BAC Fluorescent Fingerprinting workflow at Institute of Applied Genomics, Udine (Italy)

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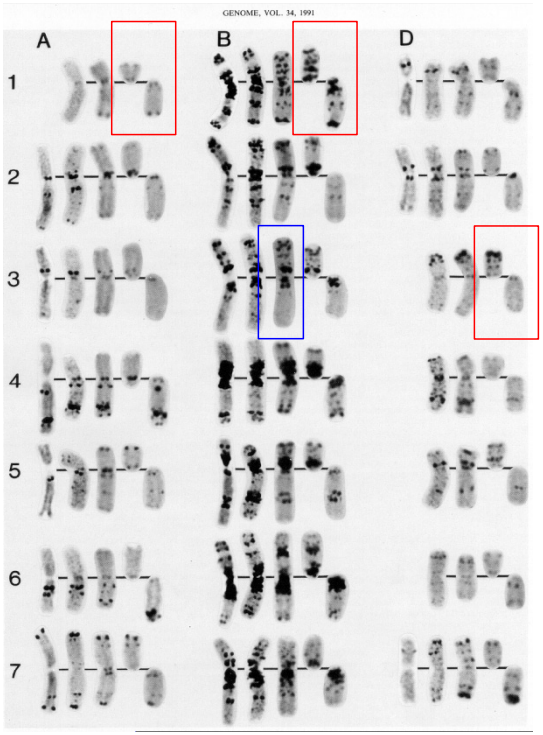
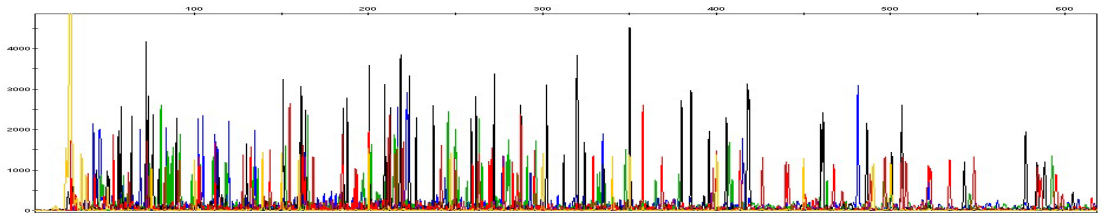
IWGSC: Physical Mapping Standard Protocols Workshop
FINGERPRINTING
PAG Meeting, San Diego, January 2010

TriticeaeGenome Project



Chromosome_(arm)	Clones #	Status dic. 2009	Recipient partner
3Bv1_MTP	7740	completed	INRA
3Bv2	82176	completed	INRA
3DL	64512	completed	JIC
3DS	36864	completed	IEB
1AS	31104	completed	UZH
1BS	55296	completed	HU
1BL	92160	30720 to fingerprint	INRA
1AL	92554	Gen-Feb 2010	SABA
TOTAL	**462406**		

CURRENT STATUS DECEMBER 2009



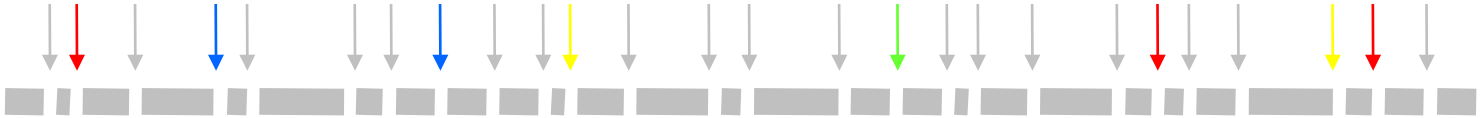
Fluorescent fingerprinting improvements

automated miniprep protocol

1. BAC DNA extraction and purification

miniaturized one-tube fingerprinting reaction protocol

2. Multiple restriction endonuclease digestion
(4 rare cutters, ***Eco*RI**, ***Xba*I**, ***Bam*HI**, ***Xho*I** and 1 frequent cutter, ***Hae*III**)



3. Fragment labeling using fluorescent SNaPshot chemistry
(**ddATP**, **ddCTP**, **ddGTP**, **ddTTP**)

novel speedy cleanup protocol

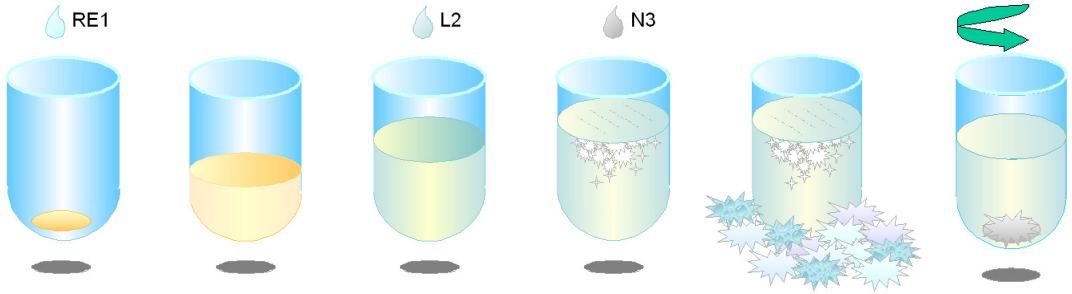
4. Post extension cleanup of unincorporated dyes

5. Electrophoresis separation and peak detection
(**ABI3730** capillary automated sequencer)

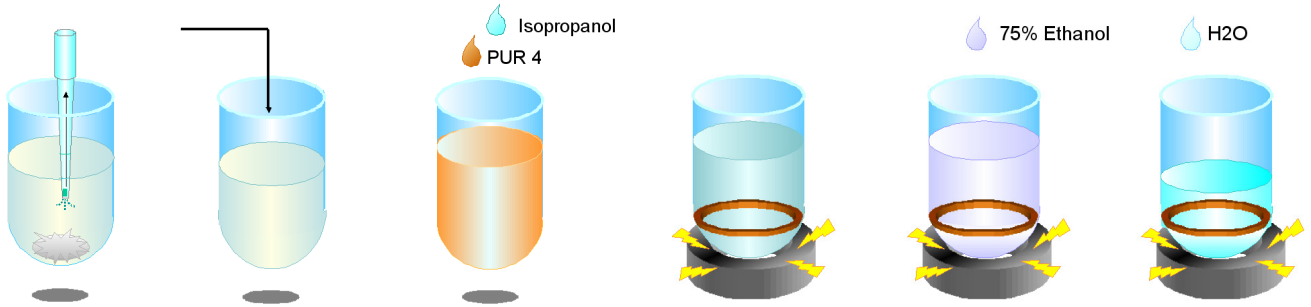
1. BAC DNA extraction & purification

- alcaline lysis DNA extraction protocol based on **AGENCOURT® CosMCPrep® System**
- automatization of the procedure on a BECKMAN 96 head FX^{p} robot

phase1: alkaline lysis and flocculent pelletting



phase2: lysate transfer, SPRI purification and elution



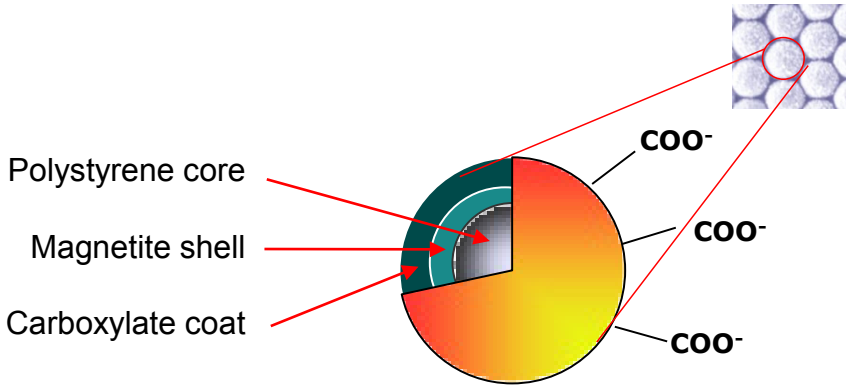
1. BAC DNA extraction & purification

Agencourt® SPRI™ technology

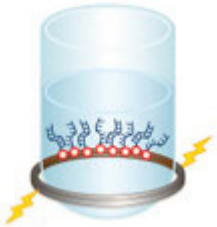
Solid Phase Reverse Immobilization

Patented technology developed at Whitehead Institute (*Nucleic Acids Res. 1995; 23:22*)

- Polystyrene: low sedimentation rate
- Magnetite: bead pellet in magnetic field
- Carboxylic groups: reversible interaction between hydrophilic beads and nucleic acids
- Elution in aqueous solution (no caotropic salts)



