PharmacoLearn

MPL103T Pharmacological and Toxicological Screening Methods I, M.Pharm Semester 1

Unit 5: Preclinical Screening: Immunomodulators, Immunoassay, Alternatives and Extrapolation

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5.1 Screening of immunomodulators

An immunomodulator shifts immune function up or down, and screening must first say which direction, in which arm (cellular or humoral), and at what cost. The standard battery in rodents combines:

- **Antibody response to sheep red blood cells (SRBC):** animals are immunised with SRBC and the haemagglutination titre or plaque forming cell count in the spleen measures humoral immunity; suppression or enhancement against control is the primary endpoint.
- **Delayed type hypersensitivity (DTH):** SRBC or another antigen is given in the footpad after sensitisation; the swelling at 24 to 48 hours measures cell mediated immunity.
- **Carbon clearance test:** intravenous colloidal carbon is cleared by the reticuloendothelial system; the clearance rate (phagocytic index) measures macrophage function, raised by immunostimulants.
- **Supporting counts:** total and differential leucocyte counts, neutrophil adhesion, spleen and thymus weights, and resistance to a standard infection challenge.

Levamisole is the classical immunostimulant reference; cyclophosphamide induced immunosuppression provides the standard suppressed background against which restorative agents are proven.

5.2 Screening of immunosuppressants and immunostimulants

Immunosuppressants are screened for activity and for selectivity: the goal is suppression of the target response at doses that do not simply poison the animal. The SRBC antibody and DTH models of section 5.1, run in the suppressive direction, are the primary screens; skin graft survival prolongation is the classical transplant model; and mixed lymphocyte reactions and lymphocyte proliferation assays (mitogen stimulated) are the in vitro tier. Cyclosporine is the reference standard, with azathioprine and corticosteroids as supporting comparators. A selectivity index against general toxicity (weight loss, marrow counts) separates immunosuppression from cytotoxicity.

Immunostimulants are screened on the same models in the enhancing direction, and against cyclophosphamide suppressed animals for restorative claims: raised antibody titres, restored DTH, faster carbon clearance, and increased resistance in infection challenge models (for example survival after a standard bacterial inoculum). Levamisole is the standard; many plant derived preparations are examined in exactly this battery.

5.3 Immunoassay: theoretical basis

An **immunoassay** measures a substance using the binding of a specific antibody as the measuring event. Its theoretical basis is the **law of mass action** applied to a reversible antigen antibody equilibrium: antigen plus antibody form a complex whose amount, at equilibrium, depends on the concentrations and on the antibody's affinity constant. Because the complex itself is invisible, one partner carries a **label** (a radioisotope historically, now usually an enzyme, a fluorescent tag or a chemiluminescent marker) whose signal can be counted.

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Two architectures follow from the equilibrium:

- **Competitive (limited reagent) assays:** labelled and unlabelled antigen compete for a fixed, limited number of antibody sites. The more analyte in the sample, the less label is bound, so signal falls as concentration rises. This design suits small molecules such as digoxin and hormones.
- **Immunometric (excess reagent) assays:** excess labelled antibody (or a capture antibody plus a labelled second antibody, the sandwich design) binds the analyte; signal rises with concentration. Sandwich designs need an analyte with two binding sites, so they suit large molecules such as insulin and protein hormones.

Sensitivity comes from the antibody's affinity and the label's detectability; specificity comes from the antibody's selectivity, which is why cross reactivity (section 5.6) is the standing weakness.

5.4 Optimisation of immunoassay

Optimisation means tuning the assay so that its dose response curve is steepest and most precise across the concentrations that matter clinically. The variables adjusted, one at a time and then together, are:

- **Antibody concentration and affinity:** lower antibody concentrations raise sensitivity in competitive designs, but too little antibody flattens the signal and worsens precision.
- **Incubation time and temperature:** long enough to approach equilibrium, short enough to be practical; non equilibrium (sequential) formats trade some equilibrium purity for speed.
- **Label and tracer quality:** specific activity, purity and stability of the labelled antigen or antibody.
- **Separation conditions:** in heterogeneous systems the bound free separation step (section 5.5) must be complete without disturbing the equilibrium being measured.
- **Matrix controls:** buffer composition, pH, ionic strength and protein content matched between standards and samples so that serum itself does not shift the curve (matrix effect).
- **Standard curve design:** enough calibrators, in the right range, fitted with the correct curve model, because every reported value is read from that curve.

