Advanced Food Microbiology

Laboratory Module A:
Experiment no.1: How to handle the instruments in microbiology lab.
Experiment no. 2: Different microbial culture media.
Experiment no. 3: Catalase , Oxidase and other common tests.

Laboratory Module B:
Experiment no. 4: Quantification of microbes using different selective media.
Experiment no. 5: Study on serial dilution in microbiology laboratory.
Experiment no. 6: Study on microbial colony morphology.
Experiment no. 7: Study on how to calculate colony forming unit(CFU).

Laboratory Module C:
Experiment no. 8: Quantification of Lactobacillus species in commercial yogurt.
Experiment no. 9: Quantification of Coliform bacteria in commercial fruit juice.
Experiment no. 10: Quantification of Salmonella species in commercial yogurt and fruit juice.

Laboratory Module D:
Experiment no. 11: Study on bacteria preservation and how to revive bacterial culture from glycerol stocks.
Experiment no. 12: Study on citrate utilization test using simmon citrate agar.

Cell lysis and dissolution	Cell lysis can be achieved using buffers or reagents containing chaotropic agents such as guanidinium isothiocyanate, guanidinium chloride, sodium dodecyl sulphate (SDS), sarcosyl, urea, phenol or chloroform. TRIzol or RNAlater or Qiazol [10] can be used to maintain RNA integrity during lysis.
Denaturation of DNA and proteins	DNase can be used to degrade DNA, while proteinase K can be added to digest proteins. Alternatively, repeated organic extraction using phenol and chloroform, or dissolving the sample in buffers containing guanidinium salts, can also be used to remove proteins.
Denaturation and inactivation of RNases	This can be achieved using any of the chaotropic agents mentioned above, such as phenol and chloroform.
Removal/separation of cellular components	RNA can be separated from other cellular components by adding chloroform and centrifuging the solution. This separates the solution into two phases: organic and aqueous phases. The aqueous phase contains RNA.
Precipitation	RNA is often recovered from the aqueous phase using isopropyl alcohol. RNA can also be selectively precipitated from DNA through the use of ammonium acetate. Alternatively, lithium chloride can be used to selectively precipitate RNA from DNA as well as proteins

Table 2. Basic steps involved in the RNA extraction using organic solvents/chaotropic agents.

Kit	RNA				DNA		
	Yield (ng/mm3)	260/280	260/230	DV200	Yield (ng/mm3)	260/280	260/230
HPRNA	2288.89	1.91	1.55	69	-	-	-
NucRNA	985.99	1.99	1.63	10	-	-	-
PuLink	3603.48	1.92	1.67	62	-	-	-
NorRNA	1662.61	1.85	1.05	70	-	-	-
EZNRNA	1845.12	2	1.55	31	-	-	-
RNeasy	2713.04	1.97	2.11	41	-	-	-
RecAll	2249.95	1.93	1.33	43	767.65	1.84	1.61
AllPrep	2512.08	2.01	1.4	54	757.2	1.77	1.2
GenJet	-	-	-	-	750.59	1.8	1.81
QIAamp	-	-	-	-	980	1.79	1.32
DNeasy	-	-	-	-	1236.03	1.91	1.45
HPDNA	-	-	-	-	536.53	1.87	2
NorDNA	-	-	-	-	468.53	1.91	2.09
NuDNA	-	-	-	-	429.71	1.99	1.87

https://doi.org/10.1371/journal.pone.0179732.t002

Method/Kit	Vendor	Num	Sample Reference
RNeasy kits (MinElute [34], miRNeasy [35, 36], Universal [37])	Qiagen	359	[34-37]
TRIzol/TRI Reagent	Thermo Fisher, MilliporeSigma	328	[38, 39]
RNase free DNase	Thermo Fisher, Promega, Qiagen	29	
RNAlater	Thermo Fisher, Qiagen	24	
Pico Pure RNA isolation kit	Thermo Fisher	12	
Nucleospin RNA kits	Macherey-Nagel	13	#740955 [40]
mirVANA microRNA isolation kit	Thermo Fisher	14	
Absolutely Kits	Stratagene, Agilent Technologies	8	
SV total RNA isolation kit	Promega	5	
RNAqueous Kit	Thermo Fisher	9	
AllPrep DNA/RNA Micro Kit	Qiagen	8	80204 [9]
GenElute Mammalian Total RNA Miniprep kit	MilliporeSigma	4	
peqGOLD RNA kits	PeqLab Biotechnologie	3	
MagMax	Thermo Fisher	3	[41, 42]
Dynabeads mRNA DIRECT Micr	Thermo Fisher	1	[43]
NucleoSpin RNA set for NucleoZOL	Macherey-Nagel	1	[44]
Quick-RNA MicroPrep	Zymo Research	1	[45]
RNAClean XP	Beckman Coulter	1	[46]

Table 3. Commonly used reagents, methods or kits for RNA extraction among formal articles surveyed by Labome. Major suppliers a listed. Num: the number of articles.

Method/Kit	Applications	Sample size & processing	Advantages
TRIzol Reagent (Thermo Fisher)	Provides high-quality RNA for RT-PCR, northern blotting, nuclease protection assays, cloning, real-time PCR and cDNA library construction.	Can be used for small samples of tissue (50-100 mg) and cells (5×10^6), and also for larger samples of tissue (≥1 g) and cells ($>10^7$)	It is a complete ready to use reagent for RNA isolation. It isolates transcriptome RNA, total RNA and micro RNA. Can simultaneously isolate RNA, DNA and protein from a sample, in approximately 1 hour.
RNeasy kits (Qiagen)	Provides purified RNA for RT-PCR, real-time RT-PCR, differential display, cDNA synthesis, northern blotting, dot and slot blot, primer extension, poly A RNA selection, RNase/S1 nuclease protection and microarrays	Can be used to isolate up to 100 µg of total RNA using the mini kit, or up to 45 µg of total RNA using the micro kit. TheRNeasy mini kit can be used for up to 1 mg of RNA and RNeasy maxi kit can be used for up to 6 mg of RNA. Isolates and provides high-quality RNA in minutes.	It employs a fast procedure, and gives consistent yields even from small samples. It does not require phenol/chloroform extraction, CsCl gradients, LiCl or ethanol precipitation.
RNase free DNase I (Thermo Fisher)	Provides purified RNA for ribonuclease protection assays, cDNA library contraction, and RT-PCR	One Molecular Biology Unit (MBU) of RNase-Free DNase I digests 1 µg of pUC19 DNA to oligodeoxynucleotides in 10 minutes at 37°C under standard assay conditions.	It removes contaminating DNA from total RNA preparations, while maintaining the integrity of the RNA. For best results, it requires bivalent cations such as $Mg^{2+}$and Ca^{2+}, and a pH of 7.8. It can also be used for characterization of DNA-protein interactions by DNase I footprinting [91].