Step 1
NA Immobilization

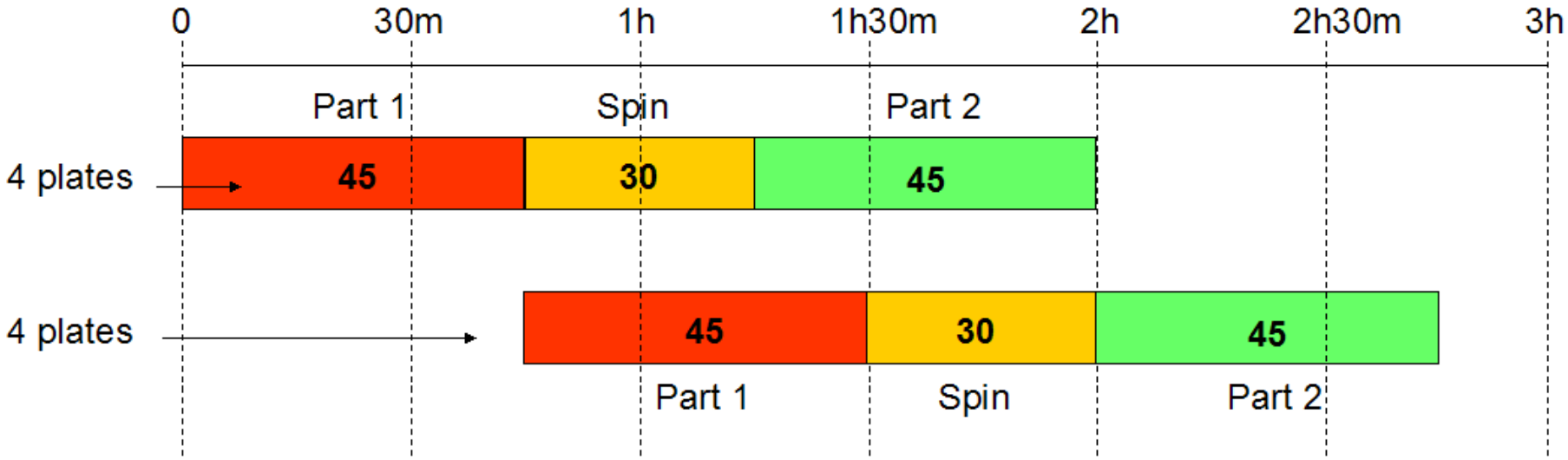


Step 2
Contaminant Removal & Wash

Step 3
NA Elution

1. BAC DNA extraction & purification

High Throughput: Overlapping Batches

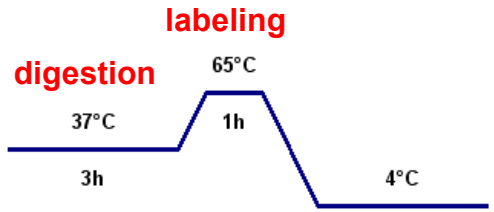


8 x 96-well plates in less than 3 hours → 4-6 384-well plates per day

BAC preparation remains the fingerprinting procedure bottleneck
(split of 384-format in 96-format)

2. One-tube digestion & labeling reactions

- Reduction of SNaPshot and restriction enzymes amounts
- SNaPshot Ready Reaction mix directly added to the digestion mixture
- Two temperature ramp incubation to perform digestion and labeling reaction in one tube



3. Post extension treatment: size standard

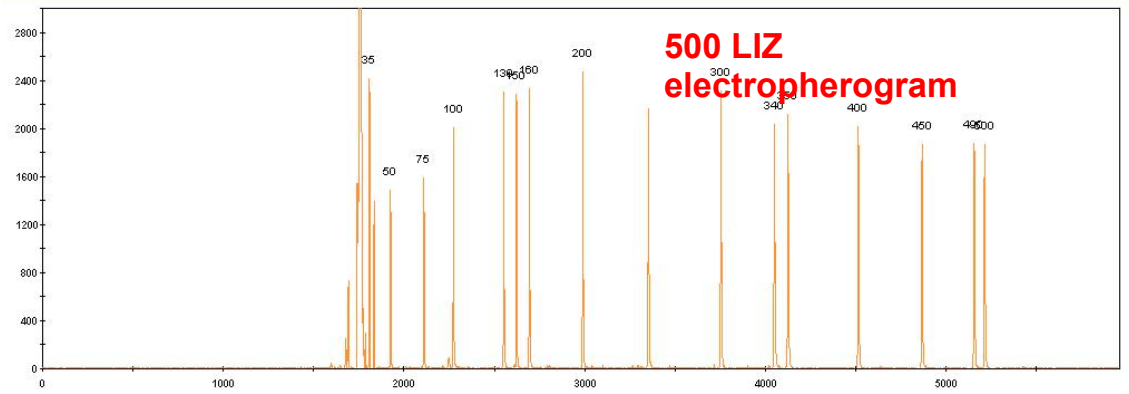
GeneScan™ 500 LIZ® internal Size Standard

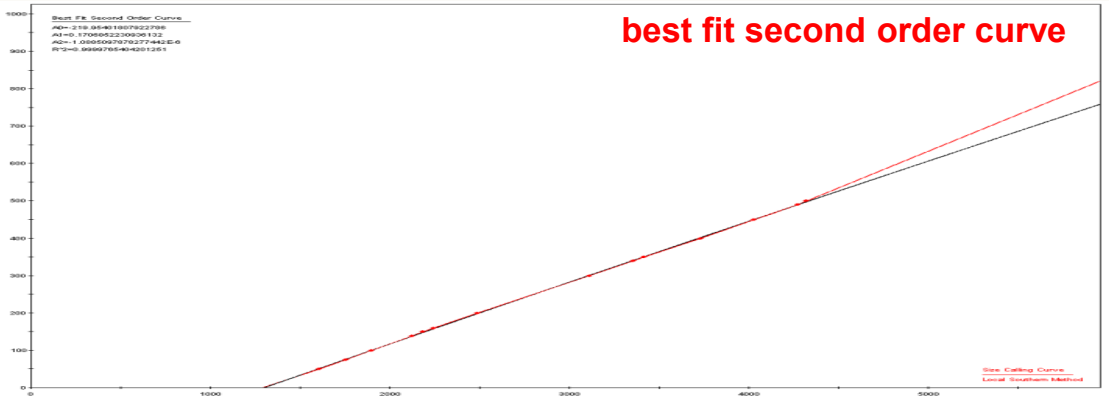
A set of DNA fragments of known sizes, labeled with an AB proprietary fluorophore (**orange**), used for determining the size of unknown DNA fragments run on ABI PRISM® DNA sequencers

GeneMapper software generate a calibration or sizing curve based upon the migration times of the known fragments in the standard.

The unknown fragments are mapped onto the curve and the sample data is converted from migration times to fragment sizes.

(The 250-bp peak shows abnormal migration and shall not be used to size samples)

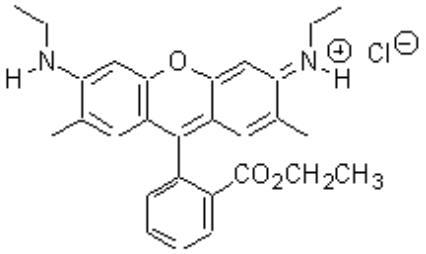




pass **check** **low quality**

3. Post extension treatment: dye clean up

Applied Biosystems BigDye® XTerminator™ system



dRhodamines, the fluorochromes in the SNaPshot mixture, are small molecules difficult to remove by ethanol precipitation in 384-well plates (dye-blobs and overall decrease of the signal strength)

The **Applied Biosystems BigDye® XTerminator™ Purification Kit** is a cleanup system for DNA sequencing reactions that removes free BigDye® terminators

A resin sequesters reagent components as salt ions and unincorporated dye terminators to prevent coinjection with dye labeled extension products and improve signal intensity

