Research centre
for toxic compounds
in the environment

Bi5596
Moderní metody v ekotoxikologii

STUDIUM RNA & DNA II.

Co nás zajímá a jak to zjistíme

RNDr. Iva Sovadinová, Ph.D.
sovadinova@recetox.muni.cz

podzim 2014

CÍLE PŘEDNÁŠKY

- ✓ Jak namnožit NK
- ✓ Jak zjistit sekvenci NK
- ✓ Co to je transkriptom a k čemu nám je
- ✓ Co to je (eko)toxigenomika
- ✓ Jak zapnout či vypnout gen
- ✓ Jak zkoumat genotoxicitu

CO NÁS, (EKO)TOXIKOLOGY, ZAJÍMÁ PŘI STUDIU NUKLEOVÝCH KYSELIN?

DNA Testing.

NK: PROČ ZKOUMÁME?

- NUKLEOTIDOVÁ SEKVENCE ⇒ analýza variací
- POLYMORFISMUS DNA ⇒ bodový nebo repetitivní
- GENETICKÝ OTISK
- OTISK MIKROBIÁLNÍHO SPOLEČENSTVA
- MUTACE
- GENOVÁ EXPRESE, REGULACE A FUNKCE
- REKOMBINACE DNA

PCR
aneb
JAK NAMNOŽIT SPECIFICKÝ ÚSEK NK?

PCR: „KOPÍRKA“ PRO DNA

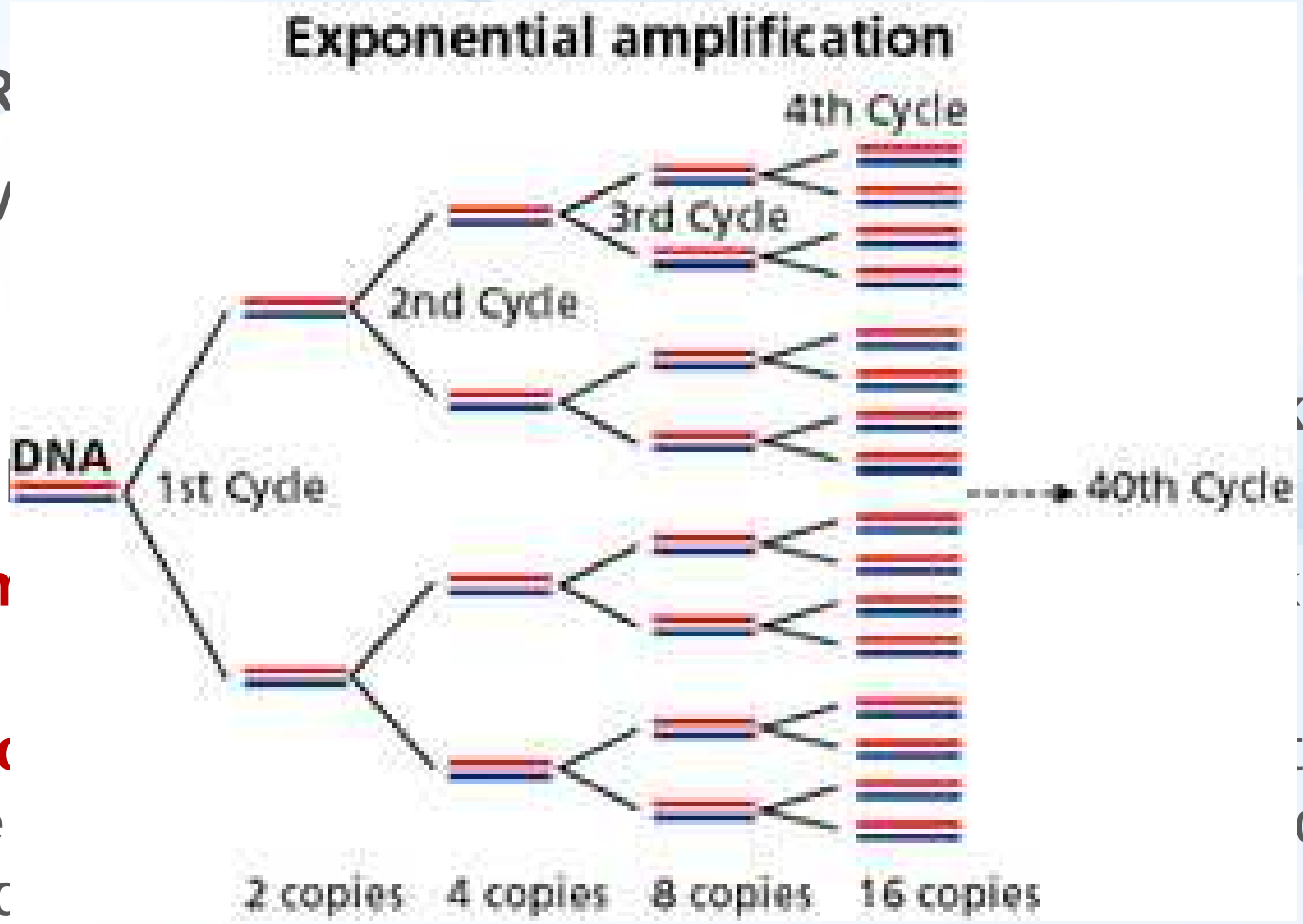
- **PCR = „Polymerase Chain Reaction“**
- **Polymerázová řetězová reakce**
  - objevena v roce 1983 ⇒ Kary Mullis
  - Nobelova cen 1993
  - **ústřední metoda v biochemii a molekulární biologii**

„polymerázová“ – využití DNA polymerázy ⇒ replikace *in vitro*

„řetězová reakce“ – více reakcí za sebou, produkt první reakce se stává „šablonou“ v další reakci atd. atd. atd. atd. atd. atd.

PCR: „KOPÍRKA“ PRO DNA

- PCR
- Poly



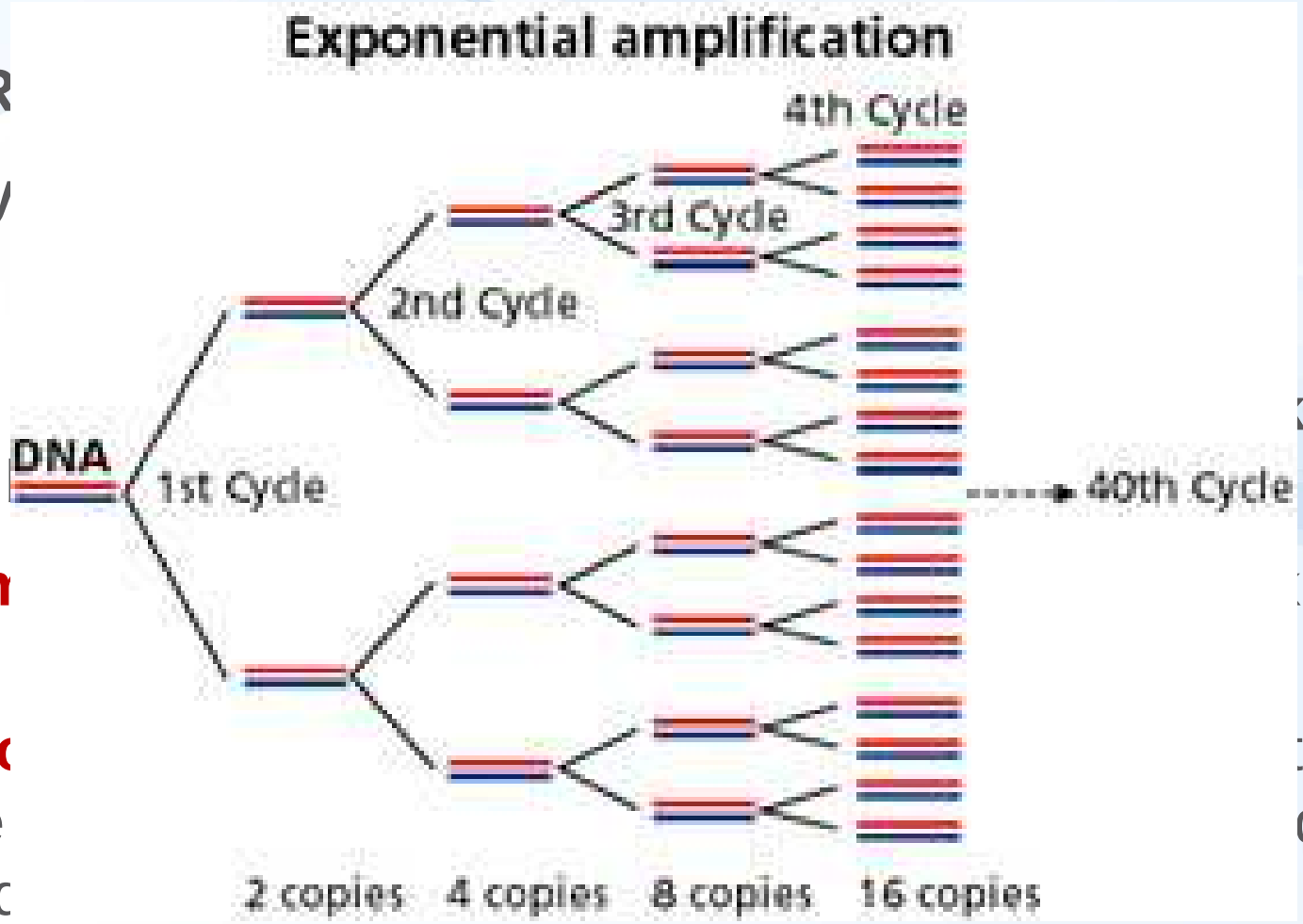
kulární

„polyn ace *in vitro*

„řetězc : první reakce d. atd. atd. atc

PCR: „KOPÍRKA“ PRO DNA

- PCR
- Poly



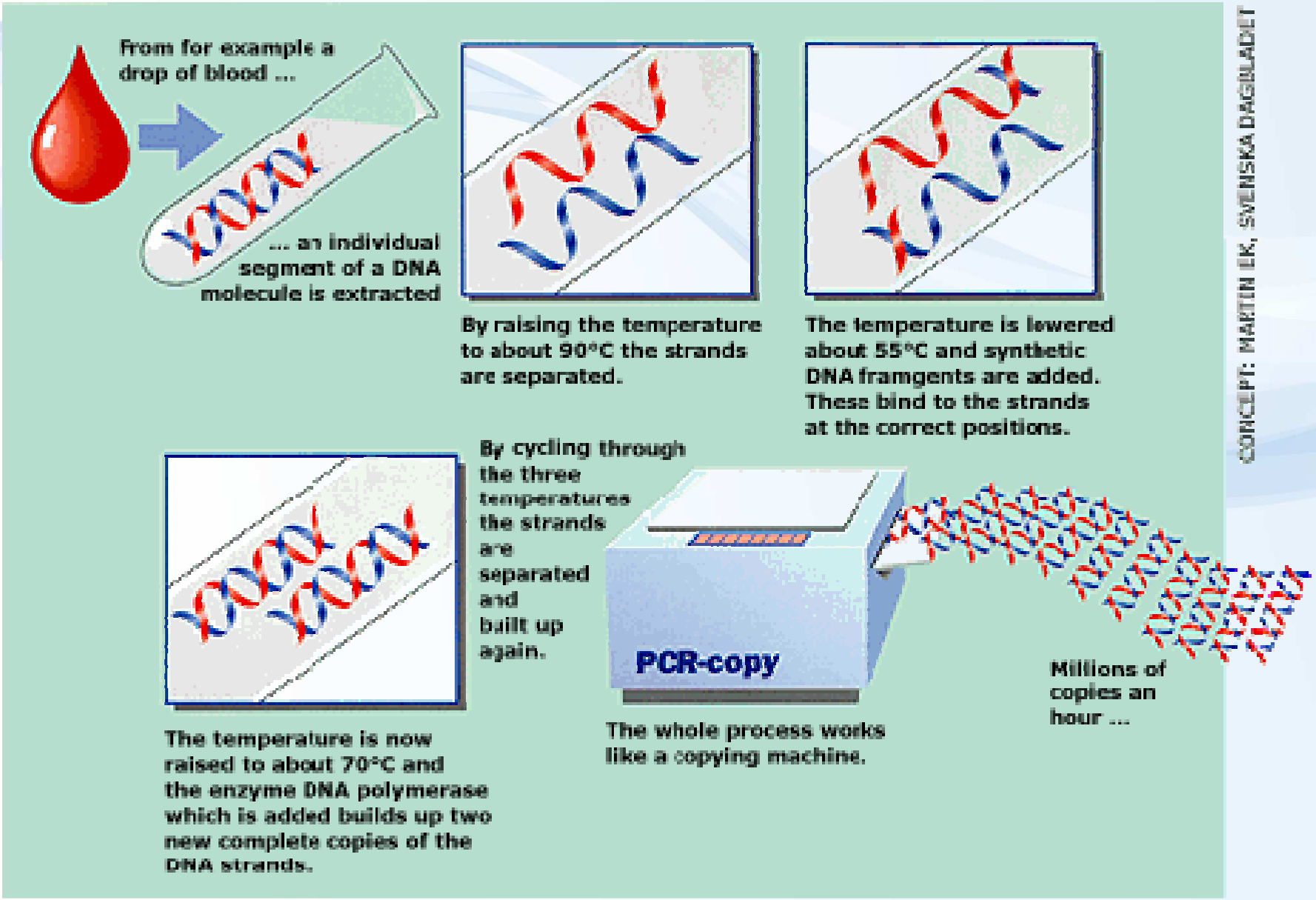
kulární

„polyn ace *in vitro*

„řetězc : první reakce d. atd. atd. atc

2^{40} = 1 099 511 627 776 kopií

Research centre for toxic compounds in the environment


From for example a drop of blood ...
... an individual segment of a DNA molecule is extracted
By raising the temperature to about 90°C the strands are separated.
The temperature is lowered about 55°C and synthetic DNA framgents are added. These bind to the strands at the correct positions.
By cycling through the three temperatures the strands are separated and built up again.
PCR-copy
Millions of copies an hour ...
The temperature is now raised to about 70°C and the enzyme DNA polymerase which is added builds up two new complete copies of the DNA strands.
The whole process works like a copying machine.
CONCEPT: MARTIN EK, SVENSKA DAGBLADET

PCR: CO POTŘEBUJEME? I.

1. TEMPLÁT

DNA

2. PRIMERY

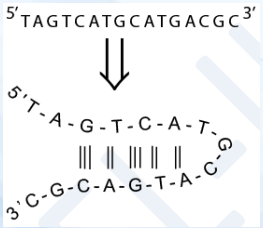
⇒ Specifické pro např.

- gen (receptor, enzym, membránový přenašeč atd.)
- kmen bakterií (16S RNA)

⇒ Nespecifické

PCR: (NE)SPECIFICKÉ PRIMERY

- ✓ 20 až 30 bazí
- ✓ Teplota nasedání závislá na sekvenci primerů (~ 50% obsahu GC)
- ✓ Neměl by tvořit sekundární struktury, zejména na 3’ konci
- ✓ Neměly by tvořit tzv. dimery ⇒ komplementarita sekvencí primerů

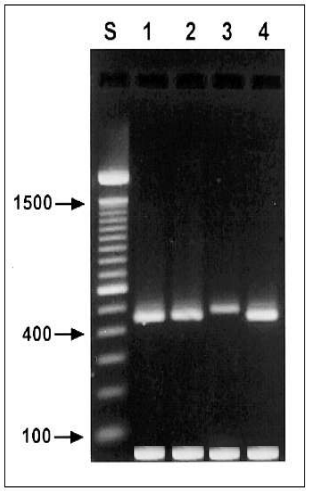


5’-ACGGATACGTTACGCTGAT-3’
3’-GACTATTCCATGTAGACCT-5’

Primer 1

5’-ACGGATACGTTACGCTGATAAGGTACATCTGGA-3’
3’-TGCCTATGCAATGCGACTATTCCATGTAGACCT-5’

Primer 2



- ✓ Sekvence primerů by měla končit GC ⇒ silnější vazba ńa templát

PCR: PROGRAMY PRO DESIGNOVÁNÍ PRIMERŮ

OLIGO
www.oligo.ne

PRIMER3
http://biotools.umassmed.edu/bioapps/primer3_www.cgi

PrimerQuest
http://www.idtdna.com/primerquest/home/index

PCR: CO POTŘEBUJEME? II.

3. PŘÍSTROJ

umožňující rychlé a precisní střídání teplot

95 °C ⇒ denaturace DNA
50-60 °C ⇒ nasednutí primerů
72 °C ⇒ prodloužení vlákna

Průběh PCR
před vynalezením thermocykleru

95º C
5 min

55º C
3 min

72º C
5 min

35 cyklů

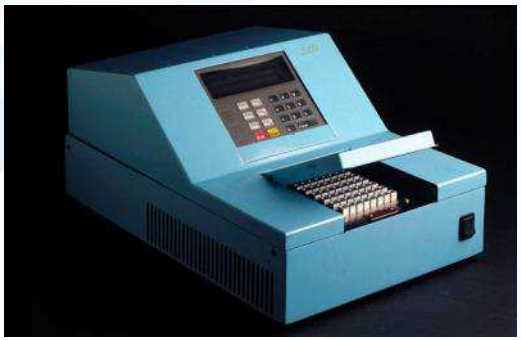
8 **NUDNÝCH** hodin !!!

Research centre
for toxic compounds
in the environment

Průběh PCR


We couldn't afford one of those cool PCR robots,
so we just got an undergrad and a cardboard box.

AUTOMATIZACE



„Baby Blue“ (1986)

Starší prototyp termocykleru

Techne TC-PLUS (2010)

Biometra T1 (1999)

Eppendorf Mastercycler EP (2003)

G-STORM GS4 (2006)

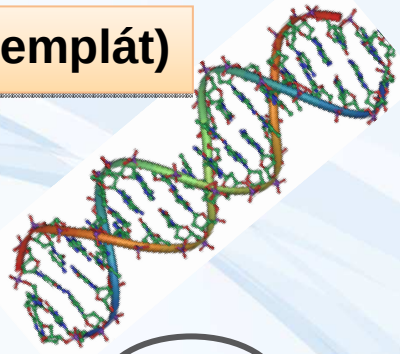
Biometra Toptical (2012)

Research centre for toxic compounds in the environment

5. REAKČNÍ SMĚS VE ZKUMAVCE

Vzorek DNA
Primery pro (ne)specifickou detekci
Nukleotidy
Enzym

DNA (templát)



Složky PCR:

T C G A

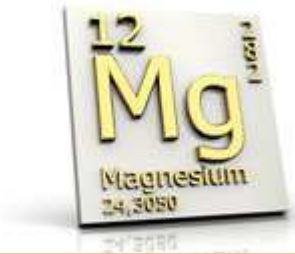
Volné deoxynukleotidy

Taq

Termostabilní DNA polymeráza

(Tag polymeráza)

Pufr

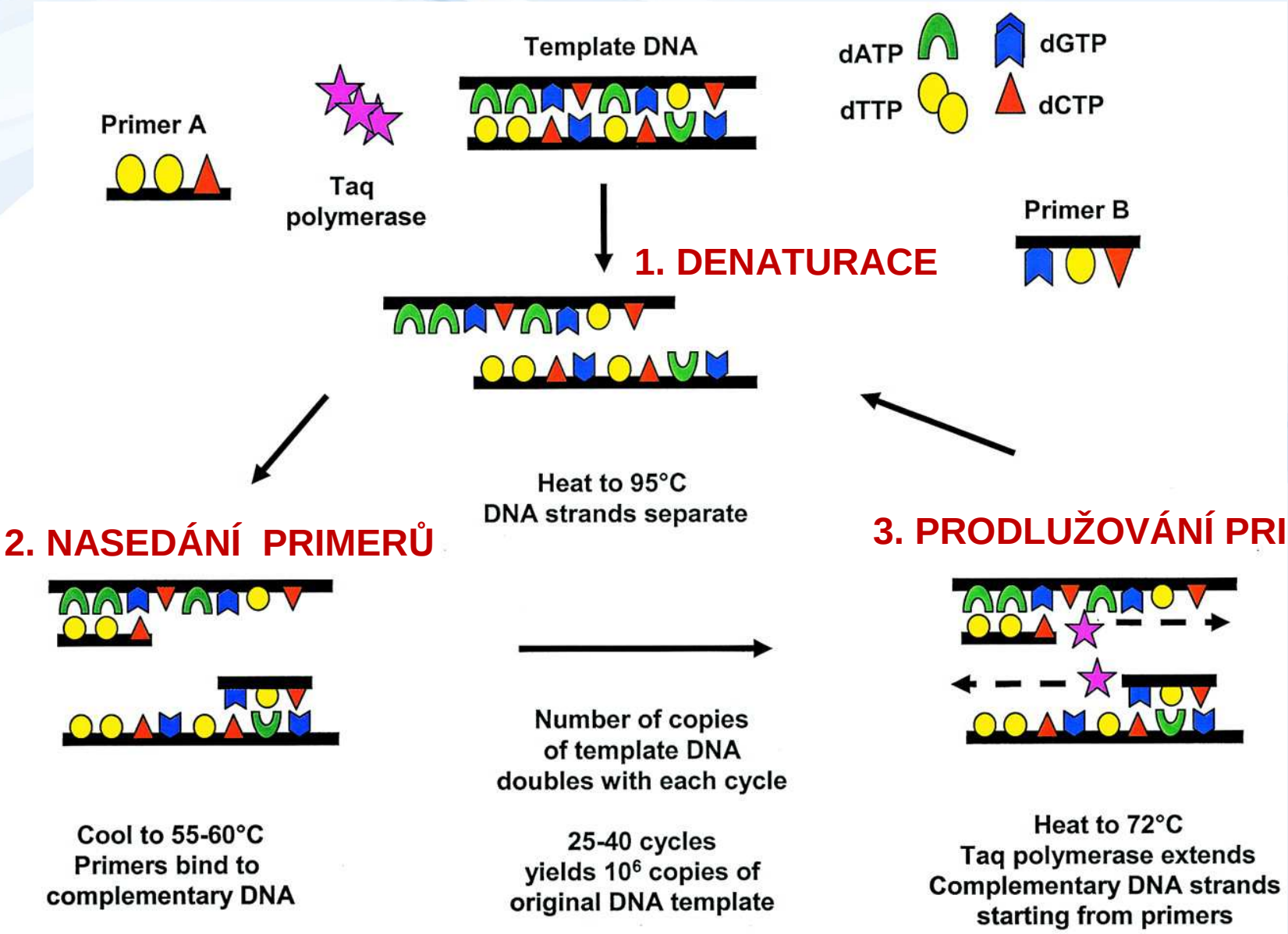


Hořčík

ddH_2O

Primery

VIDEO ⇒ http://www.dnalc.org/view/15475-The-cycles-of-the-polymerase-chain-reaction-PCR-3D-animation.html



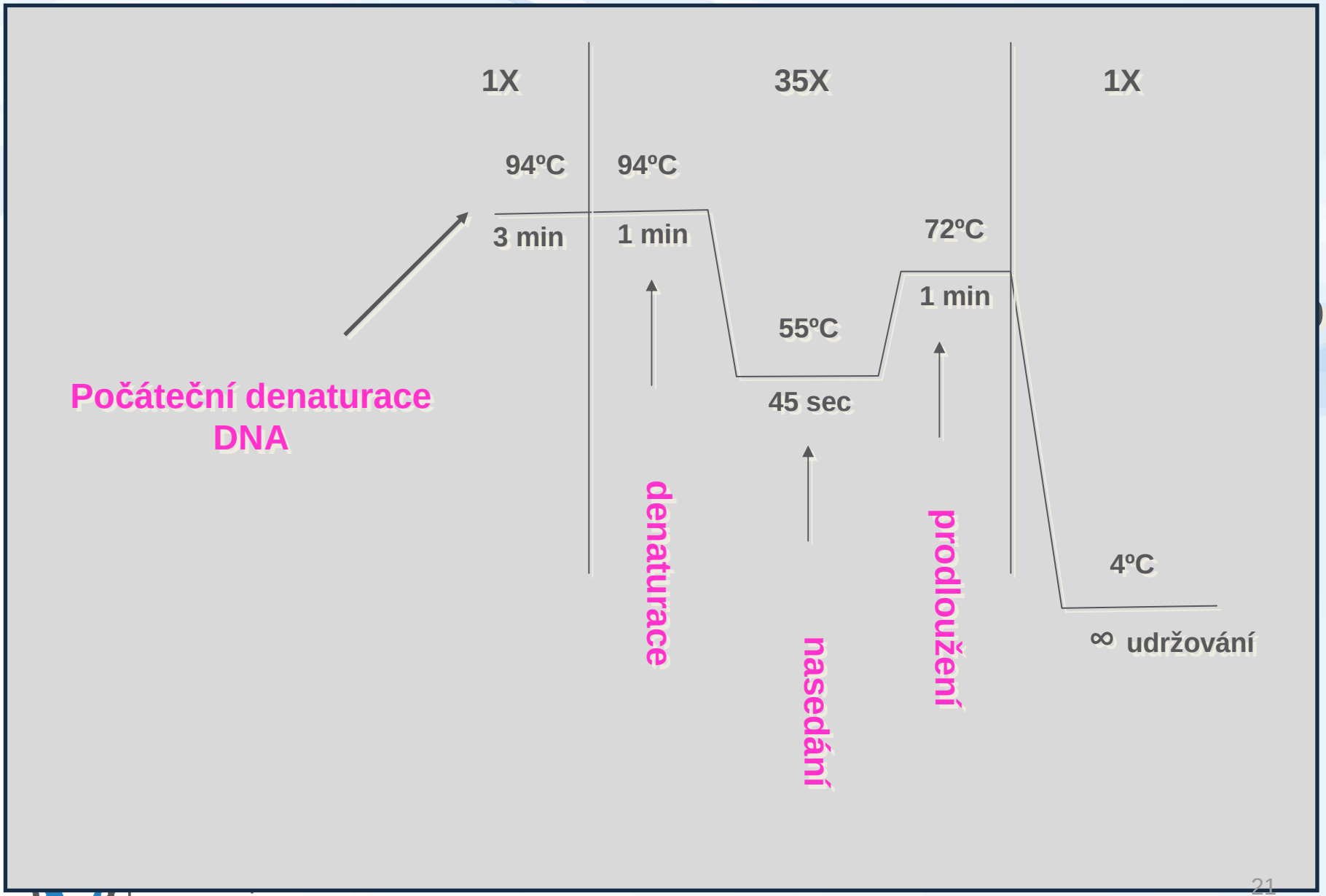
Clin. Microbiol. Rev. October 2004 vol. 17 no. 4 903-925

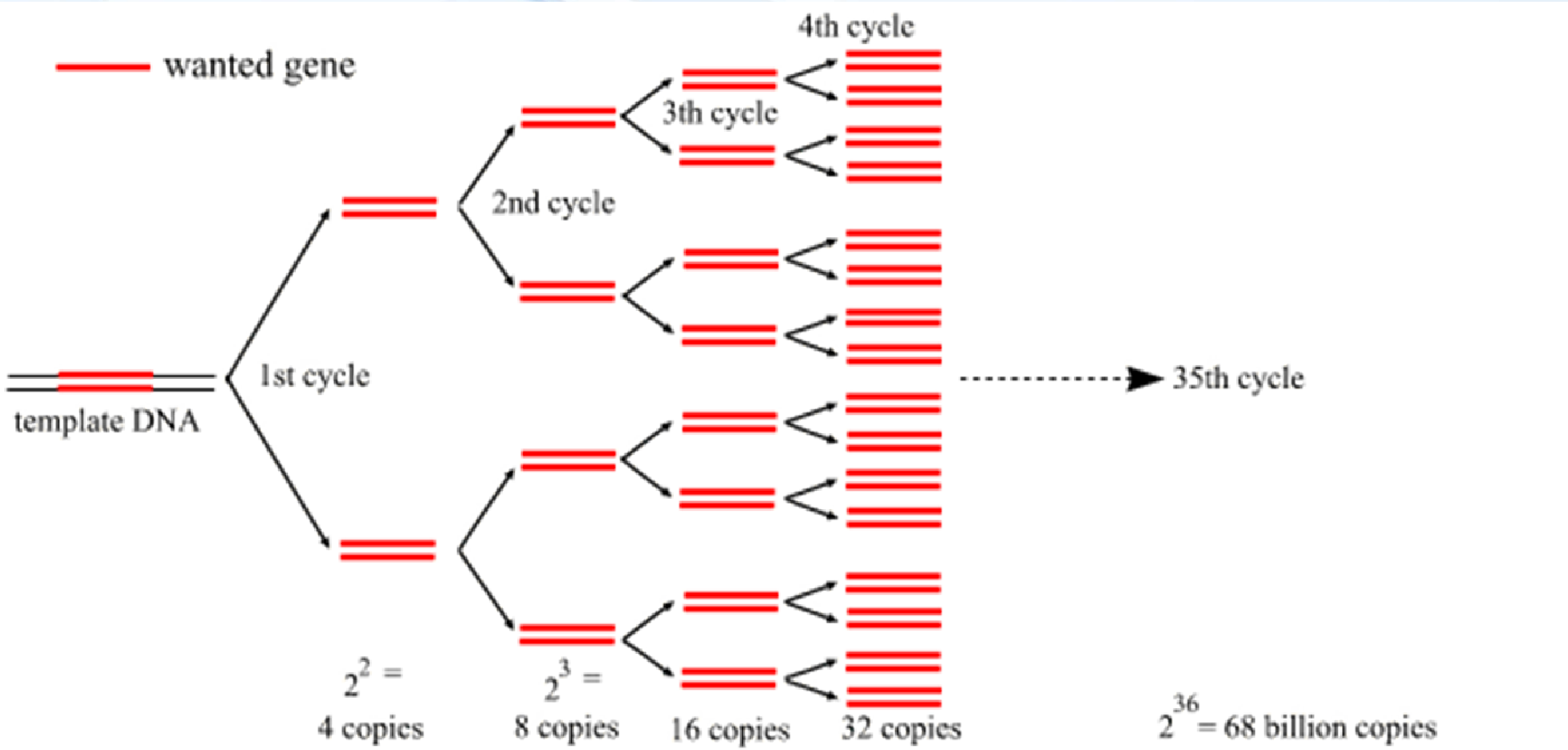
Obvyklé teploty & časy PCR

Počáteční denaturace	90º – 95º C	1 – 3 min
Denaturace	90º – 95º C	0.5 – 1 min
Nasedání primerů	45º – 65º C	0.5 – 1 min
Prodlužování primerů	70º – 75º C	0.5 – 2 min
Konečné prodlužování	70º – 75º C	5 – 10 min
Ukončení reakce	4º C	

20 – 40 cyklů

Obvyklé teploty & časy PCR



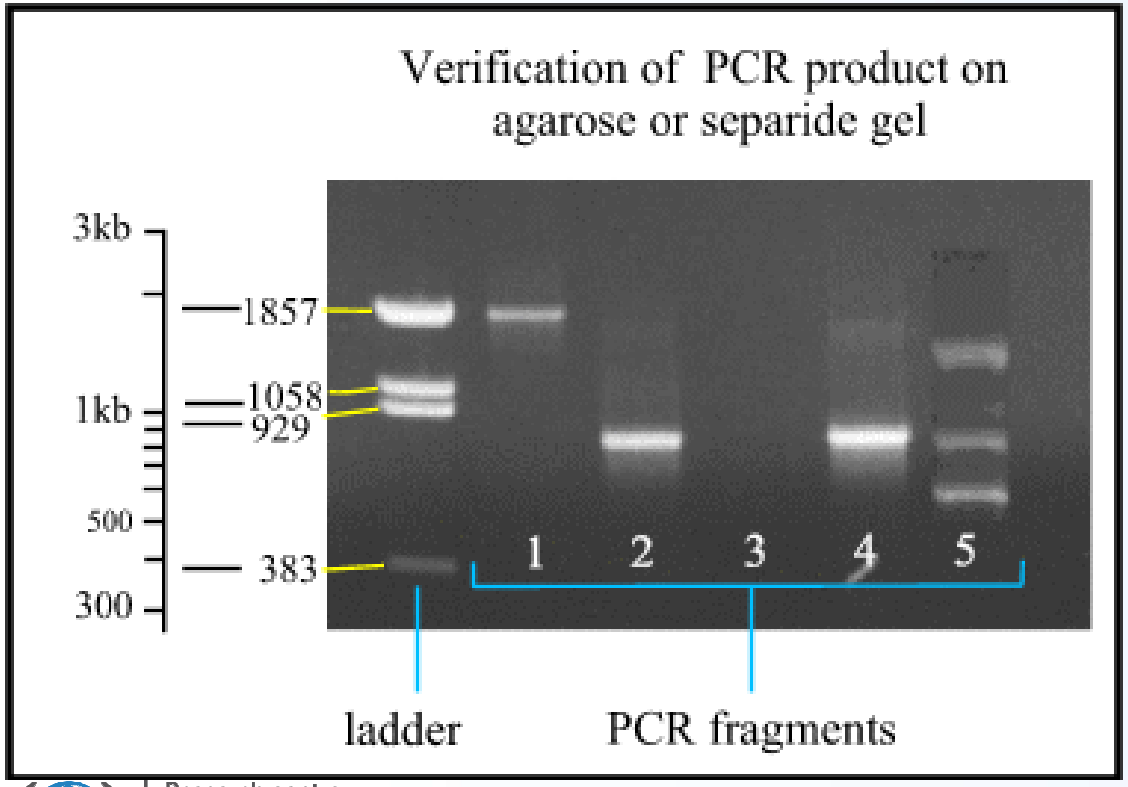


po n-tém cyklu => 2^n molekul DNA

(Andy Vierstraete 1999)

PCR: JAK DETEKUJEME NAMNOŽENÉ KOPIE DNA?