Method/Kit	Applications	Sample size & processing	Advantages
Pico Pure RNA isolation kit (Thermo Fisher)	Provides high-quality RNA for real-time PCR and microarray analysis	It gives consistent RNA recovery from as few as ten cells, using LCM (laser capture microdissection) to prepare samples.	It can be used to isolate high-quality RNA from even a single cell. It gives efficient and consistent RNA recovery. It gives small elution volumes thereby maximizing the concentration of recovered RNA. It retains low abundance RNA. Prevents sample contamination by using RNase free column lids.
mirVANA microRNA isolation kit (Thermo Fisher)	It can be used for miRNA, siRNA, shRNA, and snRNA analysis. Provides purified RNA for microRNA analysis and microarray analysis.	It isolates total RNA within a size range of kilobases to 10-mers, from samples of 10^2– 10^7 cultured cells or 0.5 – 250 mg tissue. It can isolate RNA in about 30 minutes.	It employs a simple and efficient procedure. It can be used for most types of tissues and cells. It also purifies RNA molecules of ≤ 200 nucleotides from larger RNA molecules, thereby enriching miRNAs, siRNAs, and/or snRNAs.
Absolutely Total RNA, miRNA & mRNA Purification Kits (Stratagene, Agilent technologies)	It provides high-quality RNA for northern blotting, RT-PCR, qRT-PCR, microarray target labeling, in-vitro transcription reactions,ribonuclease protection assays, and cDNA synthesis and library construction.	Purified RNA can be recovered in ≤ 30 minutes. It is available in miniprep, microprep, nanoprep and 96 well versions. The Absolutely RNA Nanoprep Kit can isolate RNA from even a single cell and can be used for small samples, in an elution volume of 10 µl	It uses a simple, safe and fast procedure that does not require organic extraction using phenol. It provides flexibility in primer design by completely removing genomic DNA.

Method/Kit	Applications	Sample size & processing	Advantages
SV total RNA isolation kit (Promega)	Provides high-quality RNA for molecular biology applications including RT-PCR, and northern blotting	60 mg of tissue can be processed per purification. RNA can be isolated in ≤ 1 hour. The sample can be purified using two methods, which include microcentrifugation (spin) and vacuum.	It uses a simple and fast procedure. The procedure includes a DNase treatment step to reduce genomic DNA contamination, without any DNase carryover in the final purified RNA. Does not require phenol-chloroform and ethanol precipitation.
RNAqueous Kit (Thermo Fisher)	Provides RNA for RT-PCR (endpoint), cDNA library construction, nuclease protection assays, northern blotting and real-time PCR.	It can isolate DNA from 0.1 g to 0.5 g of tissue or 10^7-10^8cells	The procedure used does not require phenol. It utilizes a convenient syringe-based filtration. It can be used with vacuum manifolds to process multiple samples simultaneously.
AllPrep DNA/RNA Micro Kit (Qiagen)	The purified total RNA is suitable for RT-PCR and real-time RT-PCR; differential display; cDNA synthesis; northern-, dot-, and slot-blot analyses; and microarrays. Purified genomic DNA is suitable for Southern-, dot-, and slot-blot analyses; and PCR and multiplex PCR.	It can be used with up to 10^7 cells or 30 mg tissue. Total RNA is of high quality and has a RIN value of 10 indicating that the RNA is intact.	Simultaneous purification of both genomic DNA and total RNA. Purified genomic DNA has an average length of 15–30 kb depending on homogenization conditions.
GenElute Mammalian Total RNA Miniprep kit (MilliporeSigma)	It provides purified DNA for various downstream applications including reverse transcription, PCR, labeling and microarray analysis. It does not recover RNA molecules smaller than 200 nucleotides.	It can be used for up to 10^7 cells or 40 mg of tissue, and can recover RNA from as few as 100 cells. It yields up to 150 µg of purified RNA in an elution volume of 50-100 µl, per preparation in ≤ 30 minutes.	Uses a simple and efficient procedure that can process up to 12 to 18 preps in about 30 minutes. It implies that it gives 40% more purifications per kit than the leading supplier. It does not use cesium chloride gradients, alcohol precipitation, and hazardous organic compounds such as phenol and chloroform.

Method/Kit	Applications	Sample size & processing	Advantages
Spectrum Plant Total RNA kit (MilliporeSigma)	Provides purified RNA for common downstream applications including northern blotting, RT-PCR and qRT=PCR.	It can extract up to 100 µg of total RNA from 100 mg of tissue in approximately 30 minutes. Typical yields range from 20–60 µg.	It can isolate purified RNA from plant samples containing high levels of secondary metabolites. It can be used for challenging plant tissue samples including pine needles, cotton leaves, grape leaves, and soybean leaves.
peqGOLD Total RNA kits (PeqLab Biotechnologie)	It gives purified DNA that can be used for most downstream applications.	It isolates RNA molecules ≥ 200 nucleotides, from samples of up to 10^7 cells or 40 mg tissue in ≤ 25 minutes, in an elution volume of 50-100 µl. A maximum yield of 100 µg/column can be obtained using this kit.	It utilizes a simple and fast procedure. It does not require organic extraction and ethanol precipitation. It is specifically recommended for total RNA isolation from adipose tissues. Note: adipose tissues have much less RNA and protein content than other tissue types [92].
RNAlater (Thermo Fisher, Thermo Fisher)	It can be used for tissue storage to stabilize and protect cellular RNA until it is processed. It is also used for storage of RNA after RNA isolation and purification. It is compatible with most RNA isolation procedures.	It has been used for samples of mammalian tissues, plants, *E. coli*, *Xenopus*, fish, and *Drosophila*. The reagent is economical, can be stored at room temperature and saves time.	It is an aqueous, non-toxic tissue storage reagent that immediately inactivates RNase, protecting and stabilizing an RNA sample. It avoids the need to store samples in liquid nitrogen or in freezers. It minimizes freezing and grinding.

Table 5.Overview of kits including applications and particular advantages.

- Harvest Grow Cultures to OD_{600}=0.4-0.5
- Prepare Blue Cap Falcon Tubes (sterile) containing 2.5 ml of 5% Phenol in 100% EtOH (made prior to use)
- Do not remove from H_20 bath, pipet 25 mL quickly into Falcon tubes Invert rapidly to mixSpin: 6000rpm (5524g) 5min 4ºC
- Discard supernatantFreeze tubes with pellet on dry ice, then keep at -80ºC or extract RNA(Freezing the pellet may increase RNA yield)NA Extraction (for 25ml cells, harvested at OD_{600}~0.45)
- Defrost pellet on ice~20minResuspend pellet in 960µL cell resuspension buffer (4ºC) vortex 15-30 sec
- Split sample (480µl each) into 2Epp tubes, each with an equal volume (480µl) of hot phenol solution (400µl buffer, 4µl merc-EtOH, 80µl phenol), vortex 15-30 sec
- 95ºC 1min
- Spin down cell debris in a microcentrifuge, max speed, RT for 15 min
- Transfer supernatant to 2-ml Epp tubes
- Extract with 600µL phenol/chloroform (1:1) by vortexing 10sec, spin 1min
- Extract twice with 600µL chloroform

DNA Precipitation

- Add 900µL isopropanol, invert to mix, keep (-20ºC) for 5min
- Spin in a microcentrifuge, RT (pellet should be translucent/white, visible) for 15 min
- Wash pellet with 1mL 75% EtOH (-20ºC) by vortexing for 15sec, spin for 10min
- Discard supernatant, spin remains 5sec at RT, pipette out remaining EtOH
- Dry the pellets at 42ºC for 3-5min
- Resuspend DNA in 45µL ddH_2O, vortex 5sec, 37ºC heat block for 5min, vortex for 5 sec, spin down 5sec
- Combine like tubes (90µL total), keep on ice
- Check DNA Concentration: OD260 of 1/200 dilution- should have ~100-300µg RNA+DNA

Composition of buffers used in RNA preparation:

Cell Resuspension Buffer		**200ml**
100 mM	KCl	20ml of 1M stock
5 mM	$MgCl_2$	2ml of 0.5M stock
10 mM	Tris (pH7.5)	2ml of 1M stock