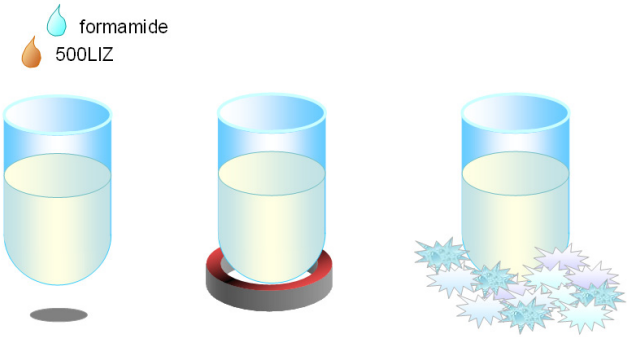
SAM buffer: slight denaturing effect

BDX resin: fast sedimentation rate

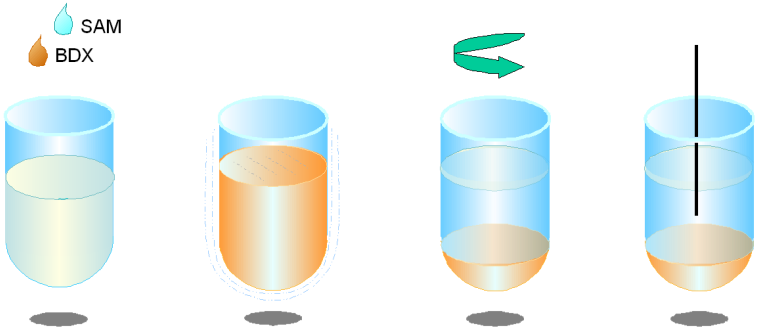
3. Post extension treatment

In collaboration with Applied Biosystems we transfer this fast and simple purification system to the fingerprinting clean up

phase1: internal standard adding and sample denaturation

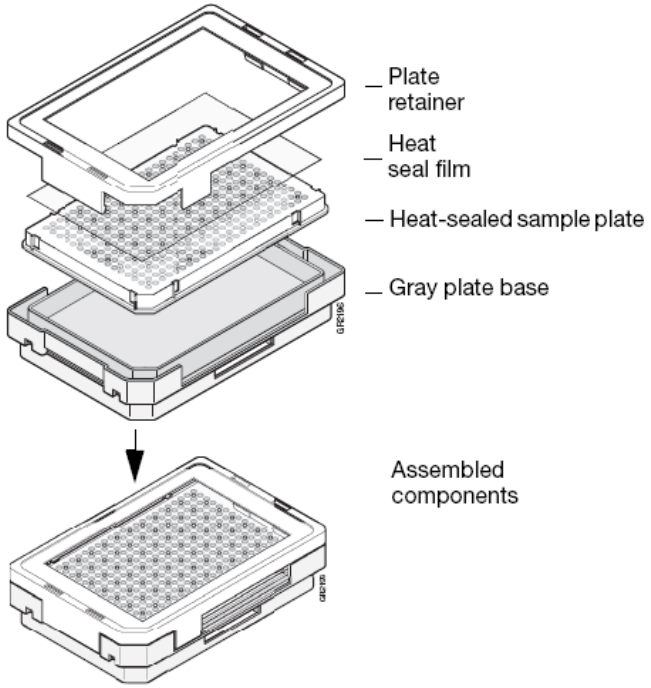


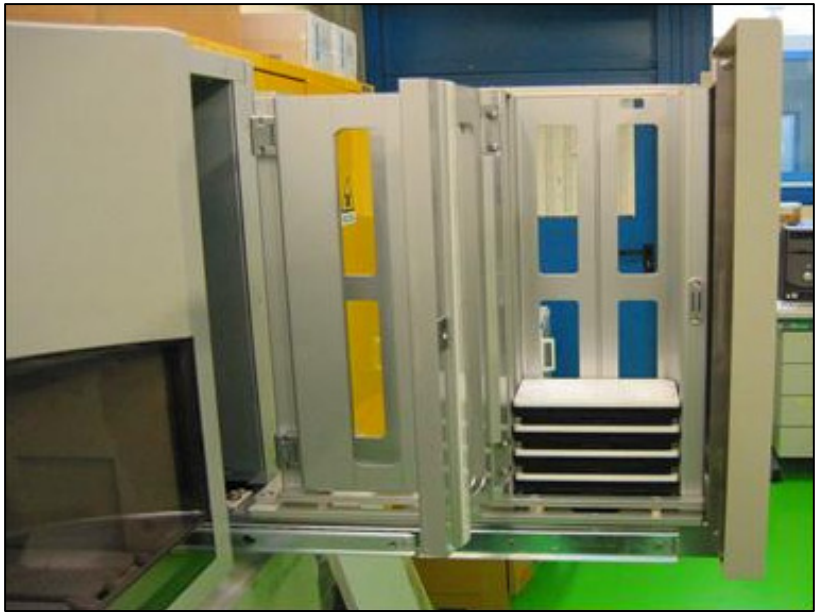
phase2: dye cleanup, resin pelletting and CE loading



4. Fingerprinting run

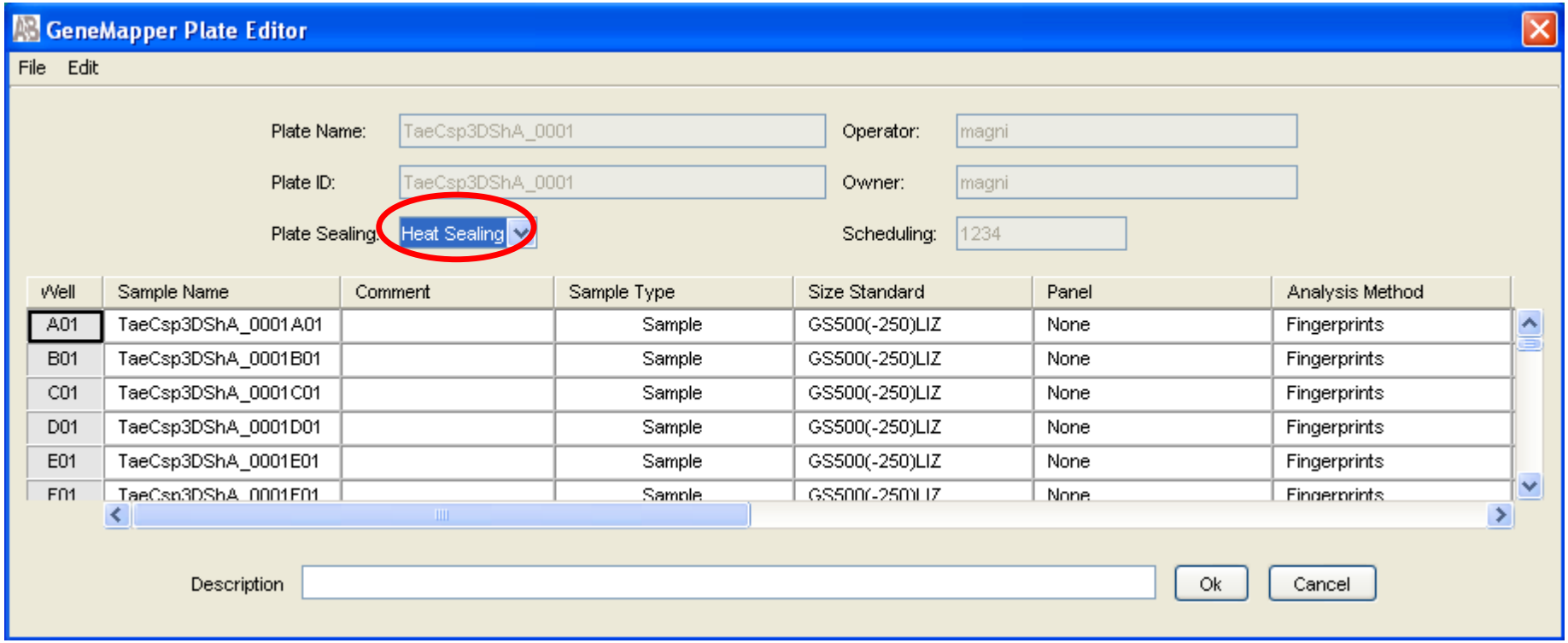
- The sample sheet is automatically generated by a barcoding system during the fingerprinting pipeline
- The heat-sealed plate is assembled in a specific base-retainer box and directly loaded on the instrument