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OPTIMISING A COMPETITIVE IMMUNOASSAY

  Fix antibody, vary tracer amount
        |   choose the lowest tracer giving
        |   a countable, stable signal
        v
  Titrate antibody against fixed tracer
        |   select the dilution binding about
        |   50 percent of tracer with no analyte
        v
  Run full standard curve; check sensitivity
  (least detectable dose) and working range
        |
        v
  Confirm precision and recovery in real samples
  before accepting the assay for use
```

5.5 Heterogeneous and homogeneous immunoassay systems

Immunoassays are classified by whether bound and free label must be physically separated before measurement.

Feature	Heterogeneous systems	Homogeneous systems
Separation step	Required: bound fraction separated from free (solid phase antibody, charcoal adsorption, precipitation, washing)	Not required: binding itself changes the label's behaviour, so the mixture is read directly
How binding is read	Signal of the separated bound (or free) fraction	Signal modulation in solution, for example enzyme activity switched by antibody binding
Classical examples	Radioimmunoassay (RIA), ELISA, immunoradiometric assay (IRMA)	EMIT (enzyme multiplied immunoassay technique), fluorescence polarisation immunoassay (FPIA), CEDIA
Strengths	High sensitivity, washing removes interference	Fast, simple, easily automated, no separation equipment
Weaknesses	More steps, longer, washing variability	More vulnerable to sample matrix interference; usually less sensitive

ELISA, a heterogeneous enzyme labelled system on a solid phase, is the workhorse format of screening laboratories as well: the same architecture that measures digoxin in a patient sample also measures antibody titres in the immunomodulator screens of section 5.1.

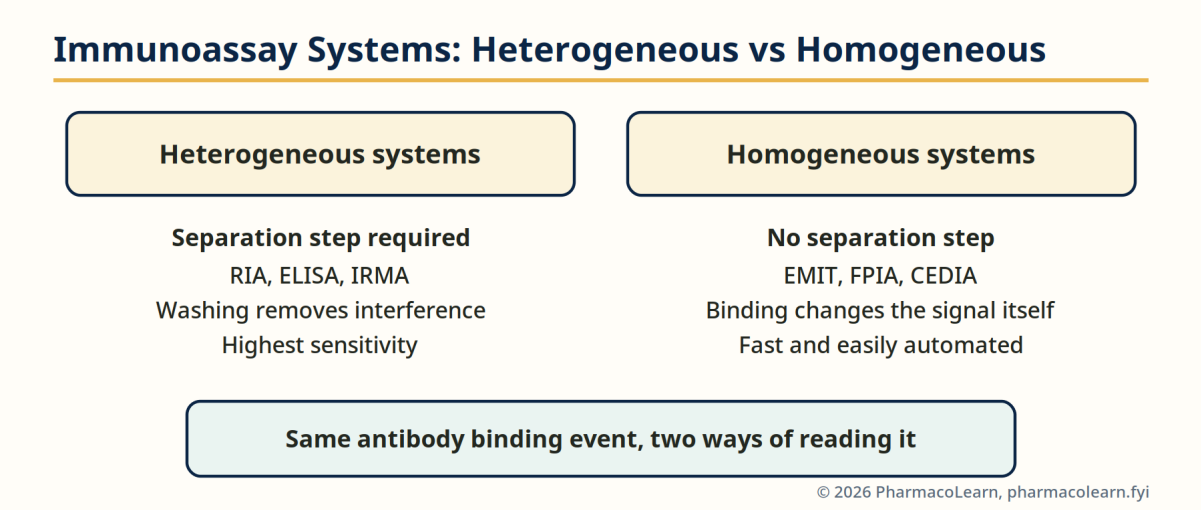

Figure: Heterogeneous and homogeneous immunoassays read the same antibody binding event in two different ways.

5.6 Evaluation of immunoassay methods: protocol outline, objectives and preparation

An immunoassay is only adopted after formal evaluation against a written protocol. The **objectives** are to prove that the method measures what it claims, accurately and reproducibly, in real samples. **Preparation** includes defining the reference method for comparison, collecting patient and control samples across the clinical range, setting acceptance criteria in advance, and training staff on the final written procedure.

The evaluation then measures:

- **Accuracy and method comparison:** results on the same samples by the new method and the reference method, assessed by correlation and bias across the range.
- **Precision:** within run and between run replication of control materials at low, medium and high levels, expressed as coefficient of variation.
- **Sensitivity:** the least detectable dose, from the variability of the zero standard.
- **Recovery and dilution linearity (parallelism):** known amounts added to samples are recovered quantitatively, and diluted samples read parallel to the standard curve; failure means matrix interference.

- **Specificity and cross reactivity:** related substances (for digoxin, its metabolites and other cardiac glycosides and steroids) are tested for false responses.
- **Interference and stability:** haemolysis, lipemia and bilirubin effects; reagent shelf life and calibration stability.

Only a method that passes its pre set criteria on all of these is released for routine use; that discipline, not the label technology, is what makes an immunoassay a measurement rather than a demonstration.

5.7 Immunoassay for digoxin