Hot Phenol Buffer		**200ml**
40mM	EDTA	16ml of 0.5M stock
1%	SDS	2g
20mM	Tris (pH7.5)	4ml of 1M stock

DNAse I Buffer		**10ml**
400mM	Tris (pH7.5)	4ml of 1M stock
60mM	$MgCl_2$	1.2ml of 0.5M stock

3M Na-Acetate	**pH5.2**	**50ml**	**1000ml**
NA-Acetate•$3H_2O$		20.4g	408.1g
adjust pH with glacial acetic acid			

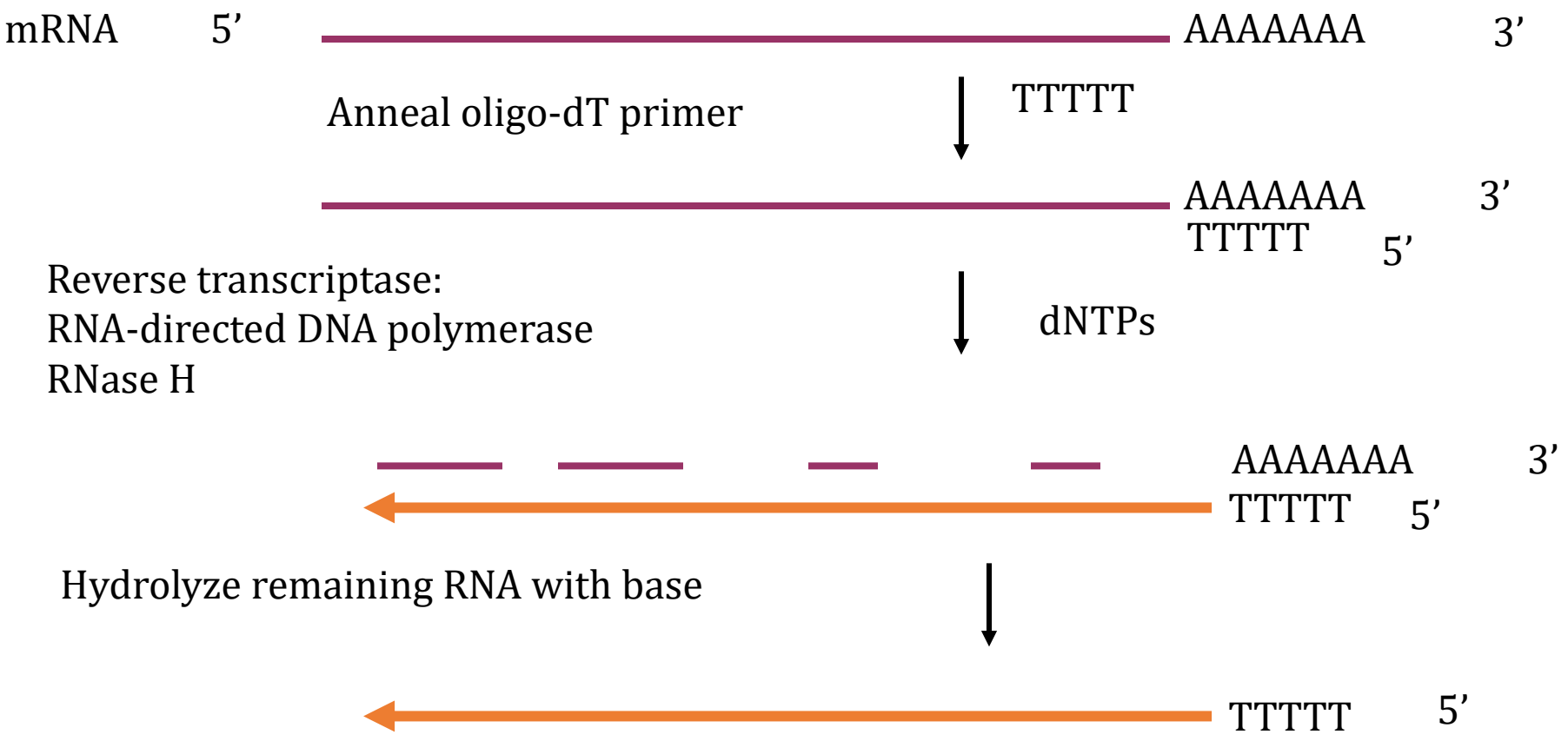
cDNA clones are copies of mRNAs

- Much of the genomic DNA is **not** expressed as mRNA
- Many issues about gene function are best addressed by examining the product that they encode.
- The cDNA copies of mRNA contain primarily sequences that encode protein.
- Therefore, cDNA clones are useful for many studies of gene function.

Construction of cDNA clones

- Use the enzyme *reverse transcriptase* to copy mRNA into complementary DNA, called cDNA. This is equivalent to the template strand of the duplex DNA.
- Use a DNA polymerase to copy that cDNA into the nontemplate (message synonymous) strand.
- Insert the duplex cDNA product into a cloning vector and propagate in a host, e.g. *E. coli.*