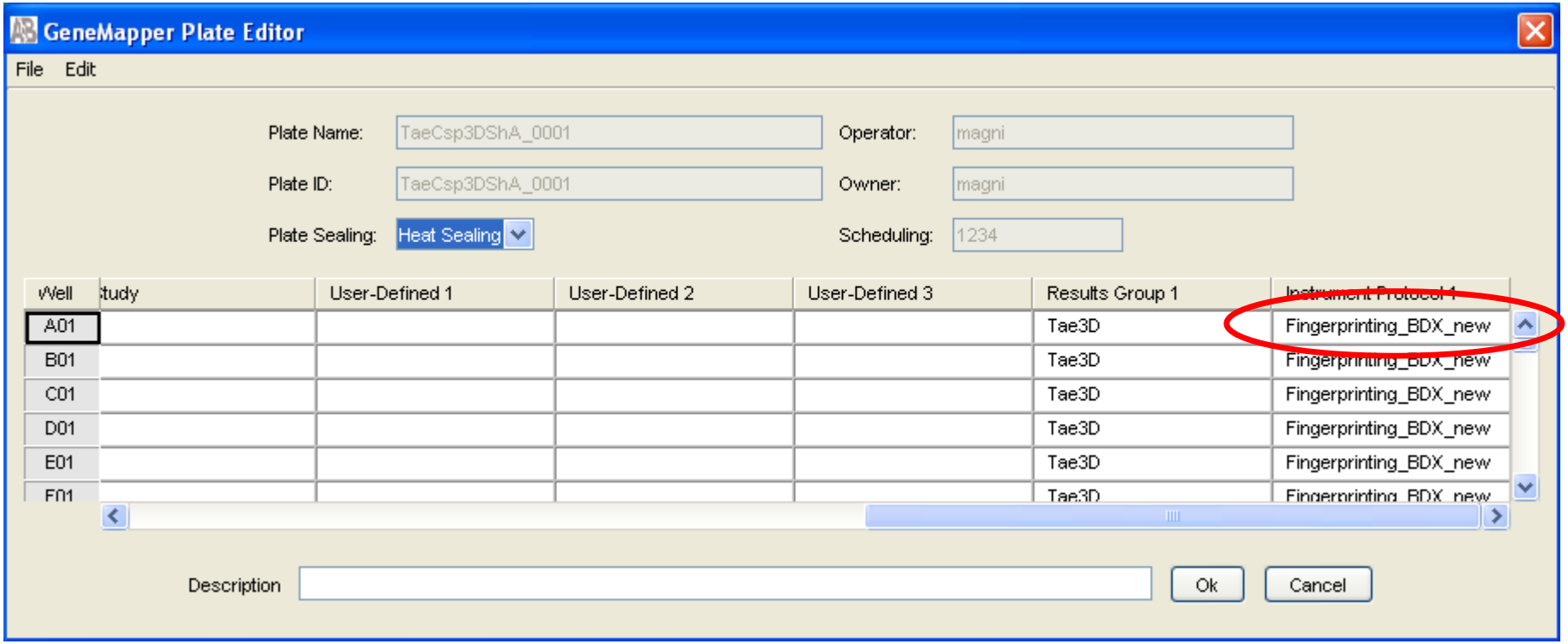
4. Fingerprinting run

The sample sheet must report the plate sealing type

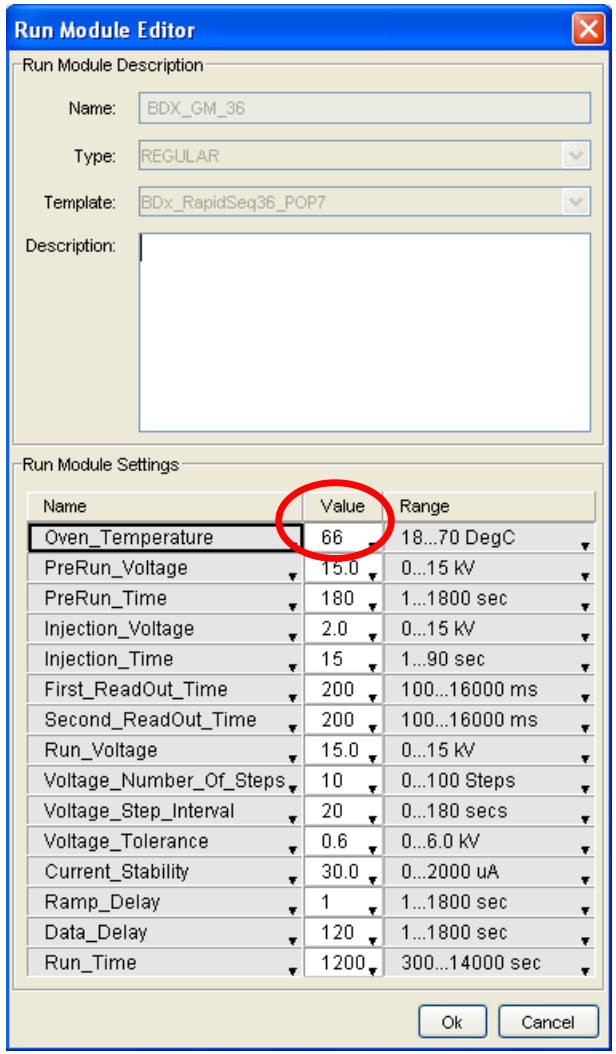


4. Fingerprinting run

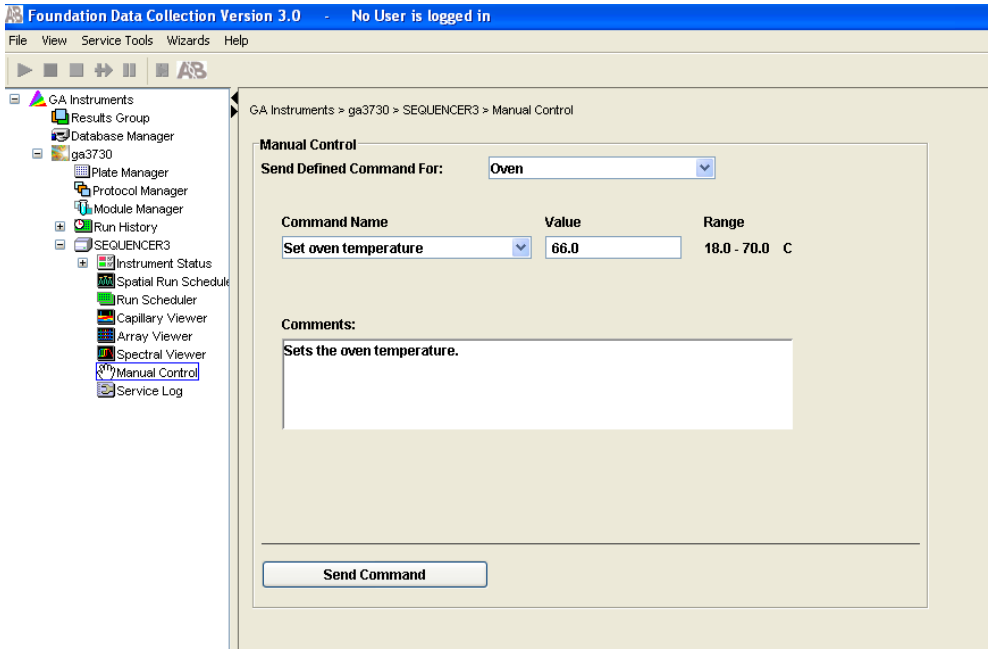
The sample sheet must report the specific instrument protocol