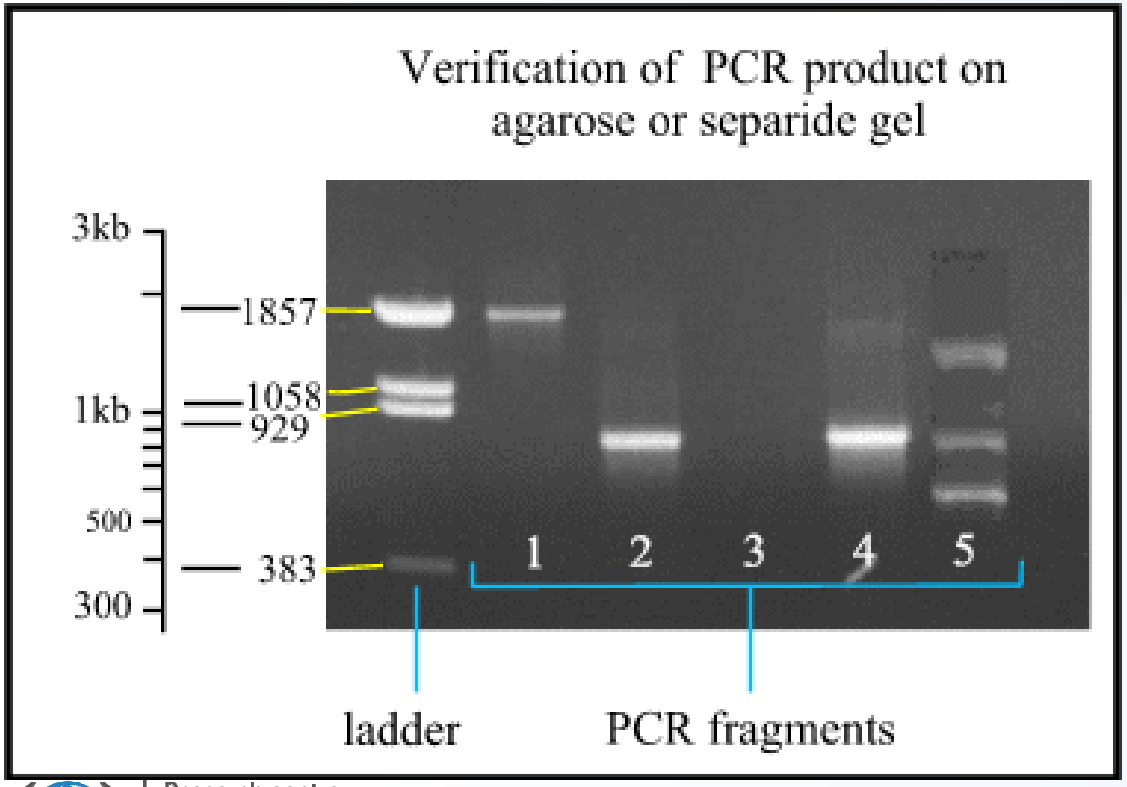
- **GELOVÁ ELEKTROFORÉZA**
  - **horizontální elektroforéza na agarózovém gelu**
  - **DNA fragmenty: 100 až 300 pb**



- potvrzení specificity fragmentu sekvenačními metodami

PCR: JAK DETEKUJEME NAMNOŽENÉ KOPIE DNA?

- **GELOVÁ ELEKTROFORÉZA**
  - **horizontální elektroforéza na agarózovém gelu**
  - **DNA fragmenty: 100 až 300 pb**



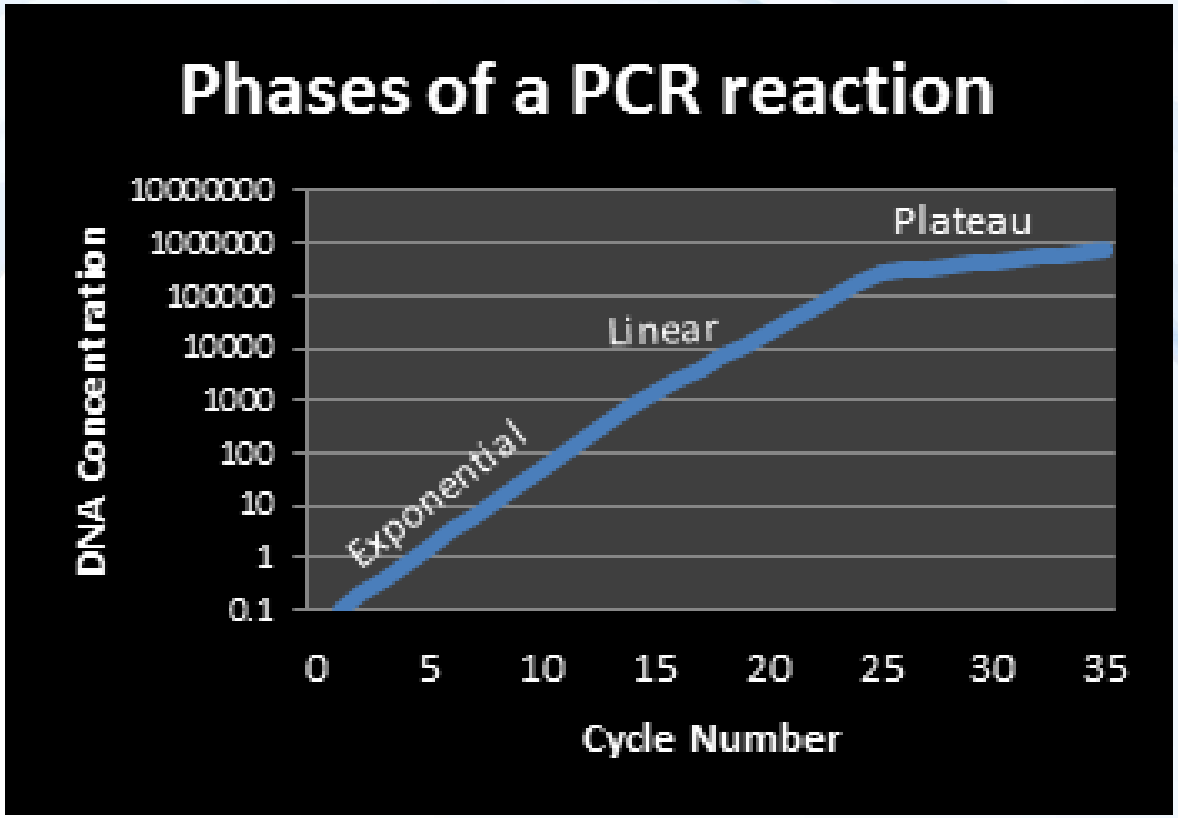
- potvrzení specificity fragmentu sekvenačními metodami

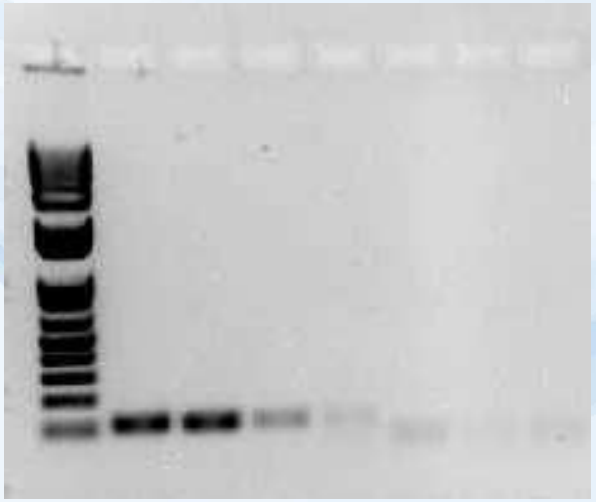
⇒KONVENČNÍ PCR („end-point“ PCR)

Research centre for toxic compounds in the environment

http://users.ugent.be/~avierstr/principles/pcr.html

KONVENČNÍ PCR: LIMITACE





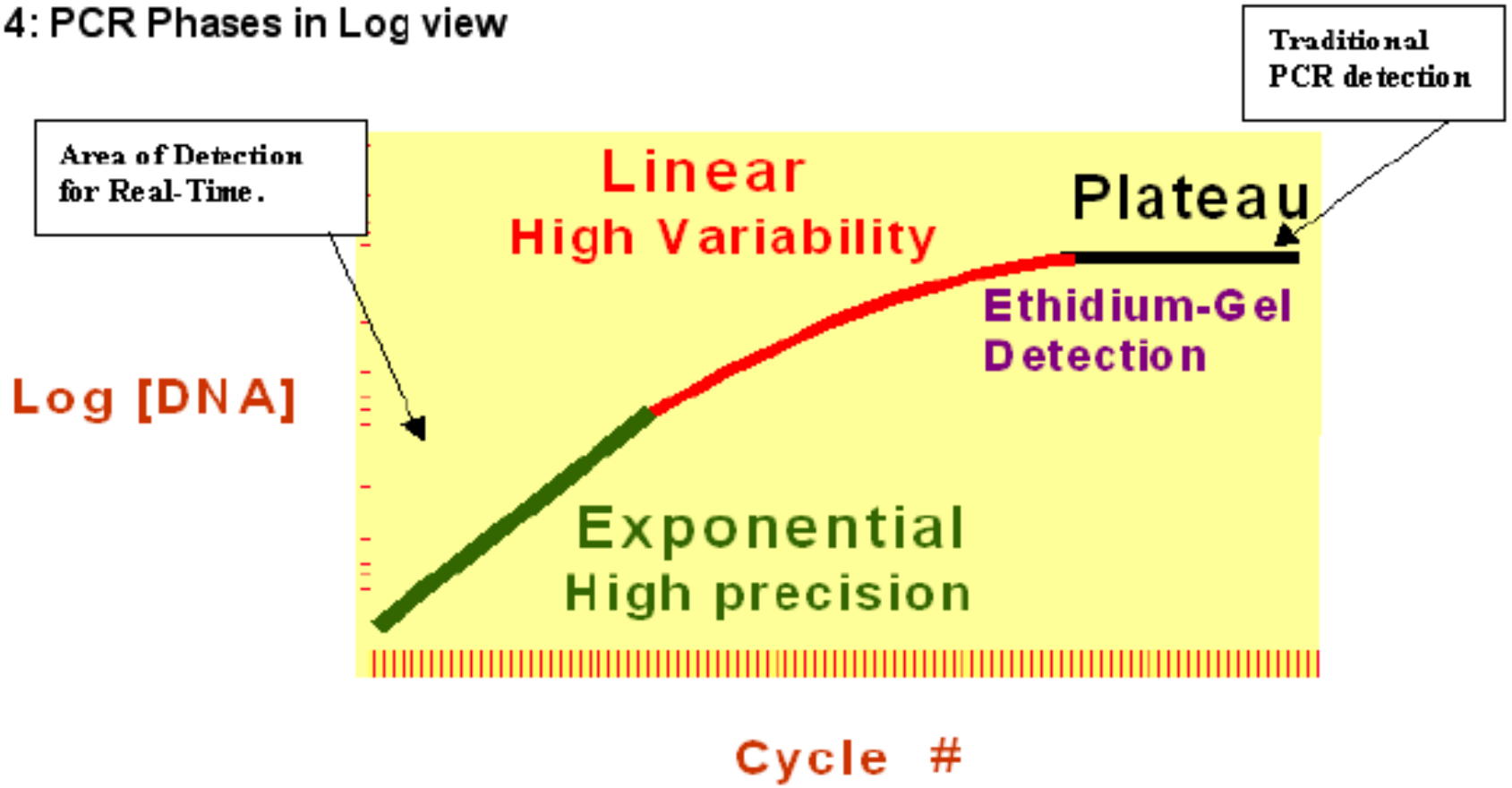
http://www.labome.com/method/Current-PCR-Methods.html

KONVENČNÍ PCR: LIMITACE

- ↓ citlivost
- ↓ rozlišení
- ↓ dynamický rozsah < 2 logs
- neautomatizovatelná
- pouze rozlišení velikosti
- ethidium bromid je spíše pro semikvantitativní barvení

qPCR: DETEKCE V REÁLNÉM ČASE
kvantitativní PCR

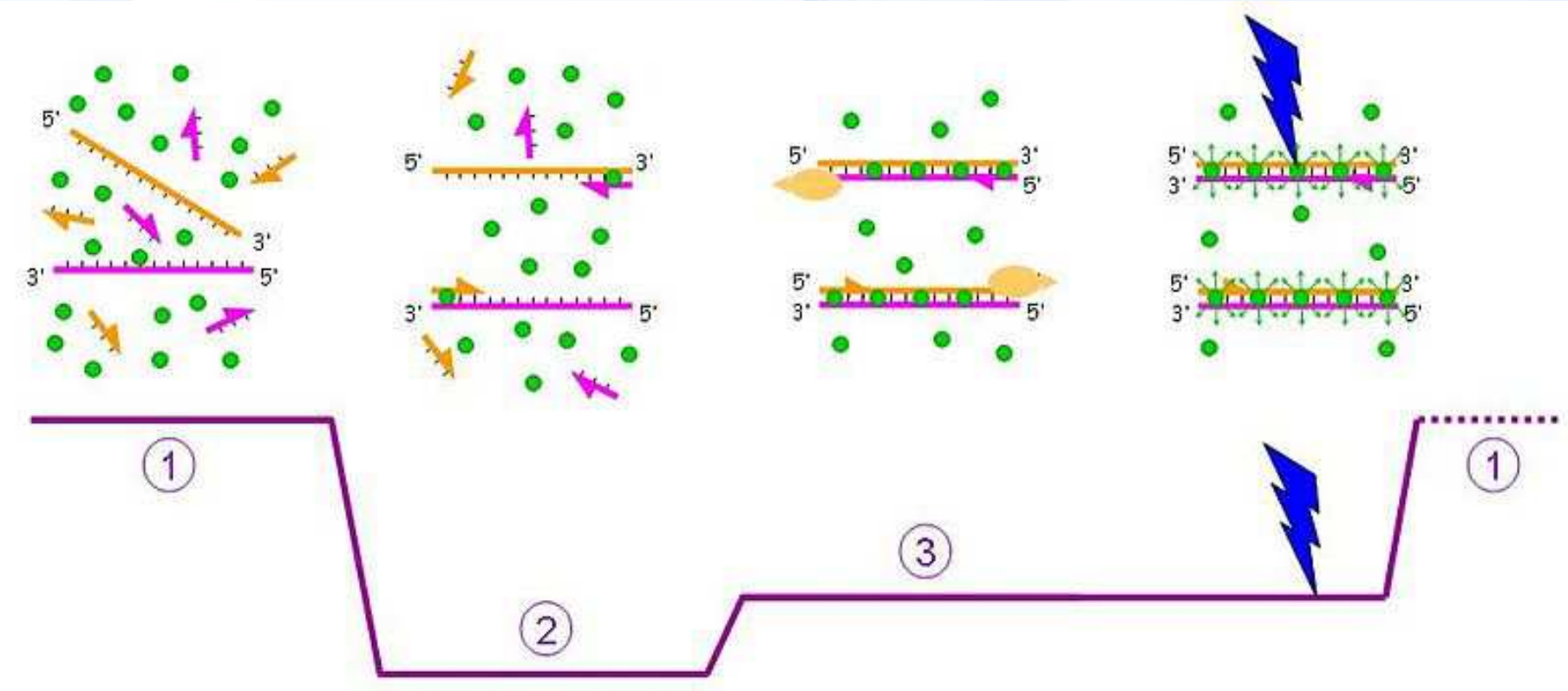
Figure 4: PCR Phases in Log view



http://www.gene-quantification.de/block.html

qPCR: SYBR GREEN I

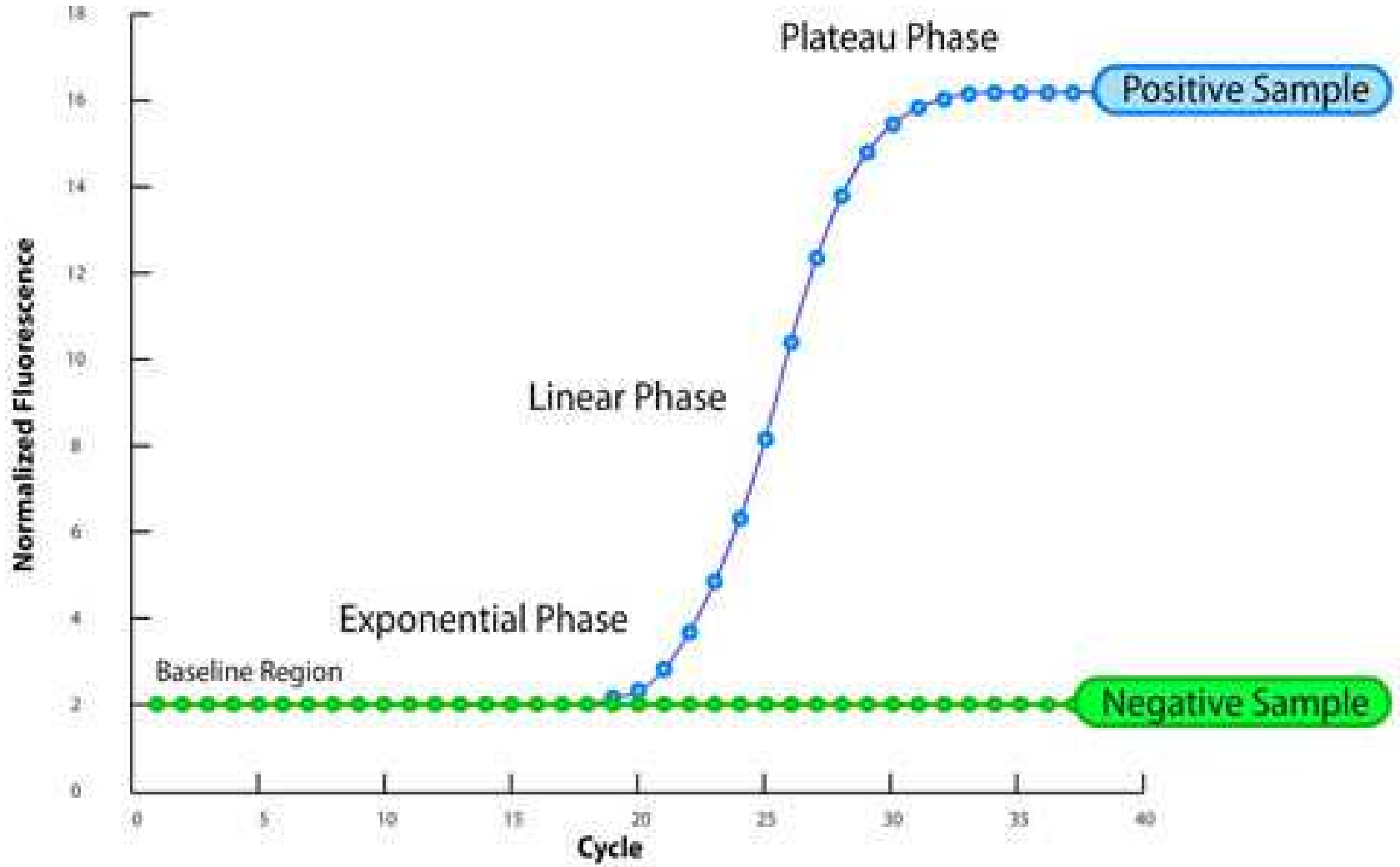
⇒ fluorescenční barva vázající se na dsDNA

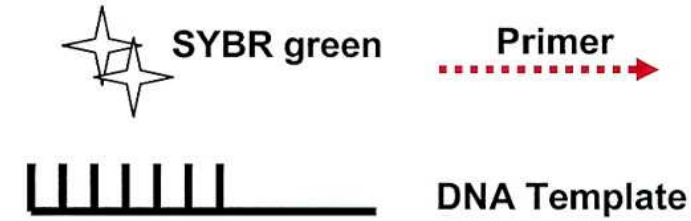


http://www.sigmaaldrich.com/life-science/molecular-biology/pcr/quantitative-pcr/sybr-green-based-qpcr/syber-green-animation.html

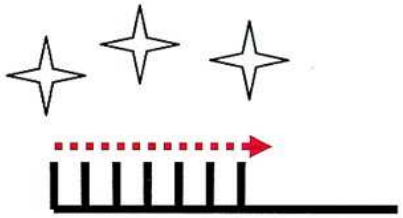
qPCR: SYBR GREEN I

⇒ fluorescenční barva vázající se na dsDNA

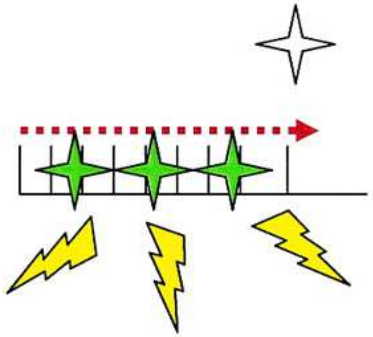




Primer Annealing



SYBR green has minimal fluorescence in the presence of single stranded DNA



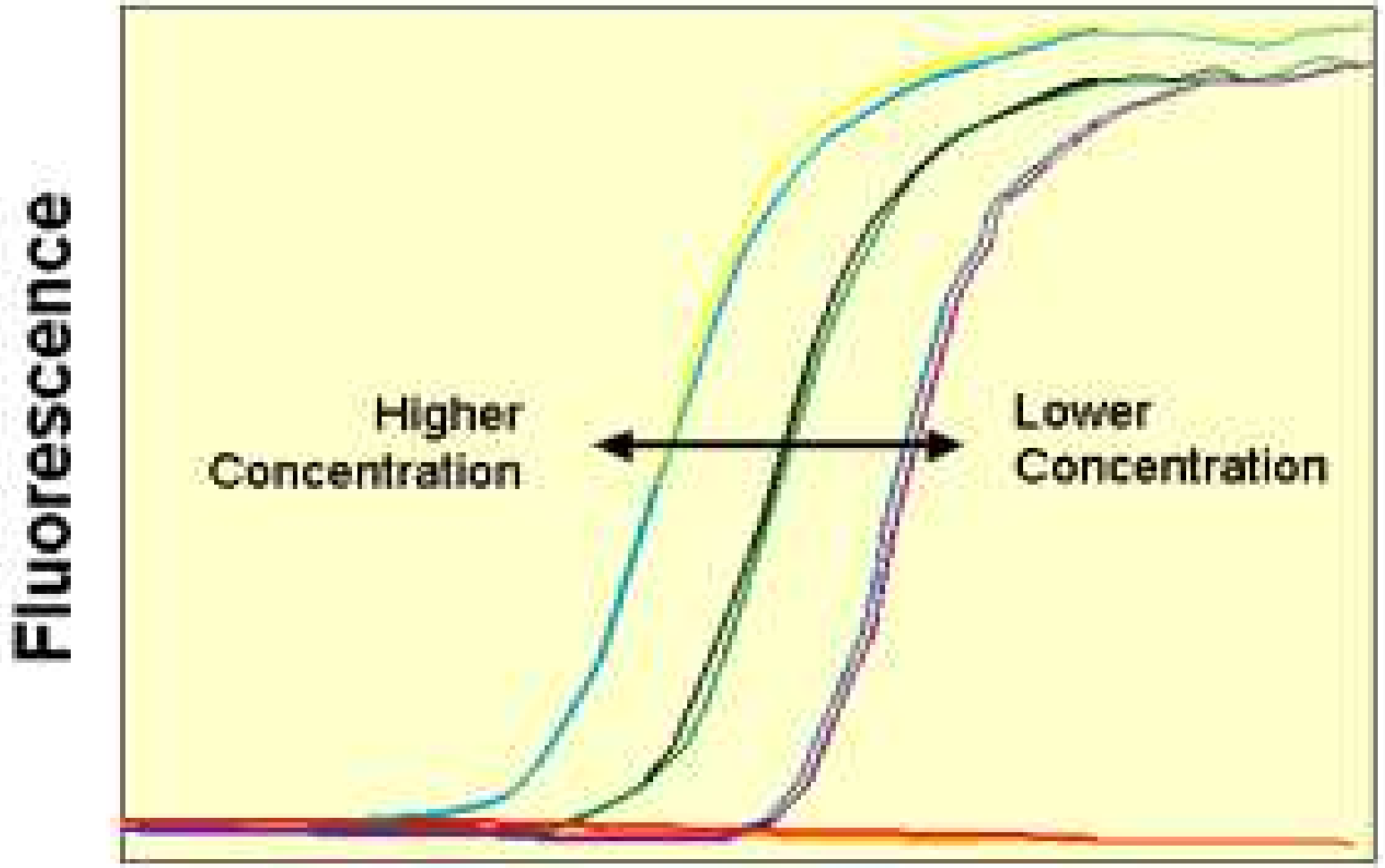
As PCR progresses, double stranded DNA is produced

SYBR green intercalates into double stranded DNA products and fluoresces

After multiple cycles, fluorescence rises above background (threshold cycle) and this is used to quantify amount of DNA template present in the original sample

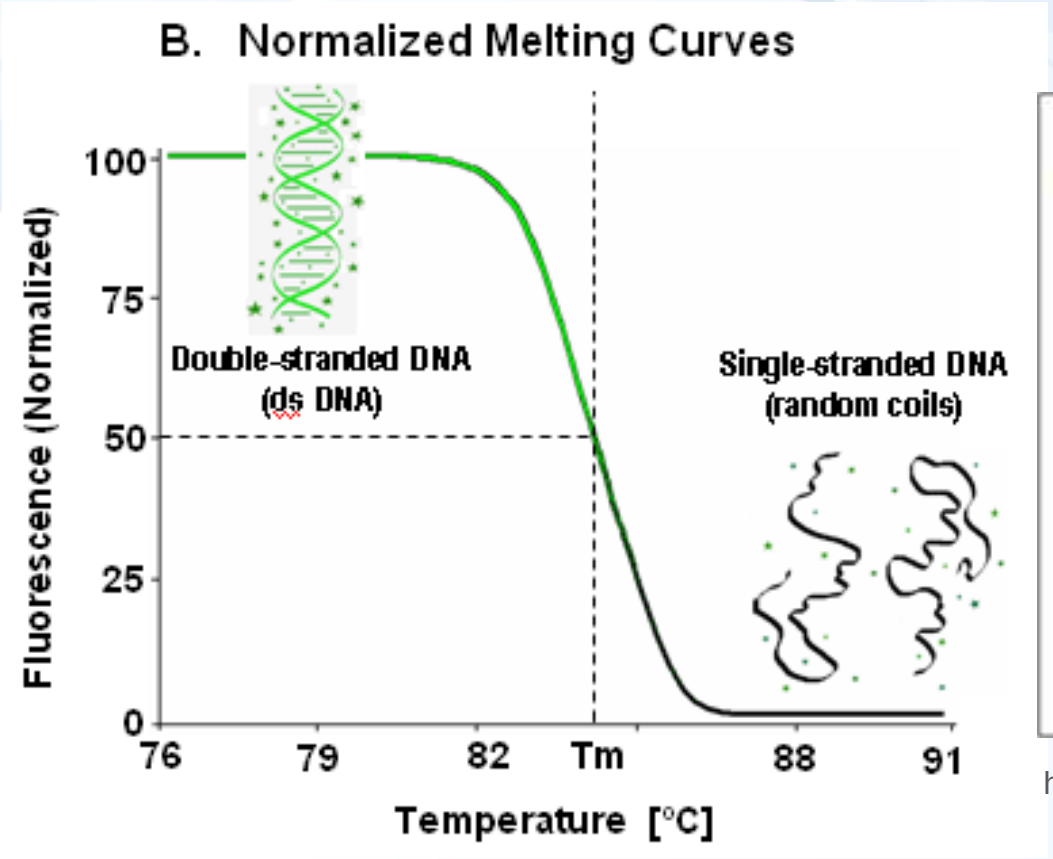
DeBiasi R L , and Tyler K L Clin. Microbiol. Rev. 2004;17:903-925

Real-Time Monitoring of PCR

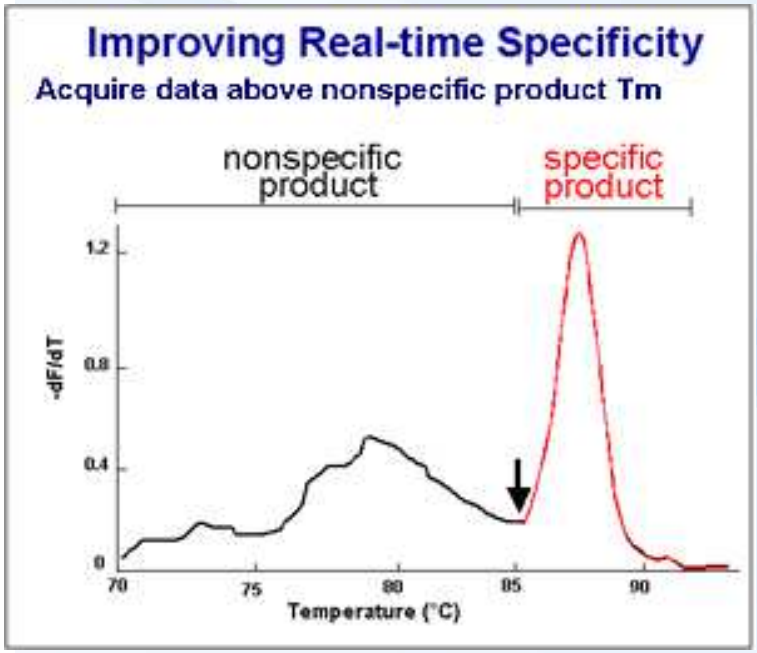


https://dna.utah.edu/LightCycler/Top_LightCycler.html

⇒ limitace ⇒ SYBRG GREEN se váže nespecificky na jakoukoliv dsDNA (“primer-dimer”, nespecifický produkt atd.)



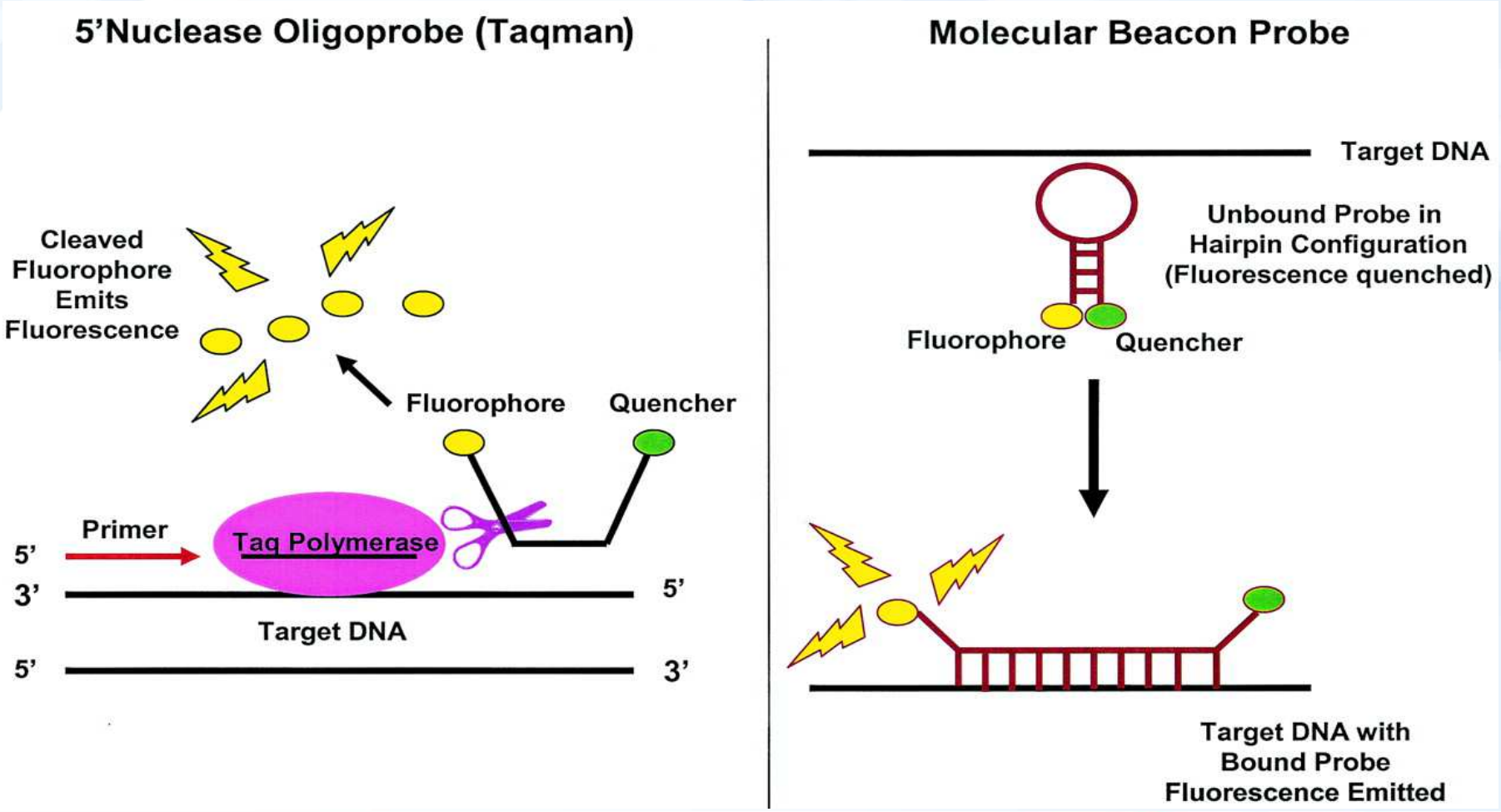
http://hrm-dyes.gene-quantification.info/