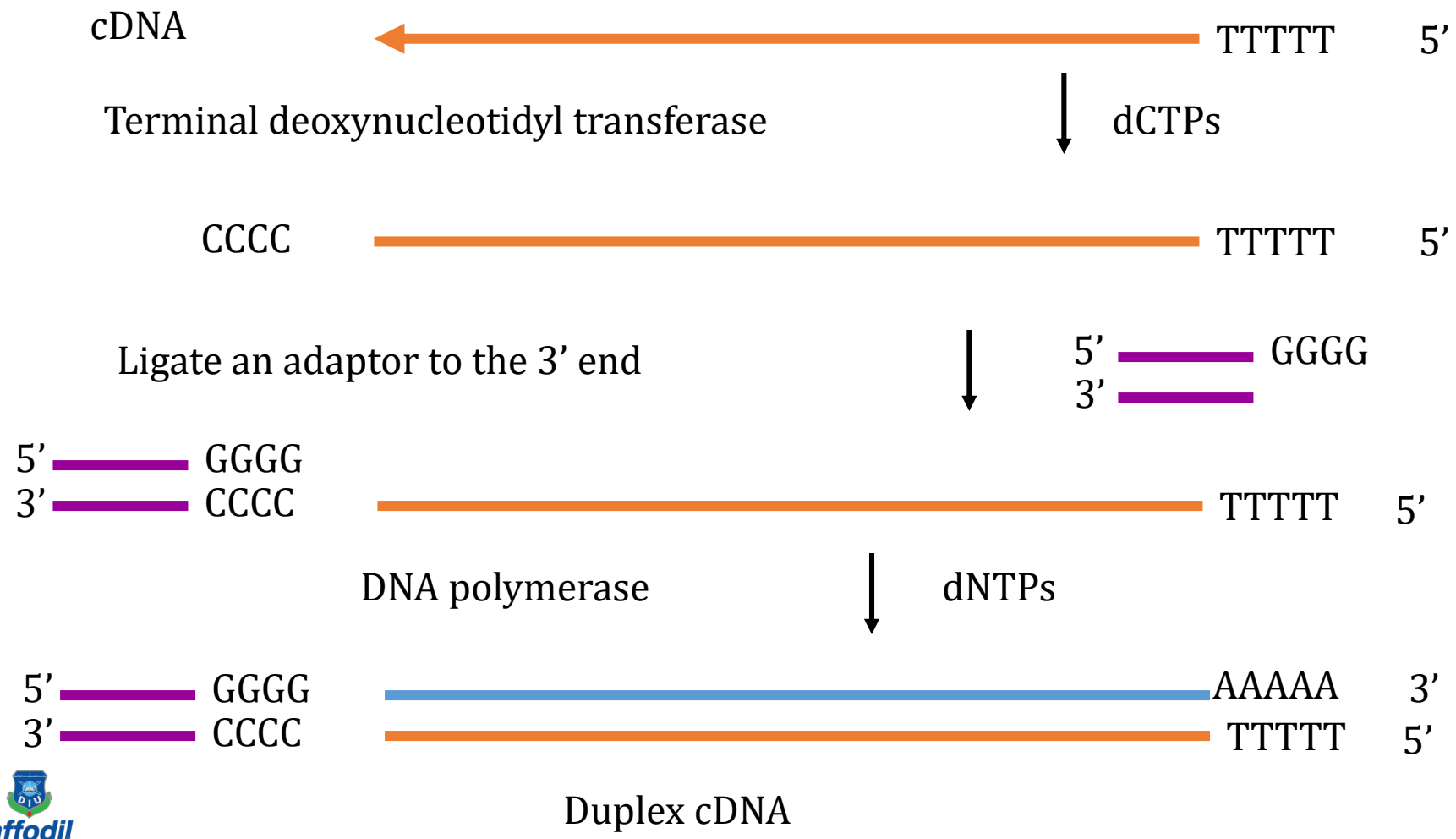
cDNA: first strand synthesis



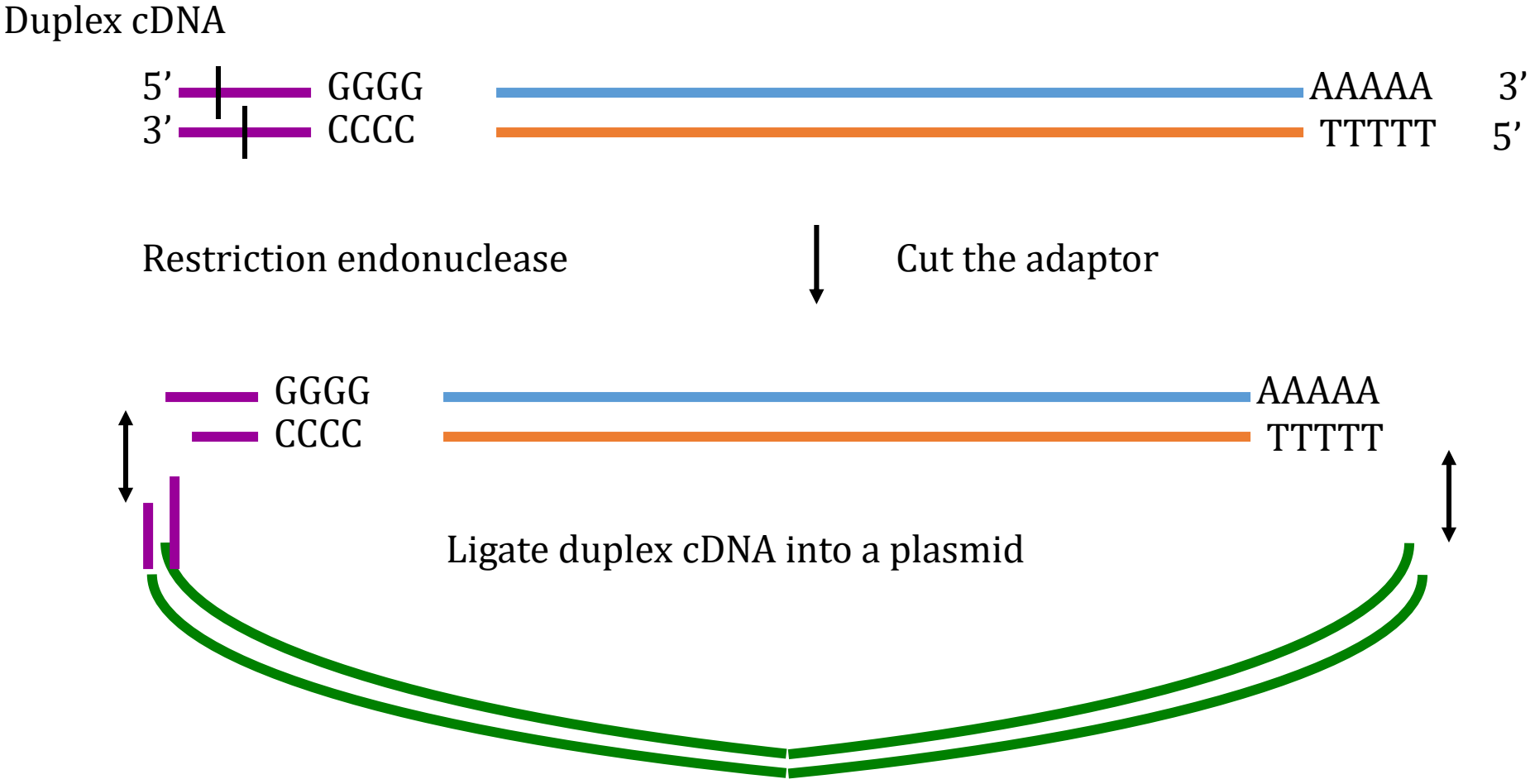
Product is complementary DNA, called **cDNA**. It is equivalent to the template strand of the duplex DNA.

cDNA: second strand synthesis

Problem: How to get a primer for 2nd strand synthesis?



Ligate duplex cDNA into a plasmid



Transform the population of cDNA plasmids into bacteria.
Result is a cDNA library.

Limitations of cDNA synthesis

First strand synthesis often does not go to completion.

- Individual cDNA clones will frequently have the reverse complement of only part of the mRNA.
- Multiple cDNA clones from a single mRNA will be present in the library

Priming second strand synthesis is inefficient

- Some methods necessarily result in the loss of sequences at the 5' end of the nontemplate strand

A cDNA library has >100,000 individual clones.

It contains copies of as many as 50,000 different mRNAs .

The frequency of occurrence of a cDNA from a given gene reflects the abundance of the mRNA for that gene.

Try to find correct 1 clone in about 100,000.

Strategies for screening cDNA clones

Brute force screen for abundant cDNAs.

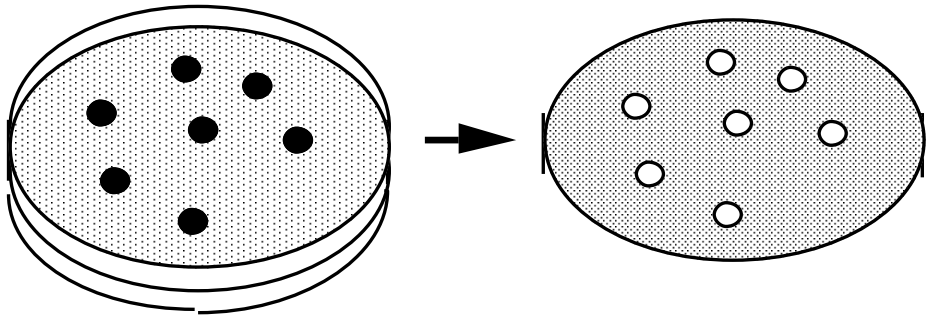
Hybridization with a gene-specific probe.