Digoxin is the model competitive immunoassay because it is a small molecule with a narrow therapeutic range (about 0.5 to 2 ng per ml in serum) that chemical methods cannot measure at clinical concentrations. The design is competitive: digoxin in the patient's serum competes with labelled digoxin (enzyme, fluorescent or chemiluminescent label in modern kits; iodine 125 in the classical RIA) for a limited amount of anti digoxin antibody, and the bound signal, read against the standard curve, falls as the sample concentration rises.

Evaluation points specific to digoxin illustrate section 5.6 in action: cross reactivity of digitoxin, digoxin metabolites and endogenous digoxin like immunoreactive substances (raised in renal failure, liver disease and pregnancy) can falsely elevate results; samples from patients given digoxin specific antibody fragments (Fab) after poisoning disturb the assay because much of the digoxin is antibody bound. The clinical use is therapeutic drug monitoring: dose adjustment in heart failure and atrial fibrillation, and confirmation of suspected digoxin toxicity.

5.8 Immunoassay for insulin

Insulin is the model immunometric assay because it is a peptide large enough to carry two antibody binding sites. The modern design is a **two site sandwich**: a capture antibody fixed to a solid phase binds one epitope of insulin, and a second, labelled antibody binds another epitope; the sandwich count is directly proportional to insulin concentration. The classical competitive radioimmunoassay of Berson and Yalow, the first immunoassay ever described, made insulin measurable at all and founded the whole field.

Specific insulin questions shape its evaluation: cross reaction with **proinsulin** (the precursor, raised in type 2 diabetes and insulinoma) falsely inflates older assays, so modern antibodies are chosen for true insulin specificity; insulin treated diabetic patients develop anti insulin antibodies that interfere; and paired measurement with **C-peptide** (secreted with insulin but not present in injected preparations) distinguishes endogenous secretion from injected insulin, a distinction used in evaluating hypoglycaemia and insulinoma. Fasting insulin and C-peptide values feed indices of insulin resistance and beta cell function in the metabolic screening of Unit 4.

5.9 Limitations of animal experimentation

Animal screening built pharmacology, but its limits must be stated as plainly as its successes.

- **Species differences:** metabolism, receptor distribution and sensitivity differ; thalidomide was quiet in rat tests yet teratogenic in humans, and penicillin is lethal to guinea pigs through gut flora disruption while being safe in humans. A clean animal result is evidence, not proof.
- **Disease models are imitations:** carrageenan oedema is not rheumatoid arthritis, and a scopolamine deficit is not Alzheimer's disease; models answer the question they embody and no other.
- **Dose and exposure differences:** animals often receive doses and durations far from clinical use, and routes differ.

- **Statistical and design limits:** small group sizes, strain peculiarities (section 1.5) and publication bias towards positive results all distort the record.
- **Cost, time and ethics:** whole animal work is slow and expensive, and every use must carry its ethical justification with it (Unit 1, CPCSEA framework).

5.10 Alternatives to animal experiments: the 3Rs

The organising idea is the **3Rs of Russell and Burch**:

- **Replacement:** substitute animals with non animal systems where the science allows: cell and tissue culture, isolated organs, computer (in silico) models of binding and toxicity, lower organisms such as zebrafish embryos and invertebrates, and human volunteer and microdosing studies for defined questions.