https://dna.utah.edu/LightCycler/Top_LightCycler.html

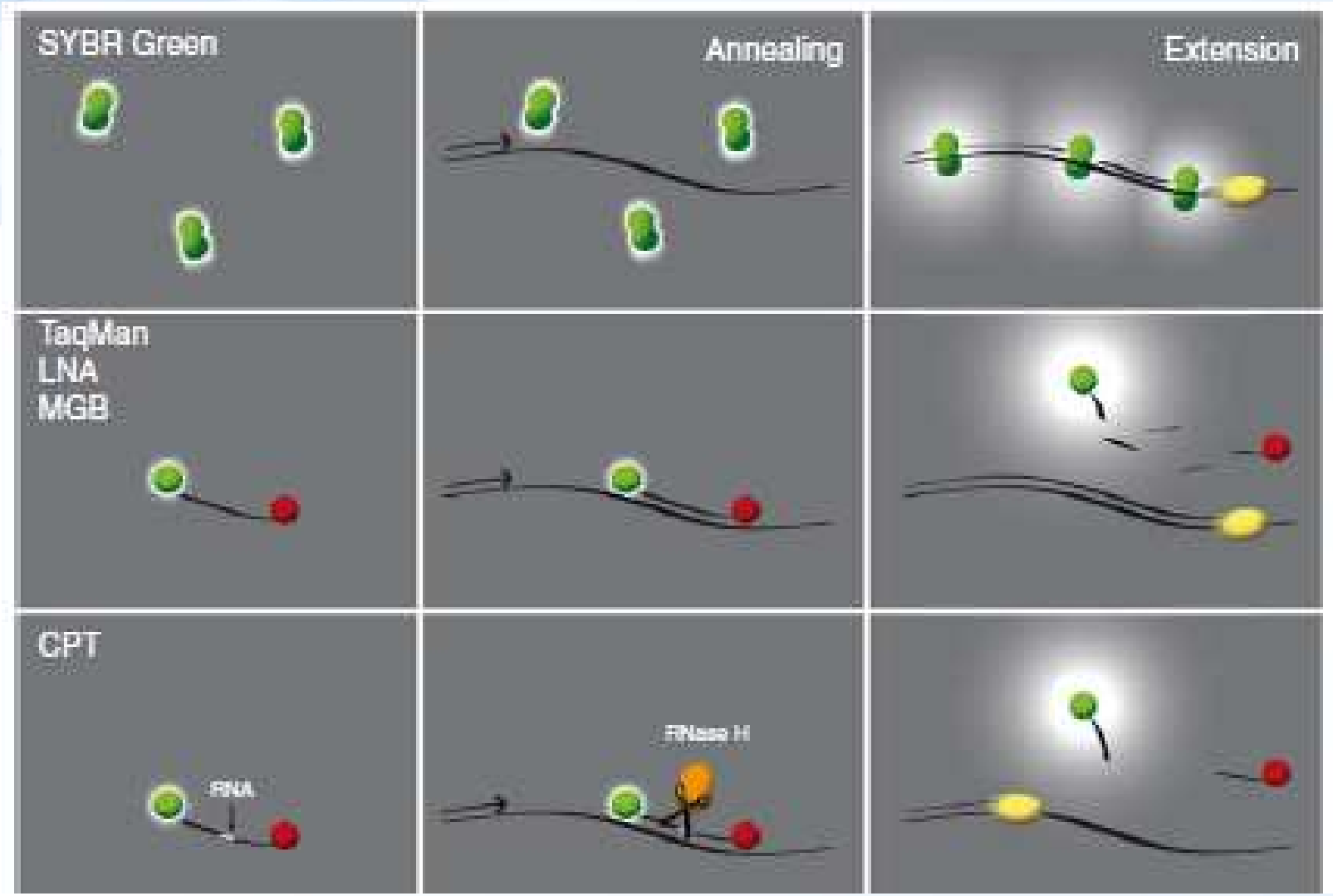
qPCR: PRÓBY

- Fluorescenčně značené próby vázající se specificky na cílový produkt PCR

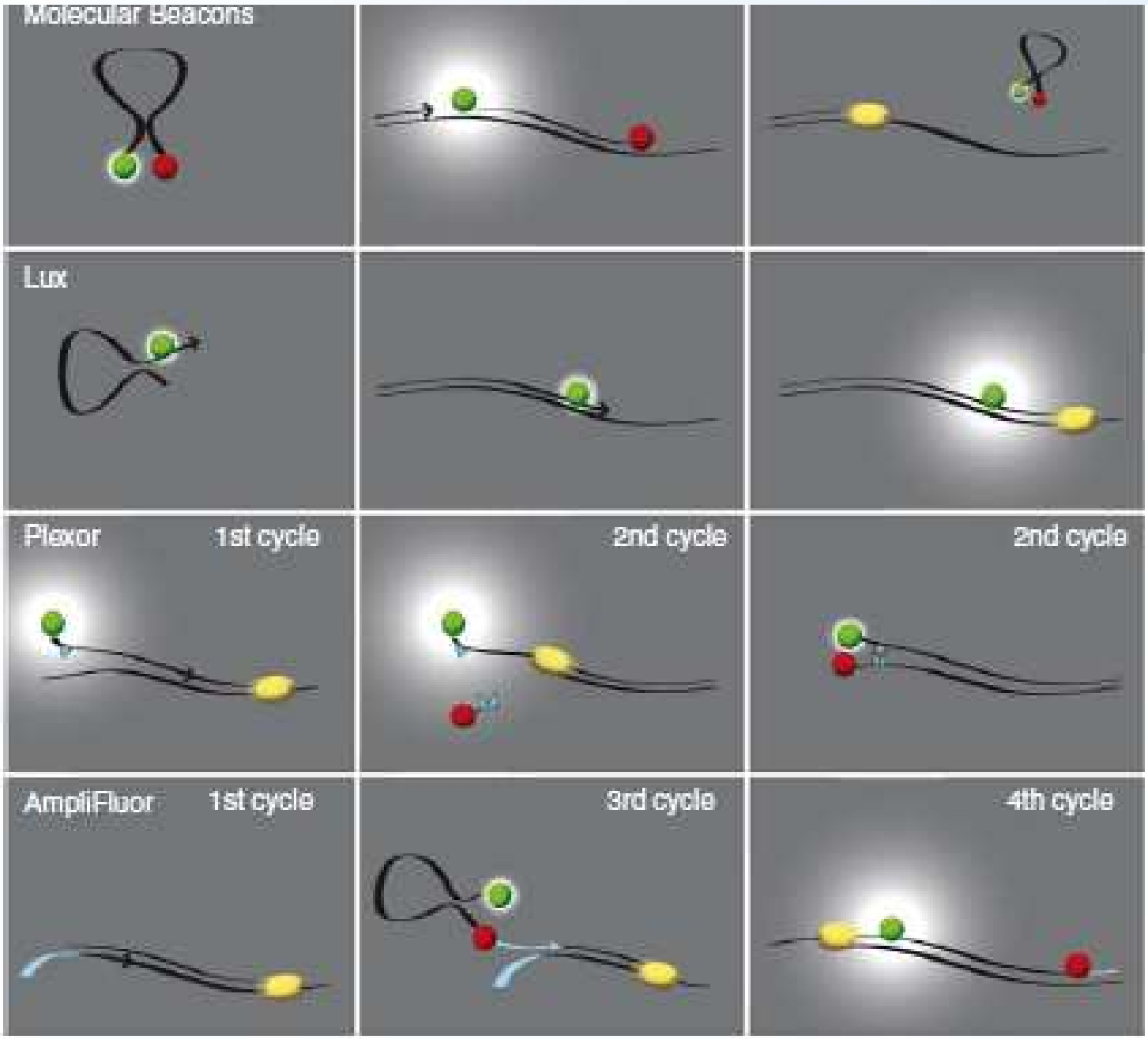


DeBiasi R L , and Tyler K L Clin. Microbiol. Rev. 2004;17:903-925

Clinical Microbiology Reviews



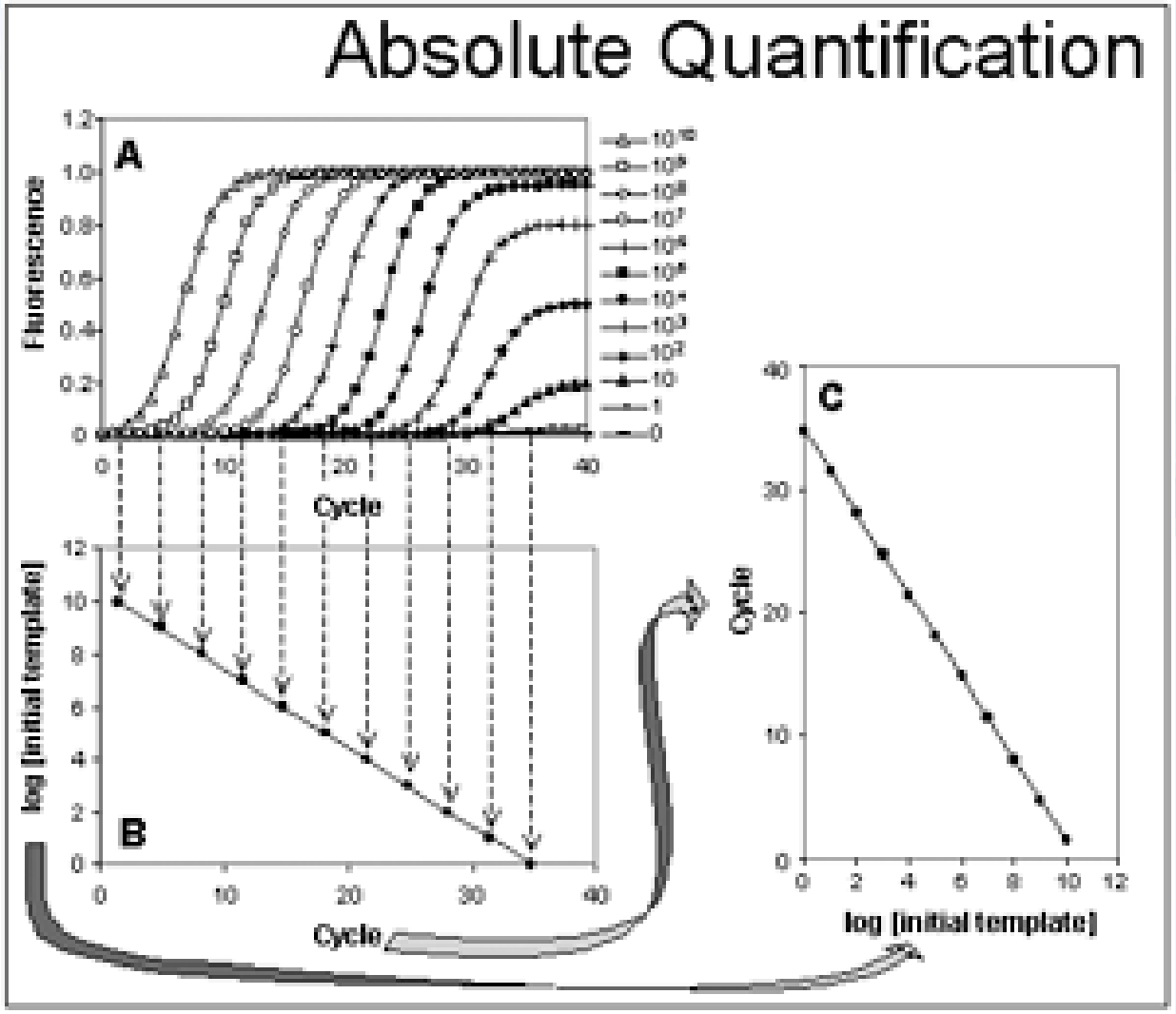
http://www.labome.com/method/Current-PCR-Methods.html



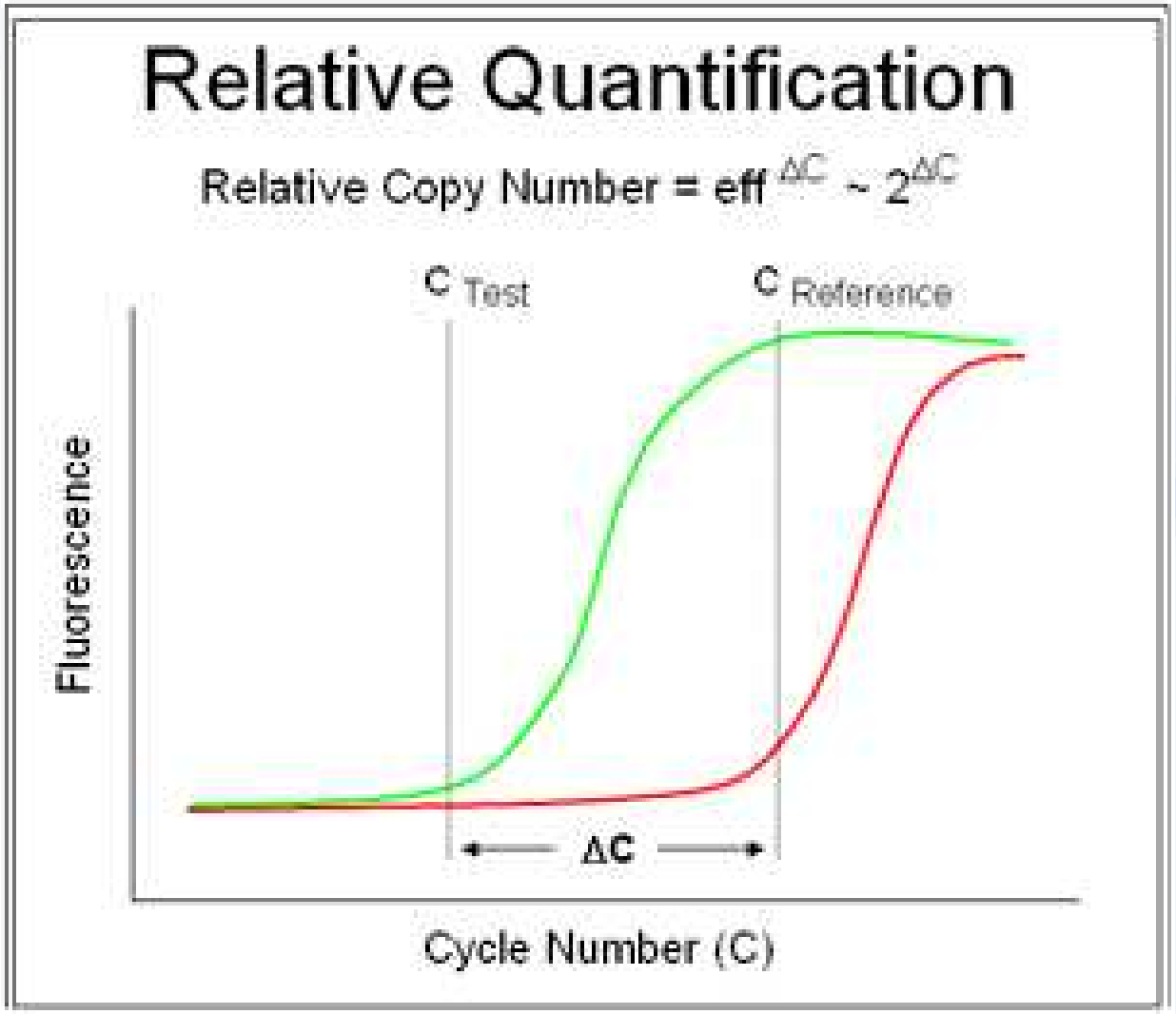
for toxic compounds
in the environment

http://www.labome.com/method/Current-PCR-Methods.html

qPCR: KVANTIFIKACE

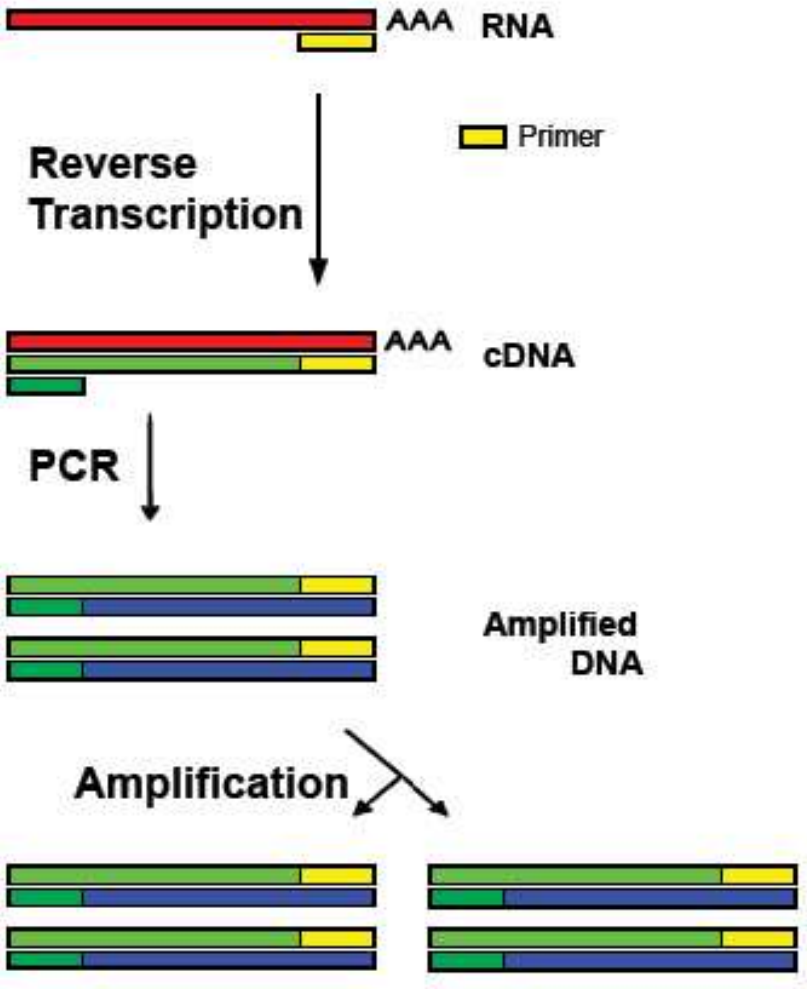


qPCR: KVANTIFIKACE



RT-(q)PCR: PCR PRO RNA

- "Reverse transcription polymerase chain reaction"



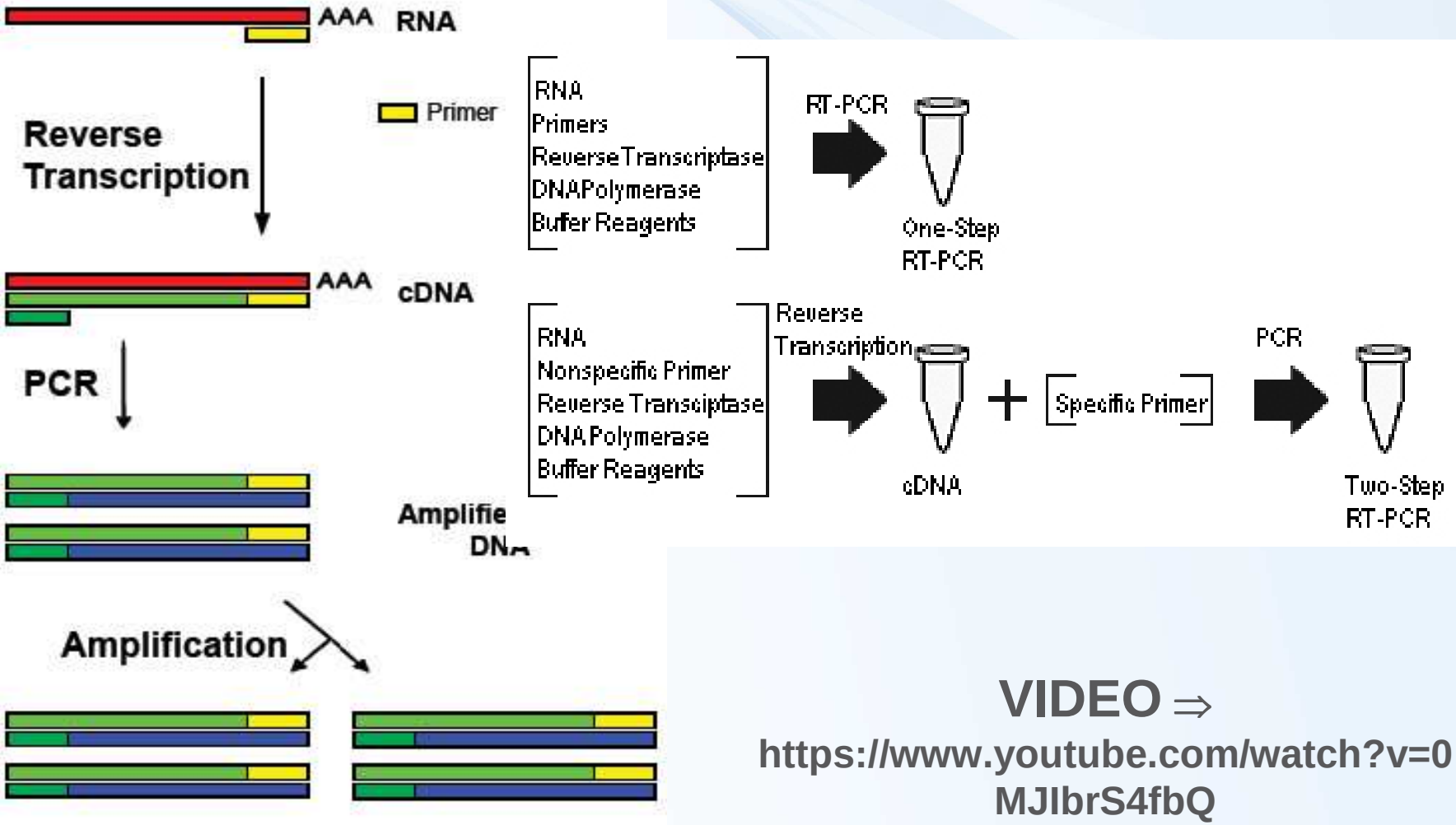
- **RNA**
(total, mRNA, miRNA)
- **Reverzní transkriptáza**
- **Primery pro RT:**
  - **oligodT**
  - **specifický primer**
  - **náhodný primer**

VIDEO ⇒
https://www.youtube.com/watch?v=0MJIbrS4fbQ

http://en.wikipedia.org/wiki/Reverse_transcription_polymerase_chain_reaction

RT-(q)PCR: PCR PRO RNA

- "Reverse transcription polymerase chain reaction"

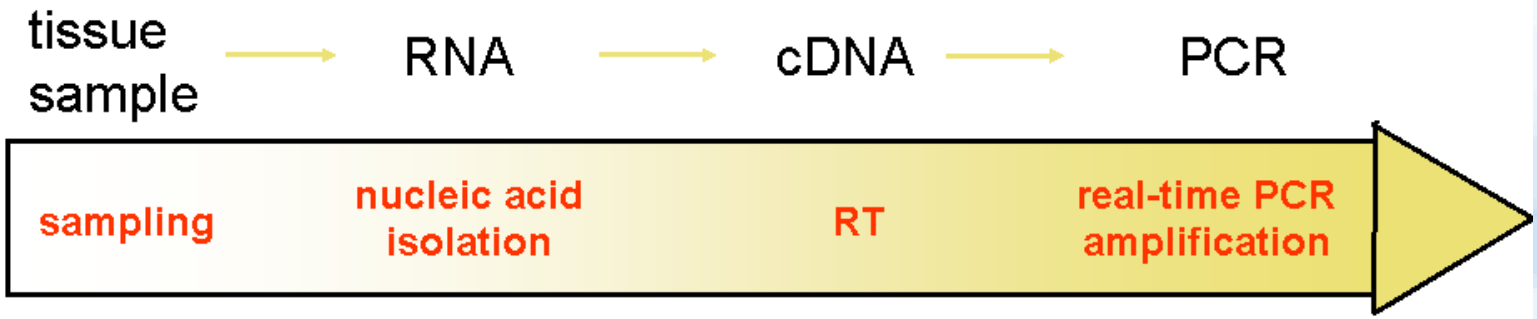


VIDEO ⇒
https://www.youtube.com/watch?v=0MJIbrS4fbQ

http://en.wikipedia.org/wiki/Reverse_transcription_polymerase_chain_reaction

RT-(q)PCR: KRITÉRIA ÚSPĚŠNOSTI

Steps and variables of a successful mRNA quantification using real-time RT-PCR (1)



Sampling method:

- Biopsy
- Fixed material
- Fresh blood
- Tissue storage
- Liquid Nitrogen
- RNA Later
- 1st extraction buffer
- RNA storage –80°C

  => native RNA

Extraction method:

- total RNA
  - mRNA
  - microRNA
- liquid-liquid
- columns
- Robot vs. hand made
- **RNA integrity:**
  - Bioanalyzer 2100
  - Experion
  - Nano-Drop
  - mFold algorithm

Efficiency of RT:

- RT enzyme type
- RT temperature
- **Primers:**
  - poly-T Primer
  - Random-hexamers
  - Specific primer
  - Primer mixtures
- **one-step qRT-PCR**
- **two-step RT-qPCR**

PCR Efficiency / Specificity:

- Primer design
  - Primer specificity
  - Consensus Primer
- mRNA abundance
- RNA / cDNA input
- Polymerase types
- Polymerase Mixtures
- PCR Inhibitors & Enhancers
- Robot vs. hand made

http://www.gene-quantification.de/optimization2.html#overview

RT-(q)PCR: KRITÉRIA ÚSPĚŠNOSTI

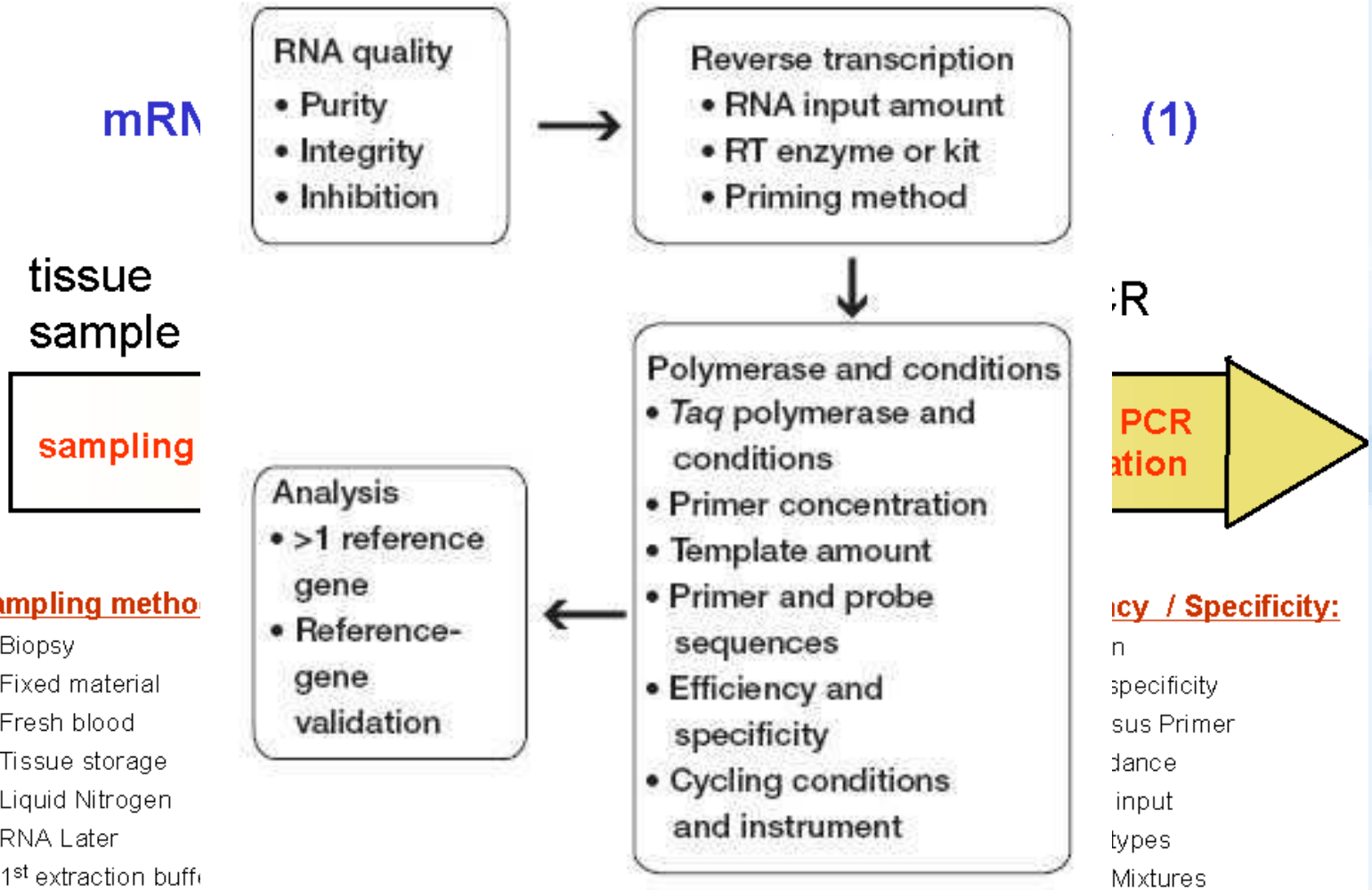


Figure 1 | Key parameters that determine the quality of qPCR data.

(RT)-(q)PCR: KONTROLY

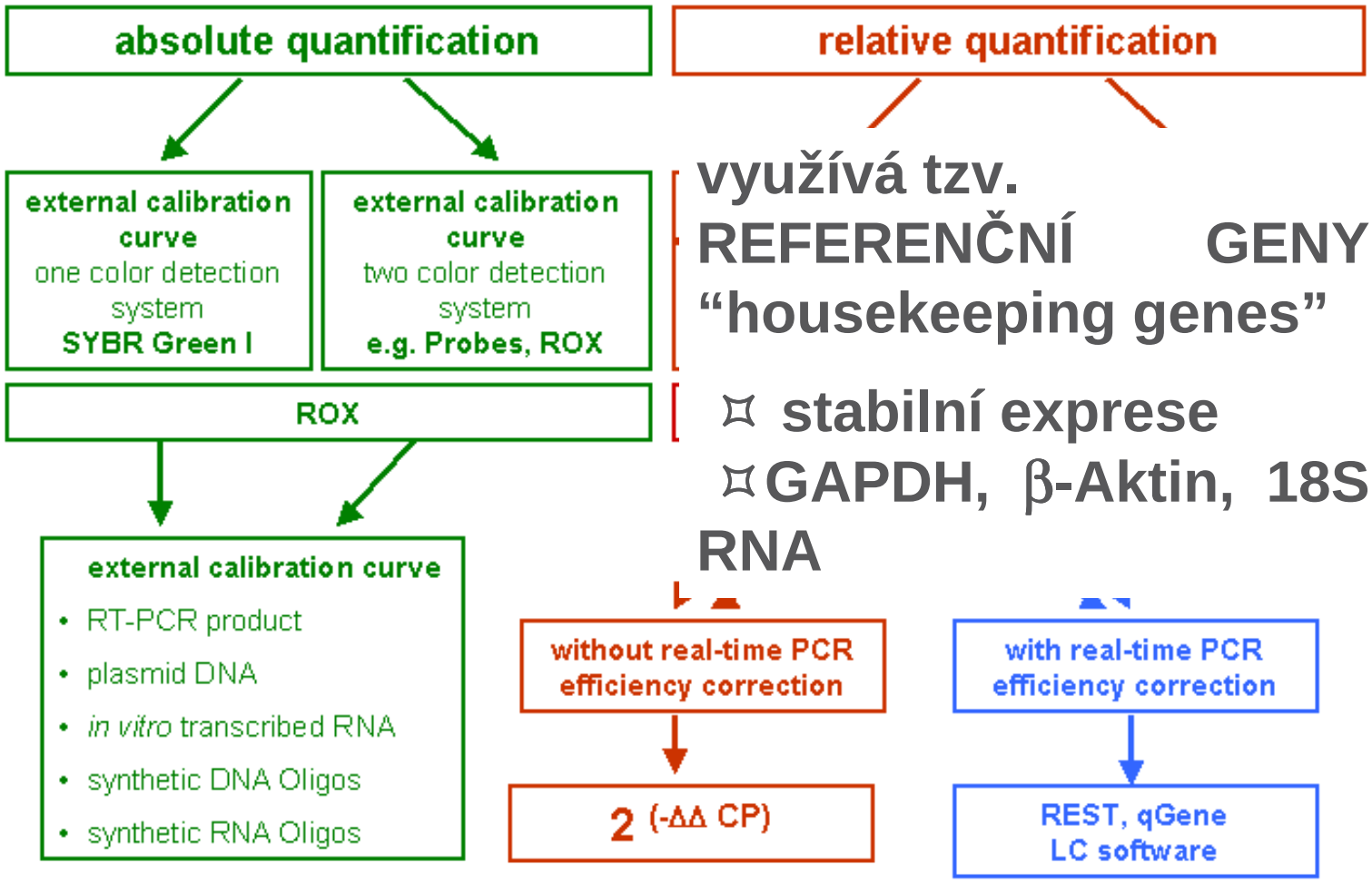
- **KONTROLA „no-RT“ ⇒ bez reverzní transkriptázy**
  - kontaminace genomovou DNA (využití primerů vázajících se na introny)
  - kontaminace DNA
- **PCR KONTROLA „NTC“⇒ bez DNA templátu (DNA, cDNA)**
  - kontaminace DNA
  - nespecifické produkty
- **POZITIVNÍ KONTROLY**

http://bitesizebio.com/4074/the-pcr-controls-you-must-use/

RT-(q)PCR: KVANTIFIKACE

Quantification Strategies in real time qRT-PCR

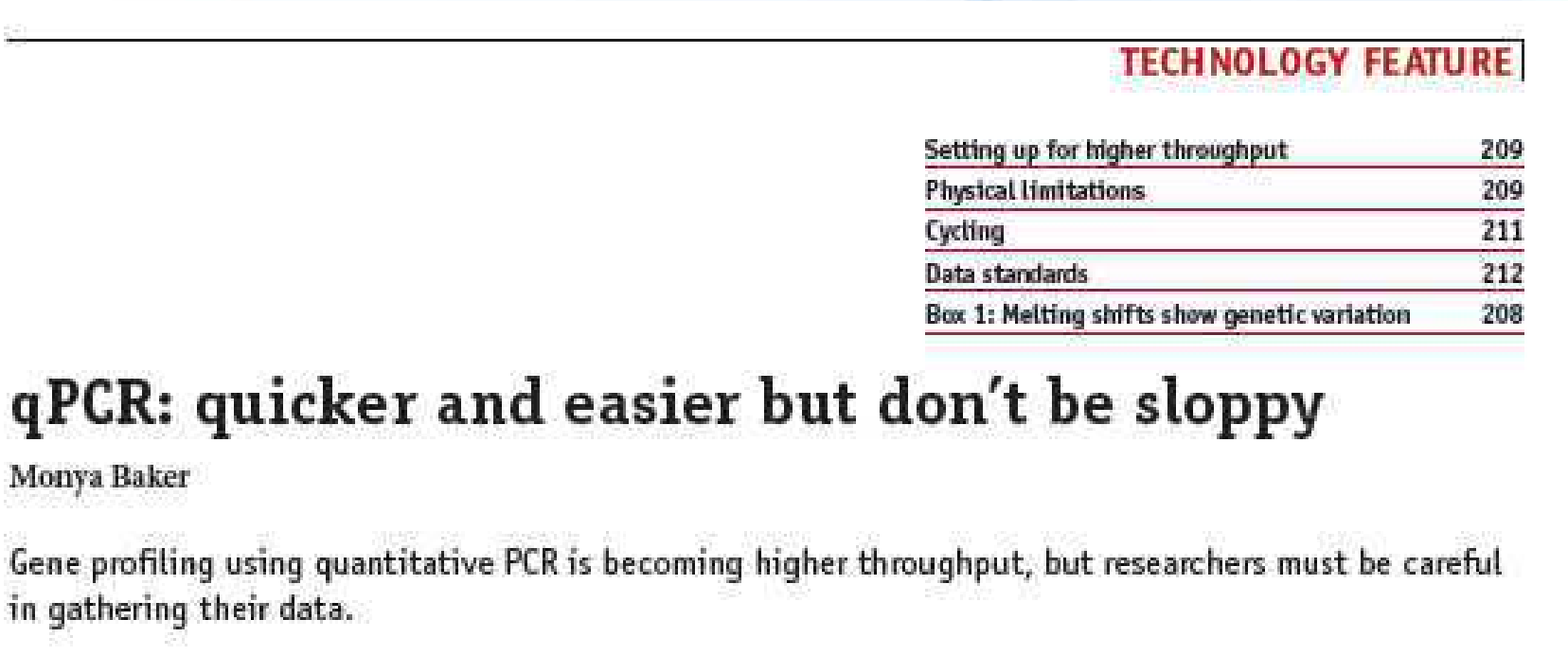
M.W. Pfaffl, BioSpektrum 2004 (Sonderausgabe PCR)



RT-(q)PCR: PUBLIKOVATELNOST DAT

The MIQE Guidelines - Minimum Information for Publication of Quantitative Real-Time PCR Experiments

Clinical Chemistry 2009, 55(4): 611-622
http://miqe.gene-quantification.info/
http://www.rdml.org/MIQE_checklist.pdf



TECHNOLOGY FEATURE

qPCR: quicker and easier but don't be sloppy

Monya Baker

Gene profiling using quantitative PCR is becoming higher throughput, but researchers must be careful in gathering their data.