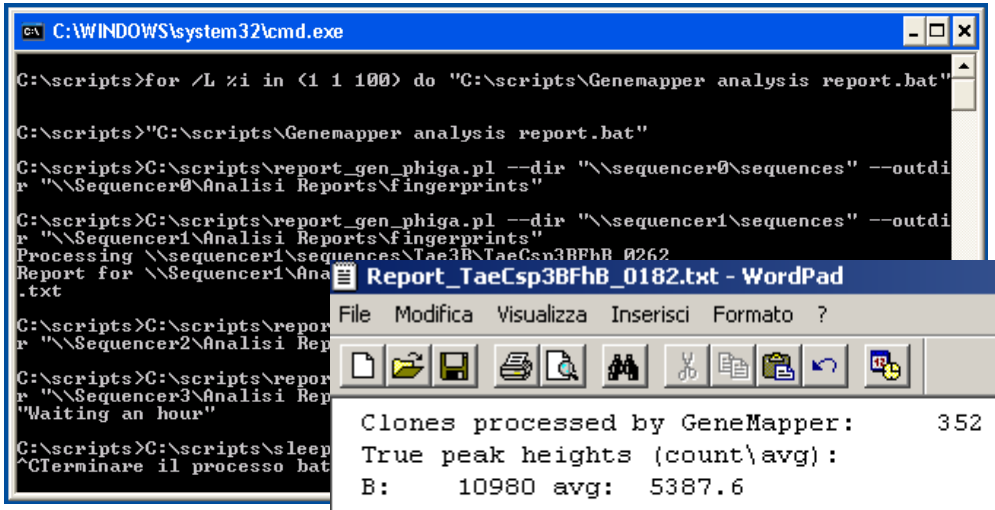
4. Fingerprinting run



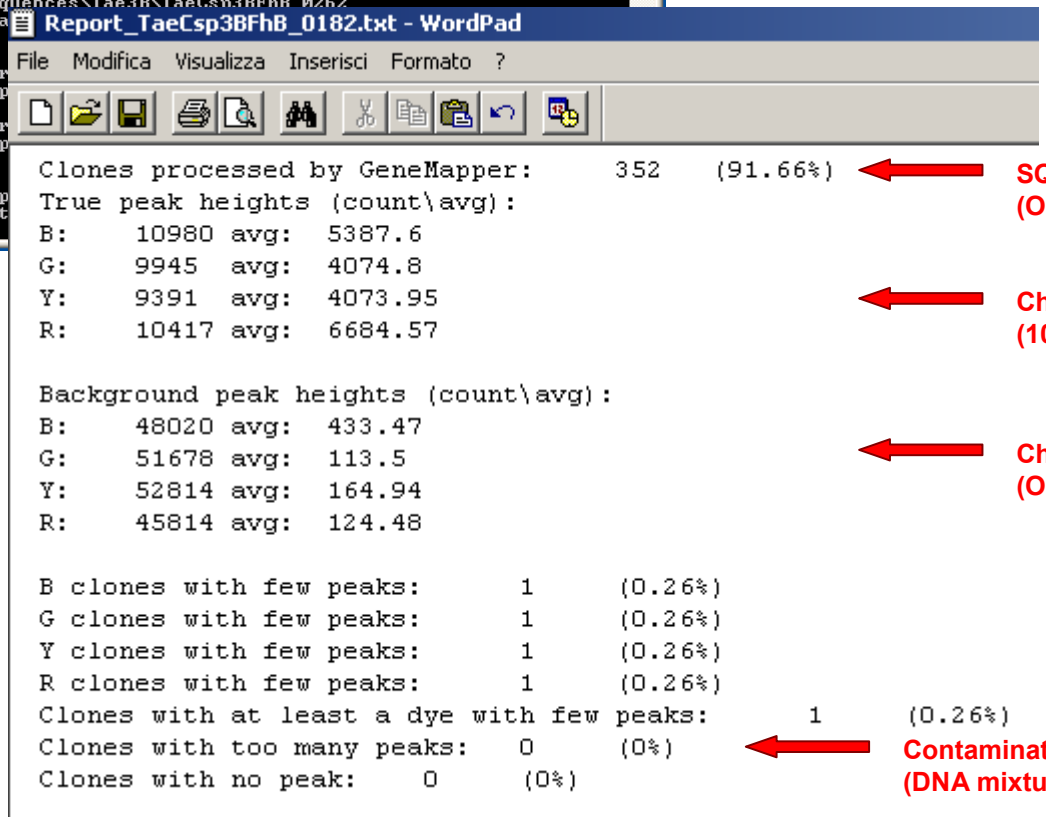
- An appropriate run module is needed for sample injection (from ABI)
- Oven temperature must be manually set



5. Fingerprinting quality check



The summaries of the automatic analysis performed by GeneMapper and FPBscript are saved for each plate in txt format



SQ passed: GM_SizeCalling goodness measure (OK < 10-15%)

Channel Intensities: true peak measure (10000rfu>OK>200rfu; 1000rfu>optimal>3000rfu)

Channel Intensities: background peak measure (OK if 10% of the true peaks)

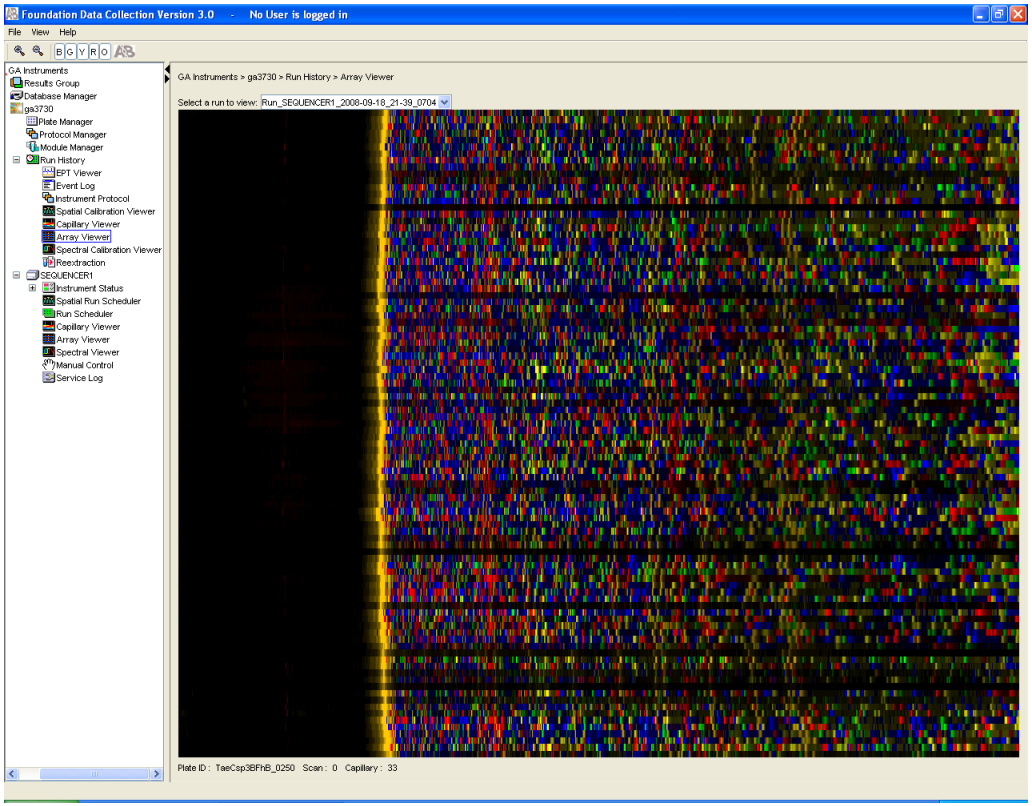
Failed samples (no DNA; no fingerprinting rxn)

Contaminated samples (DNA mixture)

improved HICF statistics

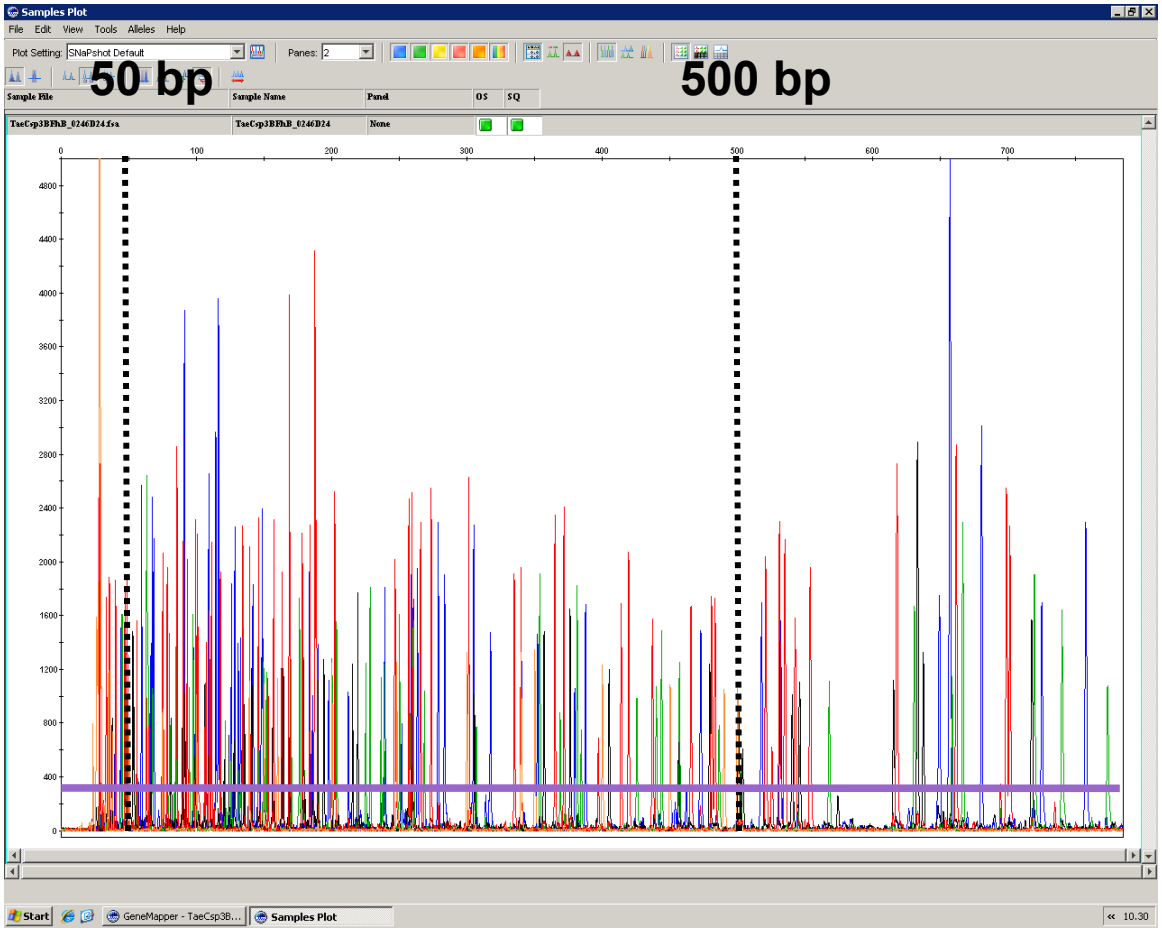
On 82176 processed fingerprints (wheat TaeCsp3BFhB library):