- **Reduction:** use the fewest animals that keep the experiment statistically valid: better design, sharing control groups, in vitro pre screening so animals meet only promising compounds (the funnel of Unit 2), and full use of each animal's data.
- **Refinement:** reduce suffering in the animals still used: anaesthesia and analgesia, humane endpoints, better housing and handling, and non invasive measurements such as telemetry and imaging.

Validated alternatives already in routine use include reconstructed human skin models for irritation, cell based assays replacing some pyrogen and potency tests (the monocyte activation test replacing much rabbit pyrogen work), and in silico prediction in early toxicity screening. An alternative replaces an animal test only after validation shows it predicts as well, which is why the replacement programme itself is a screening science.

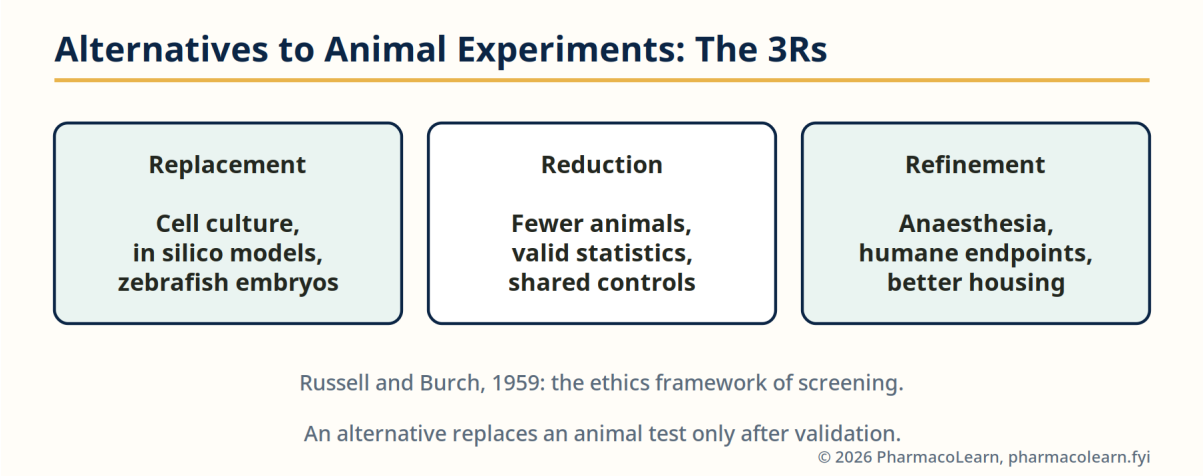

Figure: The 3Rs of Russell and Burch. Every CPCSEA protocol is expected to show how each of the three was considered.

5.11 Extrapolation of in vitro data to preclinical studies

Moving from the test system to the animal is the first extrapolation, and it succeeds or fails on concentration. The discipline is comparison: the concentration active in vitro must be reachable, as free drug, in the animal's plasma or tissue at tolerated doses. A compound potent at nanomolar levels in a receptor assay but never achieving even micromolar free levels in vivo will fail, and recognising that early is the point of the exercise. Binding to plasma proteins and tissue components lowers the free fraction in the animal in a way buffer systems do not reproduce; metabolism in the intact animal can destroy an in vitro winner or create the real active agent from an inactive looking parent (the prodrug pattern).

Pharmacokinetic measurement in the screening species therefore accompanies the first in vivo efficacy tests, so that a negative result can be read correctly: no exposure, or exposure without effect. These are different verdicts and lead to different decisions.

5.12 Extrapolation from animals to humans

The final extrapolation crosses to the clinic, and its working tool is **allometric scaling**: physiological quantities such as clearance and dose relate to body weight by power laws across species, so a human equivalent dose can be estimated from animal doses by body surface area based conversion factors (the basis of first in human starting dose calculations, further discounted by safety factors from the no observed adverse effect level in toxicology). Beyond arithmetic, three judgement rules govern the crossing:

- **Weight concordance:** an effect seen in several species, at overlapping exposures, is more likely to appear in humans than a single species curiosity.
- **Prefer mechanism over phenomenology:** if the animal effect is explained by a receptor or enzyme that humans share, confidence rises; if it rides on a species specific pathway, confidence falls.
- **Treat safety signals conservatively:** toxicity in animals is assumed relevant to humans until shown otherwise, while efficacy is assumed provisional until the clinic confirms it.

Preclinical screening therefore does not predict the clinic; it prices the risk of entering it. That honest framing, from the CPCSEA protocol of Unit 1 to the first human dose, is the subject as a whole.

Frequently Asked Questions

Q: Which laboratory animals are most commonly used in pharmacological screening, and why? Mice and rats dominate because they are small, cheap, quick breeding and need only small amounts of a new compound. Guinea pigs serve bronchospasm and ileum work, rabbits pyrogen and eye studies, dogs cardiovascular and emetic work, and primates only where no lower species can answer the question.