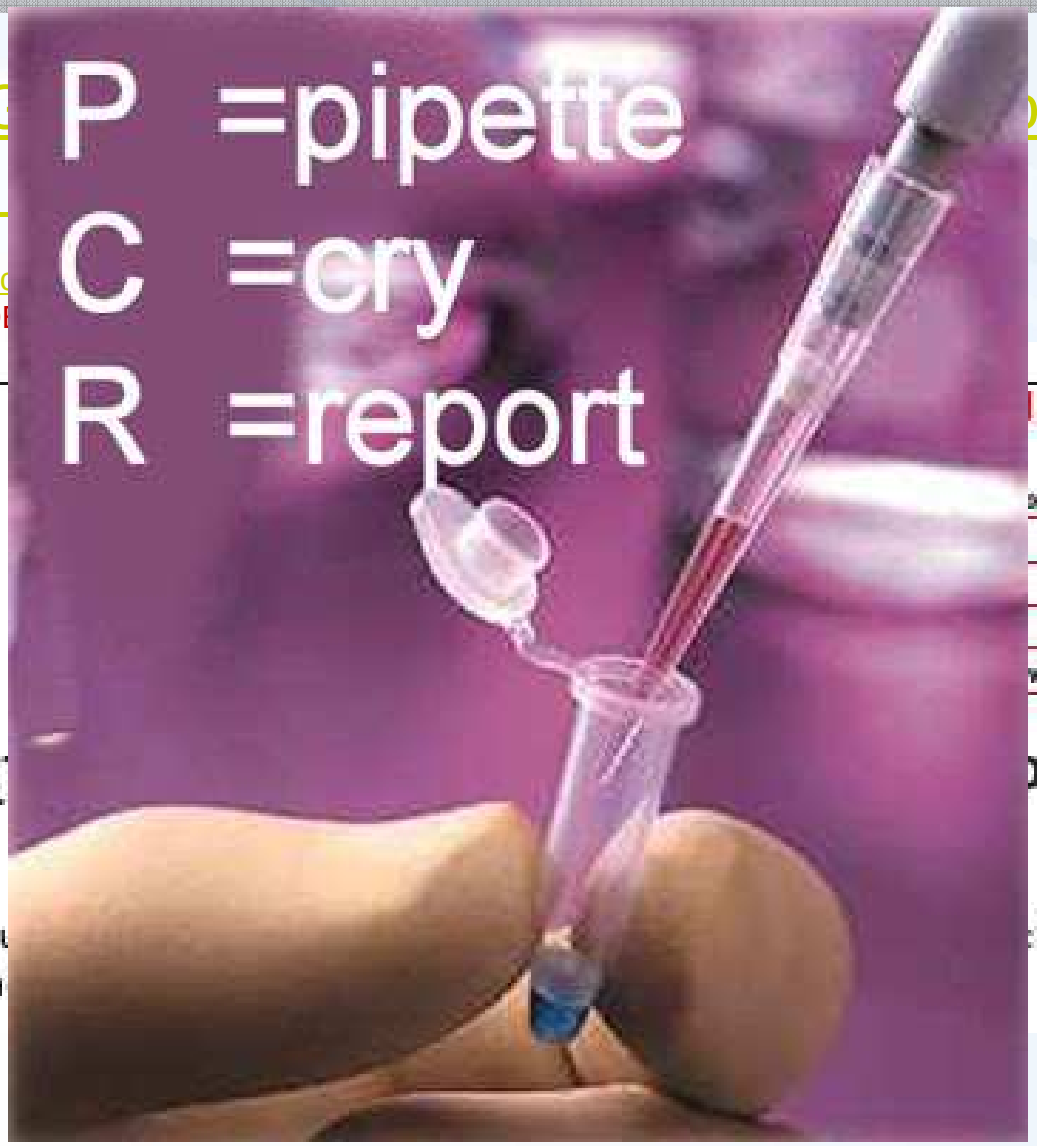
RT-(q)PCR: PUBLIKOVATELNOST DAT

The MIQE G … or Publication of Quantitative …

Clinical Chemistry 2009, …

http://miqe.gene-quantific…

http://www.rdml.org/MIQE…



…NOLOGY FEATURE

…ughput	209
	209
	211
	212
…w genetic variation	208

qPCR: q… …ppy

Monya Baker

Gene profiling … …chers must be careful in gathering th…

(q)PCR: MODIFIKACE

❑ PCR ARRAY

- více sad primerů ve 96j nebo 384 desce
- exprese až 88 genů (96j deska)
- stanovuje specifické produkty v jednotlivých jamkách desky
- referenční geny
- biologické dráhy ⇒ oprava DNA, buněčný cyklus, oxidativní stres, apoptóza, cytokiny & zánět, signální dráhy atd.

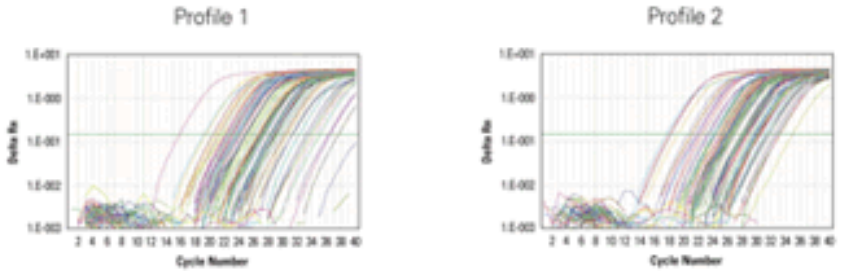
❑ PCR ARR

- více sad pr
- exprese až
- stanovuje s mkách desky
- referenční g
- biologické cyklus, oxidativní st ignální dráhy atd.

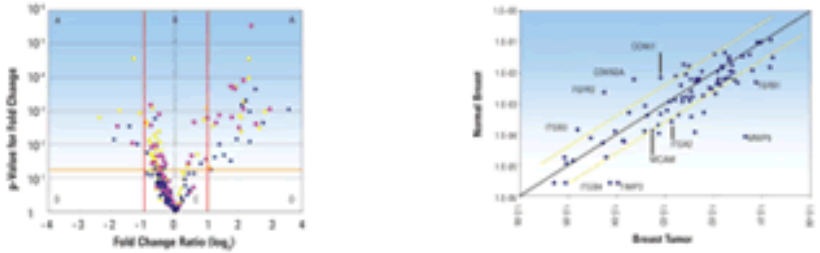
1. Convert Total RNA to cDNA.

2. Add cDNA to RT² qPCR Master Mix & Aliquot Mixture Across PCR Array.

3. Run in Your Real-Time PCR Instrument.



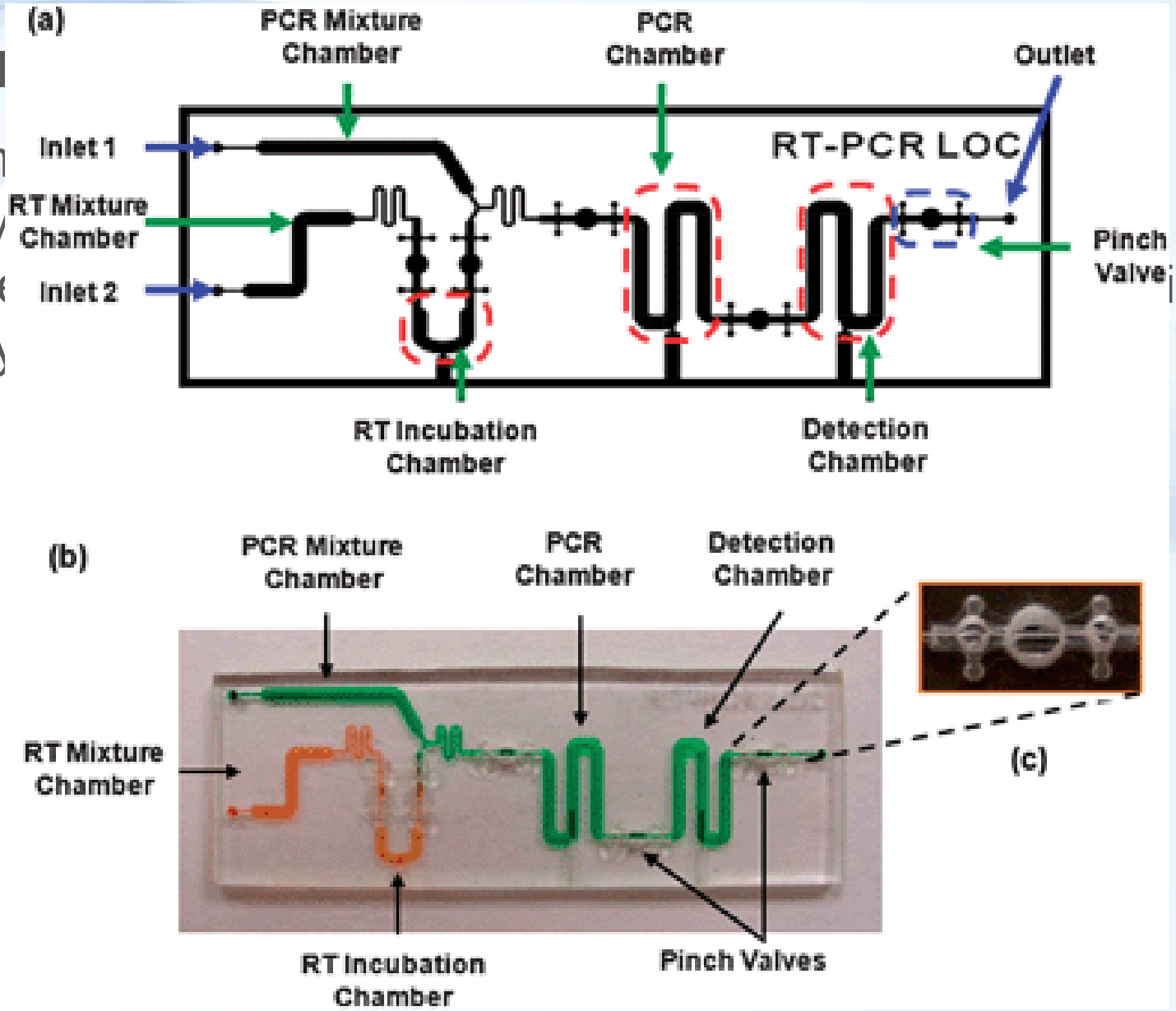
4. Data Analysis.



❑ PCR čip

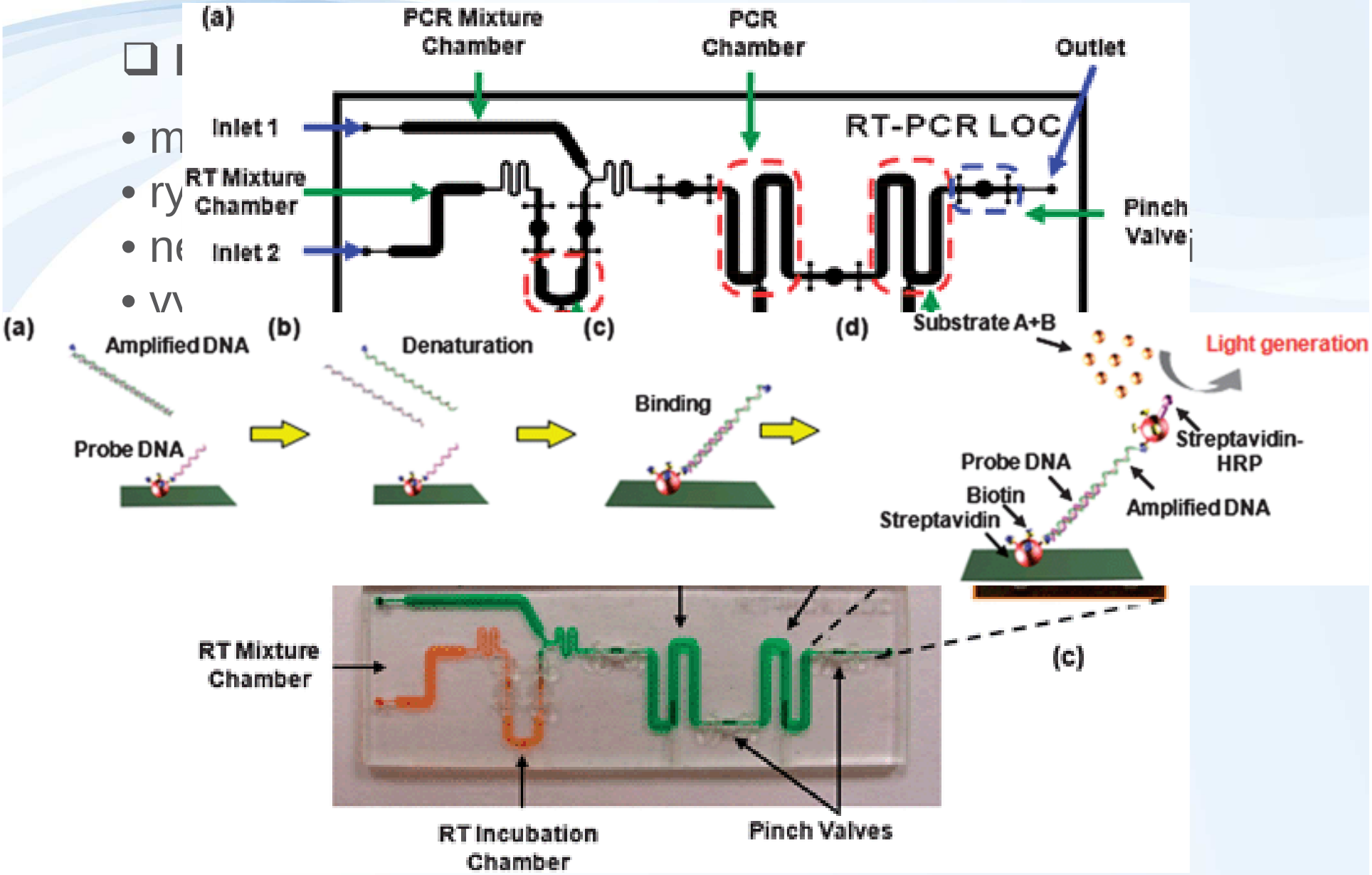
- mikrofluidní čip
- rychlejší
- není nutná standardní křivka při absolutní kvatifikaci
- vysoká citlivost

- m
- ry
- ne
- vy



Lab Chip, 2008,8, 2121-2127

- m
- ry
- ne
- vi



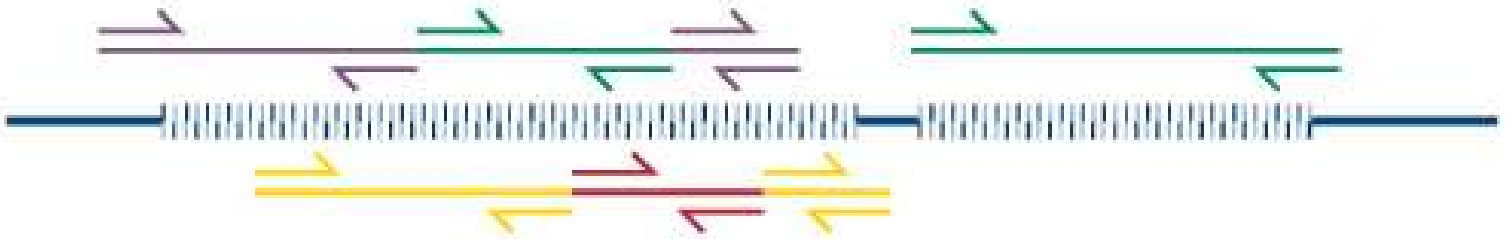
Lab Chip, 2008,8, 2121-2127

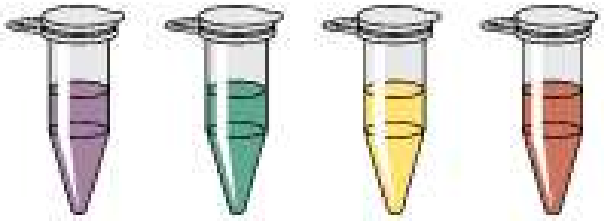
Experimental needs	PCR method	advantages	limitations
determine if a target NA sequence is present or absent in a few samples	standard or conventional	•easy access to equipment •minimal cost	•qualitative results only •post reaction handling
determine quantities of target NA in many samples	real-time	•can generate quantitative results •can be sequence specific	•more expensive than conventional •PCR speed is mid-level
determine quantities of many target NA for a few samples	PCR arrays	•up to 88 genes can be measured per sample at a time	•one array is needed per sample •costly for many samples
detect target NA in the field	microfluidic chip	•fast results •small size •portable	•specialized equipment •costly
detect very low abundance NA targets or need extremely accurate quantitation	digital and drop digital	•very precise absolute quantitation	•specialized equipment •Costly

http://www.labome.com/method/Current-PCR-Methods.html

❑ multiplex PCR

- v jedné PCR směsi vícero primerových párů

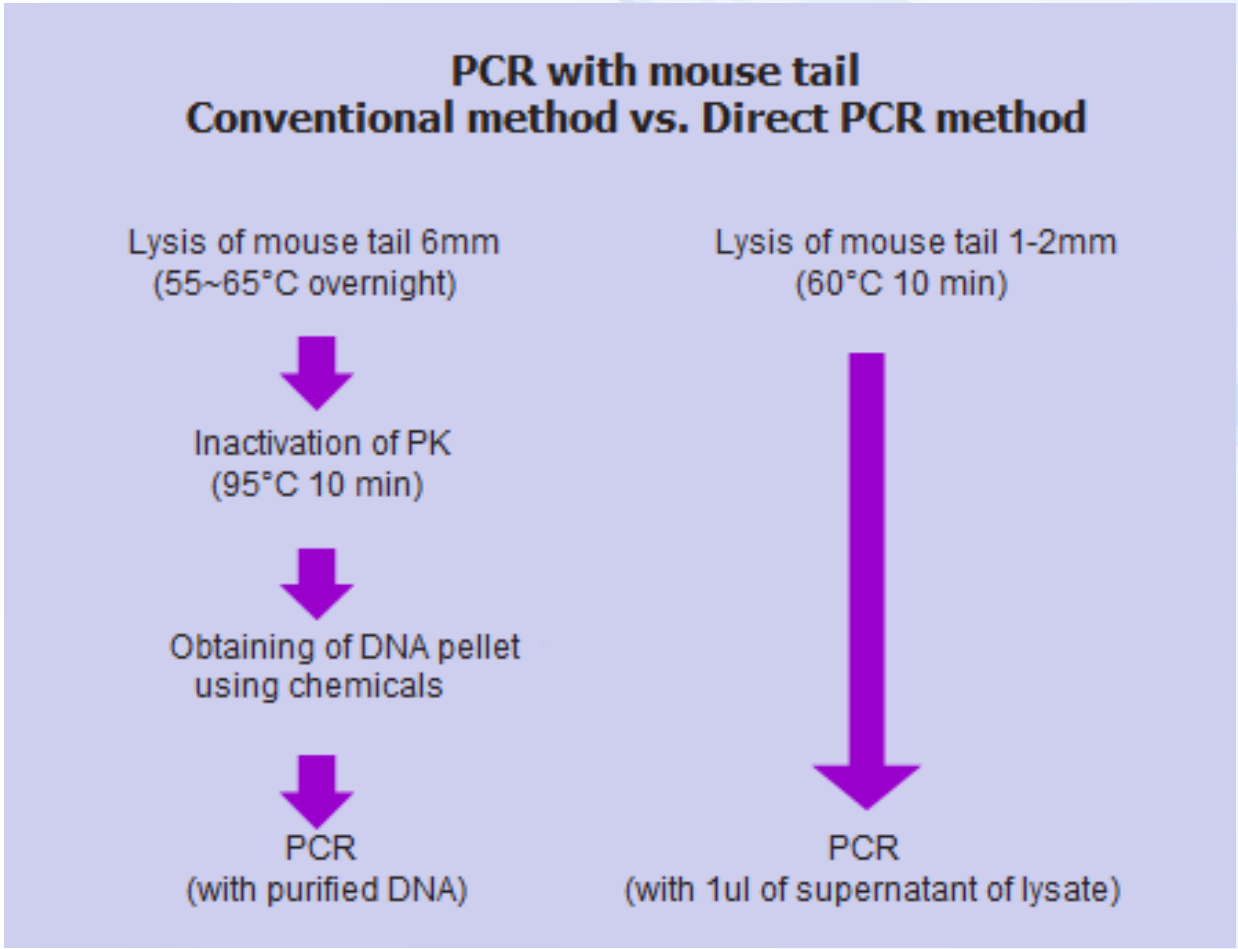




Multiplex PCR primer sets

❑ „direct“ PCR

- PCR bez přečištěné DNA nebo RNA



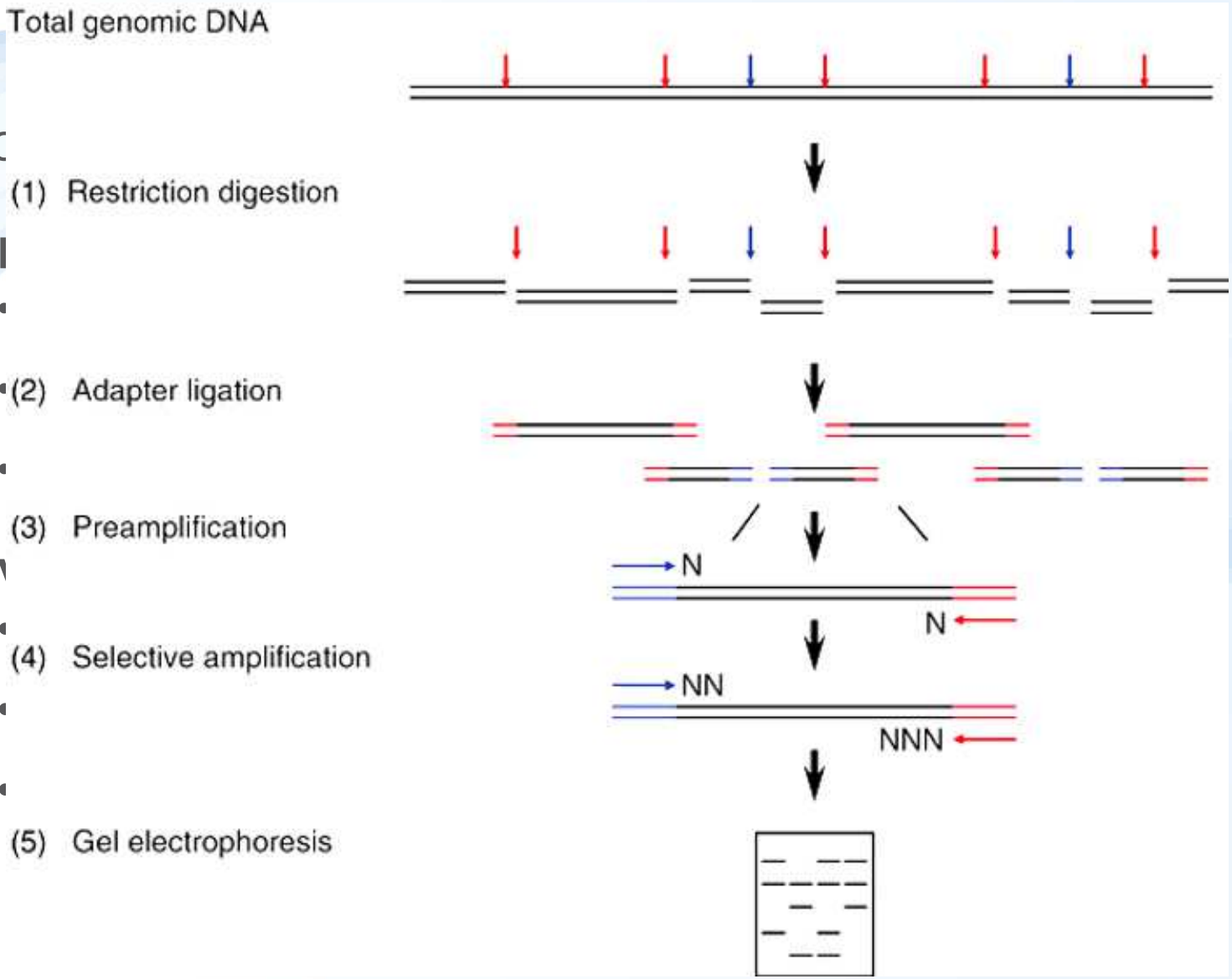
❑ **AFLP** („Amplified fragment length polymorphism“) ⇒ Polymorfismus délky amplifikovaných fragmentů

Princip

- metoda založená na restrikci DNA dvěma enzymy
- selektivní namnožení jen některých proužků
- vizualizace proužků na gelu

Využití

- polymorfismus DNA
- genetická variabilita
- fylogenetické studie



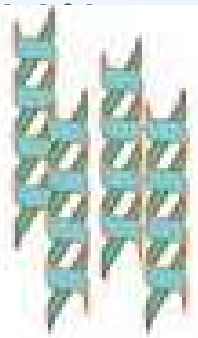
http://www.nature.com/scitable/content/outline-of-the-aflp-procedure-41047
https://botany.natur.cuni.cz/dna/index.php?option=com_content&view=article&id=47:aflp-princip&catid=35:aflp&Itemid=55

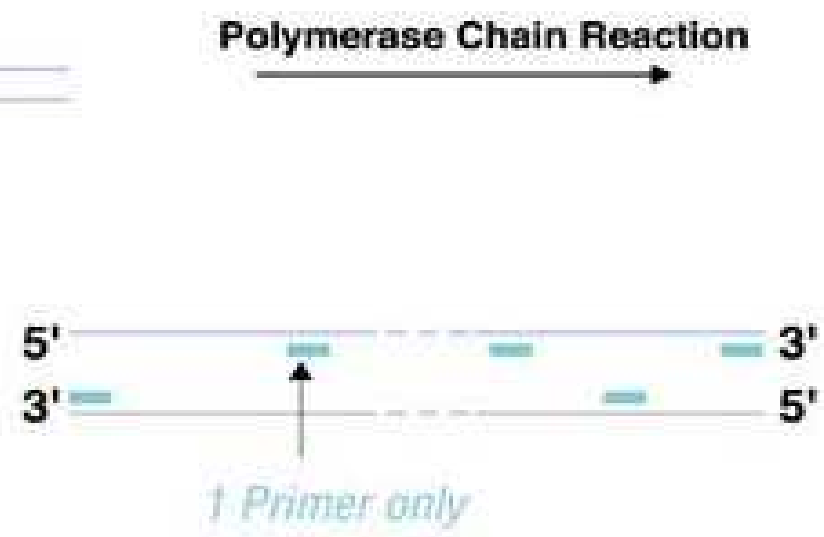
❑ RAPD-PCR („Random Amplified Polymorphic“ DNA)

- krátké nespecifické primery (8–12 nukleotidů)
- není potřeba znát sekvenci celé genomové DNA nebo studovaného úseku
- **VYUŽITÍ**
  - DNA polymorfismus
  - genetický otisk
  - otisk mikrobiální komunity

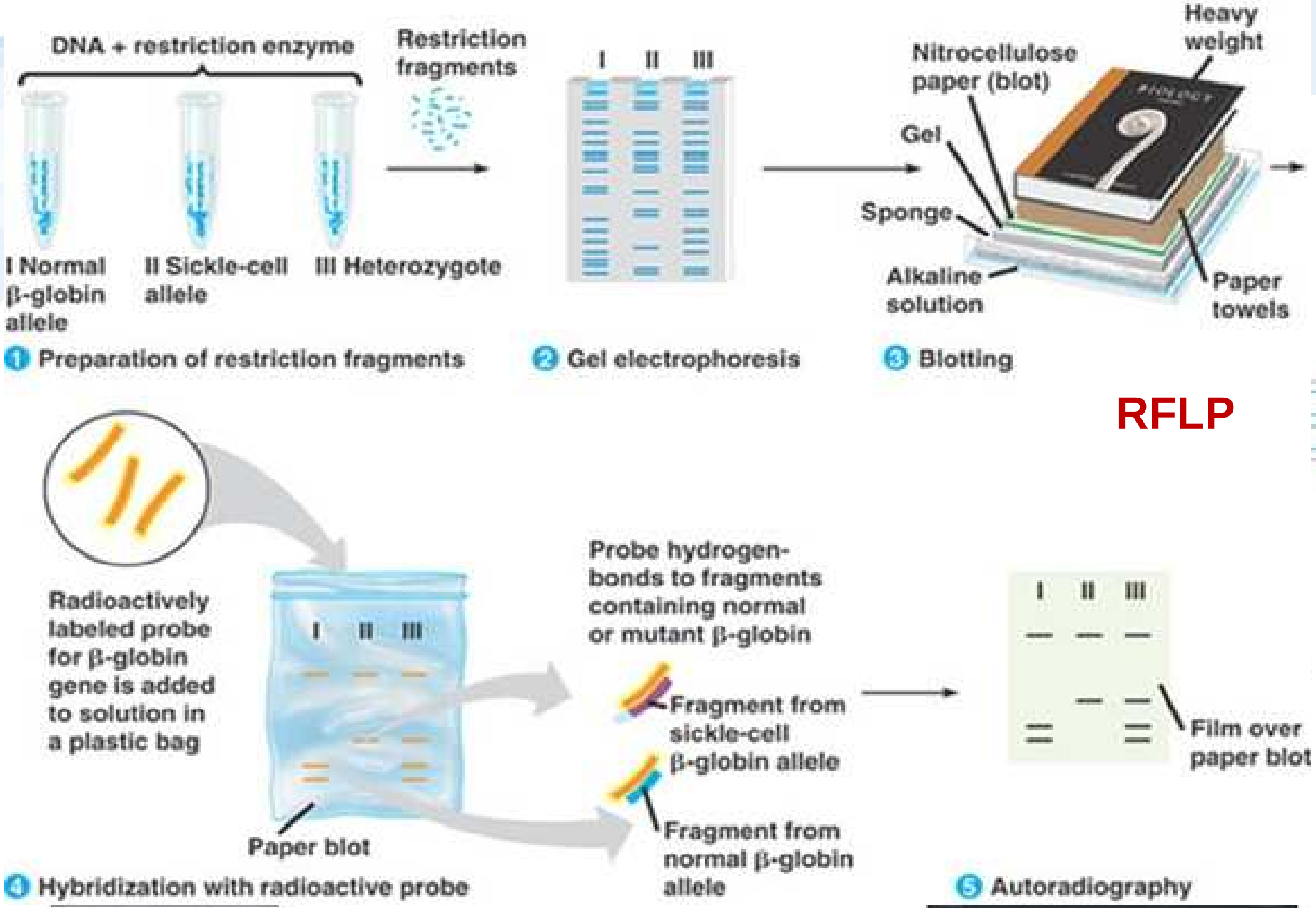
❑ RAPD-PCR („Random Amplified Polymorphic“ DNA)

- **RAPD**





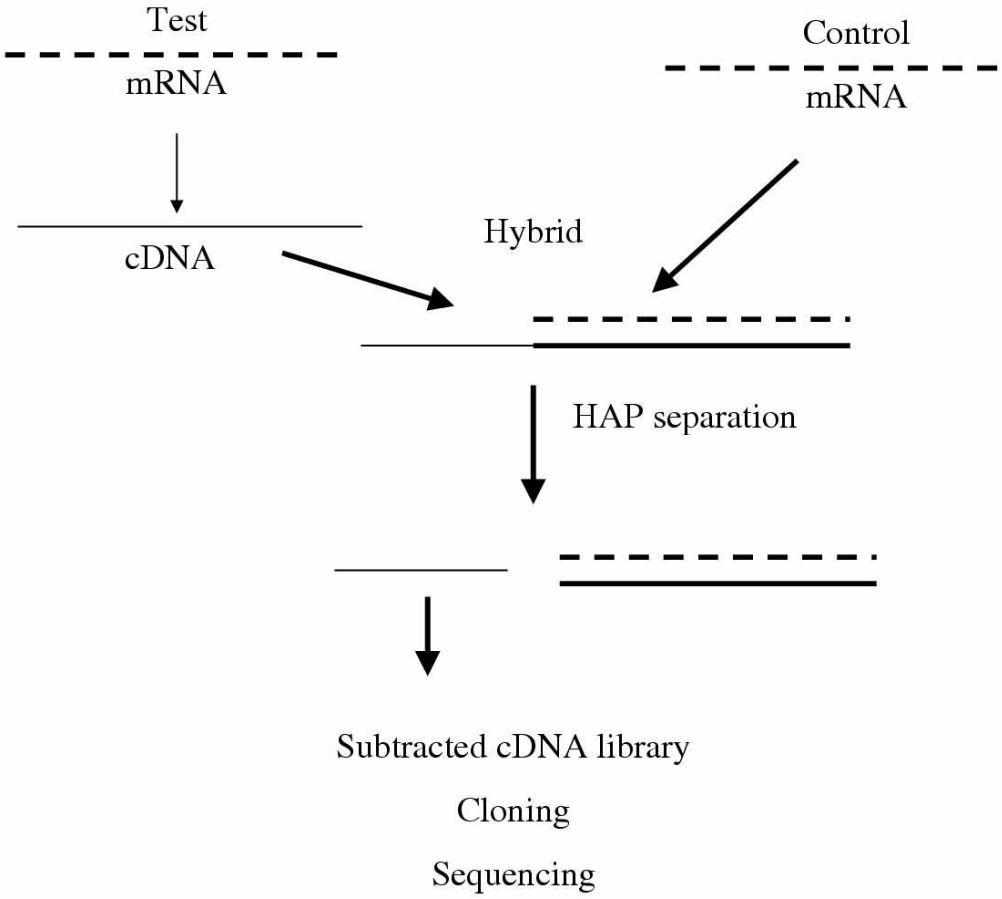
http://www.majordifferences.com/2013/02/difference-between-rapd-and-rflp.html#.VDD5SBawRSM



http://www.majordifferences.com/2013/02/difference-between-rapd-and-rflp.html#.VDD5SBawRSM

❑ SSH-PCR („Suppression subtractive hybridization“ PCR)

• dovoluje namnožit pouze cDNA fragmenty, které se liší mezi kontrolou („driver“) a experimentální skupinou („test“)