Express the cDNA in the host cell (i.e. make a functional protein product)

- Specific antisera
- Labeled ligand to a receptor
- Assay for a function (complementation)

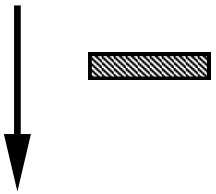
Differential analysis

Screening by hybridization

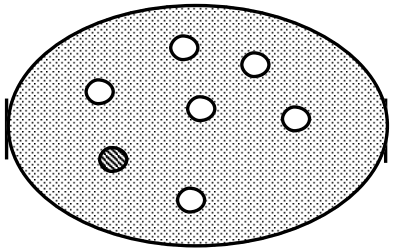


Each bacterial colonies contains a single type of cDNA plasmid

Filter replica of DNA in colonies

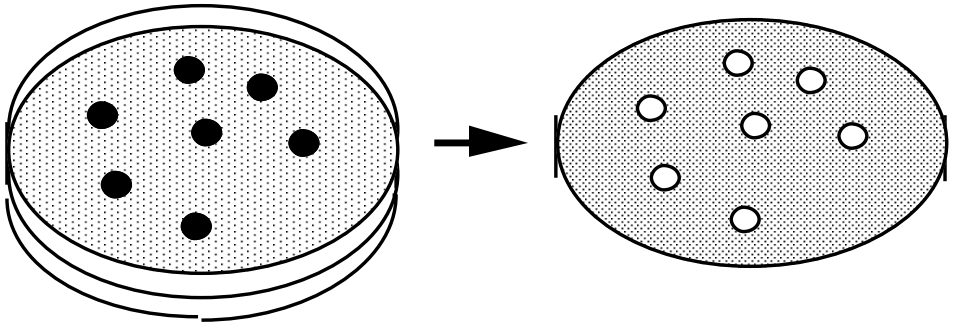


Hybridize with a labeled DNA from gene of interest

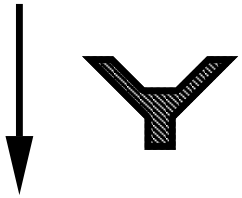


Detect by autoradiography

Screening for an expressed product

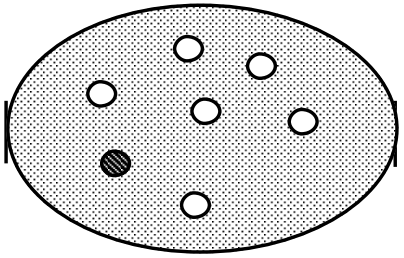


Filter replica of protein in colonies

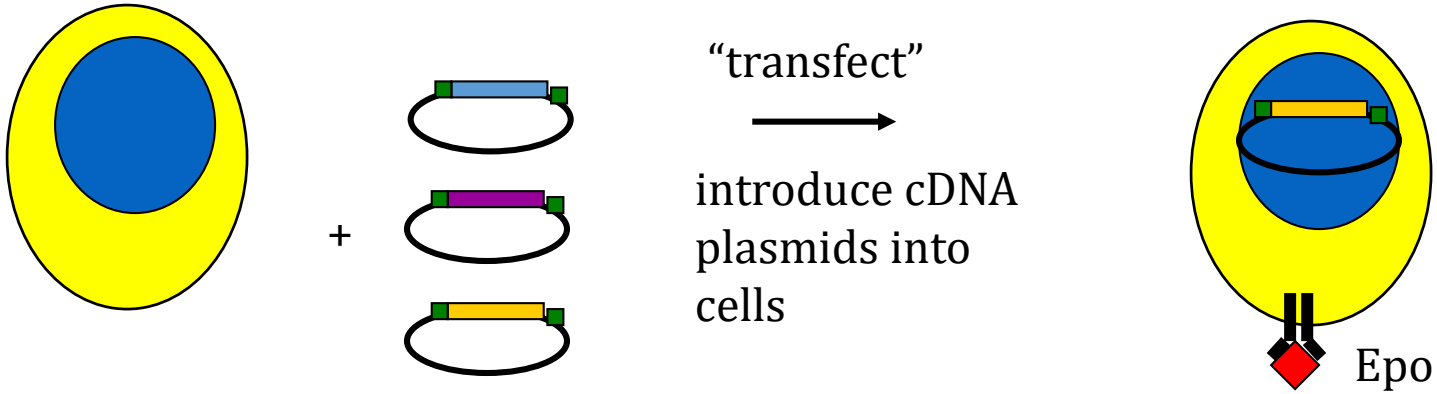


Bind an antibody specific for the protein of interest

Detect the bound antibody with an enzymatic assay (generating color or light).



Expression screening in eukaryotic cells



Cell line that needs a cytokine (e.g. IL-3) to grow. Has no Epo receptor, will not grow in Epo.

Expression library: cDNA inserts in a vector that will drive expression in eukaryotic cells

A transformed cell line that expresses the Epo receptor will now grow in Epo without IL-3. The plasmid with the Epo receptor cDNA can be isolated from this cell line.

Differential analysis

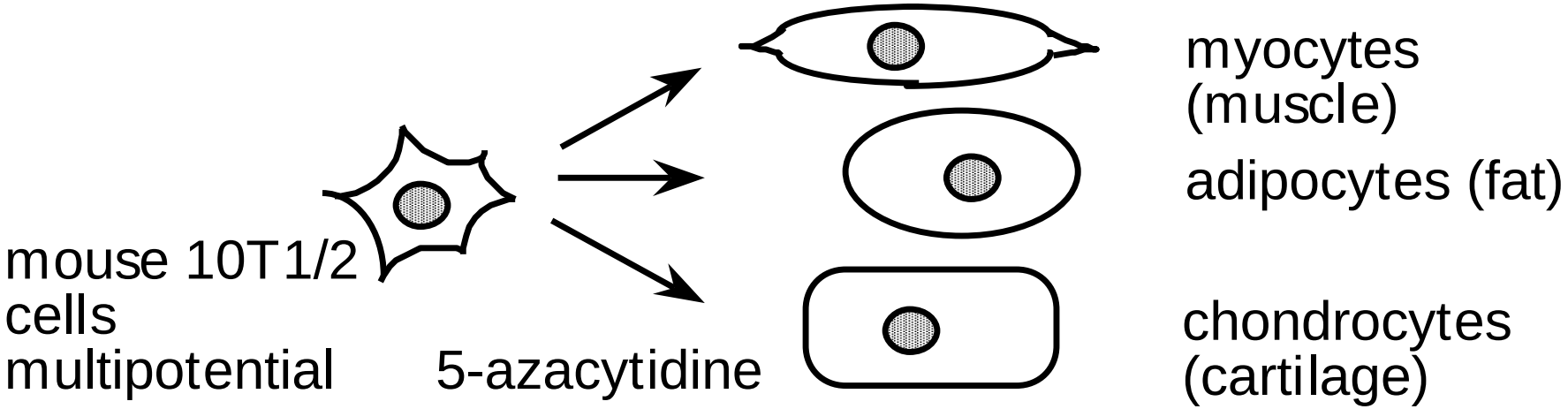
Instead of looking for one particular cDNA, look for cDNAs from **all** genes whose expression differs in the process under study

- Differentiation from mesoderm to muscle
- Response to different nutrients
- Progression through S phase of the cell cycle