- Good sized clones: **91%** (GeneMapper SQ threshold 0.5)
- Empty/failed clones: **1.6%** (FPB script – minBands 40)
- Cross contaminated clones: **<5%** (GenoProfiler size matched 50%)



improved HICF quality

True signal *vs.* background



Fluorescence of the true peaks
1000-5000 rfu
(BDX purification improves the signal intensities)

Background threshold
(<10% of the true peaks)

Fingerprinting core facility

- CosPrep (Agencourt) for BAC DNA extraction
- Restriction enzyme digestion
- SNaPShot (ABI) labelling

Weekly (5 days)	Monthly (20 days)	Annually (240 days)
7,680-9,600	30,720-38,400	368,640 - 460,800

384-well PCR

Biomek FX
(96-head)

ABI3730 xl

Centrifuge for microplates

Matrix WellMate Microplate Dispenser

Standard protocols

Same method, different workflows: same enzyme combination + SNAPShot labelling

UD Davis	IGA
Qiagen miniprep (filter)	CosPrep (SPRI technology)
96-well format	384-well format
Dig+labelling	Dig/labelling in the same well
LIZ 1200	LIZ 500
Etanol precipitation	BDX Technology

1. Miniprep

Miniprepping is the most delicate phase of the workflow

Set-up very well the bacterial growing conditions to avoid too many or too low bacterial cells:

an accurate optimization need to be done
in each lab with the aid of the kit manufacturer
(i.e. Qiagen or Beckman)

Do not prepare all the minipreps at a time: it's better to finish the entire workflow for each batch of plates

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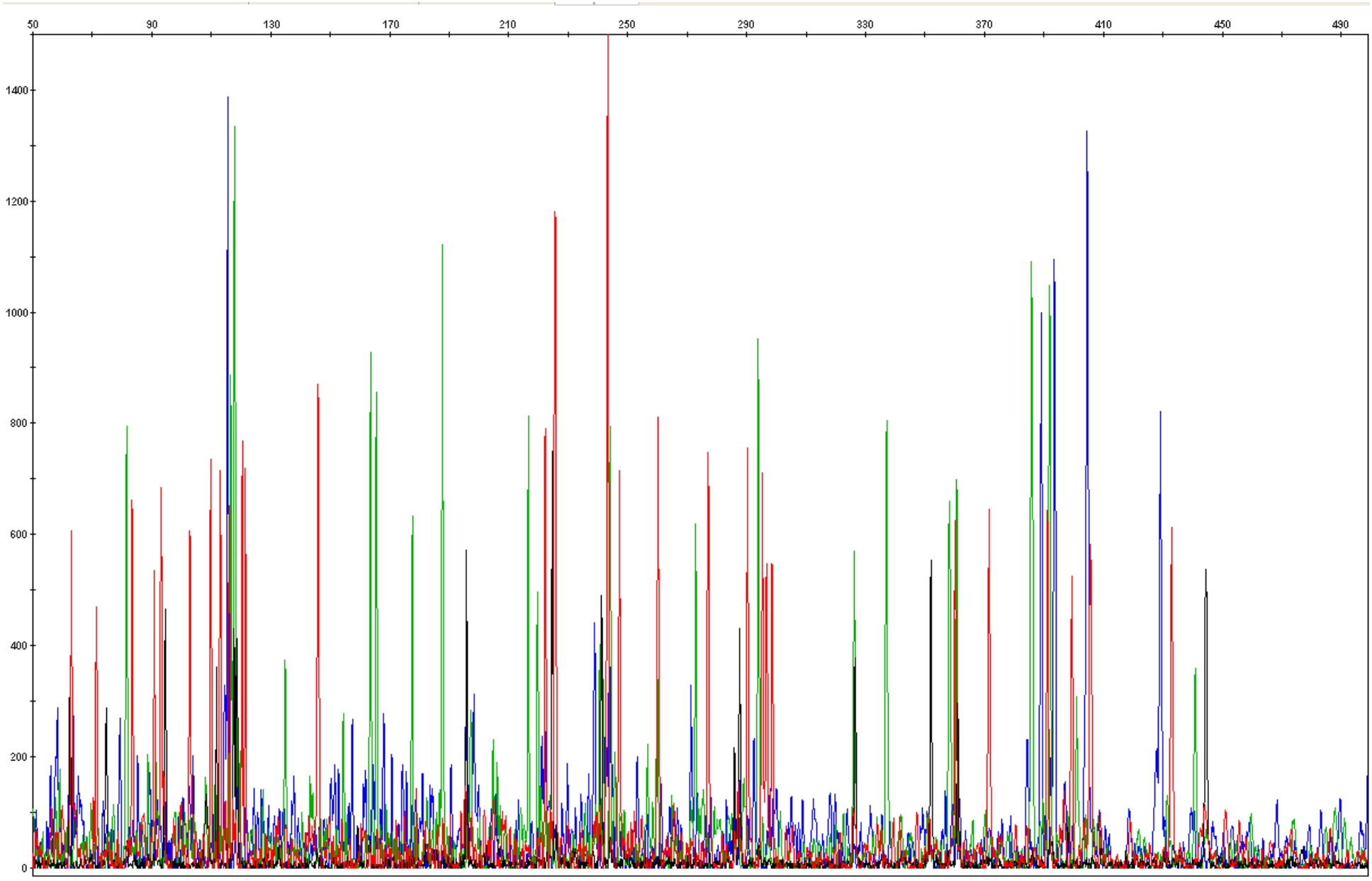
1. CONTAMINATED PATTERN: high background