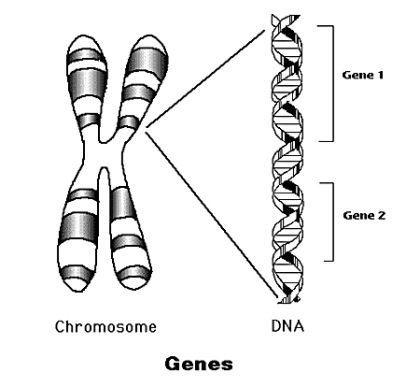


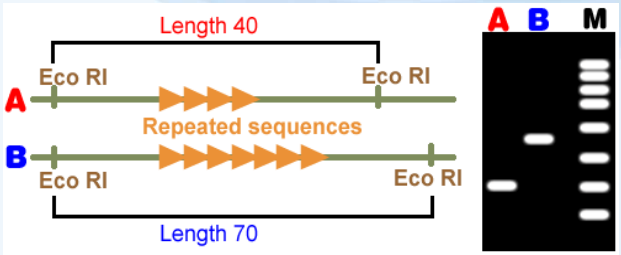
https://cellularphysiology.wikispaces.com/Suppression+subtractive+hybridization+%28SSH%29

(q)PCR: APLIKACE

- klonování DNA
- sekvenace DNA
- fylogenetické analýzy založené na DNA
- genetická diverzita
- genetický otisk
- detekce genu
- exprese genu a jejich funkce
- mutace

(q)PCR: POLYMORFISMUS DNA



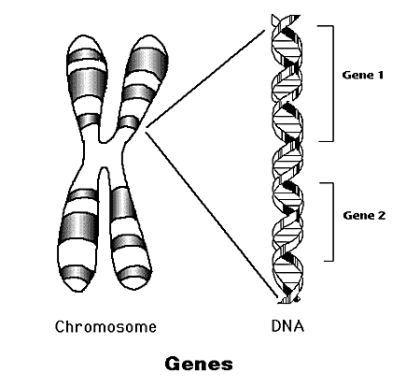


VNTR – variabilní počet tandémových repetitivních sekvencí

http://www.thenakedscientists.com/HTML/uploads/tx_naksciimages/dalya8_vntr_diagram.gif

(q)PCR: POLYMORFISMUS DNA

❑ Typy sekvencí DNA



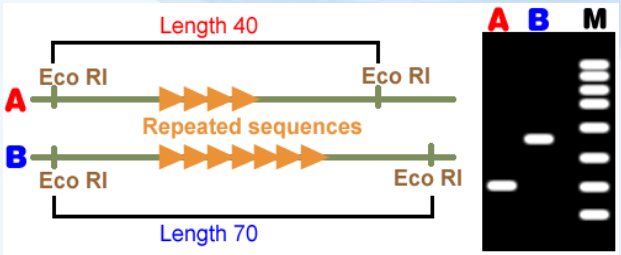
- jedinečné sekvence

  ⇒ geny (člověk – 80-100 000)

- repetitivní sekvence

  ⇒ *tandemové* - bloky opakujících se repetitivních sekvencí uspořádaných za sebou

  - satelitní DNA
  - minisatelitní DNA
  - mikrosatelitní DNA



VNTR – variabilní počet tandémových repetitivních sekvencí

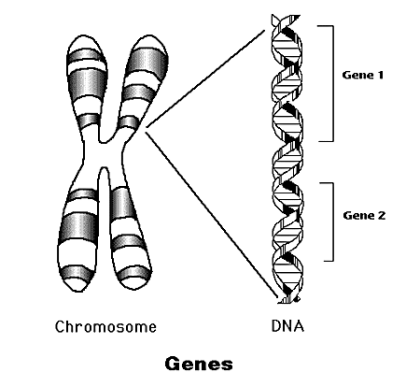
http://www.thenakedscientists.com/HTML/uploads/tx_naksciimages/dalya8_vntr_diagram.gif

  ⇒ *rozptýlené*

  - SINE – krátké rozptýlené repetice
  - LINE – dlouhé rozptýlené repetice

(q)PCR: POLYMORFISMUS DNA

❑ Typy sekvencí DNA



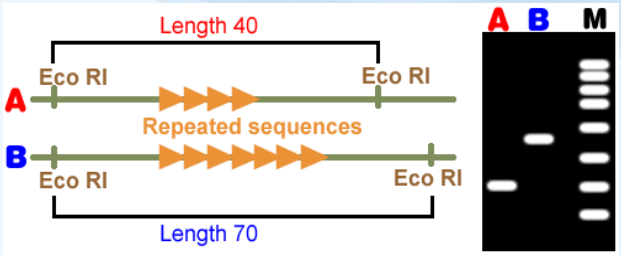
- jedinečné sekvence

  ⇒ geny (člověk – 80-100 000)

- repetitivní sekvence

  ⇒ *tandemové* - bloky opakujících se repetitivních sekvencí uspořádaných za sebou

  - satelitní DNA
  - minisatelitní DNA
  - mikrosatelitní DNA



VNTR – variabilní počet tandémových repetitivních sekvencí

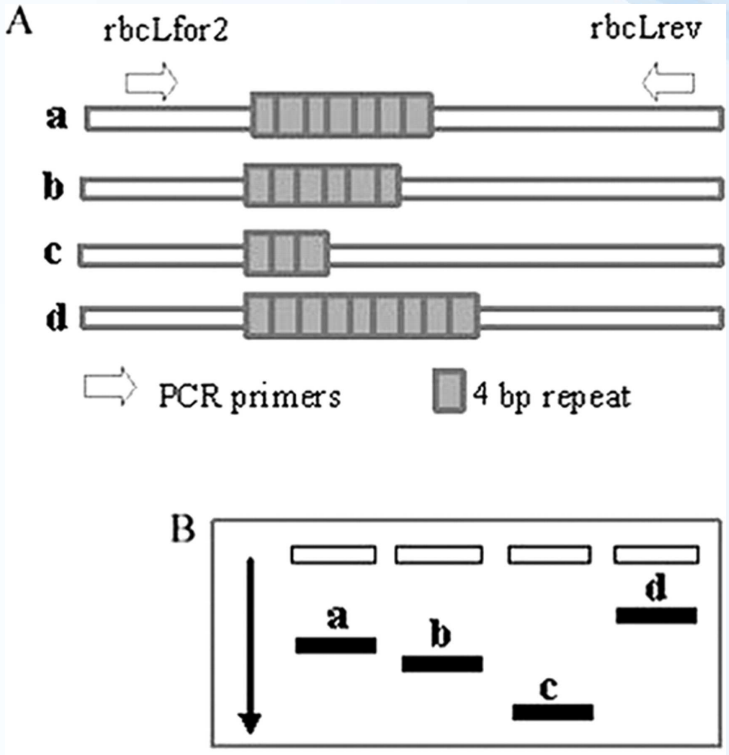
http://www.thenakedscientists.com/HTML/uploads/tx_naksciimages/dalya8_vntr_diagram.gif

  ⇒ *rozptýlené*

  - SINE – krátké rozptýlené repetice
  - LINE – dlouhé rozptýlené repetice

❑ Mikrosatelity

- krátké tandemové repetice STR („short tandem repeats“)
- repetice jednoduchých sekvencí SSR („simple sequence repeats“)
- kódující i nekódující oblasti
- hlavní zdroj vysoké proměnlivosti – sklouznutí nukleotidového řetězce během replikace („replication slippage“)
- alely se liší delkou



PCR: UŽITEČNÉ ODKAZY I.

Osobní stránky K. Mullise, obsahují popis objevu a principu PCR (vč. videa)

http://www.karymullis.com/pcr.shtml

Video o průběhu PCR

http://www.youtube.com/watch?v=2KoLnIwoZKU

PCR song, povedená píseň firmy BioRad

http://www.youtube.com/watch?v=x5yPkxCLads

PCR song, text

http://bio-rad.cnpg.com/lsca/videos/ScientistsForBetterPCR/assets/BioRad_PCRsong_Lyrics.pdf

The PCR Song

There was a time when to amplify DNA,
You had to grow tons and tons of tiny cells.

Then along came a guy named Dr. Kary Mullis,
Said you can amplify in vitro just as well.

Just mix your template with a buffer and some primers,
Nucleotides and polymerases, too.

Denaturing, annealing, and extending.
Well it's amazing what heating and cooling and heating will do.

PCR, when you need to detect mutations.
PCR, when you need to recombine.
PCR, when you need to find out who the daddy is.
PCR, when you need to solve a crime.

(repeat chorus)

PCR: UŽITEČNÉ ODKAZY II.

Kvantifikace qPCR

http://www.gene-quantification.de

Fóra, diskusní skupiny

http://www.researchgate.net/topics

http://www.bio.net/

www.molecularstation.com

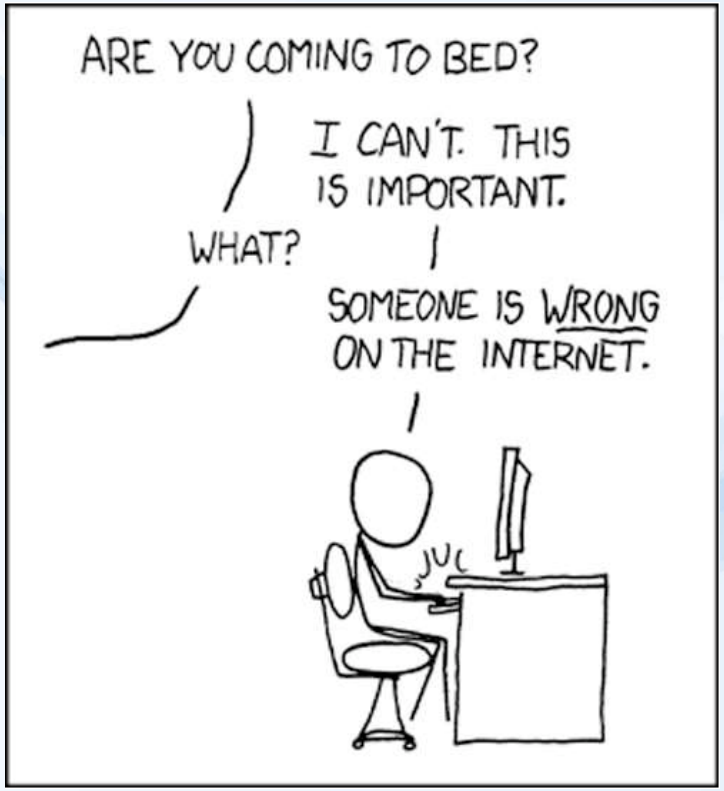
www.protocol-online.org

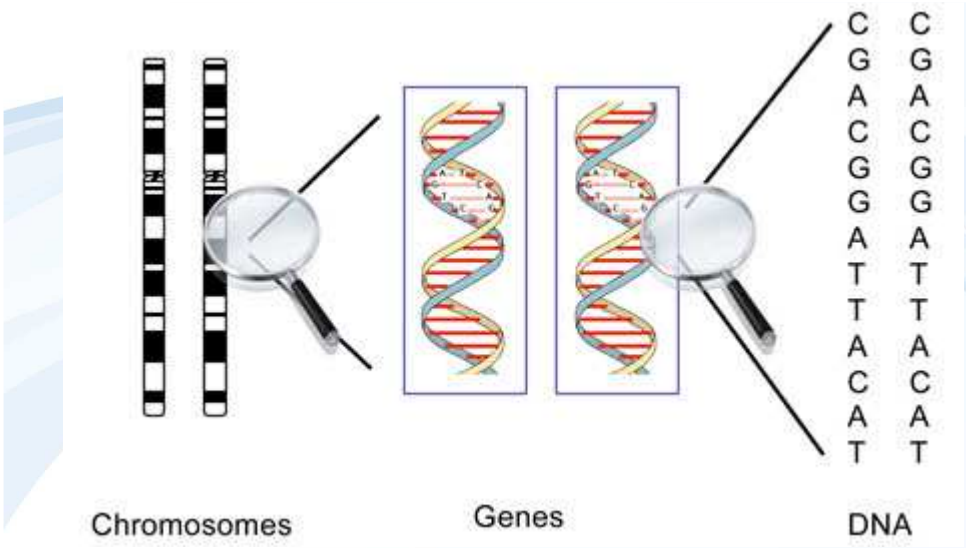
molecularbiology.forums.biotechniques.com

www.scienceforums.net

www.biotechnologyforums.com

www.biology-online.org





JAK ZJISTÍME SEKVENCI NK?

NK: SEKVENOVÁNÍ

1) Předstupeň: PCR s použitím dvojice primerů
   - namnožení studovaného úseku DNA

2) Sekvenační reakce
   - použití pouze jednoho primeru
   - produkce fragmentů lišících se přesně o 1 bázi

3) Elektroforetická separace fragmentů na gelu

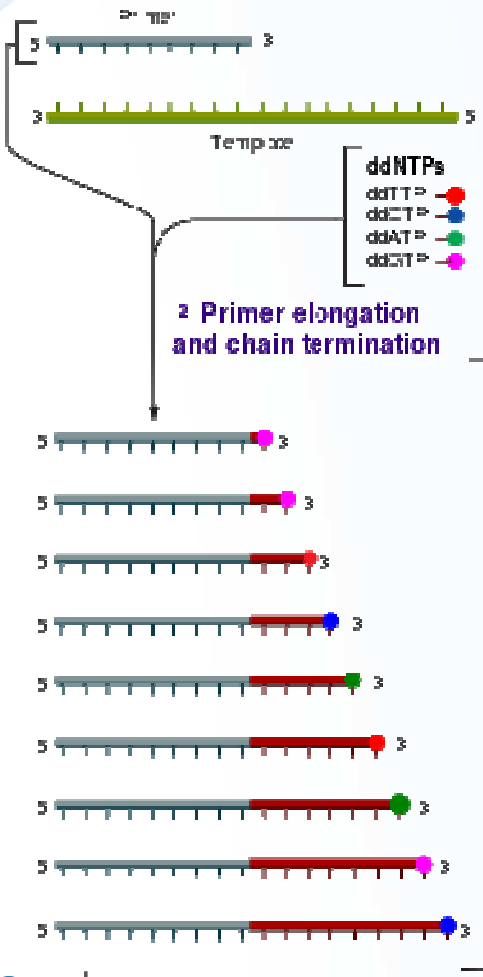
SEKVENOVÁNÍ: SANGEROVA METODA

Sangerova metoda terminace řetězce

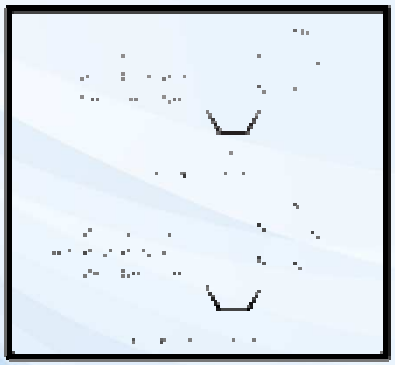
- založena na terminaci replikace nového řetězce podle matrice zkoumané sekvence dideoxynukleozidtrifosfátem (ddNTP)
- na 3'-uhlíku deoxyribózy chybí OH - skupina a proto k nim DNA polymeráza nemůže navázat další nukleotid
- pokud během replikace dojde k náhodné inkorporaci dideoxynukelotidu (ddA, ddC, ddG, ddT), replikace se zde zastaví

https://www.youtube.com/watch?v=oYpIlbI0qF8
http://biologie.upol.cz/metody/Sekvenovani%20DNA.htm

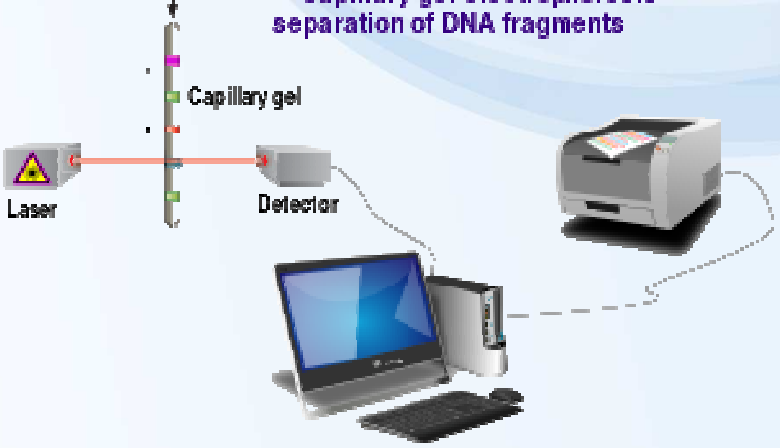
1 Reaction mixture
- Primer and DNA template
- DNA polymerase
- ddNTPs with flourochromes
- dNTPs (dATP, dCTP, dGTP, and dTTP)

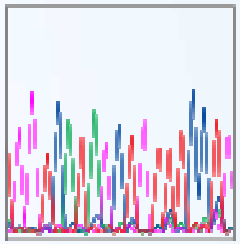


2 Primer elongation and chain termination



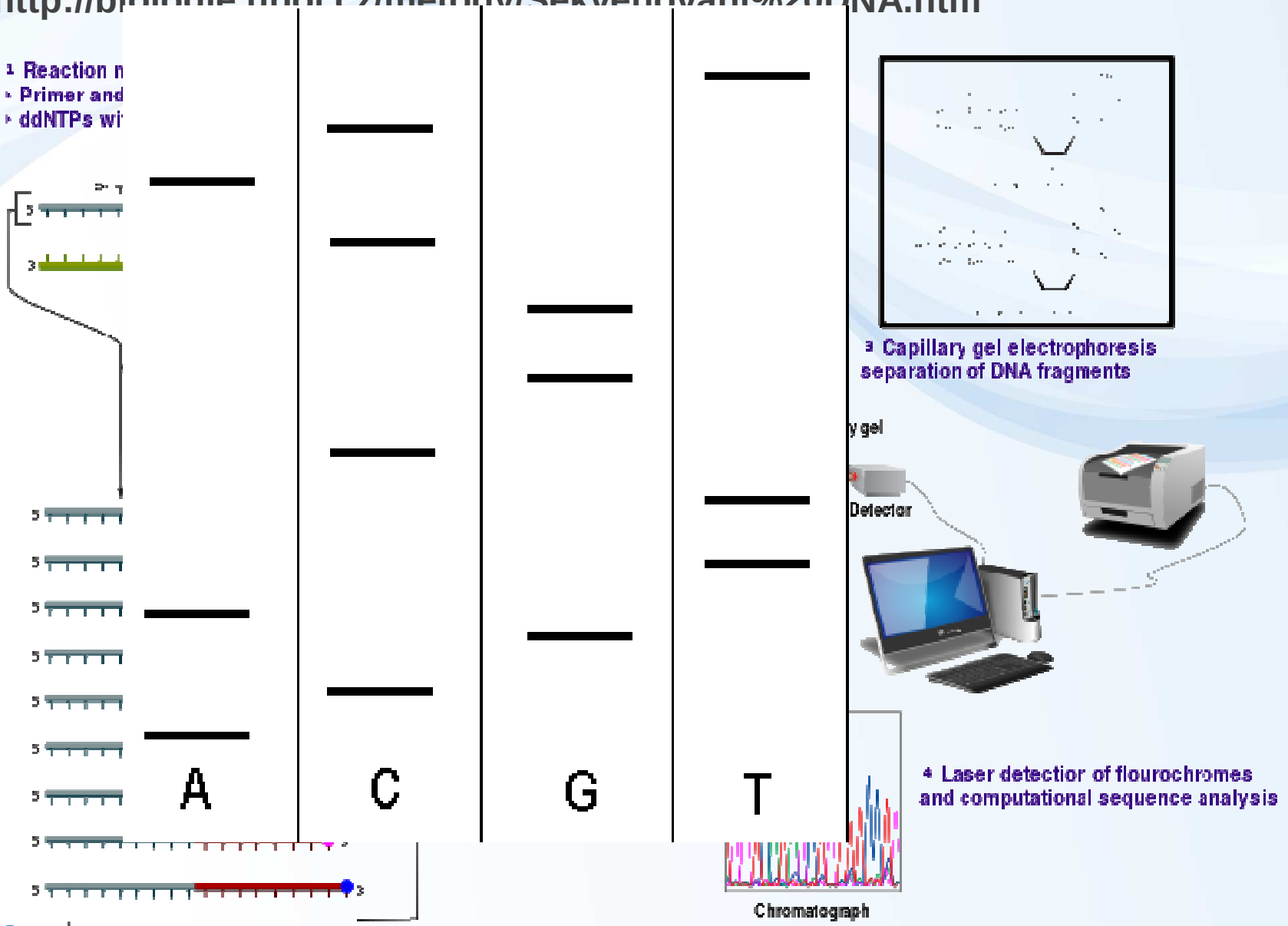
3 Capillary gel electrophoresis separation of DNA fragments





4 Laser detection of flourochromes and computational sequence analysis

https://www.youtube.com/watch?v=oYpllbI0qF8
http://biologie.upol.cz/metody/Sekvenovani%20DNA.htm



1 Reaction n
Primer and
ddNTPs wi

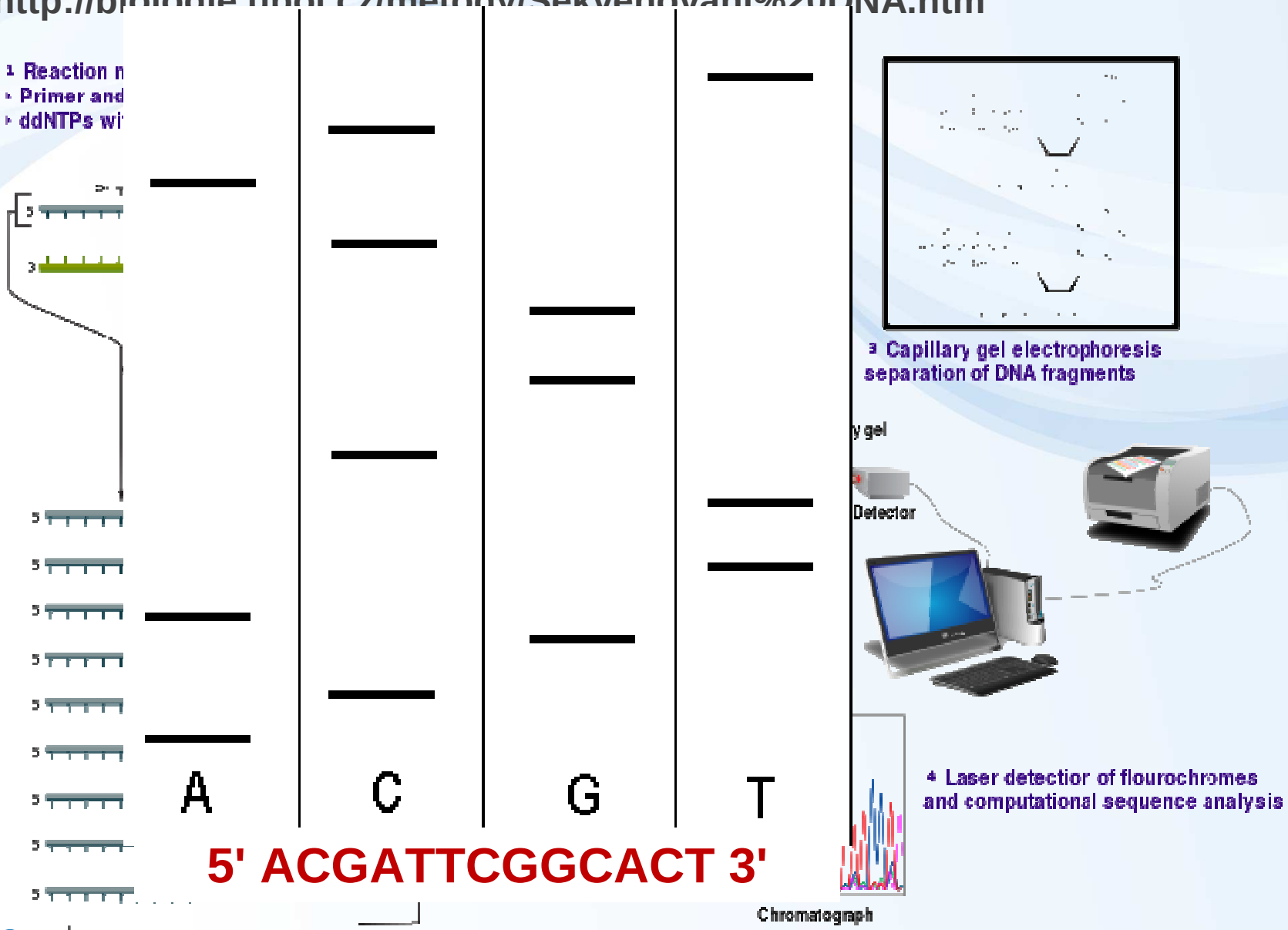
3 Capillary gel electrophoresis separation of DNA fragments

y gel

4 Laser detection of flourochromes and computational sequence analysis

https://www.youtube.com/watch?v=oYpIlbI0qF8

http://biologie.upol.cz/metody/Sekvenovani%20DNA.htm



5' ACGATTCGGCACT 3'

SEKVENOVÁNÍ: DATABÁZE DNA

http://www.ncbi.nlm.nih.gov/

SEKVENOVÁNÍ: MODERNÍ METODY

http://web.natur.cuni.cz/zoologie/biodiversity/prednasky/GenetickeMetodyVZoologii/Prednasky_2012/NextGenerationSequencing_2012.pdf

Pyrosekvenování

- detekce aktivity DNA-polymerázy během syntézy DNA

SEKVENOVÁNÍ: MODERNÍ METODY

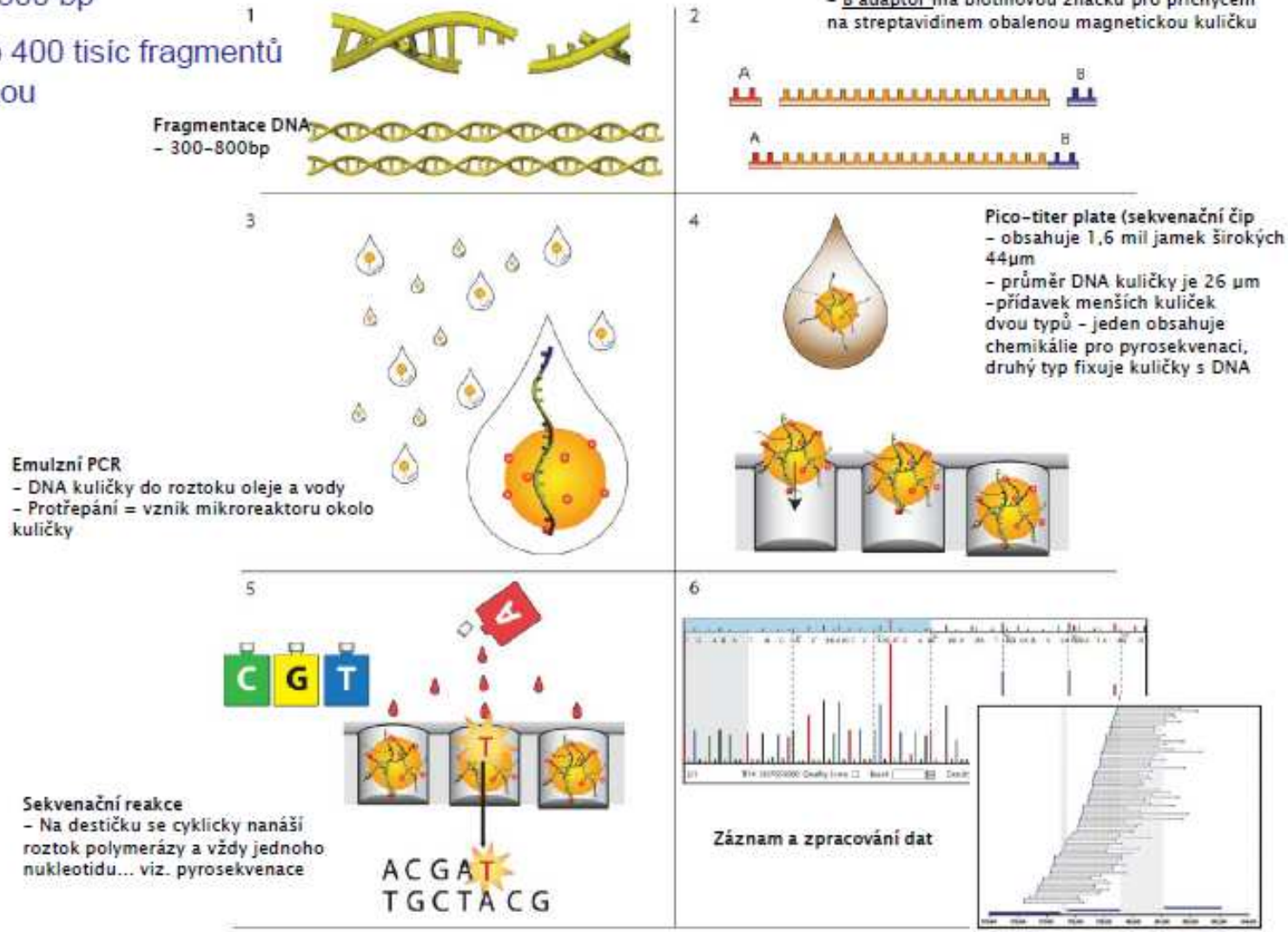
http:/ ...olo
gii/Pr

Py
• d

454 sekvenování

úseky 300 bp

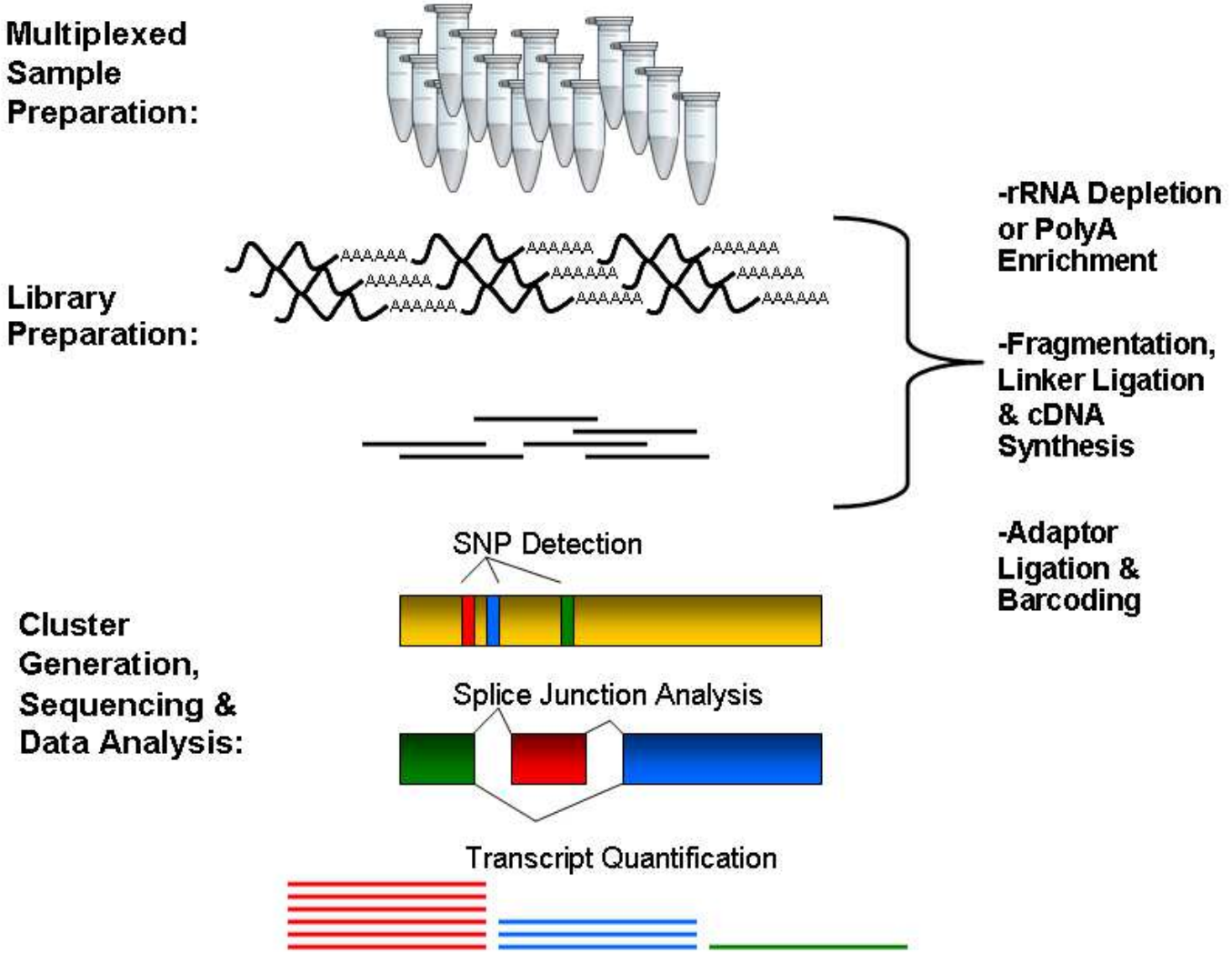
- čteno 400 tisíc fragmentů najednou



RNA-seq

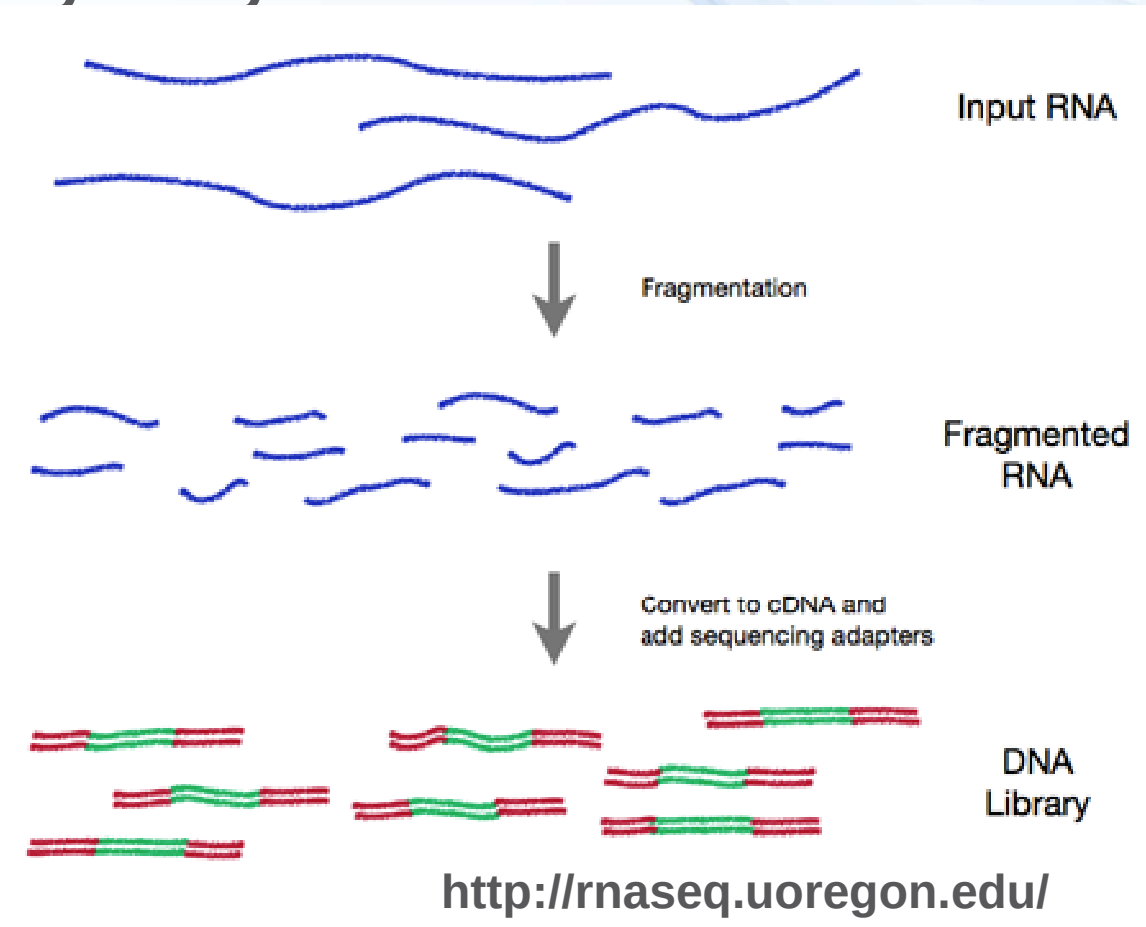
(RNA Sequencing), "Whole Transcriptome Shotgun Sequencing"("WTSS")