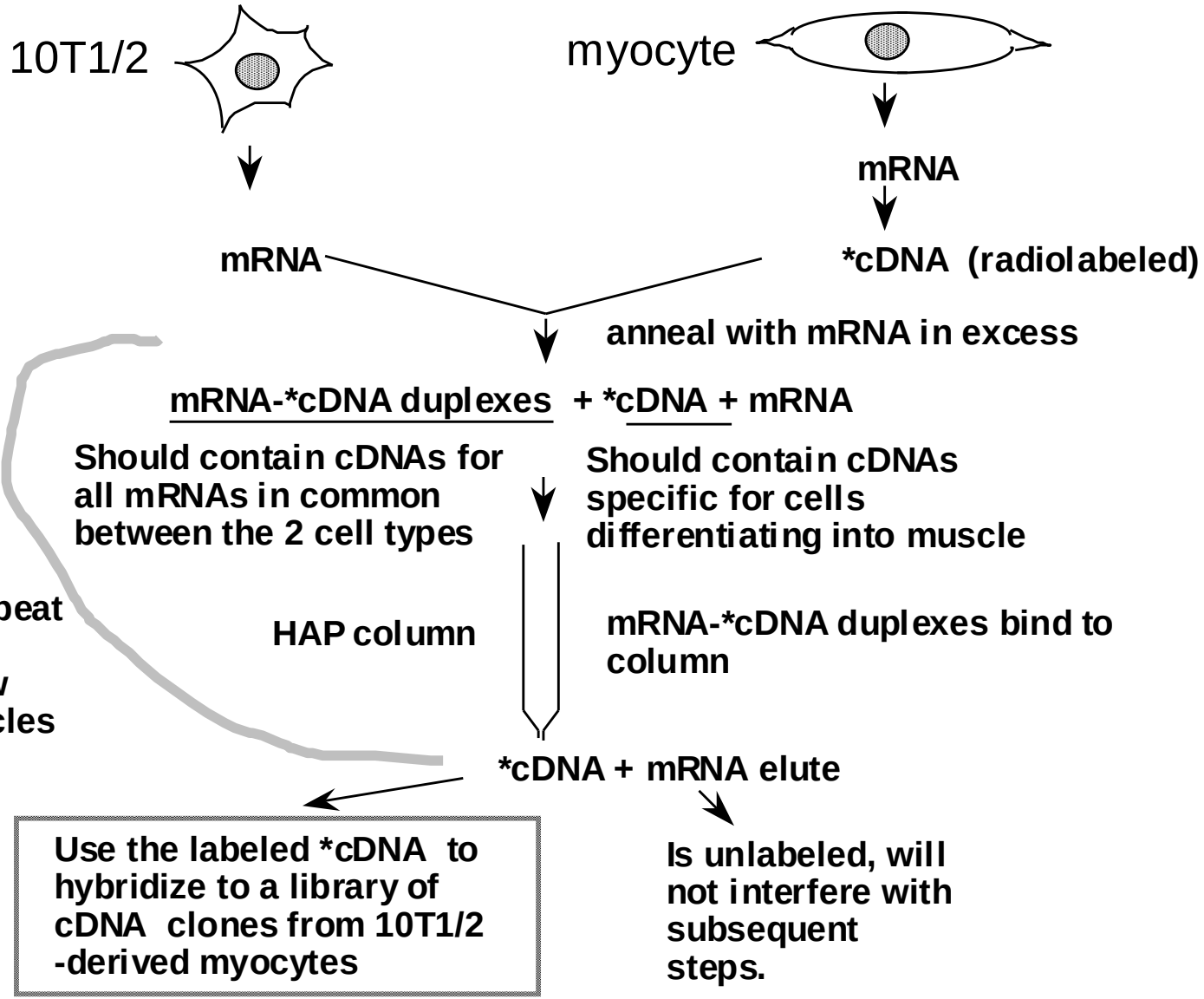
Methods:

- Subtractive hybridization
- Differential display
- Hybridization to massively parallel arrays of cDNAs.

Differential analysis applied to muscle differentiation



Subtractive hybridization



Differential display of RT-PCR products

Make cDNA from all mRNA in the two different cellular states (RT = reverse transcriptase).

Use several sets of PCR primers to amplify a representative sample of all the cDNAs.

Resolve those RT- PCR products on a gel.

Find the products that are *present in only one* of the two cellular states being compared.

Try to isolate the corresponding gene.

Sequence everything, find function later

- Determine the sequence of hundreds of thousands of cDNA clones from libraries constructed from many different tissues and stages of development of organism of interest.
- Initially, the sequences are partials, and are referred to as expressed sequence tags (ESTs).
- Use these cDNAs in high-throughput screening and testing, e.g. expression microarrays (next presentation).

Genomic DNA clones

Clones of **genomic DNA** contain fragments of chromosomal DNA. They are used to:

obtain detailed structures of genes

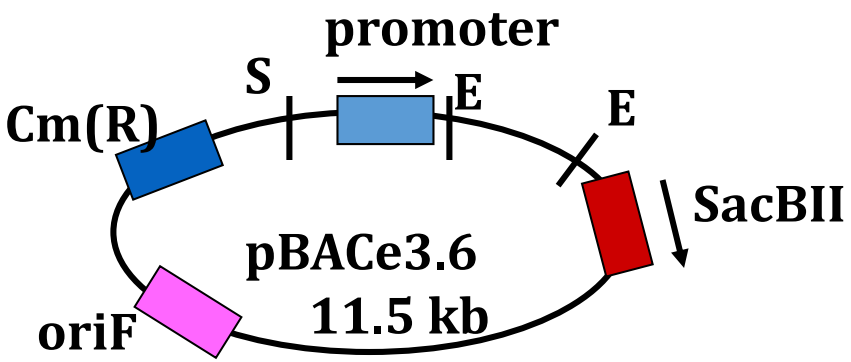
identify regulatory regions

map and analyze alterations to the genome, e.g. isolate genes that when mutated cause a hereditary disease

direct alterations in the genome

sequence the genome.

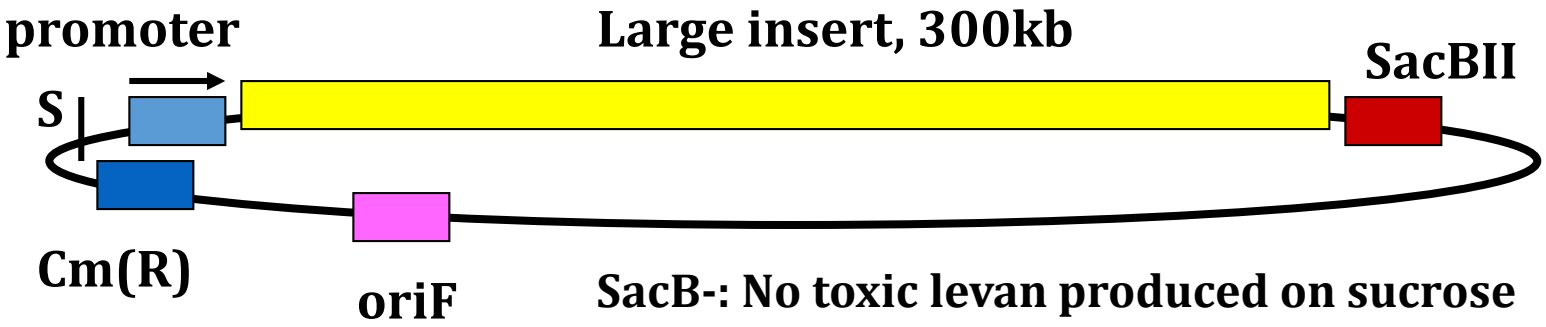
BAC vectors for large DNA inserts



SacB+: SacBII encodes levansucrase, which converts sucrose to levan, a compound toxic to the bacteria.