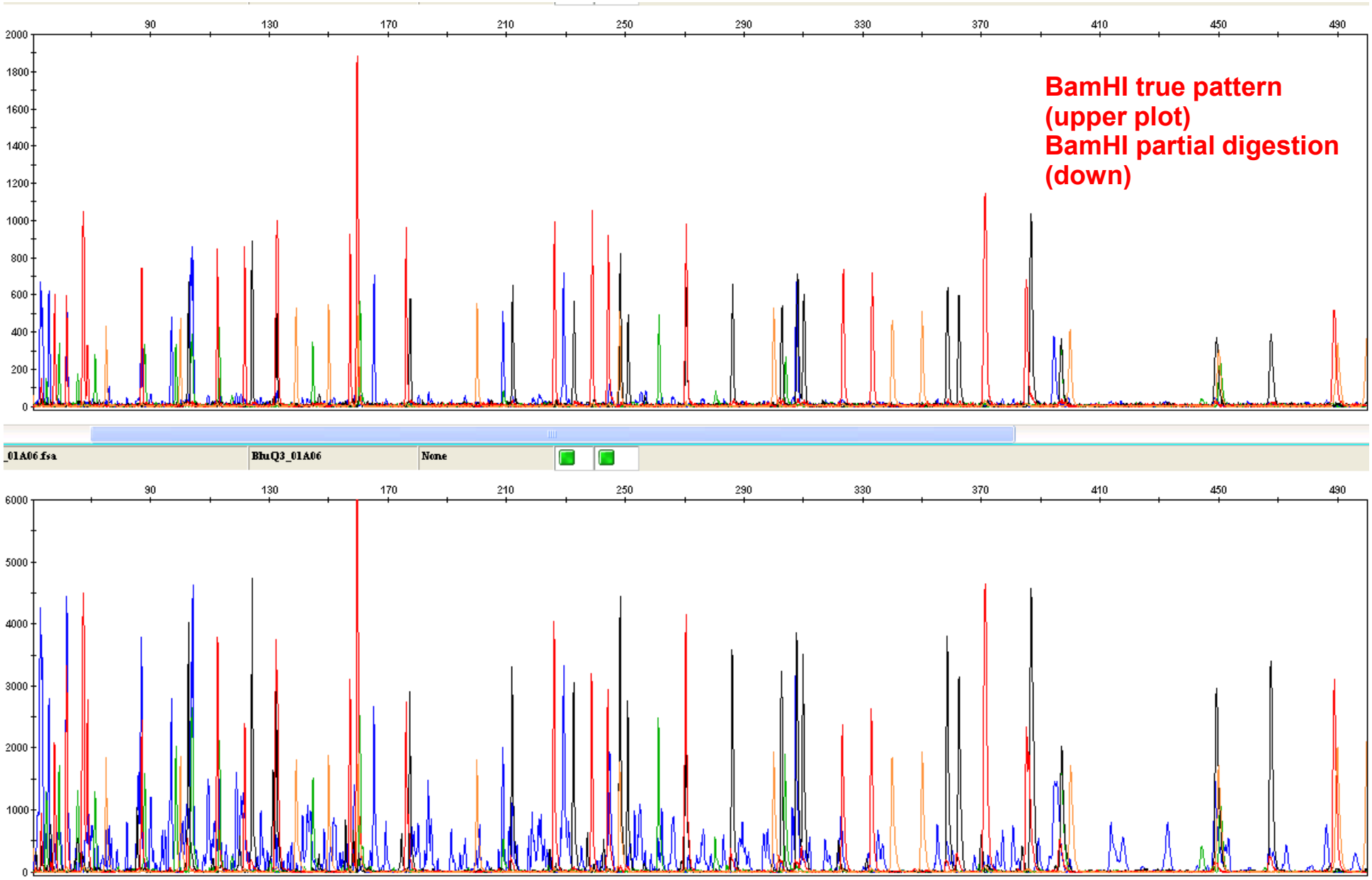


2. Restriction enzyme digestion

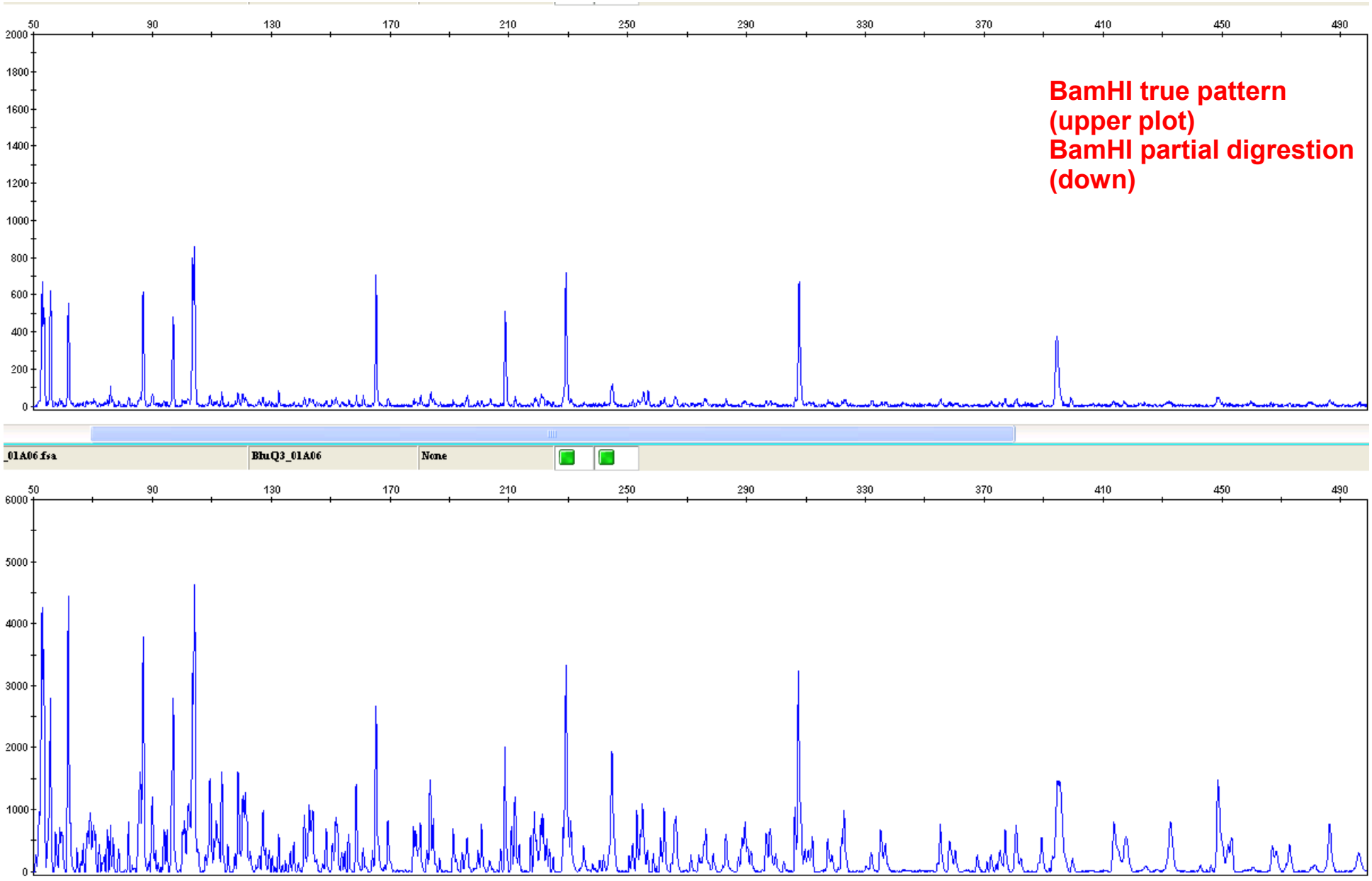
Use high fidelity restriction enzymes (i.e. New England Biolabs) to avoid star activity

Star activity=activity of restriction endonuclease under non-standard conditions that resulted in cleavage at sequences similar but not identical to their defined recognition sequences