Q: What is CPCSEA and what does an IAEC do? CPCSEA is the statutory committee under the Prevention of Cruelty to Animals Act, 1960 that registers establishments and sets norms for animal experiments in India. The Institutional Animal Ethics Committee of each establishment, which must include the CPCSEA nominee, approves every protocol before work begins and monitors welfare during it.

Q: What is the difference between inbred and outbred strains? Inbred strains come from at least 20 generations of brother to sister mating, so animals are almost genetically identical, which removes genetic noise but narrows generalisation. Outbred stocks are bred to keep genetic variation, so results generalise better across genotypes, and they are the routine screening default.

Q: What is bioassay and when is it preferred over chemical assay? Bioassay measures potency by the response of a living system, always relative to a standard preparation. It is preferred when the active principle is unknown, when no chemical method is sensitive or specific enough, or when biological activity itself is the property being measured, as with vaccines and hormones.

Q: How do the MES and PTZ tests divide antiepileptic drugs between them? The maximal electroshock test, judged by abolition of tonic hindlimb extension, detects drugs for generalised tonic clonic seizures such as phenytoin. The pentylenetetrazol test detects drugs for absence and myoclonic seizures such as ethosuximide and valproate. Phenytoin fails the PTZ test, exactly as it fails absence seizures clinically.

Q: Which models screen analgesics, and how are central and peripheral agents separated? The hot plate and tail flick tests respond to central, opioid analgesics such as morphine but not to NSAIDs. The

acetic acid writhing and formalin late phase and Randall Selitto tests respond to peripheral agents such as diclofenac as well. Running both families, with naloxone reversal as confirmation, classifies a new analgesic.

Q: What is the carrageenan paw oedema model and what does its time course show? Carrageenan injected into the rat paw produces swelling in two phases: an early histamine and serotonin phase and a late prostaglandin phase. NSAIDs suppress the late phase, which is why percentage inhibition at about 3 hours, measured by plethysmometer against a diclofenac or indomethacin standard, is the reportable endpoint.

Q: Which animal models are used for screening antidiabetic drugs? Insulin deficient models (streptozotocin or alloxan treated rats) suit insulin and secretagogues; genetic models (db/db mice, Zucker fatty rats) and high fat diet models suit insulin sensitisers such as metformin. Glucose tolerance tests are the first line screen across classes.

Q: What is the difference between heterogeneous and homogeneous immunoassays? Heterogeneous assays such as RIA and ELISA must physically separate bound from free label before measuring, which gives high sensitivity at the cost of extra steps. Homogeneous assays such as EMIT need no separation because binding itself changes the label's signal, making them faster and easier to automate.

Q: What are the 3Rs in animal experimentation? Replacement of animals with non animal systems where possible, Reduction of animal numbers to the statistical minimum, and Refinement of procedures to reduce suffering, as framed by Russell and Burch. Indian CPCSEA review expects every protocol to show how the 3Rs were considered.

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