- **sekvenování RNA (total, mRNA, siRNA, miRNA atd.)**



http://www.labome.com/method/RNA-seq-Using-Next-Generation-Sequencing.html#ref2

- Stanovení genové exprese
- Objevení a popsání všech transkriptů
- Charakterizace alternativního sestřihu (hranice exon-intron) a polyadenylace



http://rnaseq.uoregon.edu/

Front. Plant Sci., 28 August 2012

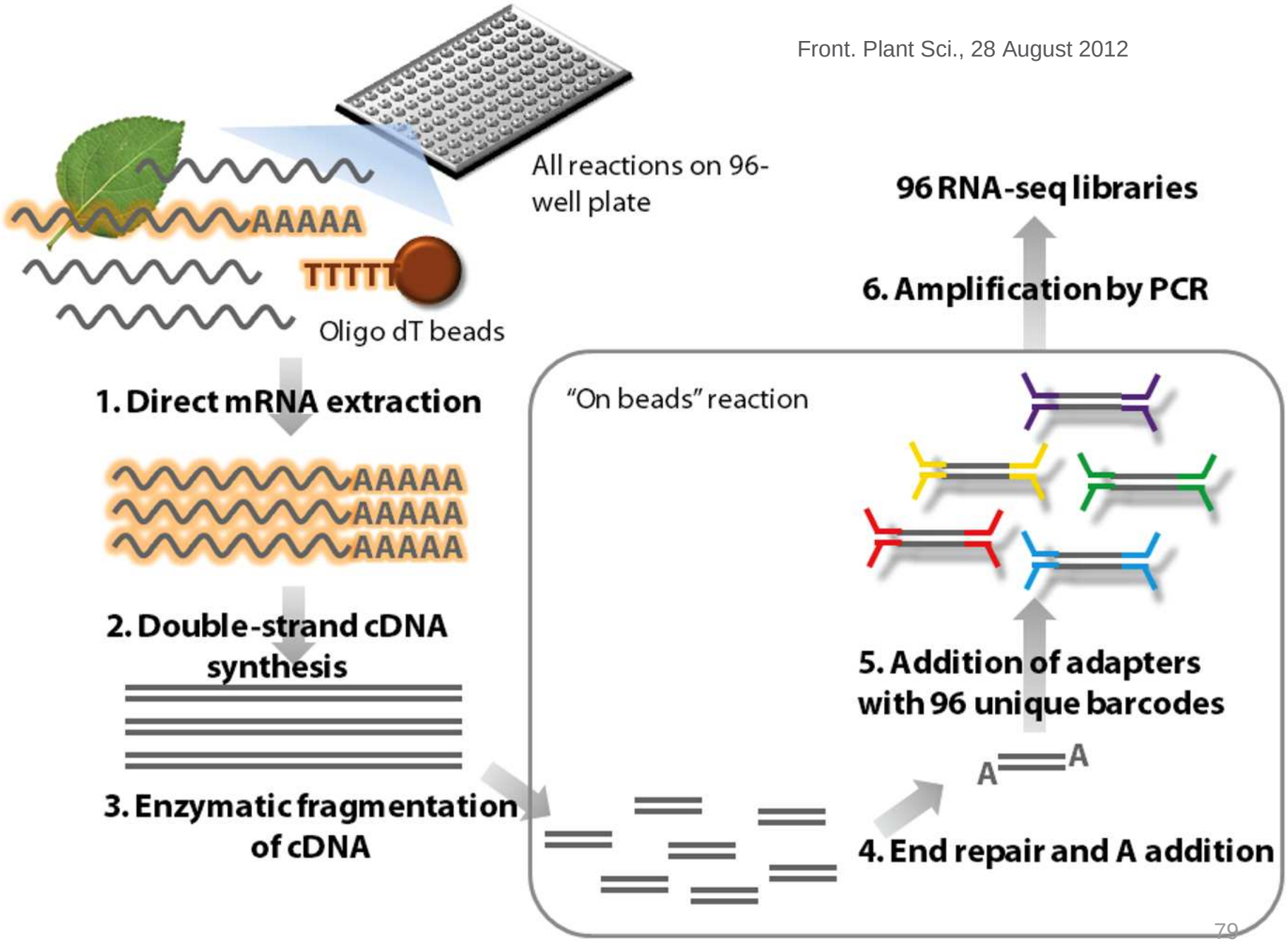
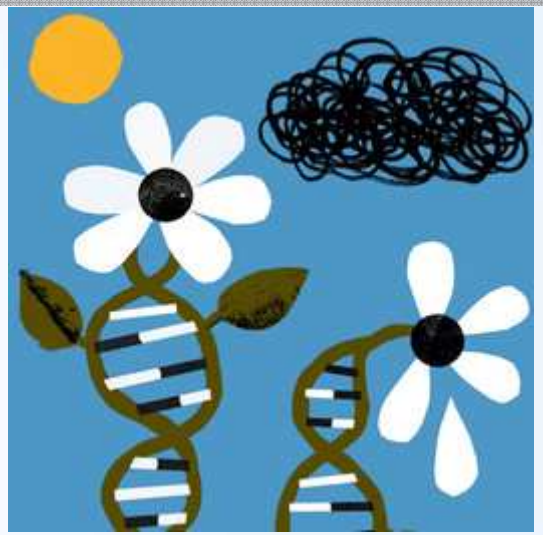


Table 1 Advantages and disadvantages of RT-PCR and RNAseq

Technique	Advantages	Disadvantages
RT-PCR	➢ High sensitivity ➢ High sequence-specific	➢ Time consumed ➢ Analyze one gene each time ➢ Variability of the results depending the laboratory ➢ Necessary to know the gene sequence ➢ Require many amount of RNA
RNA-seq	➢ A single experiment can provide information about all the genes (translocation, …) ➢ High reproducibility ➢ Require low amount of RNA	➢ Needs a bioinformatician for the analysis of the results ➢ High cost for one analysis

Transl Lung Cancer Res 2013;2(2):87-91

JAK STUDUJEME INTERAKCE GENŮ A PROSTŘEDÍ?



NK: TRANSKRIPTOMIKA

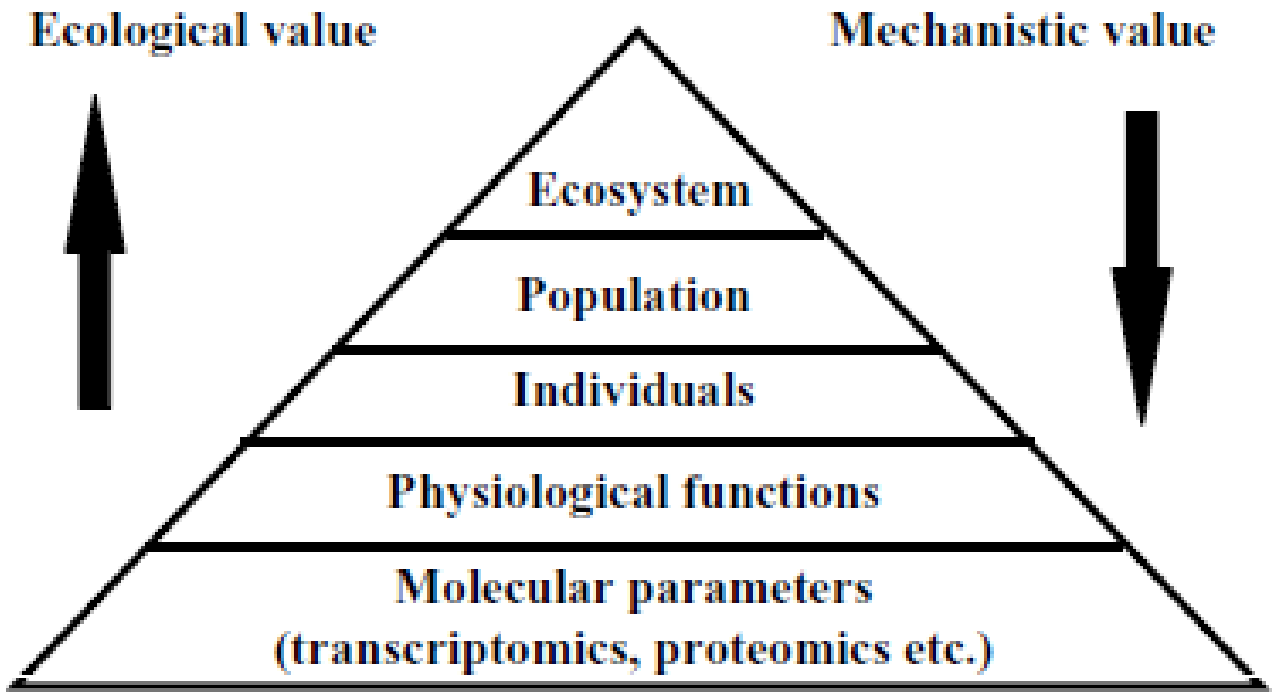
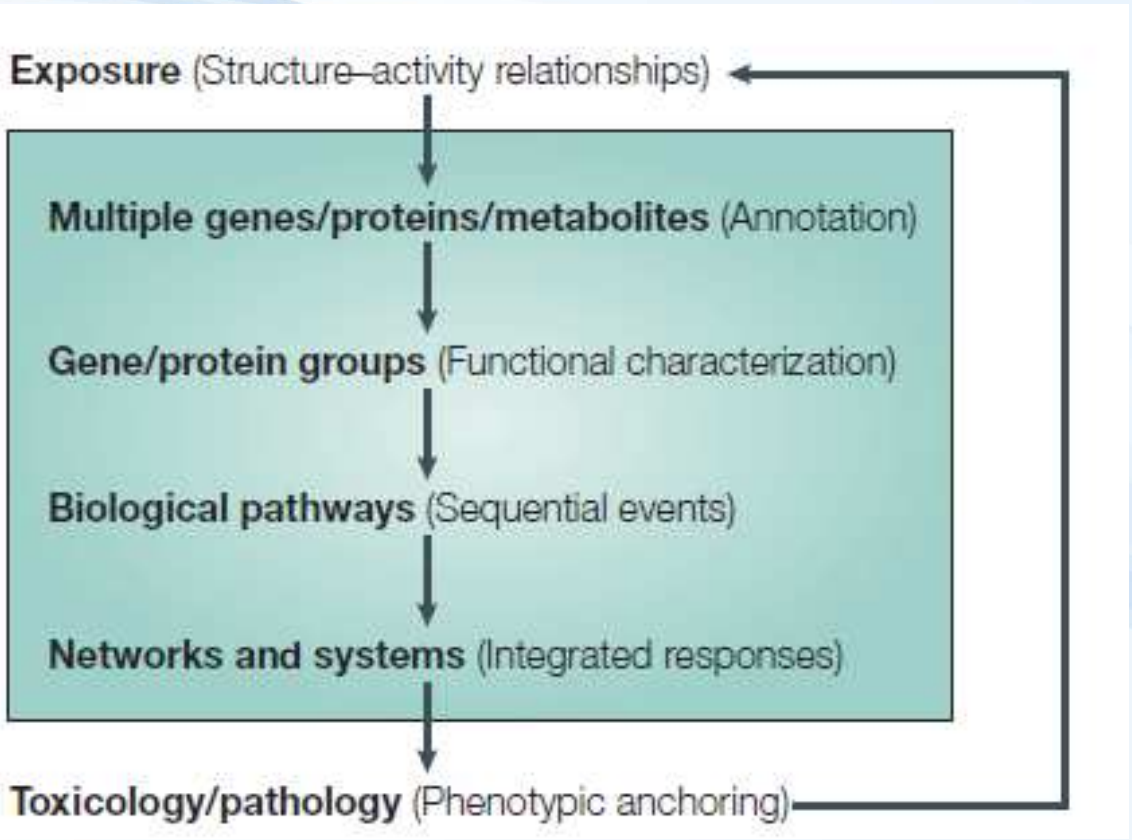
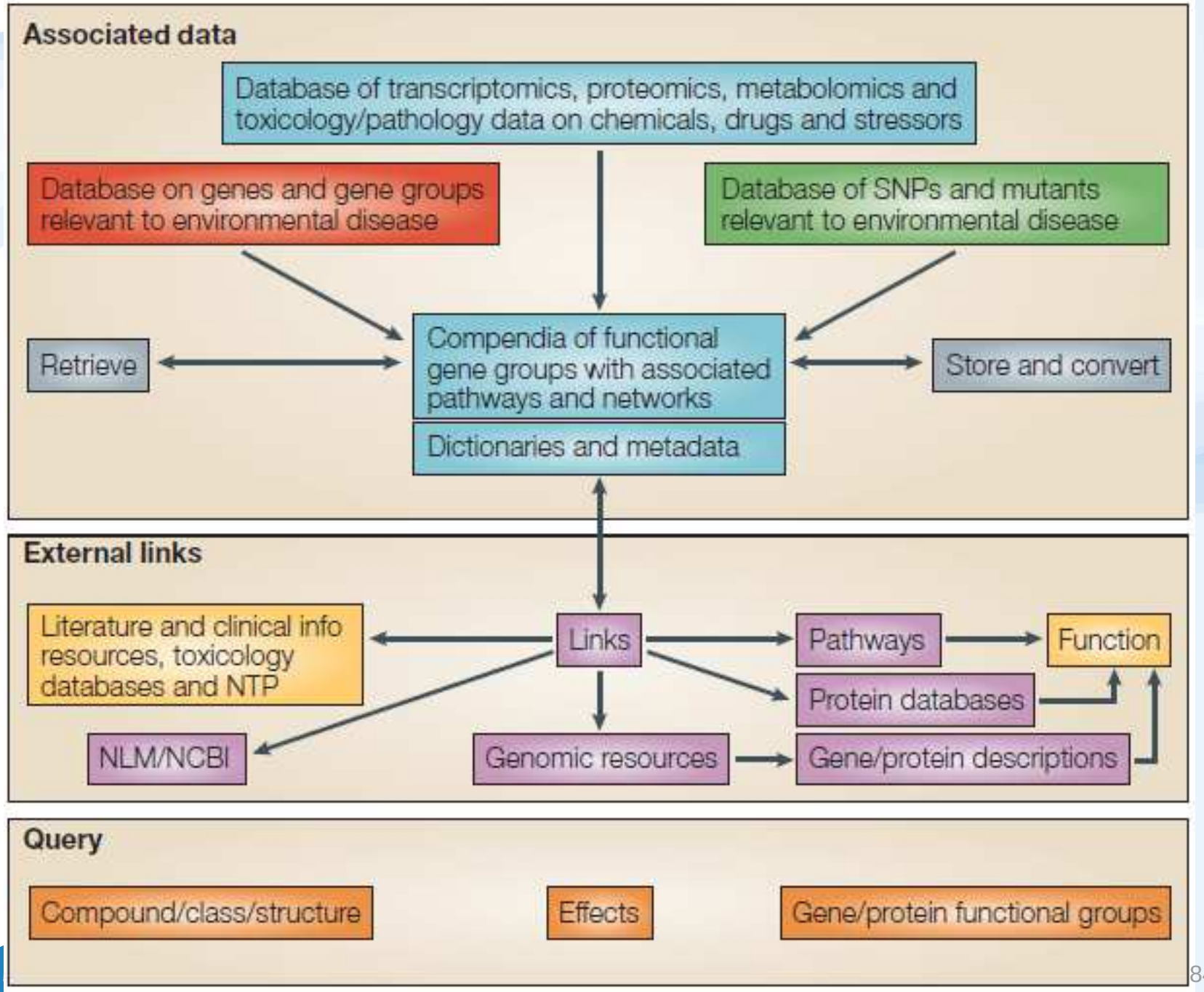


Fig. 3. Conceptual framework for ecotoxicogenomics.

Aquatic Toxicology 67 (2004) 143–154


Exposure (Structure–activity relationships)
Multiple genes/proteins/metabolites (Annotation)
Gene/protein groups (Functional characterization)
Biological pathways (Sequential events)
Networks and systems (Integrated responses)
Toxicology/pathology (Phenotypic anchoring)


Associated data
Database of transcriptomics, proteomics, metabolomics and toxicology/pathology data on chemicals, drugs and stressors
Database on genes and gene groups relevant to environmental disease
Database of SNPs and mutants relevant to environmental disease
Retrieve
Compendia of functional gene groups with associated pathways and networks
Store and convert
Dictionaries and metadata
External links
Literature and clinical info resources, toxicology databases and NTP
Links
Pathways
Function
Protein databases
NLM/NCBI
Genomic resources
Gene/protein descriptions
Query
Compound/class/structure
Effects
Gene/protein functional groups

TRANSKRIPTOMIKA: METODY

TRANSKRIPTOM ⇒ výčet a kvantifikace RNA v buňce, tkáni nebo organismu (mRNA, microRNA, siRNA, dlouhá nekódující RNA)

- **METODY**
  - **⇒ qPCR**
  - **⇒ RNA-seq**
  - **⇒ microarray**

TRANSKRIPTOMIKA: DNA array a čip

❑ DNA ARRAY a čip

• dvouřetězcové úseky molekul cDNA (komplementární DNA) vzniklé reverzní transkripcí mRNA

• oligonukleotidové sondy sekvenčně specifické pro každý gen z genomu

• hybridizační technika

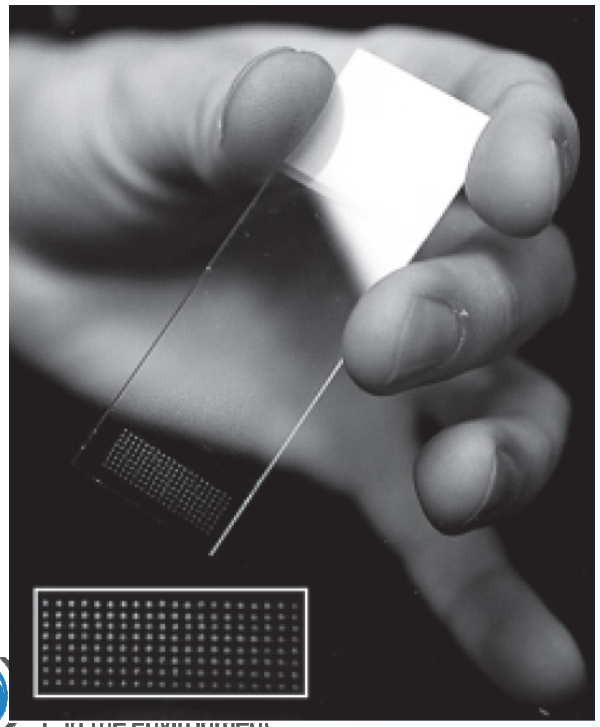
• vychází z tzv. Northern blotu ⇒ imobilizována mRNA se hybridizuje se značenou probou reprezentující jeden gen

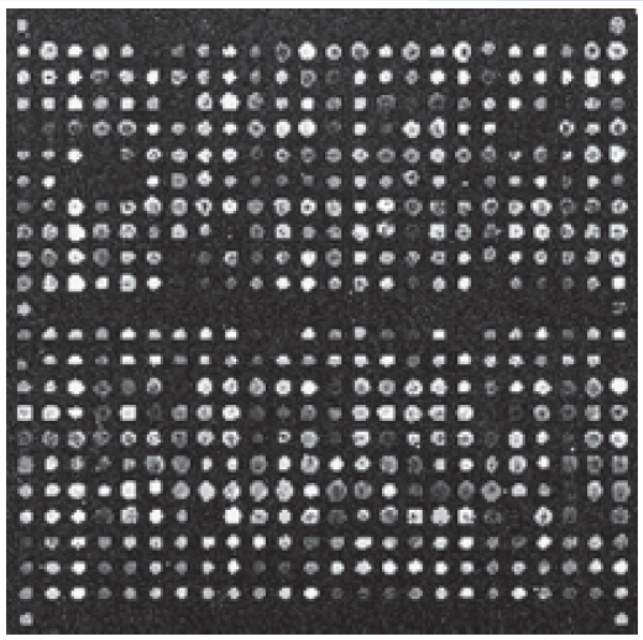
• geny nebo fragmenty genů (cDNA, EST) jsou roboticky v přesně daných souřadnicích umístěny na mikroskopické sklo (plast)

❑ PRINCIP

• funguje na principu specifické hybridizace ⇒ na principu párování komplementárních bází nukleotidů (spojení vodíkovými můstky)

• destička (skleněná nebo silikonová) s mnoha (běžně desetitisíci, statisíci, výjimečně až miliony) vzorky jednovláken DNA oligonukleotidů nebo cDNA





Klin. Biochem. Metab., 14 (35), 2006, No. 2, p. 89–95.

POSTUP:

1. Vytvoření DNA čipu

- Navázáním oligonukleotidu (sondy) kovalentní vazbou na destičku

2. Příprava vzorku (cDNA nebo genomová DNA)

- nezbytný krok označení molekul (fluorescentní, radioaktivní) a denaturace

3. Hybridizace vzorku s čipem

- nanesení vzorku na čip

4. Omytí čipu

- sondy pevně přichycené kovalentní vazbou k povrchu čipu a molekuly vzorku přichycené dostatečně pevně na sondách.

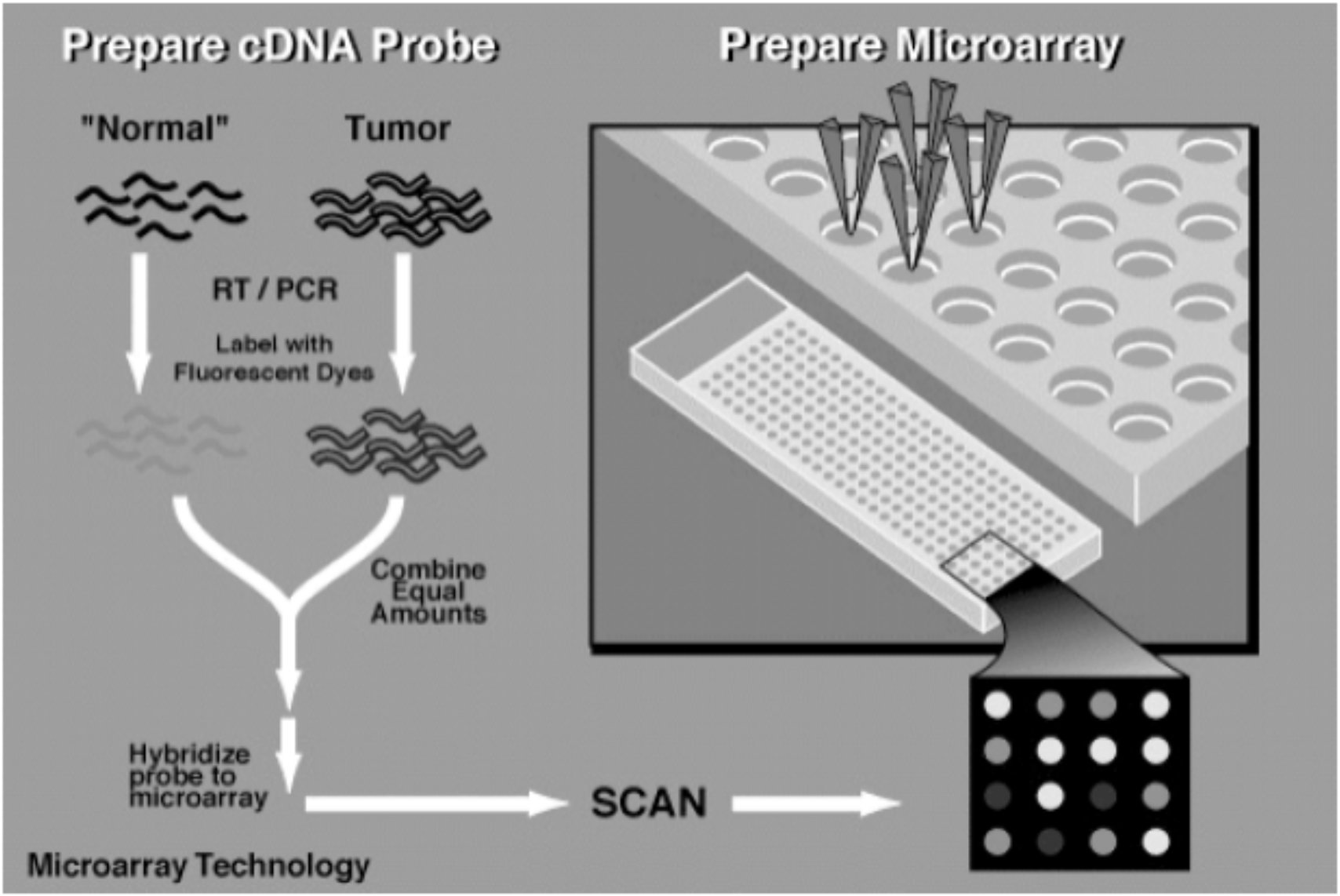
5. Skenování čipu

- Vícekanálový skener

6. Zpracování výsledků

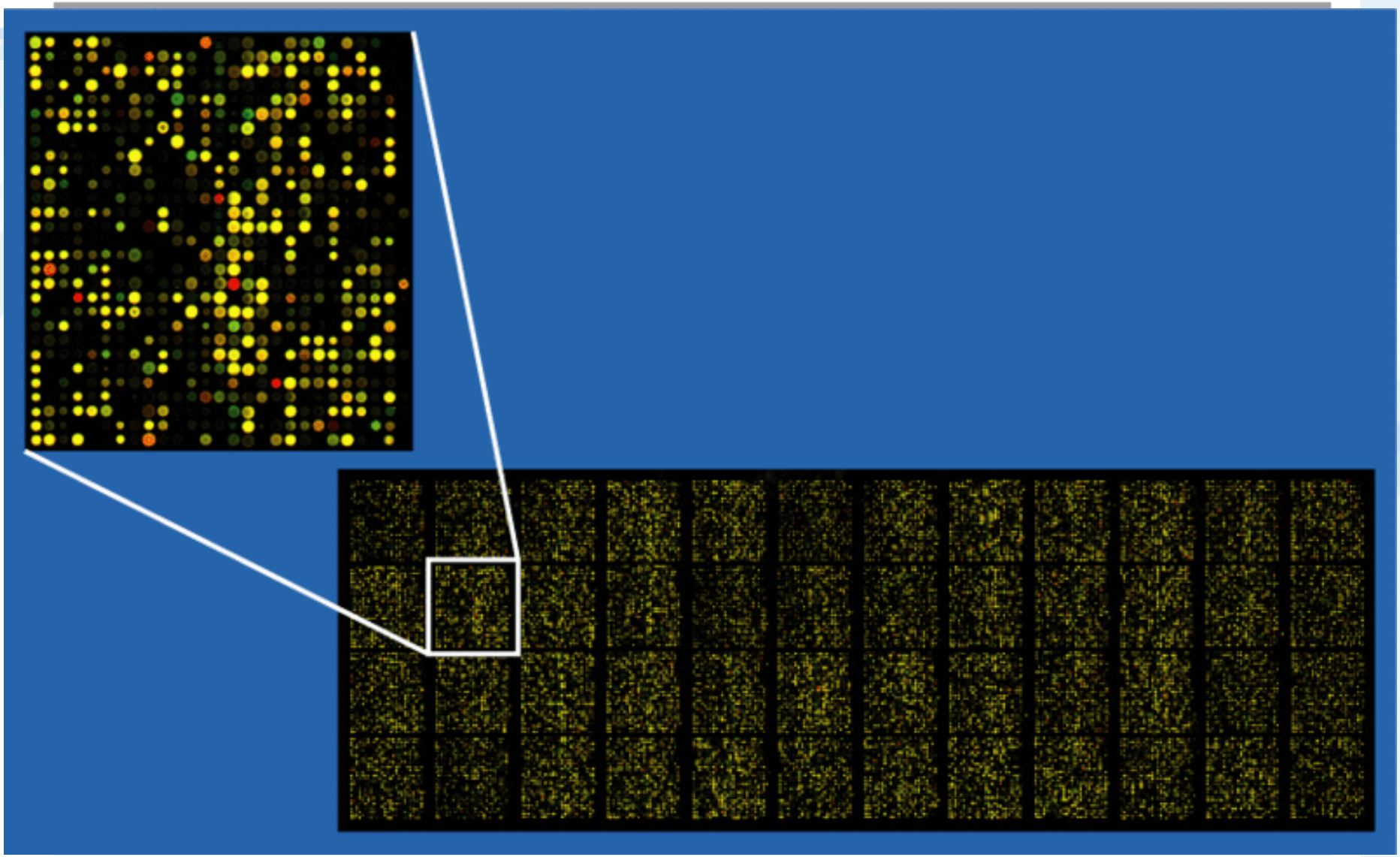
- podle intenzity vyzářeného světla lze určit množství komplementárních molekul přítomných ve vzorku

http://www.molbio.upol.cz/stranky/vyuka/cgi/5.pdf



- podle intenzity vyzářeného světla lze určit množství komplementárních molekul přítomných ve vzorku

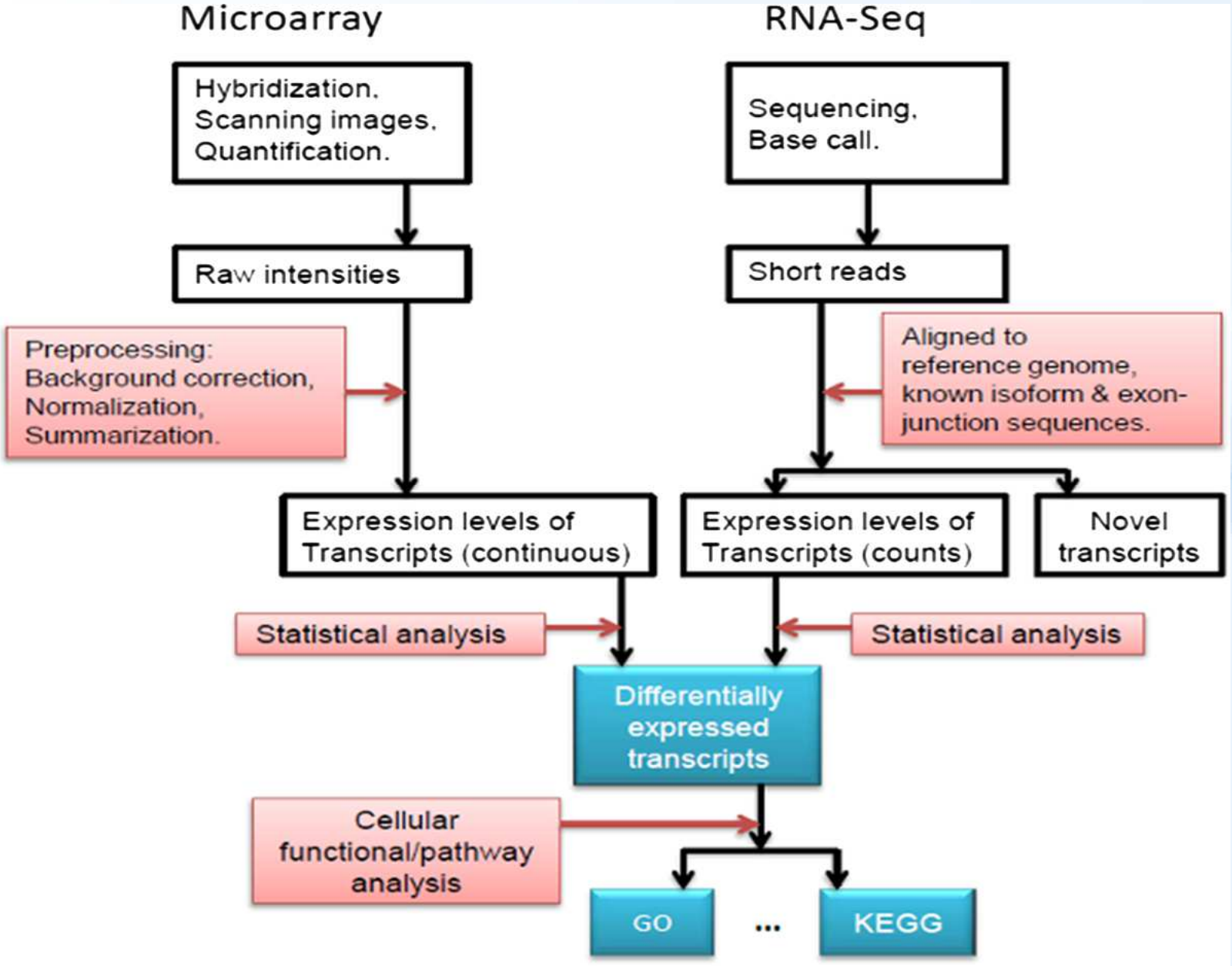
http://www.molbio.upol.cz/stranky/vyuka/cgi/5.pdf



