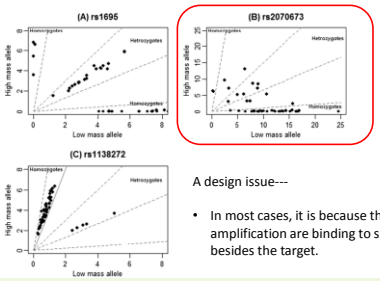


only unextended probes are observed

Figures adapted from sequenom's iPLex application guide

(#2)

Indeterminate SNP Typing Results



MassArray-- typer

A design issue---

- In most cases, it is because the primers for amplification are binding to something else besides the target.

L. Jensen et al. / Clinical Biochemistry 49 (2016) 1299–1301

(#3)

Disagreement Between Methods

Variant[1,2]	Quality metrics (reference reads, alternate reads, genotype quality)	WES	iPLEX® ADME PGx Panel	Sanger sequencing
rs1902023	0, 5, 15	Heterozygous variant	Homozygous variant	Heterozygous variant
rs72552763	6, 4, 99	Heterozygous variant	Homozygous variant	Heterozygous variant
rs3740066[3]	15, 16, 99	Heterozygous variant	Homozygous variant	Heterozygous variant
	17, 15, 99	Heterozygous variant	Homozygous variant	Heterozygous variant
rs9928286[3]	7, 0, 18	Homozygous variant	Heterozygous variant	Homozygous variant
	8, 0, 24	Homozygous variant	Heterozygous variant	Homozygous variant
	4, 0, 12	Homozygous variant	Heterozygous variant	Homozygous variant
	13, 0, 39	Homozygous variant	Heterozygous variant	Homozygous variant

[1]WES genotype calls were required to have a read depth≥4 and a genotype quality score ≥10.
[2]Genotype calls generated by the iPLEX ADME PGx to have a call rate >85%
[3]These discordant calls originated from different samples

- a concordance rate of 98.89%.

Chua, et.al., 2016, Frontier in Pharmacology
Carney Centre for Pharmacogenomics, Department of Pathology, University of Otago, Christchurch, New Zealand

Cost

TABLE 4. Cost (in U.S. Dollars) Comparison of Genotyping Using Mass Spectrometry Versus Alternative Methods

		Mass spec typing			RFLP typing		Taqman assay	
	Total rxn vol.	Single locus	4-plex	7-plex	Total rxn vol.	Single locus	Total rxn vol.	Single locus
PCR	4 µl	$0.16	$0.04	$0.02	20µl	$0.48	20µl	$0.93
Enzyme digest	6 µl	$0.08	$0.02	$0.01	25µl	$0.60	–	–
Post PCR	10 µl TS	$0.92	$0.23	$0.13	2%	$0.13	–	–
	10 µl Tth	$0.73	$0.18	$0.10	gel			
Purification		$0.14	$0.04	$0.02				
Total		$1.11–$1.30	$0.28–$0.33	$0.15–$0.18		$1.21		$0.93

Genotyping methods to detect known SNP	Instrument mean costs§	Approximate reagent costs per SNP$	Approxim time-labour per SNP#
D-HPLC	++++	moderate	very fast
SSCP	+	Low	very laborious
Allele Specific Amplification (ASA)	+	very low	moderate
Restriction Fragment Length Polymorphism (RFLP)	+	very low	very laborious
FRET probe Allelic Discrimination (Hyb Probe® TaqMan®, Beacons® Scorpions®)	++	moderate	moderate
Locked Nucliec Acid (LNA) probe	++		
Oligo ligation assay (SNPlex®)	+++		
PCR.Invader® Assay	+++		
High resolution melting (HRM)	++		
Pyrosequencing*	+++	high	fast
Peptide nucleic acid-mediated Clamping PCR*	+	moderate	moderate
Gene Chip technology (LabOnChip)*	+++/++++	very high	moderate
Maldi-TOF	++++	very high	moderate
Conventional sequencing*	++	low	moderate

Multiplexing is Key!

‡Approximate instrumentation list price were scored as + (<10000€), ++ (<50000€), +++ (<1000000€), ++++ (>1000000€).
§Reagent costs were scored as very low (<1k), low (<10k), moderate (<30k), high (<50k), very high (>50k).
#Time-labour refers input needed to perform a single test of multiple samples. It were scored as very fast (<1 hour), fast (<4 hours), moderate (<1 day), laborious (<2 day) very laborious (>2 working day).
*No address to allelic discrimination (mutant vs. wild type).

Table 1: Most common platforms used for genotyping at molecular level.

Pharmacogenomics Pharmacoproteomics 5: 123. doi:10.4172/2153-0645.1000123

HUMAN MUTATION 17:296.304 (2001)

Interdisciplinary Collaboration

- No FDA approved MS platforms
- Need extensive technical training
- LIS team support
- R&D team support
- PGx results interpreter

Clin Pharmacol Ther. 2013