Cut with restriction enzyme E, remove "stuffer"

Ligate to very large fragments of genomic DNA



SacB-: No toxic levan produced on sucrose media: positive selection for recombinants.

Not to scale.

How many clones make a representative library?

P = probability that a gene is in a library

f = fraction of the genome in a single recombinant

f = insert size/genome size

For N recombinants, 1-P = (1-f)expN

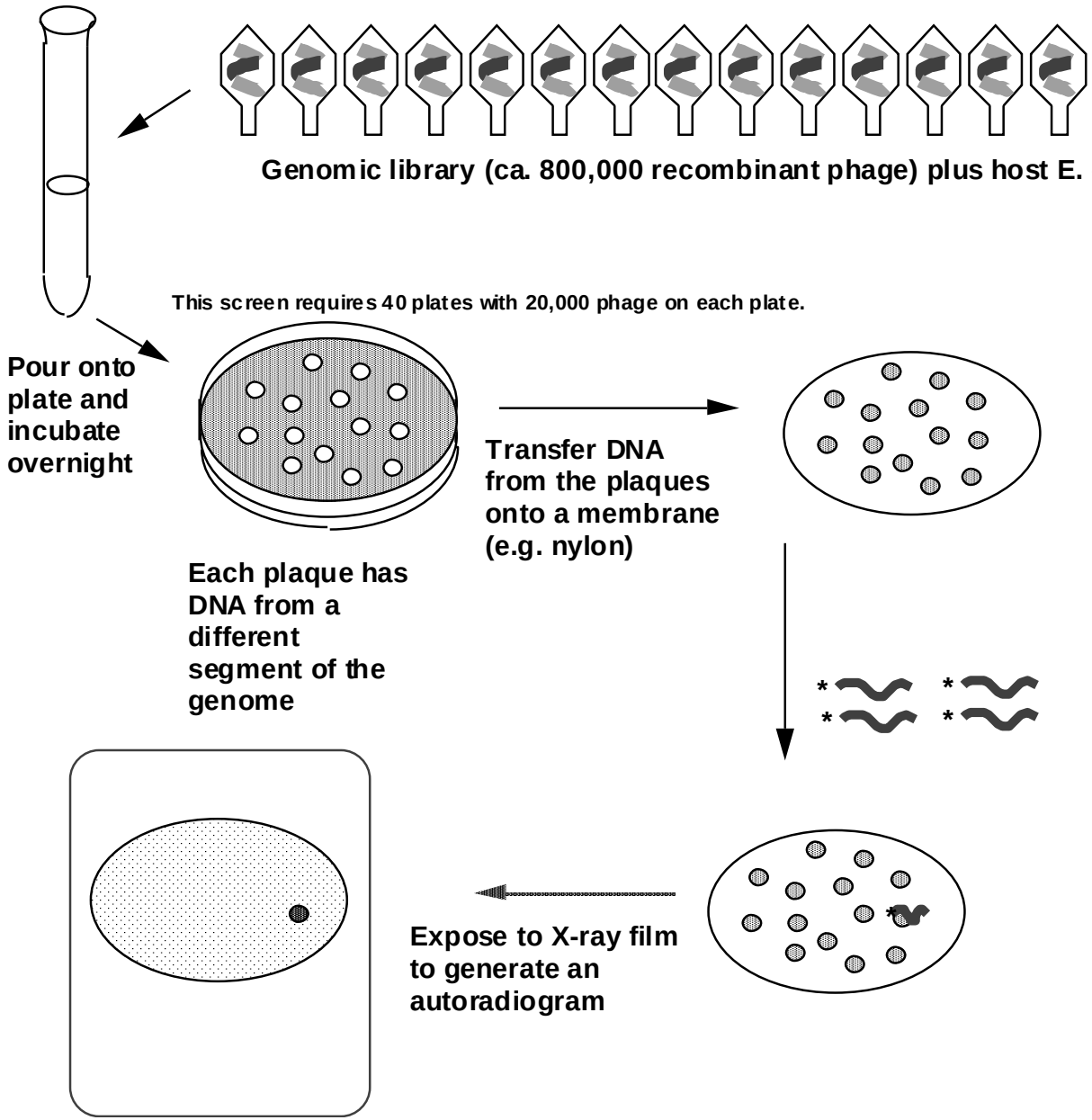
ln(1-P) = N ln(1-f)

N = ln(1-P) / ln(1-f)

For a lambda library with an average insert size of 17 kb and a genome size of 3 billion bp, then one needs a library of 800,000 clones to have a probability of 0.99 of having all genes in the library.

For a BAC library, with an average insert size of 300 kb and a genome size of 3 billion bp, then the library size required for P=0.99 is reduced to about 46,000 clones.

Screening libraries of genomic clones



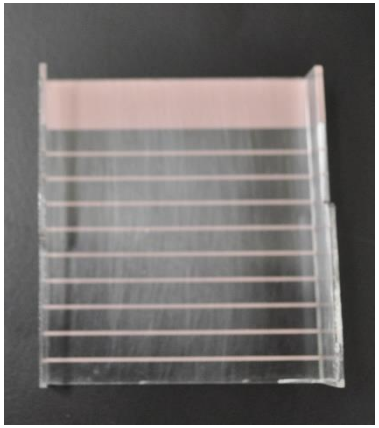
Sequence everything: genomics

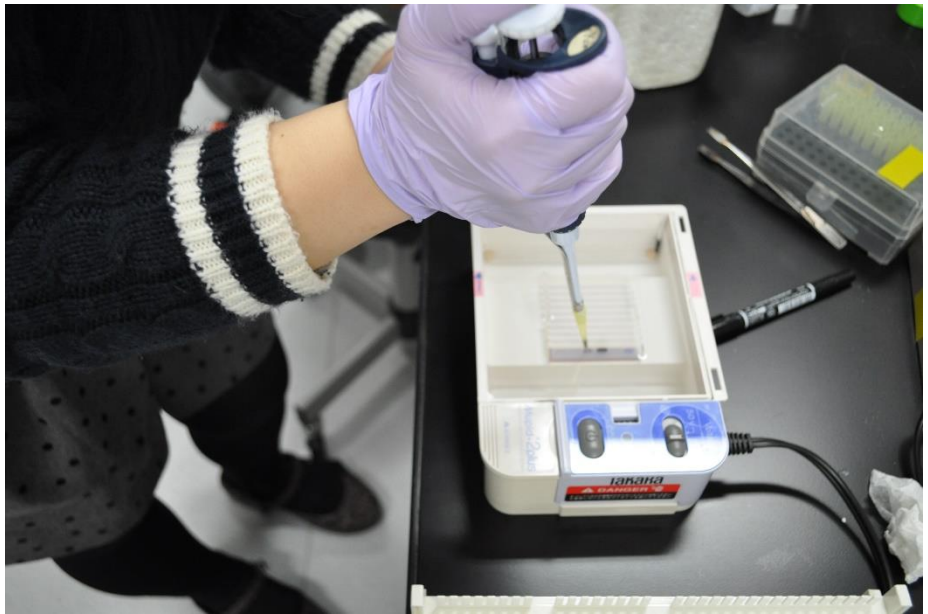
Instead of screening for one gene at a time, an entire genome can be sequenced, and one can use experimental and bioinformatic approaches to find many (all?) genes of interest.