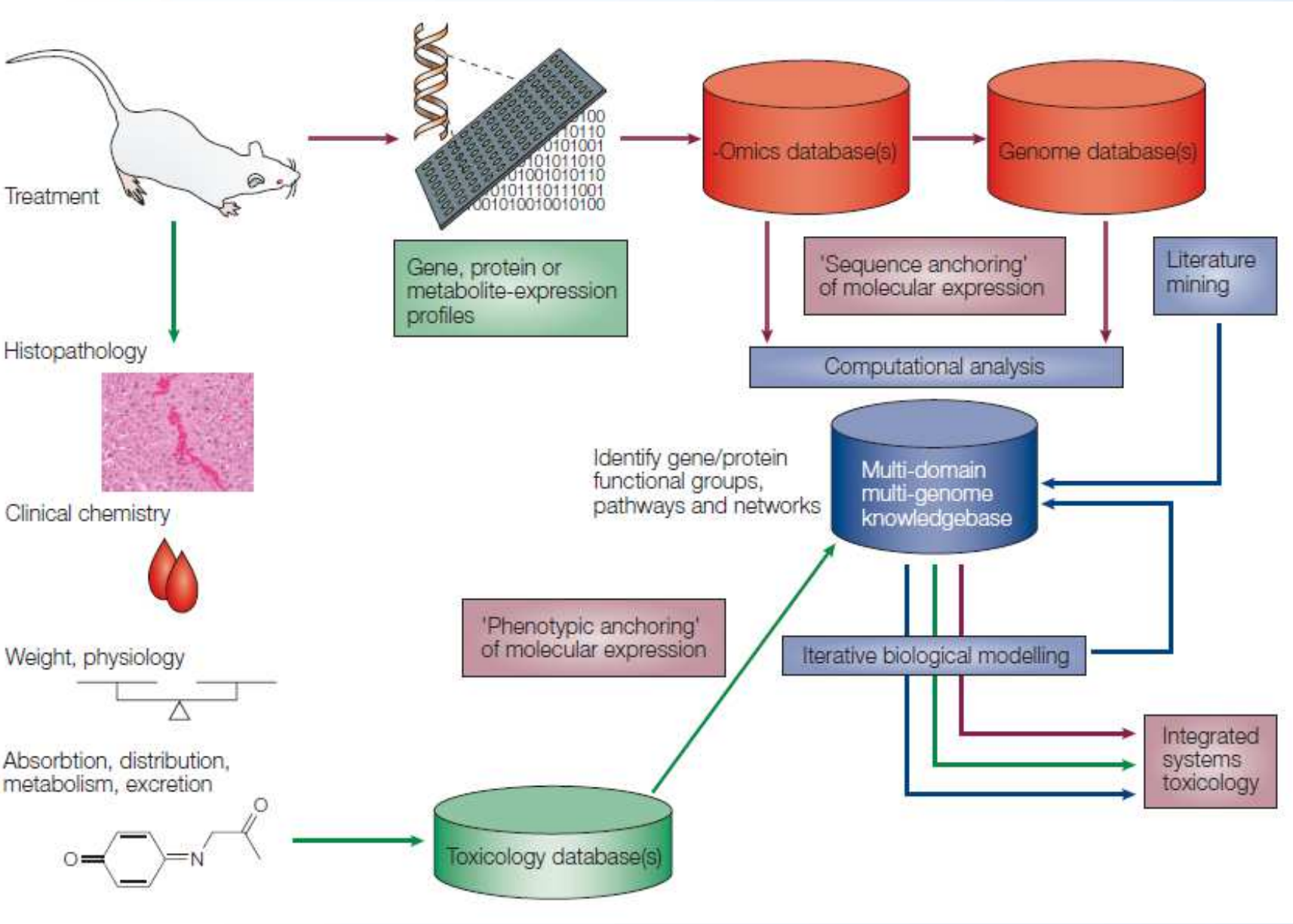
- podle intenzity vyzářeného světla lze určit množství komplementárních molekul přítomných ve vzorku

Research centre for toxic compounds in the environment

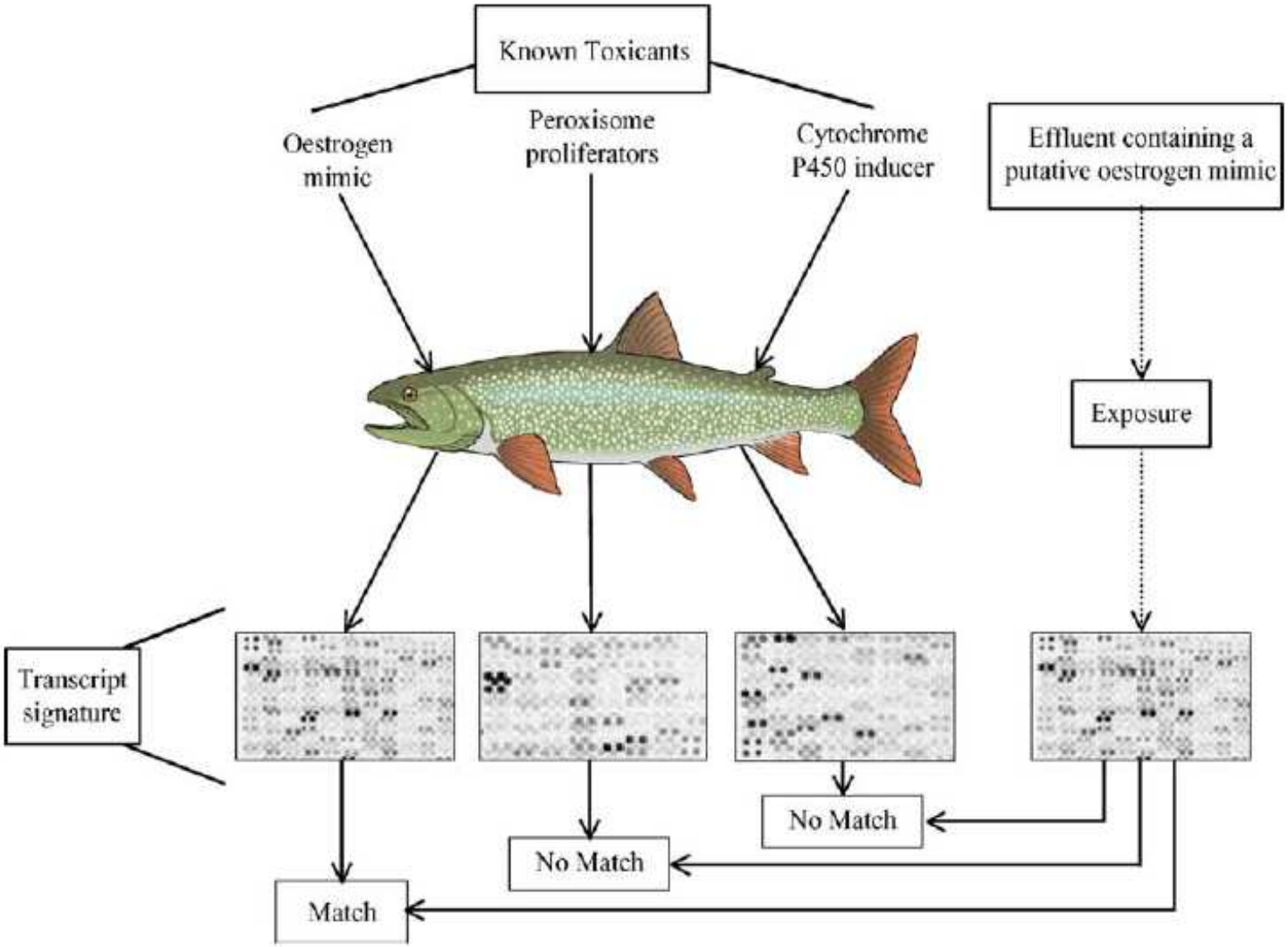
http://www.molbio.upol.cz/stranky/vyuka/cgi/5.pdf



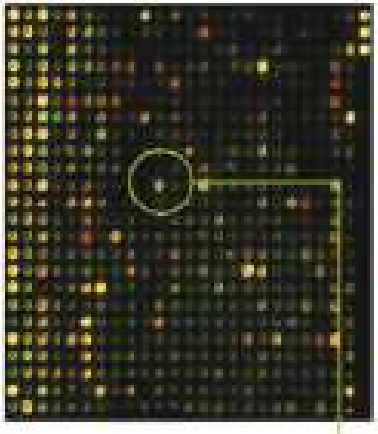
Cell Biosci. 2012;2:26



NATURE REVIEWS | GENETICS VOLUME 5 | DECEMBER 2004 | 937



Aquatic Toxicology 67 (2004) 143–154



(a) Identification of toxicant specific key genes

Toxicant

Light

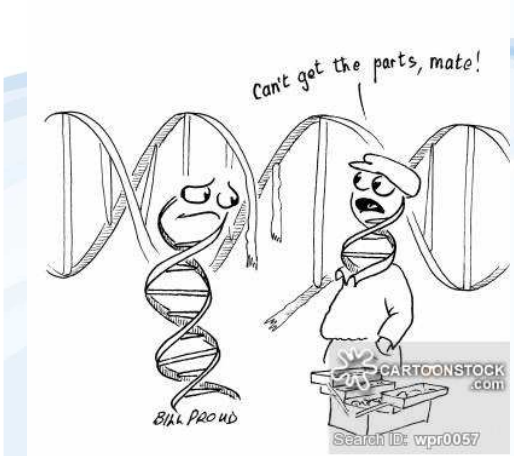
Activator

Promotor | Regulatory element | Bioreporter gene

(b) Development of a toxicant specific reporter

TRENDS in Biotechnology

TRENDS in Biotechnology

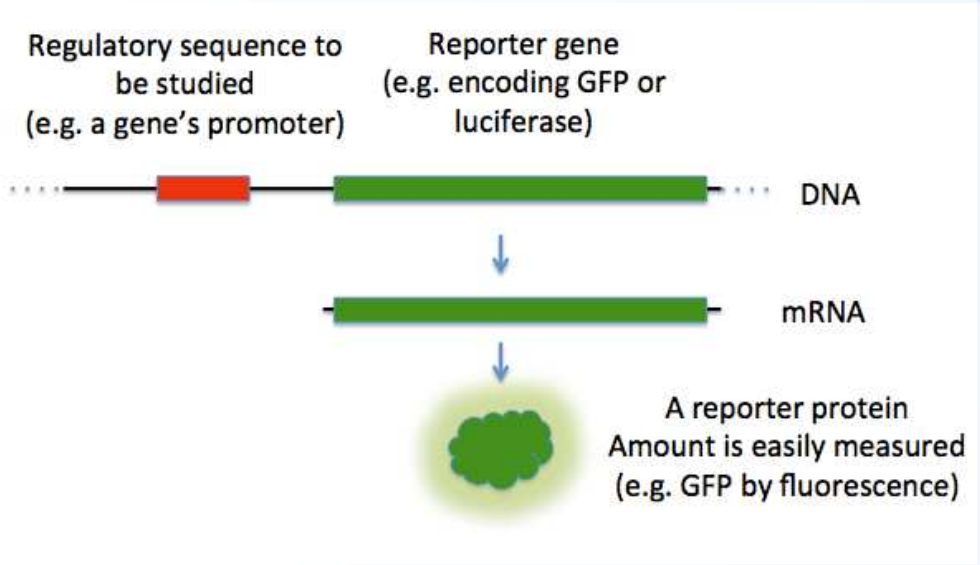


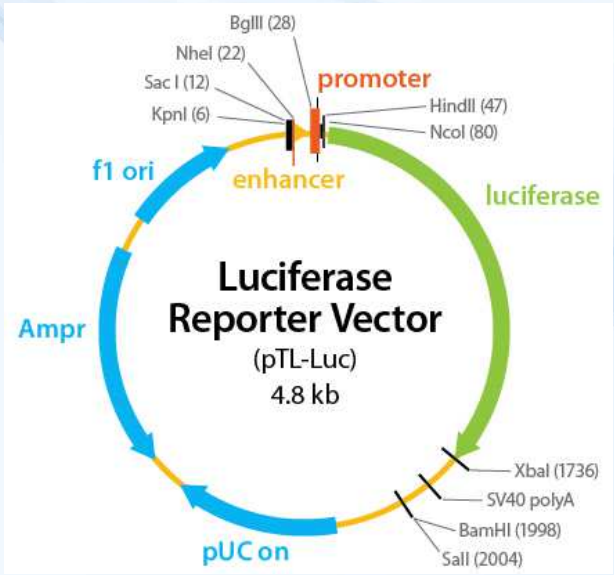
Genetic Engineer.

JAK VYTVOŘIT REKOMBINANTNÍ MODEL PRO (EKO)TOXIKOLOGII?

REKOMBINACE DNA: REPORTÉROVÉ MODELY

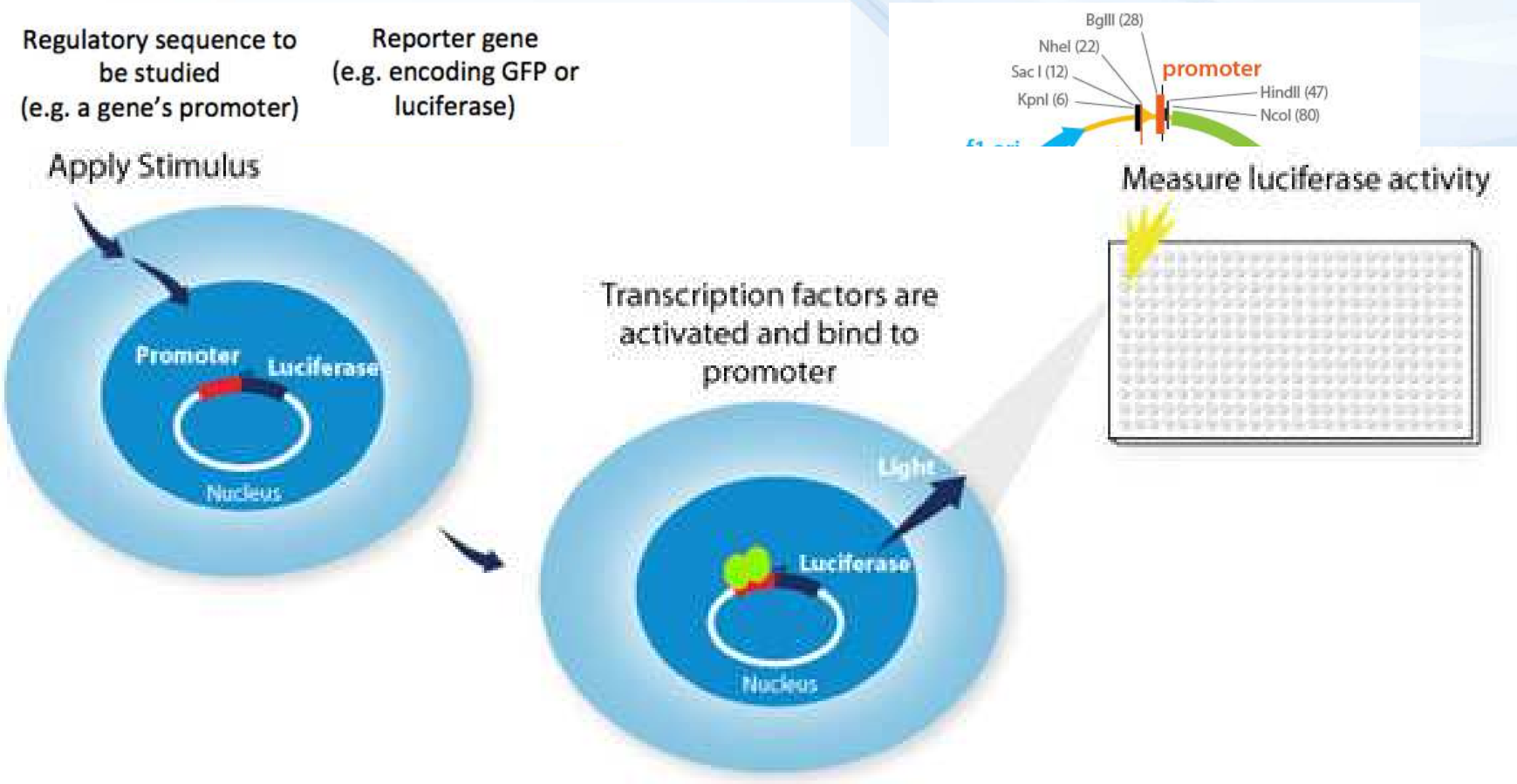
http://worldwide.promega.com/resources/multimedia/reporter-assays-and-transfection/introduction-to-reporter-gene-assays/

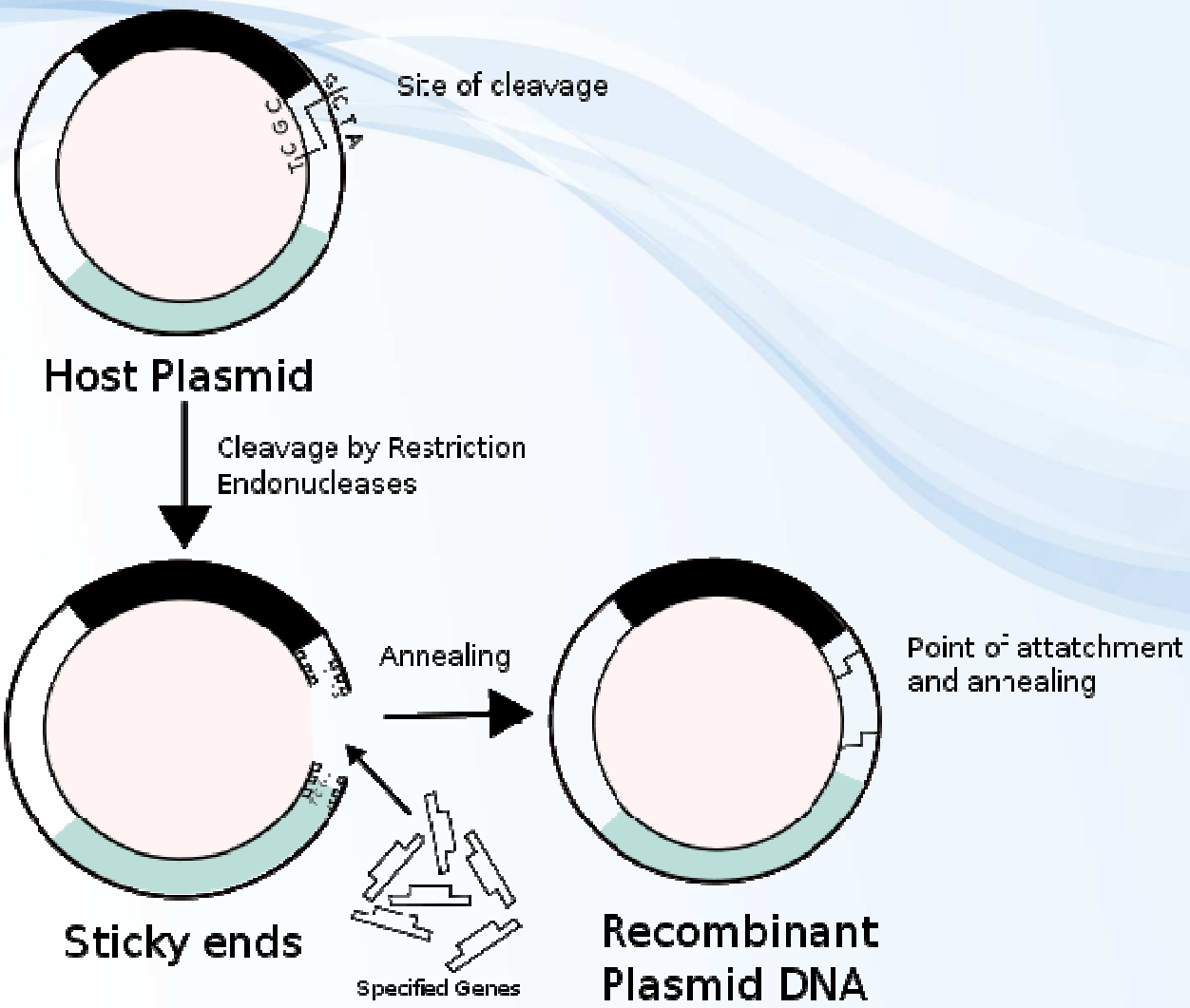




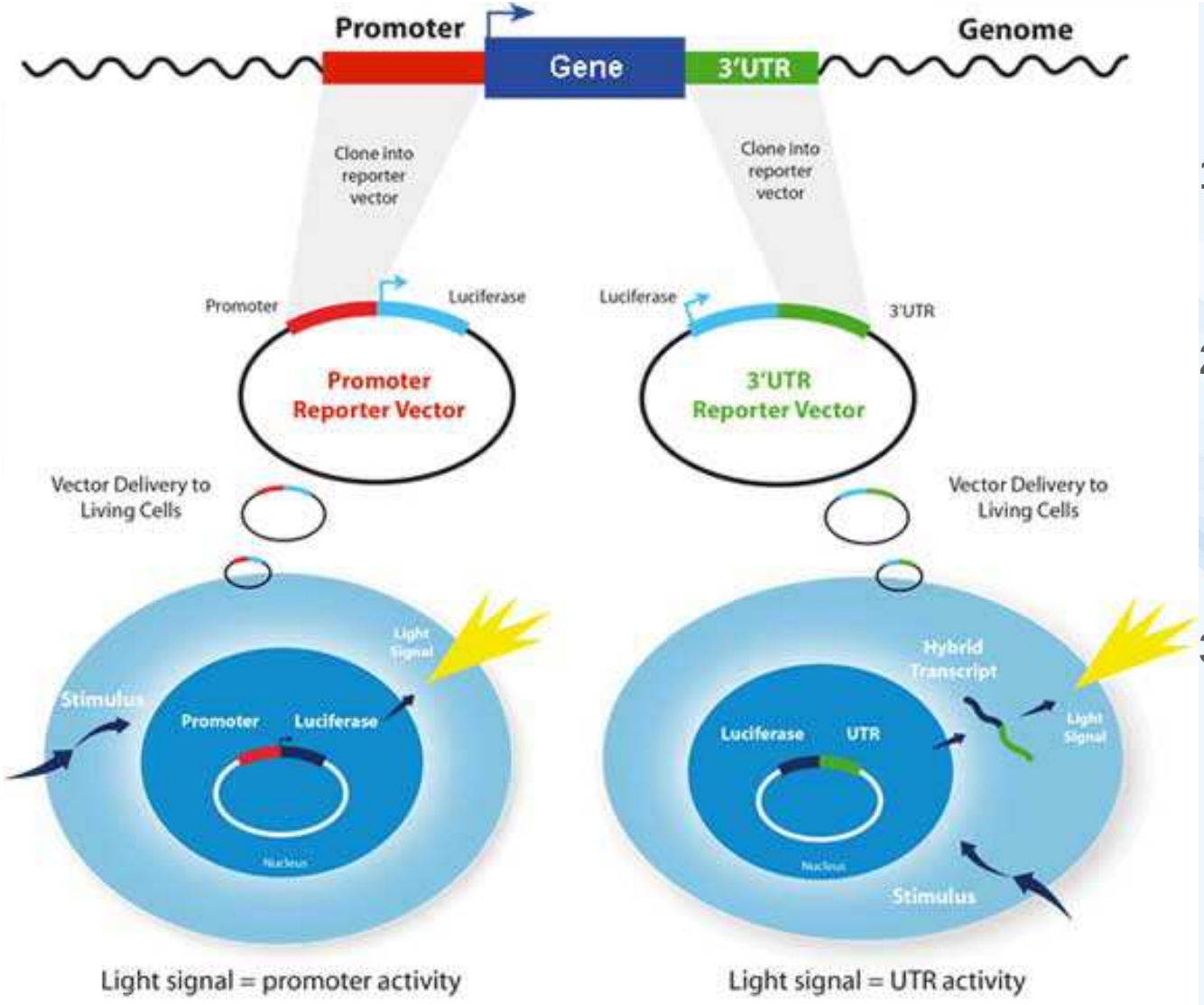
REKOMBINACE DNA: REPORTÉROVÉ MODELY

http://worldwide.promega.com/resources/multimedia/reporter-assays-and-transfection/introduction-to-reporter-gene-assays/



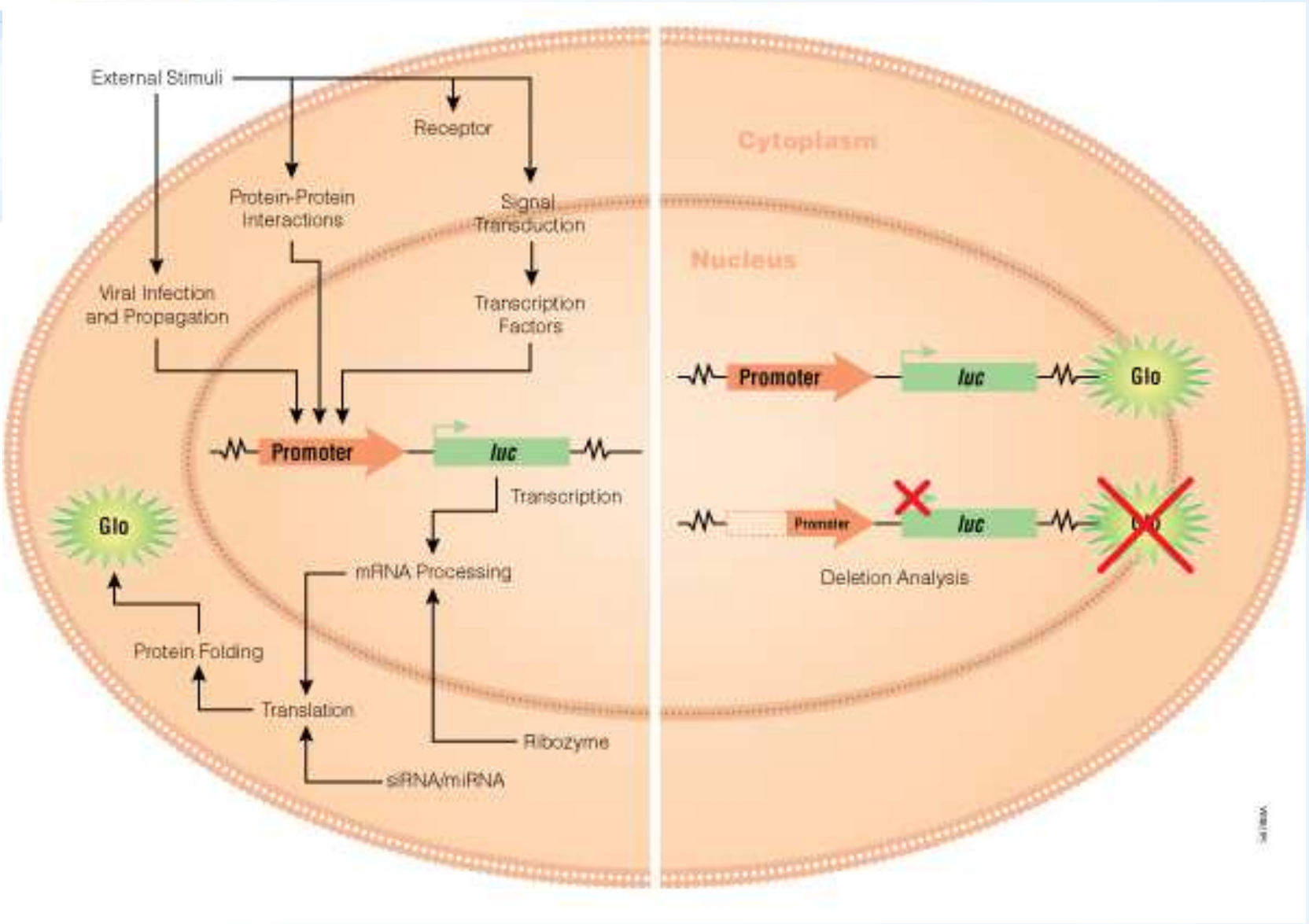


http://en.wikipedia.org/wiki/Biomolecular_engineering

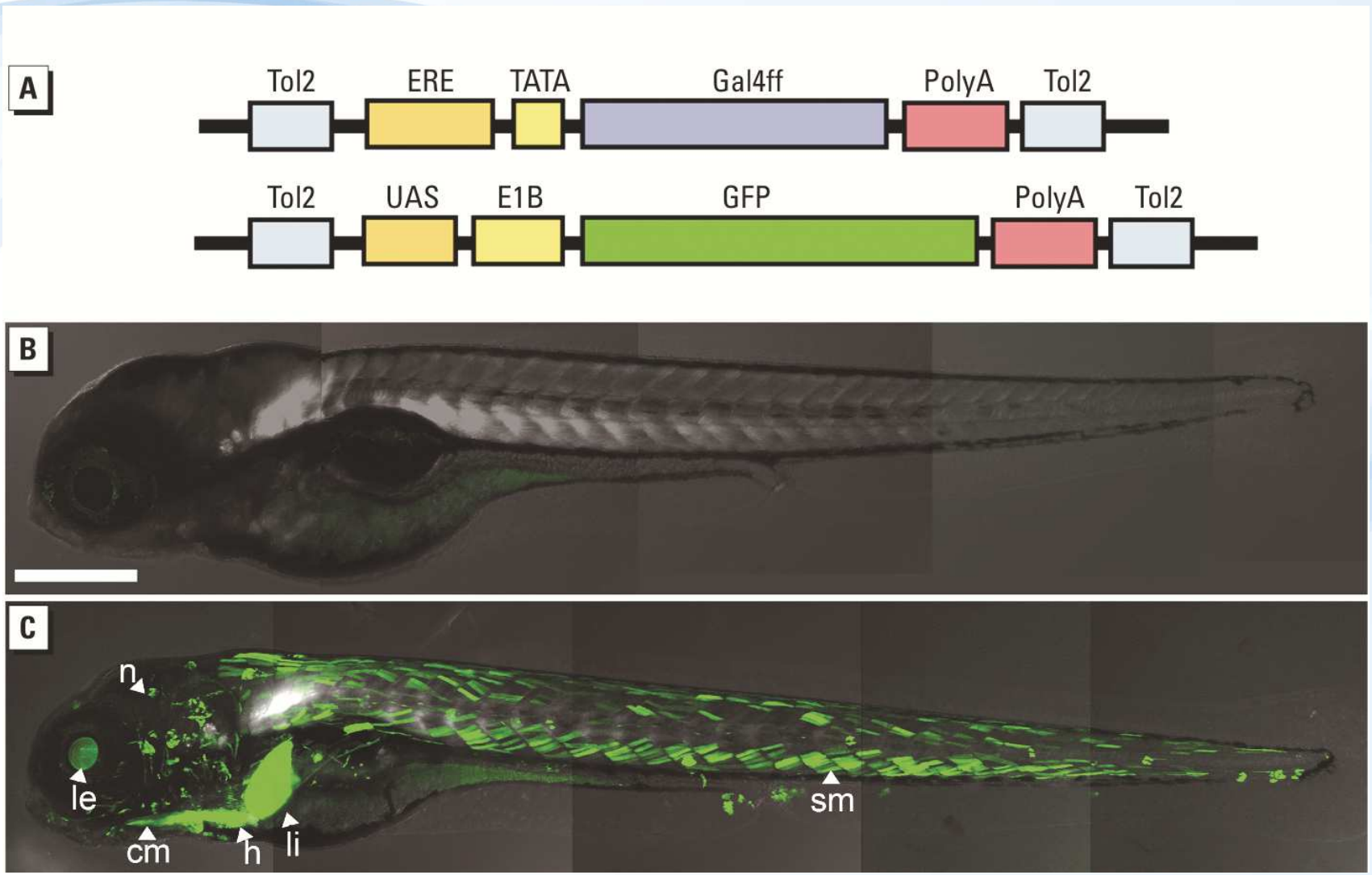


1. **Promoter nebo 3'UTR responzivního elementu**
2. **Tvorba plazmidu a jeho namnožení (restrikční enzymy, klonování, selekce a izolace plazmidů)**
3. **Přenesení konstruktu (plazmidu) do buněk = transfekce**

http://www.activemotif.com/catalog/900/lightswitch-luciferase-assay-system



http://worldwide.promega.com



EHP 120 (2012): 990

REKOMBINACE DNA: JAK VYPNOUT GEN?

- **“GENE KNOCK OUT”**

⇒ kombinace technik vede k vyřazení genu z provozu

⇒ DNA konstrukt přenesen do buněčné kultury

⇒ u zvířat jsou embryonální kmenové buňky geneticky upraveny a vneseny do ranního stádia embrya

- **„GENE KNOCK-IN“** jedná se o nahrazení genu, ne o jeho vymazání
- **„GENE KNOCK-DOWN“** jedná se o snížení či inhibici genové exprese

REKOMBINACE DNA: JAK VYPNOUT GEN?