2. PARTIAL DIGESTION: BamHI star activity (blue)



2. PARTIAL DIGESTION: BamHI star activity (blue)

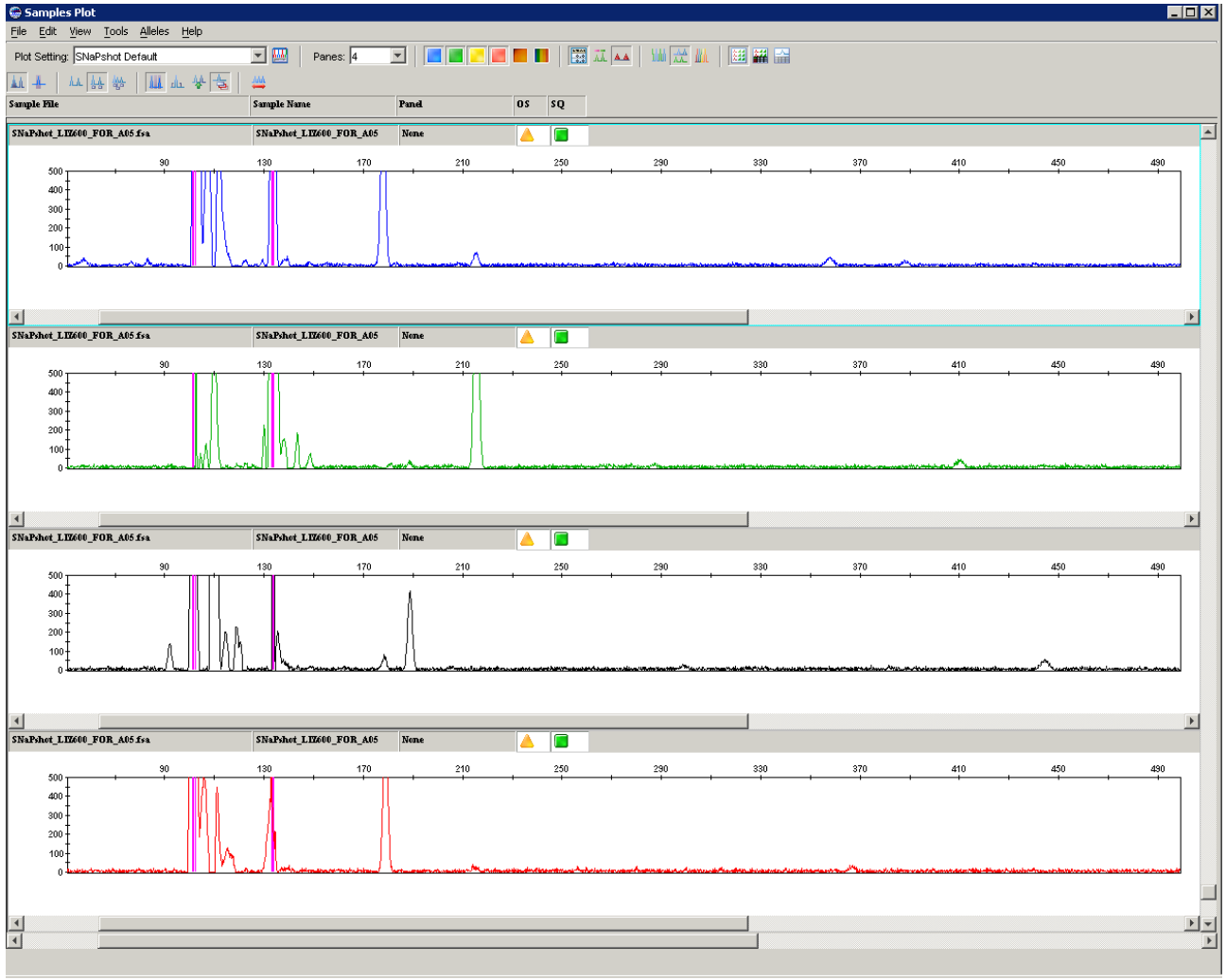


3. Dye-blob cleaning

In 96-well format the Etanol precipitation is sufficient to clean from Dye-blob

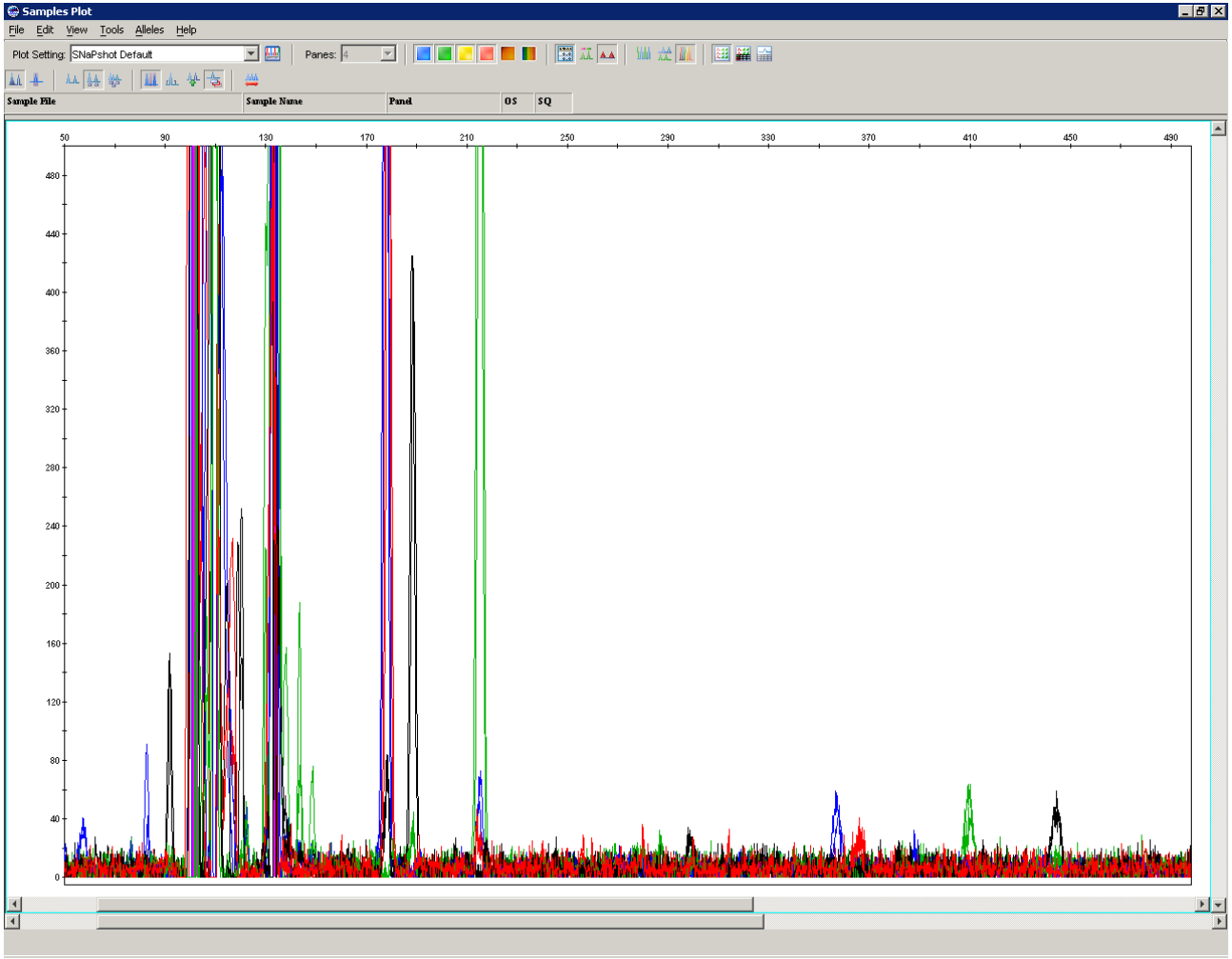
In 384-well format it's necessary to clean better from d-Rhodamine

3. Dye-blobs: SNaPshot free fluoresceinated dNTPs



Dye blobs due to free (i.e. unincorporated) electrophoretic migration of SNaPshot fluoresceinated dNTPs

3. Dye-blobs: overlay all view



Rhodamine dye blobs migrate quite precisely

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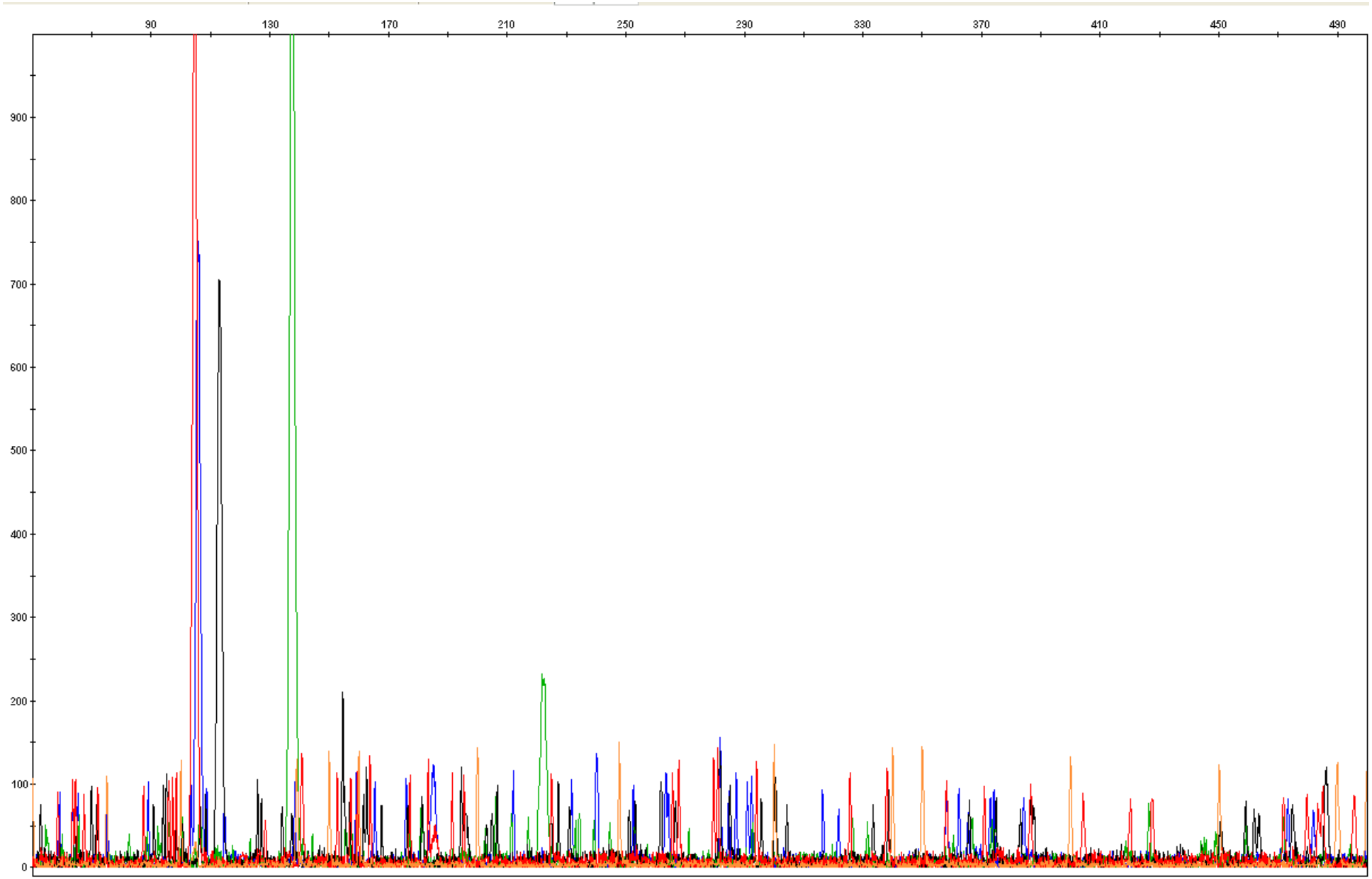
3. Dye-blobs: BAC fingerprint



Conclusions

It is not easy to set-up a fingerprinting workflow (it is easier to do sequencing!)

Money investments need to be done to acquire robotics and for a first phase of optimization

A preliminary managing work need to be done to obtain the best discounts: cost per sample can be very variable, a high-troughput facility have more discount on the reagents!

WE ARE AVAILABLE TO EVALUATE THE OPPORTUNITY TO DO FOR YOU THE FINGERPRINTS IN SERVICE