Made possible by

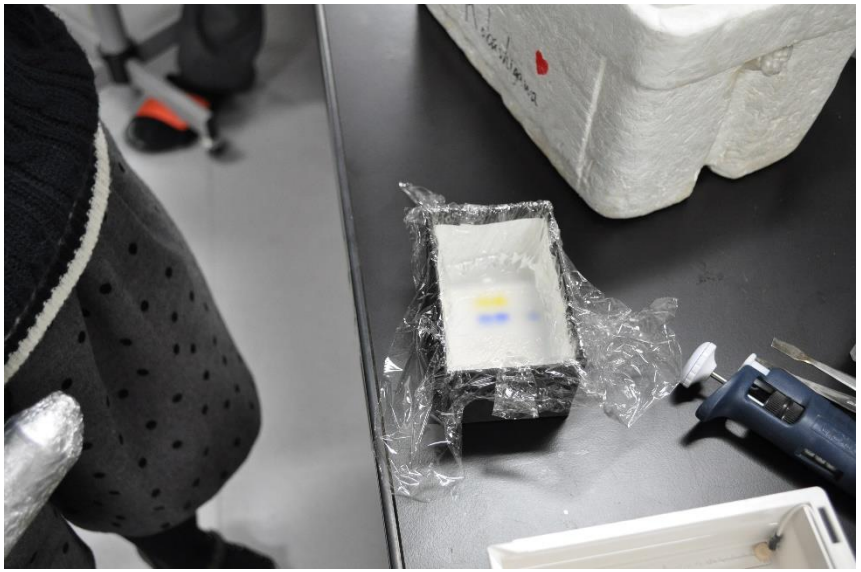
- Substantial increases in speed of sequencing
- Larger insert libraries for larger genomes
- Combination of hierarchical sequencing (based on maps) and whole genome shotgun sequencing

Biochemistry and Molecular Biology





Biochemistry and Molecular Biology



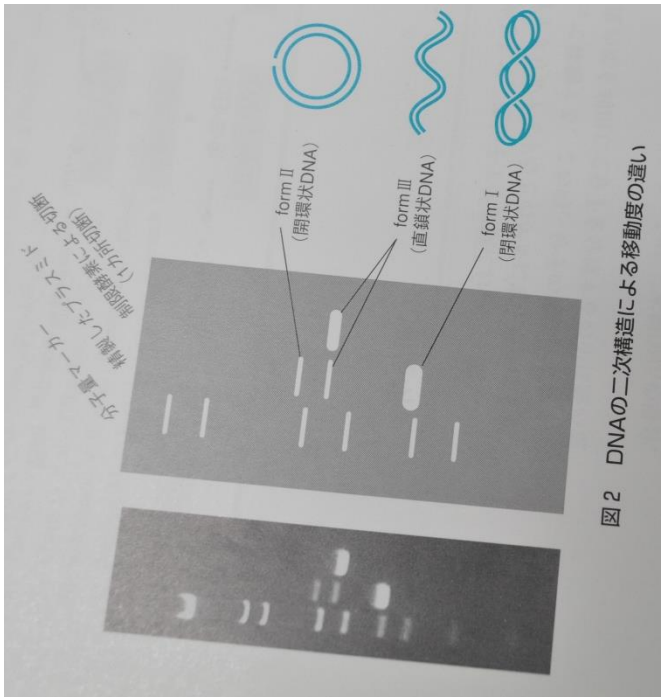
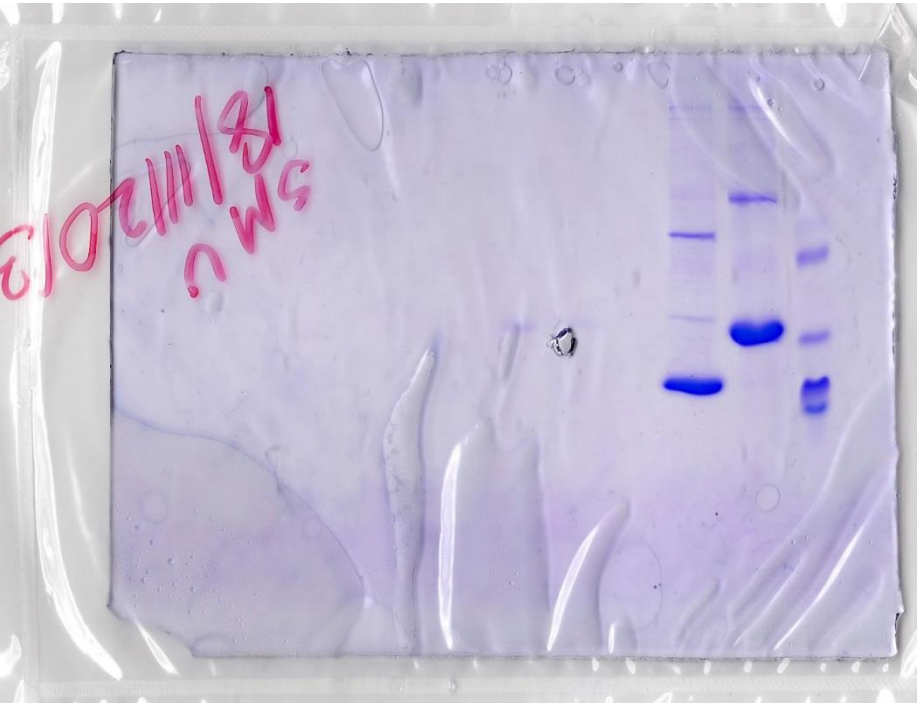


図2 DNAの二次構造による移動度の違い



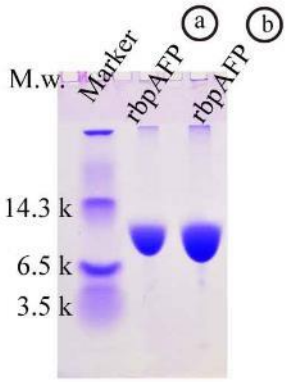


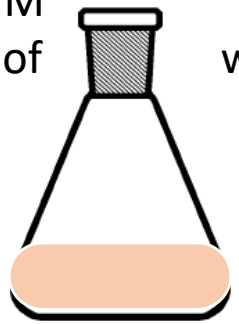
Figure 8. Electrophoretogram of pure rbpAFP.

Expression of Metzincin in Mammalian Cell

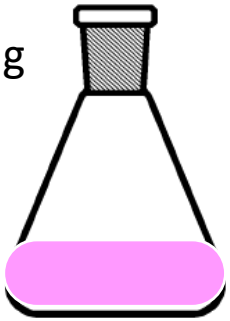
Human MMP9 (hMMP9)

HEK293 cells

Culture to get 0.5× 10^6 M cells in desired volume of media



Transient transfection with the cDNA encoding hMMP9



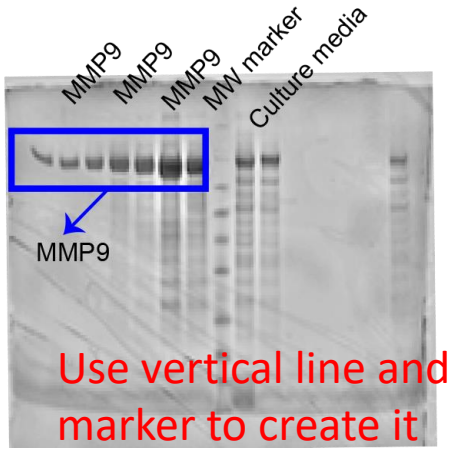
Incubate at 37 ˚C and 125 rpm

Cells are allowed to prepare hMMP9 for next 6 days

Centrifugation and filtration

Media containing hMMP9

Ni-NTA affinity purification



Electrophoretogram of hMMP9 purification process.