How it works:

in vivo Model

Knock-in Mouse Generation

1. Embryos with attp sites are collected from TARGATT™ mouse.

2. TARGATT™ Vector with Gene of interest (eg. GFP)

3. Pronuclear injection into TARGATT™ embryos.

4. Screening pups for site-specific gene integration

Knock-in Mouse within 3 months

Applications

in vivo screenings
Generation of humanized models
Generation of disease models
Generation of drug / genome interaction models

in vitro Model

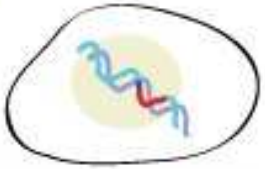
Stable Cell Line Generation

1. Generate master TARGATT™ Cells with attp sites (6 months)

2. TARGATT™ Vector with Gene of interest (eg. GFP)

3. Transfection

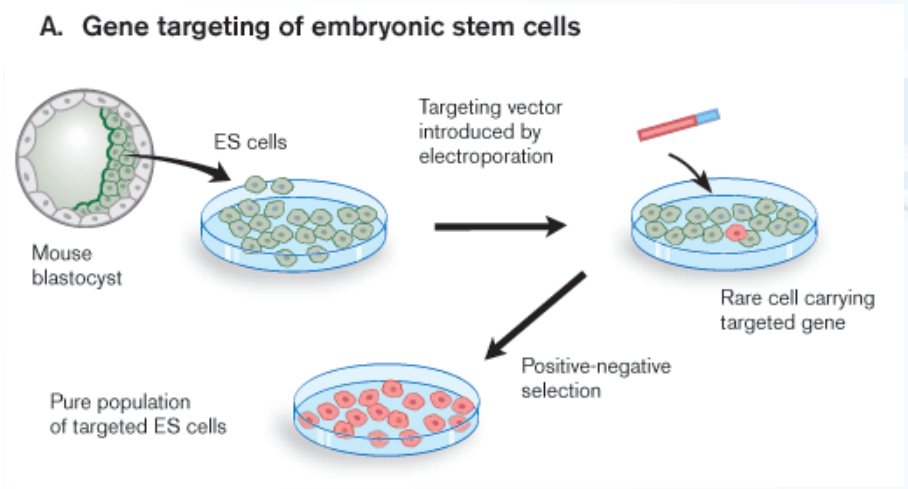
4. TARGATT™ Cell Lines Expressing Gene of Interest

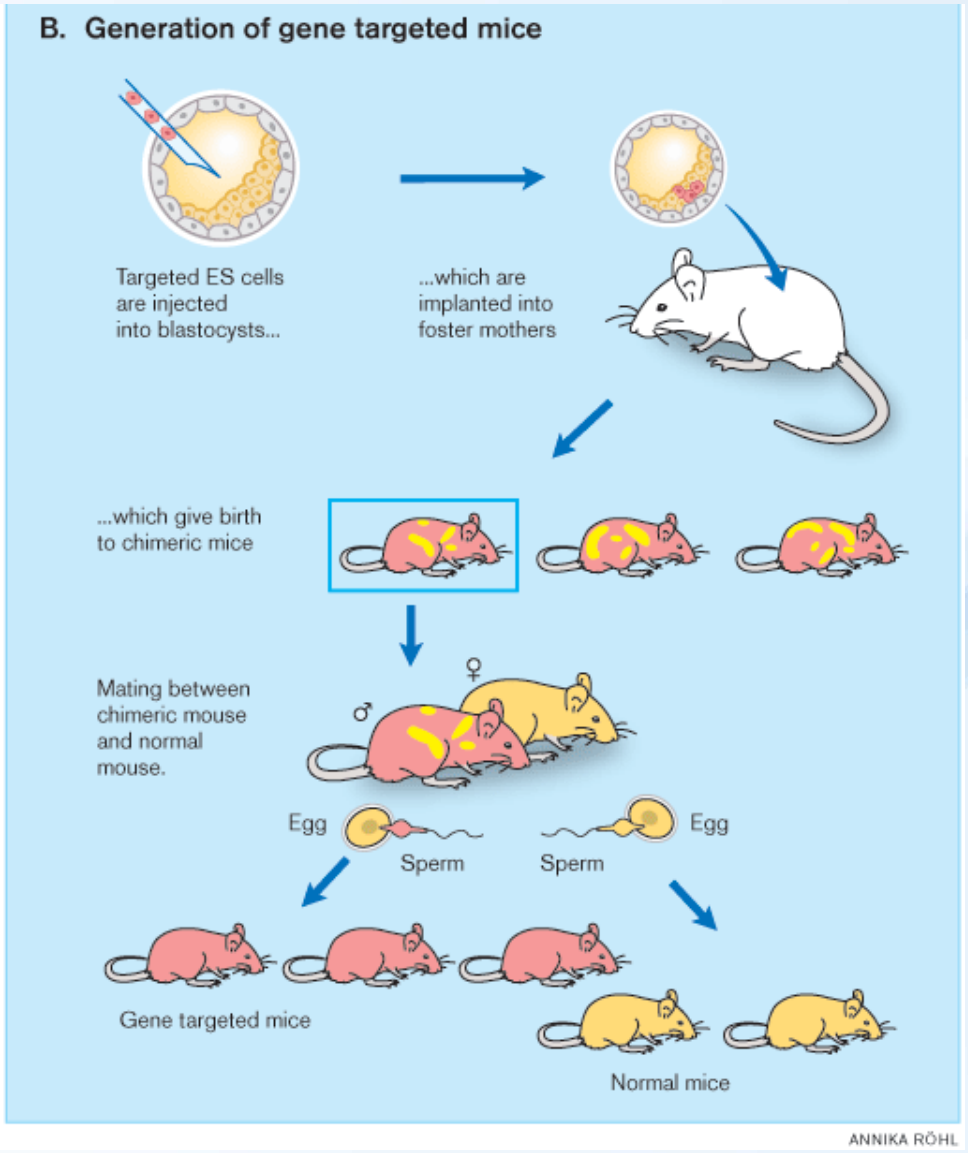


Knock-in Cell Line within 1 - 3 months

Applications

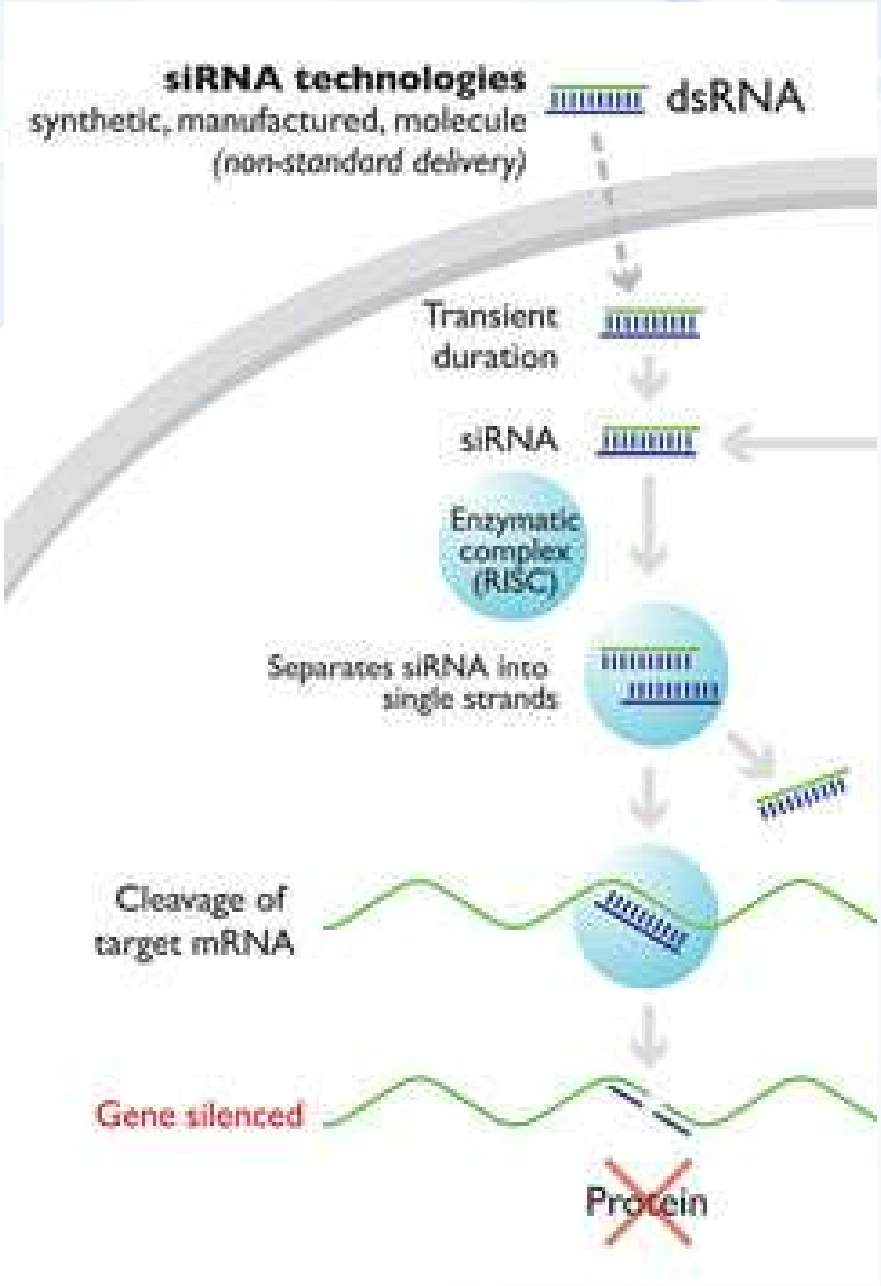
in vitro screenings
Drug discovery
Study drug interactions
Toxicity Study
Personalized medicine

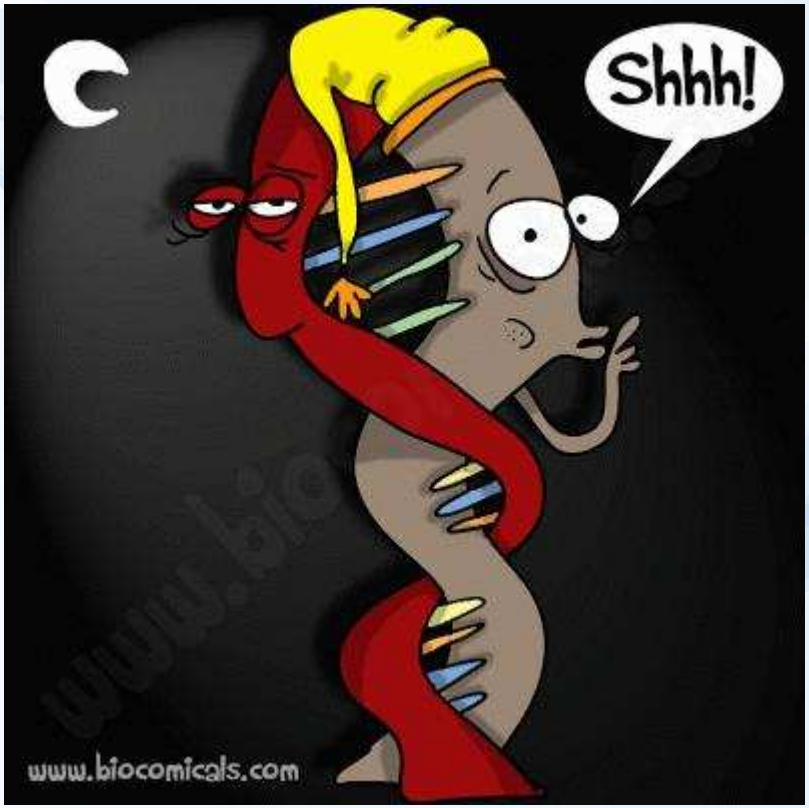




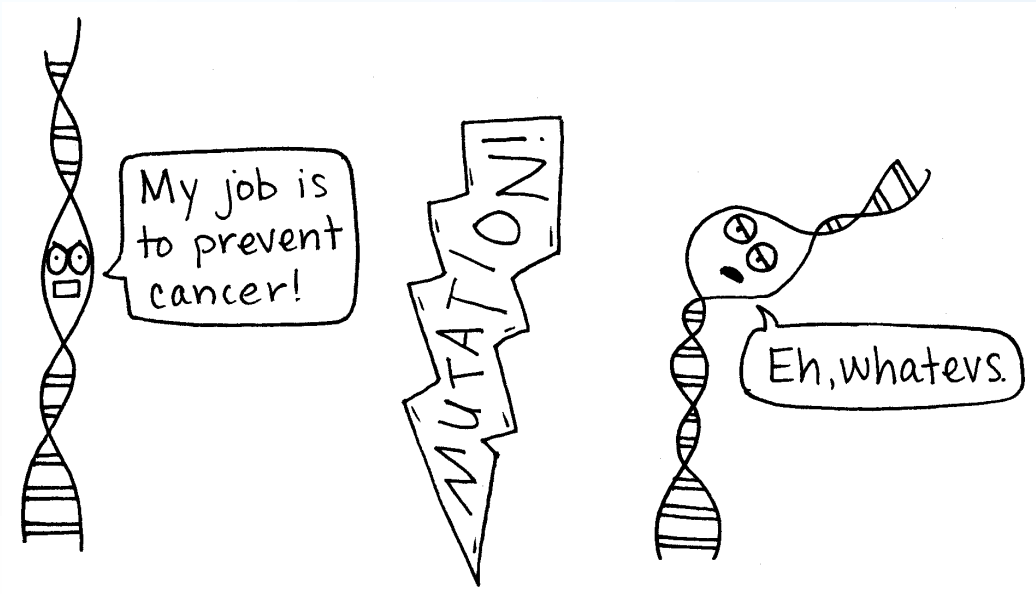
http://www.nobelprize.org/nobel_prizes/medicine/laureates/2007/advanced.html

- “siRNA”, ribozymy,

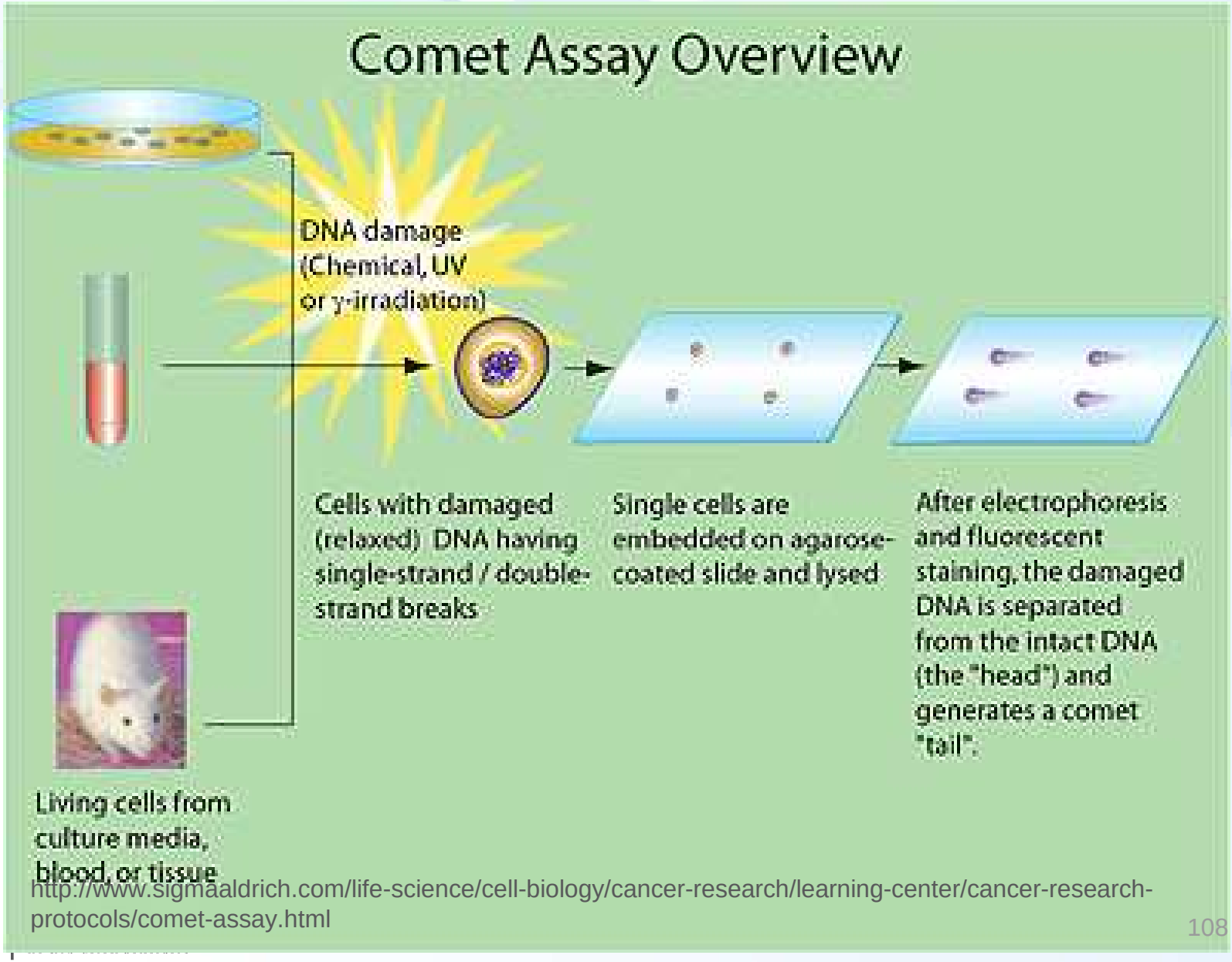




JAK STUDUJEME GENO(EKO)TOXICITU?



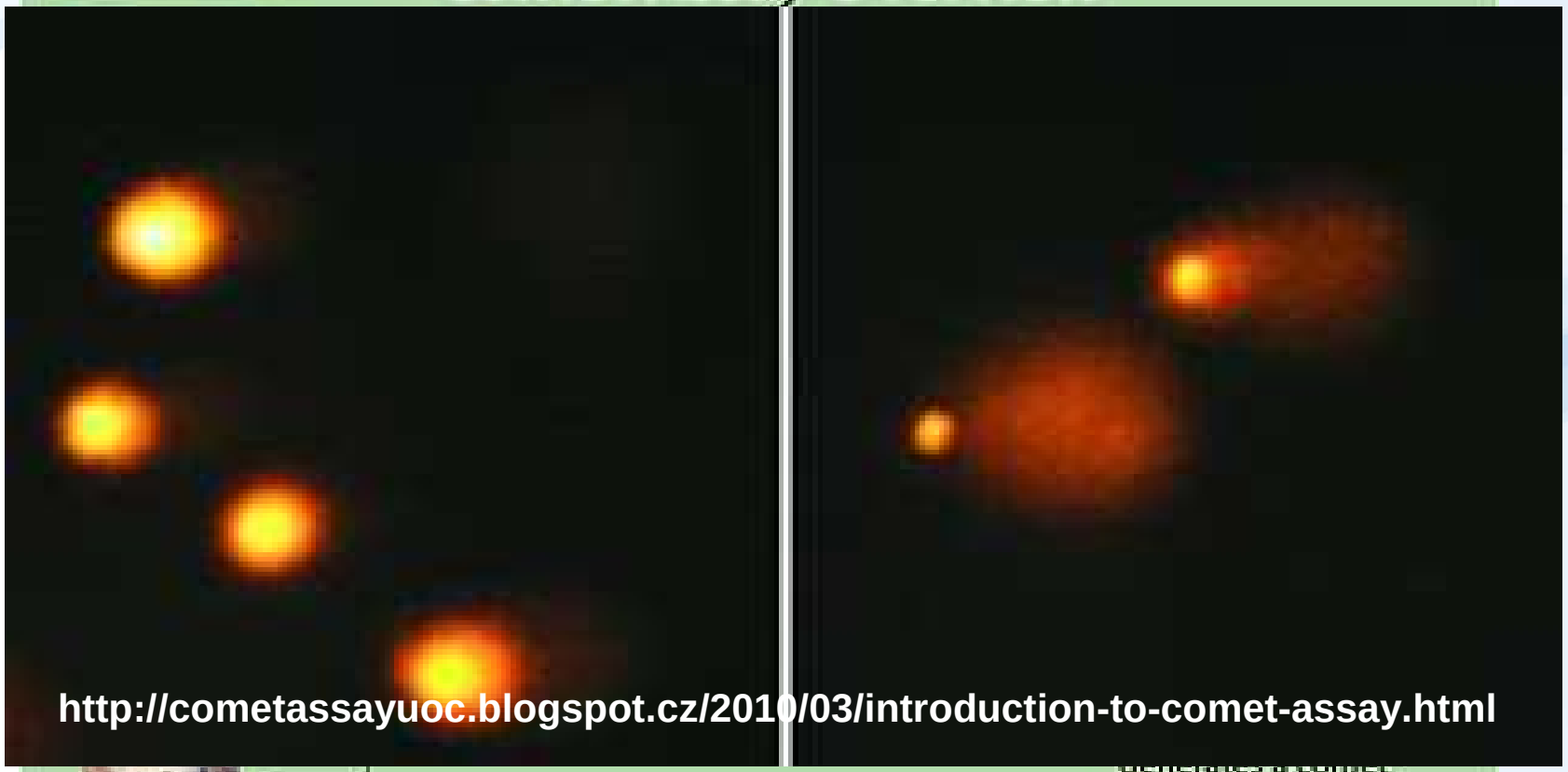
GENOTOXICITA: COMET ASSAY



http://www.sigmaaldrich.com/life-science/cell-biology/cancer-research/learning-center/cancer-research-protocols/comet-assay.html

GENOTOXICITA: COMET ASSAY

Comet Assay Overview



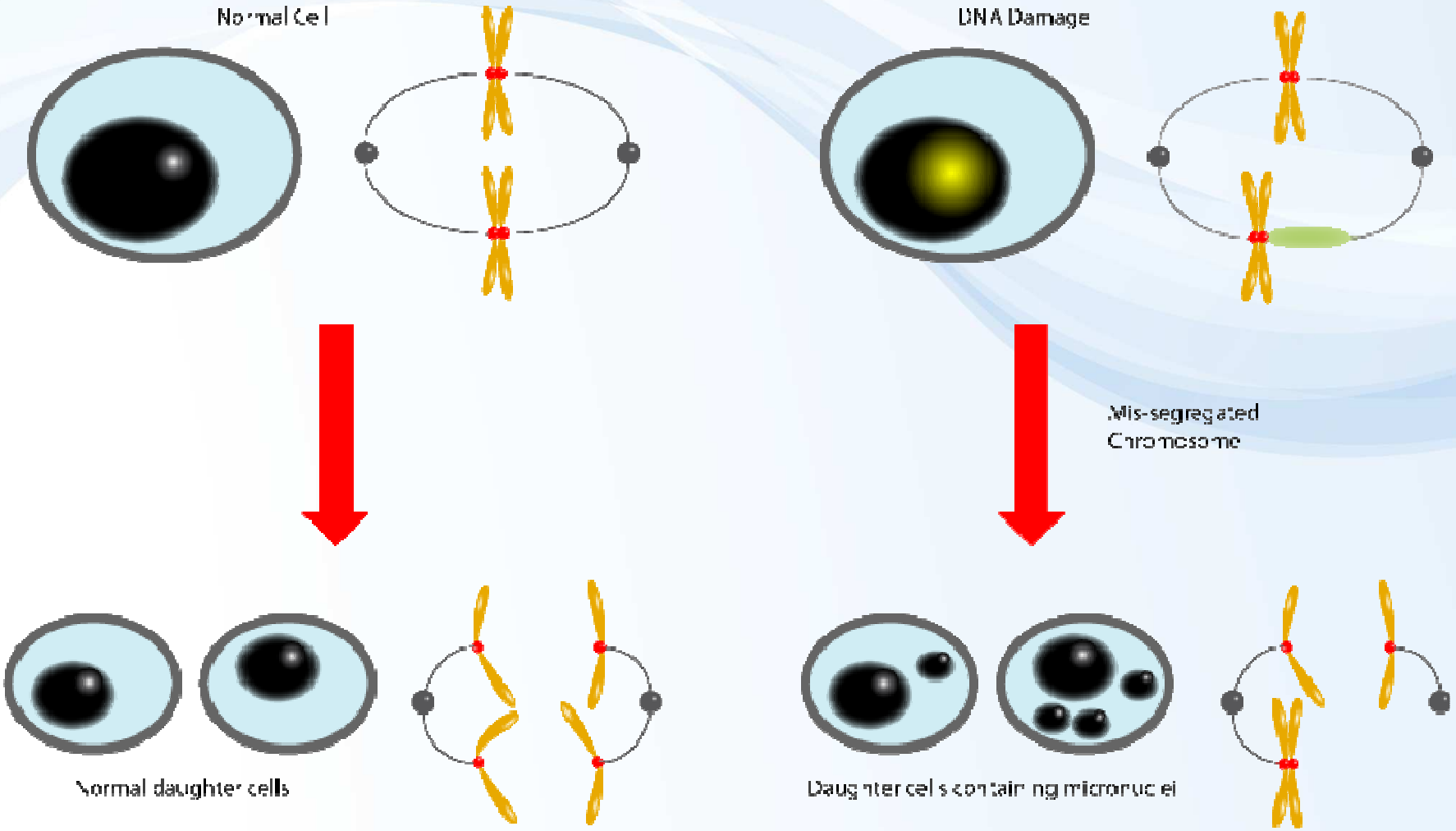
http://cometassayuoc.blogspot.cz/2010/03/introduction-to-comet-assay.html

"tail".

Living cells from culture media, blood, or tissue

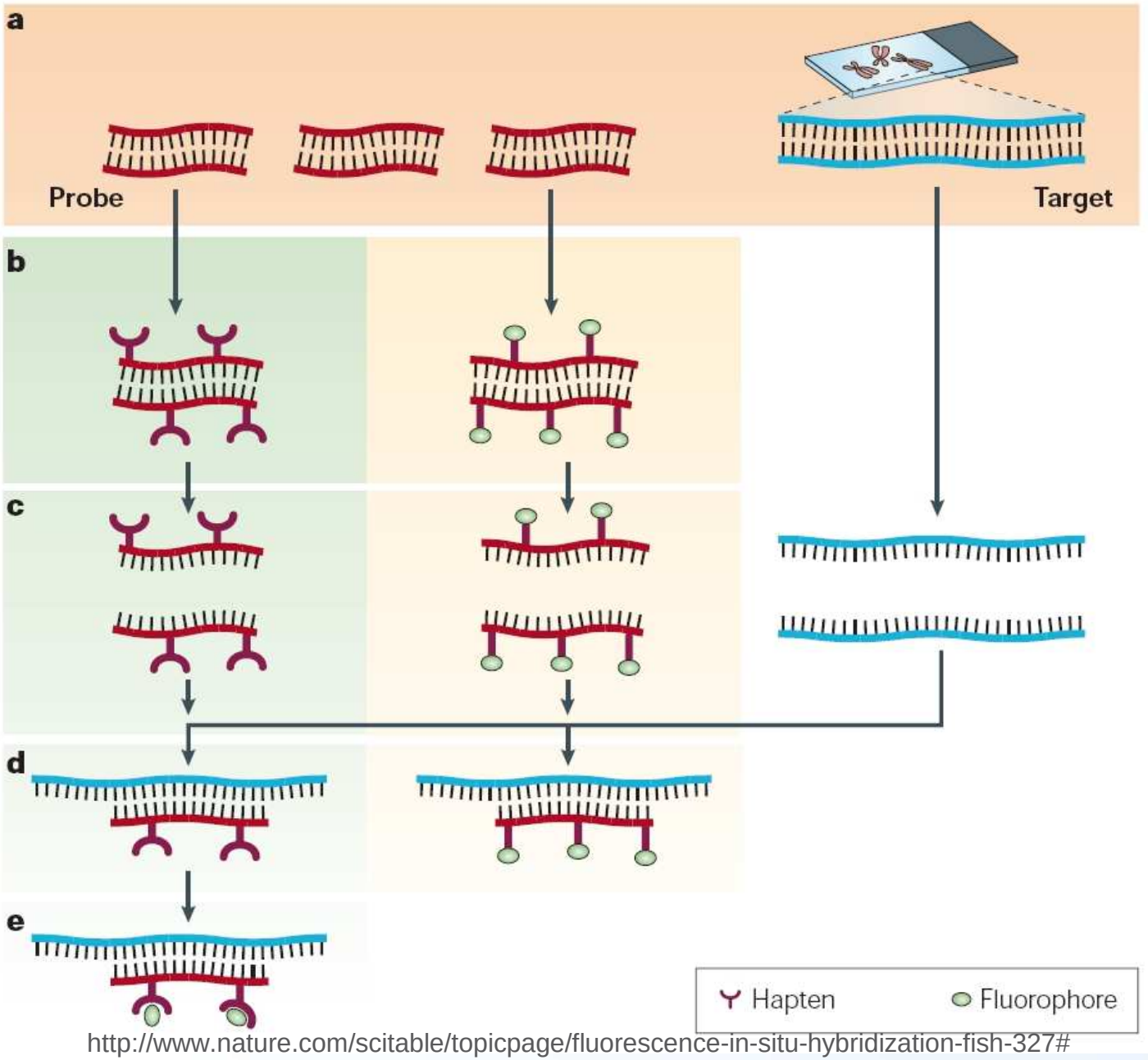
http://www.sigmaaldrich.com/life-science/cell-biology/cancer-research/learning-center/cancer-research-protocols/comet-assay.html

GENOTOXICITA: MIKROJÁDROVÝ TEST

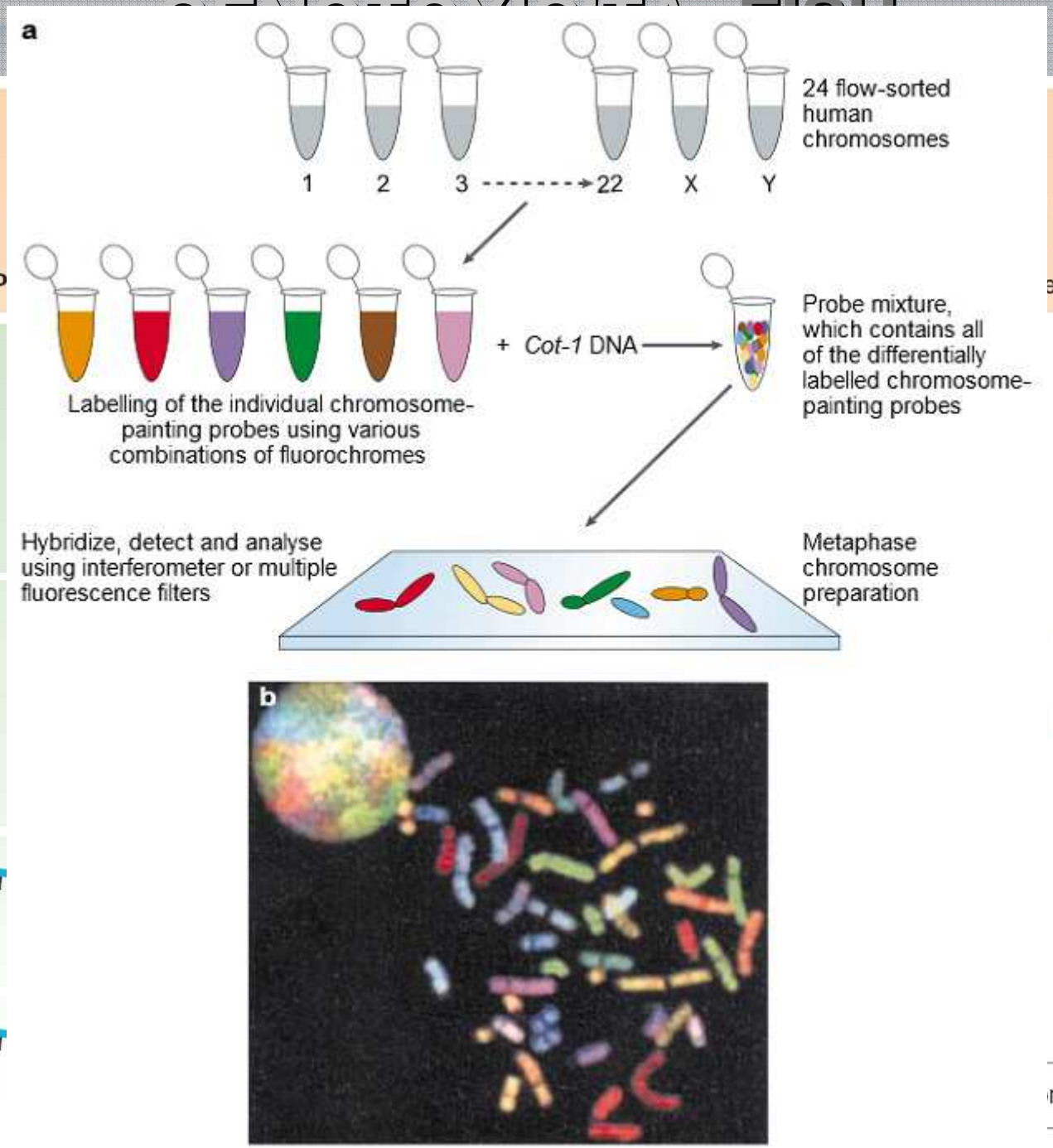


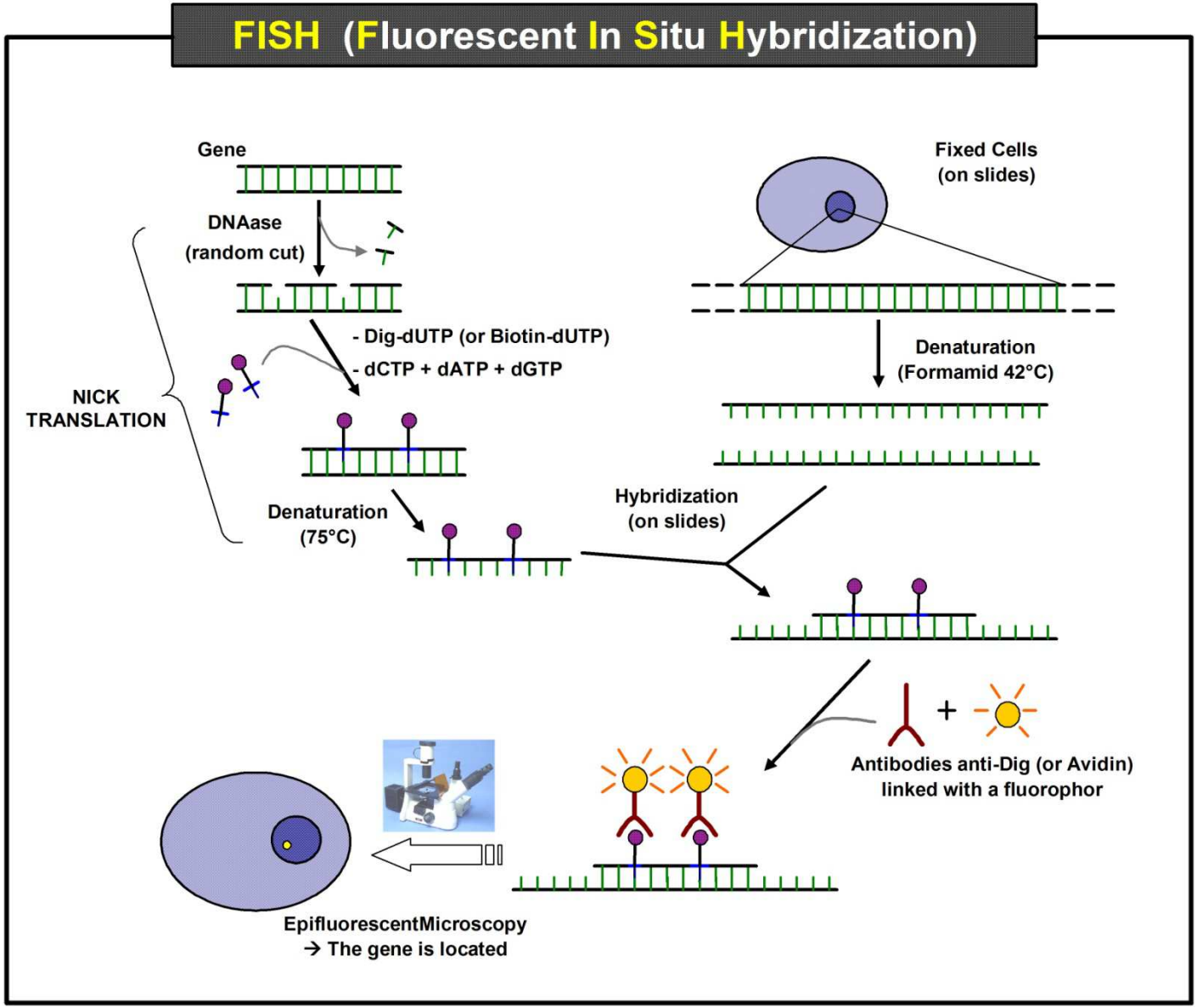
http://www.gentronix.co.uk/product/micro-nucleus-test/

GENOTOXICITA: FISH



http://www.nature.com/scitable/topicpage/fluorescence-in-situ-hybridization-fish-327#


a
1
2
3
22
X
Y
24 flow-sorted human chromosomes
Labelling of the individual chromosome-painting probes using various combinations of fluorochromes
+ Cot-1 DNA
Probe mixture, which contains all of the differentially labelled chromosome-painting probes
Hybridize, detect and analyse using interferometer or multiple fluorescence filters
Metaphase chromosome preparation
b



http://commons.wikimedia.org/wiki/File:FISH_%28Fluorescent_In_Situ_Hybridization%29.jpg